ORIGINAL ARTICLE

Evaluation *in vitro* of cytotoxicity of dentin desensitizers on human gingival fibroblasts.

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Abstract: The purpose of this study is to compare the cytotoxic effect of three materials, which have been used for treating dental hypersensitivity. Material and method: *In vitro* study. Clinpro (3M Co, St. Paul, MN. USA), Seal & Protect (Dentsply, DeTrey GmbH. Germany) and UltraEZ (Ultradent Products, Inc., S. South Jordan UT. USA) were used at concentrations of 0.1, 0.05, 0.01 and 0.001g/ml on human gingival fibroblasts. Furthermore, Clinpro and Seal & Protect were applied to this cell culture as polymerized disks. Toxicity was assessed at 24 and 48 hours by the use of the cell viability assay (MTT). Statistical analysis for cell viability was performed using two-way ANOVA and Tukey's post hoc test. Statistical significance was set at 5%. Results: Seal & Protect and Clinpro were found to be highly toxic at 24 and 48 hours, reaching 70% toxicity at concentrations over 0.01g/ml. Seal & Protect and Clinpro polymerized disks were toxic at 24 and 48 hours. UltraEZ showed an increased between 46% and 67% in cell viability at 24 hours and between 8% and 45% at 48 hours. Statistical analysis showed differences between these three desensitizers when comparing concentration and control group (p<0.05). Discussion: UltraEZ did not have a cytotoxic effect and may be considered a compatible and safe material, whereas polymerized and non-polymerized Clinpro and Seal & Protect should be used with caution.

Keywords: *Dentin Desensitizing Agents, Cytotoxicity, Human Gingival Fibroblasts, Dentin Sensitivity.*

INTRODUCTION.

Dentine hypersensitivity is defined as a short and sharp pain arising after the exposure of dentin in response to external stimuli (osmotic, thermal, chemical or tactile), which cannot be ascribed to any other disease or dental defect[1,2]. This condition affects between 4.1 % and 74% of the population, being more frequent in women between 20 and 49 years old[3].

Dentine hypersensitivity has been attributed to the stimuli, which act on exposed dentinal tubules[4]. The most effective treatment to eliminate its symptoms is sealing of exposed dentinal tubules[5,6]. This seal has been achieved through the use of desensitizing agents such as toothpastes[7], fluoridated varnishes[8] and resin or glass ionomer-based agents with high rates of success at the beginning which decrease in time[9,10].

Resinous dentine desensitizers are composed of glutaraldehyde, 2-hydroxyethyl methacrylate (HEMA), bisphenol A-glycidyl methacrylate (BIS-GMA), photo-initiators and acetone, which have proven to be toxic in human gingival fibroblasts (HGF) [11,12]. When applying a desensitizing agent to the affected tooth in a cervical area, the contact of these compounds with the gum is inevitable. However, there are no studies indicating the effect of these agents on the periodontium in clinical practice. That is why the cytotoxic effect of desensitizing agents on human gingi-

val fibroblasts have been studied, finding a high cytotoxicity and causing reactions in the gingival tissue due to the lack of protection of the gingiva during the application of the agent[13]. The main purpose of this study is to compare the cytotoxic effect of three dentinal desensitizers, Clinpro (3M ESPE), Seal & Protect (Dentsply) and UltraEZ (Ultradent) on cell cultures of human gingival fibroblasts.

MATERIALS AND METHODS.

Human Gingival Fibroblasts Culture

For this *in vitro* experimental study, a primary cell culture was performed using healthy gingival tissue associated to an unerupted third molar which was removed for orthodontic reasons from three young patients between 18 and 25 years of age, without use of medications and systemic diseases. Patients accepted the informed consent previously approved by the Ethics Committee of the Faculty of Medicine of the Universidad Austral de Chile (Authorization Number 2101.2014). A layer of gingival epithelial tissue was eliminated from the gingival connective tissue. This connective tissue was divided into 1-2mm fragments with a surgical scalpel.

Unless otherwise specified, the materials used for the preparation of culture medium were from Gibco® (Life Technologies Inc., Grand Island, NY, USA.). The fragments of connective tissue were cultured in 60 mm culture plates in a culture medium supplemented with 10% fetal bovine serum (FBS), 20% penicillin and 70% Dubelco's modified Eagle's medium (DMEM) (HyClone Laboratories Inc., Utah, USA) during three days at 37°C, 95% humidity with 5% CO_2 until a confluent cell monolayer was formed. Once the cells reached the desired confluence, they were recovered through trypsinization method using 0.25% trypsin and 1mm ethylenediaminetetraacetic acid (EDTA) solution. Then, they were placed into 75cm^2 culture flasks and cultured in complete culture medium (86.3% DMEM, 10% SBF, 2mm L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin) at 37°C and 95% humidity and 5% CO_2 until the new formation of confluent cell monolayers. The medium was changed every 24 hours.

Two lines of human gingival fibroblasts were obtained from different passages. The cells were stored in liquid nitrogen with 90% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, MO, USA.) until the toxicity test.

Materials and study groups.

Three desensitizing agents (Table 1) were used to create fourteen intervention groups and two control groups. Twelve groups corresponded to a solution of each desensitizer in complete culture medium (86.3% of DMEM, 10% FBS, 2mm L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin), getting four different concentrations per agent (0.1, 0.05, 0.01 and 0.001g/ml). Two other groups corresponded to Clinpro and Seal & Protect polymerized disks. These disks were prepared in 2x3mm metallic matrices and polymerized for 40 seconds with curing light Gnatus Optilight Max (1200mW/cm^2) (Gnatus, SP, Brazil). All experimental groups were cultured in the same complete culture medium previously used. A cell group in a culture medium without stimuli was used as negative control group and another with 10% of DMSO as positive control group.

Table 1. Composition of the materials used in this study.

Materials	Components	Manufacturer
Clinpro™	40-50% acrylic copolymer and itaconic acid and 30-40% water, 15-25% 2-hydroxyethyl methacrylate (HEMA), 1-10% calcium glycerophosphate.	3M Co, St. Paul, MN. USA
Seal & Protect	25-50 % acetone, 25-50% di and trimethacrylate resins, 2.5-10% dipentaerythritol penta acrylate monophosphate, 2.5-10% triclosan, phosphoric acid, functionalized amorphous silica, photoinitiators, butylated hydroxytoluene, celtilamina hydrofluoride	Dentsply, DeTrey GmbH. Germany
UltraEZ®	0.25 % sodium fluoride, 3% potassium nitrate	Ultradent Products, Inc., S. South Jordan UT. USA.

Table 2. Cytotoxicity of dentin desensitizers on human gingival fibroblasts assessed at 24 and 48 hours.

	Clinproxt varnish™		Seal & Protect™		UltraEZ®	
	24H	48H	24H	48H	24H	48H
	% (S.D)	% (S.D)	% (S.D)	% (S.D)	% (S.D)	% (S.D)
0.001g/ml	58.1(3)[a]	29.8(7)[a]	28.4(3)[a]	58.4(9)[a]	146 (7)[a]	108(2)[a]
0.01g/ml	15.9(3)[a]	5.0(5)[a]	12(2)[a]	14.5(3)[a]	141 (2)[a]	118(2)[a]
0.05g/ml	3(2)[a b]	2.7(2)[a b]	6.4(3)[a b]	13.7(2)[a]	167 (9)[a]	145(7)[a]
0.1g/ml	2(1)[a b]	1(1)[a b]	4(1)[a b]	2(2)[a b]	137 (3)[a]	134(8)[a]
Polymerized Disk	75.5(16)	11.4(10)[a]	21.7(5)[a]	11.2(7)[a]		
Negative Control	100	100	100	100	100	100
Positive Control	4.5(2)[a]	10(3)[a]	5(2)[a]	8(8)[a]	2(2)[a]	7(4)[a]

Results are presented according to the percentage of cell viability obtained from the negative control. S.D.: Standard Deviation. a: Indicate significant differences between the experimental groups and negative control (p<0.05). b: Indicates no significant differences between the experimental groups and positive control (p>0.05)

Evidence of cytotoxicity.

Cytotoxicity of the desensitizer was assessed by cell viability using dimethyl-thiazolyl-diphenyl tetrazolium-bromide (MTT) assay, which is converted to insoluble formazan by the action of dehydrogenases enzymes.

HGFs were seeded in 24-well plates at a concentration of 20,000 cells/well, with complete culture medium for 24 hours at 37°C to allow proliferation and cell adhesion. Then, the culture medium was replaced by a new complete culture medium containing the desensitizing concentration and the polymerized disk. During this procedure, the medium was protected from light to prevent polymerization of resin-based agents. The observation and control period for each desensitizer was repeated in triplicate. Each group was observed at 24 and 48 hours when the culture medium was removed and 100µl of MTT (Sigma Aldrich, St. Louis, MO, USA.) were added at a concentration of 0.55mg/ml per well for 4 hours. After completing the period of time, this medium was removed and each plate was washed with phosphate buffered saline (PBS), pH 7.4 (NaCl 124mmol/l, Na_2HPO_4 10mmol/l and KH_2PO_4 3mmol/l). Then, the formazan was solubilized in a mixture of 1000µl isopropanol (Merck KGaA, Darmstadt, Germany) and hydrochloric acid (Merck KGaA, Darmstadt, Germany) in a proportion of 2:10. After 5-10 minutes incubation, optical density (O.D.) was read at a wavelength of 570nm using a spectrophotometer (Bio-Mate™ 3S Waltham, MA USA, Thermo Fisher Scientific Inc.). The percentage of viability was obtained according to the International Organization for Standardization (ISO) for biological evaluation of medical products, MTT cytotoxicity assay.

Statistical analysis

Cell viability was assessed for the different materials, concentrations and times using two-way ANOVA, followed by Tukey tests to establish differences between the groups (GraphPad InStat®, Version 6.0.5. San Diego, CA, USA). The level of statistical significance was set at 5%.

RESULTS.

Clinpro and Seal & Protect dental desensitizers significantly decreased cell viability of human gingival fibroblasts at 24 and 48 hours reaching a toxicity greater than 70% at concentrations higher than 0.01g/ml. Clinpro and Seal & Protect, in its polymerized state, decrease cell viability at 24 hours to 75% and 21%, respectively, while at 48 hours, both desensitizer decreased to 11%.

In contrast, UltraEZ was capable of increasing cell via-

bility compared to the negative control group (p=0.021). The results for each desensitizer are detailed in the following table (Table 2).

The positive control of 10% DMSO achieved a 3% cell viability at 24 hours, and a 5% at 48 hours.

Statistical analysis shows statistically significant differences for the three desensitizers when comparing concentration *v/s* negative control group (p<0.05). At concentrations above 0.01g/ml, Clinpro and Seal &Protect did not show statistically significant differences compared with the positive control group at 24 hours (p>0.05).

DISCUSSION.

Assessing toxicity using MTT assay allows comparing cell viability of gingival fibroblasts exposed to different concentrations of desensitizers used in dental practice. Clinpro and Seal & Protect showed a highly cytotoxic behavior on human gingival fibroblasts, indicating that the higher the desensitizer concentration used is, the less cell viability.

At concentrations of 0.05 and 0.01g/ml, cell viability of human gingival fibroblasts treated with Clinpro and Seal & Protect was very low, less than 10% at 24 and 48 hours. Therefore, the highest concentration used for these desensitizers did not show statistically significant differences compared with the positive control (p=0.295).

The values of cell viability of Seal & Protect increase between 24 and 48 hours, while the values of Clinpro continued to decline at 48 hours. Polymerising the material to reduce the release of their components into the culture medium succeeded in reducing cytotoxic effects. However, the decrease in cell viability can be compared at the lowest concentration of each desensitizing used (0.001g/ml) at 24 hours, but it progressed over time and it was about 0.01g/ml at 48 hours. Clinpro presented a higher cell viability in measurement twice than polymerized Seal & Protect. On the contrary, UltraEZ was capable of promoting cell viability between 46% and 67% at 24 hours and between 8% and 45% at 48 hours. Since UltraEZ is a desensitizing gel and cannot use curing light or be set, the comparison with the polymerized

disks of the other two desensitizers was not possible. It is not possible to compare UltraEZ with polymerized discs of the other desensitizing agents as it is a gel without the possibility of light curing or hardening.

UltraEZ is a desensitizer based on two components, potassium nitrate and sodium fluoride. Studies on the cytotoxicity of sodium fluoride at concentrations of 0.0095% and 0.07% have indicated that cell viability decreases to 11% when assessed at 12 hours[13]. Despite having 0.25% sodium fluoride, UltraEZ did not generate cytotoxicity in this study. Duraphat®, a material which presents 5% sodium fluoride and is indicated for the treatment of dentinal hypersensitivity and dental caries prevention was evaluated in a study by Hoang-Dao *et al.*[14] along with two other materials, Isodan® and Shellac F. The toxicity test using MTT assay revealed that Duraphat® was the least toxic material followed by Shellac F. and Isodan®, evaluating the same concentrations used in this work for each desensitizer. This shows that cell viability increases when the concentration of the materials is reduced and methacrylate components act as toxic in these cell groups. The increase in cell viability when applying UltraEZ on HGF cannot be explained on the basis of the components listed by its manufacturer.

Camps *et al.*[15] compared cell viability of resin-based desensitizing agents on L-929 mouse fibroblast cell lines using MTT assay by interpositioning a dentin disk as a barrier between fibroblasts and the desensitizer, concluding that all of the materials used in this study had a low cytotoxicity. In that study, Seal & Protect showed a cell viability of 88% at 48 hours. These data are in contrast with the results of Sengün *et al.*[16], who conducted a study of cytotoxicity in human gingival fibroblasts directly exposed to the desensitizers at three concentrations: 0.1, 0.3, and 0.5μl/ml, assessing cell viability through MTT assay at 48 hours, in addition to a count of viable cells at 24 and 48 hours, reporting that all the desensitizers were cytotoxic. In the study by Sengün, Seal & Protect showed a range of cell viability between 40% and 85% at the different concentrations evaluated. The count of viable cells for Seal & Protect was 50% at 24 hours and

10% at 48 hours, demonstrating a high cytotoxicity at all concentrations evaluated and compared with the results obtained in this research. Seal & Protect has a many components. From among them, the cytotoxicity of di and trimethacrylate resins, dipentaerythritol penta acrylate monophosphate[17,18] and triclosan[19] have been studied. Also, its composition is similar to adhesive systems which reported a high cytotoxicity[20]. It has been shown that the combined use of different types of monomers has a synergistic effect in cellular toxicity of human gingival fibroblasts[21].

The cytotoxicity of Clinpro has not been reported yet. Since it is composed of acrylate copolymers, itaconic acid and 2-hydroxyethyl methacrylate (HEMA), it is considered as a resin-modified glass-ionomer[22]. Studies on resin-modified glass-ionomer cements, have shown to release HEMA in solution for being photopolymerized and even hyperpolymerized[23].

HEMA is a monomer present in a large number of dental biomaterials[24]. This monomer affects proliferation, apoptosis and cell cycle[25,26]. Aditionally, it has been studied the inflammatory response in gingival fibroblasts, causing an increase in the levels of reactive oxygen species, cyclooxygenase-2, tumor necrosis factor-alpha gene expression and prostaglandin E2 release[27].

While the clinical behavior of desensitizer on the gingiva cannot be ensured because there are no studies evaluating cellular toxicity in humans, such factors as the clearance from the gingival sulcus and the saliva, which diluIes the concentrations and reduces the lenght of time for contact of these materials with the periodontium, could mitigate the cytotoxic effects of the desensitizers and not generate evident lesions at the clinical level.

It is necessary to carry out studies in three-dimensional oral mucosa cell cultures or in animal to identify toxicity and inflammatory changes through histopathological sections, in order to better quantify the periodontal tissue response to the exposure of these biomaterials. In the absence of this kind of studies, the clinician should be careful when handling these materials in cervical areas, as being in contact with the gum, could generate responses in the periodontium, causing migration of periodontal attachment, exposing dental tissue and producing dentinal hypersensitivity again.

The results of this study indicate that UltraEZ has no cytotoxic effect, but it increases cell viability. However, Seal & Protect and Clinpro, both in their polymerised and non-polymerised form, were highly toxic at the concentrations evaluated in this study.

Evaluación *in vitro* **de la citotoxicidad de desensibilizantes dentinarios en fibroblastos gingivales humanos.**

Resumen: Introducción: El propósito de este estudio es comparar el efecto citotóxico de tres materiales que se han utilizado para el tratamiento de la hipersensibilidad dental. Material y método: Estudio *in-vitro*. Los desensibilizantes dentinarios Clinpro (3M ESPE), Seal&Protect (Dentsply) y UltraEZ (Ultradent) fueron utilizados a concentraciones de 0,1; 0,05; 0,01 y 0,001 g/ml sobre cultivos celulares de fibroblastos gingivales humanos. Además, Clinpro y Seal&Protect se aplicaron a este cultivo celular como discos polimerizados. La toxicidad se evaluó a 24 y 48 horas mediante ensayo de viabilidad (MTT). El análisis estadístico para la viabilidad celular se realizó mediante ANOVA de dos vías seguido de análisis Tukey. La significancia estadística se fijó al 5%. Resultados: Clinpro y Seal&Protect resultaron ser altamente tóxicos a las 24 y 48 horas, alcanzando un 70% de toxicidad a concentraciones superiores de 0,01 g/ml. Los discos polimerizados de Clinpro y Seal&Protect fueron tóxicos a 24 y 48 horas. UltraEZ produjo un aumento de la viabilidad celular entre un 46% y 67% a las 24 horas y entre un 8% y 45% a

las 48 horas. El análisis estadístico mostró diferencias entre estos tres desensibilizantes al comparar la concentración y su grupo control (p<0,05). Discusión: UltraEZ no tuvo efecto citotóxico y puede ser considerado como un material compatible y seguro para ser utilizado, mientras que Clinpro y Seal&Protect en su estado polimerizado y no polimerizado deberían ser utilizados con precaución.

Palabras clave: *Desensibilizante Dentinario, Citotoxicidad, Fibroblastos Gingivales Humanos, Sensibilidad dentinaria.*

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