The effects of different concentrations of Alum solutions on Mutans streptococci (in vitro study)

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ABSTRACT

Background: Alum has been used as a treatment medication in cases of oral and gingival ulcers, and also as antiseptic mouthwash. This study aimed to examine the effects of different concentrations of Alum on inhibition zone, viability counts and adherence ability of Mutans streptococci compared with deionized water and chlorhexidine gluconate in vitro.

Materials and methods: The study dealt with an in vitro study to establish a concentration of Alum mouthrinse that would have the minimum inhibitory concentration and minimum bacteriocidal concentration. The second part evaluated the anti-adherence ability of the experimental agents.

Results: This study found that the antibacterial effect of Alum increases with its concentration from 50 to 10000 PPM but still weaker than 0.1% chlorhexidine gluconate. Only concentrations of 5000 and 10000 PPM showed negative adherence of Mutans streptococci to the tooth surface.

Conclusions: This study found that the antibacterial effect of Alum increases with its concentration from 50 to 10000 PPM but still weaker than 0.1% chlorhexidine gluconate. Only concentrations of 5000 and 10000 PPM showed negative adherence of Mutans streptococci to the tooth surface.

Key words: Alum, chlorhexidine, Mutans streptococci. (J Bagh Coll Dentistry 2013; 25(Special Issue 1):146-151).

INTRODUCTION

Potassium alum, $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$, crystallizes in regular octahedra and is very soluble in water. The solution reddens litmus and is an astringent.

Alum is used in cases of gingivitis [(1,2)], in management of mucositis [(3)] and oral ulcers when it was noticed that the treatment of recurrent aphthus ulcerations with alum in concentrations of 1000, 2000, and 4000 PPM was significant in enhancing the healing procedure of the ulcers [(4)].

Aluminum salts have demonstrated anticaries activity in a number of laboratory and animal studies [(1,5)] and was proved to be significant as alum mouthrinse in prolonged daily use of the mouthrinse for decreasing the level of Mutans streptococci in saliva (three and six weeks) [(6)], and also the effect of an alum mouthrinse on dental caries formation both by itself and in combination with an ADA-approved sodium fluoride dentifrice [(7)]. It was concluded that daily supervised use of an alum mouthrinse inhibited caries development in decay-prone children at least as effectively as a fluride dentifrice [(1,5)].

Olmez et al [(8)] assessed the effect of daily supervised rinsing with a specially formulated, alum-containing mouthrinse on plaque and salivary levels of S. mutans, S. mitis and S. salivarius in caries susceptible children (12-14 years old) and to monitor the effect on the oral tissues and acceptability to subjects.

He concluded a daily use of an alum-containing mouthrinse was safe and produced significant reduction effect on plaque and salivary levels of oral streptococcus and can be used in children for the preventive dentistry.

Some authors introduced alum in dentifrices and investigated a comparative three-year caries protection from an aluminum-containing and a fluoride-containing toothpaste and concluded that after 3 years, the mean caries increment was significantly higher in the group using the aluminum-containing toothpaste measured both clinically and radiographically [(9)].

The effects of solutions containing various aluminum concentrations (Alum) on formation of smooth surface and sulcal caries in cariogenically challenged rats infected with Streptococcus sobrinus was investigated using $AIK(SO_4)_2$ solutions containing 100, 1000, 2000, or 4000 PPM. Results showed that topically applied aluminum reduced the formation and progression of both smooth surface and sulcal caries and showed evidence of a dose response in a rat model infected with S. sobrinus [(10-12)].

It was found that aluminum reduced enamel dissolution in a pH 4 acetate buffer most effectively when used in the pH range of 3 to 4, at concentrations above 0.005 mol/L, and for treatment times of more than four minutes [(13)]. However, significant activity was observed with concentrations as low as 0.0005 mol/L aluminum and treatment times as short as 15 seconds. The effects of aluminum solution pH and concentration on enamel dissolution were related to the hydrolysis of aluminum above pH 4 and to

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the accelerated dissolution of apatite below pH 3 [14-16].

A sodium-potassium aluminum silicate cleaning and polishing agent was compared with conventional prophylaxis abrasives and was found to be highly compatible with fluoride. When formulated into a fluoride prophylaxis paste, especially with stannous fluoride, a larger reduction in enamel solubility and greater fluoride uptake were obtained with representative commercial prophylaxis pastes [17].

The study aimed to evaluate the effect of different concentrations of Alum (50 - 10000 PPM) on the inhibition zone, viability counts and adherence ability of Mutans streptococci in vitro.

MATERIALS AND METHODS

Media preparation:

a) Preparation of Phosphate buffer saline (PBS):

A phosphate buffer saline of pH 7.0-7.2 was prepared by dissolving a tablet of phosphate buffer (LDH) in 100 ml of deionized water and spread in ten test tubes (9.9ml) and sealed with a piece of cotton and tin foil and then sterilized in the autoclave. Then it was left to cool down and finally stored in the fridge until the time of its use.

b) Mitis salivarius agar (MSA):

It is a selective medium was used for the cultivation of total streptococci. The medium was prepared and sterilized according to the manufacturer's directions (HiMedia, India). Its pH was adjusted to 7.2 by adding NaOH or HCl and then sterilized by autoclave. After the media cooled to 45-50°C, 20 ml was poured in each plate and left to cool down to room temperature. The plates were collected and turned upside-down and sealed, and finally stored in the fridge until time of use.

c) Mitis salivarius Bacitracin Agar (MSBA):

This medium is selective for the cultivation of Mutans streptococci. The selective property is determined by the addition of the selective agents, sucrose and bacitracin (AppliChem, Germany), at the optimal levels determined to the MSA composition to be effective in inhibiting bacteria other than Mutans streptococci since the relative resistance of Mutans streptococci to high concentration of both sucrose and bacitracin had been reported [18].

Bacitracin stock solution was prepared by dissolving 0.364 gm powder in 100 ml of deionized water to give 200 IU/litre concentration (1 unit bacitracin= 0.0182 mg). Then it was sterilized by 0.45µm millipore filtering [19] and kept in the fridge. A new fresh solution was prepared every 2-3 weeks.

MSA was prepared as described above. Sucrose was added to obtain a concentration of 150 gm per one liter of the medium [18]. The media pH was adjusted to 7.2 by adding NaOH or HCl and then sterilized by autoclave.

After the media cooled to 45°C, bacitracin stock solution was added under aseptic conditions. Then the media was poured into plates and left it to cool for 24 hours at room temperature and store it in the fridge until use.

d) Mueller Hinton Agar (MHA):

This was prepared according to manufacturer instruction, which involved the suspension of 35 gm in one liter of distilled water. After being completely dissolved with boiling, it was autoclaved at 121ºC for 15 minutes. Finally, kept tightly closed in a cool dry place.

e) Tryptose Phosphate Broth (TPB):

It is composed from the following ingredients:

- Tryptose 20 gm (Oxide)
- Dextrose 2.0 gm (Difco)
- Sodium chloride 2.0 gm (BDH)
- Di-sodium hydrogen phosphate 2.5 gm

These were dissolved in one liter of distilled water (pH 7.2) distributed in screw-capped bottles (10 ml each) sterilized by autoclave, then stored in the fridge till use.

Isolation and purification of Mutans streptococci

Collection of the stimulated salivary samples:

Stimulated saliva was collected from a 26 year old healthy male under the conditions following the criteria described by Tenovuo and Lagerlof [20]; by asking the volunteer not to take antibiotic for the last two week before the collection of the samples and not to eat or to drink (except water) for an hour before the collection of the samples. The volunteer did not have any illnesses or diseases.

The volunteer was seated in a relaxed position on an ordinary chair. The saliva collection includes the following steps:

- chewing a piece of Arabic gum (0.35–0.4 gm) for 1 minute and then expectorate to remove all saliva.
- chewing a small piece of the gum (0.35-0.4 gm) for 10 minutes and collecting the saliva.
- After collection and disappearance of salivary foam, 0.1 ml of saliva is transferred to 0.9ml of sterile phosphate buffer saline (pH 7.0) for microbiological analyses.
- within less than 15 minutes, the pH of the saliva was measured by the digital pH meter and the volume and the rate of secretion were measured.

Isolation of Mutans streptococci:

Ten-fold dilutions of saliva were performed by transferring 0.1 ml of from each suspension to 0.9 ml of sterile phosphate buffer saline (pH 7.0). Each suspension was agitated well using vortex mixer for two minutes. From dilutions 10-3 and 10-5 of salivary samples, 0.1 ml was taken and spread in duplicate on the selective media mitis salivarius bacitracin agar (MSB). Plates were incubated anaerobically using a gas pack (BD BBLTM, USA) or 48 hours at 37°C, then aerobically for 24 hours.

The examination and diagnosis of colonies incubated was done according to the following criteria:

1- **Morphological characteristics:** Mutans streptococci were examined under dissecting microscope (magnification x15). Diagnosis was according to their morphological characteristics on MSB agar plates (Fig. 1), according to the criteria described by Edwardsson [21].

2- **Gram's stain (**Crescent diagnostics, KSA**):** Colonies were picked up from the MSB agar plates under the microscope and subjected to Gram's stain method [22]. Mutans streptococci are gram positive, appear small ovoid or spherical in shape in small or medium chains [23] (Fig. 2).

3- **Biochemical tests:** A colony of Mutans streptococci was elevated from each MSB agar plate under a microscope inoculated in 10 ml of sterile TPB. When two types of colonies were observed in one plate, each colony was picked up and inoculated separately. Broths were incubated aerobically at 37°C for 18 hours, there after the following tests were conducted (Fig. 3).

Carbohydrate fermentation test:

The ability of Mutans streptococci to ferment sugars was tested by the addition of a selected carbohydrate (sorbitol and mannitol) in a concentration of 1% to BHI broth (HiMedia, India) in a presence of an indicator (Bromocresol purple, BDH).

Carbohydrate utilization broth was prepared by dissoliving 10 gm carbohydrate in one liter Brain heart Infusion broth and adding 1 ml bromocresol purple (1.6% in 95% ethanol) (Fig. 4a & b).

The broth was distributed into test tubes (3ml each) and autoclaved. Each incubated aerobically at 37ºC for four days. Change in the color from red to yellow indicated a positive reaction, in comparison to control positive (broth and bacteria only) and control negative (broth and sugar with out bacteria) [24].

Maintenance of bacterial isolates:

Isolates of Mutans streptococci were checked for purity by re-inoculation on MSB agar plates incubated anaerobically for 48 hours at 37ºC followed by another incubation aerobically for 24 hours. A selected colony was transferred to 10 ml of sterile TPB (pH 7.0), incubated for 24 hours aerobically at 37ºC. Broth was stored in fridge until use. This procedure was repeated monthly.

Part One

This involved bacteriological analyses of an in vitro study to establish a concentration of Alum (BDH- Great Britain) mouthrinse that has the MIC and MBC.

Bacterial inoculum used in all sets of in vitro experiments were prepared by addition of pure isolates of Mutans streptococci in concentration of 1% to 10 ml of sterile BHI broth (pH 7.0), incubated aerobically for 18 hours [25].

Determination of MIC and MBC

A serial dilution method was applied to identify the lowest concentration of Alum that inhibited the growth of Mutans streptococci (26). The experiment was carried out on eleven isolates of Mutans streptococci with different concentrations of Alum (50, 100, 250, 500, 1000, 2000, 5000, 10000 PPM) compared with chlorhexidine (0.1% and 0.2%) and deionized water as control.

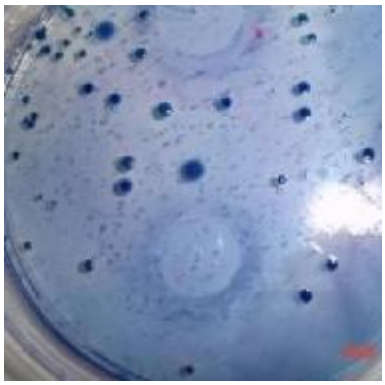

Figure 1: Colonies of Mutans streptococci.

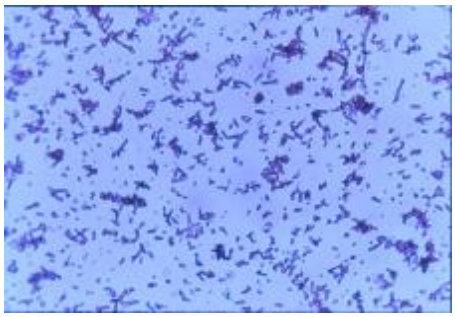

Figure 2: Gram's stain positive view of a slide.

Figure 3: TPB (on the right control without agent and on the left with Alum 1000 PPM).

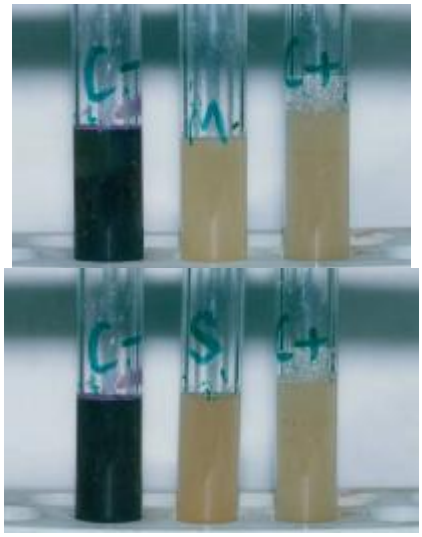

Figure 4: Carbohydrate fermentation test using (a) mannitol and (b) sorbitol.

Procedure:

1- Brain Heart Infusion broth was prepared and distributed in test tubes by 8.9 ml each (pH 7.0), sterilized by autoclave.
2- One ml of the tested agent was added to both control and study groups.
3- 0.1 ml inoculum was added to each broth of the study group giving the total volume for each test tube 10 ml of BHI broth plus tested agent plus bacterial inoculum.
4- Both study and control groups were incubated aerobically at 37ºC for 24 hours.
5- The least concentration that lacks a visual turbidity matching the negative control (deionized water) was considered as the MIC.
6- The MBC was assessed, by inoculating 0.1 ml of the concentration assigned as MICs on MSB agar, then incubated anaerobically for 24 hours at 37ºC. The least concentration that inhibited growth of Mutans streptococci on MSB agar was considered as MBC.

Sensitivities of Mutans streptococci to different concentration of Alum mouthrinses:

Agar well technique was applied to study the antibacterial effect of different concentrations of Alum (50, 100, 250, 500, 1000, 2000, 5000, 10000 PPM) compared with chlorhexidine (0.1% and 0.2%) and deionized water as control on Mueller Hinton Agar media. The experiment was conducted on 11 isolates of Mutans streptococci.

Procedure:

1- A volume of 25 ml of MHA (pH 7.0) was poured into sterile Petri dish, left at room temperature for 24 hours.
2- To each plate 0.1 ml of Mutans streptococci inoculum was spread, left at room temperature for 20 minutes.
3- Four wells of equal size and depth were prepared in each agar plate. Each well was filled with 0.1 ml of the test agent (Alum, chlorhexidine and deionized water).
4- Plates were left at room temperature for one hour then incubated anaerobically for 24 hours at 37ºC. Zones of inhibition were measured across the diameter of each well. No zone indicated a complete resistance of bacteria to the tested agent (Fig. 5).

Effects on Viability Counts of Mutans streptococci:

The viability counts of Mutans streptococci inoculated from broth media, to which different concentrations of Alum rinses were added, have been estimated in comparison to the control. The procedure was carried out on eleven isolates, eight from different concentration of Alum mouthrinses (50, 100, 250, 500, 1000, 2000, 5000 and 10000 PPM), and two with chlorhexidine gluconate mouthrinse 0.1% and 0.2%, and deionized water as control. The followed method was in accordance to that described by Baron et al. [26].

Figure 5: Well agar technique.

Procedure:

1- Brain Heart Infusion broths were prepared (pH 7.0) and distributed in screw- capped bottles by 8.9 ml to each bottle.
2- One ml of the test agent was added to each bottle.
3- Then 0.1 ml of bacterial inoculum was added to both study and control bottles. From the control tube, 0.1 ml was transferred to 0.9 ml of sterile PBS (pH 7.0) and a ten-fold dilution

was performed. From dilutions 10^{-3} and 10^{-5}, 0.1 ml was taken and spread on duplicates of MSB agar plates which were incubated anaerobically at 37ºC for 48 hours. This value was considered as the initial count of bacteria.

4- Study and control cultures were incubated aerobically at 37ºC for 24 hours. From the each tube, 0.1 ml was transferred to 0.9 ml of sterile PBS and a ten-fold dilution was performed. From dilutions 10^{-3} and 10^{-5}, 0.1 ml was taken and spread on duplicates of MSB agar plates which were incubated anaerobically at 37ºC for 24 hours. The colony-forming units per milliliter of innoculum was counted (CFU/ml) for all the plates.

RESULTS

Sensitivity tests of MS:

The results of the in vitro bacteriological tests revelaed that the MIC of Alum was 500 PPM whereas the MBC was 1000 PPM. The sensitivity of MS to the different concentrations of Alum and chlorhexidine in comparison to control were performed by two tests.

Inhibition zone:

The inhibition zone (mm) for the control and test agents are presented in table 1. The inhibition zone for Alum increased with the increase of concentration of the solution starting from 0mm for 50 PPM to 5mm for 10000 PPM. While, chlorhexidine showed a considerably larger inhibition zone of 8mm for 0.1% concentration and 10mm for 0.2%.

Viability count of MS:

The colony- forming units per millilitre of the inoculum for the control and test agents are presented in table 2 and figure 6. Low Alum concentrations were close to the count obtained for deionized water, while stronger concentrations approximated the readings for chlorhexidine.

Adherence test

The results of the adherence test for the control and test agents are displayed in table 3. Low Alum concentrations (50, 100, 250, 500, 1000 and 2000 PPM) resembled the negative control (deionized water) in showing positive adherence of the MS to the tooth surface, while a stronger concentration of 5000 PPM resembled the action of 0.1% chlorhexidine in showing negative adherence at 2 minutes only. Whereas, the strongest Alum tested concentration of 10000 PPM resembled the action of 0.2% chlorhexidine in showing negative adherence at 1 and 2 minutes.

DISCUSSION

The inhibition zone increased with the increase of concentration of Alum solution from 50 to 10000 PPM; while, chlorhexidine showed a considerably larger inhibition zone for 0.1% and 0.2% concentration.

Viability count of MS also decreased with higher alum concentration, with stronger concentrations approximating the readings for chlorhexidine, which agrees with Mourughan and Suryakanth [6].

This indicates that alum has an antibacterial action somewhat weaker than that of chlorhexidine. This action increases with increased alum concentration which agrees with the findings of Putt and Kleber [12] who found that the protective effect of the 100 PPM Alum solution was less than solutions containing 1000 PPM Alum or more.

Therefore it was decided to use the alum concentration of 1000 PPM for the in vivo study as a mouth rinse as there was a scant difference between 1000 PPM and higher concentration regarding the inhibition zone and the MS viability count in agreement with Putt and Kleber [12].

Low Alum concentrations showed positive adherence of the MS to the tooth surface, while stronger concentrations of 5000 and 10000 PPM resemble the action of 0.1% and 0.2% chlorhexidine in showing negative adherence, this was with agreement to the findings of Bihani and Damle [2] and Olmez et al [8]. This indicated that alum has a weak effect on the adherence of MS to the tooth surface.

Table 1: Results of the inhibition zone (mm) for different test agents.

Agent	CHX		DW	Alum (PPM)							
Concent-ration	0.1%	0.2%		50	100	250	500	1000	2000	5000	10000
Inhibition zone (mm)	8	10	0	0	0	0	1	3	3	4	5

Table 2: The viability count of MS in different media.

Agent	CHX		DW	Alum (PPM)							
Concent-ration	0.1%	0.2%		50	100	250	500	1000	2000	5000	10000
CFU ml x10^5	5	3	36	31	25	22	18	10	9	7	4

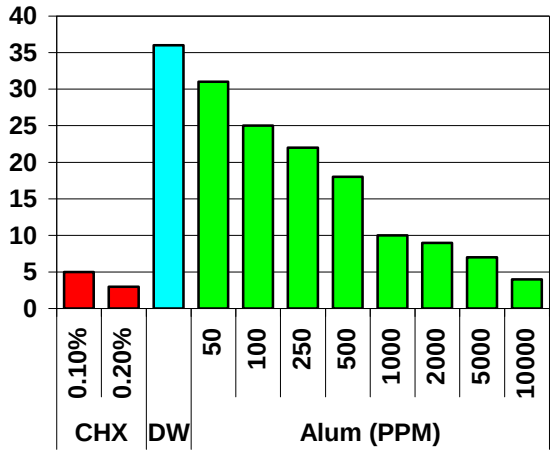

Figure 6: The viability count (colony-forming units per millilitre) of MS ($X10^5$/ml) in different media.

Table 3: Results of the adherence test for different test agents by time.

Agent	CHX		DW	Alum (PPM)							
Concent-ration	0.1%	0.2%		50	100	250	500	1000	2000	5000	10000
1 min	+	-	+	+	+	+	+	+	+	+	-
2 min	-	-	+	+	+	+	+	+	+	-	-

+ means presence of adherence
- means absence of adherence

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