CHITOSAN IMPREGNATED GUTTA-PERCHA POINTS: ANTIMICROBIAL *IN VITRO* EVALUATION AND MECHANICAL PROPERTIES

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Abstract

Chitosan-impregnated gutta-percha points (ChitGPP) were tested for their ability to inhibit the growth of microorganisms usually involved in root canal infections. Their mechanical properties were also studied and compared with the commonly used commercial points in endodontics. ChitGPP were more efficient in reducing the microbial load than those without chitosan. ChitGPP also possess better tensile and elastic properties than commercial ones. After 6 months of storage, ChitGPP's were still able to reduce the bacterial load by 1 log, suggesting that impregnation of gutta-percha points with chitosan could be a good alternative to obtain gutta-percha points with improved antimicrobial properties.

Keywords: Endodontic treatment, Gutta-percha, Chitosan, Root canal filling materials, Antimicrobial activity, Mechanical properties

1. Introduction

Microorganisms are the major etiological agents in pulpal and periapical disease. In case of primary endodontic infections the main etiological agents consists mainly of strict anaerobes. However, in cases of failed endodontic treatment, the intracanal microflora is altered, and facultative anaerobes predominate [1,2]. The efficient control and elimination of microorganisms are very important during endodontic treatment because of their role in these diseases. Biomechanical preparation of infected root canals using chemical substances with antimicrobial activity can eliminate most of the microorganisms present, although some may remain in the root canal system, dentinal tubules, or apical resorption craters, thus forming an apical bacterial biofilm. In these cases, intracanal medication is necessary to completely eliminate them [3,4]. Thus, two of the main goals of the endodontic therapy are the elimination of microorganisms from the root canal system and the prevention of subsequent reinfection [5]. For this purpose, gutta-percha cones are the most universally accepted filling material due to their desirable properties such as biological compatibility, pliability, dimensional stability, radiopacity and easy removal from the canal when required. Evidence of slight antibacterial activity for gutta-percha cones exists and it is known to be due to the zinc oxide, the major component of the cones [6]. However, the effect is too weak and limited for this material to be an effective antimicrobial agent. Otherwise, most currently used root canal filling materials do not possess a complete seal, which may contribute to treatment failures via re-colonization by bacteria and recontamination of the canal system. *Enterococcus faecalis* has been classified as one of the most resistant oral pathogens, especially in secondary and persistent root canal infections [7]. Anaerobic species such as *Peptostreptococcus micros*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia* and *Prevotella nigrescens* have also been found in teeth with failing endodontic treatment [8].

On the other hand, *Candida albicans* has been the fungal species most often detected in these infections [9].

Different compositions of gutta-percha cones have been previously studied by several authors: although $Ca(OH)_2$ and ZnO are widely recognized in the dental practice as the antimicrobials of choice, $Ca(OH)_2$, ZnO/chlorhexidine, or ZnO/ iodine-polyvinyl-pyrrolidone-containing gutta-percha cones failed to effectively inhibit endodontic pathogens in root canals [10]. In other studies, gutta-percha cones containing chlorhexidine presented antimicrobial activity whereas gutta-percha cones containing calcium hydroxide did not [11]. Similar results were attained in the studies by Decurcio et al. [12] and Öztan et al. [13]. The latter revealed that gutta-percha points containing a mixture of calcium hydroxide and chlorhexidine diacetate have higher efficacies than calcium hydroxide or chlorhexidine diacetate alone against *C. albicans*, *C. tropicalis,* and *Saccharomyces cerevisiae*. According to Vijay et al. [14], tetracycline-impregnated gutta-percha offers maximum antibacterial advantage over calcium hydroxide and traditional gutta-percha against *E. faecalis*. Shur et al. [15] showed that iodoform-containing gutta-percha cones had an *in vitro* inhibitory effect against *S. aureus* and *Fusobacterium nucleatum* but not against *E. faecalis*, *E.coli* and *Pseudomonas aeruginosa*.

Chitosan, a natural and non-toxic polymer, is produced by the deacetylation of chitin and has received considerable attention in a wide range of applications due to their biological (anti-microbial, bio-adhesive, bio-compatible and binding agent) properties [16]. Applications of the antimicrobial activity of chitosan are currently investigated in food, textile and cosmetic industries and in medicine, including dentistry [17,18,19, 20,21]. In addition, natural bioactive materials have recently been investigated as promising agents to prevent oral diseases such as dental caries. Chitosan showed antibacterial properties against oral bacterial pathogens [22,23,24,25]. Furthermore, recent studies demonstrated

that water-soluble reduced chitosan, used as a mouth rinse solution, displayed an antibacterial and plaque-reducing action [26,27,28,29]. Chewing gum containing chitosan and chitosan derivatives, also effectively inhibited the growth of cariogenic bacteria in saliva [30,31,32]. Chitosan nanoparticles were also tested for the loading of toothpaste bioactive compounds and adhesion on tooth analogs. Results showed that chitosan has a great potential to be used for the *in situ* release of the active compounds in a sustained manner [33,34].

These advantages and applications of chitosan suggest its potential in root canal treatment and some authors have already been carried out some preliminary assays in this field. Thus, Ahmed et al. [35] developed chitosan films containing ciprofloxacin and diclofenac sodium for the topical treatment of periodontitis. However, in these studies chitosan was used as release material and not as the active ingredient. More recently, DaSilva et al. [36] and Carpio-Perochena et al. [37] have reported the use of chitosan nanoparticles to inhibit biofilm formation and as an antibacterial agent when combined with root canal sealers. In this study, chitosan was directly used to impregnate gutta-percha cones and the antimicrobial and mechanical properties of ChitGPC were demonstrated and compared with that of commercial ones.

2. Materials and methods

2.1. Microorganisms

Gram-negative (*Prevotella buccae* CCUG 15401 (Culture Collection University of Göteborg, Sweden) and *Porphyromonas gingivalis* (gently donated by Dr. Cristina Pina, Universidade Fernando Pessoa) and Gram-positive (*Peptostreptococcus stomatis* CCUG 51858) strict anaerobes, a Gram-positive facultative anaerobic bacteria (*Enterococcus faecalis*, from our culture collection) and a yeast (*Candida albicans* ATCC 18804), were

used in the present study. All obligate anaerobic bacteria were grown in Wilkins-Chalgren agar (WC agar, Oxoid, Basingstoke, UK), *E. faecalis* was grown in M17 agar (Difco, Detroit, MI) and *C. albicans* was grown in potato dextrose agar (PDA, Lab M, Bury, UK). All bacterial strains were cultured at 37 °C, whereas *C. albicans* was cultured at 30 °C. Anaerobes were incubated under anaerobic atmosphere in a GasPak jar (Becton-Dickinson, Heidelberg, Germany).

2.2. Gutta-Percha Points and Chitosan

For the experiments, ISO 20 and IS0 60-sized gutta-percha points (Dentsply, UK) were used. Composition was previously described by Gurgel-Filho et al. [38] as follows: 14.5 % (w/w) gutta–percha, 1.2 % (w/w) resin, 28.0 % (w/w) metal sulphates, 2.8 % (w/w) zinc chloride and 56.3 % (w/w) zinc oxide. Chitosan (LMW, Low molecular weight, Sigma-Aldrich, St. Louis, MO, USA) were used to impregnate the commercial gutta-percha points. To carry out the coating, a chitosan solution was prepared as follows: chitosan (1.4-2 %) was dissolved in 1% acetic acid (v/v) and mixed with a plasticizer, specifically with 40 % (v/v) of a sorbitol solution. Then, the pH was adjusted to 5.8 to ensure that the antimicrobial effect caused by chitosan is not due to the low pH or the acid effect brought about by the acetic acid solution. The mixture was placed in a petri plaque and dried at 37 ºC until a dense solution was obtained, which was then used to coat the commercial gutta-percha points. After manual coating, the points were left to dry to obtain a film around the gutta-percha points and stored in the dark until further analysis. The film was prepared as aforementioned, to obtain after drying, 0.14, 0.20 and 0.40 mg of chitosan surrounding the points. These were weighed before and after impregnation.

2.3. Antimicrobial activity

The antimicrobial activity of gutta-percha points impregnated with chitosan was evaluated by determining the inhibition of microbial growth using a modified method developed by Podbielski et al. [10]. For this purpose, experimental inocula of the different microorganisms (*P. buccae, P. gingivalis, P. stomatis, E. faecalis* and *C. albicans)* were obtained after overnight growth (ca. 10^7 CFU/mL) at 37 ºC in the appropriate growth conditions. Cells were then harvested by centrifugation at 2000 x *g* for 10 min and re-suspended in the same volume of sterile 0.9% NaCl solution. Aliquots (100 µL) were then added to sterile 0.2 mL Eppendorf tubes containing three pieces of chitosan impregnated gutta-percha points (ChitGPP), previously cut and sterilized under UV light. Eppendorf tubes containing only microbial suspensions served as controls and different tubes were used for each sampling time. Un-impregnated gutta-percha points (GPP) were also evaluated in the same way than those impregnated with chitosan. In order to avoid contaminations, separate tubes containing ChitGPP and GPP as well as controls were used for each sampling time. The tubes were maintained at 37 ºC and under aerobic or anaerobic conditions (GasPak, Heidelberg, Germany) according to the microorganism. The antimicrobial assay was carried out with *E. faecalis* and ISO 60-sized gutta-percha points impregnated with a solution containing 0.14 mg of chitosan during 48 h and sampled at 0, 1, 3, 5, 7, 18, 24 and 48 h. Later, different amounts of chitosan (0.14, 0.2 and 0.4 mg) contained in the ISO 60 sized ChitGPP were tested against *E. faecalis* in 24 h assays to evaluate the effect of the amount of chitosan.

The assays were repeated for *P. buccae*, *P. gingivalis P. stomatis* and *C. albicans*, using the ISO 60 sized gutta-percha points impregnated with 0.4 mg of chitosan during 24 h and sampled at 0, 3, 7 and 24 h. The reinfection event was simulated, by inoculation with 10^9 CFU/mL to attain a final concentration of 10^7 CFU/ mL. Samples were taken at 0, 3, 7 and 24 h after re-infection. When the tubes were removed at each sampling time, they were

vortexed for 15 s and aliquots (10 μL) of the bacterial suspensions were serially diluted in 90 μL sterile 0.9% NaCl solution. Aliquots (20 μL) of each dilution were plated onto the aforementioned solid medium depending of the microorganism. After incubation at 37 ºC for 48 h, colonies were enumerated and CFU/mL of suspension were calculated. Each experiment was performed in duplicate and on two independent experiments.

Efficacy of gutta-percha points after storage period was also evaluated. Thus antimicrobial activity of GPP and ISO 60-sized ChitGPP (0.14 mg LMWC) was evaluated against *E. faecalis* after 0, 1, 3 and 6 months of storage. Antimicrobial activity was evaluated as aforementioned after 7 h of infection (at the time of maximum effect of chitosan). All assays were carried out in triplicate.

2.4. Mechanical properties

ChitGPP and GPP were subjected to tensile tests using a Universal Testing machine (model 4501, Instron Corporation, Canton MA, USA) equipped with fixed grips and a 5 kN-static load cell operated according to the reference method [39]. The initial grip separation was set at 50 mm, and the crosshead speed at 4.8 mm/min. Tensile strength, elongation at break and Young's modulus were determined in 18 independent cones using the Series IX Automated Materials Testing System software, v. 809.00 (Instron). Additionally, the maximum force required to bend the ChitGPP and GPP to a 20-degree angle at a test diameter of 10 mm was recorded in 15 independent cones using a L&W bending tester (Lorentzen & Wettre, Kista, Sweden).

2.5. Statistical analysis

Statistical analyses were performed using SPSS v.24 software (SPSS, Chicago IL, USA) via one-way analysis of variance. The viable numbers of microorganisms/mL were

transformed into the $\log_{10}$ of the CFU/mL. Differences obtained at the $p<0.05$ level were considered to be significant.

3. Results

3.1. Antimicrobial activity

E. faecalis was the selected microorganism to determine the best concentration to be used in impregnation, because it is a prevailing microorganism in the oral cavity and one which is very resistant to antimicrobials, besides sometimes being the only isolated microorganism from the oral cavity [40]. The first study encompassing the antimicrobial activity of ISO20 sized ChitGPP against *E. faecalis* was carried out during 14 days to evaluate the antimicrobial efficacy (0.14 mg of chitosan was used for impregnation). The results obtained (data not shown) indicated that, after 8 h the ChitGPP caused an important reduction (5 log-units) on bacterial cells in comparison with the control that showed an increase of bacterial cells of almost 3 log-units whereas and the un-impregnated gutta-percha points showed an increase of 0.5 log-units. After that, no significant reduction was observed with the bacterial counts remaining at ca. 2×10^7 CFU/mL. After 8 hours, bacterial counts in GPP slightly increased reaching ca. 11 log-units, and after 1 day this value decreased down to 8 log-units and only after 2 days it reached the minimum value of 5 log-units (below that reached by ChitGPP). By 3 days, the viable cell numbers obtained for the 3 conditions reached the same value (6.5 log), suggesting cell death under the conditions here reported.

Although the antimicrobial effect of ISO 20-sized ChitGPP during the first 48 h of contact was clearly demonstrated, it was evident that the thin surface of the used gutta-percha point could only support on its surface a limited amount of chitosan (0.14 mg), assuming the maximum dissolved concentration of 2%. This amount induced a rapid

reduction of 5 log CFU/mL in the first 8 h so, it was interesting to evaluate if increasing the total gutta-percha surface would promote higher and faster antimicrobial effect. Hence, a new experiment was developed with ISO 60-sized points to establish the improved antimicrobial efficiency with the increased impregnation area. Additionally, it should be noted that these points are the basis to seal the root canal, since the technique implies the introduction in the first place of a major gutta-percha point more representative of the filling area and then filling the canal with those with a smaller size just to completely seal the spaces usually associated with cements. The results are featured in Figure 1; the treatment differences were all statistically significant ($p<0.05$). The number of viable cells increased in the assays where gutta-percha points were not included, starting to decrease after 7 hours of inoculation. In the experiments where GPP were included, a lower value of bacterial cells was observed in comparison with control indicating that *E. faecalis* growth was inhibited in the presence of commercial gutta-percha points, remaining constant along the experiment with a value of ca. 6.5 log-units. In view of these results (14 days/ISO 20-sized gutta-percha points and 48h/ ISO 60-sized gutta-percha points), another experiment was conducted using the bigger sized gutta-percha points and timed experiments of 24 hours, since this is the period where major reductions were observed. It is well known that re-infection of root canal is a frequent problem in dentistry and for that, the use of materials that could prevent this reinfection, is very important. Thus, to simulate a re-infection in the root canal, the 24 h samples from the first experiment were inoculated in order to attain a bacterial concentration of ca. 6-7 log-units. Different amounts of chitosan (0, 0.14, 0.2 and 0.4 mg) included in the film covering the point were tested (Figure 2). Chitosan impregnated gutta-percha points showed higher antimicrobial activity than commercial ones; the antimicrobial activity increased with the amount of chitosan used for the impregnation and impregnated gutta-percha points with 0.4 mg of chitosan showed a

229 microbial reduction of 6 log-units during the first 24 h of the assay. After reinfection, no
230 significant differences ($p>0.05$) were found between GPP and ChitGPP with 0.14 and 0.2
231 mg of chitosan. Gutta-percha points with 0.4 mg of chitosan showed, however, significant
232 differences ($p<0.05$) and continued to show antimicrobial activity especially after 24 h of
233 reinfection.

234 In view of this, points with 0.4 mg of chitosan were selected as displaying the best
235 amount for the impregnation, and similar assays were carried out with *P. buccae*, *P.*
236 *gingivalis* and *P. stomatis* as well with the yeast *C. albicans,* which usually causes
237 infection in the root canal. Figure 3a shows the results obtained for *P. buccae*. In this case,
238 results obtained from the assays with ChitGPP, GPP and the control were statistically
239 different (except at 7h were the differences between GPP and ChitGPP were not
240 significant). An important reduction (~5 log-units) of the numbers of *P. buccae* was found
241 for GPP and ChitGPP after 24 h after inoculation, contrasting with almost no decrease for
242 the control. After reinfection, differences between ChitGPP and GPP were more
243 accentuated and significantly different ($p<0.05$) with ChitGPP showing greater
244 effectiveness than commercial points.

245 During the *in vitro* antimicrobial assays carried out against *P. gingivalis* (Figure 3b)*,*
246 GPP only started to show activity after 7 h of contact with the bacteria, however the
247 difference with respect to the control was not significant ($p>0.05$). In contrast, ChitGPP
248 showed to be active immediately after infection. After 7 h, a reduction of 7 log-units in the
249 bacterial counts was achieved. After reinfection, both types of gutta-percha points,
250 (ChitGPP and GPP) showed a similar pattern in bacterial reduction for *P. gingivalis* with
251 no significant differences between the two treatments.

252 Results obtained for *P. stomatis* (Figure 3c), showed a noticeable antimicrobial
253 activity of ChitGPP*;* a 3 log-unit reduction was obtained during the first 7 h of infection

and 6 logs after 24 h. GPP only showed a slight decrease in bacterial counts. After reinfection ChitGPP maintained its antimicrobial activity ($p<0.05$), and a 5 log-unit reduction in the bacterial counts was achieved by 30h.

Figure 3d shows the antifungal *in vitro* assays carried out with *C. albicans* and ChitGPP with 0.4 mg of chitosan. Results showed a significant decrease throughout time, up to 27h on fungal survival with respect to the assays done with non-impregnated points. However, by 48h no significant differences were found when compared to the control. Maximum reduction was obtained 7 hours after impregnation and only a 2 log-unit reduction was achieved.

3.2. Effect of storage in the antimicrobial activity of chitosan impregnated gutta-percha points

The effect of the storage on the antimicrobial activity of ISO 60-sized ChitGPP and GPP against *E. faecalis* was also evaluated. Figure 4 shows the results obtained for 0, 1 and 6 months of storage, respectively. ChitGPP (non-stored), showed to possess a reduction capacity on the bacterial charge of ca. 3.5 log-units whereas GPP did not show a significant decrease of the viable cell numbers. After 1 month of storage, ChitGPP was able to reduce the microbial load by only 2 logs with respect to the control. However, GPP showed a slight increase in its capacity to reduce the microbial load and showed a reduction of 0.5 logs with respect to the control, perhaps due to liberation of ZnO upon storage ($p<0.05$). After 6 months, the ChitGPP was able to reduce the bacterial load by 1 log, whereas GPP maintained its reduction capacity at 0.5 logs ($p<0.05$). These results suggest that partial degradation of gutta-percha points might be occurring [41] or in the film surrounding the gutta-percha points [42] and probably other assays should be carried

out to confirm this. Despite this, results were always better than those obtained for commercial points.

3.2. Mechanical properties

The main mechanical properties (tensile strength, elongation and bending force) were evaluated to determine if chitosan could affect these properties in commercial gutta-percha points and, consequently, become more suitable to be used in root canal treatments. The impregnation of commercial gutta-percha points with chitosan provided enhanced resistance to deformation/rupture. As can be observed in Table 1, significantly higher values for tensile strength (ultimate and yield strength) were found in ChitGPP. When ChitGPP was placed under tensile stress they elongated more as shown by the significantly high maximum elongation values obtained. Thus, they retained the most ductility, a useful clinical characteristic for filling materials. To examine flexural rigidity, a bending test was conducted, although no significant differences were obtained with respect to the force required to bend the points to a 20-degree angle. However, ChitGPP showed statistically significant differences for elasticity when compared to commercial ones. Young modulus was found to be significantly higher for GPP than for ChitGPP, indicating that the former are less elastic than those impregnated with chitosan.

4. Discussion

4.1. Antimicrobial activity

The first screening encompassing the antimicrobial activity of ISO 20-sized ChitGPP were carried out against *E. faecalis*, the microorganism most frequently found in endodontic infections, especially in secondary and persistent root canal infections [43]. The fact that GPP showed a higher antimicrobial activity than ChitGPP can be due to the

partial dissolution of the components of the ISO 20 -sized gutta-percha points with antimicrobial activity such as zinc oxide (ZnO). This is apparent, once the effect is not immediate as for chitosan, since ZnO requires a certain time to get dissolved, explaining the absence of effect until 8 h and the increasing inhibition effect from 8 h onwards. The ISO 20-sized points are very thin and the contact with the saline solution during the experiment allowed the release of such components, a scenario that could also occur *in vivo* conditions in the root canal. The literature reports that, although added to gutta-percha points primarily for mechanical stability reasons, ZnO alone possesses antimicrobial activity, thus decreasing bacterial counts [44]. Antimicrobial activity of ZnO alone was described for some endodontal pathogens such as *Peptostreptococcus micros*, *Veillonella parvula*, *Prevotella melaninogenica*, *Streptococcus mutans*, and *Pseudomonas aeruginosa*, a result similar to that found in this study. This effect can be attributed to the toxicity of the metal ion Zn^{2+} [45]. In the case of ChitGPP, the film around the point could be acting as a protective barrier thus hindering the liberation of ZnO, but assuring the antimicrobial effect through chitosan. As a result, the preliminary screening showed a fast bactericidal effect (reductions higher than 3 log cycles) in less than 8 h. Results obtained with ChitGPP were similar to those obtained by Podbielski et al. [46] with the other gutta-percha points containing calcium hydroxide, a mixture of ZnO and chlorhexidine (ZnO/CHX), iodine-polyvinylpyrrolidone (ZnO/I-PVP), or a mixture of CHX and I-PVP and ZnO (ZnO/CHX/I-PVP). This indicates that chitosan, incorporated in lower amounts than the other medications provided the same antimicrobial activity against the tested microorganisms than the other studied gutta-percha points, thus reflecting greater efficacy. Chlorhexidine and chitosan have the same mechanism of antimicrobial activity, as both disrupt the bacterial cell membrane, leading to cell death [22,47].

C. albicans is the most common commensal and pathological yeast found in the oral cavity [48] and, for this reason, it was included in this study. It has been demonstrated that *Candida* species are resistant to some medications commonly used in endodontics, such as calcium hydroxide. Ferguson et al. [49] evaluated the antifungal activity of aqueous $Ca(OH)_2$ and found that $Ca(OH)_2$ was only effective as a paste in direct contact with *C. albicans*. Other assays carried out with saturated $Ca(OH)_2$ solution showed that the alkalinity of saturated calcium hydroxide solution may not have a sufficient effect on *C. albicans*. In addition, $Ca(OH)_2$ solution may readily display the Ca^{2+} ions necessary for the growth and morphogenesis of *Candida* [50,51]. These mechanisms may explain why $Ca(OH)_2$ has been found to be ineffective against *C. albicans*.

Chitosan however, is known to be effective against a wide variety of fungi including *C. albicans*. *C. albicans* has shown to be susceptible to high and low molecular weight chitosans having MW > 32 kDa [52,53,29] and even to sub-mic concentrations of oligochitosan [54], which suppressed the formation of hyphal structures, causing severe cell wall alterations. Results obtained by Öztan et al. [13] showed that only the points containing $Ca(OH)_2$ and chlorhexidine were effective against the *Candida* species used in their study. Specifically for *C. albicans* results showed a total reduction after 2 days of incubation. After 24 h, 4 log units were reduced, twice the reduction obtained in our study with chitosan-impregnated gutta-percha points. In comparison with bacteria, very little is known about the ways in which fungi can circumvent the action of antimicrobials.

4.2. Mechanical properties

The study of the mechanical properties allowed establishing that the impregnation of gutta-percha points with chitosan improved its mechanical properties (higher values for tensile strength and elongation) providing enhanced resistance to fracture. An increase in tensile strength has been reported as a clinically desirable characteristic so that it reflects

the ability to retrieve a snugly inserted point completely. Cones with low percentages of gutta percha have poorer plasticity, allowing for a poorer apical sealing as reported by Gurgel-Filho et al. [55]. Gutta percha cones also present higher elastic modulus than other materials (eg. Resilon) [41], which translates in less flexibility under the same loading force (needing more force to adapt to root canal walls). The lack of rigidity and adhesivity of gutta cones have been quoted as some of the disadvantages for its use, hindering its use in small canals. From early on, Friedman et al. [57] reported that root canal filling material should possess the proper combination of flexibility and rigidity to permit the negotiation of almost any root canal. Mechanical properties of individual brands were found to be a function of the gutta-percha and zinc oxide concentration [58]. The impregnation with chitosan, as reported here, seems to improve some of the drawbacks in the mechanical properties of gutta percha points.

4. Conclusions

From the results of the present study, it can be concluded that chitosan impregnated gutta-percha points showed a higher (and significant) antimicrobial and antifungal activities and improved mechanical properties when compared to commercial gutta-percha points. The *in vitro* simulations of infection in the root canal showed that chitosan impregnated gutta-percha points were able to maintain their antimicrobial capacity throughout time. This activity was more pronounced towards *P. buccae, P. stomatis* and *P. gingivalis.*

In brief, these results show, that the use of chitosan impregnated gutta-percha points could be an interesting alternative to commercial gutta-percha points. Chitosan demonstrated an important antimicrobial effect in this application reinforcing the action of gutta-percha points. However, more studies including chitosan in the formulation of gutta-

377 percha points should be developed, especially exploiting its action against fungi and future
378 research needs to be conducted to evaluate the antimicrobial efficacy of medicated gutta-
379 percha points under clinical conditions.
380
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387
388 **Conflict of Interest**
389 The authors declare no conflict of interest.
390
391 **References**
392 1. Molander, A.; Reit, C.; Dahl, N.G.; Kvist, T. Microbiological status of root-filled teeth
393 with apical periodontitis. *Int. Endod. J.* **1998**, *31*, 1.
394 2. Lana, M.A.; Ribeiro-Sobrinho, A.P.; Stehling, R.; Garcia, G.D.; Silva, B.K.C.; Hamdan,
395 J.S.; Nicoli, J.R.; Carvalho, M.A.R.; Farias, L.M. Microorganisms isolated from root
396 canals presenting necrotic pulp and their drug susceptibility *in vitro. Oral Microbiol.*
397 *Immun.* **2001**,*16*, 100.
398 3. Torabinejad, M.; Turman, M. Revitalization of Tooth with necrotic pulp and Open Apex
399 by using platelet-rich plasma: a case report. *J. Endodont.* **2011**, *37*, 265.
400 4. El-Hadi, T.; Sorrentino, T.; Calmels, M.N.; Fraysse, B.; Deguine, O.; Marx, M.
401 Spontaneous Tegmen Defect and Semicircular Canal Dehiscence: Same

Etiopathogenic Entity? *Otol. Neurotol.* **2012**, *33*, 591.

5. Bodrumlu, E.; Alacam, T. Evaluation of antimicrobial and antifungal effects of iodoform-integrating gutta-percha. *J. Can. Dent. Assoc.* **2006**, *72*, 733.

6. Gurgel, E.D.; Feitosa, J.P.A.; Teixeira F.B.; de Paula R.C.M.; Silva, J.B.A.; Souza, F.J. Chemical and X-ray analyses of five brands of dental gutta-percha cone. *Int. Endod. J.* **2003**, *36*, 302.

7. Stuart, C.H.; Schwartz, S.A.; Beeson, T.J.; Owatz, C.B. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. *J. Endodont.* **2006**, *32*, 93.

8. Blome, B.; Braun, A.; Sobarzo, V.; Jepsen, S. Molecular identification and quantification of bacteria from endodontic infections using real-time polymerase chain reaction. *Oral Microbiol. Immun.* **2008**, *23*, 384.

9. Siqueira, J.F.; Sen, B.H. Fungi in endodontic infections. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **2004**, *97*, 632.

10. Podbielski, A.; Boeckh, C.; Haller, B. Growth inhibitory activity of gutta-percha points containing root canal medications on common endodontic bacterial pathogens as determined by an optimized quantitative *in vitro* assay. *J. Endodont.* **2000**, *26*, 398.

11. Tanomaru, J.M.; Pappen, F.G.; Tanomaru Filho, M.; Spolidorio, D.M.; Ito, I.Y. *In vitro* antimicrobial activity of different gutta-percha points and calcium hydroxide pastes. *Braz. Oral Res.* **2007**, *21*, 35.

12. Decurcio, D.A.; Crosara, M.B.; Silva, J.A.; Amorim, L.F.G.; Estrela, C.R.A. Avaliação antimicrobiana de cones de gutta-percha associados ao hidroxido de cálcio ou clorexidina. *Rev. Odont. Brasil Central.* **2010**, *18*, 30.

13. Öztan, M.D.; Kiyan, M.; Gerçeker, D. Antimicrobial effect, in vitro, of gutta-percha points containing root canal medications against yeasts and *Enterococcus faecalis.*

Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **2006**, *102*, 410.

14. Vijay, R.; Suman, M.; Shashikala, K. Evaluation of antimicrobial efficacy of tetracycline gutta-percha and calcium hydroxide impregnated gutta-percha against *Enterococcus faecalis* - an in vitro study. *Indian J. Dent. Adv.* **2010**, *2*, 248.
15. Shur A.L.; Sedgley, C.M.; Fenno, J.C. The antimicrobial efficacy of 'MGP' gutta-percha *in-vitro. Int. Endod. J.* **2003**, *36*, 616.
16. Kong, M.; Chen, X.G.; Xing, K.; Park, H.J. Antimicrobial properties of chitosan and mode of action: a state of the art review. *Int. J. Food Microbiol.* **2010**, *144*, 51.
17. Tsai, G.J. Su, W.H. Antibacterial activity of shrimp chitosan against *Escherichia coli. J. Food Prot.* **1999**, *62*, 239.
18. Khor, E.; Lim, L.Y. Implantable applications of chitin and chitosan. *Biomaterials* **2003**, *24*, 2339.
19. Cooksey, K. , Effectiveness of antimicrobial food packaging materials. *Food Addit. Contam.* **2005**, *22*, 980.
20. Kenawy, E.R.; Worley, S.D.; Broughton, R. The chemistry and applications of antimicrobial polymers: a state-of-the-art review. *Biomacromolecules* **2007**, 8, 1359.
21. Park, B.K.; Kim, M.M. Applications of chitin and its derivatives in biological medicine. *Int. J. Mol. Sci.* **2010**, *11*, 5153.
22. Helander, I.M.; Nurmiaho-Lassila, E.L.; Ahvenainen, R.; Rhoades, J.; Roller, S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. *Int. J. Food Microbiol.* **2001**, *71*, 235.
23. Rabea, E.I.; Badawy, M.E.T.; Stevens, C.V.; Smagghe, G.; Steurbaut, W. Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules* **2003**, *4*, 1457.
24. Tang, H.; Zhang, P.; Kieft, T.L.; Ryan, S.J.; Baker, S.M.; Wiesmann, W.P.; Rogelj, S.

Antibacterial action of a novel functionalized chitosan-arginine against Gram-negative bacteria. *Acta Biomaterials* **2010**, *6*, 2562.

25. Costa, E.M.; Silva, S.; Pina, C.; Tavaria, F.K.; Pintado, M.M. Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. *Anaerobe* **2012**, *18*, 305.

26. Sano, H.; Shibasaki, K.; Matsukubo, T.; Takaesu, Y. Effect of rinsing with phosphorylated chitosan on four-day plaque regrowth. *Bull. Tokyo Dent. Coll.* **2001**, *42*, 251.

27. Sano, H.; Shibasaki, K.; Matsukubo, T.; Takaesu, Y. Effect of chitosan rinsing on reduction of dental plaque formation. *Bull. Tokyo Dent. Coll.* **2003**, *44*, 9.

28. Bae, K.; Jun, E.J.; Lee, S.M.; Paik, D.I.; Kim, J.B. Effect of water-soluble reduced chitosan on Streptococcus mutans, plaque regrowth and biofilm vitality. *Clin. Oral Invest.* **2006**, *10*, 102.

29. Costa, E.M.; Silva, S.; Madureira, A.R.; Cardelle-Cobas, A.; Tavaria, F.K.; Pintado, M.M. A comprehensive study into the impact of a chitosan mouthwash upon oral microorganism's biofilm formation *in vitro*. *Carbohyd. Polym.* **2014**, *101*, 1081.

30. 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*, 871.

31. Hayashi, Y.; Ohara, N.; Ganno, T.; Yamaguchi, K.; Ishizaki, T.; Nakamura, T.; Sato, M. Chewing chitosan-containing gum effectively inhibits the growth of cariogenic bacteria. *Arch. Oral Biol.* **2007**, *52*, 290.

32. Miao, D.; Blom, D.; Zhao, H.; Luan, X.; Chen, T.; Wu, X.; Song, N. The antibacterial effect of CMCTS-containing chewing gum. *J. Nanjing Med. University* **2009**, *23*, 69.

33. Kockisch, S.; Rees, G.D.; Tsibouklis, J.; Smart, J.D. Mucoadhesive, triclosan-loaded

polymer microspheres for application to the oral cavity: preparation and controlled release characteristics. *Eur. J. Pharm. Biopharm.* **2005**, *59*, 207.

34. Liu, H.; Chen, B.; Mao, Z.; Gao, C. Chitosan nanoparticles for loading of toothpaste actives and adhesion on tooth analogs. *J. Appl. Polym. Sci.* **2007**, *106*, 4248.

35. Ahmed, M.G.; Harish, N.M.; Charyulu, R.N.; Prabhu, P. Formulation of chitosan-based ciprofloxacin and diclofenac film for periodontitis therapy. *Trop. J. Pharm. Res.* **2009**, *8*, 33.

36. DaSilva, L.; Finer,Y.; Friedman, S.; Basrani, B. Biofilm formation within the interface of bovine root dentin treated with conjugated chitosan and sealer containing chitosan nanoparticles. *J. Endod.* **2013**, *39*, 249.

37. Carpio-Perochena, A.; Kishen, A.; Shrestha, A.; Bramante, C.M. Chelating and antibacterial properties of chitosan nanoparticles on dentin. *J. Endod.* **2015**, *41*, 1353.

38. Gurgel-Filho, E.D.; Andrade Feitosa, J.P.; Teixeira, F.B.; Monteiro de Paula, R.C.; Araújo Silva, J.B.; Souza-Filho, F.J. Chemical and X-ray analyses of five brands of dental gutta-percha cone. *Int. Endod. J.* **2003**, *36*, 302.

39. ASTM-D412-98a. Philadelphia: The American Society for Testing and Materials (ASTM) Standards, 2002.

40. Lui, J.N.; Sae-Lim, V.; Song, K.P.; Chen. N.N. *In vitro* antimicrobial effect of chlorohexidine-impregnated gutta percha points on *E. faecalis*. *Int. Endod. J.* **2004**, *37*, 105.

41. Khedmat, S.; Aghajani, F.; Zaringhalam, S. The effect of storage temperature on mechanical properties of gutta-percha and resilon. *J. Dent. Tehran Univ. Med. Sci*. **2013**, *10*, 548.

42. Szymańska, E.; Winnicka, K. Stability of Chitosan—A Challenge for Pharmaceutical and Biomedical Applications. *Mar. Drugs* **2015**, *13*, 1819.

43. Peciuliene, V.; Balciuniene, I.; Eriksen, H.M.; Haapasalo, M. Isolation of *Enterococcus faecalis* in previously root-filled canals in a lithuanian population. *J. Endodont.* **2000**, *26*, 593.

44. Tchaou, W.S.; Turng, B.F.; Minah, G.E.; Coll, J.A. Inhibition of pure cultures of oral bacteria by root canal filling materials. *Pediatr. Dent.* **1996**, *18*, 444.

45. Silver, S. Bacterial resistances to toxic metal ions – a review. *Gene* **1996**, *179*, 9.

46. Podbielski A.; Spahr, A.; Haller, B. Additive antimicrobial activity of calcium hydroxide and chlorhexidine on common endodontic bacterial pathogens. *J. Endodont.* **2003**, 29, 340.

47. Castillo, J.A.; Clapés, P.; Infante, M.R.; Comas, J.; Manresa, Á. Additive antimicrobial activity of calcium hydroxide and chlorhexidine on common endodontic bacterial pathogens. *J. Antimicrob. Chemother*. **2006**, *57*, 691.

48. Fotos, P.; Hellstein, J. Candida and candidiasis: epidemiology diagnosis and therapeutic management. *Dent. Clin. North Am.* **1992**, *36*, 857.

49. Ferguson, J.W.; Hatton, J.F.; Gillespie, M.J. Effectiveness of intracanal irrigants and medications against the yeast *Candida albicans*. *J. Endodont.* **2002**, *28*, 68.

50. Waltimo, T.M.T.; Sirén, E.K.; Orstavik, D.; Haapasalo, M.P.P. Susceptibility of oral *Candida* species to calcium hydroxide *in vitro*. *Int. Endod. J.* **1999**, *32*, 94.

51. Sen, B.H.; Akdeniz, B.G.; Denizci, A.A. The effect of ethylenediamine-tetraacetic acid on *Candida albicans*. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **2000**, *90*, 651.

52. Tayel, A.A.; Moussa, S.; El-Tras, W.F.; Knittel, D.; Opwis, K.; Schollmeyer, E. Anticandidal action of fungal chitosan against *Candida albicans*. *Int. J. Biol.*

Macromol. **2010**, *47*, 454.

53. Costa, E.M.; Silva, S.; Madureira, A.R.; Cardelle-Cobas, A.; Tavaria, F.K.; Pintado, M.M. A novel direct contact method for the assessment of the antimicrobial activity of dental cements. *J. Microbiol. Methods* **2013**, *93*, 168.

54. Kulikov, S.N.; Lisovskaya, S.A.; Zelenikhin, P.V.; Bezrodnykh, E.A.; Shakirova, D.R.; Blagodatskikh, I.V.; Tikhonov, V.E. Antifungal activity of oligochitosans (short chain chitosans) against some *Candida* species and clinical isolates of *Candida albicans*: molecular weight-activity relationship. *Eur. J. Med. Chem.* **2014**, *74*, 169.

55. Gurgel-Filho, E.D.; Silva, E.J.; Gomes, B.; Ferraz, C.; Paula, R.; Coutinho-Filho, T.; Souza-Filho, F. Correlation between chemical composition and sealing ability of various gutta-percha brands using different filling techniques. *Rev. Port. Estomatol. Med. Dent. Cir. Maxilofac.* **2012**, *53*, 153.

56. Friedman, C.E.; Sandrik, J.L.; Heuer M.A.; Rapp, G.W. Composition and physical properties of gutta-percha endodontic filling materials. *J. Endodont.* **1977**, *3*, 304.

57. Venturi, M.; Di Lenarda, R.; Breschi, L. An *ex vivo* comparison of three different gutta-percha cones when compacted at different temperatures: rheological considerations in relation to the filling of lateral canals. *Int. Endod. J.* **2006**, *39*, 648.

LEGENDS OF THE FIGURES

Figure 1. Antimicrobial activity of ISO 60 sized chitosan-impregnated gutta-percha points with 0.14 mg of LMWC upon contact with *E. faecalis* during 48 hours. Vertical bars represent Standard Deviation (n=4).

Figure 2. Antimicrobial activity of ISO 60 sized impregnated gutta-percha points with different amounts of LMWC (0.14, 0.20 and 0.40 mg) upon contact with *E. faecalis* during 48 hours. Reinfection was applied 24h after the first infection. Vertical bars represent standard deviation (n=4).

Figure 3. Survival of *E. faecalis (a), P. buccae (b), P. gingivalis (c), P. stomatis (d) and C. albicans (e)* when in contact with ISO 60 sized impregnated gutta-percha points with 0.4 mg of LMWC during 48 hours. Reinfection was applied 24h after the first infection. Vertical bars represent standard deviation (n=4).

Figure 4. Effect of storage on ISO 60 sized chitosan (0.14 mg LMW)-impregnated gutta-percha points against *E. faecalis* in quantitative *in vitro* assays set for 7 hours (initial viable cell counts of 10^6-10^7 CFU/mL). a) 0 months, b) 1 month, c) 3 months and d) 6 months of storage at room temperature and dark conditions. Vertical bars represent standard deviation (n=4).