Melittin Treatment for Biofilm of MRSA (Methicillin Resistant *Staphylococcus aureus*)

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ABSTRACT

Background: According to the Centers for Disease Control and Prevention (CDC) there were estimated to be 94,360 MRSA invasive infections in the US with approximately18,650 deaths annually since 2005.Other organizations estimate the true numbers to be over one million infected in the US with MRSA and over 100,000 deaths from 2005-2010.These infections attack all ages, from the elderly to the young, mainly because their immune system is suppressed. In acute care hospitals, MRSA colonization is a common cause of nosocomial infection and increased hospital costs (Huh, Kim, &Chae, 2012). MRSA universally attach to surfaces and produce extracellular polysaccharides, resulting in the formation of abiofilm. Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause infections in patients with indwelling medical devices.An appreciation of the role of biofilms in infection should enhance the clinical decision-making process.Currently, biofilm is considered as a major mediator of infection with an estimated 80% incidence of infection associated with the formation of biofilms. Biofilm resistance is usually multifactorial, which makes it difficult to eradicate biofilms. Therefore, this study will focus on creating*Methicilin ResistantStaphylococcus aureus* biofilms in culture and on testing the effects on that biofilm of the antibacterial peptide: Melittin.
Method: This study is an experiment and the strain of Methicillin Resistant *Staphylococcus aureus*(MRSA) was WBG 8287 which was donated by France O Brien who is from the medical research facility of royal Perth hospital.
Result: The biofilm formation produces large amounts of non-cellular material with very few visible cells. The thickness of film shows that MRSA produced biofilm well. Melittin able to treat MRSAwith 60 minutes having stable color
Conclusion: The resultsshowed that the procedures used were capable of inducing this MRSA strain to form a biofilm and melittin able to treat MRSA.
Suggestion: However, it is still not a perfect trial. Therefore, for the foreseeable future will be carried out the modified experiment and the experiment should be done with the variation of treatment and increasing the time of treatment. It aims to get the best results in the healing process of the disease which is caused by MRSA.

Key words:MRSA, biofilm, Melittin.

Introduction

*Staphylococcus aureus*is a major human pathogen and has many virulence factors that allow the infection of humans and animals, from superficial lesions to life-threatening systemic conditions such as endocarditis, osteomyelitis, pneumonia, meningitis and sepsis (Sorum *et al*., 2013).*Staphylococcus aureus*is a gram-positive bacterium, with a cell wall that contains two major components;peptidoglycan and teichoic acid. The speciesis found on the surface of the skin and upper respiratory tract, (Iwatsuki, *et al*., 2006). These organisms evolved methicillin resistance by acquiring the mecA gene and are known widely as methicillin resistant *Staphylococcus aureus* (MRSA). MecA expression results in the production of a penicillin-binding protein (PBP2a), which has reduced affinity for β-lactam antibiotics and confers resistance to all β-lactams, including the extended spectrum β-lactams, in practical use as antimicrobial agents (Lulitanond *et al*., 2013). According to the Centers for Disease Control and Prevention (CDC) there were estimated to be 94,360 MRSA invasive infections in the US with approximately18,650 deaths annually since 2005.Other organizations estimate the true numbers to be over one million infected in the US with MRSA and over 100,000 deaths from 2005-

2010.85% of all invasive MRSA infections were from healthcare facilities with patients with two-thirds showing symptoms of infections after their stay and one-third while in the facility.14% of all infections occurred in the community with no exposure to healthcare facilities and this number is continuing to grow. Increasing antibiotic resistance is defined by an increase in the number of hospitalizations in in the last 15 years, with almost 5% of hospitalized patients acquiring an infection (Klevens - 2007). These infections attack all ages, from the elderly to the young, mainly because their immune system is suppressed. In acute care hospitals, MRSA colonization is a common cause of nosocomial infection and increased hospital costs (Huh, Kim, &Chae, 2012). Biofilm resistance is usually multifactorial, which makes it difficult to eradicate biofilms. Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms
to antimicrobial agents and the potential for these organisms to cause infections in patients with indwelling medical devices.
An appreciation of the role of biofilms in infection should enhance the clinical decision-making process.Therefore, this study will focus on creatingmethicillin resistant*Staphylococcus aureus* biofilms in culture and on testing the effects on that biofilm of the antibacterial peptide: Melittin.

Method

Preparation of MRSA
1 colony of MRSA was suspended in 200µL Bacteriological Peptone. The concentration of bacteria was estimated to be 5×10^7 bacteria per mL (calculation: 1 colony has previously been shown to contain approximately 10^7 cells (J. Ravensdale, personal). One colony was suspended in 200µL Bacteriological Peptone to produce a suspension of 5×10^7 cells/mL.

Preparation of MRSA Biofilm
30 µL of human serum was spread on the surface of 12 electron microscope stubs which were incubated at 37ºC for 30 minutes. After 30 minutes, all stubs were rinsed with 50 µL water. Then 2 mL of Bacteriological Peptone and 10 µL of an MRSA suspension (5×10^7 bacteria per mL) were added to the well containing the stub. Cultures were shaken for 48 hours at 70 rpm and 37ºC.

Treatment of Biofilm MRSA with Melittin and Melittin Fragment
The biofilm in each well was rinsed with Bacteriological Peptone, 100 µL Melittin (5 µg / mL) was placed on the stubs, which were incubated for 15, 30, or 60 minutes.

Fixing of MRSA Biofilm
Stubs were rinsed with HpH_2O, PBS, or Bacteriological peptone and then 50 µL Glutaraldehyde (2.5%) was placed onto the biofilm for 3 hours. Bacteria on the stubs were then dehydrated by sequential treatment withethanol at 70%, 90% and 100% for 10 minutes each, dried in desiccator until overnight.

Measurement of MRSA Viability
200µL of bacteriological peptone containing 1%was placed into the wells of the culture plate containing biofilm cultures.

Process of bacteria shooting
All images were taken by scanning electron microscope. Zeiss Neon EsB focussed ion beam scanning electron microscope (FIBSEM) located within the Centre for Materials Research, at Curtin University. The bacteria were coated in a way, evaporative deposition of platinum at 3 or 10 nm thickness.

Result

There are three types of biofilm formed in this experiment. In the first type of biofilm shown in figure (1, 2, & 3) shows that the film is produced thick enough. Figure 3 is taken from a low magnification, showing the number of cells that are not too much but movies are pretty much. This is reinforced, when the pictures are taken from different sides (shown by figure 2) looks very thick blobs but no visible cell there. This phenomenon raises a question in this trial, whether the bacteria is below the blob or no bacteria at all there.Biofilm on the second and third types is shown in figure 4 and 5. Figure 4 is a biofilm formed by use of the Bacteriological peptone medium were added ethanol. The result shows that a lot of cells are formed but a little film. With a low magnification, the cell looks

almost cover the entire surface of the stub. Figure 5 shows the presence of more cells of figure 4 it is clear that even looks like a blob of biofilm. This type of biofilm using Bacteriological peptone medium were added sucrose. But the figure 5, if carefully observed seem obvious presence of other bacteria in the biofilm (looks different forms of type MRSA cells, foreign cells and elongated oval).

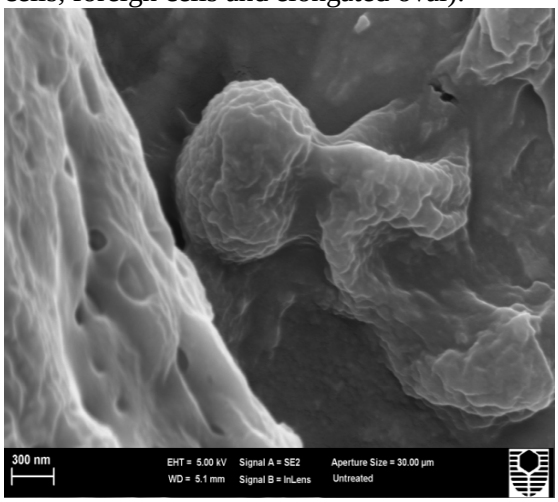

Fig.1. In this figure, from the high magnification shows that the cell is coated by a film thick enough

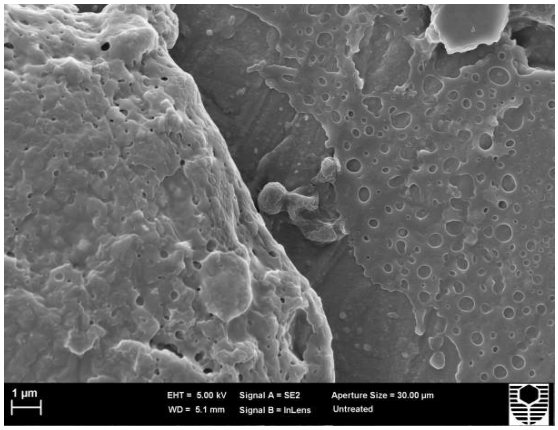

Fig.2In this figure, from the low magnification shows the blobs. Possibility, cells was coated in a thick film.

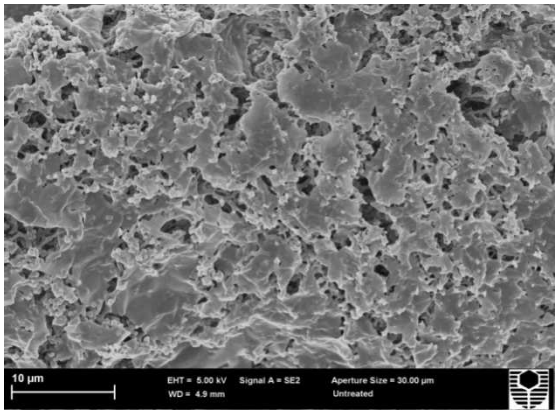

Fig.3. In this figure, from the low magnification shows many of these possible cells are present on this part of the film.

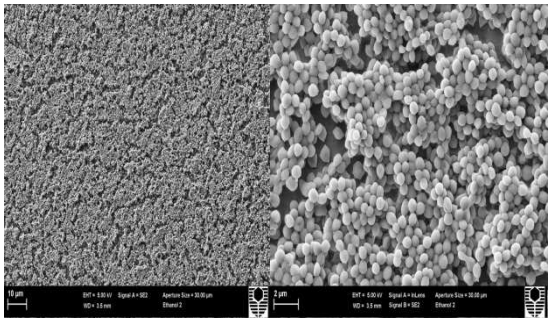

Fig.4. Representative, biofilm which was created using bacteriological peptone plus ethanol as a medium. In this figure, from the low magnification shows many of cells. Bacteria were prepared as described above with each of the two media described.

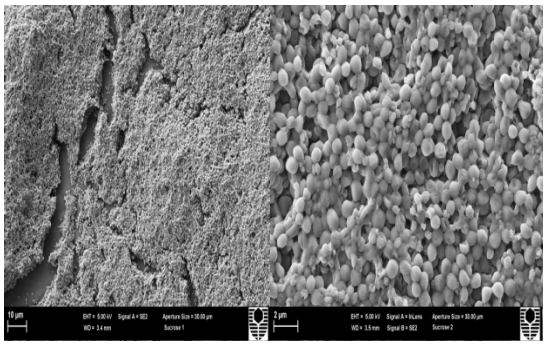

Fig.5. Representative, biofilm which was created using bacteriological peptone plus sucrose as a medium. In this figure, from the low magnification shows the number of cells is bigger than biofilm which was created by third method and using bacteriological peptone plus ethanol as a medium.

Treating of Biofilm with Melittin on stubs

Figure 6 is a biofilm with the same type obtained from the first method. From the figure shows that the biofilm was broken and the drying process is not perfect. Damage to the biofilm is still a big question, whether caused by melittin treatment or other factors that occur during shooting process which uses SEM.

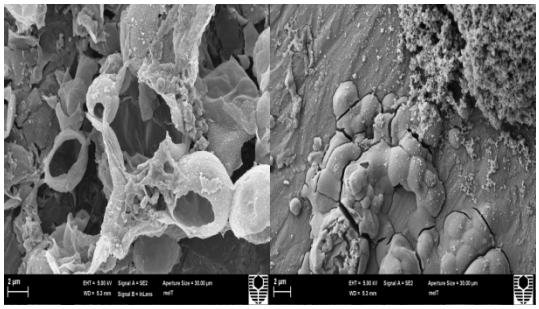

Fig.6. Representative, biofilm which was treatedwith Melittin(3µg/mL). This figure showed some areas of biofilm incomplete desiccation.

Treating of Biofilm with Melittin in well

Figure 7 shows the biofilms treated with melittin(5 µg/mL) in the well. C is a positive control samples, MA is a sample in treatment with melittin for 15 minutes, MB is in the sample treatment with melittin for 30 min and an MC sample in treatment with melittin for 60 minutes. Last color (shown in figure 7) was produced in accordance with the expectations of this experiment, namely;

- Red color changed to yellow, indicating the presence of cell
- Red color or does not change color, indicating nonexistence cells.

On other hand, if observed in tables 1 and 2 (the color change for each hour) it will show oddities (the sample MA, MB and MC). Peculiarities that arise, namely, the resulting red color of red as the primary color (after the addition of Bacteriological peptone medium were added phenol red) changed to orange and even some samples that have changed color to yellow back to red after standing for 24 hours. All samples (MA, MB and MC) back into the red.

Discussion

The images (Fig. 1, 2, & 3) above show that the biofilm formation produces large amounts of non-cellular material with very few visible cells. The thickness of film makes it impossible to know how many cells may be embedded within. When viewed from the biofilm formation processing, this method using human serum in order to make the cells can stick to each other. Basically,MRSA cells able to produce polysaccharides by themself to make their inherent between cells with other cells. Human serum is a protein which is found in human blood plasma. Essentially, human serum has the same elements (hydrogen, oxygen, carbon, and nitrogen) with polysaccharides which is produced by cells MRSA. Based on these explanations, possibility that the film formed on the first experiments is human serum,so it is notfilm which is produced by MRSAcells. In figure 5, growth in medium supplemented with sucrose resulted in a deeper biofilm than with Bacteriological peptone as a regular medium, or with ethanol added. Based on previous research (Welch *et al*., 2012) describe rapid growth in a culture in the presence of sucrose in base medium. Therefore, sucrose was chosen as an additive to the medium in this experiment.

On biofilm treatment process using melittin (3 µg/mL), the images show that the iofilm has been visibly damaged. However, this cannot be assumed to occur as a result of melittin. This form of damage could be a result of incomplete desiccation prior to the vacuum process associated with evaporative metal coating.According to Welch, Ken et al., (2012), the viability assay used in this experiment based on a metabolic activity assay (MAA), evaluates the amount of viable bacteria with the help of a pH indicator. The accumulation of metabolic acid products causes a drop in pH of the assay, which is indicated by a change in color of phenol red from red to yellow (Welch, Ken et al., 2012). The tables showing color-changes following melittin treatment showed irregularities. Controls showed a rapid change in color, with melittin treatments showing less rapid changes. At all-time variations melittin treatment process (15, 30 and 60 minutes) all showed weird occurrence. However, there is still one sample on melittin treatment with 60 minutes having stable color is red. This is consistent with the hypothesis of the experiment. But for another samples, the color is not stable. They changed, from red to orange to yellow and they became stable on red after overnight. From the latter observation suggests that the cells in the biofilm dead. Basically, this metabolic test is used to assess the number of viable bacteria in the test medium through production of metabolites. Metabolites produced by the

biofilm or planktonic cultures will depend on both, the individual bacterial metabolic activity and the number of bacteria that are marked with red color changes to yellow.When reviewed in images which were produced from the process of biofilm formation using sucrose medium, biofilm has formed two different cell shapes. It has the largest number of cells which were shaped similar to MRSA cells, but many kinds of other cells form unknown. Based on that information, the opinion raises that two different cell typesmust be have different metabolic activities. Therefore unstable discoloration caused by the metabolic activity of cells other than the metabolic activity of MRSA.

Conclusion

The resultsshowed that the procedures used were capable of inducing this MRSA strain to form a biofilm and melittin (5µg/mL) able to treat MRSA with treating of time 15, 30, or 60 minutes.

Suggestion

However, itis still not a perfect trial due to process of checking the viability of cells which were observed for each hour was still a lot of samples that unstable discoloration.Therefore, for the foreseeable future will be carried out the modified experiment and the experiment should be done with the variation of treatment and increasing the time of treatment. It aims to get the best results in the healing process of the disease which is caused by MRSA.

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