

Comparison Of The Antibacterial Efficacy Of Calcium Hydroxide When Mixed With Zamzam Water, Normal Saline And 2% Chlorhexidine - An *In Vitro* Study

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ABSTRACT:

Aim: To investigate the antimicrobial properties of calcium hydroxide when mixed with three vehicles namely Zamzam water, Normal saline and 2% Chlorhexidine.

Materials & Method: 6 plates of nutrient broth and *S. aureus* culture were prepared. 2 (5*3 mm) wells were prepared in one plate and is used as control. 3 wells each were prepared in the remaining 5 plates and filled with freshly prepared mix of calcium hydroxide with the three vehicles (5 wells were filled with each mix). The same process was repeated using 6 plates containing nutrient broth and *B. subtilis* culture. The plates were then incubated for 24 hours and the Zone of inhibition (ZOI) was measured. The pH of each mixture was also measured by using a pH meter. The data was analyzed by ANNOVA test and compared by using Tukey HSD test.

Result: The mean value of ZOI for Ca(OH₂) and Zamzam was statistically significant when tested against both the bacteria. The pH of 12.26, 11.90 and 12.10 was achieved for Ca(OH₂) and Zamzam, Ca(OH₂) and Normal saline and 2% Chlorhexidine and Ca(OH₂).

Conclusion: Calcium hydroxide powder mixed with Zamzam water exhibited the highest pH of 12.26 and showed statistical significant antimicrobial difference when compared to other medicaments. However further in depth scientific investigations regarding the properties and safety of Zamzam water and World health organization (WHO) approval would be required before considering its clinical use.

Clinical significance: Persistence of infection after cleaning and shaping of the canals is attributed to the presence of bacteria which necessitates the use of an antimicrobial agent

as a inter appointment dressing. Calcium hydroxide was considered gold standard intra canal medicament due to its high alkalinity. A proper vehicle is necessary to activate the calcium hydroxide by releasing the Ca^+ and OH^- ions. The results of this study showed that Calcium hydroxide powder mixed with Zamzam water exhibited the highest pH of 12.26

KEYWORDS: *Antimicrobial activity; Bacillus subtilis; Calcium Hydroxide; Chlorhexidine; Staphylococcus aureus; Zamzam water.*

1. INTRODUCTION:

Endodontic failure due to persistent infection is usually due to the perseverance of microorganisms in the root canal.^{1,2} Reduction in the volume of microorganisms can be achieved by shaping the canals using larger instrument sizes and thoroughly cleaning the canals with the aid of an irrigant.³ However achieving perfectly clean canals may not always be possible due to varying canal anatomy and occasionally due to the relative inability of the operator to visualize this anatomy based on radiographs.⁴

Calcium hydroxide [$\text{Ca}(\text{OH})_2$] used as an inter appointment dressing has been shown to minimize reinfection.⁵⁻⁷ Its mechanism of action is basically dependent on the ionic dissociation of $\text{Ca}(\text{OH})_2$ into Ca^{2+} and OH^- ions.^{7,8} This dissociation is considered to be much quicker when an aqueous vehicle is used to prepare the paste than viscous or oily fluids.⁹ Numerous aqueous vehicles in the form of distilled water, normal saline, dental anesthetics, Ringer's solution and Chlorhexidine (CHX) have been used in the past with varying results.¹⁰

This study used Zamzam water, Normal saline and 2% Chlorhexidine as a vehicle to make the paste. Zamzam water is consumed by millions of Pilgrims who visit Mecca. Its consumption is based on religious belief of having medicinal and healing properties. It is alkaline in nature and a pH value of 8 have been reported.¹¹ Studies have shown that Zamzam water has unique oncolytic properties and has a strong anti-inflammatory effect due to the presence of a strong antitumor necrosis factor ($\text{TNF}\alpha$) and effect on interleukin I. Its oncolytic action is due to its influence on endocrine immunology and the growth system of the body.^{12,13} Due to the higher level of fluorides in Zamzam water studies have shown that the use of zamzam water reduces the incidence of dental caries.¹⁴ This in-vitro study focuses on the antimicrobial properties of Zamzam water, Normal saline and 2% CHX when used as a vehicle to mix $\text{Ca}(\text{OH})_2$ powder to form a paste.

2. METHODOLOGY:

This study was conducted in Microbiology Lab, College of Medical Sciences, Prince Sattam bin Abdulaziz university, Al Kharj. The pH meter (SensoDirect pH 200, Lovibond, Germany) was calibrated with standard pH solutions of pH 4, 6, and 9. The test tip was washed with distilled water and dried with tissue paper to prevent any contamination between the tests. The PH apparatus precision was constantly checked by using standard buffer solution (Lovibond, Germany) of known pH value, so that errors due to malfunctioning of the

apparatus would be minimized as much as possible. An average of 3 pH value readings was taken for each medicament of freshly prepared mixture of Ca(OH)_2 (Merck KGaA, Darmstadt, Germany) with Zamzam, Normal Saline and 2% CHX2 (chlorhexidine gluconate - -CHX, Henry Schein, USA). [Table 1]

This study included 8 groups.

Group I- calcium hydroxide mixed with Zamzam against *S aureus*.

Group II- calcium hydroxide mixed with Zamzam against *B subtilis*.

Group III- calcium hydroxide mixed with chlorhexidine against *S aureus*.

Group IV- calcium hydroxide mixed with chlorhexidine against *B subtilis*.

Group V- calcium hydroxide mixed with saline against *S aureus*.

Group VI- calcium hydroxide mixed with saline against *B subtilis*.

Group VII - Positive and negative culture for *S aureus*.

Group VIII -Positive and negative culture for *B subtilis*.

Standard strains of *Staphylococcus aureus* (*S.aureus*) and *Bacillus subtilis* (*B.subtilis*) was obtained from Department of Microbiology. Bacterial suspension with standard opacity equivalent to standard concentration of barium sulfate was prepared. Totally, 12 culture plates were considered. Six petri plates were prepared containing nutrient agar broth and inoculated with *S.aureus*. Dilutions of the *S aureus* 1:50,000 were made and 0.3 ml. dilution was used for plating over the nutrient agar broth. In one plate two holes 5*3 mm (5 mm in diameter and 3 mm in depth) were created. The first hole was filled with Vancomycin discs which acted as a positive control and the other hole was kept empty to act as a negative control. In the remaining 5 plates three holes of 5*3 mm were created. Freshly prepared creamy mixtures of Ca(OH)_2 and Zamzam, Ca(OH)_2 and Saline and Ca(OH)_2 and 2% CHX were inserted into the holes. Each mix was placed in one hole of every plate i.e. 5 holes for each material.

Six more petri plates were prepared containing nutrient broth and inoculated with *B.subtilis*. 1:50,000 were made and 0.3 ml. dilution was used for plating over the nutrient agar broth. In one plate two wells 5*3 mm were created. The first well was filled with Penicillin G discs which acted as a positive control and the other well was kept empty to act as a negative control. In the remaining 5 plates three wells of 5*3 mm were created. Calcium hydroxide – 6 scoop of powder was used and was mixed with the chemical agents until a smooth creamy consistency was achieved. Freshly prepared creamy mixtures of Ca(OH)_2 and Zamzam, Ca(OH)_2 and Saline and Ca(OH)_2 and 2% CHX were inserted into the wells. Each mix was placed in one well of every plate i.e. 5 wells for each material. The plates were incubated at 37⁰C for 24 hours and then the diameter of microbial zone of inhibition (ZOI)

was measured. ZOI was measured from the edge of the well filled with calcium hydroxide paste to the edge of area with zero growth with the help of a ruler in millimeters.

Statistical Analysis: The ANOVA was used to test for differences among the extracts. The Tukey test was carried out in parallel ($p=0.05$) to make pairwise comparisons of means of the treatments. The regression analysis together with Pearson correlation method were done with the extracts in response to the performed assays

3. RESULTS:

The results of ZOI in positive control group for both the groups was zero and in negative control group for *S. aureus* was 7 mm and *B. subtilis* was 6 mm. The mean value of microbial ZOI for *S. aureus* and *B. subtilis* for different medicaments are shown in Table II and III. $\text{Ca}(\text{OH})_2$ when mixed with Zamzam water showed statistical significant difference when compared to other medicaments against *Staphylococcus aureus* and *Bacillus subtilis*. $\text{Ca}(\text{OH})_2$ when mixed with Zamzam water showed a ZOI mean value of 2.70 against *S. aureus* and mean value of 2.69 against *S. aureus* which was greater than that of saline and chlorhexidine.

Using Tukey HSD for comparing between different medicaments against *S. aureus*, $\text{Ca}(\text{OH})_2$ mixed with Zamzam water showed statistical significant difference when compared with $\text{Ca}(\text{OH})_2$ mixed with Saline and CHX. There was no statistical significant difference when $\text{Ca}(\text{OH})_2$ mixed with Saline was compared to $\text{Ca}(\text{OH})_2$ mixed with CHX [Table IV]. When comparing between different medicaments against *B. subtilis* there was statistical significant difference between all the medicaments [Table V]

This study was intended to investigate the antimicrobial properties of calcium hydroxide when mixed with three vehicles namely Zamzam water, Normal saline and 2% Chlorhexidine. The results of this study show that the mean value of ZOI for $\text{Ca}(\text{OH})_2$ and Zamzam was statistically significant when tested against both the bacteria. The pH of 12.26, 11.90 and 12.10 was achieved for $\text{Ca}(\text{OH})_2$ and Zamzam, $\text{Ca}(\text{OH})_2$ and Normal saline and 2% Chlorhexidine and $\text{Ca}(\text{OH})_2$.

FIGURE1: THE BUFFERING SOLUTIONS USED IN THE STUDY



FIGURE 2: ARMAMENTARIUM USED IN THE STUDY



FIGURE 3: THE THREE SOLUTIONS USED IN THE STUDY



FIGURE 4: PLATES SHOWING ZONES OF INHIBITION

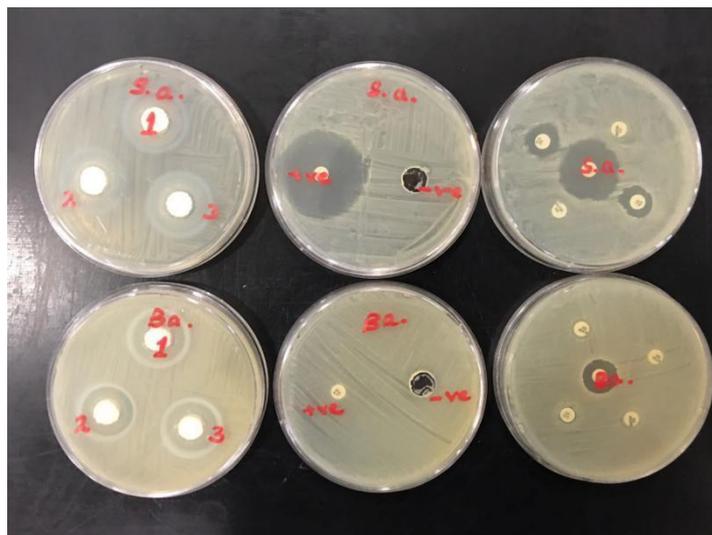


TABLE 1
AVERAGE PH VALUE AS MEASURED BY PH METER.

Sl.no	Content	pH
1	Ca (OH) ₂ + Zamzam	12.26
2	Ca (OH) ₂ + Saline	11.90
3	Ca (OH) ₂ + CHX	12.10

TABLE II
COMPARISON OF ZONE OF INHIBITION OF MEDICAMENTS AGAINST S. AUREUS

	Mean	SD	F value	P value
Ca (OH) ₂ + Zamzam	2.70	0.25	5.066	0.025*
Ca (OH) ₂ + Saline	2.28	0.08		
Ca (OH) ₂ + CHX	2.54	0.25		

p < 0.05 * - Significant

TABLE III
COMPARISON OF ZONE OF INHIBITION OF MEDICAMENTS AGAINST BACILLUS SUBTILIS

	Mean	SD	F value	P value
Ca (OH) ₂ + Zamzam	2.69	0.047	94.957	< 0.001**
Ca (OH) ₂ + Saline	2.30	0.040		
Ca (OH) ₂ + CHX	2.58	0.049		

p < 0.05 ** - Highly Significant

TABLE IV
DIFFERENT MEDICAMENT COMPARISON AGAINST S.AUREUS

Multiple Comparisons Tukey HSD						
Group	Compared with	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Ca (OH) ₂ + Zamzam	Ca (OH) ₂ + Saline	0.42200*	0.13386	0.021	0.0649	0.7791
	Ca (OH) ₂ + CHX	0.16000	0.13386	0.478	-0.1971	0.5171
Ca (OH) ₂ + Saline	Ca (OH) ₂ + CHX	-0.26200	0.13386	0.165	-0.6191	0.0951

*. The mean difference is significant at the 0.05 level.

TABLE V
DIFFERENT MEDICAMENT COMPARISON AGAINST B.SUBTILIS

Multiple Comparisons Tukey HSD						
Group	Compared with	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Ca (OH) ₂ + Zamzam	Ca (OH) ₂ + Saline	0.39200*	0.02930	.000	0.3138	0.4702
	Ca (OH) ₂ + CHX	0.11200*	0.02930	.006	0.0338	0.1902
Ca (OH) ₂ + Saline	Ca (OH) ₂ + CHX	-0.28000*	0.02930	.000	-0.3582	0.2018

*. The mean difference is significant at the 0.05 level.

4. DISCUSSION:

Calcium hydroxide is generally the most commonly used intra canal medicament in Endodontics.¹⁵ Its extensive use is attributed to properties such as antimicrobial activity, tissue dissolving ability, inhibition of tooth resorption and induction of repair by hard tissue formation.¹⁶ Traditionally, it is available in the form of powder. Limitations in the application of powder in the canal in addition to the lack of an agent to activate the powder by disintegrating the ions has led to the use of different vehicles to mix it into a creamy consistency.¹⁷

Generally, clinicians prefer to use a freshly mixed preparation. However numerous readymade paste are also available which are a mixture of Ca(OH)₂ powder premixed with aqueous liquid like isotonic saline solution or viscous solutions like Propylene glycol to which barium sulphate is added for radio opacity.¹⁸

Zamzam water is found to be continuously flowing since 2000BC in the valley of Makkah; a city located in the western part of Saudi Arabia. Zamzam water is found to have higher concentrations of both cations and anions than normal water.¹⁹ Studies revealed that Zamzam water has anticancer properties, antioxidant properties, anti-inflammatory characteristics, and stimulates aquaporins, endometrial growth, and formation of immunoglobulin.^{19,20,21}

In the present study, three different aqueous vehicles namely Zamzam water, Normal Saline and 2% Chlorhexidine were used to prepare the paste and tested for antimicrobial activity against *S. aureus* and *B. subtilis*. The use of Normal saline and Chlorhexidine to mix the calcium hydroxide powder has been previously reported.²² Chlorhexidine kills bacteria by disrupting the membrane integrity and inducing the precipitation of cytoplasm. It is used in endodontics either as a medicament.^{23,24}

Combinations of CHX and Ca(OH)₂ have been shown to have antimicrobial activity against obligate anaerobes and this combination has also been shown to enhance the antibacterial effect of either medicament on certain species.^{25,26} Although sufficient scientific

published data is lacking for the use of Zamzam water the intention to use it in this in vitro study was to take advantage of its higher alkalinity and natural availability. The need for a multidisciplinary approach in addition to the difficulty in obtaining zamzam water were the limitations of this study.

5. CONCLUSION:

Under the limitations of this study, Calcium hydroxide powder mixed with Zamzam water exhibited the highest pH of 12.26 and showed statistical significant difference when compared to other medicaments. This could be attributed to the preservation of the alkaline pH and enhancement in disintegration of $\text{Ca}(\text{OH})_2$ into Ca^+ and OH^- ions. Further studies on the properties of Zamzam water are needed before it can be considered to be used clinically.

Conflict of interest: None declared.

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