Original Article

Cytotoxic Potential of Vasoconstrictor Experimental Gingival Retraction Agents - in Vitro Study on Primary Human Gingival Fibroblasts

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

D. NOWAKOWSKA¹, J. SACZKO², J. KULBACKA², A. CHOROMANSKA², Z. RASZEWSKI³

¹Department of Dental Materials, ²Department of Medical Biochemistry, Wroclaw Medical University, Wroclaw, Poland
³Zhermapol® Dental Materials, Warszawa, Poland

Abstract. The aim of this in vitro study was to evaluate the cytotoxic effects of the vasoconstrictor experimental gingival retraction agents (VEGRAs) in a dynamic setting. The strong cytotoxic effects of the astringent-based conventional gingival retraction agents (ACGRAs) on human gingival fibroblasts (HGFs) in vitro was our motivation to evaluate the biocompatibility of the vasoconstrictor-based experimental gingival retraction agents (VEGRAs) for the selected minimally invasive chemical agent. These agents were used to create three self-made retraction gels. Human gingival fibroblasts (HGFs) were treated with two groups of retraction agents: 1) three α- and β-adrenergic agents (VEGRA-αβ-s) based on 0.1%, 0.01% and 0.05% HCl-epinephrine, and 2) seven α-adrenergic agents (VEGRA-α-s), including two commercially available 0.05% HCl-tetrahydrozoline solutions, one 0.05% HCl-oxyphenmetazoline solution, 10% HCl-phenylephrine solution, and three new self-made experimental 0.05% HCl-tetrahydrozoline-based gels. The methyl thiazolyl tetrazolium (MTT) colorimetric assay was performed to determine the oxidoreductive mitochondrial function after 3, 5, 10 min and 24 h of incubation. The cytotoxic effect, measured by cell viability lower than the 50% threshold, was not observed at any time period, even 24 h after application of 0.05% HCl-tetrahydrozoline-based self-manufactured retraction gels. High cell viability values of human gingival fibroblasts after the treatment with the three self-made 0.05% HCl-tetrahydrozoline-based gels may serve as a basis for further studies aimed at selecting the best retraction agents biocompatible with gingival margin tissues.

Introduction

Gingival margin retraction techniques create optimal conditions for diagnostic, preventive and treatment procedures requiring access to the gingival sulcus space in contemporary dentistry. Vertical and horizontal sulci larger than necessary for conventional impression techniques are needed when making direct optical impressions for fixed prosthodontic restorations on natural teeth or/and implants supported (Bennani et al., 2008). The chemo-mechanical gingival margin retraction methods are still most popular in dental practice, using, beside retraction materials, also various gingival retraction agents (GRAs) (Hansen et al., 1999; Nowakowska et al., 2006a; Al Ani et al., 2010). Different clinical forms of the retraction chemicals are applied in situ: fluids (GRFs), gels (GRGs) or pastes (GRPs) (Nowakowska and Panek, 2007). The retraction fluids are used as ex tempore soaked or as manufacturer’s impregnation in various types of retraction cords. Gels and pastes can be immediately injected into the gingival sulcus. Clinical effects of the retraction fluids and gels support different retraction materials, while the effect of retraction pastes is reinforced by mechanical fillers contained in them (Poss, 2002; Phatale et al., 2010). All these chemicals
remain on average from 3 to 10 min in direct contact with periodontal tissues and prepared teeth structures (Nowakowska et al., 2006b).

These commonly accepted chemicals are based on two different pharmacological action categories: astringents (blood coagulation factors) and vasoconstrictors (adrenergic agents) (Porzier et al., 1991; Felpel, 1997). The astringent-based conventional gingival retraction agents (ACGRAs) class contains various metal salts; mainly aluminum chloride, aluminum sulphate and ferric sulphate. The vasoconstrictor experimental gingival retraction agents (VEGRAs) class was divided into two groups: α- and β-adrenergics (racemic epinephrine group VEGRA-αβ-s) and α-adrenergics (sympathomimetic amines group (VEGRA-α-s)) (Nowakowska, 2008a).

The usefulness of the different ACGR class was confirmed in numerous clinical studies; however, under clinical conditions some undesirable local side effects on the teeth structures and surrounding periodontal tissues were identified (Nowakowska, 2009a). Also in vitro studies by the 3-(4,5-dimethyl-2-thiazollyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay revealed cytotoxicity of astringent-based solutions on various cell cultures (Kopač et al., 2002a; Liu et al., 2004, 2009; Lodetti et al., 2004; Nowakowska et al., 2010). Nowakowska et al. (2010) evaluated the dynamic oxidoreductive potential on human gingival fibroblasts (HGFs) after treatment with liquid- and gel-based retraction astringents (ACGRAs) and concluded that cell viability increases with decreasing concentration of the retraction agents and cell exposure time. The values of cytotoxicity of these chemicals were the lowest for aluminum sulphate, higher for aluminum chloride and the highest for ferric sulphate-based agents, and the clinical form of retraction agents additionally influenced their impact on cell cultures.

In the vasoconstrictor experimental gingival retraction agents (VEGRAs) class, the racemic epinephrine (VEGRA-αβ-s) alone, or in combination with different astringents, is available as commercially impregnated retraction cords (Pogue and Harrison, 1967; Houston et al., 1978; Kellam et al., 1992; Nowakowska and Galewski, 2008b). The HCl-epinephrine had previously been used in higher concentrations such as 32 % and 16 % (Gogerty et al., 1957); later Pelzner et al. (1978) proposed to lower these concentrations from 8 % to 4 %. In dentists’ preferences surveys in the United States in 1991, 20 % respondents preferred racemic epinephrine-impregnated cords (Shaw and Krejci, 1986), while in 1999 this proportion changed to 14 % (Hansen et al., 1999), and in Poland in 2004 it was 21.1 % (Nowakowska et al., 2006a). However, 20 % of 33 % dentists observed different unfavourable clinical side effects (Shaw et al., 1987; Hansen et al., 1999; Nowakowska et al., 2006a). The main studies on animal and human models proved associated significant local and systemic side effects (Nowakowska et al. 2009b). The “epinephrine syn-drome” involved a collapse, heart rate increase, tachycardia, cords palpitation, systolic and diastolic blood pressure, dyspnea, pale skin, excessive stimulation or cold sweat (Gogerty et al., 1957; Woycheschin, 1964; Phatak and Lang, 1966; Forsyth et al., 1969; Stark et al., 1977; Pelzner et. al., 1978; Houston et al., 1978; Buchanan and Thayer, 1982; Hatch et al., 1984; Bowles et al., 1991; Polat et al., 2007). The risk of epinephrine use with retraction cords in hypertensive patients is unacceptable (Bader et al., 2002). In dental practice adverse drug interactions with epinephrine-based vasoconstrictors were observed (Yagiela, 1999) and fatality associated with combined use of halothane and epinephrine-impregnated gingival retraction cords was noticed (Hilley et al., 1984). Local unfavorable influences such as hyperaemic response, trauma of crevicular and junctional epithelium were reported, with complete healing after the period from 7 to 10 days (Harrison, 1961; Loë and Silness, 1963; de Gennaro et al., 1982; Kopač et al., 2002b). Furthermore, in vitro study showed a strong cytotoxic effect of extracts from commercial retraction cords impregnated with DL-racemic epinephrine after 10 min and 24 h incubation on human gingival fibroblasts, as reported by Liu et al. (2004).

For these reasons it has become crucial to develop new clinical retraction agents that would guarantee higher biocompatibility and safety levels. Recommendation for the use of lower concentrations of agents from the VEGRA-αβ-s class, 0.1 % epinephrine proposed by Fazekas et al. (2002) and 0.01 % epinephrine by Csillag et al. (2007) considerably lowered the risk of systemic side effects. Liu et al. (2009), in comparison of cytotoxicity between chemical retraction agents, showed that 0.1 % HCl-epinephrine displayed statistically significant strongest cytotoxic effect on cell cultures than 0.01 % HCl-epinephrine; however, after gingival retraction with 0.1 % HCl-epinephrine, prolonged crevicular fluid flow and active level of aspartate amino-transferase (AST) were reported (Sun, et al., 2008).

As an alternative to HCl-epinephrine, Bowles et al. proposed three commercially available medicaments, used commonly in ophthalmology and laryngology; 0.05 % HCl-tetrahydrozoline, 0.05 % HCl-oxymetazoline and 0.25 % HCl-phenylephrine as new experimental gingival retraction agents (VEGRA-α-s) (Bowles et. al., 1991). These synthetic sympathomimetic agents are more effective and safer than epinephrine. Kopač et al. (2002a) determined the in vitro surviving fraction of V-79 fibroblasts after 1 min exposure to 0.05 % HCl-tetrahydrozoline in 1 : 10 dilution (Visine®, Pfizer, Warszawa, Poland) with colorimetric assay (Mosmann, 1983) as 70 %.

The aim of this in vitro study was to evaluate, in a dynamic setting, the cytotoxic effects of vasoconstrictor experimental gingival retraction agents (VEGRAs) on human fibroblasts isolated from patients’ healthy gingival tissues.
Material and Methods

Experimental vasoconstrictor-based retraction agents

Ten retraction agents from experimental vasoconstrictors class (VEGRAs), including three from the α- and β-adrenergics group (VEGRA-αβ-s) 0.1%, 0.01% and 0.05% HCl-epinephrine solutions, and seven from the α-adrenergics group (VEGRA-α-s), including four solutions: 0.05% HCl-tetrahydrozoline (Visine® classic and starazolin® (Polpharma, Warszawa, Poland)), 0.05% HCl-oxymetazoline (Afrin®), Schering-Plough, Brussels, Belgium) and 10% HCl-phenylephrine (Neosynephrin POS® (Ursapharm, Saarbrücken, Germany)) and three self-manufactured gels based on 0.05% HCl-tetrahydrozoline, were subject of this study. The chemicals were diluted with distilled water to 1 : 10 and 1 : 20 dilutions. The components and pH value of the tested retraction adrenergics are presented in Table 1.

Cell cultures

The tissue cultures of human gingival fibroblasts (HGFs) were obtained from patients with healthy periodontium tissues undergoing the extraction procedure. The gingival biopsies were provided by the Department of Dental Surgery of Wroclaw Medical University. The experiments were conducted in accordance with the requirements of the Bioethics Commission of Wroclaw Medical University. The HGFs were isolated from healthy gingival tissues according to the procedure described previously by Saczko (Saczko, 2008, Patent No: P 3812045, Saczko et al., 2008). The cells were grown routinely in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO).

Cytotoxicity test (MTT assay)

The MTT assay (In Vitro Toxicology Assay; Sigma-Aldrich) was used to evaluate the cytotoxicity of the gingival retraction agents. 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 PBS and treated according to the manufacturer’s protocol. The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Multiscan MS microplate reader, Labsystem, Helsinki, Finland). The results were expressed as the percentage of untreated control cells.

Statistical analysis

Statistical analysis was performed using commercial Statistica version 9.0 software. 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 ≤ 0.05 taken to imply statistical significance.

Results

The dynamics of the oxidoreductive mitochondrial function of the human primary cells is shown in Fig. 2. In the group of HCl-epinephrine-based retraction agents (VEGRA-αβ-s), 0.1% HCl-epinephrine was most cytotoxic after 3, 5, 10 min and 24 h of incubation, whereas self-made 0.01% and 0.05% HCl-epinephrine dilutions showed higher and comparable HGF oxidative mitochondrial function after all the incubation periods (Fig. 2 A). The values of cell viability ranged from 100 % to

<table>
<thead>
<tr>
<th>Chemical group</th>
<th>Retraction agents</th>
<th>Manufacturer</th>
<th>Lot/Batch</th>
<th>Active ingredients</th>
<th>Clinical form</th>
<th>pH level in dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α and β-adrenergics</td>
<td>Injec. Adrenalin 0.1%</td>
<td>Polfa, Warszawa, Poland</td>
<td>03BL0807</td>
<td>0.1% HCl-epinephrine</td>
<td>solution</td>
<td>3.62 4.09</td>
</tr>
<tr>
<td></td>
<td>Injec. Adrenalin 0.01%</td>
<td>Self-made dilution of Injec. Adrenalin 0.1%</td>
<td>x</td>
<td>0.01% HCl-epinephrine</td>
<td>solution</td>
<td>3.90 5.36</td>
</tr>
<tr>
<td></td>
<td>Injec. Adrenalin 0.05%</td>
<td>Self-made dilution of Injec. Adrenalin 0.1%</td>
<td>x</td>
<td>0.05% HCl-epinephrine</td>
<td>solution</td>
<td>3.85 5.25</td>
</tr>
<tr>
<td></td>
<td>Visine® classic</td>
<td>Pfizer, Warszawa, Poland</td>
<td>07064</td>
<td>0.05% HCl-tetrahydrozoline</td>
<td>solution</td>
<td>6.85 7.15</td>
</tr>
<tr>
<td></td>
<td>Afrin®</td>
<td>Schering-Plough, Brussels, Belgium</td>
<td>6APMB</td>
<td>0.05% HCl-oxymetazoline</td>
<td>solution</td>
<td>4.85 5.58</td>
</tr>
<tr>
<td></td>
<td>Neosynephrin POS® 10%</td>
<td>Ursapharm, Saarbrücken Germany</td>
<td>003077</td>
<td>10% HCL-phenylprine</td>
<td>solution</td>
<td>4.30 5.18</td>
</tr>
<tr>
<td>α-adrenergics</td>
<td>Starazolin®</td>
<td>Polpharma, Warszawa, Poland</td>
<td>1031109</td>
<td>0.05% HCl-tetrahydrozoline</td>
<td>solution</td>
<td>5.67 5.70</td>
</tr>
<tr>
<td></td>
<td>Experimental gel 1</td>
<td>Self-made</td>
<td>x</td>
<td>0.05% HCl-tetrahydrozoline</td>
<td>gel</td>
<td>5.73 6.08</td>
</tr>
<tr>
<td></td>
<td>Experimental gel 2</td>
<td>Self-made</td>
<td>x</td>
<td>0.05% HCl-tetrahydrozoline</td>
<td>gel</td>
<td>6.16 6.64</td>
</tr>
<tr>
<td></td>
<td>Experimental gel 3</td>
<td>Self-made</td>
<td>x</td>
<td>0.05% HCl-tetrahydrozoline</td>
<td>gel</td>
<td>5.26 5.68</td>
</tr>
</tbody>
</table>
110 % for both lower concentrations of the HCl-epinephrine (0.01% and 0.05%).

In the sympathomimetic amine-based experimental retraction agents (VEGRA-αβ-s) group the results of cell viability ranged from 80 % to 115 % and statistically non-significantly after 3, 5 and 10 min treatment in both (1 : 10 and 1 : 20) dilutions (Fig. 2 B). All the evaluated medicaments (Visine® classic, Afrin®, Neosynephrin®-POS and Strazolin®) achieved high oxidoreductive mitochondrial function, especially 0.05% tetrahydrazoline-based agents (VEGRA-α-s) (gels). Results are expressed as the mean ± SD. * P < 0.05.

![Fig. 2. Human gingival fibroblast viability after exposure to A) epinephrine-based retraction agents (VEGRA-αβ-s) (solutions); B) sympathomimetic amine-based retraction agents (VEGRA-α-s) (solutions); C) self-made experimental 0.05% tetrahydrazoline-based agents (VEGRA-α-s) (gels). Results are expressed as the mean ± SD. * P < 0.05.](image-url)
line-based agents. The series of cytotoxicity of evaluated VEGRAs decreased in the order: 0.1% HCl-epinephrine > parallel 0.01% and 0.05% HCl-epinephrine > α-sympathomimetic amine solutions > 0.05% HCl-tetrahydrozoline gels. The highest levels of oxidoreductive mitochondrial potential were demonstrated by three self-made experimental 0.05% HCl-tetrahydrozoline based gels in all evaluated time periods, including 24 h, whereas the differences in cell viability in these gel groups were not statistically significant (Fig. 2 C). The oxidoreductive HGF mitochondrial function after 5 and 10 min incubation increased in 1:10 dilution and after 5 min in 1:20 dilution.

Discussion

Guaranteeing the safety and effectiveness of the chemo-mechanical gingival margin retraction procedures has for long been the aim of research on chemical retraction agents. In vitro studies using MTT colorimetric assay were focused on cytotoxic effects of the conventional astringent-based retraction solutions on different cell cultures (Kopac et al., 2002a; Liu et al., 2004, 2009; Lodetti et al., 2004). Nowakowska et al. (2010), using Saczko et al. (2008) methods, evaluated the dynamic oxidoreductive potential of commonly used ACGRA solutions and later developed astringent-based retraction gels in HGFs. The cytotoxic effect increased in the order of aluminum chloride-< aluminum sulphate-< ferric sulphate-based astringents. This in vitro study concerned the experimental retraction agents and used the same, by Saczko et al. (2008) proposed method, to compare the cytotoxic effects of VEGRA-based solutions and three new self-made retraction gels in HGFs.

Under clinical conditions the use of retraction agents from the epinephrine group (VEGRA-αβ-s) was always connected with a certain risk that undesirable systemic effects might occur (Nowakowska et al., 2009b). Apart from the “epinephrine syndrome”, its interactions with other medicaments were noted in the literature, including one case of patient’s death after general anesthesia using halothane (Hilley et al., 1984; Yagiela, 1999). The results of clinical experiments with lower concentrations of these chemicals, 0.1% and 0.01% HCl-epinephrine, considerably lowered the risk of systemic side effects (Fazekas et al., 2002; Csillag et al., 2007).

Clinical trials were conducted by Fazekas et al. (2002) with 0.1%, and Csillag et al. (2007) with 0.01% racemic epinephrine, in microcirculation of the free gingival margin in a group of young and healthy volunteers using Periotron 6000 and Laser Doppler Flowmeter. The results showed that racemic epinephrine in these low concentrations produced satisfactory local clinical effects in gingival margin retraction procedures, without prolonged increase in crevicular fluid production and hyperaemic response after cord removal. Also, systemic effects could be prevented by application of 0.01% HCl-epinephrine solution. Polat et al. (2007) demonstrated in a clinical study that 0.1% HCl-epinephrine-soaked cords were effective retraction agents if patient stress and gingival trauma are avoided. Racemic epinephrine in 0.1% and 0.01% concentrations is currently also considered an experimental gingival retraction agent (VEGRA-αβ-s).

Sparse studies of the effects of different vasoactive chemical retraction agents on cell cultures in vitro by MTT colorimetric assay can be found. Liu et al. (2004), reported that eluates from DL-racemic HCl-epinephrine-impregnated cords (Gingi-Pak, Belpont Co., Inc. Camarillo, CA) in direct contact with human gingival fibroblasts reduced the cell viability after 10 min to 21 %, and after 24 h to 58 %. In addition, their cytotoxicity was higher than that of eluates from aluminum sulphate-impregnated cords (Gingi-Aid, Belpont Co.) (Liu et al., 2004). Liu et al. (2009) selected the best retraction agents for the clinical use, comparing cytotoxicity of 0.1% and 0.01% HCl-epinephrine and four different astringents. All of chemical retraction agents caused cell damage and proliferation inhibition. The least toxic was 0.01% HCl-epinephrine, then 0.1% HCl-epinephrine, and astringents were found to have the strongest cytotoxic effect on human gingival fibroblasts.

From the VEGRAs group, only Kopac et al. (2002a) evaluated experimental 0.05% HCl-tetrahydrozoline (Visine, Pfizer) and three conventional AGRAs on Chinese hamster diploid lung fibroblasts (V-79-379 A). The surviving fraction of V-79 fibroblasts reduced the cell viability after 10 min to 21%, and after 24 h to 58%. In addition, their cytotoxicity was higher than that of eluates from aluminum sulphate-impregnated cords (Gingi-Pak, Belport Co., Inc., CA). In direct contact with human gingival fibroblasts, the cell viability was reduced to 21% after 10 min exposure with 0.05% HCl-tetrahydrozoline in 1:10 dilution by colorimetric assay (Mosmann, 1983) was 78% (Kopac et al., 2002a). The cytotoxic effect of other agents from the VEGRA-α-s group on cultured human cells, however, was not reported in the literature. This motivated the authors to undertake a study aimed at comparison of these α and β-adrenergics versus α-adrenergic experimental gingival retraction agents containing different active chemical substances for selection of the minimally invasive chemicals. These chemicals were used to create a series of experimental gels, which were later subjected to MTT evaluation of their oxidoreductive potential dynamics.

In vitro cytotoxicity evaluation of the HCl-epinephrine solution on HGFs an undesirable impact of its use in this concentration was proved (Liu et al., 2009). The present study also confirms this result, as the 0.1% HCl-epinephrine solution turned out to be cytotoxic for...
HGFs in all evaluated time periods. The best results were obtained for the 0.01% and 0.05% HCl-epinephrine, where stable and high oxidoreductive mitochondrial potential was preserved after 3, 5 and 10 min and 24 h. These two experimental VEGRs can be considered as relatively safe gingival retraction agents, but exogenous HCl-epinephrine may be cumulated during the gingival retraction procedure with endogenously produced epinephrine resulting from clinical stress (Fazekas et al., 2002; Csillag et al., 2007; Polat et al. 2007).

The experimental α-s sympathomimetic amine solution group (VEGRA-α-s) in both concentrations (1:10 and 1:20) presented satisfactorily high cell viability after 3, 5 and 10 min. Only after 24 h incubation for all evaluated α-adrenergic-based solutions the cytotoxic effect was significantly stronger. Improved results obtained with 0.05% HCl-tetrahydrozoline versus conventional gingival retraction agents (ACGRAs) were already reported by Kopač et al. (2002a) in a study on Chinese hamster diploid lung fibroblasts.

The current study goes beyond this early finding and extends the analysis to several experimental retraction agents, some of them self-manufactured. The authors compared the oxidoreductive potential of the α- and β-adrenergics (VEGRA-αβ-s) group versus α-adrenergics (VEGRA-α-s) group (all experimental agents) and obtained better results for the latter, especially for Visine ® classic and Strazolin®, both as active substance. According to several comparative studies evaluating the pH value of different retraction medicaments, 0.05% HCl-tetrahydrozoline achieved the highest and thus most neutral pH values among all experimental agents (Nowakowska and Raszewski, 2010). Additionally, 0.05% HCl-tetrahydrozoline fulfills the criterion of compatibility with the majority of elastomer impression materials (Sabio et al., 2008). All these advantages of 0.05% HCl-tetrahydrozoline suggested the choice of this chemical as a basis for proposing three new self-manufactured gels, which were further tested in this study.

The cytotoxicity of these three self-made gels was determined to be low and the HGF oxidoreductive mitochondrial potential remained high in all evaluated time intervals. These results hold for both considered concentrations. The cytotoxic effect, measured by cell viability lower than the 50% threshold, was not observed at any time period, even after 24 h. For self-manufactured gels Nos. 1 and 2 after 5 and 10 min incubation in both dilutions (1:10 and 1:20), the oxidoreductive potential of HGFs increased from 120 to 150 %, which may suggest activation of their defensive mitochondrial action after treatment with these experimental retraction gels. They can thus serve to select the best, minimally invasive retraction agents.

Conclusions

The high cell viability values of human gingival fibroblasts after treatment with three self-manufactured 0.05% HCl-tetrahydrozoline-based gels may serve as a basis for further studies aimed at selecting the best retraction agents, most biocompatible with gingival marginal tissues.

References


