Research Paper: The Effects of Melittin Coding Gene of Bee Venom on *Bcl-2* and *Bax* Genes Expression in ACHN Cells

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ABSTRACT

Introduction: Venom therapy is a traditional procedure for cancer treatment. Various types of natural compositions in bee venom indicate an area of research for therapeutic and anti-cancer purposes. Melittin is a cationic and amphipathic peptide that interferes with lipid-protein molecular interactions in cell membranes leading to cell lysis. Melittin also prevents metastasis in hepatocarcinoma through inhibition of cell migration. This study aimed to investigate the expression of *Bcl-2* and *Bax* genes in ACHN cells transfected with a recombinant vector carrying bee melittin gene by real-time RT-PCR technique.

Methods: In the present study, the recombinant plasmid of pcDNA3.1(+)-melittin was designed. Then, the synthesized gene was cloned into the pcDNA3.1(+) vector. Following plasmid amplification, PCR assay and enzymatic digestion method were used to approve the accuracy of plasmid amplification. ACHN cells were cultured, and sham and control groups were also prepared. RNA was extracted from the treatment and control groups using RNX-Plus solution. cDNA was generated using a specific laboratory kit. A real-time RT-PCR assay was performed to evaluate the expression of *Bcl-2* and *Bax* genes in ACHN cells transfected with recombinant vector. Finally, the obtained data were analyzed using SPSS.

Results: The expression of the *Bax* gene significantly (P=0.03) increased in pcDNA3.1(+)-melittin compared to the pcDNA3.1(+) group. *Bcl-2* gene expression showed a significant decreasing trend in the experimental group of pcDNA3.1(+)-melittin (P=0.001) compared to the pcDNA3.1(+).

Conclusion: According to the obtained results, the expression of *Bax* and *Bcl-2* genes in the ACHN cell line can be affected by the melittin gene of bee venom. Thus, bee venom as a natural substance with its anti-cancer properties can be considered for further experimental investigations.

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1. Introduction

Renal Cell Carcinoma (RCC) is one of the most lethal types of urinary tract cancers, comprising approximately 3% of adult cancers and 90-95% of all primary malignant tumors. RCC is more common in men than women. Although RCC can occur at any age, the maximum age of onset is 50-70 years. Globally, more than 270000 new cases of RCC are diagnosed each year. Also, 116000 patients die because of this disease, which is 2-fold more significant in men than women [1]. One-third of patients with this cancer have metastases, but this tumor initially invades the lungs, bones, liver, brain, and kidneys. Smoking and obesity are the most critical risk factors of RCC [2]. Three classic symptoms of RCC are hematuria, flank pain, and a palpable abdominal mass [3]. Therapies used for RCC include surgery, hormone therapy, immunotherapy, chemotherapy, and biological response modulators. Generally, there are three types of RCC: clear cell (ccRCC), papillary (pRCC), and chromophobic (chRCC) [4]. Hemorrhage and necrosis are commonly found in ccRCC, resulting from mutations in the 3p chromosome and von Hippel-Lindau (*VHL*) gene. This type of RCC has a worse prognosis than pRCC and ccRCC [5].

As a common cellular mechanism in multi-cellular and even single-cellular organisms, apoptosis is one of the main reactions of unwanted cells elimination. Many viruses generate specific products to control this biological process. In response, the immune system hires the pathway of apoptosis against many pathogens, including viruses [6]. Today, the molecular process of apoptosis can be inhibited by applying apoptosis-inducing or apoptosis-inhibitory genes. This method can direct the cell activity to the desired targets of cellular and humoral immune responses. Also, the effect of these genes has been investigated on cancer treatment, autoimmune diseases, and allergies [7].

Bee Venom Therapy (BVT) is defined as the application of live bee venom for therapeutic purposes. BVT is a traditional medicine widely used to treat many diseases, including arthritis, gout, multiple sclerosis, and infections [8]. Recently, BVT has been studied for the treatment of various cancers [9]. Bee venom contains different enzymes, including phospholipase, hyaluronidase, peptides (including melittin, apamin, adolapine), and biological amines (like histamine and epinephrine) [10]. The natural compounds in bee venom indicate the need for further studies on its potential therapeutic and anti-cancer properties. Thus, it seems necessary to study the effects of bee venom on cancer stem cells differentiation. Normal stem cells have unique biological properties, such as the capability to self-renew and differentiate into whole types of tissues and organs [11].

Melittin is a cationic and amphipathic peptide containing 58 amino acids. Its first 57 amino acids are mainly hydrophobic, while the last ones on the carboxyl end (amino acid 56 to 58) have hydrophilic features with a positive electric charge. This dual structure of melittin provides the specific feature to react with or destroy the phospholipid cellular membranes. Research has shown that antimicrobial peptides can potentially disrupt cell membranes and all membrane-associated transportation. Also, assessments on the effect of bee venom, melittin peptide, and phospholipase A2 on caries-causing bacteria indicate its considerable high lethal effects on oral pathogens [12]. Melittin interferes with the lipid-protein interaction of cell membranes leading to cell lysis. In liver cancer, melittin also prevents metastasis by reducing the rate of cell migration [13]. With the development of more efficient melittin delivery mechanisms such as nanoparticles to specific cells, this agent has become a major target for cancer treatment.

Bcl-2 is one of the essential genes involved in apoptosis. Bcl-2 proteins are part of the mitochondrial and endoplasmic membranes and play a vital role in inducing and inhibiting apoptosis. Some family members, including Bcl-2 and Bcl-xl, are involved in apoptosis inhibition, while others can induce apoptosis, such as Bad, Bal, and Bcl-xs (Bax). The ratio of inducers and inhibitors of apoptosis and their balance determine the apoptosis rate or preservation of cell life. The Bcl-2 protein, as a proteococcus in germ cells, is involved in the regulation of cell apoptosis. This protein is also generally involved in apoptosis inhibition. Following the reduction of *Bcl-2* gene expression, the programmed cell death or apoptosis is initiated by activating other oncogenes, such as *p53*.

Based on the role of caspases in different types of cells, the Bcl-2 can also prevent apoptosis by inhibiting caspases synthesis. More specifically, the Bcl-2 proteins in the mitochondrial membrane can prevent apoptosome formation and caspase cascade by blocking the cytochrome-c release or binding to the Apaf-1 complex [14]. *Bax* gene is located on chromosome 19. Many Bax-related studies on mitochondria have shown that this protein can potentially create ion channels and holes by modifying mitochondrial membrane, causing cytochrome-C intracytosolic release [15]. In addition to affecting mitochondria, the Bax-related compounds can affect lysosomes leading to direct changes in membrane

and materials in lysosomes. This critical phenomenon can lead to lysosomal enzymes release, a series of cellular structural changes, and the onset of cell death.This study aimed to evaluate the expression of *Bcl-2* and *Bax* genes in ACHN cells transfected with a recombinant vector carrying bee melittin gene through the real-time RT-PCR technique.

2. Materials and Methods

Plasmid design and replication

Recombinant plasmid pcDNA3.1(+)-melittin was designed to carry the gene encoding of bee venom. Then, the synthesized gene was cloned into a pcDNA3.1(+) vector using BamHI/EcoRV enzyme (Chinese company). The recombinant vector was transferred to E. coli by heat shock procedure. For heat-shock protocol, the microtubes containing bacterial and plasmid cells were placed in cold water for 20 min. In this procedure, the plasmids were absorbed by bacterial cells. Then, they were placed on a heater (42 s, 42 °C) and followed immediately by cold water (2 min). After plasmid amplification, its accuracy was confirmed by PCR and enzymatic digestion assays.

Cell Line proliferation and lipofection

ACHN cells were provided from the Stem Cell Unit of the Islamic Azad University of Shahrekord, Shahrekord City, Iran. The cells were cultured in DMEM or RPMI 1640 medium with 10% fetal bovine serum, penicillin 100 µg/mL, and streptomycin 100 µg/mL. The cells were incubated in an incubator in standard conditions of 37°C, 90% humidity, and 5% CO_2. ACHN cells proliferated with the recombinant vector were transfected. The lipofraction method was used for the transfusion of cells. In this procedure, the plasmid was inserted into the cells using lipofectamine solution. The culture medium was supplemented with neomycin for the proliferation of plasmid received cells. To produce stable cells resistant to neomycin, they were kept in a neomycin-containing culture medium for two weeks [16]. Two other groups of cells were also prepared as sham and control. The control group was treated with no plasmid transfer, and the sham group was tested with empty plasmid transfer of the target gene. All three cell groups of treatment, control, and sham were cultured in a medium containing neomycin. Control cells (with no plasmids) were expected to be destroyed by the effect of antibiotic neomycin.

RNA extraction and real-time RT-PCR technique

RNA was extracted (in 48 h) using RNX-Plus solution (SinaClon Company, Iran) from the remaining two groups of cells (treatment and sham groups). cDNA was prepared using the related specific kit (Thermo Fisher, USA). A real-time RT-PCR assay was used to evaluate the expression of *Bcl-2* and *Bax* genes in ACHN cells transfected with recombinant vector and sham cells.

Data analysis

Following data extraction, the normal distribution of data was confirmed by the Kolmogorov-Smirnov test. The Independent t-test was used to assess the expression of each gene (SPSS v. 19). P values less than 0.05 were regarded statistically significant, and data were expressed as Mean±SD.

3. Results

Results of plasmid accuracy using enzymatic digestion

Dual enzymatic digestion was hired to confirm the presence of the melittin gene in the pcDNA3.1(+) plasmid. There were enzyme cleavage sites for both BamHI/EcoRV restriction enzymes on either side of the melittin gene. Enzymatic digestion on recombinant plasmid pcDNA3.1(+)-melittin using two restriction enzymes of BamHI/EcoRV resulted in the formation of 96-bp bands belonging to the melittin gene (Figure 1).

Results of plasmid accuracy using PCR technique

PCR technique was performed to confirm the presence of melittin gene in plasmid pcDNA3.1(+) using specialized primers. The result was obtained on 1% gel electrophoresis. The size of the melittin gene with a length of 81 bp is shown in Figure 2.

Assessment of the accuracy of cDNA synthesis

In the present study, to determine the quality of the produced cDNA, the PCR technique was performed using the GAPDH primer as a housekeeping gene. Then the products were transferred on electrophoresis gel (1% agarose gel). Figure 3 represented the GAPDH gene with a length of 183 bp as the housekeeping gene for cDNA quality.

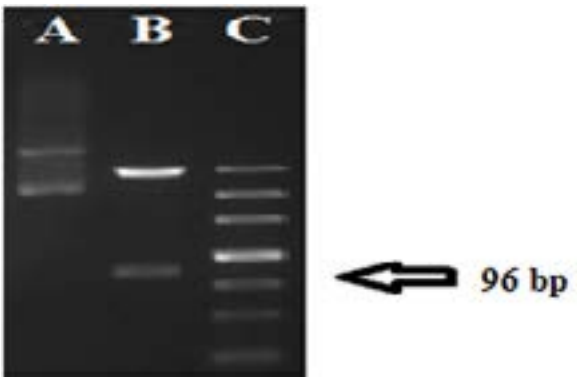

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Figure 1. Enzymatic digestion of a vector containing the melittin gene

96-bp band size was associated with the melittin gene. Well A is a vector not cleaved by the restriction enzyme, well B showed the size of the melittin gene band, and well C was the 50-bp marker.

Results of gene expression analysis

To perform the gene expression analysis, the Ct value of each sample (desired and the internal control gene samples) was recorded separately, and the expression fold-change was obtained. Following data normalization using housekeeping gene, the levels of gene expression were obtained. *Bax* gene expression increased significantly (P=0.03) in the pcDNA3.1(+)-melittin group compared to the PCDNA3.1(+)- meaning that the gene expression of melittin could accelerate *Bax* gene expression (Figure 4). On the other hand, the *Bcl-2* gene expression in the experimental group of pcDNA3.1(+)-melittin significantly decreased (P=0.001) in comparison with the pcDNA3.1(+) group meaning that the expression of the melittin gene could reduce the expression of the *Bcl-2* gene (Figure 5).

4. Discussion

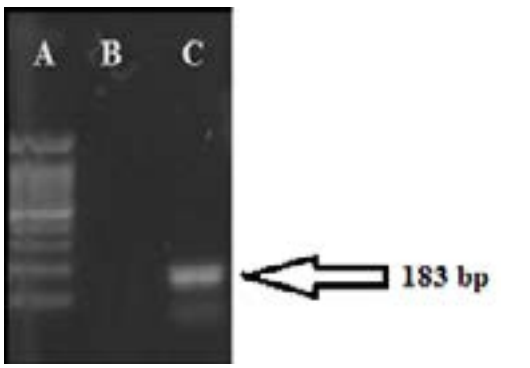

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Figure 3. Assessment of the cDNA synthesis accuracy

Well A is a marker of 100 bp; well B is negative control (lacked DNA pattern), and well C was the 183-bp band of the GAPDH gene.

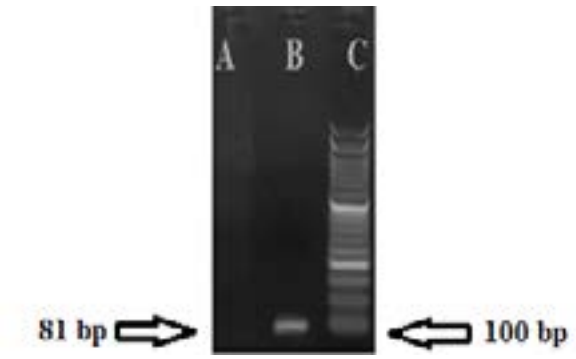

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Figure 2. Results of plasmid extraction accuracy using PCR assay

Well A represents a negative control sample; well B represents the presence of 81-bp band related to melittin gene, and well C is a marker of 100 bp.

The present study results showed that *Bax* gene expression increased in the pcDNA3.1(+)-melittin group compared to the pcDNA3.1(+) group. Thus, the expression of the melittin gene could increase *Bax* gene expression. On the other hand, the gene expression of *Bcl-2* decreased in the pcDNA3.1(+)-melittin group compared to the pcDNA3.1(+) group. Therefore, the expression of the melittin gene could potentially reduce the expression of the *Bcl-2* gene.

Moon et al. investigated the role of critical controllers of apoptosis in bee venom in regulating the *Bcl-2* and caspase-3 gene expression in U937 human leukemia cells [17]. The results showed that bee venom could reduce *Bcl-2* and *ERK* genes expression. In addition, bee venom initiated apoptotic processes by decreasing the expression of the apoptotic inhibitor IAP protein family. These results were inconsistent with the results of the present study on *Bcl-2* gene expression. Yang et al. examined the role of natural elements in bee stings, especially melittin, on inhibiting prostate cancer proliferation. Their results showed that the melittin could increase *Bax* and decrease *Bcl-2* genes expression as an anti-apoptotic protein leading to activation of apoptotic pathways in prostatic cancer cells [18]. Jo et al. also studied the anti-tumor role of melittin on ovarian cancer [19]. They found that the melittin can increase the gene expression of death receptor-3, -6, *Bax*, and caspases-3 and -8, while *Bcl-2*, *Jak2*, and *STAT3*'s expression was considerably reduced.

In addition, Hur et al. assessed the inhibitory role of bee venom on lung cancers. They concluded that the apoptosis process could be induced using the acceleration of the expression of apoptosis-related factors, including TNF-R1, TNF-R2, and FAS, in samples treated with toxins of bee venom. Also, the gene expression of *Bax*, caspases-3,

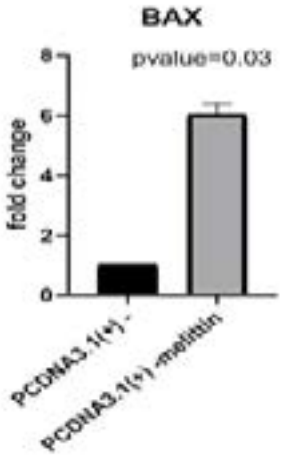

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Figure 4. Statistical analysis of *Bax* gene expression
Bax gene expression significantly (P=0.03) increased in the experimental group of pcDNA3.1(+)-melittin compared to the pcDNA3.1(+) group

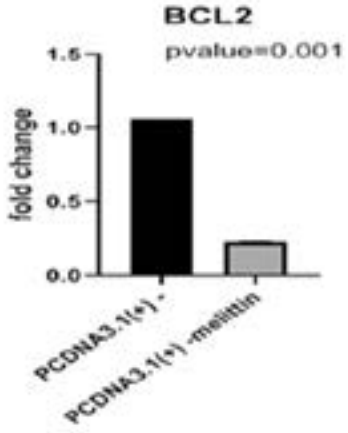

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Figure 5. Statistical analysis of the *Bcl-2* gene expression
Bcl-2 gene expression significantly (P=0.001) reduced in the experimental group of pcDNA3.1(+)-melittin compared to the pcDNA3.1(+) group

-8, and -9 increased in the treated samples, but the expression of anti-apoptotic factors of Bcl-2 showed a reducing trend [20]. These results were consistent with our findings. El Sharkawi et al. examined the anti-tumor effects of melittin in bee venom on two cell lines of liver and breast cancers. They found that the compounds in venom could increase *Bax* and decrease *Bcl-2* genes expression.

However, the results of this study showed that the melittin treatment decreased the *Bcl-2* gene expression with no increasing effects on *Bax* levels in cancer cells [21]. But in the present study, we detected the decreasing trend of *Bcl-2* expression and elevated levels of *Bax*. Zheng et al. assessed the anti-cancer effects of bee venom on the growth of colon cancer cells. They concluded that bee venom could induce apoptosis in colon cancer cells. In addition, these compounds can increase the expression of death receptors of p53, Bax, and caspases [22]. Those findings were inconsistent with the present study results. Wang et al. examined the role of melittin in the reduction of CVB3-induced myocarditis. The changes in gene expression also showed that the melittin could decrease *Bax* and caspase-3 and increase *Bcl-2* genes expression in myocardial tissue. Finally, the results showed that melittin could improve the function of the cardiovascular system [23]. The results of the present study also confirm the results of Wang and associates. Shu et al. investigated the role of melittin of bee venom in apoptotic activation in renal tubular epithelial cells and its effects on the expression of *Bax* and *Bcl-2* genes and activation of the TNF-α signaling pathway. The results showed that *Bax* gene expression was increased significantly in the presence of melittin, while *Bcl-2* gene expression decreased. Finally, this study showed that the apoptosis in renal tubular epithelial cells in the presence of melittin was mediated by increasing the *Bax*/*Bcl-2* ratio and activation of the TNF-α signaling pathway [24]. This study also showed that *Bax* gene expression in the pcDNA3.1(+)-melittin group increased considerably compared to the pcDNA3.1(+) group. So, the expression of the melittin gene can increase the *Bax* gene expression. On the other hand, statistical analysis of *Bcl-2* gene expression in the experimental group of pcDNA3.1(+)-melittin showed significantly decreased levels than the pcDNA3.1(+) group meaning that the expression of the melittin gene could reduce the *Bcl-2* gene expression.

5. Conclusion

The present study results showed that the expression of *Bax* and *Bcl-2* genes can be affected by the melittin gene of bee venom and can potentially alter the expression of *Bax* and *Bcl-2* genes in the ACHN cell line.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Shahrekord Islamic Azad University (Code: IR.IAU.SHK.REC.1400.011).

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Authors' contributions

Both authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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