Interdiscip Toxicol. 2021; **Vol. 14**(1): 20–31.
doi: 10.2478/intox-2021-0004

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Biocompatibility of cleaning agents for ocular prostheses

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ITX140124A04 • Received: 01 August 2020 • Accepted: 04 May 2021

ABSTRACT

This study aimed to access the cytotoxicity, on the human conjunctival cell line, of different agents (neutral soap, 4% chlorhexidine, Efferdent effervescent tablets, 1% triclosan, and citronella essential oil) used for cleaning ocular prosthesis. Human conjunctival cell line was cultivated on acrylic resin specimens and the cytotoxicity of the studies cleaning agents was evaluated by MTT, Neutral Red, and RT-PCR (Real-time reverse transcription polymerase chain reaction) for the gene expression of type IV collagen, transforming growth factor β, myeloid cell leukemia sequence 1, matrix metalloproteinase 9, and caspases 3 and 9. Data were submitted to the two-way analysis of variance (ANOVA) and Bonferroni ($p<0.05$) tests. All cleaning agents showed biocompatibility for the human conjunctival cells. This, immersion in 4% chlorhexidine, effervescent tablets, and 1% triclosan could be the best protocols indicated for ocular prosthesis cleaning due to their biocompatibility.

KEY WORDS: artificial eye; acrylic resins; toxicity; materials testing, conjunctiva

Introduction

Ocular prosthesis is an important rehabilitation treatment for patients who suffered eye loss (Goiato *et al.,* 2007; Paranhos *et al.,* 2007). The prosthesis not only restore the aesthetic, but also protects the anophthalmic cavity and avoids the eye lid collapse (Perrone & Azambuja, 1996). Acrylic resins are the most used material for fabrication of ocular prosthesis making them much more versatile, resistant, and comfortable to use (De Caxias *et al.,* 2019).

Daily cleaning to control of biofilm formation is very important to maintain the health of the anophthalmic cavity of ocular prosthesis wearers, and can be performed with different cleaning agents, such as neutral soap (Goiato *et al.,* 2012; Pesqueira *et al.,* 2011), chlorhexidine (Goiato *et al.,* 2012; Moreno *et al.,* 2014; Altieri *et al.,* 2013), triclosan (Aiello *et al.,* 2007), effervescent tablets (Goiato *et al.,* 2012; Moreno *et al.,* 2014; De Andrade *et al.,* 2007; Goiato *et al.,* 2013) and citronella essential oil, which is an innovative method.

Although disinfection of the prosthesis is indispensable, the patient, after using the disinfectant, may not properly rinse the prosthesis before placing it back in the anophthalmic cavity. Also, acrylic resin is a material that can become rough or porous over time, and can accumulate substances on its surface. These substances can be released into the anophthalmic cavity, possibly causing some damage to the mucosa of cavity (Pavarina *et al.,* 2003). Most chemicals in contact with the skin or mucosa can be potentially irritating. Toxic potential of cleaning agents has already been tested in many types of cells (Le Coz & Bezard 1999; Lachapelle, 2014; Arabaci *et al.,* 2013; Kpoviessi *et al.,* 2014). Studies showed some of the irritation and sensitization effects of these materials which are: chlorhexidine can cause rare allergic reactions such as contact dermatitis, angioedema, anaphylaxis (Lachapelle 2014), and can induce necrosis and apoptosis (Gianneli *et al.,* 2008); triclosan is non-cytotoxic, non-irritating, and not a chemical pyrogen (Barbolt, 2002); and citronella essential oil has cytotoxic effect in epithelial cells (Cunha *et al.,* 2020). Data regarding irritation and sensitization of neutral soap and effervescent tablets still lack in literature. Despite of available data regarding

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cellular toxicity of these materials, there are no data in the literature on the human conjunctival cell lines, which is one of the only tissues that remains after surgical resection of the eyeball.

Chang human conjunctival cell line has been used for *in vitro* studies that assess, for example, the biocompatibility of substances on the conjunctiva (Walter *et al.,* 2010). *In vitro* biocompatibility tests are important to guarantee the safety and text cytotoxicity of substances in humans. The cell culture method is simple to be performed, reproductible, can be controlled and have an adequate cost-benefit ratio (Poggio *et al.,* 2010; Bural *et al.,* 2011).

Based on the above information, this study can be justified by the importance of assessing the cytotoxicity of previously mentioned cleaning agents used for disinfection of ocular prosthesis before their indication, since many studies demonstrated their side effects in several types of cells, but not on human conjunctival cells. Thus, this study aimed to access the cytotoxicity, on the human conjunctival cell line, of different agents (neutral soap, 4% chlorhexidine, Efferdent effervescent tablets, 1% triclosan, and citronella essential oil) used for cleaning ocular prosthesis. The hypothesis was that the cleaning agents tested would not produce toxic effects on the human conjunctival cell line.

Material and methods

Manufacturing of acrylic resin specimens

The acrylic resin specimens were prepared with a metallic matrix containing 10 circular compartments with 10 mm in diameter and 3 mm in thickness according to manufacturer's recommendations. An N1 heat-polymerized resin (white color) was manipulated and placed into a flask, specifically for microwave polymerization (VIPI STG, VIPI Indústria, Pirassununga, SP, Brazil), which was placed in a hydraulic press (VH, Midas Dental Produtos Ltda, Araraquara, SP, Brazil) with a force of 1200 kg/F for 2 minutes. A bench polymerization was performed for 30 min, followed by microwave-energy polymerization for 10 minutes and then the specimens were removed from the flask (Santos *et al.,* 2016; Monteiro *et al.,* 2012). Polishing was made for 3 minutes using sandpapers with different granulations (Buehler, Lake Bluff, IL, USA) and a colloidal silica solution (1 micron-granulometry, Buehler, Lake Bluff, IL, USA). Then, an ultrasonic cleaning (Arotec, Odontobrás, Ribeirão Preto, Brazil) was performed for 20 minutes in distilled water to remove possible debris from the resin surface. After that, the specimens were left on the bench for drying.

Cytotoxicity test

Specimens were submitted to treatment processes described in Table 1. Each treatment was performed daily for different periods of time: 1, 7, 15, 30, 60, and 90 days, using three different test specimens for each period (totaling 180 specimens). After each period, the cytotoxicity was assessed by using the eluates of the hydrosoluble substances present in the previously disinfected specimens (Jorge *et al.,* 2007; Lefebvre *et al.,* 1995).

Specimens from each group were placed in a tube containing 6 ml of 199 culture medium (Gibco, New York, USA) supplemented with 5% concentration of fetal bovine serum (FBS), previously determined by a pilot study, and incubated in an oven at 37 °C for 24 hours. Subsequently, 0.22 µm Millex filters (Merck Millipore Corporation, Darmstadt, Germany) were used to filter the eluates.

For culture and maintenance of cells, the human conjunctival cell line (Wong Kilbourne derived from the Chang conjunctiva, clone 1-5c-4), obtained from the American Type Culture Collection (CCL-20.2, Manassas, VA, USA), was used and expanded in culture medium supplemented with 10% FBS. Cells were incubated at 5% CO_2 and 37 °C (Badowin *et al.,* 2007; Da Silva *et al.,* 2016a; Da Silva *et al.,* 2016b; Debbasch *et al.,* 2011)

Table 1. Procedures performed on acrylic resin specimens and cleaning agents' composition

Antibiofilm solution	Treatments	Manufacturer	Composition
Saline	Immersion in 1 ml for 10 min	Apothicario Manipulation Pharmacy, Araçatuba, SP, Brazil	0.9% sodium chloride (NaCl)
Neutral soap	Digital friction for 30 sec	Johnson & Johnson, São José dos Campos, São Paulo, Brazil	Aqua, Cocamidopropyl Betaine, PEG-80 Sorbitan Laurate, Glycerin, PEG-150 Pentaerythrityl Tetrastearate, PPG-2 Hydroxyethyl Cocamide, Decyl Glucoside, Phenoxyethanol, Sodium Methyl Cocoyl Taurate, Sodium Benzoate, Citric Acid, Parfum, Ethylhexylglycerin
4% Chlorhexidine	Immersion in 1 ml for 10 min (Goiato *et al.,* 2012; Pesqueira *et al.,* 2011)	Apothicario Manipulation Pharmacy, Araçatuba, SP, Brazil	Chlorhexidine diglyconate, distilled water
Effervescent tablets - Efferdent Original Denture Cleanser	Immersion in sterile distilled water containing a tablet at 37 °C for 15 min (Moreno *et al.,* 2014; Goiato *et al.*, 2013)	Pfizer Consumer Health, Morris Plains, New Jersey, USA	Potassium monopersulfate, sodium perborate, sodium bicarbonate
1% triclosan	Immersion in 1 ml for 10 min	Apothicario Manipulation Pharmacy, Araçatuba, SP, Brazil	Triclosan, propylene glycol
Citronella essential oil	Immersion in 1 ml for 10 min	Apothicario Manipulation Pharmacy, Araçatuba, SP, Brazil	Citronella, distilled water

until they reached confluence (Lachapelle, 2014; Ayaki *et al.,* 2012). Then, cell suspensions of 1x105 cells/ml were used and 1 ml was pipetted into each well of a 24-well plate. After 24 hours, the culture medium was discarded and 500 µl of each previously obtained eluate containing 5% FBS was added to each well. After incubation, MTT and Neutral Red (NR) assays were performed.

The MTT is a colorimetric analysis used to measure mitochondrial metabolic activity. After 24 hours, eluates were replaced with medium without FBS, containing 0.5 mg/ml of MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide), and incubated at 37 °C for 2 hours (Santos *et al.,* 2016; Abena *et al.,* 2007). After incubation, the medium was aspirated and 500 µl of isopropyl alcohol was added to each well. Absorbances were measured at the 570 nm wavelength using a UV-visible spectrophotometer (SpectraMax 190, Molecular Devices, San Jose, CA, USA) (Bural *et al.,* 2011; Bal *et al.,* 2009). The NR assay measures lysosomal activity in a culture (Siguschi *et al.,* 2009). After 24 hours, eluates were replaced with 0.5 ml of NR dye and plates were incubated at 37 °C for 2 hours. Then, NR and medium were replaced with 500 µl of 1% formic acid for washing. The formic acid was removed and 1 ml of 1% acetic acid was added to each well. After extracting the dye from the cells, cell viability was assessed by the optical density of the resulting solution at 540 nm using a UV-visible spectrophotometer.

For MTT and NR assays, negative control consisted of untreated cell cultures (1 ml of culture medium with 5% FBS), while positive control consisted of medium with Tween 20 (Sigma-Aldrich, Saint Louis, MO, USA). Both were submitted to the same conditions of incubation and temperature used to obtain eluates.

The RT-PCR (Real-time reverse transcription polymerase chain reaction) was performed for quantitative analysis of gene expression of tissue repair markers, type IV collagen (COL IV; COL4A3BP: Hs00178621_m1), transforming growth factor β (TGF-β; TGFB1: Hs0099133_m1), matrix metalloproteinase 9 (MMP9; Hs00234579_m1), and of markers related to apoptosis and cell death, myeloid cell leukemia sequence 1 (MCL-1; MCL1: Hs01050896_m1), and caspases 3 (CASP3: Hs0023487_m1) and 9 (CASP9: Hs00609647_m1) (Bernabe *et al.,* 2011, Oliveira & Santos 2011). The assay was performed according to the manufacturer's recommendations using a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA, USA). The β-actin (ACTB: Hs030223880_g1) was used as an endogenous control. Results were analyzed using the comparative CT (Cycle Threshold).

Cytotoxicity analyses were carried out in compliance with ISO standard 10993-5 for *in vitro* toxicity analysis. Cell proliferation percentages of the controls represented 100% and cell viability (%) was calculated for all groups (Jorge *et al.,* 2007; Jorge *et al.,* 2007).

Data analysis

Normality test showed normal distribution of the obtained data, which were submitted to two-way analysis of variance (ANOVA) and Bonferroni tests ($p<0.05$). The variation factors were cleaning agents (neutral soap, 4% chlorhexidine, Efferdent effervescent tablets, 1% triclosan, and citronella essential oil) and periods (1, 7, 15, 30, 60, and 90 days).

Results

Two-way ANOVA showed a significant relationship between variation factors (cleaning agents and periods) interfering in the results of cytotoxicity assays (MTT, NR, and RT-PCR) ($p<0.001$ for all factors). In the MTT assay, the lowest cell proliferation values were observed for the neutral soap group, which were statistically lower than the non-stimulated group in 5 of the 6 evaluated periods (1, 7, 15, 60, and 90 days), with the lowest percentage (84.35%) at 60 days (Figure 1, Table S1). On the other hand, the highest cell proliferation values were found for the citronella group, which were statistically higher than the non-stimulated group in 3 of the 6 evaluated periods (30, 60, and 90 days) (Figure 1). In addition, although significant differences were observed in the comparisons among treatment periods within the same group, there was no pattern of increase or decrease in cell proliferation in both MTT and NR assays for any of the cleaning agents and periods evaluated. However, for citronella, periods of 15, 30, 60, and 90 days presented statistically higher proliferation values than the period of 1 day, and especially at 30 days (Figure 1).

In the NR assay, although some groups showed a significant difference when compared to the non-stimulated group, it was observed that none of the evaluated cleaning agents showed a potential cytotoxic activity, since all percentages were higher than 90% (Figure 2, Table S2). The analysis showed that the highest percentage was found for the citronella group (109.3% - day 1), and this was the only group statistically different from the untreated group at 1 day, and also at other periods evaluated (Figure 2).

For both assays (MTT and NR), although the interaction between factors (cleaning agents and periods) interfered in the results, and there were statistically significant differences between treated groups and the control group within a period, all values were higher than 84%, indicating no cytotoxicity of the agents tested (Figures 1 and 2).

Figures 3 to 8 and tables S3 to S8 show the RT-PCR results. For the COL IV (Figure 3, Table S3), the triclosan presented higher gene expression, with statistical difference from the control group, except when higher gene expression was found for neutral soap at 60 days and for citronella at 90 days (Figure 3).

In the analysis of TGF-β (Figure 4, Table S4), triclosan (1 and 15 days) and neutral soap (7 and 60 days) showed the highest gene expression, with statistical difference from the control group. At 30 days, all groups showed lower gene expression with statistical difference from the control group, except for the saline group. On the other hand, citronella presented higher gene expression at 90 days.

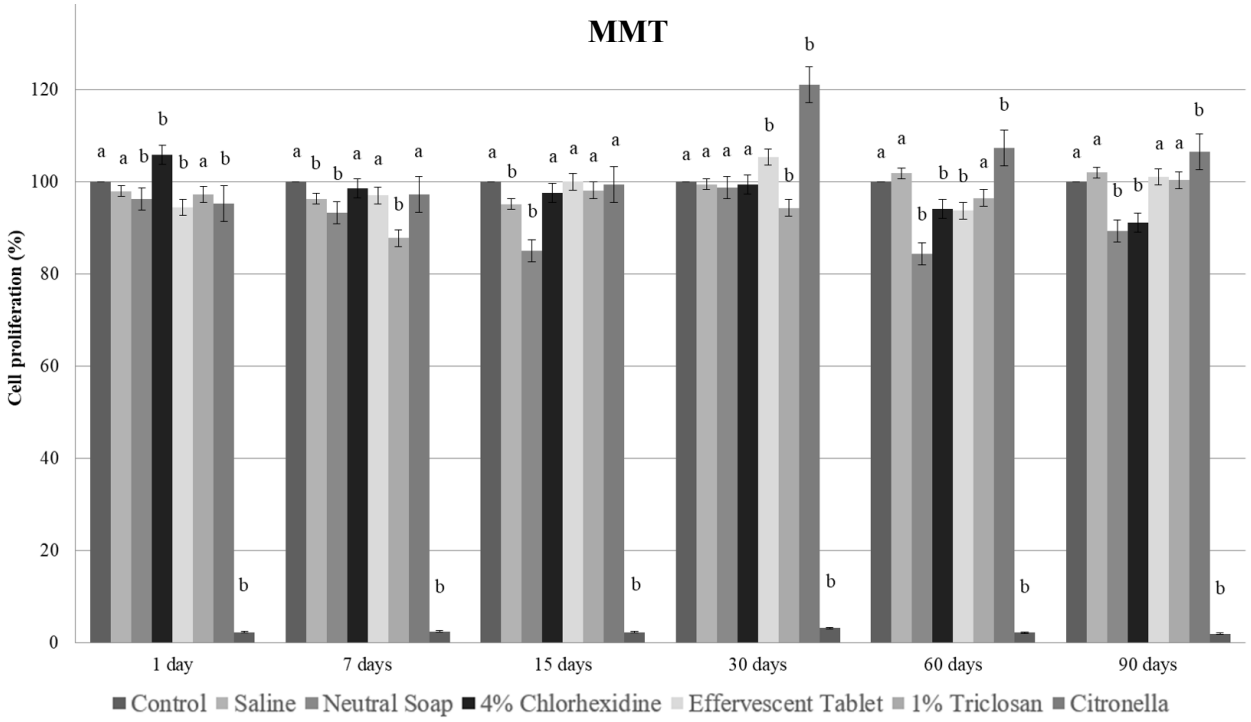

Figure 1. Mean ± standard deviation of cell proliferation percentages obtained by MTT analysis for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

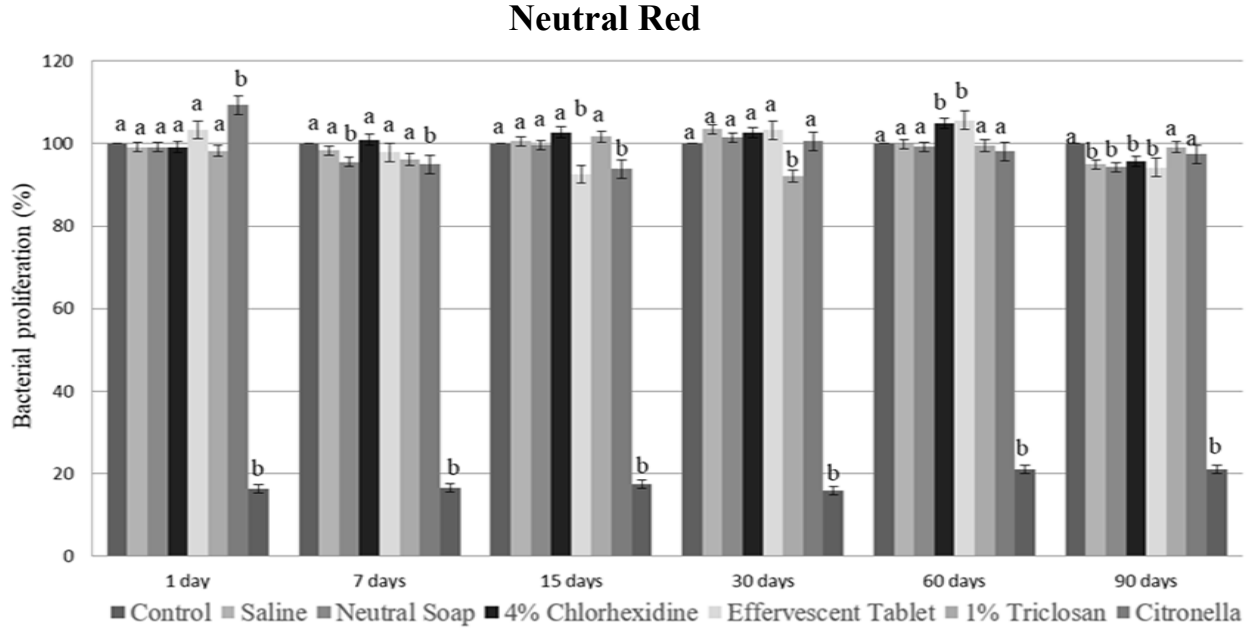

Figure 2. Mean ± standard deviation of cell proliferation percentages obtained by NR analysis for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

For the MCL-1 (Figure 5, Table S5), the gene expression varied over time. At 1 day, all groups had gene expression smaller and statistically different from the control group. At 7 and 30 days, the triclosan group showed the highest gene expression. At 15 days, higher gene expression was observed for the citronella group. The group treated with neutral soap had the highest gene expression at 60 days, and the chlorhexidine group at 90 days.

Figure 6 and table S6 show the MMP-9 gene expression of the tested groups. There was a variation among groups over the analysis periods. At 1 day, the citronella group showed the highest gene expression and was

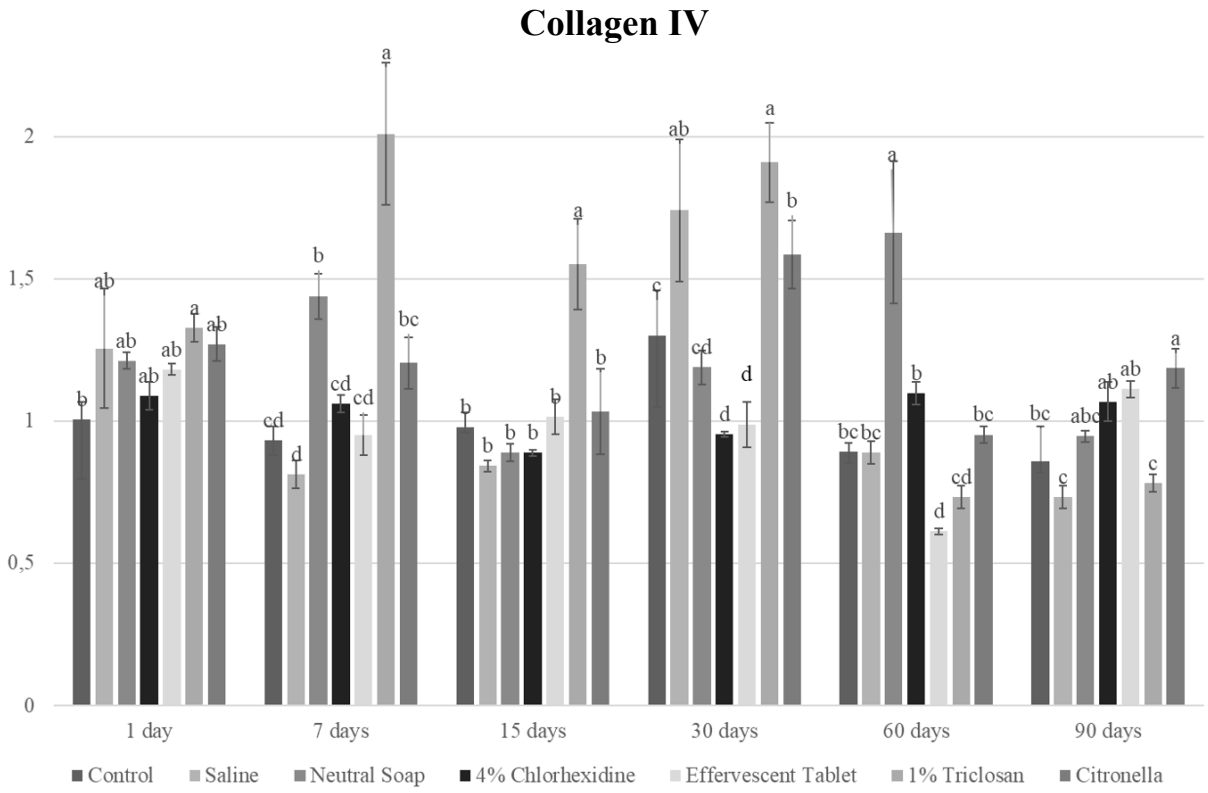

Figure 3. Relative quantification of mRNA for COL IV ± standard deviation for evaluated groups in different periods Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

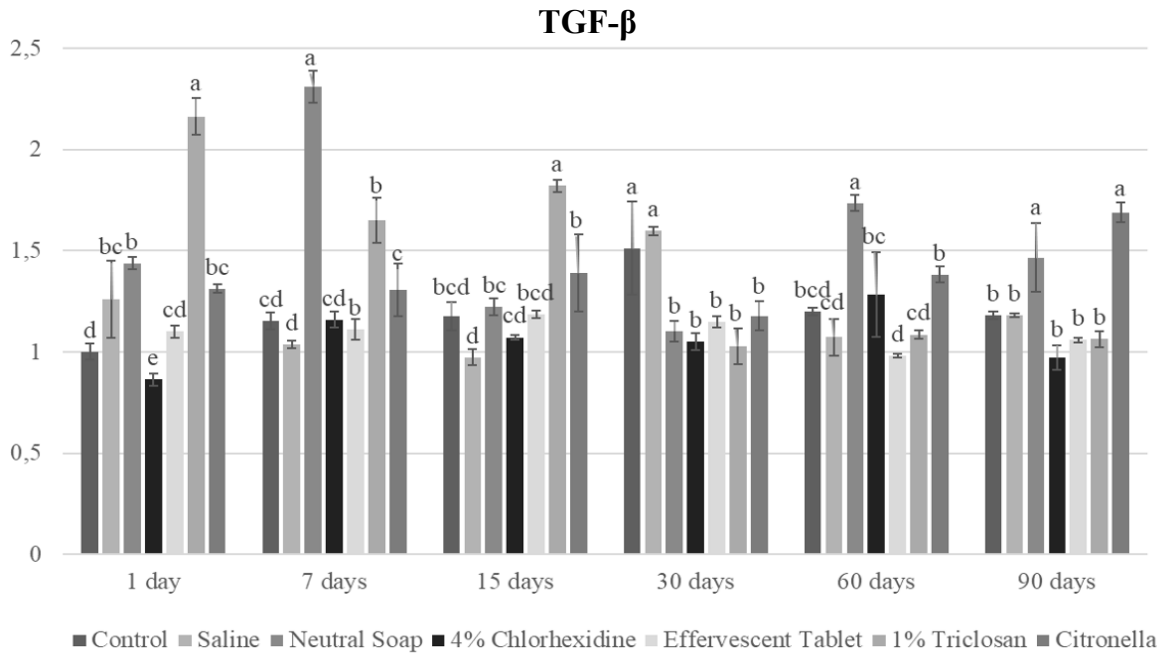

Figure 4. Relative quantification of mRNA for TGF-β ± standard deviation for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

statistically different from the control group. At 7 and 30 days, the triclosan group showed the highest gene expression. At 15 days, only the effervescent tablet group presented statistical difference from the control group and with a lower value. At 60 days, all groups exhibited statistically lower gene expression of MMP-9 than the control group, except for the saline and effervescent tablet groups. Finally, at 90 days, the chlorhexidine group showed a higher gene expression, with statistical difference from the control group.

Regarding the CASP-3 (Figure 7, Table S7), the triclosan group showed a higher gene expression with

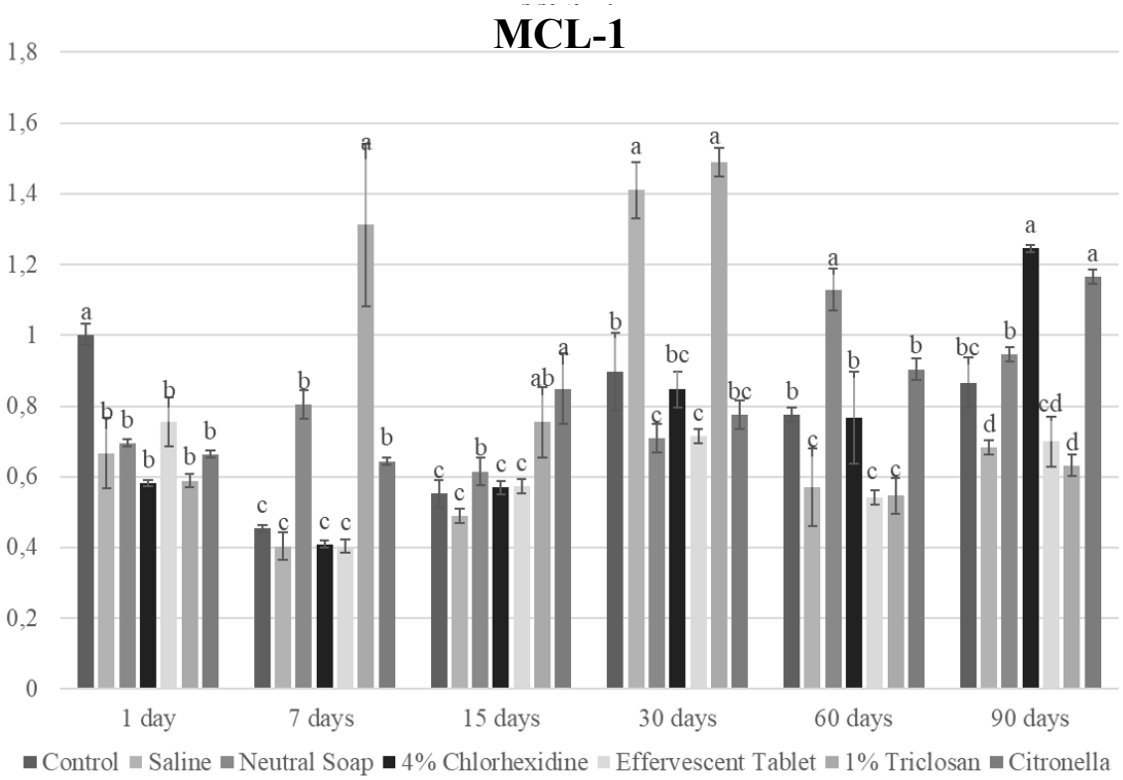

Figure 5. Relative quantification of mRNA for MLC-1 ± standard deviation for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

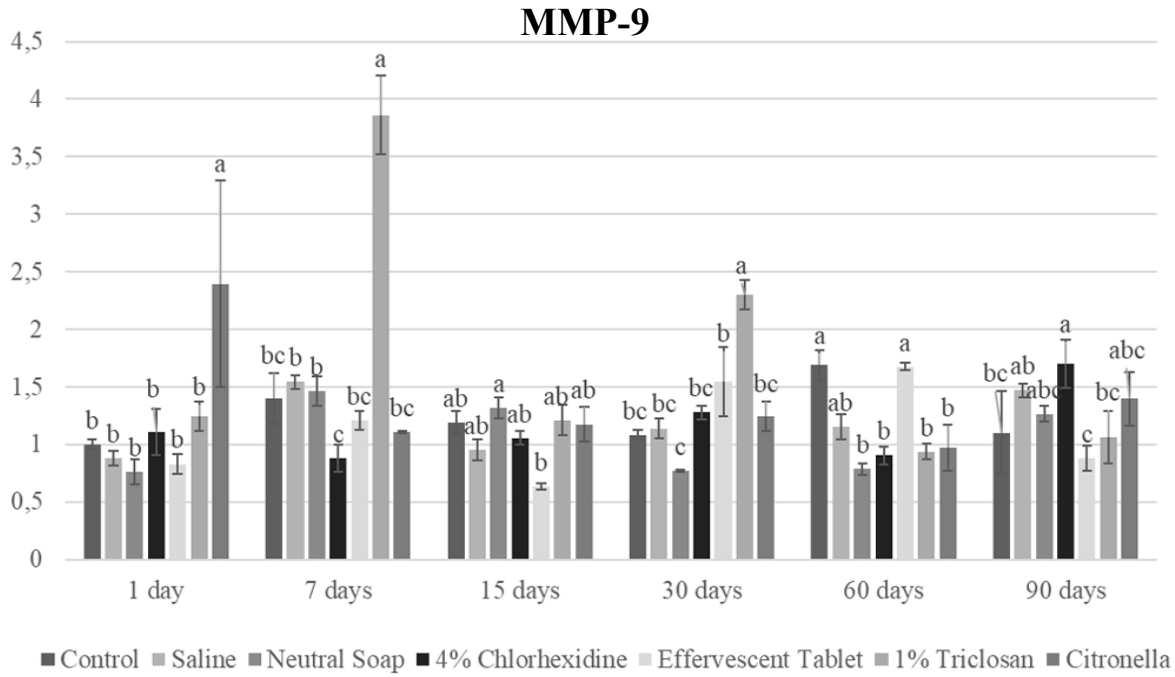

Figure 6. Relative quantification of mRNA for MMP-9 ± standard deviation for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

statistically significant difference from the control group at 1, 7, and 30 days. The citronella group presented the same characteristics of triclosan at 15 and 90 days. However, the chlorhexidine group had the highest gene expression and a significant difference from the control group at 60 days.

For CASP-9 (Figure 8, Table S8), the triclosan exhibited higher gene expression and a statistically significant difference from the control group at 1 and 15 days. The neutral soap group showed the same characteristics as triclosan at 7 and 60 days. At 30 days, no group presented statistical difference from the control group.

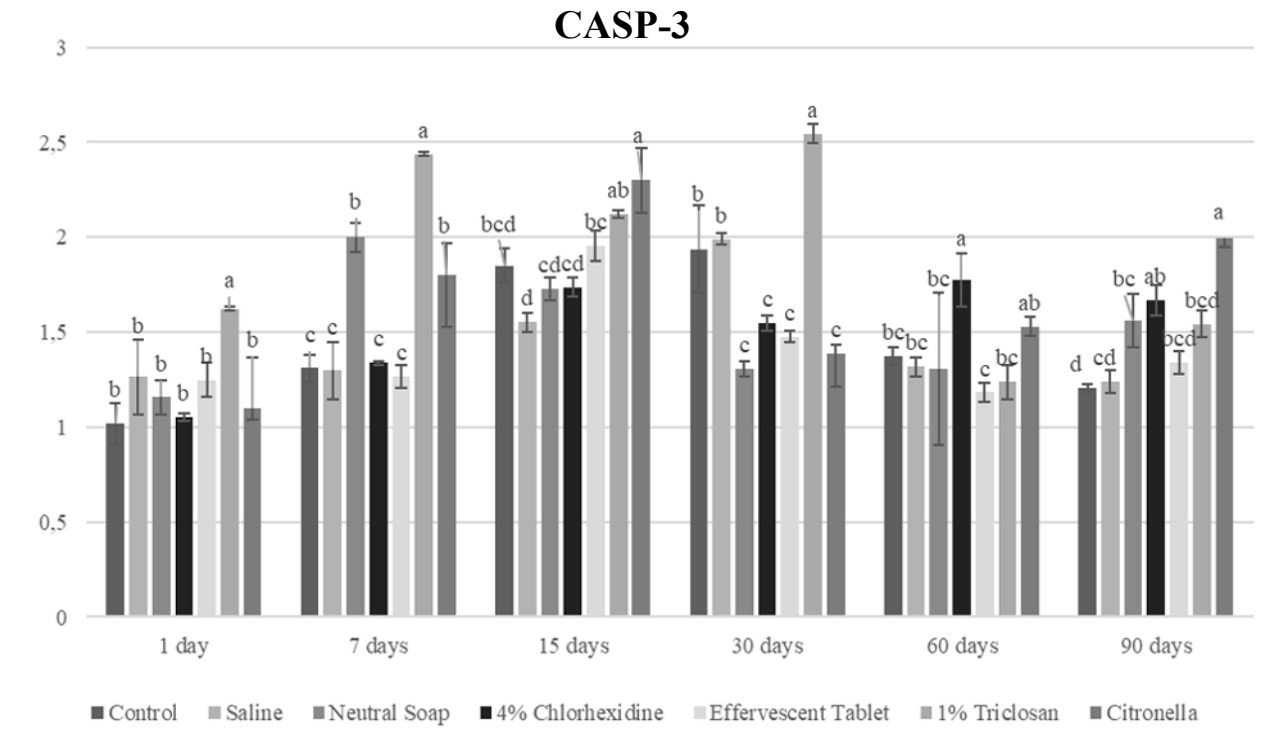

Figure 7. Relative quantification of mRNA for CASP-3 ± standard deviation for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

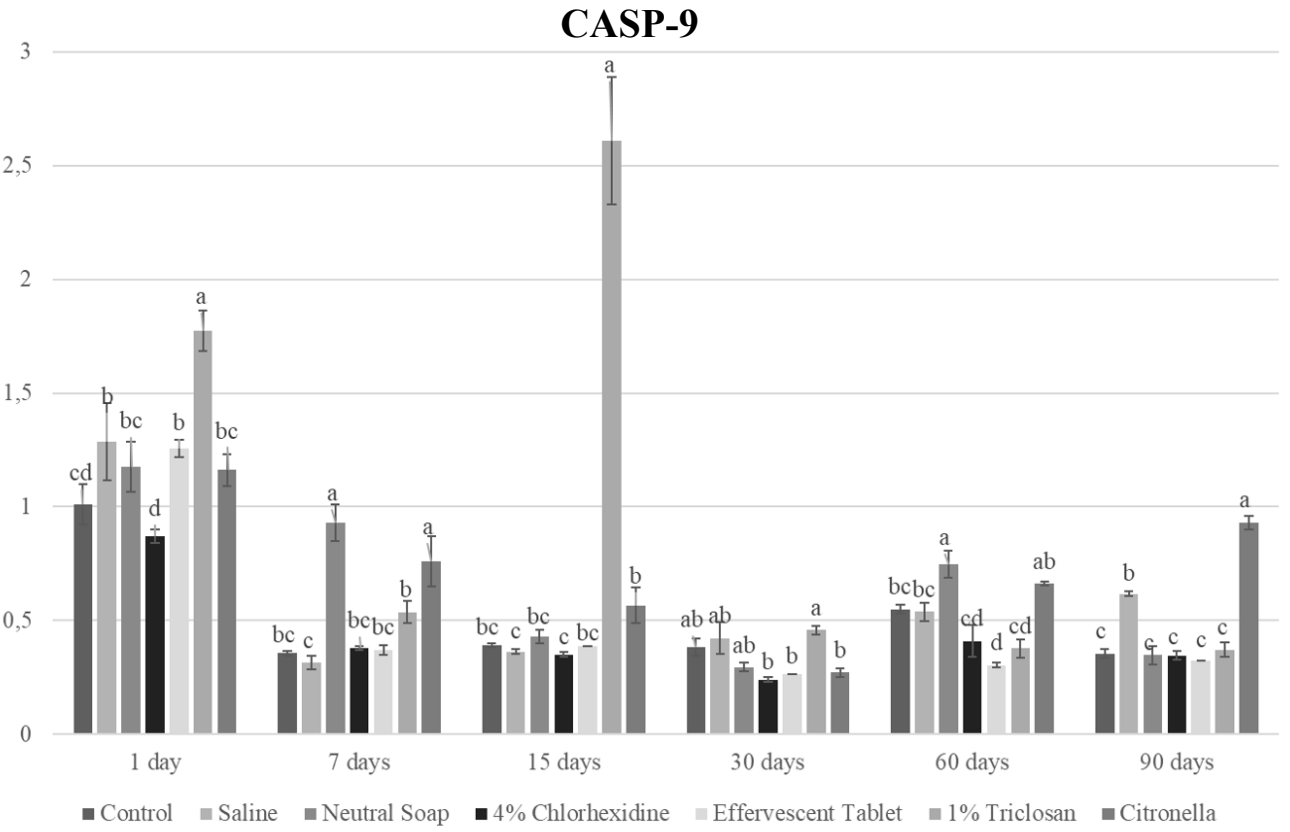

Figure 8. Relative quantification of mRNA for CASP-9 ± standard deviation for evaluated groups in different periods. Different letters for each period indicate statistical difference between each group and respective negative control (non-stimulated group) (Bonferroni, $p<0.05$).

ISSN: 1337-6853 *(print version)* | 1337-9569 *(electronic version)*

Finally, the citronella group showed the highest gene expression at 90 days, with a statistical difference when compared to the control group.

Discussion

The hypotheses that cleaning agents would not produce toxic effects on the human conjunctival cell line, was accepted, since none of the groups show cytotoxicity in human cells.

The present study demonstrated that all cell proliferation percentages were greater than 84%. According to ISO-standard 10993-5, a substance evaluated *in vitro* can be classified as non-cytotoxic (cell proliferation higher than 75%), slightly cytotoxic (between 50 and 75%), moderately cytotoxic (between 25 and 50%), and highly cytotoxic (lower than 25%) (Gjermo, 2020). Thus, the antibiofilm agents evaluated did not exhibit cytotoxic activity, regardless of the period tested.

According to Fardal & Turnbull 1986, chlorhexidine is active against a wide variety of gram-positive and gram-negative organisms, fungi, aerobes, and facultative anaerobes. It is used as cleaning agent for ocular prosthesis and systemic side effects of this solution are rare and have not been demonstrated by long-term clinical studies, indicating a low toxicity potential (Pavarina *et al.,* 2003). This corroborates with the present study, since there was no cytotoxicity of this material (Figures 1 and 2).

Triclosan has been used in skin care products for more than 30 years, promoting a broad-spectrum antibiofilm action when used in suitable formulations (Jones *et al.,* 2000; Bhargava & Leonard, 1996). Clinical and laboratory studies suggest that formulations containing 1% triclosan offer the ideal balance between antibiofilm effectiveness and safety (Jones *et al.,* 2000). A study on acute toxicity of skin care products containing 0.1% to 1% triclosan in their composition was conducted and the authors verified that products containing a 1% concentration were not irritating to the skin after remaining in contact with the skin for 14 days (Bhargava & Leonard, 1996). These reports corroborate with the present study, where the 1% triclosan eliminated bacterial biofilms and showed no cytotoxic activity in conjunctival cells (Figures 2 and 3).

Regarding effervescent tablets, Le Coz *et al.,* 1999 found that the main compound of the tablet that could possibly cause reactions would be the potassium perborate. However, the manufacturer reports that there are no remnants of this substance on the prosthesis if the patient uses these tablets as recommended (rinsing the prosthesis abundantly after disinfection protocol). This recommendation was followed in the experiment, where all specimens were rinsed adequately in running water for 30 seconds after each disinfection, favoring the removal of residues from the surface.

Despite being biocompatible neutral soap presented, in general, the lowest values of cell proliferation. The authors of this study are unaware of any reports of a cytotoxic potential of this substance, although the manufacturer reports that 'it is delicate and safe for eyes and skin'. Thus, it is possible to suggest that, since this was the only group submitted to mechanical cleaning with gauze friction and soap, the specimens may have undergone greater roughness alteration. This may have increased the solution impregnation on the surface, resulting in the higher concentration in the eluates. Lira *et al.,* 2012 evaluated the surface roughness of denture ARs submitted to mechanical (brushing) and chemical disinfection (effervescent tablets and hypochlorite), and only mechanical treatment had an increase in the surface roughness. Pellizzaro *et al.,* 2012 reported that although mechanical cleaning effects are positive, friction on the acrylic resin surface can damage it, increasing wear and roughness.

Contrary to neutral soap, citronella showed, in general, higher cell proliferation percentages (Figures 1 and 2). It is known that the use of medicinal plants in bactericidal, antiparasitic, insecticidal, and cosmetic applications has increased extensively, since most of the essential oils obtained from these plants are known to be non-toxic (Bakkali *et al.,* 2008; Costa *et al.,* 2011; Fandohan *et al.,* 2011). Regarding toxicity, some studies found safety in oral administration and topical application of citronella (Costa *et al.,* 2011; Opdyke, 1976). In the present study, the fact that the group immersed in citronella presented higher cell proliferation percentages may be associated with its antioxidant characteristic. As shown in some studies, natural chemical derivatives of plants have a protective effect against genotoxicity induced by oxidative stress (Glei & Pooll-Zobel, 2006; Plazar *et al.,* 2008; Sinha *et al.,* 2001). Thus, the conjunctival cells evaluated in our study may have undergone some protective action, resulting in greater cell proliferation.

"The saline solution was used aiming to simulate the tear fluid condition in which prostheses are used under normal conditions. A biocompatibility was observed in all evaluated periods (Figures 1 and 2). According to Retamoso et al 2014 and Ebrahimi *et al.,* 2012, the maintenance of the acrylic resin in an aqueous medium promotes the leaching of monomers to this medium, reducing the deleterious effects from the chemical irritation caused by the methyl methacrylate. Thus, this may have occurred in the present study in specimens immersed in saline, favoring the biocompatibility in contact with conjunctiva cells.

During the immersion in an aqueous medium, the acrylic resin tends to absorb an amount of liquid through diffusion, and molecules of that liquid penetrate the acrylic resin matrix and position themselves between polymer chains (Rawlf, 2003). Kalyon & Olgun 2001 evaluated the antibiofilm efficacy of a polymer incorporated with an antibiofilm agent and reported that it remains encapsulated within the polymer and does not effectively diffuse to the surface. Based on this, it is suggested that the acrylic resin may have absorbed part of the solution, and an amount sufficient to cause some cytotoxic reaction in cells was not released to the medium. Regarding the expression of COL-IV, TGF-β, MCL-1, MMP-9, CASP-3, and CASP-9, triclosan had higher mRNA expression in

most analyses. The COL IV is an essential component of the basal membrane of epithelial cells (Simon *et al.,* 1993), and the balance between its synthesis (related to TGF-β) and degradation (related to MMP9) is important to avoid a fibrous reaction (Tirado-Rodriguez *et al.,* 2009) and for preservation of the cellular structure (Da Silva *et al.,* 2016b). In turn, MCL-1 is an anti-apoptotic protein that promotes cell survival, whereas CASP-3 and -9 are proteases involved in apoptosis (Clouzeu *et al.,* 2012; Yamada *et al.,* 2009), and their activation induces the increase of reactive oxygen species (Chan *et al.,* 1991). The balance of these markers is important for the health of conjunctival tissue since the ocular prosthesis and its cleaning agent should maintain tissue health, preventing the formation of fibrosis and unbalanced cell death, avoiding inflammatory reactions in the remaining tissue.

A limitation of the present research is it being an *in vitro* study since it does not reflect all clinical conditions of the use of the ocular prosthesis. Further studies should be performed with different concentration of cleaning agent solutions and their effect on human conjunctival cell line. As well as the accumulation on cleaning agent in the acrylic resin surface regarding polishing methods.

Conclusion

It can be concluded that, among the cleaning agents tested for ocular prosthesis cleaning, the most indicated are immersion in 4% chlorhexidine, effervescent tablets, and 1% triclosan, since their biocompatibility for human conjunctival cells, regardless the period of treatment.

Acknowledgements

This research received financial support from National Council for Scientific and Technological Development/ CNPQ #304462/2016-0; CAPES (Coordination for the Improvement of Higher Education Personnel) and FAPESP (Sao Paulo Research Foundation) #2015/04695-9, #2014/25714-9. The funding soured had no participation in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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Supplemetary material

Suppl. Table 1. Mean±standard deviation of cell proliferation percentages obtained by MTT analysis for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	100.00±2.28 Aa	100.00±3.70 Aa	100.00±2.69 Aa	100.00±2.63 Aa	100.00±3.97 Aa	100.00±1.23 Aa
Saline	97.92±2.67 Abc	96.33±0.86 Bbc	95.17±2.51 Bc	99.46±0.67 Aab	101.81±0.92 Aa	102.02±2.22 Aa
Neutral soap	96.19±2.35 Bab	93.33±3.25 Bb	85.09±1.63 Bd	98.70±0.28 Aa	84.35±3.60 Bd	89.39±3.32 Bc
4% Chlorhexidine	105.89±6.77 Ba	98.64±2.48 Ab	97.61±0.92 Abc	99.47±2.23 Ab	94.05±4.95 Bcd	91.08±5.92 Bd
Efferdent tablet	94.44±2.10 Bd	97.01±1.33 Acd	100.02±1.38 Abc	105.35±3.75 Ba	93.72±6.05 Bd	101.06±3.12 Ab
1% Triclosan	97.26±3.09 Aabc	87.75±1.92 Bd	98.13±6.73 Aab	94.31±4.33 Bc	96.47±6.66 Abc	100.32±3.16 Aa
Citronela	95.33±0.79 Bd	97.25±2.68 Acd	99.33±4.47 Ac	121.12±2.56 Ba	107.39±4.10 Bb	106.56±1.40 Bb
Tween	2.30±0.08 Ba	2.43±0.15 Ba	2.27±0.04 Ba	3.17±0.14 Ba	2.18±0.18 Ba	1.93±0.11 Ba

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 2. Mean±standard deviation of cell proliferation percentages obtained by NR analysis for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	60 days	90 days
Control	100.00±2.21 Aa	100.00±2.81 Aa	100.00±2.58 Aa	100.00±4.07 Aa	100.00±3.11 Aa
Saline	99.20±2.81 Ab	98.34±2.78 Abc	100.60±3.38 Aab	99.91±2.69 Aab	94.98±3.83 Bc
Neutral soap	99.13±5.61 Aab	95.53±3.00 Bbc	99.70±2.13 Aa	99.31±1.33 Aab	94.31±4.10 Bc
4% Chlorhexidine	99.20±2.14 Abc	100.98±3.06 Ab	102.80±3.59 Aab	104.92±3.02 Ba	95.76±4.48 Bc
Efferdent tablet	103.40±7.27 Aa	97.90±3.52 Ab	92.61±3.28 Bc	105.71±3.44 Ba	94.25±3.08 Bbc
1% Triclosan	98.30±5.52 Aab	96.19±2.51 Ab	101.70±5.62 Aa	99.55±2.53 Aab	99.17±4.66 Aab
Citronela	109.33±4.03 Ba	95.04±4.92 Bcd	93.91±2.72 Bd	98.14±2.62 Abc	97.43±2.46 Abcd
Tween	16.25±0.67 Bb	16.48±0.81 Bb	17.40±0.20 Bab	20.99±0.73 Ba	21.01±1.16 Ba

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 3. Relative quantification of mRNA for COL IV±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.006±0.06 Bb	0.931±0.05 CDb	0.979±0.05 Bb	1.300±0.16 Ca	0.893±0.03 BCDb	0.860±0.12 BCb
Saline	1.255±0.21 ABb	0.812±0.05 Dc	0.843±0.02 Bc	1.740±0.25 ABa	0.890±0.04 BCDc	0.734±0.04 Cc
Neutral soap	1.212±0.03 ABbc	1.439±0.08 Bab	0.889±0.03 Bd	1.189±0.06 CDbc	1.663±0.25 Aa	0.946±0.02 ABCcd
4% Chlorhexidine	1.088±0.05 ABa	1.060±0.03 CDa	0.888±0.01 Ba	0.953±0.01 Da	1.098±0.04 Ba	1.068 ±0 .07 ABa
Efferdent tablet	1.181±0.02 ABa	0.951±0.07 CDa	1.015±0.06 Ba	0.988±0.08 Da	0.612±0,01 Db	1.112±0.03 ABa
1% Triclosan	1.327±0.05 Ab	2.009±0.25 Aa	1.551±0.16 Ab	1.909±0.14 Aa	0.734±0.04 CDc	0.781±0.03 Cc
Citronela	1.270±0.06 ABb	1.204±0.09 BCbc	1.033±0.15 Bbc	1.585±0.12 Ba	0.952±0.03 BCc	1.185±0.07 Abc

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 4. Relative quantification of mRNA for TGF-β±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.002±0.04 DEb	1.154±0.04 CDb	1.178±0.07 BCDb	1.513±0.23 Aa	1.200±0.02 BCDb	1.182±0.02 Bb
Saline	1.260±0.19 BCb	1.038±0.02 Dbc	0.974±0.04 Dc	1.598±0.02 Aa	1.073±0.09 CDbc	1.182±0.01 Bbc
Neutral soap	1.438±0.03 Bcd	2.310±0.08 Aa	1.223±0.04 BCde	1.102±0.05 Be	1.736±0.04 Ab	1.466±0.17 Ac
4% Chlorhexidine	0.864±0.03 Ec	1.159±0.04 CDab	1.072±0.01 CDabc	1.051±0.04 Babc	1.284±0.21 BCa	0.974±0.06 Bbc
Efferdent tablet	1.102±0.03 CDa	1.112±0.05 CDa	1.187±0.02 BCDa	1.149±0.03 Ba	0.981±0.01 Da	1.059±0.01 Ba
1%Triclosan	2.163±0.09 Aa	1.651±0.11 Bb	1.821±0.03 Ab	1.028±0.09 Bc	1.086±0.02 CDc	1.064±0.04 Bc
Citronela	1.313±0.02 BCb	1.306±0.13 Cb	1.390±0.19 Bb	1.179±0.07 Bb	1.383±0.04 Bb	1.690±0.05 Aa

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 5. Relative quantification of mRNA for MLC-1±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.002±0.03 Aa	.454±0.01 Cc	0.552±0.04 Cc	0.897±0.11 Bab	0.776±0.02 Bb	0.866±0.07 BCab
Saline	0.667±0.10 Bb	0.404±0.04 Cc	0.489±0.02 Cc	1.410±0.08 Aa	0.571±0.11 Cbc	0.684±0.02 Db
Neutral soap	0.695±0.01 Bcd	0.805±0.04 Bbc	0.615±0.04 BCd	0.710±0.04 Ccd	1.129±0.06 Aa	0.945±0.02 Bb
4% Chlorhexidine	0.582±0.01 Bc	0.409±0.01 Cc	0.569±0.02 Cc	0.847±0.05 BCb	0.768±0.13 Bb	1.246±0.01 Aa
Efferdent tablet	0.755±0.07 Ba	0.404±0.02 Cc	0.573±0.02 Cbc	0.715±0.02 Cab	0.541±0.02 Cbc	0.699±0.07 CDab
1%Triclosan	0.589±0.02 Bcd	1.312±0.23 Ab	0.754±0.10 ABc	1.490±0.04 Aa	0.546±0.05 Cd	0.632±0.03 Dcd
Citronela	0.663±0.01 Bc	0.644±0.01 Bc	0.849±0.10 Ab	0.776±0.04 BCbc	0.904±0.03 Bb	1.165±0.02 Aa

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 6. Relative quantification of mRNA for MMP-9±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.002±0.04 Bb	1.401±0.22 BCab	1.187±0.10 ABab	1.084±0.04 BCb	1.688±0.13 Aa	1.103±0.36 BCb
Saline	0.881±0.06 Bc	1.541±0.06 Ba	0.954±0.09 ABbc	1.140±0.09 BCabc	1.156±0.11 ABabc	1.469±0.06 ABab
Neutral soap	0.762±0.11 Bb	1.463±0.13 Ba	1.316±0.09 Aab	0.775±0.01 Cb	0.787±0.05 Bb	1.265±0.07 ABCab
4% Chlorhexidine	1.109±0.20 Bb	0.879±0.12 Cb	1.057±0.06 ABb	1.279±0.06 BCab	0.904±0.08 Bb	1.697±0.21 Aa
Efferdent tablet	0.830±0.09 Bb	1.207±0.08 BCab	.634±0.03 Bb	1.548±0.30 Ba	1.675±0.03 Aa	0.880±0.11 Cb
1%Triclosan	1.243±0.13 Bc	3.862±0.34 Aa	1.211±0.13 ABc	2.302±0.13 Ab	.938±0.07 Bc	1.062±0.23 BCc
Citronela	2.396±0.96 Aa	1.112±0.01 BCb	1.173±0.15 ABb	1.244±0.13 BCb	0.969±0.20 Bb	1.397±0.23 ABCb

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.

Suppl. Table 7. Relative quantification of mRNA for CASP-3±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.017±0.11 Bc	1.311±0.07 Cbc	1.849±0.09 BCDa	1.936±0.23 Ba	1.373±0.05 BCb	1.208±0.02 Dbc
Saline	1.263±0.20 Bb	1.297±0.15 Cb	1.552±0.05 Db	1.991±0.03 Ba	1.318±0.05 BCb	1.241±0.06 CDb
Neutral soap	1.157±0.09 Bd	1.998±0.08 Ba	1.726±0.06 CDab	1.307±0.04 Ccd	1.307±0.40 BCcd	1.560±0.14 BCbc
4% Chlorhexidine	1.055±0.02 Bc	1.338±0.01 Cbc	1.737±0.05 CDa	1.547±0.04 Cab	1.775±0.14 Aa	1.668±0.08 ABab
Efferdent tablet	1.248±0.09 Bb	1.267±0.06 Cb	1.955±0.08 BCa	1.475±0.03 Cb	1.183±0.05 Cb	1.339±0.06 BCDb
1%Triclosan	1.622±0.01 Ac	2.439±0.01 Aab	2.120±0.21 ABb	2.543±0.05 Aa	1.237±0.09 BCd	1.542±0.07 BCDcd
Citronela	1.098±0.06 Be	1.799±0.27 Bbc	2.301±0.17 Aa	1.386±0.17 Cde	1.530±0.05 ABcd	1.996±0.05 Aab

Different uppercase letter in columns indicate statistical difference ($p<0.05$ regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$ between treatment periods.

Suppl. Table 8. Relative quantification of mRNA for CASP-9±standard deviation for evaluated groups in different periods.

Antibiofilm solution	1 day	7 days	15 days	30 days	60 days	90 days
Control	1.010±0.09 CDa	0.356±0.01 BCc	0.391±0.01 BCbc	0.383±0.04 ABbc	0.550±0.02 BCb	0.355±0.02 Cc
Saline	1.285±0.17 Ba	.315±0.03 Cd	.363±0.01 Ccd	.423±0.07 ABcd	0.538±0.04 BCbc	0.618±0.01 Bb
Neutral soap	1.175±0.11 BCa	0.931±0.08 Ab	0.430±0.03 BCc	0.295±0.02 ABc	0.748±0.06 Ab	0.348±0.04 Cc
4% Chlorhexidine	0.869±0.03 Da	0.379±0.01 BCb	0.350±0.01 Cb	0.240±0.01 Bb	0.410±0.07 CDb	0.346±0.02 Cb
Efferdent tablet	1.256±0.04 Ba	0.371±0.02 BCb	0.387±0.00 BCb	0.265±0.00 Bb	0.304±0.01 Db	0.323±0.00 Cb
1%Triclosan	1.773±0.09 Ab	0.537±0.05 Bc	2.608±0.28 Aa	0.458±0.02 Ac	0.378±0.04 CDc	0.372±0.03 Cc
Citronela	1.161±0.07 BCa	0.759±0.11 Abc	0.567±0.08 Bc	0.271±0.02 ABd	0.663±0.01 ABc	0.931±0.03 Ab

Different uppercase letter in columns indicate statistical difference ($p<0.05$) regarding the respective non stimulated group. Different lowercase letters in line indicate statistical difference ($p<0.05$) between treatment periods.