Immunomodulatory efficacy of nisin – a bacterial lantibiotic peptide

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Nisin is a peptide bacteriocin, grouped under the category of lantibiotics. It is naturally produced by *Lactococcus lactis* to eliminate other competing gram-positive bacteria from its vicinity. Moreover under certain conditions it is reported to be effective against a broad range of gram-negative bacteria as well. Thus, it has been widely used as a safe food preservative especially in the dairy industry. Because of its wide-scale consumption, its effect on eukaryotic cells should be of great concern. Here we examine the immunomodulatory efficacy of nisin *in vitro*. MTT-based cytotoxicity assay demonstrated nisin's cytotoxicity on human T-cell lymphoma Jurkat cells, Molt-4 cells and freshly cultured human lymphocytes at over 200 μM concentration (IC$_{50}$ 225 μM). The cell death mechanism induced by nisin in all these lymphocyte types was independent of oligonucleosomal DNA fragmentation, as analyzed by agarose gel electrophoresis and comet assay. Additionally, scanning electron microscope and fluorescence microscopy demonstrated the ability of nisin to activate human PMNs *in vitro*. Nisin-activated neutrophils extruded intact nuclear chromatin to form NETs, well known for neutralization of virulence factors and extermination of bacterial pathogens. Nisin's presence also elevated neutrophil intracellular superoxide levels, normally produced by activated NADPH oxidase and prerequisite to NET formation. These nisin-induced responses in cellular representatives of two separate branches of human immune system – adaptive and innate – although leading to cell death, did not include DNA fragmentation. From these findings, we propose that nisin might trigger similar AICD mechanisms in lymphocytes and neutrophils, different from conventional apoptosis which involves DNA fragmentation. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: lantibiotic; nisin; cytotoxicity; NETs; activation-induced cell death

Introduction

Nisin is a well-known bacteriocin belonging to the category of lantibiotics, produced by *Lactococcus lactis* [1,2]. Nisin, like all other lantibiotics has some unusual thioether linkages which provide it with remarkable stability [3,4]. It exhibits a broad-spectrum antibacterial activity especially against the gram-positive bacteria [5], which has made it a popular food preservative. As it is also included in GRAS (generally recognized as safe) its utility in food preservation has grown extensively [6–8], especially in the dairy industry [1] all over the world.

Structurally, nisin consists of 34 amino acid residues and has five thioether bridges [9]. These thioether bridges are deliberately synthesized as a part of the post-translational modification of lantibiotic prepeptide. The dehydrated Ser and/or Thr residues in the prepeptide are stereo and regiospecifically linked via a thioether bridge to a nearby Cys residue and the residues thus synthesized are referred as lanthionine and/or methyl lanthionine residues [5,9,10]. This unique structural modification allows nisin to effectively kill variety of microorganisms through different strategies. It can kill the bacterium through the inhibition of cell wall synthesis, cell membrane insertion and pore formation [11,12], inhibition of spore germination, induction of premature lysis of some dividing cells etc. [13]. It is probably this pleiotropic activity of lantibiotics that limits the gain of resistance by other bacteria against them.

Involvement of peptides in human immune response is not a new concept. There are several amphiphilic cationic peptides of 20–50 amino acids which include the α- and β-defensins, cathelicidins, cecropins, magainins, bactenecins and protegrins, collectively called host-defense peptides [13]. These not only are bactericidal but can also effectively modulate the host immune functions. Thus they could be a part of innate as well as adaptive immune responses for effective clearance of bacterial pathogen. T-lymphocytes form the major part of adaptive immune system while neutrophils being a leader of innate host defense. Both these cells have there own strategies to deal with the invading pathogen. Neutrophils being the front liners tend to invade and digest the entire bacterium through phagocytosis [14,15]. While T-cells being more specialized, deal with only processed bacterial proteins to remember the pathogens' identity, so that quicker defense response could be elicited during the secondary infection. An invading bacterium could produce some extracellular structures or molecules to escape these host-defense strategies.

Nisin being a bacterial extracellular product might have some effect on human immune response. And as it is being extensively used as a food additive this study might be of great concern. Although some studies on fishes and mice have indicated short-term induction of immune response [16,17] in presence of nisin,

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no such studies have been conducted on human immune system cells.

Materials and Methods

Lymphocyte and PMN Isolation from Whole Blood

Whole blood was collected from normal healthy young males (20–25 years of age) and the mononuclear cells were collected by standard centrifugation over Ficoll-paque from the lymphocyte buffy coat. The red blood cell (RBC) and granulocyte pellet obtained was resuspended in HBSS and overlayed on 5% dextran in PBS to separate the PMNs. Both the lymphocytes and the PMNs were washed with HBSS (Invitrogen, Carlsbad, CA, USA) and suspended in RPMI 1640 (Invitrogen) for further assays. The cell viability measured by trypan blue (MP Biochemicals, Vannes, France) exclusion test was over 95%.

MTT-Based Cytotoxicity Assay

The cytotoxicity of nisin on human T-cell lymphoma Jurkat cells was determined *in vitro*. Freshly passaged Jurkat cells were centrifuged and washed with HBSS. The cell count was adjusted to 2.5×10^5 cells/ml by suspending the cells in fresh RPMI with 5% FBS (Invitrogen). This cell suspension was then transferred to a 96-well TC plate for the assay. A gradient of nisin (Himedia, Mumbai, MS, India) concentration supplemented in the medium for the assay had the final concentration 75, 150, 225, 300, 375, 450 and 525 μM. The plate was incubated at 37 °C in a humidified environment with 5% CO_2 in air for 48 h. After incubation the cells were washed and resuspended in fresh medium containing MTT (Himedia) and were further incubated for 3 h. The MTT formazan formed in the viable cells was extracted in DMSO (BIOGENE Reagents Inc., CA, USA) and read at 570 nm in the Bio-Rad ELISA plate reader. Similar test was repeated with freshly isolated and cultured human peripheral blood lymphocytes and Molt-4 cells.

Apoptosis Assay

DNA fragmentation analysis

DNA fragmentation is the biochemical hallmark of apoptosis. Jurkat, Molt-4 cells and human lymphocytes were exposed to nisin at the cytotoxic concentration and the cells were incubated at 37 °C for 6 h as well as for 48 h. The degree of DNA fragmentation was determined by performing electrophoresis, as per the protocol detailed by Barry *et al*. [18]. The treated and untreated cells were directly subjected to agarose gel electrophoresis. The cells were loaded into the wells casted in the cell lysis gel – 0.9% agarose in TBE buffer at pH 8.0 with 2% SDS and 64 μg/ml proteinase K (SIGMA-ALDRICH, ST. LOUIS, MO, US). The cell lysis gel was followed by the 2% agarose separating gel. The electrophoresis was carried out in TBE buffer pH 8.0 overnight at 35 V.

Single cell gel electrophoresis (SCGE)/comet Assay

To further assess apoptosis and DNA fragmentation, a more specific and sensitive comet assay was performed [19–21]. The Jurkat, Molt-4 cells and human lyphocytes were treated with 600 μM concentration of nisin in RPMI without serum for 3 h at 37 °C. Cells exposed to 60 μM H_2O_2 [22] were treated as positive control and untreated cells as negative control. After incubation the cells were embedded in 1% low melting agarose in PBS and were lysed in

lysis buffer at 4 °C for 2 h. The lysed cells were then incubated in 0.3 N NaOH, 1 mμ EDTA solution for 30 min at room temperature and further electrophoresed at 25 V for 20 min in the same buffer. After electrophoresis the agarose embedded cells were stained with propidium iodide (PI) (20 μg/ml) in PBS washed with chilled distilled water and observed under Motic fluorescent microscope.

In vitro Hemolytic Activity of Nisin on Human RBCs

The assay was performed as detailed by Reddy *et al*. [23]. Freshly isolated human RBCs were washed and diluted in PBS ($\sim$10^6 cells/ml). Each 1 ml aliquot of RBCs was exposed to varying nisin concentrations (0, 75, 150, 300, 450, 600 and 750 μM) and was incubated at 37 °C for 30 min. The cell free supernatants were used for spectrometric quantitation (at 540 nm) of hemoglobin released from lysed RBCs. The extent of hemolysis was compared to that of 0.1% Triton X-100.

NET Induction Assay

Whether nisin can induce NET formation was assessed by performing two parallel assays. As NET have chromatin as its major structural component and is also not membrane bound, a fluorescent DNA staining dye PI was used to visualize NETs by fluorescent microscopy. The NETs were also visualized by scanning electron microscopy.

In purified PMNs suspended in RPMI 1640 NET formation was induced by 25 μM PMA (MP Biochemicals) which was treated as the positive control [24]. An uninduced fraction of PMNs in RPMI 1640 was treated as negative control. And the NET induction ability of nisin was tested by exposing PMNs to 75 and 150 μM nisin in RPMI 1640 with and without 2% heat inactivated donor serum. The cells were incubated at 37 °C in a humidified environment with 5% CO_2 in air for 30–60 min. For fluorescence microscopy, following incubation, the neutrophils were immediately fixed with methanol, stained with 10–20 μg/ml PI (MP Biochemicals) in PBS (BIOGENE Reagents Inc.) for 5–10 min washed to remove excess stain and observed under Motic fluorescent microscope. A separate batch of PMNs after activation by PMA (MP Biochemicals) and nisin was briefly exposed to DNase I prior to methanol fixation. The extent of NET staining was compared to DNase I untreated cells.

For scanning electron microscope (SEM), after incubation, the cells were fixed in 2.5% gluteraldehyde and post fixed with 1% osmium tertroxide/1% tannic acid. Following fixation neutrophils were dehydrated with graded ethanol series, critical point dried and coated with 2 nm platinum and analyzed in JOEL analytical scanning electron microscope.

Quantitation of Neutrophil NADPH Oxidase

Spectrometric quantitation of superoxide production was used as an index of intracellular NADPH oxidase activity. Superoxide radical formation results after activation of neutrophil NADPH oxidase. Neutrophil activation by bacterial pathogens leads to intracellular oxidative burst which ultimately kills the pathogen. Neutrophil NADPH oxidase plays a major role in the production of ROC involved in oxidative burst. Thus activated neutrophils produce intracellular superoxide radicals which can be quantitated by Yellow-Nitroblue Tetrazolium (Y-NBT) reduction into an insoluble blue formazan. The formazan is extracted and solubilized in 2 M KOH and DMSO and further quantified spectrometrically at 620 nm as per the method proposed by Choi *et al*. [25]. Isolated PMNs were

resuspended in RPMI and transferred to 24-well TC plate (2.5×10^5 cells/well). The growth medium was supplemented with 100 µl of saturated NBT in PBS. PMNs stimulated with PMA (50 µM) were treated as positive control, untreated PMNs as negative control whereas nisin 150 µM exposure worked as the test. The cells were incubated at 37 °C in a humidified environment with 5% CO_2 in air for 45 min following which formazan was extracted and quantified.

Effect of Exogenous Catalase on Nisin-Induced ROS Production and NET Formation

Freshly isolated human PMNs were exposed to 150 µM nisin in presence and absence of 0.5 mg/ml catalase (∼1750 units/ml) (Himedia). 50 µM PMA-induced cells were treated as positive control whereas untreated cells were considered as negative control. Additionally an LPS (1 µg/ml) (Fluka, Kassel, Germany) stimulus was also used to induce NET formation in presence and absence of exogenous ROS scavenger-catalase and the extent of NET induction was compared. The cells were incubated at 37 °C in a humidified environment with 5% CO_2 in air for 60 min and were stained with PI. A parallel quantitative NBT assay was also carried out to show a correlation of NET induction and intracellular superoxide production in presence and absence of catalase.

Statistical Analysis

The Student's *t*-test was performed using SYSTAT Software (Systat Software Inc., Chicago, IL, USA). Data are expressed as mean ± SD and a *P* value <0.001 was considered significant.

Results

MTT-Based Cytotoxicity Assay

The assay was performed to assess the cytotoxicity of nisin on human lymphocytes *in vitro*. The model cell lines chosen for the purpose were Jurkat and Molt-4 which were exposed to a gradient of nisin concentration (Figure 1A), supplemented in the medium. The IC_{50} value of nisin as determined from the graph (Figure 1B) was 225 µM, and was found to be the same for Jurkat, Molt-4 and cultured human lymphocytes.

Apoptosis Assay

Nisin's ability to induce apoptosis was tested by performing two parallel assays. The extent of DNA fragmentation was determined by subjecting the nisin-treated and untreated Jurkat, Molt-4 and human lymphocytes (not shown) to agarose gel electrophoresis. As indicated in Figure 2 there was absolutely no difference in the DNA band pattern obtained from normal untreated cells and nisin-treated cells. To further confirm the results of electrophoresis, comet assay was performed using same cytotoxic concentration of nisin to treat the cells as was used for agarose gel electrophoresis. But even much higher nisin concentration exposure failed to induce actual comets with substantial tail length, as were induced by 60 µM H_2O_2 (Figure 3A). All the comets were graded as per Choucroun *et al.* [26] and a count of C > 1 was determined to derive a percentage of cells with DNA damage from a total of 50 randomly scored cells. There was no significant difference in the percentage of C > 1 cell in the batch of nisin-treated and untreated cells (Figure 3B).
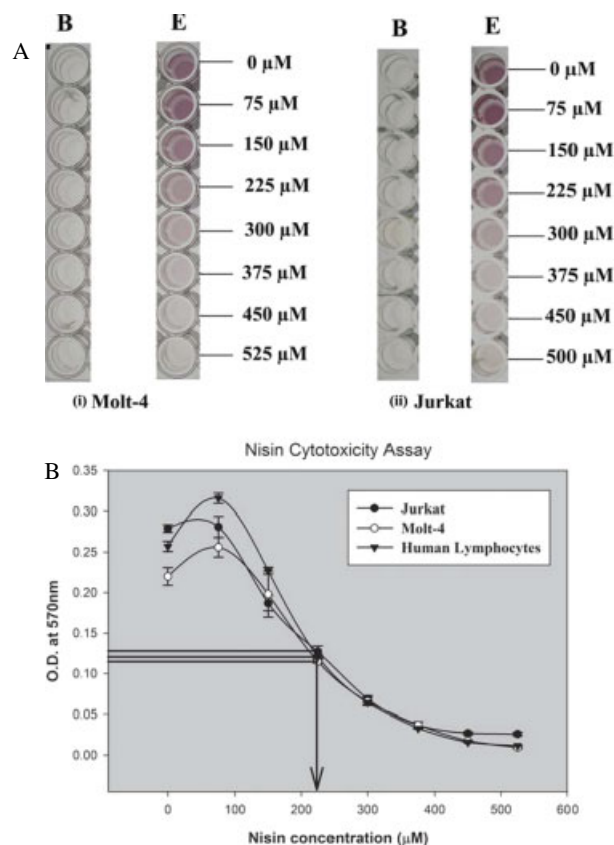

Figure 1. (A) Comparative image of wells from TC plate showing cell viability after 3 h incubation with MTT and reduced-MTT formazan extracted with DMSO. Each well was seeded with (i) Molt-4 cells in the medium supplemented with a gradient of nisin concentration (Panel E). or (ii) Jurkat. Panel B: uninoculated growth medium. (B) MTT-based cytotoxicity evaluation of nisin on lymphocytes. Respective curves showing decrease in cell viability with increase in nisin concentration marked by drop in the absorption due to reduced-MTT formazan. All absorbance values plotted are the mean of three independent assays ± SD. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

Hemolytic Activity of Nisin

Nisin was found to be only mildly hemolytic in the tested concentration range (Figure 4).

NET Induction Assay

A comparatively recent strategy employed by neutrophils to exterminate bacterial pathogens is through NET formation. Nisin being a bacterial product was tested for induction NETs *in vitro*. Nisin stimulated NET formation in isolated human PMNs at 75 and 150 µM concentration. Although it triggered NET formation both in presence and in absence of serum, better induction was observed in absence of serum (Figure 5A), which was further confirmed by SEM analysis (Figure 5B). After stimulation of neutrophils with nisin some of them were incubated briefly with DNase I prior to their methanol fixation. These DNase-treated activated neutrophils were then stained and observed. Neither PMA nor nisin-activated neutrophils showed any NET-like structures after DNase I treatment (Figure 5A). This not only confirmed the presence of NETs in nisin-induced cells but also demonstrated that a simple PI staining method can be used well for staining NETs *in vitro*.
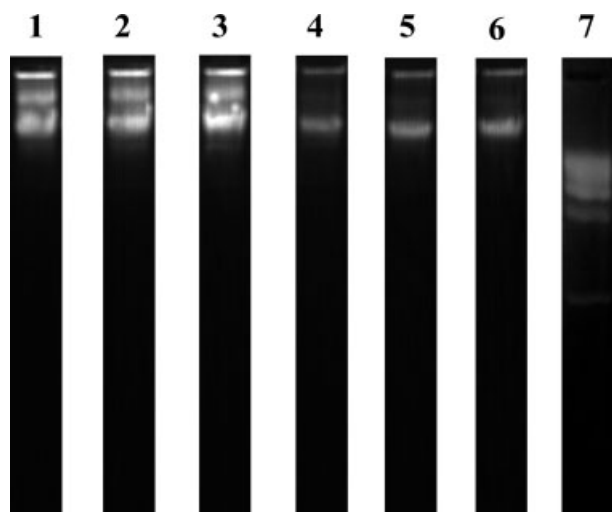
Figure 2. DNA fragmentation analysis by agarose gel electrophoresis. Lanes 1 and 2 show the pattern of DNA bands obtained from Molt-4 cells treated with 300 and 225 µM concentration of nisin for 6 and 48 h respectively, whereas lane 3 had untreated cells incubated for 48 h. Similarly, lane 4, 5 and 6 contained Jurkat cells exposed to 300 and 225 µM nisin, and untreated cells, respectively. Lane 7 contained a 500 bp DNA ladder.

**Effect of Exogenous Catalase on Nisin-Induced ROS Production and NET Formation**

Intracellular NADPH oxidase is prerequisite for activation of neutrophils and for NET formation. Superoxide radicals being the product of NADPH oxidase reaction, if quantified, could give an index of neutrophil activation. Quantification of reduced NBT formazan clearly demonstrated the ability of nisin to activate neutrophils *in vitro*. Presence of nisin caused a modest increase in the intracellular superoxide levels as compared to that in untreated neutrophils. These elevated levels of superoxides were comparable to those generated in presence of LPS. In presence of exogenous catalase there was a significant drop in the superoxide levels of stimulated and untreated neutrophils (Figure 6). Although there was this reduction in superoxides, substantial level of NET formation was still observed in nisin and LPS-induced neutrophils in the presence of exogenous catalase (Figure 7).

Discussion

Lantibiotics are unique antimicrobial peptides produced preferentially by gram-positive bacteria to kill other gram-positive bacteria and to maximize their chances of survival. Nisin is one of the best characterized lantibiotics and has a high commercial utility. Nisin is a potent bacteriocin that is reported even to inhibit bacterial spore germination [2]. Its wide use in food industry has recently evoked interest in elucidation of nisin's effect on animal cells including mammalian cells. There are a few reports about tissue macrophage phagocytosis activation by nisin in some fishes [17]. Also its oral administration has been reported to elevate T-cell population (CD8 and CD4) and deplete B-cell number in mice [16]. However, these were also shown to return back to normal level after a few days of oral intake.

In present study, nisin was found to remarkably influence some human immune system cells. The first assay determined the cytotoxicity of nisin on Jurkat and Molt-4 cells *in vitro* which could be used as a model to study its effect on human T-cells. It was shown to be cytotoxic to Jurkat and Molt-4 over 200 µM concentration. To check whether this activity of nisin is due to membrane pore formation ability of nisin as is demonstrated in gram-positive bacteria, its hemolytic activity was also assessed in the cytotoxic concentration range. As Figure 4 indicates, nisin did not exhibit any substantial hemolytic effect on human RBCs *in vitro*. This result seemed to be in good agreement with some similar
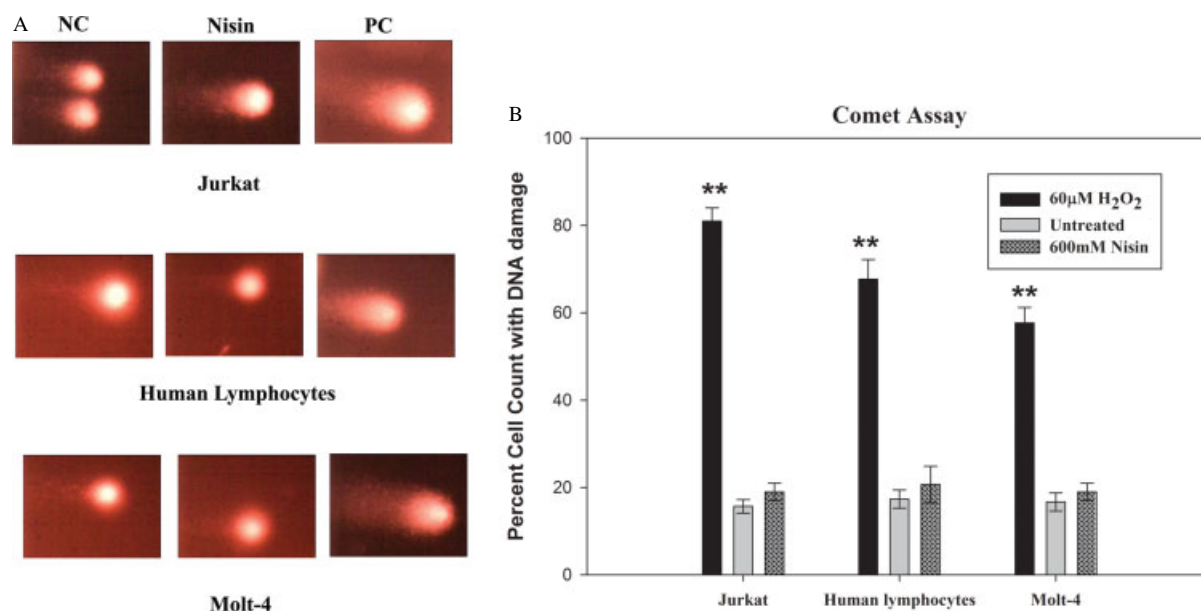
Figure 3. (A) Comet assay. The NC (Negative Control) column represents the untreated Jurkat, Human lymphocytes and Molt-4 cells. Similarly, Nisin and PC (Positive Control) column represent 600 µM nisin and 60 µM H_2O_2 exposed Jurkat, human lymphocytes and Molt-4 cells, respectively. (B) Percentage of C > 1 comets obtained from Jurkat, human lymphocytes and Molt-4 cells after exposure to 600 µM nisin and 60 µM H_2O_2 compared to those obtained from normal untreated control cells. Data expressed as mean percentage $\pm$ SD ($n = 3$) **$P < 0.001$ (control vs H_2O_2 treated cells). This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.
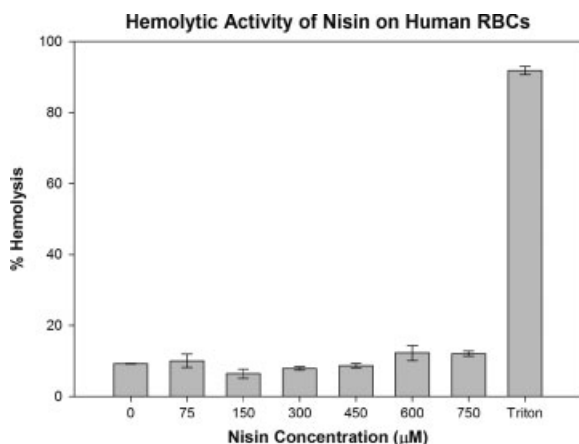
Figure 4. *In vitro* screening of hemolytic activity of nisin on human RBCs. The percent hemolysis was determined according to the spectrometric estimation of hemoglobin released from lysed RBCs in cell free supernatants. 5 µl/ml 0.1% Triton X-100 was used as positive control with ~90% hemolysis. Data expressed as mean ± SD of three independent experiments.

previously documented studies [23]. Although toward a high concentration side, what was interesting about nisin's cytotoxicity was that it induced cell death without DNA fragmentation. Thus the mechanism of death induction by nisin could be either DNA fragmentation independent apoptosis or necrosis or a totally new mechanism of cell death. Also this mode of cell death is not new to Jurkat, or isolated T-cells because some plant products like curcumin have been shown to induce such DNA fragmentation independent cell death mechanisms *in vitro* [27]. In addition to this, human lymphocytes can also undergo what is referred to as AICD in response to certain stimuli, e.g. PHA [28]. In short, nisin-induced cell death does not resemble typical apoptosis.

To test the response of innate immune system toward nisin, its effect was checked on human PMNs *in vitro*. A relatively recent,

neutrophil-driven bacterial killing mechanism was found to be activated in presence of nisin. Neutrophils were conventionally known to kill bacterial pathogens through phagocytosis followed by the formation of ROS. In this process, activated neutrophils extend their membrane protrusions to invade the bacterium which is further exposed to a burst of ROS and eventually killed. But this mechanism of bacterial killing seems to be inefficient as the neutrophils can only deal with a little number of bacteria at a time. Thus, probably, neutrophils have evolved this new mechanism which not only ensures bacterial killing but also restricts spreading of infection even after neutrophils' death. The structures produced by activated neutrophils which carry out this unique function are called NETs [24]. NET formation is known to be induced by bacterial LPS, IL-8 and PMA. In addition to these, conventional stimuli nisin was also found to induce NET formation *in vitro* in a dose-dependent manner. Patients with chronic granulomatous disease carrying mutations in NADPH oxidase do not form NETs [29]. Thus, activation of intracellular NADPH oxidase is considered prerequisite for NET formation [30] and hence induction of neutrophil intracellular NADPH oxidase by nisin was also tested. Nisin-activated neutrophil NADPH oxidase in a dose-dependent manner further supports its role in NET induction. Moreover both NET induction and NADPH oxidase activation by nisin were found to be optimal in the absence of serum which actually mimics the conditions at inflammatory sites [30].

Exogenous catalase is reported to inhibit PMA-induced NET formation so the effect of catalase on nisin-induced NET formation was also assessed. In three independent assays, nisin treatment was found to induce NET formation even in the presence of catalase (Figure 6) so a parallel NBT assay was carried out to quantitate intracellular superoxide levels in nisin-treated neutrophils in presence and absence of catalase. There was only a moderate elevation of superoxides in nisin-treated neutrophils in absence of exogenous catalase but a significant decrease in superoxides was observed in the presence of catalase in comparison to the untreated cells (Figure 7). LPS (1 µg/ml) is also reported to induce NET formation with only moderate elevation of intracellular
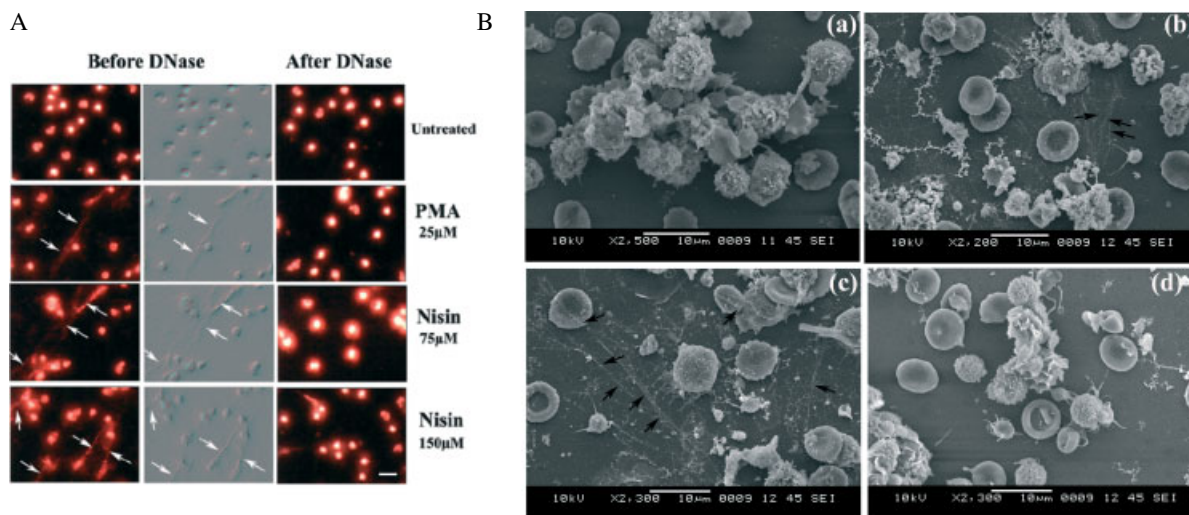
Figure 5. (A) Fluorescent microscopic examination of NET induction by nisin. The top most row represents untreated human neutrophils, where as the second, third and fourth row represents PMA, 75 and 150 µM nisintreated neutrophils, respectively. Panel (i) and (ii) are the images of NETs (white arrow) obtained in the absence of DNase I, panel (ii) presenting embossed images of panel (i) for better NET visibility. Panel (iii) shows disappearance of NETs after DNase I exposure. Bar= 10 µm. (B) Induction of NET formation by nisin. Scanning electron micrographs of isolated human neutrophils with black arrows indicating NETs (a) Untreated (b) PMA induced (c) 150 µM nisin treated in the absence of serum and (d) 150 µM nisin treated in the presence of donor serum.
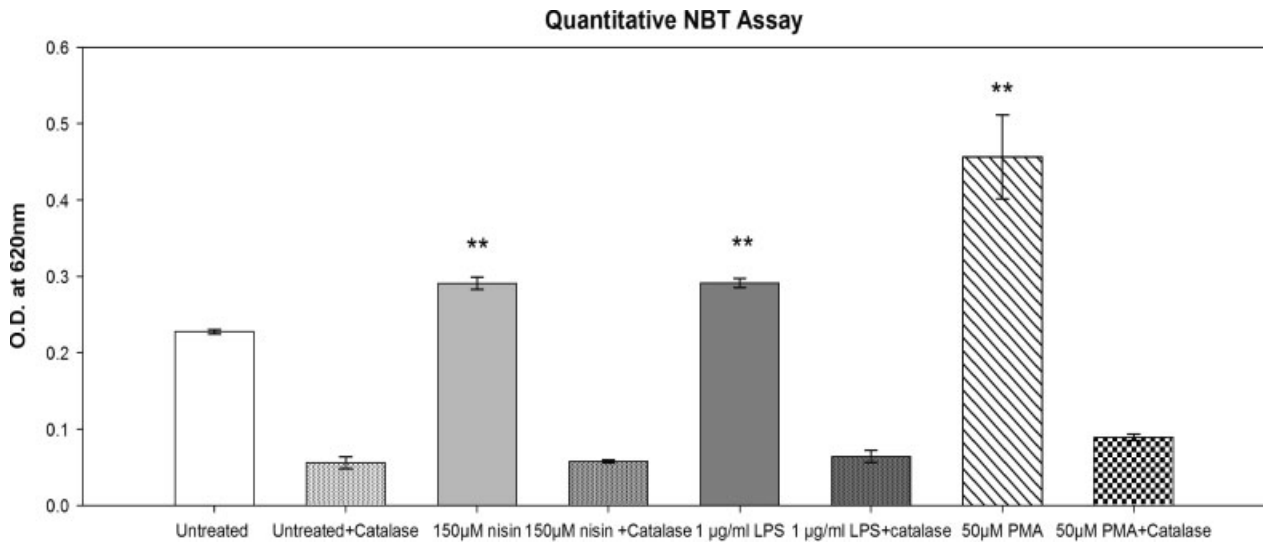
Figure 6. Intracellular superoxide quantitation in nisin- and LPS-induced human neutrophils in presence and absence of exogenous catalase by quantitative NBT assay. Data expressed as mean $\pm$ SD ($n = 3$) **$P < 0.001$ (vs negative control).
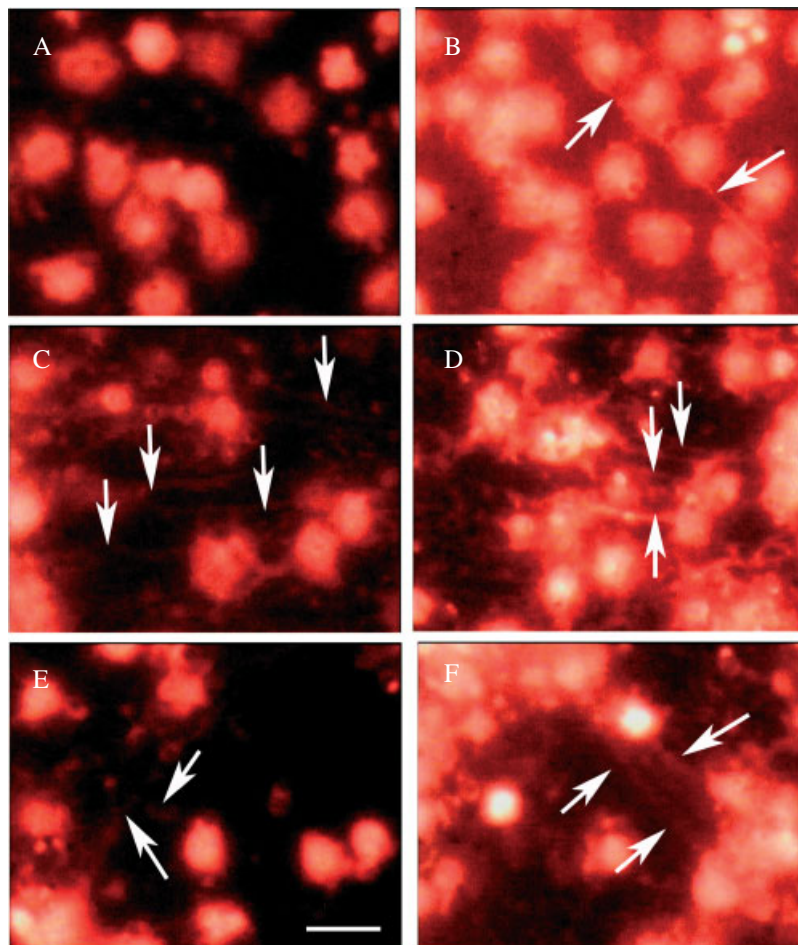
Figure 7. Comparative fluorescent microscopic examination of NET induction by nisin and LPS in presence and absence of exogenous catalase. (A) Untreated human neutrophils (B) 50 μM PMA-induced cells (C) 150 μM nisin-induced cells – catalase (D) 150 μM nisin-induced cells + 0.5 mg/ml catalase (E) 1 μg/ml LPS-induced cells – catalase (F) 1 μg/ml LPS-induced cells + 0.5 mg/ml catalase. Bar = 10 μm.

superoxides in neutrophils [31]. Thus, LPS-induced NET formation was assessed in presence and absence of exogenous catalase and was compared with that of nisin. There was a striking similarity in nisin- and LPS-induced NET formation as both triggered NET formation even in the presence of exogenous catalase. Recent reports also demonstrate that in addition to intracellular superoxides some other signals may also play critical role in NET formation [32,33]. Formation of NET marks the early events of neutrophil death as these structures are formed by soluble nuclear chromatin. Therefore, it can be said that nisin is ultimately involved in killing the neutrophils.

A striking similarity in T-cell and PMN cytotoxicity caused by nisin is, in both cases, there was no DNA fragmentation. The results of the comet assay as well as agarose gel electrophoresis assay clearly demonstrate the same, at least in case of Jurkat and isolated peripheral blood mononuclear cells (PBMCs). NET formation requires intact DNA so the possibility of DNA fragmentation in presence of nisin is obviously ruled out and is also supported by previous studies [30]. This type of neutrophil death mechanism is included in active cell death category. It is clearly different from apoptosis or necrosis and has been reported to depend upon the generation of ROS by NADPH oxidase. Thus, it is probable that even lymphocytes like neutrophils, might undergo the same mechanism of cell death which is independent of DNA fragmentation or the mechanisms might be different. If however, the same death mechanism is induced by nisin in both these cells representing two separate branches of immune system, then from the bacterial perspective, nisin is hitting two targets in the same shot. But from the human perspective the bacteria producing nisin will still be eliminated because of the neutrophil's unique strategy of bacterial killing through NET formation.

Acknowledgements

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