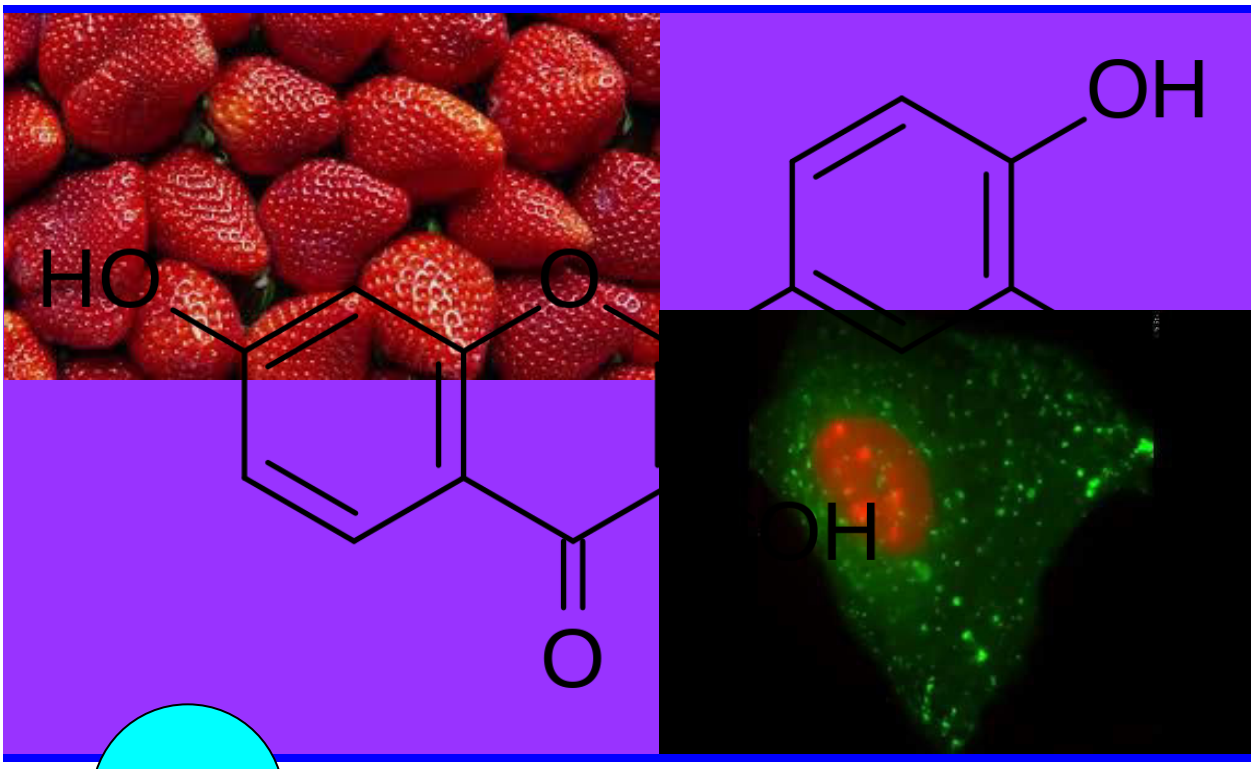

BJP

Bangladesh Journal of Pharmacology

Research Article

Fisetin, a dietary flavonoid induces apoptosis via modulating the MAPK and PI3K/Akt signalling pathways in human osteosarcoma (U-2 OS) cells

A Journal of the Bangladesh Pharmacological Society (BDPS) **Bangladesh J Pharmacol 2015; 10: 820-29**
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index; **ISSN:** 1991-0088

Fisetin, a dietary flavonoid induces apoptosis via modulating the MAPK and PI3K/Akt signalling pathways in human osteosarcoma (U-2 OS) cells

Jian-Ming Li[1], Wu-Yin Li[1], Man-Yu Huang[1] and Xiao-Qiang Zhang[2]

[1]Department of Hip Traumatology and [2]Orthopedics, Luoyang Orthopedic-Traumato Logical Hospital, Luoyang 471 002, China.

Article Info

Received: 20 April 2015
Accepted: 23 May 2015
Available Online: 2 October 2015

DOI: 10.3329/bjp.v10i4.23039

Cite this article:
Li JM, Li WY, Huang MY, Zhang XQ. Fisetin, a dietary flavonoid induces apoptosis via modulating the MAPK and PI3K/Akt signalling pathways in human osteosarcoma (U-2 OS) cells. Bangladesh J Pharmacol. 2015; 10: 820 -29.

Abstract

Human osteosarcoma is the most prevalent primary malignant bone tumor with high frequency of invasion and metastasis. Strong resistance coupled with toxicity of the currently available chemotherapeutic drugs poses challenge in treatment. The study aimed to investigate if fisetin, a dietary flavonoid induced apoptosis in human osteosarcoma (U-2 OS) cells. Fisetin at 20-100 µM effectively reduced the viability of OS cells, and induced apoptosis by significantly inducing the expression of caspases (Caspases- 3,-8 and -9) and pro-apoptotic proteins (Bax and Bad) with subsequent down-regulation of Bcl-xL and Bcl-2. While fisetin inhibited PI3K/Akt pathway and ERK1/2, it caused enhanced expressions of p-JNK, p-c-Jun and p-p38. Fisetin-induced ROS generation and decrease in mitochondrial membrane potential would have also contributed to rise in apoptotic cell counts. The observations suggest that fisetin was able to effectively induce apoptosis of U-2 OS cells through ROS generation and modulation of MAPK and PI3K/Akt signalling cascades.

Introduction

Osteosarcoma, most frequent of the bone tumors, occurs predominantly in adolescents and children. It is characterized by high recurrence and metastasis of bone and soft tissue (Kager et al., 2003; Kim et al., 2004). Treatment includes multimodal approaches including surgery, radiotherapy and chemotherapy (Guijarro et al., 2014; Luetke et al., 2014). However, drug resistance and severe side effects associated with conventional therapeutics (Chou and Gorlick, 2006) have urged for novel and more effective therapeutics.

Studies have reported dysregulation of several signalling pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Zhang et al., 2015). PI3K/Akt pathway is negatively regulated by phosphatase and tensin homolog (PTEN) can activate downstream targets such as mammalian target of rapamycin (mTOR) (Zhang et al., 2015). As a vital pathway in various physiological and pathological processes it is well established in human cancers (Yuan and Cantley, 2008; Porta et al., 2014).

Extracellular signal regulated kinase (ERK1/2), c-Jun N -terminal (JNK) and p38 MAPK, main members of the mitogen-activated protein kinases (MAPKs) family regulate several of cellular responses (Genestra, 2007; Wagner and Nebreda, 2009). MAPK cascades exert crucial roles in drug-induced apoptosis in osteosarcoma (Chen et al., 2009; Noh et al., 2011). Evidences suggest that cancer cells are under increased oxidative stress (Pelicano et al., 2004). Further excessive reactive oxygen species (ROS) affect MAPK signals (Shen and Liu, 2006; Avisetti et al., 2014). Accordingly, targeted inhibition of the MAPK and PI3/Akt pathways may be effective in cancer treatment.

Studies have demonstrated anti-cancer effects of various phytochemicals (Lin et al., 2011; Li et al., 2014,

2015; Zhao et al., 2015). Recent research have reported anti-proliferative (Chen et al., 2002; Haddad et al., 2006) and anti-metastasic effects (Chou et al., 2013) of fisetin, a polyphenolic flavonoid present in many fruits such as strawberries and cucumbers (Kim et al., 2012). Mechanisms through which fisetin exerts its action is yet to be elucidated completely, although it has been demonstrated to increase expressions of p53, down-regulate cyclin D1 and cyclin E levels (Chen et al., 2002; Lee et al., 2002; Lu et al., 2005b; Haddad et al., 2006). Present study investigates whether fisetin induces apoptosis of human osteosarcoma cells and modulats the MAPK and PI3/Akt signalling pathways.

Materials and Methods

Cell lines

The human osteosarcoma cell line U-2 OS (HTB-96TM, ATCC) was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 µg/mL), 2 mM glutamine and penicillin (100 U/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Reagents and chemicals: Fisetin, glutamine, RPMI1640 medium, fetal calf serum (FCS), penicillin, streptomycin, PBS and 0.25% trypsin were purchased from Sigma Aldrich (St.Louis, MO, USA). Antibodies against ERK1/2, phospho- ERK1/2, p38, phospho-p38, JNK, phospho-JNK, c-Jun, phospho-c-Jun, Akt, phospho-Akt, GSK3β (glycogensynthase kinase 3β), phospho-GSK3β), NF-κB, IκB, caspase-3, caspase-8, caspase-9 (Cell Signaling Technology, Beverly, MA), Bcl-xL, Bcl-2, Bad, Bax, mTORC1, cyclinD1 and PTEN (Santa Cruz, Biotechnology, Inc. Santa Cruz, CA, USA) were used in the study. Other chemicals and reagents used in the study were obtained from Sigma Aldrich (St.Louis, MO, USA) unless otherwise specified.

Cell viability assay

The anti-proliferative effect of fisetin on osteosarcoma cells was determined using MTS kit (Promega, Madison, USA). Briefly, cells at a density of 3-7 × 10^3 were seeded per well in 96-well plates. After 12 hours of incubation at 37°C, the cells were treated with various concentrations of fisetin (20-100 µM) for 12, 24 or 48 hours. The cells were further incubated with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and phenazine methosulfate (PMS) for 2-4 hours at 37°C as per manufacturer's instructions. The color developed was read at 490 nm using a MR7000 microplate reader (Dynatech, USA).

Analysis of apoptosis by annexin V assay

Following incubation with various concentrations of fisetin (20-100 µM) for 24 and 48 hours, cells were trypsinized and collected for detection of apoptosis using annexin V-FITC (Fluorescein isothiocyanate) apoptosis detection kit (Santa Cruz Biotechnology, USA). Briefly, 1 × 10^6 cells treated with fisetin were subjected to annexin V staining. The cells were washed in PBS and resuspended in binding buffer containing (100 µL) FITC-conjugated anti-annexin V antibody and analyzed for flurosence using a flow cytometer (FACS Calibur, BD Biosciences).

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was measured with JC-1 fluorescent probe (Cayman Chemical). In brief, 2 × 10^5 cells exposed to fisetin (20 - 100 µM) for 24 hours were incubated with JC-1 for 20 min at 37°C. The stained cells were washed twice with PBS and analyzed by a flow cytometer. Mitochondrial depolarization was indicated by a decrease in the red/ green fluorescence intensity ratio.

Measurement of ROS

Generation of intracellular ROS was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Beyotime). Briefly, 2 x 10^5 cells were plated in each well of six-well plates. Following incubation with various concentrations of fisetin (20-100 µM) for 12 hours and 24 hours, cells were incubated with DCFH-DA (10 µM) for 30 min at 37°C. The level of ROS was determined by fluorescence microscopy and flow cytometer (FACS Calibur, BD Biosciences).

Western blotting

The U-2 OS were seeded at a density of 5 × 10^5 cells in 60-mm dishes and exposed to fisetin (20 - 100 µM) for 24 hours. The cells were collected by centrifugation and the pellets were lysed in RIPA lysis buffer containing protease inhibitor cocktail for 30 min on ice. To determine the cytoplasmic IkB, cytoplasmic extracts were prepared (Lee et al., 2006). To analyze NF-kB (p65), nuclear extract was prepared using a previously described method (Lee et al., 2006). Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). Proteins were then separated by SDS-PAGE, electro-transferred to nitrocellulose membranes, blotted with respective antibodies (Cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, ERK1/2, p-ERK1/2, p-38, p-p38, JNK, p-JNK, c-Jun, p-c-Jun, Akt, phospho-Akt, GSK3β, phospho-GSK3β,NF-kB (p65), IkBα,Bcl-xL, Bcl-2, Bad, Bax, mTORC1, cyclinD1, PTEN and β-actin). The immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare).

Statistical analysis

The data are presented as means ± SD obtained from three or six individual experiments. The values were analysed by one-way ANOVA (analysis of variance).

All statistical analyses were performed using the SPSS software (version 17.0, SPSS, USA).

Results

Fisetin induces apoptosis of osteosarcoma cells

U-2 OS osteosarcoma cancer cells exposed to fisetin showed considerable susceptibility to different concentrations. The cell viability gradually decreased in a concentration and time-dependent manner. Treatment with fisetin at 80 and 100 µM presented more pronounced decreases in cell viability than lower doses (Figure 1).

To further assess apoptosis induced by fisetin, annexin-V/PI staining followed by FACS analysis was

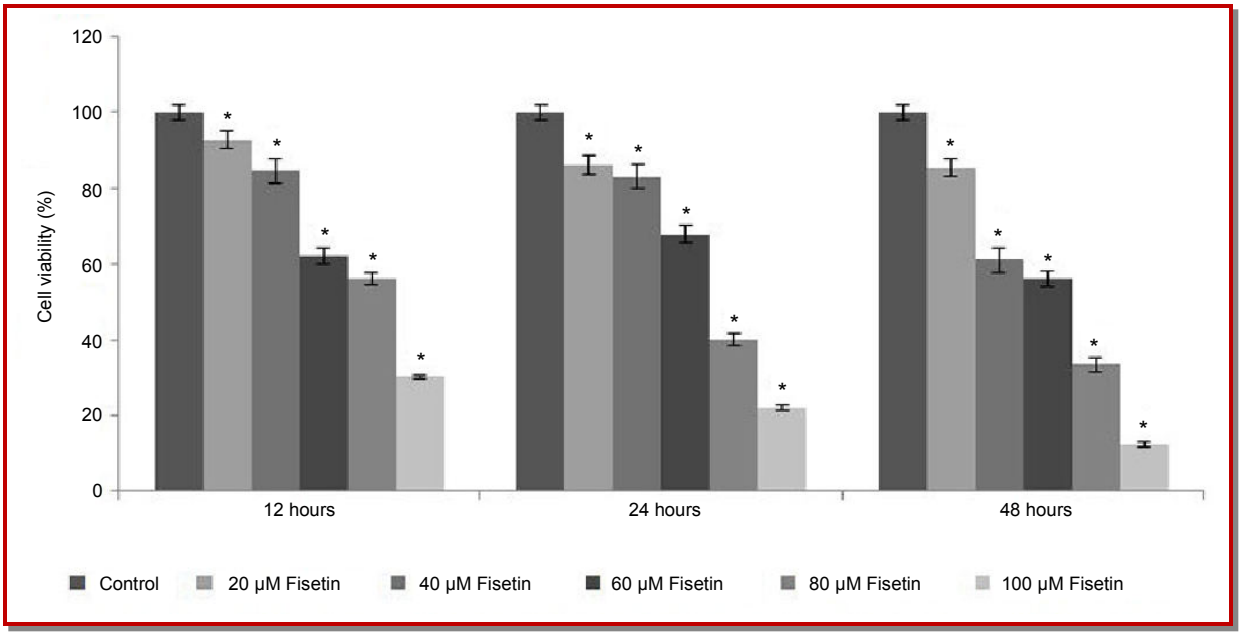

Figure 1: Effect of fisetin on the cell viability of U-2 OS cells

Values are represented as mean ± SD; n=6; *represents p<0.05 compared with control as determined by one way-ANOVA

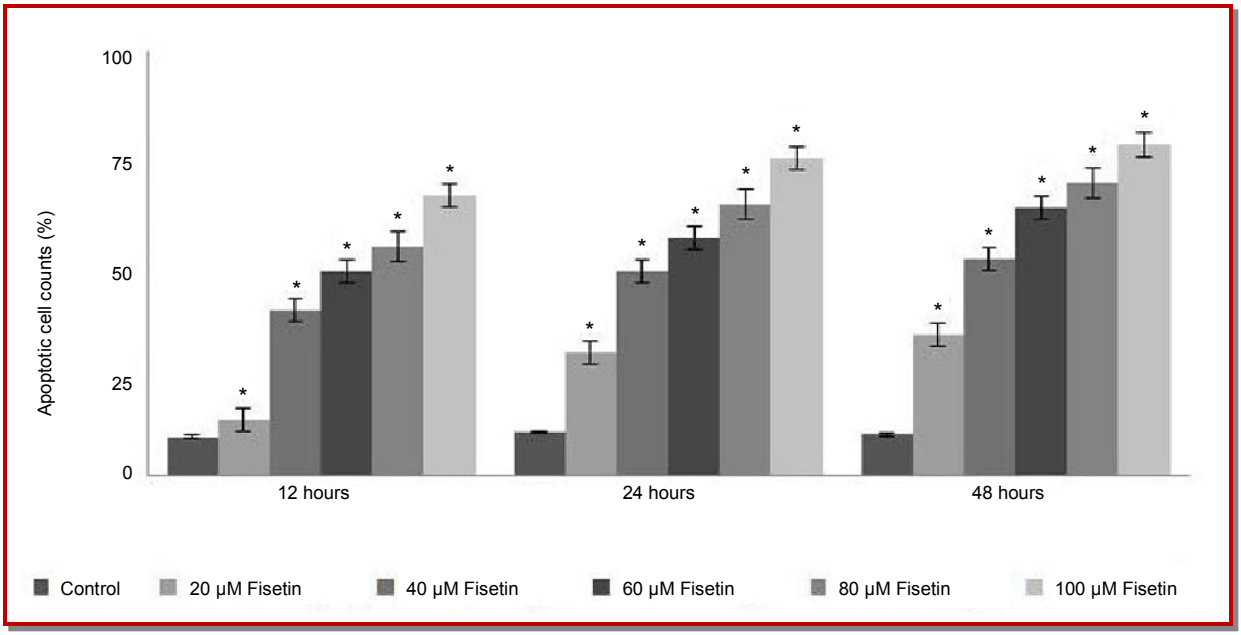

Figure 2: Influence of fisetin on apoptotic cell counts

Fisetin induced apoptosis of the U-2 OS cells in a dose-dependent manner as determined by Annexin V staining. Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one way-ANOVA

performed. Fisetin caused significant ($p<0.05$) increases in apoptotic cell counts (Figure 2). Fisetin at 100 µM exhibited more profound effects than the lower concentrations. Moreover, the time of exposure to fisetin also influenced the effects. Incubation with fisetin for 48 hours brought about more apoptosis than 24 or 12 hours of exposure. Significant ($p<0.05$) difference were observed between the apoptotic cell counts following 24 and 48 hours of fisetin treatment.

Effects of fisetin on apoptotic protein expression

Caspases-3,-9 and -8 were down-regulated in U2-OS cells, suggesting suppression of apoptosis in cancer cells. However, fisetin effectively caused significant ($p<0.05$) up-regulation in the expression of cleaved caspase -3,-8 and -9, thus promoting the apoptotic cascades (Figure 3). Further, fisetin at 100 µM dose was more effective in enhancing the expression of caspases than 20-60 µM. Significant ($p<0.05$) increase in the levels of Bcl-2 and Bcl-xL expressions in the U-2 OS cells was observed, nevertheless following fisetin exposure caused significant down-regulation in the expression. In addition, the level of pro-apoptotic proteins such as Bax and Bad that mainly modulate apoptosis, were enhanced observably in the U2-OS cells exposed to fisetin. Up-regulation of Bax and Bad correlated with the down-regulation of inhibitors of apoptosis- Bcl-2 and Bcl-xL.

Decrease in mitochondrial transmembrane potential indicates early apoptosis. Fisetin exposure resulted in a noticeable decline of mitochondrial transmembrane potential ($\Delta\Psi m$) in a dose-dependent manner (Figure 4). Bcl-2 and Bcl-xL proteins modulate apoptosis at the mitochondrial outer membrane and control the initiation of mitochondrial outer membrane permeabilization (Anilkumar and Prehn, 2014). In our study, Bcl-

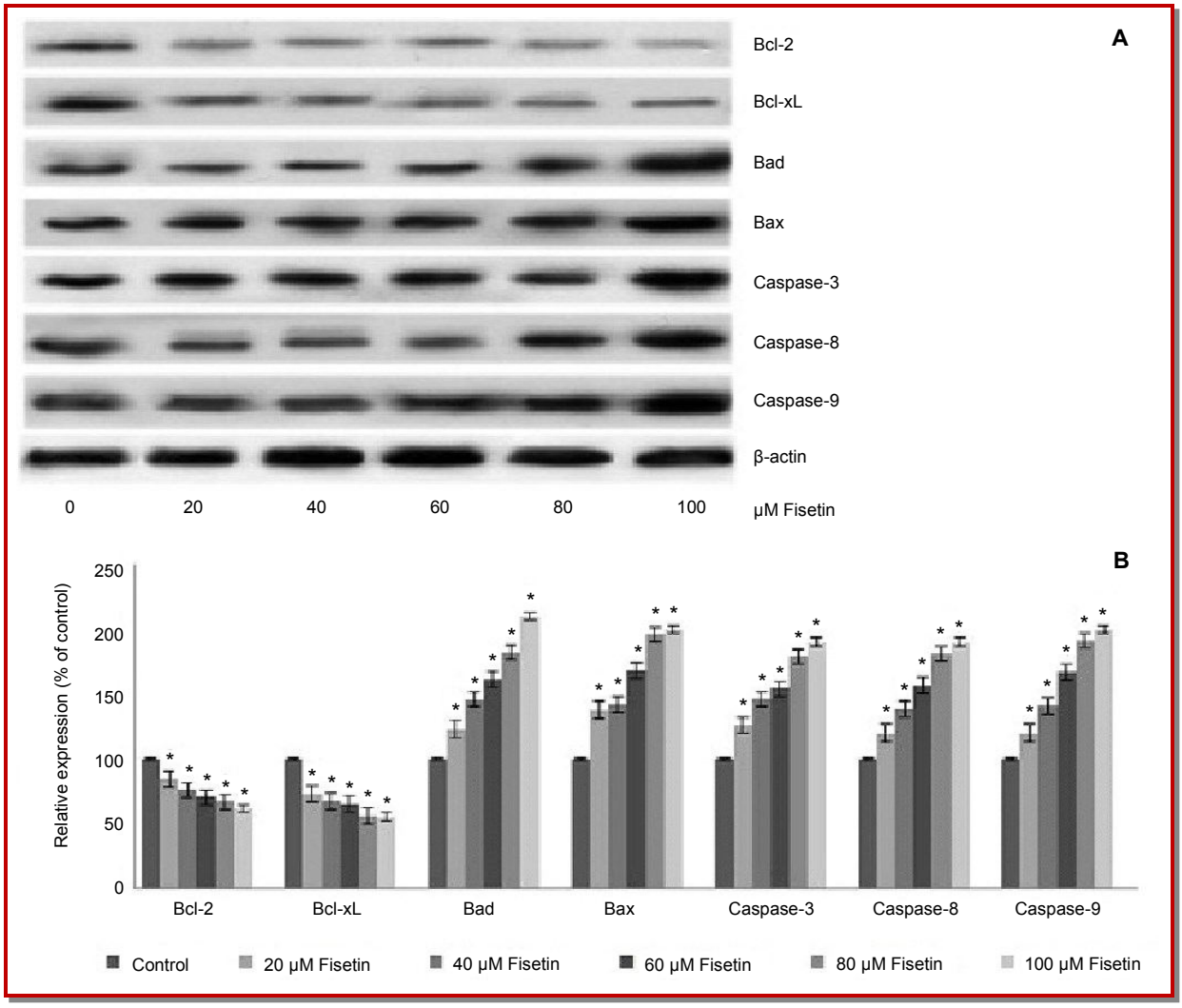

Figure 3: Influence of fisetin on the expression of apoptosis pathway proteins

(A) Fisetin markedly up-regulated the pro-apoptotic proteins and reduced the expressions of Bcl-2 and Bcl-xL. (B) Relative expression of the proteins. Values are represented as mean ± SD; n=3; *represents $p<0.05$ compared with control as determined by one way-ANOVA

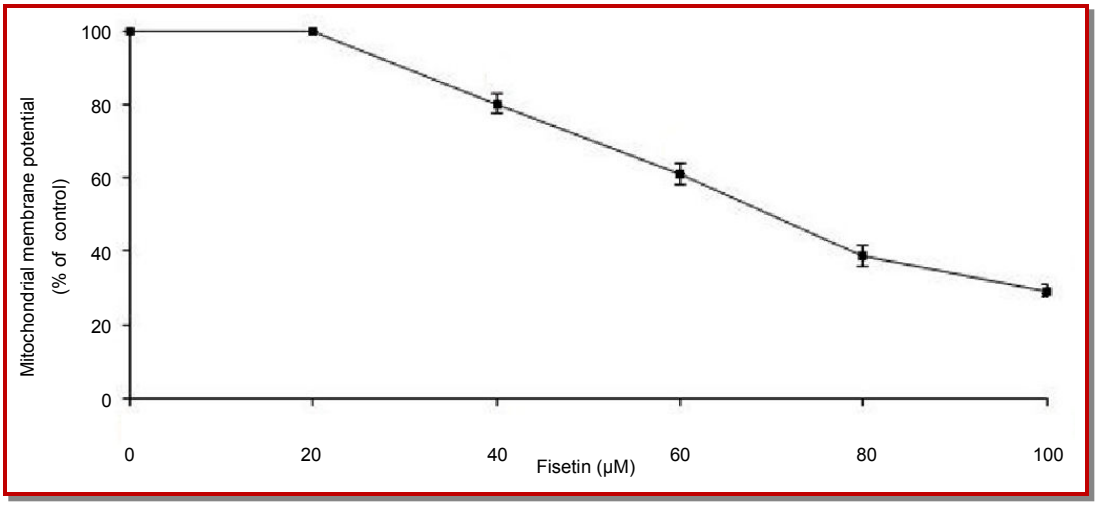

Figure 4: Effect of fisetin on the mitochondrial membrane potential of U-2 OS cells

Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one way-ANOVA

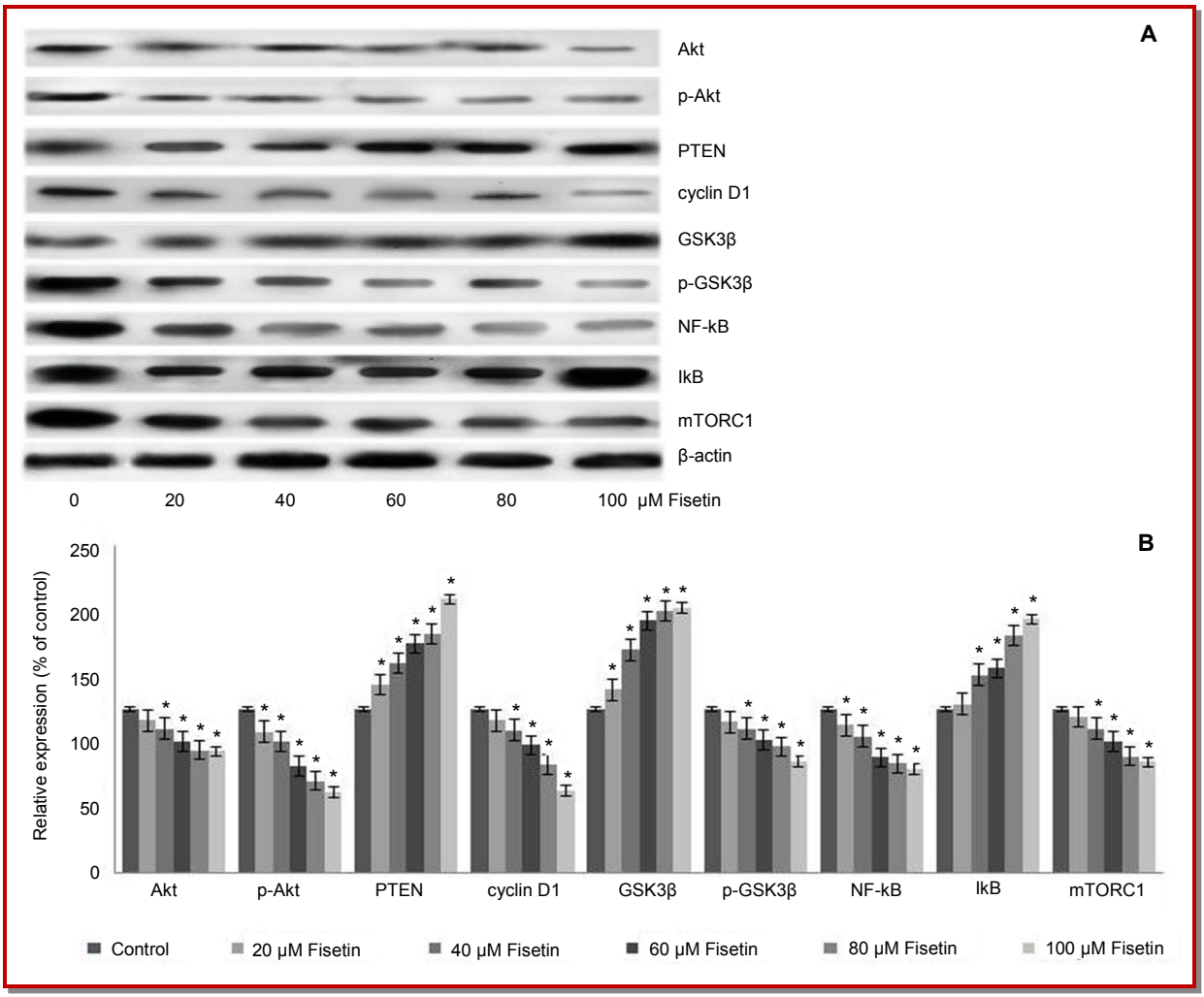

Figure 5: Fisetin regulates the expression of PI3K/Akt pathway proteins

(A) Fisetin effectively modulates the expressions of the PI3K/Akt signalling proteins. Fisetin dose-dependently inhibits the pathway. (B) Relative expressions of the PI3K/Akt pathway proteins. Values are represented as mean ± SD; n=3; *represents p<0.05 compared with control as determined by one-way ANOVA

xL and Bcl-2 were down-regulated in a concentration-dependent manner after fisetin treatment. This could have caused alteration in the mitochondrial membrane potential and thus promoting apoptosis.

Effect of fisetin on PI3/Akt pathway proteins

Activation of the PI3K/Akt pathway plays a critically oncogenic role in the initiation and progression of OS (Zhang et al., 2015). Effect fisetin over the expressions of PI3/Akt pathway proteins in U2-OS cells were assessed. Akt is a critical signalling junction down-stream of the PI3K pathway which is activated through phosphorylation. p-Akt levels are raised in OS cells, whereas PTEN, an important regulator of the pathway was found to be down-regulated in U2-OS cells not exposed to fisetin (Figure 5). However, fisetin at various concentrations (20-100 µM) caused a significant ($p<0.05$) decrease in the level of p-Akt and mTORC1 (an important effector protein of Akt), while up-regulated PTEN. Level of phosphorylated glycogensynthase kinase 3β (GSK3β), (a serine/threonine kinase) and cyclin D1 were potentially decreased by fisetin which is in line with raised non-phosphorylated levels of GSK3β. Another important target activated by Akt is nuclear factor-κB (NF-κB), the levels of which are raised in OS cells. Down-regualtion of NF-κB along with significant up-regulations in IκB upon fisetin treatment correlates with the down-regulation of p-Akt levels. These observations suggest the effective blocking of the PI3K/Akt pathway, an important target in cancer therapy.

Influence of fisetin on generation of ROS

It is well documented that excessive generation of ROS could interfere with DNA, lipids and proteins and cause cellular damage (Simon et al., 2000; Chen et al., 2007). U-2 OS cells presented raised levels of ROS. Fisetin exposure at various concentrations resulted in a noticeable increase in ROS levels in a dose-dependent way (Figure 6). This increase though observable, was not significant.

Fisetin modulates the ERK/JNK/p38MAPK signalling cascade

As MAPKs are important regulators of stress responses including the induction of apoptosis (Johnson and Lapadat, 2002) we assessed the influence of fisetin over JNK, ERK1/2 and p38 expression level. Significant up-regulation in the phosphorylated levels of JNK was observed (Figure 7). Incubation of OS cells in the presence of fisetin (20-100 µM) considerably ($p<0.05$) down-regulated the activation and decreased the levels of p- ERK1/2 and ERK1/2 as well. However, higher expression of phosphorylated p38 was observed. Further, fisetin brought multi-fold raise in the levels of p-c-Jun. While 20-60 µM of fisetin was effective in decreasing the expression of the phosphorylated forms of ERK1/2, 100 µM fisetin presented additional down-regulation.

Discussion

Most cancer therapeutic regimens including chemotherapy, inhibit tumors by activating apoptosis (Zhang et al., 2013). In our study, dietary flavonoid, fisetin at concentrations 20-100 µM induced a decrease in the viability of U-2 OS cells. Further, increase in apoptotic cell counts were observed following fisetin exposure as determined by annexin-V/PI staining.

Mitochondria play a crucial role in apoptosis (vanLoo et al., 2002). Disruption of mitochondrial integrity has been established as one of the earliest intracellular events that occur following initiation of apoptosis (Qi et al., 2010). Decreases in the mitochondrial transmembrane potential (Δψm,) is associated with mitochondrial dysfunction, that activates the efflux of cytochrome C to

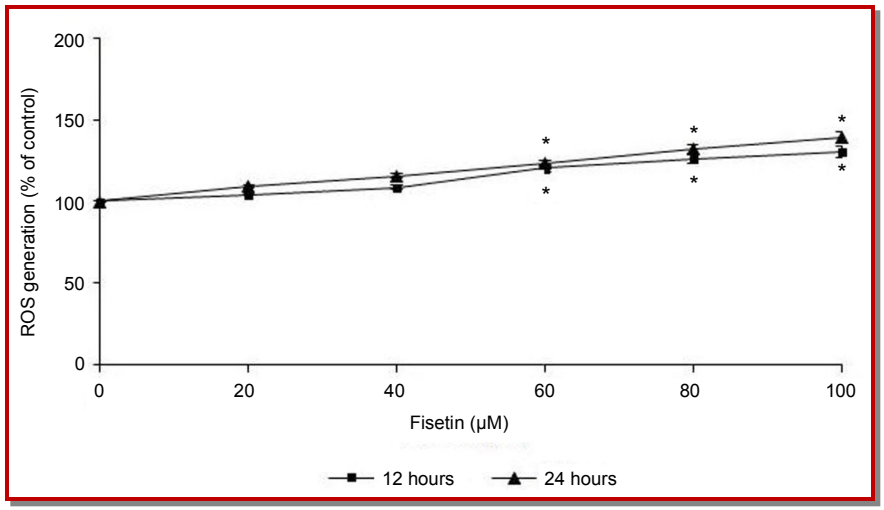

Figure 6. Influence of fisetin on intracellular ROS generation in U-2 OS cancer cells

Values are represented as mean ± SD; n=3. *represents $p<0.05$ compared with control as determined by one-way ANOVA

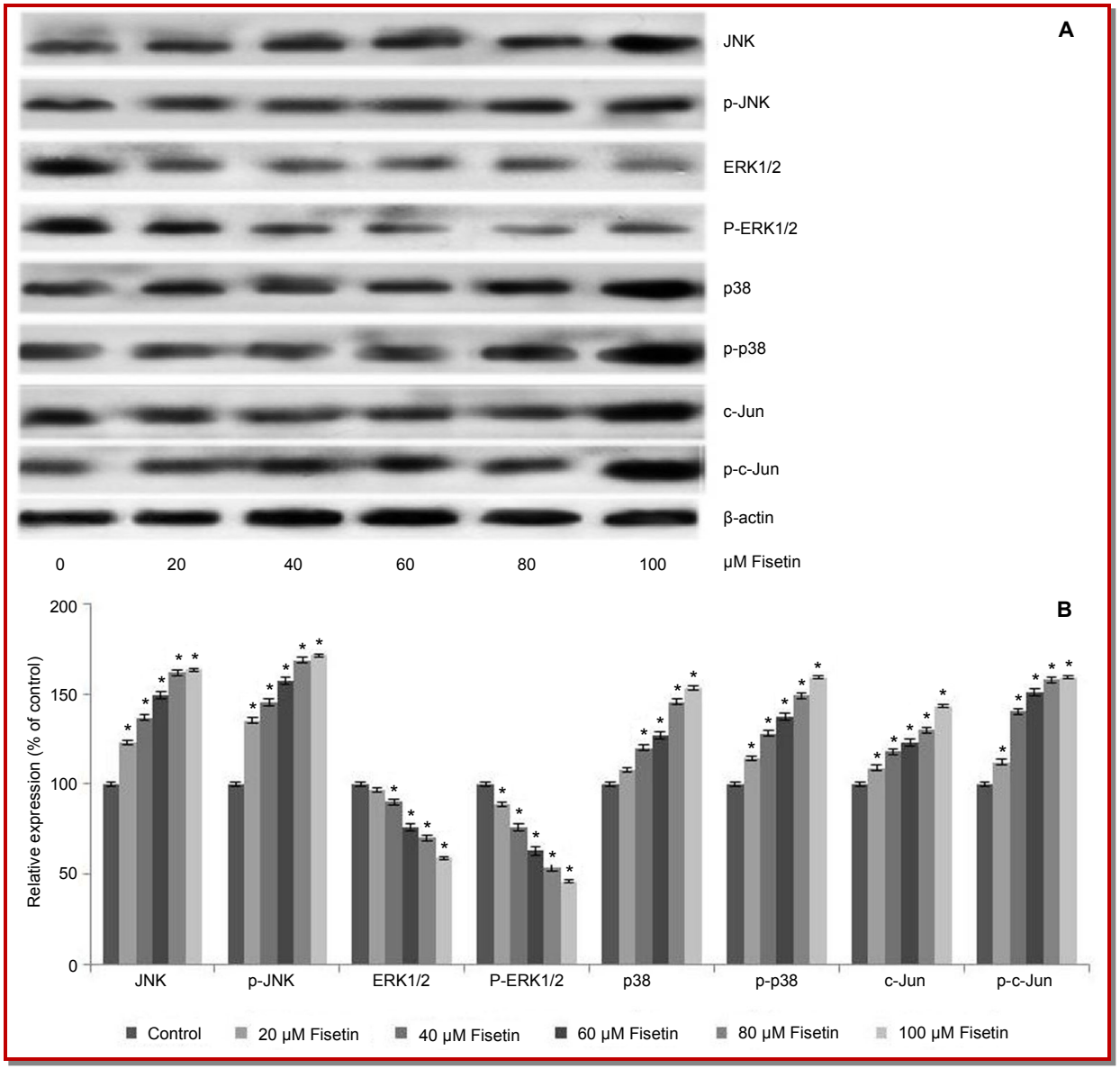

Figure 7: Fisetin regulates the MAPK pathway proteins

(A) Significant down regulations of ERK1/2 with marked increase in the expressions of JNK and c-Jun were observed following fisetin exposure. Marked up-regulations in the phosphorylated forms of JNK, c-Jun and p38 were also observed. (B) Relative expressions of the MAP kinases. Values are represented as mean ± SD; n=3; *represents $p<0.05$ compared with control as determined by one-way ANOVA

the cytosol and initiates the caspase cascade leading to apoptosis (Han et al., 2006; Kroemer et al., 2007). Therefore, the observed decrease of $\Delta\psi m$ following treatment with fisetin, suggests mitochondrial dysfunction and disruption in mitochondrial integrity that could have possibility be responsible for the raised apoptotic cell counts.

To assess the activation of caspase cascade, expression levels of caspases-9, -8 and -3 were determined. Activation of caspase-9 and -8 suggests the involvement of both intrinsic and extrinsic pathways of apoptosis that sequentially lead to the activation of caspase-3 (Hartojo et al., 2010). Fisetin was observed to cause dose-dependent multifold activation of caspase-3, -8 and -9. These findings indicate that fisetin induces apoptosis in human osteosarcoma cells by activating caspase cascades.

Bcl-2 family proteins are involved in the regulation of apoptosis and mitochondrial membrane potential (Hunter et al., 2007; Heath-Engel et al., 2008; Gyrd-Hansen and Meier, 2010; Huttemann et al., 2011). The pro-apoptotic Bcl-2-family proteins such as Bax and Bak form pores in the outer mitochondrial membrane and stimulate apoptosis, while the anti- apoptotic proteins including Bcl-2 and Bcl-xL inhibit pore formation (Gross et al., 1999). It has been demonstrated that Bcl-xL

has been highly expressed in some hematopoietic and solid tumors (White et al., 2005). Down-regulation of Bcl-xL and Bcl-2 observed in fisetin exposure could have contributed to disruption of the membrane integrity leading to intrinsic apoptosis (Gottlieb et al., 2000). Down-regulation of Bcl-xL was accompanied with a marked up-regulation in the expression of the pro-apoptotic proteins Bax and Bad, suggesting that fisetin modulates the expressions of not only caspases but also the apoptotic proteins to trigger apoptosis in the osteosarcoma cells.

MAPK signalling cascades are important regulators of stress responses, including the induction of apoptosis (Johnson and Lapadat, 2002; Park, 2011; You and Park, 2011). Previous studies have suggested that JNK, p38, and ERK1/2 pathways have critical roles in the induction of apoptosis (Tournier et al., 2000; Kim and Chung, 2008). Activation of ERK has been shown to promote proliferation and survival of most of the cell types (Lewis et al., 1998; Wang et al., 1998) and as well regulate cell apoptosis (Ishikawa and Kitamura, 1999). Nevertheless, the MAP kinases- JNK and p38 are often activated by oxidative stress and xenobiotics, and have been reported to subsequently induce apoptosis (Lewis et al., 1998; Obata et al., 2000). Studies suggest that p38 and/or JNK directly activate the caspase cascade, and also cause the activation of the apoptotic transcription factor c-Jun (Davis, 2000). In our study, fisetin brought about marked down-regulation of the phosphorylated forms of ERK1/2, whereas up-regulated the phosphorylated levels of JNK, and p-p38 in a dose-dependent manner. This observed activation of JNK could have caused up-regulation of p-c-Jun in the U-2 OS cells.

Excessive generation of ROS could interfere with cellular signalling pathways (Simon et al., 2000; Trachootham et al., 2009) and is one of the contributing factors in the malignant transformation of normal cells via inducing oxidative DNA damage (Klaunig et al., 2010; Lee et al., 2012). However, induction of ROS plays a significant role in the chemotherapeutic activity of several anticancer drugs and anticancer compounds (Fruehauf and Meyskens, 2007; Trachootham et al., 2009). Drug-induced ROS mediates the activation of MAP kinases, disrupts the mitochondrial membrane potential and subsequently activates apoptotic caspases in cancer cells (Zhang et al., 2000; Raza et al., 2011). JNK could be activated by stimuli as cytokines, ROS, pathogens, toxins, drugs, and metabolic changes (Seki et al., 2012). Thus the observed raised levels of ROS could have also contributed to the activation of JNK leading to apoptosis. Fisetin thus could have promoted the generation of ROS and caused the activation of JNK via ROS-induction or could have acted directly.

PI3K/Akt pathway is another major signalling cascade that is deregulated in many cancers (Porta et al., 2014). Dysregulation of this pathway plays a vital role in multiple pathological processes of OS including cell cycle progression, tumorigenesis, invasion, angiogenesis, metastasis, apoptosis and chemoresistance (Zhang et al., 2015). Activation of PI3K leads to the activation of major effector Akt through phosphorylation. Activated Akt translocates to the cytoplasm and nucleus and further cause phosphorylation of many downstream effector proteins that regulate various cellular functions such as mTOR and GSK3β, which further leads to cell cycle progression. Additionally, Akt increases the activity of inhibitor of κB (IκB) kinase (IKK), that leads to phosphorylation and thus degradation of IκB causing the subsequent release of NF-κB, a central signalling factor that has been reported to be involved in tumorigenesis of various cancers (Ahmad et al., 2013).

Fisetin at various concentrations caused down-regulation of p-Akt in a dose-dependent way. This decrease in the activation of p-Akt could have contributed the reduced expression levels of p-GSK3β that further inhibited cyclin D1 expression. Moreover, fisetin effectively inhibited NF-κB and as well modulated the levels of IκB and mTORC1. PTEN levels were enhanced significantly by fisetin. PTEN is a main negative regulator of the PI3K/Akt pathway, and loss of PTEN activity has been frequently observed in OS (Nielsen-Preiss et al., 2003). Thus, PTEN activators may be an alternative approach for suppression of the pathway in OS. Fisetin induced PTEN levels contribute to the negative regulation of the PI3K/Akt pathway thus reducing cell cycle progression and promoting apoptosis.

Conclusion

Fisetin at 20-100 μM concentration potentially induced apoptosis of the U-2 OS cells by up-regulating the apoptotic proteins and also modulating the expression of MAPK and PI3K/Akt signalling cascades proteins.

References

Ahmad A, Biersack B, Li Y, Kong D, Bao B, Schobert R, Padhye SB, Sarkar FH. Targeted regulation of PI3K/Akt/mTOR/NF-kappaB signaling by indole compounds and their derivatives: Mechanistic details and biological implications for cancer therapy. Anticancer Agents Med Chem. 2013; 13: 1002-13.

Anilkumar U, Prehn JH. Anti-apoptotic BCL-2 family proteins in acute neural injury. Front Cell Neurosci. 2014; 8: 281.

Avisetti DR, Babu KS, Kalivendi SV. Activation of p38/JNK pathway is responsible for embelin induced apoptosis in lung cancer cells: Transitional role of reactive oxygen species. PLoS One 2014; 9: e87050

Chen YC, Shen SC, Lee WR, Lin HY, Ko CH, Shih CM, Yang LL. Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1.

Arch Toxicol. 2002; 76**:** 351-59.

Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Mitochondrial electron-transport chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. J Cell Sci. 2007; 120: 4155-66.

Chen YC, Chang CN, Hsu HC, Chiou SJ, Lee LT, Hseu TH. Sennoside B inhibits PDGF receptor signaling and cell proliferation induced by PDGF-BB in human osteosarcoma cells. Life Sci. 2009; 84: 915-22.

Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther. 2006; 6: 1075-85.

Chou RH, Hsieh SC, Yu YL, Huang MH, Huang YC, Hsieh YH. Fisetin inhibits migration and invasion of human cervical cancer cells by down-regulating urokinase plasminogen activator expression through suppressing the p38 MAPK-dependent NF-κB signaling pathway. PLoS ONE 2013; 8: e71983.

Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000; 103: 239-52.

Fruehauf JP, Meyskens FL Jr. Reactive oxygen species: A breath of life or death? Clin Cancer Res. 2007; 13: 789-94.

Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal 2007; 19: 1807-19.

Gottlieb E, Vander Heiden MG, Thompson CB. Bcl-xL prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. Mol Cell Biol. 2000; 20: 5680-89.

Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Develop. 1999; 13: 1899-911.

Guijarro MV, Ghivizzani SC, Gibbs CP. Animal models in osteosarcoma. Front Oncol. 2014; 4: 189.

Gyrd-Hansen M, Meier P. IAPs: From caspase inhibitors to modulators of NF-kappaB inflammation and cancer. Nature Rev Cancer. 2010; 10: 561-74.

Haddad AQ, Venkateswaran V, Viswanathan L, Teahan SJ, Fleshner NE, Klotz LH. Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines. Prostate Cancer Prostatic Dis. 2006; 9**:** 68-76.

Han J, Goldstein LA, Gastman BR, Rabinowich H. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. J Biol Chem. 2006; 281: 10153-63.

Hartojo W, Silvers AL, Thomas DG, Seder CW, Lin L, Rao H, Wang Z, Greenson JK, Giordano TJ, Orringer MB, Rehemtulla A, Bhojani MS, Beer DG, Chang AC. Curcumin promotes apoptosis, increases chemosensitivity, and inhibits nuclear factor kappaB in esophageal adenocarcinoma. Transl Oncol. 2010; 3: 99-108.

Heath-Engel HM, Chang NC, Shore GC. The endoplasmic reticulum in apoptosis and autophagy: Role of the BCL-2 protein family. Oncogene 2008; 27: 6419-33.

Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 2007; 12: 1543-68.

Huttemann M, Pecina P, Rainbolt M, Sanderson TH, Kagan VE, Samavati L, Doan JW, Lee I. The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian cell: From respiration to apoptosis. Mitochondrion 2011; 11: 369-81.

Ishikawa Y, Kitamura M. Dual potential of extracellular signal-regulated kinase for the control of cell survival. Biochem Biophy Res Commun. 1999; 264: 696-701.

Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298: 1911-12.

Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, Branscheid D, Kotz R, Salzer-Kuntschik M, Winkelmann W, Jundt G, Kabisch H, Reichardt P, Jurgens H, Gadner H, Bielack SS. Primary metastatic osteosarcoma: Presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol. 2003; 21: 2011-18.

Kim BM, Chung HW. Desferrioxamine (DFX) induces apoptosis through the p38-caspase8- Bid-Bax pathway in PHA stimulated human lymphocytes. Toxicol Appl Pharmacol. 2008; 228: 24-31.

Kim SJ, Choi JA, Lee SH, Choi JY, Hong SH, Chung HW and Kang HS. Imaging findings of extrapulmonary metastases of osteosarcoma. Clin Imaging. 2004; 28: 291-300.

Kim HJ, Kim SH, Yun J-M. Fisetin inhibits hyperglycemia-induced proinflammatory cytokine production by epigenetic mechanisms. Evid Based Complement Alternat Med. 2012; 2012: 639469

Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicologic Pathol. 2010; 38: 96-109.

Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev. 2007; 87: 99-163.

Lee WR, Shen SC, Lin HY, Hou WC, Yang LL, Chen YC. Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase 3 and Ca^{2+}-dependent endonuclease. Biochem Pharmacol. 2002; 63**:** 225-36.

Lee HY, Jeon HS, Song EK, Han MK, Park SI, Lee SI, Yun HJ, Kim JR, Kim JS, Lee YC, Kim SI, Kim HR, Choi JY, Kang I, Kim HY, Yoo WH. CD40 ligation of rheumatoid synovial fibroblasts regulates RANKL-mediated osteoclastogenesis: Evidence of NF-kappaB-dependent, CD40-mediated bone destruction in rheumatoid arthritis. Arthritis Rheum. 2006; 54: 1747-58.

Lee JC, Son YO, Pratheeshkumar P, Shi X. Oxidative stress and metal carcinogenesis. Free Radic Biol Med. 2012; 53: 742-57.

Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res. 1998; 74: 49-139.

Li J, Zhang F, Wang S. A polysaccharide from pomegranate peels induces the apoptosis of human osteosarcoma cells via the mitochondrial apoptotic pathway. Tumour Biol. 2014; 35: 7475-82.

Li HY, Zhang J Sun LL, Li BH, Gao HL, Xie T, Zhang N, Ye ZM. Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcomacells: An *in vitro* and in vivo study. Cell Death Dis. 2015; 6: e1604

Lin CC, Kuo CL, Lee MH, Lai KC, Lin JP, Yang JS, Yu CS, Lu CC, Chiang JH, Chueh FS, Chung JG. Wogonin triggers apoptosis in human osteosarcoma U-2 OS cells through the endoplasmic reticulum stress, mitochondrial dysfunction and caspase-3-dependent signalling pathways. Int J Oncol. 2011; 39: 217-24.

Lu X, Jung J, Cho HJ, Lim DY, Lee HS, Chun HS, Kwon DY, Park JH. Fisetin inhibits the activities of cyclin-dependent kinases leading to cell cycle arrest in HT-29 human colon cancer cells. J Nutr. 2005b; 135**:** 2884-90.

Luetke A, Meyers PA, Lewis I, Juergens H. Osteosarcoma treatment: Where do we stand? A state of the art review. Cancer Treat Rev. 2014; 40: 523-32.

Nielsen-Preiss SM, Silva SR, Gillette JM. Role of PTEN and Akt in the regulation of growth and apoptosis in human osteoblastic cells. J Cell Biochem. 2003; 90: 964-75.

Noh K, Kim KO, Patel NR, Staples JR, Minematsu H, Nair K, Lee FY. Targeting inflammatory kinase as an adjuvant treatment for osteosarcomas. J Bone Joint Surg Am. 2011; 93: 723-32.

Obata T, Brown GE, Yaffe MB. MAP kinase pathways activated by stress: the p38 MAPK pathway. Crit Care Med. 2000; 28: N67-N77.

Park WH. MAPK inhibitors differentially affect gallic acid induced human pulmonary fibroblast cell growth inhibition. Mol Med Report. 2011; 4: 193-204.

Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Update. 2004; 7: 97-110.

Porta C, Paglino C, Mosca A. Targeting PI3K/Akt/mTOR signaling in cancer. Front Oncol. 2014; 4: 64.

Qi F, Li A, Zhao L, Xu H, Inagaki Y, Wang D, Cui X, Gao B, Kokudo N, Nakata M, Tang W. Cinobufacini, an aqueous extract from Bufo bufo gargarizans Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. J Ethnopharmacol. 2010; 128: 654-61.

Raza H, John A, Benedict S. Acetylsalicylic acid-induced oxidative stress, cell cycle arrest, apoptosis and mitochondrial dysfunction in human hepatoma HepG2 cells. Eur J Pharmacol. 2011; 668: 15-24.

Seki E, Brenner DA, Karin M. A liver full of JNK: Signalling in regulation of cell function and disease pathogenesis, and clinical approaches. Gastroenterology 2012; 143: 307-20

Shen HM, Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radical Biol Med. 2006; 40: 928-39.

Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 2000; 5: 415-18.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000; 288: 870-74.

Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov. 2009; 8: 579-91.

vanLoo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P. The role of mitochondrial factors in apoptosis: A Russian roulette with more than one bullet. Cell Death Differ. 2002; 9: 1031-42.

Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 2009; 9: 537-49.

Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: Influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J. 1998; 333: 291-300.

White C, Li C, Yang J, Petrenko NB, Madesh M, Thompson CB, Foskett JK. The endoplasmic reticulum gateway to apoptosis by Bcl-XL modulation of the InsP3R. Nat Cell Biol. 2005; 7: 1021-28.

You BR, Park WH. The effects of mitogen-activated protein kinase inhibitors or small interfering RNAs on gallic acid induced HeLa cell death in relation to reactive oxygen species and glutathione. J Agric Food Chem. 2011; 59: 763-71.

Yuan TL, Cantley LC. PI3K pathway alterations in cancer: Variations on a theme. Oncogene 2008; 27: 5497-510.

Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. Science 2000; 290: 989-92.

Zhang C, Yang L, Wang XB, Wang JS, Geng YD, Yang CS, Kong LY. Calyxin Y induces hydrogen peroxide-dependent autophagy and apoptosis via JNK activation in human non-small cell lung cancer NCI-H460 cells. Cancer Lett. 2013; 340: 51-62.

Zhang J, Yu XH, Yan YG, Wang C, Wang WJ. PI3K/Akt signaling in osteosarcoma Clin Chim Acta. 2015; 444: 182-92.

Zhao X, Ma S, Liu N, Liu J, Wang W. A polysaccharide from Trametes robiniophila inhibits human osteosarcoma xenograft tumor growth *in vivo*. Carbohydrate Polymers. 2015; 124: 157-63.

Author Info
Jian-Ming Li (Principal contact)
e-mail: ljm63630@gmail.com; Tel & Fax: 008637963546521