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Apigenin Induced MCF-7 Cell Apoptosis-Associated Reactive Oxygen Species

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Summary: Apigenin is a flavonoid, which has been proved to possess effective anti-cancer bioactivities against variety of cell lines. However, little is known about its effect on the cell-surface and the interaction between cell-surface and the reacting drug. In this study, human breast cancer line (MCF-7) was selected to be as a cell model to investigate the effects of apigenin on cell growth, proliferation, apoptosis, cellular morphology, etc. MTT assay showed that the growth inhibition induced by apigenin was in a dose-dependent manner when treated with different concentrations of apigenin while had little cytotoxic effects on human normal cells (MCF-10A). Fluorescence-based flow cytometry was used to detect cellular apoptosis and ROS production. The results showed that 80 μM apigenin could effectively induce apoptosis and overproduction of ROS in MCF-7 cells. Here, atomic force microscopy (AFM) was utilized to detect the shapes and membrane structures of MCF-7 cells at cellular or subcellular level. The results showed that the control MCF-7 cells presented typical elongated-spindle shapes with abundant pseudopodia, while after treated with apigenin, the cells shrunk and became round, the pseudopodia diminished. Moreover, the images of ultrastructure indicated that the cell membrane was composed of nanoparticles of 49 nm, but with the treated concentrations of apigenin increasing, the sizes of membrane particles significantly increased to 400 nm. These results can improve our understanding of apigenin, which can be potentially developed as a new agent for treatment of cancers. SCANNING 36:622–631, 2014.

Key words: apigenin, reactive oxygen species, MCF-7 cells, AFM

Introduction

Human breast cancer is the most common cancer in the world, with more than one million new cases being diagnosed every year. It is potentially curable by surgical resection and chemotherapy. But surgery is the treatment of choice for only the small fraction of patients with localized disease and chemotherapy drugs can damage normal cells, as well as some other side effects (Budhraja *et al*., 2012) However, chemotherapy is only effective to a few patients with cancer, especially, chemotherapy always cause serious damages to normal tissues, and induce side effects as well. Thus, it is very imperative to design new methods or drugs to prevent, diagnose, and treat breast cancers.

Recent attention is focused on phytochemicals as anticancer agents. Flavonoids have long been recognized as having potential anticancer, anti-inflammatory, antioxidant, and antimicrobial properties, serving as important nutraceutical components of our diet (Di Carlo et al., '99; Middleton et al., 2000; Rice-Evans et al., 2000; Havsteen, 2002). Apigenin (4′,5,7-trihydroxyflavone) (the chemical formula was shown in Fig. 1), a common plant dietary, flavonoid, which was found at high levels in parsley and celery, abundant vegetables in the Mediterranean's diet, is emerging as an alternative anticancer compound (Stafford, '91).

Previous studies have shown that apigenin can inhibit carcinogenesis in various cancer cell lines such as gastric, colon, breast, cervical, hepatic, and ovarian cancer (Shukla and Gupta, 2010). Compared with some chemotherapy drugs, apigenin could selectively inhibit and kill tumor cells but cause a little damage to normal

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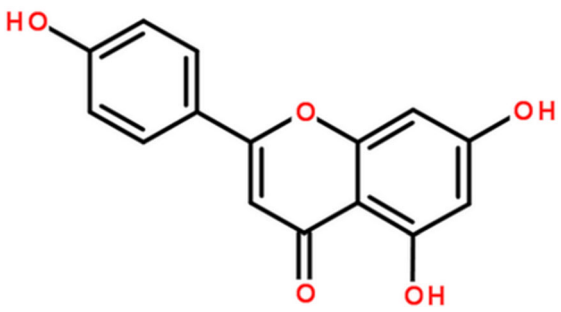

Fig 1. Chemical structure of apigenin.

cells (Xu *et al.*, 2011). Thus, it's a potential compound for drug development against human breast cancer.

Although there are many studies of anticancer effect about of apigenin, little is known about membrane morphology and mechanism induced by it. Plasma membrane plays a very important role on cell physiology. It's a boundary between live cell and external environment and protects cell from harm (Puech *et al.*, 2006). Also it regulates cell functions and transportation of nutrition inside and outside cell (Heidemann and Wirtz, 2004; Alarmo *et al.*, 2009). Changes of membrane structure have a direct influence on cell functions (Voïtchovsky *et al.*, 2006; Sato *et al.*, 2007). In recent years, studies of the cellular, subcellular and molecular mechanical changes on human disease states, including cancer, have emerged as a topic of rapidly expanding scientific interest. A particular focus is to explore the connections among the cell ultrastructure, cellular and cytoskeletal mechanical properties, biological function, and human health/disease (Suresh, 2007). In this paper, atomic force microscopy (AFM) was used to visualize cell morphology and the membrane ultrastructure.

Recent studies have demonstrated that cell apoptosis is related to reactive oxygen species (Raj *et al.*, 2011), especially for the cancer cells. In this paper, the ROS level was evaluated in apigenin-induced human breast cancer cell line (MCF-7). And the apoptosis mechanism was investigated.

Materials and Methods

Materials

Human breast cancer (MCF-7) cells and MCF-10A cells were donated by College of Medicine, Jinan University. DMEM medium and fetal bovine serum were purchased from Gibco (Brazil, South America). Apigenin was purchased from Shanxi Huike Botanical Development Co., Ltd. (China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Beyotime Institute of Biotechnology (Shang Hai, China). Annexin V-FITC/PI apoptosis detection kit, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE), DAPI, Tubulin-Tracker-Red were purchased from Beyotime Institute of Biotechnology (Nanjing, China). All reagents used in the experiments were of analytical grade.

Cell Lines and Culture

Apigenin was dissolved in dimethylsulphoxide. Final apigenin concentrations of 20–100 μmol/L were obtained by dilution in culture media such that the final concentration of dimethylsulphoxide was not >0.1%. Controls containing 0.1% dimethylsulphoxide were included in all experiments. Human breast cancer MCF-7 cells and MCF-10A cells were cultured in DMEM medium (Gibco), 10% (v/v) fetal bovine serum (FBS, Gibco) at 37°C in 95% air and 5% CO_2. The medium was refreshed every 2 or 3 days.

Cell Growth Inhibition Detected by MTT Assay

The effect of apigenin on the cell viability was detected by the MTT assay. MCF-7 cells and MCF-10A cells were transferred into the wells of 96-well plates at a density of 5×10^3 cells/ml, MCF-7 cells were incubated with various doses of apigenin (0, 20, 40, 60, 80, 100 μmol/L) for 24 and 48 h, separately. A total of 10 μl MTT was added to each well and incubated under darkness for 4 h at 37°C. Then, culture medium was removed and 150 μl DMSO was added to each well. Then the absorbance of the colored product was detected at 570 nm using a dual-beam microplate reader.

Intracellular ROS Measurement

Levels of intracellular O_2^- and H_2O_2 were assessed spectro-fluorimetrically by oxidation of specific probes: DHE and DCFH-DA, respectively. Briefly, cells were seeded on 6-well tissue culture plates, After 24 h treatment with or without apigenin, cells were harvested and washed twice in cold PBS, then resuspended in DHE and DCFH-DA (2 μM) for 30 min in dark. Detection of intracellular ROS was carried by Flow cytometer at 490 and 535 nm excitation wavelength.

Cell Apoptosis Assay

Apoptosis was determined by translocation of phosphatidylserine to the cell surface using an Annexin V-FITC and PI apoptosis detection kit. After 24 h of treatment with or without apigenin, cells were harvested and washed twice in cold PBS, and resuspended in

Annexin V-FITC and PI for 30 min in the dark. Cell apoptosis was analyzed by using Cell Quest software on a FACSAria Flow Cytometer (BD, Inc.). Fluorescence was detected with an excitation wavelength of 480 nm.

Atomic Force Microscopy Visualization

Incubating MCF-7 cells with apigenin (0–80 μmol/L) for 24 h were fixed for 15 min with 4% paraformaldehyde after monolayer-cultured, then washed twice to triple in distilled water and air-dried in room temperature.

After putting the prepared sample on the XY scanning station of AFM (Thermomicroscope, Bruker), the monitor was used to locate scanning area and contact mode was applied for imaging. In our study, we adopted 100 μm scanner and UL20B Si_3N_4 probe of which the elasticity coefficient was 2.8 N/m. All of the acquired images (256 × 256 pixels) were smoothed by onboard software in order to remove the background noise at low frequency in scanning direction.

Expression of Tubulin

The characterization of nuclear and cytoskeleton was evaluated by staining with DAPI, and Tubulin-Tracker-Red, separately. The cells with or without apigenin treatments were fixed with 4% paraformaldehyde for 30 min and incubated with 200 μM of Tubulin-Tracker-Red and DAPI for 60 min in the dark at room temperature, separately. After that, the cells were washed twice with PBS. Then, the nuclear morphology and cytoskeleton organization were imaged by a Carl Zeiss LCM 510 Meta Duo Scan laser scanning confocal microscope (LCM). Moreover, the resulting fluorescence of was measured by flow cytometer at 488 nm excitation wavelength, 530 nm emission wavelength to quantitatively compare the alterations of cytoskeleton proteins.

Statistical Analysis

Each experimental group was repeated for more than three times. Statistical analysis was performed using Student's *t*-test. $p < 0.05$ was regarded as statistically significant.

Effect of Apigenin on Breast Cancer Cell Viability

The MTT assay was performed to detect the inhibited effects of apigenin on MCF-7 cells and MCF-10A cells. As shown in Figure 2, with the concentration of apigenin increasing, cell viability was decreased significantly after treated with apigenin for 24 and 48 h, respectively. Particularly, when the concentration of apigenin was increased from 20 to 60 μmol/L, the cell viability was significantly decreased from 90.24 ± 2.5% to 53.31 ± 1.5% (48 h). The 50% inhibiting concentration (IC50) (109.3 ± 3.7 μmol/L for 24 h treatment and 65.8 ± 4.3 μmol/L for 48 h treatment) was located in this area. However, cell viability decreased slowly between 80 and 100 μmol/L. At 60 μmol/L, the viability of MCF-7 cells was about four times lower than that of the control cells. But interestingly, with the increasing concentration of apigenin, MCF-10A cell viability was only decreased from 97.24 ± 2.5% to 82.53 ± 1.5%. Therefore, the results indicated that apigenin could efficiently inhibit the growth of MCF-7 cells in a concentration-dependent manner but without cytotoxic effects to normal breast cells.

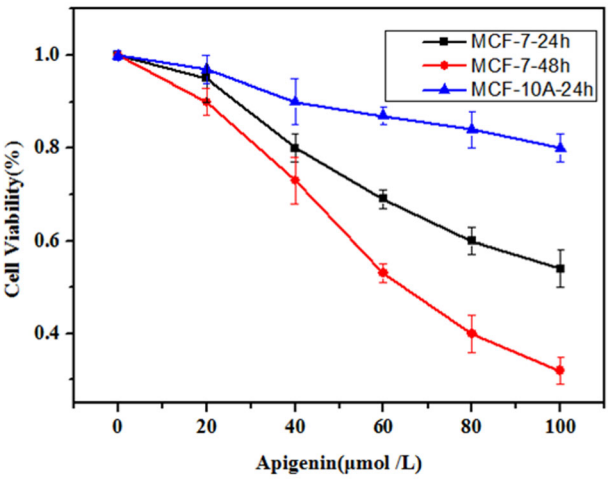

Fig 2. Effect of apigenin on MCF-7 and MCF-10A cell viability. Cell viability was determined by a MTT assay as described in the text. Results are expressed as means ± SD of data obtained in three independent experiments.

Effect of Apigenin to MCF-7 Cell Morphology and Ultrastructure

As shown in Figure 3(A1), the cells in control group revealed a typical long spindle-shaped morphology. The cell tails were unrolled and wider than that of the treated group. Between cells, the pseudopodium connected with each other for material exchange and information transfer. The AFM ultrastructure showed that cell membrane was made up of many nanoparticles (Fig. 3 (B1)). Previous work has been reported that the visible protruding particles are clusters of membrane proteins (Christian *et al.*, '98). After treated with 20 μmol/L apigenin, the cell morphology and ultrastructure were similar to the control group (Fig. 3(A2–C2)). But, when

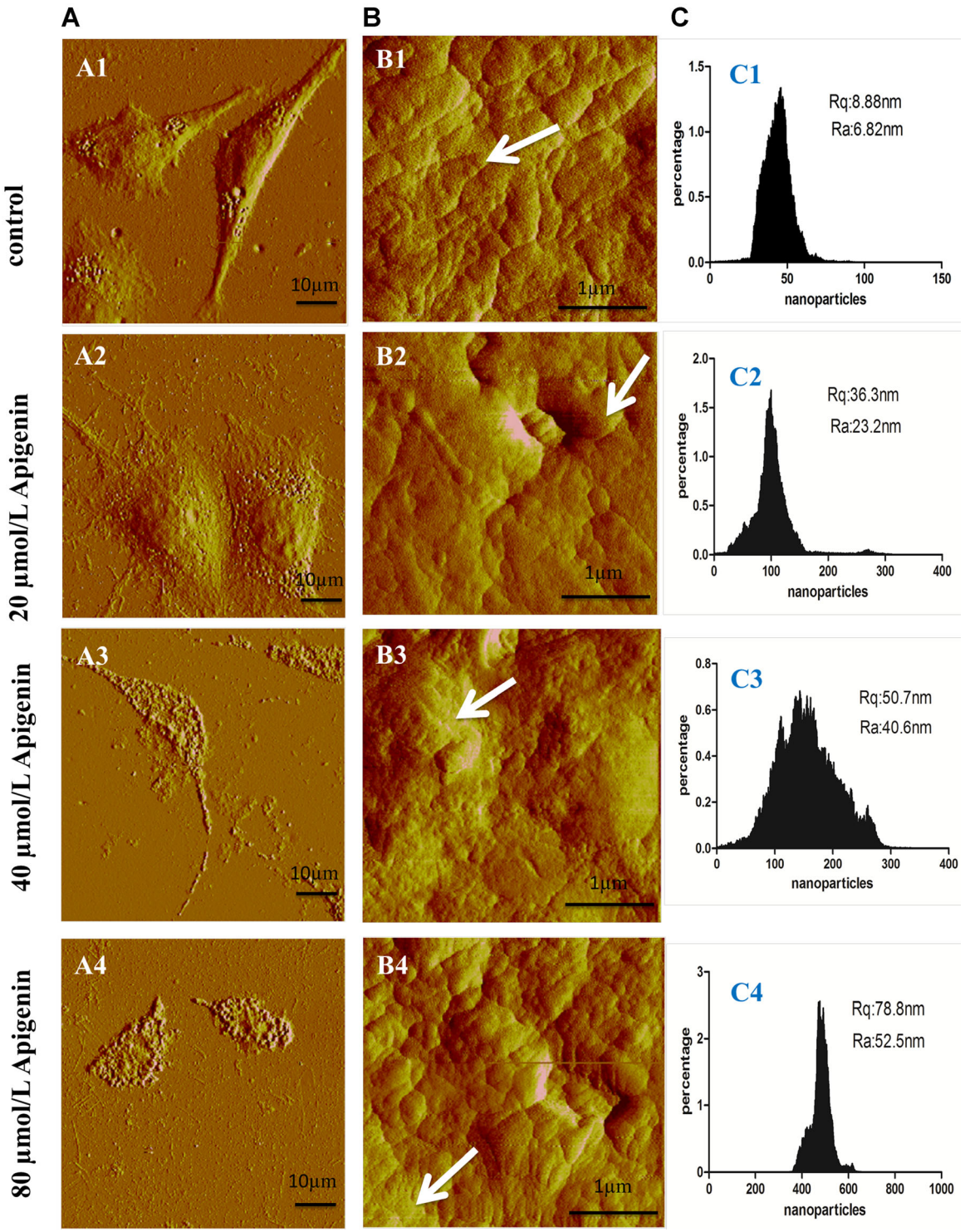

Fig 3. AFM images of MCF-7 cells before and after apigenin treatment for 24 h. (A) Topography of MCF-7, scanned area in A1–A4 is 60 μm; (B) cell membrane ultrastructure of MCF-7 cells, scanned area in B1–B4 is 3 μm; (C) the average roughness and particles distribution of MCF-7 (C1–C4) control group (A1, B1, and C1); 20 μmol/L apigenin treated group (A2, B2, and C2); 40 μmol/L apigenin treated group (A3, B3, and C3); 80 μmol/L apigenin treated group (A4, B4, and C4).

apigenin concentration increased to 40 μmol/L, the cell morphology was deformed (Fig. 3(A3)). As increased to 80 μmol/L, the cells were become flat and shrank. These indicated that the intercellular communication reduced. The ultrastructure showed the average roughness and size of nanoparticles on membrane were bigger than that of control group (Fig. 3(C1–C4)). It indicated a disorganized topographic structure of the membrane. Figure 3(B4) showed that the nanoparticles on cell membrane were not of uniform size.

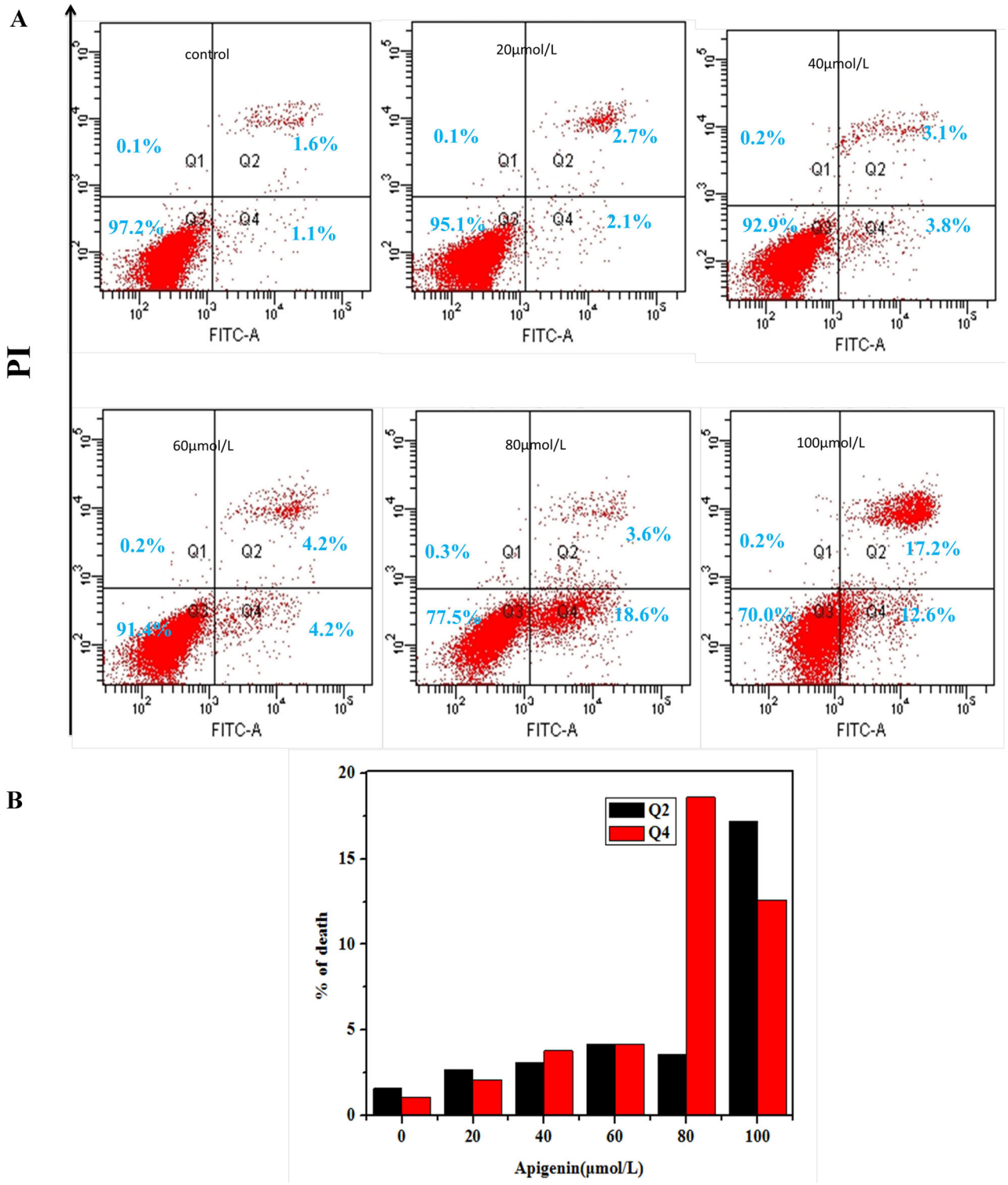

Fig 4. Effect of apigenin to MCF-7 cells apoptosis after treated for 24 h. (A) Annexin V-FITC and PI staining for apoptosis detected by flow cytometry; (B) the graph shows the apoptosis rate of (A): Q2 represents the late apoptosis and dead cells, and Q4 represents the early apoptosis. Control group means the cells are not treated with apigenin.

Effect of Apigenin on MCF-7 Cell Apoptosis

Cell apoptosis was determined by Annexin V-FITC/PI apoptosis detection kit. In the apoptosis map (Fig. 4 (A)), the Q2 means the late apoptosis and necrosis, the Q3 was presented the live cells and the Q4 was the early apoptosis cells. As shown in Fig. 4(A and B), which indicated that at 100 μmol/L apigenin treatment induced a significant increase late apoptosis and necrosis of MCF-7 cells at 24 h, from 1.6% to 17.2% for 100 μmol/L

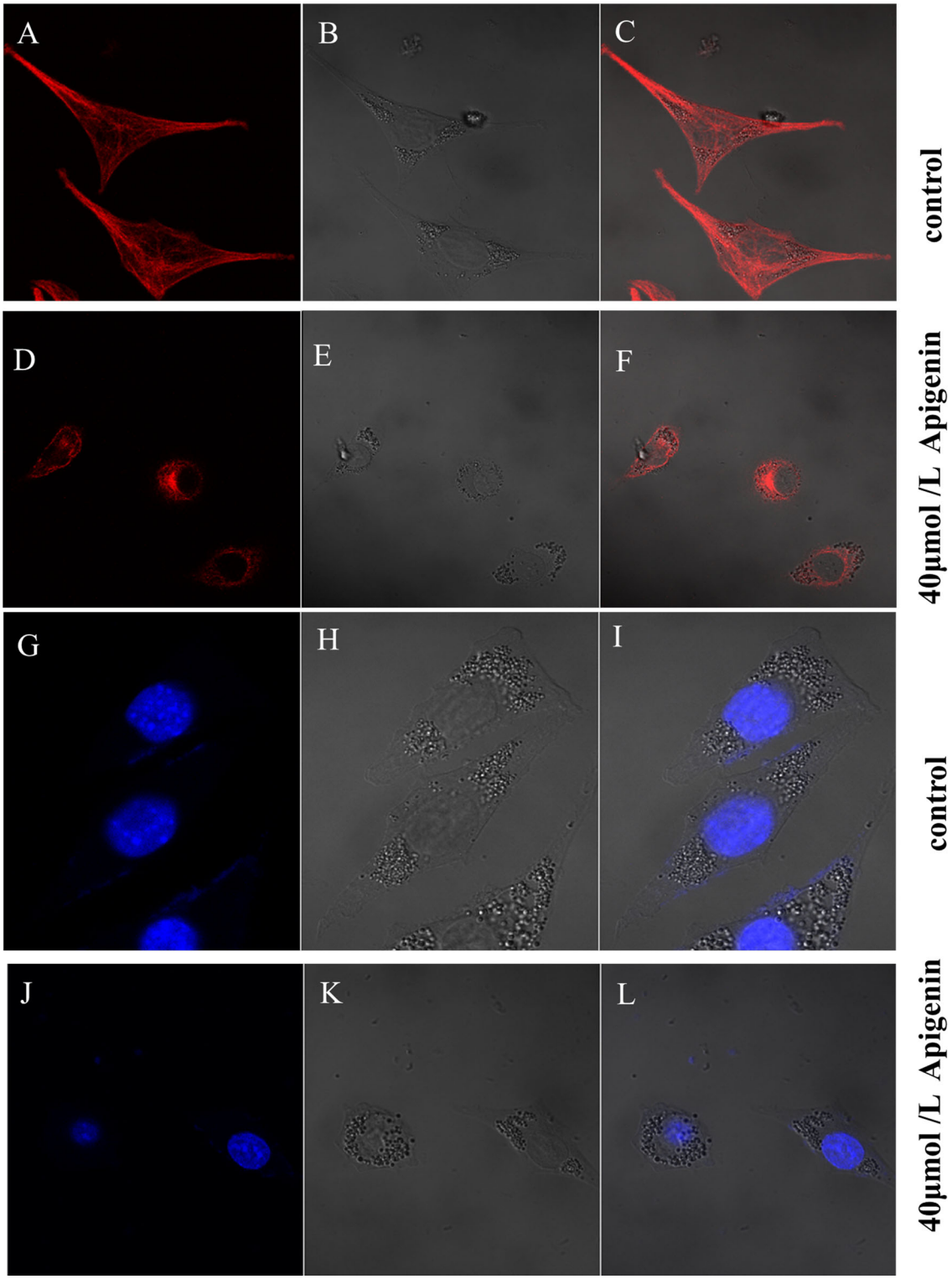

Fig 5. Alterations in cell cytoskeleton and nuclei of MCF-7 cells treated with different concentrations of apigenin. Immunofluorescence data of MCF-7 cells treated with different concentrations of apigenin. (A–F) Reorganization of cytoskeleton in cells stained with α-tubulin. (G–L) Nuclei of normal and apoptotic cells stained with DAPI.

apigenin exposure. In addition, the ratio of early apoptosis MCF-7 cells changed from 1.1% to 18.6% for 80 μmol/L apigenin treatment. These results indicated that apigenin could not only induce apoptosis as reported, but could also induce necrosis in cancer cells.

Characterization of Cytoskeleton

The fluorescence images shown in Figure 5 indicate that the assembly of tubulin in control cells is regular (A–C). These cells are typically well-spread, and tubulin

display regular networks and distribute extensively in pseudopodium regions. While after 40 μmol/L apigenin treatment (D–F), the cell became shrank and the cytoskeleton regular networks disappeared and presented a gathered state. Cotter et al. reported that tubulin polymerization is a necessary step in the process of apoptotic body formation (Cotter *et al*., '92). Besides, the morphology of cell nuclei, which stained with DAPI, also indicated the specified feature of apoptosis, i.e. nuclei chromatin condensation, nucleus shrinkage.

Moreover, the qualitative and quantitative expression of cytoskeletal proteins were investigated using fluorescence based-flow cytometry. The resulting mean fluorescence intensity (MFI) of α-tubulin antibody-FITC was the indicator of expression levels of α-tubulin. The data showed that the level of the α-tubulin decreased obviously (Fig. 6). It suggested that the microtubules had significant influence on apigenin-induced cell apoptosis.

Apigenin Induces Apoptosis in a ROS-Dependent Manner

ROS has been reported as an important regulator for mitochondrial function (Dhanasekaran *et al*., 2005) and the production of ROS is for the amounts required for microdomain cell signaling. But in the imbalance act of aerobic metabolism, the excessive production of ROS can damage cellular components such as proteins, lipids and DNA, and further induce cell death. Here, using the fluorescent probe DCFH-DA and DHE, the ROS level in MCF-7 cells was measured using flow cytometry. Figure 7 showed that there was a dose-dependent increase in ROS levels in MCF-7 cells under exposure to apigenin. DHE fluorescence, reflecting O_2^- accumulation, also increased during apigenin exposure, apigenin (100 μmol/L) induced a 20% increase in DHE fluorescence after 24 h.

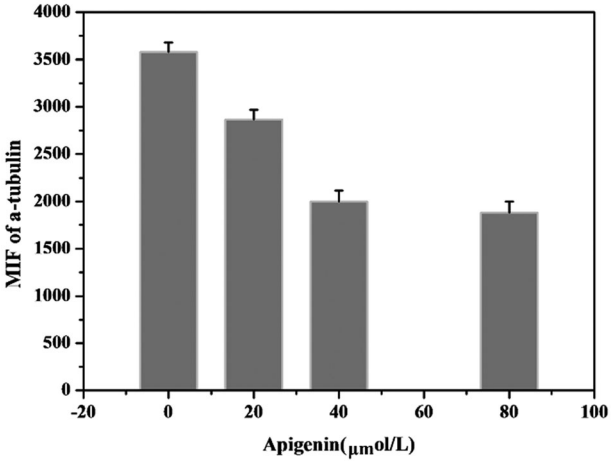

Fig 6. Expression of α-tubulin. Analysis of tubulin expression on MCF-7 cells by flow cytometer. Apigenin-treated MCF-7 cells with concentration of 0, 20, 40, and 80 μmol/L for 24 h, respectively.

Discussions

Tumor cell resistance to apoptosis is an inherent part of the carcinogenic process and is also implicated in resistance to chemotherapeutic drugs (Johnstone *et al*., 2002). Therefore, phytochemicals such as apigenin is applied to target resistant cells and improves efficacy without toxicity on normal cells (Gupta *et al*., 2001). Previous studies have reported that apigenin can't induce apoptosis in normal cells including primary cultures (Gupta *et al*., 2001). Gupta *et al*. (2008) studied the effect of apigenin on human prostate carcinoma cells. Their results indicated that apigenin had no toxicity on normal cells, which showed by no superoxide generation and therefore no cell death. Several studies have suggested that apigenin can induce apoptosis in malignant cells (Liang *et al*., '99). But the mechanisms responsible for apoptosis induced by apigenin appear to be unknown. Most of the studies declared that apoptotic cell death was related to the stability of oncogene protein p53, the release of cytochrome c, and the generation of reactive oxygen species (Tsvetkov *et al*., 2005). More recently, treatment with apigenin has been reported to induce autophagic cell death in malignant cells (Horinaka *et al*., 2006). Our results (shown by Fig. 2) indicated that there was an increase in the cytotoxicity in MCF-7 cells with increasing concentration of apigenin treatment but only had little impact on normal cells (MCF-10A). Thus, apigenin has very low toxicity on normal cells but can significantly induce cancer cells apoptotic. Existed evidence supported apigenin used in cancer prevention through its antiproliferative and anticarcinogenic properties or as an adjunct in overall cancer treatment.

In our study, apigenin could inhibit growth and induce apoptosis in MCF-7 cells. Cell viability and cell apoptosis had dose-dependent and time relationship with apigenin concentration, respectively. With the apigenin concentration increased, cell viability decreased significantly and the apoptosis increased steadily (as shown in Fig. 3). The results strongly suggested that apigenin possessed high apoptotic induction effect in cancer cells, especially in high dosages.

AFM can serve as a valuable tool to elucidate ultrastructural changes in cell-surface topography at the nanoscale level. The realization of the full potential of high-resolution AFM imaging has revealed some very important biological events such as exocytosis and endocytosis (Samsuri *et al*., 2010). These changes on the cell-surface topography may potentially provide novel insights into the control of cell shape and interaction with pericellular matrix during activation of chemical

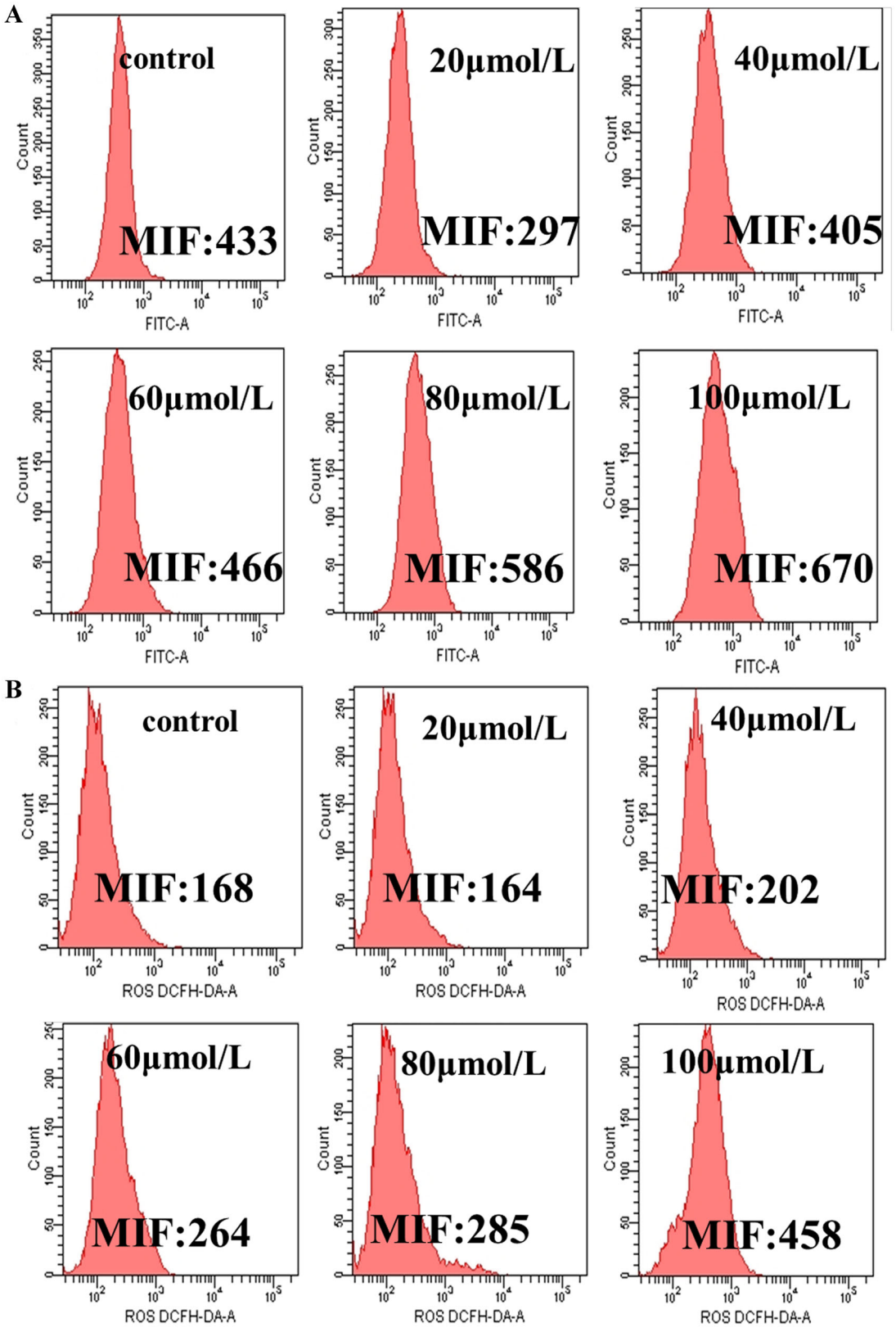

Fig 7. Effect of apigenin on ROS in MCF-7 cells after treated for 24 h, (A) cells were determined by flow-cytometric analysis stained with DCFH-DA for 30 min, (B) cells were determined by flow-cytometric analysis stained with DHE for 30 min. Results are expressed as relative fluorescent intensity.

and mechanical signaling pathways (Iscru *et al*., 2008). To address this point, we analyzed the cell membrane morphology and ultrastructure in order to investigate effect of apigenin to MCF-7 cell. Through AFM direct AFM measurement of MCF-7 cells (Fig. 3), the cell in control was in a typical long spindle-shaped morphology at the single cellular. After treated with apigenin, the cell morphology turned into round, cell tails shrank and presented threadiness. These changes implied apigenin could decrease the capacity of MCF-7 cell motility and intracellular communication. The cell-surface ultrastructure of control cells (Fig. 3(A1) and (B1)) was homogeneous and displayed granular morphology with uniform particles on it. By a concrete analysis, the average roughness of cell-membrane (located) surface was 8.88 nm. Particles were distributed uniformly on cell surface. The size of most particles was in a range of 40–60 nm (Fig. 3(C1)). But, after being treated with 20 μmol/L apigenin for 24 h, cell morphology changed to some extent, and the cell surface also became rougher (Fig. 3(B2) and (C2)), the average roughness increased to 36.3 nm, the size of surface particles increased to 70–150 nm (Fig. 3(C3)). In the 80 μmol/Lapigenin treated group, the cell changed into round (Fig. 3(A4)), the size of most granules was in 400–600 nm, which was almost ten times of that in the control group (Fig. 3(C4)). Meanwhile, the average roughness also increased to 78.8 nm. With AFM at subcellular level, we visualized that after treated with apigenin, particles on cell membrane were bigger than that in control group. It has been reported that the visible protruding particles are clusters of membrane proteins (Christian *et al*., '98), which means some biological events such as change of ion channels might have occurred.

For further detecting the relationships between the cytoskeleton and cell stiffness, the characterization of cytoskeleton was performed using immune fluorescence staining. The cytoskeleton of cells, an intricate polymer network under cell membrane, which mainly contains actin and tubulin proteins, is the structural framework that predominantly shapes a cell and provides its mechanical rigidity. Figure 5 showed the reorganization of α-tubulin of MCF-7 cells stained with Tubulin-Tracker Red. The assembly of tubulin fibers in control cells represented regular mesh networks (Fig. 5(A–C)) while these microfilaments disrupted after apigenin treatments, which also induced the damage of cell integrity (Fig. 5(D–F)). To further investigate how apigenin induced changes in the morphology of nuclei, DAPI specific staining experiments were performed. When DAPI binds to natural double-stranded DNA, the fluorescence is strongly enhanced so that the morphology of the nuclei can be clearly visualized and the apoptotic cells can be identified. Figure 5(G–L) revealed the typical nuclei morphology in DAPI-stained control MCF-7 cells and 40 μmol/L apigenin treated MCF-7 cells. Control MCF-7 cells showed intact and plump nuclei (Fig. 5(G)). But the nuclei of apoptotic MCF-7 cells induced by apigenin represented the segmentation of nuclei and the gatheration of condensed chromatin at the periphery of the nuclear membrane (Fig. 5(J)). Taken together, these results indicated that apigenin could significantly change the nuclei morphology of MCF-7 cells through induction of apoptosis in MCF-7 cells.

For quantitative analysis of the amounts of α-tubulin in MCF-7 cells, we determined (as is shown in Fig. 6) the fluorescent signal of tubulin-tracker red in MCF-7 cells by flow cytometry and found that apigenin also decreased the amounts of intracellular α-tubulin in MCF-7 cells, which also meant that apigenin could induce the depolymerization of α-tubulin. Thus we could conclude that apigenin treatment could decrease the stiffness of MCF-7 cells through disturbing structure and amounts of intracellular α-tubulin. Additionally, α-tubulin has been viewed as the critical component of cytoskeleton that is necessary for cell motility and sustained cell signaling because it provides the capability of cells to move organelles within the cytoplasm (Natsume *et al*., 2012). This indicated that apigenin induced changes of α-tubulin in MCF-7 cells might also be closely related to apigenin induced apoptosis in MCF-7 cells.

Reactive oxygen species (ROS) plays an important role in apoptosis induction under both physiologic and pathologic conditions. Early study had shown that hydrogen peroxide is able to induce apoptosis, which is prevented by catalase (Pierce *et al*., '91). For instance H_2O_2 induced apoptosis in neutrophils, which can be prevented by catalase (Kasahara *et al*., '97). ROS are thought to mediate the toxicity of oxygen because of their greater chemical reactivity with regard to oxygen (Autréaux and Toledano, 2007). Here, we study the ROS level induced by apigenin, as is shown in Figure 7, apigenin increased the ROS level in cells. The increasing of ROS induced by apigenin demonstrated that apigenin could disturb the balance of ROS in MCF-7 cells to induce cell apoptosis. There are numerous examples of the inhibition of apoptosis through antioxidative drugs or enzymes (Simon *et al*., 2000). That induced the cell-surface molecule distribution and biochemical or biomechanical signal changes, which were reflected in the ultrastucture of cell-surface. Since the ROS-dependent process is one of the common features in the cell death, apigenin may be also useful for treatment of some diseases such as cancer.

Conclusion

In this work, we demonstrated that apigenin could effectively inhibit the growth and induce apoptosis of tumor cells (MCF-7 cell), but at the same concentration, apigenin only caused a little damage to normal cells (MCF-10A cell), indicating that apigenin possessed a

good killing selectivity on tumor cells. There was a dose-dependent relationship between the drug concentration and cell apoptosis after treated with apigenin for the same time (24 or 48 h). Cell morphology shown apigenin could damage the cell membrane and suggested that the degree of damage to a tumor cell membrane has a certain positive correlation with drug concentration. AFM detected ultrastructure indicated that the membrane protein granules gathered to cluster after apigenin treated. All these changes disturbed the cell homeostasis and affected cell function. As the apigenin concentration increased from 40 to 100 μmol/L, intracellular ROS level increased by nearly two times. These resulted in cell apoptosis, and were reflected in the ultrastructure of cell surface. This research provided us detailed insights into new agents' development for treatment of Human breast cancer and mechanism study of apigenin. Meanwhile, it could contribute to visual diagnosis of early stage apoptosis in tumor cells in response to anti-cancer drugs, as well as in the studies of the interaction between drugs and cells. However, it required further more research to fully understand the specific anti-cancer mechanism of apigenin.

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