The Effects of *Streptococcus gordonii* Derived H_2O_2 on RAW 264.7 Macrophage Cells

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Abstract

This study determined if orally derived *Streptococcus gordonii* bacteria is capable of inducing macrophage cell death as a result of hydrogen peroxide production (H_2O_2). Other orally derived *Streptococcus* strains have been shown to induce macrophage cell death via H_2O_2 production; however, there is no information on *S. gordonii*. This study also sought to determine if the addition of catalase (an enzyme that decomposes H_2O_2) to *S. gordonii* infected macrophage samples could significantly reduce or prevent macrophage cell death. Multiplicity of Infection (MOI) quantities of 0, 50, 100, and 200 of *S. gordonii* cells were used to infect cultured macrophage cells. Samples of macrophage cells infected with a 200 MOI were also treated with either 10U/μL or 100 U/μL of catalase enzyme. Macrophage cell viability was determined post-bacterial infection using trypan blue exclusion dye and counting the dead and live cells. It was found that the 200 MOI and 100 MOI treatments had significantly higher dead/live ratios than the 0 MOI (control). The addition of catalase to 200 MOI treated macrophage cells did not improve the dead/live ratio when compared to the control. This experiment provided evidence that infected macrophage cell death was a result of *S. gordonii* derived H_2O_2.

Introduction

Dental plaque is a type of biofilm that is composed of thousands of microorganisms and is known to be the causative agent of human periodontal disease and dental caries (Marsh, 1995). In a healthy oral cavity, the plaque biofilm is non-pathogenic and exists in a mutualistic relationship with the host (Do, 2013). In this relationship, the biofilm provides support for the host's innate and adaptive immune response to eliminate foreign bacteria (Do, 2013). However, during the process of oral disease development, the non-pathogenic dental biofilm is converted into a pathogenic biofilm, and the host's immune response becomes ineffective at eliminating the pathogenic bacteria (Seneviratne, 2011). The conversion into a pathogenic biofilm is due to the accumulation and proliferation of pathogenic bacterial strains in a formerly non-pathogenic biofilm (Seneviratne, 2011). The accumulation of pathogenic oral bacterial strains is a complex process that involves many changes within the oral cavity. However, initial colonizing bacteria that start a biofilm on the enamel of a tooth surface are responsible for binding to these pathogenic bacteria (Zhu, 2012).

When microbe populations adhere to one another on a stationary surface, it is known as a biofilm. In the oral cavity, these neighboring cells form a biofilm layer on dental and sub-gingival surfaces (Marsh, 1995). The bacterial population within a biofilm collectively provides efficient nutrient usage, chemical communication, and horizontal gene transfer to develop and sustain a mature biofilm community (Zhu, 2012). In a healthy human oral cavity, the biofilm is effective at protecting the individual from foreign pathogens. In humans, biofilm formation begins with the attachment of a colonizing *Streptococcus* bacterial species to salivary proteins that are commonly found on dental enamel. This attachment of the colonizing *Streptococcus* species results in a continuous addition of various bacterial species to the biofilm (Zhu, 2012).

It has been found that *Streptococcus gordonii* is a common initial colonizing bacteria found in oral biofilms (Kreth, 2009). This bacterium initially binds to the dental enamel and promotes cell-to-cell adhesion by producing extracellular DNA and other molecules that provide for adhesion. This DNA provides stability for the biofilm and allows for horizontal gene transfer (Montanaro, 2011). *S*. *gordonii* provides binding sites for other colonizing bacteria, which can lead to the generation of a mature biofilm. Unfortunately, *S*. *gordonii* commonly co-cultures with and supports the growth of *P. gingivalis*, the bacteria responsible for periodontal disease, as well as other pathogenic oral microbes (Huang, 2014). S. *gordonii* is also a causative agent of dental plaque and is likely to contribute to the development of infective endocarditis through shedding from the oral cavity (Okahashi, 2013).

Researchers have discovered that many *Streptococcal* bacterial strains involved in oral biofilm formation are producers of hydrogen peroxide (H_2O_2) (Okahashi, 2013). It is believed that production of H_2O_2 aids in in the initial construction of a biofilm by selectively inhibiting H_2O_2 sensitive bacterial species (Zhu, 2012). H_2O_2 reacts with iron molecules within cells to generate hydroxyl radicals, which causes cellular damage by enzyme inactivation and nucleic acid oxidation (Jakubovics, 2008). The production of H_2O_2 by oral *Streptococci* has cytotoxic effects upon certain other invading and competing bacterial strains and functions to select specific bacteria to include in the biofilm

(Okahashi, 2013). The large amount of H_2O_2 produced by some strains of colonizing bacteria also has a cytotoxic effect upon human macrophage cells (Okahashi, 2013). However, the effects of H_2O_2 produced by *Streptococcus gordonii* upon host immune system cells are largely unknown.

Macrophages are a type of white blood cell involved in host immune systems that ingest and kill various invading microorganisms and other foreign substances in a process called phagocytosis (Karavitis, 2011). Additionally, macrophages synthesize multiple compounds called cytokines that are involved in the activation and termination of an inflammatory response (Fujiwara, 2005). Examples of cytokines include prostaglandins, IL-1, and TNF-alpha. During a bacterial infection, certain bacterial products may also activate macrophage cells. These activated macrophages can then kill various microbes or tumors (Speer, 1989).

Researchers have been able to show that certain H_2O_2 concentrations are able to induce human (THP-1) and mouse (RAW 264.7) macrophage cell death (Zou, 2013). Furthermore, certain oral *Streptococci* strains produce amounts of H_2O_2 that are significant enough to induce human macrophage cell death (Okahashi, 2013). For example, the H_2O_2 that is produced by bacterial strains *S*. *oralis* and *S*. *sanguinis* have cytotoxic effects and induce cell death in host macrophage cells (Okahashi, 2014). This is significant because inducing macrophage cell death allows oral *Streptococcal* strains to escape phagocytosis and travel through the blood stream, where they can colonize in heart valves. Once colonized in the heart valve, oral *Streptococcal* species can induce foam cell formation or macrophage cell death (Okahashi, 2011). Dead macrophage and foam cells can lead to the development of atherosclerotic plaque and result in an increased risk for stroke or heart attack in the host (Tabas, 2010).

The addition of catalase (an enzyme that decomposes H_2O_2) to human macrophage cells infected with H_2O_2 producing *S*. *oralis* was shown to counter the cytotoxic effects of the H_2O_2. Therefore, infected macrophage cell death was reduced after the addition of the H_2O_2 mitigating catalase (Okahashi, 2013). In addition, research has shown that the addition of catalase to oral biofilms reduces the production of extracellular DNA by S. *gordonii*. This in turn reduces the rate of biofilm formation and therefore dental plaque formation (Kreth, 2009).

Due to the dangers associated with accumulating large amounts of dead macrophage cells, it is important to have an understanding of the different strains of oral bacteria that have the capability to induce macrophage cell death. Currently, only two oral bacteria are known to have this capability. While *S*. *oralis* and *S*. *sanguinis* induce macrophage cell death, similar studies have not been performed with S. *gordonii*. Because S. *gordonii* is another biofilm colonizing bacteria, knowledge of its ability to contribute to oral disease and plaque development because of H_2O_2 production could prove to be very useful. Therefore, a strain of S. *gordonii* isolated from a human oral cavity was selected for this study.

The purpose of this study was to determine if the amount of H_2O_2 produced by S. *gordonii* is sufficient to induce cell death in a macrophage cell line (RAW 264.7). Furthermore, catalase was added to the infected macrophages in order to determine if macrophage cell death can be prevented or reduced by the addition of catalase. It was hypothesized that the infection of macrophage cells with S. *gordonii* would induce

macrophage cell death due to the production H_2O_2, and that macrophage cell deaths would be reduced by the addition of catalase. If *S*. *gordonii* successfully induced macrophage cell death, it would mean that there are multiple oral bacterial strains that can cause macrophage cell death. While *S*. *gordonii* is involved in oral biofilm formation and subsequently, host oral pathogen defense, it has also been shown to have negative impacts on oral and cardiac health. Therefore, the inhibition of *S*. *gordonii* and other oral H_2O_2 producing bacteria in people with severe periodontal disease or cardiac issues could potentially have positive health benefits.

Methods

A bacterial sample of *Streptococcus gordonii* (ATCC 51656) isolated from a human oral cavity was be used in this experiment. The sample was cultured in Brain/Heart Infusion (BHI) agar and broth, and grown aerobically at 37 ^oC. To start a culture, the frozen pellet sample of *S*. *gordonii* was hydrated with 1 mL of Brain/Heart Infusion (BHI) broth, and then transferred to 6 mL of BHI broth and vortexed. This sample was used to inoculate a 100 mL BHI broth sample and a BHI agar plate. These samples were grown aerobically for 24 hours at 37 ^oC (ATCC 51656). The number of bacterial cells used to infect the macrophage samples was quantified with a hemocytometer. One mL of the *S*. *gordonii* broth sample grown overnight was pipetted into a tube containing one mL of 1.0% crystal violet dye to give a 1:1 dilution of the sample. A pipette was then used to fill the hemocytometer counting chamber with 10 µL of the dilution sample. Bacterial cell numbers were determined using a common protocol and calculation procedure (JoVE, 2015).

In order to quantify the amount of H_2O_2 produced by the *S. gordonii* sample before addition to the macrophage culture, the bacterial medium was assayed using a Hydrogen Peroxide Assay Kit (Abova). *S. gordonii* was cultured in 6 mL of BHI broth for 18 hours prior to the assay (Okahashi, 2013). The cell culture supernatant was then diluted 50-fold in PBS, and 50 µL of this sample was assayed. The H_2O_2 concentrations were then determined according to the manufacturer's instructions (Abnova) using fluorescence-linked analysis at 540 nm with emission at 590 nm.

RAW 264.7 (ATTCC TIB-71RAW) macrophage cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum and antibiotics (1% penicillin and streptomycin) at 37 ^oC and 5% CO_2 (Okahashi, 2013). The cells were handled and sub-cultured according to the manufacturer's product sheet. Adherent macrophage cells were mechanically lifted using a cell scraper. The culture medium was changed every 2 days. In order to confirm that H_2O_2 causes macrophage cell death and to serve as a positive control, macrophage cultures were grown in the presence of 1mM or 10 mM H_2O_2 for 18 hours (Okahashi, 2013). A hemocytometer and 0.4% trypan blue dye were used to count macrophage cell before treatment with H_2O_2 (methods as described above). An equal number of cells were then plated in each well of a 6 well plate prior to the treatment. Cell death was then quantified after H_2O_2 treatment using 0.4% trypan blue staining and a hemocytometer.

To test the effect of H_2O_2 produced by *S. gordonii* on macrophages, a 24 well plate with equal numbers of macrophage cells in each well was infected with *S. gordonii* for two

hours in the absence of antibiotics. The amount of *S. gordonii* used to infect the sample was determined by a multiplicity of infection (MOI) calculation of 0 (control), 50, 100, and 200 (Okahashi, 2013). The MOI was determined by using the macrophage cell count to infect the macrophage culture with multiples of 0, 50, 100, and 200 *S. gordonii* cells. The control contained only Brain/Heart Infusion broth and no bacteria. In addition, 10U/µl or 100U/µl of catalase was added to different macrophage cultures before infecting the cultures with S. gordonii for two hours. After infection for two hours, the bacterial medium was removed, and the adherent macrophage cells were washed with sterile PBS to remove extracellular bacteria and cultured for 18 hours in fresh medium and antibiotics. The viable macrophage cell count was then determined by lifting the adherent macrophage cells using a cell scraper, staining the cells with 0.4% trypan blue exclusion dye, and counting with a hemocytometer.

The data collected included the dead to live ratio of macrophage cells (dependent variable) and the amount of *S. gordonii*, or *S. gordonii* and catalase used to infect a macrophage sample (independent variable). A one-way ANOVA was performed between these variables and the significance between the variables was analyzed with a Tukey Post-Hoc Test.

Results

The mean dead/live ratio of the 1mM H_2O_2 macrophage samples was 0.142(max= 0.146, min= 0.139). The mean dead/live ratio of the 10mM H_2O_2 samples was0.642 (max= 0.737, min= 0.560) (Figure 1). H_2O_2 signficantly induced more death at the 10 mM concentration (p=0..02).

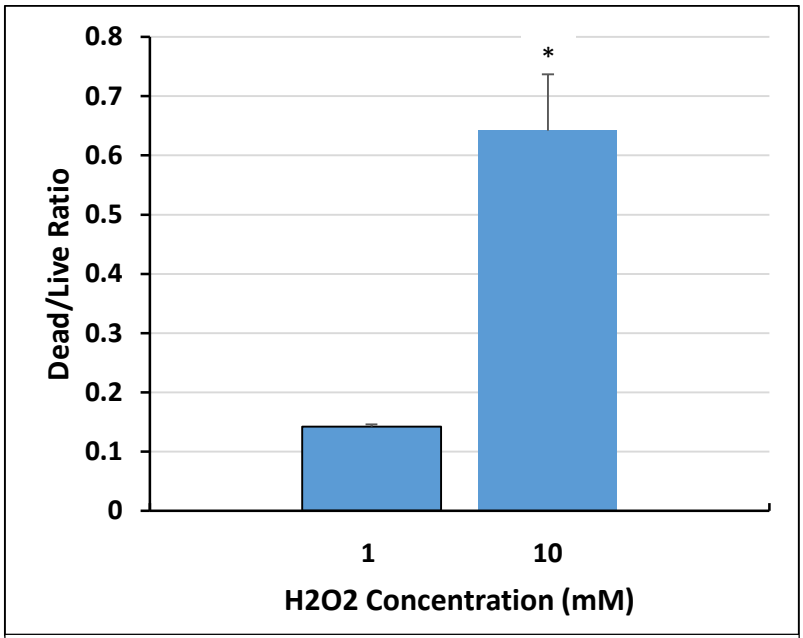

Figure 1. Effects of 1 and 10mM peroxide treatments on the dead/live ratio in RAW 264.7 macrophages. * significantly different than 1mM

The dead/live ratios from the bacterial infection experiment were analyzed using SPSS software. The dead/live ratios differed between multiple treatments ($F_{7,16} = 7.99$, $p < 0.05$). There was no significant difference between the 50 Multiplicity of Infection (MOI) treatments and the control ($p= 0.059$).The 50 MOI treatments had a mean dead/live ratio of 8.5 (SD= 2.65, max= 11.0, min=6.0). The 100 MOI treatments had a significantly higher dead/live ratio than the control (Figure 1). Macrophage cell dead/live ratios in response to treatments of 1mM or 10mM of H_2O_2 alone ($p < 0.05$). The 100 MOI treatments had a significantly higher dead/live ration than the control ($p <0.05$). The 100 MOI treatment has a mean dead/ratio of 8.5 (SD= 4.33, max=11, min=3.5). The 200 MOI treatments also had a significantly higher dead/live ratio than the control ($p < 0.05$). The 200 MOI treatments had a mean dead/live ratio of 12.33(SD= 3.21, max=16, min=10) (Figure 2).

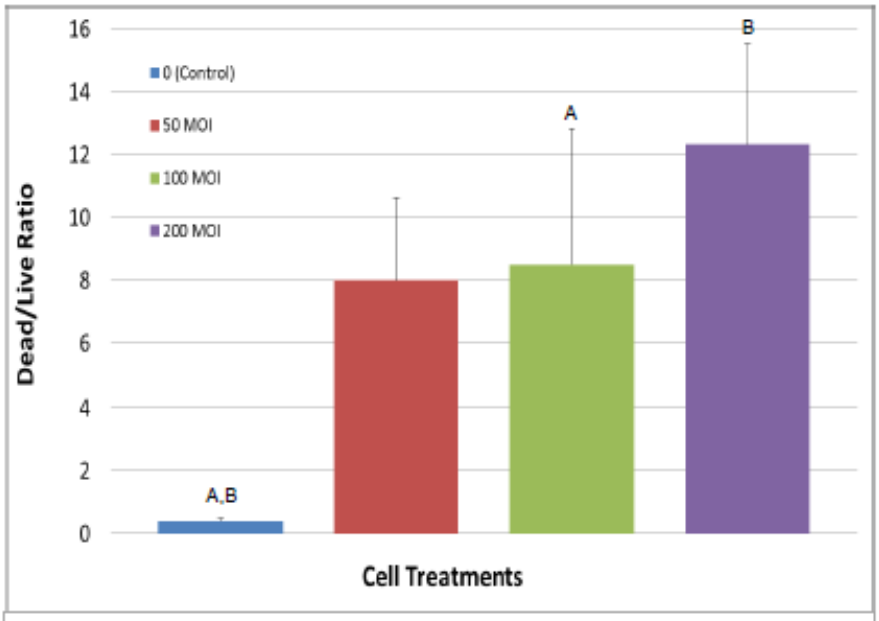

Figure 2: Macrophage death ratios in response to various bacterial treatments. (A) 100 MOI significantly higher than control, (B) 200 MOI significantly higher than control ($p< 0.05$).

There was no significant difference between any of the catalase treatments and the 0 MOI + 100U/µL catalase control (Figure 3). The 200 MOI + 10U/µL catalase treatments were not statistically different than the control ($p= 0.135$). The 200 MOI +10U/µL catalase treatments had a mean dead/live ratio of 7.0 (SD= 2.31, max= 11.0, min= 3.0) (Figure 3).

The 200 MOI + 100U/µL catalase treatments were also not statistically different than the 0 MOI + 100U/µL catalase control ($p= 0.215$). The 200 MOI + 100U/µL catalase treatments had a mean dead/live ratio of 6.4 (SD= 2.94, max= 8.2, min=3.0).

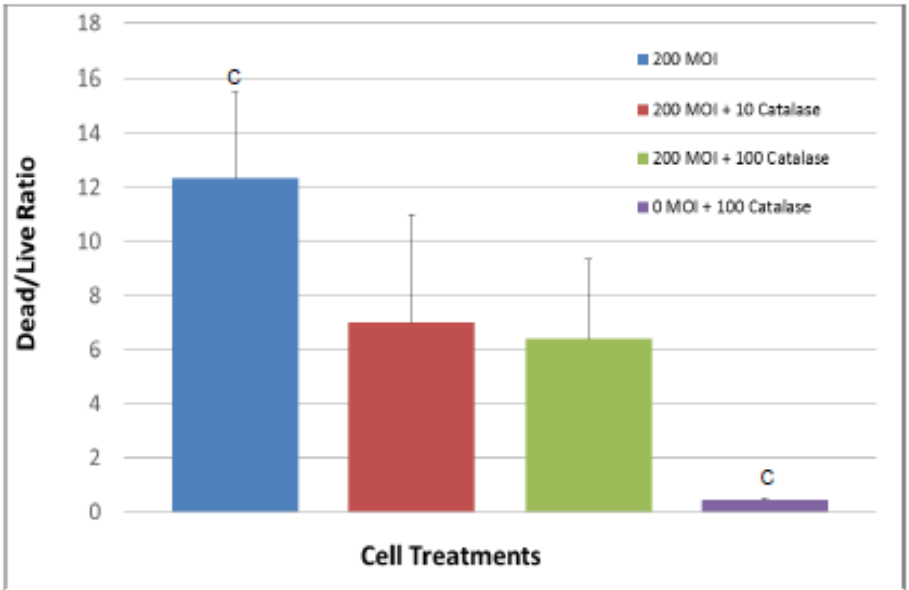

Figure 3: Macrophage dead/live ratios in response to bacterial treatment and various amounts of catalase enzyme. Letters (C) indicate a significant difference ($p<0.05$) between dead/live ratio of catalase only and 200 MOI.

Discussion

It has been confirmed in previous research that H_2O_2 alone is sufficient to induce macrophage cell death (Okahashi, 2013). As was expected, in this experiment the10mM H_2O_2 treated macrophage cells exhibited an increased dead/live ratio in comparison to the 1mM H_2O_2 treated macrophage cells (Figure 1).

Similar to results found in other studies with different H_2O_2 producing oral *Streptococcal* strains, infecting RAW 264.7 macrophage cells with increasing amounts of *S. gordonii* resulted in extensive macrophage cell death (Figure 2). However, due to inconclusive hydrogen peroxide assay results, the amount of H_2O_2 produced *by S. gordonii* is unknown. Therefore, it cannot be definitively concluded that the macrophage cell death was a result of the H_2O_2 produced by *S. gordonii*. Nonetheless, research has shown that infection of macrophage cells with oral *Streptococcal* strains that lack the production of H_2O_2 results in significantly increased macrophage cell viability (Okahashi, 2013). Consequently, because macrophage cell viability significantly decreased as the infecting amount of H_2O_2 producing *S. gordonii* was increased, there is evidence that macrophage cell death was induced by H_2O_2. .

Unexpectedly, the addition of various concentrations of catalase enzyme did not reduce macrophage cell death of any statistical significance (Figure 3). However, it was observed that the addition of increasing concentrations of catalase to infected macrophage samples resulted in an increased number of live cells in comparison to sole bacterial treatments.

The hypothesis that macrophage cell death is mediated by *S. gordonii* derived H_2O_2 could be further supported by determining the concentration of H_2O_2 produced by *S. gordonii* from a successful H_2O_2 assay. Furthermore, it is possible that allowing the macrophage cells to incubate overnight in the presence of catalase after bacterial infection instead of only during infection could significantly prevent macrophage cell death. In another study that used a similar protocol but instead allowed the infected macrophage cells to incubate overnight in the presence of catalase after oral *Streptococcal* infection, it was found that macrophage cell death was significantly decreased in comparison to the control (Okahashi, 2013). Furthermore, other research has found that a similar concentration of catalase that was added to oral *Streptococcal* cultures remained effective for the entire duration of a 6 hour experiment by preventing H_2O_2 mediated bacterial cell death (Thomas, 1994). It is possible that allowing the macrophage cells to incubate overnight in the presence of catalase after bacterial infection will allow the catalase enzymes more time to prevent cellular damage by reducing enzyme inactivation and nucleic acid oxidation (Jakubovics, 2008).

As a whole, this study presents evidence that the oral *Streptococcus* strain *S. gordonii* can induce macrophage cell death as a result of H_2O_2 production. Further studies may wish to confirm the cytotoxic properties of *S. gordonii* derived H_2O_2 on human macrophage cells, and further explore the use of catalase as a preventative tool in macrophage cell death, in order to develop a targeted treatment for individuals with severe periodontal disease and high risk for various cardiac diseases.

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Protocols

Abnova H_2O_2 Assay Protocol:

http://www.abnova.com/products/products_detail.asp?catalog_id=KA1322

ATCC TIB-71 RAW 264.7 Culture Methods:

http://www.atcc.org/products/all/TIB-71.aspx#culturemethod

ATCC 51656 Culture Methods:

http://www.atcc.org/products/all/51656.aspx#documentation.