Fisetin induces apoptosis in human cervical cancer HeLa cells through ERK1/2-mediated activation of caspase-8/caspase-3 dependent pathway

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Abstract

Fisetin is a naturally occurring flavonoid that has been reported to inhibit the proliferation and to induce apoptotic cell death in several tumor cells. However, the effects of fisetin on the induction of apoptosis in human cervical cancer HeLa cells were never investigated and unclear. In this study, we found that fisetin induced apoptosis of HeLa cells in a dose- and time-dependent manner, as evidenced by nuclear staining of DAPI, flow cytometry assay and Annexin-V/PI double-labeling. In addition, fisetin triggered the activations of caspases-3, and -8 and the cleavages of poly (ADP-ribose) polymerase, resulting in apoptosis induction. Moreover, treatment of HeLa cells with fisetin induced a sustained activation of the phosphorylation of ERK1/2, and inhibition of ERK1/2 by PD98059 (MEK1/2 inhibitor) or transfection with the mutant ERK1/2 expression vector significantly abolished the fisetin-induced apoptosis through activation of caspase-8/-3 pathway. The in vivo xenograft mice experiments revealed that fisetin significantly reduced tumor growth in mice with HeLa tumor xenografts. In conclusion, our results indicated that fisetin exhibited anti-cancer effect and induced apoptosis in HeLa cell lines both in vitro and in vivo.

KEY WORD: Fisetin; Apoptosis; Cervical cancer; Caspase; ERK1/2. HeLa cells

Introduction

Cervical carcinoma is the second most common cancer in women, and is the most prevalent female malignancy in many developing countries (Smith et al., 2000) and in Taiwan. Chemotherapy, surgery and drugs are always the first choice for those patients with cervical cancer, where the prognosis remains very poor. Recently, cancer therapy also used herbal medicine or natural food to treatment for different of cancers to replace the conventional chemotherapy. In present, many researcher studies support herbal medicine or nature food as potent chemopreventive drug (da Rocha et al., 2001).

Flavonoids are polyphenolic and phytochemicals that are ubiquitous in plants and present in the common human diet. It has various biological activities, including apoptosis induction, cell cycle arrest, anti-proliferation, and anti-oxidation or a combination of these activities (Birt et al., 2001; Le, 2002; Ren et al., 2003). Many flavonoids have anti-cancer activity against various human tumor cell lines and xenograft systems of human tumors (Deep et al., 2010; Hanneken et al., 2006; Mohammad et al., 2006). Recently, some in vitro and vivo evidences suggested that flavonoids are generally safe and associated with low toxicity, so there have been concentrated efforts to develop novel dietary substances as cancer chemopreventive agents (Hassan et al., 2007; He et al., 2009). Fisetin is a naturally occurring flavonoid found in various vegetables and fruits such as cucumber, onion, persimmon, strawberry and apple, then exhibits a wide variety of biological functions including anti-oxidant, anti-inflammatory, anti-proliferative and apoptosis effects (Moon et al., 2006). Several studies have demonstrated that the fisetin have anti-cancer effects against several cancer types (Haddad et al., 2006; Sung et al., 2007; Murtaza et al., 2009). Fisetin can induce apoptosis and suppress the growth of HT-29 human colon cancer cells, via inhibition of COX2 and Wnt/EGFR/NF-κB-signaling pathways (Suh et al., 2009). Fisetin suppressed androgen receptor signaling pathway and tumor growth in nude mice (Khan et al., 2008b), in addition to the induction of LNCap cell apoptosis and cell cycle arrest (Khan et al., 2008a). Fisetin also induced cells performing their autophagic cell death through inhibition of both mTORC1 and mTORC2 pathways (Suh et al., 2010). Fisetin had effective cytotoxity and apoptosis induction in SK-Hep1 and Huh-7 cells, via its activation of caspase-3 accompanied by DNA fragmentation, induction increased expressions of p53 and p21(Waf/Cip-1) proteins (Chen et al., 2002) and down regulation of BIRC8 and Bcl_2L_2 protein (Kim et al., 2010). These results suggested the potential use of fisetin as an anti-tumor or chemotherapeutic reagent.

Apoptosis is a selective process of physiological cell deletion that plays a fundamental role in the balance between cellular survival and death, these changes including cell morphological changes, chromatin condensation, apoptotic bodies, DNA fragmentation, and caspase family activation. Apoptotic signaling can proceed two main apoptotic pathways, which have been identified as the extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The former is the death receptor pathway involved Fas and other members of the tumor-necrosis factor (TNF) receptor family activating caspase-8 (Thorburn, 2004), which in term activated Bid, and then triggered the mitochondrial pathway or directly activated caspase-3 (Yin, 2000). The later apoptotic pathway is the mitochondrial pathway, which is triggered from the cytochrome c released from mitochondria, bound with Apaf1 and pro-caspase-9 to activate caspase-9, which in turn proteolytically activated caspase-3. Activated caspase-3 cleaves a lot of substrates, including poly (ADP-ribose) polymerase (PARP) and leads to trigger cell death (Jiang et al., 2004; Kuwana and Newmeyer, 2003).

The mitogen-activated protein kinase (MAPK) family regulated the cellular responses to different extracellular stimuli and played as a multi-functional mediator of signal transduction processes, including cell death, differentiation, proliferation and migration (Dong et al., 2002; Matsukawa et al., 2004). Three major MAPK subfamilies have been define the extracellular regulated kinases 1/2 (ERK1/2), the p38 and the c-Jun N-terminal kinases 1/2 (JNK1/2). Each MAPK is activated through a specific phosphorylation cascade. The ERK1/2 cascade is associated with cell differentiation, proliferation and survival (Cobb, 1999; Xia et al., 1995). Activation of ERK1/2 phosphorylation can induce cell cycle arrest and apoptosis, such as DNA damage. Sustained activation of ERK1/2 can induce apoptosis in neurons via a caspase-8-dependent pathway that is independent of Fas or FADD signal pathway (Cagnol et al., 2006). In contract, the JNK1/2 and p38 cascades that are activated by cytokines and various external stresses appeared to be closely related to cell growth arrest and cell death (Chen et al., 1996). However, some reports have shown that the balance between ERK1/2 and stress activated JNK/p38 pathways had been proposed to be a fundamental determinant of cell apoptosis or survival (Cagnol and Chambard, 2010; Johnson and Lapadat, 2002). To further detect its anti-cancer effect in vitro and in vivo and explore precise mechanism, we chose the human cervical cancer HeLa cells as model.

In the present study, it was demonstrated that fisetin suppressed the cell viability in human cervical cancer HeLa cells. The human cervical cancer HeLa cell was chosen for further examining of

the anti-cancer effect of fisetin and investigating its molecular mechanism both in vitro and in vivo. The results showed that fisetin can indeed induce the apoptosis of HeLa cells in a dose- and time-dependent manner, and the activation of ERK1/2 and caspase-8/-3, not p38 or JNK1/2, was the major intracellular pathway in charge of the fisetin-induced apoptosis in HeLa cells. We tempted to speculate that fisetin would be a potent chemotherapeutic drug against human cervical cancer through its activation of the ERK1/2 and caspase-8/-3 signaling pathway.

Materials and methods

Reagents

Fisetin (3,3',4',7-tetrahydroxyflavone) of 99% purity was purchased from Sigma (St. Louis, MO). Stock solution of fisetin was made at 100 mM concentration in dimethyl sulfoxide (DMSO) (Sigma, St. Louis Co.) and stored at -20℃. The final concentration of DMSO for all treatments was less than 0.1%. Antibodies against cleaved caspase-3, caspase-8, caspase-9, PARP, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-ERK1/2, p-p38, p-JNK, ERK1/2, p38 and JNK1/2 were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Promega (Madison, WI). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), DAPI (4'-6-Diamidino-2-phenylindole) was purchased from Sigma (Louis, MO, USA). The p38 MAPK inhibitor SB203580, MEK1/2 inhibitor PD98059, and JNK1/2 inhibitor SP600125 were purchased from Calbiochem (San Diego, CA). Caspase-3 inhibitor Z-DEVE-fmk, caspase-8 inhibitor Z-IETD-fmk, and caspase-9 inhibitor Z-LEHD-fmk were purchased from BioVision (Mountain View, CA). Dominant negative (DN)-ERK1/2 mutant plasmid was kindly donated by Dr. C.H. Tang (Department of Pharmacology, School of Medicine, China Medical University and Hospital, Taichung, Taiwan).

Cell culture

HeLa (human cervical adenocarcinoma), A549 (human lung adenocarcinoma), RL95-2 (human endometrial carcinoma), MDA-MB-231 (human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma), U2OS (human osteosarcoma), SW480 (Human colon adenocarcinoma), Hs68 (human normal foreskin fibroblast), and WI-38 (human normal lung fibroblast) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in RPMI 1640, MEM or DMEM/F12 supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 mM non-essential amino acid, 2mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Cambrex, Walkersville, MD). The cultures were incubated at 37℃ in a humidified atmosphere with 5% CO_2. Cells were passaged every 2–3 days to obtain an exponential growth.

In vitro cytotoxicity assay

The cytotoxicity was assessed by MTT assay. Cells were plated into 24-well plates at 3×10^4 cells/well, grown for 24 h, and treated with 0.1% DMSO or different concentrations of fisetin (0, 10, 20, 40, 60, and 80 μM). After 24 h incubation MTT was added to each well (0.5 mg/ml final concentration), and the mixture of MTT and cells were further incubated for 4 h. The viable cell number was directly proportional to the production of formazan following the solubilization with isopropanol. The color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate.

Flow cytometer

The cells (2×10^6/ml) were seeded in 10 cm dishes and cultured with 0.1% DMSO or fisetin (10, 20, 40, 60 and 80 μM) for 24 h. At the end of incubation, the cells were collected and fixed with 70% ethanol. The cells were stained with propidium iodide (PI) buffer (4 μg/ml PI, 1% Triton X-100, 0.5 mg/ml RNase A in PBS) for 30 min in the dark at room temperature and then filtered through a 40-μm nylon filter (Falcon, USA). The cell cycle distribution was analyzed for 10000 collected cells by a FACS Vantage flow cytometer that uses the Cellquest acquisition and analysis program (Becton Dickinson FACS Calibur, San Joes, CA, USA). The apoptotic cells with hypodiploid DNA content were detected in the sub-G1 region. All the results were obtained from three independent experiments.

Annexin V/PI staining assay

Apoptosis-mediated cell death of tumor cell was examined using a double staining method with FITC-labeled Annexin-V/propidium iodide (PI) Apoptosis Detection kit (BD Biosciences, CA). For PI and Annexin-V double staining, cells were suspended with 100 μl of binding buffer (10 mM HEPES/ NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with 5 μl of FITC-conjugated Annexin V and 5 μl of PI (50 μg/ml) for 30 min at room temperature in dark place and then added 400 μl binding buffer. Apoptotic cells were analyzed via flow cytometry, by use of a FACScan system Flow cytometric analysis. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using Cell Quest software.

DAPI staining

HeLa cells were plated in six-well dishes and treated for 24 h with fisetin (0, 20, 40 and 80 μM). The apoptotic morphological changes were assessed via DAPI staining, as previously described

(Hwang et al., 2009). The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted, and minimum of 150 cells/field and at least 3 fields in each well were counted.

Transient transfection

Transfections were done using LipofectAMINE 2000 Transfection Reagent (Invitrogen). Cells seeded at 60-mm dish were cultured in DMEM/F12 supplemented with 10% FBS at 37℃ for 24h. Briefly, lipofectamine (5 μl) and DNA (3 μg) were diluted in 100 μl of DMEM/F12 followed by equilibration at room temperature for 5 min after mixing. The lipofectamine-DNA complex was added to HeLa cells and incubated for 6 h. Cells were then washed with PBS and replenished with DMEM/F12 containing 20% serum. At 6 h after transfection, the cells were incubated with 40 μM of fisetin for 24 h. The cells were then lysed for Western blotting

Western blotting

The cell lysates were prepared as previously described (Hsieh et al., 2007). Equal amounts of protein extracts (35 μg) was subjected to 10% or 12.5% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore, Belford, MA). After blocking, the membrane was incubated with the anti-cleaved caspase-9 (1:1000), anti-cleaved caspase-3 (1:1000), anti-cleaved caspase-8 (1:1000), anti-cleaved PARP (1:1000), anti-caspase-8 (1:1000), anti-ERK1/2 (1:1000), anti-phosphorylated ERK1/2 (1:500), anti-p38 (1:1000), anti-phosphorylated p38 (1:500), anti-JNK1/2 (1:1000), anti-phosphorylated JNK1/2 (1:500) and β-actin (1:5000). The blots were then incubated with HRP-conjugated anti-mouse or anti-rabbit antibody (1:5000) at room temperature for 2 h. Signals were detected via enhanced chemiluminescence by using Immobilon Western-HRP Substrate (Millipore, Billerica, USA).

Measurement of tumor growth in vivo

Immuno-deficient nude mice (BALB/c nu/nu male mice, 5-6 weeks old, 18-22 g) were used for in vivo assay. Mice were housed under pathogen-free conditions with a 12-h light/12-h dark schedule and fed with an autoclaved diet ad libitum. All experimental protocols conducted in the present study were approved by the Committee of Animal Research at Chung Shan Medical University (IACUC Approval No. 429). We chose HeLa cells for determining the in vivo effects of fisetin based on the fact that these cells form rapid and reproducible tumors in nude mice. Eighteen animals were randomly and evenly

divided into three groups, and thus each group has 6 mice. For xenograft implantation, we injected HeLa cells (5× 10^6 cells/0.1 ml/mice) subcutaneously into the right posterior flank of mice by use of a 1 ml syringe with 24-gauge needle. After 10 days, when tumors became visible (approximately 5×4 mm in size). At this point, the mice were randomly assigned to three groups of six mice, and treatments fisetin given twice weekly at 2 mg/kg and 4 mg/kg. The first group of animals received i.p. injection of DMSO (30 µl) and served as control. The animals of groups 2 and group 3 received i.p. injection of fisetin (2 and 4 mg/kg body weight) in 30 µl of DMSO twice weekly, respectively. The tumor volume was calculated by the formula: $0.5236 \times L1(L2)^2$, where L1 is long diameter, and L2 is short diameter. All procedures conducted were in accordance with the guidelines for the use and care of laboratory animals. Tumors were measured once every 5 days for a total of 35 days during the treatment

Statistical analysis

The results were expressed as mean±SEM of three independent experiments. The data was analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey's multiple comparison test was used for parametric data. $P<0.05$ was considered to be statistically significant.

Results

The chemical structure of fisetin was shown in Fig. 1A. The cytotoxic effects of fisetin were examined in seven cancer cell lines and in two normal cell lines using MTT assays. Each cell line was treated with 0~80 μM fisetin for 24 and 48 h, and the cytotoxic effects were assessed using 50% growth inhibition concentration (IC50) values (Table 1). The results showed that fisetin reduced HeLa cell viability dose-dependently and time-dependently (Fig. 1B), with an IC50 of 52±0.9 and 36±0.5 μM at 24 h and 48 h, respectively. No significant change of cell viability in normal cells (Hs68 and WI-38) was observed in cells treated with highest dose of fisetin (80 μM). The concentrations of 20, 40, and 80 μM incubating for 24 h were chosen for the subsequent studies.

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosome degradation of DNA, and apoptotic body formation. The next specific aim was to determine whether the cell viability inhibitory effect of fisetin is associated with induction of cell apoptosis. For cells treated with fisetin (0, 20, 40 and 80 μM) for 24 h, DAPI staining assay revealed that fisetin caused nuclei of condensing and apoptotic bodies in a dose-dependent manner in HeLa cells (Fig. 2A). To further characterize fisetin-induced apoptosis, analysis of the cell cycle by flow cytometer showed the dose-dependent increased Sub-G1 phase in fisetin-treated HeLa cells (Fig. 2B). Similarly, as shown in Fig 2C, we assessed the translocation of phosphatidylserine (PS) using Annexin-V and PI double staining. The proportion of cells in lower-right quadrants, which corresponded to early apoptosis cells (Annexin-V positive), were increased up to 27.89% and 40.72% after treating the HeLa cells with 40 and 80 μM of fisetin, respectively. These results are all hallmarks of apoptotic cell death, and demonstrated the ability of fisetin to induce apoptosis in HeLa cells.

The apoptotic process is executed by a member of highly conserved caspases, then modulating the mechanisms of caspase activation and suppression is a critical molecular target in chemoprevention since these processes lead to apoptosis (Khan et al., 2007). To identify the mechanism involved in fisetin induced apoptosis and activation of caspase-8, -9, -3 and the cleavage of PARP. Treatment with fisetin result in a dose-dependent manner increases the activation of caspase-8, -3 and the cleavage-PARP, but not activation of caspase-9 (Fig. 3A). In order to further explore the significance of caspase activation in fisetin-induced apoptosis, the specific inhibitors for caspase-3, -8, and -9, namely Z-DEVE-fmk, Z-IETD-fmk and Z-LEHD-fmk, were used to suppress the effects of fisetin.

Pretreatment with Z-DEVE-fmk and Z-IETD-fmk both could effectively attenuate fisetin-induced cell apoptosis (Fig. 3B). In contrast, Z-LEHD-fmk did not perform the similar effect. Thus, our data showed that fisetin-induced apoptosis is dependent on the activation of caspase-8 and caspase-3, but not that of caspase-9.

To investigate the possible role of MAPK pathways in fisetin-induced apoptosis, we examined the expression of the phosphorylated forms of ERK1/2, p38MAPK, and JNK1/2 after treating HeLa cells with fisetin for 24 h by Western blotting. Fisetin only sustained activation of ERK1/2 in a dose-dependent manner, but not activation of p38 and JNK1/2, suggesting that ERK1/2 may be specifically activated in fisetin-induced apoptotic pathway (Fig. 4A). Next, to assess whether these MAPK kinases were involved in fisetin-induced apoptosis, the cells were pre-treated for 1 h with PD98059 (MEK1/2 inhibitor), SP600125 (JNK1/2 inhibitor), and SB203580 (p38 inhibitor). Thereafter, the cells were exposed to 40 μM of fisetin for 24 h and then analyzed by MTT assay. The results showed that fisetin-induced apoptosis was attenuated by inhibiting ERK1/2 activation with PD98059 pre-treatment, but not by those of SP600125 and SB203580 (Fig. 4B), suggesting that the action of fisetin on HeLa cells was specifically via ERK1/2, but not JNK1/2 or p38MAPK pathway.

To investigate the involvement of caspase-3 and -8 downstream after the activation of ERK1/2 by fisetin, HeLa cells were pretreated with 50 μM PD98059 for 1 h, then treated with 40 μM fisetin for another 24 h, and analyzed by Western blotting and MTT assay. Our data suggested PD98059 reduced fisetin-induced caspase-8 and caspase-3 activation (Fig. 5A). Furthermore, MTT assay showed that PD98059 pretreatment also completely prevented fisetin-induced apoptosis of HeLa cells (Fig. 5B, 5C). On the basis of these findings, we hypothesized that activation of ERK1/2 plays a critical role in fisetin-induced apoptosis. We transiently transfected HeLa cells with DN-ERK1/2 and examined it effects on fisetin-induced caspase signaling events and apoptosis. HeLa cells expressing DN-ERK1/2 slightly reduced basal ERK1/2 phosphorylation and significantly blocked fisetin-mediated ERK1/2 phosphorylation. Moreover, HeLa cells expressing DN-ERK1/2 also blocked fisetin-induced caspase-8 and caspase-3 activation (Fig. 5D). However, DN-ERK1/2 transfectants showed significantly inhibited fisetin-induced apoptosis (Fig. 5E, 5F). These data indicating that the fisetin-induced cell apoptosis was dependent on ERK1/2 activation and subsequent caspase-8/-3 pathway.

To further explore the anti-tumor effects of fisetin in vivo, we studied the tumor-inhibitory effect of fisetin on HeLa human cervical cancer cells using a tumor-xenografted nude mice model. Fourteen

days after implantation of HeLa cells, mice bearing HeLa tumor xenografts were treated with DMSO as the compound was dissolved into DMSO and different concentrations of fisetin (2 mg and 4 mg/kg). Our data resulted in a significant decrease in the growth rate of tumors compared with control group ($P<0.05$), with the inhibition rates of 82.65%, and 92.62%, for 2 mg and 4 mg/kg, respectively (Fig. 6).

Discussion

Many flavonoids possess anti-tumor or growth suppressive capacities against various human tumor cell lines, which lead to arrest the tumor promotion and progression, presumably by affecting or disturbing crucial factors that control cell proliferation, invasion or apoptosis. Previous studies have demonstrated that fisetin had extensively anti-tumorigenic ability in various cancer cells (Haddad et al., 2010; Lee et al., 2009; Moon et al., 2006). Therefore, induction of apoptosis in malignant cells by fisetin may be one of its critical features as a chemopreventive agent. In the present study, we demonstrated that fisetin suppressed the growth and induced apoptosis of HeLa cells in vitro and in vivo. The mechanism of fisetin-induced apoptosis of HeLa human cervical cancer cells through activation of ERK1/2 signal pathway was shown in the present study. In addition, the activation of ERK1/2-induced cell apoptosis by fisetin was linked to the caspase-8 and caspase-3 activation, but not that of caspase-9, in HeLa cervical cancer cells.

Base on the present study, we indicated that fisetin may activate the MAPK signaling pathway, which has been reported to be important for both the induction of apoptosis and mitogenic response during a variety of stress responses in many tumor cells (de Sousa et al., 2007). The results showed that fisetin treatment caused a sustained activation of ERK1/2 in a dose-dependent manner, but had no effect on the alternative p38 and JNK MAPK pathways. Also, the pre-treatment of MEK inhibitors PD98059 effectively attenuated the apoptotic effects of fisetin suggested that ERK1/2 is involved in this cell apoptosis signaling. The findings are in agreement with the using of PD98059 to abolish quercetin-induced apoptosis in A549 cells, and similar to the effects of flavonoid of trifolin (Torres et al., 2008) and apigenin (Shin et al., 2009) on ERK1/2 and subsequently cell death. From our data gathered from MTT, nucleus condensation, and Western blotting assays, fisetin-induced apoptosis and the activation of caspase-8 and casepase-3 downstream were all confirmed to be controlled by ERK1/2.

The detail relationship among ERK1/2, caspases, and apoptosis were still controversial. Some previous evidence indicated that ERK1/2 signaling is involved in protection against apoptosis (Wang et al., 2000), while some may suggest that the induction of ERK activation was association with caspase-8 activation (Cagnol et al., 2006; Snyder et al., 2010). In Jurkat cells, the activating of ERK1/2 would suppress the Fas-mediated apoptosis (Wilson et al., 1999). On the contrary, the activation of ERK1/2 may promote apoptosis. In literature, ERK1/2 activation would induce caspase-8 activation, via extrinsic and intrinsic pathways. In one study, the caspase-8 activation was independent of FADD or

Fas, and no involvement of caspase-9 in the induction of apoptosis (Cagnol et al., 2006); while another study reported that the HIV-1 viral protein r would induced the apoptosis of renal tubular epithelial cells, activating not only the caspase-8 and t-BID, but also the caspase-9 (Snyder et al., 2010). In our study, caspase-8 and caspase-3 were involved in the fisetin-induced apoptosis. On the contrary, neither caspase-9 was involved. Our findings were different from all the studies mentioned above, and this may be caused by cell specificity and different apoptotic pathway involved. Further studies of the interactions and detail mechanisms among ERK1/2 and the caspases-8 in the fisetin-induced apoptosis may contribute to the knowledge of apoptotic network.

The fisetin anti-proliferative activity determined in this study on HeLa and other adenocarcinoma cell lines as shown in Table 1. These result confirmed its cytotoxic activity have been reported on other adenocarcinoma cell lines, such as in prostate (Haddad et al., 2006; Khan et al., 2008a), bladder (Li et al., 2011), liver (Chen et al., 2002) and colon (Suh et al., 2009) cancer cells. In this study, in vitro cytotoxicity assay indicated that adenocarcinoma cell lines are more sensitive to fisetin-mediated inhibition of cell viability than the osteosarcoma cell lines (U2OS) and normal cells. It is of interest that the fisetin relative selectivity towards adenocarcinoma cell lines, compared to osteosarcoma cell lines and normal cells. A number of flavonoids have previously been reported to exert inhibitory effects on sulfotransferase (SULT) activity (Moon et al., 2006), and fisetin may inhibit human SULT activity in varity of tumor cells (Eaton et al., 1996). It is possibility that the osteosarcoma cell lines (U2OS) have low SULT activity (Dubin et al., 2001), whereas the human adenocarcinoma cell lines have high SULT activity, such as HepG2 cells (Shwed et al., 1992) and MDA-MB231 cells (Pasqualini, 2009). We speculate that possible correlation between SULT activity and proliferation in adenocarcinoma cells, not in osteosarcoma cells. This relatively osteosarcoma cells and normal cells selectivity could therefore confer a valuable advantage of fisetin for in adenocarcinoma cells treatment. However, further we need additional choose more human osteosarcoma cells should also be execute to compare and confirm the selective effects of fisetin.

In conclusion, this is the first report demonstrating the effect of fisetin, a naturally occurring flavonoid, of its inhibitory effect on human cervical cancer cell growth and its capacity in promoting cell death. The investigation of the mechanisms revealed that as for the MAPKs, the ERK1/2, but not p38 or JNK was involved; as for the caspases, the caspase-8 and caspase-3, but not caspase-9 was involved. Our findings revealed that fisetin is very potent to be developed into a chemotherapeutic drug

for human cervical cancer, with the preclinical investigations in cellular and animal models conducted in the study.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Effect of fisetin on the cell viability of HeLa cells. (A) Structure of fisetin. (B) HeLa cells were treated with fisetin (0-80 μM) for 24 and 48 h. Cell viability was determined by the MTT assay. The results were expressed as the percentages of cell viability. Data were represented as mean±SE of three independent experiments performed in triplicate. *, $P<0.05$, compared with the control (0 μM).

Fig. 2. Effect of fisetin on HeLa cells apoptosis. (A) HeLa cells treated with different concentration of fisetin (0-80 μM) for 24 h were analyzed by fluorescence microscope after DAPI staining. (B) Flow cytometry using PI staining to detect cell death in sub-G1 phase. (C) Quantitative analysis of cell apoptosis by Annexin-V and PI double-stained flow cytometry. Data were represented as mean±SE of three independent experiments performed in triplicate. *, $P<0.05$, compared with the control (0 μM).

Fig. 3. Fisetin induced the caspase activation in HeLa cells. (A) The expression levels of caspase-3, -8, -9, and PARP, was assessed by Western blot analysis at various concentration after fisetin treatment (0-80 μM) for 24 h. β-actin was used as an internal control for protein equal loading. (B) Effects of caspase inhibitors on fisetin-induced cell apoptosis. Cells were treated with 40 μM fisetin for 24 h in the presence or absence of 2 μM Z-DEVE-FMK, 20 μM Z-IETD-FMK and 20 μM Z-LEHD-FMK. Cell viability was determined by MTT assay. Data were represented as mean±SE of three independent experiments performed in triplicate. *, $P<0.05$, control versus fisetin; #, $P<0.05$, fisetin versus z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK plus fisetin.

Fig. 4. Effect of Fisetin on MAPKs pathway. (A) The effect of fisetin on the phosphorylation of ERK1/2, p38 and JNK1/2 was investigated by Western blot analysis. β-actin was used as an internal control for protein equal loading. (B) Effects of MAPK inhibitors on fisetin-induced cell death. Cells were treated with 40 μM fisetin for 24 h in the presence or absence of 50 μM PD98059, 20 μM SB203580 and 20 μM SP600125. Cell viability was estimated by MTT assay. The results were expressed as the percentage of cell viability. Data were represented as mean±SE of three independent experiments performed in triplicate. *, $P<0.05$, control versus fisetin; #, $P<0.05$, fisetin versus MAPK inhibitor plus fisetin.

Fig. 5. ERK1/2 is essential for the apoptosis induced by fisetin. HeLa cells were pretreated with PD98059 (50 μM) for 1 h or transfected with empty vector or DN-ERK1/2, followed by fisetin (40 μM)

for an additional 24 h. (A, D). The expression levels of activation of ERK1/2, caspase-8 and caspase-3 were determined by Western blot was assessed by Western blot analysis after PD98059 or transfected with empty vector or DN-ERK1/2. β-actin served as internal control (B, E). Cell viability was determined by MTT assay (C, F). Quantitative assessment of the percentage of apoptosis cells was determined by using DAPI stain by fluorescence microscopy. Data were represented as mean±SE of three independent experiments performed in triplicate. *, $P<0.05$, control versus fisetin; #, $P<0.05$, fisetin versus PD98059 or DN-ERK1/2 plus fisetin.

Fig. 6. Inhibitory effects of fisetin on tumor growth in vivo. Nude mice (ICR nu/nu) were treated with fisetin (2 and 4 mg/kg) after an initial subcutaneously injection of HeLa cells. The growth of xenograft tumors were measured daily using vernier calipers to measure long and short dimensions of the tumors and calculated as described in Materials and Methods. The difference between fisetin and control groups was analyzed by Student's t-test (**, $P<0.001$).