Antibiofilm Activities of the Self-Prepared Silver Solution Against *Streptococcus Mutans* Biofilm: *In Vitro*

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Abstract

Dental caries is a common chronic disease which is one of many factors, caused by *Streptococcus mutans* biofilm. This study aimed to determine the ability of the self-prepared silver solution toward *S. mutans* biofilm formation and eradication. The study design contained 2 parts; biofilm inhibition test along with susceptibility test of the self-prepared silver solution. The experiment design for biofilm inhibition test was microplate assay with crystal violet. The testing solutions were two-fold serial dilution from the self-prepared silver solution 300 µg/mL and 1.5% TSB as positive control. The biofilm susceptibility test of the self-prepared silver solution was done with 2 different methods; CFU assay and Live/Dead assay. The CFU assay was done with testing solutions at 300, 150, 50,15, and 5 µg/mL of the self-prepared silver solution and 1% NaOCl as positive control. Live/Dead assay was done with the same testing solutions and the biofilms were observed under Confocal Laser Scanning Microscope (CLSM) and percentage of dead bacteria was calculated. All experiments were repeated three times, Data were analyzed with one-way ANOVA and Tukey's multiple comparison.

The results showed that the lowest concentration of the self-prepared silver solution that could inhibit *S. mutans* biofilm formation was 2.34 µg/mL (p-value $\leq$ 0.0001) and the lowest concentration that could eradicate *S. mutans* cell in biofilm form was 300 µg/mL (p-value $\leq$ 0.05). The percentage of dead bacteria under CLSM of 300 µg/mL of the self-prepared silver solution was 51 $\pm$ 28.18. This study concluded that the minimum biofilm inhibition concentration and eradication concentration of the self-prepared silver solution is 2.34 and 300 µg/mL, respectively.

Keywords**:** *Silver, Biofilm inhibition, Biofilm susceptibility, Confocal Laser Scanning Microscope*

1. Introduction

Dental caries is a common chronic disease in patients worldwide. A national survey showed that Thai preschoolers aged 3 and 5 years had dental caries prevalence at 31.1% and 31.3% respectively. The high prevalence of dental caries in young patients can affect children's quality of life. The most common microorganism that is related to initiating dental caries is *Streptococcus mutans* (*S. mutans*). *S. mutans* is one of many important pioneer bacteria in dental biofilm formation. Ramalingam and Messer (2004) reported a relationship between high caries incidence and high salivary counts of *S. mutans* in young children (Ramalingam & Messer, 2004; van Houte, 1994). Silver diamine fluoride (SDF) is one of many forms of topical fluoride that has evidence on arresting caries progression. SDF not only promotes remineralization of dentin but also inhibits the growth of common cariogenic bacteria (Horst, 2018). Clinical use of SDF has been recommended to apply 38% SDF on dentinal caries biannually in order to arrest caries progression (Chu et al., 2015; Gao et al., 2020). However, SDF can cause black staining after applying on the lesion which vastly affects the esthetic aspect (Gluzman et al., 2013; Moazami et al., 2018; Vinson et al., 2018; Zhao et al., 2017).

Silver have been an interesting antimicrobial material. *S. mutans* has been reported to be more susceptible to silver than zinc oxide and gold. The antimicrobial efficiency of silver depends on the contact surface area with microorganism; the smaller the size of silver, the better antimicrobial effect (Hernandez-Sierra et al., 2008).The self-prepared silver solution was not only prepared for the purpose of decreasing size for better antimicrobial activity, but also prepared from pharmaceutical grade material to minimize possible toxicity in human cells. There was a study reported that applying silver solution on the carious lesion did not demonstrate any discoloration on dentin caries after application.(Hernandez-Sierra et al., 2010). The study in 2020 mentioned that SDF and silver solution showed the similar antimicrobial potential against cariogenic flora including *S. mutans* (Fakhruddin et al., 2020). However, the availability of silver for dental application

is limited. In order to minimized tooth discoloration from SDF, we try to develop our silver solution that can arrest dental caries by inhibiting biofilm formation and eradicate cariogenic bacteria without causing tooth discoloration.

2. Objectives

1) To determine the lowest concentration of the self-prepared silver solution that can inhibit formation of *S. mutans* biofilm.

2)To determine the lowest concentration of the self-prepared silver solution that significantly kill *S. mutans* in the biofilm.

3. Materials and Methods

3.1 Materials

3.1.1 The self-prepared silver solution

The self-prepared silver solution was synthesized with a chemical reduction method in a laboratory at the Pharmacology department, Faculty of Dentistry, Mahidol University, Thailand. After synthesis, final concentration of the self-prepared silver solution was around 300 µg/mL and the self-prepared silver solution was kept at -80°C until use.

3.1.2 Bacterial culture

S. mutans in the study was obtained from strain ATCC 35688. The bacteria were obtained in BHI agar and culture for 48 hours at 37°C in incubator. Three to four single colonies were picked from agar and transferred to BHI broth. The bacterial suspension was incubated at 37°C for 24 hours and adjusted to 1.5 x 10^8 CFU/mL according to the 0.5 McFarland standard.

3.2 Methods

This study contained 3 experiments

3.2.1 Biofilm inhibition of the self-prepared silver solution

The experiment was performed according to the protocol of Barbara M. Coffey *et al.*(Coffey & Anderson, 2014). The study included 10 concentrations of the self-prepared silver solution for test group (two-fold dilution from 300 µg/mL with 1.5% sucrose TSB; 150, 75, 37.5, 18.75, 9.375, 4.688, 2.344, 1,172, 0.586 and 0.293 µg/mL) and 2 control group. A positive control was 1.5% sucrose TSB and negative control was 1.5% sucrose TSB with *S. mutans*.

3.2.2 Biofilm susceptibility by CFU assay

The experiment included 5 concentrations of the self-prepared silver solution for the test group (5, 15, 50, 150 and 300 µg/mL) with 1% NaOCl as a positive control and BHI broth as a negative control. The specific concentrations were determined to cover the full range of the self-prepared silver solution concentration and we can do further study for more specific concentration to develop new material. The self-prepared silver solution was diluted with deionized water from original concentration of 300 µg/mL. Flat-bottom 96-well plates were used to form *S. mutans* biofilm. After incubating for 24 hours, biofilms were washed and treated with the self-prepared silver solution at different concentrations and controls for 5 minutes then removed and washed with BHI broth. The surviving bacteria were scraped with sterile pipette tips and transferred into glass tube containing glass bead and vortex for 1 minute. Serial decimal dilutions were made with BHI broth and 15 µg/mL of suspensions were pipetted on BHI agar. Number of *S. mutans* colonies was determined and CFU per milliliter were calculated. All experiments were performed in triplicate.

3.2.3. Biofilm susceptibility by Live/Dead assay

The experiment included 5 concentrations of the self-prepared silver solution for the test group (5, 15, 50, 150 and 300 µg/mL) with 1% NaOCl as a positive control and BHI broth as a negative control. The biofilms were grown in a 24 well plate flat-bottom with cover slip in each well for 24 hours. After biofilm formation, culture medium was removed and gently washed with BHI three times prior to loading the self-prepared silver solution and control solutions for 5 minutes then removed and washed with BHI broth. Live/Dead assay was performed using SYTO 9 and Propidium iodide to stain the biofilm. Confocal Laser

Scanning Microscope was used to capture pictures of live bacteria and analyze the percentage of dead bacteria by Leica Application Suite X software. All experiments were performed in triplicate.

The rationale for individual concentrations between biofilm inhibition and biofilm susceptibility tests was that the researcher determined to cover full range of the self-prepared silver solution concentration (from 300 µg/mL) and the preparation of silver solution in biofilm inhibition was done with two-fold dilution technique, so it contained subtle concentrations in this experiment. While a biofilm susceptibility test could not follow the subtle concentrations due to the limitation of the biofilm assay, we selected the concentrations that were easily prepared to eliminate error but still cover the full range of the self-prepared silver solution concentration. The statistical analysis was tested with one-way ANOVA with post-hoc Tukey's HSD method using GraphPad software Prism version 9.4.1. The *p*-value less than 0.05 was considered as statistical significance.

4. Results and Discussion

4.1 Result

4.1.1 Biofilm inhibition of the self-prepared silver solution

The lowest concentration of the self-prepared silver solution that could inhibit *S. mutans* biofilm formation more than 50 percent was 2.34 µg/mL, see Table 1.

Table 1 Percentage of *S. mutans* biofilm inhibition ability of the self-prepared silver solution (* *p*-value $\leq$ 0.0001 compared with control**)**

Groups	Mean ± SD (%)
Negative control	0 ± 0
Positive control	95.63 ± 2.14 *
150 µg/mL	96.41 ± 0.89 *
75 µg/mL	95.92 ± 1.14 *
37.5 µg/mL	92.67 ± 1.5 *
18.75 µg/mL	95.92 ± 0.79 *
9.38 µg/mL	96.35 ± 0.8 *
4.69 µg/mL	95.90 ± 0.84 *
2.34 µg/mL	74.24 ± 18.47 *
1.17 µg/mL	8.45 ± 13.12
0.59 µg/mL	0.78 ± 30.09
0.3 µg/mL	2.9 ± 25.05

4.1.2 CFU assays of the self-prepared silver solution

The experiment showed that the highest and lowest bactericidal activity against *S. mutans* in the biofilm was observed in 1% NaOCl and BHI group respectively, and the treatment of the self-prepared silver solution showed to kill *S. mutans* in the biofilm in a dose dependent manner with at 300 µg/mL of the self-prepared silver solution exhibiting more dead bacteria than BHI significantly ($p \leq 0.05$). Although the survival bacteria at the lower concentration of the self-prepared silver solution were less than BHI, but they were not statistically significant ($p > 0.05$), see Figure 1.

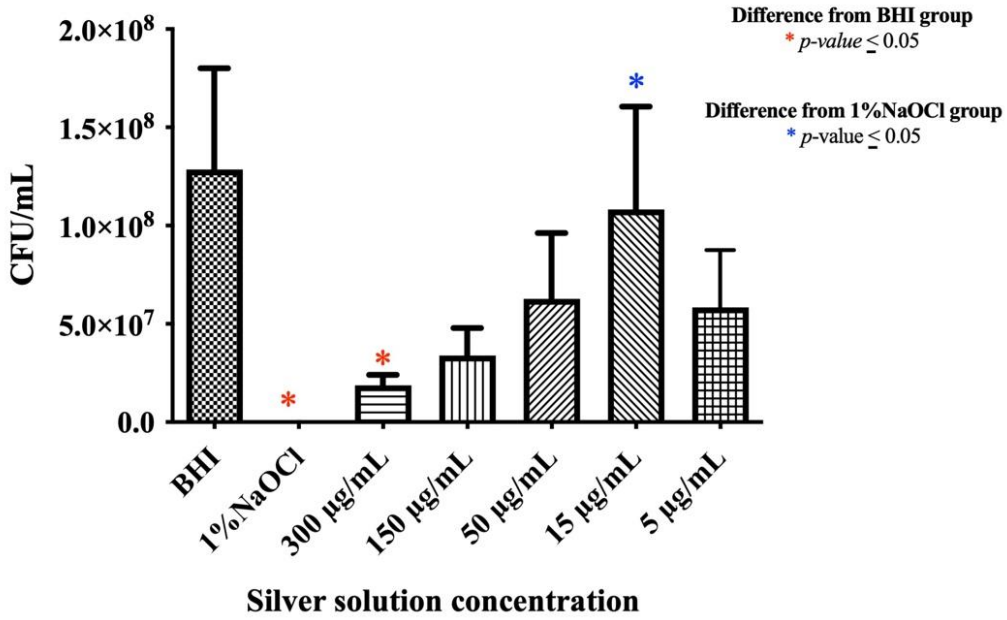

Figure 1 Quantitative analysis of the viable *S. mutans* in the biofilm by counting the colony forming unit

4.1.3 Live/Dead assay by Confocal laser scanning microscope

Biofilm susceptibility was determined in parallel with colony forming unit assay to confirm viability of *S. mutans* in the biofilm after 5 minutes exposure of the self-prepared silver solution at different concentrations. Fluorescence patterns of live and dead bacteria after treatment showed in Figure 2. Red areas represented fluorescence from dead bacteria and green areas were fluorescence from live bacteria. The highest percent dead cell was 65.93 ± 4.491 from 1% NaOCl group which represented as positive control and the lowest percent dead cell was 6.771 ± 3.308 from BHI group which represented as negative control. The percent of dead bacteria gradually increased when a higher concentration of the self-prepared silver solution was used. At 300 µg/mL silver solution and 1%NaOCl showed a statistically significant higher percentage of dead bacteria than in the BHI treatment, see Table 2.

Table 2 Percentage of dead bacteria in each group compared with BHI (* *p*-value ≤ 0.001)

Groups	Mean ± SD (%)
BHI	6.771 ± 3.308
1% NaOCl	65.93 ± 4.491*
300 µg/mL	51.34 ± 28.18*
150 µg/mL	37.19 ± 16.01
50 µg/mL	23.30 ± 13.00
15 µg/mL	7.92 ± 0.7
5 µg/mL	10.04 ± 1.42

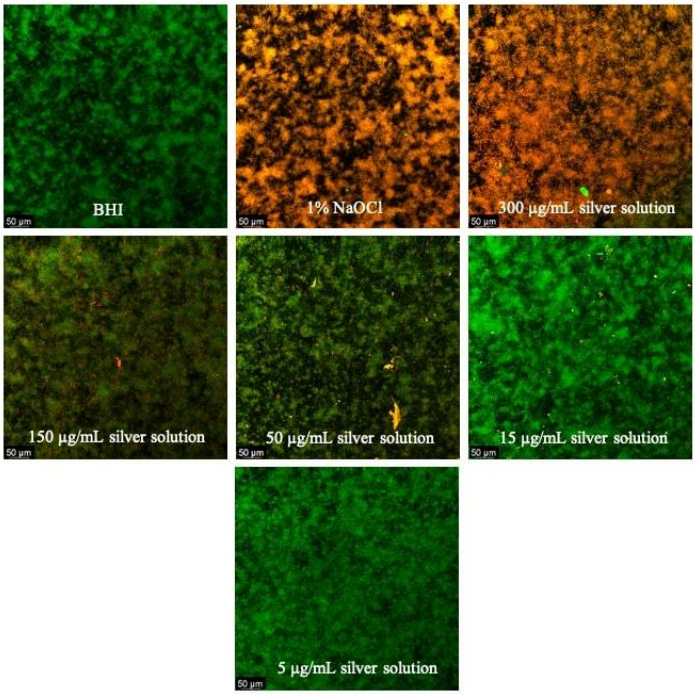

Figure 2 Confocal laser scanning microscopy images of *S. mutans* biofilms following different treatments

4.2 Discussion

Management of carious lesion in primary dentition has multiple approaches. Arresting caries with topical treatment have been proved to be one of the effective methods. SDF is antimicrobial agent constituting of silver ion that can reduce the development of *S. mutans* biofilm, and the kill *S. mutans* residing in the caries, especially in primary dentition (Zhao et al., 2018). However, oxidizing reaction of silver ion and oxygen in SDF can stain the carious infected area of the tooth causing a black appearance on tooth, which might affect the sociopsychological development during childhood of the young patients. Silver solution is developed with a potential of killing *S. mutans* without leaving the tooth stained (Butron Tellez Giron et al., 2020). Recently, we have developed silver solution using chemical reduction method with a new reducing agent and stabilizer. However, it has never been investigated for the antibacterial effects. Thus, this study aimed to observe the efficiency of the self-prepared silver solution on the biofilm inhibition and biofilm susceptibility of the *S. mutans* biofilm. Our result showed that the self-prepared silver solution can inhibit *S. mutans* biofilm formation and kill *S. mutans* residing in the biofilm in a dose dependent manner with 300 µg/mL silver solution as the most suitable concentration.

S. mutans biofilm is a major concern in various oral diseases. To prevent biofilm formation, biofilm inhibitors have been developed widely. Silver solution as a biofilm inhibitor has been reported to be affected by various characteristics of the particles. Many studies reported that size and shape of particles are important characteristics for bactericidal effects. In 2005, there was a study of the bactericidal effect of silver solution at different diameter sizes of particle and concluded that bactericidal properties of silver solution are size dependent, the smaller, the better (Morones et al., 2005). Our self-prepared silver solution is spherical, monodisperse particles with 4±2.25 nm. in diameter under transmission electron microscopy (TEM). In this study, the lowest concentration of the self-prepared silver solution that could inhibit *S. mutans* biofilm formation was 2.34 µg/mL, which was similar to a study by Hernandez-Seirra in 2008 (Hernandez-Sierra et al., 2008). However, Espinosa-Cristobal *et al* reported a minimal inhibition concentration of 66.87 µg/mL silver solution, which was a higher concentration compared to our study. They reported of using silver solution with spherical shape with diameter at around 8.4 nm (Espinosa-Cristóbal et al., 2009). This might be

due to the smaller size of our self-prepared silver solution that could attach to the cell surface of bacteria in a greater number resulting in disturbing membrane permeability (Freire et al., 2017). Although the self-prepared silver solution satisfactorily showed less biofilm formation, it could not be concluded whether the silver solution interfered at which biofilm formation stage or just killed planktonic *S. mutans* before developing the biofilm.

In this study, biofilm inhibitory concentration was determined using a microtiter plate assay with crystal violet, which is the most widely used method (Cremet et al., 2013). Considering its benefits, this assay is cheap, easy to use, require small volume of testing agents, and easy to control growing conditions enabling testing at various biofilm development. However, microtiter assay cannot differentiate between eradicating of planktonic cells and inhibiting biofilm formation at different stages since the antibacterial agent was mixed with bacteria before the inoculation (An et al., 2021). Further experiment to understand the mechanism might be useful to explain the involvement of silver solution in bacterial biofilm formation.

Topical anti-caries agent provides the noninvasive approach for dental therapy. The use of SDF has been documented since last decade to overcome carious lesions by releasing of the silver ion molecules to attack the bacteria. SDF is commonly available as a 38% solution containing 253,900 µg/mL of silver and 44,800 µg/mL fluoride ions(Yan et al., 2022). On the other hand, silver solution is a small sized particle that carries greater antibacterial effects than the silver ion. The susceptibility tests of silver solution showed that it could kill *S. mutans* in the biofilm in dose-dependent manner at the concentration between 15-300 µg/mL. At 5 µg/mL, the number of survival bacteria increased conversely to the tendency of a graph but there was no statistically significance with the control group ($p \geq 0.05$). This contrary pattern at low concentration might be due to the change of pharmacological response at molecular level. There are a few studies reported that at a very low dose, some materials can initiate hormetic phenomenon (Bell et al., 2014; Iavicoli et al., 2010; Nascarella & Calabrese, 2012) which is a biphasic nonlinear dose-response relationship when a low dose of agents can stimulate high dose of inhibitory effect (Mattson, 2008). Further study on hormetic phenomenon may help us understand more about the mechanism of action of silver solution at low dose. Furthermore, the susceptibility tests of the self-prepared silver solution showed that at 300 µg/mL is the only concentration with a statistically significant difference to the control group. Although a previous report by Perez-Diaz could show lower silver solution concentration at 100 µg/mL to efficiently eradicate *S. mutans* in the biofilm at more than 99.99% kill rate, their application required over 24 hours of exposure time, which is different from this study that determined only 5 minutes exposure time (Perez-Diaz et al., 2015).

Live/dead assay was performed by using confocal laser scanning microscope (CLSM) to study the viability of *S. mutans* biofilm after treatment with different silver solution concentrations. Biofilms were labeled using two fluorescent probes (SYTO-9 and propidium iodine). After examined biofilm under CLSM, negative control group (BHI) demonstrated a majority of green area, while the positive control group (1% NaOCl) demonstrated a majority of red area. The micrographs and percent dead cells of *S. mutans* biofilm after being treated with silver solution each concentration showed dose-dependence relationship. At higher concentrations of silver solution, more red area and higher percent dead cell were shown, whilst green area majority and lower percent dead cell were shown at lower concentrations, except for 5 µg/mL silver solution shown higher percent dead cell than 15 µg/mL silver solution which might be from hermetic phenomenon of silver solution as mentioned above. The results from quantity and quality methods to determine susceptibility of silver solution were in endorsement that the self-prepared silver solution at 300 µg/mL had significantly bactericidal effect toward *S. mutans* biofilm.

The current applications of silver solution in dentistry are widely used. The silver solution was added to nanocomposites for antimicrobial and antifungal effects, to dental implants to stimulate osseointegration and fibroblast proliferation and used as treatment of cancer due to its antitumor properties (Noronha et al., 2017). From this present study, silver solution could be developed into varnish, toothpaste and mouth rinse in order to arrest dentinal caries and promote remineralization of teeth. Despite the benefits of silver solution, further studies on toxicity to human cells and pharmacokinetics of antimicrobial agents are crucial for the purpose of safe use.

5. Conclusion

In conclusion, the minimum biofilm inhibition concentration of the self-prepared silver solution is 2.34 µg/mL and the minimum biofilm eradication concentration of the self-prepared silver solution is 300 µg/mL. Further *in vitro* and *in vivo* study about toxicity and pharmacokinetics of silver solution should be done before developing this antimicrobial agent to use in patients.

6. Contribution for academic research field

The self-prepared silver solution could be added to some dental materials such as resin composite, soft liner to prevent dental caries or could be developed to solution or varnish to arrest dental caries.

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8. References

An, A. Y., Choi, K. G., Baghela, A. S., & Hancock, R. E. W. (2021). An Overview of Biological and Computational Methods for Designing Mechanism-Informed Anti-biofilm Agents. *Frontiers in Microbiology*, *12*, 640787. https://doi.org/10.3389/fmicb.2021.640787

Bell, I. R., Ives, J. A., & Jonas, W. B. (2014). Nonlinear effects of nanoparticles: biological variability from hormetic doses, small particle sizes, and dynamic adaptive interactions. *Dose Response*, *12*(2), 202-232. https://doi.org/10.2203/dose-response.13-025.Bell

Butron Tellez Giron, C., Hernandez Sierra, J. F., DeAlba-Montero, I., Urbano Pena, M. L. A., & Ruiz, F. (2020). Therapeutic Use of Silver Nanoparticles in the Prevention and Arrest of Dental Caries. *Bioinorganic Chemistry and Applications*, *2020*, 8882930. https://doi.org/10.1155/2020/8882930

Chu, C. H., Gao, S. S., Li, S. K., Wong, M. C., & Lo, E. C. (2015). The effectiveness of the biannual application of silver nitrate solution followed by sodium fluoride varnish in arresting early childhood caries in preschool children: study protocol for a randomised controlled trial. *Trials*, *16*, 426. https://doi.org/10.1186/s13063-015-0960-2

Coffey, B. M., & Anderson, G. G. (2014). Biofilm formation in the 96-well microtiter plate. *Pseudomonas Methods and Protocols*, *1149*, 631-641. https://doi.org/10.1007/978-1-4939-0473-0_48

Cremet, L., Corvec, S., Batard, E., Auger, M., Lopez, I., Pagniez, F., Dauvergne, S., & Caroff, N. (2013). Comparison of three methods to study biofilm formation by clinical strains of Escherichia coli. *Diagnostic Microbiology and Infectious Disease*, *75*(3), 252-255. https://doi.org/10.1016/j.diagmicrobio.2012.11.019

Espinosa-Cristóbal, L., Martínez-Castañón, G., Martínez-Martínez, R., Loyola-Rodriguez, J., Patino-Marin, N., Reyes-Macías, J., & Ruiz, F. (2009). Antibacterial effect of silver nanoparticles against Streptococcus mutans. *Materials Letters*, *63*(29), 2603-2606.

Fakhruddin, K. S., Egusa, H., Ngo, H. C., Panduwawala, C., Pesee, S., & Samaranayake, L. P. (2020). Clinical efficacy and the antimicrobial potential of silver formulations in arresting dental caries: a systematic review. *BMC Oral Health*, *20*(1), 160. https://doi.org/10.1186/s12903-020-01133-3

Freire, P. L. L., Albuquerque, A. J. R., Sampaio, F. C., Galembeck, A., Flores, M. A. P., Stamford, T. C. M., & Rosenblatt, A. (2017). AgNPs: The New Allies Against S. Mutans Biofilm - A Pilot Clinical Trial and Microbiological Assay. *Brazilian Dental Journal*, *28*(4), 417-422. https://doi.org/10.1590/0103-6440201600994

Gao, S. S., Chen, K. J., Duangthip, D., Wong, M. C. M., Lo, E. C. M., & Chu, C. H. (2020). Preventing early childhood caries with silver diamine fluoride: study protocol for a randomised clinical trial. *Trials*, *21*(1), 140. https://doi.org/10.1186/s13063-020-4088-7

Gluzman, R., Katz, R. V., Frey, B. J., & McGowan, R. (2013). Prevention of root caries: a literature review of primary and secondary preventive agents. *Special Care in Dentistry*, *33*(3), 133-140. https://doi.org/10.1111/j.1754-4505.2012.00318.x

Hernandez-Sierra, J. F., Ruiz, F., Pena, D. C., Martinez-Gutierrez, F., Martinez, A. E., Guillen Ade, J., Tapia-Perez, H., & Castanon, G. M. (2008). The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold. *Nanomedicine*, *4*(3), 237-240. https://doi.org/10.1016/j.nano.2008.04.005

Hernandez-Sierra, J. F., Salas-Lopez, E. K., Martinez-Gutierrez, F., Ruiz, F., Pierdant-Perez, M., Mandeville, P., & Pozos-Guillen, A. J. (2010). Bactericidal capacity of silver nanoparticles associated with Gantrez S-97 on Streptococcus mutans. *Journal of Clinical Pediatric Dentistry*, *35*(2), 183-185. https://doi.org/10.17796/jcpd.35.2.c61l421mj0655lgm

Horst, J. A. (2018). Silver Fluoride as a Treatment for Dental Caries. *Advances in Dental Research*, *29*(1), 135-140. https://doi.org/10.1177/0022034517743750

Iavicoli, I., Calabrese, E. J., & Nascarella, M. A. (2010). Exposure to nanoparticles and hormesis. *Dose Response*, *8*(4), 501-517. https://doi.org/10.2203/dose-response.10-016.Iavicoli

Mattson, M. P. (2008). Hormesis defined. *Ageing Research Reviews*, *7*(1), 1-7. https://doi.org/10.1016/j.arr.2007.08.007

Moazami, F., Sahebi, S., & Ahzan, S. (2018). Tooth Discoloration Induced by Imidazolium Based Silver Nanoparticles as an Intracanal Irrigant. *Journal of Dentistry (Shiraz)*, *19*(4), 280-286. https://www.ncbi.nlm.nih.gov/pubmed/30680300

Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramirez, J. T., & Yacaman, M. J. (2005). The bactericidal effect of silver nanoparticles. *Nanotechnology*, *16*(10), 2346-2353. https://doi.org/10.1088/0957-4484/16/10/059

Nascarella, M. A., & Calabrese, E. J. (2012). A method to evaluate hormesis in nanoparticle dose-responses. *Dose Response*, *10*(3), 344-354. https://doi.org/10.2203/dose-response.10-025.Nascarella

Noronha, V. T., Paula, A. J., Duran, G., Galembeck, A., Cogo-Muller, K., Franz-Montan, M., & Duran, N. (2017). Silver nanoparticles in dentistry. *Dental Materials*, *33*(10), 1110-1126. https://doi.org/10.1016/j.dental.2017.07.002

Perez-Diaz, M. A., Boegli, L., James, G., Velasquillo, C., Sanchez-Sanchez, R., Martinez-Martinez, R. E., Martinez-Castanon, G. A., & Martinez-Gutierrez, F. (2015). Silver nanoparticles with antimicrobial activities against Streptococcus mutans and their cytotoxic effect. *Materials Science and Engineering: C*, *55*, 360-366. https://doi.org/10.1016/j.msec.2015.05.036

Ramalingam, L., & Messer, L. B. (2004). Early childhood caries: an update. *Singapore Dental Journal*, *26*(1), 21-29. https://www.ncbi.nlm.nih.gov/pubmed/15736838

van Houte, J. (1994). Role of micro-organisms in caries etiology. *Journal of Dental Research*, *73*(3), 672-681. https://doi.org/10.1177/00220345940730031301

Vinson, L. A., Gilbert, P. R., Sanders, B. J., Moser, E., & Gregory, R. L. (2018). Silver Diamine Fluoride and Potassium Iodide Disruption of In Vitro Streptococcus mutans Biofilm. *Journal of Dentistry for Children*, *85*(3), 120-124. https://www.ncbi.nlm.nih.gov/pubmed/30869588

Yan, I. G., Zheng, F. M., Gao, S. S., Duangthip, D., Lo, E. C. M., & Chu, C. H. (2022). Ion Concentration of Silver Diamine Fluoride Solutions. *International Dental Journal*, *72*(6), 779-784. https://doi.org/10.1016/j.identj.2022.04.005

Zhao, I. S., Gao, S. S., Hiraishi, N., Burrow, M. F., Duangthip, D., Mei, M. L., Lo, E. C., & Chu, C. H. (2018). Mechanisms of silver diamine fluoride on arresting caries: a literature review. *International Dental Journal*, *68*(2), 67-76. https://doi.org/10.1111/idj.12320

Zhao, I. S., Mei, M. L., Burrow, M. F., Lo, E. C., & Chu, C. H. (2017). Effect of Silver Diamine Fluoride and Potassium Iodide Treatment on Secondary Caries Prevention and Tooth Discolouration in Cervical Glass Ionomer Cement Restoration. *International journal of molecular sciences*, *18*(2). https://doi.org/10.3390/ijms18020340