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Chitosan-Based Film of Tyrothricin for Enhanced Antimicrobial Activity against Common Skin Pathogens Including *Staphylococcus aureus*

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Chitosan-based film-forming gel is regarded as a promising vehicle for topical delivery of antimicrobial agents to skin wounds, since it protects from microbial infection and the cationic polymer itself possesses antibacterial activity. In this study, possible synergistic interaction against common skin pathogens between the cationic polymer and tyrothricin (TRC), a cyclic polypeptide antibiotic, was investigated, by determining the concentration to inhibit 90% of bacterial isolates (MIC). The addition of the polysaccharide to TRC dramatically reduced the MIC values of TRC by 1/33 and 1/4 against both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*, respectively. The synergism of TRC and chitosan combination against both strains was demonstrated by the checkerboard method, with a fractional inhibitory concentration index below 0.5. Moreover, co-treatment of TRC and chitosan exhibited antibacterial activity against *Pseudomonas aeruginosa*, due to the antibacterial activity of chitosan, whereas TRC itself did not inhibit the gram-negative bacterial growth. These findings suggested that the use of chitosan-based film for topical delivery of TRC could be an alternative to improve TRC antimicrobial activity against strains that are abundant in skin wounds.

Keywords: Tyrothricin, chitosan, antimicrobial activity, *Staphylococcus aureus*, skin infection

Introduction

Tyrothricin (TRC), an antimicrobial peptide consisting of 70~80% tyrocidins and 20~25% granicidins, is clinically used for the topical treatment of infected skin and oro-pharyngeal mucus membranes [26]. Numerous investigations demonstrated that the polypeptide exhibits a broad spectrum of antibacterial activity against gram-positive cocci and rods, by forming a hydrophilic ion channel into the bacterial membrane, thus causing cell leakage and cell death [9, 22, 23]. The long-term use of TRC-loaded topical preparations in the infected skin has not raised any concerns about the resistance against gram-positive bacteria and/or yeasts, even in the case of methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) strains of *Staphylococcus aureus* [24]. The original formulation consisted of 0.1% (w/v) TRC in the carbomer-based hydrogel along with ethanol, propylene glycol, and cetylpyridinium chloride (CPC) as preservatives (Tyrosur gel; Engelhard Arzneimittel GmbH & Co. KG, Germany). The cationic detergent CPC is effective against both gram-positive and gram-negative bacteria, by strongly binding to an anionic compound on the bacterial surface and altering the cytoplasmic membrane integrity [1, 17].

Chitosan, a linear polysaccharide composed of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units, has been widely explored in biomedical and pharmaceutical areas, owing to its biocompatibility, non-toxicity, and high stability [2, 8, 10, 19, 28]. Chitosan-based films are considered a promising local delivery carrier, providing mechanical barrier function to protect from microbial infection and preserve low relative humidity in the wound site [4]. In addition, chitosan itself possesses antibacterial and antifungal activities against various strains that are

abundant in infected skin and tissues [3, 18, 20]. The positively charged polymer reportedly interacts with negatively charged microbial cell membranes, disturbing the outer membrane with subsequent leakage of proteinaceous and other intracellular constituents [12, 21]. Taken together, we assumed that the chitosan-based film system could be a promising external base for topical delivery of TRC, with the additive and/or synergistic antibacterial activity. We previously demonstrated the superior healing effects of chitosan-based TRC gel (Dodana gel, Korea), as compared with Tyrosur gel in various wounds, including burn, abrasion, incision, and excision wounds in rats [11]. However, to the best of our knowledge, there are no reports on the possible synergism between TRC and the cationic polymer.

Therefore, the aims of the study were to comparatively evaluate the in vitro antimicrobial activity of the chitosan-based TRC preparation (Dodana gel) and the marketed product (Tyrosur gel) and to investigate the possible synergism between the individual ingredients such as TRC, CPC, and chitosan. The antimicrobial activity of the individual components and their combinations was assessed by determining the minimal concentration that inhibits 90% of bacterial isolates (MIC) against several strains that are abundant in infected skin such as MSSA, MRSA, *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeroginosa*). Subsequently, the possible interactions between the compounds were determined by the checkerboard method.

Materials and Methods

Materials

TRC (purity >98%) and chitosan (molecular mass of 200,000 dalton, degree of deacetylation of 90%) were purchased from Xellia Pharmaceuticals Ltd. (Budapest, Hungary) and Koyo Chemical Co. (Sakaiminato, Japan), respectively. Ethanol, propylene glycol, lactic acid, and CPC were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Mueller-Hinton agar (MHA) and Mueller-Hinton broth (MHB) were purchased from BD Difco (Franklin Lakes, NJ, USA). All other chemicals and reagents were of analytical grade and purchased from commercial sources. Doubly distilled water was used for all experiments.

Bacterial Strains

Four bacterial strains of MRSA (ATCC M126), MSSA (ATCC 25923), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection (Manassas, VA, USA). ATCC 25923, a clinical isolate with the designation Seattle 1945, has been reported to be susceptible to methicillin and oxacillin [13, 14, 27]. All strains were cultured on MHA and incubated at 37°C for 24 h. Isolated colonies were subsequently diluted with autoclaved MHB to obtain the final bacterial inoculum of 10^8 CFU/ml. Aliquots of bacterial suspension were further diluted with MHB to adjust to 2×10^6 CFU/ml in each well prior to the experiment.

Determination of MIC Values

The MIC values of the marketed products and microbial substances were determined using the microdilution method according to the National Committee of Clinical Laboratory Standards (2004). One gram of each marketed product, namely, Dodana gel (TRC 0.1%, CPC 0.05% in chitosan film), Tyrosur gel (TRC 0.1%, CPC 0.05% in carbomer gel), and Fucidin ointment (sodium fusidate 2%; Dongwha Pharm. Co., Korea), was weighed and diluted with 4 g of broth medium to a concentration of 20% (w/w). Fucidin ointment was used as a positive control to estimate the degree of antibacterial activity of the TRC preparations. The solution was 2-fold serially diluted with MHB to obtain final concentrations in the ranges from 0.00002% to 0.2%.

A stock solution of each active compound and their combinations was prepared as follows: the solution of 0.1% TRC, CPC 0.05%, and their combination was prepared by dissolving the substances in 1 g of ethanol and then diluting appropriately with ultrapure water. The chitosan solution (4.5% (w/v)) was prepared by dissolving the cationic polysaccharide in lactic acid (2.25% (w/v)) solution. The TRC/chitosan, CPC/chitosan, and TRC/CPC/chitosan solutions were prepared by mixing 1 g of 10% TRC and 5% CPC ethanolic solution with 100 ml of chitosan solution. Then each solution was serially diluted with MHB to adjust the final concentrations in the ranges from 0.00002% to 0.2%.

A 50 µl bacterial suspension and 50 µl of test solution were added to each 96-well plate and incubated at 37°C for 24 h. The final concentration of the test solution in the test ranged from 0.00001% to 0.1% (w/v). The absorbance at 560 nm was measured with a microplate spectrophotometer (SpectraMax Plus 384; Molecular Devices, LLC). The lowest concentration of product or substance solutions showing visible growth <10% was recorded as the MIC. Each assay was carried out in triplicates.

Determination of Synergistic Interaction between Compounds

The synergistic, additive, or antagonistic interaction between each compound (TRC/CPC, TRC/chitosan, CPC/chitosan, and TRC/CPC/chitosan) was determined using the checkerboard test [8, 15, 16]. Using the previously determined MIC values of individual compounds and their combinations, the fraction inhibitory concentration (FIC) index was calculated according to the following equation:

$$FIC = MIC_{a/b} / MIC_a + MIC_{b/a} / MIC_b$$

where MIC_a is the MIC of compound a alone, MIC_b is the MIC of compound b alone, $MIC_{a/b}$ is the MIC of compound a in the presence of compound b, and $MIC_{b/a}$ is the MIC of compound b in

the presence of compound a. Synergism is defined as a FIC index of 0.5 or less; additivity as a FIC index of more than 0.75 and less than 2; and antagonism as a FIC index of more then 2.

Results and Discussion

Comparison of Antimicrobial Activity of TRC Topical Formulations

Wound healing, a biological process to restore the damaged skin structure and its function to normal state, is recurrently obstructed by microbial infection. Therefore, several antibiotics, including TRC, are administered topically to treat infection or inflammation in skin wounds. However, external preparations such as ointments, creams, and hydrogels are easily washed out from the application site in the absence of occlusion, which hinders antibacterial remedy [11]. In this study, a chitosan-based film-forming gel of TRC was constructed to complement the drawbacks of conventional preparations. The TRC-containing hydrogel is transformed to a film layer after topical application by solvent evaporation and/or diffusion. The formed chitosan film offers barrier function to protect the wound site from the infiltration of microbials, as well as to prevent washout of the antibiotics. Moreover, we expected that the antiseptic effect of the cationic polymer against a broad spectrum of strains abundant in skin and tissues would impart better therapeutic effects with TRC after topical application.

The MIC values of the chitosan- or carbomer-based TRC gels and the positive control, Fucidin ointment, against several strains are shown in Table 1. The bacterial strains tested are widely distributed in nature and carried by most injured skin layers. According to antimicrobial surveillance database records on bacteria associated with skin and soft tissue infections in hospitalized patients, *S. aureus*, *P. aeruginosa*, *E. coli*, and *Enterococcus* spp. were the predominant pathogens in all geographical regions [6]. The stronger microbial activity against these strains was obtained from the treatment of the chitosan-based TRC gel, as compared with the carbomer-based TRC gel in all strains tested, despite the same active ingredients. The MIC values of the chitosan-based TRC gel were only 1/16, 1/2, and 1/4 of those from the Tyrosur gel against MSSA, MRSA, and *E. coli*, respectively. Because both TRC and fusidic acid are effective primarily on gram-positive bacteria, the Tyrosur gel and Fucidin ointment could not suppress the growth of *P. aeruginosa,* a gram-negative bacterium. On the other hand, the chitosan-based TRC gel was effective against the gram-negative bacteria (MIC 1,563 μg/ml as entire cream, 1.563 μg/ml for TRC). These results suggested that the incorporation of TRC and/or CPC in the chitosan-based base could eradicate both gram-positive and gram-negative bacteria, by additive and/or synergistic antimicrobial interactions. Several authors proposed a synergistic action of antibiotic and/or preservatives with the cationic polysaccharide. Decker *et al*. [5] reported a synergistic activity of the chlorhexidine/chitosan combination for antiplaque therapy. Tin *et al*. [25] also reported that the combination of sulfamethoxazole and chitosan showed synergistic action against *Pseudomonas* strains. Based on these findings, we expected that the use of chitosan as an external base for topical delivery of TRC would be beneficial in boosting the antimicrobial activity of TRC and/or CPC, and further investigated these antimicrobial interactions against various strains that are abundant in infected skin.

Efficacy of Individual and Combined Treatments against *S. aureus*, *E. coli*, and *P. aeruginosa*

The antimicrobial effects of individual compounds (TRC, CPC, and chitosan) and their combinations against MSSA are presented in Table 2. TRC and CPC showed strong antibacterial activity with the MIC values of 3.12 and 0.78 μg/ml, respectively. The MIC values obtained closely coincided with previously reported values [24]. The chitosan solution showed weaker antibacterial activity (17.59 μg/ml) against MSSA, as compared with TRC and CPC. The cationic polymer is only soluble in acidic medium, hence lactic acid

Table 1. Comparison of MIC values of a chitosan-based TRC preparation (Dodana gel) with that of a carbomer-based preparation (Tyrosur gel).

	MSSA		MRSA		*E. coli*		*P. aeruginosa*	
	ASPP[a]	AS[b]	ASPP	AS	ASPP	AS	ASPP	AS
Dodana gel	98	0.098	781	0.781	6,250	6.250	1,563	1.563
Tyrosur gel	1,563	1.563	1,563	1.563	25,000	25	No inhibition	
Fucidin ointment	12	0.234	12	0.234	No inhibition		No inhibition	

[a]MIC of the active substance as part of the product (ASPP) expressed as μg/ml.

[b]MIC of the active substance calculated by multiplying the MIC for the product by the concentration of the active substance in the cream.

Table 2. MIC and FIC index for TRC, CPC, chitosan and their combinations against *MSSA* strain.

Composition	MIC (μg/ml)	FIC[a]
TRC	3.125	–[c]
CPC	0.781	–
Chitosan solution[b]	17.59	–
Lactic acid	2,250	–
TRC + CPC	1.563 + 0.781	1.50
TRC + chitosan	0.098 + 4.410	0.28
CPC + chitosan	0.195 + 17.59	1.25
TRC + CPC + chitosan	0.098 + 0.049 + 4.410	0.34

[a]FIC index ≤ 0.5: synergism; 0.5 < and < 1: partial synergism; 1 < and < 2: addition; and 2 ≤: indifference or antagonism [16].

[b]Prepared by dissolving chitosan in 2.25% (w/v) lactic acid.

[c]Not determined.

Table 3. MIC and FIC index for TRC, CPC, chitosan and their combinations against *MRSA* strain.

Composition	MIC (μg/ml)	FIC[a]
TRC	3.125	–[c]
CPC	0.781	–
Chitosan solution[b]	281.2	–
Lactic acid	2,250	–
TRC + CPC	1.563 + 0.781	1.50
TRC + chitosan	0.781 + 35.14	0.37
CPC + chitosan	0.781 + 70.33	1.25
TRC + CPC + chitosan	0.781 + 0.390 + 35.14	0.87

[a]FIC index ≤ 0.5: synergism; 0.5 < and < 1: partial synergism; 1 < and < 2: addition; and 2 ≤: indifference or antagonism [16].

[b]Prepared by dissolving chitosan in 2.25% (w/v) lactic acid.

[c]Not determined.

was used as the solvent; the MIC value of lactic acid was negligible at 2,250 μg/ml, which was 128-times higher than that of the chitosan. The co-treatments of TRC/CPC or chitosan/CPC did not exhibit synergistic effect, with a FIC index over 1.25. On the other hand, the synergistic antimicrobial activity was obvious when TRC and chitosan were combined, as the MIC of the compound in the combinations were lessened to only 1/33 and 1/4 of the individual MIC values, respectively. Based on the FIC calculations (Table 2), the combinations of TRC/chitosan exhibited synergistic effect with a FIC index of 0.28. The combination of three compounds was also tested, but there was no difference between the TRC/chitosan and TRC/chitosan/CPC treatments.

The antibacterial activities of TRC and CPC against the methicillin-resistant strain were equal to those against methicillin-susceptible strains, with MIC values of 3.12 and 0.78 μg/ml, respectively (Table 3). On the other hand, the chitosan exhibited weaker antibacterial activity against MRSA, as compared with MSSA (281.3 μg/ml). The effect of combination treatment on the viability of MRSA was quite analogous to those obtained from MSSA. The combined treatments of TRC/CPC or chitosan/CPC did not exhibit synergistic effect, with a FIC index of over 1.25. On the other hand, co-treatment of TRC with chitosan had a significant effect on enhancing the antibacterial activity of TRC against MRSA. The MIC value of TRC was remarkably reduced to 1/4 in the presence of chitosan (Table 3), suggesting the synergistic effect of TRC and chitosan against the reference strain of MRSA (FIC value of 0.37).

The antibacterial activity against *E. coli*, an important gram-negative strain in skin and soft tissue infections, are shown in Table 4. These compounds exhibited weak antibacterial activity against the gram-negative strain with higher MIC values in the range of 12.5 to 562.5 μg/ml, as compared with those against gram-positive strains. The TRC/CPC and TRC/chitosan combinations showed no synergistic antimicrobial effects, with a FIC value of over 1.1. However, a partially synergistic effect was observed with the chitosan/CPC combination, with MIC values reduced by 1/2 and 1/4 of each compound, respectively. Correspondingly, the combination of chitosan/CPC had a remarkably low FIC value of 0.75, as compared with the TRC/CPC and TRC/chitosan combinations. The partially synergistic effect of the chitosan/CPC combination supports the stronger antiseptic activity of the chitosan-based gel (Dodana gel) against *E. coli*, as compared with the carbomer-

Table 4. MIC and FIC indexes for TRC, CPC, and chitosan and their combinations against *E. coli* strain.

Composition	MIC (μg/ml)	FIC[a]
TRC	100.0	–[c]
CPC	12.5	–
Chitosan solution[b]	562.5	–
Lactic acid	No effect	–
TRC + CPC	25.0 + 12.5	1.25
TRC + chitosan	12.5 + 562.5	1.13
CPC + chitosan	3.125+ 281.2	0.75
TRC + CPC + chitosan	6.25 + 3.125 + 281.2	0.81

[a]FIC index ≤ 0.5: synergism; 0.5 < and < 1: partial synergism; 1 < and < 2: addition; and 2 ≤: indifference or antagonism [16].

[b]Prepared by dissolving chitosan in 2.25% (w/v) lactic acid.

[c]Not determined.

Table 5. MIC and FIC indexes for TRC, CPC, and chitosan and their combinations against *P. aeruginosa* strain.

Composition	MIC (μg/ml)	FIC[a]
TRC	No inhibition	–[c]
CPC	No inhibition	–
Chitosan solution[b]	17.59	–
Lactic acid	2,250	–
TRC + CPC	No inhibition	–
TRC + chitosan	3.125 + 140.6	–
CPC + chitosan	1.562 + 140.6	–
TRC + CPC + chitosan	1.562 + 0.781 + 70.33	–

[a]FIC index for ≤ 0.5: synergism; 0.5 < and < 1: partial synergism; 1 < and < 2: addition; and 2 ≤: indifference or antagonism [16].

[b]Prepared by dissolving chitosan in 2.25% (w/v) lactic acid.

[c]Not determined.

based gel (Tyrosur gel).

The susceptibility of *P. aeruginosa* to each antimicrobial agent and their combinations is shown in Table 5. In the case of TRC, CPC, or their combination, no bactericidal activity was observed against the gram-negative strain, whereas the cationic polysaccharide exhibited antibacterial activity with the MIC value of 17.6 μg/ml. Tin *et al*. [25] reported that chitosan and its oligosaccharide exhibited antibacterial activity against several *P. aeruginosa* strains (32 μg/ml); they suggested that the binding of the cationic polymer to teichoic acids located in the cell membrane predominantly causes membrane alterations and deficit of barrier function, subsequently leading to bacterial death [25]. On the other hand, the susceptibility of *P. aeruginosa* to chitosan was remarkably diminished in the presence of TRC or CPC, probably hindering the electrostatic interaction of the cationic polymer with the bacterial outer membrane. Nevertheless, the use of chitosan as an external base for TRC delivery would be beneficial to broaden the antimicrobial spectrum against gram-negative bacteria.

In summary, the primary finding of the study was that the use of chitosan as an external base for topical TRC delivery is advantageous to improve antibacterial activity against common skin pathogens. The combination of TRC with the positively charged polymer was synergistically effective against both MSSA and MRSA, lessening the MIC values of TRC by 1/33 and 1/4, respectively. Chitosan also exhibited partially synergistic bactericidal effects with CPC, a preservative, against *E. coli*. Moreover, the TRC-loaded chitosan gel exhibited antibacterial activity against *P. aeruginosa* due to the antibacterial activity of chitosan, whereas the TRC-loaded carbomer-based gel did not inhibit bacterial growth. Thus, a chitosan-based film-forming system could be favorable to formulate external preparations of antimicrobial agents, including TRC, with improved wound healing effect.

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