

Dynamic Oxidoreductive Potential of Astringent Retraction Agents

(gingival margin retraction agents / cytotoxicity / human gingival fibroblasts)

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Abstract. The aim of this study was to evaluate the dynamics of the cytotoxicity of gingival margin retraction astringents based on aluminium chloride, aluminium sulphate, and ferric sulphate (solutions and gels) in human fibroblasts isolated from gingiva. The cytocompatibility of ten astringent-based chemical retraction agents: Gingiva Liquid, Alustin, Racestypine, Orbat sensitive, Astringedent®, Alustat, Hemostat, Racécord, Gel cord and ViscoStat®, in dilutions of 1 : 10 and 1 : 20, with human gingival fibroblasts was investigated. The MTT assay was performed to determine oxidoreductive mitochondrial function after 3, 5, 10 min and 24 h of incubation. Cell viability was determined according to the chemical group, concentration, exposure time, and the clinical form of the gingival retraction agents. Ferric sulphate-based agents were the most cytotoxic, followed by aluminium chloride and aluminium sulphate. The form of the astringents influenced cell viability. The evaluated astringents may have cytotoxic potential for gingival margin tissues under clinical conditions.

Introduction

Gingival margin retraction is a commonly accepted procedure in modern restorative dentistry. Providing visibility and easy access to a clean and dry gingival sulcus, it creates optimal conditions for performing direct and indirect tooth restoration. This is especially important for subgingival finish-line imaging using con-

ventional impression materials or CAD/CAM digital/optical techniques, for fixed dental restoration, and for adhesive methods very useful in aesthetic dentistry (Bennani et al., 2008).

Gingival retraction agents (GRAs) are used in clinical practice in the form of gingival retraction fluids (GRFs) or gingival retraction gels (GRGs) (Nowakowska and Panek, 2007). With respect to the pharmacological effects of the active substance, they belong either to class 1 (vasoconstrictors, adrenergics) or class 2 (haemostatics, astringents) (Nowakowska, 2008). Chemical retraction agents based on aluminium chloride, aluminium sulphate, ferric sulphate, and, less frequently, zinc chloride and aluminium potassium sulphate are astringents (Shillingburg et al., 1980). The above-mentioned survey demonstrated that over 80 % of dentists applied astringents for gingival margin retraction in clinical practice (Donovan et al., 1985; Hansen et al., 1999; Nowakowska et al., 2006b). Chemically, all the retraction agents containing astringents are characterized by a relatively high level of acidity, with their original concentrations ranging from pH 1 to pH 3 for solutions (Woody et al., 1993; Land et al., 1994, 1996; Ayo-Yusuf et al., 2005). Our previous study of the pH levels of commonly used astringents in solution and gel form found that the pH values of these agents both in the original concentrations and in dilutions of 1 : 10 and 1 : 20 were surprisingly low (Nowakowska and Raszewski, 2009).

Astringents containing conventional non-injectable (packing) materials and the newly developed injection-type retraction materials to be placed in the gingival sulcus remain in direct contact with free gingival margin tissues for some time and are also in contact with mineralized tooth structures prepared by cutting. The practical application time of these substances reported in clinical studies were from 2 to 30 min (De Gennaro et al., 1982; Akca et al., 2006).

In numerous studies, the effectiveness of astringents under clinical conditions was evaluated positively. However, *in vivo* and/or *in vitro* observations showed that they induce undesirable local side effects on gingival margin tissues (De Gennaro et al., 1982; Azzi et al.,

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Abbreviations: ANSI – American National Standards Institute, DMEM – Dulbecco's Modified Eagle's medium, GCF – gingival crevicular fluid, GRA – gingival retraction agent, GRF – gingival retraction fluid, GRG – gingival retraction gel, MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, PBS – phosphate-buffered saline.

1983; Nemetz et al., 1984; Weir and Williams, 1984; Benson et al., 1986; Kopač et al., 2002a,b,c; Akca et al., 2006; Kumbuloglu et al., 2007; Al Hamad et al., 2008). These authors demonstrated studies with human and animal models using various research methods that confirmed inflammatory response of the surrounding soft tissues. This was demonstrated by different methods: histomorphometric (De Gennaro et al., 1982; Kopač et al., 2002b,c; Akca et al., 2006), gingival crevicular fluid (GCF) flow measurements (Feng et al., 2006; Wöstmann et al., 2008), and of GCF analysis, for example TNF- α proinflammatory cytokine levels (Feng et al., 2006). The inflammatory response was normally transitory and its severity depended on the type and concentration of the retraction agent. Results obtained by SEM-EDX techniques reported an altered morphology of prepared human dentine surface after exposure to conventional astringents containing gingival retraction fluids (Land et al., 1994, 1996; Ayo-Yusuf et al., 2005).

Cytotoxicity evaluation of human cell colonies is one of the most objective methods for assessing the biocompatibility of dental materials and agents (Phillips, 1973; Mosman, 1983). Only Kopač et al. (2002a) studied this on Chinese hamster diploid lung fibroblasts (V-79-379 A) and Lodetti et al. (2004) evaluated keratinocyte viability after treatment with astringent-based agents. In an attempt to determine the safety level of retraction agents by human fibroblast viability evaluation, a newly developed method by Saczko et al. (2008) seems most valuable and appropriate.

The aim of this *in vitro* study was to evaluate the dynamic cytotoxic effects of different gingival retraction astringents, both solutions and gels, on human fibroblasts isolated from patients' gingival tissues.

Material and Methods

Retraction astringents

Ten gingival retraction agents from three different chemical groups (aluminium chloride, aluminium sul-

phate, and ferric sulphate), including five solutions and five gels, were selected for this study. Experiments with the original concentrations of all the gingival astringents, cell culture viability from 0 to 2 % were determined. The commercially available agents were diluted 1 : 10 and 1 : 20 with deionized water. Their characteristics and pH values are presented in Table 1.

Cell cultures

The tissue cultures of human gingival fibroblasts (Fig. 1) were obtained from patients with healthy periodontium undergoing tooth extraction. The gingival biopsies were provided by the Department of Dental Surgery of Wrocław Medical University. The cells were isolated from the healthy gingival tissues according to the procedure described by Saczko et al. (2008). The cells were grown routinely in Dulbecco's Modified Eagle's medium (DMEM). DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS and glutamine with penicillin/streptomycin (Sigma) in 25-cm² flasks (Falcon, Franklin Lakes, NJ). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO₂. For experimental purposes, the cells were removed by trypsinization (0.25% Trypsin-EDTA, Sigma).

Cytotoxicity test

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay (Sigma) was used to evaluate the cytotoxicity of the gingival retraction astringents. Cells were seeded onto 96-well plates at a concentration of 5×10^3 cells/well. For the viability assay the cells were exposed to different gingival retraction agents. Following incubation for 3, 5, and 10 min and 24 h at 37 °C, the cells were washed twice in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) and treated according to the manufacturer's protocol. The absorbance was determined using a multi-well scanning spectrophotometer at 570 nm (Multiscan MS, Helsinki, Finland). The results were expressed as the percentage of untreated control cells.

Table 1. Characteristics of the evaluated gingival retraction astringents

Chemical group	Retraction agents	Manufacturer	Lot/Batch	Active ingredients	Clinical form	pH level in dilution	
						1:10	1:20
Aluminium chlorides	Gingiva Liquid	Roeko, Langenau, Germany	1980200	10% AlCl ₃	solution	2.82	3.33
	Alustin	Chema, Rzeszów, Poland	061204	20% AlCl ₃	solution	2.33	2.84
	Alustat	Cerkamed, Nisko, Poland	31082009	20% AlCl ₃	gel	1.99	2.23
	Hemostat	Chema, Rzeszów, Poland	120609	20% AlCl ₃	gel	1.78	2.19
	Racestypine	Septodont, Saint-Maur-des-fossés Cedex, France	35928	25% AlCl ₃	solution	1.99	2.72
	Racécord	Septodont, Saint-Maur-des-fossés Cedex, France	35416	25% AlCl ₃	gel	1.76	2.48
Aluminium sulphates	Orbat sensitive	Lege artis, Germany	1481207	25% Al ₂ (SO ₄) ₃	solution	3.25	3.85
	Gel cord	Pascal, Bellevue, WA	0086	25% Al ₂ (SO ₄) ₃	gel	3.47	3.32
Ferric sulphates	Astringedent®	Ultradent, South Jordan, UT	B3338	15.5% Fe ₂ (SO ₄) ₃	solution	1.83	2.50
	ViscoStat®	Ultradent, South Jordan, UT	B31BK	20% Fe ₂ (SO ₄) ₃	gel	1.65	2.31

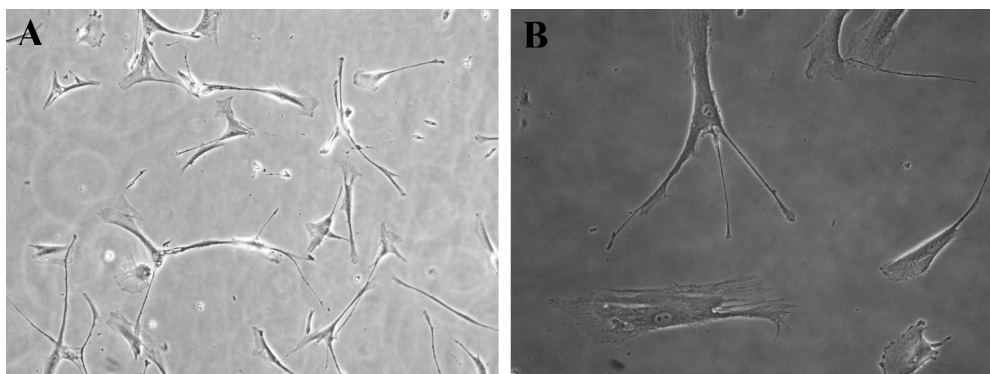


Fig. 1. Human gingival fibroblasts, primary cells: **A)** $\times 100$; **B)** $\times 200$.

Statistics

The significance of differences between the mean values of different groups of cells compared with the control group (untreated cells) was assessed by Student's *t*-test, with values of $P \leq 0.05$ taken to imply statistical significance.

Results

The influence of three retraction astringent groups on gingival fibroblasts was investigated. Oxidoreductive mitochondrial function is shown in Fig. 2. In the group of retraction astringents that contained aluminium chlo-

ride (solution- and gel-based), the oxidative mitochondrial function of the fibroblasts was similar at a dilution of 1 : 10. After 3 min of incubation, the levels of viability were about 100 %, i.e. comparable to that of the control cells (Fig. 2 A). The 10% aluminium chloride agent (Gingiva Liquid) was the least cytotoxic of all the agents in the 1 : 20 dilution. Cells treated for 5 min with these compounds displayed significantly lower oxidative mitochondrial function at both dilutions than the control cells (1 : 10, 1 : 20) (Fig. 2 A), whereas after 10 min of incubation an increase in oxidative mitochondrial function in Gingiva Liquid-treated cells was observed, higher than in the control cells. The level of viability was

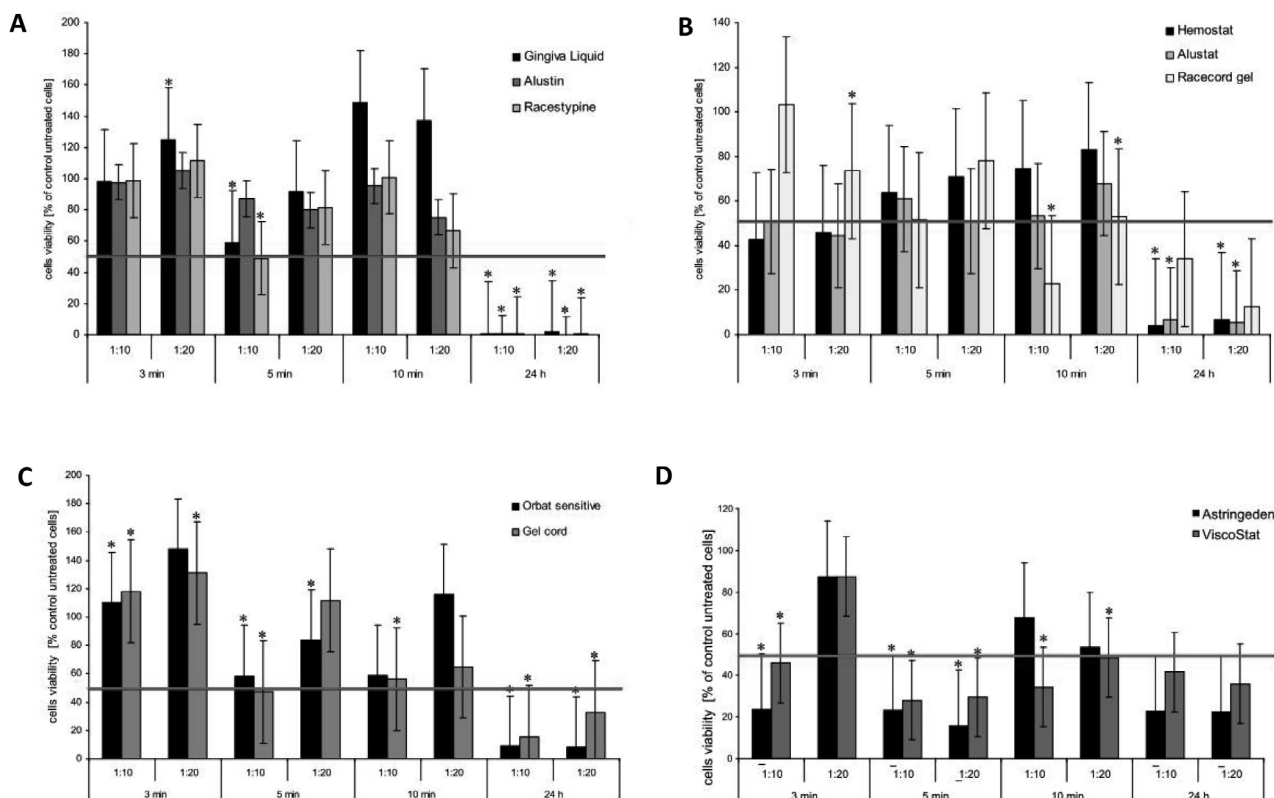


Fig. 2. Human gingival fibroblast viability after exposure to **A)** aluminium chloride-based retraction agents (solutions); **B)** aluminium chloride-based retraction agents (gels); **C)** gingival retraction astringents with aluminium sulphate groups (solution and gel); **D)** gingival retraction astringents with ferric sulphate groups (solution and gel). Results expressed as the mean \pm SD. * $P < 0.05$.

comparable to that of the control cells for cells incubated with 20% (Alustin) and 25% aluminium chloride (Racestypine) at both dilutions (Fig. 2 A). The greatest damage to mitochondrial function was observed in cells treated with 25% aluminium chloride in gel form (Racé-cord gel). The results for the 20% aluminium chloride gels (Hemostat and Alustat) indicated cell viability (40 to 70 %) for both 3 and 5 min incubation (Fig. 2 B). Twenty-four-hour incubation with the retraction astringents resulted in the highest level of damage to mitochondrial function (Fig. 2 B).

For the agents containing aluminium sulphate we noted a significant increase in mitochondrial function compared with those based on aluminium chloride. Oxidative mitochondrial function was 110% for the 1 : 10 dilution and 140% in the cells treated with 25% aluminium chloride in liquid form (Orbat sensitive) and from 120% (1 : 10) to 130% (1 : 20) in the gel form (Gel cord) (Fig. 2 C). The level of viability decreased significantly in cells after 5 min of incubation and was similar to that of the cells after 10 min (Fig. 2 C). The levels of fibroblast viability were higher with the 1 : 20 dilutions and increased similarly to the control cells for sulphate aluminium, but were on the same level as that of sulphate aluminium in the gel form. Both forms of the astringents were cytotoxic after 24 h of incubation.

The agents based of ferrous sulphate demonstrated the statistically significant lowest level of viability (Fig. 2 D). After 3 min of incubation, oxidative mitochondrial function was below 50 % in the 1 : 10 dilution and at 1 : 20 viability increased to 90 %. Oxidative mitochondrial function decreased to below 50 % after 5 min and was on the same level for both dilutions, but after 10 min it rose to above 50 % for both ferrous sulphate retraction agents (Astringedent[®] solution and ViscoStat[®] gel, Ultradent Product, South San Francisco, CA).

Discussion

According to the guidelines of the American National Standards Institute (ANSI) and the Technical Report ISO-TR 7405 of the ISO Technical Committee concerning dentistry (TC 106), *in vitro* cytotoxic screening investigations of different cell cultures is commonly accepted as adequate for dental devices for the primary determination of their biocompatibility (Kopač et al., 2002a). In clinical practice, retraction agents are applied with retraction materials or incorporated in retraction materials directly into the gingival sulcus. They remain there until effective shrinkage and displacement of free gingiva away from tooth structures and haemostasis is obtained. Hence they remain in direct contact with the thin monolayer of epithelial cells in the gingival sulcus and the connective epithelium (epithelial attachment) at the bottom of the sulcus. Many authors observed an inflammatory response or even necrosis of the sulcular epithelium and subepithelial connective tissue induced by gingival margin retraction agents with an astringent base (De Gennaro et al., 1982; Azzi et al., 1983; Nemetz

et al., 1984; Weir and Williams, 1984; Benson et al., 1986; Akca et al., 2006; Kumbuloglu et al., 2007; Al Hamad et al., 2008). Under these conditions, chemical agents influence the gingival connective tissues directly. The choice of primary cells cultured from fibroblasts obtained from patients with healthy periodontal tissue undergoing tooth extraction seems to be the most appropriate for constructing an adequate *in vitro* study model.

Only Kopač et al. (2002a) and Lodetti et al. (2004) studied the cytotoxic effects of gingival retraction fluids on cell cultures using the MTT assay. Kopač et al. (2002a) evaluated the viability of fibroblasts obtained from Chinese hamster diploid lung (V-79-379 A) treated with astringents based on aluminium chloride and sulphate. After 1 min of exposure, all chemical agents in the original concentrations caused stronger cytotoxic effects than in 1 : 10 dilution. At a 1 : 10 dilution of the agents, the viability of Chinese hamster lung fibroblasts treated with 25% aluminium chloride was significantly lower than that of fibroblasts incubated with 10% aluminium chloride and 20% aluminium sulphate. The study of Lodetti et al. (2004) demonstrated the cytotoxic effects of astringent retraction solutions on human oral keratinocytes. The most damaging was the agent Astringedent X[®], which contains ferric sulphate and ferric subsulphate.

Kopač et al. (2002c) also observed changes in primary cell cultures of rat keratinocytes after 10 minutes of treatment with 25% aluminium chloride used for gingival retraction. The cells, examined by scanning and transmission electron microscopy, differed significantly from those of a control group.

Chemo-mechanical methods based on two-element systems may pose the additional danger of accumulation of the cytotoxic effects of the gingival retraction agent and material. Liu et al. reported that even non-impregnated cords were cytotoxic for human gingival fibroblasts cultured from gingival explants. Evaluation after 10 min and 24 h of exposure to retraction cords impregnated with aluminium sulphate also demonstrated a significant potential for gingival toxicity (Liu et al., 2004).

In clinical conditions, the duration of the chemo-mechanical retraction procedure should range from 3 to 10 min (Nowakowska et al., 2006c). Our experiments took place in four time intervals: from 0 to 3 min, 3 to 5 min, 5 to 10 min, and 10 min to 24 h after treatment with three chemical groups of astringents in different concentrations and clinical forms. The results after 3 min showed that aluminium sulphate-based retraction agents and aluminium chloride-based fluids and gels ensure a relatively high oxidoreductive potential of fibroblasts. The statistically significant lower oxidoreductive functions of cells cultured with ferric sulphate-based astringents in the first 3 min of incubation suggest limitations in their use in clinical practice. The cytotoxic effects on fibroblasts after 5 min incubation to all evaluated retraction astringents exhibited the lowest viability. The in-

crease of the viability of fibroblasts after 10 min of exposure to all of the evaluated chemical groups provided the interesting insight that oxidoreductive mitochondrial potential was activated, which may suggest a reactive defensive action of the cells to the impact of the retraction agents. The observation after 24 h showed that all the retraction agents (except for the ferric sulphate agents) caused a cytotoxic effect. According to the results it can be stated that cell viability increases with decreasing concentration of the astringents and decreases with increasing exposure time. Retraction agents composed of ferric sulphate proved to be the most cytotoxic, followed by aluminium chloride and aluminium sulphate. It seems that the lower pH of the agent, the higher the cytotoxicity.

The agent's form proved to have a significant influence on human gingival fibroblast viability. This experiment is most probably the first examination of the cytotoxic effects of gel-based retraction astringents on gingival cells. The results obtained at the shortest exposition, i.e. 3 min, on fibroblasts (except for the ferric sulphate gel-based agent) revealed that the agents do not induce any significant increase of the cells' mitochondrial oxidoreductive functions. The use of gel-type astringents allows reducing the area of gingival tissue exposure to the effect of the retraction agent. Additionally, gel-type agents diminish the scratching effect involved when applying and removing the retraction material into and from the gingival sulcus (Nagler et al., 2002; Nowakowska et al., 2006a).

Our results can be directly extrapolated to clinical conditions, but they are predictive of the probability of the behaviour of these agents under *in vivo* conditions. Healthy gingival epithelium and epithelial attachment constitute a natural barrier protecting the connective gingival tissues and reducing the level of damage. Additionally, the aggressive clinical action of chemical retraction agents may be less intense because their concentration is diluted by water spray, human saliva, and natural gingival fluids (Edgar, 1990; Nagler et al., 2002). A systematic *in vivo* review of the impact of retraction astringents on gingival margin tissues reported that the healing period after retraction with chemical agents in their original concentrations was from seven to ten days (Nowakowska, 2009).

The presented results may also suggest the need for reducing the use of retraction astringents in their original concentrations, especially ferric sulphates. This is particularly important when damage to the gingival margin tissues occurs during mechanical tooth preparation. In this case, retraction with the use of chemical retraction agents should be postponed until the tissues have recovered in order to reduce the potential cytotoxic effect on human gingival fibroblasts. These investigations suggest that the evaluated chemical retraction agents can have cytotoxic potential towards gingival tissues under clinical conditions. It can be concluded that there is a need to obtain oxidoreductive stress markers

and determine the type of cell death induced by the retraction agents.

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