**Cytotoxic Effect of Astringent Agents on Human Gingival Fibroblasts**

**Abstract**

**Aims**: During fixed prosthesis fabrication, gingival margin retraction is an important step before making an impression. Astringent agents should provide sufficient free gingival margin displacement, and must be free of systemic and local harmful adverse effects. This study aimed to determine the biocompatibility of three different astringent agents on human gingival fibroblast.

**Materials and Methods**: In order to evaluate cytotoxicity of 25% aluminum chloride, 25% aluminum sulphate and 20% ferric sulphate, in 24 well culture plates containing human gingival fibroblasts, RPMI media, antibiotic and 10% fetal bovine serum was added. Cell cultures were incubated in a CO2 incubator. After 1, 5 and 15 minutes, optical absorption of each plate was determined by MTT assay. Cytotoxicity of each astringent at 1, 5 and 15 minutes was compared with each other by student *t*-test. A p-value <0.05 was considered as significant level.

**Results**: The cytotoxicity of aluminum chloride at all periods of time was significantly greater than the other two astringents (p < 0.05). At 1 minute application, cytotoxicity of ferric sulphate was significantly lower than aluminum sulphate (p = 0.01). At 5 minutes, the effect of ferric sulphate and aluminum sulphate was similar and at 15 minutes, aluminum sulphate had significantly lower cytotoxicity compared to ferric sulphate (p = 0.043).

**Conclusions**: At all tested time periods, 25% aluminum chloride exhibited greater cytotoxicity than aluminum sulphate and ferric sulphate. At 1 minute, cytotoxicity of ferric sulphate compared to aluminum sulphate was lower, at 5 minutes similar, and at 15 minutes was greater.

**Key words**: cytotoxicity; astringent agents; MTT assay; fibroblast

**Introduction:**

Gingival margin retraction is an approved procedure during fixed prosthodontic construction. By providing visibility, easy access to a clean and dry gingival sulcus, it creates optimal conditions for performing direct and indirect tooth restoration, especially for those with subgingival finish lines. The impression procedure for fixed prosthodontic restorations requires careful management of the soft tissues. The gingival tissues must be displaced to allow sufficient bulk of impression material or tooth scan in the gingival sulcus [1].For this purpose, various methods and techniques have been used, including mechanical, chemical, mechanical-chemical and surgical methods. Of these four categories, the mechanical-chemical is the most commonly used technique for gingival tissue retraction. Although chemo-mechanical method is an effective and predictable technique, previous studies have shown that some gingival retraction cords tend to produce transient damage to the gingival sulcular epithelium and underlying connective tissues [2-6].Additionally, the use of retraction cord can be time-consuming, difficult and uncomfortable for patients in the lack of anesthesia [7,8].

Recently various chemical retraction agents have been introduced. The claimed advantages are: saving of time, patient comfort and minimal invasion. All retraction agents can be selected as astringents or vasoconstrictors. All retraction agents using astringents have high acidity that varies from pH of 1 to pH of 3 in their original and diluted concentrations. Therefore, tissues and tooth structure could be influenced by this acidity [9-12].Kopac et al. [13] , showed that chemical retraction agents are cytotoxic to Chinese hamster lung fibroblasts. Harrison et al. [4] concluded that temporary changes induced in the gingival sulcular epithelium by gingival retraction agents can result in damage in junctional epithelium and original connective tissues. According to de Gennaro et al. [10], although the effectiveness of astringents under clinical conditions is desirable, *in vivo* and *in vitro* observations showed undesirable local side effects on gingival margin tissues.

To study cytotoxicity of dental materials and agents, human fibroblast cell cultures have become commonly accepted in recent years, because these cells are comparable to those of the oral cavity in their reaction pattern. Therefore, in most of the studies that determined cytotoxicity, these types of fibroblast were used [14,15].Dental materials can be assessed by cell culture as it is replicable, economical, and controllable. To determine the biocompatibility of retraction agents by human fibroblast viability evaluation, MTT assay is one of the most valuable and appropriate method. Mosmann [16] first performed MTT cytotoxicity analysis and the results of this test are directly dependent on the number of viable remaining cells after a period of incubation time.

Today, although aluminum chloride, aluminum sulfate and ferric sulfate are the commonly used astringents in clinical practice, only a few studies have been conducted to compare the biologic characteristics of these agents. Hence, the aim of this study was to evaluate the effects of: 25% aluminum chloride, 25% aluminum sulphate and 20% ferric sulphate on human gingival fibroblasts at 1, 5 and 15 minutes, which are considered reasonable periods of time for adequate gingival displacement.

**Materials and Methods:**

Human gingival fibroblasts (HGF1-PI1) were cultured for cytotoxicity evaluation (Table 1). Regarding the aim of this experiment and the three time periods to compare the three astringents with each other, 225 wells of a microplate in 15 groups were used. Plates with no astringent, containing only fibroblast and RPMI media, made up the negative control group and the plates in which RPMI was replaced with distilled water had the role of positive control. For the blank control group the plates only contained RPMI media. All experiments were conducted twice.

The tested astringents in this experiment were:

1. 25% aluminum sulphate gel (Light Blue Gel, Pascal- USA)

2. 20% ferric sulphate gel (ViscoStat, Ultradent-USA)

3. 25% aluminum chloride gel (Hemosthase Gel, FGM-Brazil).

The cytotoxic effects of each astringent were tested after 1, 5 and 15 minutes of exposure to the fibroblast cells.

For culturing the cells, fibroblasts HGF1-PI were passaged in culture flasks. After obtaining an adequate volume of cultured cells, EDTA-trypsin solution was used to separate the cells. In order to raise the number of cells to 3×10 [14], after collecting the cells, they were counted using a neobar lam and the cell volume in the flask was increased to the desired level. Fibroblasts were cultured in RPMI supplemented with streptomycin, penicillin and 10% fetal bovine serum (FBS). Cells were maintained at 370C in a 5% CO2 incubator. Viability of the cells was evaluated by Trypan blue solution staining and light microscope with ×40 magnification. The number of viable cells required for succeeding stages of experiment should be more than 90% (stained area) or evidence of cell death and necrosis less than 10% (non-stained area). Each well held one sample solution which was numbered randomly.

The MTT assay, that measures mitochondrial function as a surrogate for cell cytotoxicity, was performed separately after 1, 5 and 15 minutes of astringent application. For MTT test, 10μL of MTT solution was added to each micro plate followed by incubation at 370c incubator. After 4 hours, if the cells were able to reduce MTT, they produced formazan crystals that changed the color of the media and made it dark. When the color changed, 200μL of acid-alcohol solution (0.04 M HCL in isopropanol) was added and mixed thoroughly to dissolve formazan crystals. Finally, the intensity of staining was determined in an ELISA plate reader at 630nm. The absorbance represents the total number of viable cell.

For statistical comparison of cell viability in different culturing conditions, the student T-test was used (α=0.05).

**Results:**

The cell viability as measured by the MTT assay, at different periods of time exposed to astringent agents is illustrated in Figure 1 and Table 2. The most aggressive agent was aluminum chloride which at all periods of time destroyed almost all fibroblasts. Cytotoxicity of aluminum chloride at 1, 5 and 15 minutes was significantly greater than the ferric sulphate and aluminum sulphate (p < 0.05). Cytotoxicity of aluminum sulphate and ferric sulphate after 1 minute showed significant difference (p = 0.01) with ferric sulphate less toxic than aluminum sulphate. After 5 minutes of applying ferric sulphate and aluminum sulphate, there was no significant difference in their cytotoxic effect (p = 0.053). However; aluminum chloride at the same time showed significant differences with two other astringents (p = 0.023). After 15 minutes, aluminum sulphate showed lower cytotoxicity than ferric sulphate and the difference was significant (p = 0.043).

**Discussion:**

Results of this experiment indicated that the tested astringents exhibited different levels of cytotoxicity at different time periods of exposure to culture human gingival fibroblasts. Regardless of incubation time period, 25% aluminum chloride was significantly more cytotoxic than the other two astringents. However 25% aluminum sulphate at 1 minute showed higher cytotoxicity than 20% ferric sulphate, the cytotoxicity of both was similar at 5 minutes, while at 15 minutes, aluminum sulphate was less cytotoxic than ferric sulphate.

In vitro cytotoxic assessment as a principal factor of biocompatibility is determined by different cell culture. The guidelines from the American National Standards Institute (ANSI), the American Dental Association, and the Technical Report ISO-TR 7405 of the International Standards Organization Technical Committee in regard to dentistry (TC 106) have accepted in vitro methods. Due to humane and practical reasons, cell culture techniques are more appropriate than in vivo studies on animals or humans [17,18].Cell culture methods allow a precise quantitative and qualitative assessment of the results. However, in vivo models are more favorable for qualitative evaluation of cytotoxicity effects [19]. For *in vitro* determination of biomaterial cytotoxicity, several tests may be employed. This study was performed using the MTT assay due to its appropriateness and accessibility [16].

In clinical practice, gingival retraction agents come in different forms such as fluids or gels and their active substance based on their pharmacological effects can be categorized into two classes, namely class 1 (vasoconstrictors, adrenergic) and class 2 (hemostatic, astringents) [20,21].Chemical retraction agents are astringent if they contain aluminum chloride, aluminum sulphate, ferric sulphate, zinc chloride and aluminum potassium sulfate. These astringents are used routinely for gingival margin retraction by many practicing dentists [22] and there is direct contact between prepared tooth structure and free gingival margin tissue with ordinary non-injectable (packing) astringents and injected retraction materials in the gingival sulcus. It has been shown that all astringents have chemically high acidity in the range of pH1 to pH3. The low pH value of agents was observed in both original concentrations and a dilution in both fluid and gel forms [23].

Although the results of some investigations indicate that astringent use in clinical conditions has a positive effect, other in vitro and in vivo studies demonstrated unfavorable local side effects of astringents on gingival tissues [13,24-26]. The current study concluded that the tested astringents were destructive to fibroblasts and the degree of destruction depends on the exposure length. Twenty five percent aluminum chloride was the most toxic agent because of the destruction of almost the entire cell culture at all tested periods of time, even within 1 minute of exposure. These findings support those of Kopac et al. [13] who showed that all astringents in their original form were cytotoxic and that the toxic effect of 25% aluminum chloride was significantly higher than that of other chemical agents. Following on, Kopac et al. [26] indicated that 25% aluminum chloride caused changes in rat keratinocytes’ primary cell culture after 10 minutes of treatment. However, contrary to our study, Lodetti et al. [27] reported that astringents retraction solutions produced damage on human oral keratinocytes due to ferric sulphate and ferric sub sulphate. Nowakowska et al. [28] showed that ferric sulfate agents were the most toxic , followed by aluminum chloride and aluminum sulphate.

To simulate clinical conditions, although 3 to 10 minutes is often allowed for the process of chemo-mechanical retraction [29], it has been demonstrated that keeping the astringent in place for up to 15 minutes yields both a good tissue response and prolonged opening of gingival sulcus, thus guaranteeing a very high percentage of impression success [30,31]. Therefore, our study was implemented using original concentration for 3 periods of time of 1 min, 5 min, and 15 min. Also, in the current study we evaluated biocompatibility of astringent agents on human gingival fibroblasts because when these agents are applied clinically, they come in close contact with gingiva.

The result of our study showed that after 15 min incubation of retraction astringents, the lowest viability of fibroblasts was observed. Therefore, cytotoxicity is time-dependent with longer exposures leading to greater cytotoxicity and less cell viability. Another finding is that the most cytotoxic retraction agents are those made up of aluminum chloride followed by aluminum sulphate and ferric sulphate.

From the findings of this in vitro study it cannot be automatically assumed that these and other astringents will have identical effects in clinical conditions. It is proposed that there is a decrease in the direct negative effect of the mentioned chemicals in clinical situation. This can occur due to the barrier formed between astringent and fibroblasts by the epithelium of the healthy gingiva. While different factors such as water spray, human saliva and natural gingival sulcular fluid flow may dilute the concentration of astringent, there is less intensity in negative clinical performance of chemical retraction agents [32]. Furthermore, since this study was performed in vitro, we considered the cytotoxic effects of chemicals only on cultured cells. Therefore, it is necessary to consider all other relevant factors to gingival retraction that might occur in clinical situations and future in vivo studies are recommended.

**Conclusions:**

Based on the limitations of the present study it is concluded that:

1. 25% aluminum chloride was the most toxic agent at all time periods of exposure tested.

2. After 1 minute of applying the tested agents, ferric sulphate showed lower cytotoxicity effects compared to aluminum sulphate.

3. After 5 minute of applying the tested agents, both aluminum sulphate and ferric sulphate had similar cytotoxic effects.

4. After 15 minutes of applying the tested agents, aluminum sulphate showed lower level of cytotoxicity compared to ferric sulphate.

**References**:

1. Kurbad A. Impression –free production techniques. *International Journal of Computerized Dentistry.* 2011;**14**:59-66.

2. Bennani V, Schwass D, Chandler N. Gingival retraction techniques for implants versus teeth. *Journal of the American Dental Associasion*. 2008;**139**:1354-1363.

3. Hansen PA, Tina DA, Barlow J. Current methods of finish-line exposure by practicing prosthodontists. *Journal of Prosthodontics*. 1999;**8**:163-170.

4. Harrison JD. Effect of retraction materials on the gingival sulcus epithelium. *Journal of Prosthetic Dentistry*. 1961;**11**:514-521.

5. Ruel J, Schuessler PJ, Malament K, Mori D. Effect of retraction procedures on the periodontium in humans. *Journal of Prosthetic Dentistry* .1980;**44**:508-515.

6. Woycheshin FF. An evaluation of the drugs used for gingival retraction. *Journal of Prosthetic Dentistry*. 1964;**14**:769-776.

7. Azzi R, Tsao TF, Carranza FA, Kenney EB. Comparative study of gingival retraction methods. *Journal of Prosthetic Dentistry*. 1983;**50**:561-565.

8. Feng J, Aboyousef H, Weiner S, Singh S , Jandinski J. The effect of gingival retraction procedures on periodontal indices and crevicular fluid cytokine levels: a pilot study. *Journal of* *Prosthodontics*. 2006;**15**:108-112.

9.Ayo-Yusuf OA, Driessen DH, Botha AJ. SEM-EDX study of prepared human dentine surfaces exposed to gingival retraction fluids. *Journal of Dentistry*. 2005;**33**:731-739.

10. de Gennaro GG, Landesman HM, Calhoun JE, Martinoff JT. A comparison of gingival inflammation related to retraction cords. *Journal of Prosthetic Dentistry*. 1982;**47**:384-386.

11. Nowakowska D, Panek H, Bogucki ZA. Clinical and microscopic evaluation of retraction set Racecord Systeme ®. *Dental and Medical Problems*. 2006;**43**:85-86. (in Polish)

12. Nowakowska D. The impact of retraction astringents on gingival margin tissues from literature review of in vivo studies. *Protetyka* *Stomatologiczna*. 2009;**59**:119-124. (in Polish)

13. Kopac I, Batista U, Cvetko E, Marion L. Viability of fibroblasts in cell culture after treatment with different chemical retraction agents. *Journal of Oral* *Rehabilitation*. 2002;**29**:98-104.

14. Chang YC, Chou MY. Cytotoxicity of fluoride on human pulp cell cultures in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*. 2001;**91**:230-234.

15. Chang YC, Tai KW, Huang FM, Huang MF. Cytotoxic and nongenotoxic effects of phenolic compounds in human pulp cell cultures. *Journal of Endodontics*. 2000;**26**:440-443.

16. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983;**65**:55-63.

17. Pucher JJ, Daniel CJ. The effects of chlorhexidine digluconate on human fibroblasts in vitro. *Journal of Periodontology*. 1992;**63**:526.

18. Tipton AD, Braxton DS, Dabbous KM. Effects of a bleaching agents on human gingival fibroblasts. *Journal of Periodontology*. 1995;**66**:7.

19. Schedle A, Samorapoompichit P, Rausch-Fan XH, Franz A, Fureder W, Sperr WR, [Ellinger](http://jdr.sagepub.com/search?author1=A.+Ellinger&sortspec=date&submit=Submit) A,  [Slavicek](http://jdr.sagepub.com/search?author1=R.+Slavicek&sortspec=date&submit=Submit) R,  [Boltz-Nitulescu](http://jdr.sagepub.com/search?author1=G.+Boltz-Nitulescu&sortspec=date&submit=Submit) G,  [Valent](http://jdr.sagepub.com/search?author1=P.+Valent&sortspec=date&submit=Submit) P. Response of L-929 fibroblasts, human gingival fibroblasts, and human tissue mast cells to various metal cations. *Journal of Dental Research*. 1995;**74**:1513-1520.

20. Nowakowska D, Panek H. Classification of retraction materials in the aspect of biocompatibility with gingival sulcus environment. *Polish Journal of Environmental Studies*. 2007;**16**:204-208.

21. Nowakowska D. Classification of chemical retraction agents. *Protetyka* *Stomatologiczna.* 2008;**58**:202-208. (in Polish)

22.Nowakowska D, Panek H, Nowakowska M, Nowakowska A. Gingival retraction- survey results of Polish dentists. Part 1. Method, materials and chemical retraction agents preferences. *Protetyka* *Stomatologiczna*. 2006;**56**:352-360. (in Polish)

23. Nowakowska D, Raszewski Z. Evaluation of pH levels in gingival retraction agents. *Protetyka* *Stomatologiczna.* 2009;**59**:25-31.

24. Al Hamad KQ, Azar WZ, Alwaeli HA, Said KN. A clinical study on the effects of cordless and conventional retraction techniques on the gingival and periodontal health. *Journal of Clinical Periodontology.* 2008;**35**:1053-1058.

25.Kopac I, Cvetko E, Marion L. Gingival inflammatory response induced by chemical retraction agents in Beagle dogs. *International Journal of Prosthodontics.* 2002;**15**:14-19.

26. Kopac I, Sterle M, Marion L. Electron microscopic analysis of the effects of chemical retraction agents on cultured rat keratinocytes. *Journal of Prosthetic Dentistry*. 2002;**87**:51-56.

27. Lodetti G, D'Abrosca F, Fontana P, Pavoni E, Gigola P. Set up of in vitro methods able to detect the safety of astringent liquids. *Minerva* *Stomatologica*. 2004;**53**:361-367.

28. Nowakowska D, Saczko J, Kulbacka J, Choromanska A. Dynamic oxidoreductive potential of astringent retraction agents. *Folia Biologica* (Praha). 2010;**56**:263-268.

29.Nowakowska D, Panek H, Nowakowska M, Nowakowska A. Gingival retraction- survey results of Polish dentists. Part 2. Clinical habits related to retraction procedures. *Protetyka* *Stomatologiczna*. 2006;**56**:361-366. (in Polish)

30. Ramadan FA, Harrison JD. Literature review of the effectiveness of tissue displacement materials. [*Egyptian Dental Journal*](http://europepmc.org/search?page=1&query=JOURNAL:%22Egypt+Dent+J%22&restrict=All+results). 1970;**16**:271-282.

31. Weir DJ, Williams BH. Clinical effectiveness of chemical tissue displacement methods. *Journal of Prosthetic Dentistry*.1984;**51**:326-329.

32. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick AZ. Characterization of the differentiated antioxidant profile of human saliva. [*Free Radical Biology and Medicine*](http://www.sciencedirect.com/science/journal/08915849)*.* 2002;**32**:268-277.

**Table 1. Properties of the cultured media**

|  |  |
| --- | --- |
| Designation | 1HGF1-PI |
| Species | Human |
| Tissue | Gingiva |
| Morphology | Fibroblast-like |
| Culture Medium | 2RPMI 1640 + 10% 3FBS |
| Preservation Medium | FBS + 10% 4DMSO |
| Isoenzymes | 5LDH,6G6PD,7NP |
| Passage No | 7 |
| Viability | 89%, 1.2 × 10^6 cells/vial |
| Chromosome Frequency Distribution (Cells /Chromosomes) | 1 | 1 |28  44|45|46 |
| NCBI8 Code | C165 |

1-Human gingival fibroblast

2-Roswell Park Memorial Institute (culture medium)

3-Fetal Bovine Serum (culture media)

4-Dimethylsulfoxide

5-Lactate Dehydrogenase

6-Glucose-6-Phosphate Dehydrogenase

7-Nucleoside Phosphorylase

8-National Center of Biotechnology Information

|  |  |  |  |
| --- | --- | --- | --- |
| 1min | 5 min | 15 min | groups |
| 3.6±0.5 | **2.7±0.5** | **1.4±0.3** | 25% aluminium sulphate |
| 0.1 | **0** | **0** | 25% aluminium chloride |
| 4.3±0.2 | **2.8±0.5** | **1.1±0.2** | 20% ferric sulphate |
| 9.9±0.1 | **9.9±0.1** | **9.9±0.1** | Normal saline (positive control) |
| 10±0.2 | **10±0.2** | **10±0.2** | Media only (negative control) |

**Table2.Mean and standard deviation of optical density of groups in this experiment**

Figure legend:

Figure.1. Comparison of cell viability based on optical density after applying astringents at different periods of time