An in Vitro Biocompatibility Study of Conventional and Resin-modified Glass Ionomer Cements

Ismael A. Rodriguez[a] / Carlos A. Rozas Ferrara[b] / Fernando Campos-Sanchez[c] / Miguel Alaminos[d] / Jorge Uribe Echevarría[e] / Antonio Campos[f]

Purpose: To evaluate the biocompatibility of a glass-ionomer (GIC) and a resin-modified glass-ionomer cement (RM-GIC), cell viability was examined in a model of human gingival fibroblasts using morphological, biochemical, and ionic patterns by means of phase contrast microscopy, lactate dehydrogenase (LDH) release, and quantitative x-ray microanalysis (EPXMA).

Materials and Methods: The GIC Ketac-Molar Easymix (3M ESPE) and the RM-GIC Vitrebond (3M-ESPE) were compared in human gingival fibroblasts exposed to the cements for 72 h. As controls, fibroblasts cultured with DMEM culture medium (negative control) and with 1% triton × (positive control) were used.

Results: Light microscopic findings showed greater morphological alterations in cells exposed to RM-GIC than to GIC. The relative percentage of LDH released from the cells to the supernatant was significantly higher in RM-GIC cultures than in the control. Quantitative x-ray microanalysis showed that cultures exposed to RM-GIC were characterized by an increase in intracellular Na and a decrease in intracellular Cl and K. These changes in ion composition were significant compared to control and GIC cultures.

Conclusion: The three indicators of cellular biocompatibility after 72 h of exposure showed that RM-GIC led to more marked alterations than GIC in human gingival fibroblasts.

Keywords: glass-ionomer cements, biocompatibility, intracellular, electron probe microanalysis, human gingival fibroblast, LDH.

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The ability of glass-ionomer cements to adhere to dental substrates, specifically dentin, has made them a material of choice when the pulp-dentin complex needs to be protected.[19] As a biomaterial, glass ionomers adhere to dental structures via chemical bonds between carboxylic groups and the calcium of hydroxyapatite in dentin and enamel. Because they are polar molecules, they are also able to exchange ions with the dental structure. Thus, enamel incorporates fluoride ions from the biomaterial, remineralizing its structure and therefore helping to prevent caries.[30] However, despite these advantages, several authors have noted that glass-ionomer cements may be cytotoxic in vitro, and that their cytotoxicity varies depending on whether conventional glass-ionomer (GIC) or resin-modified glass-ionomer cements (RM-GIC) are used.[9,15,18,28]

The results obtained by different authors with different protocols to investigate the cytotoxic action of GIC and RM-GIC have failed to clarify the mechanism of cell death that is induced by these materials, especially if these materials are evaluated at low concentrations. One technique shown to be efficacious for the analysis of cell death mechanisms is electron probe x-ray microanalysis (EPXMA), the results of which have been identified by different authors as highly specific for normal cells, apoptotic cells, or necrotic cells.[2] This cytochemical technique makes it possible to study the microscopic changes and ion composition simultaneously.[13] Although this method has been used to investigate the biocompatibility of resin monomers such as 2-hydroxyethylmethacrylate (HEMA),[23,24] it has not been used to

[a] *Professor, Department of Histology, School of Dentistry, National University of Cordoba, Cordoba, Argentina. Conceived, designed, and performed the experiments, analyzed data, contributed reagents/materials/analysis tools, wrote the paper.*

[b] *Professor, Department of Operative Dentistry, School of Dentistry, National University of Cordoba, Cordoba, Argentina. Performed experiments, contributed reagents/materials/analysis tools, wrote the paper.*

[c] *Undergraduate Student, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain. Performed experiments.*

[d] *Professor, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain. Conceived and designed the experiments, analyzed data, contributed reagents/materials/analysis tools, wrote the paper.*

[e] *Professor, Secretary of Science and Technologic, School of Dentistry, National University of Cordoba, Cordoba, Argentina. Conceived and designed the experiments, contributed reagents/materials/analysis tools.*

[f] *Professor, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain. Conceived and designed the experiments, analyzed data, wrote the paper.*

Correspondence: *Prof. Ismael A. Rodriguez, Department of Histology, National University of Cordoba, Avenida Haya de la Torre S/N, Ala Izquierda, Pabellón Argentina. C.P. 5000, Ciudad Universitaria, Córdoba, Argentina. Tel: +54-3582-48-1735, Fax: +34-958-24-4034. e-mail: ismaelrodriguez18@hotmail.com*

evaluate glass-ionomer cements. The present study was designed to analyze GIC and RM-GIC biocompatibility in an in vitro experimental model in human gingival fibroblasts. Cell viability was determined using morphological, biochemical, and EPXMA criteria. We anticipated that the results would shed light on the possible mechanisms of cell death induced by these biomaterials. As an apoptotic response elicits less inflammatory response in the surrounding tissues than does a necrotic process, elucidating the cell death mechanism that is associated with each material could be scientifically and clinically important for the evaluation of the biocompatibility of dental materials.[5]

MATERIALS AND METHODS

Cell Culture and Treatment

Primary cell cultures of gingival fibroblasts were generated from 3 small biopsies corresponding to normal human oral mucosa obtained from different healthy donors at the School of Dental Sciences, University of Granada. All donors agreed to participate in the study and informed consent was obtained from each donor. After enzymatic digestion of the samples using collagenase I, fibroblasts were collected by centrifugation and expanded in culture flasks containing DMEM medium rich in glucose (Sigma-Aldrich; St. Louis, MO, USA) and supplemented with antibiotics/antimycotics (100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) and 10% fetal bovine serum (FBS) (Sigma-Aldrich).

In this study, 20,000 human oral fibroblasts per well corresponding to the third cell passage were cultured in 24-well plates with 500 µl of culture medium supplemented with 10% FBS and antibiotics/antimycotics. The cells were incubated at 37°C in 5% carbon dioxide for 24 h. They were then washed twice in phosphate-buffered saline (PBS) and incubated in DMEM (supplemented with glutamine) without phenol red or antibiotics. The biomaterials Ketac-Molar Easymix (GIC) (3M ESPE; St Paul, MN, USA) and Vitrebond (RM-GIC) (3M ESPE) were hand mixed according to the manufacturer's recommendations and inserted into silicone molds with cylindrical apertures (1 mm thick, 2 mm in diameter) to manufacture samples of each cement. RM-GIC samples were light activated with a halogen light-curing unit (Coltolux LED, Coltène/Whaledent; Altstätten, Switzerland) for 40 s. All biomaterials were completely set in a humidified incubator at 37°C with 5% CO_2. In the case of GIC samples (Ketac Molar), a setting time of 8 min was employed. Then, the biomaterials were extracted from the molds and placed on the membrane of Transwell culture inserts with a 0.4-µm-pore polyester membrane (Costar, Corning; Corning, NY, USA). These inserts were set on each well containing the cultured cells. Subsequently, 1500 µl of culture medium were added to completely cover the cells cultured at the bottom of the well and the biomaterial on the porous membrane of the insert. The pH of all culture media was 7.4. By using this system, particles of the biomaterial could freely flow through the membrane and make contact with the cells. The area of each set material was 12.56 mm^2, with a weight of 3.58 mg and an estimated HEMA content of 0.31 mg per RM-GIC cylinder.[18] Cells in contact with the different biomaterials were incubated at 37°C in 5% carbon dioxide for 72 h.

As negative controls (no toxicity), cells were incubated under the same conditions without biomaterials. As positive controls (100% toxicity), cells were incubated in the same medium with the addition of 1% (vol/vol) Triton X-100 (Sigma-Aldrich). For each one of the 3 cell cultures, 6 independent experiments were carried out, meaning that 18 individual experiments were performed per method and condition (controls or ionomers). The results of each of these individual experiments were then analyzed in triplicate (3 different measures of the same experiment).

Phase Contrast Microscopy

To analyze possible morphological alterations in human gingival fibroblasts after exposure to different glass-ionomer cements, cells were examined in well plates with light microscopy (Nikon Eclipse Ti-U; Tokyo, Japan) supported by NIS-Elements imaging software (Nikon), and the morphology of the cells was characterized as normal (spindle shaped) or altered (rounded) in each group. The percentage of normal and altered cells was then calculated for each experimental group. Means, standard deviations, and median values were calculated for each group.

Lactate Dehydrogenase (LDH) Release Assay

To quantify the amount of intracellular lactate dehydrogenase (LDH) released into the culture medium, a commercial immunological Cytotoxicity Detection Kit (Roche Applied Science; Mannheim, Germany) was used. Briefly, 100 µl of the cell culture supernatant was added to 100 µl of the detection kit solution, and the presence of the cytoplasmic enzyme was detected by colorimetric assay using a ELX-800 plate reader (Biotek; Winooski, VT, USA). To determine the percentage of LDH release referred to the control samples, the following formula was used: percentage release = 100 × (A_{bs}-CM)/(CT-CM), where A_{bs} is the reading under each specific condition, CM is the reading for negative controls, and CT is the reading for positive controls.

Quantitative Electron-probe X-ray Microanalysis (EPXMA)

For EPXMA, subconfluent human gingival fibroblast cells were subcultured using trypsin-EDTA on plated gold grids covered with a thin layer of Pioloform (polyvinyl butyral) (Ref.G100 – G3) (Ted Pella; Redding, CA, USA) and sterilized overnight under UV light before cell culture.

Cells were seeded at a density of 20,000 cells per grid and cultured in DMEM supplemented with 10% FBS and antibiotics. After 24 h of culture, support grids containing the human gingival fibroblast cells were left in contact with RM-GIC or GIC for 72 h using the same protocol employed for cell treatment (see above).[23] After 72 h, cells were washed in ice-cold distilled water for 5 s to remove the extracellular medium.[36] After washing, excess water was drained from the surface and the grids were immediately plunge frozen in liquid nitrogen.[1,33] After cryofixation, the grids were placed in a precooled aluminum specimen holder at liquid nitrogen temperature and freeze dried at increasing tempera-

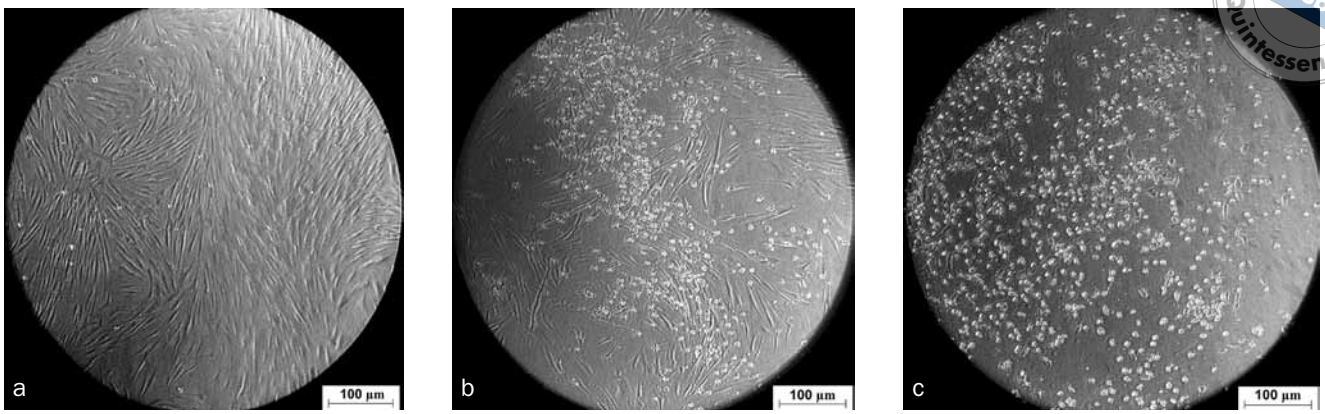

Fig 1 a) Untreated control cells. Phase contrast microsocopy image of orthotypic spindle-shaped gingival human fibroblasts. b) Effect of GIC. Two cell forms, one showing control cells and one showing a second group of cells characterized by their rounded morphology. c) Effect of RM-GIC. All cells are characterized by their rounded shape. Scale bar: 100 µm.

tures for 24 h in an E5300 Polaron freezer-drying device equipped with a vacuum rotatory pump system. Freeze-dried gold grids were carbon coated in a high-vacuum coating system and microanalyzed within 6 h. EPXMA of the specimens was performed with a Philips XL30 scanning electron microscope (Philips; Eindhoven, The Netherlands) equipped with an EDAX DX-4 microanalytical system and a solid-state backscattered electron detector. For EPXMA, the analytical conditions were: tilt angle 0 degrees, take-off angle 35 degrees, and working distance 10 mm. The acceleration voltage was 10 kV. All spectra were collected in spot mode at 10,000X (equivalent to 50 nm spot diameter) for 200 s live time, and the number of counts per second recorded by the detector was approximately 500.[11,23] All determinations were performed on the central area of the cell nucleus. To determine total ion content, the peak-to-local-background (P:B) ratio method[2,6,10] was employed with reference to standards composed of 20% dextran containing known amounts of inorganic salts.[32] For each sample (controls, GICs, and RM-GICs), 5 different grids were analyzed, and 30 different cells corresponding to the 5 grids were randomly microanalyzed using EPXMA.

Statistical Analysis

To determine overall differences among all experimental groups, the Kruskal-Wallis test was used. The Mann-Whitney U test was used to compare the LDH release levels corresponding to two different groups of study (pair-wise comparisons). This test was also used to compare the intracellular ionic contents as determined by microanalysis and the cell morphology as determined by phase contrast microscopy between two different study groups. P values lower than 0.05 in two-tailed tests were considered statistically significant.

RESULTS

Phase Contrast Microscopy

As shown in Fig 1a, most control human gingival fibroblasts showed a typical, elongated spindle shape (mean 98.4% ± 3.3 of all cells; median 100%). When cells were exposed to GIC, two cell populations were observed: one that maintained the characteristic spindle shape of normal, living cells (52.2% ± 20.4; median 52.9%), and a second group of altered cells characterized by a rounded morphology (Fig 1b). In cultures exposed to RM-GIC, most of the fibroblasts were characterized by their rounded shape, with only a small population of cells showing normal morphology (3.9% ± 5.0; median 1.5%) (Fig 1c). The Kruskal-Wallis test revealed statistically significant differences ($p < 0.001$), as did all individual pair-wise comparisons (control vs GIC, control vs RM-GIC, and GIC vs RM-GIC) ($p < 0.001$).

Lactate Dehydrogenase Release

As shown in Fig 2, the percentage of LDH released from the cells into the supernatant was significantly higher after exposure to RM-GIC (mean 38.46% ± 7.29; median 31.6) ($p < 0.001$) than in negative controls and samples exposed to GIC (11.04% ± 21.69; median 3.0). The percentage of LDH release was also significantly higher in GIC cultures than in negative controls ($p < 0.05$). In the same sense, the global analysis revealed statistically significant differences among all the groups compared ($p < 0.001$, Kruskal-Wallis test).

Quantitative Electron-probe X-ray Microanalysis (EPXMA)

The Kruskal-Wallis test revealed statistically significant differences among all the groups compared ($p < 0.001$) for Na, Cl, and K. The results of EPXMA (Fig 3) showed that the highest intracellular levels of Na and Cl were found with RM-GIC samples (mean 187.03 mmol/kg ± 113.11, median 147.50; and 153.12 mmol/kg ± 57.28, median 150.01, respectively). In contrast, the lowest values were found in negative control cells (68.07 mmol/kg ± 26.66, median 57.64 of Na and 115.40 mmol/kg ± 32.30, median 116.10 of Cl), whereas cells incubated in GIC showed an average intracellular concentration of 85.79 mmol/kg ± 58.03, median 64.43 of Na and 115.89 mmol/kg ± 75.69,

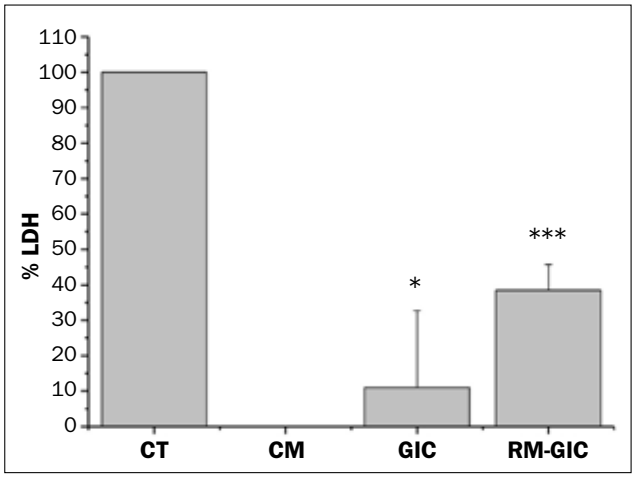

Fig 2 Lactate dehydrogenase (LDH) release by human oral fibroblasts incubated for 72 h in GIC and RM-GIC. All results are shown as percentages of LDH release in reference to controls. CT: cells incubated in 1% Triton-X (positive control); CM: cells incubated in culture medium without glass-ionomer cements (negative control); GIC and RM-GIC refer to the different glass-ionomer cements. All values correspond to percentage of LDH release in reference to positive controls (100%) and negative controls (0%). *p < 0.05, ***p ≤ 0.001 compared to negative controls.

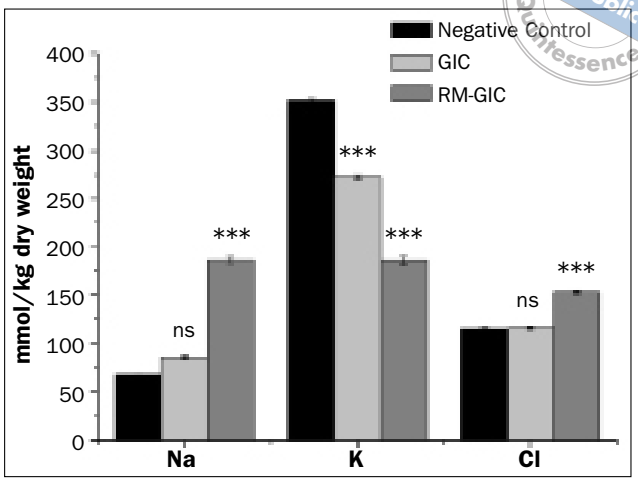

Fig 3 Intracellular ionic concentrations of sodium (Na), potassium (K), and chlorine (Cl) (mmol/kg dry weight) as determined by electron-probe x-ray microanalysis in human gingival fibroblasts incubated in different glass-ionomer cements. Negative controls correspond to human gingival fibroblast incubated in DMEM. Data are shown as the mean ± standard error of the mean. ***p < 0.001; ns p > 0.05 compared to negative controls.

Table 1 Intracellular contents of Na, K, Cl (in mmols per kg of dry weight) as determined by EXPMA in human gingival fibroblasts incubated in different biomaterials

	Control	GIC	RM-GIC
Na	68.07 ± 26.6 (57.64)	85.79 ± 58.03 (64.43)	187.03 ± 113.11*** (147.5)
K	351.18 ± 77.44 (332.75)	272.72 ± 75.69*** (285.07)	186.74 ± 132.06*** (192.52)
Cl	115.40 ± 32.30 (116.1)	115.89 ± 75.69 (104.53)	153.12 ± 57.28*** (150.01)

All values are shown as means ± standard deviations and median values (in brackets). ***p < 0.001 for the statistical comparison versus control cells.

median 104.53 of Cl. Differences were statistically significant for the comparison of RM-GIC to controls, but not between GIC and controls. In contrast, the concentration of K was significantly higher in control cells (351.18 ± 77.44; median 332.75) than in both the GIC group (272.72 ± 75.69; median 285.07) ($p < 0.001$) and the RM-GIC group (186.74 ± 132.06; median 192.52) ($p< 0.001$), with the lowest values found for RM-GIC (Table 1). Finally, the highest K:Na ratios (5.15) were found in control samples incubated in culture medium with no glass-ionomer cement, whereas the K:Na ratio was 3.17 for cells treated with GIC and 0.99 for cells incubated with RM-GIC.

DISCUSSION

Human gingival fibroblasts have been shown to be useful in evaluating the cytotoxicity of biomaterials used in dentistry[21,24] and have also been used to assess the cytotoxicity of glass-ionomer cements.[27] Due to their presence in the oral cavity and their sensitivity to drugs as well as toxic and chemical agents, gingival fibroblasts are excellent models for studying the in vitro biocompatibility of materials used in dentistry, although the results should not be directly transferred to a clinical situation. In the present work, the effects of GIC and RM-GIC were compared to assess cytotoxicity and possible mechanisms of cell death, which earlier studies with different cell types did not clearly establish.[3,9,17,22,29] This study was carried out using low concentrations of the GIC and RM-GIC materials (12.56 mm^2 per 1.5 ml of cell culture medium) in order to determine the cytotoxic effects of these ionomers when used in water-rich environments, such as the human mouth, where particles eventually released by dental materials would be diluted by saliva.

The first line of evidence of biocompatibility examined here is based on light microscopy observations of fibroblasts in culture. In cells exposed to GIC, two cell forms were observed: one which conserved the characteristics of control cells (the spindle shape) and another characterized by a rounded shape. In contrast, cells exposed to RM-GIC appeared as a single cell form, characterized by a rounded shape. Our observations agree with those Costa et al[9] and Aranha et al,[3] who used SEM and found that RM-GIC (Vitremer, Vitrebond) produced alterations characterized by the presence of cells with a rounded shape and plasma membrane disruption. However, when Costa et al[9]

analyzed GIC (Fuji IX and Ketac Molar), they observed the persistence of cells resembling those in the control group, with fewer cells adhered to the glass substrate.

The second indicator we used to evaluate biocompatibility was LDH release into the culture medium. Lactate dehydrogenase is a stable cytoplasmic enzyme stored in viable cells. An increase in LDH levels in the culture medium shows that membrane stability has been disrupted.[35] In this study, we observed that cells exposed to RM-GIC released significantly more LDH than control cells and those exposed to GIC. In cells treated with GIC, we observed that LDH release was significantly higher than in control cells.

The results of both morphological analysis and LDH quantification showed that RM-GIC was more cytotoxic than GIC under these conditions, possibly because of the presence of the HEMA monomer or other cytotoxic components in its composition. In this regard, previous works suggested that certain RM-GIC components such as fluoride, aluminum, silver, silica, strontium, zinc, and silicate, may be released during the setting reaction of the cement in a wet environment, although it is unlikely that these components can induce cytotoxicity by themselves, except for Zn^{2+}.[29] In the present work, the exact amount of HEMA monomer that the RM-GIC materials may release into the culture medium is unknown. However, several studies demonstrated that HEMA is released by these materials even when they are properly polymerized following the manufacturer's recommendations.[3] Previous studies demonstrated that HEMA monomers are cytotoxic even at low concentrations, especially after long incubation times. Palmer et al[18] noted that the HEMA monomer may be a major contributor to the high toxicity of resin-modified glass-ionomer cements. Issa et al,[14] who studied 24-h LDH release in human gingival fibroblasts, demonstrated that resin composite monomers such as HEMA produced toxicity in a time and dose-dependent manner. In addition, Aranha et al[3] demonstrated that the HEMA monomer causes morphological alterations, characterized by round cells, within 72 h in an odontoblast cell line (MDPC-23).

On the other hand, cytotoxicity associated with GIC has been related to the presence of small aluminum particles and other metallic ions in this type of cement.[27] Stanislawski et al[29] reported that zinc was found in high concentrations and considered this element to be responsible for inducing the cytotoxic effects. Our findings agree with those of Costa et al,[9] whose in vitro assays with odontoblast cell line MDPC-23 also demonstrated that during a 72-h performance evaluation with a methyltetrazolium assay, RM-GICs (Vitrebond, Vitremer, and Fuji II LC) and GICs (Fuji IX GP and Ketac Molar) had a cytotoxic effect, although GICs were less cytotoxic. Moreover, Oliva et al[17] showed GICs to be less cytotoxic to osteoblastic cells than RM-GICs.

Although establishing the pattern of cell death can be important in evaluating the biocompatibility of dental materials,[5] and may be clinically useful, earlier research on glass-ionomer cement cytotoxicity did not produce clear evidence of which mechanism of cell death is induced by these materials. In this regard, EPXMA not only allows the simultaneous determination of the morphology and the intracellular ionic profile of biological samples, it has also been demonstrated to be a very useful approach to evaluating cell death processes.[23,31] Quantitative energy dispersive x-ray microanalysis combined with electron microscopy is a well-established procedure to evaluate the viability and the physiological status of cultured cells, especially when these cells are intended for clinical purposes.[11] In contrast with other methods based on the exclusion of dyes (such as trypan blue), propidium iodide, LDH, or DNA,[7,8,24] EPXMA is a highly sensitive technique for determining cell viability. Previous studies by our group have demonstrated a strong correlation between the morphological changes that take place in cells during death and the intracellular levels of different ionic components in human U937 cells,[4,23] K562 cells,[33] and normal primary cell cultures.[2,24] Thus, this method makes it possible to evaluate cytotoxicity of cells incubated in the presence of toxic agents or drugs, yielding information on cell death mechanisms.[24]

In this regard, our EPXMA results confirmed that human gingival fibroblasts exposed to two glass-ionomer cements showed different ion profile alterations. Interestingly, the K:Na ratio decreased from control > GIC > RM-GIC, which we interpret as evidence of alterations in intracellular levels of K and Na and decreasing cell viability. This is consistent with Roomans and von Euler's[25] suggestion that the K:Na ratio is one on the most sensitive and reliable criteria of cell viability.

The microanalytic data for RM-GIC indicated that intracellular Na and Cl increased while K decreased, and these changes in ion composition were significantly different from the changes seen both in control cultures and cells exposed to GIC. Previous experiments with cells undergoing induced necrosis confirmed that this ionic profile is specific to necrotic cells, and these ionic alterations are followed by a reduction in the intracellular ATP.[2,16] In contrast, cells undergoing death by apoptosis showed a different pattern of ionic alterations: an increased concentration of sodium, and the depletion of potassium and chlorine.[2,11,12,24,26] In this context, Rodriguez et al[23] also noted that exposure of the U937 cell line to HEMA at the LD50 (lethal dose 50%) led to an increase in Na levels within 120 min, and suggested that cation channels were activated in association with increasing cell volume. The increase in Cl concentration during the last period of these experiments (120 min) also supports this mechanism. Reeves and Shah[20] showed that during the late stage of necrotic lesions, Cl enters the cell after intracellular ATP depletion and after profound alterations in intracellular cation homeostasis. Moreover, Waters and Sharellman[34] suggested that Cl could be used as a late indicator of necrotic lesions. All these results lead suggest that a process of cell necrosis could be responsible for the cytotoxic effects induced in vitro by RM-GIC in the present study. Further research using increasing incubation times and biomaterial concentrations should determine whether this process is time or concentration dependent.

When human gingival fibroblasts were exposed to GIC, we observed no significant changes in Na and Cl levels in comparison to control cultures, although K decreased significantly. In general, this ionic profile was not very dif-

ferent from that of the normal control cells, except for the K depletion. The lack of global, severe ionic alterations may indicate that these cells are not undergoing a process of cell death by either apoptosis or necrosis, suggesting that GIC is not very cytotoxic at these concentrations and incubation times. The morphological alterations that we observed with light microscopy in a subset of cells could be associated with the K reduction found in cells incubated in the presence of this ionomer.

CONCLUSION

The morphological, biochemical, and microanalytical indicators of cell biocompatibility used here suggest that RM-GIC could cause greater alterations than GIC in human gingival fibroblasts kept in culture after 72 h of exposure under these conditions, and that these alterations point toward necrosis as the mechanism of cell death in vitro. Further in vivo studies are necessary to determine whether these results can be extrapolated to clinical situations.

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Clinical relevance: GICs could be recommended for use in areas deep in the dentin-pulp complex as sealants or for protective purposes, while RM-GICs may be a more suitable material for restorations.