Cytotoxicity of substances leached from a conventional and a self-etching adhesive system on human pulp fibroblasts

Citotoxicidade de substâncias liberadas de sistemas adesivos convencional e auto-condicionante sobre fibroblastos de polpa humana

Bruno Neves CAVALCANTI
PhD - Department of Dentistry - University of Taubaté – Taubaté - SP - Brazil.

Lorena Ribeiro BÄRSCHNEIDER
Márcia Martins MARQUES
PhD - Department of Restorative Dentistry - School of Dentistry – Universidade de São Paulo - São Paulo – SP - Brazil.

Summary

The use of adhesives for direct dental pulp capping is not advisable, due to its harmful effects to the tissue. However, new adhesive systems are often released, and self-etching systems seem to be less toxic than conventional ones. The purpose of this study was to compare the in vitro cytotoxicity of substances leached from calcium hydroxide and two adhesive systems on human dental pulp fibroblasts. Cell culture media conditioned by Calcium Hydroxide (CH), Single Bond (SB), Clearfill Protect Bond primer (CP) or Clearfill Protect Bond resin (CB) were applied to human pulp fibroblasts. Fresh cell culture medium was used in the Control group. The number of viable cells was obtained through the MTT reduction assay. Data were compared by ANOVA and Tukey's test ($p \leq 0.05$). The mean number of viable cells was 3.9x103(±0.75) for the control group, which was similar to those found in the CH group (4.31x103±0.87). Statistical differences were found among the groups ($p < 0.001$), with the cell viability decreasing significantly with SB (0.09x103±0.06) and CP (0.28x103±0.08) when compared to CH and control groups. CB (2.37x103±0.72) was significantly less cytotoxic than CP and SB, but more cytotoxic than CH. It was concluded that Single Bond and Clearfill Protect Bond primer release substances that decrease cell viability of human dental pulp cells in culture. According to this study the use of bonding systems for direct pulp capping is not recommended, since they are cytotoxic.

Uniterms

Calcium hydroxide; dental pulp capping; dentin bonding agents.

Introduction

Calcium hydroxide has become a widely accepted material for direct pulp capping, due to its biological properties related to its high pH [1-3]. Although it is the standard material for this procedure, some studies have shown its weak mechanical properties, particularly solubility through the time, which could allow bacterial leakage [4,5].

In the last decade, researchers have evaluated dental adhesive systems to be used for direct pulp capping. This is controversial since most of the literature has supported the use of calcium hydroxide given its properties [1-3,6]. This is still a matter of discussion, since studies have demonstrated that adhesive systems can avoid bacterial leakage and seal the cavity [5,7,8]. On the other hand, studies have found that dental adhesives are cytotoxic to cells and harmful to dental pulp tissue [9-11], what seems to be the standard performance of adhesives regarding biocompatibility.

Recently, with the development of some self-etching adhesive systems, a number of papers relating its biological properties have been published. These studies have found lower cytotoxicity and better tissue response in histological evaluations than those related to conventional adhesive systems, but at this time they are still harmful to the pulp [12-17].

To assess the controversy regarding biocompatibility of dental adhesives, the use of cell cultures is indicated, since this method is easier controlled, eliminating the factors related to the subject reaction [15,18-21]. Moreover, the use of cell cultures allows the analysis of the substances leached

from dental adhesives when placed in contact with a humid environment [11,22].

The purpose of this study was to compare the response of cultured human dental pulp fibroblasts to substances leached from a conventional and a self-etching adhesive system. It was hypothesized that, according to the recent literature, a self-etching primer could be less harmful to the pulp tissue than a conventional bonding system.

Materials And Methods

The response of cultured human dental pulp fibroblasts induced by substances leached or dissolved from four dental materials was analyzed by an in vitro cell culture method. For this, five groups were established, as follows: C: Control (fresh cell culture medium); CH: Calcium hydroxide; SB: Single Bond® (3M ESPE, St. Paul, MN, USA); CP: Clearfill Protect Bond® primer (Kuraray, Japan) and CB: Clearfill Protect Bond® resin (Kuraray).

Preparation of the tested materials

Calcium hydroxide was prepared by mixing 1 g of calcium hydroxide [Ca(OH)2] powder in 350 µL of sterile distilled water on a sterile glass plate. The adhesive systems were prepared following the manufacturer's instructions. The adhesive systems were light-cured for 15 seconds with a visible light-curing unit with an output of 400 mW/cm2.

Culture medium conditioning

In order to obtain the conditioned media (e.g. media containing the substances leached or dissolved from the test materials), the Ca(OH)2 as well as the adhesive materials were applied into the bottom of 50 mL centrifuge tubes. These tubes were filled with fresh Eagle modified culture medium (DMEM, Sigma, St. Louis, MO, USA) in a proportion of 0.2 g of materials per mL of culture medium. Then, the adhesives were light-cured allowing dissolution of substances before and during the polymerization procedure. The medium conditioning process was carried out for one hour at 37°C. The stock conditioned media obtained during this step were diluted (10%) and then applied into the cell cultures.

Cell culture

The FP5 cell line, fibroblasts derived from a human third molar, was cultured as previously described [11]. Briefly, the cultured medium was the Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% antimycotic-antibiotic solution (10.000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL in 0.9% sodium chloride; Sigma). The cells were maintained in an incubator at 37ºC and a humidified 5% CO2 atmosphere. Cultures were supplied with fresh medium every other day. Cells between the fifth and tenth passages were used in all experimental procedures.

Experiments

FP5 cells were plated into 96-well microtitration plates (1 x 103 cells/well), in a total of 4 wells per group. After 24 h, when the cells were attached to the bottom of the wells, the culture medium was replaced by the conditioned medium of each group. Then, 24 h later, the conditioned media were replaced by fresh DMEM. The cultures were incubated again at 37ºC and a humidified 5% CO2 atmosphere for other 24 h, when the cell viability was assessed.

Cell viability analysis

The cell viability was determined by the mitochondrial activity analysis. This analysis was carried out using the MTT-based cytotoxicity assay. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan salt. This process will occur only by viable cells. Then, the formazan is solubilized, and the concentration determined by optical density at ≈570 nm. A MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR, USA) was used. Immediately after the end of the assay procedures the absorbance was read in a micro plate reader (Biotrak II, Biochrom Ltd, Eugendorf, Austria) using a 562 nm filter. The absorbance data was transformed into number of viable cells that was used to plot the cell growth curves.

Statistical analysis

All experiments were conducted in triplicate, resulting in 12 samples per group (4 wells x 3 assays). Data were compared by ANOVA complemented by the Tukey's test ($P<0.05$).

Results

Figure 1 illustrates the results of cytotoxicity assay. The mean number of viable cells was 3.9x103(±0.75) for the control group, which was similar to those found in the CH group (4.31x103±0.87). Statistical differences were found among the groups as shown in Table 1 ($p<0.001$), showing a decrease of cell viability significantly with SB (0.09x103±0.06) and CP (0.28x103±0.08) when compared to CH and control groups. CB (2.37x103±0.72) was significantly less cytotoxic than CP and SB, but more cytotoxic than CH. According to the statistical analysis, the average number of cells obtained in the control group (3.9 x 103 ± 0.75) was statistically similar to that found in the calcium hydroxide group (4.31 x 103 ± 0.87). The Clearfill Protect Bond® resin (2.37 x 103 ± 0.72) was more cytotoxic than calcium hydroxide, but less than the Clearfill protect Bond® primer and the Single Bond®, which were similar to each other.

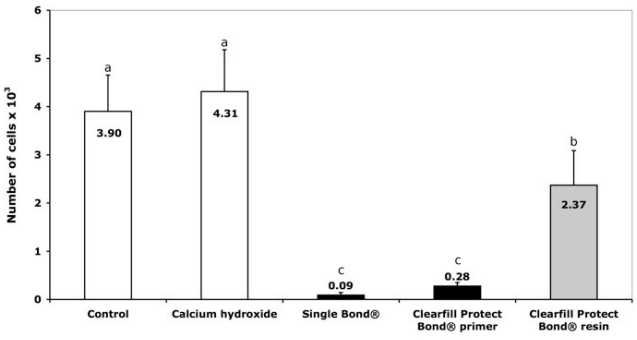

Figure 1 - Graphic representation of the mean number of viable cells after 24 hours in contact with the conditioned media. Statistical differences are presented by different letters.

Table 1 - ANOVA

Source of Variation	SS	df	MS	F	P-value
Between Groups	186.61	4	46.653	126.598	0.000
Within Groups	20.27	55	0.369		
Total	206.88	59			

Tukey's critical value = 0.73

Discussion

The adhesive systems are able to avoid bacterial leakage and seal dental cavities [5,7,8], which are desirable properties for direct pulp capping materials. However, there are adverse effects on the dental pulp elicited by the cytotoxicity of the adhesive systems, which would contraindicate these materials for direct pulp capping. Thus, this is still a matter of discussion, once some authors have shown that new adhesives present less cytotoxicity than the conventional ones [12-17]. For this reason, this study compared the cytotoxicity of different adhesive systems on human dental pulp fibroblasts in culture, using the calcium hydroxide as gold standard, once this material is of choice for direct pulp capping. Although this study showed that the adhesive resin from a self-etching system is less cytotoxic than the conventional adhesive resin, all adhesive systems tested are more cytotoxic than calcium hydroxide.

Cell culture was chosen, since this method allows bias control, eliminating the factors related to the subject reaction and is easier controlled [6,18-20]. Moreover, it can provide information about the cytotoxicity of substances leached or dissolved into the culture media, which can simulate the humid clinical condition generally found when the pulp tissue is exposed [11]. The presence of humidity is particularly important when adhesive systems are used, since part of the monomer present in the composition of these materials cannot be converted to polymer and can cause harmful effects to the cells and consequently to the tissue [10].

As expected, substances leached from calcium hydroxide showed low cytotoxicity, allowing cell growth similar to that of control cultures that were maintained in the best culture conditions for this type of cells. This result is consonant with the literature, where several authors have demonstrated that this material, both in cement and dry powder forms, is biocompatible [3,6,11,15,19]. On the other hand, adhesive systems were cytotoxic. There are controversies in the literature about the use of this kind of material in direct contact with the exposed pulp. Some authors affirm that this adhesive material causes no harm to the tissue [5,7,8,19,23]. Nonetheless, many other studies showed the negative effects of the pulp capping with adhesive systems, either in the cell culture or in vivo studies [3,9-11,13,19].

With the development of the self-etching adhesive systems and the new studies regarding their biocompatibility, promising results were found, particularly when these systems were compared with conventional ones [12-17]. In this study, the self-etching system was partially biocompatible, since the

primer was cytotoxic. On the other hand, the adhesive resin was positioned in an intermediate level of biocompatibility, information also found in literature [12].

The results observed in this study cannot support the immediate clinical use of the systems in exposed pulp tissues. Thus, complementary studies at in vitro and in vivo levels should be done in order to provide complete information on the use of these systems and the ones that are constantly developed.

Conclusions

Under the conditions of this study, it was concluded that Single Bond and Clearfill Protect Bond primer release substances that decrease cell viability of human dental pulp cells in culture. The Clearfill Protect Bond resin is less cytotoxic than its primer, but more cytotoxic than calcium hydroxide. The use of bonding systems for direct pulp capping is not recommended, since they are usually cytotoxic.

Resumo

O uso de adesivos dentinários sobre a polpa não é recomendável, devido aos efeitos deletérios provocados sobre o tecido. Entretanto, novos sistemas adesivos são frequentemente lançados, e sistemas autocondicionantes parecem ser menos tóxicos que os sistemas convencionais. O objetivo deste estudo foi comparar in vitro, a citotoxicidade de substâncias liberadas pelo hidróxido de cálcio e dois sistemas adesivos utilizando fibroblastos de polpa humana. Meios de cultura celular condicionados por Hidróxido de cálcio (CH), Single Bond (SB), Clearfill Protect Bond primer (CP) ou Clearfill Protect Bond resin (CB) foram testados em fibroblastos de polpa humana. Meio fresco de cultivo celular foi utilizado no grupo centrole. O número de células viáveis foi obtido através do teste da redução do MTT. Os dados foram comparados por ANOVA e teste de Tukey ($p \leq 0,05$). O número médio de células viáveis foi de 3,9x103(±0,75) para o grupo controle, que foi similar ao encontrado para o grupo CH (4,31x103±0,87). Houve differences estatísticas entre os grupos ($p<0.001$), sendo que a viabilidade celular decresceu significantemente com SB (0,09x103±0,06) e CP (0,28x103±0,08), quando comparados ao CH e controle. CB (2,37x103±0,72) foi menos citotóxico que CP e SB, mas mais citotóxico que CH. Conclui-se que o Single Bond e o Clearfill Protect Bond primer liberam substâncias que diminuem a viabilidade celular de fibroblastos humanos em cultura. De acordo com este estudo, o uso de sistemas adesivos para capeamento direto não é recomendado, uma vez que são citotóxicos.

Unitermos

Adesivos dentinários; capeamento da polpa dental; hidróxido de cálcio.

References

1. Stanley HR, Pameijer CH. Dentistry's friend: calcium hydroxide. Oper Dent. 1997;22(1):1-3.
2. Gwinnett AJ, Tay FR. Early and intermediate time response of the dental pulp to an acid etch technique in vivo. Am J Dent. 1998;11:S35-44.
3. Hebling J, Giro EM, Costa CA. Biocompatibility of an adhesive system applied to exposed human dental pulp. J Endod. 1999;25(10):676-82.
4. Tam LE, Pulver E, McComb D, Smith DC. Physical properties of calcium hydroxide and glass-ionomer base and lining materials. Dent Mater. 1989;5(3):145-9.
5. Cox CF, Suzuki S. Re-evaluating pulp protection: calcium hydroxide liners vs. cohesive hybridization. J Am Dent Assoc. 1994;125(7):823-31.
6. Huang FM, Chang YC. Cytotoxicity of dentin bonding agents on human pulp cells in vitro. Int Endod J. 2002;35(11):905-9.
7. Cox CF, Hafez AA, Akimoto N, Otsuki M, Mills JC. Biological basis for clinical success: pulp protection and the tooth-restoration interface. Pract Periodontics Aesthet Dent. 1999;11(7):819-26.
8. Akimoto N, Momoi Y, Kohno A, Suzuki S, Otsuki M, Suzuki S, et al. Biocompatibility of Clearfil Liner Bond 2 and Clearfil AP-X system on nonexposed and exposed primate teeth. Quintessence Int. 1998;29(3):177-88.
9. Trope M, McDougal R, Levin L, May KN Jr, Swift EJ Jr. Capping the inflamed pulp under different clinical conditions. J Esthet Restor Dent. 2002;14(6):349-57.

10. Mantellini MG, Botero TM, Yaman P, Dennison JB, Hanks CT, Nör JE. Adhesive resin induces apoptosis and cell-cycle arrest of pulp cells. J Dent Res. 2003;82(8):592-6.
11. Cavalcanti BN, Rode SM, Marques MM. Cytotoxicity of substances leached or dissolved from pulp capping materials. Int Endod J. 2005;38(8):505-9.
12. Tarim B, Hafez AA, Suzuki SH, Suzuki S, Cox CF. Biocompatibility of Optibond and XR-Bond adhesive systems in nonhuman primate teeth. Int J Periodontics Restorative Dent. 1998;18(1):86-99.
13. de Souza Costa CA, Lopes do Nascimento AB, Teixeira HM, Fontana UF. Response of human pulps capped with a self-etching adhesive system. Dent Mater. 2001;17(3):230-40.
14. Medina VO 3rd, Shinkai K, Shirono M, Tanaka N, Katoh Y. Histopathologic study on pulp response to single-bottle and self-etching adhesive systems. Oper Dent. 2002;27(4):330-42.
15. Vajrabhaya L, Pasasuk A, Harnirattisai C. Cytotoxicity evaluation of single component dentin bonding agents. Oper Dent. 2003;28(4):440-4.
16. Kitasako Y, Shibata S, Tagami J. Migration and particle clearance from hard-setting Ca(OH)2 and self-etching adhesive resin following direct pulp capping. Am J Dent. 2006;19(6):370-5.
17. Koulaouzidou EA, Helvatjoglu-Antoniades M, Palaghias G, Karanika-Kouma A, Antoniades D. Cytotoxicity of dental adhesives in vitro. Eur J Dent. 2009;3(1):3-9.
18. Schmalz G. Use of cell cultures for toxicity testing of dental materials – advantages and limitations. J Dent. 1994;22:S6-11.
19. Demarco FF, Tarquinio SB, Jaeger MM, de Araújo VC, Matson E. Pulp response and cytotoxicity evaluation of 2 dentin bonding agents. Quintessence Int. 2001;32(3):211-20.
20. Schmalz G, Schuster U, Koch A, Schweikl H. Cytotoxicity of low pH dentin-bonding agents in a dentin barrier test in vitro. J Endod. 2002;28(3):188-92.
21. Freshney RI. Culture of animal cells: a manual of basic technique. 5th ed. Indianapolis: Wiley-Liss; 2005.
22. Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Périanin A. TEGMA-induced citotoxicity in human fibroblasts is asoosciated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J Biomed Mat Res A. 2003;66(3):476–82.
23. Nayyar S, Tewari S, Arora B. Comparison of human pulp response to total-etch and self-etch bonding agents. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007;104(2):e45-52.

Recebido: 10/03/2011
Aceito: 18/04/2011

Corresponding author:
Bruno Neves Cavalcanti
Departamento de Odontologia - UNITAU
Rua dos Expedicionários, 09 - Centro
Taubaté, SP
12020-330
Brazil
E-mail: bncavalcanti@uol.com.br