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Antibacterial Effects of Chitosan, Formocresol and CMCP as Pulpectomy Medicament on *Enterococcus faecalis*, *Staphylococcus aureus* and *Streptococcus mutans*

Zhila Imani [a], Zahra Imani [b*], Leila Basir [c], Mohsen Shayeste [c], Effat Abbasi Montazeri [d], Vahid Rakhshan [e]

a Private Practice, Tehran, Iran; b Department of Pediatric Dentistry, Birjand University of Medical Sciences, Birjand, Iran; c Department of Pediatric Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; d Department of Microbiology, Medical School, Jundishapur University of Medical Sciences, Ahvaz, Iran; e Private Practice, Tehran, Iran

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**Corresponding author*: Zahra Imani, Department of Pediatric Dentistry, Birjand University of Medical Sciences, Birjand, Iran. Postal code: 3146688934

Tel: +98-912 465 4973
E-mail: z.imani1365@yahoo.com

ABSTRACT

Introduction: During pulpectomy of primary teeth, cytotoxic medicaments such as formocresol or camphor mono-chlorophenol (CMCP) are used as medicaments. For the first time it is theorized that chitosan can substitute these traditional materials used in pulpectomy of infectious primary teeth. **Methods and Materials**: This preliminary *in vitro* study consisted of two separate phases (*n*=75), each of which assessed the antibacterial effects of chitosan *versus* formocresol and CMCP and positive/negative controls (*n*=15) on three bacteria types [*Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mutans*, (*n*=5 per subgroup)]. Phases 1 and 2 concerned respectively with 1- and 7-day effects of these materials. Bacteria were cultured and injected into sterilized canals and colonies were counted. Medicaments were applied and colonies were re-counted after 1 day of treatment (phase 1). Specimens were re-sterilized and re-randomized, and used for phase 2, in which the same procedures were performed for a 7-day period. Effects of agents on bacteria were analyzed statistically (Kruskal-Wallis α=0.05 and Mann-Whitney α=0.017). **Results**: Treatments reduced bacterial count either after 1 or 7 days (P=0.000). Their effects on different bacteria types were not significant either after 1 or 7 days (P>0.48). Antibacterial efficacies of treatments (indicated by colony reduction) were significantly different, after 7 days (P=0.045). Antibacterial efficacy of chitosan was similar to that of formocresol or CMCP, in both phases [either after 1 or 7 days of treatment (P>0.017). Formocresol and CMCP had similar efficacies in either phase (P>0.017). **Conclusions**: This preliminary study confirmed the appropriate antibacterial efficacy of chitosan as a medicament in pulpectomy of infectious primary teeth.

Keywords: Antibacterial Agents; Camphor Mono-Chlorophenol; Chitosan; CMCP; *Enterococcus faecalis*; Formocresol; Medicament; Pulpectomy; *Staphylococcus aureus*; *Streptococcus mutans*

Introduction

The purpose of root canal treatments (including pulpectomy in primary dentition) is to remove bacteria and their products as well as contaminated tissues from the canal through mechanical and chemical cleansing and debridement [1, 2]. Mechanical preparation accounts for about only half of the reduction in the number of bacteria. Because of anatomical variations and complex morphology of canals, isthmuses, apical accessory canals and incomplete root canal connections, these variations and anatomical limitations are more pronounced in primary teeth, especially in primary molars [3, 4]. Therefore, it is recommended to use chemical methods and antimicrobial agents that can access such anatomical variations and reduce microorganisms more effectively [5].

Due to the possibility of microorganisms' survival after mechanical and chemical canal preparation and cleaning, the use of medicaments or antimicrobial dressings is recommended between

sessions in order to reduce bacteria as well as pain and swelling after treatment [6]. Such agents should possess desirable antimicrobial effects [2, 7]. Although various agents have been proposed for this purpose, none of them have shown considerable superiority over the others [8]. Formocresol and camphor mono-chlorophenol (CMCP) have high antimicrobial activity [9-11]. However, the use of formocresol which is the most common used material and also the golden standard for medicament is controversial due to its potential cytotoxicity [12-16]. It has been associated with carcinogenicity, immunological changes, cytotoxicity, teratogenicity, mutagenic effects and causing enamel defects in permanent teeth and systemic changes in internal organs such as the kidneys and the liver [11-13, 15-19]. CMCP is also a phenolic derivative that can stimulate periapical tissues at higher concentrations. Therefore, an alternative material with high efficacies would be of utmost clinical interest.

Local and systemic distribution of medicaments [20] calls for seeking safer and yet adequately effective agents. A new material recently proposed for root canal therapy is chitosan, which is an appropriate antimicrobial agent, with anti-inflammatory properties, proper biocompatibility, and does not cause irritation [21-23]. Chitosan is a cationic polysaccharide derived from chitin (a major component of crustaceans) which has attracted great attention due to its excellent biocompatibility and non-toxicity [23-30]. Its positive charge facilitates its attachment to surfaces with negative charge and exert its anti-bacterial and anti-fungal properties, through binding to the negatively charged bacterial and increasing its permeability [31]. Besides being effective against a wide range of microorganisms, chitosan also possesses anti-inflammatory effects and facilitates tissue regeneration, and also can enhance the anti-erosive/anti-abrasive effects of the Sn^{2+} [32-37]. Chitosan has been used in different forms including gums, dentifrices, toothpastes, *etc*, and has shown to be effective in reducing enamel decalcification and caries as a result of reduced bacterial activity [24, 38-40] and inhibiting the release of mineral elements [41]. In terms of intracanal agents, few studies have evaluated effects of chitosan on microorganisms, and have found promising results compared to traditional canal irrigating medicaments such as sodium hypochlorite [42-45].

However, such concentrations were not optimized for primary root canal therapy, and no studies have assessed the efficacy of chitosan as a "medicament" for root canal treatment of infectious primary teeth (*i.e.* pulpectomy). Therefore, this two-phase study aimed to assess comparatively the efficacy of a new concentration of chitosan optimized for pulpectomy dressing in comparison with the most effective materials in the field (Formocresol and CMCP).

Materials and Methods

This *in vitro* experimental study was performed in two phases (each with its independent data) on 150 observations from 75 primary canines (65 maxillary and 10 mandibular) extracted for clinical purposes. The first phase concerned with the bacterial growth over a 24 h period, while the second phase was about bacterial growth over a one-week period. Each of these phases had its own baseline colony counts, to be compared with the colony counts after either 1 or 7 days. The inclusion criteria were intact roots or health of more than 3/4 of the root. The exclusion criteria were fractures or external resorptions on the root.

The teeth were first debrided and stored in 0.9% normal saline + 0.1% sodium hypochlorite for 6 h, and then washed and stored in 0.9% normal saline until the examination day. The teeth were cut using paper discs from the cementoenamel junction (CEJ) [46] in order to exclude the coronal length and bur preparation variables as well as for a better access to the root canals for medicament placement. A K-file #15 (Mani, Tochigi, Japan) was used to ensure the patency of canals. The working length was then estimated by subtracting 1 mm from the length of the K-file in a tip-to-tip position. The canals were cleaned and prepared to the #40 K-file (Mani, Tochigi, Japan) while being irrigated between filing sessions, with 2 mL of normal saline.

Producing chitosan

An experimental concentration of chitosan solution was determined as 1.5% through discussion of experts in pediatric dentistry. It was produced by blending chitosan (ChitoClear, Primex, Siglufjordur, Iceland) with 1% acetic acid (Merck, Darmstadt, Germany).

Phase 1: 24h effects

Grouping

The teeth were randomly divided into 15 subgroups of 5 each, according to the following descriptions:

Group 1 (*n*=25): *Enterococcus faecalis* (ATCC 29212) was applied to this group, which would consist of 5 subgroups (1A to 1E). Group 2 (*n*=25): *Staphylococcus aureus* (ATCC 29213) would be applied to this group, which consisted of 5 subgroups (2A to 2E). Group 3 (*n*=25): *Streptococcus mutans* (PTCC 1683) would be applied to this group (3A to 3E subgroups).

Group A (*n*=15): comprised roots that would be filled with bacteria (three subgroups of 5 each corresponding to the groups 1, 2, and 3 [*i.e.*, 1A, 2A, 3A]) but were not subjected to antimicrobial treatments. Group B (negative control) (*n*=15): consisted of sterilized roots which were not subjected to bacterial addition or antimicrobial application. This group was used to assess the efficacy of sterilization and aseptic protocols, during the study (102). Group C (chitosan, *n*=15): this group would include roots filled with three different bacteria (*n*=3) and later filled with chitosan, as a potential antimicrobial agent. Groups D (formocresol, PD, Switzerland),

(*n*=15): the same as group C but with formocresol for the antimicrobial agent. Group E (CMCP, PD, Switzerland), (*n*=15): similar to groups C and D, but with CMCP as the antibacterial agent.

Mounting the roots in containers

Before being mounted in acrylic resin, the apices were sealed using light-cured glass ionomer (Fuji II LC, GC Corporation, Tokyo, Japan), to prevent leakage of acrylic resin into the canal. The roots of each subgroup (*n*=5) were mounted upright by their apices on a glass plate filled with 5 mm of transparent acrylic resin (Meliodent, UK). Therefore, there were 15 plates for the 15 subgroups.

Sterilization the roots before the examination

The plates were autoclaved for 15 min at 121°C and a 15 Pascal pressure.

Microbial suspension

Standard strains of bacteria used include *Streptococcus mutans* (PTCC 1683), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), were purchased from the Center for Collection of Fungi and Bacteria as a collection of lyophilized vials [47]. To prepare the bacterial suspension and induce the viability of the bacteria, 2 cc of sterile liquid medium (Brain Hearth Infusion (BHI) Broth) (Merck, Darmstadt, Germany) was added into the lyophilized vials and mixed completely, so that the powder was completely solved and a homogenous suspension produced. A sterilized loop was used to obtain a drop of the above suspension and culture it on a blood agar medium (Merck, Darmstadt, Germany). Finally, the plates were stored at 37°C for 48 h. *S. mutans* plates were stored at 37°C in 5-10% CO_2. The bacteria were cultured in BHI Broth to achieve a sufficient volume of 0.5-McFarland (1.5×10^8 CFU/mL) concentration.

Injection of bacterial suspension into the root canals

Except for subgroups B (negative control), each root in other subgroups was injected with 10 µL of bacterial suspension within BHI Broth. In the negative control group, BHI Broth alone was injected.

Incubation

Plates were incubated for 72 h at 37°C. In order to prevent root dehydration, 10 cc distilled water was added to each plate. As bacterial nutrition, 10 µL of BHI-Broth medium was added daily to each of the roots.

Baseline colony count

After removing the specimens from the incubator, a #25 K-file was drawn to the canal wall to accumulate debris. Then, 20 microliters of BHI-Broth medium were placed the roots using specific samplers. After ensuring the release of bacteria, 10 µL of canal content was extracted using the sampler and transferred to 990 µL of BHI-Broth medium, in order to produce an initial concentration of 1:100. Owing to the high number of colonies, the content was again diluted 1:10000. Using a standard loop, 0.01 mL was collected from the suspension and transferred to the solid BHI agar medium. Plates containing these media (except those containing *S. mutans*) were then incubated at 37°C for 24-48 h. *S. mutans* plates were cultured on blood agar, at 37°C and 5-10% CO_2 for 24 to 48 h.

Afterwards, the colonies were counted with the naked eye, and the number of viable bacteria was estimated according to the following formula: colony-forming unit (CFU)/mL=colony count × suspension dilution (10000) × loop coefficient (100).

Application of antimicrobial agents

In the next step, a cotton wool was impregnated with 20 µL of antimicrobial agents including formocresol (PD, Swiss), CMCP (PD, Swiss), and chitosan 1.5% (Chitoclear, Island). They were placed in the canal orifices. Then the canal was sealed using and the orifice of the canal was sealed with a dressing (Zonalin, Kemdent, UK).

Incubation

The roots were incubated (as mentioned earlier) for 24 h.

Colony count after 1 day of incubation

After removing the dressing with a dental excavator, the colonies were counted as explained earlier, with the only difference that the bacteria-incorporated BHI Broth was diluted to 1:100 only. Afterwards, a standard loop was used to pick 0.01 µL of the suspension and culture it on BHI agar and blood agar for 24 to 48 h at 37°C. The colonies were counted as CFU/mL =colony count × suspension dilution (100) × loop coefficient (100).

Phase 2: one-week effects

The 7-day effects of materials were not tested in the same sample, because the assessment of bacterial count in the first day could confound the results pertaining to the 7-day examination. Therefore, we re-performed the whole study to estimate the 7-day effect of the materials. For this purpose, all of the plates of teeth were randomized and sterilized again and used for procedures which were identical to the phase 1, with the only difference that there were 7 days of incubation after the placement of antibacterial agents (instead of 1 day). All the other procedures were identical to the phase 1. During the period of treatment (either the 1-day treatment in phase 1 or the 7-day treatment in phase 2), the canals were not injected with culture medium, as they were sealed for treatment purposes. The two phases did not share the same baseline colony counts, as they were two different studies. The negative control specimens in this phase were as well all clear of any bacteria.

Calculation the percentage of reduction in colony count (%RCC)

For each of the two phases, the colony count after the placement of antimicrobial agent (either after 1 day in phase 1 or after 7 days in phase 2) was subtracted from the baseline colony count in that phase, in order to calculate the delta-count. A positive delta-count value would indicate a reduction in bacterial numbers, while a negative delta-count value meant bacterial growth. The delta-count was divided by the baseline count to calculate the %RCC.

Statistical analysis

The negative control confirmed the complete aseptic conditions (as no bacterial growth in any negative controls), in both phases. Since the two phases did not share the same baseline and 1-day colony counts and since the roots had been randomized before phase 2, we did not merge the data obtained from both phases. Instead we analyzed each phase separately, as an independent study. In each phase, means and medians were calculated for each material, before and after the treatment. Comparisons were performed using the Kruskal-Wallis, Mann-Whintey U and Wilcoxon tests with SPSS 25 (IBM, Armonk, NY, USA) within each phase and between the short-term (in phase 1, after 1 day of treatment) *versus* long term exposures (in phase 2, after 7 days of treatment). Level of significance was predetermined as 0.05 for all tests except post-hoc Mann-Whitney U tests which had levels of significance equal to 0.017.

Table 1. Descriptive statistics for pre- and post-treatment colony counts, delta colony counts, and percent of colony counts (%RCC) in each subgroup of phase 1, and the results of the Kruskal-Wallis test

		Chitosan	Formocresol	CMCP	Control	*P*-value
Bacteria	**Parameter**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (Median)**	
Enterococcus	**Pre treatment**	11×10^{6} (10×10^{6})	484×10^{5} (610×10^{5})	232×10^{5} (250×10^{5})	388×10^{5} (190×10^{5})	0.116
	Post treatment	306×10^{3} (100×10^{3})	0 (0)	0 (0)	436×10^{5} (230×10^{5})	0.007
	Delta-Colony	10694×10^{3} (9900×10^{3})	484×10^{5} (610×10^{5})	232×10^{5} (250×10^{5})	-48×10^{5} (-40×10^{5})	0.093
	%RCC	98 (99)	100 (100)	100 (100)	-19 (-16)	0.011
Staphylococcus	**Pre treatment**	194×10^{6} (170×10^{6})	25×10^{7} (25×10^{7})	228×10^{6} (240×10^{6})	25×10^{7} (25×10^{7})	0.300
	Post treatment	4926×10^{3} (6000×10^{3})	330 (0)	0 (0)	257×10^{6} (260×10^{7})	0.003
	Delta-Colony	189074×10^{3} (162000×10^{3})	249999670 (25×10^{7})	228×10^{6} (240×10^{6})	-12×10^{6} (-10×10^{6})	0.184
	%RCC	97 (96)	100 (100)	100 (100)	-380 (-5)	0.003
Streptococcus	**Pre treatment**	44400 (42000)	74360 (10×10^{4})	57360 (65×10^{3})	5×10^{4} (5×10^{4})	0.296
	Post treatment	40 (0)	0 (0)	0 (0)	52600 (55000)	0.116
	Delta-Colony	44360 (42000)	74360 (10×10^{4})	57360 (65×10^{3})	-2600 (-2000)	0.296
	%RCC	100 (100)	100 (100)	100 (100)	-6 (-4)	0.117

Table 2. Descriptive statistics for pre- and post-treatment colony counts, delta colony counts, and percent of colony counts (%RCC) in each subgroup of phase 2, and the results of the Kruskal-Wallis test

		Chitosan	Formocresol	CMCP	Control	*P*-value
Bacteria	**Parameter**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (Median)**	
Enterococcus	**Pre treatment**	264×10^{6} (200×10^{6})	248×10^{5} (210×10^{5})	142×10^{5} (100×10^{5})	372×10^{5} (200×10^{5})	0.008
	Post treatment	31×10^{4} (30×10^{4})	0 (0)	0 (0)	34×10^{6} (19×10^{6})	0.007
	Delta-Colony	26369×10^{4} (19970×10^{4})	248×10^{5} (210×10^{5})	142×10^{5} (100×10^{5})	32×10^{5} (20×10^{5})	0.008
	%RCC	100 (100)	100 (100)	100 (100)	11 (8)	0.007
Staphylococcus	**Pre treatment**	1728×10^{5} (1400×10^{5})	1088×10^{5} (700×10^{5})	42×10^{5} (20×10^{5})	3×10^{8} (3×10^{8})	0.007
	Post treatment	1196×10^{3} (900×10^{3})	0 (0)	0 (0)	272×10^{6} (270×10^{6})	0.007
	Delta-Colony	171604×10^{3} (139870×10^{3})	1088×10^{5} (700×10^{5})	42×10^{5} (20×10^{5})	28×10^{6} (30×10^{6})	0.327
	%RCC	99 (100)	100 (100)	100 (100)	9 (10)	0.000
Streptococcus	**Pre treatment**	13×10^{4} (3×10^{4})	10×10^{4} (10×10^{4})	58×10^{3} (65×10^{3})	48×10^{3} (50×10^{3})	0.042
	Post treatment	0 (0)	0 (0)	0 (0)	44×10^{3} (47×10^{3})	1.000
	Delta-Colony	13×10^{4} (3×10^{4})	10×10^{4} (10×10^{4})	58×10^{3} (65×10^{3})	40×10^{2} (30×10^{2})	0.042
	%RCC	100 (100)	100 (100)	100 (100)	9 (10)	1.000

Results

Except few subgroups which showed an increase in colony count, all other subgroups showed reductions in colony count in either phase. The Kruskal-Wallis test showed significant differences among treatments, in many of subgroups' pre- and post-treatment colony counts as well as delta-counts and %RCCs (Tables 1 and 2).

Phase 1

In phase 1, Wilcoxon test showed a significant effect on colony counts (*P*=0.000, positive mean rank for comparison=20.17, negative mean rank=33.35) (Figure 1). The Kruskal-Wallis test did not show a significant difference between %RCC values of different bacteria types (*P*=0.485, mean ranks for bacteria: *Enterococcus faecalis*=28.89, *Staphylococcus aureos*=27.65, *Streptococcus mutans*=33.40). The Kruskal-Wallis test indicated a significant difference between %RCC values of treatment types (*P*=0.000, mean ranks for treatments: Chitosan=12.13, Formocresol=26.80, CMCP=29.00). According to the Mann-Whitney U test, difference between chitosan and formocresol was significant with chitosan showing a smaller reduction (*P*=0.001, mean ranks for treatments: Chitosan=10.27, Formocresol=20.73); difference between chitosan and CMCP was significant as well again with chitosan showing a smaller reduction (*P*=0.000, mean ranks: Chitosan=9.87, CMCP=20.50); however, there was not a significant difference between formocresol and CMCP (*P*=0.561, mean ranks: Formocresol=14.07, CMCP=16.00).

Comparing delta-colony values (changes in colony counts over time), Kruskal-Wallis test did not show a significant difference between delta-colony counts among treatment types (*P*=0.548).

Phase 2

In phase 2, Wilcoxon test showed a significant effect on colony counts in 7 days (*P*=0.000, positive mean rank for comparison=0.00, negative mean rank=30.50) (Figure 1). The Kruskal-Wallis test did not show a significant difference between %RCC values of different bacteria types (*P*=0.581, mean ranks for bacteria: *Enterococcus faecalis*=29.25, *Staphylococcus aureos*=28.85, *Streptococcus mutans*=33.40). The Kruskal-Wallis test showed a significant difference between %RCC values of different treatment types (*P*=0.000, mean ranks for treatments: Chitosan=15.00, Formocresol=27.00, CMCP=27.00). According to the Mann-Whitney U test chitosan acted weaker than other two, difference between chitosan and formocresol was significant (*P*=0.011, mean ranks for treatments: Chitosan=11.50, Formocresol=19.50); difference between chitosan and CMCP was significant as well (*P*=0.011, mean ranks: Chitosan=11.50, CMCP=19.50); however, there was not a significant difference between formocresol and CMCP (*P*=1.0, mean ranks: Formocresol=15.00, CMCP=15.00).

Comparing delta-colony counts, the Kruskal-Wallis test indicated a significant difference between delta-colony values of treatment types (*P*=0.045, mean ranks for treatments: Chitosan=28.20, Formocresol=24.30, CMCP=16.50). According to the Mann-Whitney U test, difference between chitosan and formocresol was not significant (*P*=0.233); difference between chitosan and CMCP was marginally significant, with chitosan showing a greater reduction (*P*=0.041, mean ranks: Chitosan=18.77, CMCP=12.23); and there was a marginally significant difference between formocresol and CMCP (*P*=0.045, mean ranks: Formocresol=18.73, CMCP=12.27).

Table 3. Asymptotic *P*-values calculated using Mann-Whitney U test between both phases

Bacteria		Pretreatment Colony Count	Post treatment Colony Count	Delta Colony Count	%RCC
Enterococcus faecalis	Chitosan	0.009	0.834	0.009	0.094
	Formocresol	0.175	1.000	0.175	1.000
	CMCP	0.600	1.000	0.600	1.000
	Control	1.000	0.347	0.009	0.009
Staphylococcus aureos	Chitosan	0.458	0.175	0.465	0.173
	Formocresol	0.035	0.136	0.036	0.136
	CMCP	0.009	1.000	0.009	1.000
	Control	0.228	0.674	0.009	0.009
Streptococcus mutans	Chitosan	0.915	0.134	1.000	0.136
	Formocresol	0.521	1.000	0.521	1.000
	CMCP	0.916	1.000	0.916	1.000
	Control	1.000	0.602	0.009	0.008

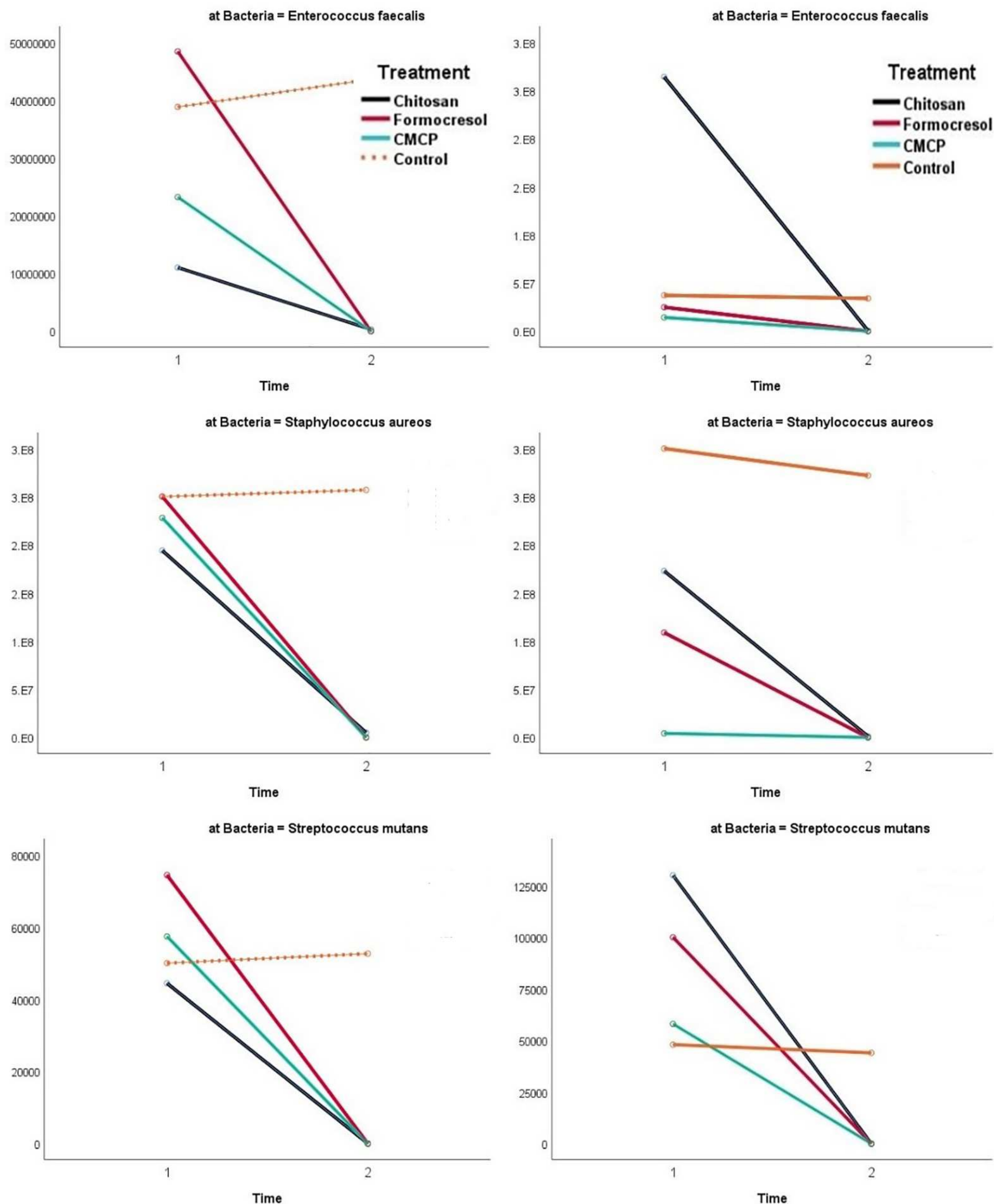

Figure 1. Colony counts in the pre- and post-treatment sessions, in phases 1 (left) and 2 (right)

Comparisons between short-term and long-term exposures (phases 1 and 2)

Differences between the %RCC and delta-count values of phase 1 versus phase 2 were observed in few cases only (Table 3). When all bacteria subgroups were aggregated, pre-treatment values were not different in phase 1 *versus* phase 2, in any of the 4 treatment groups (all 4 Mann-Whitney *P*-values>0.2). The same held for post-treatment colony counts (all 4 Mann-Whitney *P*-values>0.4). When comparing the delta-colony counts of each treatment between two phases (when all bacteria groups were aggregated), only the control group showed a significant difference between the two phases (*P*=0.000) while the other groups [CMCP, formocresol and chitosan] did not have a significant difference between the short-term and long-term phases (*P*>0.2). When comparing the %RCC values between the short- and long-term phases, only the control group showed a significant difference (*P*=0.000). CMCP, formocresol and chitosan did not have different %RCC values between two phases (*P*>0.07).

Discussion

Findings of this study indicated that compared with the control, all three medicaments were effective in reducing bacterial count, either after 1 day or 7 days of treatment. Chitosan was as effective as formocresol and CMCP in short term, but was slightly more effective than CMCP, in 7 days period. Formocresol and CMCP acted similarily in short and long terms (although formocresol was slightly better than CMCP in 7 days). The 1-day and 7-day effects of the agents on various bacteria were not different except for few agents in the case of some bacteria. The control group showed a slight increase in bacterial count after 1 day; however, in phase 2, controls showed a slight reduction in bacterial count, which might be due to sealing of canals and lack of nutritional materials necessary for bacterial growth. However, this is not the case in clinical situations where sufficient nutrition is provided. Comparison of both phases showed differences between the colonies counted in the first and seventh days, only in the case of control group. This might be due to the post-treatment colony counts being close to zero in both phases (either after 1 day or after 7 days).

Our results in terms of the efficacy of chitosan -being comparable to that of CMCP and formocresol in 1 or 7 days- can suggest promising results for this experimentally produced concentration of chitosan. Studies on other uses of chitosan as well found it successful. Moghadas *et al.* [42] reported similar antimicrobial effects of nano-chitosan and sodium hypochlorite (used as canal irrigators) on *Staphylococcus aureus* and *Enterococcus faecalis* [42]. Also Suzuki *et al.* [43] confirmed the antibacterial effects of chitosan solution against *Enterococcus faecalis*. Hayashi *et al.* [39] examined antibacterial effects of chitosan-incorporated chewing gum, and observed reduced amounts of bacteria compared to their control group who had used a mouth rinse. Jaiswal *et al.* [44] comparatively assessed the efficacy of chitosan as a canal irrigation solution against *Enterococcus faecalis* and concluded that Chitosan + Chlorhexidine, Chlorhexidine and Propolis can be as efficacious as sodium hypochlorite. Camacho-Alonso *et al.* [45] examined the antibacterial efficacy of photodynamic therapy and chitosan against *Enterococcus faecalis* in experimentally infected root canals and reported that combination of PDT and chitosan can be effective.

Uysal *et al* [38] evaluated the effect of chitosan dentifrice on enamel decalcification around orthodontic brackets, and reported that the chitosan mouthwash was able to reduce decalcification in patients with poor oral hygiene. Another study on chitosan-containing mouthwashes was performed by Costa *et al.* [48] who observed superior results of chitosan mouthwash compared with two commercial mouth rinses. Mirhashemi *et al.* [49] added chitosan nanoparticles to composite bracket-bonding agents and verified its antibacterial effect. Targino *et al.* [24] assessed the antimicrobial effects and biocompatibility of a formulation containing silver nanoparticles, chitosan, and fluoride; and concluded that this formula is a promising anti-caries agent with proper antimicrobial effects, low toxicity to living cells, and not staining teeth black [24].

Since there was no study on the efficacy of chitosan as a medicament in infectious primary teeth, we are limited to discussing results pertaining to more general aspects of this study. The findings of this study in terms of similar efficacies of CMCP and formocresol were similar to the study of Rosa *et al.* [9] who reported these two materials as similarly the best ones among others including chlorhexidine and calcium hydroxide. Menezes *et al.* [10] as well reported $Ca(OH)_2$ + CPMC paste as the most effective intracanal drug for the elimination of the two microorganisms *Candida albicans* and *Enterococcus faecalis*. Ferreira *et al.* [50] as well did not observe any significant difference between the antimicrobial effects of formocresol and CMCP, which again was similar to the present study. In the study of Meshki *et al.* [8], antimicrobial effects of formocresol, CMCP, sodium hypochlorite and 0.2% chlorhexidine were compared. According to them, formocresol and CMCP were superior to the other two. In another study, among formocresol, 2% glutaraldehyde and iodine-potassium, formocresol and glutaraldehyde showed the highest antimicrobial activity [51].

This pilot study was limited by some factors. Although the sample size was adequate to draw numerous significant results, it should have been determined based on pilot studies. Another limitation was that we could not technically sample from the same canals both in the first and seventh days; therefore, we had to either limit the study to one phase only or repeat the study in two phases. This was advantageous, as the accuracy of many of the practices (such as the negative and positive controls, or the pre-treatment colony counts) were verified by being conducted twice in separate studies (phases). Still, future studies should evaluate longer periods. We checked a 1-week period because it was relevant to the clinical situations, when there is a 1-week interval between the two sessions. Also future studies should evaluate various concentrations of chitosan in terms of optimum antibacterial efficacy and biocompatibility, even though chitosan is already known as a biocompatible material for other uses [21, 22]. Since results of *in vitro* studies cannot be generalized to clinical situations, future animal and

human studies are needed to verify the current study. As an advantage, we used three different bacteria which are mostly held responsible for recurrent root canal infections. Finally, since pulpectomy as well needs antibacterial dressings [52], chitosan can be a proper alternative; therefore, future studies should assess the antibacterial efficacy and biocompatibility of this material as a pulpectomy dressing.

Conclusion

This preliminary study confirmed for the first time the appropriate efficacy of chitosan as a medicament for pulpectomy. Both phases indicated a decrease in colony counts over time. After 24 h of treatment, the antibacterial effect of chitosan might be comparable to or better than formocresol and CMCP, all being superior to the effect of positive control which might not show a decrease. After 7 days of treatment as well, chitosan might act similar to CMCP and formocresol, both of which were significantly effective compared to the control.

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References

1. Ansari G, Tabari M, Kazemi B. Assessment of pathogens microorganisms of periapical abscess in primary teeth. Shahid Beheshti University Dent J. 2005;22(4):567-73.
2. Cerqueira DF, Volpi Mello-Moura AC, Santos EM, Guedes-Pinto AC. Cytotoxicity, Histopathological, Microbiological and Clinical Aspects of an Endodontic Iodoform-Based Paste Used in Pediatric Dentistry: A Review. J Clin Pediatr Dent. 2007;32(2):105-10.
3. Thomas AM, Chandra S, Chandra S, Pandey RK. Elimination of infection in pulpectomized deciduous teeth: A short-term study using iodoform paste. J Endod. 1994;20(5):233-5.
4. Dean JA. McDonald and Avery's Dentistry for the Child and Adolescent. Maryland Hights: Elsevier Health Sciences; 2015.
5. Leonardo MR, Silveira FF, Silva LABd, Tanomaru Filho M, Utrilla LS. Calcium hydroxide root canal dressing. Histopathological evaluation of periapical repair at different time periods. Braz Dent J. 2002:17-22.
6. Queiroz AMd, Nelson Filho P, Silva LABd, Assed S, Silva RABd, Ito IY. Antibacterial activity of root canal filling materials for primary teeth: zinc oxide and eugenol cement, Calen paste thickened with zinc oxide, Sealapex and EndoREZ. Braz Dent J 2009;20(4):290-6.
7. Harini P, Bhat S, Sundeep Hegde K. Comparative Evaluation of Bactericidal Potential of Four Root Canal Filling Materials against Microflora of Infected Non -Vital Primary Teeth. J Clin Pediatr Dent. 2010;35(1):23-9.
8. Meshki R, Nekourad M, Amin M, Goudarzi H. A Comparative in vitro Study on the Antimicrobial Efficacy of Formocresol, CMCP and 0.2% Chlorhexidine against some Bacteria of Infected Primary Teeth. Jundishapur Sci Med J. 2015;14(5):515-22.
9. Rosa OPdS, Torres SA, Ferreira CM, Ferreira FBdA. In vitro effect of intracanal medicaments on strict anaerobes by means of the broth dilution method. Pesquisa Odontológica Brasileira. 2002;16(1):31-6.
10. Menezes MM, Valera MC, Jorge AOC, Koga-Ito CY, Camargo CHR, Mancini MNG. In vitro evaluation of the effectiveness of irrigants and intracanal medicaments on microorganisms within root canals. Int Endod J. 2004;37(5):311-9.
11. MASILLAMONI CR, Kettering J, Torabinejad M. The biocompatibility of some root canal medicaments and irrigants. Int Endod J. 1981;14(2):115-20.
12. Chandrashekhar S, Shashidhar J. Formocresol, still a controversial material for pulpotomy: A critical literature review. J Restor Dent. 2014;2(3):114.
13. Lewis BB, Chestner SB. Formaldehyde in dentistry: a review of mutagenic and carcinogenic potential. J Am Dent Assoc. 1981;103(3):429-34.
14. Seale NS. The formocresol controversy: we have the answer. Pediatr Dent. 2012;34(5):370-1.
15. Samiei M, Asgary S, Farajzadeh M, Bargahi N, Abdolrahimi M, Kananizadeh U, Dastmalchi S. Investigating the mutagenic effects of three commonly used pulpotomy agents using the Ames test. Adv Pharm Bull. 2015;5(1):121.
16. Gahyva SMM, Siqueira Junior JF. Direct genotoxicity and mutagenicity of endodontic substances and materials as evaluated by two prokaryotic test systems. J App Oral Sci. 2005;13(4):387-92.
17. Ranly D, Horn D. Assessment of the systemic distribution and toxicity of formaldehyde following pulpotomy treatment: Part two. ASDC J Dent Child. 1987;54(1):40-4.
18. Sun HW, Feigal R, Messer H. Cytotoxicity of glutaraldehyde and formaldehyde in relation to time of exposure and concentration. Pediatr Dent. 1990;12(5):303-7.
19. Nishimura H, Higo Y, Ohno M, Tsutsui TW, Tsutsui T. Ability of root canal antiseptics used in dental practice to induce chromosome aberrations in human dental pulp cells. Mutat Res Genet Toxicol Environ Mutagen. 2008;649(1-2):45-53.
20. Bartelstone HJ. Radioiodine Penetration through Intact Enamel with Uptake by Bloodstream and Thyroid Gland. J Dent Res. 1951;30(5):728-33.
21. Tharanathan RN, Kittur FS. Chitin — The Undisputed Biomolecule of Great Potential. Crit Rev Food Sci Nutr. 2003;43(1):61-87.
22. Ikeda T, Yanagiguchi K, Viloria I, Hayashi Y. Relationship between lysozyme activity and clinical symptoms following the application of chitin/chitosan in endodontic treatment. Chitosan per os: from dietary supplement to drug carrier. Grottammare: Atec. 2000:275-92.

23. Skoskiewicz-Malinowska K, Kaczmarek U, Malicka B, Walczak K, Zietek M. Application of Chitosan and Propolis in Endodontic Treatment: A Review. Mini Rev Med Chem. 2017;17(5):410-34.
24. Targino AGR, Flores MAP, dos Santos Junior VE, Bezerra FdGB, de Luna Freire H, Galembeck A, Rosenblatt A. An innovative approach to treating dental decay in children. A new anti-caries agent. J Mater Sci Mater Med. 2014;25(8):2041-7.
25. Tharanathan RN, Kittur FS. Chitin—the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr . 2003;43:61-87.
26. Muzzarelli R. Chitins and Chitosans as Immunoadjuvants and Non-Allergenic Drug Carriers. Marine Drugs. 2010;8(12):292-312.
27. Robert ME, Eric JB. Chitin Chemistry, The. 1992.
28. Roberts GA. Structure of chitin and chitosan. Chitin chemistry: Springer; 1992. pp. 1-53.
29. Dutta PK, Dutta J, Tripathi V. Chitin and chitosan: Chemistry, properties and applications. 2004.
30. Rinaudo M, Domard A. Solution Properties of Chitosan'in Chitin and Chitosan,(Skjak-Braek, G., Anthonsen, T., Sandford, P., eds). Elsevier Applied Science, London, UK; 1989.
31. Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W. Chitosan as Antimicrobial Agent: Applications and Mode of Action. Biomacromolecules. 2003;4(6):1457-65.
32. Xue-lian S, Ying-ying S, Yang L, Qing C, Xin-xin B, Jing-tao Y. An in vitro evaluation of the effect of carboxymethyl chitosan and its composites against Enterococcus faecalis in the root canal. Shanghai J Stom. 2013;22(3):265-9.
33. Muzzarelli R, Mattioli-Belmonte M, Pugnaloni A, Biagini G. Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. Birkhauser: Verlag; 1999. 251-64 pp.
34. Ueno H, Yamada H, Tanaka I, Kaba N, Matsuura M, Okumura M, Kadosawa T, Fujinaga T. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. Biomaterials. 1999;20(15):1407-14.
35. Matsunaga T, Yanagiguchi K, Yamada S, Ohara N, Ikeda T, Hayashi Y. Chitosan monomer promotes tissue regeneration on dental pulp wounds. J Biomed Mater Res A. 2006;76(4):711-20.
36. Schlüter N, Klimek J, Ganss C. Effect of a chitosan additive to a Sn 2+-containing toothpaste on its anti-erosive/anti-abrasive efficacy—a controlled randomised in situ trial. Clin Oral Invest. 2014;18(1):107-15.
37. Costa EM, Silva S, Pina C, Tavaria FK, Pintado MM. Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. Anaerobe. 2012;18(3):305-9.
38. Uysal T, Akkurt MD, Amasyali M, Ozcan S, Yagci A, Basak F, Sagdic D. Does a chitosan-containing dentifrice prevent demineralization around orthodontic brackets? Angle Orthod. 2011;81(2):319-25.
39. Hayashi Y, Ohara N, Ganno T, Ishizaki H, Yanagiguchi K. Chitosan-containing gum chewing accelerates antibacterial effect with an increase in salivary secretion. J Dent. 2007;35(11):871-4.
40. Stamford TCM, Stamford-Arnaud TM, de Medeiros Cavalcante HM, Macedo RO, de Campos-Takaki GM. Microbiological chitosan: Potential application as anticariogenic agent. Practical Applications in Biomedical Engineering: InTech; 2013.
41. Arnaud TMS, de Barros Neto B, Diniz FB. Chitosan effect on dental enamel de-remineralization: an in vitro evaluation. J Dent. 2010;38(11):848-52.
42. Moghadas L, Narimani T, Shahmoradi M. Antimicrobial activity of a new nanobased endodontic irrigation solution: In vitro study. Dent Hypotheses. 2012;3(4):142-6.
43. Suzuki S. The Study of Chitosan-Citrate Solution as a Root Canal Irrigant: A Preliminary Report. J Oral Hygiene & Health. 2014;02(04):1-4.
44. Jaiswal N, Sinha DJ, Singh UP, Singh K, Jandial UA, Goel S. Evaluation of antibacterial efficacy of Chitosan, Chlorhexidine, Propolis and Sodium hypochlorite on Enterococcus faecalis biofilm : An in vitro study. J Clin Exp Dent. 2017;9(9):e1066-e74.
45. Camacho-Alonso F, Julian-Belmonte E, Chiva-Garcia F, Martinez-Beneyto Y. Bactericidal Efficacy of Photodynamic Therapy and Chitosan in Root Canals Experimentally Infected with Enterococcus faecalis: An In Vitro Study. Photomed Laser Surg. 2017;35(4):184-9.
46. Mathew V, Madhusudhana K, Sivakumar N, Venugopal T, Reddy R. Anti-microbial efficiency of silver diamine fluoride as an endodontic medicament - An ex vivo study. Cont Clin Dent. 2012;3(3):262-4.
47. de Magalhães Silveira CF, Cunha RS, Fontana CE, de Martin AS, de Almeida Gomes BPF, Motta RHL, da Silveira Bueno CE. Assessment of the antibacterial activity of calcium hydroxide combined with chlorhexidine paste and other intracanal medications against bacterial pathogens. Eur J Dent. 2011;5(1):1.
48. Costa E, Silva S, Madureira A, Cardelle-Cobas A, Tavaria F, Pintado M. A comprehensive study into the impact of a chitosan mouthwash upon oral microorganism's biofilm formation in vitro. Carbohydr Polym. 2014;101:1081-6.
49. Mirhashemi A, Bahador A, Kassaee M, Daryakenari G, Ahmad-Akhoundi M, Sodagar A. Antimicrobial effect of nano-zinc oxide and nano-chitosan particles in dental composite used in orthodontics. J Med Bacterio. 2015;2(3-4):1-10.
50. Ferreira FB, Torres SA, Rosa OP, Ferreira CM, Garcia RB, Marcucci MC, Gomes BP. Antimicrobial effect of propolis and other substances against selected endodontic pathogens. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007;104(5):709-16.
51. Lele GS, Subba Reddy VV. Comparison of antibacterial efficacy of intracanal medicaments in multiple visit pulpectomies in primary molars-anin vivostudy. J Indian Soc Pedod Prev Dent. 2010;28(1):18-24.
52. Ansari G, Morovati SP, Asgary S. Evaluation of Four Pulpotomy Techniques in Primary Molars: A Randomized Controlled Trial. Iran Endod J. 2018;13(1):7-12.

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