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Cytotoxicity effect of orthodontic miniscrew-implant in different types of mouthwash: An in-vitro study

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Abstract:

CONTEXT: Orthodontic miniscrew implants (OMIs) are widely used as anchorage alternatives, but recent studies revealed the corrosion behavior of OMIs when they come in contact with mouthwashes. The corrosion materials that are released can cause toxicity, allergy, and mutagenicity.

AIMS: This study aims to analyze the cytotoxicity effects of OMIs exposed to different types of mouthwash using human gingival fibroblast (HGFs).

SETTINGS AND DESIGN: Experimental laboratory research.

METHODS AND MATERIAL: Twenty-eight samples of Ti alloy OMIs immersed separately in four groups of different types of mouthwash (chlorhexidine gluconate 0.2% mouthwash (CHX), fluoridated (sodium fluoride 0.2%) mouthwash, chitosan mouthwash 1.5%, and aquadest) for 28 d. Elution of each group and the mouthwash itself were added to the cell culture and incubated for 24 h. Changes in cell viability were performed by MTT Assay.

STATISTICAL ANALYSIS USED: Data were tested for normality with Shapiro–Wilk, homogeneity with Levene test, and analyzed using an independent T-test ($P < 0.05$).

RESULTS: The differences between the cytotoxicity of the elution of MIO and the mouthwash solution itself in the group of CHX and Fluoride were statistically significant ($P < 0.05$). No significant differences were found in the group of chitosan and aquadest ($P > 0.05$).

CONCLUSIONS: The 1.5% chitosan mouthwash can be offered to patients with Ti alloy-based OMIs rather than the 0.2% chlorhexidine gluconate and 0.2% sodium fluoride mouthwashes.

Keywords:

Chitosan, chlorhexidine, cytotoxicity, mouthwash, orthodontic mini-implant, Ti alloy

Introduction

Biomaterials used in dentistry, or orthodontics particularly, must have good biocompatibility. Ti alloy is one of the materials currently used for orthodontic miniscrew-implants (OMIs) or temporary anchorage devices (miniscrews). Miniscrews are orthodontic devices that are used as an intraoral skeletal anchorage. This compliance-independent device is known to be easy to install and remove, relatively affordable, and able to provide absolute anchorage.[1,2]

Chlorhexidine and fluoride mouthwashes are common choices of mouthwash used to maintain oral health in orthodontic patients.[3,4] However, several studies have reported the effect of the exposure of both types of mouthwash on the properties and biocompatibility of orthodontic appliances, including miniscrew implants.[5-7] A study using scanning electron microscopy (SEM) also stated that fluoride mouthwash could cause corrosion in the form of crevices

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and pits on the surface of miniscrews after 28 d of immersion.[8] These findings need some attention because the use of mouthwashes is often recommended for patients to maintain their oral health.

Chitosan, on the other hand, as a natural biomaterial has been studied as an alternative mouthwash because of its antibacterial properties and biocompatibility.[9-12] Its antibacterial activity against total bacteria and *E. faecialis* has been found to be comparable to chlorhexidine mouthwash.[10,13] Therefore, this study sought to evaluate the cytotoxicity effect of miniscrews exposed to different types of mouthwash using human gingival fibroblast (HGFs).

Subjects and Methods

Samples

Miniscrews used in this study were dual-top composed of Ti alloy (Ti_6Al_4V), (Jeil Medical, Korea). The four types of solutions used are commercially available: 0.2% digluconate mouthwash (MINOSEP®, PT. Minorock Mandiri, Depok, Indonesia), 0.2% sodium fluoride mouthwash (Pepsodent Pro Complete, Unilever, Indonesia), 1% chitosan with 0.25% acetic acid (KITOBE™, Berkah Inovasi Kreatif Indonesia, Bogor, Indonesia), and aquadest (Aqua Pro Injection Sterile, PT. Ikapharmindo Putramas, Jakarta, Indonesia). This protocol was approved by Ethics Committee of Research at the Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia (ref. No. 11/Ethical Approval/FKGUI/VII/2020 Date of approval: 21-July-2020.

Preparation of eluates from miniscrews

Each type of treatment solution (n = 7) was randomly filled in Eppendorf tubes and randomly labeled as red, blue, green, and yellow by the first author's supervisor.

The amount of solution was calculated by the ratio of 1 mL for 0.2 g of miniscrew weight according to DIN ED ISO 10271. Twenty-eight specimens of eluates were obtained by individually immersing miniscrews in 385 µl of each type of treatment solution (seven eluates of miniscrews in each type of solution) and incubated for 28 d at 37°C [Figure 1]. Each type of treatment solution without TAD was also incubated for 28 d as a control group.

Human primary gingival fibroblast cell culture

Human primary gingival fibroblast (HGF) cells were obtained from the stored biological material of the previous study. The cells were cultured in alpha-modified eagle medium (α-MEM) pH 7.2 (Gibco, Grand Island, NY, USA) with 10% of fetal bovine serum (FBS) (Biosera, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% amphotericin B (Gibco, Grand Islan, NY, USA).

Figure 1: Preparation of eluates from miniscrews in different types of mouthwash

Cultures were maintained at 37°C, 5% CO_2, and 95% air until reach 70%–80% confluence [Figure 2].

In vitro cytotoxicity by MTT assay

Aliquots of 100 µl of HGF cell suspension (4×10^4 cells/well) were pipetted into 96-well flat-bottom plates (Corning Costar Corporation, Cambridge, MA, USA) and incubated for another 48 h to obtain a cell monolayer. After the monolayer growth was confirmed by an inverted light microscope, the culture medium was removed and 20 µl of each eluate or 20 µl of each treatment solution with 100 µl of fresh culture medium were added to the correspondent well. Each eluate was tested in quadruple and incubated at 37°C, 5% CO_2, and 95% air for 24 h. Aliquots of 20 µl 2% chlorhexidine gluconate solution and an additional 20 µl of the medium culture were also added to the well as positive and negative control, respectively [Figure 3].

After 24 h, the toxic effect of miniscrews exposed in the mouthwashes was tested against the mouthwash itself by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. 10 µl aliquots of MTT solution (5 mg/ml) were added to each well and incubated for another 3 h at 37°C, 5% CO_2, and 95% air. Next, 100 µl of acidified isopropanol (HCL and isopropanol) were added to dissolve the formazan crystals and the absorbance was read by using an enzyme-linked immunosorbent assay (ELISA) plate reader at 600 nm. The percentage of cell viability was calculated as

$$\text{Cell viability}\,(\%) = \frac{\text{Treated cells}}{\text{Negative control cells}} \times 100$$

Statistical analysis

All data were processed in Excel and tested with the Statistical Package for Social Science (SPSS) version 23.0 software program (IBM Corp., Armonk, NY, USA).

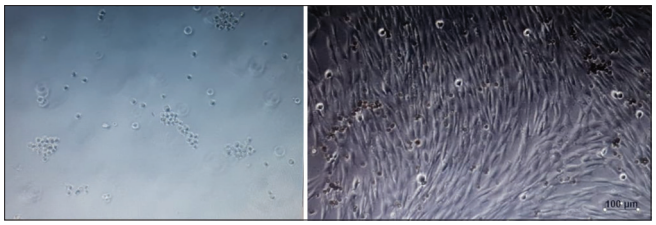

Figure 2: Human gingival fibroblast cells (HGFs), initial stage (left) and after reached 80% confluence (right)

Percentage of cell viability in each group was tested for normality with Shapiro–Wilkis test and homogeneity with Levene test. The difference in the percentage of cell viability between the eluates and the treatment solutions in the chlorhexidine, fluoride, chitosan, and aquadest groups were tested using an independent T-test. Statistical significance was determined at the level of $P < 0.05$.

Results and Discussion

There are four groups of cytotoxicity based on the percentage of cell viability, namely not cytotoxic if there are more than 90% of cells that are viable, mild cytotoxic if 60%–90% of viable cells are found, moderate cytotoxic if 30%–59% of viable cells are obtained, and severe cytotoxicity when less than 30% of cells are viable.[14]

The four test solutions without OMI immersion showed different toxicities [Table 1]. Aquadest and 1.5% chitosan solution showed less than 30% toxicity, meaning that aquadest and 1.5% chitosan solution were not toxic to gingival fibroblasts. This is in accordance with previous research by Chellat *et al*.[15] that chitosan does not show toxicity in fibroblast cell lines. This finding is different from the research of Uğur Aydin *et al*.,[16] which revealed a moderate toxic effect of chitosan solution on gingival fibroblast cell line. The possibility of the differences in these results is due to the greater concentration of the chitosan solution used as well as the different test methods.

A solution of 0.2% chlorhexidine and 1.5% fluoride (sodium fluoride 0.2%) was found to be highly toxic to gingival fibroblast with cell viability of less than 30%. The toxicity of the two solutions has been a concern in recent decades. Chlorhexidine was found to be toxic to gingival fibroblast cells[17,18] and several other cells,[19] as well as fluoride which was also found to be toxic to gingival fibroblasts with the level of toxicity depending on the concentration and time of exposure.[20]

The four test groups in this study showed an increase in the toxicity of the mouthwashes [Figure 4]. The increase in toxicity is most likely due to the toxic effect of the release of OMI Ti alloy metal ions in the mouthwashes due to decreased resistance of OMI. The released

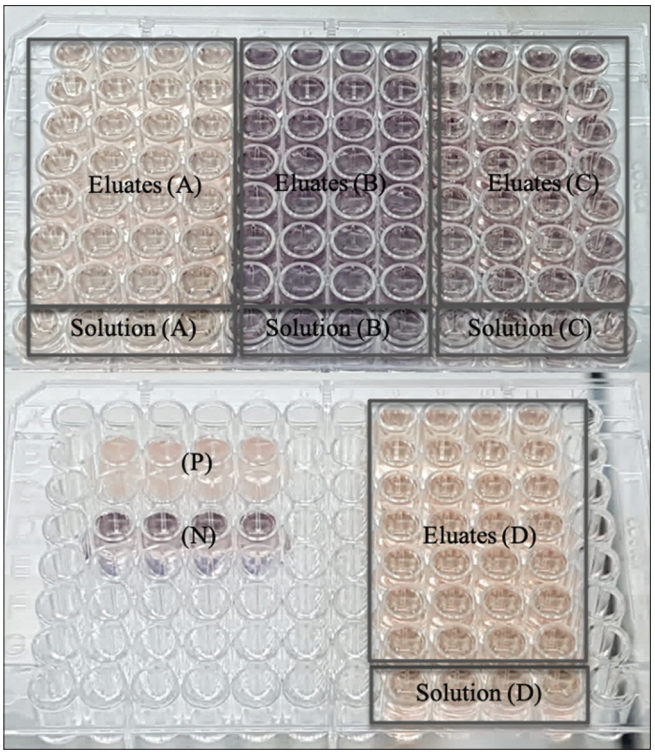

Figure 3: Configuration of the 96-well flat-bottom plates. Eluates of Miniscrews on the treatment solutions; (A) 0.2% Chlorhexidine gluconate mouthwash, (B) 1% Chitosan solution, (C) Aquadest, (D) 0.2% Sodium fluoride mouthwash. 2% chlorhexidine gluconate as positive control (P) and complete medium without treatment solution as negative control (N)

Table 1: The comparison of cell viability between groups

Groups	Solution	Elution	*P* (independent *t*-test)
Aquadest	95.91±0.61	97.01±4.42	0.520
Chlorhexidine	5.15±0.35	1.96±0.51	0.000*
Fluoride	5.05±0.69	1.99±0.23	0.000*
Chitosan	93.97±6.45	101.25±4.11	0.076

*P<0.05=significant

metal ions can induce toxic effects depending on the type, size, and concentration. Okazaki *et al*.[21] in their research revealed that the concentration of aluminum ions 0.2–0.5 ppm and 0.002–0.2 ppm vanadium in the culture medium could significantly decrease the viability of L929 and MC3T3-E1 cells, while the concentration of Ti ions was 0.2–0.3 ppm in the medium culture is not toxic to the two cells. Another study revealed that Ti ions are toxic at concentrations of more than 11 ppm in the culture medium.[22] However, we cannot conclude the relationship between the type and concentration of metal ions released and the toxicity of gingival fibroblast cells in this study. This can be further investigated by quantitatively evaluating the number and types of metal ions released in the test solution by using the inductively coupled plasma-optical emission spectrometry (ICP-OES) method.

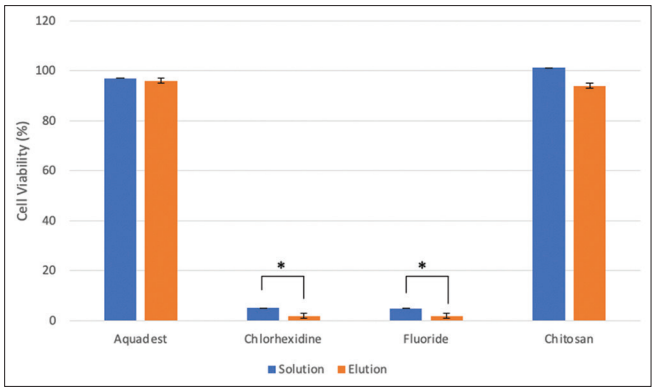

Figure 4: Cell damage caused by solution without TADs (blue) and caused by elution or solution conditioned by miniscrews for 28 d (orange). (*): Cell survival was significantly decreased in chlorhexidine and fluoride groups ($P < 0.05$)

Two test groups in this study showed a significant increase in toxicity, which are the 0.2% chlorhexidine gluconate solution and 0.2% fluoride solution. This result is in line with the research of Quaranta *et al.*,[23] which showed the effect of exposure to 0.2% chlorhexidine mouthwashes on Ti alloy in the form of signs of corrosion on Ti alloy discs when associated with OMI corrosivity. The electrochemical study by Bhola *et al.*[24] revealed that chlorhexidine 0.1% can increase the corrosivity of the Ti material by increasing the dissolution of the Ti oxide protective layer. In addition, chlorhexidine can bind with chloride ions, which can hinder the process of repassivation of the protective layer of the Ti material.

The increase in toxicity in the fluoride group in this study is thought to be closely related to the ability of fluoride ions to damage the TiO_2 protective layer of Ti alloy. This finding is in line with the SEM research by Aboodi *et al.*[8] which shows that fluoride solution can trigger corrosion of OMI Ti alloy in the form of signs of corrosion in the form of dots and niches. Previous research by Anwar *et al.*[25] regarding the effect of fluoride on the corrosion properties of cpTi and Ti alloy dental implants also revealed that the use of fluoride therapy (NaF) above 0.1 M could significantly decrease the corrosion resistance of Ti and Ti alloy. Huang *et al.*[26] also revealed that the higher the fluoride content in a mouthwash, the lower the corrosion resistance of Ti alloy. This study used a 0.2% sodium fluoride mouthwash, which was the standard concentration and was sufficient to significantly increase the toxic effect of OMI Ti alloy. The phenomenon of corrosion of Ti alloy in fluoride solution is described by the formation of dissolved Ti–F complexes in the form of Na_2TiF_6[27] or in the form of $TiCl_6$ and TiF_6,[28] which leads to a decrease in the resistance of the protection layer.

Immersion of OMI Ti alloy in aquadest and chitosan solution did not significantly increase the toxicity. This is in accordance with several previous studies which proved that Ti alloy was not toxic and did not cause a decrease in the viability of gingival fibroblast cells with the extraction medium of culture medium and saline solution.[29,30] Toxicity of OMI immersed in aquadest and chitosan solution (95.91% and 93.97%) in this study was slightly larger than those found in the research of Finke *et al.*,[31] which was 89.4%. Although using the same OMI brand, this difference may occur due to differences in the extraction medium and cell types used. This study by Finke *et al.*,[31] used a culture medium (α-MEM) as an extraction media with gingival fibroblast cell line, whereas this study used gingival fibroblast primary cells.

Nonetheless, the results of this study must be interpreted with caution because like other *in vitro* studies, it does not describe the actual condition of the mouth. The results obtained may show lower toxicity than the actual oral conditions due to the presence of saliva as a buffer. In contrast, it can also show higher than actual toxicity, probably due to the dynamic conditions of the oral cavity with varying salivary pH and oral temperature.

This study shows that the elution of OMI Ti alloy in chlorhexidine and fluoride mouthwashes can increase its toxic effect on gingival fibroblasts. This study also showed that OMI Ti alloy elution in aquadest and chitosan solution was not toxic to gingival fibroblasts. This finding can provide added value in the development of chitosan solution as a mouthwash for patients with OMI Ti alloy or other Ti alloy-based devices, apart from its effectiveness which is also still under development.

Conclusion

1. The 0.2% chlorhexidine gluconate and 0.2% sodium fluoride mouthwashes are highly toxic to HGF cell cultures, while the 1.5% chitosan solution and aquadest are not toxic to HGF cell cultures.
2. Miniscrews significantly increased the toxicity of the 0.2% chlorhexidine gluconate and 0.2% sodium fluoride mouthwashes to HGF cell cultures, but it did not increase the toxicity of 1.5% chitosan solution and aquadest.

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Conflicts of interest
There are no conflicts of interest.

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