Research Article

Rice bran derived pentapeptide-induced apoptosis in human breast cancer cell models (MCF-7 and MDA-MB-231)

Ruiqi Li[1], Navam Hettiarachchy[1*] and Mahendran Mahadevan[2]

[1]Department of Food Science, Dale Bumpers College of Agricultural, Food & Life Science, University of Arkansas, 2650 n Young Ave., Fayetteville, AR 72704.
[2]Department of Obstetrics and Gynecology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205.

*Correspondence Info:
Navam Hettiarachchy,
Department of Food Science,
Dale Bumpers College of Agricultural,
Food & Life Science, University of Arkansas,
2650 n Young Ave., Fayetteville, AR 72704
E-mail: nhettiar@uark.edu

Abstract

Studies have focused on the characterization of a single peptide from natural sources that contribute specific health benefits. A pentapeptide derived from rice bran has shown anti-proliferative property on human breast cancer cells. The objective of this study was to investigate the apoptotic features of pentapeptide-induced apoptosis in breast cancer cell models (MCF-7 and MDA-MB-231). The MTS (phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2, 5-diphenyl tetrazolium bromide)assay was used to evaluate the growth inhibition activities of pentapeptide in a time-dependent manner. The apoptotic features of pentapeptide-induced apoptosis in cancerous breast cells were evaluated by morphological changes, DNA fragmentation, and caspases-3/7 activities. The levels of molecular targets (COX-2, and p53) were evaluated by ELISA kits. Pentapeptide has shown growth inhibition activity on MCF-7and MDA-MB-231 cells. The morphological changes and DNA fragmentation were observed in pentapeptide treated MCF-7 and MDA-MB-231 cells. Significant decreases in levels ($p < 0.05$) of COX-2 and increases in levels ($p < 0.05$) of p53 were detected after treatment with pentapeptide from 72 to 96 h. The results suggest that pentapeptide inhibits growth of human breast cancer cells by introducing apoptosis and can regulate the death signal by up-regulating the level of p53 in both cells lines and down-regulating COX-2inER-positive MCF-7 cells.

Keywords: pentapeptide, breast cancer, apoptotic properties, COX-2, p53

1. Introduction

Breast cancer is one of the leading causes of cancer related deaths and illnesses in the United States. In 2013, 39,620 estimated deaths for breast cancer (females) were reported according to the American Cancer Society[1]. Traditional cancer therapies include surgery, radiation, hormone therapy, and chemotherapy which may result in side effects such as nausea, vomiting, loss of appetite, fever, and fatigue[2]. Hernandez and others concluded that lunasin (a promising chemo-preventive peptide derived from soybean, wheat, barley, and other plant seeds) has shown breast cancer-preventive properties[3]. Researchers also reported that breast cancer proliferation was inhibited by peptides found in rice bran protein and fish protein hydrolysates[4,5]. Studies have shown that anti-cancer agents derived from foods maybe promising new candidates for breast cancer therapy.

Rice (*Oryza sativa*) bran is the hard external outer layer of the brown rice kernel. Bran is removed from the kernel by friction during the production of white rice and it accounts for about 10% of rough rice[7]. It contains high content of dietary fiber and antioxidants such as polyphenolics (phenolic acids and flavonoids), α-tocopherols (vitamin E), tocotrienols, and oryzanols, all of which have proven bioactivities including lowering the cholesterol, decreasing the incidence of obesity, diabetes, intestinal system disorders, and coronary heart diseases[8]. In recent years, rice bran protein derived hydrolysates and peptides have received considerable attention and there are lines of evidence shown their anti-oxidative, anti-mutagenic, and anti-carcinogenic properties[9-11,4].

Apoptosis, or programmed cell death (PCD), is a genetically regulated process that happens in every cell and is initiated by physiological and pathological stimuli. The apoptosis is characterized by caspases cascade, morphological changes, and DNA fragmentation[6]. The morphological changes of cells include condensation of chromatin, cellular atrophy, and blebbing[6]. Caspases are a group of cysteine-dependent aspartate-directed proteases and play an important role in controlling cell apoptosis. During early stages of apoptosis, the activation of initiator caspases through a signal transduction cascade leads to the cleavage of cellular components, which finally results in the PCD. In the process of tumorigenesis, the ratio of proliferated cell and apoptotic cells is altered and uncontrolled.

Kannan et al (2010)[4] have prepared a novel pentapeptide with a sequence Glu-Gln-Arg-Pro-Arg that showed nearly 80% of growth inhibition activity on human breast cancer cell (MCF-7). However, the apoptotic features and mechanistic insight of apoptosis in breast cancer cells induced by pentapeptide remains unknown. In this study, MCF-7 and MDA-MB-231 cell lines were selected as breast cancer cell models. MCF-7 cells are useful for *in vitro* breast cancer studies due to their characteristic to process estrogen via estrogen receptors in the cell cytoplasm, which makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line. The MDA-MB-231 cells have an epithelial-like morphology, which appear phenotypically as spindle shaped cells. The differences between two cell lines are MCF-7 is an ER-positive breast cancer line derived from an in situ carcinoma, meaning that the cancerous cells had not yet invaded surrounding tissues; however, the MDA-MB-231 is an ER-negative breast cancer line derived from a metastatic carcinoma. Selection of both cell lines helps in better illustrating and understanding the mechanism of pentapeptide-induced growth inhibition on human breast cancer. The objective of this study was to investigate the effect of pentapeptide-induced apoptosis on human breast cancer cell models (MCF-7 and MDA-MB-231). The growth inhibition activity of pentapeptide was determined by MTS assay. The apoptotic properties of pentapeptide on human breast cancer cells were evaluated by morphological changes, DNA fragmentation, and caspases activity assay. The levels of COX-2 and p53 in pentapeptide treated breast cancer cells were also investigated.

2. Materials and Methods

2.1 Materials

Pentapeptide (amino acids sequence: EQRPR) was purchased from Biomatik LLC. (Wilmington, Delaware, USA). Human breast cancer cell lines (MCF-7and MDA-MB-231), Eagle's Minimum Essential Medium and Leibovitz's L-15 Medium were purchased from ATCC (Manassas, VA, USA).Fetal bovine serum (FBS), insulin and trypsin-EDTA solution were purchased from Sigma (MO, USA). The phenazine methosulfate 3-(4,5-dimethyl thiazole-2-yl)]-2, 5-diphenyl tetrazolium bromide (MTS) one solution kit and Caspase-Glo®3/7kit were obtained from Promega Corporation (Madison, WI, USA). The human COX-2 and p53 ELISA were supplied by Enzo Life Science Inc. (Farmingdale, NY, USA) and Abcam Plc. (Cambridge, MA, USA), respectively.

2.2 Methods

2.2.1 Cell culture

The human breast cancer cell lines (MCF-7and MDA-MB-231)were cultured following the recommend protocols from ATCC (Manassas, VA, USA). The MCF-7 cells were cultured in Eagle's Minimum Essential Medium with 0.01mg/mL bovine insulin and 10% FBS. Cells were grown at 37°C in a humidified 5% CO_2 incubator. The MDA-MB-231cells were cultured in Leibovitz's L-15 Medium supplement with 10% FBS and incubated at 37°C withoutCO_2.

2.2.2Cell viability assay (MTS assay)

The cytotoxic effects of pentapeptide on the breast cancer cell lines (MCF-7and MDA-MB-231) were evaluated by the MTS assay[4]. The pentapeptide at a concentration of 1000μg/mL was used to induce cell death in all assays. The concentration was determined by the preliminary study on the dose-response of pentapeptide on growth of human breast cancer cell lines, which pentapeptide at 1000μg/mL have shown maximum growth inhibition on both cell lines (MCF-7and MDA-MB-231). Human breast cancer cells were collected from a 75 cm^2 flask using trypsin-EDTA and the cells density was determined using a hemocytometer. The cells were plated on a 96-well plate at a density of 5x10^3 cells per well. The cells were allowed to attach for24hr following by removing the spent media. The pentapeptide (1000μg/mL), medium alone (negative control), and genistein at 400μg/mL (positive control) were added to respective well with 200μL of final volume and incubated for various time periods (24, 48, 72, and 96hr). Then, the MTS one solution reagent (40μL/well) was added and incubated for 1hr. The absorbances of samples were measured at 490nm using a microplate reader after adding 10% SDS for reaction termination. The cytotoxic effect of the pentapeptide was expressed as a relative percentage of inhibition calculated as follows:

Relative inhibition (%) = (A_{490}Experiment - A_{490}Control) ×100

2.2.3 Observation of morphological changes

Cells (MCF-7 and MDA-MB-231) were trypsinized from a 75 cm^2 flask and the cells number was determined by a hemocytometer. Cells were seeded into a 12-well plate with a density of 5×10^4 cells per well. After 24hr incubation, the cells were treated with pentapeptide (1000μg/ml) and observed after incubated for 72hr. The cellular morphological changes were observed using a phase contrast microscopy (magnification: 200) and the photos were taken using AmScope Color Digital Camera (MD 1000-CCD). The photos of cells treated media were taken and referenced as the negative controls. The morphological changes of apoptotic cells include the shrinkage of cells, blebbing nuclei, and floating cells.

2.2.4 DNA fragmentation

The detection of DNA fragmentation in pentapeptide treated human breast cancer cells was determined by fluorometric DNA fragmentation detection kit III (F-dUTP) following the company protocol (PromoKine, Germany). Cells (MCF-7 and MDA-MB-231) were cultured following the same method described above and treated with pentapeptide for 72hr. The cells cultured with media were used as controls. All the cells were fixed using 1.0% (w/v) paraformaldehyde in PBS and ice-cold 70% (v/v) ethanol. Then, the cells were stained with Staining Solution supplied by the company (PromoKine, Germany) and propidium iodide/RNase solution. A confocal microscopy was used to detect the DNA fragmentations in apoptotic cells.

2.2.5Assay for caspase-3/7 activity

The activity of caspase-3/7 was determined bycaspase-Glo®3/7 assay kit (Promega Cop., USA). The caspase-3/7 kit reagents were equilibrate to room temperature before use and prepared following the company instruction. Cells (MCF-7 and MDA-MB-231) were cultured and transferred into the white-walled 96-well plate with a density of 5 x 10^3 per well and allowed to attach for 24hr. Then, cells were treated with pentapeptide (1000 μg/mL) for 72 and 96hr. The cells treated with media alone and genistein (400μg/mL) in media were referred as negative and positive controls, respectively. One hundred μl of Caspase-Glo®3/7 reagent was added into each well and gently mixed contents using a plate shaker at 300rpm for 30 seconds, followed by 2hr incubation at room temperature. The luminescence of each sample was measured using a plate-reading luminometer. The levels of activated caspase-3/7 in cells were expressed as relative luminescence intensities.

2.2.6 Determination of levels of COX-2 and p53

The levels of COX-2andp53 in pentapeptide-treated MCF-7 and MDA-MB-231 cells were determined using respective human ELISA kits following the company protocols with similar principle. Cells (MCF-7 and MDA-MB-231) were cultured and treated with pentapeptide (1000μg/mL) for 72 and 96hr. The cell lysates were prepared using lysis buffer and transferred in 96-well plates that coated with monoclonal detective antibodies and incubated for 2hr. The unbound materials were removed by washing buffer. Then, the horseradish peroxidase conjugated streptavidin was added to bind to the antibodies in each wells and incubated for 1hr. The substrate reagent was added to each well and the absorbances were measured at 450nm using a microplate reader. The concentrations of COX-2 and p53 were determined by standard curves plotted using standard proteins.

2.3Analysis of Data

All the treatments in assays were conducted in triplicates and the values were reported as means ± standard deviation. The cytotoxic effects of pentapeptide on human breast cancer cell lines were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS 9.2 2000, SAS Institute Inc., Cary, N.C., U.S.A.). The Fisher's protected least significant difference (LSD) test was conducted to separate the means at $P \leq 0.05$.

3. Results

3.1 Pentapeptide Induced Growth Inhibitions on MCF-7 and MDA-MB-231 Cells

To determine the growth inhibitions of pentapeptide on human breast cancer cell lines, the MTS assay was used to evaluate the anti-proliferative activities after pentapeptide (1000μg/mL) treatment and incubated for various times (24, 48, 72, and 96hr). Figure 1shows that the survival rates of pentapeptide treated MCF-7 and MDA-MB-231 cells decreased significantly ($p < 0.05$) in a time-dependent manner compared to negative controls (cells cultured using media alone).The maximum inhibitory activities were found onMCF-7 (90.9%) and MDA-MB-231 (87.0%) after incubating with pentapeptide for 72 and 96hr, respectively. However, a relatively lower growth reduction on MDA-MB-231 cells suggested it was more resistant to pentapeptide treatment than MCF-7 cells.

Figure 1.The growth inhibition effects of pentapeptide (1000µg/mL) on MCF-7 and MDA-MB-231 cells after various incubation times (24, 48, 72, 96hr). The cell lines cultured with media alone are negative controls. Values are means ± standard deviations of three determinations. Values with the same letter are not significant different ($P>0.05$). The standard deviations are shown by the error bars.
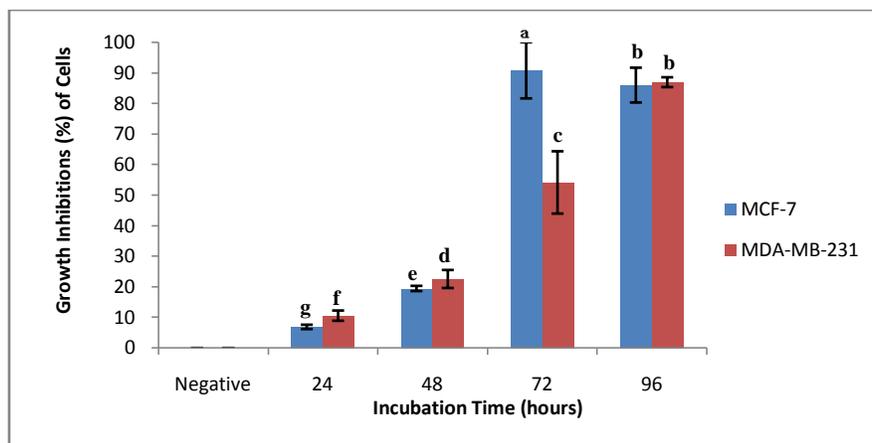
3.2 Morphological Changes of MCF-7 and MDA-MB-231 Cells Induced by Pentapeptide

The morphological change is considered one of the most remarkable features of apoptotic cells. Thus, in order to evaluate whether the pentapeptide-induced cell death was caused by apoptosis, the morphological changes of pentapeptide-treated MCF-7 and MDA-MB-231 cells were observed using a phase contrast microscopy (magnification: 200x) and compared with the untreated controls. After 72hr of pentapeptide (1000µg/mL) treatment on MCF-7 and MDA-MB-231 cells, the morphological changes (pointed out by arrows) including cell floating and shrinkage, nucleic blebbing, and the display of granular apoptotic bodies were observed (Figures2b and 2d); however, the control groups without pentapeptide treatment did not show any detectable morphological changes (Figures 2a and 2d).

**Figure 2 (a,b,c,d): Effect of pentapeptide on the morphology of MCF-7 and MDA-MB-231 cells**
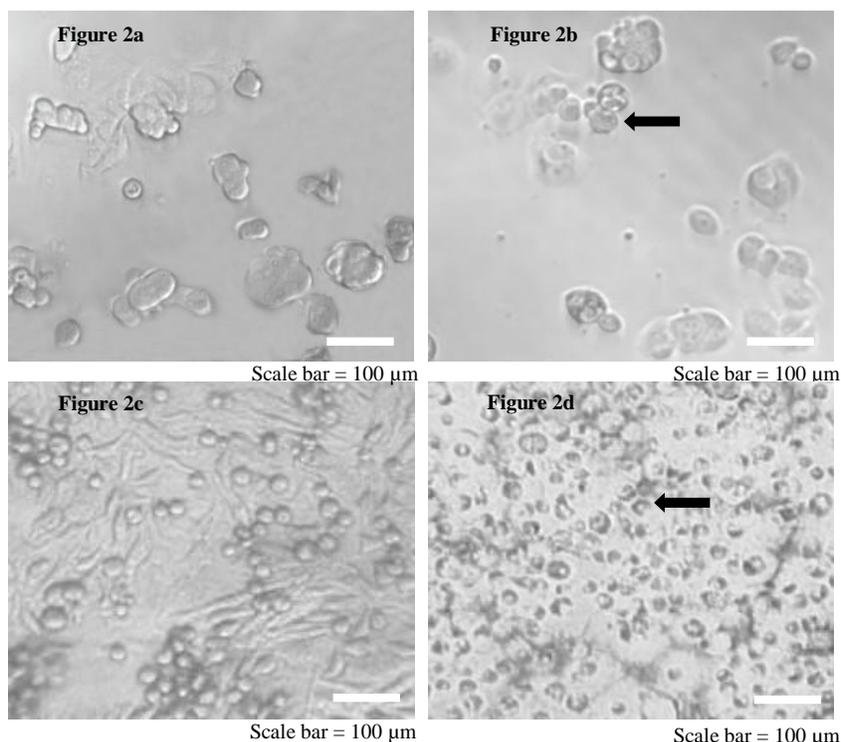
All cell morphology were observed using a phase contrast microscopy (magnification: 200) and photos were taken using AmScope Color Digital Camera (MD 1000-CCD). Figure 2a and 2c show morphology of MCF-7 and MDA-MB-231 cells respectively when cultured in media only and observed after 72 hr incubation (controls). Figure 2b and 2d show morphology of MCF-7 and MDA-MB-231 cells respectively when cultured in the presence of pentapeptide (1000 µg/mL) and observed after 72 hr incubation. Arrows point to the retracted and round cells which were undergoing apoptosis.

3.3 Pentapeptide Induced DNA fragmentation in MCF-7 and MDA-MB-231 Cells:

DNA fragmentation is well recognized as one of the features of cells death caused by apoptosis. In the current study, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL)-based kit was used to detect the DNA fragmentation in apoptotic cells. The principle of this method is that the TdT catalyzes the fluorescein-12-dUTP incorporation located at the free 3'-hydroxyl ends of the fragmented DNA[13]. Then, a confocal microscopy could be used to observe the fluorescein-labeled fragmented DNA from the stained apoptotic cells that emits green fluorescence at 520nm against an orange red propidium iodide (PI) counter-staining (fluorescence at 620nm). In the current study, MCF-7 and MDA-MB-231 cells were treated

with pentapeptide incubated for 72hr and the negative controls were cells cultured with media only. Figures 3b and 3d displayed strong green color against red counter-staining, which suggested a clearly fragmented DNA pattern compared to negative controls (Figures 3a and 3c). These results suggested that pentapeptide inhibited the growth of both human breast cancer cells through apoptosis.

Figure 3 (a,b,c,d): DNA Fragmentation of pentapeptide treated MCF-7 and MDA-MB-231 cells.
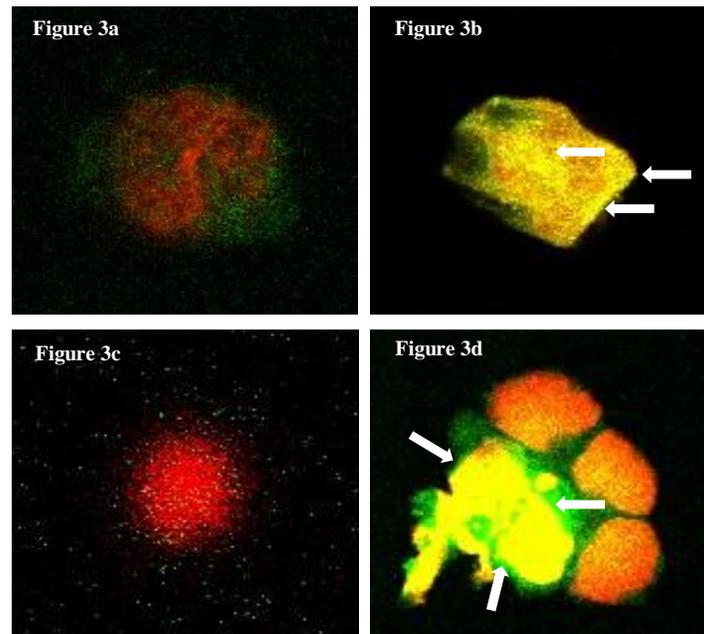

All cells were stained using a Terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL)-based DNA fragmentation kit and observed using a confocal microscope (Ex/Em = 488/520nm for FITC/green/apoptotic, and 488/623 nm for PI/red/rhodamine). Figures 3a and 3c are MCF-7 and MDA-MB-231 cells grown in media and observed after 72hr. Figures 3b and 3d were pentapeptide (1000μg/mL) treated MCF-7 and MDA-MB-231 cells after 72hr incubation. Arrows point to the areas emit green light which reflect the fragmented DNA.

3.4 Activation of Caspase-3/7 in Pentapeptide Treated human breast cancer cells
Caspase cascade is generally recognized as a hallmark of cell undergoing apoptosis. To verify the role of caspases in pentapeptide-induced growth inhibition on human breast cancer cells, the levels of caspase-3/7 was evaluated after pentapeptide (1000μg/mL) treatments. Genistein (400μg/mL) was used to induce apoptosis in MCF-7 and MDA-MB-231 cells and considered positive controls. Significantly increased ($p< 0.05$) levels of active caspase-3/7 fragments were detected in both cell lines in the presence of pentapeptide (1000μg/mL) from 72 to 96hr (Figure 4). Besides, MDA-MB-231 cells have shown comparatively higher caspase-3/7 levels than MCF-7.

Figure 4.The relative intensity of activated caspase-3/7 in pentapeptide (1000 μg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96hr.
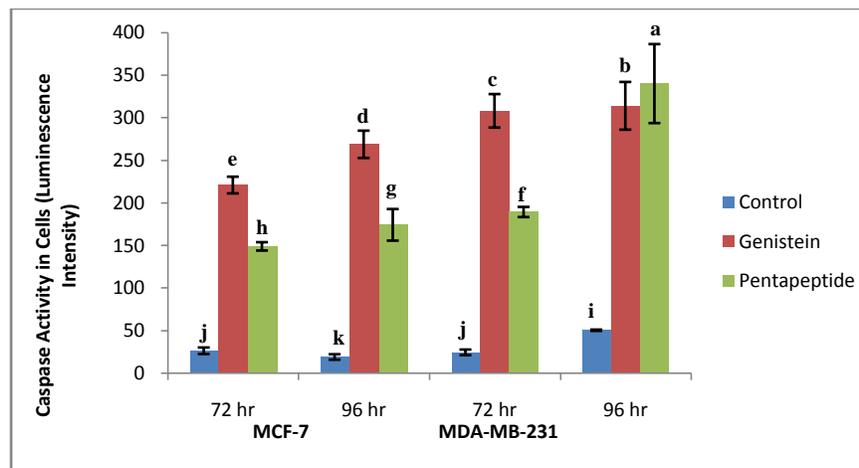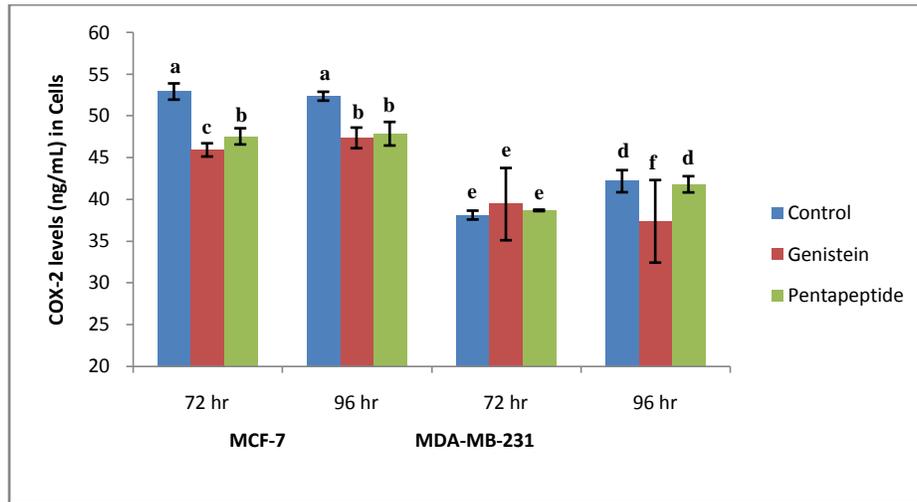

The cells cultured with media alone and genistein (400μg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different ($P>0.05$). The standard deviations are shown by the error bars.

3.5 Expression of p53 and COX-2 in pentapeptide treated human breast cancer cell lines
To further investigate the possible mechanisms of pentapeptide-induced apoptosis in MCF-7 and MDA-MB-231 cells, the expressions of p53 (a tumor suppressor) and COX-2 (potential target for prevention and treatment of breast cancer) were assessed using ELISA assays in the presence of

pentapeptide (1000µg/mL). Significantly ($p< 0.05$) increased levels of p53 were observed after 72 and 96hr of incubation with pentapeptide in both cell lines (Figure 5). On another hand, pentapeptide shown significant ($p< 0.05$) down-regulate on COX-2 levels in MCF-7 cells while no significant ($p> 0.05$) decreased levels of COX-2 in MDA-MB-231 were observed (Figure 6).

Figure 5.The levels of COX-2 in pentapeptide (1000µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96 hr.

The cells cultured with media alone and genistein (400µg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different ($P>0.05$). The standard deviations are shown by the error bars.

Figure 6.The levels of p53 in pentapeptide (1000µg/mL) treated MCF-7 and MDA-MB-231 cells after incubation for 72 and 96hr.
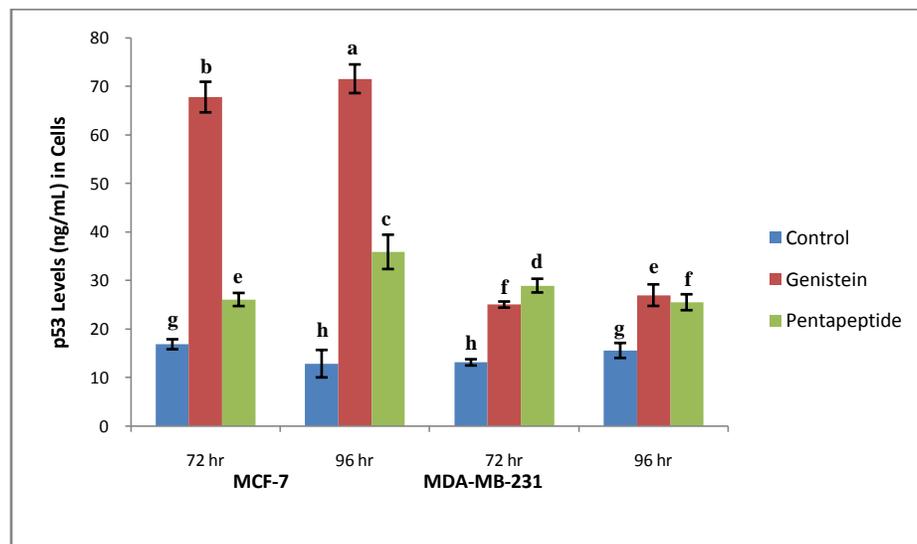
The cells cultured with media alone and genistein (400µg/mL) are negative and positive control, respectively. Values are means ± standard deviation of three determinations. Values with the same letter are not significant different ($P>0.05$). The standard deviations are shown by the error bars.

4. Discussion

Previous research has shown that pentapeptide caused growth inhibitions on several cancer cell lines including Caco-2, MCF-7, HepG-2, and A549[4]. In this study, media alone and genistein (400µg/mL) were used as negative and positive controls and were carried out as parallel study. Genistein causes growth inhibition on human breast cancer cells via apoptosis and is used as a positive control[12]. The above results demonstrated that pentapeptide significantly inhibited the growth of MCF-7 and MDA-MB-231 cells in a time-dependent manner which suggested that cell death may be caused by membrane disruption through apoptosis.

Generally, abnormal and unhealthy cells such as cancerogenesis cells are removed through apoptosis which is a programmed cell death physiological mechanism[14]. Apoptosis could be recognized by several biological features including morphological changes, DNA fragmentation and caspases activation[15]. Two major apoptotic mechanisms including death receptor-dependent and mitochondria-dependent pathways involve the activation of caspase-8 and -9, respectively[16]. Then, the caspase-3 or -7 is activated to cleave various cytoplasmic or nuclear substrates including DNAse which lead to the typical morphological changes and DNA fragmentation[17-19]. The observation of fragmented DNA has been widely used for identifying and monitoring the apoptosis cells[20]. The DNA fragmentation assay revealed a typical fragmented DNA pattern in pentapeptide-exposed MCF-7 and MDA-MB-231 cells and suggested that the cell deaths were caused by apoptosis. Figure 4 indicated that the caspase-3/7 activities in cells treated with pentapeptide increased significantly ($p< 0.05$) compared to control treatments with medium alone. Since MCF-7 is a caspase-3-deficient breast cancer cell line, the results suggest that effector caspase-7, a sub-family member of caspase-3, plays an indispensible role in MCF-7 cells during apoptosis. Previous studies have shown its role

in apoptosis of caspase-3-deficient MCF-7 cells[21-23]. The activations of caspase-3/7 were observed in both cell lines after treatment with pentapeptide from 72 and 96hr which consistent with the results of morphological changes and the DNA fragmentation. Therefore, the results suggested that pentapeptide-induced MCF-7 and MDA-MB-231 cells death were involved in a mechanism of apoptosis.

In an attempt to identify the possible mechanism of apoptosis in MCF-7 and MDA-MB-231 cells in response to pentapeptide, the expressions of p53 and COX-2 were evaluated after pentapeptide treatment. It is widely known that p53 is a tumor suppressor gene involved in cell-cycle regulation and the induction of apoptosis by mediating the ratio of Bax (pro-apoptotic protein)to Bcl-2 (anti-apoptotic protein)[24, 25].The p53 protein helps in repairing the damaged DNA to cause G1 arrest and avoiding the cancerous cell proliferation and neoplastic transformation. Studies have suggested that various anti-cancer agents suppress the growth of cancerous cells via up-regulating the level of p53 protein[26-30].In our study, the expression of p53 increased significantly ($p< 0.05$) in the pentapeptide treated MCF-7 and MDA-MB-231 cells and suggested that pentapeptide up-regulates the p53 which may either trigger the onset of DNA repair or induced the apoptosis via mitochondrial-dependent pathway by regulating its downstream molecules such as Bax, Bcl-2, and Fas. Thus, further investigation is needed for indentifying in which pathways the p53 is activated in pentapeptide-mediated apoptosis in MCF-7 and MDA-MB-231 cells.

COX-2 (Cyclooxygenase-2) is being intensively evaluated as a pharmacologic target for both the prevention and treatment of cancer. Studies have shown that high expression of prostaglandin (PG) level is detected in many types of cancers and enhanced PG synthesis may contribute to carcinogenesis via stimulation of cancer cell proliferation[31]. Since COX-2 is the primary syntheses in PG production, studies have been carried out to design COX-2 inhibitors in treating experimental breast tumors that have yielded promising results[32]. Other studies also suggested that COX-2 over-expression may result in the suppression of proapoptotic proteins such as Bax and Bcl-xLduring apoptosis[33]. In our research, pentapeptide displayed a down-regulating effect on COX-2 level in MCF-7 cells; while the COX-2 level remains unchanged in a MDA-MB-231 cell line. Those results suggested that pentapeptide may suppress the level of COX-2 which leads to the cell growth inhibition via decreasing estrogen levels in an estrogen receptor (ER)-positive MCF-7 cell line; however, it has no effect in regulating COX-2 level in an ER-negative MDA-MB-231 cell line. This result is in agreement with the previous study by Harris and others, who suggested that PGs may also stimulate proliferation indirectly via increased estrogen biosynthesis in breast tissue[34].

In conclusion, the current study demonstrated that pentapeptide showed anti-tumor activities on human breast cancer cells (MCF-7 and MDA-MB-231) through apoptosis. Pentapeptide stimulated the levels of p53 in both cells lines and may also suppress the COX-2 in ER-positive breast cancer cells such as MCF-7 by regulating the estrogen synthesis. Pentapeptide maybe a potential anti-breast cancer agent or future replacement for current expensive drug when the molecular mechanisms will be better characterized.

Acknowledgments

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