

***In Vitro* Effects of Bleaching Agents on L929 Cell Line**

Efectos *In Vitro* de Agentes de Blanqueamiento sobre la Línea Celular L929

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ABSTRACT: Bleaching agents can potentially cause damage to dental and gingival tissues. Due to the use of bleaching agents close to gingival tissues, the objective of the present study was to test the hypothesis that the higher the concentration of hydrogen peroxide, the greater is the cytotoxicity to fibroblast cells. Two concentrations of hydrogen peroxide (6%, and 7.5%) used in home bleaching techniques were evaluated regarding their possible cytotoxic effect on gingival tissues. The materials were divided into two groups as follows: Group P6 (White Class 6%, FGM), and Group P7.5 (White Class 7.5%, FGM). The cytotoxicity essay was carried out using cell cultures (mouse fibroblast L929 cell line) in which the viable cells were determined by means of the dye-uptake method performed at 2, 4, and 8 hours. Analysis of variance (ANOVA) with multiple comparisons and Tukey's test were employed ($P < 0.05$). The results showed statistically significant differences between Groups P6 and P7.5, and the cell control at 2, 4, and 8 hours ($P < 0.05$). The amount of cell lysis increased proportionally to the exposure time to the materials studied. One can conclude, therefore, that the 6 and 7.5% hydrogen peroxide demonstrated high cytotoxic character for all times studied.

KEY WORDS: toxicity, biocompatibility, materials.

INTRODUCTION

Coffee, tea, juices, wines and cola-based soft drinks are potential dark or coloring beverages, which could stain or discolor the bleached enamel surface. In addition, certain beverages, artificial food colorations and smoking used with a high frequency are considered responsible for primary staining, dark and discoloration of teeth (Arens, 1989; Attin *et al.*, 2003).

Dental bleaching has been used for more than one hundred years (Haywood *et al.*, 1992), and since then several techniques and chemicals have been employed to whiten the teeth. The use of hydrogen peroxide to bleach teeth with pulp vitality began in the early 1900's (Ames, 1937). Nowadays, two main bleaching techniques exist: the in-office bleaching and supervised at-home bleaching techniques (Haywood, & Heymann, 1989).

The at-home bleaching technique, which was introduced by Haywood & Heymann in 1989, consists

of using carbamide peroxide-based bleaching agents at concentrations lower than those used in the office (16% and 22%) or hydrogen peroxide (from 1.5% to 7.5%). The patients themselves use these chemical agents daily (1-8 hours) by means of a personal tray during periods ranging from 2 to 4 weeks (Haywood, 1997). In general, bleaching agents comprise hydrogen peroxide or products releasing hydrogen peroxide, such as 10% carbamide peroxide, which breaks down into 6.4% urea and 3.6% hydrogen peroxide. The hydrogen peroxide dissociate into water and oxygen, whereas urea breaks down into carbon dioxide and ammonia (Feinman, 1987).

All dental bleaching agents, from carbamide peroxide to hydrogen peroxide, ionize and decompose to produce free radicals. However, these free-radical reactions are not specific to the pigment molecules of the tooth only, as they can react with other organic structures as well (Kawamoto, K. & Tsujimoto, 2004).

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The reactive oxygen-derived species are known to cause damage to living cells due to the oxidative stress they provoke. This oxidative stress can cause apoptosis, DNA damage (genotoxicity), and cell cytotoxicity (Kanno *et al.*, 2003). Therefore, the possible alterations caused by the use of bleaching agents indiscriminately can potentially cause damage to all dental tissues (Potocnik *et al.*, 2000).

By definition, cytotoxicity of a given agent means destructive effect on cells (Li, 1996). So far, there are very few studies reporting cytotoxicity effects of bleaching agents on pulp cells (Anderson *et al.*, 1999), fibroblasts (Kanno *et al.*), and odontoblastic cells. Because the application of such bleaching agents occurs close to gingival tissue, the objective of the present study was to test the hypothesis that the higher the concentration of hydrogen peroxide, the greater is the cytotoxic effect on fibroblast cells.

MATERIAL AND METHOD

Two concentrations of hydrogen peroxide (6% and 7.5%) commonly used in the at-home bleaching technique were assessed in the present study in order to determine their possible cytotoxic effect on gingival tissues. The materials were divided into two groups: Group P6 (White Class 6%, FGM Produtos Odontológicos, Joinville, Santa Catarina, Brazil) and Group P7.5 (White Class 7.5%, FGM Produtos Odontológicos, Joinville, Santa Catarina, Brazil) (Table I). To verify the cell response to extreme situations, other three groups were included in the study: Group CC (cell control), consisting of cells not exposed to any material; Group C+ (positive control), consisting of Tween 80 (Polyoxyethylene-20-sorbitan); and Group C (negative control), consisting of PBS solution (phosphate-buffered saline) in contact with the cells.

Culture of L929 cells (mouse fibroblasts), obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were used in the present study and maintained in Eagle's minimum essential medium (Cultilab, Campinas, Brazil). To the cell culture were added 0.03 mg/ml glutamine (Sigma, St. Louis,

Missouri, USA), 50 mg/ml of garamicine (Schering Plough, Kenilworth, New Jersey, USA), 2.5 mg/ml fungizone (Bristol-Myers-Squibb, New York, USA), 0.25% sodium bicarbonate solution (Merck, Darmstadt, Germany), 10 mM HEPES (Sigma, St. Louis, Missouri, USA), and 10% bovine foetal serum (Cultilab, Campinas, Brazil) as growth medium or no bovine foetal serum for maintenance medium only. The cell culture was incubated at 37o C for 48 hours.

The so-called "dye-uptake" technique, which is based on the incorporation of neutral red by the living cells, was employed to determine the cytotoxicity of the materials (Santos *et al.*, 2008). These bleaching agents were evaluated at 2, 4, and 8 hours because according to their manufacturers they are commonly used during periods ranging from 2 to 8 hours a day. This period of time represents the contact of hydrogen peroxide with the cells for 2, 4, and 8 hours for analysis of cytotoxicity.

Dye-Uptake. Volumes of 100 µl of L929 cell suspension were distributed into 96-well microplates. After 48 hours, the growth medium was replaced with 100 µl of the culture medium (Eagle's MEM) obtained from the mixture (1 ml of culture medium) with 0,5 ml of hydrogen peroxide. The positive and negative control cells comprised culture mediums obtained from their mixture (1 ml) with, respectively, 0.5 ml Tween 80 and 0.5 ml PBS solution. The experiment was performed 4 times, with a total of 15 samples for each group.

After 2, 4, and 8 hours of incubation, 100 ml of 0.01% neutral red solution (Sigma, St. Louis, Missouri) were added to each well of the microplaque that was then incubated at 37oC for 3 hours for allowing the living cells to absorb the dye. Next, after discarding the dye, 100 µl of 4% formaldehyde solution (Vetec, Rio de Janeiro, Brazil) were added to PBS (130 mM NaCl; 2 mM KCl; 6 mM Na2HPO4 2H2O; 1 mM K2HPO4; pH = 7.2) for 5 minutes to promote cell fixation. Next, 100 ml of 1% acetic acid solution (Vetec, Rio de Janeiro, Brazil) with 50% methanol were added in order to remove the dye. After 20 minutes, a spectrophotometer (BioTek, Winnoski, Vermont, USA) was used for data reading at wavelength of 492 nm (l=492 nm).

Table I. Description of the composition of the bleaching agents.

Bleaching agents	Basic Composition	Manufacturer
White class 6%	Hydrogen peroxide, carbopol, potassium nitrate, sodium fluoride, aloe Vera, deionized water	FGM
White class 7.5%	Hydrogen peroxide, carbopol, potassium nitrate, sodium fluoride, aloe Vera, deionized water	FGM

Data were compared by using analysis of variance (ANOVA) and then Tukey's test was used to assess any difference between groups at a significance level of 5%.

RESULTS

The results showed statistically significant differences between the Groups P6 (6% hydrogen peroxide) and P7.5 (7.5% hydrogen peroxide) in

relation to the Group CC (cell control) at 2, 4, and 8 hours ($P < 0.05$). No statistically significant differences were observed between Groups P6 and P7.5 at 2 and 8 hours ($P > 0.05$) (Table II). The amount of cell lysis increased in direct proportion to the exposure time of the materials to cell cultures.

Group P6 had the lowest cytotoxicity values for all periods of time studied (Table II), thus suggesting that the level of cytotoxicity is directly related to the hydrogen peroxide concentration in the bleaching agents.

Table II. Descriptive statistics for optical density of Bleaching agents. $n=15$. Values followed by same letters are not significantly different ($p>0.05$) for the same time. SD: standard deviation.

Groups	n	Time (2 h)				Time (4 h)				Time (8 h)			
		Mean	Median	S. D.	Viable Cells (%)	Mean	Median	S. D.	Viable Cells (%)	Mean	Median	S. D.	Viable Cells (%)
CC	15	0.634 ^a	0.652	0.084	100	0.695 ^a	0.726	0.074	100	0.740 ^a	0.755	0.094	100
C-	15	0.616	0.650	0.109	97.2	0.683	0.710	0.098	98.3	0.725	0.734	0.072	98.1
C+	15	0.055	0.068	0.008	8.7	0.066	0.082	0.010	9.5	0.073	0.080	0.012	9.9
P6	15	0.438 ^b	0.468	0.063	69.1	0.373 ^b	0.390	0.062	53.8	0.311 ^b	0.324	0.059	42.1
P7,5	15	0.410 ^b	0.422	0.083	64.8	0.350 ^c	0.360	0.079	50.5	0.299 ^b	0.318	0.052	40.5

DISCUSSION

In dentistry, more specifically, a comprehensive evaluation of the dental material needs to follow a series of tests established by ANSI/ADA, ISO, and FDI, which are divided into three categories: initial tests, secondary tests, and pre-clinical tests. The initial tests are aimed at determining the toxic effects of a given material by using several methods, such as the cell cultures.

Tooth whitening has been increasingly sought by the patients in the dentist's office, however, the effect of these bleaching agents on gingival and dental tissues has been questioned. Because this bleaching agents act close to gingival tissue, the objective of the present study was to test the hypothesis that the higher the concentration of hydrogen peroxide, the greater is the cytotoxicity to fibroblast cells.

Some *in vitro* studies have evaluated the cytotoxic effects of the components of bleaching products by means of cell cultures (Kanno *et al.*; Aren, 2003). Cytotoxicity tests in cell cultures can help evaluate the effects resulting from a specific agent concentration through the damage caused to either dental structures or biochemical pathways within the cells (Wataha *et al.*, 1994).

Koulaouzidou *et al.* (1998) who assessed the cytotoxicity of hydrogen peroxide in fibroblastic cells cultivated *in vitro*, reported that all products studied had caused definite cytotoxic effects on the cells.

In the present study, one can observe the cytotoxic aspect of the materials after their exposure to cell culture. Hydrogen peroxide concentration of 7.5% caused more cell death in comparison to the 6% hydrogen peroxide studied during the three experimental periods, thus suggesting that the level of cytotoxicity is directly related to the hydrogen peroxide concentration existing in the bleaching agent as all the materials studied have carbopol (carboxypolyethylene polymer) that, in turn, may influence the cytotoxicity. Woovelton *et al.* (1993) determined that adding thickening agent (Carbopol) to the bleaching agent reduces the potential cytotoxic effect of this material on the cells, possible due to its capacity to increase the viscosity and delay the release of hydrogen peroxide. This supports the idea that hydrogen peroxide without thickening agent can have a more cytotoxic effect compared to that found in the present study.

The amount of cell lysis observed in the present study increased in direct proportion to exposure time

of the materials to cell cultures, a finding also corroborated by Koulaouzidou, et al. and González-Ochoa (2002), who performed *in vitro* studies on the cytotoxicity of bleaching agents and reported that the cytotoxic effects on viable cells are both dose-dependent and time-dependent.

The metabolic processes in animal systems are more complex and dynamic than in cell cultures (Li). In addition, when an agent is added to the cell culture it becomes readily available to the cells, which does not occur in the living systems. It is important to understand that highly cytotoxic dental materials, when applied directly to cell cultures, may not cause significant risks to the pulp-dentine complex as the dentine acts as a biological barrier that reduces or dilutes the soluble components of the materials (Pashley *et al.*, 1987). However, in contact with gingival tissues, cytotoxic

dental materials may cause more severe damages.

However, there are in the oral cavity enzyme, protein and tamponade systems that reduce such effects. These cytotoxic effects are directly related to the concentration and time of use of the bleaching agent.

CONCLUSION

Altogether, one can conclude that the use of low concentrations of such agents is less cytotoxic than the bleaching agents containing higher concentrations of hydrogen peroxide, and the risk of damage to gingival tissues depends on hydrogen peroxide concentration, bleaching agent composition and exposure time of the gingival tissues.

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RESUMEN: Los agentes blanqueadores, pueden potencialmente causar daños a los tejidos gingivales y dentarios. Debido a la utilización de estos agentes blanqueadores próximos a los tejidos gingivales, el objetivo del presente estudio fue comprobar la hipótesis que, en cuanto mayor sea la concentración del peróxido de hidrógeno, mayor es la citotoxicidad provocada en las células fibroblásticas. Fueron utilizadas en este estudio 2 concentraciones de peróxidos de hidrógeno (6% y 7,5%), utilizados en la técnica de blanqueamiento casero, dividiéndolos en 2 grupos: grupo P6 (White Class 6%, FGM) y grupo P7,5 (White Class 7,5%, FGM). El ensayo de citotoxicidad fue realizado utilizando cultivos celulares (línea celular L929, fibroblastos de ratón) sometidos a pruebas para viabilidad celular mediante el método de tinción "dye-uptake", en tiempos de 2, 4 y 8 horas. Fueron realizados el análisis de varianza, comparación múltiple (ANOVA) y la prueba de Tukey ($p < 0.05$). Los resultados mostraron diferencias estadísticamente significativas entre los grupos P6 y P7,5 con el grupo CC (Control de células) a las 2, 4 y 8 horas ($p < 0.05$). La cantidad de lisis celular aumentó directamente proporcional al tiempo de exposición de los materiales estudiados en los cultivos celulares. Se puede concluir que el peróxido de hidrógeno al 6 y 7,5% demostró un alto carácter citotóxico en todos los tiempos estudiados.

PALABRAS CLAVE: toxicidad; biocompatibilidad; Materiales.

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