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Original article

IN VITRO EFFECT OF NISIN ALONE AND IN COMBINATION WITH AMIKACIN, CEFTAZIDIME AND IMIPENEM ON POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS

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Abstract

The objective of this study was to investigate the effect of antimicrobial cationic peptide nisin (32µg/mL) at minimal inhibitory concentration (MIC) alone in combination with amikacin (21µg/mL), ceftazidime (42µg/mL) and imipenem (50µg/mL) at therapeutic concentrations and their combinations with nisin on polymorphonuclear leukocyte (PMN) functions (phagocytosis and intracellular killing activity) were examined in vitro on 20 healthy volunteers whose mean age was 25.

PMNs ($1x10^7$cells/mL) were isolated by ficoll-hypaque gradient centrifugation method from venous blood with ethylene diaminetetraacetic acid (EDTA). Phagocytosis and intracellular killing activity were assayed by modifying Alexander's method. It was observed that nisin, nisin+amikacin, nisin+ceftazidime and nisin+imipenem combinations significantly decreased phagocytosis and intracellular killing activity of PMNs in healthy volunteers when compared with the control ($p<0.001$).

Consequently, while nisin alone and in combination with amikacin, ceftazidime, and imipenem showed inhibitory activity on PMN functions, amikacin, ceftazidime and imipenem at therapeutic concentraions does not impair normal PMN function.The finding of our study shows the importance of PMN functions for its potential influence in decision of rational antimicrobial selection is extremely important for success of therapy. Additional studies are necessary to elucidate the nature of these immunologic phenomenon.

Key words: *Nisin, Amikacin, Ceftazidime, Imipenem, Phagocytosis, Intracellular killing activity.*

Nisinin Tek Başına ve Amikasin, Seftazidim ve İmipenemle Olan Kombinasyonlarının Polimorf Nüveli Lökosit Fonksiyonlarına Etkisi

Çalışmamızda antimikrobik etkili katyonik peptitlerden minimal inhibitör konsantrasyonlarındaki nisinin (32µg/mL) tek başına ve terapötik konsantrasyonlarındaki amikasin (21µg/mL), seftazidim (42µg/mL) ve imipenem (50µg/mL) ile olan kombinasyonlarının sağlıklı gönüllü PNL fonksiyonları (fagositoz ve hücre içi öldürme aktivitesi) üzerine etkisi in vitro koşullarda, yaş ortalaması 25 olan 20 sağlıklı gönüllüde araştırılmıştır.

PNL'ler ($1x10^7$hücre/mL) EDTA'lı venöz kandan ficoll-hypaque gradient yöntemi ile ayrılmıştır. Fagositoz ve hücre içi öldürme aktivitesi tayininde Alexander ve arkadaşlarının yöntemi değiştirilerek kullanılmıştır.

Tek başına nisin ve nisinin, amikasin, seftazidim ve imipenem ile olan kombinasyonlarının, sağlıklı gönüllü PNL'lerinin fagositik ve hücre içi öldürme aktivitesini kontrole göre anlamlı olarak azalttığı saptanmıştır ($p<0.001$). Sonuç olarak; tek başına nisin ve nisinin, amikasin, seftazidim ve imipenem ile olan kombinasyonları PNL fonksiyonları üzerine inhibitör etki gösterirken, terapötik konsantrasyonlarındaki tek başına amikasin, seftazidim ve imipenem PNL fonksiyonlarını olumsuz etkilememiştir. Bulgularımız, infeksiyon hastalıklarının tedavisi başarısı açısından son derece önemli olan, rasyonel antimikrobiyal ajan seçiminde PNL fonksiyonlarının önemli bir etkisinin olabileceğini göstermektedir. Bu immünolojik olayın açıklanması için ilave çalışmalar yapılmalıdır.

Anahtar Kelimeler: *Nisin, Amikasin, Seftazidime, İmipenem, Fagositoz, Hücre içi öldürme aktivitesi.*

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INTRODUCTION

Antimicrobial peptides (AMPs) are important component of the natural defences of most living organisms against invading pathogens. All peptides contain the rare amino acid lanthionine. Best examples of such peptides are those produced by the bacteria. Nisin, a lantibiotic, is a peptide produced by *Lactococcus lactis* and is composed of rare amino acids like lanthionine, 3-methyllanthionine, dehydroalanine and dehydrobutyrine (1,2). The most studied member of lantibiotics is nisin. This peptide inhibits the vegetative growth of a range of gram-positive bacteria, since nisin inhibits the food-borne pathogens *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus* (3,4). Recently, it has been recognized that their function is essential to the immune response. AMPs have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells, influencing diverse processes such as cell proliferation, wound healing, cytokine release, chemotaxis, immune induction (5).

Several AMPs are currently undergoing laboratory testing but few have already reached clinical trials. The lantibiotic nisin has been developed commercially by Astra and Merck for treatment of gastric helicobacter infections and ulcers, while other nisin variants have entered preclinical trials for treating vancomycin-resistant enterococci. Also they have been shown to be protective against topical and systemic infections in combination with conventional antibiotics. AMPs look promising with their activity against especially multidrug resistant (MDR) bacteria and synergistic reactions with antibiotics (1).

Aminoglycoside antibiotics have been reported by several investigators to induce suppression of polymorphonuclear leukocyte (PMN) functions, including inhibition of cancidacidal activity, impairment of the chemotactic response and enhanced adherence (6).

Currently, β-Lactam antibiotics are widely used for the treatment of various infectious diseases because of their potent antimicrobial activities as well as their favorable adverse effect profiles (7).

Imipenem has an unusual broad spectrum, high potency and no cross-resistance with other beta-lactam antibiotics. Susceptible gram-negative species include *Pseudomonas aeruginosa*, Serratia and Enterobacter. Activity is high against *Staphylococcus aureus*, most group D streptococci, and *Staphylococcus epidermidis* but is variable against methicillin-resistant *S. aureus* (MRSA) (8).

Since levels of antibiotic resistance have been increasing at an alarming rate worldwide, AMPs seems to be the most preferable class of antimicrobial substances as therapeutic agents. Antibiotics are widely used as bacteriostatic or bactericidal drugs in the therapy of bacterial infections. When a given antibiotic causes immune depression, it may counteract its own bactericidal effect. The impact of antimicrobial drugs on the immune system already important in the patient with intact immune function and may become even more substantial in patients with immunological disease or deficiency (9).

The function of phagocytosis in the immune response has been under evaluation for a long time. The process appears to be more and more important in our defence against infection (9).

Recent clinical finding are reviewed, focusing on evaluation of efficacy, emerging resistance, potential toxicities, and combination therapy. The rapid spread of MDR bacterial pathogens necessitates the search for alternative antibacterial agents (10). To combat against rapidly emerging bacterial resistance rational approaches must be pursued to the use of older lantibiotics such as nisin. The immunomodulating activity of several antimicrobial agents was investigated in the past. Unfortunately, clinical studies with human demonstrating the relevance of these in vitro findings have yet to be reported (11). There is no much knowledge about the in vivo and in vitro immunomodulatory efficacy of the lanthionine-containing peptide nisin on PMN functions. Nisin is a potent bactericidal agent against pathogenic strains of gram-positive bacteria including MDR *S. aureus*, methicillin-resistant coagulase-negative staphylococci, a necrotizing strain of Group A streptococci, and MDR pneumococci (10).

In this study, we aimed to evaluate the in vitro effect of nisin alone and in combination with clinically used antimicrobial agents such as amikacin, ceftazidime and imipenem on polymorphonuclear leukocyte function in healthy volunteers.

EXPERIMENTAL

Subjects

The study included 20 healthy volunteers whose average age was 25. Ten milliliters of blood were taken from volunteers, all of whom were drug-free individuals. The research protocol was approved by the local Ethics Committee of Marmara University.

Antibiotics

Nisin was purchased from Sigma–Aldrich (Lot:115K1021, St Louis, Missouri, US;1.020.000 IU/g). Amikacin and ceftazidime were kindly provided by I.E. Ulagay Pharmaceutical Inc. (Istanbul-Turkey), imipenem by Merck Sharp&Dohme Pharmaceutical Inc. (Istanbul-Turkey).

Drug stock solutions were prepared using millipore (Sigma), sterilized by 0.22µm pore-diameter filter and then stored at 4°C before usage. The range of concentrations studied before were included for all drugs that take place this study. The known upper limits of safely achievable concentrations in plasma are as follows: amikacin, 21µg/mL; ceftazidime, 42µg/mL; imipenem, 50µg/mL. The susceptibility breakpoints for nisin were established as 32µg/mL, based on previous studied (12-17).

Preparation of PMN

The PMNs were isolated from the venous blood by the Ficoll-Hypaque gradient centrifugation method. Briefly, whole blood (10mL) of healthy volunteers in ethylene diaminetetraacetic acid (EDTA:0.1g/mL, Sigma) was centrifuged at 2500 rpm for 30 min, the buffy coat layer was removed, added to Ficoll-Hypaque (Histopaque-1077, Sigma-Aldrich, Inc., St. Louis, MO, USA) plus Polymorphprep (1.113±0.001g/mL, Nycomed Pharma AS, Oslo, Norway) solution and was centrifuged at 3000 rpm for 30 min. The PMN layer was removed and washed three times in Phosphate Buffered Solution (PBS). Finally, PMNs were adjusted to $1x10^7$cells/mL in Hanks' Buffered Salt Solution (HBSS). Viability of PMNs was tested by trypan blue (0.5%, in 0.9% saline solution) exclusion method by counting the stained (dead) versus unstained (alive) cells on a hemacytometer (6,18-26).

Phagocytosis and intracellular killing activity

In order to measure the uptake of microorganisms by PMNs, a clinical strain of *Candida albicans* was used. The isolate was grown on Sabouraud agar plate for 24 h at 37ºC before the experiments. Under these conditions the *C. albicans* forms only blastoconidial phase without any germ tubes or pseudohyphae. *C. albicans* viability was evaluated by exclusion of methylene blue (0.01%, Sigma) greater than 99%. These cells were suspended in HBSS and their concentration was adjusted to 10^7cfu/ml with a hemacytometer. In order to opsonize, this suspension in HBSS, to which a pool of fresh human serum (from ten samples) was added at a proportion of 4:1, was incubated at 37ºC for 30 min. in a shaker incubator. Prior to assay, 10^7 PMNs in HBSS were incubated at 37ºC for 30 min. with and without the antibiotic compounds (6,18-27).

After incubation, the suspension of PMNs and opsonized yeast cells were mixed and incubated at 37ºC for 30 min. in a shaker incubator. The mixture contained $5x10^6$PMNs/mL and $5x10^6$yeasts/mL. Five min. before incubation was complete, 1mL of methylene blue was added to each tube 1:2 (v/v) to stain the dead yeast cells. Wet mounts were prepared, and phagocytosis was determined by counting PMNs that ingested alive and dead yeast cells and the intracellular killing activity was determined by counting PMNs that included killed yeast cells on a slide

under a microscope and the result was expressed as a percentage. All assays were performed in triplicate (6,18-27).

Statistical analysis

Data were expressed as means ± standart deviation (SD). Repeated measures of ANOVA and Students Newman Keuls multiple comparisons test were performed by comparing the PMN activities of control and antibiotic-exposed PMN to calculate the value of significance (p) of the differences obtained in this study. P values less than or equal to 0.05 were considered significant.

RESULTS

Twenty different healthy volunteers served as PMN donors for testing the effect of each antibiotic on the PMN function, and the results are expressed as the mean ± SD.

Viability of PMN

The viability of the PMNs tested by trypane blue exclusion was >99% for all the drugs in the range of concentrations used.

Viability of C. albicans

Yeast viability was evaluated by exclusion of methylene blue greather was than 99%.

Phagocytosis and intracellular killing activity of C. albicans blastoconidia

The phagocytic and intracellular killing activity of PMNs was tested after pretreatment with antibiotic and was compared with the control without antibiotics. The results of the effects of nisin alone and in combination with amikacin, ceftazidime and imipenem on human PMNs in vitro are shown in Table 1 and 2.

Table 1. In vitro effects of nisin, amikacin, ceftazidime and imipenem on phagocytosis and intracellular killing activity of healthy volunteers's PMNs.

Drugs µg/mL	Phagocytosis %	Intracellular killing activity %
Nisin		
0	100 (80.65±4.51)	100(5.15±3.12)
32	38.05±10.71*	1.15±1.31*
Amikacin		
0	100 (80.65±4.51)	100(5.15±3.12)
21	80.35±5.21	6.35±3.08
Ceftazidime		
0	100 (80.65±4.51)	100(5.15±3.12)
42	81.20±5.27	5.55±2.54
Imipenem		
0	100 (80.65±4.51)	100(5.15±3.12)
50	79.05±3.79	5.35±3.35

Values are expressed as percentage of (test/control) ± SD and are means of 20 individual experiments. The control (without drug) is shown is brackets.

*$p<0.001$, As compared with the control (Repeated measures ANOVA and Student Newman Keuls multiple comparison test)

Table 2. In vitro effects of the combination of nisin with amikacin, ceftazidime and imipenem on phagocytosis and intracellular killing activity of healthy volunteers's PMNs.

Drug combination µg/mL	Phagocytosis %	Intracellular killing activity %
Nisin+Amikacin		
0	100(86.10±3.84)	100(6.75±2.45)
64+42	41.45±12.48*	1.20±0.89*
Nisin+Ceftazidime		
0	100(86.10±3.84)	100(6.75±2.45)
64+84	43.40±10.27*	1.50±1.00*
Nisin+Imipenem		
0	100(86.10±3.84)	100(6.75±2.45)
64+100	42.00±11.55*	1.05±0.83*

Values are expressed as percentage of (test/control) ± SD and are means of 20 individual experiments. The control (without drug) is shown is brackets.
*$p<0.001$, As compared with the control (Repeated measures ANOVA and Student Newman Keuls multiple comparison test)

Amikacin, ceftazidime and imipenem did not affect either phagocytosis or intracellular killing activity at concentration of 21µg/mL, 42µg/mL and 50µg/mL respectively.

Nisin alone and nisin+amikacin, nisin+ceftazidime and nisin+imipenem combination significantly decreased phagocytosis and intracellular killing activity of healthy volunteers, when compared with the control ($p<0.001$).

DISCUSSION

A serious side effect of antibiotic therapy is the development of resistance to the antibiotic used (28). Resistance among bacteria is on the rise, both in the hospital and in the community (29).

P. aeruginosa shows a particular propensity for the development of resistance. There are a limited number of antimicrobial agents with reliable activity against *P. aeruginosa*, including antipseudomonal penicillins and cephalosporins, carbapenems and fluoroquinolones. Aminoglycosides are frequently used as part of combination regimens in treatment of serious pseudomonal infections but are generally not recommended as single drugs. For each of these agents, emergence resistance during therapy has been described and has been recognized as a cause of treatment failure (30,31).

Important differences between antibiotics were evident. The clinical emergence of resistant *P. aeruginosa* has been described during imipenem therapy, ranging from 14 to 53% and occasionally leading to treatment failures. In other gram-negative pathogens, such as Enterobacter spp., resistance to broad-spectrum cephalosporins, including ceftazidime, may occur frequently, while resistance to imipenem is extremly rare (30).

Imipenem is antibacterial agents which has activity against many gram-negative, gram-positive, and anaerobic microorganisms and is often used as a last resort in infections due to MDR isolates (28).

The ability of various antibiotics to enter human PMNs and other immune system cells differs. It has been reported that imipenem binds rapidly to phagocytes (32).

Adalati et al (33) showed that imipenem (30μg/mL) affected the phagocytic function of PMNs but this was not statistically significant.

Pasqui et al (34) showed that phagocytic activity of diabetic patients after preincubation of PMNs with the highest concentration of imipenem (30 and 60μg/mL) was found to increase in vitro.

In another study which was done in our laboratory, imipenem (50μg/mL) in had no effect on the phagocytic activity of healthy volunteers PMNs but increased the intracellular killing activity of PMNs significantly (35).

In the present investigation imipenem (50μg/mL) alone did not affect either phagocytosis or the intracellular killing activity of healthy volunteers's PMNs. The combination of imipenem with nisin significantly decreased both the percentage of phagocytosis and the intracellular killing activity ($p<0.001$).

Aminoglycoside antibiotics play an important role in the therapy of serious staphylococcal infections, although emerging resistance against staphylococci is widespread (36).

Amikacin has high resistance against bacterial inactivation. To prevent the development of bacterial resistance to this very powerful antibiotic, its usage strictly is regulated. Amikacin may be combined with an "old" and more potent selective antimicrobial agents (37,38).

Many antimicrobial agents have been demonstrated to impair host immune activity, including delayed-type hypersensivity, lymphocyte transformation, and various neutrophil functions. In particular, the effects of aminoglycosides on PMN function have caused much controversy. Venezio et al (6) demonstrated that amikacin at 32 μg/mL had no significant effect on killing of *Candida* by PMNs after intravenous infusion.

In our study, amikacin at concentration 21μg/mL did not affect either phagocytosis or intracellular killing activity in vitro. Our data demonstrated that increase of intracellular killing activity for amikacin was not statistically significant. Our results supports those which have been previous published (33,6).

We found that combination of nisin+amikacin at the concentration of 64+42μg/mL significantly decreased phagocytosis and the intracellular killing activity.

Ceftazidime is a well-known paranteral cephalosporin, which is characterized by a broad antimicrobial spectrum, including *P. aeruginosa* (39). The MIC of ceftazidime for *P. aeruginosa is* 2-8μg/mL, and the plasma concentration of ceftazidime should be maintained above 4-5xMIC (10-40μg/mL) (13).

Gnarpe et al (40) found that ceftazidime at concentration of 10μg/mL did not show significant alteration on phagocytosis.

We found that ceftazidime alone, at therapeutic concentration of 42μg/mL chosen for this study, generally did not suppress phagocytosis and killing of *C. albicans* by healthy volunteers's PMNs; however, the combination of ceftazidime with nisin decreased the percentage of phagocytosis and intracellular killing activity ($p<0.001$).

The emergence of gram-negative bacteria that develop resistance to most available antibiotics and the lack of development of new antimicrobial agents have prompted the medical community to reuse of "old" drugs as an alternative therapy for patients infected MDR strains (41).

Since the discovery of polymyxins, a number of cationic peptides have been isolated from a wide range of bacterial, plant, and animal species (29).

They are a recently emerged class of antibiotics with therapeutic potential. Recent reports have shown that a synergistic effect was observed in several clinically isolated bacterial strains when some antimicrobial peptides were combined with several clinically used antibiotics. Therefore, the presence of this synergistic effect makes the cationic peptides potentially valuable as an adjuvant for antimicrobial chemotherapy against antibiotic resistant bacterial strains (29).

Nisin is a natural antimicrobial peptide of 34 amino acids and is suggested to be effective against a wide range of gram-positive bacteria, including mastitis pathogens. As an active

ingredient, nisin has been formulated into some commercially available products for teat dipping in order to prevent mastitis (42). Wu et al (42) reported that intramammary administration of nisin at a dose of 2,500,000 IU once daily for 3 days was effective in treatment of subclinical mastitis caused by several different mastitis pathogens in lactating dairy cows on a commercial dairy farm, and nisin therapy resulted in the bacteriological cure rates of 90.1% for *Streptococcus agalactiae*, 50% for *S. aureus.*

Severina et al (10) examined the efficacy of the antibiotic nisin against 56 MDR isolates of *S. pneumoniae*, 33 *S. aureus* and 29 vancomycin-resistant *Enterococcus faecium* (VRE) and *Enterococcus faecalis* isolates. The bacterial isolates used for testing are part of the large collection of resistant strains deposited at the Laboratory of Microbiology of The Rockefeller University. The investigators reported that most antibiotic-resistant pneumonococcal strains tested underwent rapid lysis during treatment with 1mg/L nisin for 20 min. and in the majority of the staphylococcal test strains, exposure to 10mg/L of nisin at 37°C for 3h resulted in an extensive drop in viable titre accompanied, and all of the 29 VRE isolates underwent on at least 10^4 fold loss of viable titre during exposure from 10 to 20mg/L nisin at 37°C for 4h. These results indicate that nisin has the powerful bactericidal activity against MDR gram-positive pathogens.

Murdock et al (43) investigated the synergistic effect of nisin (10 IU/mL, 250 IU/mL) and lactoferrin (250µg/mL, 500µg/mL) on the inhibiton of *Listeria monocytogenes* and *Escherichia coli* O157:H7. They reported that lactoferrin and nisin act synergistically to inhibit the growth of *L. monocytogenes* and *E. coli* O157:H7. This study demonstrates that lactoferrin and nisin work synergistically in reducing the levels required independently in inhibiting growth of two major foodborne pathogens.

The examples of successful use of nisin as a therapeutic agent in the treatment of bovine mastitis were reported by Delves-Broughton et al (44). In another study, Howell et al (45) reported the use of nisin to prevent gingival inflammation in a dog model. In human medicine nisin was found to be effective against *Helicobacter pylori* (46).

Giacometti et al (47) have reported that the activity of cationic peptides (e.g. cecropin PI, indolicidin and nisin) against *P.aeruginosa* and have observed that the activity of these peptides in enhanced when they are used in combination with clinically used antibiotics like polymyxin E and clarithromycin at concentrations close to their mean serum level in humans.

Recent reports have shown that the peptides may act by inserting into the cytoplasmic membrane and triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of the peptidoglycan and have direct mambrane permeabilizing activity. These damages probably allows the entry of hydrophobic compunds (15).

Giacometti et al (15) in another study showed that nisin and ranalexin alone and combination with amoxycillin, amoxycillin-clavulanate, imipenem, clarithromycin, ciprofloxacin, rifampin and vancomycin at concentration of 1 to 32µg/mL were effective against 40 nosocomial isolates of MRSA. They also reported that synergy was observed when the peptides were combined with other agents, except β-lactams. These results indicate that nisin and ranalexin are active against MRSA and exhibit a rapid bactericidal effect. Their activity is enhanced when they are combined with several antimicrobial agents.

From the above studies it is understood that AMPs are an important component of innate host defence in a wide range of organisms, from bacteria to humans. It is encouraging to know that few peptides have shown potential and desirable therapeutic properties like antimicrobial, antiviral, anticancer and contraceptive activities. Also they have been shown to protect against topical and systemic infections in combination with conventional antibiotics (1).

In spite of all the positive facts associated with AMPs, there is no data about immunomodulatory activities of the peptide.

CONCLUSION

In summary, in our study we have observed that nisin at the MIC alone and in combination with amikacin, ceftazidime and imipenem at concentrations that are safely achievable in serum and has an inhibitory activity on phagocytosis and intracellular killing activity. Our finding indicated that amikacin, ceftazidime and imipenem does not impair normal PMN functions as threapeutic levels in blood. This depressive effect on phagocytosis may be due to the binding of nisin to sterols of the PMN and concomitant cell membrane injury. It has been demonstrated that cationic peptides by disintegrating the biological membranes yield to uncoupling of the oxidative respiration (15).

It is conceivable that a drug with a stimulatory effect on phagocytosis and intracellular killing activity would be useful in the treatment of severe infections due to MDR bacteria. Therefore, additional studies are required to investigate the immunomodulating effect of AMP drugs on PMN of patients. Cationic peptides might be valuable as new antimicrobial agents.

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