The Antibacterial Activity of Natural Product Black Tea Theaflavins On *Streptococcus Mutans*

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**Abstract.** **Objective** The aim of the study is to investigate the antibacterial effect of the black tea theaflavins (TFs) on *Streptococcus mutans* (*S. mutans*) which cause the dentin caries. **Methods** (1) Broth dilution method was used for the determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). (2) Mixed bacterial suspension with three different concentrations of TFs and recorded pH values before and after anaerobic incubation. (3) Scanning electron microscopy was used for observing bacteria morphology. **Results** (1) The MIC and MBC were 0.5mg/ml and 1.0mg/ml respectively. (2) With the increasing concentration of TFs, the ability of bacteria to produce acid gradually decreased. (3) TFs changed the shape of *S. mutans*. **Conclusions** Theaflavins inhibits *S. mutans* effectively.

1. Introduction

Dental caries, also known as tooth decay, is the breakdown of enamel and dentin due to the acids produced from bacteria. The presence of cariogenic bacteria is a crucial for the develop of caries, and *Streptococcus mutans* (*S. mutans*) is one of the most important bacteria that causes caries [1-2].

Natural product, theaflavins (TFs) is the main component of black tea, which is a benzoquinone compound oxidized by tea polyphenols. It is known for its widely proven anti-oxidation, antibacterial and anti-inflammatory effects. The potential of theaflavins in the prevention and treatment of oral diseases has received increasing attention from other researchers. [3-4].

However, the researches on the effects of black tea is mainly seen in the use of black tea water extract or alcohol extract, and not much studies have been done on pure theaflavins. In this study, highly purified theaflavins were used to investigate the antibacterial and bactericidal effects of the *S. mutans*, the effects on the acid production of the *S. mutans*, as well as the morphological changes of TFs treated bacteria under scanning electronic microscope (SEM).

2. Materials and methods

2.1. Bacterial resuscitation and culture

*Streptococcus mutans* ATCC 25175 (Shanghai Beinuo Biotechnology Co., Ltd. China) was cultured on 3% sucrose-containing tryptone soy agar (TSA) medium anaerobically (80% N₂, 10% CO₂, 10% H₂) at 37 °C for 48 hours. After resuscitation, gram stain was done to confirm that no other bacteria is present.
in the sample culture. The non-contaminated strand is then cultured and passaged in TSA medium anaerobically for 48 hours at 37 °C. Bacterial colonies were picked up and suspended in sterile saline.

2.2. **The minimum inhibitory concentration and the minimum bactericidal concentration of theaflavin**

The minimum inhibitory concentration (MIC) of TFs against the *S. mutans* was determined by microplate dilution method. According to the American Society for Clinical and Laboratory Standards (CLSI) M07-A9 standard [7], TFs (Jiangsu Dehe Biotechnology Co., Ltd. China) are dissolved in dimethyl sulfoxide (DMSO) and sterilized distilled water. TSB liquid medium is used according to the double dilution method and made the TF solution with concentrations of 8, 4, 2, 1, 0.5, 0.25, and 0.125 mg/ml. 100 μL of different concentrations of theaflavin solution were added into each individual wells of the sterilized microplate using a sterile micro-sampler. Then, 100 μL of the bacterial solution was added to mix. In the experimental group, 3 sets of repetitions were set up parallelly.

Through visual observation, the concentration of the samples with no bacterial growth (solution appeared to be clear) was identified as the MIC of TF against *S. mutans*. These samples are smeared on another TSA plate to allow anaerobic growth at 37 °C for 24 hours. The concentration of the samples that did not see any bacterial growth was identified as the MBC.

2.3. **The effect of theaflavins on the acid production of the *S. mutans***

According to the MIC of theaflavin against the *S. mutans*, TSB medium is used to prepare theaflavin solutions with the concentrations of 1 / 2 MIC, 1 / 4 MIC, 1 / 8 MIC. 2 mL of those concentrations are added into a sterile test tube along with the same amount of bacteria suspensions to mix. METTLER TOLEDO FE20 pH meter (METTLER TOLEDO, Switzerland) is calibrated and the initial pH is measured. A control group of only bacterial liquid and medium was used as a control. Each group was set to 3 parallel repetitions. After 24 hours of anaerobic growth at 37 °C, the final pH value was measured and the change in pH (ΔpH) is calculated as final pH minus initial pH.

2.4. **Scanning electron microscopic observation of the effect of theaflavin on the *S. mutans***

Place sterile slides in a 24-well plate, add 0.5 ml of bacterial solution, and allow anaerobic growth for 24 hours at 37 °C. After bacterial growth was observed on the glass slides, the supernatant was discarded and carefully rinsed 3 times with PBS buffer. TFs were double diluted with TSB medium, 0.5 mL was added to the well plate, and the control group had the same number of bacteria, incubated at 37 °C for 24 h.

The supernatant was discarded again, rinsed 3 times with PBS, and left overnight with 2.5% glutaraldehyde at 4 °C. The soaked samples were washed 3 times with PBS for 15 min each time. The sample was treated with 50%, 75%, 90%, and 100% ethanol solutions for 10 min each. They were then observed by a Scanning electron microscopy (HITACHI SU8010, Japan).

2.5. **Statistical analysis**

The data were analyzed with SPSS 22.00 statistical software. The multiple groups were compared by one-way ANOVA test. P ≤ 0.05 was considered as statistically significant.

3. **Results**

3.1. **MIC of TFs against the *S. mutans* are shown in Tables 1.**

| Theaflavin Concentrations | MIC | MBC |
|---------------------------|-----|-----|
|                           | 0.5 | 1   |

Table 1. MIC and MBC of TFs against the *S. mutans* (mg/mL)
3.2. The effects of TFs on the acidogenic ability of the S. mutans are shown in Tables 2 Compared with the control group.

Table 2. The effect of Theaflavins on S. mutans (x±s)

| Theaflavin Concentrations | n  | ΔpH           | P      |
|---------------------------|----|---------------|--------|
| Control                   | 3  | 2.1867±0.1850 | 0.136  |
| 1/8 MIC                   | 3  | 1.9200±0.2128 | 0.002**|
| 1/4 MIC                   | 3  | 1.4400±0.1249*| 0.000***|
| 1/2 MIC                   | 3  | 0.6667±0.2458*|        |

*p<0.05. **p<0.01, ***p<0.001

3.3. Scanning electron microscopy (SEM) of the effect of TFs on the morphologic changes of the S. mutans is shown in Fig. 1.

Fig 1. SEM images of S. mutans (X5000). (A) Untreated control group of S. mutans. (B) S. mutans treated with 0.5 mg/ml TFs after 24 hours. (C) S. mutans after 24 hours exposure to TFs of 1 mg/ml.

4. Discussion

In the past, researchers have studied the inhibition of chlorhexidine and fluoride on S. mutans, but these two chemicals have certain limitations due to their own toxicity or adverse side effects. For example, chlorhexidine causes tooth staining and produces a large amount of fluoride, which may potentially cause fluorosis. As the result of their low toxicity and side effects, natural plant polyphenols are much more advantages and have recently received many attentions from researchers worldwide [5-7].

Theaflavins (TFs) is one of the many important flavonoids in black tea along with theaflavin-3'-gallate (TF-3-G), theaflavin-3' - Gallic acid ester (TF-3'-G) and theaflavin digallate (TF-DG) [2,6].

In this study, the MIC measured by the microplate dilution method was 0.5 mg/mL and the MBC was 1 mg/mL. This is very similar to the results from previous studies on the extraction of black tea with methanol, which they obtained a MIC of 0.6 mg/mL [7-8]. Other studies were suggesting that the hydroxyl group of TFs can be hydrogen-bonded to the lipid bilayer of the cell membrane [2], which may cause damage to the bacterial cell membrane and cause cytoplasmic outflow in the cell membrane. This mechanism also has an antibacterial effect since the enzyme are prevented from binding to its receptor site [7].

In this experiment, we found that with the increase in concentration of TFs, the acid-producing ability of the S. mutans was gradually weakened. As one of the flavonoids, TFs can destroy the acidogenic action of the S. mutans [8], which increases the pH of the local oral cavity. TFs can slowly degrade in the oral environment with a pH of 7.0-7.4 with a half-life in saliva of 49-76 min [9]. When scanning electron microscope was used to observe the morphological changes of the S. mutans (Fig 1), it is found that the bacteria were no longer closely arranged in a chain after 24 hours of theaflavin treatment. As
the concentration of theaflavins increased, the phenomenon of bacterial chain scission became more obvious, there are even cases where single cells were present. Moreover, the shape of the *S. mutans* also changed from the original spherical shape to the long rod shape, and some of the bacterial cells also showed a concave split on the surface. At the same time, we also found that the growth of the *S. mutans* after the theaflavin treatment was sparse, while the untreated rules grew tightly to form dense colonies. We believe that theaflavins may slow the formation of plaque biofilm by preventing the initial attachment of the *S. mutans*, which may be related to its inhibition of glucosyltransferase (GTF). In addition, flavonoids can inhibit the activity of GTF and impede bacterial adhesion [8-10]

The Theaflavin in this study demonstrated its good antibacterial properties, which provides a theoretical basis for potential clinical anti-caries applications. At the same time, it also provides new possibilities for the development of new anti-caries methods such as mouthwashes, toothpastes, chewable tablets, etc. by using this natural plant.

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