A new approach for treatment of deep skin infections by an ethosomal antibiotic preparation: an in vivo study

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Objectives: Dermal and subdermal bacterial infections, caused mainly by Staphylococcus aureus, are currently treated by systemic antibiotics. The aim of the present study was to investigate a new approach to treat deep skin and soft tissue bacterial infections by dermal application of erythromycin in an ethosomal carrier.

Methods: A model for deep dermal S. aureus infection in mice was developed. The efficiency of ethosomal erythromycin applied to the skin-infected site was compared with intraperitoneal erythromycin administration and with local application of hydroethanolic erythromycin solution. The parameters evaluated were the development of dermal wound, histological sections and bacterial count of the infected tissue.

Results: The in vivo experiments demonstrated a very efficient healing of S. aureus-induced deep dermal infections when the mice were treated with ethosomal erythromycin. Bacterial counts and histological evaluation of the skin treated with ethosomal antibiotic revealed no bacterial growth and normal skin structure. On the contrary, no subdermal healing was observed in infected animals treated with topical hydroethanolic erythromycin solution. In this group, animals developed deep dermal abscesses and the dermal structures were destroyed where S. aureus colonies were present. Bacterial counts of the infected tissues were $1.06 \times 10^7$ and $0.27 \times 10^7$ cfu/g of tissue, respectively, on days 7 and 10.

Conclusions: Therapy with ethosomal erythromycin applied to the skin of S. aureus-infected mice was as effective as systemically administered erythromycin, suggesting a new possibility to treat deep dermal infections by local application of antibiotic in ethosomal carrier.

Keywords: Staphylococcus aureus, dermal infections, ethosomes, erythromycin

Introduction

Microbial infections of the skin and underlying tissues are among the most frequent conditions encountered in acute ambulatory care.1 Staphylococcus aureus is the causative agent for the majority of primary skin infections.1,2 Skin infections (such as cellulitis, erysipelas and trauma- and wound-related infections), especially when associated with co-morbid conditions and/or bacteraemia, may lead to severe complications and hospital admission. In some cases they can be a cause of extensive morbidity and mortality.2

Owing to lack of permeation of most antibiotic agents into the deep skin layers and subdermal tissues from conventional topical preparations, deep skin infections generally do not respond to external therapy with antibiotics. These conditions are therefore usually treated by oral or parenteral antibiotics with high doses of macrolides, β-lactams or other antibacterial agents.3,4 During the course of systemic antibiotic treatment there is a significant incidence of side-effects, allergic reactions and patient inconvenience, all of which may highly influence the treatment efficiency.

In this study we proposed dermal delivery of antibiotics by means of an enhancing skin permeation carrier, the ethosome, as a new approach to treating deep skin bacterial infections. Ethosomes are specially tailored phospholipid vesicles with fluid bilayers containing ethanol.5,6 In contrast to liposomes,
Ethosomes have been shown to allow transport of significant concentrations of active agents to the deep layers of the skin transdermally. Highly efficient delivery of anti-infective drugs to the deep dermal strata and subcutaneous tissues by means of this therapeutic system has been reported previously. For this study, erythromycin ethosomes were designed and tested in vivo in an animal model for deep dermal S. aureus infections.

Materials and methods

Materials

Erythromycin base and Phospholipon 90 were gifts from Trima (Israel) and Natterman GmbH (Germany), respectively. Ethanol and formaldehyde were purchased from Frutarom (Israel). Haematoxylin and eosin stains were obtained from Sigma (Israel). Other materials were of analytical or pharmaceutical grade.

Experimental animals

All experiments were conducted on 4- to 5-week-old (body weight 16–20 g) immunocompetent ICR male mice obtained from Lowenstein Farms, Yokneam, Israel. Mice were housed under standard conditions of light and temperature, and were fed standard laboratory chow and water ad libitum. The experiments were carried out in accordance with institutional guidelines for animal care, by a protocol approved by the animal ethical care committee of the Sheba Medical Center of the Tel Aviv University, Faculty of Medicine.

Bacterial strains

Three S. aureus strains used in this study were: S. aureus ATCC 29213, S. aureus ATCC 29616 (ATCC, Manassas, VA, USA) and a clinical strain isolated from a patient with a skin wound infection (Infectious Diseases Unit, Sheba Medical Center, Tel Hashomer, Israel). Bacteria were subcultured from brain–heart infusion (BHI; Difco, USA) agar plates to 10 mL of BHI broth supplemented with 5% bovine serum albumin (BSA) and incubated at 37°C until the concentration of bacteria reached 10^6 cfu/mL. The bacteria were kept frozen at −80°C until use. Before use, the bacterial solution was thawed, washed in sterile saline and diluted to a final concentration of 10^7–10^8 cfu/mL.

Preparation and characterization of erythromycin ethosomes

Ethyosomal erythromycin containing 100 mg/g erythromycin in a carbomer ethosomal gel formulation was prepared at room temperature as described previously. Beside the antibiotic, the formulation also contained ethanol, carbopol 974 and water.

The physicochemical characteristics of erythromycin ethosomes were assessed by dynamic light scattering, transmission and scanning electron microscopy, confocal laser scanning microscopy, differential scanning calorimetry (DSC) and ultracentrifugation, as reported previously. Erythromycin ethosomes are unilamellar vesicles with a narrow size distribution and average diameter of 123.1 ± 6.9 nm. DSC results indicated that the bilayers in antibiotic ethosomes are in a fluid state, resulting in a soft vesicular structure.

Animal model for in vivo deep dermal infection

In initial experiments carried out to set up the animal model of deep dermal staphylococcal infection, three strains of S. aureus (ATCC 29213, ATCC 29616 and the clinical isolate) were used. Three groups of ICR mice, with 18 mice in each group, were inoculated intracutaneously with the bacteria. The intracutaneous injections containing live bacteria were applied to the back of each animal previously shaved with electrical clippers (Oster, Germany). Six mice from each group were inoculated with 0.1 mL of saline containing either 10^7, 10^8 or 10^9 cfu/mL of S. aureus. The mice were followed daily for the development of deep dermal abscesses, inflammatory reaction of the inoculated area and wound size for a total of 3 weeks, and were sacrificed by cervical dislocation at the end of the experiment.

Experimental protocol

The experiments were run on a total of 60 animals submitted to two treatments.

In the first experimental set, 24 animals were inoculated with S. aureus ATCC 29213 at a concentration of 10^8 cfu/mouse and were randomized to be administered either dermal ethosomal erythromycin, intraperitoneal injection of erythromycin solution or left untreated. Approximately 100 mg (containing 10 mg of erythromycin base) of the ethosomal preparation were topically administered to the skin area above the inoculation site in two daily doses. Animals received an accepted intraperitoneal dosage for erythromycin in rodents of 60 mg/kg erythromycin twice daily (2.4 mg/day).

The treatments were started 72 h after the challenge and lasted for 10 days. Animals were clinically monitored, photographed daily and sacrificed at the end of the experiment, at which time their skins and subdermal tissues were processed for histopathological examination.

In the second experimental set 36 mice were randomly divided into three groups: mice topically treated with ethosomal erythromycin, mice topically treated with hydroethanolic solution of erythromycin and controls (untreated mice).

The animals were challenged with 10^7 cfu/mouse S. aureus ATCC 29213. Treatment was conducted by application of either the ethosomal erythromycin preparation or erythromycin hydroethanolic solution. A daily dose of 20 mg erythromycin was administered to the skin from each system in two divided doses. The treatment was started 72 h following inoculation of the bacteria and lasted for 2 weeks. The progress of intradermal wounds was clinically monitored for local inflammatory reaction and photographed once daily. The skin area corresponding to the infection site and underlying tissues from the animals sacrificed on days 0, 3, 7 and 10 after the challenge were removed and processed for bacterial count and histopathological evaluation. At least three mice from each treatment group were sacrificed at each time interval.

Histopathological examination

Skin samples were examined after routine fixation with 4% formaldehyde, paraffin block processing and staining with haematoxylin–eosin or Gram stain. Microscopic evaluation was performed by a blinded evaluator to characterize and grade the inflammatory patterns and bacterial colonies. At least five skin sections per mouse were performed.

Bacterial counts

After disinfection with 70% alcohol, samples of skin and adjacent tissues corresponding to the inoculation sites were homogenized under liquid nitrogen in a sterile pestle with a mortar, deposited in sterile plastic tubes and suspended in 2 mL sterile saline. Suspensions were plated in appropriate dilutions on tryptic soy agar (Difco) plates at 37°C for 24 h in ambient air. Bacterial counts were expressed as numbers of S. aureus colony forming units per gram (cfu/g) of tissue.
**MIC determination**

MICs were determined by the microdilution technique according to the NCCLS criteria for *S. aureus*.10 Erythromycin base was dissolved in phosphate-buffered saline (pH 7.2). Two-fold dilutions of the antibiotic were used in concentrations ranging from 0.015 to 1024 mg/L diluted in 100 μL of Mueller–Hinton broth and poured into wells of flat-bottomed microtitre plates (Nunc 96-well flat-bottomed microtitre plates; Nunc, Denmark). A 10 μL volume containing 10^5 cfu was then added. Following incubation of the plates for 18 h at 37°C in ambient air, the MIC was determined. The MIC was recorded as the lowest concentration that completely inhibited visible growth of the bacteria.

**Results and discussion**

To conduct these studies we first developed a model for deep dermal staphylococcal infection. For this purpose, we monitored the course of infection in ICR mice intradermally inoculated with *S. aureus* ATCC 29616, *S. aureus* ATCC 29213 or the clinical isolate of *S. aureus* at 10^6, 10^7 or 10^8 cfu/animal. Irrespective of the inoculation dose, none of the animals inoculated intradermally with *S. aureus* ATCC 29616 or with the clinically isolated strain developed local or systemic signs of infection. Thus these microorganisms were not considered virulent enough for the purpose of this study in ICR mice. In all groups of animals inoculated with *S. aureus* ATCC 29213, development of notable abscesses within the skin was observed within 24–48 h. *S. aureus* was isolated from these abscesses 24–48 h after challenge and persisted for at least 3 weeks. At an inoculum of 10^6 cfu, five of six mice developed mild abscesses (~10 mm diameter). Larger abscesses of a diameter of ~20 mm formed in all recipients of 10^7 cfu *S. aureus* ATCC 29213. The group that received 10^8 cfu developed severe abscesses that involved not only dermal and subdermal structures, but also vital organs, such as liver and lungs. In this inoculation group four out of six mice died on day 3–5 after induction of the infection.

Based on these results, we concluded that the suitable pathogen for a deep dermal staphylococcal infection in these animals is *S. aureus* ATCC 29213 at an inoculum of 10^6 or 10^7 cfu for mild and aggressive infectious patterns, respectively. The MIC of erythromycin for *S. aureus* ATCC 29213 was 12.5 mg/L.

The ability of ethosomal erythromycin to modify the course of intradermal *S. aureus* infection was primarily evaluated in mice challenged with 10^6 cfu *S. aureus* ATCC 29213 that developed a mild abscess. In these experiments we monitored the progress of the dermal infection in three animal groups: those treated by skin application of ethosomal erythromycin, a group treated by intraperitoneal administration of erythromycin and untreated mice (controls). The treatment in all groups started 72 h following the induction of the infection and lasted for 2 weeks. The infection course of the untreated control animals was similar to that observed in the infected mice in the experiments designed for the development of this model. Five of six mice had mild skin abscess of ~10 mm in diameter with clear signs of dermatonecroses at the infection site. In addition, abscess formation in the superficial musculature was noted in these animals. The typical histological pattern consisted of necrotic cells, destroyed skin structures with no differentiation between epidermis and dermis, and a dense infiltrate of neutrophilic granulocytes with visible bacteria in the deep dermis layers and subcutaneous fat (Figure 1a). Animals treated with skin application of the ethosomal erythromycin preparation fully recovered from the infection and re-grew hair on their backs by the end of the 10 day treatment. These animals did not demonstrate dermatonecroses (Figure 1b). In the group treated with systemic erythromycin a similar clinical picture to the animals treated with ethosomal erythromycin was observed. However, in the systemic erythromycin group of animals, one of six mice showed slight signs of dermatonecroses by the end of the treatment (Figure 1c). From the above results it is clear that dermal application of ethosomal erythromycin is similar to systemic administration of erythromycin in its potential to cure deep dermal bacterial infections.

![Figure 1. Histological images taken from skin of mice intradermally inoculated with 0.1 mL × 10^6 cfu/mL (10^6 cfu/mouse) *S. aureus* ATCC 29213: (a) untreated mice; (b) mice treated with skin application of ethosomal erythromycin; and (c) mice treated with 120 mg/kg/day erythromycin by intraperitoneal injection. In both cases (b and c), the treatment started 72 h after challenge and lasted for 10 days.]
In a further investigative step, the efficiency of erythromycin to eradicate bacteria located in the deep dermal strata by ethosomal erythromycin or hydroethanolic solution of erythromycin, both applied to the skin, as well as an untreated control group, was tested. In these experiments the animals were challenged with a higher inoculum of $10^7$ cfu/animal, to test the ability of ethosomal erythromycin to cure severe infectious pattern. The treatments were started 72 h following the induction of the infection and lasted for 2 weeks. The average *S. aureus* count immediately before the beginning of antibiotic therapy was $1.5 \times 10^7$ cfu/g of tissue. Visible differences in the appearance of the animals from various treatment groups were observed starting from the fourth day of treatment. In mice given ethosomal erythromycin, clear signs of regression of the infection were observed and no dermatonecroses appeared on both days 7 and 10 after challenge. No *S. aureus* was isolated from the inoculation sites at both time intervals. In contrast, in two other groups of animals (untreated and treated with hydroethanolic erythromycin system), the infection progressed, resulting in a significant dermatonecroses of the skin/adjacent tissues and an initial crust formation over the necrotic area. The histopathological examination (Figure 2) confirmed the macroscopic observation and revealed necrosis, destroyed skin structures and a dense infiltrate of neutrophils and macrophages within the abscess, mostly situated in the subcutaneous layer. Abundant Gram-positive bacterial colonies were also present. There were no clear differences in the characteristics of the inflammatory infiltrates between these two groups. Bacterial counts of $0.90 \times 10^7$ and $1.06 \times 10^7$ cfu/g of tissue were assayed on day 7 in the wounds of untreated mice and those receiving hydroethanolic erythromycin solution topically, respectively. At day 10, the *S. aureus* count in skin and adjacent tissues was $0.57 \times 10^7$ versus $0.27 \times 10^7$ cfu/g of tissue in the same animal groups, respectively.

The hydroethanolic solution of erythromycin was chosen as a control in these experiments in order to examine possible artefacts owing to the fact that ethanol is a component of the ethosomal system. It was important to find out if the observed curative effect is due to the presence of ethanol or is related to the mechanism of action of the ethosomes—the specially tailored soft vesicles. The results of this study revealed that hydroethanolic solution of erythromycin topically applied to the skin covering the infected area had no effect whatsoever on the progress of deep dermal staphylococcal infection. On the other hand, erythromycin applied from ethosomes resulted in efficient inhibition of the infection, thus stopping its development. As

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**Figure 2.** Histological images taken from skin of mice intradermally inoculated with $0.1 \text{ mL} \times 10^8$ cfu/mL ($10^7$ cfu/mouse) *S. aureus* ATCC 29213 on days 0, 3, 7 and 10 after challenge. Mice groups: untreated control (left panels), ethosomal erythromycin applied on the infected skin (middle panels) and hydroethanolic erythromycin solution applied on the infected skin (right panels).
Ethosomal erythromycin for deep skin infections

A further investigative step we plan to conduct a pharmacokinetic study, in which levels of antibiotic in the infected deep dermal strata will be monitored.

There are a number of publications on the use of liposomes for the treatment of skin infections.11,32 However, these studies describe the benefits of liposomes for treating either superficial or open post-operative wound infections. For example, tobramycin liposomal encapsulation for local treatment of infected post-operative wounds leads to controlled drug release at the infectious site and to a more prolonged effect in comparison with treatment with free tobramycin. In another study, subcutaneous injection of gentamicin encapsulated in liposomes was needed to bring about a more efficient treatment of the infection.13 The need for injection could be explained since, using classic liposomes, the quantity of drug absorbed from the skin surface into the deep dermal layers and subdermal tissues is often too low and is not adequate to achieve the required therapeutic effect.14–16

To our knowledge, no previous reports have been published evaluating skin antibiotic administration as a substitute for the systemic antibiotic treatment of deep dermal wounds. Thus, we are proposing a novel approach to effectively treat bacterial dermal infections involving the deep dermal strata by delivery of the macrolide antibiotic using a special lipid vesicular carrier, the ethosome. Ethosomes can penetrate the skin barrier and exhibit enhanced delivery of drugs into and beneath the deep dermal layers.5,7 A proposed mechanism of action explaining the permeation-enhancing effect of the ethosomal carrier is the dual fluidizing effect of ethanol on the ethosomal lipid bilayers and on the stratum corneum lipids. The soft ethosome penetrates the disturbed skin lipid bilayers creating a pathway through the skin, and fuses with cell membranes in the deeper skin layers releasing the active agent.5,7 The effectiveness of ethosomal erythromycin for treatment of S. aureus-infected animals may be related to the property of the ethosomes as an enhancing carrier for delivery of the therapeutic agent. In our study, ethanolic solution of erythromycin was not effective in curing the deep dermal infection. Thus, it is assumed that the efficient therapeutic effect of ethosomal erythromycin was enabled by the acting of the soft vesicles.

The ethosomal carrier was previously tested for dermal delivery of other anti-infective drugs. For example, a two-armed, double-blind, randomized clinical trial demonstrated the efficiency of an antiviral ethosomal 5% aciclovir system compared with a 5% aciclovir cream (Zovirax®, ZC) for the topical treatment of herpetic infection.5 Based on the results of that study an ethosomal cream of aciclovir was developed and is now in clinical use.

In another recent study, the efficiency of ethosomes to enhance delivery of the polypeptide antibiotic bacitracin to deep layers of the skin was examined by confocal laser scanning microscopy.7 In contrast to bacitracin liposomes, which remained confined on the skin surface, bacitracin ethosomes allowed a significant penetration of this antibiotic into the deep skin layers.

It is noteworthy that ethosomes are safe carriers and do not irritate the skin. Human tolerability experiments with ethosomes and control systems were performed in healthy volunteers using a non-invasive technique of reflectance spectrophotometry. The authors of these studies reported no signs of erythema following 12, 24 and 48 h application of ethosomes. Moreover, no significant difference in erythema index was measured between skin areas treated with ethosomes and with saline.17

In conclusion, in the present study we have shown that ethosomal erythromycin was highly efficient in eradicating S. aureus-induced intradermal infections. The treatment with ethosomal erythromycin applied on the skin was as effective as the intraperitoneal systemic administration of this antibiotic. The clinical implications of the data presented here suggest the possibility of substituting systemic antibiotic treatment with local treatment. This would result in decreased drug exposure and the associated side-effects, thereby potentially increasing patient compliance. In addition to these possible therapeutic benefits, a rapid bacterial kill and short therapy courses with ethosomal erythromycin could ultimately result in reducing treatment costs and minimizing bacterial resistance. These findings may open new avenues for the treatment of deep dermal infections by local application of tailored antibiotic ethosomes.

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References