Iranian Biomedical Journal 13 (4): 229-236 (October 2009)

Apoptotic and Necrotic Effects of Pectic Acid on Rat Pituitary GH3/B6 Tumor Cells

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Received 8 May 2009; revised 15 August 2009; accepted 19 August 2009

ABSTRACT

Background: Pectin is composed of complex polysaccharides that can inhibit cancer metastasis and proliferation with no evidence of toxicity. In the present study, the apoptotic and necrotic effects of pectic acid (PA) on the rat pituitary GH3/B6 tumor cells has been investigated. **Methods:** GH3/B6 cells were cultured in the Ham's F12 medium enriched with 15% horse serum and 2.5% fetal bovine serum for 3 days. Then, they were treated by various amounts of PA in different periods (6, 24 and 48 hours). Bromocriptine was used as positive control and the cell viability was detected by MTT test. The nuclear morphology of cells was explored by florescent stains including acridine orange (AO)/ethidium bromide (EB). In addition, percentages of apoptotic and necrotic cells were studied with triphosphate nick-end labeling (TUNEL) assay, cell cycle analysis and propidium iodide (PI) staining. **Results:** Long-term incubation with PA results in increased cell death and DNA damage as detected by MTT assay and AO/EB staining. TUNEL assay showed that PA (100 µg/ml to 1 mg/ml) could induce apoptosis in a dose-dependent manner, while higher concentrations of PA (2.5 and 5 mg/ml) induced necrosis which was confirmed by PI staining. Furthermore, cell cycle analysis indicated that PA induced sub G_1 events, and DNA fragmentation was also correlated with the number of the apoptotic cells. **Conclusion:** It can be concluded that PA is responsible for apoptosis in the rat pituitary tumor cells. Therefore, one may suggest that this group of polysaccharides can be used in treatment of pituitary tumors. *Iran. Biomed. J. 13 (4): 229-236, 2009*

Keywords: Pectic Acid (PA), Apoptosis, Necrosis, GH3/B6 cells

INTRODUCTION

The pituitary plays a major role in regulating metabolic, developmental and reproductive functions. Lactotrophs produce prolactin (PRL) and represent about 20-50% of the cellular population of the anterior pituitary. PRL production and release are tightly regulated processes, and their disruption lead to several pathological conditions [1]. The majority of pituitary adenomas are benign; however, invasiveness of adjacent tissues may serve as an important factor adversely affecting the outcome of tumor and compromising the disease-free survival of patients [2]. Bromocriptine is commonly chosen as a therapeutic agent for patients with prolactinomas or other pituitary adenomas [3]. Also, bromocriptine binds to the dopamine D2 receptors on pituitary epithelial cells to inhibit PRL secretion [4]. A rat pituitary prolactinoma cell line, GH3, is commonly used as a cellular model for studying prolactinoma formation. It has been reported that long-term incubation of GH3 cells with bromocriptine induces cell apoptosis [3].

Pectin is a complex polysaccharide found in virtually all plant cell walls to help cells binding together [5]. It has been shown that pectins have inhibitory effects on cancer cell growth and metastasis [6]. It has firstly indicated that the presence of galactoside-specific binding lectins (galectin) on tumor cells is responsible for targeting carbohydrate-binding proteins on surface of tumor cells [7]. Other cancer researchers have found that pectin can suppress several types of cancers including breast, prostate, colon and lung [6, 8, 9]. Although the usage of pectins in cancer therapy is being to be appreciated, the mechanism of induction

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of apoptosis by pectin is not understood. It seems that the different parameters such as the structure and the pectin modification by heat or pH affect pectin-induced apoptosis [10].

In this study, we examined the effect of apple pectin (pectic acid [PA]) on rat pituitary tumor cells, GH3/B6. Indeed, it has been investigated whether PA in its intact form, as well as modified pectins, inhibits pituitary tumor cell proliferation and induces apoptosis.

MATERIALS AND METHODS

Cell culture. The somatomammotroph cell line, GH3/B6 cells, was grown as a monolayer in Ham's F12 medium (Gibco, USA) supplemented with 2.5% fetal bovine serum (Hi media, India) and 15% horse serum (Hi media, India) in a humidified 37°C incubator with a controlled 5% CO_2 atmosphere. Cultures were divided after forming a monolayer by harvesting and diluting to a concentration of 5×10^5 cells/ml. Cell counts were assessed with a hemacytometer and cell membrane integrity was determined using Trypan blue exclusion technique. The effect of PA (Fluka, USA) on apoptosis and cell cycle was evaluated either in the cells cultured in complete medium in the absence or presence of different doses of PA (100, 250, 500, 750 µg/ml and also 1, 2.5, 5 mg/ml). Bromocriptine (35 µM, Sigma Aldrich, USA) was used as the positive control [3]. Assessment of apoptosis by triphosphate nick-end labeling (TUNEL) assay or DNA fragmentation was done after 24 h of culture.

Cell viability. The effects of PA on cell proliferation were measured using MTT-based assay [11]. Briefly, the cells were incubated in triplicate in a 96-well plate in the presence of various concentrations of PA in a final volume of 200 µl for the indicated times. Thereafter, 20 µl of MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Aldrich, USA) solution (5 mg/mL) was added to each well and then incubated for 2 h and the supernatant was removed from each well. The colored formazan crystals which were produced from MTT were dissolved in DMSO (Sigma, USA) and then the OD value was measured at 570 nm by a multi-well spectrophotometer (Rayto, China).

Acridine orange (AO) and ethidium bromide (EB) double staining. DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells [12]. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double-stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different concentrations of PA for 24 h, the cells were detached, washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope (Zeiss, Germany) at 400 × magnifications. The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei). In each experiment, more than 300 cells/sample were counted.

Propidium iodide (PI) uptake. PI (sigma, USA) uptake is a permeability assay which identifies membrane damage [13]. The incubated cells were harvested and washed once with PBS and received PI (3 µg/ml) followed by flow cytometry analysis to determine the population positive for PI staining. The cells that displayed a high permeability to PI were necrotic one. A minimum of 1×10^4 cells were analyzed in each sample.

TUNEL assay. Apoptotic studies were performed using TdT-mediated deoxyuridine TUNEL method [3]. Cells (5×10^5) were seeded using 12-well multidishes for 72 h in order to treat with different concentrations of PA as mentioned above. After 24 h, the cells were fixed in 4% paraformaldehyde in PBS at room temperature for 60 min and then incubated in a permeablizing solution (0.1% Triton X-100) at 4°C for 2 min. TUNEL assay was performed using the *in situ* cell death detection kit (Roche Diagnostics, Germany) according to the manufacture's protocol, and flow cytometry was performed to determine the apoptotic cells. For each analysis, 1×10^4 events were recorded.

Cell cycle analysis. GH3/B6 cells were grown in the absence or presence of PA for 24 h. Afterward, they were trypsinized, washed with PBS and fixed with cold ethanol at 4°C. After 2 h, the cells were washed and resuspended in PBS containing 50 µg/ml of RNase A and PI solution (Sigma, USA). Cell cycle distribution was analyzed by a flow

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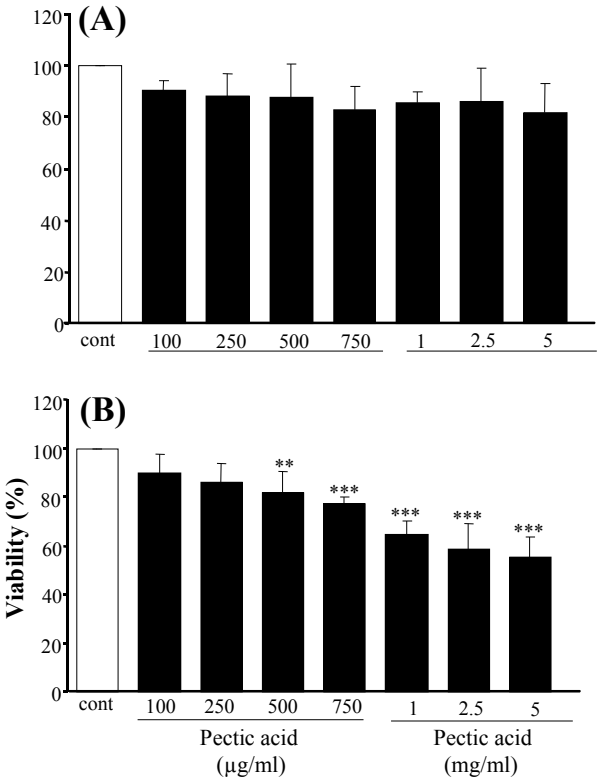

Fig. 1. Effect of PA on GH3/B6 cells viability. MTT assay were done **(A)** after 6 h and **(B)** 24 h treatment of PA. Results are expressed as mean ± SEM (n = 4), $^{**}P<0.01$ and $^{***}P<0.001$.

cytometer (FACS Calibur, USA) and the data were analyzed by WinMID software [14]. A minimum of 10^4 cells in each sample was analyzed to determine the percentage of cells in each phase of the cell cycle and three independent experiments were performed.

DNA fragmentation: analysis by agarose gel electrophoresis. This procedure is based on internucleosomal DNA cleavage, a characteristic biochemical hallmark of the apoptotic mode of cell death [15]. When DNA extracted from apoptotic cells is subjected to gel electrophoresis, a typical internucleosomal ‘‘ladder’’ of DNA fragments is produced. Briefly, cellular DNA was extracted using Genomic DNA kit (Fermentas, USA). The DNA samples were analyzed on 1% agarose gel (Merck, Germany) containing 0.2 µg/ml EB at 80 V for 120 min. The DNA was visualized using 312 nm UV radiation.

Statistical analysis. The data are expressed as means ± S.E.M. The statistical analyses were performed using one-way analysis of variance (ANOVA). Following a significant *P* value, post-hoc analysis (Tukey) was performed for assessing specific group comparisons. $P<0.05$ was considered statistically significant.

RESULTS

Viability reduction in PA-treated cells. GH3/B6 cells were treated with different concentrations of PA (100, 250, 500, 750 µg/ml and 1, 2.5, 5 mg/ml) and 35 µm bromocriptine. The viability was assessed by using the MTT assay. As shown in Figure 1, PA has no significant effect on GH3/B6 cell viability in 6 h [$F(7, 24) = 1.6$, $P>0.05$], while PA treatment for 24 h reduced cell viability in a dose-dependent manner [$F(7, 24) = 25.31$, $P<0.0001$].

Morphological changes by optical microscope. After treatment with PA for 24 h, the cells shrank and developed bubble-like blebs on the membrane (Fig. 2). The apoptotic cells were broken into smaller pieces which are named apoptotic bodies. These morphological features were obviously seen in the high doses of PA (1, 2.5 and 5 mg/ml).

Apoptosis and necrosis evaluation by fluorescent double staining. To assess the type of cell death induced by PA in GH3/B6 cells, the morphological changes after double staining cells with AO and EB were investigated. These dyes emit different shades of fluorescence and possess a different ability to penetrate cells. AO penetrates into living cells, emitting green fluorescence after intercalation into DNA. The second dye, EB emits red fluorescence in the cells with an altered cell membrane. Morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, alterations in the size and the shape of cells, as revealed by fluorescence microscopic analysis, were observed predominantly after PA treatment in 24 h. As shown in Figures 3 and 4A, PA treatment in the different concentrations (100, 250, 500 and 750 µg/ml) has no effect on necrosis, while the maximum percentage of necrotic cells was observed in 5 mg/ml concentration of PA (27.47 ± 1.49%). The maximum increase in the number of apoptotic cells (about 53.7 ± 1.82%) was observed in 1 mg/ml of PA (Fig. 4B) which was significantly more than the number of apoptotic cells induced with bromocriptine [$F(8, 27) = 36.15$, $P<0.0001$].

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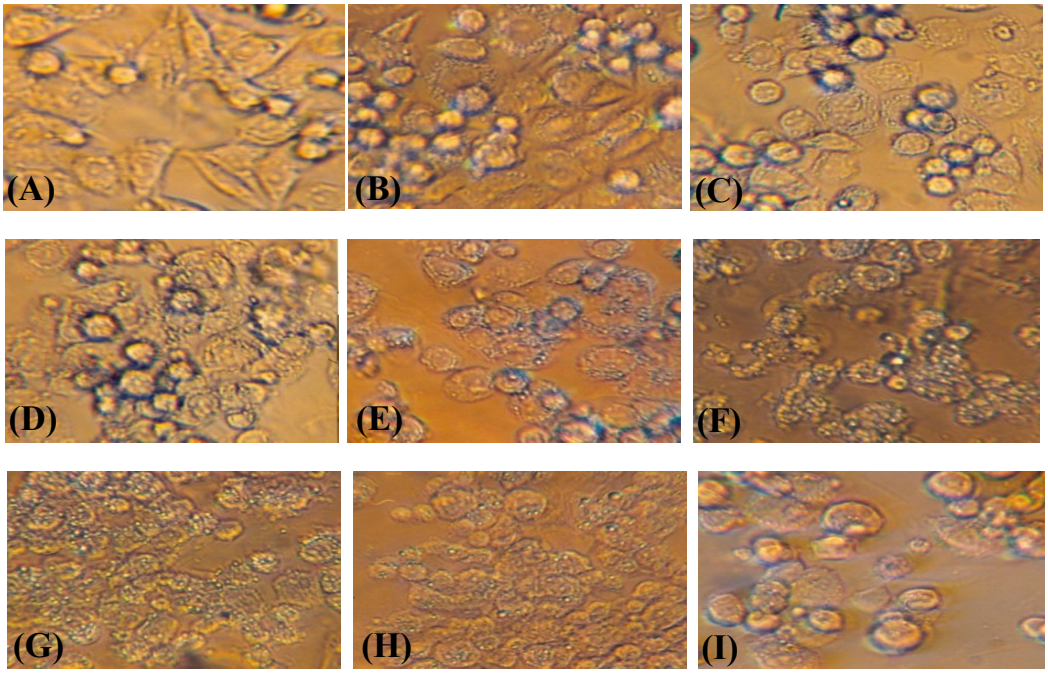

Fig. 2. Phase contrast microscopy image of GH3/B6 cells after 24 h. **(A)** Control, **(B)** 100 µg/ml PA, **(C)** 250 µg/ml, **(D)** 500 µg/ml, **(E)** 750 µg/ml, **(F)** 1mg/ml, **(G)** 2.5 mg/ml, **(H)** 5 mg/ml and **(I)** bromocriptine 35 µM.

Necrosis assay by PI uptake. PI is a fluorescent nucleic acid dye which enters into the cells with the low membrane integrity. Loss of membrane integrity is a main characteristic of necrotic process. As demonstrated in Figure 5, PI uptake increased in 5 mg/ml of PA (37.9%) which was significant in comparison with the control group.

Apoptosis detection with TUNEL assay. To confirm whether the PA-induced cell death is apoptosis, apoptotic cells were assessed by measurement of DNA fragmentation using TUNEL staining. The fragmented nuclei were labeled with TUNEL signal, confirming the apoptotic nature of the cell death (Fig. 6). Under basal conditions, a few cells were TUNEL positive. Incubation with different concentrations of PA for 24 h significantly increased the number of apoptotic cells in a dose-dependent manner up to 1 mg/ml concentration. The highest percentage of apoptotic cells was observed in 1 mg/ml concentration of PA in comparison to the control group, but the decrease of apoptosis percentage was observed in the highest dose of PA (5 mg/ml) compared to 1 mg/ml of PA.

Cell cycle analysis. After treatment of GH3/B6 cells with 1 mg/ml of PA, the cells were stained with PI, which were then analyzed by flowcytometer to

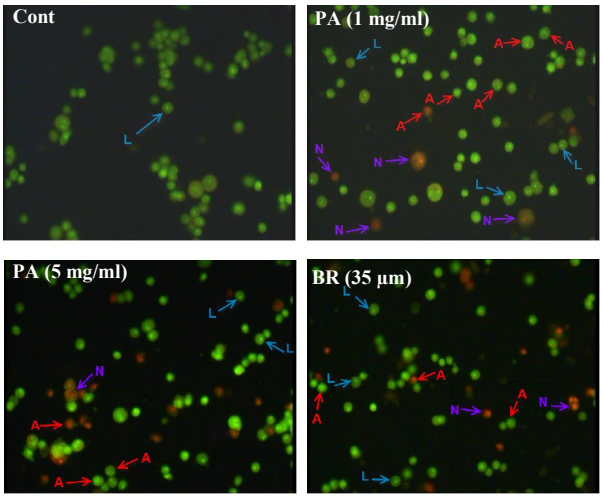

Fig. 3. Morphological changes in GH3/B6 cells after 24 h treatment with PA and bromocriptine .The cells were stained with acridine orange and ethidium bromide. Blue arrows next to "L" point to live cells; Red arrows next to "A" indicate apoptotic cells; and purple arrows next to "N" indicate necrotic cells (magnification at 400×).

determine the effect of PA on the cell-cycle progression. As shown in Figure 7, PA clearly induced subdiploid (sub G1) events which are a hallmark of apoptosis. The percentage of apoptotic cells increased from 3.2% in control group to

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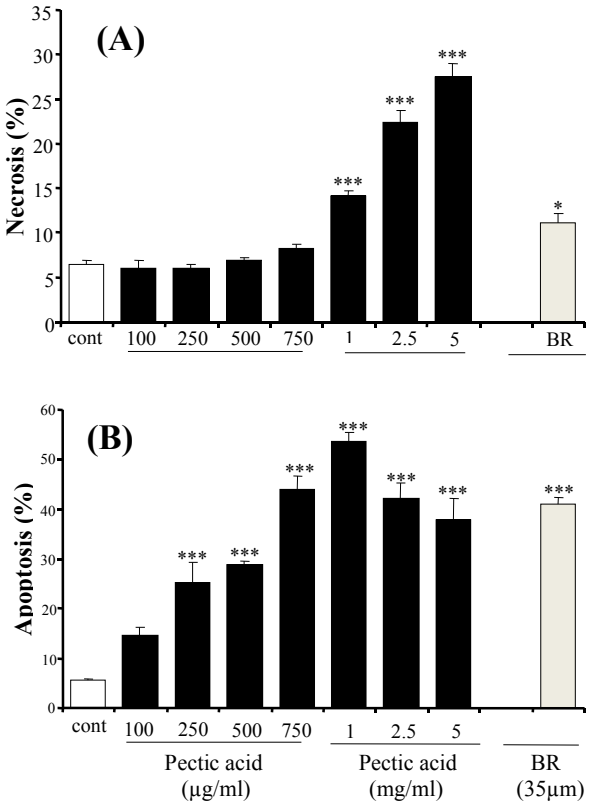

Fig. 4. The percentage of apoptotic **(A)** (early and late) and necrotic **(B)** cells after 24 h incubation with PA and bromocriptine (BR) by AO/EB staining method. The figures show the mean percentages ± SEM from three independent experiments, *P<0.05 and ***P<0.001.

30.22% in 1 mg/ml of PA treatment (P<0.001); however the amount of apoptotic cells in bromocriptine group was 13.77 % (P<0.05).

Internucleosomal DNA fragmentation. To examine whether PA induced-apoptosis was associated with the internucleosomal DNA fragmentation in GH3/B6 cells, the quality of extracted DNA was investigated on the agarose gel. As shown in Figure 8, the smear-like pattern of internucleosomal DNA cleavage was detected. DNA fragmentation was positively correlated with the number of apoptotic cells. Under the same conditions, no DNA fragmentation was found in untreated (control) cells.

DISCUSSION

Pectin, a natural plant polysaccharide, which presents in all higher plant cell walls, fruits and vegetables, is a substance that appears to be able to inhibit cancer metastasis [6, 9, 16]. In most of the previous studies, the modified pectins which were made by pH or temperature alteration have been used in order to investigate the anti-cancer effects of pectin [16-18]. It is important that the inhibitory effects of pectin are mediated through their binding to galectin-3 (a galactoside-binding lectin) [19]. Galectins are specific carbohydrate binding proteins present on the surface of cancer cells and contain a conserved sequence of the carbohydrate binding domain, which have an affinity to β-galactosides [20, 21]. In the present study, the role of PA as a potential inducer of apoptosis has been investigated in GH3/B6 cells.

Our data showed that the administration of PA dose-dependently decreased the viability of GH3/B6 cells in 24 h treatment, but not in 6 h treatment. The results also indicated that PA inhibited the proliferation of GH3/B6 cells as determined with the MTT assay. Therefore, there is a correlation between cell proliferation and the viability measured by the MTT assay [22]. Furthermore, morphological

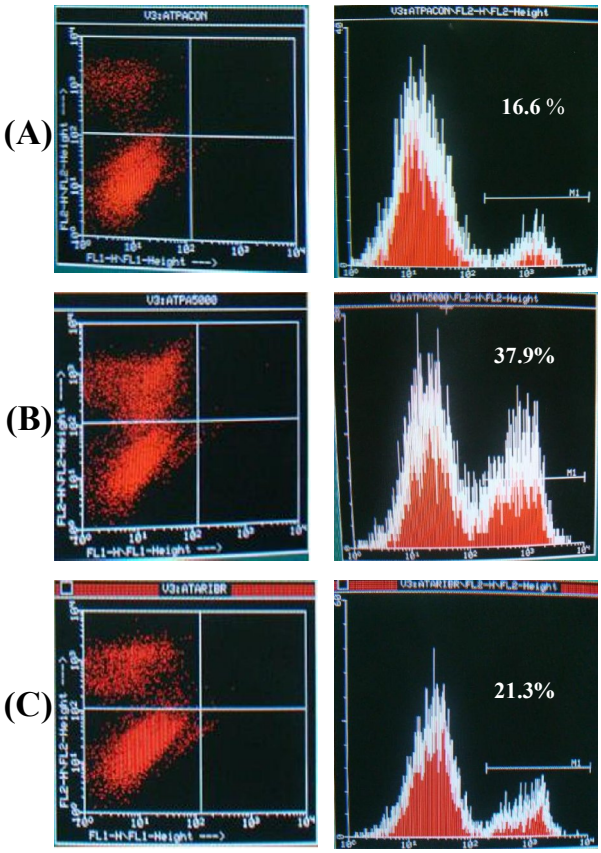

Fig. 5. Induction of necrosis in GH3/B6 cells by PA and bromocriptine using PI staining and flow cytometry method. The left peak of each diagram shows the viable cells and the right peak shows the necrotic cells. **(A)** Control, **(B)** 5 mg/ml PA and **(C)** 35 µM bromocriptine.

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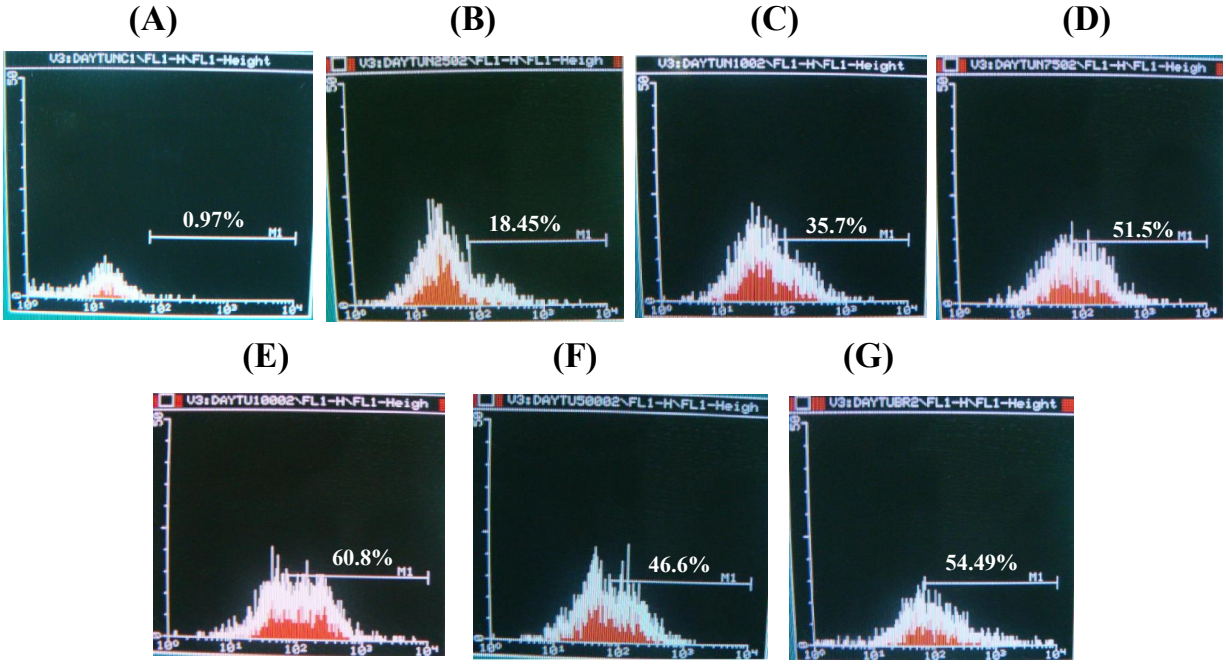

Fig. 6. Detection of apoptosis with TUNEL assay. The left side of each diagram shows the viable cells and the right side shows the apoptotic cells. GH3/B6 cells were treated **(A)** without (control), or with PA, **(B)** 100 µg/ml, **(C)** 250 µg/ml, **(D)** 750 µg/ml, **(E)** 1 mg/ml, **(F)** 5 mg/ml and **(G)** 35 µm bromocriptine.

analysis of GH3/B6 cells treated with PA revealed cell shrinkage and loss of membrane integrity, a phenotype that is commonly seen in cells undergoing apoptosis.

On the other hand, the influence of PA on GH3/B6 cell death mode was studied by AO/EB double staining in the present experiments. It should be considered that healthy cells have green nuclei and uniformly chromatin with intact cell membrane. Also, the cells undergoing apoptosis have orange or green nuclei with condensed chromatin and the necrotic cells have red nuclei with damaged cell membrane [23]. As shown in Figure 3, most cells treated with 1 mg/ml of PA were apoptotic with green or orange fragmented nuclei (53.7 ± 1.82%), which were also in accordance with relatively low cell viability. The data suggested that PA could induce cell death through apoptosis. Recently, some studies have proved the ability of pectin in inducing of apoptosis. It has been shown that the dietary use of pectin enhances colonocyte apoptosis in a rat model of experimentally induced colon cancer [24]. Similarly, pectin and pectic oligosaccharides treatment in HT-29 colon adenocarcinoma cells caused a significant increase in caspase 3 activity and apoptotic rate [15]. Moreover, it is reported that GCS-100 (modified citrus pectin [MCP]) induced apoptosis in various multiple myeloma cell lines including chemo-resistant ones [11]. It has also been suggested that pectin may be able to induce apoptosis in androgen-responsive (LNCaP) and androgen independent (LNCaP C4-2) human prostate cancer cells [10].

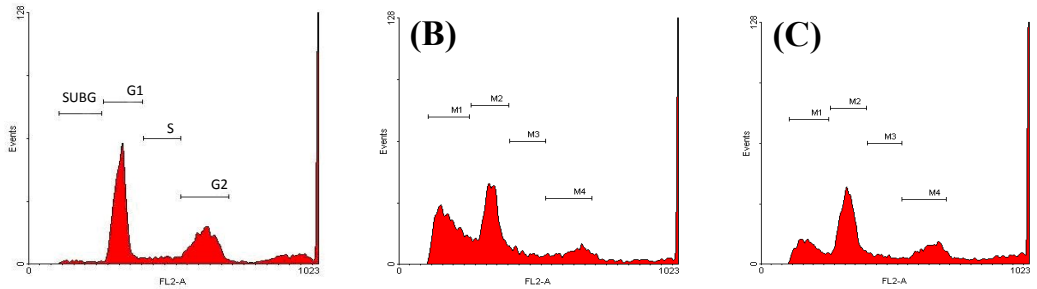

Fig. 7. The histograms showing the effect of PA on the cell cycle of GH3/B6 cells with flow cytometry analysis of **(A)** control cells, **(B)** 1 mg/ml PA and **(C)** 35 µm bromocriptine.

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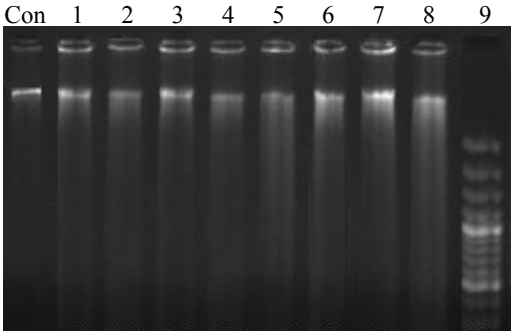

Fig. 8. Internucleosomal DNA fragmentation was observed in PA treatments. Con, control group; lane 1, 100 µg/ml PA; lane 2, 250 µg/ml PA; lane 3, 500 µg/ml PA; lane 4, 750 µg/ml PA; lane 5, 1 mg/ml PA; lane 6, 2.5 mg/ml PA; lane 7, 5 mg/ml PA; lane 8, 35 µm bromocriptine; lane 9, molecular size marker.

To investigate whether PA induced apoptosis in pituitary tumor cells, TUNEL and PI staining to quantitate apoptosis were used in the present experiments. The fluorescent microscopic findings showed that PA (up to 1 mg/ml) had a significant effect on apoptosis (Fig. 6). In addition, DNA fragmentation strongly indicated that the observed cell death in response to PA was apoptotic in nature, agreeing with published data showing MCP treatment of human prostatic JCA-1 cells reduced cell growth and DNA synthesis [25]. It is important to note that DNA degradation is a relatively late event in apoptotic cascade [26], which can not be applied to all apoptotic conditions [27].

Our results showed that the rate of apoptosis percentage decreased in higher concentrations of PA (2.5 and 5 mg/ml) in comparison to 1 mg/ml density (Fig. 4B). It seems that the reduction of apoptosis rate in 5 mg/ml and 2.5 mg/ml groups of PA was due to increasing of necrosis amount (Fig. 4A). The necrotic results were confirmed with PI staining (Fig. 5). The rate of bromocryptine (35 µm)-induced apoptosis was about 41.47 ± 1.33%, while 1 mg/ml of PA had greater apoptotic effect on GH3/B6 cells compared with bromocryptine which is an inducer of apoptosis in these cells. Finally, the treatment of GH3/B6 cells with PA increased the percentage of cells in sub G_1 phase, indicating apoptotic induction.

In conclusion, our results showed that the intact form of apple pectin (PA) has the apoptotic effects on lactotroph pituitary tumor cell line, GH3/B6. Furthermore, the administration of bromocriptine or PA had the same apoptotic effects on GH3/B6 cells. It should be noted that PA-induced apoptosis was higher than bromocriptine. It is noteworthy that the anti-metastic effects of pectins occurred in the absence of cell toxicity [19] suggest that PA can be used as an appropriate substitute for chemical drugs such as bromocriptine.

ACKNOWLEDGMENTS

This study was supported by a research grant from the College of Sciences, University of Tehran (Iran). We would like to express thanks to Dr. D. Gourdji for her gift of GH3/B6 cells and also wish to acknowledge the useful advice of Dr. B. Goliaei and Mrs. K. Siamaki. The authors would like to express thanks to Dr. A. Rezoyf for her skillful assistance.

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[DOR: 20.1001.1.1028852.2009.13.4.7.3]

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