Chapter 3

Synergism between maggot excretions and antibiotics

Maggot excretions interact with antibiotics

Submitted

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Abstract

Maggots are successfully used to treat severe, infected wounds in trauma patients. This study investigated whether maggot excretions/secretions (ES) influence the antibacterial activity of different antibiotics. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were determined of gentamicin and flucloxacillin for *Staphylococcus aureus*, of penicillin for *Streptococcus pyogenes*, of amoxicillin and vancomycin for *Enterococcus faecalis*, of gentamicin for *Enterobacter cloacae*, and of gentamicin, tobramycin and ciprofloxacin for *Pseudomonas aeruginosa* by checkerboard titration. A range of concentrations of antibiotics in combination with ES was examined to investigate the potential of ES to influence antibacterial activity. The results showed a dose-dependent increase of the antibacterial effect of gentamicin in the presence of ES on *S. aureus*. MIC and MBC of gentamicin decreased respectively 64- and 32-fold. The MBC's of gentamicin/ES against *E. cloacae* and flucloxacillin/ES against *S. aureus* were also decreased. The other antibiotic/ES combinations defined an indifferent effect. ES alone did not have any antibacterial effect. The synergism between gentamicin and maggot ES could be of direct importance in clinical practice, because it could permit the use of lower doses of gentamicin and thus minimize the risk of gentamicin related side-effects.

Introduction

Infected wounds can be treated by maggots of the blowfly *Lucilia sericata*.[1] Since Maggot Debridement Therapy (MDT) is very effective in clinical practice and increasing antimicrobial resistance is a serious common problem nowadays, MDT is widely used in many countries in Europe.[1-3] William Baer, an orthopaedic surgeon, introduced maggots as a new therapy for osteomyelitis in the 1930's.[4] The results of his treatment were very successful,[4] but a few years later, MDT was replaced by the discovery of penicillin by Alexander Fleming and the improved surgical procedures.[5] In the 1980's, maggots were reintroduced in Europe, because of the increasing bacterial resistance against antibiotics.[6,7] Recent literature has shown, especially after trauma surgery, improved wound healing induced by MDT and prevention of major amputations.[2,8,9] In 2004 MDT was approved by the US Food and Drug Administration (510[k] # 33391).[10]

The underlying mechanism of the beneficial effects of MDT on wound healing is unknown. MDT is supposed to have a debriding and disinfecting effect and to stimulate wound healing.[11-16] Previous research,[17] which focused on the disinfecting effect, focused on whether maggots and/or their excretions/secretions (ES) had a bacteriostatic and/or bactericidal effect on different bacterial species, because literature suggested that they possess antimicrobial properties.[18-20] Direct antibacterial activity has not been found, neither from the live maggots nor from the ES, despite various testing methods.[17] In our clinical practice, better healing of wounds by treatment of MDT in combination with antibiotics has been observed. Based on this fact we hypothesized that maggot ES enhance the antibacterial effect of antimicrobial agents.

Therefore, in this study we investigated whether maggot ES could influence the minimal inhibitory concentration (MIC) and/or the minimal bactericidal concentration (MBC) of different antimicrobial agents to five bacterial species.

Methods

We determined the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of gentamicin and flucloxacillin for *Staphylococcus aureus*, of penicillin for *Streptococcus pyogenes*, of amoxicillin and vancomycin for *Enterococcus faecalis*, of gentamicin for *Enterobacter cloacae*, and of gentamicin, tobramycin and ciprofloxacin for *Pseudomonas aeruginosa*. All bacterial species were isolated from patients of our departments for Trauma and Orthopaedic Surgery. The MIC is defined as the lowest concentration of antimicrobial agent that inhibits visible bacterial growth and the MBC requires significant 99.9% killing of a particular bacterium by an antimicrobial agent.[21]

Bacteria were grown overnight in Tryptic Soy Broth (TSB) medium (Becton,

Dickinson and Company, Franklin Lakes, NJ, USA). In the first experiment, the MIC and MBC were determined of every bacterium and antibiotic alone. Then, by checkerboard titration, a range of concentrations of antibiotics was combined with ES, after which MIC and MBC were compared (Fig. 1).

An overnight culture of the bacterial suspension (BS) was diluted in TSB medium to a turbidity of McFarland 0.5 (1.5×10^8 Colony Forming Units (CFU)/ ml). Then, the BS was diluted further to 5×10^6 CFU/ml. The target MIC for each antimicrobial agent was defined, based on the article of Andrews.[22] To observe the occurrence and magnitude of synergism or antagonism, a serial range of concentrations antibiotics diluted with PBS from four times the expected MIC to at least 1/8 to 1/16 times the expected MIC was tested in a 96-well round-bottom microtiter plate (Greiner Bio-one BV, Monroe, NC, USA).[22] The total volume in each well was 150 ml, which included 50 ml BS, 50 ml of the antimicrobial solution and 50 ml PBS.[22]

Sterile Instar-3 larvae (*Lucilia sericata*) were produced and provided by BioMonde GmbH (Barsbüttel, Germany). Instar-3 maggots are full-grown and have a size of 8 mm after 4 to 5 days. For maturation the larvae were fed with sterile blood agar. The ES were obtained as described previously.[23] Briefly, the maggots were incubated in sterile tubes for one hour at 35°C in darkness. Then, ES were removed by pipette and stored at -80°C. The protein concentration was determined with the Pierce Bicinchonic Acid Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). For the experiments, ES were set to a protein concentration of 1000 mg/mL and serially diluted with PBS (1:1) to a maximum of 1:32 for our experiments. Sterility of ES was tested in duplicate.

To determine the influence of maggot ES on the MIC and/or MBC, we added ES in different concentrations (Fig. 1). Every possible combination of antibiotics and ES was pipetted in the wells (Fig. 1). In these experiments, the total volume also was 150 ml, which included 50 ml BS, 50 ml of the antimicrobial solution and 50 ml of the ES solution.

The turbidity of the wells, which is a measure of bacterial growth, was measured by Optical Densitometry (OD) at a wavelength of 570 nm at the start of the experiment and after 20 hours of incubation of the microtiter plates at 37°C. The background of the OD-values at the beginning of the experiment, the day before, was subtracted from the values after incubation. The MIC corresponds to the concentration of antibiotic in the well with an OD-value of <0.06, which represents invisible bacterial growth in a (seemingly) clear well.[23] OD-values higher than 0.06 show visible growth (turbid wells). For determination of the MBC, a volume of 100 ml from each well was subcultured overnight on nutrient agar plates (Biotrading Benelux BV, Mijdrecht, The Netherlands). The concentration of antibiotic that shows a decrease in bacterial colonies from the start inoculum of 5×10^6 CFU/ml to less than 5×10^3 CFU/ml on the agar plate represents the MBC.

Antibiotics:	4 x MIC	2 x MIC	MIC	½ x MIC	¼ x MIC	1/8 x MIC	1/16 x MIC	No antibiotics	No antibiotics
50 µg ES	BS+antibiotic +ES							BS+ES+PBS	BS+PBS
25 µg ES									
12.5 µg ES									
6.25 µg ES									
3.125 µg ES									
1.5625 µg ES									
No ES	BS+antibiotic+ PBS								

Figure 1 Schematic overview of the checkerboard titration. The columns represent the range of concentrations antibiotics and the rows represent the range of concentrations of maggot ES in µg/well. The ninth column is the control of the bacterial growth in presence of ES, but without antibiotics and the last column is the control of the bacterial growth without ES and antibiotics. The last row shows the bacterial growth in presence of antibiotics without ES. MIC = minimal inhibitory concentration; ES = excretions and secretions; BS = bacterial suspension; PBS = phosphate buffered saline.

Agents were defined bactericidal if the MIC and MBC-value did not differ more than four serial concentrations.[21] All experiments were done in triplicate.

In each experiment, wells for growth control and sterility control were included. All MIC-values were compared to those described in recent literature, as a quality control. For the inoculum control at the start of the experiment, viable counts were determined by inoculating agar plates with 10-mL volumes of serial 1:10 dilutions of the suspension. The plates were incubated overnight in 37⁰C, after which colonies were counted.

For each combination interaction, the fractional inhibitory concentration (FIC) index of the antimicrobial agent was calculated as follows:

FIC of antibiotic = (MIC of antibiotic and ES / MIC of antibiotic alone).[21]

The FIC of ES cannot be calculated, because single ES do not have antibacterial activity and therefore neither have a MIC. All FIC-values of the antibiotics equal to or below 0.5 were defined as synergism between the agents, values between 0.5 and 4 as indifferent effects and values higher than 4 as antagonism.[21] Likewise, for each combination the fractional bactericidal concentration (FBC) index was calculated.

Statistical analysis

Where appropriate, the Student's t-test for independent groups was used. For all combinations of antibiotics and ES, the mean OD-value of the MIC of antibiotic/ES was compared with the mean OD-value of that specific concentration for the antibiotic alone. The mean MBC of the combination antibiotic/ES was compared with the mean count of bacterial colonies of that specific concentration for the antibiotic alone.

Results

Maggot ES decreased the MIC and MBC of gentamicin for *S. aureus* at 50 µg ES/well 64- and 32-fold, to 0,125 mg/L (p<0.0001) and to 0,5 mg/L (p<0.0001) respectively (Fig. 2). The corresponding FIC and FBC-indexes were 0,0015625 and 0,03125. The reduction of the MIC and MBC was dose-dependent (Fig. 2). Maggot ES did not reduce the MIC of flucloxacillin for *S. aureus*, though the MBC was four times lower in the presence of 50 µg ES/well (p<0.0001). No interaction between penicillin and ES against *S. pyogenes* was noted. The results of the growth of *E. faecalis* in combination with amoxicillin/ES and vancomycin/ES did not show alteration of the MIC. However, the turbidity of the wells with amoxicillin/ES and vancomycin/ES below the MIC was higher as well as the turbidity of the wells with single ES, compared to the control bacterial growth, which means that ES increased the growth of *E. faecalis* (at 50 µg ES/well compared to the control bacterial growth: p<0.0001). This increase explained the higher MBC of amoxicillin/ES at 50 µg ES/well (p=0.0051). The MBC of vancomycin was equal to the MBC of vancomycin/ES for *E. faecalis*. For the combinations of gentamicin/ES against *E. Cloacae* and of gentamicin/ES, tobramycin/ES and ciprofloxacin/ES against *P. aeruginosa*, no difference of the MIC or the MBC was detected. As mentioned in previous research, single ES did not have an antibacterial effect on any of the bacterial species.[20] An overview of the results is shown in Table 1 and Table 2.

The MIC-values of each combination of antibiotics and bacterial species were compared with the MIC-values, described in literature, to control the reliability of the tests. Table 1 indicates that all values fell in the expected concentration ranges.[22]

Discussion

In this study we showed that maggot ES act synergistically with some antimicrobial agents against *S. aureus*. The synergistic effect was noted for gentamicin/ES and flucloxacillin/ES against *S. aureus*. These results may explain part of the accelerated wound healing that is observed when MDT is combined with antibiotics in comparison with the use of MDT alone. The growth of *S. pyogenes*, *E. cloacae* and *P. aeruginosa* was not affected in the presence of ES.
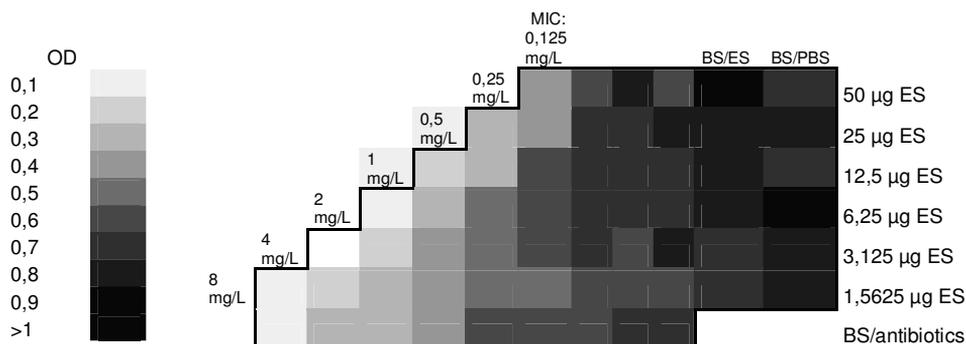

Fig. 2 This figure shows the synergism between gentamicin and maggot ES against *S. aureus*. The different gray hues represent the mean quantity of the bacterial growth of three independent experiments. The index on the left shows the OD-values that relate to the gray hues and an OD-value of <0.06 is defined as MIC. The overview corresponds with the checkerboard as presented in Fig. 1. In the columns, the various concentrations antibiotics are demonstrated and in the rows, the different concentrations ES are displayed. The typical stepwise decrease of the MIC in presence of ES, comparing to the MIC of the controls (BS/antibiotics, BS/ES and BS/PBS), indicates the increase in antibacterial activity of gentamicin. ES = excretions and secretions; OD = optical density; MIC = minimal inhibitory concentration; BS = bacterial suspension; PBS = phosphate buffered saline.

The interaction between gentamicin and ES could be explained by the antibacterial mechanism of action of gentamicin. Gentamicin, an aminoglycoside, kills bacteria by inhibiting their protein synthesis, which restrains their vital growth, and by disrupting the structure of the bacterial cell wall.[24] However, bacterial cell walls are relatively impermeable to gentamicin.[25] The ES could increase the permeability of the cell wall and therefore improve the penetration of the aminoglycoside in the cell, and hence the bactericidal effect. The Gram negative cell wall of *P. aeruginosa* and *E. cloacae* has a bilayer structure and is therefore even more impermeable to aminoglycosides than the cell wall of *S. aureus*, which is Gram positive. Possibly, this could clarify the differences in the results for both Gram negative microorganisms and *S. aureus*.

Flucloxacillin and ES showed a reduced MBC of *S. aureus.* Flucloxacillin inhibits bacterial cell wall synthesis, which causes cell lysis,[26] and perhaps ES also influence this process. Further research has to verify the underlying mechanism of these interactions.

	MIC of antibiotic (mg/L)	MIC of antibiotic/ES (mg/L)	FIC Index	MIC range of antibiotics in literature (mg/L)	MBC of antibiotic (mg/L)	MBC of antibiotic/ES (mg/L)	FBC Index
Gentamicin/ *S. aureus*	8	0,125	0,001563	0,008-128	16	0,5	0,03125
Flucloxacillin/ *S. aureus*	0,125	0,125	1	Not found	0,5	0,125	0,5
Penicillin/ *S. pyogenes*	0,125	0,125	1	Not found	0,125	0,125	1
Amoxicillin/ *E. Faecalis*	2	2	1	0,12-128	4	8	2
Vancomycin/ *E. Faecalis*	2	2	1	0,12-128	>8	8	< 1
Gentamicin/ *E. Cloacae*	2	2	1	0,03-128	2	2	1
Gentamicin/ *P. aeruginosa*	0,5	0,5	1	0,06-128	2	2	1
Tobramycin/ *P. aeruginosa*	0,25	0,25	1	0,03-128	1	1	1
Ciprofloxacin/ *P. aeruginosa*	0,125	0,125	1	0,015-128	1	1	1

Table 1 Overview of all the results.
MIC = minimal inhibitory concentration; MBC = minimal bactericidal concentration;
ES = excretions and secretions; FIC = fractional inhibitory concentration; FBC = fractional bactericidal concentration.

ES did not influence the MIC's of vancomycin and amoxicillin against *E. faecalis*, but ES (with or without antibiotics) induced increased growth of *E. faecalis.*
The MBC of amoxicillin was slightly increased by the addition of ES. Apparently ES contain nutrients for *E. faecalis.*

No synergism or antagonism was observed between the other antibiotics and ES. This is the first time that the interaction between antibiotics and maggot ES has been investigated. In other studies, direct antibacterial properties of ES were described, but neither in our earlier study, nor in this one was any direct antibacterial effect of ES found. All methods in literature, that were used for examination of antibacterial activity of maggots and/or ES were accurately compared and tested in our previous study.[20] The procedure of collecting ES might be a factor involved to explain the inconsistent results. We collected ES with as few external factors as possible and adapted the conditions to clinical practice, to not alter consistency and influence the outcome.[23] The other studies had a more complex procedure

of collecting the ES. A recent randomised clinical trial confirms the results of our previous study.[27] This trial compares the application of hydrogel versus maggots for the treatment of leg ulcers and does not find a reduction of the bacterial load in the wound during MDT, however the study proves effective wound debridement by larvae. In view of the lack of a direct antibacterial effect of ES, the beneficial effect

Combination	Interaction	p-value
Gentamicin/ *S. aureus*	Synergism:	p < 0.0001
	FIC Index = 0,0015625	p < 0.0001
	FBC Index = 0,03125	
Flucloxacillin/ *S. aureus*	Indifferent MIC: FIC Index = 1	ns
	Synergistic MBC:	
	FBC index = 0,5	p < 0.0001
Penicillin/ *S. pyogenes*	Indifferent MIC and MBC:	
	FIC/FBC Index = 1	ns
Amoxicillin/ *E. faecalis*	Indifferent MIC and MBC	ns
	FIC Index = 1	
	FBC Index = 2	p = 0.0051
Vancomycin/ *E. faecalis*	Indifferent MIC and MBC	
	FIC/FBC Index = 1	ns
Gentamicin/ *E. cloacae*	Indifferent MIC and MBC	
	FIC/FBC Index = 1	ns
Gentamicin/ *P. aeruginosa*	Indifferent MIC and MBC:	
	FIC/FBC Index = 1	ns
	Below MIC growth reduction	p = 0.0083
Tobramycin/ *P. aeruginosa*	Indifferent MIC and MBC:	
	FIC/FBC Index = 1	ns
Ciprofloxacin/ *P. aeruginosa*	Indifferent MIC and MBC:	
	FIC/FBC Index = 1	ns

Table 2 The interaction and corresponding significance of all combinations antibiotics/ES. MIC = minimal inhibitory concentration; MBC = minimal bactericidal concentration; FIC = fractional inhibitory concentration; FBC = fractional inhibitory concentration; ns = non significant (p > 0.05)

of MDT that is observed in clinical studies must be an indirect antibacterial activity of maggots and/or their ES, such as an immune-related effect. A report of van der Plas et al. shows evidence for inhibition of multiple neutrophil pro-inflammatory responses by maggot ES.[28]

Several limitations can be noted in this study. The interaction of maggot ES and antibiotics has been tested in vitro and could differ from the results in vivo, because of other influences, such as the immune system, that possibly play a role in this interaction. However, the conditions of the experiments were adjusted as much as possible to the clinical situation. For example, we incubated the maggots in darkness at a temperature of 35°C and used bacterial strains that were isolated from infected wounds of our trauma and orthopaedic patients. This study made only use of the checkerboard titration as their main focus, though synergism can also be determined by the killing curve method.[29] In literature the checkerboard assay is advised and described to be the most accurate test to search for synergism between different substances.[22]

The treatment of acute and chronic infections in these times is more often complicated by antibiotic resistance, which increases fast.[30] Therefore we need to search for new antibacterial treatment possibilities. In clinical practice, MDT shows successful effects in the healing of severe, infected wounds.[1-3] We also observed that MDT and antibiotics as a combined therapy might accelerate wound healing even more than single maggot therapy.

The synergism between gentamicin and maggot ES could be of direct importance in clinical practice, because it could permit the use of lower doses of gentamicin. A low concentration of gentamicin is already bactericidal in the presence of maggot ES and could therefore provide better patient security by reducing the risks of severe gentamicin related side-effects, such as nefrotoxicity and hearing loss. The exact mechanism of the interaction between gentamicin and ES, as well as the clarification of the underlying mechanism of MDT in severe, infected wounds and the possible role herein of the immune system, are our current topics of interest for further research.

Acknowledgements

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