

The Antimicrobial Peptide, Nisin, Synergistically Enhances the Cytotoxic and Apoptotic Effects of Rituximab Treatment on Human Burkitt's Lymphoma Cell Lines

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Abstract

Background: Non-Hodgkin's lymphomas comprise the most common hematological cancers worldwide and consist of a heterogeneous group of malignancies affecting the lymphoid system. Treatment of non-Hodgkin's lymphoma has been significantly enhanced with the addition of Rituximab to the standard chemotherapy regimen. However, even with the advancement of treatment patients continue to relapse and develop resistance to Rituximab, rendering subsequent treatments unsuccessful. The use of novel drugs with unique antitumor mechanisms has gained considerable attention. In this study, we explored the *in vitro* anti-cancer effects of the combined therapy of Rituximab and Nisin on human Burkitt's lymphoma cells.

Methods: The human Burkitt's lymphoma cells lines, Raji and Daudi, were treated with Nisin, Rituximab, or a combination of the two agents at various concentrations. Cytotoxicity following treatment was determined using cell viability assay. The degree of apoptosis was verified via flow cytometric analysis using FITC annexin V/PI staining.

Results: Our findings show that the combined treatment of Rituximab and Nisin results in a more significant reduction in the survival of Raji and Daudi Burkitt's lymphoma cells, compared to Nisin or Rituximab treatment alone. Additionally, our results indicate that Nisin can induce a significant degree of apoptosis in the Burkitt's lymphoma cells compared to the negative controls. However, the addition of Nisin to the Rituximab treatment synergistically enhances the apoptotic antitumor effect.

Conclusions: This study demonstrates the synergistic antitumor effect of Nisin treatment *in vitro* to enhance tumor cell apoptosis and the potential value of Nisin as an adjunct therapy in the treatment of lymphoma.

Keywords: Apoptosis, Burkitt's lymphoma, Cell viability, Combination, Nisin, Rituximab.

Introduction

Non-Hodgkin's lymphomas (NHLs) are one of the most common malignancies worldwide and include a heterogeneous group of diseases that arise from malignant lymphocytes. One of the many subtypes include Burkitt's lymphoma, which is a highly aggressive form of B cell NHL (1). Although patients with NHLs have a high initial response rate to chemotherapy, it is common for patients to ultimately relapse and develop drug resistance. Thus, the subsequent

chemotherapy regimen is unable to successfully eliminate the tumor cells (2). The use of antibody-mediated immunotherapy, alone or in combination with chemotherapy, has been shown to improve the overall survival rate. Anti-CD20 antibodies are a promising candidate in the treatment of NHLs. Rituximab is the first chimeric anti-CD20 monoclonal antibody (mAb) used to treat B cell disorders, including NHLs such as chronic lymphocytic leukemia. The use of Rituximab has also been demonstrated in the

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Received: 18 Feb, 2020; Accepted: 29 Mar, 2020

treatment of autoimmune diseases, such as multiple sclerosis (3,4). Rituximab acts via binding to CD20 which induces antibody-dependent cell-mediated cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and to a lesser extent, apoptosis of malignant cells (5–7). Rituximab is frequently used alone or in combination with chemotherapy to treat lymphoma, however patients often develop a resistance to Rituximab and the cancer may relapse. These findings highlight the importance of enhancing existing treatments to improve the clinical outcome for these patients.

A growing number of studies have revealed the antitumor properties of antimicrobial peptides which has led to considerable interest in their use as an alternative cancer treatment (8–14). These small molecules have potent tissue penetration and uptake by cancer cells. They exert their cytotoxic effects through both their intrinsic activity or synergistically interacting with existing therapeutics, enhancing their anticancer properties (15). Nisin is a type of antimicrobial peptide that is 34 amino acid residues in size and produced the *Lactococcus lactis* bacterium (16). Bacteriocins, including Nisin, have become a promising candidate showing great potential in combating various infectious diseases (15,17). Nisin was approved by the Food and Drug Administration (FDA) in 1988 as a food preservative safe for human use. The World Health Organization (WHO) has also confirmed its safety and it has been shown to have no cytotoxic effects on the human body. While antimicrobial peptides have a long history of use in the food industry to prevent the growth of bacteria, they have recently gained considerable attention for their antitumor activity. Several studies have shown the antitumor properties of Nisin, including its ability to induce apoptosis in gastrointestinal, hepatic, and K562 cancer cell lines (8), inhibit head and neck squamous cell carcinoma (HNSCC) tumorigenesis (11), enhance cell death and apoptosis in colon cancer cell lines (10), and decrease cell proliferation and induce apoptosis in melanoma cells (9). Nisin exerts its effect by creating pores in the cell membrane, altering the electrical potential of the cell (18). The exact role of calcium in Nisin dependent cell death has yet to

be fully elucidated. However, since the pores created by Nisin lead to a net influx of calcium ions, calcium may hold a critical role in the induction of apoptosis and inhibition of cell proliferation (17). In this study, we investigated the cytotoxic effect of Nisin in human Burkitt's lymphoma cell lines and explored the potential synergistic effect of Nisin on Rituximab-induced apoptosis and cytotoxicity *in vitro*.

Materials and methods

Cell culture

Human Raji and Daudi Burkitt's lymphoma cells were prepared from the National Cell Bank of Iran (Tehran, Iran). Antibiotic-free RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) was used for maintaining the cells at 37 °C in a humidified incubator with 5% CO₂.

Cell viability assay

To determine the effect of Nisin Z (Handary, Belgium) on Burkitt's lymphoma cell viability alone or in combination with Rituximab treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) colorimetric assay was used. Briefly, 5x10⁴ Daudi cells, Raji cells, or peripheral blood mononuclear cells (PBMCs) were plated in a 96-well cell culture plate. Following plating, 10 µl of Nisin or Rituximab was added to each well containing 90 µl RPMI 1640. The effect of treatment was tested as various concentrations including 4, 40, 400, and 4000 µg/mL for Nisin, and 0.1, 1, 10, and 100 µg/mL for Rituximab in a final mixture of 100 µl/well. The concentrations used for the combined Nisin and Rituximab treatment were the IC₅₀ concentration for each agent, which is the concentration needed to reduce cell viability by 50%. For the negative controls, Daudi and Raji cells were treated with phosphate buffer saline (PBS). Next, MTT (0.5 mg/mL) was added 72 hours after treatment, and incubated for 3 hours at 37 °C. After centrifugation at 2000 g for 5 minutes and discarding the supernatant 100 µl dimethyl sulfoxide (DMSO) was added. The optical densities of each well were recorded using a 96-well microplate reader (Bio-Rad Laboratories

Inc. Philadelphia, PA) at 570 nm. The following equation was used to estimate the percentage of cell viability: Cell viability (%) = $[A_{570}(\text{sample})/A_{570}(\text{control})] \times 100$. All experiments were performed at least three times.

Apoptosis assay

The effect of Nisin Z alone or in combination with Rituximab on Burkitt's lymphoma cell apoptosis was determined using a direct staining method and a flow cytometry assay using FITC annexin V apoptosis detection kit with PI (BioLegend, San Diego, CA). In brief, cells were plated in 12-well plates at 1×10^6 cells/mL. Subsequently, 100 μ l of Nisin or Rituximab was added to each well containing 900 μ l of RPMI 1640 medium, and the plates were incubated at 37 °C for 48 h. The concentration of Nisin and Rituximab were 4000 and 100 μ g/mL in 1 mL mixture, respectively. These concentrations correspond to the IC₅₀ of each agent. The effect of Nisin in combination with Rituximab was determined by adding 100 μ l of Nisin at a concentration of 4000 μ g/mL and 100 μ l of Rituximab at a concentration of 100 μ g/mL. The negative controls were incubated with PBS. For the direct staining method, Annexin V/PI staining was used according to the manufacturer's instructions. The stained cells were visualized under a Nikon fluorescence microscope. The amount of apoptosis was assayed using flow cytometry analysis of cells stained with Annexin V/PI. Stained cells were analyzed using the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific). FlowJo software version 7.6.1 was used for analysis of the results.

Statistical analysis

Data were analyzed by one-way ANOVA using SPSS software 19.0 (SPSS, Inc., Chicago, IL). Results were considered statistically significant if p-value < 0.05.

Results

The combined treatment of Nisin and Rituximab reduces cell viability

The cytotoxicity of Nisin, Rituximab, and the combined treatment of both agents on the Burkitt's lymphoma cell lines was investigated by MTT

cell viability assay. The effect of Nisin and Rituximab on cell viability were observed to be dose dependent, as the viability decreased progressively with increasing concentrations of Nisin and Rituximab. The maximum decrease in percentage cell viability was observed following treatment with Nisin at a concentration of 4000 μ g/mL and Rituximab at a concentration of 100 μ g/mL in both Raji and Daudi cell lines (Figs. 1A and 1B). The IC₅₀ of Nisin and Rituximab was estimated to be 4000 μ g/mL and 10 μ g/mL, respectively. For the combined treatment, the IC₅₀ concentration of each agent was used. In the cells that received the combined treatment of Rituximab and Nisin, we observed a significant decrease in the percentage cell viability compared to treatment with Rituximab and Nisin alone at their respective IC₅₀ concentrations ($p < 0.001$). These findings indicate that the addition of Nisin to Rituximab treatment can enhance the degree at which cell viability is reduced (Fig. 1C). Treatment with Nisin or the combination of Nisin and Rituximab showed no cytotoxic effects on normal human B cells.

Nisin and Rituximab combination induces significant apoptosis

We further examined whether the effect of Nisin on Rituximab-mediated cytotoxicity was synergistic. As demonstrated in Figure 2A, flow cytometric analysis clearly showed that treatment with either Nisin or Rituximab at their IC₅₀ concentrations induces moderate apoptosis in both Raji and Daudi cells, compared to the negative controls. In contrast, the combination of Nisin (4000 μ g/mL) and Rituximab (10 μ g/mL) resulted in a synergistic increase in apoptosis of the Burkitt's lymphoma cells (Fig. 2). Following combination treatment, about > 20% and > 40% of the Raji and Daudi cells underwent apoptosis, respectively. Flow cytometry analysis is shown in Figure 2 and the results of the Annexin V/PI staining assay are illustrated in Figure 3. Greater PS exposure and cell death were induced by the Nisin and Rituximab combined treatment compared to Nisin or Rituximab alone.

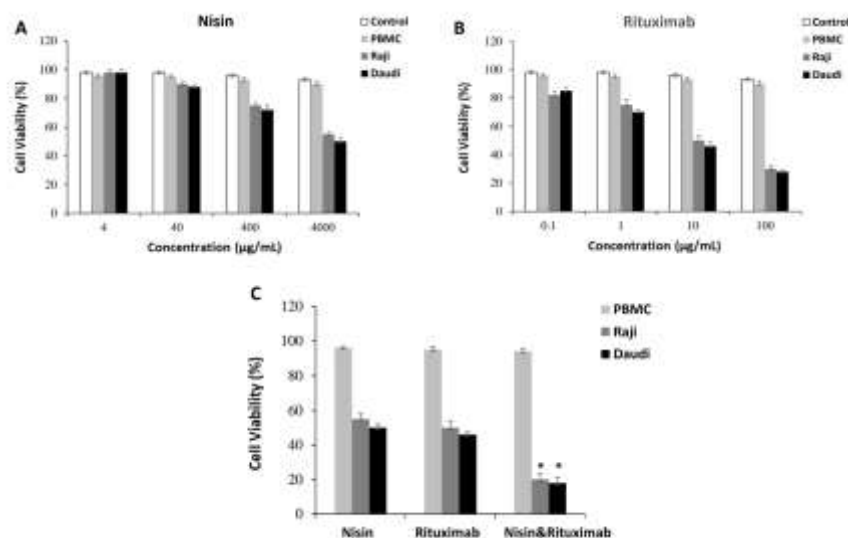


Fig. 1. Nisin significantly reduces cell viability in a dose-dependent manner and potentiates the cytotoxic effect of Rituximab. The effect of (A) Nisin, (B) Rituximab, and (C) Nisin in combination with Rituximab (* $p < 0.001$ compared with Nisin or Rituximab alone) on the viability of Raji and Daudi cells and PBMCs, as measured by the MTT assay. The concentration used for combination treatments with Nisin and Rituximab corresponds to the IC₅₀ of each agent. Controls were Raji or Daudi cells incubated with PBS. Comparisons between groups were analyzed by one-way ANOVA.

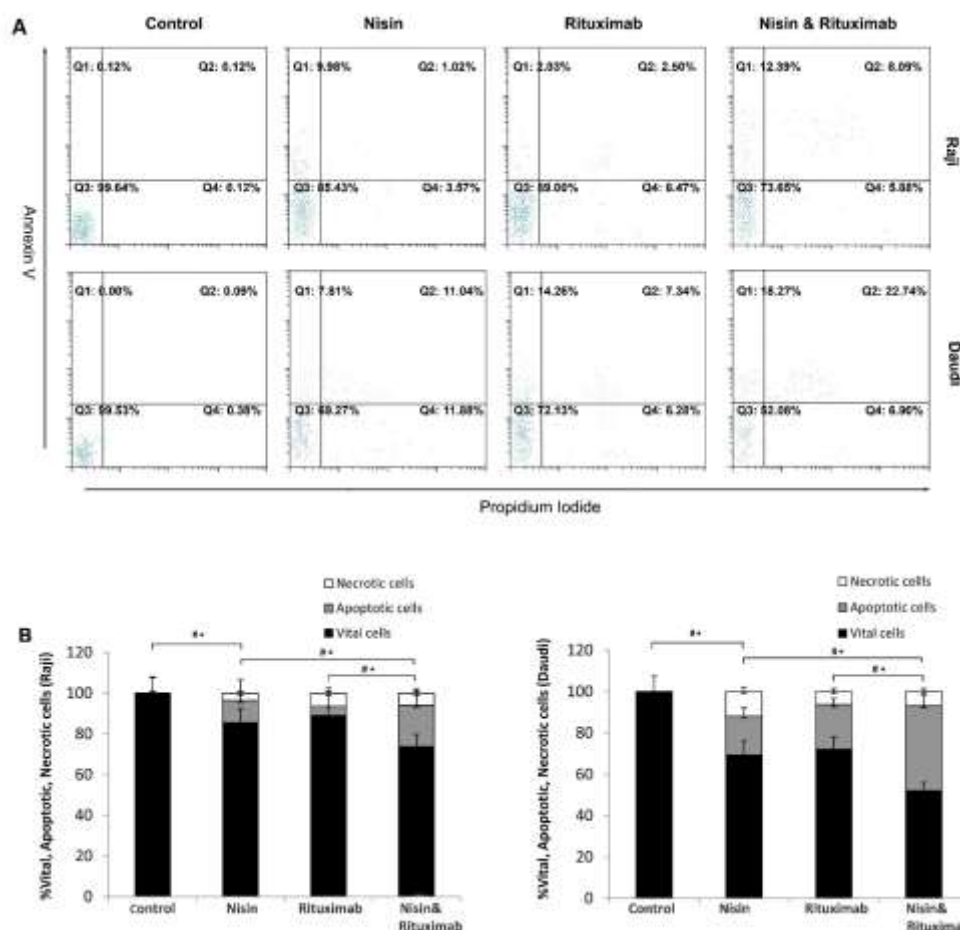


Fig. 2. Nisin induces significant cell apoptosis and potentiates the apoptotic effect of Rituximab. (A) Flow cytometry analysis was performed to determine the impact of Nisin, Rituximab, or the combined treatment on Raji and Daudi cell apoptosis. (C) Percentage of vital, apoptotic, and necrotic cells (Raji and Daudi) treated with control (PBS), Nisin (4000 µg/mL), Rituximab (10 µg/mL), and Nisin (4000 µg/mL) in combination with Rituximab (10 µg/mL). Comparisons between groups were analyzed by one-way ANOVA (# $p < 0.001$ vital cells; #* $p < 0.001$ apoptotic cells).

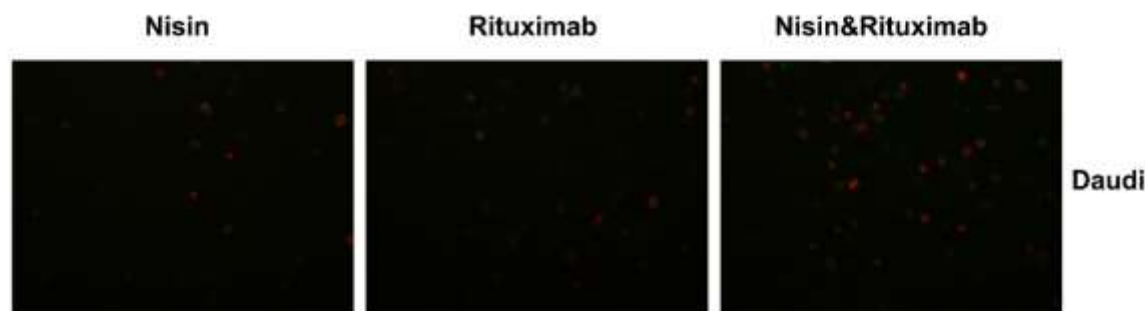


Fig. 3. Microscopic analysis of direct FITC annexin V/PI staining of Daudi cells treated with Nisin, Rituximab, and Nisin in combination with Rituximab. Greater PS exposure and cell death were induced by the combination of Nisin and Rituximab compared to Nisin or Rituximab alone.

Discussion

In this study, we provide novel evidence that Nisin can potentiate the cytotoxic effects of Rituximab and lead to a significant increase in the level of apoptosis in human Burkitt's lymphoma cell lines. The purpose of the present study was to evaluate the *in vitro* anti-cancer effects of the combined treatment of Nisin and Rituximab on human Burkitt's lymphoma cells. Cells received treatment with either Nisin, Rituximab, or a combination of the two agents. Our findings showed that Nisin decreases the viability of Raji and Daudi Burkitt's lymphoma cells at a concentration of 4000 $\mu\text{g/mL}$, while the combined treatment of Nisin and Rituximab reduced cell viability significantly more than that of Nisin or Rituximab alone at their respective IC_{50} concentrations. These findings indicate that the addition of Nisin to Rituximab treatment may have the ability to further reduce the cell viability of Burkitt's lymphoma cell lines. To verify the level of apoptosis, flow cytometry analysis using FITC annexin V/PI staining was performed. Nisin was found to induce significantly more apoptosis compared to the negative controls. However, the combined treatment of Nisin and Rituximab was observed to synergistically increase the level of apoptosis of each Burkitt's lymphoma cell line compared to Nisin or Rituximab alone. This synergistic effect appears to be a result of the combined function of both Nisin and Rituximab in modulating apoptotic regulatory proteins. B-cell lymphoma (Bcl)-extra-large (Bcl-xL) and Bcl-2 are critical anti-apoptotic genes that promote cell survival, while Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) are both pro-apoptotic genes that induce cell death. The expression of BAX and BAK increase the

apoptotic index (bax, bak/ Bcl-xL, Bcl-2), leading to the induction of apoptosis. Nisin can induce apoptosis through increasing the apoptotic index (bax/bcl-2 ratio) both at the mRNA and protein level (10), while Rituximab selectively down-regulates the expression of the anti-apoptotic Bcl-xL gene (7). The regulatory role of Rituximab on apoptosis, along with those exerted by Nisin, demonstrate that the combination of Nisin and Rituximab treatment may be an effective therapy for patients with treatment resistant Burkitt's lymphoma.

A growing number of studies have highlighted the antitumor properties of antimicrobial peptides (8–14). In a recent study conducted by Goudarzi et al. (8), Nisin was observed to have a cytotoxic effect on K562, AGS, HepG2, and KYSE-30 cancer cell lines. The use of Nisin-loaded nanoparticles was found to have a more cytotoxic effect than free Nisin. In a separate study, Nisin Z was shown to induce selective toxicity in the A375 melanoma cell line in comparison to non-malignant keratinocytes. These findings suggest that the combined therapy of Nisin Z and anti-melanoma agents may be an effective treatment option for patients with melanoma (9). Zainodini et al. (19) reported that Nisin treatment significantly increases cell death and apoptosis in the astrocytoma cell line, SW1088, compared controls. In a study conducted by Ahmadi et al. (10), treatment with Nisin was found to significantly increase the cell viability and apoptosis in the SW480 colon cancer cell line. The anti-proliferative and apoptotic effects of Nisin were reported to occur at a concentration 2000–4000 $\mu\text{g/mL}$. This is similar to our findings in which the

IC50 concentration of Nisin obtained in our study was about 4000 $\mu\text{g/mL}$. Consistent with our findings, Ahmadi *et al.* reported that the anti-proliferative effect of Nisin was dose dependent. Moreover, they reported that Nisin induces apoptosis by increasing the apoptotic index (bax/bcl-2 ratio). Furthermore, a separate study has shown that Nisin can significantly increase apoptosis by mediating calcium flux via the activation of calpain and decrease cell proliferation through cell cycle arrest in head and neck squamous cell carcinoma cells both *in vitro* and *in vivo* (11).

Although the antitumor ability of Nisin to induce apoptosis and its cytotoxic effects have been previously described, our study provides novel evidence showing that Nisin can potentiate the cytotoxic effect of Rituximab, leading to a significant increase in apoptosis in Raji and Daudi Burkitt's lymphoma cell lines.

In conclusion, our findings have shown that Nisin can reduce the viability of Raji and Daudi Burkitt's lymphoma cell lines *in vitro* and confirmed that Nisin reduces cell viability in a dose-dependent manner. Furthermore, the combined treatment of Nisin with Rituximab at

their respective IC50 concentrations results in a significant reduction in cell viability compared to Nisin or Rituximab alone. These findings suggest that the addition of Nisin can enhance the ability of Rituximab treatment to reduce the viability of Burkitt's lymphoma cell lines. Furthermore, our findings show that Nisin can induce apoptosis in Burkitt's lymphoma cell lines. Similarly, the combined treatment of Nisin with Rituximab works synergistically to enhance the level of apoptosis in these lymphoma cells. Our findings demonstrate the importance of Nisin as a possible adjuvant therapy capable of increasing the *in vitro* antitumor properties of Rituximab via apoptosis. However, further research is required to determine the *in vivo* antitumor effects of Nisin treatment.

Acknowledgment

We would like to thank all members of the Medical Biology Research Center for their discussion and support. This work was supported by Vice Chancellor for Research and Technology, Kermanshah University of Medical Sciences (97770).

The authors declare no conflict of interest.

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