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Cytotoxicity of hemostatic agents on the human gingival fibroblast

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Abstract. – BACKGROUND: Achieving of moisture control especially gingival bleeding control is great challenge in clinical practice. Various hemostatic agents and techniques have been promoted for bleeding control during dental operation. But few studies have focused on the cytotoxicity of hemostatic solutions.

AIM: The aim of this study was to evaluate cytotoxic effect of hemostatic agents on human gingival fibroblast cells by using real-time cell analysis method.

MATERIALS AND METHODS: Two hemostatic solutions, Hemoban (Sultan Healthcare, Hackensack, NJ, USA) and Hemasatic Solutions (W.P. Dental, Hamburg, Germany) that includes mainly aluminum chloride were used with different concentration. Gingival fibroblasts were isolated from gingival connective tissue during crown lengthening surgery of systemically healthy subjects. Gingival fibroblasts were maintained with Dulbecco's modified eagle medium containing 10% fetal bovine serum. A real-time cell analyzer (RT-CES, xCELLigence; Roche Applied Science, Mannheim, Germany, and ACEA Biosciences, San Diego, CA, USA) was used to evaluate cell survival. After seeding 200 mL of the cell suspensions into the wells (10,000 cells/well) of the E-plate 96, gingival fibroblasts were treated with hemostatic solutions (1/2, 1/4 and 1/8 dilutions) and monitored every 15 minutes for 72 hours. For the proliferation experiments, the statistical analyses used were 1-way analysis of variance (ANOVA) and Tukey HSD multiple comparisons tests.

RESULTS: According to statistically analysis, when evaluated at 48 and 72 hours, there were significant differences between the cell indexes of the control and all hemostatic agents groups ($p < 0.001$). Agent reduced cell index value significantly when compared to untreated control group.

CONCLUSIONS: The results indicate that using of Hemoban or Hemostatic Solutions as astringent solutions have a significant cytotoxic effect on gingival fibroblast cells.

Key Words:
Hemostatic agent, Real-time cell analyzer, Cytotoxicity.

Introduction

Achieving good moisture control is great challenge in daily clinical practice. Operative dentistry cannot be performed properly unless the moisture is controlled. Moisture control refers to excluding sulcular fluid, saliva and gingival bleeding from the operating field[1]. During the operation process of restorations at the cervical area, gingival bleeding sometimes appears as a result of the trauma from cavity preparation when caries lesions are located near or below the gingival margin[2]. Since adhesive systems are very vulnerable to contamination by blood, contamination control is an important factor to obtain a successful and durable bond of composite resin to tooth structure[3,4]. As well known, any blood or saliva contamination had negative effects on other dental materials like amalgam, cements especially glass ionomer's properties.

Various hemostatic agents and techniques have been promoted for bleeding control during dental operation. Aluminum chloride is a convenient topical hemostatic solution because of its effectiveness to bleeding control, low cost, easy to use and storage at room temperature[5]. The mechanism of action of aluminum chloride solution is thought to be secondary to its hydrolysis hydrogen chloride and results in tissue coagulation, vasoconstriction or activation of the extrinsic coagulation pathway[6]. Aluminum chloride has been reported to be irritant in moderate concentrations or even cause caustic damage at higher concentrations[7]. In clinical practice various hemostatic agents have been found to produce various degrees of tissue damages.

In vitro cytotoxic screening as a primary factor of biocompatibility is determined by cell cultured should be selected for in vitro toxicity tests[8]. Real-time and continuous monitoring allows label-free assessment of cell proliferation, viability and

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cytotoxicity, revealing the physiological state of the cells and at the same time saves expensive reagents used in conventional cell analysis. In the xCELLigence system, the kinetic control of cellular status during entire experiment runs reveals continuous information about cell growth, morphological changes and cell death[9].

Few studies have focused on the cytotoxicity of hemostatic solutions on human cells[8]. Hence, the studies that evaluate the cytotoxic effects of hemostatic solutions are very limited. The aim of this study was to evaluate cytotoxic effect of two commercial astringent, which included in aluminum chloride solutions with respect to the concentration used.

Materials and Methods

Two hemostatic solutions, Hemoban (Sultan Healthcare, Hackensack, NJ, USA) and Hemasatic Solutions (W.P Dental, Hamburg, Germany) were used in this study. Material details are listed in Table I. The hemostatic solutions were used with different concentrations as 1/2, 1/4 and 1/8 dilutions.

Cell Culture

Human gingival fibroblasts were obtained (with informed consent) from a healthy patient subjected to biopsies. Briefly, the gingival tissues were cut into small pieces, rinsed with the biopsy medium, placed in tissue culture dishes, and incubated in the biopsy medium in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C overnight. The following day, the biopsy medium was replaced with the culture medium (Dulbecco's modified eagle medium [Biological Industries, Beit Haemek, Israel] with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin). After reaching confluency, the cells were passaged with 0.25% trypsin and 0.1% ethylene diaminotetraacetic acid. Cells at passage 20 were used for the experiments. After seeding 200 µL (10.000 cells/well) of the cell suspensions into the wells the E-plate 96, cells were treated with tested hemostatic solutions (1/2, 1/4 and 1/8 dilutions) and monitored every 15 min for 72 hours.

Cytotoxicity Assay Using xCELLingence System

The xCELLigence system (Roche Diagnostics GmbH, Mannheim, Germany and ACEA Biosciences, Inc., San Diego, CA, USA) was used to evaluate cell survival according to the instructions of the supplier. The xCELLigence system consists of 4 main components: the impedance-based real-time cell analyzer (RTCA), the RTCA single-plate station, the RTCA computer with integrated software, and a disposable E-plate 96. The RTCA single-plate station fits inside a standard tissue-culture incubator. The electronic impedance of the sensor electrodes was measured to allow monitoring and detection of physiologic changes in the cells on the electrodes. The voltage applied to the electrodes during the RTCA measurement was approximately 20 mV (root mean square). The impedance measured between the electrodes in each well depends on the electrode geometry, ion concentration in the well, and attachment of the cells to the electrodes. In the absence of cells, the ion environment both at the electrode-solution interface and bulk solution mainly determines electrode impedance. In the presence of cells, cells attached to the electrode sensor surfaces act as insulators and thereby alter the local ion environment at the electrode-solution interface, leading to increased impedance. Thus, the obtained values of electrode impedance increase as the number of cells growing on the plate increases. The data expressed in cell index units can be exported to the Excel for any type of mathematical analysis.

Statistical Analysis

All calculations were performed using the RTCA integrated software of the xCELLigence system. The RTCA software performs a curve fitting of selected sigmoidal dose-response equations to the experimental data points. The data are presented as means (mmol/L) ± SD deviation (n =

Table I. Tested materials.

Brand	Chemical composition	pH	Manufacturer
Hemoban	25% Aluminum chloride	< 1	Sultan Healthcare, Hackensack, NJ, USA
Hemostatic solution	24.8% Aluminum chloride Hexahidrat	1.84	W.P. Dental, Hamburg, Germany

Table II. Cell index values by real-time cell analysis and comparisons of 48 and 72 hours with ANOVA and Tukey HSD multiple comparison tests (means and standard deviations).

	Concentration	48 hours	72 hours	pH
Control		3.921 (0.2694)*	3.575 (0.1718)*	
Hemoban	1/2	0.0797 (0.04278)	0.1179 (0.04817)	3.21
	1/4	0.2622 (0.03716)	0.2980 (0.03457)	3.36
	1/8	0.2014 (0.02176)	0.1774 (0.02233)	3.30
Hemostatic solutions	1/2	0.6029 (0.04320)	0.5975 (0.04391)	4.1
	1/4	0.9288 (0.12440)	0.5708 (0.08132)	4.4
	1/8	1.4750 (0.07644)	1.3630 (0.05400)	5.8

*Means statistically different group in same column, $p < 0.001$).

6). For the proliferation experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey honestly significantly difference (HSD) multiple comparison tests ($p < 0.01$).

Results

According to ANOVA, when evaluated at 48 and 72 hours, there were significant differences between the cell indexes of the control and all hemostatic agents groups ($p < 0.001$). Table II represented the means and standard deviations for all groups. After 48 hours and 72 hours, different dilutions of Hemoban had statistically different from Control group for both immersion times. Same results had shown in different dilutions of Hemostatic Solutions groups (Figure 1).

Discussion

Gingival hemostasis is defined as control of sulcular hemorrhage so that bleeding does not recur when gingival and/or pulpal tissues are touch or manipulated. This referred to as active hemostasis, as distinguished from passive hemostasis, in which seepage could occur at any time, even just before or during placement of dental materials' procedures. Active hemostasis are necessary for successful restorative dentistry whether

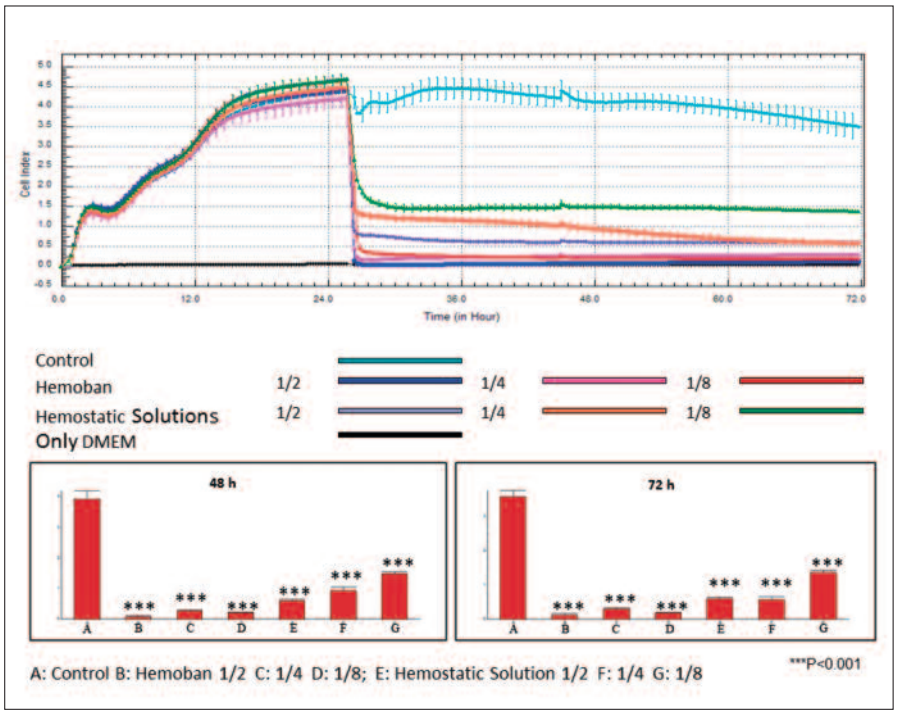

Figure 1. Dynamic monitoring of cell adhesion and cell proliferation for 48, 72 hour and cell indexes of tested materials during 48, 72 hours.

dentin bonding or other restorative procedures[10]. For bleeding control, there are so may techniques include the applying with pellets soaked in saline, hydrogen peroxide, sodium hypochlorite, anesthetic solutions containing epinephrine, chlorhexidine, ferric sulfate, ferric chloride, aluminum chloride, aluminum chloride and ferric sulfate combinations in clinical practice[5, 11-14]. One of the most commonly used solutions in dentistry is ferric sulfate, which also has cytotoxicity and causes tissue necrosis[15]. von Arx et al[5] reported that it was found to be ferric sulfate less affective than aluminum sulfate.

There are several investigations about aluminum chloride solutions. Forsyth et al[16] indicated that aluminum chloride caused no increase in blood pressure and pulse rate. Other studies determined that aluminum chloride led to a slight increase in inflammatory cells in connective tissue and claimed that aluminum chloride medicament is the most acceptable gingival retraction medicament[17]. On the other hand, de Gennaro et al[18] reported that a statistically significant difference in gingival inflammation was not observed between control and aluminum chloride groups. However, the study was carrying out on human volunteers. It has been shown that aluminum chloride elicits inflammatory tissue reactions. In a clinical comparative study on gingival retraction, aluminum chloride (25%) showed slower healing and more inflammatory reactions compared with a Nd:YAG-laser treatment[19]. A dermatologic report on four cases demonstrated that aluminum chloride could cause a proliferative histolytic reaction when used as a topical cauterizing agent[20]. Kopac et al[21] demonstrated in an experimental research in evaluating four different retraction agents on Chinese hamster lung fibroblasts and it was shown that containing 25% aluminum chloride was the most aggressive inflammatory infiltrate in gingival connective tissue. These reports have focused on the local effects of gingival retraction methods that include aluminum chloride and its cytotoxic effects. In this work we evaluate the cytotoxic effects of two topical hemostatic solutions on human gingival fibroblast cells. In agreement with the previous studies, both solutions showed significantly cytotoxic effect even if lowest concentration groups.

These commonly used hemostatic agents are highly acidic solutions with pH values from 0.5 to 2. Land et al[22] reported that higher acidity of retraction solutions had potential danger to the periodontal and dental tissues. The cytotoxicity of hemostatic solutions used in present study may occur resulting from lower pH values of solutions.

Human gingival fibroblasts were obtained as primary culture from explants of biopsy in this study. The use of human gingival fibroblasts permits enhanced relevance as such cells are exposed to topical hemostatic solutions. This was the reason why we chose primary human gingival fibroblasts in this study.

Different biocompatibility test methods such as cell growth, effects on membrane or cytoplasmic marker cytolysis, mitochondrial dehydrogenase of active cells and changes in metabolic activity have been used to evaluate the cytotoxicity of dental materials[23]. We conducted experiments with a new real-time system that investigated the cytotoxicity of the hemostatic solutions on human gingival fibroblasts by real-time and continuous monitoring of cell growth, proliferation and viability. Furthermore, the real-time cell analysis system allowed for calculation of time-dependent physiologic values, which can give more informative than single-value end points of classical toxicity testing[9,24]. Compared with conventional endpoint cell-based assays, dynamic monitoring of cell response, such as cell adhesion, spreading, proliferation, and cell death, is an advantage of the real-time system to optimize the cell concentration for *in-vitro* assays; it also allows both cell and assay conditions to be constantly obtained before and during the experiments. Furthermore, the response of live cells to, for example, a chemical exposure can be monitored in real time; this is impossible with current end-point assays, such as the XTT-based viability assay[25].

Cytotoxic effect of topical hemostatic solutions needs to be evaluated further because concentration used and exposure time to the agent is important factors. It has been reported that hemostatic agents that include aluminum chloride, with a concentration between 20%-25%, is frequently used[22]. Application time of hemostatic agents was extended to 1 min, a sufficient amount to achieve hemostasis. In present study, cell cultured screened after 48 hour and 72 hour in order to reveal cytotoxicity of materials completely. We suggest that final flushing with water should be sufficient to remove residual chemical hemostatic agents. Careful management of topical hemostatic solutions would help lower the risk of potential dental tissue damage during clinical application procedure and thus increase the success of restorative procedure.

Conclusions

Within the limitations of this in vitro laboratory study, it can be concluded that use of Hemoban or Hemostatic Solutions as an astringent solutions does have a significant cytotoxic effect on gingival fibroblast cells. Hemostasis becomes of utmost importance in maintaining the ideal, contaminat-free working field. The ideal hemostatic agents also should be free of cytotoxicity, but the ability of hemostasis is more important; however, after hemostasis is achieved, unused hemostatic material should be eliminated, leaving as little hemostatic agent as possible in order to avoid postoperative complications.

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