The Effect of a Homoeopathic and Herbal Mouthwash Formulation on the Growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*

Samba Adelaide De Oliveira

(Student number: 820413284)

Postal address: University of Johannesburg, PO BOX 524, Auckland Park, 2006 South Africa

Abstract

There are a few hundred different types of bacterial species within the oral cavity which amount to more than several 100 millions in number within dental plaque. Some bacteria are commensal and others play a key role in the progression of periodontal disease. Periodontal disease can manifest as gingivitis which is seen as infection of the gingival tissue, or periodontitis which results in loss of collagen attachment of tooth to bone and loss of bone tissue. *Streptococcus salivarius* is the principal commensal bacterium in the oral cavity of humans. *Fusobacterium nucleatum* is commonly found in the dental plaque of humans and is frequently associated with periodontal disease. The mouthwash formulation is sea salt based together with homoeopathic remedies and herbal extracts. It is indicated for the treatment of sensitive irritated gums, halitosis, sore throat and mouth ulcers.

The aim of this study was to determine the *in vitro* effect of a homoeopathic and herbal mouthwash formulation on *Streptococcus salivarius* and *Fusobacterium nucleatum*. This study forms part of a three part *in vitro* study to determine the effect of the mouth wash formulation. The effect was evaluated by measuring zones of inhibition around discs impregnated with the mouthwash solution, saline solution and distilled water.

Lypholized ATCC 25586 *Fusobacterium nucleatum* and ATCC 13419 *Streptococcus salivarius* were obtained from Quantum Biotechnologies. *Fusobacterium nucleatum* was cultured on chocolate blood agar and nutrient agar and incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. *Streptococcus salivarius* was cultured on Tryptic Soy Agar (TSA) agar with 5% sheep blood and incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. Agar plates were refrigerated at 2-8 ^{o}C for two weeks and used as stock cultures. In the Kirby-bauer disk diffusion method, agar plates incubated with *Fusobacterium nucleatum* and *Streptococcus salivarius* respectively were streaked with the mouth wash solution, and the control substances distilled water and saline solution respectively. The agar plates were incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours. In Adaptation experiment one, a five times, and two times strength mouth wash solution was prepared and tested following the Kirby-bauer streaking method. In the Adaptation experiment two, a volume of 100 µl of one time, two times and five times strength mouth wash solution was mixed with 10 µl cultures of *Streptococcus salivarius* and *Fusobacterium nucleatum* in a 1ml eppendorf tube. The mixture was incubated for 5 minutes at room temperature and streaked on TSA agar with 5% sheep blood and nutrient agar plates respectively, agar plates were incubated at 35 ^{o}C in 5-7% CO_2 for 48 hours.

Results showed no zones of inhibition in the Kirby-bauer disk diffusion method, Adaptation experiment one and Adaptation experiment two. The results showed that the homoeopathic and herbal mouth wash formulation had no significant effect on *Streptococcus salivarius* and *Fusobacterium nucleatum* respectively.

Introduction

Periodontal disease

Oral diseases initiate with the growth of dental plaque on the tooth surface and accumulation of bacteria on the dental plaque (Marsh, 2006). In periodontal disease the areas at or below the gingival margin become infected causing an inflammatory response of the gums and surrounding connective tissues (Palombo, 2011).

Pathology

Gingivitis and periodontitis are inflammatory conditions which arise from infection of the gingival tissue. Gingivitis is a reversible inflammatory condition whereas periodontitis is non-reversible, destructive and results in loss of connective tissue attachment to bone (Tatakis and Kumar, 2015).

Diagnosis

Clinical diagnosis of periodontal disease is based on visual and radiographic assessments of periodontal tissues and measurements of the spaces between tooth and gingiva (Armitage, 2004b).

Treatment

Root planing and scaling are the most common non-surgical methods used to treat moderate periodontal disease such as periodontitis before surgery is employed. The procedure involves cleaning between the gums and teeth down into the roots. This is done when the gums have started to recede or there is plaque accumulation (NIH, 2013). Scaling and root planing carries the risk of introducing opportunistic pathogenic bacteria into the blood stream during the procedure, therefore, antibiotics are administered prior to and post procedure (NIH, 2013).

Materials and method

Research design

This was a quantitative *in vitro* study conducted over 2 weeks.

Stock cultures

Lyophilized *Fusobacterium nucleatum (*ATCC 25586) and *Streptococcus salivarius (*ATCC 13419) were obtained from Quantum Biotechnologies (Randburg, South Africa).

Subculturing

For experimental test, fresh culture was prepared by sub-culturing *Fusobacterium nucleatum* and *Streptococcus salivarius* from stock culture into fresh media plates. *Fusobacterium nucleatum* and *Streptococcus salivarius* were sub-cultured on chocolate blood agar, nutrient agar and TSA with 5% sheep blood agar respectively. The plates were incubated at 35 °C in 5 to 7% CO_2 for 48 hours.

Mouth wash formulation and saline preparation

The mouth wash solution was prepared by adding 1 scoop which was equivalent to 62,5 grams when measured, in 125 ml sterile warm distilled water. Saline was prepared by adding 9 grams of table salt to 1L of sterile distilled water and shaken until the granules dissolved.

Disk diffusion method

The antimicrobial activity of the mouth wash formulation was determined by using the disc diffusion procedure recommended by the Clinical and Laboratory Standards Institute (Wikler *et al*., 2006). Using a sterile swab TSA agar with 5% sheep blood and chocolate blood agar plates were streaked respectively with a fresh culture of *Streptococcus salivarius* and *Fusobacterium nucleatum* that had been adjusted to a turbidity of 0.5 McFarland standards.

To obtain uniform growth, the agar plates were streaked with a sterile swab in one direction and then the agar plates were rotated by 90° and streaked again in that direction. The rotation was repeated three times. After streaking, the plates were allowed to dry for approximately 5 minutes. Sterile discs were placed one at a time on the streaked plates using a flame-sterilized forceps. The discs were impregnated with the mouthwash solution (10 µl), distilled water (10 µl) and saline (10 µl).
The agar plates were inverted and incubated at 35°C in 5% to 7% CO_2 for 48 hours. Each agar plate was numbered before incubation to allow identification of the contents progress.

After 48 hours incubation the TSA agar with 5% sheep blood and chocolate blood agar were checked for a zone of inhibition around each of the impregnated discs. This zone marks the effective inhibition of microbial growth. The diameter of each zone was measured in millimeters (mm) and recorded to determine the extent of antimicrobial activity. The experiments were done in triplicates and repeated three times.

Adaptation experiment one

A five and two times strength mouthwash solution was prepared. The five times and two times strength mouthwash solutions were tested following the disk diffusion method. The same controls, which are saline and distilled water, were included in the experiments. The experiments were done in triplicates and repeated three times. For this adaptation experiment *Fusobacterium nucleatum* was cultured and grown on chocolate agar and *Streptococcus salivarius* was grown on TSA 5% sheep blood agar.

Adaptation experiment two

A volume of 100 µl (one time, two time and *five* time strength) mouthwash formulation was mixed with 10 µl fresh cultures of *Streptococcus salivarius* and *Fusobacterium nucleatum* in 1 ml eppendorf tube and incubated for 5 minutes at room temperature. *Streptococcus salivarius* and *Fusobacterium nucleatum* were adjusted to a turbidity of 0.5 McFarland standards. In parallel a 10 µl *Streptococcus salivarius* and *Fusobacterium nucleatum* that were adjusted to 0.5 McFarland standards were incubated in 100 µl saline and distilled water for 5 min at room temperature. After incubation the mixtures were streaked on TSA agar with 5% sheep blood and nutrient agar plates for *Streptococcus salivarius* and *Fusobacterium nucleatum* culturing respectively. The TSA agar with 5% sheep blood and nutrient agar plate were streaked with a sterile swab in one direction and then the agar plates were rotated by 90° and streaked again in that direction. The rotation was repeated three times. After streaking, the plates were allowed to dry for approximately 5 minutes. The agar plates were inverted and incubated at 35°C in 5% to 7% CO_2 for 48 hours. Each agar plate was numbered before incubation to allow identification of the contents progress.

After 48 hours incubation the TSA agar with 5% sheep blood and nutrient agar was checked for a zone of inhibition around each of the impregnated discs. This zone marks the effective inhibition of microbial growth. The diameter of each zone was measured in millimeters (mm) and recorded to determine the extent of antimicrobial activity. The experiments were done in triplicates and repeated three times.

Data analysis

It was anticipated that the ANOVA Statistical method was to be used to analyse the data gathered (Devey, 2013). The ANOVA method was not used because there was no effect of the mouth wash on the bacteria.

Results

Experiment one: Kirby-bauer disk diffusion method

The antimicrobial activity of the mouthwash formulation, saline solution and distilled water was determined by using the disc diffusion procedure. The mouthwash was prepared as per manufactures instructions. *Fusobacterium nucleatum* and *Streptococcus salivarius* cultures were adjusted to 0.5 McFarland standard before being used in the experiment. The experiments were done in triplicates and repeated three times. As shown in Figure 4.1 all the plates for all the solutions tested (mouthwash formulation, saline solution and distilled water) had good growth for *Streptococcus salivarius* and *Fusobacterium nucleatum*. This indicated that the three solutions tested at the specific concentration do not have antimicrobial capabilities for *Streptococcus salivarius* and *Fusobacterium nucleatum* at a concentration of 0.5 McFarland standard.

Adaptation experiment one

An increased strength (two times and five times strength) of mouthwash solution was tested with *Streptococcus salivarius* and *Fusobacterium nucleatum* at a concentration of 0.5 McFarland standard. The aim of this experiment was to determine whether increasing the strength of the mouthwash solution will have an effect on the inhibition of *Streptococcus salivarius* and *Fusobacterium nucleatum.* The same controls, which are saline and distilled water, were included in the experiments. The experiments were done in triplicates and repeated three times. The increasing strength of mouthwash solutions did not have any effect on the good growth of *Streptococcus salivarius* and *Fusobacterium nucleatum* (Figure 4.2). The results for saline and distilled water were still consistent with the first experiment as expected. There were no zones of inhibition across all experimental plates.

Adaptation experiment two

With the current experiment the mouthwash exposure time was increased to 5 minutes. A volume of 100 µl (one time, two time and five time strength) mouthwash solutions was mixed

with 10 µl fresh cultures of *Streptococcus salivarius* and *Fusobacterium nucleatum* in a 1 ml eppendorf tube. *Streptococcus salivarius* and *Fusobacterium nucleatum* were adjusted to a turbidity of 0.5 McFarland standards. The mixture was incubated for 5 minutes and streaked on TSA agar with 5% sheep blood and Nutrient agar plates for *Streptococcus salivarius* and *Fusobacterium nucleatum* culturing respectively. The aim of this experiment was to determine the effect of incubating *Streptococcus salivarius* and *Fusobacterium nucleatum* cultures for 5 minutes as compared to the 2 minutes described by the mouthwash manufacture. The bacteria were immersed in the mouthwash solution therefore, allowing direct contact with the active ingredients of the mouthwash solution. Incubating *Streptococcus salivarius* and *Fusobacterium nucleatum* cultures for 5 minutes in different concentration of the mouthwash solution did not have any effect on the growth of the two bacteria as shown by good growth of chocolate blood agar and TSA agar in (Figure 4.3). The same results were found for saline and distilled water.

Discussion

Results showed that the homoeopathic and herbal mouthwash formulation containing salt (119.5g), *Arnica montana 30cH*, *Echinacea purpurea 30cH*, *Staphysagria 30cH*, *Phosphorus 30 cH*, *Hepar sulphuris calcarea 30 cH*, Cinnamon (0.27g) and Liquorice (0.27g) had no effect on *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*. Comparisons between different concentrations of the mouthwash showed no significant difference in results.

Conclusion

Using the Kirby-bauer disk diffusion method and the two adaptation methods it was determined that the mouth wash formulation displayed no action against the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

The results support the null hypothesis that the mouth wash formulation has no effect on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro* and rejects the hypothesis that states the mouth wash formulation has an effect on the growth of *Streptococcus salivarius* and *Fusobacterium nucleatum in vitro*.

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