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Isolating Melittin from Bee Venom and Evaluating its Effect on Proliferation of Gastric Cancer Cells

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ABSTRACT

Background: Gastric cancer (GC) is one of the most common cancers worldwide and in Iran. As conventional therapies such as surgery and chemotherapy are invasive with adverse effects, current studies are important as they are conducted to find natural compounds with few adverse effects. In this study, melittin with 26 amino acids was isolated and purified from bee venom and its effect on the viability and proliferation of gastric cancer cells was investigated.

Methods: At first, melittin was purified from honeybee venom by a reversed-phase high performance liquid chromatography (RP- HPLC) and using C18 column. The biologic activity of melittin was evaluated by hemolytic test on red blood cells to melittin standard. To investigate the effect of melittin on viability and proliferation of AGS human gastric adenocarcinoma cells, the related cells were cultured in a 96-well plate and treated with serially diluted concentrations of melittin for 6 and 12 hours and their mortality was determined by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method at 540 nm wavelengths.

Results: The obtained chromatogram from RP-HPLC showed that melittin comprised 50% of the studied bee venom. SDS-PAGE analysis of melittin fraction confirmed purity of isolated melittin. Hemolytic assay showed that the extracted melittin indicates a strong hemolytic activity (HD50=0.55µg/ml). MTT assay showed that melittin strongly inhibits proliferation of gastric cancer cells at concentrations more than 2µg/ml. This inhibitory effect depends on melittin concentration and time.

Conclusion: The results of this study showed that melittin is a strong inhibitor of proliferation of the gastric cancer cells. Also, it was observed that this inhibitory effect is increased with increasing concentrations of melittin and incubation time.

Keywords: Melittin, Gastric Cancer, Reverse-Phase Chromatography.

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Introduction

In traditional eastern medicine, honey bee venom has been always used as a drug in the treatment of various diseases such as rheumatoid arthritis and also to reduce muscular pains.[1, 2] It contains 18 different compounds such as enzymes, peptides and biological amines. The major peptides in bee venom are melittin, apamin, adolapine and mast cell degranulating (MCD) peptide and enzymes such as phospholipase A2. Also of biologically active amines of honey bee venom, histamine and epinephrine are named.[3,4] Melittin is a peptide of 26 amino acid, cationic and amphipathic and is considered as one of the main ingredients in honey bee venom. 5 Melittin forms 40% to 50% dry weight of bee venom and the amount of its secretion in the venom varies depending on the feeding and bee species. Of 26 amino acids in melittin, 20 amino acids at the end of peptide are mainly hydrophobic while amino acids at the carboxyl-terminal (amino acids 21 to 26) are more hydrophilic. This amphipathic structure gives melittin the possibility to react to phospholipid membranes and destroy those.[5,6]

So far, various properties have been reported for melittin such as antibacterial, antiviral, anti-inflammatory and anticancer effects.[7] In recent years, inhibition of ovarian, breast, liver and lung cancer cells by honey bee venom and melittin have been reported.[8,9] In addition, studies have shown that the toxic effects of melittin on cancer cells are more than on normal cells of the body. [10] Gastric cancer was the second most lethal cancer in 2000 and the fourth most common cancer worldwide. Each year, 650,000 deaths due to gastric cancer, and 880,000 new cases of the disease are reported. About two-thirds of these cases are related to developing countries.[11] In Iran, gastric cancer is the second most common cancer and is the most common cancer among men and the third common cancer in women.[12] Distribution of the disease is not uniform across the country, so that the most gastric cancer cases are observed in the Northern and Northwestern Provinces and the fewest cases are seen in Central provinces in Iran.[13] Surgery, chemotherapy and radiotherapy are among methods used to treat gastric cancer that in most cases as this disease is diagnosed in advanced stages of the disease the mentioned methods fail and are also invasive methods with many complications.[14]

Given the disadvantages of existing treatment methods, the need to use new compounds and methods, particularly natural compounds to control and treat gastric cancer is felt more than ever, so in this study the effect of melittin isolated from honey bee venom native to Iran was examined on proliferation of gastric cancer cells.

Materials and Methods

In this study, trifluoroacetic acid (TFA), acetonitrile (ACN), MTT, dimethyl sulfoxide (DMSO) and trypan blue were purchased from Sigma Company (Sigma-Aldrich, St. Louis, MO, USA) and materials used in cell culture such as culture medium RPMI 1640, penicillin and streptomycin, FBS and trypsin were purchased from Gibco Company (Gibco-BRL, Paisley, UK). Human gastric adenocarcinoma AGS cells were purchased from the cell bank of Pasteur Institute of Tehran. The current study was performed by following steps in vitro in Venom and Toxin Laboratory, Pasteur Institute of Tehran from summer 2011 to spring 2012.

Honey bee venom was collected by electroshock and was prepared according to Benton Protocol 15. For this purpose, a wired glass plate was placed in front of the hive entrance and the bees that wanted to enter the hive had to land on this glass plate. After sufficient number of bees were on the plate, the wires were electrified with a weak electric current. In response to the weak electric shock, the bees stung the plate. Venom quickly dried on the glass plate in exposure to air and then it was scraped off the plate and transferred to the laboratory.

In this venom collection method no harm is done to bee. The collected venom was dissolved in sterile deionized water and it was prepared for injection into the HPLC device after being filtered with a 0.2 micrometer filter.

Isolation of melittin by RP-HPLC: To separate melittin a reversed-phase high performance liquid chromatography (RP-HPLC) (Knauer, Berlin, Germany) with a pump K-1000, UV detector model 2550, (Knauer, Berlin, Germany), manual injector, 20 microliter loop, column C18 (100 C18, 250 × 4.6 mm, Knauer, Berlin, Germany, Eurosfer) and analysis software Chrom Gate version 3.3.2 were used. The separation was done by creating a gradient between two solutions A and B. Deionized water

containing TFA 0.05% and pure acetonitrile containing TFA 0.05% were selected as solution B and for isolation of melittin linear gradient was applied from 0% to 60% of solution B for 60 min. Column eluate was monitored at 214 nm wavelengths.

Retention time of melittin was determined by using a standard purchased from Sigma Corporation and the fraction was manually collected. After being lyophilized and when the purity was confirmed, the concentration was determined by SDS-PAGE and it was kept in -20 ^{0}C for subsequent experiments.

To evaluate the hemolytic activity of melittin, one of the properties that have been reported so far related to melittin is about the ability of melittin lysis of red blood cells (hemolytic activity) 16. In this study, hemolytic activity test was performed in accordance with AL-Badri protocol 17.

Erythrocytes were isolated from fresh heparinized blood by centrifuge (10 minutes, 3000 rpm) and were rinsed with phosphate buffer solution (PBS). Then precipitated red blood cells (RBC) were diluted in PBS so that 2% RBC suspension was achieved. Suspension prepared was transferred to eight wells of a 96-well plate (100 µl per wall) and they were treated by eight serially diluted concentrations of melittin (8, 4, 2, 1, 0.5, 0.25, 0.125, 0.625 µg/mL). As positive and negative control, the amount of RBC treated with triton X-100 and PBS were used, respectively.

The 96-well plate containing the samples tested and positive and negative controls were incubated at 37 ^{0}C for two hours and then they were centrifuged (10 min, 3000 rpm) and 60 µl of supernatant of each well was transferred to another plate. Optical density (OD) was measured by a micro-plate reader at 450 nm wavelengths (Biotech, EPOCH, USA). The percentage of hemolytic activity of the samples compared to the positive control was calculated from the following equation:

Percentage of hemolytic = [(ODnegative control – ODsample)/(ODnegative control – ODpositive control)×]100

This experiment was repeated three times and data are expressed as Mean ± SD.

Cell culture: Human Gastric Adenocarcinoma Cells (AGS) was cultured in culture medium RPMI 1640 enriched with 10% fetal bovine serum (FBS), penicillin 10000 units per ml and streptomycin 10 mg per ml in incubator 37^{0}C with CO2 5%. After proper cell growth, cells attached to the bottom of the cell culture flask were separated by a solution containing 0.25 trypsin and EDTA 0.1% and were counted with stained trypan blue.

Evaluation of melittin effect on proliferation of AGS cells by MTT: To perform MTT test, AGS cells with a population of 4 × 104 cells in each well were transferred to 96-well plates for 24 hours in the incubator CO2 and were kept at 37^{0}C. Then, the cells were treated with various concentrations (8, 4, 2, 1, 0.5, 0.25 µg/mL) of melittin for 6 and 12 hours.

Four hours before the end of incubation, 20 µl of MTT 2.5 mg/ml solution was added to each well. In this method, salt MTT is reduced by living cells and is converted to a purple precipitate called formazan. The intensity of the produced color is directly related to the number of viable cells.

After incubation (for 6 and 12 hours), plates were centrifuged for 10 minutes at 3000 rpm and the supernatant was discarded. To dissolve colored formazan dye precipitate 100µl DMSO was added to each well and the intensity of absorbed light was measured at 540 nm wavelengths. Untreated cells with melittin were used as negative control. The following formulas were used to calculate the percentage of melittin's inhibitory effect on proliferation of AGS:

A) % Viability= (ODsample/ ODnegative control) × 100

B) % Inhibition= 100- Viability

In this study all experiments were repeated three times and data were expressed as Mean±SD. In all analyses, the significance level was considered $P<0.05$. The graphs were plotted by SigmaPlot Version 11 software.

Results

The results of chromatography: Based on the chromatogram obtained from the analysis of honey bee venom sample used in this study (RP-HPLC, Knauer, Berlin, Germany) over a hundred large and small peaks were observed with column C18 at 214 nm wavelengths. Of this number, about 20 main peaks could be detected in the main chromatogram. Retention time of melittin was determined 42 min by Sigma standard.

As melittin began to elute from the column, it was collected manually. Area under the curve of melittin fraction was about 50% (49.85%) of honey bee venom's total composition that is consistent with previous reports on the amount of melittin in honey bee venom 18,19 (**Figure 1**).

As it is observed in gel electrophoresis, melittin was extracted without impurity. The collected melittin fraction was lyophilized and its purity was confirmed by gel SDS-PAGE 15% (**Figure 2**). After the concentration was determined, melittin was kept at-20^{0}C to be used in subsequent experiments.

Hemolytic activity of melittin: Melittin increases light absorption through the destruction of red blood cell membrane and the release of hemoglobin at 450 nm wavelengths. The increased intensity of light absorption can indicate the level of lytic activity of melittin.

Based on the results of hemolytic test performed in this study, it was observed that melittin has no remarkable activity at very low concentrations, 0.25, 0.125 µg/mL, whereas it has full hemolytic activity at higher concentrations. The concentration of melittin, which causes lysis of 50% of red blood cells compared to positive control (triton X-100) (HD50) was determined equal to 0.5 µg/ml (**Figure 3**).

Inhibition of proliferation of AGS by melittin: The in-

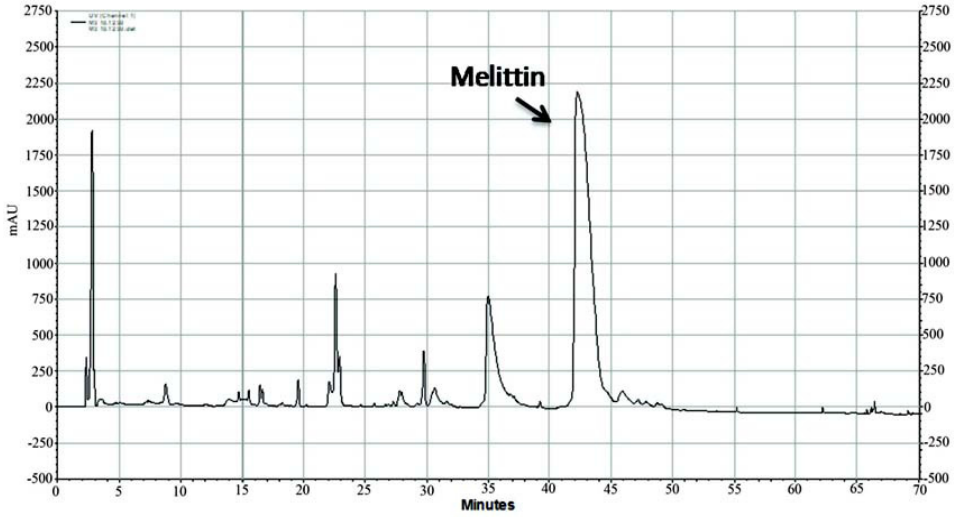

Figure 1: Chromatogram obtained from the analysis of honey bee venom sample native to Iran used in this study by RP-HPLC with column C18 at 214 nm wavelengths

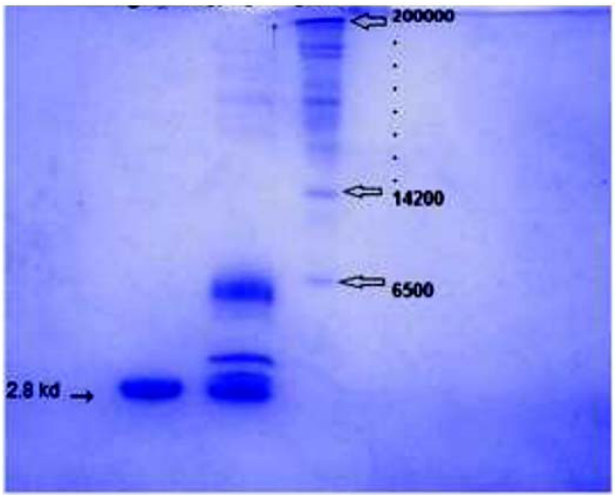

Figure 2: Electrophoretic analysis (SDS-PAGE 15%) of melittin fraction

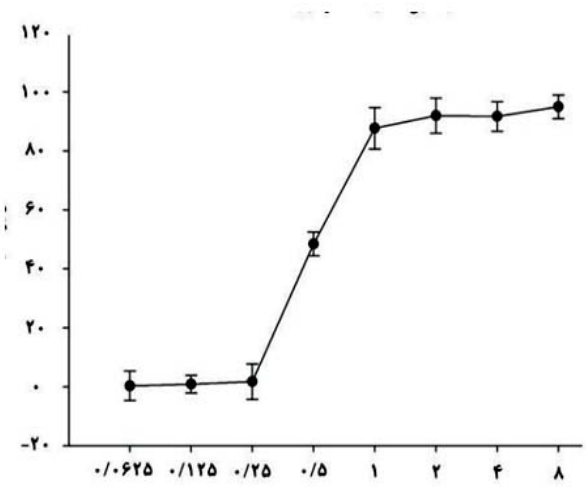

Figure 3: The effect of melittin concentration on its hemolytic activity

hibitory effect of melittin on proliferation of gastric cancer cells (AGS) was determined by MTT method. Based on the results, melittin controlled proliferation of cancer cells. This inhibitory effect is fully evident at both 6 and 12 hours. Concentration of melittin that killed cancer cells by 50% (IC50) is 1.2 µg/ml for 6 hours and 1 µg/ml for 12 hours (**Figure 4**).

The results show that melittin has a strong effect on the proliferation of gastric cancer cells. The inhibitory effect increases with increasing concentrations of melittin and duration of cell treatment.

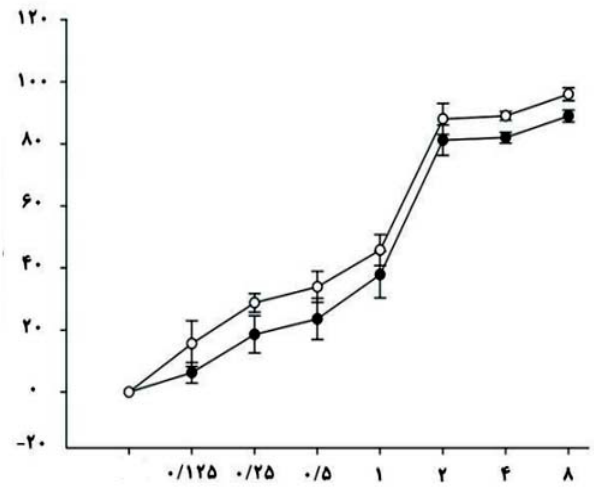

Figure 4: The effect of melittin concentration on growth of gastric cancer cells

Discussion

This study was conducted to examine the melittin effect isolated from honey bee venom on proliferation of gastric cancer cells AGS. Although some earlier reports on inhibition of a variety of cancer cells by honey bee venom or its major component (melittin) have been provided, a report regarding the melittin effect on gastric cancer cells (AGS) has not been observed by the researchers of this study.

The results of the tests in this study clearly indicate that melittin inhibits the proliferation of gastric cancer cells. Gastric cancer is considered as one of the most deadly cancers worldwide. 20, 21 Unsuccessful treatment methods of the disease shows that more studies are needed to introduce more effective drugs and make more efficient treatment methods. 22

So far, the effect of various compounds on different cancer cells has been studied and there are many compounds that their anti-cancer properties have been reported. Among the compounds tested, we can refer to natural ingredients such as some types of plants and edibles. Some of these natural compounds whose anti-cancer properties have been widely studied are honey bee venom and its main compound, melittin.

Traditionally, honey bee venom has been used as a drug in traditional eastern medicine for the treatment of rheumatoid arthritis and joint pain relief. Moreover, many therapeutic effects of honey bee venom on various diseases have been reported in past years. Several properties of melittin have been reported so far including the anti-pain, anti-inflammatory, antimicrobial, hemolytic properties, etc.

Research on honey bee venom and melittin has been noticed for many years. About 60 years ago, Havas investigated the effect of honey bee venom on a type of tumor induced by colchicine. He showed that honey bee venom has toxic effects on tumor cells. In recent years, the anti-cancer properties of melittin has attracted scholars and many reports have been presented in this regard. It has been reported that melittin inhibits cancer cells such as lung and prostate cancers through apoptosis. 23

It seems that induced apoptosis occurs in cancer cells by melittin through caspase-dependent pathway, proteases involved in apoptosis. 24 Most drugs used in chemotherapy induce apoptosis in cancer cells and according to above reports, it seems that melittin will be a suitable option for treatment of cancer. The amphiphilic property of melittin makes this peptide react with phospholipid membranes and destroy them. The destructive effects of melittin are performed by creating channels in membrane surface. 6, 25, 26

Melittin is a very strong compound and if it is used to treat cancer through a variety of methods with fewer complications, there will be a revolution in the human struggle against cancer. The results of evaluation of hemolytic activity test proved that melittin used in this study has hemolytic activity. Based on these results, it can be said that the main obstacle in the treatment of cancer by melittin is its hemolytic activity. Therefore, for designing cancer treatments, it should be noted that melittin will

cause hemolysis if it enters blood stream. Hence, it seems that more appropriate methods to treat cancer by using melittin is gene therapy (melittin gene transfer to cancer cells) or melittin-specific delivery to cancer cells as for liposomes.

Determination of growth inhibition of AGS cells treated with melittin by MTT method showed that melittin has a high toxicity on these cells and the inhibitory effect depends on time and concentration. This dependence means that by increasing the concentration of melittin and duration of cells treatment, cell growth is inhibited more. In cells treated with higher concentrations than 2 µg/ml, more inhibited cell growth (over 80%) was observed which indicates the higher strength of melittin in destruction of gastric cancer cells.

In summary, this research showed that melittin has a strong inhibitory effect on the proliferation of gastric cancer cells and this inhibitory effect is dependent on the concentration of melittin and duration of cell treatment. This study can be considered as a beginning for further studies in line with using melittin to provide more effective methods for gastric cancer treatment.

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