Antibacterial Effect of Three Kinds of Methylene Blue Photosensitizers Mediated Photodynamic Therapy on Streptococcus Mutans

Zhixuan Liu, Yuezi Li, Dian Huang, Longhua Chen, Longyu Zhu, Xiaozin Chen and Fahu Yuan *

School of medicine, Jianghan University, Wuhan, China

* Corresponding author: Fahu Yuan (Email: 1308712856@qq.com)

Abstract: In order to study the antibacterial effect of methylene blue (MB) photosensitizer-mediated photodynamic therapy (PDT) on Streptococcus mutans in aqueous solvent form, SDS solution form and their mixed form, the influence of MB-PDT on antibacterial effect of Streptococcus mutans in different dosage forms was analyzed. The number of colonies after and before photodynamic therapy mediated by MB-mediated photodynamic therapy was compared by in vitro viable bacterial colony counting method. Negative control group, light alone group, water dosage MB group, mixed dosage MB group, SDS dosage MB group, water dosage MB-PDT group, mixed dosage MB-PDT group and SDS dosage MB-PDT group were set up respectively to analyze the effect of different dosage forms of photosensitizers on the antibacterial effect of Streptococcus mutans was analyzed by counting and comparing in vitro for 24 hours. Through in vitro culture experiment, it was found that the number of colonies in water dosage form, mixed dosage form and SDS dosage form MB-PDT group was less than that in water dosage form, mixed dosage form and SDS dosage form MB. The proportion of viable bacteria in SDS MB group was significantly lower than that in water dosage form, mixed MB-PDT group and mixed MB-PDT group. These results indicated that MB-PDT could significantly inhibit the proliferation and growth of Streptococcus mutans. Among them, SDS MB-PDT group had more obvious antibacterial effect on Streptococcus mutans than water MB-PDT group and mixed MB-PDT group, but there was no significant difference in antibacterial effect between water MB-mediated PDT group and mixed MB-mediated PDT group.

Keywords: Streptococcus Mutans; Photodynamic Therapy; Methylene Blue; Antibiosis.

1. Introduction

Photosensitizer-mediated photodynamic therapy (PDT), as an international interdisciplinary subject that is currently developing, was first reported as an anti-microbial infection in the early part of the last century, but it was gradually forgotten due to the discovery of antibiotics in the middle of the last century. PDT stands out among many anti-infection strategies due to its advantages of effectiveness, safety, low toxicity and reusable effects. With the development of more and more photosensitizers and the improvement of experimental conditions, PDT has also made great progress in the field of stomatology research, and has shown broad application prospects. The main antibacterial mechanism of PDT is that the photosensitizer reacts with Oxygen molecules to produce Reactive Oxygen Species (ROS) under the activation of light source, which acts on target cells, thus causing the lysis and death of bacteria. Its basic elements are mainly composed of light source, photosensitizer and oxygen, of which photosensitizer is the core material of PDT.

At present, PDT is mainly used as the second generation photosensitizer represented by Toluidine Blue O (TBO) or Methylene Blue (MB) in the fight against oral microbial infection. Both of them belong to phenothiazine alkaline dyes and have strong affinity with DNA in the nucleus and RNA in the cytoplasm. As a hydrophilic photosensitizer, MB has been shown to be effective against G+ and G- bacteria. However, the existing in vitro studies have shown that although PDT mediated by it can achieve significant efficacy against root canal infection bacteria, it still cannot achieve complete sterilization effect. The polymerization state of MB in aqueous solution is a very important research content in the physical properties of MB. When the water dosage form MB interacts with bacteria, it is easy to aggregate on the surface of bacteria to produce dimers, and the formation of dimers directly affects the production of ROS, thus reducing the photosensitive effect of MB. Therefore, this study mainly compared the antibacterial effect of different dosage forms of MB-mediated PDT on Streptococcus mutans.

2. Materials and Methods

(1) Preparation of MB-based photosensitizers in three dosage forms

For the preparation of water-soluble MB, 96 mg MB powder with a molecular weight of 319.85 was dissolved in 10mL PBS to make a solution with a concentration of 30mmol/L. For the preparation of MB-SDS solution, 23mg sodium dodecyl sulfate was dissolved in 10mL PBS to make SDS solution, and then 96mg MB powder was dissolved in SDS solution to make a solution with a concentration of 30mmol/L. For the preparation of mixed dosage form MB, 96mg MB powder was dissolved in 10mL of the mixture composed of glycerol, alcohol and PBS (in which the volume ratio of glycerol, alcohol and PBS was 30:20:50) to make a solution with a concentration of 30mmol/L.

(2) Preparation of bacterial suspensions of Streptococcus mutans

Streptococcus mutans were inoculated in BHI medium and diluted 1:10 in 40mL of freshly prepared BHI medium. The OD value was adjusted to make the OD600 equal to 0.4, and the bacterial solution with 108 colonies /mL was obtained.

(3) Antibacterial test

International Journal of Biology and Life Sciences
ISSN: 2957-9511 | Vol. 4, No. 1, 2023
The antibacterial test was divided into 8 groups according to the following Settings. The prepared Streptococcus mutans suspension was added to the 96-well plate according to the experimental requirements, and each group had 3 compound Wells:

1) Negative control group: 100 μL bacterial solution was added into the Wells, and then 100μL PBS was added. After mixing, 100μL mixed bacterial solution of Streptococcus mutans solution and PBS was extracted, diluted 1:10000, and 100μL diluted sample was extracted and evenly spread on BHI AGAR plate, and cultured anaerobically for 24-48 hours, and the colonies were counted.

2) Single light group: 100μL bacterial solution and 100μL PBS were added to the Wells, then mixed, and illuminated vertically by a semiconductor laser with a wavelength of 650 nm from above the well plate for 8 minutes. After laser irradiation, the bacterial solution in the Wells was mixed, and 100μL of the mixed bacterial solution of Streptococcus mutans and PBS was extracted. After 1:10000 dilution, 100μL diluted samples were extracted and evenly spread on BHI AGAR plates, and cultured anaerobically for 24-48 hours, and the colonies were counted.

3) Water dosage MB group: Then 100μL of freshly prepared aqueous dosage form MB was added into the well, and 100μL of bacterial solution was added respectively. After 8 minutes of interaction between the two in the dark, 100μL of the mixed bacterial solution of Streptococcus mutans and aqueous dosage form MB was extracted, diluted 1:10000, and the 100μL diluted sample was evenly spread on the BHI AGAR plate. Colonies were counted after 24 to 48h of anaerobic incubation.

4) Mixed dosage form MB group: 100μL freshly prepared mixed dosage form MB was added into the hole, and then 100μL bacterial solution was added respectively. After the interaction between the two under dark conditions for 8 minutes, 100μL of Streptococcus mutus bacterial solution and mixed dosage form MB were extracted. After 1:10000 dilution, 100μL of diluted samples were extracted and evenly spread onto BHI AGAR plates, and after 24 to 48 h of anaerobic cultivation, colonies were counted.

5) SDS type MB group: 100μL freshly prepared SDS type MB was added into the hole, and then 100μL bacterial solution was added respectively. After interaction between the two for 8 minutes in the dark, 100μL mixed bacterial solution of Streptococcus mutans and SDS type MB was extracted. After 1:10000 dilution, 100μL of diluted samples were extracted and evenly spread onto BHI AGAR plates, and after 24 to 48 h of anaerobic cultivation, colonies were counted.

6) Water-based MB-PDT group: 100μL freshly prepared water-based MB-PDT was added into the hole, then 100μL bacterial solution was added, and a semiconductor laser with a wavelength of 650 nm was illuminated vertically from the top of the hole plate for 8min. The output power of the laser was 50 mW, and the illumination energy was 15J. After laser irradiation, the bacterial solution in the Wells was mixed, and 100μL of the mixed bacterial solution of Streptococcus mutans and MB was extracted. After 1:10000 dilution, 100μL diluted samples were extracted and evenly spread on BHI AGAR plates, and the colonies were counted after 24-48 hours of anaerobic culture.

7) Mixed dosage MB-PDT group: 100μL freshly prepared mixed dosage MB-PDT was added into the hole, then 100μL bacterial solution was added respectively, and a semiconductor laser with a wavelength of 650 nm was illuminated vertically from the top of the hole plate for 8min. The output power of the laser was 50 mW, and the illumination energy was 15J. After laser irradiation, the bacterial solution in the Wells was mixed, and 100μL of the mixed bacterial solution of Streptococcus mutans and MB was extracted. After 1:10000 dilution, 100μL diluted samples were extracted and evenly spread on BHI AGAR plates, and the colonies were counted after 24-48 hours of anaerobic culture.

8) SDS MB-PDT group: 100μL freshly prepared SDS MB-PDT was added into the well, and then 100μL bacterial solution was added respectively. A semiconductor laser with a wavelength of 650 nm was illuminated vertically from the top of the well plate for 8min. The output power of the laser was 50 mW, and the light energy was 15J. After laser irradiation, the bacterial solution in the Wells was mixed, and 100μL of the mixed bacterial solution of Streptococcus mutans and SDS MB was extracted. After 1:10000 dilution, 100μL diluted samples were extracted and evenly spread on BHI AGAR plates, and the colonies were counted after 24-48 hours of anaerobic culture.

Each experiment was repeated three times and the mean value was taken. The antibacterial rate was calculated according to the following formula:

antibacterial rate (%) = \( \frac{N - n}{N} \times 100\% \)

(N is the mean number of bacteria in the negative control group, n is the mean number of bacteria in the experimental group)

3. Results

In the negative control group and the light alone group, the number of colonies in the visible light group in the medium was less than that in the control group (Figure 1).

![Figure 1. Negative control group (left) and light alone group (right)](image)

The number of colonies in the water-based MB-PDT group was less than that in the water-based MB group and the water-based MB-PDT group (Figure 2).

![Figure 2. Water-based MB (left) and water-based MB-PDT (right) groups](image)
PDT group, the number of colonies in the mixed dosage MB-PDT group was less than that in the mixed dosage MB group (Figure 3).

![Figure 3. Mixed dosage MB (left) and mixed dosage MB-PDT (right) groups](image)

The number of colonies in the SDS MB-PDT group was less than that in the SDS MB group and the SDS MB-PDT group (Figure 4).

![Figure 4. SDS MB group (left) and SDS MB-PDT group (right)](image)

SDS-mediated PDT had the least viable bacteria. Among the three formulations of methylene blue, the amount of viable bacteria in the SDS formulation was less than that in the other two formulations (aqueous and mixed formulations). The aqueous form of MB has a certain antibacterial effect, and the mixed form of MB also has antibacterial effect, but the antibacterial advantage is not obvious, and SDS has the strongest advantage (Table 1).

### Table 1. Effect of different dosage forms of MB-mediated photodynamic therapy on the viability of Streptococcus mutans

<table>
<thead>
<tr>
<th>Group</th>
<th>Viable bacteria (CFU/100mL) Mean (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Negative control</td>
<td>37.33±6.28</td>
</tr>
<tr>
<td>2. Light alone</td>
<td>33.00±7.80</td>
</tr>
<tr>
<td>3. The water dosage form MB</td>
<td>18.17±4.49</td>
</tr>
<tr>
<td>4. Mixed dosage MB</td>
<td>19.83±3.87</td>
</tr>
<tr>
<td>5. The SDS type MB</td>
<td>3.33±1.21</td>
</tr>
<tr>
<td>6. Water-based MB-PDT</td>
<td>10.17±2.64</td>
</tr>
<tr>
<td>7. Mixed dosage MB-PDT</td>
<td>10.33±1.75</td>
</tr>
<tr>
<td>8. SDS-type MB-PDT</td>
<td>0.67±0.82</td>
</tr>
</tbody>
</table>

4. Conclusion

Dental caries is one of the common and frequently occurring diseases that endanger human health. The incidence of dental caries ranks at the forefront among various diseases. Bacterial infection is still one of the main factors in the pathogenesis of multiple dental caries. Although there are hundreds of species in oral plaque biofilm, Streptococcus mutans is still the most closely related pathogen. Streptococcus mutans has been proved to promote the occurrence and development of dental caries through acid production, acid resistance, and synthesis of exopolysaccharides and virulence factors [1]. In oral cavity, S. mutans colonize the tooth surface mainly through the formation of dental plaque biofilms.

Photodynamic therapy is a new method of noninvasive or minimally invasive, non-thermogenic, and photochemical effect of photoactivated photosensitizer to selectively kill pathogenic microorganisms. It has very important application value in the treatment of dental caries and periodontal diseases [2,3]. The treatment principle is that after the photosensitizer enters the body, it is stimulated to transition from the ground state to the excited state under the irradiation of appropriate wavelength light, and the energy is transferred to the oxygen molecules. The oxygen molecules that receive the energy undergo a series of photochemical reactions to produce singlet oxygen and a variety of reactive oxygen species with strong oxidation [4]. Studies have shown that PDT-mediated treatment of periodontal disease can eliminate plaque formation without involving normal periodontal mucosal tissue [5]. It has also been reported that by culturing Streptococcus mutans in vitro and pretreating them with PDT method, the growth and proliferation of bacteria can be effectively inhibited [6]. PDT can inhibit the proliferation of bacteria in complex environments, such as subgingival plaque, which is resistant to microbial agents, but PDT can inhibit plaque formation.

MB is a hydrophilic photosensitizer. Its cationic surface and small molecular weight make it often selected for anti-microbial infection. However, MB-PDT treatment can reduce the acid-producing capacity of the biofilm on the surface of S. mutans, and PDT treatment can reduce the proportion of live bacteria in the plaque biofilm. Therefore, methylene blue-mediated photodynamic therapy was used in this study. The dosage form of MB is also closely related to its photosensitizing effect. In this study, an in vitro model of Streptococcus mutans was established, and they were divided into MB group, MB-PDT group and control group according to different methods. Finally, the obtained colonies were counted, and the statistical analysis showed that the conclusions were as follows:

1. Through pairwise comparison of the proportion of viable bacteria in each group, it can be seen that the proportion of viable bacteria in the PDT group was significantly lower than that in the MB group and the negative control group, while the proportion of viable bacteria in the MB group and the negative control group was not statistically different, indicating that MB-PDT can significantly inhibit the proliferation and growth of Streptococcus mutans.

2. By calculating and comparing the proportion of viable bacteria in the samples of each group, it can be found that the number of colonies in the three dosage forms groups was less than that in the control group. There was no significant difference in the proportion of viable bacteria in the samples of water dosage form MB-mediated PDT and mixed dosage form MB-mediated PDT. There was a significant difference in the proportion of viable bacteria in the samples of SDS MB-mediated PDT, aqueous MB and mixed MB mediated PDT, indicating that MB formulations had antibacterial effect on Streptococcus mutans. The antibacterial effect of SDS MB-mediated PDT was more obvious than that of aqueous MB and mixed MB mediated PDT. However, there was no significant difference in antibacterial effect between the water-based and mixed-based MB-mediated PDT groups.
Acknowledgments

This work is supported by the Innovative Training Program for College Students in Hubei Province (No. S202211072041).

References


