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SEA CUCUMBER ENHANCES APOPTOTIC ACTIVITY OF LOVASTATIN

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ABSTRACT

Apoptosis or programmed cell death is an essential process for elimination of damaged cells. Induction of apoptosis in cancer cell has a pivotal role in cancer therapy. The screening for agents that induce apoptosis in tumor cells help in the development of novel agents for cancer treatment. Numerous studies suggest that the exposure of tumor cells to sea cucumber extract can lead to apoptosis. Sea cucumber consumes widely in Asia and comprises potent antioxidant and anticancer properties. The aim of this study was to determining the apoptosis induced by sea cucumber methanolic extract with or without lovastatin in human colon tumor cells by using neutral comet assay. Severe fragmentation of DNA during apoptosis can be readily measured by neutral comet assay. HT29 cells were grown in DMEM medium then exposed to different concentrations of sea cucumber extract at 24 and 48h. The results showed that at 24 hours, for all of concentrations used, there were no significant differences with the control group. At 48 hours exposure, concentrations of 100,200, and 400µg/ml demonstrated significant difference with control group ($P<0.05$). When HT29 cells were exposed to both sea cucumber (200 µg/ml) and lovastatin, apoptosis was detected higher as compared to sea cucumber (200 µg/ml). The results showed that methanolic extract of sea cucumber time-dependently induced apoptosis in human colon tumor cells. Effective concentration of sea cucumber when combined with lovastatin induced considerable apoptosis relatively at shorter incubation time.

Keywords: Apoptosis, Persian gulf, Comet assay, Sea cucumber.

INTRODUCTION

Cancer has become one of the leading causes of human death in the last 20 year [1] According to the American Cancer Society, total cancer deaths were 7.6 million people in 2007 [2]. Colon cancer is the third most common cause of cancer related death in both genders in the United States. Besides inherited predisposition, colon cancer developed through different combinations of endogenous and exogenous carcinogens. Modifying colon cancer risk factors such as food, individual's lifestyle, genetic and environmental factors can be helpful in improving this condition [3]. Dietary factors, the intestinal flora (bacteria), and endogenously produced metabolites contribute to the production of free radicals, which are involved in the colon cancer development [4]. One of the contributing factors with cancer development is impairment of apoptosis control pathways, besides abnormal cell proliferation. Apoptosis or programmed cell death is a primary mechanism that control cell death [5-6] and any impairment of its function or regulation could leads to various pathological conditions including Alzheimer, cancer, and Parkinson's disease [[7]. To date, studies have shown that there are two main apoptotic pathways: the extrinsic, extracellular pathway, or cytoplasmic pathway that begins with death receptors (Fas), a subset of a large family of tissue necrosis receptors of TNFα [8] and intrinsic or mitochondrial pathway which release cytochrome c from the mitochondria.

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Bcl2 and Bax proteins are involved in the apoptosis pathway, and inhibit or activate caspases, respectively. The bax/bcl2 ratio is known as determining factor for cell survival and death. In a study conducted on human pancreatic cancer cells, extract of sea cucumber frondoside A was led to reduction of Bcl2 protein expression, increase in expression of Bax protein; activation of 3, 6, 9 caspases, reduction of P21, and apoptosis induction through mitochondrial pathways [9-10]. Bax is an apoptotic protein that increase mitochondrial membrane permeability, release of cytochrome c from mitochondria, activation of caspases, and increased induction of apoptosis. There are several medications and factors that potentially can induce apoptosis in cancer cells, which upon identification improve cancer treatment [5].

Holothuria leucospilota or black sea cucumber belongs to Aspidochirotida order and class of *Holothuriidae* that is a common species in the Persian Gulf and Southwest Asia. Sea cucumbers have worm like cylindrical shape, soft bodied and leathery skin [11-13]. Sea cucumber is an important source of natural antioxidants in diet , which typically play an important role against the harmful effect of free radical produced mostly by metabolism. As an economically valuable aquatic, sea cucumber use widely in food, pharmaceuticals and medical industries and extrinsic factors [14]. Sea cucumbers have cytotoxic [15] antioxidant and hepatoprotective [16-17] anti-virus, bacterial, and fungi [18-19] anti-tumor [20] anti-inflammatory, and anticancer properties [21]. Sea cucumber has many applications in the pharmaceutical industry [11]. In this study we tested whether lovastatin would strengthen the apoptotic properties of sea cucumber extract in colon carcinoma cell line, HT29.

MATERIALS AND METHODS

Colon cancer HT29 cell was purchased from Pasteur institute of Iran, lovastatin obtained from Biocon, Fetal bovin serum (FBS) from Gibco, Anisomycin from Sigma and DMEM from Applichem.

Preparation of sea cucumber extract

The sea cucumbers were cutout and the abdominal contents were removed, then the muscular tissues were cut into small slices and were homogenized by a mixer. The tissue was put in a sterile container containing 96% methanol at room temperature for a week at the ambient temperature, and then filtered by a filter paper. The methanolic crude extract was evaporated using rotary device under vacuum conditions and 45°Ctemperature. Then methanolic extract was freeze-dried and the resulting powder was stored at -20 ° [19].

Cell culture

HT29 (obtained from Pasteur Institute of Iran) were maintain in a 95% air, 10% FBS ,5% CO2 incubator in the DMEM medium supplemented with 100 µg/ml streptomycin and 100U/ml penicillin. For assay, the cells were removed by trypsinization, and washed with PBS [5, 22].

Cell Viability

In first cell viability was determined by using the trypan blue exclusion assay. Viable cells (cells that trypan blue are not impermeable) and nonviable cells (stained cells) were counted using a hemocytometer and cell viability was calculated [5].

Lovastatin Treatment

To activate lactone form of Lovastatin, the powder was dissolved in ethanol and NaOH at 50˚C for 2 hours. HT29 cells (cells/ml) were exposed to different concentration of lovastatin (10, 20, 40,100 µM) for 48 and 72 hours in 6-well plates (data not shown).

Sea cucumber extraction treatment

HT29 cells (cells/ml) were exposed to different concentration of sea cucumber extraction (0, 50, 100, 200, 400µM) and (200 µM sea cucumber extraction with 100 µM lovastatin) for 24 and 48 hours. As positive controls, cells were treated with anisomycin (2 µg/ml) for 2 hours.

Neutral Comet Assay

Neutral comet assay method was used to evaluate DNA fragmentation associated with apoptosis. In order to prepare slide, HT29 cells at a concentration of 10^6 cells/ml were mixed with 1% low temperature melting agarose (LMPA) in PBS (phosphate buffered saline) at a ratio of 1:10 (v/v). Slides were placed in precooled lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, 10% DMSO and 1% Triton X-100) at 4˚C for 30 minutes. Electrophoresis was performed for 20 minutes at 25 v and 300 mA. Slides were stained with ethidium bromide solution (20 mg/ml) for 5 minutes and covered with a cover slip. To assess the comet pattern, 50 cells were counted per slide, and comets were scored from 0 to 3 based on the head size and tail length. The apoptotic cells were classify by scoring them as 2 or 3. Comet of score 0 and 1 reflected intact cells (figure1) [5].

Statistical Analysis

Statistical analysis was performed using the non-parametric chi-squared test to compare groups of data.

Results

The HT29 cell line was cultured in DMEM medium. As a positive control, 1 ml of cultured medium containing 10^6 cells were exposed to Anisomycin (2 µg/ml) and incubated for 2 h at 37 °C. There were 58% apoptotic cells in cultures exposed to Anisomycin (fig 1 and 2).

Cells were exposed to different concentrations of methanolic extract of sea cucumber (0, 50, 100,200,400µg/ml) and the combination of sea cucumber

extract (200 µg/ml) and 100 µg/ml lovastatin. In our previous work the proper apoptotic effects has been demonstrated at 24 and 48 hours time interval for lovastatin [5]. HT29 cells were incubated for 24 h (figure 2) and 48 h (figure3) at 37 °C. The amount of apoptotic cells at mentioned concentrations of sea cucumber were 2%, 8%, 16%, 22%, 18% respectively in 24 hours of incubation and 2%, 50%, 52%, 60%, 86% in 48 hours incubation. At the next step,1 ml of cultured medium were exposed to 200 µg/ml of sea cucumber extract and 100 µg/ml of lovastatin. Then, they were incubated at 37 °C for 24 and 48 hours. Percentage of apoptotic cells was 40% at 24 hours and 42% at 48 hours incubation.

Fig 1. Comet pattern scores: score 0: A, score 1: B, score 2: C, score 3: D

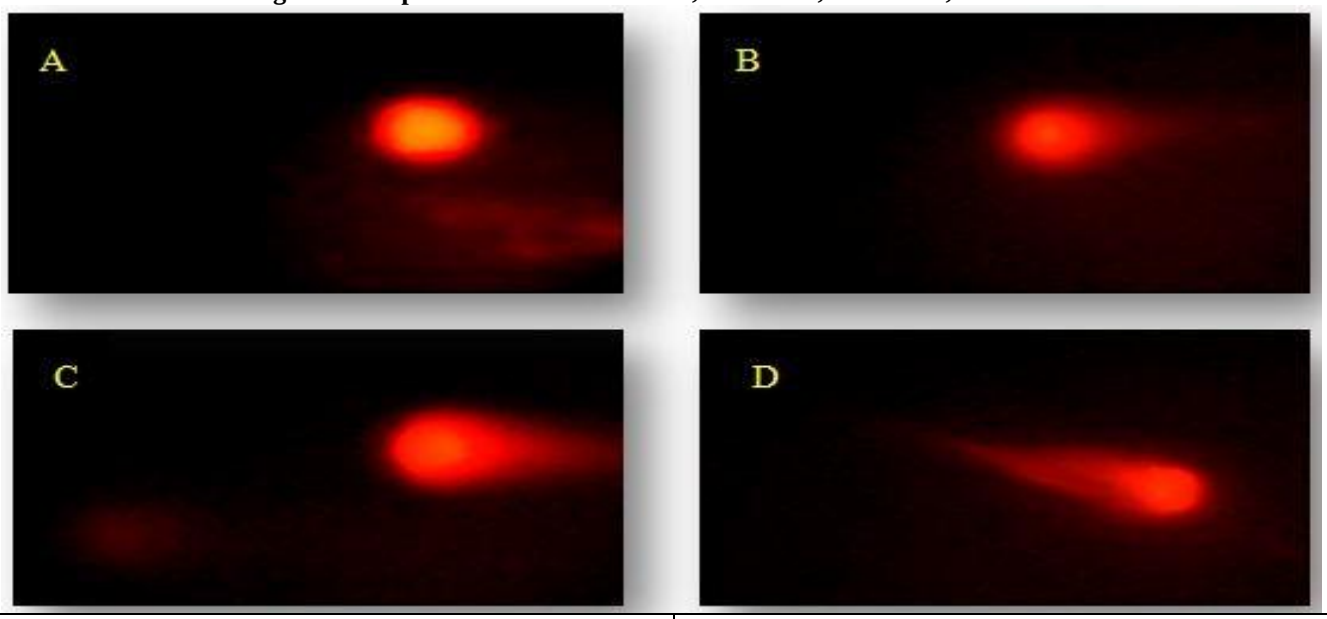
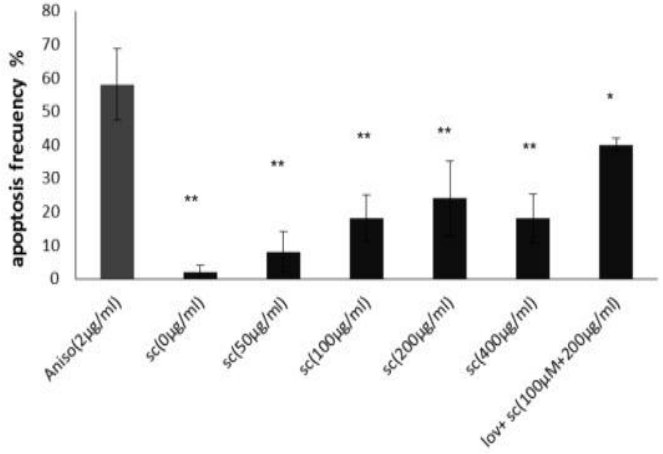
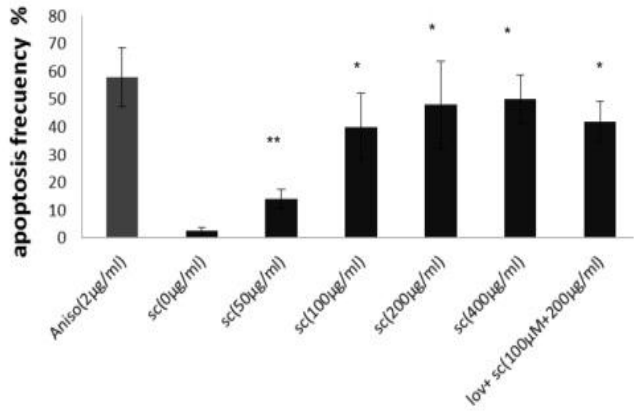


Fig 2. Rate of apoptotic cells in HT29 carcinoma cell line exposed to different concentration of sea ucumber extract for 24 hour. Aniso: anisomycin, sc: sea cucumber, lov: lovastatin



Significant difference as compared to the control (P<0.05)*
Significant difference as compared to the anisomycin (P<0.05) **

Fig 3. Rate of apoptotic cells in HT29 carcinoma cell line exposed to different concentration of sea cucumber extract for 48 hour. Aniso: anisomycin, sc: sea cucumber, lov: lovastatin



Significant difference as compared to the control (P<0.05)*
**Significant difference as compared to the anisomycin (P<0.05)

DISCUSSION

Cancer arises from a change in the nature of normal cells. By altering the nature of normal cells, their growth rate became out of control, self-governing and continuously without normal interruption [6, 23]. Dietary factors, the intestinal flora (bacteria), and endogenously produced metabolites contribute to the production of free radicals, which stated to be involved in the colon cancer

development. The food antioxidants reduce the amount of these harmful oxidative products [4]. Apoptosis is physiologic and programmed cell death, which would remove damaged and harmful cells in normal states. Any functional or regulating disturbances of this process lead to various pathological condition and development of numerous diseases [24, 7]. The results of current study (Figure2 and 3) showed that 2 µg/ml anisomycin could induces apoptosis with rate of 58%. Anisomycin is a protein synthesis inhibitor. Studies have shown that anisomycin induces apoptosis through increase in caspase 3 activation. Possible mechanisms of anisomycin-induced apoptosis is penetration of anisomycin through the cytoplasmic membrane and influence on the S28 subunit of the ribosomal RNA [25].

Our results showed (figure 2and 3) that methanolic extract of sea cucumber with 200µg/ml could induced cell death at 24 and 48 h incubation with rate of 22% and 48%,respectively; which indicated time-dependent manner in apoptosis induction. In a study conducted by Petter Collin on cucumaria frandosa, Frondanol A5 extract have reduced phosphorylation of P38 kinase and induced apoptosis in pancreatic cancer cells line. In another study by Tatsuya the sphingiod compounds that extracted from sea cucumber led to increased caspases activity and apoptosis induction, and also were cytotoxic in human cancerous cells (Sugawara et al, 2006). Studies showed that the extract of Japonicum sea cucumber has potent tumor suppressor effect on lung, liver, and stomach cancer cells [26]. The results of our study (Figure 2 and 3) have shown that the effective concentration of sea cucumber extract and lovastatin increased apoptosis (40%) after 24 hours of exposure. Lovastatin has increased apoptotic effects of sea cucumber extract. In a study have been conducted on the lymphoblast, the role of statins in induction of apoptosis through the mitochondrial pathway was investigated. The results showed that statins has cytotoxic effects on lymphocytes B, T and myeloma cell through regulation of HMG-coA enzyme, and inhibition of Farnesylpyrophosphate and Geranylpyrophosphate; these protein alterations involved in the regulation of cellular signals led to the induction of apoptosis [27] as well, statins induce apoptosis through the mitochondrial pathway in the muscle cells and thus cause myopathy [28-29] and lovastatin enhances apoptosis in colon cancer cells [5]. In a study conducted by Jim Dimitroulakos, the effects of lovastatin on different tumor cell lines have been examined.Results have shown the induction of apoptosis by lovastatin in these cell lines [30]. As our results showed, lovastatin enhanced apoptotic effect of sea cucumber extract in 24 hours. Some other mechanism for sea cucumber activity could be involved in this additive pattern of action. In this regard, mice ear inflammation model showed that sea cucumber has anti-inflammatory effects through inhibition of COX-1, COX-2 and lipooxygenase; and has antiangiogenic effects through blocking prostaglandin EP-1 receptors. Therefore, it can be postulated that sea cucumber extract treatment leads to inhibition of COX-2 [31-32], activation of caspases and induction of apoptosis in different mechanism compare to lovastatin; In conclusion it seems that the induction of apoptosis triggered by different mechanisms would be more helpful particularly for reducing the incubation period.

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