Evaluation of antimicrobial peptide nisin as a safe vaginal contraceptive agent in rabbits: *in vitro* and *in vivo* studies

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Abstract

In the midst of the global epidemics of both unwanted pregnancies and sexually transmitted infections (STIs), options that provide protection are ideal. In the present study, nisin, a known antimicrobial peptide, was evaluated for safety and contraceptive potential *in vitro* and *in vivo* in the rabbit. A concentration of 400 µg nisin per ml was found to be spermicidal *in vitro*, and the effect was dose and time dependent. *In vivo* studies indicated that intravaginal application of 1 mg nisin blocked conception in rabbits. Repeated application of nisin (50 mg/animal per day) in rabbits for 14 consecutive days did not cause local inflammation or damage to the vaginal epithelium. In addition, the rate of diffusion of nisin into the blood via the vaginal mucosal epithelium, and its clearance from the circulation was found to be rapid. No treatment-related changes were observed in the reproductive performance of rabbits after cessation of treatment. Furthermore, no changes were observed in the gestation period, subsequent growth and survival of neonates in these animals. When male rats were given nisin orally for 13 consecutive weeks, no effect was observed on reproductive performance. The number of pups born, survival and growth of pups were unaltered. The affinity studies of nisin revealed that spermatozoa are more susceptible to nisin than red blood cells and vaginal epithelial cells. We suggest that nisin with spermicidal and antimicrobial properties could serve as a safe vaginal contraceptive for future therapeutic interventions in STIs.


Introduction

Overpopulation, particularly in developing countries, is complicated by the pandemic of sexually transmitted infections (STI) and human immunodeficiency virus (HIV) infections (Fenton 1996). The high incidence of these infections is owing to heterosexual intercourse, and the infections spread more readily from men to women than from women to men (Saracco 1994, D’Cruz & Uckun 2001). Male or female condoms used correctly and consistently is the only available method shown to be effective in preventing both unwanted pregnancies and ST/HIV infections (Deschamps et al. 1996, Hardy et al. 1998). But women often have little power to negotiate the use of condoms with their partners and are unable to protect themselves from nonconsensual coercive sex (Elias & Heise 1994, Anderson et al. 2002). Hence, a desperate need exists to provide people with new, easy to use, safe and affordable methods of protection that will allow women to take the necessary measures themselves without having to negotiate with their partners. Microbicides may be the answer to this problem; therefore, the development of such compounds is now recognized as an urgent global priority.

Vaginal contraceptive products have been available for many years and usually contain the membrane surfactant nonoxynol-9 (N-9) as one of the main ingredients (Zaneveld et al. 2002). However, the major drawback of using N-9 or other surfactants is their detergent-type cytotoxic effect on vaginal cells (Zaneveld 1994). Besides, N-9 is also known to inactivate lactobacilli, which form the normal flora in vaginal tissues (Richardson et al. 1998). Disturbance of the vaginal microflora can lead to vaginal infections, which in turn increase the chances of STI/HIV transmission (Martin et al. 1999).

Therefore, development of vaginal spermicidal microbicides lacking membrane toxicity may offer a significant clinical advantage over the currently marketed detergent-type spermicides (Pivot 2000). Since these compounds would probably be used repeatedly over decades, an ideal spermicidal microbicide should have an established safety record and lack genital epithelial toxicity (Zaneveld et al. 2002). It is not known when a safe, affordable and effective vaccine will be developed, or even when such a vaccine will become available. Therefore, in the interim, a dual protection vaginal microbicide capable of reducing...
the risk of STIs/HIV infection as well as controlling population is urgently needed. One such group of compounds exhibiting the above properties are antimicrobial peptides (AMPs).

AMPs are widely distributed in nature, being found in various species from bacteria to man (Mor 2000). Nisin, a bacteriocin produced by Lactococcus lactis, is one such peptide. It is a 34 amino acid, cationic peptide having a molecular mass of ~3.5 kDa. It contains several unusual amino acids such as dehydrobutyrine, dehydroalanine, lanthionine and β-methyl lanthionine (Gross & Morell 1971, Liu & Hansen 1990). Over the last 50 years, nisin has been used as a food preservative throughout the world; hence, the World Health Organization (WHO) and the US Food and Drug Administration (FDA) have conferred GRAS (generally regarded as safe) status on the peptide (Delves-Broughton 1990, Hansen 1997). In addition, nisin is reported to be nontoxic to humans (Breukink & Kruijff 1994) and possesses high bactericidal activities. Hence, it is believed to hold great promise as an antimicrobial peptide (Breukink et al. 1998, 1999). Phase I safety trials of Nisaplin (commercial nisin) are under progress to check gastrointestinal infections caused by Helicobacter pylori (Hancock 1997). We have demonstrated for the first time that, in addition to its antimicrobial effect, nisin is a potent contraceptive agent in vivo. The objective of the present study is to evaluate the safety and contraceptive efficacy of nisin, using rabbit as an experimental animal, as it would be immensely beneficial to develop a dual function vaginal compound against unwanted pregnancies and STIs.

Materials and Methods

Animals

Rabbits

Sexually mature male (mean age, 9 ± 1 months; mean body weight, 4.2 ± 0.48 kg) and female (mean age 7 ± 1 months; mean body weight, 3.8 ± 0.40 kg) New Zealand White (NZW) rabbits were maintained under standard laboratory conditions (temperature 20 ± 1 °C, relative humidity 50 ± 10% and 12h light:12h darkness cycle). Animals were housed individually in stainless steel cages. Food and water were available ad libitum.

Rats

Mature, 70-day-old male (mean body weight 100 ± 10 g) and female Holtzman strain rats (mean body weight, 80 ± 10 g) were used in the present study. The animals were maintained under standard laboratory conditions (12h light:12h darkness cycle). The Animal Care and Ethics Committee, National Institute for Research in Reproductive Health, Mumbai (Bombay, India) had approved the experimental protocols for the study. Handling of rats and rabbits was according to the guidelines for care and use of laboratory animals.

Methods

In vitro studies

Nisin Nisin (N-5764, Sigma) was dissolved in glass-distilled water, centrifuged at 10 000 g for 10 min at 4 °C and passed through a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column to separate nisin from denatured milk solids and salts. The absorbance of each fraction was measured at 280 nm with a spectrophotometer (Shimadzu, UV-160, Shimadzu, Japan). The presence of nisin was confirmed by SDS–PAGE. The fractions positive for nisin were pooled, dialyzed and lyophilized.

In vitro effect of nisin on sperm motility in rabbits

Semen samples were collected from three NZW rabbits of proven fertility via an artificial vagina (Wales et al. 1965). Sperm count and motility were assessed microscopically to ensure that males were ejaculating good-quality semen. Spermicidal activity of nisin was determined by Sander-Cramer (1941) assay. Briefly, twofold serial dilutions of nisin (5, 10, 20, 40, 80, 160, 320 and 640 µg) were prepared in physiological saline and tested for their effect on sperm motility. After mixing for 10 s, ten fields were rapidly examined under high-power magnification (×400) with a phase-contrast microscope (Olympus, 50 BX, Singapore) (×400). In each field a total of 100 spermatozoa were counted. The highest dilution of nisin that displayed complete immobilization of sperm within 20 s was taken as the minimum effective concentration (MEC).

Sperm revival test Test samples were washed with saline and centrifuged at 600 g for 20 min to remove the traces of spermicide. To the samples, which showed complete arrest of sperm motility at the 20 s time point, 500 µl buffered glucose saline were added and incubated at 37 °C for 30 min. Sperm motility was observed by placing a drop of mixture on a slide under a phase-contrast microscope (×400). Even if only a single sperm in the ten fields examined showed any sign of jerking or viability, the test was recorded as ‘fail’. If none of the spermatozoa showed any sign of viability, it was recorded as ‘pass’. Sperm viability was determined by eosin-nigrosin staining (WHO 1999).

In vitro effect of nisin on red blood cells (RBCs)

The effect of nisin on RBCs was determined with freshly isolated rabbit erythrocytes (Mandal & Nagaraj 2002). Heparinized blood in isotonic PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.0) was initially centrifuged at 2000 g for 10 min to remove the buffy coat. Aliquots of erythrocyte suspension (≈10¹⁶ cells/tube) were incubated at 37 °C for 30 min along with twofold serial dilutions of nisin (0, 5, 10, 20, 40, 80, 160, 320 and 640 µg/ml). After centrifugation, 100 µl of the supernatant from each dilution were transferred to a 96-well microtitrater plate. The hemoglobin
release was monitored by measuring the absorbance (A) at 450 nm with an ELISA reader (ELX-800, BioTek Instruments, Winooski, VT, USA). Percent hemolysis was calculated by the following formula: % hemolysis = [(A450 in the peptide solution – A450 in PBS) / (A450 in 0.1% Triton X-100 – A450 in PBS)] × 100. Zero and 100% hemolysis was determined with isotonic PBS and 0.1% Triton X-100 respectively.

In vitro effect of nisin on viability of sperm, RBCs and vaginal cells  The viability of rabbit spermatozoa, RBCs and endocervical vaginal epithelial HeLa-S3 cells (National Center for Cell Science, Pune, India) in the presence and absence of nisin was determined by (3-[4-5-dimethylthiazol-2-4]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay (Korting et al. 1994). This assay is based on the reduction of MTT by the mitochondria and/or cytoplasmic enzyme dehydrogenases – succinate dehydrogenase in particular – to form an insoluble, dark blue formazan product. Only viable cells having dehydrogenase activities are able to reduce significant amounts of MTT dye to formazan.

RBCs were obtained after fractionation of blood by the Ficoll-Hypaque gradient method. After washing (three times) in RPMI-1640 medium 10⁶ RBCs/well were transferred to a 96-well microtiter plate.

Rabbit spermatozoa were separated from seminal plasma by the swim-up method. The cells were washed in RPMI medium, and 10⁴ spermatozoa were added per well in a 96-well microtiter plate.

Exponentially growing HeLa epithelial cells were seeded into a RPMI 1640 well plate at a density of 10⁶ cells/well and incubated for 24 h at 37°C prior to exposure to a contraceptive dose of nisin. On the day of treatment, RPMI medium was replaced with fresh medium. Twofold serial dilutions of nisin (0, 5, 10, 20, 40, 80, 160, 320 and 640 µg/ml) were prepared in RPMI medium and added to the wells containing spermatozoa, RBCs and vaginal cells. Plates were incubated for 1 h before adding 10 µl MTT solution (5 mg/ml in 0.1 M PBS, pH 7.2). Cells containing only medium and MTT were used as negative controls. The formazan reaction was allowed to proceed for 1 h at 37°C in a 5% CO₂ atmosphere. MTT reaction medium was discarded by centrifugation, and the dark crystals remaining were dissolved in 100 µl solubilizing buffer (10% sodium dodecyl sulfate in 0.1 mol/l HCl). The optical density (OD) at 570 nm was measured with a 96-well multispecimen ELISA reader with the solubilizing buffer serving as blank. To translate the OD570 values into the number of live cells in each well, the OD570 vs cell number curves were generated. The percent viability was calculated by the following formula: % cell viability = OD of the test sample/OD of the control sample × 100. The results were expressed as mean ± S.M. of three independent experiments. The minimum effective concentration (MEC) was defined as the highest dilution that displays 100% reduction in cell viability.

The viability of these cells was also tested by propidium iodide (PI) (Sigma) (Anderson et al. 2002). Briefly, HeLa cells, RBCs and spermatozoa were incubated for 1 h as above in the presence of various concentrations of nisin. The cells were removed from the wells with 0.1% trypsin and treated with PI (5 µg/ml) for 5 min. Red fluorescence emission of PI was detected by fluorescence microscopy (Olympus, 50 BX, Singapore). Cells that had high PI fluorescence were considered membrane damaged and nonviable.

In vivo studies

Experiment 1: effect of intravaginal application of nisin on fertility in rabbits  For this study, a total of 24 does and six bucks was used in three fertility trials. Eight healthy does were divided into two groups (control and treated) of four animals each. Ovulation was not induced in rabbits before mating. Although several investigators have used ovulated rabbit models, our experience is that this is not necessary, as we observe conception rates close to 100% in control animals. Moreover, natural mating is less invasive to the animal and most closely mimics that in humans. From our preliminary studies, 1 mg intravaginal nisin was selected for vaginal application. Nisin (1 mg) was deposited by inserting 5–6 cm of a 1 ml tuberculin syringe into the vagina, and the does were held in the supine position for a period of 10–15 min. These does were kept under observation for a period of 35 days.

Experiment 2: bioavailability of nisin in the vaginal lavage and assessment of fertility in rabbits  The purpose of this experiment was to determine the bioavailability of nisin in the vaginal lavage collected at different time intervals after single intravaginal administration of contraceptive dose of nisin. Healthy and mature NZW rabbits were divided into five groups consisting of four animals each. Two does in each group were used to measure residual nisin levels present in the vaginal lavage following the intravaginal application of nisin. The remaining two does in each group were used for fertility studies as mentioned below. ELISA was used to measure the levels of nisin in the vaginal lavage. Briefly, 200 µl saline were introduced with a 1 ml tuberculin syringe by inserting 5–6 cm into the vagina. A volume of 300–400 µl of vaginal lavage collected from each rabbit was diluted to a required concentration with sodium carbonate bicarbonate buffer (0.1 M, pH 9.6) and coated onto a polystyrene microtiter plate (Nunc, Roskilde, Denmark). Polyclonal antibodies to nisin were raised in NZW rabbits, and the titers were found to be 1:10⁴. The detection limit for nisin in the competitive direct ELISA with generated antibodies was 1 µg/ml. Antisera raised against nisin Z cross-reacted with nisin A peptide but did not recognize other peptides from the lantibiotic family. After overnight incubation at 4°C, the plate was washed (three times) with PBS-T and

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reaction was stopped with 1 M H2SO4 and the absorbance at room temperature in darkness for exactly 20 min. The absorbance was measured at 492 nm with an ELISA reader (ELX 800, Biotek Instruments Inc., Winooski, VT, USA). Preimmune rabbit serum was used as negative control.

**Effect of delayed mating after intravaginal application of nisin on pregnancy outcome in rabbits** This experiment was similar to experiment 1, except that the mating was delayed for various lengths of time after intravaginal administration of nisin (1 mg) to test the in vivo stability and retention of the peptide. For each fertility trial, does in subgroups of two were allowed to mate at various time intervals after placing the peptide into the vagina; that is, at 15 min (group 1), 30 min (group 2), 1 h (group 3), 3 h (group 4) and 6 h (group 5). Mating was confirmed by the presence of spermatozoa in the vaginal lavage.

**Experiment 3: safety studies of nisin in rabbits** Dermal irritation test Amounts of 50 µg nisin and saline (placebo) were applied topically once daily for 5 consecutive days, to one intact and one abraded test site per rabbit. Each group consisted of three mature rabbits. The test site was occluded by covering each site with a gauze pad and over wrapping the site with plastic wrap. On day 6, dermal irritation was scored according to the Draize scoring system (Draize 1965).

**Effect of 14-day (subacute) vaginal administration of high dose of nisin on local and systemic toxicity in rabbits** To evaluate the local and systemic toxic effect of nisin, 12 mature NZW rabbits were divided into two groups (control and treated) of six animals each. Nisin at the dose of 50 mg/day was administered intravaginally for 14 consecutive days. All the six animals of the placebo group received 1 ml of vehicle. On day 15, three animals from each group were autopsied, and the remaining three animals were used for fertility studies.

**Effect on fertility in rabbits** Twenty-four hours after the completion of 14-day treatment, three animals each from control and treated groups were mated with proven fertile bucks. The females were allowed to complete their pregnancies. The litter size, as well as the weight and general condition of the offspring, was monitored for a period of 3–4 weeks to measure the perinatal effect of nisin.

**Effect on hematologic and serum biochemical parameters in rabbits** At autopsy, 20–25 ml blood were collected from the control and treated rabbits to estimate the hematologic and serum biochemical profiles. Hematologic parameters were analyzed with the 920 Autocounter (Swelab Instruments, Stockholm, Sweden), which was standardized for rabbit blood. The hematocrit, hemoglobin, erythrocyte count (RBC) and differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils and basophils) and clinical chemistry profiles (total proteins, albumin, blood urea nitrogen, creatinine, aspartate aminotransferase (AAT), alanine aminotransferase (AlAT), glucose, sodium, potassium and calcium) were determined in the serum samples of control and treated animals.

**Effect on vaginal morphology in rabbits** The rabbits were necropsied, vaginal tissue was slit open ventrally between the urethral orifice and fornix, and representative samples of the proximal, middle and distal portions were collected and fixed in Bouin’s solution. Paraffin sections 5 µm thick were cut and stained with hematoxylin-eosin. Vaginal sections were evaluated semiquantitatively by four basic criteria: epithelial ulceration, leukocyte infiltration, edema formation and vascular congestion. The magnitude of vaginal changes was rated according to the treatment-related tissue abnormalities (Eckstein et al. 1969). A score of 0–11 (none to intense) was given to each of them and compared with controls. A total score between 0 and 4 was considered acceptable, an intermediate score of 6–8 was marginal (either acceptable or unacceptable) and 9–11 was unacceptable.

**Experiment 4: quantification of nisin in vaginal lavage and blood in rabbits** This experiment was designed to measure nisin levels in the circulation (rate of diffusion and clearance) by ELISA. Blood levels after administration of 5 mg nisin or less were below the sensitivity of the ELISA. For this study, a total of 18 rabbits was used. After intravaginal application of a single dose of nisin (50 mg/ml), the animals were divided into six groups of three animals each. A total of 300–400 µg vaginal lavage and 3–4 ml blood was collected at the same time but at different time intervals (0.30, 1, 3, 6, 12 and 24 h). Serum was separated by centrifugation and kept at –70°C until use. The rest of the procedure was same as described in experiment 2.

**Experiment 5: effect of oral administration of nisin for 13 weeks (subchronic) on general health and reproductive performance of male rats** This experiment was designed to study the effect of nisin on the general health and reproductive capacity of male rats upon oral administration of nisin for 13 consecutive weeks. Mature male rats were randomly divided into two groups.
Acute oral toxicity of nisin was determined by the limit test (Gad & Chengelis 1998). Nisin was dissolved in distilled water and administered by oral gavage once daily, at a dose level of 10 mg/kg body weight for 13 weeks. The animals were monitored for appearance, behavior, pharmacotoxic signs and reproductive performance for 3 weeks after administration. On day 92, three animals from each group were killed by inhalation of anesthetic ether. Prior to autopsy, 3–4 ml blood were collected directly by cardiac puncture for hematologic and serum biochemical analysis. The vital nonreproductive (liver, kidney and brain) and reproductive tissues (testes, epididymis, prostate and seminal vesicles) were removed and blotted, and weights were recorded. The remaining three animals from each group were cohabited with proven fertile females (1:2) in the proestrus–estrus transition phase during the first week after treatment. Duration of gestation, number of pups and weight of pups were recorded. After successful mating, the male rats were autopsied, and the epididymis was removed. Spermatozoa were retrieved in physiological saline, and total sperm count and motility were recorded. The experiment was repeated twice with a similar number of rats.

**Tissue somatic indices (TSI) of reproductive and nonreproductive tissues in rat after 13-week oral administration of nisin**

The weights of the rats and various reproductive tissues (testes, epididymis, prostate and seminal vesicle) and nonreproductive tissues (liver, brain and kidney) were recorded 24 h after the completion of 13-week oral administration of nisin. The TSI (percent organ weight in relation to body weight) were determined.

**Statistical analysis**

All data points are expressed as mean±S.D. Differences in sperm motility and viability between control and nisin-treated samples were analyzed by Student’s paired t-test (Sokal & Rohlf 1996). Serum hematologic and biochemical profiles were expressed as mean and standard deviation (S.D.). A P value of <0.01 was considered statistically significant.

**Results**

**In vitro effect of nisin on sperm motility in rabbits**

Nisin was highly spermicidal with a mean MEC value of 400 μg/ml as determined by the Sander–Cramer test (Fig. 1). At this concentration, nisin causes complete immobilization of spermatozoa within 20 s. The effect was found to be dose and time dependent. Motility was not restored in the nisin-treated immotile spermatozoa, even after incubation in buffered glucose solution for 30 min. Immediately after nisin-induced sperm immobilization, sperm viability decreased significantly (P < 0.001) as compared with control. The viable sperm count further declined with time and was less than 1% after 4 min incubation.

**In vitro effect of nisin on RBCs**

The effect of nisin (10–640 μg/ml) on RBCs was tested in vitro. Hemolysis was not observed up to a concentration of 600 μg, but it increased thereafter with increasing dose of nisin (Fig. 2).

**In vitro effect of nisin on the viability of spermatozoa, RBCs and vaginal cells**

Selectivity and/or affinity of nisin towards spermatozoa, RBCs and vaginal epithelial cells was studied in vitro by the MTT assay. The results indicated that the toxic effect of nisin on RBCs and vaginal epithelial cells was minimal when compared with its effect on spermatozoa (Fig. 3). The membrane affinity of nisin towards these cells was found to be in the order of HeLa < RBCs < spermatozoa. These results were further confirmed by measuring the
nisin did not show any change in cell viability.


Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of mating after treatment (h)</th>
<th>Number of does used</th>
<th>Bioavailability of nisin in vaginal fluid after treatment (µg)</th>
<th>Number of does becoming pregnant (%)</th>
<th>Number of pups delivered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15</td>
<td>4</td>
<td>836 ± 17.18</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.30</td>
<td>4</td>
<td>420 ± 11.34</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4</td>
<td>94 ± 3.69</td>
<td>1/4</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>4</td>
<td>52 ± 3.01</td>
<td>2/4</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>4</td>
<td>40 ± 2.30</td>
<td>3/4</td>
<td>8</td>
</tr>
</tbody>
</table>

The susceptibility of cells to nisin was found to be in the order of sperm (< RBCs (< vaginal cells (>). Control wells without nisin did not show any change in cell viability.

Contraceptive efficacy of nisin in rabbits

When nisin (1 mg) was applied intravaginally to a group of four rabbits just before mating, no pregnancies followed. The study was repeated three times using the same number of rabbits, and similar results were obtained.

Bioavailability of nisin in the vaginal fluid and assessment of fertility in rabbits was determined after intravaginal administration of 1 mg nisin. The results showed that at 15 min, the concentration of nisin was 836 µg/ml, while it was 40 µg/ml at 6 h. The animals that mated at 15 min (group 1) did not become pregnant, whereas the animals of the remaining three groups became pregnant (Table 1). The experiment was repeated twice and similar results were obtained.

Delayed mating of rabbits after intravaginal application of nisin and its effect on pregnancy outcome

The contraceptive efficacy of intravaginally administered spermicides is also dependent on the time interval between delivery of the test compound and mating. Therefore, in this experiment, rabbits in subgroups of four were allowed for mating with proven fertile bucks at 15 min, 30 min, 1 h, 3 h and 6 h after intravaginal administration of nisin (1 mg). Nisin provides variable but significant inhibition of fertility in vivo. Moreover, the observed reduction in fertility by nisin was consistent over the course of 3 h. The results indicated that >50% activity was evident even when mating was delayed until 30 min after intravaginal administration of nisin (Table 1).

Effect of 14-day (subacute) treatment of high dose of nisin on structural changes in the vaginal epithelium and systemic toxicity in rabbits

Mortality did not occur, and there were no toxic signs attributed to intravaginal exposure of rabbits to a high dose of nisin for 14 consecutive days. At necropsy, no gross abnormalities in the vaginal tissue were seen after treatment with 50 mg nisin. In dermal sensitization studies, no edema or irritation was observed in any of the rabbits after treatment with 50 µg of contraceptive dose for 5 consecutive days. Light microscopic examination of vaginal epithelium of rabbits was evaluated before and after nisin treatment. Intravaginal administration of the contraceptive dose of nisin (1 mg) for 14 consecutive days did not induce inflammatory reactions in the vaginal epithelium, and no effect was seen on tissue histopathology. There was no edematous thickening of the submucosal layer or infiltration of polymorphonuclear leukocytes into the mucosa. The overall mean semiquantitative score of the microscopic analysis showed that the changes did not exceed the definition for acceptability (data not shown).

The maximum amount of nisin administered was detected by ELISA in the serum samples drawn 60 min after treatment. At this point of time, the levels were low in the vaginal lavage. This shows an inverse relation of nisin levels between vaginal lavage and blood (Fig. 4). The retention time of nisin in the circulation declined rapidly thereafter and reached control levels by 12 h.

Effect of 14-day treatment of high dose of nisin on fertility in rabbits

When vehicle controls and nisin-treated rabbits were evaluated for their fertility within 24 h after the completion of 14-day intravaginal treatment, we confirmed that the high dose of nisin (50 mg/day/animal) had no adverse effect on subsequent fertility. No treatment-related changes in gestation (31 ± 1 day), number of pups delivered (4–6) or weight of pups (60 ± 10 g) were observed. The growth of the pups was observed for 30 days and was found to be normal and similar to the control groups.

Effect of 14-day treatment of high dose of nisin on hematologic and serum biochemical profiles

Nisin did not cause any abnormalities in hematologic and serum biochemical profiles after the treatment. The values of hematologic parameters, including hemoglobin percentage and total RBC, white blood cells, neutrophils, monocytes, lymphocytes and eosinophils, were within the normal range and did not vary after the treatment (Table 2). Analysis of serum biochemical profiles revealed no significant treatment-related differences between treated and control animals (Table 3).

Effect of oral administration of nisin for 13 weeks on general health and reproductive performance of male rats

Tissue somatic indices (TSI) of various reproductive and nonreproductive tissues in control and nisin-administered rats for 13 weeks (5 mg/kg body weight). Each value is the mean ± s.d. of three observations.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3.69 ± 0.264</td>
<td>3.73 ± 0.264</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.964 ± 0.07</td>
<td>0.954 ± 0.06</td>
</tr>
<tr>
<td>Brain</td>
<td>0.412 ± 0.03</td>
<td>0.420 ± 0.03</td>
</tr>
<tr>
<td>Testis</td>
<td>0.452 ± 0.038</td>
<td>0.461 ± 0.03</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.699 ± 0.07</td>
<td>0.687 ± 0.06</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.241 ± 0.01</td>
<td>0.235 ± 0.01</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>0.397 ± 0.02</td>
<td>0.383 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4 Tissue somatic indices (TSI) of various reproductive and nonreproductive tissues in control and nisin-administered rats for 13 weeks (5 mg/kg body weight). Each value is the mean ± s.d. of three observations.

RBC, red blood cells; WBC, white blood cells.

Table 3 Serum biochemical profiles of rabbits administered nisin (50 mg/day) for 14 consecutive days. No significant difference between control and treated was observed. Each value is the mean ± s.d. of six observations from three animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg %)</td>
<td>102 ± 8.16</td>
<td>109.96 ± 8.84</td>
</tr>
<tr>
<td>Calcium (mg %)</td>
<td>14.36 ± 1.64</td>
<td>16.26 ± 3.26</td>
</tr>
<tr>
<td>Phosphorus (mg %)</td>
<td>4.8 ± 0.26</td>
<td>4.31 ± 0.19</td>
</tr>
<tr>
<td>Uric acid (mg %)</td>
<td>4.36 ± 0.31</td>
<td>4.18 ± 0.23</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg %)</td>
<td>16.87 ± 1.63</td>
<td>20.0 ± 1.17</td>
</tr>
<tr>
<td>Creatinine (mg %)</td>
<td>1.35 ± 0.02</td>
<td>1.40 ± 0.026</td>
</tr>
<tr>
<td>Total proteins (gm %)</td>
<td>6.9 ± 0.32</td>
<td>7.03 ± 0.41</td>
</tr>
<tr>
<td>Albumin (gm %)</td>
<td>23.81 ± 0.04</td>
<td>2.81 ± 0.3</td>
</tr>
<tr>
<td>Sodium (meq/l)</td>
<td>142.0 ± 11.06</td>
<td>139.0 ± 10.63</td>
</tr>
<tr>
<td>Potassium (meq/l)</td>
<td>5.15 ± 0.32</td>
<td>4.37 ± 0.41</td>
</tr>
<tr>
<td>Chloride (meq/l)</td>
<td>110.0 ± 9.26</td>
<td>99.94 ± 6.67</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/l)</td>
<td>44.0 ± 3.01</td>
<td>43.16 ± 3.17</td>
</tr>
<tr>
<td>AAT (IU/l)</td>
<td>26.02 ± 2.07</td>
<td>30.11 ± 3.67</td>
</tr>
<tr>
<td>Total proteins (gm %)</td>
<td>6.9 ± 0.32</td>
<td>7.03 ± 0.41</td>
</tr>
</tbody>
</table>

Table 2 Hematologic parameters for rabbits administered nisin (50 mg/day) for 14 consecutive days. No significant difference observed in any of the parameters between control and treatment animals. Each value is the mean ± s.d. of six observations from three animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (&gt;10^6/mm^3)</td>
<td>5812 ± 338</td>
<td>6413 ± 515</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>43.26 ± 0.88</td>
<td>43.87 ± 0.82</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>WBC (&gt;10^3/mm^3)</td>
<td>7053 ± 477</td>
<td>6998 ± 394</td>
</tr>
<tr>
<td>Total neutrophils (%)</td>
<td>55 ± 3.0</td>
<td>56 ± 3.6</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>1.71 ± 0.16</td>
<td>1.65 ± 0.12</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>38.22 ± 2.7</td>
<td>39.0 ± 2.91</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.08 ± 0.17</td>
<td>1.02 ± 0.16</td>
</tr>
</tbody>
</table>

Figure 4 Levels of nisin in the vaginal lavage (A) and serum (B) after intravaginal administration in rabbits (50 mg/day per 14 days) as determined by ELISA. Peak levels of nisin were detected at 1 h in serum and were found to be low in the vaginal fluids. The levels declined rapidly in the serum and reached baseline level by 12 h. Each bar represents the mean ± s.d. of three determinations.

Effect of oral administration of nisin for 13 weeks on general health and reproductive performance of male rats

TSI in rats after 13-week oral administration of nisin (5 mg/kg per day)

In the oral toxicity studies in rats, no abnormal weight changes in the body (Fig. 5) or reproductive (testes, epididymis, prostate and seminal vesicle) and nonreproductive tissues (liver, kidney and brain) and TSI were observed (Table 4). No change was observed in the total sperm count retrieved from cauda epididymis. The results also indicated that there were no biologically significant differences between nisin dosed and placebo in their hematologic and serum biochemical profiles (data not shown). The reproductive performance of the treated rats remained unaffected. Furthermore, nisin treatment did not reduce the number of pups born (6–10 in both groups), weight (3–4 g) and general health of the pups with no perinatal or postnatal repercussions. Growth of the pups was
required to completely immobilize spermatozoa within 20 s. The effect was found to be dose and time dependent (Fig. 1). This concentration is 20-fold higher than the minimum inhibitory concentration (MIC) required to inhibit the growth of various pathogens (MIC of nisin for pathogens: 10–50 μg/ml) (Breukink et al. 1999). Nisin possesses spermicidal activity to a certain extent even at much lower concentrations (<100 μg/ml) but at longer times of exposure (4 min). The loss of motility was completely irreversible, since the immobilized sperm resuspended in buffered glucose solution did not regain motility. The exact mechanism by which nisin interacts with sperm has yet to be elucidated.

An interesting observation of the present study is the selective action of nisin on spermatozoa, RBCs and HeLa vaginal epithelial cells. The results indicate that nisin does not show a cytotoxic effect on RBCs and vaginal cells at a concentration (400 μg/ml) that is toxic to spermatozoa. This suggests that nisin interacts with different cell membranes in a selective manner, and this could be attributed to the following hypotheses: 1) Nisin can interfere with sperm motility by inhibiting the activity of mitochondrial enzyme, succinate dehydrogenase (SDH). Since SDH is known to be involved in the conversion of MTT into formazan, the low levels of formazan in nisin-treated spermatozoa (as measured by ELISA) could reflect reduced SDH activity. In view of these observations, suppressed oxidative metabolism in sperm may be envisaged (Reddy et al. 1998). 2) Since most AMPs are cationic and amphipathic in nature, they are known to interact with cell membranes rich in acidic phospholipids and possessing a negative transmembrane potential (De Wall et al. 1991, Tytler et al. 1995, Wieprecht et al. 1997, Rocca et al. 1999). The higher affinity of nisin for spermatozoa may be due to the presence of anionic phospholipids such as phosphatidylglycerol and phosphatidylserine in the plasma membrane. In contrast, the RBC membrane consists predominantly of zwitterionic phospholipids (such as phosphatidylcholine and sphingomyelins) which have neutral charge (Rocca et al. 1999) and therefore can escape from nisin attack at a concentration that is spermicidal. A higher concentration of nisin may be required to lyse RBCs.

In contrary to sperm and RBCs, vaginal cell membranes are generally characterized by a low content of negatively charged phospholipids (such as phosphatidylinositol, phosphatidylglycerol and phosphatidylserine), a high amount of cholesterol (Reddy & Aranha 2000) and an inner high negative transmembrane potential (De Wall et al. 1991), making them more resistant to nisin. However, further study is needed.

In the present study, we also evaluated the in vivo contraceptive efficacy of nisin, since compounds that impair sperm motility in vitro are not necessarily contraceptive in vivo (D’Cruz et al. 1998). For example, compounds such as heparin and dextrin sulfate immobilize sperm in vitro but are not contraceptive in vivo (Parrish et al. 1989). The spermicidal dose required for the control of fertility varies between species (Reddy et al. 1996). For the present study, the contraceptive efficacy of nisin was evaluated using rabbit as an in vivo model. Since rabbits are similar to humans in several reproductive physiological features, they serve as useful animal models to study compounds that have not been shown to be safe for human intravaginal use (Castle et al. 1998). Our results indicated that 1 mg nisin is sufficient to arrest sperm motility and protect

Discussion

Nisin has been widely used as a food preservative worldwide for the past 50 years. The fact that it has attained GRAS status reflects its relative safety in humans. Though its antimicrobial properties have been extensively studied (Breukink & Kruifj 1994), its potential as a microbicide/spermicide has hardly been explored. This is the first report indicating the spermicidal activity of nisin. The results from the above experiments clearly demonstrate that nisin possesses contraceptive properties both under in vitro and in vivo conditions. Such contraceptive antimicrobial compounds are of particular clinical interest because consumer preference studies suggest that most women worldwide prefer a vaginal prophylactic product that is both antimicrobial and contraceptive (Zaneveld et al. 2002). Earlier in vitro studies by other investigators and our own studies revealed that magainin A is spermicidal (Edelstein et al. 1996, Reddy et al. 1996) and an effective contraceptive agent in rats and rabbits (Reddy et al. 1996, Reddy & Manjramkar 2000).

Generally compounds that fail to immobilize sperm within 20 s are unacceptable because of the rapidity with which sperm migrate into the cervix and upper reproductive tract. Hence the Sander–Cramer test was used to identify the minimum concentration of spermicide required to completely immobilize spermatozoa within 20 s. In vitro studies show that nisin at a concentration of 400 μg/ml causes complete immobilization of sperm within 20 s. The effect was found to be dose and time dependent (Fig. 1). This concentration is 20-fold higher than the minimum inhibitory concentration (MIC) required to inhibit the growth of various pathogens (MIC of nisin for pathogens: 10–50 μg/ml) (Breukink et al. 1999). Nisin possesses spermicidal activity to a certain extent even at much lower concentrations (<100 μg/ml) but at longer times of exposure (4 min). The loss of motility was generally

Figure 5 Changes in body weight of control (−) and treated (●) rats during the 13-week oral administration of nisin (5 mg/kg body weight). The weight gain of rats was normal, and no treatment related abnormalities were observed when compared with saline treated controls. Values are the mean of six animals.

observed until the end of lactation and was found to be normal and similar to the control group.

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against pregnancy. To evaluate the relationship between availability of nisin in the vagina and pregnancy outcome, mating was delayed for various periods after intravaginal application of nisin (Table 1). Since efficacy of vaginal spermicidal products is dependent on the time interval between application and mating (D'Cruz & UcKun 2003), the present results suggested that nisin blocks conception even when mating is delayed for 30 min. Furthermore, complete arrest of sperm motility was seen within 20 s with 400 μg nisin compared with 1 mg magainin A, indicating that the spermicidal efficacy of nisin is significantly higher than that of magainin A (Reddy et al. 1996).

For the evaluation of the safety of new vaginal formulations, it is important to study the toxic effects resulting from repeated intravaginal application. Rabbits have a simple cuboidal or columnar epithelium, which is highly sensitive to mucosal irritants when compared with the stratified squamous epithelium of the human vagina. For vaginal toxicity studies in rabbits, tissue irritation is usually evaluated by gross examination of the entire vaginal area as well as complete histopathologic evaluation of the vagina (D'Cruz & UcKun 2001). In the present study, repeated vaginal administration of nisin (50 mg/animal per day) for 14 consecutive days caused no adverse effects on the vaginal cell morphology and vaginal epithelium, indicating no damage. Furthermore, no adverse effects were seen on subsequent fertility after repetitive intravaginal application of nisin in these rabbits. After cessation of treatment, when these animals were allowed to mate, they conceived and delivered normal offspring, implying that the genital tract was not affected despite repeated exposure to high dosages of peptide for 14 days. On a molar basis, the concentration represents 50 times its in vivo contraceptive potency. Unlike the detergent-type spermicide, N-9, the spermicidal dose of nisin did not show toxicity to vaginal epithelial cells. The levels of various hematologic and biochemical profiles were measured, and the values were found to be similar to the controls (Tables 2 and 3). In general, there was no correlation between treatment-related increase or decrease of parameters.

In addition to any local reaction, potential systemic effects were also investigated. Absorption of nisin through vaginal epithelium into the circulation and its subsequent clearance suggests rapid systemic turnover of nisin. Maximum levels of nisin were detected in blood samples drawn 60 min after treatment (Fig. 4B). At this point of time, the levels were significantly low in the vaginal lavage (Fig. 4A), suggesting an inverse relation between vaginal and blood levels of nisin. In the serum at 3 h, there was still a detectable concentration. But the levels declined rapidly thereafter, reaching baseline levels by 12 h.

Subchronic studies on repeated oral administration of nisin to rats for 13 weeks showed no adverse effect on the growth and TSI (organ weights in relation to body weight) (Table 4). The TSI, which marks the functional status of the tissue under various experimental conditions (Reddy et al. 1984), were found to be similar to the controls. After nisin treatment, no adverse effect was seen on the subsequent reproductive performance of treated animals, perinatal growth and development of offspring (data not shown).

In conclusion, under conditions of its intended use as a dual function spermicidal microbicide, nisin appears to be a safe and effective contraceptive compound for future use in humans. Experiments are currently underway with gel formulation to determine the effective contraceptive dose in rabbits.

Acknowledgements

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