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Silk Fiber Films for the Slow and Continuous Release of Tetracycline and Doxycycline

A Thesis

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ABSTRACT

Aim: To assess the potential use of silk-based films for the slow and continuous release of tetracycline and doxycycline for use in periodontal or other surgical procedures.

Hypothesis: We hypothesize that silk-based films will allow for long-term release of antibiotics.

Material and Method: Tetracycline and doxycycline were mixed, at various concentrations, with the silk solution. Antibiotic loaded silk fiber films were incubated with *Streptococcus mutans* cultures to assess the release of the antibiotics. Chlorhexidine was used as a positive control and plain silk films without antibiotic as a negative control. Bacterial growth inhibition was measured spectrophotometrically after each consecutive 24-hour incubation period. The number of colony forming units (CFUs) was determined to validate growth inhibition.

Results: Tetracycline and doxycycline-loaded silk fiber films inhibited *S. mutans* growth in a concentration and time-dependent manner. Tetracycline with the concentrations of 0.375, 0.75 and 1.5 mg/mL inhibited *S. mutans* growth with noticeable release up to three, four and five days, respectively. Doxycycline with the concentrations of 0.375, 0.75 and 1.5 mg/mL inhibited *S. mutans* growth up to six, ten and eleven days, respectively. The colony forming units for the 1.5 mg/mL doxycycline and tetracycline exposed *S. mutans* cultures were $1.2x10^6$ and $1.8x10^6$ CFUs/mL, respectively, which are markedly less compared to the uninhibited control cultures which contained $3.5x10^9$ CFUs/mL.

Conclusions: We concluded that tetracycline and doxycycline can be successfully loaded into silk fiber films and inhibited bacterial growth with significant release up to five days for tetracycline and eleven days for doxycycline. Our data implies that silk fiber loaded films can be used as a medium for the localized delivery of antibiotics during periodontal and oral surgeries.

DEDICATION

I would like to dedicate my work to my mother, whose unconditional love and support throughout the years was my main source of motivation to overcome obstacles and who was always there when I needed her.

To my father for his support and continues care.

To my brother Sina, whose wisdom and guidance never let me get lost along the way.

I also like to dedicate this work to my beloved fiancé, Sarmad who has provided me with strong love shield and was always there to hold me and never let any sadness enter me.

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Introduction

Oral Microbiome and Periodontal Disease

Periodontium, which is the underlying structure that supports teeth, is a common site of inflammation. Should the inflammation of periodontium persist over a prolonged period of time, a group of conditions that together are called periodontal diseases, may arise [1]. The major contributors to periodontal diseases are bacteria, which can infect the periodontium and cause inflammation. Osseointegrated implants can also be the target for bacterial infection and, when get infected, can result in an inflammatory disease called peri–implantitis. In this condition, both the hard and the soft tissue around the implants are destructed and the prognosis is often poor. Peri-implant pockets can form as a result of this destruction, which can over time decrease the integrity of the supporting hard tissue [2, 3]. Since microorganisms have been shown to be involved in development of peri-implantitis, successful treatment of such infectious agents have been the topic of many recent scientific inquiries.

The oral cavity contains an extremely diverse bacterial flora in its healthy state [4, 5] . A new class of pathogenic bacteria is often introduced to the oral cavity within an hour of installing a dental implant and a complex biofilm is usually observed within 2 weeks of the procedure [6, 7]. Adult periodontitis is the result of periodontal destruction followed by a complex interaction between the host and the microbe [8-10]. Susceptible individuals undergo an elongated immunological response against bacteria in their periodontium, as the bacteria, which are considered as foreign agents by the body's immune system, stimulate the activity of matrix-degrading metalloproteinases (MMPs). These are protein-degrading

enzymes that are produced by the connective tissue and the inflammatory cells. When activated, MMPs degrade collagen, the main compound that is important for the structural integrity of the periodontium [8-11]. Destruction of collagen weakens the connective tissue and its surrounding bony structure [8, 11]. The role of different bacterial infections in recurrent periodontal diseases has made antimicrobial therapy a common practice for treating these diseases [12]. Of these cariogenic bacteria, *Streptococcus mutans* is one of the most common species that is present in the human oral microbiome [13, 14].

Antibiotics, Common Treatments for Oral Infections

Tetracycline, doxycycline and minocycline are antibiotics that can treat a broad range of infectious diseases and have been shown effective against obligate and facultative anaerobes [12] . When delivered systemically, these antibiotics are bacteriostatic, or simply stop the growth of bacteria, at different concentrations [12, 15]. It is important to note that 12% of the human oral flora is resistant to tetracycline [15, 16, 17]. On the other hand, metronidazole is commonly used to inhibit obligate anaerobes and its hydroxymetabolite can affect facultative anaerobes. Therefore, metronidazole is used as an anti-anaerobic agent [18]. The growing interest in clinical use of such antibiotics has increased the demand for determining the antibiotic concentrations in the specimen obtained from gingival crevices [19]. The dosage and administration schedules for antimicrobial treatments can be optimized by relating the local concentration of the drug at the site of infection to the least antibiotic concentrations needed to inhibit the microbial agent [19] .

Treatment of Periodontal Disease Using Systemic Antibiotics

Rate of metabolism, degree of absorption, and duration of antimicrobial activity are among critical pharmacological characterizes of antibiotics. These criteria are used to determine the use, dosage, and route and frequency of administration of antibiotics.

Periodontal disease has been traditionally treated via systemic antibiotics. For example, Atilla et al found that, in periodontal pockets greater than 6 mm, systemic treatment using minocycline significantly decreased the number of salivary proteases and epithelial cells [15, 20]. Similarly, systemic therapy with spiramycin or tetracycline for 14 days were found to reduce the rate of gingival crevicular flow [15, 20]. Although they are generally very effective in treating infections, systemic antibiotics carry the risk of developing resistant microbes as well as destroying the patient's natural flora in locations that were not intended for treatment [15, 20].

Since antibiotic resistance is a major growing issue that has outpaced the development of novel antibiotics, a selective and conservatives approach is suggested for treatment of infections [20]. One method for preventing this ongoing global challenge is localized antibiotic delivery using novel, bio-friendly media such as naturally obtained silk [20].

Local Drug Delivery

The ability of a drug delivery system to deliver the antimicrobial agents to the base of the pocket at a bacteriostatic or bactericidal concentration when treating periodontal infections is desired is a measure of its success [1]. The higher specificity of drug delivery

increases the desirability of that mechanism, as it increases the efficiency of the treatment and minimizes its side effects on non-desired sites.

In addition to specificity, biodegradability is a desirable characteristic of local delivery products, as it resolves the need to remove the drug delivery device upon completion of the treatment [1, 21]. Patients with recurrent diseases or those with non-reachable/non-responding sites are the best candidates for local drug delivery, which, in addition to conventional care, can greatly improve the quality of their treatment. When the goal is to reduce pocket depth and regain gingival attachment, locally applied antimicrobial agents, either an antibiotic or antiseptic, can significantly improve traditional scaling and root planning success[1].

Local antimicrobial agents are biodegradable and slow-release products that are placed as a repository into a prepared periodontal pocket. Locally applied antimicrobial agents help reduce or prevent reinfection during the healing process of the pocket [22] . Subgingival medications, which work by providing a high concentration of an antimicrobial agent directly to the diseased tissues, contain some of these properties and are proven effective against periodonto–pathogenic bacteria [22].

Since peri–implantitis oral lesions usually have a clear boundary and are well demarcated, controlled delivery devices can locally treat periodontal infections. This is because these devices are capable of sustaining a high local antimicrobial concentration for several days. This can in turn inhibit the bacteria that were protected in the incompletely-cleared biofilm [23, 24, 25].

Silk as a mean for local antibiotic delivery

Silk biomaterials are optimal vehicles for antibiotic delivery because of their tunable biodegradation, biocompatibility, effective antibiotic stabilizing properties, water-based processing, and various material formats [26-30]. Silkworms and spiders are the natural producers of silk fibers [31, 32] . The silk fibers produced by the *Bombyx mori* silkworm have for long been the primary silk material used for producing sutures [32-35]. In addition to making sutures, silk can provide scaffolds for tissue engineering and is commonly used in biomaterials and for controlled release purposes [21, 34, 36]. These fibers are biocompatible, mechanically strong, and have been shown to release certain drugs, like antibiotics, within a controllable interval [26, 31, 35]. Silk films have been found to improve antibiotic stability, which is another important benefit of using this substance [26, 35].

Reduced side effect and increased efficacy are among the favorable properties of controlled-release polymers [26, 37-40]. This is specifically important in cases such as bone infections and abscesses, where systemic drug penetration into the target is limited [41-43].

Regulation of the beta sheet content of the silk implants can tune their degradation rate from days to years. The bioactivity and sensitivity of the silk can be maintained by processing it in mild, ambient conditions [26, 44, 45] . The developments in tissue engineering has increased the demand for these biomaterials, as they can be used to synthesize tissues *in vitro*, which can be thoroughly studied prior to being implanted in vivo [26].

Silk loaded with different concentrations of antibiotics can potentially slow down the ongoing problem of antibiotic resistance, which is a growing problem that is mainly due to the use of systemic antibiotics. In this study, we evaluated the efficacy of silk as a medium for the slow and continuous release of tetracycline and doxycycline.

Aim

To assess the potential use of silk-based films for the slow and continuous release of tetracycline and doxycycline for use in periodontal or other surgical procedures.

Hypothesis

We hypothesize that silk-based films will allow for slow and continuous release of tetracycline and doxycycline.

Research Design and Methods:

Materials. The bacterial strain used was *S. mutans* ATCC 25175 and was obtained from American Type Culture Collection (Manassas, Virginia). The Bacto™ Brain Heart Infusion and BBL™ Brain Heart Infusion Agar were purchased from VWR (Radnor, Pennsylvania). Doxycycline hydrochloride, tetracycline hydrochloride, chlorhexidine digluconate solution and CellCrown™ inserts were purchased from Sigma Aldrich (St. Louis, Missouri).

Preparation of silk solution. The silk from the *B. mori* cocoons was extracted into liquid solution in Dr. Kaplan's lab (Department of Chemical Engineering and Department of Biomedical Engineering, Tufts University, Medford, MA) and was used without further purification. Following the extraction process, the silk solution was prepared using a 30-minute boil technique and was diluted appropriately with distilled water so that the final amount of silk protein in solution was 7.2%. Once the stock silk solution was obtained, antibiotic film solution, containing either tetracycline or doxycycline, were prepared by serial dilution as described below.

Preparation of antibiotic-loaded silk films. Stocks solutions of tetracycline and doxycycline with 10 mg/mL concentrations were prepared and kept in the freezer. A serial dilution of the antibiotic was made to prepare films with the desired concentrations. Final antibiotic concentrations in silk films were 0.375, 0.75, 1.5 and 3.0 mg/mL in 2% silk protein. Once the appropriate solutions of tetracycline or doxycycline and silk were made, 1.7 mL of each concentration of antibiotic-loaded silk was pipetted onto silicone molds that

were previously cleaned with tape. The silicone molds were then left to dry at room temperature for 24 hours. Following the drying process, the films were removed from the silicone molds using adhesive tape and then placed in a water-annealing vacuum chamber for 24 hours. After the water annealing process, each film was cut in half using a sterile scalpel and mounted in CellCrown^{TM} inserts. These inserts held each silk film in place during liquid culture incubation and kept the film from folding over on itself.

Preparation of *S. mutans* cultures. Broth and agar plates were prepared 2-3 days before the experiment and were stored at 4ºC. In 1 L of distilled water, 37 g of Brain Heart Infusion (BHI) broth was added and the solution was mixed. The solution was then autoclaved at 121ºC and stored at 4ºC. On the same day that films were placed in the vacuum, fresh broth culture of bacteria were prepared by inoculating 5 mL of BHI broth in a test tube with a single colony from an agar plate and incubating for 24 hours at 37ºC. The amount of *S. mutans* in liquid culture broth was standardized to 10^6 CFU/mL using serial dilutions and optical density (590 nm) measurements. After 24 hours, the optical density of the liquid culture was measured for each well. The silk films mounted in the inserts were then placed into a 12-well plate with fresh liquid culture broth containing 10^6 CFU/mL *S. mutans*. This process was repeated every 24 hours until the bacteria started to grow again in the presence of the antibiotics.

Measurement of viable counts. Serial dilution and plate counting method were used to measure the total viable counts. Seven tubes were obtained and were labeled 10^1 to 10^7 to

represent the dilution factors for *S. mutans*. Similarly, 7 agar plates were labeled with same dilution factors. To each tube 900 µL of the broth were added. To the tube labeled 10^1, 100 µL bacterial culture were added and the entire solution was mixed by pipetting up and down. From tube 10^1, 100 µL of solution was pipetted to tube 10^2 and the solution was similarly mixed. Using the same method, all 7 solutions were prepared. From each tube, 100 µL of solution was pipetted out and spread onto the surface of its corresponding agar plate, which was then incubated at 37ºC. When the colonies were large enough to count the number of colonies in each plate was determined using the ImageJ software.

Results

Inhibition of *S. mutans* growth in the presence of different concentrations of tetracycline and doxycycline loaded onto silk films was studied by measuring the optical density (O.D. at 590 nm) of the cultures at different time-points post-incubation. We used chlorhexidine as a positive control, representing 100% growth inhibition and silk films containing no antibiotic as a negative control. The data showing the growth inhibition of *S. mutans* by tetracycline are depicted in Figure 1. Silk films, containing increasing concentrations of tetracycline were prepared for the study. Initially we studied the 0.01, 0.075, and 0.15 mg/mL concentrations as options for loading low doses of antibiotic for local release. Figure 1A illustrates that the 0.01 mg/mL sample showed a rapid decrease in inhibition starting from day 1 and was not considered useful for further study. On the other hand, the 0.15 mg/mL and the 0.075 mg/mL samples showed 98% inhibition until the 2nd day and the 1st day, respectively. Since these low concentrations inefficiently inhibited *S. mutans* growth, the concentrations of tetracycline were increased to 0.375, 0.75, and 1.5 mg/mL. As the data on Figure 1 show, a higher percent inhibition of growth was achieved for samples with a higher concentration of tetracycline, with the 0.375 mg/mL and the 0.75 mg/mL samples inhibition was achieved only until the 3rd and 4th days, respectively. This is while the 1.5 mg/mL sample showed inhibition of greater than 98% for up to 5 days.

Taken together, these data suggest that at relatively higher doses of tetracycline, *S. mutans* growth was inhibited up to 5 days.

We then investigated the efficacy of doxycycline to inhibit the growth of *S. mutans*. Based on the results of the tetracycline experiments, doxycycline was used at 0.375, 0.75,

and 1.5 mg/mL. As can be seen in Figure 2, the samples with a higher concentration of antibiotic had a higher day-by-day and longer overall period of growth inhibition compared to the less concentrated samples. For example, the sample with 1.5 mg/mL doxycycline had a greater than 98% inhibition of growth for 8 days, after which it slowly decreased to 90% by the end of the 12th day. The sample with 0.75 mg/mL doxycycline showed a growth inhibition of greater than 97% until the 7th day which then decreased to 75% by the end of the 12th day. Finally, the lowest concentration of doxycycline, 0.375 mg/mL, showed a growth inhibition of 95% until the 6th day, followed by a decrease to only 31% by the end of the 12th day.

In another series of experiments, we determined the CFUs in the cultures from the final day that the 1.5 mg/mL concentrations of doxycycline and tetracycline showed nearly 100% inhibitions. Figure 3 shows representative images of the agar plates containing negative controls with dilution factors of 10^4 and 10^6 as well as the cultures with tetracycline and doxycycline with a dilution factor of 10^4. The positive control, chlorhexidine was also plated without any dilution and showed that there was complete 100% inhibition at each time point (data not shown). As can be seen in Figure 3, the negative control with a 10^4 dilution had too many colonies and was not useful as a baseline, while the negative control with a 10^6 dilution was found to have 2,106 colonies, relating to a CFUs/mL of 3.51×10^9. The doxycycline and tetracycline samples with a dilution factor of 10^4 were found to have 73 and 109 colonies, respectively which correspond to 1.22×10^6 and 1.82×10^6 CFUs/mL, respectively. These CFUs are very similar to the initial inoculation suggesting inhibition of growth (bacteriostatic) but not killing (bactericidal) by both antibiotics.

Taken together, our data suggest that doxycycline is more effective in inhibiting *S. mutans* growth for a longer period of time when compared to tetracycline.

Discussion

Comparing the silk samples containing 1.5 mg/mL of antibiotic, inhibition of *S. mutans* growth of greater than 98% lasted for 7 days with doxycycline and for 5 days with tetracycline. Up to the 8th day of the experiment, growth inhibition for doxycycline was still above 95% while that of the tetracycline was closer to 25%. This observation can be explained by either proposing that doxycycline is a more potent antibiotic compared to tetracycline or that the release of doxycycline from the silk films was slower and it remained in the solution for a longer period of time.

A possible explanation for the hypothesized difference in release rate of doxycycline and tetracycline would be the difference in the physical properties of these two drugs that may affect their interaction with the silk [26]. In addition to the difference in interacting with the silk, their slightly different properties could potentially change their solubility in aqueous environment, which would in turn influence their release rate. Doxycycline and tetracycline belong to the same class of antibiotics and have similar, but non-identical, physical properties. Another common antibiotic that belongs to this family is minocycline. This is in line with what was observed by Pritchard, et al. showing antibiotics from the same class had very different efficacies in silk film preparations [26] . Thus, since doxycycline and tetracycline behaved differently in identical conditions despite of having minor physical differences, it is possible that minocycline would have its own unique release rate and

efficacy in inhibiting bacteria. Thus, the reactivity and release rate of minocycline can be a subject of future studies.

An interesting phenomenon that limited the experiments was the lack of formation of the silk films when 3 mg/mL of either antibiotic was added to the silk solution. As a result, the experiments were run with maximum antibiotic concentrations of 1.5 mg/mL. In order to overcome this limitation, either the silk or the antibiotics can be changed. Since the silk has been shown in other studies to be an effective medium for localized antibiotic release [31, 36], the best option would be using a different family of antibiotics and determining whether or not the silk film formation happens at higher antibiotic concentrations [46]. If the silk were stable in the presence of the new antibiotics, higher concentrations can be tested. If not, more potent antibiotics that can be slowly released from the silk can be used. Studies have been done on silk-mediated release of a wide range of penicillin concentrations including concentrations much higher than 3 mg/mL, which have been shown to result in a stable silk film [46]. Thus, the concentration limitation may only be due to the properties of the antibiotics that were used in our experiments.

Although antibiotics at 3 mg/mL concentration resulted in an unstable silk fiber formation and could not be used for the experiments, the samples with 1.5 mg/mL of either tetracycline or doxycycline showed a strong inhibition of *S. mutans* growth for several days. As was expected, the growth inhibition was a function of concentration of either antibiotic as well as the exposure time to the drug. Among the tetracycline solutions, the sample with 1.5 mg/mL of antibiotic had the strongest inhibition for 5 days and among the doxycycline solutions, the sample with the same concentration had a strong inhibition until the 11th day.

Knowing that a typical treatment course for oral infections is between less than 1 and up to 2 weeks, it can be argued that both antibiotics showed favorable results for locally providing antibiotics over an extended period of time.

As was previously observed, antibiotics in the same family behaved differently when they were loaded on silk. Since a wide variety of antibiotics may be utilized for the treatment of oral infections, it would be beneficial if other families of antibiotics could be studied as well. Rifampicin, gentamycin, penicillin, and ampicillin have been previously studied as a way of showing the efficiency of silk as a slow-release medium [26]. Future studies should investigate which family of antibiotics is more effective when loaded on silk or what is the release duration of each antibiotic. Additionally, different species of bacteria that are common in oral infections can be used to expand the results and create a reference dataset for treating different oral infections.

Conclusion

With the limitations of our studies, we concluded that tetracycline and doxycycline can be successfully loaded into silk fiber films with various concentrations and inhibiting bacterial growth with the significant release up to five days for tetracycline and eleven days for doxycycline. Our data implies that silk fiber loaded films can be used as a medium for localized delivery of antibiotics during periodontal and oral surgeries.

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APPENDICES

Appendix A: Figures

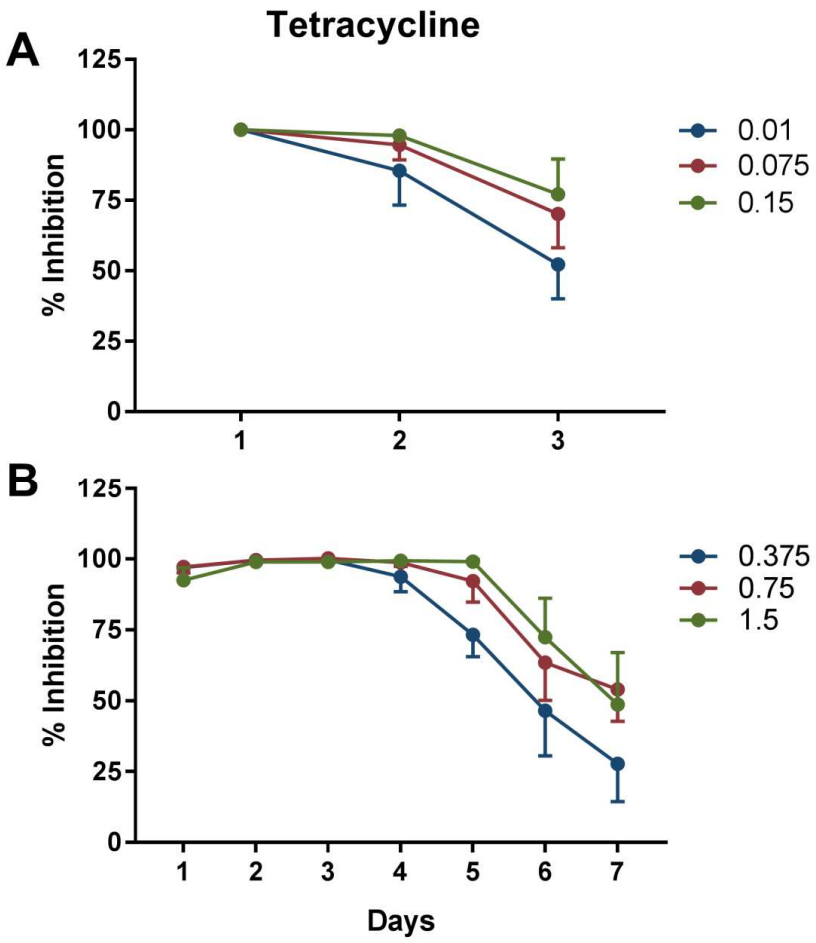

Figure1. Percent inhibition of *S. mutans* in the presence of different concentrations of tetracycline over a given number of days of exposure.

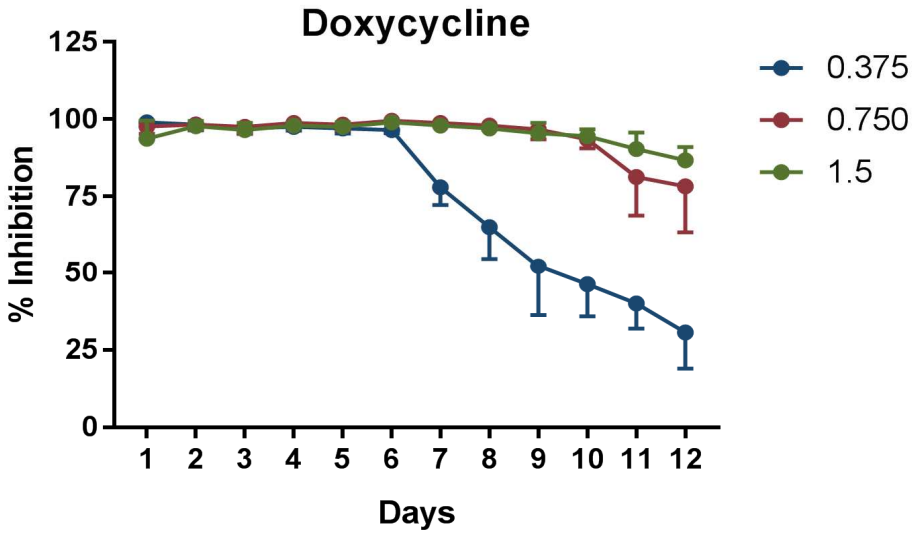

Figure2. Percent inhibition of *S. mutans* in the presence of different concentrations of doxycycline over a given number of days of exposure.

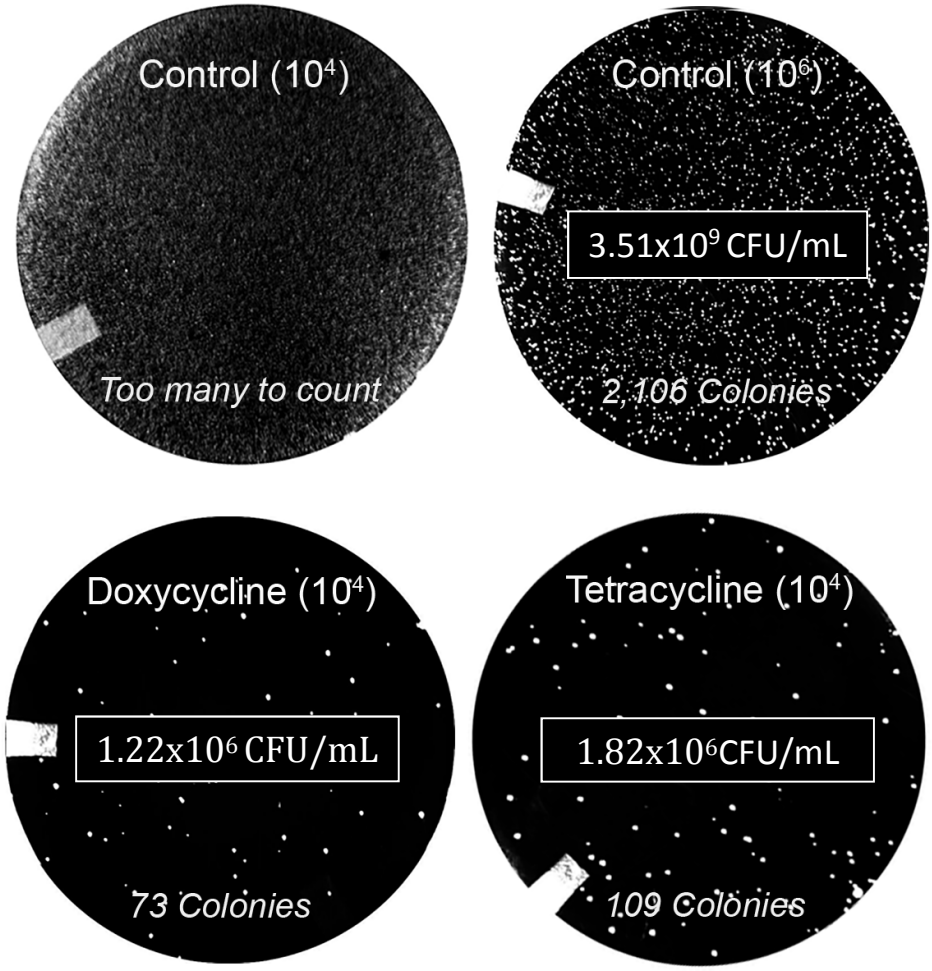

Figure3. Colony Forming Unite (CFU) Assay (Total Viable Count) before and after Treatment of Antibiotic and with Different Dilutions of the *S. mutans*.