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Endodontics

Cytotoxic effects of cyanoacrylates used as retrograde filling materials. An *in vitro* analysis

Efeitos citotóxicos de cianoacrilatos usados como material de obturação retrógrada. Uma análise in vitro

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ABSTRACT: Cyanoacrylate has been used in medicine and dentistry for many years. It has been used as a postextraction dressing and retrograde filling material in endodontic surgery. The aim of this study was to evaluate the cytotoxic effects of Histoacryl and other two homologue ethyl cyanoacrylates, Super Bonder and Ultrabond, on cultured fibroblasts, using the Trypan blue dye exclusion assay. The cyanoacrylates were applied to round glass coverslips, which were placed in contact with NIH 3T3 cells. After 0, 6, 12 and 24 h (short-term assay; viability) and 1, 3, 5 and 7 days (long-term assay; survival), the cells were examined under phase light microscopy and counted. The data were compared by the Kruskal-Wallis test. In the short-term experiments, only the cultures of the Ultrabond group (GIV) presented significant smaller percentages of cell viability than the cultures of the other groups (GI: control; GII: Super Bonder; GIII: Histoacryl). Although the cultures of the Super Bonder group (GII) presented smaller percentages of cell viability than cultures of the other groups (GI, GIII, GIV) at the long-term assay, this group was the only experimental group presenting a continuous and progressive cell growth. Our results have shown an *in vitro* biocompatibility of Histoacryl and ethyl cyanoacrylate homologues. These cyanoacrylates could therefore be of importance for endodontic purposes.
DESCRIPTORS: Biocompatible materials; Cell culture; Cyanoacrylates.

RESUMO: Os cianoacrilatos tem encontrado aplicabilidade tanto na Medicina como na Odontologia há muitos anos. Têm sido usados como curativo após exodontias, bem como para obturação retrógrada em cirurgia parendodôntica. O objetivo deste estudo foi o de avaliar o efeito citotóxico do Histoacryl e outros dois homólogos etil cianoacrilatos: Super-Bonder e Ultrabond, em cultura de fibroblastos, empregando ensaios de viabilidade pela exclusão de células coradas pelo azul de Trypan. Os cianoacrilatos foram aplicados em lamínulas de vidro circulares, que foram colocadas sobre cultura de fibroblastos NIH - 3T3. Após 0, 6, 12 e 24 horas (resposta celular imediata - curto prazo) e 1, 3, 5 e 7 dias (sobrevivência celular - longo prazo), foram efetuadas contagens celulares em microscópio de fase. Os dados obtidos foram analisados valendo-se do teste estatístico de Kruskal-Wallis. No experimento de curto prazo, somente as culturas do grupo Ultrabond (GIV) apresentaram porcentagens de viabilidade celular significantemente menores que a dos outros grupos (GI: controle; GII: Super Bonder; GIII: Histoacryl). Embora as culturas do grupo Super Bonder (GII) apresentassem porcentagens de viabilidade celular menores que as dos outros grupos (GI, GIII, GIV) no experimento de longo prazo, esse grupo foi o único que mostrou crescimento celular progressivo e contínuo. Nossos resultados mostraram biocompatibilidade *in vitro* tanto do Histoacryl como dos outros dois homólogos etil cianoacrilatos. Esses cianoacrilatos podem ser importantes para finalidades biológicas.
DESCRITORES: Materiais biocompatíveis; Cultura de células; Cianoacrilatos.

INTRODUCTION

Endodontic treatment is based on disinfection and enlargement of root canals. As a result of this treatment, the dentine permeability increases. The opening of dentine tubules is important for improving the final sealing of the root canal (Taylor *et al.*[17], 1997). However, when highly permeabilized, the dentine becomes more vulnerable to reinfection (Michelich *et al.*[15], 1980). Furthermore, when conventional treatment is unsuccessful, periapical surgery may be necessary. Then, the retrograde root canal filling is done to hermetically seal the root canal system against leakage of irritants from the root canal system into periapical tissue (Barkhordar *et al.*[2], 1988).

For many years, amalgam has been accepted as the material of choice for retrofillings in endodontic surgery. However, poor results in leakage as well as concern about corrosion products, and electrochemical reactions have led researchers to

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look for alternative materials (Dorn, Gartner[9], 1990). The suggested materials include cyanoacrylates, Cavit, gold foil, composites, glass ionomers, IRM, Super EBA (Dorn, Gartner[9], 1990) and bone cement (Holt, Dumsha[13], 2000).

Cyanoacrylate has been used in medicine and dentistry for many years (Barkhordar *et al.*[2], 1988). It has been used as a postextraction dressing, a tissue adhesive, and for pulp capping (Bhaskar *et al.*[5,6,7], 1972, 1969, 1966; Bhaskar, Frisch[8], 1968). Moreover, because of its bonding property, cyanoacrylate was evaluated as both root canal sealer (Isaac, Bombana[14], 1999) and a retrograde filling material (Barkhordar *et al.*[2], 1988; Bhaskar *et al.*[5], 1972; Torabinejad *et al.*[18], 1984; Azevedo *et al.*[1], 1996). These studies indicated that cyanoacrylates have potential use in endodontics.

Biocompatibility of root-end filling materials has been the subject of numerous studies in the past (Zhu *et al.*[20], 1999). However, there are only a few studies on the cytotoxic effect of cyanoacrylates using the cell culture technique (Galil *et al.*[11], 1984). With this in mind, and based on the prospect that cyanoacrylate could be used as both a dentine impermeabilizing substance and a retrograde filling material, improving the final root canal sealing, we analyzed the cytotoxic effects of three different commercially available cyanoacrylates in cultured fibroblasts.

MATERIAL AND METHODS

The toxicity of three different commercial presentations of cyanoacrylate was measured *in vitro*. NIH 3T3 cells (CRL 1658) obtained from American Type Culture Collection (Rockville, MD, USA) were grown at 37°C in Dulbecco's Modified Eagle medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA) in a humid 5% CO_2 atmosphere. Experimental groups were as follow: GI: control, cultures that received plain round coverslips, GII: cultures that received round coverslips coated by Super Bonder (Loctite Brasil, São Paulo, SP, Brazil), GIII: cultures that received round coverslips coated by Histoacryl (B. Braun Melsungen AG, Melsungen, Germany), GIV: cultures that received round coverslips coated by Ultrabond (Garin Cia. Ltda., São Paulo, SP, Brazil). Both an immediate or short-term response and a long-term survival that measure the retention of the self-renewal capacity of the cells were analyzed.

Short-term assay (cell viability)

Cells (2×10^4) were plated on 60 mm Petri dishes. Three days later, the experimental cultures received coverslips coated by the different cyanoacrylates, and the control cultures received plain coverslips. After 0, 6, 12 and 24 h the cells were counted and viability curves were made.

Long-term assay (cell survival)

Cells (1×10^4) were plated on 60 mm diameter culture dishes. Treated cultures received coverslips coated by cyanoacrylates. Control cultures received plain coverslips. One, 3, 5 and 7 days after seeding the cells were counted and growth curves were plotted.

Growth and viability cell curves

Growth curves were carried out as described elsewhere (Freshney[10], 2000; Zaccaro Scelza *et al.*[19], 2001). Briefly, cell counts were determined by counting the viable cells in a hemocytometer using the Trypan blue dye exclusion assay. For each time period, three dishes of each group were counted. The number of viable cells harvested from each Petri dish was obtained by the following mathematical equation: UC x D x 10^4/#SQ, where UC = unstained cell count (viable cells), D = the dilution of the cell suspension, and #SQ = number of squares of the hemocytometer counted.

The viability percentage of the cell population of each Petri dish was obtained by the following mathematical equation: UC/TC x 100, where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells).

Statistical analysis

Each data point corresponded to the mean ± SEM (standard error of the mean) of either cell count or percentage of cell viability from 3 dishes. The data were compared by the Kruskal-Wallis test. The level of significance was 5% ($p < 0.05$).

Morphological analysis

The morphology and the distribution of cells were monitored throughout the experimental time. Using phase light microscopy, the relationships between the cells grown in the Petri dishes and the coverslips of all groups were studied. Additionally, the individual morphology of the cells, as well as the presence of both living cells and dead cells was

analyzed. Phase photomicrographs were obtained from a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberköchen, Germany).

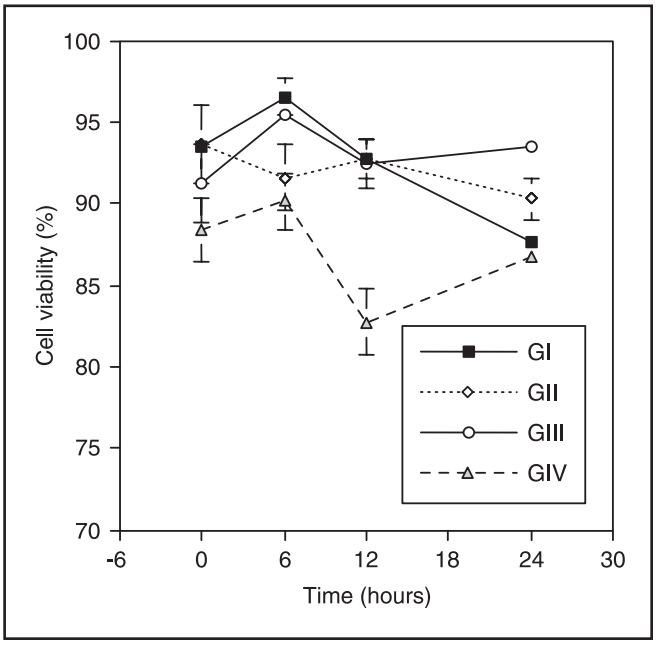
RESULTS

Short-term assay

Throughout the experimental time (0-24 h), controls and treated cultures maintained stable cell viability (87.67% to 96.53%), except for the cultures treated with Ultrabond (GIV) (Graph 1). These cultures presented a viability that ranged from 82.73% to 90.13%, which was significantly smaller than the viability rates of the other groups, especially at 12 h. The other two groups (GII: Super Bonder, GIII: Histoacryl) mostly behaved as the control cultures (GI).

Long-term assay

There was a progressive cell growth in the control cultures (GI) from day 1 to 7 (Graph 2A). Cultures treated with Super Bonder (GII) also had progressive increase in cell numbers. However, this group presented significantly fewer cells than control cultures (GI) throughout the experimental time. Cultures treated with Histoacryl (GIII) and Ultrabond (GIV) presented significantly higher numbers of cells when compared to the control cultures until 3 days after seeding. From this point on, cultures from group III still increased cell numbers until day 5, when a decrease was observed, reaching values significantly smaller than those of control cultures at the end of the experimental time. Cultures from group IV (Ultrabond) presented a progressive decrease in cell numbers after day 3, with values ($p < 7.302$) significantly smaller than those of control cultures (GI).



GRAPH 1 - Viability curves of NIH 3T3 cells in the short-term assay. Note that cultures of Ultrabond group (GIV) present significantly smaller percentages of cell viability than the cultures of the other groups. ($p < 0.05$).

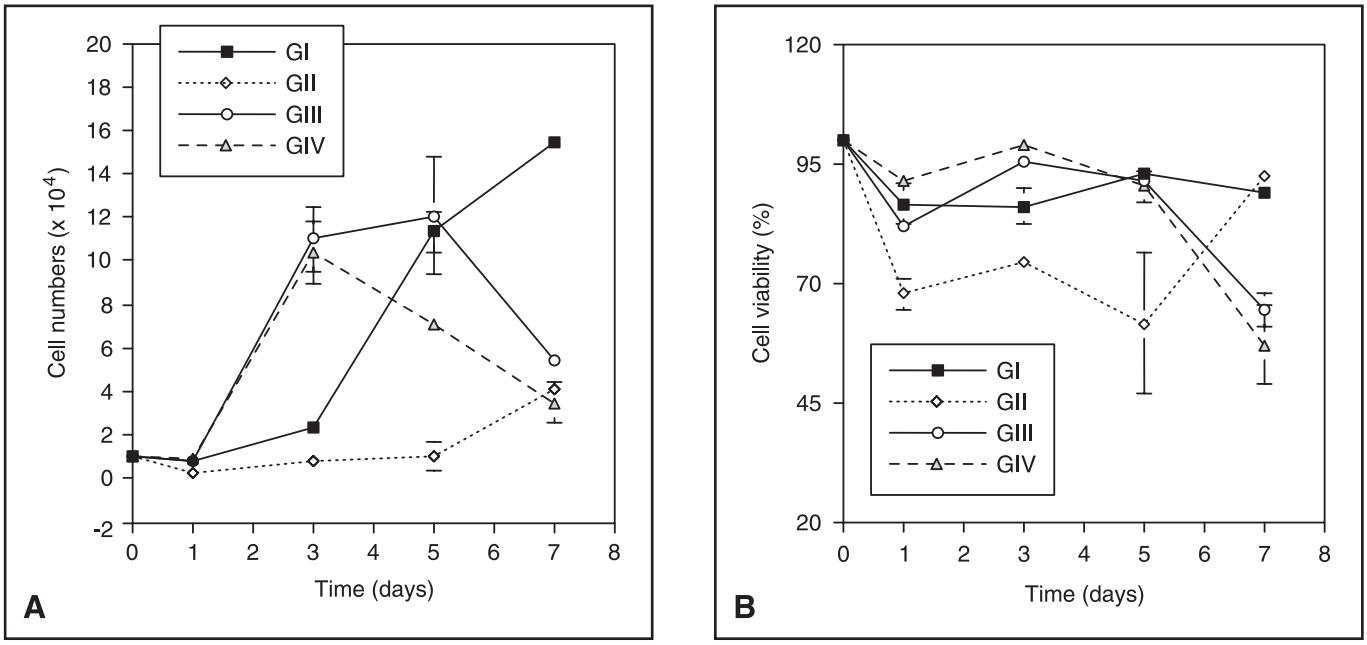
The percentage of cell viability of control cultures (GI) was stable around 95% during all of the experimental time (Graph 2B). Until day 5, cultures of group II (Super Bonder) presented significantly smaller percentages of cell viability ($p < 11.860$) than those of the other groups. Then, there was a recovery of the percentage of cell viability in this group (GII), reaching the viability values of the control cultures at the end of the experimental time. The percentage of cell viability of cultures treated with Histoacryl (GIII) and Ultrabond (GIV) after 5 days in culture dropped at day 7, reaching values of 64.1% and 56.87%, respectively.

Phase microscopy

Representative phase micrographs of NIH-3T3 cells, control and treated with cyanoacrylates, are shown in Figure 1. Control cultures (GI; Figures 1A, B) and cultures treated with Super Bonder (GII; Figures 1C, D) showed the morphological findings of this study. Control cells at day 1 (Figure 1A) were spindle-shaped or stellate, and grew in intimate contact with the plain glass coverslips. Several cells in division were observed at this time. Three days later (Figure 1B) most of the plastic culture surface was covered by fibroblasts. Cultures treated with Super Bonder (GII) also were spindle-shaped or stellate. At day 1 (Figure 1C) there were smaller cell counts in treated cultures (GII) than in controls (GI), but the cells were also in close contact with the coverslips coated by cyanoacrylate. Three days later in GII the cells presented a change in shape, becoming more polygonal and epithelial-like. There was an intimate contact of cells with the cyanoacrylate coated glass coverslips. Clusters of dead cells were present. Throughout the cell monolayers, unattached round cells were observed, resembling cells in division.

DISCUSSION

Measurements of cytotoxicity and viability *in vitro* are specifically designed to assay viability or survival as the major parameter of response to a substance under study, thereby establishing a



GRAPH 2 - Graphs of cell growth and cell viability in the long-term assay. **A:** growth curves of control (GI) and experimental (GII-GIV) cultures. Note that only control cultures (GI) and cultures treated with Super Bonder (GII), although exhibiting a delay in growing, present progressive growth during all experimental times. **B:** viability curves of NIH 3T3 cells. Control cultures (GI) present a stable viability around 95%. Cultures from group II (Super Bonder) present smaller percentages of cell viability than the cultures of the other groups only until day 5. The other two groups (GIII and GIV) present a continuous drop in the percentage of cell viability after day 5 in culture.

cheaper, more reproducible substitute for a test otherwise performed in animals. These assays can be divided into two classes: an immediate or short-term response and a long-term survival usually measured by the retention of the self-renewal capacity of cells (Freshney[10], 2000). Using these assays, we have demonstrated that all cyanoacrylates tested are biocompatible. Ultrabond presented significantly smaller percentages of cell viability than the viability of the cultures of other groups. Although Super Bonder had shown smaller percentages of cell viability than those of the other groups at the long-term assay, this group (GII) was the only experimental group presenting a continuous and progressive cell growth. The cultures treated with Histoacryl (GII) and Ultrabond (GIV) exhibited a diminishing of cell numbers after day 3 in culture.

For testing biomaterials in cell culture, especially solids, it is recommended to apply these materials to the cells as they will be used in future clinical procedures (Spångberg, Pascon[16], 1988). For this reason, we covered glass coverslips with the different cyanoacrylates and then immersed these coated coverslips upside down into the culture dishes, to obtain a direct contact of the testing substances with the cultured fibroblasts. To rule out the effect of cell physical damage caused by the coverslips themselves, we used plain coverslips in the control cultures.

Initial uses of cyanoacrylates for endodontic purposes focused on the pulpal response to these materials. Clinical and histological analyses of exposed pulpal tissue treated with cyanoacrylate spray showed immediate hemostasis, low inflammation and the formation of a dentinal bridge deposited directly on the cyanoacrylate dressing (Bhaskar, Frisch[8], 1968; Berkman *et al.*[4], 1971). Later on, cyanoacrylate was evaluated as both root canal sealer (Torabinejad *et al.*[18], 1984) and a retrograde filling material (Barkhordar *et al.*[2], 1988; Isaac, Bombana[14], 1999; Azevedo *et al.*[1], 1996). These studies indicated that cyanoacrylates have a potential use in endodontics.

The ideal material for the perfect retrofilling should have many properties that allow a hermetic seal and guarantee biocompatibility. Hence, such a material should exhibit minimal leakage, low cytotoxicity, be easily manipulated, allow connective tissue attachment, be bacteriostatic, and tolerate a moist environment (Holt, Dumsha[13], 2000).

Azevedo *et al.*[1] (1996) analyzed by comparison the sealing quality of n-butyl-2-cyanoacrylate (Histoacryl) and of amalgam as retrofilling materi-

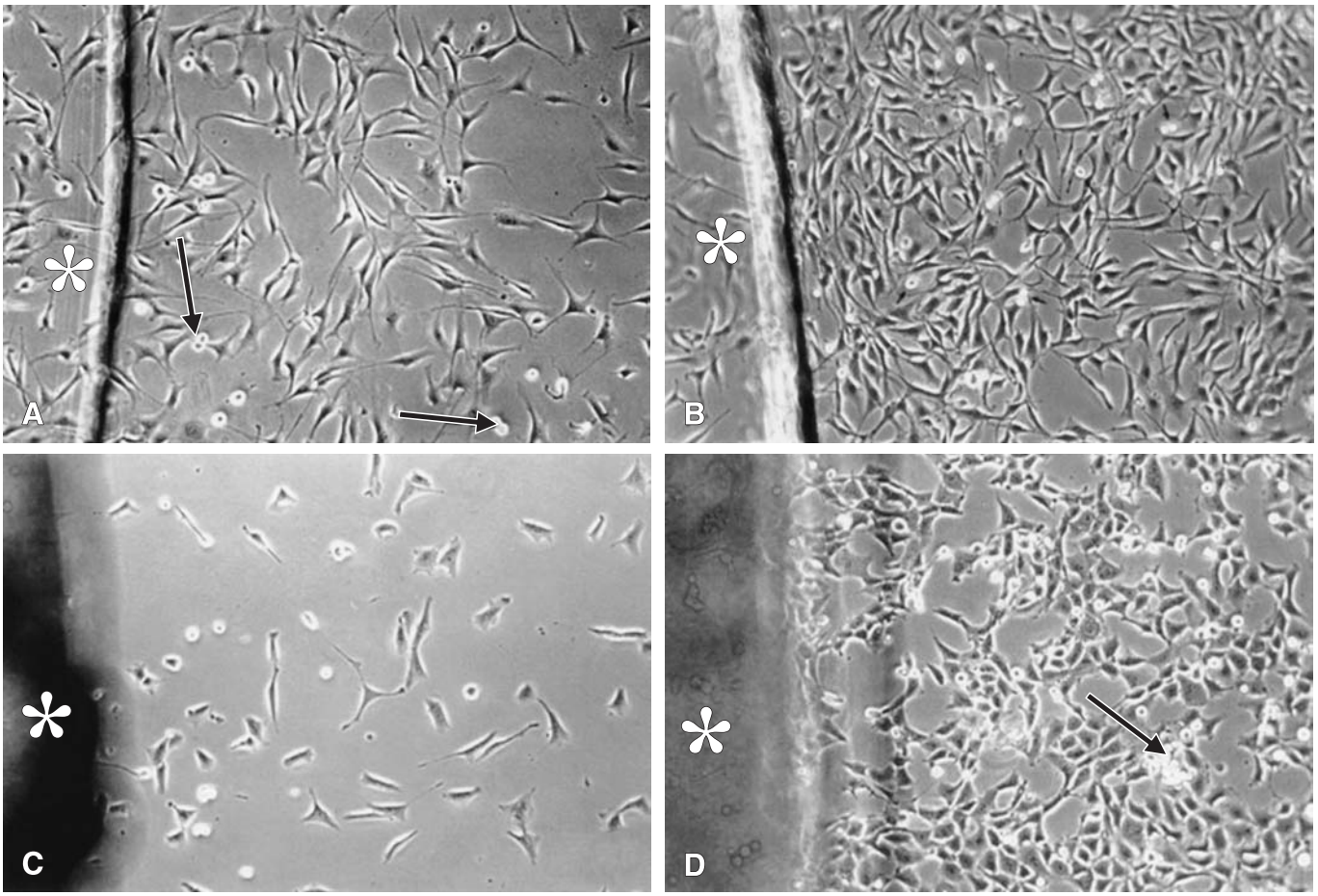


FIGURE 1 - Representative phase micrographs of NIH 3T3 cells of the control group (GI, **A**, **B**) and of those treated with cyanoacrylate (GII, **C**, **D**) show the morphological findings of this study. **A:** Control cells at day 1 grow in intimate contact with the plain glass coverslips (*). Several cells in division are observed at this time (arrows). **B:** Three days later most of the plastic culture surface is covered by fibroblasts. **C:** Cultures treated with Super Bonder (GII) at day 1 present smaller cell numbers than control cultures (GI), but the cells are also in close contact with the coverslips coated by cyanoacrylate (*). **D:** Three days later treated cells present a polygonal and epithelial-like shape. There is an intimate contact of cells with the Super Bonder coated glass coverslips (*). Clusters of dead cells are present (arrow; *). Throughout the cell monolayers, unattached round cells are observed resembling cells in division.

als. Dye penetration, as well as scanning microscopy examination showed that Histoacryl presented better sealing quality than did amalgam. Issac, Bombana[14] (1999) compared the root canal apical sealing obtained by several different filling materials, including an n-butyl-2-cyanoacrylate (Histoacryl). The authors concluded that Histoacryl used either as filling material or as filling material coating presented better sealing quality than did the other materials.

Several studies have been conducted to evaluate the bonding capacity of cyanoacrylates to dentin and enamel (Herod[12], 1990). Beech[3], 1972, found that the methyl, ethyl and isobutyl-2-cyanoacrylate formed strong bonds with dentin, acid treated enamel and two polymeric restorative materials under aqueous conditions. The adhesion to dentin was probably a result of covalent bonds being formed with the organic constituents of dentin.

Studies exploring the biologic receptivity of some cyanoacrylate homologues, such as ethyl, propyl and butyl monomers were conducted by Bhaskar *et al.*[7] (1966). All the homologues produced immediate hemostasis in tongue injuries in rats and a typical cicatrization occurred. In 1966, an examination of the hexyl, heptyl and octyl cyanoacrylates showed that the higher homologue compounds were better tolerated by tissues than the lower homologues (Bhaskar *et al.*[7], 1966). Following these initial studies, research on biocompatibility focused primarily on the butyl

cyanoacrylate homologue with promising results (Herod[12], 1990).

The only cell culture cytotoxicity analysis of cyanoacrylates until now was performed by Galil *et al.*[11], 1984. By applying polymerized cyanoacrylates into Petri dishes containing monolayers of L-929 mouse fibroblasts, these authors showed some evidence of cytotoxicity. Their results suggested that soluble degradation products of cyanoacrylates are responsible for the cytotoxic effect observed. All cyanoacrylates tested in our study left more than 82% of the cells alive within the first 24 h. However, Galil *et al.*[11] (1984) showed total cell death within the first 12 h. This discrepancy of results could be due to the cyanoacrylate formulation that might have been more toxic in the eighties than it is nowadays. On the other hand, our study showed a greater deal of cell death after 5 days in culture in the Ultrabond group (GIV) in the long-term assay. This result is in accordance with the observations of Galil *et al.*[11] (1984) where one type of cyanoacrylate reached its maximum cytotoxicity after 4 days.

The close contact between the fibroblasts and the cyanoacrylate coated coverslips demonstrated some degree of biocompatibility of these substances. Additionally, the presence of dividing cells three days after seeding in the group treated with Super Bonder also indicates the preservation of the self-renewal capacity of these cells. These results together with the clinical findings reported in the literature are encouraging for the use of cyanoacrylates in endodontics.

CONCLUSIONS

Our results have shown an *in vitro* biocompatibility of both butyl and ethyl cyanoacrylate homologues. Thus, these materials could be of importance for endodontic purposes.

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