

Cytotoxicity in fibroblasts from young and elderly donors from two mouthwashes used to prevent the spread of SARS-CoV-2

Citotoxicidade em fibroblastos de doadores jovens e idosos de dois enxaguatórios bucais usados para prevenir a propagação de SARS-CoV-2

Citotoxicidad en fibroblastos de donantes jóvenes y ancianos de dos enjuagues bucales utilizados para prevenir la propagación del SARS-CoV-2

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Abstract

In cases of injuries in the oral cavity, the mouthwash comes in contact with the underlying gingival connective tissue and should have its cytotoxicity assessed. However, there is no available evidence if cells of elderly donors react differently during *in vitro* assessments of mouthwashes. This study aimed to compare the cytotoxicity evaluation of two different mouthwash types when assessed with primary gingival fibroblasts from either young and older donors. Primary cells were collected from two elderly patients (mean age 66.5 years old) and two young patients (mean age 27.5 years old). The primary cell culture was produced from gingival fragments and exposed for 24h in Peroxidid[®] and Oral B[®]. A control group was exposed to unconditioned culture media, representing 100% of cell survival (negative control), and 200mg/mL solution of latex fragments was used as a positive control due to its well-known toxicity. Both products presented similar dose-dependent cytotoxicity. In the toxic range, from 0.035% to 0.00035% for Peroxidid[®] and 0.06 to 0.0006% for Oral B[®]. The calculated IC₅₀ values were very similar, with the exception of Oral B[®] tested with young cells, which presented a slightly higher toxic concentration (0.0523 mM). The statistical analysis shows no significant difference between tests with cells from young our elderly donors ($p > 0.05$). These mouthwashes should be used sparingly to prevent the spread of SARS-CoV-2. However, the use of age-matched cells during *in vitro* tests may not be necessary to predict differences in the biological response of the elderly to these products.

Keywords: Dentistry; Toxicity tests; Fibroblasts; Mouthwashes; Coronavirus.

Resumo

O enxaguatório pode entrar em contato com o tecido conjuntivo gengival subjacente em tecidos lesionados, devendo ter sua citotoxicidade avaliada. No entanto, não há evidências disponíveis se as células de doadores idosos reagem de forma diferente durante as avaliações *in vitro* de enxaguatórios bucais. Este estudo objetivou comparar a avaliação da citotoxicidade de dois tipos de enxaguatórios bucais avaliados com fibroblastos gengivais primários de doadores jovens e idosos. As células primárias foram coletadas de dois pacientes idosos (idade média de 66,5 anos) e dois pacientes jovens (idade média de 27,5 anos). A cultura celular primária foi produzida a partir de fragmentos gengivais e exposta por 24h em Perioxidin® e Oral B®. Um grupo controle negativo foi exposto a meios de cultura não condicionados, representando 100% de sobrevivência celular, e solução de fragmentos de látex 200mg/mL foi utilizada como controle positivo devido à sua toxicidade. Ambos os produtos apresentaram citotoxicidade dependente da dose semelhante. Na faixa tóxica, de 0,035% a 0,00035% para Perioxidin® e 0,06 a 0,0006% para Oral B®. Os valores de IC50 calculados foram muito semelhantes, com exceção do Oral B® testado com células jovens, que apresentou uma concentração tóxica ligeiramente superior (0,0523mM). Não houve diferença estatística significativa entre os testes com células de doadores jovens ou idosos ($p > 0,05$). Concluiu-se que o uso de células da mesma idade durante os testes *in vitro* pode não ser necessário para prever diferenças na resposta biológica dos idosos a esses produtos.

Palavras-chave: Odontologia; Testes de toxicidade; Fibroblastos; Antissépticos bucais; Coronavírus.

Resumen

El enjuague bucal puede contactar con el tejido conectivo gingival subyacente en los tejidos lesionados y debe evaluarse su citotoxicidad. No hay evidencia disponible si las células de donantes de edad avanzada reaccionan de manera diferente durante las evaluaciones de enjuague bucal *in vitro*. Este estudio objetivó comparar la evaluación de la citotoxicidad de dos tipos de enjuagues bucales evaluados con fibroblastos gingivales primarios de donantes jóvenes y ancianos. Se recolectaron células primarias de dos participantes ancianos (edad media 66,5 años) y dos jóvenes (edad media 27,5 años). El cultivo de células primarias se produjo a partir de fragmentos gingivales y se expuso durante 24 horas en Perioxidin® y Oral B®. Un grupo de control negativo fue expuesto a medios de cultivo no acondicionados, lo que representa un 100% de supervivencia celular, y se utilizó una solución de 200 mg/mL de fragmentos de látex como control positivo debido a su toxicidad. Ambos productos mostraron una citotoxicidad dependiente de la dosis similar. En el rango tóxico, 0.035% a 0.00035% para Perioxidin® y 0.06 a 0.0006% para Oral B®. Los valores de CI50 calculados fueron muy similares, a excepción del Oral B® en células jóvenes, que presentó una concentración tóxica ligeramente superior (0,0523mM). No hubo diferencia estadísticamente significativa entre las pruebas con células de jóvenes o ancianos ($p > 0,05$). Se concluyó que el uso de células de la misma edad durante las pruebas *in vitro* puede no ser necesario para predecir diferencias en la respuesta biológica de los ancianos a estos productos.

Palabras clave: Odontología; Pruebas de toxicidad; Fibroblastos; Antisépticos bucales; Coronavirus.

1. Introduction

In conjunction with routine mechanical methods, mouthwashes are widely used as an extra tool for oral hygiene preservation. These products have synthetic and/or natural compounds that act on microorganisms by inhibiting their growth and blocking some of their enzymatic reactions, thus playing a useful role in biofilm control and decreasing the severity of oral disease (Saad et al., 2011).

Recently, with the SARS-CoV-2 pandemic, publications have suggested that rinsing the oral cavity may control and decrease the risk of viral transmission (Ather et al., 2020; Peng et al., 2020). There are reports that some mouthwashes currently on the market have ingredients that can contribute to the reduction of SARS-CoV-2 viral load and, thus, facilitate the fight against oral transmission (Carrouel et al., 2020; Salminen et al., 2012). Chlorhexidine mouthwash was effective in reducing the SARS-CoV-2 viral load in the saliva for a short-term period (Yoon et al., 2020). Therefore, the American Dental Association (ADA) (American Dental Association, 2020) has endorsed the use of mouthwashes before oral procedures, which may probably increase its recommendation and use.

Therapeutic mouthwashes are usually composed of active ingredients such as essential oils, chlorhexidine and cetylpyridinium chloride, water, alcohol, surfactants, and humectants. An acceptable oral hygiene procedure includes products that effectively remove, control, or reduce these pathogens without producing damage to host tissues (De Oliveira et al., 2018).

Therefore, regardless of their composition, mouthwashes should be biocompatible, i.e., do not cause undesired biological responses and be non-cytotoxic to oral tissues.

However, the literature presents some reports of *in vitro* cytotoxicity of commonly used mouthwashes during pre-clinical assessments. A study has demonstrated that moderate to strong cytotoxic effects were observed for oral rinses containing cetylpyridinium chloride, 0.2% chlorhexidine, or cocamidopropyl betaine (Müller et al., 2017). Other studies indicate that higher concentrations of ethanol present on mouthwashes may increase their cytotoxicity (Calderón-Montaña et al., 2018). In cases of injuries present in the oral cavity, the mouthwash also comes in contact with the underlying gingival connective tissue.

Since human gingival fibroblasts are among the most abundant resident cells from the oral mucosa (Mah et al., 2014), these cells are often used on pre-clinical *in vitro* assessments of mouthwash toxicity. Mouthwashes containing essential oil (E.O.), chlorhexidine (CHX), and amine fluoride/stannous fluoride (AFSF) presented variable effects on human gingival fibroblast proliferation and gene expression of extracellular matrix components of oral mucosa (Balloni et al., 2016). Other reports indicate cytotoxic effects of various enzymatic and chlorhexidine-based mouthwashes on human gingival fibroblasts (Coelho et al., 2020; Ghabanchi et al., 2012).

Oral fungal infections are a clinical problem among the elderly, and the use of mouthwashes is a good choice due to debilitating conditions that limit their adequate oral hygiene, along with a suppressed immune system (Scheibler et al., 2017). However, regardless of this age group's specific needs, the safety tests performed for oral products are usually the same adopted for young patients, even there is accumulating evidence that the elderly experience different inflammatory responses in the gingiva (Fransson et al., 1999). It has been also demonstrated that cells from older adults might show genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion, and altered intercellular communication (Kanasi et al., 2016; Salminen et al., 2012). Furthermore, a recent study has shown that gingival fibroblasts from young and elderly donors may react differently to dental products such as denture adhesives during *in vitro* testing. (Soares et al., 2018)

Considering the current moment when the elderly, as a risk age group on the COVID-19 pandemics, may be exposed to these products more frequently, this use must be safe and not damage the oral mucosa. Therefore, this study's objective was to compare the standardized cytotoxicity evaluation of two different types of mouthwash (0.12% Chlorhexidine Digluconate - Periogard® and Cetylpyridinium Chloride – Oral B®) when assessed in gingival fibroblast primary cells from either a young and an older donor.

2. Methodology

This work consists of an experimental laboratorial *in vitro* research, though a quantitative approach. (Ferreira et al., 2005)

2.1 Collection of human gingival fibroblasts

This study was part of a project approved by the Antonio Pedro Hospital Research Ethics Committee, protocol no. CAAE 05647613.1.0000.5243. Informed consent was obtained from all participants included in the study.

Primary cells were collected from two elderly patients (mean age 66.5 years old) and two young patients (mean age 27.5 years old). The four participants were patients of the Dentistry Clinic at the University Federal Fluminense, who met the following criteria: subjects indicated for surgery that allowed the collection of a gingival fragment without affecting the original surgical plan had no chronic disease, made no continuous use of drugs and had no gingival bleeding.

After collecting the gingival tissue, fibroblasts' isolation was performed according to a previously described protocol (Damante et al., 2009). Fragments were immersed on polypropylene tubes containing the culture medium (Dulbecco's Modified Eagle's Medium – DMEM high glucose) with 3% antibiotics (penicillin and streptomycin) and washed with Phosphate Buffered Saline (PBS) in a sterile hood. After washing, the fragments were immersed in 70% ethylic alcohol for 1 min and washed again in PBS. To separate connective from epithelial tissue, the fragments were sectioned with a scalpel into sections of approximately 2 mm. The connective tissue fragments were treated with trypsin for enzyme digestion, with final inactivation by the addition of Fetal Bovine Serum (FBS) (GIBCO/Invitrogen, Grand Island, Nebraska, USA). The digested sections were transferred to 6-well cell culture plates and allowed to adhere for 3 minutes. After this period, 1 mL of DMEM high glucose with 1% antibiotic was added to each well and incubated at 37°C for 48 hours, and then the culture medium was changed and cells cultured until confluence (about ten days).

Finally, the fragments were removed from the plate, and the cell culture was established according to standard protocols for adherent cells. The resulting cells presented fibroblast morphology (elongated fusiform nuclei organized in a parallel pattern) and positive staining for vimentin and type I collagen, markers of fibroblast origin cells (Damante et al., 2009).

2.2 Sample Preparation

Two types of mouthwash were tested: Chlorhexidine Digluconate 0.12% (Perioxidin® Gross/Lacer Laboratory, Rio de Janeiro, Brazil) and 0.053% Cetylpyridinium Chloride (Oral B®, Procter & Gamble, Cincinnati, Ohio, USA). Data on their lots and composition is presented in Table 1. The cytotoxicity assay to detect cell viability was performed following the recommendations of the OECD Guide 129 (OECD, 2010), which presents a standardized test method for estimating the starting dose for an acute oral systemic toxicity test.

This method proposes an assessment of the linear range of the toxic concentrations of the tested solution using log dilutions, thus allowing the estimation of the IC₅₀ value, i.e., the concentration which can inhibit growth or cause death on half of the exposed cell population (Mannerström et al., 2017; Soares et al., 2018), which was used in this study as the parameter for comparison of the biological response of the young and elderly donor fibroblasts. Therefore, each mouthwash solution was submitted to seriated dilutions in sterile DMEM, ranging from 1:10 to 1:10¹⁰.

Table 1. Description of the mouthwashes.

Material	Lot	Manufacturer	Composition
Perioxidin®	P142	Gross/Lacer Laboratory, Rio de Janeiro, Brazil	Chlorhexidine Digluconate 0.12%, Propylene Glycol, Glycerin, Hydrogenated Castor Oil, Polyethylene Glycol 40, Xylitol, Poloxamer, Acesulfame Potassium, Menthol, Saccharin Sodium, Neohesperidine DC, Lactic Acid, Dyes CI 16185 E 16855 E85 Mint and Water
Oral B®	0139B670C1	Procter & Gamble, Cincinnati, Ohio, USA	Water, Glycerin, Aroma, Cetylpyridinium Chloride, Poloxamer 407, Methylparaben, Sodium Saccharin, Cinnamal, Propylparaben, Eugenol, CI42090, 0,053% monohydrated cetylpyridinium chloride and 0,050% sodium fluoride

Source: Authors.

2.3 Cytotoxicity assay

Cells from the young and elderly donors at the second passage were cultured at 37°C under 5% CO₂ in DMEM high-glucose containing 10% fetal bovine serum (GIBCO/Invitrogen, Grand Island, Nebraska, USA) and 1% antibiotic. The cultures were then seeded at a density of 3×10⁴ cells mL⁻¹ in a 96-well plate, followed by incubation for 24h at 37°C / 5% CO₂. The cells were exposed to the mouthwashes by replacing 200 µL of the medium in each well with 200 µL of one of the mouthwash diluted samples described previously, followed by incubation for 24 h. A control group was exposed to unconditioned culture media, representing 100% of cell survival (negative control), and 200mg/mL solution of latex fragments was used as a positive control due to its well-known toxicity. (Lourenço et al., 2014)

Following the OECD Guide 129(OECD, 2010), cell viability was assessed through the Neutral Red Uptake test (NRU) using a commercial kit (In Cytotox, Xenometrics, Germany). After 4 hours of exposure to the Neutral Red dye, cells were fixed, the dye extracted, and the optical density (O.D.) measured on a microplate reader (Sinergy II, Biotek Inst., USA) at 540nm, corresponding to the density of viable cells with intact membranes. The tests were performed in two biological and five technical replicates.

2.4 Statistical Analysis

All cell densities were calculated as a percentage of the O.D. of the control group. The dose-response of the cytotoxic effects for each solution and cell-system was calculated after a linear regression from plotted curves on a logarithmical scale, producing the line equation [β]:

$$\text{Cell survival (\% Control)} = a.[\text{Mouthwash}] + b \quad [\beta]$$

Where *a* represents the angular and *b* the linear coefficients, respectively.

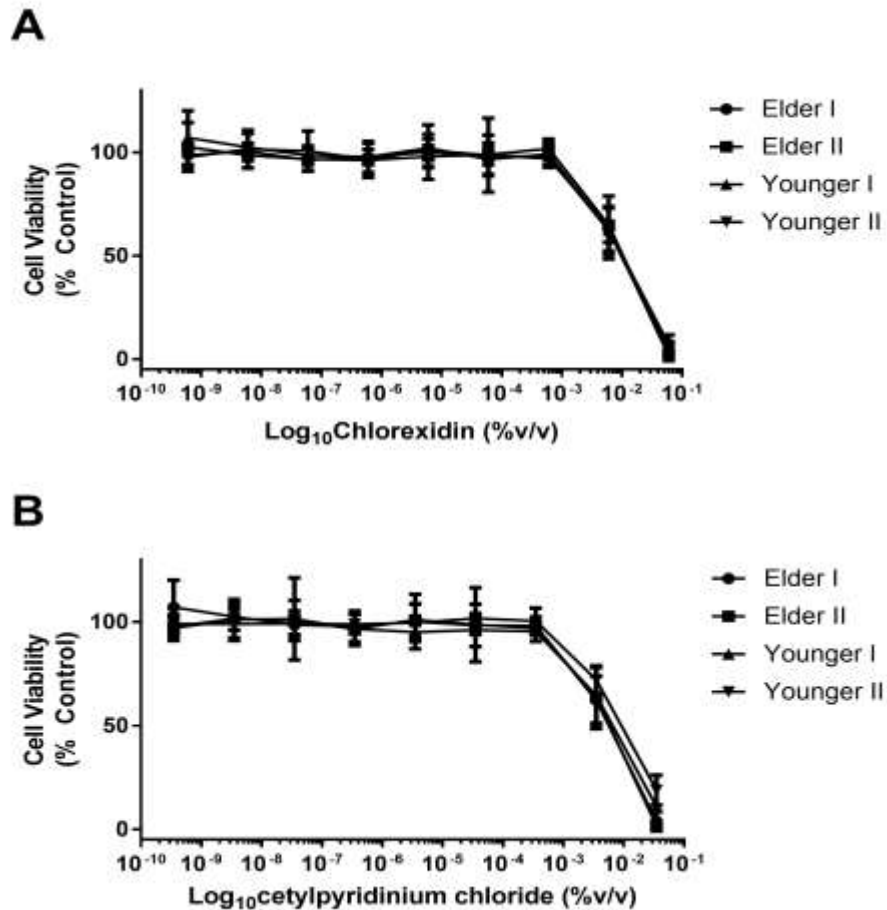
The equations were used to estimate the IC₅₀ by extrapolating the mouthwash concentration that induces 50% Cell survival. A Mann-Whitney U test was performed to compare the IC₅₀ values calculated for the elderly and young donor curves. A non-parametric Kruskal-Wallis test with Dunn post-test was used to compare the different cell systems in each mouthwash dilution. Statistical significance was established at α= 0.05. All statistical analyzes were performed with GraphPad Prism 7 (Graphpad Software, Inc., San Diego, CA, USA).

3. Results

The test was internally validated by the behavior of the positive and negative controls, which promoted the expected levels of cell death and survival, respectively (O.D. readings around 0.8 for the negative controls, and mean cell survival of 12% induced by the positive control – data not shown).

All materials were toxic to the cells when at the highest concentration, regardless of whether the cell system was originated from young or old donors, as shown in Figure 1A and 1B, but the cytotoxicity is completely suppressed with dilutions above 1:10⁷.

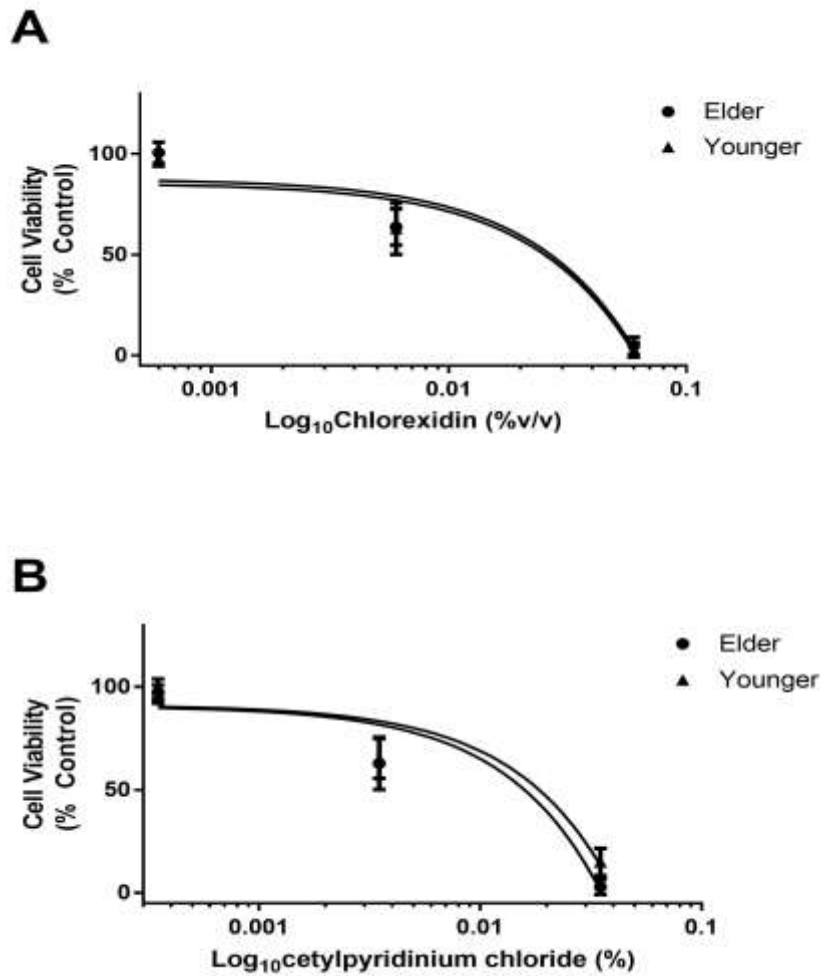
Figure 1. Cytotoxic effects of Chlorexididn (A) and Cetylpyrдинium chloride-based mouthwashes (B) tested on human gingival fibroblasts from younger and elder donors, as assessed by the NRU assay, and expressed as a mean percentage of the control (cells exposed only to culture medium). Points indicate mean \pm SD.



Source: Authors.

Figure 2 shows the dose-response of the cytotoxic effects of the mouthwashes. In the toxic range, from 0.035% to 0.00035% for Chlorexididn and 0.06 to 0.0006% for cetylpyrдинium chloride, it was possible to estimate the IC₅₀, i.e., the concentration capable of killing 50% of the cells. The calculated IC₅₀ values were very similar, with the exception of Oral B® tested with young cells, which presented a slightly higher toxic concentration (0.0523 mM), which could suggest a lower sensitivity of the cells to this product. However, statistical analysis shows that there is no significant difference between any group of mouthwashes and cells ($p > 0.05$) (Table 2).

Figure 2. Representation of cell survival in the toxic range for the calculation of the linear equation to estimate the IC₅₀ (the concentration capable of killing 50% of the cells) for Chlorhexidin (A) and Cetylpyridinium chloride-based mouthwashes (B).



Source: Authors.

Table 2. Results from the Regression analysis of the cytotoxicity assay.

		Goodness of fit (R ²)	Line Equation	IC ₅₀	p-value ^a
Chlorhexidine	Young	0.92	Y = -1399*X + 85,46	0.02535%	0.6857
	Elder	0.91	Y = -1402*X + 87,55	0.02678%	
Cetylpyridinium chloride	Young	0.90	Y = -2228*X + 91.18	0.01848%	0.6857
	Elder	0.92	Y = -2563*X + 90.69	0.01588%	

IC₅₀ = concentration of test solution that causes the death of 50% of the exposed cells. ^acalculated by a Mann-Whitney U test.
 Source: Authors.

4. Discussion

Currently it is believed that an antimicrobial mouthwash reduces the number of oral microorganisms, reducing infections (Borenfreund & Puerner, 1985). With the recent SARS-CoV-2 pandemic, a significant increase in the use of

mouthwashes is expected to prevent the spread of the disease.(Vergara-Buenaventura & Castro-Ruiz, 2020) Therefore, the investigation of the cytotoxicity of these products becomes relevant in the current context. However, as instructed by the Guideline for the Diagnosis and Treatment of New Coronavirus Pneumonia (the 5th edition) released by the National Health Commission of the People's Republic of China (China, 2020), chlorhexidine, which is commonly used as a mouthwash in dental practice, has not yet been demonstrated to be capable of eliminating 2019-nCoV (Fallahi et al., 2020; Peng et al., 2020). Furthermore, the main available mouthwashes should be assessed for their cytotoxicity to elderly cells and tissues.

There are different standards available to assess the *in vitro* biocompatibility of dental materials, including ISO 10993-5:2009 (ISO 10993-5:2009, 2009) and ISO 7405:2008 (Standardization, 2008). However, most of these protocols take into account dental materials, implants, and other solid products, and they do not directly adapt to the testing of liquid products or products that may eventually be ingested, such as mouthwashes. Therefore, *in vitro* cell tests for these products are closer to protocols such as the OECD 129 Guide for estimating the starting doses for acute oral systemic toxicity tests (OECD, 2010). That guide proposes a test based on cells' exposure to different dilutions, followed by the determination of viability by neutral red, and was designed and validated to identify the initial concentration to be applied on an *in vivo* test. However, since it is based on the calculation of IC₅₀, which is a determinant of cell sensitivity to different substances, we considered this test to understand whether cells of the elderly or young people would be sensitive to these products. The assessment was performed with human primary gingival cells, which may provide more useful data to clinicians when compared to immortalized cell lines (often from tumor origin), since primary cells present the natural ploidy, the same regulation of gene expression, the response to stress and other biological parameters observed in humans, *in vivo* (Czekanska et al., 2012, 2014).

The test is based on a fixed time of 24 hours (previously validated for animal testing), which does not exactly simulate the time that this product remains in contact with the mouth, considering the regular use of these products as a complement to oral hygiene. In addition to increased exposure time, the enormous confinement and increased exposure of cells tested in 2D models (monolaminar cultivation models) may explain the large toxicity levels found at the highest initial concentrations of the products. Flemingson et al. (2008) using concentrations equal to or higher than 0.02%, the metabolic activity was reduced by more than 90% in all of the tested groups (Flemingson, Emmadi Pamela, Ambalavanan N, Ramakrishnan T, 2008). Oral mouthwashes containing Cetylpyridinium Chloride were also identified as highly cytotoxic *in vitro* (Müller et al., 2017), in agreement with the findings of the present study. Despite previous evidence of toxicity from *in vitro* tests with fibroblasts (Balloni et al., 2016; Coelho et al., 2020; De Oliveira et al., 2018; Flemingson, Emmadi Pamela, Ambalavanan N, Ramakrishnan T, 2008; Ghabanchi et al., 2012), it is worth noting that the chosen products are already known to be safe and approved for use worldwide. Therefore, the present result did not necessarily imply that these materials are necessarily cytotoxic, but rather that in exacerbated conditions, these materials would cause cell death. Furthermore, considering the potential toxicity of chlorhexidine or Cetylpyridinium Chloride, and the fact that 2019-nCoV is vulnerable to oxidation, it may be recommended a pre-procedure mouth rinsing containing oxidizing agents, such as 1% hydrogen peroxide or 0.2% povidone, with the goal of reducing the salivary load of oral microbes, including potential transport of 2019-nCoV (Peng et al., 2020).

According to some evidence, fibroblasts from older individuals are likely to be more sensitive to adverse effects related to cellular senescence, loss of telomeric structures, mitochondrial activity, production of reactive oxygen species, and DNA repair capability (Walston et al., 2006). In the present study, it was shown that the donor's age did not affect the result of the cytotoxicity tests of the mouthwashes using a primary gingival fibroblast cell model. Therefore, these findings provide the confirmation that the *in vitro* biological response of elderly cells to available commercial mouthwashes is quite similar to younger cells systems, often employed in dental materials testing.

In this study, the comparison was made with two donors for each age group. It is worth mentioning that the possibility that individual factors, more than factors related to age, may have affected the results of cytotoxicity tests is not ruled out.

However, the general results indicate relevant similarities in the performance of human gingival fibroblasts from an older donor when compared to a younger donor during the *in vitro* evaluation.

5. Conclusion

The present findings indicate that both 12% Chlorhexidine Digluconate - Periogard® and Cetylpyridinium Chloride – Oral B® present dose-dependent cytotoxicity to human gingival fibroblasts and should be used sparingly to prevent the spread of SARS-CoV-2, following manufacturers' instructions. However, there is no difference in the biological response of young or elderly fibroblasts to these products. Additional evaluations can be carried out to verify whether these young/elderly cell models differ in other biological endpoints, in addition to cytotoxicity, when testing new mouthwashes or oral solutions.

Conflict of Interest

The authors declare no conflict of interest.

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