

# Cytotoxicity of carbamide peroxide bleaching gel on L929 cells

## Citotoxicidade do gel clareador peróxido de carbamida em células L929

### Abstract

**Purpose:** To test the hypothesis that the higher the concentration of carbamide peroxide, the greater is its cytotoxicity to fibroblast cells.

**Methods:** Three concentrations of carbamide peroxide (10%, 16%, and 22%) used in home bleaching techniques were evaluated regarding their cytotoxic effect on gingival tissues. The materials were divided into three groups as follows: Group C10 (White Gold Home 10%, Dentsply), Group C16 (White Gold Home 16%, Dentsply, and Group C22 (Nite White 22%, ACP Discus Dental). 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. Data were analyzed by analysis of variance (ANOVA) with multiple comparisons and Tukey's test ( $P < 0.05$ ).

**Results:** The results showed statistically significant differences between Groups C10, C16, C22, 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.

**Conclusion:** The 22% carbamide peroxide group was more toxic than the other two groups (16% and 10% concentration) regardless of the exposure time.

**Key words:** Toxicity; hydrogen peroxide; dental bleaching

### Resumo

**Objetivo:** Testar a hipótese que quanto maior a concentração do peróxido de carbamida, maior a citotoxicidade provocada em células fibroblásticas.

**Metodologia:** Foram avaliadas 3 concentrações de peróxidos de carbamida (10%, 16% e 22%) usados na técnica de clareamento caseiro, divididos em 3 grupos: grupo C10 (White Gold Home 10%, Dentsply), grupo C16 (White Gold Home 16%, Dentsply) e grupo C22 (Nite White 22%, ACP Discus Dental) quanto ao efeito citotóxico nos tecidos gengivais. O ensaio de citotoxicidade foi realizado utilizando cultura de células (linhagem L929, fibroblastos de camundongos) e submetidos ao teste para células viáveis em vermelho neutro ("dye-uptake") no tempo de 2, 4 e 8 h. Os dados foram analisados estatisticamente por análise de variância e teste de Tukey ( $P < 0,05$ ).

**Resultados:** Houve diferença estatisticamente significativa entre os grupos C10, C16 e C22 com o grupo CC (controle de células) nos tempos de 2, 4 e 8 h ( $P < 0,05$ ). A quantidade de lise celular aumentou diretamente proporcional ao tempo de exposição dos materiais com as culturas de células.

**Conclusão:** O peróxido de carbamida 22% foi mais citotóxico que os peróxidos de carbamida nas concentrações de 16 e 10% independentemente do tempo avaliado.

**Palavras-chave:** Toxicidade; peróxido de hidrogênio; clareamento dentário

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## Introduction

Dental bleaching has been used for more than one hundred years (1), 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 (2). Nowadays, two main bleaching techniques exist: the in-office bleaching and the supervised at-home bleaching techniques (3).

The at-home bleaching technique, which was introduced by Haywood and Heymann (3) 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 (4). 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 (5).

All dental bleaching agents, from carbamide peroxide to hydrogen peroxide, ionize and decompose to produce free radicals, which are highly unstable and react very easily with other organic substances (6). The bleaching principle is based on the fact that these agents react with highly conjugated organic molecules by breaking down their electronic conjugation and altering their molecular energy absorption, thus resulting in changes in their optical structure (7). This can affect the absorption spectrum of the composite, transforming its long wavelength (dark) into a shorter wavelength, that is, resulting in a lighter shade (8). 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 (6). The reactive oxygen-derived species are known to cause damage to living cells due to the oxidative stress they provoke (9). This oxidative stress can cause apoptosis, DNA damage (genotoxicity), and cell cytotoxicity (9) observed in bacteria and cultured cells (10).

Hydrogen peroxide was shown to have a weak local carcinogenic-inducing potential (10). The mechanism is unclear (10), but it appears unlikely that oral health products which hydrogen peroxide release will enhance cancer risk in individuals except in those who have an

increased risk of oral cancer due to tobacco use, alcohol abuse, or genetic predisposition (11). Therefore, the possible alterations caused by the use of bleaching agents indiscriminately can potentially cause damage to dental tissues (12).

By definition, cytotoxicity of a given agent means destructive effect on cells (13). So far, there are very few studies reporting cytotoxicity effects of bleaching agents on pulp cells (14), fibroblasts (9), 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 carbamide peroxide, the greater the cytotoxic effect on fibroblast cells.

## Methods

Three concentrations of carbamide peroxide (10%, 16%, 22%) commonly used in the at-home bleaching technique were assessed in order to determine their possible cytotoxic effect on gingival tissues. The materials were divided into three groups: Group C10 (White Gold Home 10%, Dentsply, Petrópolis, Rio de Janeiro, Brasil), Group C16 (White Gold Home 16%, Dentsply, Petrópolis, Rio de Janeiro, Brasil), and Group C22 (Nite White 22%, ACP Discus Dental, Culver, CA, USA) (Table 1). 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 37° C for 48 hours.

**Table 1.** Description of the composition of the tested bleaching agents.

Bleaching agents	Basic Composition	Manufacturer
White Gold Home 10%	Carbamide peroxide, thickening agent, flavour, deionised water	Dentsply
White Gold Home 16%	Carbamide peroxide, thickening agent, flavour, deionised water	Dentsply
Nite White 22%	Propylene glycol, C <sub>3</sub> H <sub>8</sub> O <sub>3</sub> , carbamide peroxide, sílica, hydrogen peroxide, KNO <sub>3</sub> , emulsifying wax, NF, hydroxypropyl cellulose, flavour, deionised water, KOH, dimethicone	ACP Discus Dental

## Dye-Uptake

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 (15,16). The 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 carbamide peroxide with the cells for 2, 4, and 8 hours for analysis of cytotoxicity.

Volumes of 100  $\mu$ L of L929 cell suspension were distributed into 96-well microplates. After 48 hours, the growth medium was replaced with 100  $\mu$ L of the culture medium (Eagle’s MEM) obtained from the mixture (1 mL of culture medium) with 0.5 mL of carbamide 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  $\mu$ L of 0.01% neutral red solution (Sigma, St. Louis, Missouri, USA) were added to each well of the microplaque that was then incubated at 37°C for 3 hours for allowing the living cells to absorb the dye. Next, after discarding the dye, 100  $\mu$ L of 4% formaldehyde solution (Vetec, Rio de Janeiro, Brazil) were added to PBS (130 mM NaCl; 2 mM KCl; 6 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ; 1 mM  $\text{K}_2\text{HPO}_4$ ; pH=7.2) for 5 minutes to promote cell fixation. Next, 100  $\mu$ L 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 ( $\lambda=492$  nm).

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

## Results

The results showed statistically significant differences between the Groups C10 (10% carbamide peroxide), C16 (16% carbamide peroxide), and C22 (22% carbamide 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 C10 and C16 as well as between Groups C16 and C22 at 2 hours, and the same occurred between Groups C10, C16, and C22 at 4 and 8 hours (Table 2). The amount of cell lysis increased in direct proportion to the exposure time of the materials to cell cultures. Group C10 had the lowest cytotoxicity values for all periods of time studied. Group C22 was notoriously more cytotoxicity compared to Groups C10 and C16 (Table 2), thus suggesting that the level of cytotoxicity is directly related to the carbamide peroxide concentration in the bleaching agents.

## Discussion

All materials or chemical agents developed for application to dental or medical areas have to be submitted to previous tests for cytotoxicity and biocompatibility in order to evaluate their biological properties and risk factors before being introduced to the market. In Dentistry, 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 dental office, however, the effect of these bleaching agents on gingival and dental tissues has been questioned. Because these bleaching agents act close to the gingival tissue, the objective of the present study was to test the hypothesis that the concentration of carbamide peroxide affects its cytotoxicity to fibroblast cells.

Benetti et al. (17) have shown that the higher the concentration of bleaching agent applied to the dental enamel, the greater is the penetration of hydrogen peroxide into the pulp chamber. According to Robertson and Melf (18), heat seems to have a direct relation to the increased diffusion of bleaching agents through the dental structures, including the pulp. Both hydrogen peroxide and heat are important components of the technique used for dental bleaching. Heat is applied in order to accelerate the chemical reaction between hydrogen peroxide and dental structures, that is, the cytotoxic effect of the bleaching agent will be enhanced if the material is in contact with the oral tissues.

**Table 2.** Descriptive statistics\* for the optical density of the tested bleaching agents.

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	0.756 <sup>a</sup>	0.782	0.064	100	0.561 <sup>a</sup>	0.582	0.064	100	0.638 <sup>a</sup>	0.644	0.064	100
C-	15	0.741	0.748	0.089	98.1	0.548	0.574	0.089	97.7	0.630	0.660	0.089	98.9
C+	15	0.069	0.082	0.009	9.2	0.048	0.066	0.009	8.6	0.057	0.062	0.009	9.0
C10	15	0.509 <sup>b</sup>	0.536	0.057	67.4	0.284 <sup>b</sup>	0.302	0.057	50.8	0.250 <sup>b</sup>	0.272	0.057	39.2
C16	15	0.475 <sup>bc</sup>	0.498	0.073	62.9	0.275 <sup>b</sup>	0.298	0.073	49.1	0.239 <sup>b</sup>	0.254	0.073	37.5
C22	15	0.429 <sup>cd</sup>	0.454	0.045	56.8	0.262 <sup>b</sup>	0.288	0.045	46.8	0.220 <sup>b</sup>	0.248	0.045	34.5

\* Mean values followed by same letters are not significantly different ( $P>0.05$ ) for the same time. SD: standard deviation.

Some *in vitro* studies have evaluated the cytotoxic effects of the components of bleaching products by means of cell cultures (9,19). 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 (20). Koulaouzidou et al. (21), who assessed the cytotoxicity of hydrogen peroxide in fibroblastic cells cultivated *in vitro*, reported that all products 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. Carbamide peroxide concentration of 22% caused more cell death in comparison to other concentrations during the three experimental periods, thus suggesting that the level of cytotoxicity is directly related to the carbamide peroxide concentration existing in the bleaching agent as all the materials studied have thickening agent that, in turn, may influence the cytotoxicity. Woovelton et al. (22) determined that adding a thickening agent (ex. Carbopol – carboxypolyethylene polymer) to the bleaching agent reduces the potential cytotoxic effect of this material on the cells, possibly due to its capacity to increase the viscosity and delay the release of hydrogen peroxide. This supports the idea that carbamide peroxide without thickening agent may 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. (21) and González-Ochoa (23), 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.

In clinical trials that used 10% carbamide peroxide in custom made trays, 25 to 40% of the patients reported gingival irritation during treatment (24). The damage to the gingival tissue caused by the carbamide peroxide in the oral cavity can manifest itself in the form of mild hyperemia to ulceration. It is therefore advisable that the tray be designed to prevent gingival exposure by the use of a tray that has firm contact with the teeth.

The metabolic processes in animal systems are more complex and dynamic than in cell cultures (13). 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 (25). However, in contact with gingival tissues, cytotoxic dental materials may cause more severe damages.

## Conclusions

This study suggests that the use of low concentrations of carbamide peroxide is less cytotoxic than the bleaching agents containing higher concentrations of such agent, and the risk of damage to gingival tissues depends on carbamide peroxide concentration and bleaching agent composition, besides exposure time to gingival tissues.

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