Evaluation of Apoptotic Gene Expression in Hepatoma Cell Line (HepG2) Following Nisin Treatment

Nahid Zainodini¹, Mohammad Reza Hajizadeh²,³, Mohammad Reza Mirzaei²,³* 

Abstract

Objective: The present study aims to examine the effects of nisin on the survival and apoptosis of the hepatoma cell line HepG2 and to investigate possible apoptosis pathways activated by nisin. Materials and Methods: For this purpose, viability and apoptosis of the cells were accomplished by the nisin treatment using the MTT assay and Annexin-V-fluorescein/propidium iodide (PI) double staining, respectively. Additionally, the human apoptosis PCR array was performed to determine pathways or genes activated by nisin during possible apoptosis. Results: The results of the present study showed that nisin was able to decrease cell viability (IC₅₀ ~ 40 µg/ml) in a dose-dependent manner and could induce apoptosis in HepG2 cells. PCR data indicated a considerable increase in the expression of genes, such as caspase and BCL2 families, involved in the induction of apoptosis. Conclusions: The data from this study showed that overexpression of genes involved in the intrinsic pathway of apoptosis, especially caspase-9 and BID, increased apoptosis in HepG2 cells treated by nisin, compared to the control group.

Keywords: Liver cancer- antimicrobial peptide- programmed cell death- cancer treatment- nisin

Introduction

Hepatocellular carcinoma is the third-most prevalent cause of cancer-related deaths leading to approximately half a million annual deaths across the world. Besides, it is referred to as the primary malignant neoplasm of epithelial liver cells. The prevalence of hepatocellular carcinoma is increasing in the western countries. However, this disease has been a serious health problem in Asia and Africa for a long time (Lin et al., 2017; Ayuso et al., 2018; Taskaeva and Bgatova, 2018).

In the current clinical practice, the main risk factors of hepatocellular carcinoma were primarily associated with suppressed hepatitis B virus during the treatment, sustained virologic response after hepatitis C, as well as alcoholic and non-alcoholic fatty liver disease (Kulik and El-Serag, 2019). Chemotherapy and surgical procedures are the most prevalent clinical treatments for hepatocellular carcinoma. Thus, novel and efficient medicines are required to be discovered for human hepatoma (Chen et al., 2015; Ayuso et al., 2018).

Peptides with antimicrobial activity are archaic evolutionary weapons. Besides, their broad distribution across the species of animals and plants indicates that antimicrobial peptides play a key role in the effective development of complex multicellular organisms (Zasloff, 2002). Nisin is an antimicrobial peptide composed of particular gram-positive bacteria, such as the species Streptococcus and Lactococcus (Lubelski et al., 2009). It was originally identified in milk fermentation culture in 1928 and was commercialized as an antimicrobial component in England in 1953 (Delves-Broughton et al., 1996). The safe usage of nisin was approved in 1969 as bacterial blockage in human foods (Shin et al., 2016).

Antimicrobial peptides have been studied for their therapeutic effects, such as their ability to perform several biological tasks, including antiviral properties, DNA synthesis, inhibition of membrane protein synthesis, and apoptosis or cytotoxicity of tumor cells (Cornut et al., 2008; Hamedeyazdan et al., 2012). To put it differently, antimicrobial peptides have been examined as potential therapeutic medicines for such features (Yusuf et al., 2014). This study concentrates on examining effects of nisin on the HepG2 apoptosis pathway as a cell line of hepatocellular carcinoma.

Materials and Methods

Cell culture
The National Cell Bank of the Pasteur Institute (Tehran,
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Disrupted in the 500 μl RLT buffer. Next, the cell lysis was the cells were washed twice with the PBS and then nisin 0.25 μg/ml or the control at 37°C for 24h. Besides, an array. The cells were either treated with a medium of apoptosis-related genes, housekeeping genes, as well as RNA and PCR quality controls were included in the array. The cells were either treated with a medium of apoptosis-related genes, housekeeping genes, as well as RNA and PCR quality controls were included in the array.

Statistical analysis

All tests were performed three times. Besides, the data on cell survival and apoptosis were analyzed by the one-way analysis of variance (ANOVA) using SPSS 16.0 (SPSS, Chicago, IL, USA). The mean ± standard deviation (SD) were measured, and the difference appeared to be statistically significant at P < 0.05. In addition, the web-based software of RT2 Profiler PCR Array version 3.5 (http://pcedataanalysis.sabiosciences.com/pcr/arrayanalysis.php) was employed to export and analyze the values of the resulting cycle threshold (CT) for gene expression. Besides, the ΔΔCT technique was employed to calculate fold changes.

Results

Nisin effects on cell viability

To assess potential cytotoxicity of nisin in contact with the HepG2 cell line, the MTT viability assay was employed. Accordingly, various concentrations of nisin (1, 10, 25, 50, 100, and 200 μg/ml) were employed in the present research. The viability rate of the treated cells displayed a reduction following an increase in the dose as against the control group after 24 and 48h of incubation (Figure 1). The results revealed that viability declined significantly (P < 0.05) from 1 μg/ml to 200 μg/ml with an IC50 value of around 40 μg/ml. In contrast, no significant difference was observed between the concentration of 1 μg/ml and that of the control group. Thus, nisin could be used as a growth inhibitor for HepG2 cells.

Nisin effects on apoptosis

Annexin-V-fluorescein/propidium iodide (PI) double staining was performed to examine apoptosis induction by nisin in HepG2 cells. The quantity of apoptotic cells increased as against the untreated group after being subjected to 25 μg/ml of nisin for 24h. In the untreated control tube, the population of viable cells was 92.66%, following adding 25 μg/ml of nisin, and about a quarter of the cells underwent late apoptosis (Figure 2).
Nisin effects on apoptosis-related genes

Real-time PCR was performed using the Human Apoptosis RT² Profiler PCR Array to express main genes engaged in programmed cell death, which responded to the 24h treatment (25µg/ml) of nisin. Besides, several members of the families, including the domain proteins of TNF/TNFR, BCL2/BAG, BIR, TRAF, as well as caspases were examined. In addition, genes with fold-change values of more than 2 and less than 0.5 were expressed in different manners. Table 1 shows fold changes of the genes studied on the PCR array. A total of 13 out of all chosen genes, including BAD, BAK1, BCLAF1, BNIP3L, CARD6, CASP5, CASP6, CASP10, CFLAR, FADD, HRK, LTBR, as well as TNF were upregulated. In contrast, 17 genes, including NOD1, TP53, TP73, NAIP, BIRC3, BCL2A1, BAG1, BAG3, BAG, TNFRSF25, CRADD, TNFRSF10B, CASP3, BRAF, BIK, APAF1, and BCL2 were downregulated.

Discussion

Given the low prognosis of liver cancer and inefficiency of current treatments for all types of this disorder, development of new anticancer agents for this cancer is of great importance (Anwanwan et al., 2020). Cancer cells show unlimited growth behavior; in other words, they strongly suppress apoptosis (programmed cell death). Moreover, deficiencies in the apoptotic pathway regulation, in addition to cancer development, may lead to resistance to cancer chemotherapy. One of the cancer-fighting approaches is the development of novel therapeutic agents that either upregulate pro-apoptotic molecules or downregulate anti-apoptotic molecules (Fesik, 2005; Baskić et al., 2006; Call et al., 2008).

In the late 1970s, research reported anti-cancerous characteristics for bacteriocins. They are, therefore, known to be favorable alternatives for developing anticancer composites (Breukink and de Kruijff, 1999; Bishayee and Sethi, 2016). Accordingly, potential apoptotic effects

Figure 1. The Effect of Nisin on of HepG2 Cell’s Survival. Various concentrations of nisin was applied for 24 (A), 48 (B) and then MTT assay was done for the measurement of the cell viability (%). The average of each triplicate experiment is presented in individual column as mean ± SD.
Table 1. Assessment of Apoptosis-Related Genes which are Expressed in HepG2 Cells Following Nisin Treatment for 24 h.

| Gene symbol | Protein/ gene name | Activity | Fold change |
|-------------|-------------------|----------|-------------|
| APAF1       | Apoptotic Peptidase Activating Factor 1 | Pro-apoptosis | -2.51       |
| BAD         | BCL2-associated agonist of cell death | Pro-apoptosis | 1.45        |
| BID         | BH3 interacting domain death agonist | Pro-apoptosis | 2.66        |
| BIK         | BCL2 Interacting Killer | Pro-apoptosis | -4.89       |
| BCL10       | B-Cell CLL/Lymphoma 10 | Pro-apoptosis | -1.58       |
| BCL2L11     | BCL2 Like 11 | Pro-apoptosis | -1.22       |
| BCLAF1      | BCL2 Associated Transcription Factor 1 | Pro-apoptosis | 2.23        |
| BNIP2       | BCL2 Interacting Protein 2 | Pro-apoptosis | 1.17        |
| BNIP3       | BCL2 Interacting Protein 3 | Pro-apoptosis | -1.13       |
| BNIP3L      | BCL2 Interacting Protein 3 Like | Pro-apoptosis | 2.34        |
| BRAF        | B-Raf Proto-Oncogene, Serine/Threonine Kinase | Pro-apoptosis | -4.26       |
| CARD6       | Caspase Recruitment Domain Family Member 6 | Pro-apoptosis | 3.27        |
| CARD8       | Caspase Recruitment Domain Family Member 8 | Pro-apoptosis | 1.79        |
| CASP1       | Caspase 1, Apoptosis-Related Cysteine Peptidase | Pro-apoptosis | 10.94       |
| CASP2       | Caspase 2, Apoptosis-Related Cysteine Peptidase | Pro-apoptosis | -1.51       |
| CASP3       | Caspase 3, Apoptosis-Related Cysteine Peptidase | Pro-apoptosis | -2.03       |
| CASP4       | Caspase 4, Apoptosis-Related Cysteine Peptidase | Pro-apoptosis | 1.24        |
| CASP5       | Caspase 5, Apoptosis-Related Cysteine Peptidase | Pro-apoptosis | 2.29        |
| CASP6       | Caspase 6, Apoptosis-related cysteine peptidase | Pro-apoptosis | 9.72        |
| CASP7       | Caspase 7, Apoptosis-related cysteine peptidase | Pro-apoptosis | 28.83       |
| CASP8       | Caspase 8, Apoptosis-related cysteine peptidase | Pro-apoptosis | -1.46       |
| CASP9       | Caspase 9, Apoptosis-related cysteine peptidase | Pro-apoptosis | 70.7        |
| CASP10      | Caspase 10, Apoptosis-related cysteine peptidase | Pro-apoptosis | 5.11        |
| CASP14      | Caspase 14, Apoptosis-related cysteine peptidase | Pro-apoptosis | 1.56        |
| CD27        | CD27 Molecule | Pro-apoptosis | 1.12        |
| CD40        | CD40 Molecule | Pro-apoptosis | -1.3        |
| CD40LG      | CD40 Ligand | Pro-apoptosis | 1.22        |
| CD70        | CD70 Molecule | Pro-apoptosis | 1.62        |
| CFLAR       | CASP8 and FADD-like apoptosis regulator | Pro-apoptosis | -2.99       |
| CIDEA       | Cell Death Inducing DFFA Like Effector A | Pro-apoptosis | -1.28       |
| CIDEB       | Cell Death Inducing DFFA Like Effector B | Pro-apoptosis | -1.36       |
| CRADD       | CASP2 And RIPK1 Domain Containing Adaptor With Death Domain | Pro-apoptosis | -3.61       |
| DAPK1       | Death Associated Protein Kinase 1 | Pro-apoptosis | -10.38      |
| DFFA        | DNA fragmentation factor, 45kDa, alpha polypeptide | Pro-apoptosis | 1.19        |
| FADD        | Fas Associated Via Death Domain | Pro-apoptosis | 2.49        |
| FAS         | Fas (TNF receptor superfamily, member 6) | Pro-apoptosis | -1.14       |
| FASLG       | Fas ligand (TNF superfamily, member 6) | Pro-apoptosis | -1.14       |
| HRK         | Harakiri, BCL2 Interacting Protein | Pro-apoptosis | 3.42        |
| LTA         | Lymphotoxin Alpha | Pro-apoptosis | -1.53       |
| LTBR        | Lymphotoxin Beta Receptor | Pro-apoptosis | 3.26        |
| NOD1        | Nucleotide Binding Oligomerization Domain Containing 1 | Pro-apoptosis | -2.25       |
| PYCARD      | PYD And CARD Domain Containing | Pro-apoptosis | -1.64       |
| TNF         | Tumor Necrosis Factor | Pro-apoptosis | 6.62        |
| TNFSF8      | Tumor necrosis factor (ligand) superfamily, member 8 | Pro-apoptosis | -1.14       |
| TNFSF10     | Tumor necrosis factor (ligand) superfamily, member 10 | Pro-apoptosis | 1.14        |
| TNFRSF1A    | Tumor necrosis factor receptor superfamily, member 1A | Pro-apoptosis | 10.27       |
| TNFRSF10A   | TNF Receptor Superfamily Member 10a | Pro-apoptosis | -1.14       |
| TNFRSF10B   | TNF Receptor Superfamily Member 10b | Pro-apoptosis | -2.53       |
Table 1. Continued

| Gene symbol | Protein/ gene name                                                                 | Activity     | Fold change |
|-------------|-----------------------------------------------------------------------------------|--------------|-------------|
| TNFRSF9     | Tumor necrosis factor receptor superfamily, member 9                              | Pro-apoptosis| 1.55        |
| TNFRSF11B   | Tumor necrosis factor receptor superfamily, member 11b                            | Pro-apoptosis| -1.14       |
| TNFRSF21    | Tumor necrosis factor receptor superfamily, member 21                             | Pro-apoptosis| -1.14       |
| TNFRSF25    | TNF Receptor Superfamily Member 25                                               | Pro-apoptosis| -6.612      |
| TRADD       | TNFRSF1A-associated via death domain                                              | Pro-apoptosis| -1.14       |
| BAK1        | BCL2-antagonist/killer 1                                                          | Anti-apoptosis| 2.47        |
| BAX         | BCL2 Associated X, Apoptosis Regulator                                            | Anti-apoptosis| -1.83       |
| BAG1        | BCL2 Associated Athanogene 1                                                      | Anti-apoptosis| 2.99        |
| BAG3        | BCL2 Associated Athanogene 3                                                      | Anti-apoptosis| 3.28        |
| BAG4        | BCL2-associated Athanogene 4                                                       | Anti-apoptosis| 2.36        |
| BCL2        | B-cell CLL/lymphoma 2                                                             | Anti-apoptosis| -2.59       |
| BCL2A1      | BCL2 Related Protein A1                                                            | Anti-apoptosis| 5.76        |
| BCL2L2      | BCL2 Like 2                                                                       | Anti-apoptosis| 1.48        |
| BCL2L10     | BCL2-like 10 (apoptosis facilitator)                                              | Anti-apoptosis| -2.8        |
| BFA1        | Bifunctional Apoptosis Regulator                                                  | Anti-apoptosis| -1.09       |
| BIRC3       | Baculoviral IAP repeat containing 3                                               | Anti-apoptosis| -3.56       |
| BIRC6       | Baculoviral IAP repeat containing 6                                               | Anti-apoptosis| -1.97       |
| BIRC8       | Baculoviral IAP repeat containing 8                                               | Anti-apoptosis| -1.21       |
| BNIP1       | BCL2 Interacting Protein 1                                                         | Anti-apoptosis| 1.11        |
| IGF1R       | Insulin Like Growth Factor 1 Receptor                                             | Anti-apoptosis| -1.17       |
| MCL1        | Myeloid Cell Leukemia Sequence 1 (BCL2-Related)                                   | Anti-apoptosis| 1.95        |
| NAIP        | NLR Family Apoptosis Inhibitory Protein                                           | Anti-apoptosis| -2.6        |
| NOL3        | Nucleolar protein 3 (apoptosis repressor with CARD domain)                        | Anti-apoptosis| -1.57       |
| RIPK2       | Receptor-interacting serine-threonine kinase 2                                    | Anti-apoptosis| -1.23       |
| TP53        | Tumor protein p53                                                                  | Anti-apoptosis| -1.74       |
| TP73        | Tumor protein p73                                                                  | Anti-apoptosis| -4.15       |
| TRAF2       | TNF receptor-associated factor 2                                                   | Anti-apoptosis| -2.21       |
| TRAF3       | TNF receptor-associated factor 3                                                   | Anti-apoptosis| -2.67       |
| TRAF4       | TNF receptor-associated factor 4                                                   | Anti-apoptosis| -3.52       |
| XIAP        | X-Linked Inhibitor of Apoptosis                                                   | Anti-apoptosis| 1.3         |

of nisin against HepG2 cells were investigated in this research. The PCR array technology was employed to define molecular mechanisms of nisin-induced apoptosis. According to the findings, nisin decreased cell viability and induced apoptosis. There is a small number of research conducted on the anti-tumoral activity of nisin (Lagos et

![Figure 2. Flow Cytometric Analysis of HepG2 Cells after 24 h Treatment with Medium (A), 10 µg/ml nisin (B), and 25 µg/ml nisin (C). Scatter plots consist of four quadrants: upper left (Annexin-V-/PI+, necrotic cells), upper right (Annexin-V+/PI+, late apoptotic cells), lower left (Annexin-V-/PI-, viable cells), lower right (Annexin-V+/PI-, early apoptotic cells).](image-url)
pro-apoptotic genes were increased, which included pro-apoptosis genes. As a result, a total of 14 genes of the affect this pathway, particularly by upregulation (intrinsic and extrinsic). Accordingly, nisin could of regulating and modifying the apoptosis pathway pathways of extrinsic and intrinsic apoptosis.

Following the use of the qPCR array technology, it was found out that nisin could be capable of changing the expression of apoptosis-related genes. This PCR array consisted of a number of apoptosis-involved gene families, such as caspase, TRAF, Bcl-2, and IAP, as well as TNF ligands and their receptors. In this study, the data suggested different gene expressions in the signaling pathways of extrinsic and intrinsic apoptosis.

The present study demonstrated that nisin is capable of regulating and modifying the apoptosis pathway (intrinsic and extrinsic). Accordingly, nisin could affect this pathway, particularly by upregulation of pro-apoptosis genes. As a result, a total of 14 genes of the pro-apoptotic genes were increased, which included BID, BCLAF1, BNIP3L, CARD6, caspase family members (1, 5, 6, 7, 10), FADD, HRK, LTBR, TNF, and TNFRSF1A. In contrast, 9 of other genes, including APAF1, BIK, BRAF, CASP3, CFLAR, CRADD, NOD1, TNFRSF10B, and TNFRSF2 underwent downregulation. Similarly, anti-apoptotic genes were affected by nisin, with most of which having shown decreased expression. Accordingly, BCL2, BCL2L10, BIRC3, NAIP, TP73, TRAF2, TRAF3, and TRAF4 were downregulated, while the expression of BAG1, BAG3, and BAG4 increased.

Caspases are a group of proteases produced in an inactive form, which are initiated and regulated by the apoptosis process. In general, caspases involved in apoptosis are classified into two groups, including effectors (-3, -6, and -7) and initiators (caspase-8, -9, and -10) (McComb et al., 2019; Poreba et al., 2019). When caspases are activated, they alter expression of pro-apoptotic and anti-apoptotic proteins, thereby resulting in apoptosis cells. Moreover, caspases exert their effects using extrinsic and intrinsic pathways (Poreba et al., 2019).

The present study showed that nisin plays its role in apoptosis by increasing mitochondrial pathways. Caspase-9 is the initiator of the mitochondrial or intrinsic pathway of apoptosis, which is activated by numerous cellular stresses (Mcllwain et al., 2013; Pfeffer and Singh, 2018). The intrinsic pathway is regulated by the BCL2 (B-cell lymphoma-2) protein family that contains both anti- and pro-apoptotic members, such as BAX and BH3-only proteins (BID), respectively (Zaman et al., 2014; Lopez and Tait, 2015). Our data showed that the expression of pro-apoptotic BID increased, while that of anti-apoptotic BAX decreased, which suggested that the intrinsic pathway was highly activated. The regulation of irregular growth of cancer cells is one of the ways to treat cancer. According to the results from the present study, it seems that the use of nisin as an inducer of apoptosis could play an effective role in restoring this uncontrolled condition into a normal one.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Nahid Zainodini, Mohammad Reza Mirzaei; data collection: Nahid Zainodini; analysis and interpretation of results: Nahid Zainodini, Mohammad Reza Hajizadeh; draft manuscript preparation: Nahid Zainodini, Mohammad Reza Mirzaei. All authors reviewed the results and approved the final version of the manuscript.

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Statement conflict of interest

No potential conflict of interest was reported by the authors.

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