Original Research Article

An *In-Vitro* Disk Diffusion Comparison Study of Chlorhexidine Gluconate and Salt: Application of Salt in Oral Treatments

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ABSTRACT

Oral infection is a prevalent condition that affects billions of individuals globally. This leads countries to incur significant expenses to treat oral infections. One of the most commonly used antiseptics, chlorhexidine gluconate, is a costly treatment. To assess this situation, we focused on finding a proxy for chlorhexidine gluconate under oral application. We hypothesized that a certain salt concentration would have a similar level of antiseptic effects as chlorhexidine gluconate. To compare the antiseptic properties of the chlorhexidine gluconate to different salt concentrations, we first collected the bacteria from our teeth. Then, we grew the bacterial cultures in an incubator. After bacterial colonies formed, we collected them and mixed them in water to make a bacterial solution. Then, we suspended the solution in another Petri dish and placed sterile disks that had been soaked in chlorhexidine gluconate and salt solutions. We observed the diameter of the zone of inhibition of each sterile disk as a measure of antiseptic effectiveness. Results showed that a 10% salt concentration solution produced similar effects to chlorhexidine gluconate. This result suggests that salt can be a potential alternative to chlorhexidine gluconate, as it is cost-effective, naturally occurring, and capable of producing similar effects.

Keywords: Oral; chlorhexidine; salt; bacteria; infection

INTRODUCTION

The amount of dental infections is an increasingly concerning issue, with hundreds of thousands of people being diagnosed with various oral diseases every year. The most prevalent oral conditions include dental caries (tooth decay), periodontitis (severe gum disease), tooth loss, and oral cancers. Untreated dental caries is the most common condition, affecting approximately 2.5 billion people globally. Periodontal disease, a disease that can lead to tooth loss, is estimated to affect 1 billion people. Additionally, about 380,000 new cases of oral cancers are diagnosed every year, highlighting the ever-growing issue of oral problems (1). These cases do not include undiagnosed individuals, meaning there are even more people around the world who unknowingly have dental problems. The total cost of money spent worldwide on dental infections in 2019 was 710 billion US dollars. The total costs, consisting of direct costs, were around 387 billion US dollars, while indirect costs due to dental

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diseases totaled almost 323 billion (2).

Antiseptics are chemical agents that reduce the number of microorganisms on living tissues by inhibiting growth. A wide variety of chemicals, such as alcohol, phenols, iodine, and chlorine, are active in the destruction or prevention of microbial activity (3). The effectiveness of antiseptics is closely tied to their chemical structures and properties. For instance, phenolic compounds like chloroxylenol (C_8H_9ClO) are known for their phenolic odor and are commonly used in antiseptic formulations such as Dettol (4). The introduction of antiseptics revolutionized surgical practices and everyday hygiene, marking a turning point in public health. By significantly lowering infection rates, antiseptics contributed to longer life expectancy, safer medical procedures, and improved outcomes in both military and civilian medicine (4). Despite early skepticism and resistance, the undeniable results of antiseptic use eventually led to widespread acceptance, shaping modern medicine as we know it (5).

Chlorhexidine gluconate was first created in the United Kingdom in the early 1950s while researchers at Imperial Chemical Industries (ICI) were investigating antimalarial compounds. ICI recognized its remarkable antibacterial activities and commercialized it as a disinfectant in the UK (5). By the 1970s, its use expanded. First being utilized in antiseptic soaps, then in mouthwashes (6). Chlorhexidine gluconate (CHG) is a popular antiseptic as it has become routine in infection control protocols in medical, dental, and hygiene settings. CHG is a bisbiguanide compound, giving it a powerful cationic character. This structure accounts for its antimicrobial action, as it can bind strongly to bacterial cell walls, typically anionic or negatively charged, through electrostatic interactions (7). Upon binding, CHG disrupts the microbial cell membrane structure by increasing its permeability. As a result, cytoplasmic material such as potassium ions, nucleotides, and other intracellular material leak out, ultimately causing cell death (7). CHG has concentration-dependent action: at low concentrations, it is bacteriostatic (inhibits bacterial growth), while at higher concentrations, it is bactericidal (kills bacteria outright) (8). This makes it suitable for many different applications, depending on the level of disinfection required. Additionally, an important property of CHG is its substantivity. Once applied, CHG binds to surfaces and is slowly released over time, continuing to kill bacteria long after the initial application. In dentistry, this provides for the maintenance of low concentrations of plaque and gingivitis for extended periods without the requirement for constant reapplication (8). In surgical preparation, CHG maintains sterility throughout long operations. Lastly, CHG is not easily neutralized by organic matter like blood or saliva, which is a notable advantage over some other antiseptics. It remains active in circumstances where other products might lose effectiveness (9).

CHG is frequently used in hospitals, dental offices, and mouthwashes, and its primary purpose is to prevent infections by reducing microbial presence (7). However, CHG has the potential for side effects, especially with prolonged, repeated use. Chlorhexidine can cause teeth staining, especially brown coloring, where the tooth and the gum meet. The cause of this is not fully understood, but one theory is that chlorhexidine clings to foods and drinks with a tendency to stain, such as wine, coffee, and tea (10). Additionally, while rare, reports of an allergic reaction, anaphylaxis, have increased over the years from applying chlorhexidine to the skin (11). This allergic reaction is labeled as a severe, rapidly developing, potentially life-threatening disorder caused by a sudden drop in blood pressure, with over two people having died from the reaction as a result of using chlorhexidine (12). Furthermore, when rinsing with 0.2 and 0.1% chlorhexidine gluconate and acetate for 4 months, peeling and soreness of the membrane lining of the mouth were observed, and 36% of the 50 test persons developed discolored tongues over the study period (13).

Salt is an ionic compound that is a product of an acid-base reaction. The most common example is Sodium chloride (NaCl), commonly known as table salt. It is an ionic compound that is formed by the combination of hydrochloric acid (HCI) and sodium hydroxide (NaOH), a base. Whether it is being used as a preservative, a wound-healing agent, or a seasoning for foods, salt has been an integral component of human life since the earliest civilization. Regarding the subject of this paper, the antiseptic usage of salt persisted throughout history, mainly to heal wounds, and was used for natural remedies (14, 15, 16). Sodium chloride (NaCl) kills microorganisms by disturbing the osmosis of cells (17). Sodium chloride separates into two ions when dissolved in water, sodium and chloride ions, contributing to the osmolarity. Osmosis is the movement of water molecules through the cell membrane. Water molecules move from an area with a high concentration to a low concentration until the two sides reach a state of dynamic equilibrium (18, 19). Unlike water, salt is one of the molecules that cannot pass through the semipermeable membrane of the cell wall. As a result, when a cell is put into a hypertonic solution, where there is a higher salt concentration

outside the cell, the water molecules move out of the cell to balance the concentration inside and outside the cell (20, 21). As a result, the cell would die due to shriveling. Like salt, CHG works similarly. Its mechanism involves a disturbance in the osmotic regulation of the cell. Eventually, this disturbance leads to cytoplasmic leakage and death (22).

Chlorhexidine is often considered to be the more effective agent in decreasing the total number of bacteria in the mouth. However, one study has shown that both salt water and chlorhexidine have similar antimicrobial activities. The study evaluated the effects of a 2%, 5.8%, and 20% saline solution compared to 0.1% chlorhexidine on oral bacteria counts and found reductions in both groups. They have found that salt and chlorhexidine act similarly in how they affect the oral flora and how long their antimicrobial affect persists. (23) Chlorhexidine also elicits various side effects in different applications. Another study, experimenting with the effects of chlorhexidine oral rinse on people with uncontrolled diabetes, showed that out of 140 participants, forty-four (31 percent) experienced side effects such as sour mouth/throat, staining, and taste changes (24). In another study, there was an increase in the risk of death among patients who used chlorhexidine oral rinse care. Chlorhexidine is associated with side effects, like staining of teeth, an increase in tartar, and altered taste perception. On the other hand, salt water has a variety of practical benefits, as it is natural, inexpensive, and widely accessible (25). Saltwater rinses are safe because sodium chloride is a natural component of the body's fluid and is essential for normal cellular functions. When used in small amounts for rinsing, the solution mimics the body's own saline balance. Additionally, medical-grade saline (0.9% NaCl) is commonly used in hospitals for wound care, hydration, and irrigation, which also proves its safety for oral usage (26, 27).

The objective of this study is to evaluate the effectiveness of various concentrations of sodium chloride solutions compared to chlorhexidine gluconate. We aim to determine if higher concentrations of salt solutions can produce inhibition that is comparable to that produced by chlorhexidine gluconate. To assess the antimicrobial properties of salt and CHG, we designed our experiment using the disk diffusion method. The disk diffusion method is a widely used method for studying antimicrobial susceptibility testing. It measures the zone of inhibition to measure the efficacy of the antimicrobial agent. However, the resulting data is highly dependent on the time of incubation and bacterial growth, as some aerobic oral bacteria need prolonged growth to perceive the zone of inhibition (28). Previous studies, which have suggested that salt water can have antimicrobial effects similar to chlorhexidine in oral care, but a controlled experiment that directly compares their effectiveness across different salt concentrations has not been extensively conducted. Our experiments could help validate the use of simple saltwater solutions as a practical, cost-effective alternative to commercially available antiseptics, particularly in settings where chlorhexidine is limited or undesirable. We hypothesize that certain concentrations of sodium chloride solution, specifically higher concentrations like 10% and 20%, will have similar antimicrobial effects as chlorhexidine gluconate. This would suggest that salt solutions can serve as viable substitutes for chlorhexidine under oral application.

METHODS AND MATERIALS

Materials

In this study, we used an incubator (Oen, ASIN B0BZ4T53F1), cotton swabs (JMU, ASIN B0CQ24SV24), petri dishes with agar (DalosDream, ASIN B08C546HHK), sensitivity discs (Home Science Tools), non-iodized salt (Morton Salt Inc., ASIN B0D26YSBS8), distilled water (Amazon.com Services LLC, ASIN B07VD4KN28), sterile Pipettes (moveland, ASIN B0C4JV8MZJ), House Brand 0.12% Chlorhexidine Gluconate Oral Rinse, centimeter ruler, and cryotubes (DalosDream, ASIN B08C546HHK).

Methods

To prepare the salt solutions, 250 mL beakers were labeled with different salt concentrations (0%, 1%, 5%, 10%, 20%, chg). Then, the desired amount of salt for each concentration was measured (1% = 1 g etc., for a 100 mL solution) and poured into the 250 mL beaker. Next, the beaker was filled with distilled water up to the 100 mL mark and stirred until all of the salt had completely dissolved. For 20% salt concentration solutions, we have used warm water to speed up the dissolving process.

The bacteria were collected from our teeth by gently rubbing the cotton swab against the teeth. These mixed general bacterial samples were collected using a sterile cotton swab from the oral cavity of consenting participants, who are the authors of this paper. Then, the cotton swab was used to suspend the bacteria evenly throughout the surface of the agar. Finally, the swabbed plates were put into the incubator for 1-2 days

at a temperature ranging from 37 degrees Celsius to 38 degrees Celsius. All procedures were conducted under biosafety conditions. Personal protective equipment, such as gloves, was used at all times. The work surfaces were disinfected before and after experimentation. Petri dishes were sealed during incubation to prevent contamination. All biological materials were properly disinfected and disposed of.

After the bacterial population had grown, they were collected by gently scraping the cotton swabs from the bacterial colonies. Using the marks in the sterile pipette, 2 mL of distilled water was collected by the pipette and then put into the cryotube. After that, the cotton swab that collected the bacterial colonies was stirred in the cryotube. The cotton swabs were stirred until the water became cloudy. Once the solution has been made, soak the swabber in the bacterial solution and gently spread it across the whole surface evenly. Then, take six sterile antibiotic test discs and treat each with a consistent volume of three drops of solution to ensure uniform absorption across all treatments. Place one sterile disk per solution inside its respective agar plate. Finally, incubate for 24 hours at 37-38 degrees Celsius. Each concentration was tested in three independent trials (n = 3), with separate agar plates used for each replicate. Results are measured by measuring the diameter of zones of inhibition with a centimeter ruler, which are clear circles where no bacteria grew, using a ruler in millimeters. We have repeatedly done a total of three trials of these processes.

RESULTS AND DISCUSSION

By measuring the bacterial growth inhibition zones (Figure 1), the experiment provides quantitative evidence of antimicrobial efficacy. Essentially, the inhibition zones help identify the concentration range at which NaCl begins to show significant antibacterial effects.

The experiment tested how different concentrations of salt water affect bacterial growth. First, non-iodized salt was dissolved in 100 mL of distilled water to form various salt solutions from 0% to 20%. Then, bacteria were collected from the teeth using sterile cotton swabs and evenly spread on agar plates. The plates were incubated at 37–38 degrees Celsius for 1 to 2 days to allow the bacteria to grow (Figure 2). Once bacterial

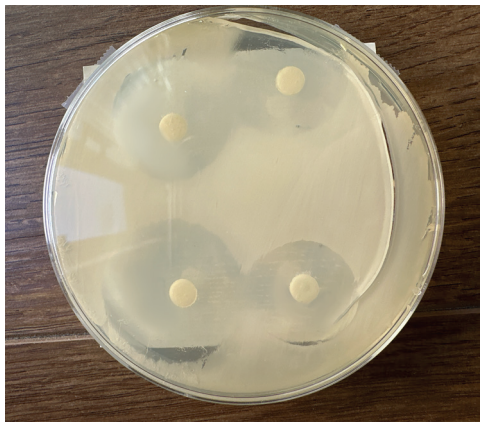

Figure 1. *Zone of inhibition of salt solutions. A uniform layer of bacterial colony grown in a petri dish in an incubator with four sterile disks, each soaked in a different salt solution (n=3).*

Figure 2. *Bacterial colonies grown in a petri dish. Using cotton swabs, bacteria were collected from the teeth and spread in a petri dish. Then the bacterial colony was grown in an incubator set to 37-38 degrees Celsius (n=3).*

Table 1. Zone of inhibition for different NaCl solution and chlorhexidine gluconate. *Data are expressed as mean ± standard deviation (SD) from three replicate trials. Standard deviations quantify experimental variability and were used to generate error bars (± SD) in graphical analyses to reflect measurement consistency and antimicrobial activity.*

NaCl Solution	0%	1%	5%	10%	20%	chlorhexidine gluconate
Zone of Inhibition (mm)	0 ± 0	14 ± 0.42	17.53 ± 0.526	22 ± 0.66	26 ± 0.78	21.5 ± 0.645

colonies formed, samples were mixed with distilled water to create a bacterial solution. Sterile disks were then soaked with 3 drops of each salt solution and chlorhexidine gluconate solution and placed onto the agar plates with an evenly covered bacterial solution. The plates were incubated again for another day (Figure 3). Lastly, the zones of inhibition were measured to measure their effectiveness.

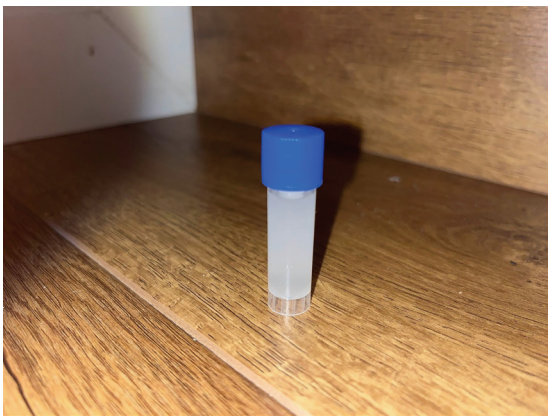

Figure 3. *Bacterial solution. Using cotton swabs, bacteria grown in the petri dish were collected and mixed with water in a cryotube (n=3).*

As seen in Table 1, NaCl solutions with 0% concentration showed no zones of inhibition in both trials, resulting in an average of 0 mm. NaCl solutions with 1% concentration showed zones of inhibition measuring 13 mm in Trial 1 and 15 mm in Trial 2, resulting in an average of 14 mm. NaCl solutions with 5% concentration showed zones of inhibition measuring 17 mm in Trial 1 and 18 mm in Trial 2, resulting in an average of 17.5 mm. NaCl solutions with 10% concentration showed zones of inhibition measuring 21 mm in Trial 1 and 23 mm in Trial 2, resulting in an average of 22 mm. NaCl solutions with 20% concentration showed zones of inhibition measuring 27 mm in Trial 1 and 25 mm in Trial 2, resulting in an average of 26 mm. Chlorhexidine gluconate solution showed zones of inhibition measuring 20 mm in Trial 1 and 23 mm in Trial 2, resulting in an average of 21.5 mm.

A series of Weltch's t-tests were conducted in to compare each NaCl concentration to chlorhexidine gluconate (CHG). Results showed that 0%, 1%, and 5% NaCl solutions had significantly smaller zones of inhibition than CHG ($p < 0.001$). The 10% NaCl solution showed no statistically significant difference compared to CHG ($t \approx 0.94$, $p > 0.05$), indicating comparable antimicrobial effectiveness. In contrast, the 20% NaCl solution produced significantly larger zones of inhibition than CHG ($p < 0.001$), suggesting greater antibacterial activity at higher concentrations.

These results show that increasing NaCl concentration leads to greater antimicrobial activity. While lower concentrations were significantly less effective than chlorhexidine gluconate, the 10% NaCl solution achieved comparable antibacterial effects, and the 20% NaCl solution exceeded CHG in inhibitory capacity. This suggests that salt solutions only become clinically relevant at sufficiently high concentrations, highlighting a threshold effect in antimicrobial efficacy.

The limitations of the experiment were controlling the bacterial species. This limitation could be attributed to the lack of methods for separating the type of bacteria. Because the collection of bacteria would acquire a mixed colony of bacteria, it is unlikely that the same salt concentrations would have a similar effect on the colonies. The lack of resources to imitate a realistic condition in a human mouth, such as humidity, may have also created a discrepancy between our data and realistic data. In additions, bacterial density was not standardized using a McFarland turbidity standard, which may have introduced variability in initial bacterial concentration across samples. Lastly, the application of saline solutions to patients with hypertension should be further researched.

The results of this study demonstrate that 10% NaCl solutions can be used as a viable alternative to chlorhexidine gluconate for oral care. NaCl solutions with 10% concentration showed zones of inhibition measuring an average of 22 mm, while the chlorhexidine gluconate solution showed zones of inhibition measuring an average of 21.5 mm. The NaCl and chlorhexidine gluconate solutions showed comparable zones of inhibition, indicating that they produce similar antimicrobial effects. NaCl solutions can be used as a more accessible and cost-effective alternative to chlorhexidine. Unlike chlorhexidine, which can cause teeth staining, altered taste perception, and allergic reactions, NaCl solutions are less likely to produce health risks, making them safer to use over time.

Our study demonstrated that sodium chloride solutions, especially at near human body temperatures, exhibited similar or even greater disinfecting qualities to chlorhexidine gluconate. For sodium chloride concentrations of 10% and 20%, they had an average

zone of inhibition of 22 mm and 26 mm, respectively. These values are both higher than CHG's average zone of inhibition of 21.5 mm, proving that sodium chloride at concentrations of 10%+ can be used as a more cost-effective and easy-to-access chemical antiseptic alternative. These findings highlight the potential that high-concentration sodium chloride has to be used as an alternative to CHG for people who cannot access it or have negative side effects while using it.

It is, however, important to note that the use of NaCl at concentrations from 10-20% raise significant concerns regarding oral tolerability because of their highly hypertonic nature. While they can provide the same level of antiseptic power as CHG, their high salt concentrations can lead to high levels of osmotic stress and would actually draw water out of oral tissues, leading to tissue dehydration, even if only swished for small periods of time, especially for 20% concentrations. It would still be best to go to a medical provider, but in situations where that's impossible, a 5-10% NaCl solution could be a good last minute, cost-effective alternative that won't cause teeth staining, altered taste perception, and allergic reactions like chlorhexidine.

The results suggest that higher NaCl concentrations exhibit antimicrobial activity comparable to or greater than that of chlorhexidine gluconate. Yet, this result alone cannot establish clinical equivalence. CHG is known to have broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and fungi, as well as substantivity, allowing it to remain active in the oral cavity for extended periods after application (29). In contrast, this study only evaluated immediate antimicrobial effects using zone of inhibition and did not assess duration of action or effectiveness across specific microbial species. Additionally, the oral environment contains complex factors such as saliva, biofilms, and diverse microbial communities that are not replicated in agar-based experiments. Therefore, while NaCl solutions may demonstrate potential as a low-cost alternative, further research is needed.

CONCLUSION

This study was to prove whether sodium chloride (NaCl) solutions could serve as a useful alternative to chlorhexidine gluconate (CHG) for general antibacterial use. By comparing zones of inhibition, we found that sodium chloride solutions may perform the same antibacterial effect as chlorhexidine gluconate. Notably, the 10% NaCl solution had an average inhibition zone of 22 mm, comparable to that of CHG of 21.5 mm, while the 20% NaCl solution produced an even larger average inhibition zone. Therefore, our hypothesis that higher concentrations of salt solutions could achieve the antibacterial property, as the chlorhexidine gluconat,e was supported. Yet, this study had limitations, including mixed bacterial species and laboratory conditions that did not fully replicate the human oral environment. Future research should aim to address and supplement these limitations.

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CONFLICT OF INTEREST

The authors: Jane Hong, Francis Hong, Jaehee Kim, Michelle Tan, Brooke Yu declare that there are no conflicts of interest regarding the publication of this article.

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