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LETTER TO THE EDITOR

EXPRESSION OF PROCOLLAGEN A1 TYPE I INDUCED BY TWO DIFFERENT DENTINE BONDING SYSTEMS IN HUMAN PULP FIBROBLASTS

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This study aimed to evaluate the effects of two different dentine bonding systems (DBSs) on primary cultures of human pulp fibroblasts (HPFs). Cell viability and procollagen α1 type I expression were investigated. Polymerised resin disks of the bonding agent from a two-step self-etch system and of the primer/bonding agent from a two-step etch-and-rinse system were used to condition culture medium for 24 or 96 h. HPFs were incubated in control (untreated) or DBSs-conditioned medium for 24 h. HPF viability was determined using the 3-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. Western blot analysis was used to analyse procollagen α1 type I expression. Statistical analyses were performed using Student's *t*-tests. The results showed that HPFs incubated with DBSs-conditioned medium for 24 h demonstrated a significant reduction in the percentage of viable cells versus cells incubated with control medium (45% for self-etch DBS and 30% for etch-and-rinse DBS; $p < 0.05$), whereas this percentage increased significantly after exposure to the 96h DBSs-conditioned medium (62% and 77%, respectively; $p < 0.05$). Procollagen α1 type I expression in HPFs was strong for control specimens, but decreased in 24 h-DBSs-conditioned medium, and was abolished with 96 h-DBSs-conditioned medium. In conclusion, HPF exposure to medium containing eluates of the different DBSs led to an early cytotoxic effect (24 h) that decreased after a conditioning time of 96 h, whereas procollagen α1 type I expression decreased at 24 h and was absent after 96 h. Procollagen α1 type I expression may be a useful parameter for evaluating DBSs biocompatibility.

Dental pulp is a loose connective tissue, composed of fibroblasts (the most abundant cell population), odontoblasts, and undifferentiated mesenchymal cells that are in contact with a complex chain of macromolecules secreted in the extracellular matrix (ECM) (1). The pulp ECM is predominately composed of types I and III collagen fibrils (56% and 41%, respectively), in contrast to the predominantly type I collagen of dentine (2, 3). As a secretory protein, fibrous collagen is synthesized as procollagen and subsequently modified and assembled into the proper collagen triple helix (4). *In vitro*, fibroblasts produce

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procollagen α1 type I in large amounts, and changes in procollagen α1 type I protein expression were reported to be related to cytotoxicity (5). Most dental restorative procedures use resin-based materials associated with dentine bonding systems (DBSs). Several studies have reported that during DBS application to the dentine substrate, monomers can be released and reach the dental pulp through the dentinal tubules, causing adverse effects, such as dental pulp inflammation (6). *In vitro*, cytotoxicity of the resin monomers contained in DBSs has been evaluated previously using cell lines and/or primary fibroblasts obtained from pulp, gingiva, and periodontal ligaments (7-12). Reduced synthesis to inhibition of procollagen α1 type I synthesis has been demonstrated when human pulp fibroblasts (HPFs) were exposed to resinous monomers, such as 2-hydroxyethyl methacrylate (HEMA) (8). However, the direct effects of commercially available DBSs on procollagen α1 type I expression has not been reported previously. Depletion of procollagen1 α1 type I synthesis could, in fact, impair HPF function, thus reducing their capacity to positively react to pulp injuries and differentiate into odontoblastic cells (13). Thus, the aim of the present study was to evaluate the possible toxic effects of commercially available DBSs on HPFs. The null hypotheses tested were that DBSs and the exposure time of DBSs in the culture medium would not affect i) HPF viability nor ii) procollagen α1 type I synthesis.

MATERIALS AND METHODS

Establishment of human pulp fibroblast culture

HPFs were isolated from third molars of healthy volunteers during routine oral surgery. Informed consent was obtained under a protocol approved by the Università Politecnica delle Marche (Ancona, Italy). Immediately after extraction, the central part of the dental papilla was cut into small pieces, washed in phosphate buffered saline (PBS), and incubated in Dulbecco's modified Eagle's medium (DMEM)/F12, containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, and 1% fungizone. Monolayer cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Confluent HPFs were then detached with 0.25% trypsin in 1 mM EDTA (Sigma-Aldrich). For cytotoxicity tests, cells were used between the 3rd and 5th passage of culture.

DBSs preparation

Polymerised resin disks were obtained using i) the bonding agent of a self-etch DBS (SE-DBS, Techbond, Isasan, Rovello Porro, Italy; $n = 12$); and (ii) the primer agent of a two-step etch-and-rinse DBS (ER-DBS, Optibond Solo, Sybron-Kerr, Orange, CA; $n = 12$). The liquid co-monomer mixtures were placed in silicone moulds 2 mm deep and 6 mm in diameter. The moulds were placed on a Mylar film-covered glass slide covered with another Mylar film glass slide, and light-cured on each side for 40 s using a quartz-tungsten-halogen unit at 600 mW/cm^2. The disks were removed from the moulds and dry-polished to a thickness of 1 mm (14).

Cell treatment

For each experiment, 3.5×10^4 cell/cm^2 HPFs were incubated for 24 h with DMEM/F12 medium conditioned as follows: group 1, DMEM/F12 maintained for 24 h (control 1); group 2, DMEM/F12 maintained for 96 h (control 2); group 3, three polymerised disks of SE (self-etch)-DBS immersed in culture medium for 24 h; group 4, three polymerised disks of ER (etch-and-rinse)-DBS immersed in the culture medium for 24 h; group 5, three polymerised disks of SE-DBS immersed in the culture medium for 96 h; and group 6, three polymerised disks of ER-DBS immersed in the culture medium for 96 h. All experiments were performed in triplicate.

MTT assay

The effects of the two DBSs on HPF viability were determined using the 3-dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT) assay. After incubation for 24 h with the control or conditioned medium, HPFs were analysed. The media were removed, and 200 μL MTT solution (Sigma-Aldrich, Milan, Italy; 5 mg/mL in DMEM without phenol red) and 1.8 mL medium were added to the cell monolayers. After incubation at 37°C for 4 h, the growth medium was removed, and 2 mL of solvent (1N HCl diluted to 4% in absolute isopropyl alcohol; Sigma) was added to each well to dissolve the purple crystals of formazan that had formed. Spectrophotometric quantification was then performed at 570 nm (Secomam, Anthelie light, version 3.8, Contardi, Italy). Results are expressed as the percentage absorbance over the control culture value. Experiments were run in triplicate and statistical analysis was performed using a Student's *t*-test.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

HPFs exposed to the control or DBSs-conditioned culture media were lysed in modified radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris-HCl, pH = 7.4, 1% Nonidet-P40, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.1% sodium dodecylsulfate,

1 mmol/L ethylene-glycol-tetraacetic acid, 1 mmol/L phenylmethane-sulfonylfluoride, 0.15% beta-mercaptoethanol) supplemented with 25 mmol/L protease inhibitor cocktail (Sigma-Aldrich). Total proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane using a wet blotting apparatus (Mini Tank Electroblotting System, Owl, Portsmouth, UK). Membranes were blocked with 2.5% dry milk (Bebilac, Sicura, France) in TBS-Tween buffer (Tween 20 0.1%, NaCl 0.15 mol/L, Tris-Base 0.01 mol/L, pH 7.5) for 1 h at room temperature and were labelled with goat anti-human procollagen α1 type I primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:5000 for 2 h at 37°C, and anti-β tubulin antibody (Sigma Aldrich, Saint Luis, Missouri) diluted 1:10000, for 2 h at 37°C as positive loading control.

After three washes in TBS-Tween buffer, the membranes were labelled with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Inc. for procollagen α1 type I, and Sigma Aldrich, Saint Luis, Missouri, for β-tubulin), diluted 1:80,000, for 90 min at 37°C. Positive bands were detected using the Enhanced Chemiluminescence System (ECL plus, Amersham Biosciences, Little Chalfont, UK). Images were obtained using an Image Station 2000R (Kodak, NY, USA).

RESULTS

Human pulp fibroblast culture

A primary culture of HPFs was established from the core of the dental pulp from the extracted healthy molars. Cells appeared near the explants after 7 days of culture (Fig. 1A). After 14 days, the adherent cells began to divide rapidly, becoming a confluent and morphologically homogeneous cell population within the next 7 days (Fig. 1B).

MTT assay

The MTT test showed a significant ($p<0.05$) inhibition of cell viability following treatment with the culture media conditioned for 24 h by polymerised disks of SE-DBS (group 3) and ER-DBS (group 4), with percentages of viable cells of 45% and 30%, respectively (significantly lower than control cells; Fig. 2). Treatment of HPFs with the culture media conditioned for 96 h with polymerised disks of SE-DBS (group 5) and ER-DBS (group 6) showed a significant decrease in inhibitory effects, with the percentage of viable cells being 62% and 77%, respectively (Fig. 2).

SDS-PAGE and Western blot

Western-blot analysis showed intense expression of procollagen α1 type I, demonstrated by a strong band at approximately 250 kDa for untreated control specimens at 24 and 96 h (groups 1 and 2; Fig. 3A, lanes 1 and 2), whereas faint bands at approximately 250 kDa were seen with specimens exposed to both DBSs for 24 h, corresponding to decreased synthesis of procollagen α1 type I in groups 3 and 4 (Fig. 3A, lanes 3 and 4, respectively). After 96 h of incubation, the DBS-treated specimens showed no expression of procollagen α1 type I, regardless of the DBS used (groups 5 and 6; Fig. 3A, lanes 5 and 6).

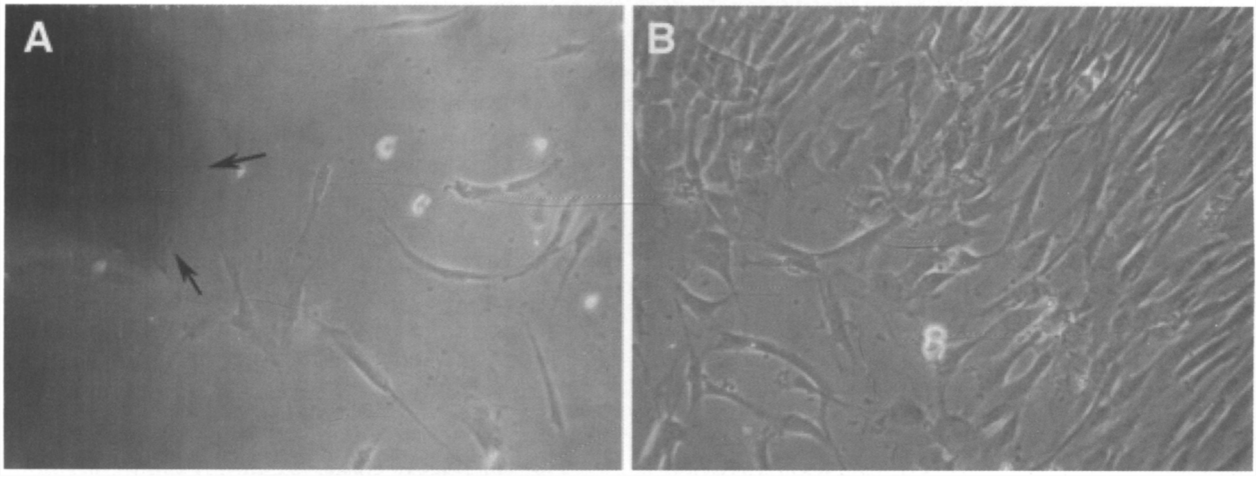

Fig. 1. *Phase contrast microscopy images of the HPF culture:* ***A)*** *after 7 days of culture, there were early HPFs near the explant (arrows);* ***B)*** *after 21 days, the cell monolayer became confluent and homogeneous, with fibroblastoid morphology.*

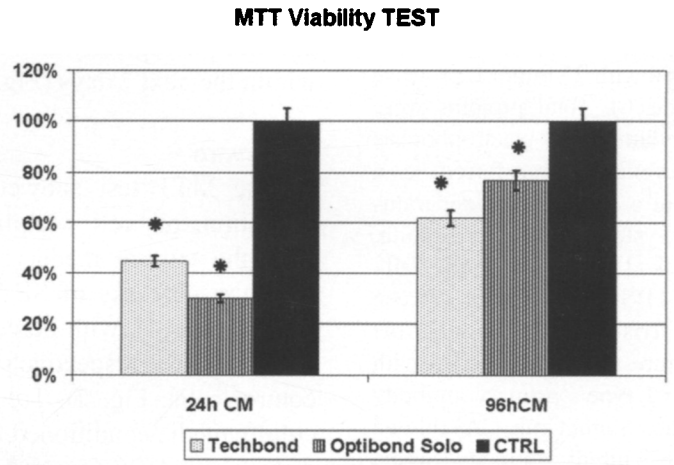

Fig. 2. *Viability of HPFs after incubation with control culture medium and culture media conditioned by polymerised disks of the two tested DBSs (Techbond or Optibond Solo) for 24 h or 96 h. Values are reported as relative percentages (y axis) measured in cells incubated with control medium (means ± standard errors, from three experiments, each performed in triplicate). Asterisks indicate significant differences ($p < 0.05$) versus cells incubated with control medium (CTRL).*

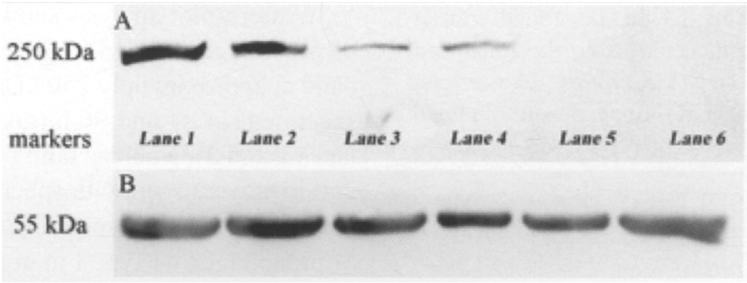

Fig. 3. *Western blot analysis of HPFs extracts after incubation with control and DBS conditioned media. **A)** Expression of procollagen α1 type I. Lanes 1-2: procollagen α1 type I expression from HPFs incubated for 24 h in DMEM/F12 previously maintained for 24 (control 1 - Lane 1) or 96 h in the incubator (control 2 – Lane 2): a strong band at approximately 250 kDa was detected in control 1 and 2. Lanes 3 and 4: procollagen α1 type I expression from HPFs incubated for 24 h with medium previously conditioned with the addition of polymerised disks of Techbond (Lane 3) or Optibond Solo (Lane 4) for 24 h showing a strong reduction in the protein signal, which can be visualized as a faint band at 250 kDa. These results were similar for both adhesives. Lanes 5-6: procollagen α1 type I expression from HPFs incubated for 24 h with media previously conditioned by adding polymerised disks of Techbond (Lane 5) or Optibond Solo (Lane 6) for 96 h revealing no band for either treatment. **B)** Western blot analysis of β-tubulin (loading control) in HPFs after incubation with control and DBSs conditioned media. β-tubulin expression confirmed similar protein loading in all lanes.*

β-tubulin expression confirmed similar protein loading in all lanes (Fig. 3B).

DISCUSSION

The present study showed that HPFs treated with both tested DBSs (i.e., two-step self-etch and two-step etch-and-rinse) resulted in decreased HPF viability, which was more pronounced after 24 h than after 96 h, and decreased procollagen α1 type I expression, that was then abolished after exposure to the 96-h conditioned culture media. Thus, the null hypotheses tested were rejected because both DBSs tested and the different conditioning times affected i) HPFs viability and ii) procollagen α1 type I expression.

The MTT assay showed a significant reduction in cell viability after treatment with DBS-conditioned media, which was higher with 24-h-conditioned

medium than with the 96-h-conditioned medium. This could be due to a possible reduction in monomers eluting over time in addition to their potential neutralization by the culture medium, suggesting that HPF alteration after exposure to the two tested DBSs may be not irreversible.

Of the two categories of DBSs tested in the present study [i.e., self-etch (SE-DBS) or etch-and-rinse (ER-DBS)], the SE two-step product showed a lower cytotoxic effect at 24 h than the simplified two-step ER product. In contrast, both DBSs induced increased cell viability at 96 h, with the ER-DBS being less cytotoxic than the SE-DBS. These findings are consistent with those of Breschi et al., who showed that simplified DBS, such as the two-step ER-DBS, exhibited a lower extent of polymerization as compared with the multi-step SE approach (15). The higher percentage of hydrophilic monomers and the presence of water in the two-step ER-DBSs may compromise their polymerization, also leading to higher permeability (15). Expression of procollagen α1 type I protein was decreased in HPFs incubated with both DBS-24-h-conditioned media versus cells cultured with control media. Procollagen α1 type I protein expression became undetectable when HPFs were incubated with the 96-h conditioned media of both DBSs. These data are consistent with previous studies that investigated the effects of different concentrations and times of exposure to HEMA on procollagen α1 type 1 expression, showing a decrease in the protein with an increase in exposure time (8-10).

Overall, the results of the present study support the hypothesis that monomer eluates of DBSs do not significantly affect HPF viability and metabolism, because recovered cellular viability was seen after 96 h; however, leaching monomers may markedly decrease the synthesis of matrix extracellular proteins, such as procollagen α1 type I.

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