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Biocidal and Bioresorbable Chitosan/Triclosan/ Collagen Matrixes

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ABSTRACT

Biomedical application of biomaterials has increased in recent years. Some preferred characteristics of these materials are biocompatibility, biodegradability and antimicrobial effect. We are facing a constant search for antimicrobial materials to be used instead of antibiotics therapy to reduce the possibility of dental surgery contamination. In this study, biocidal, bioresorbable and non-toxic matrixes were synthesized from chitosan, triclosan and collagen. Three experimental groups received different chitosan/collagen combinations, (0.5:1, 0.75:1 and 1:1), all with the same dose of triclosan (0.1%). Antimicrobial effect was measured by the inhibition of *S. aureus* growth. Moreover, matrixes were placed in a PBS-collagenase solution to measure degradation over time; matrix residues were evaluated at 1, 4, and 7 days. Finally, cell toxicity of each matrix was analyzed on NIH-3T3 fibroblast cells. As a result, inhibition of *S. aureus* growth was similar in the three established experimental groups of matrixes vs vancomycin antibiotic as control. These data suggest potent antimicrobial effect of chitosan/triclosan/collagen matrixes. Degradation over time showed that 80% of the matrix was degraded after 4 days, thus suggesting that chitosan/triclosan/collagen matrixes are bioresorbable material. On the other hand, viability of NIH-3T3 cells was between 60% to 74% after 24 h and prior to matrix exposition to culture cells. These data indicate light toxicity in the 0.5:1 and 0.75:1 matrix groups and non-toxic effect in the 1:1 matrix group. By taking together these data, we propose the application of chitosan/triclosan/collagen matrixes to prevent bacterial contamination after dental surgery.

Introduction

Antibiotics are commonly used in dentistry for prophylaxis prior to an extraction procedure, though an increasing use of antibiotics has become a concern given the possible development of resistant bacteria and adverse effects associated with the use of the drugs [1]. Sidana et al. performed a controlled trial to evaluate the role of antibiotics in the perioperative period of dental extractions in healthy patients [2]. They evaluated patients treated with antibiotics for 3 days in the postoperative period, patients without antibiotics, and patients with a prescribed single dose of antibiotics 1 hour before the extraction procedure. The results indicated that they found no significant differences with respect to pain, swelling, or post-extraction complications. Therefore, the authors concluded that prophylactic antibiotics are not required during routine dental extractions in healthy patients.

Biomaterials based on chitosan and collagen components have been developed to contribute in regenerative medicine procedures [3]. Chitosan, an amino polysaccharide deacetylated from chitin (Figure 1), has been widely used in biomedical fields given its similar structure to glycosaminoglycans found in the extracellular matrix of bone [4]. One of the main biological properties of chitosan is its antimicrobial effect against *Staphylococcus aureus, Candida albicans,* and *Escherichia coli,* among others. Moreover, chitosan is non-toxic when cell viability was evaluated [5]. On the other hand, collagen comprises the majority of the extracellular matrix (Figure 1) in most soft and hard connective tissues, whereas *in vitro* natural collagen can be formed into highly organized, three-dimensional scaffolds that are intrinsically biocompatible, biodegradable and non-toxic [6]. Other effective antimicrobial material described has been triclosan, a polychlorinated bisphenolic compound (Figure 1). In clinical practice, triclosan is used as a disinfectant and antiseptic in surgical sutures, scrubs, implants, and medical devices [7]. Triclosan has shown efficacy and safety throughout long-term application at concentrations less than 0.3% [8].

Currently, there are some studies on biopolymers with antimicrobial effect for the elaboration of matrixes with medical applications. Therefore, the purpose of this work was the elaboration of bioresorbable polymers with antimicrobial effect to make matrixes, based on a combination of chitosan/triclosan/collagen, which could act as physical barrier in the alveolar area after dental extractions to avoid potential dental infections.

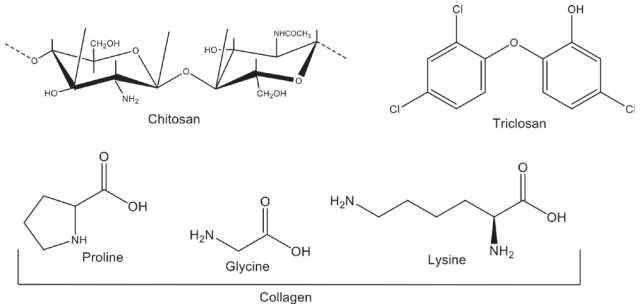

Figure 1: Molecular structure of chitosan, triclosan and type 1 collagen.

Materials and Methods

Matrix preparation: chitosan/triclosan/collagen

The following components were used: chitosan (Sigma Aldrich®, ≥75 %, deacetylated), triclosan (Corona S.A. de C.V), and porcine type 1 collagen from porcine skin prepared according to Gorlov, 2018 [9]. To prepare matrixes, 0.1 g of chitosan were dissolved in 10 mL of 1% acetic acid and mixed for 24 h at room temperature. Next, chitosan and collagen were poured into a flask according to specific ratios (Table 1) and stirred for 20 min. Subsequently, 0.1% triclosan was ground into fine powder and added to the chitosan/collagen mixture and stirred for 2 more hours. Finally, 125 µL of PBS (phosphate saline buffer, pH 7.4) were incorporated and drops of 1M NaOH was added to adjust the sample pH to 7.20 - 7.40.

The mixture was placed in a water bath at 37 °C until the components were completely diluted (approximately 1 h). Finally, the samples were frozen at -80 °C and lyophilized for 9 h at -50 °C and 37 Pa. Once the matrix was obtained following lyophilization (Figure 2), 6-mm diameter discs were made. The discs were employed both to inhibit *S. aureus* growth and in the degradation analysis.

Inhibition of S. aureus growth using chitosan/triclosan/collagen matrix

S. aureus (ATCC 1259) bacteria at 0.5 McFarland scale (1.5 x 108 CFU/mL) was prepared. For the disk diffusion test, *S. aureus* was seeded completely on the surface of Muller Hinton agar using a sterile swab. Next, discs of chitosan/triclosan/collagen (6-mm in diameter) were placed on the plate; the petri dish was sealed and incubated at 37 °C for 24 h.

Three antibiotics were used for positive experimental controls as follows: vancomycin (30 µg), clavulanic acid/amoxicillin (20/10 µg), and ampicillin (10 µg); all agents were from Becton Dickinson (BD, BBL sensi-disc antimicrobial susceptibility test). The negative control consisted of a sterile paper disk with 20 µL of collagen.

Designation	Chitosan/collagen (v/v)	Triclosan (p/v)
Group 1	0.5:1	0.1%
Group 2	0.75:1	0.1%
Group 3	1:1	0.1%

Table 1: Formulation of chitosan/triclosan/collagen matrixes

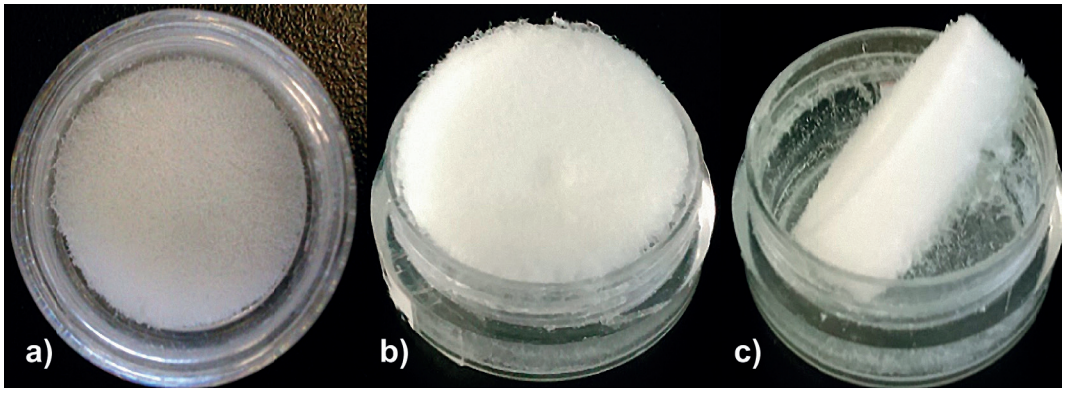

Figure 2: Shape and size of chitosan/triclosan/collagen matrixes obtained after lyophilization, a) matrix surface and shape; b) texture; c) matrix thickness.

Characterization of the chitosan/triclosan/collagen matrix

To determine functional groups or biomolecules in the chitosan/triclosan/collagen matrixes, Fourier transform infrared (FT-IR) method was applied (Bruker Vector 33 Instrument) followed by Raman dispersion spectra (Senterra Bruker Raman spectrometer coupled to an Olympus microscope with 50X objective) at 16 cm^{-1} resolution, ranging from 600-4100 cm^{-1}.

Degradation of matrix: chitosan/triclosan/collagen

Discs (6 mm in diameter) made of chitosan/triclosan/collagen matrix were weighed and the initial value (g) was recorded. Next, PBS buffer solution and type 1 collagenase (100 µg/mL concentration) were added [10]. Samples were incubated at 37 °C for 1, 4 or 7 days. After the incubation period, the supernatant was analyzed at a wavelength of 350 nm. Finally, samples were allowed to dry at room temperature for two days and weighed.

Determination of fibroblast cell viability

Cytotoxicity was tested using the MTT viability assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. NIH-3T3 mouse fibroblast cell line (ATCC; CL173) was cultured in Dulbecco´s Modified Eagle´s Medium (DMEM, Sigma), supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA) and antibiotics (10 µL streptomycin and 100 U/mL penicillin; Sigma), and kept at 37 °C and 5% CO_2 atmosphere. Subsequently, cells were trypsinized and counted. A predetermined cell concentration (1.5 x 10^4 cells/mL) was placed in a 24-well plate and incubated for 24 h. Discs of chitosan/triclosan/collagen matrix were added and incubated for 24 h. Samples were removed and 50 µL of MTT solution (5mg/mL) were added and incubated for 4 h at 37 °C. Culture medium was removed and 150 µL DMSO was added. Finally, 100 µL of the solution was placed in a 96-well plate. The absorbance was measured in a microplate reader (Bio-Rad - 680) at a wavelength of 595 nm.

Statistical analysis

Results of antimicrobial effect and cell viability were analyzed by one-way ANOVA, ±SD ($P<0.05$) and Tukey test. Statistical analysis was carried out by Prism V5 software. Results represent at least three independent experiments performed in triplicate (n=3).

Results and Discussion

Inhibition of S. aureus growth

The inhibition of *S. aureus* growth was determined by measuring the diameter of the inhibition zone in the three independent experimental groups described previously. In all cases, the inhibition of *S. aureus* growth was observed 24 h after incubation at 37 °C (Figure 3).

As it is apparent, biocidal effect of amoxicillin/clavulanic acid and ampicillin (controls) was more effective than chitosan/triclosan/collagen matrixes in all cases. In addition, it was observed that the antimicrobial activity of the matrixes was similar to vancomycin (the other control). The corresponding inhibition zones (radial distance) for groups 1, 2, 3 and vancomycin are: 7.79 ± 0.53; 8.40 ± 0.49; 7.83 ± 0.59 and 8.24 ± 0.16 mm, respectively, Group 2 showed a slightly higher inhibition zone in comparison with vancomycin. Figure 4 shows the inhibition values obtained for after 24 h incubation.

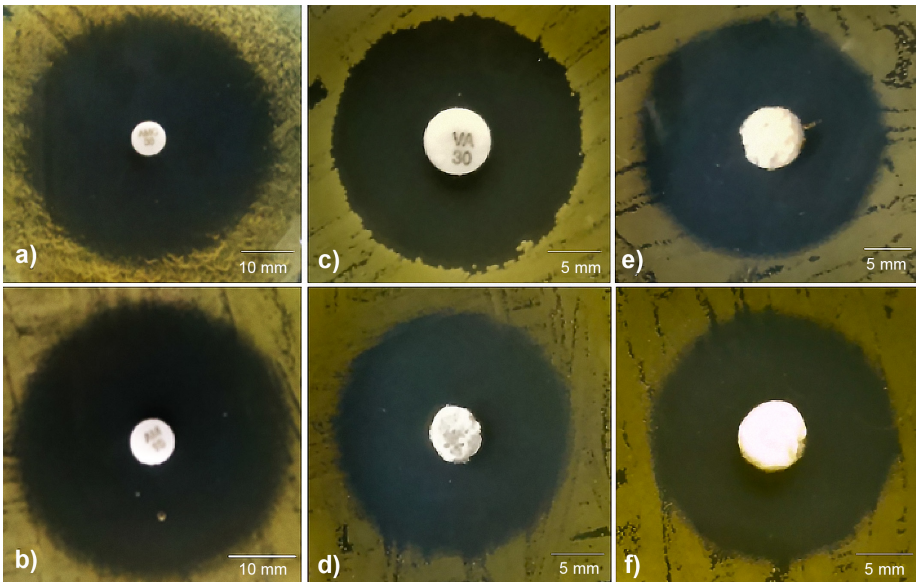

Figure 3: Inhibition zone obtained in S. aureus cultures after 24 h of incubation: a) Amoxicillin/clavulanic acid, b) Ampicillin, c) Vancomycin, d) Group 1, e) Group 2, f) Group 3.

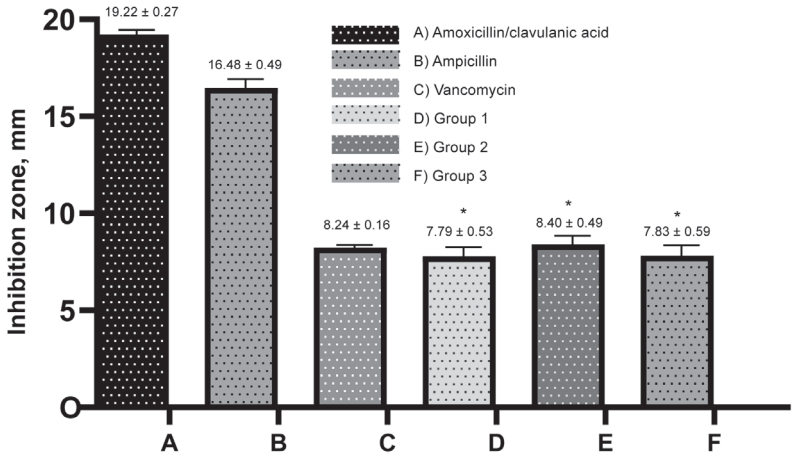

*Figure 4: Comparison of the inhibition zones (radial distance) over a 24 h period using One-way ANOVA test (P <0.05), followed by a comparison between groups using Tukey post hoc test. *P≤0.05 represents significant difference.*

Our data support the idea proposed by other authors in relation to the antimicrobial properties of the matrixes, in which the biocidal effect may be due to chitosan and triclosan properties [11]. However, for the first time, chitosan and triclosan potentiated the antimicrobial effect in the collagen composite matrix. Triclosan affects the cytoplasmatic membrane of both Gram negative and positive bacteria [12]. It has been reported that in order to prevent microbial colonization of suture materials in operative incisions, triclosan-coated materials have been developed and used. The Global Guidelines on the Prevention of surgical site infection by World Health Organization (WHO), were recently published and the use of triclosan- coated sutures was recommended to reduce the risk of infection [12, 13]. Other authors previously reported that the antimicrobial activity of chitosan is pH dependent; its activity only occurs in an acidic environment. However, this finding has not been demonstrated. Meanwhile, the results obtained in this work showed that matrixes at neutral pH exhibit an inhibitory effect on *S. aureus* growth. Similar data have been described by other authors, who assayed antimicrobial activity on scaffolds composed of chitosan/nano-hydroxyapatite/silver nanoparticles for bone tissue regeneration [14]. The inhibition zone for these scaffolds was determined using *S. aureus* ($1x10^8$ CFU/mL) and measured on average 13.34 ± 2.75 mm in diameter. Whereas, in the present study we obtained inhibition zones (radial distance) in the range of 7.8 to 8.4 mm for our chitosan/triclosan/collagen matrixes; these values indicate an increase in biocidal potential.

Molecular distribution of chitosan/triclosan/collagen matrixes

The results obtained by Raman spectroscopy are shown in Figure 5. These indicate that the band 910 cm^{-1} corresponds to the amino acid glycine (894 cm^{-1}) and N-acetyl-D-glucosamine (973 cm^{-1}). The band at 1370 cm^{-1} corresponds to the combination of two characteristic bands of N-acetyl-D-glucosamine, with medium intensity identified at 1380 cm^{-1}. In turn, the band at 2880 cm^{-1} corresponds to CH bond and at 3387 cm^{-1} to the NH_2 group. These bands must have strong intensity according to the Raman spectrum database by Gelder, 2007 [15].

According to FTIR analysis of experimental groups (Figure 6), representative bands of functional groups corresponded to -NH_2 and -OH groups (3404 cm^{-1}) present in the chitosan deacetylated solid structure [4]. The characteristic band of collagen and chitosan, which is located at a length of 2925 cm^{-1}, could be related to the asymmetrical stretch of CH [16]. The carbonyl group is also present at 1540 cm^{-1}; this group corresponds to the chitosan molecule and amide II band in collagen [17]. The salient absorption bands at around 1020 $cm^{-1}$and 1070 cm^{-1} are identified as the coupled C–C and C–O stretching vibrations, respectively. Finally, to confirm triclosan presence, the peaks in the region from 1300 to 1000 cm^{-1} and 900 to 750 cm^{-1} represent in-plane and out-of-plane bending of the aromatic ring C–H bonds, respectively [18]. Therefore, we have confirmed the presence of the three molecules after mixing chitosan, triclosan and collagen to form matrixes.

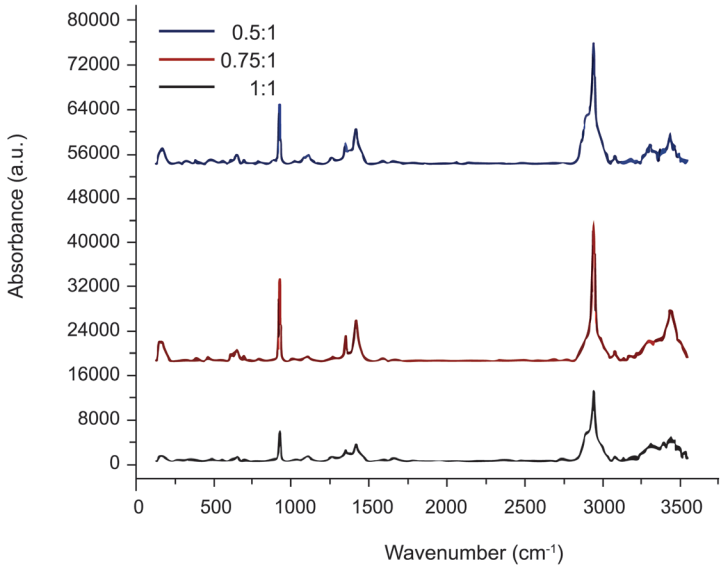

Figure 5: Raman spectroscopy results for the three experimental chitosan/triclosan/collagen matrixes.

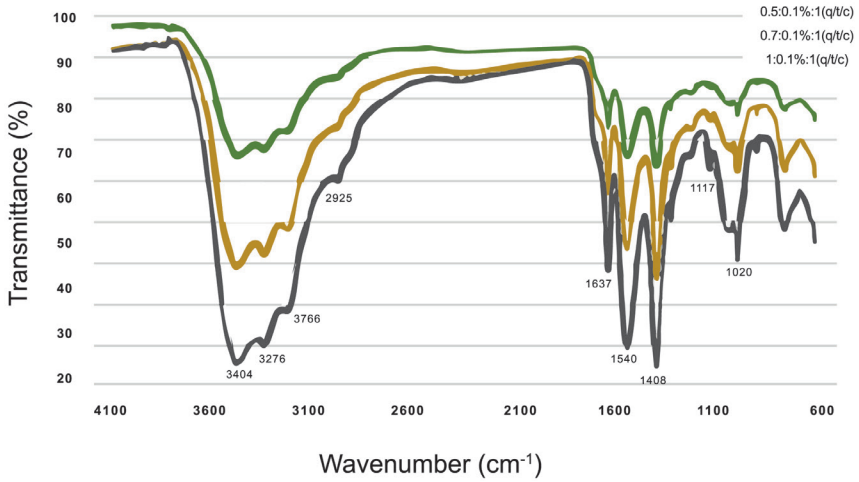

Figure 6: Results of FT-IR for the three experimental groups.

Matrix degradation by type 1 collagenase

The degradation of the matrixes was carried out by means of a solution of PBS and type 1 collagenase at 37 °C. Once the incubation time was over, the solution was measured at 350 nm. Three calibration curves were performed for each of the three experimental groups to identify the final concentration of the suspension. Table 2 shows concentration values of the degraded matrixes for periods of 1, 4 and 7 days, as well as the percentage of degradation during these periods. The results obtained showed that on day 4 the percentage of degradation was 91.00 ± 9.30% for group 1, followed by groups 2 and 3 with a percentage of 83.37 ± 8.79% and 80.37 ± 4.90 %, respectively.

Degradation was mainly observed in a period between 24 and 96 h. A previous study showed that PBS solution and type 1 collagenase (100 µg/mL) were able to completely degrade a type 1 collagen scaffold at 37 °C (12 h incubation conditions) and that 12 hours were necessary to degrade 92.1% of chitosan/collagen (1:9 ratio) scaffold exposed to PBS-collagenase solution [10]. In this context we report similar results, using the same collagenase concentration.

It has been reported that degradation of chitosan/nano-hydroxiapatite or chitosan/nano-hydroxiapatite/silver nanoparticles scaffolds in a solution of lysozyme concentration similar to circulating blood levels (10 mg/L) for 3 days at 37 °C was about 80% for the first scaffold and in the case of the chitosan/nano-hydroxyapatite/silver nanoparticles scaffold, degradation was approximately 60% [14]. However, we obtained 80% degradation in chitosan/triclosan/collagen matrixes by employing type 1 collagenase after 1 day.

Experimental Group	Time (Days)	Concentration (g/mL)	Percentage of Degradation (%)
1	1	0.35 ± 0.16	83.10 ± 4.92
	4	0.61 ± 1.77	91.00 ± 9.30
	7	0.65 ± 0.23	78.37 ± 3.50
2	1	0.46 ± 0.68	77.37 ± 4.33
	4	0.76 ± 0.56	83.37 ± 8.79
	7	0.77 ± 2.27	75.90 ± 10.70
3	1	0.27 ± 1.02	77.77 ± 5.06
	4	0.57 ± 0.48	80.37 ± 4.90
	7	0.56 ± 3.24	76.53 ± 4.91

Table 2: Concentration of matrix residues in PBS/type 1 collagenase solution at 350 nm and percentage of mass loss of the matrixes at the following incubation times: 1, 4 and 7 days.

Toxicity assay by MTT test

The biological response of the matrixes was evaluated in terms of the metabolism of fibroblast cultures with the material. For a 24-h period, the metabolic activity of mitochondria was assessed in a viability test. Our results showed that when control cells were compared with the experimental groups a significant difference was found in the comparison to experimental groups 1 and 2, but no significant differences were observed when control cells were compared to group 3 (Figure 7). High biocompatibility and intrinsic biodegradability by endogenous collagenase makes exogenous collagen ideal for use in biomedical applications [6]. Finally, the use of triclosan in surgery materials has not been prohibited, its mentioned that a secure dosis for triclosan is less than 0.3%, so that, in the present study all experimental matrixes were prepared using 0.1 % of triclosan and they showed high bactericidal effect [8].

4D-printed structures should possess smart structures and high resolution so that they can be thermo-mechanically programmed to change into functional configurations. The shape-shifting behavior is resultant from the differences in dimensional changes' ratios (coefficient of thermal expansion, modulus of elasticity) of the internal ingredients. Modification of the materials behavior in microscale can allow for their use in stem cell and tissue-engineering research for the production of scaffolds [12].

More collaborative research work needs to focus on the introduction of 4D printing in dentistry; and further prospective analyses of the potential applications of this technique need to be discussed.

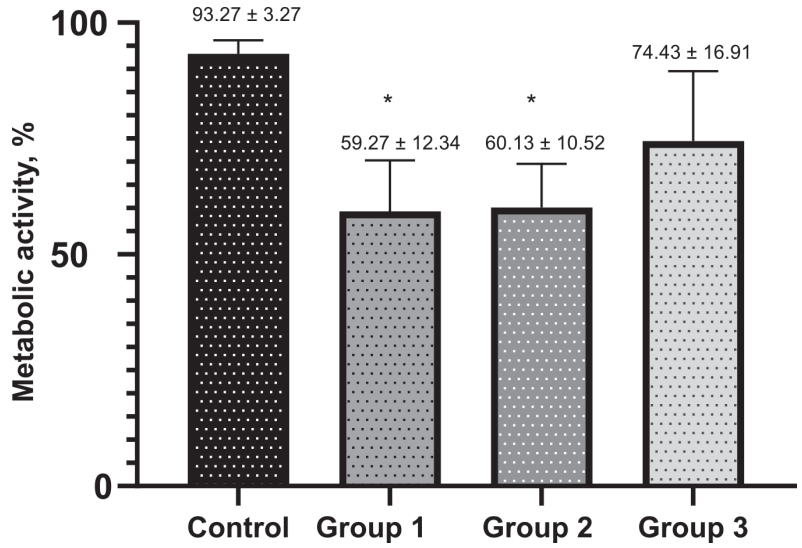

*Figure 7: Viability of NIH-3T3 cells after 24-h treatment. Comparison of the means was analyzed by a One-way ANOVA test (P <0.05), followed by Tukey's multiple comparison test (significance was set at p< 0.05. * represents significant difference.*

Conclusion

The matrix with better biological behavior was group 3, which was constituted of chitosan/collagen (1:1 v/v) and 0.1% triclosan. This combination exhibited the highest antimicrobial effect (inhibition zone = 7.83 ± 0.59 mm) against *S. aureus.* In addition, matrix degradation was approximately 80% after 7 days. Moreover, cytotoxicity was absent at 24-h incubation with fibroblast cells. Therefore, we obtained biocidal and bioresorbable chitosan/triclosan/collagen matrixes with appropriate features and potential use after dental extraction to prevent infections and to avoid antibiotic therapy.

References

1. Susarla SM, Sharaf B, Dodson TB. Do antibiotics reduce the frequency of surgical site infections after impacted mandibular third molar surgery? Oral Maxillofac Surg Clin North Am 2011;23(4):541-546.

2. Sidana S, Mistry Y, Gandevivala A, Motwani N. Evaluation of the need for antibiotic prophylaxis during routine intra-alveolar dental extractions in healthy patients: A randomized double-blind controlled trial. J Evid Based Dent Pract 2017;17(3):184-189.

3. Lim Y, Ok Y, Hwang S, Kwak J, Yoon S. Marine collagen as a promising biomaterial for biomedical applications. Mar Drugs 2019;17(8):467.

4. Xianshuo C, Jun W, Min L, Yong C, Yang C, Xiaolong Y. Chitosan-collagen/organo-montmorillonite scaffold for bone tissue engineering. Front Mater Sci 2015;9(4):405-412.

5. Rodríguez-Vázquez M, Vega-Ruiz B, Ramos-Zúñiga R. Saldaña-Koppel DA, Quiñones-Olvera LF. Chitosan and its potential use as a scaffold for tissue engineering in regenerative medicine. Biomed Res Int 2015; Article ID 821279, 15 pages.

6. Chattopadhyay S, Raines R. Collagen-based biomaterials for wound healing. Biopolymers 2014;101(8):821-833.

7. Petersen RC. Triclosan antimicrobial polymers. AIMS Mol Sci 2016;3(1):88-103.

8. Australian Government, Department of Health and Ageing NICNAS. National Industrial Chemical Notification and Assessment Scheme, Sydney Australia. 2009. Priority Existing Chemical Assessment Report No. 30, Triclosan Available from: http://www.nicnas.gov.au/publications/car/pec/pec30/pec_30_full_report_pdf.pdf

9. Gorlov IF, Titov EI, Semenov GV, Slozhenkina MI, Sokolov AY, Omarov RS, Goncharov AI, Zlobina EI, Litvinova EV, Karpenko EV. Collagen from porcine skin: a method of extraction and structural properties. Int J Food Prop 2018;21(1):1031-1042.

10. Ma L, Gao C, Mao Z, Zhou J, Shen J, Hu X, Han C. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials 2003:24:4833-4841.

11. Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: A state of the art review. Int J Food Microbiol. 2010; 144:51-63.

12. Henriksen N, Deerenberg E, Venclauskas L, Fortelny R, Garcia-Alamino JM, Miserez M, Muysoms F. Triclosan-coated sutures and surgical site infection in abdominal surgery: the TRISTAN rewiew, meta-analysis and trial sequential analysis. Hernia. 2017; 21: doi 10.1007/s10029-017-1681-0.

13. Ichida K, Noda H, Kikugawa R, Hasegawa F, Obistu T, Ishioka D, Fukuda R, Yoshizawa A, Tsujinaka S, Rikiyama T. Effect of triclosan-coated sutures on the incidence of surgical site infection after abdominal wall closure in gastroenterological surgery: a double-blind, randomized controlled trial in a single center. Surgery. 2018; 164:91-95

14. Saravanan S, Nethala S, Pattnaik S, Tripathi A, Moorthi A, Selvamurugan, N. Preparation, characterization and antimicrobial activity of a bio-composite scaffold containing chitosan/nano-hydroxyapatite/nano-silver for bone tissue engineering. Int J Biol Macromol 2011; 49:188-193.

15. GelderJ, Gussem K, Vandenabeele P, Moens L. Reference database of Raman spectra of biological molecules. J Raman Spectrosc 2007;38,1133-1147.

16. Fauzi MB, Lokanathan Y, Aminuddin BS, Ruszymah BHI, Chowdhury SR. Ovine tendon collagen: extraction, characterisation and fabrication of thin films for tissue engineering applications. Mater Sci Eng C 2016; 68:163-171.

17. Grønlien KG, Pedersen ME, Sanden KW, Høst V, Karlsen J, Tønnesen HH. Collagen from Turkey (Meleagris gallopavo) tendon: A promising sustainable biomaterial for pharmaceutical use. Sustain Chem Pharm 2019;13,100166.

18. Jug MI, Kosalec I, Maestrelli F, Mura P. Analysis of triclosan inclusion complexes with β-cyclodextrin and its water-soluble polymeric derivative. J Pharm Biomed Anal 2011;54(5):1030-9.