Original Article

The Anti-Adhesion Effect of Nisin as a Robust Lantibiotic on the Colorectal Cancer Cells

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Abstract

Background: Bacteriocins are a type of antimicrobial peptide that are produced by probiotics. They have been studied as possible therapeutic drugs and have been used to suppress bacterial development in foods. Nisin is a potent bacteriocin having the anti-microbial and anti-cancer characteristics produced by *Lactococcus lactis*. The aim of the present paper is to evaluate the influence of Nisin on cell adhesion and its two related genes, *mmp-2* and *mmp-9,* in the colorectal cancer cell line.

Materials and Methods: For this purpose, HT-29 cells were treated with various concentrations of Nisin and the cell cytotoxicity, cell adhesion, and gene expression were evaluated using the MTT assay, cell adhesion assay, and real-time PCR.

Results: Our findings showed that 32 to 1024 μg/ml of Nisin resulted in a significant reduction in cell viability ($P < 0.05$). Furthermore, 128 and 256 μg/ml of Nisin significantly reduced the cell adhesion, and *mmp*-2 and *mmp*-9 gene expressions ($P < 0.05$).

Conclusion: Our findings suggested that Nisin could prevent metastasis and cancer progression.

Keywords: Bacteriocins, colorectal cancer, *lactococcus lactis*, metastasis, nisin

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Introduction

Cancer is an intricate disease that encompasses more than 100 disorders, each with its own set of symptoms. Despite significant breakthroughs in cancer treatment, many cancer therapy strategies have negative side effects for patients.[1] Colorectal cancer (CRC) occurs when the cells that line the colon or rectum become aberrant and proliferate out of control. Regrettably, some CRCs may go undetected for years without causing any symptoms.[2,3] Mortality rates for CRC vary dramatically around the world. With 1.65 million new cases and nearly 835,000 deaths in 2015, CRC is the third most frequent cancer in men and the second most prevalent disease in women.[4] CRC is caused by a combination of genetic and lifestyle factors. Genetic variables, on the other hand, are not particularly impressive.[5] There is accumulating evidence that mutations in genes that control cell proliferation are involved in CRC initiation and progression.[6] The tumor may create secondary tumors in other areas when cells detach from the tumor and invade adjacent tissues, a process known as metastasis. Preventing and combating metastases is the best strategy for CRC treatment. The spread of this type of cancer is still a severe issue, and many people die as a result of metastatic dissemination.[7] Recent studies have focused on the use of microbial metabolites, probiotics, and toxins in cancer treatment.[8,9] Probiotics are considered live microbial species that are now being researched in the treatment of a variety of diseases, especially cancer.[10,11] Bacteriocins are a type of antimicrobial peptide that are produced by probiotics. They

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have been studied as possible therapeutic drugs and have been used to suppress bacterial development in foods.[12,13] Nisin is a potent bacteriocin having the anti-microbial and anti-cancer characteristics produced by *Lactococcus lactis*.[14] Nisin is not active against gram-negative bacteria, but liposomes of gram-negative bacteria. Nisin's anticancer effects on cancer cells have been studied in a limited number of research.[15,16] Nisin has recently been studied for cancer cell growth prevention and has been shown to cause selective apoptosis, cell cycle arrest, and inhibit cell proliferation in HNSCC cells.[17] It is an anion carrier because it can generate pores in cells, causing the release of ions, amino acids, and ATP. Calcium plays a crucial role in apoptosis, and Nisin may be mediated by changes in intracellular calcium levels.[18] The inherent features of tumor cells and the reaction of tissue invasion are two processes of metastasis. In addition, it inhibited cell growth in part by preventing cell cycle arrest caused by cdc2 phosphorylation.[19] Matrix metalloproteinase (MMPs) is a group of proteins that are momentous in cancer progression and involved in different events such as metastases and cell viability. MMP2 and MMP9 are the two main members of this family.[20]

The goal of this study was to see how Nisin, a strong lantibiotic and metabolite generated from probiotics, affected the metastatic index, cell adhesion, and changes in the expression of some key genes in the metastatic, including mmp2 and mmp9, in CRC cells.

Materials and Methods

Cell culture

The HT29, as a human colorectal adenocarcinoma cell line, was obtained from the Pasture Institute (Tehran, Iran). Cells were grew in RPMI 1640 comprising 10% FBS, penicillin/streptomycin (100 U/ml/100mg/ml), 40 µg/ml gentamicin, and L-glutamine (0.3 g/L) at 37°C and 5% CO2. All culture mediums and reagents were prepared by Ideh Zist Company (Tehran, Iran).

MTT assay

To survey the impact of Nisin on the proliferation of HT 29 cells, MTT assay was performed. In brief, 8×10^3 cells were transferred to each well of a 96-well plate containing 100 µl complete medium. After 24 h, the cells were treated with different concentrations of Nisin including 8, 16, 32, 64, 128, 256, 512, and 1024 µg/ml. After 24 h, the medium of each well was replaced with the 100 µl fresh medium without FBS, and then 10 µl of MTT (5 mg/ml) (Sigma-Aldrich, Germany) was added to each well and incubated at 37°C and 5% CO2 for 4 h. After that, the medium of each well was removed and 100 µl Dimethyl sulfoxide was added and the optical density of each well was recorded at 570 nm using ELISA reader. All tests were done three times. The PBS was tested as a negative control.

Adhesion assay

To assess the adhesion of HT29 cells, the protocol of Pereira *et al*.[21] was used. In brief, approximately 5×10^4 cells were exposed to four discrepant concentrations of Nisin including 32, 64, 128, and 256 µg/ml for 1 h and then transferred to a 96-well plate and incubated at 37°C and 5% CO2 for 3 h. In the next step, to remove un-adherent cells, the plate was washed twice with PBS. The attached cells were fixed using cold methanol for 5 min. The cells were stained with 1% Toluidine blue in sodium tetraborate 1% for 5 min. After washing with PBS, the colored cells were dissolved in SDS 1% for 20 min at 37°C and the optical density of each well was recorded at 540 nm using an Eliza reader. All tests were done three times. The PBS was tested as a negative control.

Gene expression analysis

To evaluate the effect of Nisin on the expression of *mmp2* and *mmp9* genes, a real-time PCR method was done. In brief, 5×10^6 HT29 cells were exposed to four discrepant concentrations including 32, 64, 128, and 256 µg/ml for 24 h. To isolate total mRNA, the RNX-Plus kit (Cinnagen, Iran) was utilized. After confirming the quality and quantity of mRNA by the agarose gel electrophoresis and spectrophotometer, respectively, 1 µg of each mRNA sample was used to synthesize cDNA by cDNA synthesis kit (Takara, Japan) containing two universal primers, random hexamer and oligo dT primers, and M-MLV reverse transcriptase in accordance with manufacture's guideline. In the next step, the relative expression of *mmp2* and *mmp9* genes was investigated by real-time PCR. Table 1 showed the specific primers for *mmp2* and *mmp9* genes. *β-actin* gene was analyzed as a housekeeping gene. In brief, 1.5 µl of each cDNA was added to a mixture reaction containing 2X cyber green solution and 10 pmol of specific primer with a final volume of 20 µl. The thermal program was executed by Corbett Rotor-Gene6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) as 3 min for the initial denaturation step at 94°C, 38 cycles of 15 s at 94°C, 30 s at annealing temperature, and 40 s at 72°C. The relative expression of *mmp2* and *mmp9* was computed by Rest 2009 based on $2^{-\Delta\Delta CT}$ (Qiagen, USA).

Statistical analysis

To calculate the discrepancy between test and control groups, a one-way ANOVA test was carried out. The analysis was done by SPSS Version 11 software (SPSS, Chicago, IL, USA). *P* values < 0.05 are regarded statistically significant.

Table 1: The sequence of primers

Gene name	Forward (5'-3')	Reverse (5'-3')	Product length (bp)
mmp2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC	112
mmp9	AGACCTGGGCAGATTCCAAAC	CGGCAAGTCTTCCGAGTAGT	300
β- actin	TCATGAAGATCCTCACCGAG	TTGCCAATGGTGATGACCTG	118

Results

Cytotoxicity effects

To investigate the impacts of Nisin on the proliferation of HT29 cells, an MTT assay was performed. As shown in Figure 1, 32, 64, 128, 256, 512, and 1024 μg/ml of Nisin resulted in significant reduction in the cell viability of HT29 cells (71.74, 68.72, 62.21, 49.59, 38.85, and 31.74%, respectively) compared to the negative control group (100%) ($P < 0.05$).

Cell adhesion analysis

To identify the interference properties of Nisin on the cell attachment, an adhesion assay was carried out. As illustrated in Figure 2, treating HT29 cells with 32, 64, 128, and 256 μg/ml of Nisin significantly attenuated the percentage of attached cells (88.9, 80.7, 75.7, and 70.43%, respectively) in comparison with the negative control (100%). Only 128 and 256 μg/ml of Nisin significantly decreased the cell adhesion ($P < 0.05$).

Gene expression analysis

Figure 3 illustrates the mRNA expression pattern of *mmp2* and *mmp9* genes (part (a) and (b), respectively) after treatment with Nisin. Findings from the real-time PCR assay revealed that the gene expression ratio of *mmp2* was 0.87, 0.7, 0.559, and 0.36-fold after exposure to 32, 64, 128, and 256 μg/ml, correspondingly. The level of *mmp2* expression was significantly attenuated in the cells treated with 64, 128, and 256 μg/ml Nisin relative to untreated cells ($P < 0.05$). Furthermore, the expression of *mmp9* gene were remarkably reduced after exposure to 128 and 256 μg/ml Nisin (0.626 and 0.518-fold, respectively) compared to untreated cells ($P < 0.05$).

Discussion

Metastatic cancer is an aggressive and lethal type of cancer that in spite of the great efforts of researchers, there is no proper treatment for it. Current therapeutic strategies and the synthetic chemical drug had no proper efficiency to prevent metastatic events.[22] For solving this problem, some studies offered compounds based on plants or bacteria that some of these compounds are useful to treat cancer.[23,24] In addition, it is believed that probiotics and their metabolites directly or indirectly impact the signaling with the anti-cancer properties.[25] Nisin is the FDA-approved bacteriocins with anti-cancer properties.[16] In our previous study, we observed the apoptotic effect of Nisin on the SW480 as a colon cancer cell line.[26] We showed that Nisin induced apoptosis via the intrinsic pathway by increasing the BAX/BCL-2 ratio index. In addition, we found the anti-proliferative effect of Nisin.[27] Herein, we studied the impacts of Nisin on cell adhesion and the expression of *mmp2* and *mmp9* genes involved in the detachment of cells to the extracellular matrix.

Previous studies observed the cytotoxic effect of Nisin in the discrepant cancer cell lines. The IC50 of Nisin in the current study was about 76.8 μM which is lower than previous reports. MCF7 (breast cancer cell line) and HepG2 (hepatic cancer cell line) were examined by Paiva *et al*.,[28] with the IC50 of 105.46 and 112.25 mM, respectively, which is more than our study. Moreover, Begde *et al*.,[29] reported that 225 mM is the IC50 of Nisin for the studied cell lines, Jurkat cell line, and human lymphocyte without apoptotic effects. In another study performed on colorectal cancer cells, Maher and a co-worker reported that 89.9 μM and 115 μM of Nisin showed an anti-proliferative impact on HT29 and Caco-2 cell lines, respectively.[30] The effective concentration of Nisin in that study is approximately similar to our study.

MMP family plays a momentous role in cancer cell migration, invasion, and metastasis via an effect on the extracellular matrix (ECM).[31] MMPs are involved in cancer development, cancer cell growth, angiogenesis, and cancer progression.[32] Furthermore, high expression of MMP-2 and MMP-9 is related to metastasis to lymph nodes.[33] MMP-9 are able to secrete

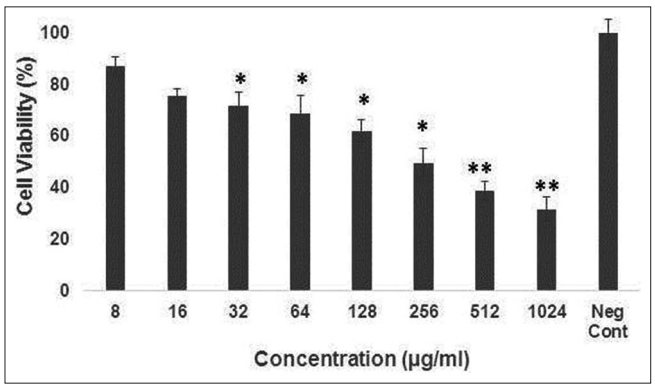

Figure 1: The cell viability percentage of HT29 cells after exposure to different concentrations of Nisin. 32, 64, 128, 256, 512, and 1024 μg/ml of Nisin caused to significant decrease in the cell viability (71.74, 68.72, 62.21, 49.59, 38.85, and 31.74%, respectively). (*) and (**) indicate significant results with *P* values less than 0.05 and 0.01, respectively. Neg Cont means the negative control group

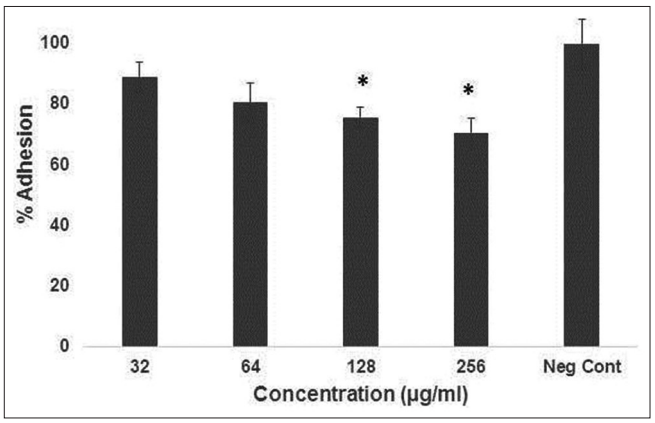

Figure 2: The percentage of attached HT29 cells after being treated with different concentrations of Nisin. A decrease in cell attachment was significantly seen at 128 and 256 μg/ml of Nisin (75.7 and 70.43%, respectively). Star (*) indicates significant results with *P* values less than 0.05. Neg Cont means the negative control group

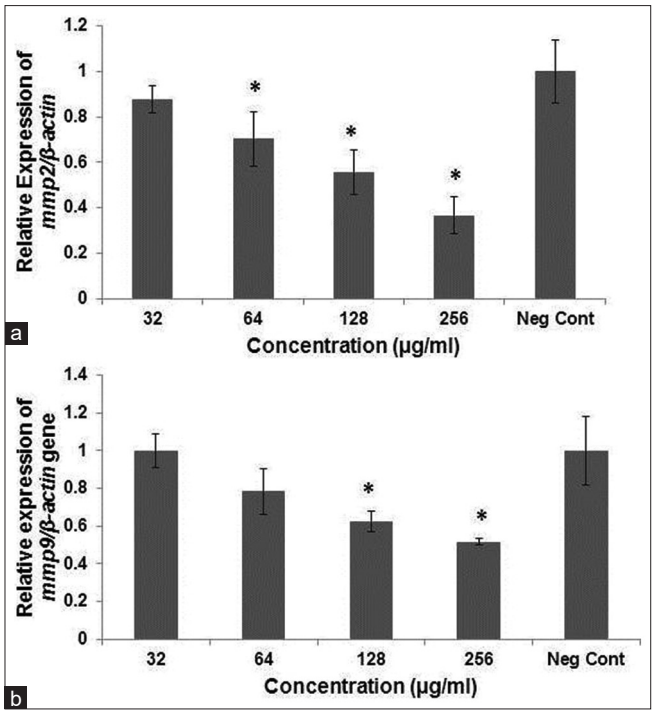

Figure 3: Relative expression of *mmp-2* gene (a) and *mmp-9* gene (b) in HT29 cells at 32, 64, 128, and 256 µg/ml Nisin. (a) Level of *mmp2* expression was significantly reduced after treating with 64, 128, and 256 µg/ml Nisin (0.7, 0.559, and 0.36 fold, respectively) compared to untreated cells. (b) Level of *mmp9* expression significantly reduced after exposing to 128 and 256 µg/ml Nisin (0.626 and 0.518 fold, respectively) compared to untreated cells. Star (*) indicates significant results with *P* values less than 0.05. Neg Cont means the negative control group

the ECM factors such as TGF-β, VEGF, and FGF-2 causing to proliferate and migrant endothelial cells and finally angiogenesis.[34]

In this study, we observed that Nisin is able to upregulate the expression of both *mmp-2* and *mmp-9* genes at 128 and 256 µg/ml. Decrease expression of these genes could be protective against tumor cell migration and metastasis. But, our finding showed that despite declining the expression of *mmp-2* and *mmp-9* genes, the cell adhesions decreased with increasing the Nisin concentration. The reason for this observation can be due to the increased cell death at higher concentrations and attenuated cell number during the time of treatment. Moreover, numerous proteins, factors, and molecular signaling are involved in cell detachment and metastasis, which has not been studied here; therefore, the conclusion about the anti-metastatic effect of Nisin is difficult.

Conclusion

Overall, Nisin is able to prevent metastasis via decreased expression of *mmp2* and *mmp9* genes in addition to cytotoxicity impacts. However, to confirm this data, further assessment of other mechanisms involved in metastatic events is essential.

Ethics approval

The study protocol was approved by the Research Ethics Committees of Baqiyatallah University of Medical Sciences. (Approval ID: IR.BMSU.REC.1396.028).

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Conflicts of interest

There are no conflicts of interest.

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