Cytotoxic Effect of Astringent Agents on Human Gingival Fibroblasts

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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 absorbance of each plate was determined by MTT assay. Cytotoxicity of each astringent at 1, 5 and 15 minutes were compared by using Student t-test. A p-value<0.05 was considered as significant level. Results: The cytotoxicity of aluminum chloride at all time periods was significantly greater than 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. Compared to aluminum sulphate, the cytotoxicity of ferric sulphate was lower at 1 minute, similar at 5 minutes, and greater at 15 minutes.

Key Words: Cytotoxicity; Astringent agents; MTT assay; Fibroblast

Introduction

Gingival margin retraction is an approved procedure during fixed prosthodontic construction. By providing visibility and easy access to a clean and dry gingival sulcus, optimal conditions for performing direct and indirect tooth restoration would be delivered, 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 methods, 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 absence of anesthesia [7,8].

Recently, various chemical retraction agents have been introduced. The proposed advantages are being time saving and providing patient comfort and minimal invasion. All retraction agents can be selected as astringents or vasoconstrictors. All retraction agents using astringents have high acidity and their pH ranges from 1 to 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 the temporary changes which were induced in the gingival sulcular epithelium by gingival retraction agents could damage 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 the cytotoxicity of dental materials and agents, human fibroblast cell cultures have become commonly accepted in recent years, because these cells are comparable to those in oral cavity regarding 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 showed that the results of this test were directly dependent on the number of viable remaining cells after a period of incubation time.

Although aluminum chloride, aluminum sulfate and ferric sulfate are currently the most frequently 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 (Pasteur Institute, Iran) 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 was used. Plates with no astringent, containing only fibroblast and RPMI media, made up the negative control group and the plates in which RPMI

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was replaced with distilled water have been employed as the 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:

- 25% aluminum sulphate gel (Light Blue Gel, Pascal-USA)
- 20% ferric sulphate gel (ViscoStat, Ultradent-USA)
- 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 3×10^5 [14], the collected cells were counted using a neobar lam and the cell volume was increased to the desired level in the flask.

Fibroblasts were cultured in RPMI supplemented with streptomycin, penicillin and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a 5% CO_2 incubator. The cells were detached with mixture of Trypsin-EDTA. Viability of the cells was evaluated by Trypan blue solution staining under light microscope with 40× magnification.

Table 1: *Properties of fibroblast cells. 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.*

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	1 | 1 |28
(Cells /Chromosomes)	44|45|46
NCBI8 Code	C165

The number of viable cells required for succeeding stages of experiment were considered to be more than 90% (non-stained area) or the evidence of cell death and necrosis less than 10% (stained area). Each well held one sample solution 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, 10 µL of MTT solution was added to each micro plate followed by incubation at 37°C 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 was 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 630 nm. The amount of absorbance represented 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 viability of cells exposed to astringent agents at different time periods was measured by MTT assay and results are summarized in *Table 2*.

The most aggressive agent was aluminum chloride which destroyed almost all fibroblasts at all time periods. 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.

Table 2. *Mean and standard deviation of optical density of groups in this experiment.*

Groups	15 min	5 min	1min
25% Aluminium Sulphate	1.4 ± 0.3	2.7 ± 0.5	3.6 ± 0.5
25% Aluminium Chloride	0	0	0.1
20% Ferric Sulphate	1.1 ± 0.2	2.8 ± 0.5	4.3 ± 0.2

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 cultured 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 agents 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 methods. The guidelines from the American National Standards Institute (ANSI), the American Dental Association (ADA), 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. Regarding moral issues 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 can 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 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]. In practice, there is a 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 with the pH range of 1 to 3. 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 indicated that astringent use in clinical conditions had 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 depended 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 the results of Kopac et al. study [13] that showed all astringents in their original form were cytotoxic and the toxic effect of 25% aluminum chloride was significantly higher than that of other chemical agents. Moreover, Kopac et al. [26] indicated that 25% aluminum chloride caused changes in rat keratinocytes' primary cell culture after 10 minutes of treatment. However, in contrast to our results, Lodetti et al. [27] reported that astringents retraction solutions produced damage on human oral keratinocytes due to ferric sulphate and ferric sub sulphate. Now akowska 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 results of our study showed that after 15 min incubation of retraction astringents, the lowest viability of fibroblasts was observed. Therefore, cytotoxicity is time-dependent and longer exposures leads 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:

- 25% aluminum chloride was the most toxic agent at all time periods of exposure tested.
- After 1 minute of applying the tested agents, ferric sulphate showed lower cytotoxicity effects compared to aluminum sulphate.
- After 5 minute of applying the tested agents, both aluminum sulphate and ferric sulphate had similar cytotoxic effects.
- After 15 minutes of applying the tested agents, aluminum sulphate showed lower level of cytotoxicity compared to ferric sulphate.

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