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Are New Bioactive Materials More Biocompatible Than Glass Ionomers? A Real-Time Cytotoxicity Assessment In Vitro

Research Article

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Abstract

Objective: As a new concept, bioactive materials are emerging for direct restorative approaches. The aim of this study was to evaluate the cytotoxicity of contemporary restorative bioactive materials and compare them with contemporary glass ionomers.

Methods: Extracts of Fuji IX GP Capsule, EQUIA Forte, Glass Fill, which are glass ionomer based materials, and Biodentine, and ActivaBioActive Restorative, which are bioactive materials, were prepared with culture medium according to ISO cytotoxicity criteria and diluted to 1/2, 1/4, 1/8, 1/16, 1/32. For negative control, dental material-free culture medium was used. The cytotoxicity of original and diluted extracts on L929 mouse fibroblasts determined by XTT assay and real-time cell analysis method. For statistical analysis one-way ANOVA, post hoc Tukey's HSD, hierarchical clustering and distance correlation methods used.

Results: As a result of the XTT assay, undiluted concentrations of all materials were found to have cytotoxic effects on L929 cells. ActivaBioActive Restorative showed less, and Glass Fill showed more cytotoxic effects than other materials. According to the results of the RTCA, ActivaBioActive Restorative showed promising results by maintaining the cell viability in all concentration groups after 144 hours. Also, due to only the lowest concentration group maintain viability, Fuji IX GP Capsule was found to be most cytotoxic material.

Conclusions: Within the limitations of this study, we can say that restorative bioactive materials are less cytotoxic than glass ionomers.

Keywords: Bioactive Materials; Cytotoxicity; Glass Ionomer Cements; RTCA; XTT Assay.

Introduction

Dental caries, also known as tooth decay, is one of the most common chronic diseases in humans worldwide, and individuals are susceptible to this disease throughout their lifetime [1]. There are many materials used in the treatment of tooth decay. However, these materials help restore the health of the tooth, and they also have the potential to produce undesirable effects on body tissues. According to primum non nocere, one of the main principles of medicine, the materials used in the treatment should not harm the body, or at least the benefits should be greater than the harm. Biocompatibility refers to the ability of a biomaterial to perform its desired function concerning a medical therapy without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy. It aims to generate the most appropriate beneficial cellular or tissue response in that specific situation and optimize the clinically relevant performance of that therapy [2]. Materials used in dentistry can be scattered to the environment by corrosion and dissolution. These components may have toxic effects on the human body. Since the components released from dental materials are very low and their LD50 (median lethal dose) values are relatively high, dental materials are not expected to produce systemic acute toxic effects. However, regional interactions in developed organisms differ from systemic toxicity; substances released from dental materials may interact with pulp, gums, alveolar bone, and oral mucosa locally. As a result of these interactions, cell metabolism may change and release inflammatory mediators, or apoptosis or necrosis may occur if the cell is damaged

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[3]. Due to these possible negative effects, the biocompatibility of restorative materials and all biomaterials used in the human body is very important.

Bioactivity refers to a unique property of a material that elicits a cellular response, such as the formation of hydroxyapatite. Compared to inert materials, bioactive materials can produce growth factors and encourage natural mineralization. Bioactivity appears to be an increasingly popular phenomenon in restorative dentistry [4]. Calcium hydroxide is a bioactive material used in pulp lining for a long time in restorative dentistry. Later, with the introduction of MTA and Biodentine, which are calcium silicate cement, bioactive materials have expanded in restorative dentistry. With the development of bioactive resins, bioactive materials have also started to be used as direct filling materials as a new concept in restorative dentistry. It can be predicted that new bioactive materials can become more popular in restoring dental tissues [5].

There are many studies on the possible damages of amalgam, which has been used for a long time, and the resin composite, the most popular restorative material of today [6]. Glass ionomers are considered more biologically acceptable than these materials. Also,theydevelop an interfacial ion-exchange layer with the tooth and show a degree of bioactivity when set [7]. Therefore, glass ionomers are considered suitable for cytotoxicity comparison with bioactive materials.

The hypothesis in this study is that bioactive restorative materials do not differ favourably from glass ionomers in terms of cytotoxicity. To test this hypothesis, regional toxicity of five different restoration materials Fuji IX GP Capsule (GC), EQUIA Forte (GC), Glass Fill (GCP Dental), which are glass ionomer-based materials, and Biodentine (Septodont), and ActivaBioActive Restorative (Pulpdent), which are bioactive materials,were evaluated in vitro by XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium] assay and RTCA (Real-Time Cell Analysis) method on L929 Mouse Fibroblasts.

Methods

Preparation of Cell Cultures

L929 cells were cultured in DMEM (Dulbecco modified Eagle's medium, Biochrom) containing 10% FBS (fetal bovine serum, heat-inactivated, non-USA origin, sterile-filtered, Merck) and 1% penicillin/streptomycin (Biochrom) at 37oC with humid air containing 5% CO_2. Cells in the exponential growth phase that reached 75-80% confluency were used for the experiments.

Preparation of Test Materials

7 samples of Fuji IX GP Capsule (GC, Tokyo, Japan), EQUIA Forte (GC, Tokyo, Japan), Glass Fill (GCP Dental), Biodentine (Septodont), and ActivaBioActive Restorative (Pulpdent) (see Table 1) were prepared with a dimension of 2 mm height and 5 mm diameteraccording tomanufacturer's instructions to obtain restor-

Table 1: Restorative materials tested in this study, manufacturers and indications.

Restorative Material and Manufacturer	Material Class	Indications (According to manufacturer's recommendations)
Fuji IX GP Capsule (GC) 	High viscosity glass ionomer cement	w Class I and II restorations in deciduous teeth. w Non-load bearing Class I and Class II restorations in permanent teeth. w Intermediate restorative and base material for heavy stress situation in w Class I and Class II cavities using sandwich laminate technique. w Class V and root surface restorations. w Core build-up
EQUIA Forte (GC) 	Glass hybrid restorative	w Class I restorations w Stress bearing Class II restorations w Non-stress bearing Class II restorations w Intermediate restorative w Class V and root surface restorations w Core build up
Glass Fill (GCP) 	Glass carbomer	w Permanent Class I and Class II restoration (non load-bearing areas) with heat w Class I and II restoration in deciduous teeth w Build-up material for crown and bridge w Cervical fillings w Class V
Biodentine (Septodont) 	Bioceramic (also classified as tricalcium silicate cement)	In the crown: w Temporary enamel restoration w Permanent dentin restoration w Deep or large carious lesions w Deep cervical or radicular lesions w Pulp capping w Pulpotomy In the root: w Root and furcation perforations w Internal/external resorptions w Apexification w Retrograde surgical filling
ActivaBioActive Restorative (Pulpdent) 	Bioactive composite	w Bioactive filling material for pits, root surface cavities and Class I, II, Ill, IV and V restorations where there is no pulpal involvement.

ative material extracts. The sample groups were placed in each well of a 6-well plate incubated at 37°C for 24 hours to ensure complete curing. 3 ml of cell culture medium (DMEM) was added to the wells to ensure that the material surface area to medium volume ratio was 91.6 mm^2/ml according to ISO (International Organization for Standardization) standards. In addition to the original extract, dilutions with the medium at a ratio of 1/2, 1/4, 1/8, 1/16, 1/32 were prepared.

Performing of the XTT Test

For the XTT assay, previously prepared cells were seeded at a density of 104 in a 96-well plate (Greiner Bio-One GmbH) and incubated at 37°C for 24 hours. After incubation, 9 wells were reserved in a 96-well platefor each concentration of each material. For the control group, 6 wells were reserved.

100 µl of different dilutions of previously obtained material extracts were added to the separated wells. Only the medium was added to the cell control group. The 96-well plates were then incubated at 37°C for 24 hours to allow the extracts to interact with the cell cultures. After incubation, extracts were discarded from the wells. Then, the reagent solution [Cell Proliferation Kit (XTT based), Biological Industries] was prepared according to the manufacturer's instructions, and 0.05 ml of the prepared reagent solution was added to each well. 96-well plates were incubated at 37°C in the incubator for 4 hours to allow the reagent to interact with the cell cultures. 96-well plates were measured by a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments, Vermont, USA) at a 460 nm wavelength.

The quantitative data obtained from the spectrophotometer were recorded in Office Excel 2016 (Microsoft). The viability percentage of the positive control group was equalled to 100%. The viability of the other groups was determined as a percentage relative to the viability of the control group. The experiment was repeated 3 times, and 9x3=27 (n) observation data were obtained for each material concentration.

Performing Real-Time Cell Analysis

Pre-warmed 50 µl of DMEM medium was added to each well of the electronic 16 well plates (E-plate 16, ACEA Biosciences), and the E-plates were kept in the safety cabinet for 30 minutes. Then, the E-plates were placed in the RTCA station (xCELLigence RTCA DP, ACEA Biosciences). The background measurement of the cell culture medium was done for a more accurate impedance measurement. After cell passaging and counting processes, 100 µl cell suspension at 10^4 ml/cell density of L929 cells was seeded into each well of the E-plates except medium control wells.The E-plates were kept in the safety cabinet for 30-60 minutes to allow the cells to adhere to the well base. The plates were then placed in the RTCA station, and an impedance measurement was taken every hour. Cells adhered to the plate bases and proliferated inside the RTCA station with 5% CO_2 and 95% humidification at 37°C for approximately 24 hours. Then, electronic cell culture plates were removed from the RTCA station to add the previously prepared material extracts.

The medium in the wells was aspirated before the cells were treated with extracts. 150 µl FBS-free DMEM was added to medium control and cell control wells. 150 µl maximum dose of material concentration was added to the material control wells, and a 150 µl volume of solution was added to the other wells at the determined concentrations (at a ratio of 1/2, 1/4, 1/8, 1/16, and 1/32). For each concentration two wells used. After adding material extract was completed, the E-plates were returned to the RTCA station. The device was programmed to take measurements every 15 minutes for 144 hours. The data obtained from the experiment were analyzed with the RTCA Software 2.0 (ACEA Biosciences). All wells'CI (Cell Index) values were equalized to 1 before adding restorative material extracts. The other CI values through the experiment were proportioned accordingly to obtain NCI (Normalized Cell Index) values to obtain more standard data between the wells.

Statistical analysis

Statistical analysis was performed usingSPSS Statistics (v. 25, IBM). For the XTT assay,the homogeneity of the data was evaluated by the Shapiro-Wilk test. Differences between the viability percentages of experimental groups and those of control groups were evaluated statistically by One-Way ANOVA and post hoc Tukey's HSD tests. P (probability) value ≤0.05 was considered for statistical significance.

In the RTCA test, the NCI values of the cell control groups of all materials were averaged to compare the toxicity between the materials. The NCI values of the material extract groups were proportioned according to this average. The distance correlation method was used to sort the toxicity degree of material concentrations.The correlation method with the Euclidean distances dissimilarity algorithm was used to analyze these time series distances. The hierarchical clustering method evaluated the significance of the differences between toxicity levels. The between-groups linkage method was used with the Euclidean distance algorithm and data standardized with the Z score for hierarchical cluster analysis.

Results

XTT Experiment Results

According to the results of the XTT experiment, it was observed that the material extracts affected the viability of L929 cells although they varied according to their concentration (see Figure1). Glass Fill was found to be the most toxic alternative restoration material and reduced survival rates of L929 cells to 27,56% and extract at 1/2 concentration were statistically cytotoxic with a 55.88% viability rate ($p < 0.05$). The difference in survival rates between Glass Fill and all other materials was statistically significant ($p \leq 0.001$). Only undiluted extracts of other tested alternative restorations materials were statistically found to be cytotoxic in L929 cells ($p<0.05$). The Fuji IX group is statistically different from the ActivaBioActive Restorative and EQUIA Forte groups ($p \leq 0.001$).Details are given in Table 2.

RTCA Results

According to the RTCA experiment results, the material extracts were observed to affect the viability of the L929 cells, although they varied according to their concentration. While most material concentrations had a toxic effect, some showed a proliferative effect (see Figure2).

Figure 1. After 24 hours in XTT experiment, the distribution of the mean viability of L929 cells by percentage according to concentrations, "*" indicates cytotoxic concentrations. (p <0.05).

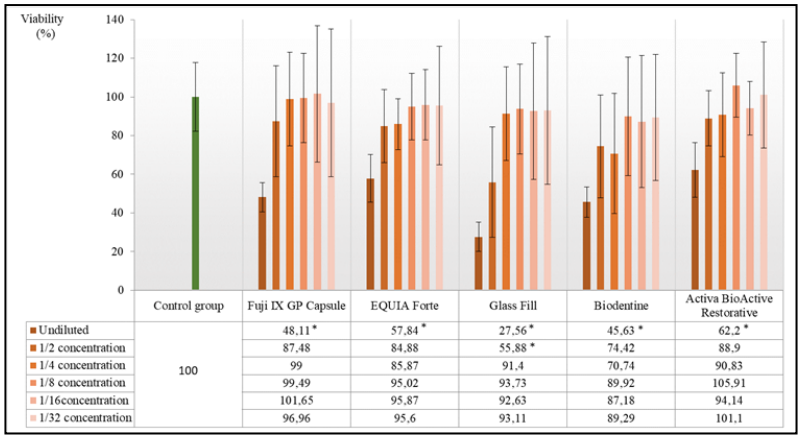


Table 2. Descriptives of statistical analysis of XTT assay.

Restorative Material	N	Mean	Std. Error	Std. Deviation	95% Confidence Interval	
					Lower Bound	Upper Bound
Fuji IX GP Capsule	36	48,10992	3,851537	7,341198	36,66328	66,95501
	28	87,48315	4,272281	8,92432	-4,3645	29,23632
	22	99,00152	4,737854	12,11544	-17,7137	19,54878
	24	99,48585	4,562447	13,5684	-17,5082	18,37468
	24	101,6472	4,562447	9,662542	-19,6696	16,21333
	23	96,96382	4,647163	6,518643	-15,3194	21,22984
EQUIA Forte	27	57,841	4,33844	12,36771	25,01749	59,13863
	27	84,87964	4,33844	18,94215	-2,02115	32,1
	27	85,87474	4,33844	13,02703	-3,01625	31,1049
	27	95,01987	4,33844	17,18807	-12,1614	21,95977
	22	95,86727	4,737854	18,18643	-14,5794	22,68303
	25	95,59691	4,483094	30,64771	-13,3073	21,95157
Glass Fill	27	27,55705	4,33844	7,54719	55,30144	89,42258
	27	55,87503	4,33844	28,62832	26,98346	61,1046
	27	91,40151	4,33844	24,21177	-8,54301	25,57813
	25	93,72508	4,483094	23,15484	-11,4354	23,8234
	27	92,62774	4,33844	35,24035	-9,76925	24,35189
	22	93,10722	4,737854	38,1721	-11,8194	25,44308
Biodentine	27	45,63469	4,33844	7,846235	37,2238	71,34494
	27	74,42387	4,33844	32,60338	8,434617	42,55576
	27	70,74088	4,33844	26,50052	12,11762	46,23876
	27	89,91549	4,33844	30,9514	-7,05699	27,06415
	27	87,184	4,33844	30,63398	-4,32551	29,79563
	19	89,29399	5,053536	34,15127	-9,24756	30,49771
ActivaBioActive	27	62,20216	4,33844	14,12688	20,65633	54,77747
Restorative	27	88,89649	4,33844	14,30625	-6,03799	28,08315
	27	90,82687	4,33844	21,61483	-7,96838	26,15277
	25	105,9053	4,483094	16,51922	-23,6156	11,64319
	25	94,13972	4,483094	13,95185	-11,8501	23,40875
	27	101,1048	4,33844	27,4164	-18,2463	15,87487

Figure 2. In RTCA experiment cytotoxicity comparison of restorative materials extracts on L929 cells according to distance correlation analysis. There is no statistical difference between groups with the same sign. For better understanding of the graph, distance correlation values are given as a percentage between the hypothetical positive control group (0%) and the cell control group (100%).

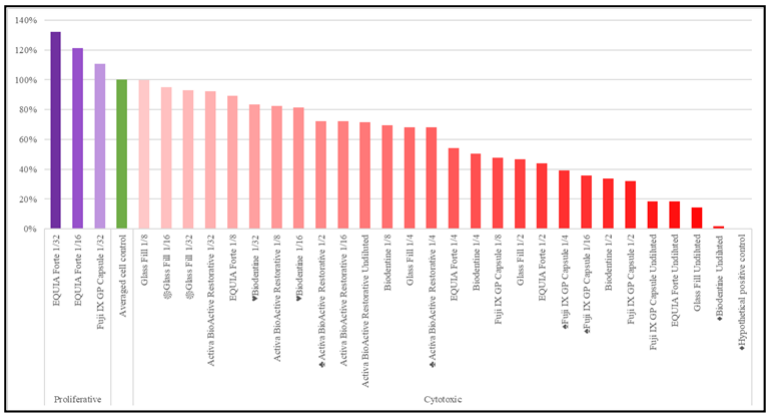
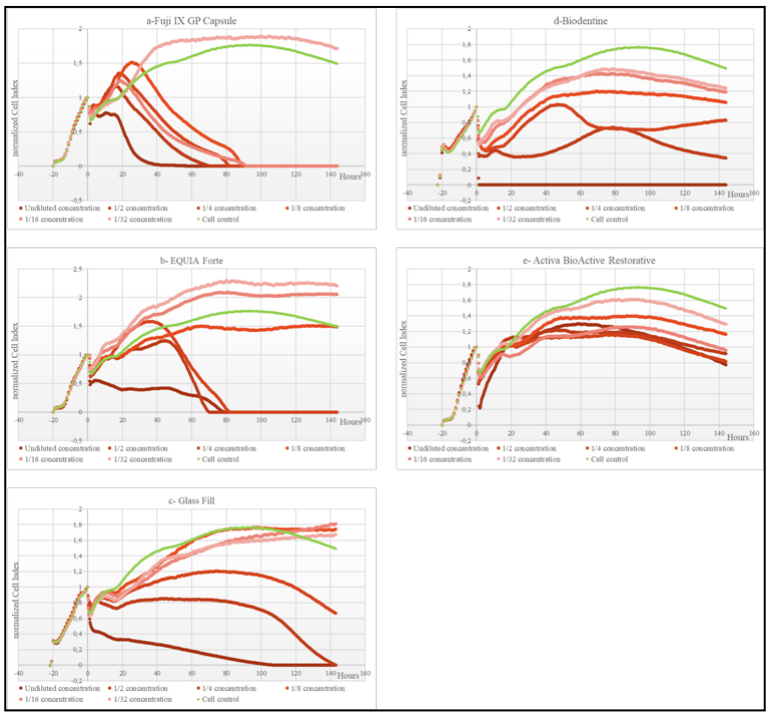


Figure 3. RTCA plot depicting change of mean cell viability in wells containing L929 cell culture. The left side of the abscissa shows the 20-24 hours incubation period of L929 cell cultures, and the right side shows the 144 hours experimental period after treatment with tested restorative material extracts with various concentrations.; a-Fuji IX GP Capsule, b-EQUIA Forte, c- Glass Fill, d-Biodentine, e- ActivaBioActive Restorative.



Viability was completely lost in nearly all cell culture groups treated with the Fuji IX GP Capsule'sextracts; at the end of the 144th hour, only control and cell culture groups treated with the extract at concentrations of 1/32 were able to survive. Also, a slightly proliferative effect was observed in the cell culture group treated with the extract at concentrations of 1/32 (see Figure3a). At EQUIA Forte, Viability was completely lost at the most potent three cell culture groups treated with extracts. Close but lower NClvalues were seen at the 1/8 concentration than the control group. Also, as in the Fuji IX GP Capsule, cell culture groups treated with extracts at low concentrations showed a proliferative effect (see Figure3b). At the Glass Fill's cell culture groups treated with undiluted and diluted extracts at concentrations of 1/2, viability was completely lost relatively late compared to EQUIA Forte and the Fuji IX GP Capsule. Although cell viability was maintained at the end of the 144th 1/4 concentration, viability significantly decreased compared to the control group. Cell viability at thinner concentrations than the control group generally remained lower, buta slightly proliferative effect was observed from the 4th day of the experiment (see Figure3c). Only the cell culture group treated with the Biodentine's undiluted extract lost viability completely; at the end of the 144th hour, all other cell culture groups survived with lower viability. Furthermore, unusual viability curve patterns were observed in cell culture groups treated with Biodentine's extracts at concentrations of 1/2 and 1/4, compared to other groups (see Figure 3d). In the ActivaBioActive Restorative's, cell culture groups treated with extracts could maintain their viability at the end of the 144th hour. Better NCl values were observed (see Figure3e), especially at higher concentrations than Biodentine, a material used in vital therapies.

Discussion

In this study, in vitro cytotoxicity of three glass ionomer-based cement and two bioactive restorative materials were tested by two different methods. Our null hypothesis was rejected because bioactive restorative materials do not differ favorably from glass ionomers in cytotoxicity.

In vitro cytotoxicity tests are an essential screening step in assessing the regional toxicity of dental materials before in vivo animal or human tests. Since L929 mouse fibroblast cells react similarly to human fibroblast cells against components released from dental materials [8] in our study, these cells were selected for use in cell cultures. Tetrazolium reduction tests, including the XTT assay, used to evaluate the viability of eukaryotic cells are suitable in vitro methods for evaluating the cytotoxicity of dental materials [9, 10]. But the XTT and other cytotoxicity determination tests, in general, give a single measurable value for cell viability at the end of each test. In addition, relatively many processing steps are required to perform these tests, which may cause variations in the measured value [11]. With the RTCA, cell viability can be read as often as desired within the specified time; thus, it is possible to monitor the viability of the cells. This provides comprehensive information during the test period. Also, in this method, no labelling is required to monitor the cells; it saves resources and workload, moreover allows for a more physiological measurement [12].

In our study, only the undiluted concentrations of the Fuji IX GP Capsule's extract, a high viscosity glass ionomer, were cytotoxic according to the XTT test results. When RTCA results are analyzed, it is seen that only the cell culture group treated with extract at concentrations of 1/2 could survive at the end of the 144th hour. How this great difference occurred between the XTT experiment of the Fuji IX GP Capsule and the RTCA method was investigated. In RTCA, the cell viability of the cell culture groups treated withthe Fuji IX GP Capsule extracts generally decreased rapidly after the first and second days. Inthe XTT test, cell cultures were evaluated for cytotoxicity one day after being treated with material extracts. However, due to the advantages of RTCA, the duration of the experiment increased to six days. Thus, the amount of data obtained for each material concentration was increased considerably, and how the material extracts changed cell viability in cultures over time could be observed in more detail.

We can call EQUIA Forte an improved high viscosity glass ionomer cement; this restorative material is also qualified as a glass hybrid restorative by its manufacturer. It is suggested that with the addition of very thin and highly reactive glass as a filler, a stronger matrix is formed in the cement, resulting in improved physical properties [13]. We can say that EQUIA Forte is less cytotoxic than the Fuji IX GP Capsule, which can be considered its predecessor. EQUIA Forte was launched in 2015, and there is a limited biocompatibility study that we can compare our findings on this material. Cosgun et al. found no difference in cytotoxicity between the Fuji IX GP Capsule and EQUIA Forte in their studies conducted with MTT assay on Vero cells. They described these two materials as slightly toxic [14]. In another study, Collado-González et al. Compared EQUIA Forte and Ionostar Molar's cytotoxicity with the MTT assay on HDPSCs (Human Dental Pulp Stem Cells)culture and found EQUIA Forte more successful [15]. In addition, it is seen that in the groups treated with the extract at the 1/16 and 1/32 concentrations, the cells gave a higher normalized cell index value at the end of the 144th hour as they proliferated more than the control group. Similar to the observations in our study, Ersahan et al. also mentioned the proliferative effect of some glass ionomer cement, including EQUIA Forte, on L929 cells [16].

Biodentine is a bioactive material that can be used in vital operations such as pulp lining, root perforation, and internal resorption repair. Since its abrasion resistance is weak, it cannot be used as a direct restorative in general. As a result of these findings, we can say that Biodentine is the least cytotoxic material after the ActivaBioActive Restorative, another bioactive material.MTA is generally used for comparison in studies related to Biodentine in the literature. In one of the limited studies comparing Biodentine with the materials we used, Zhou and his colleagues examined the effects of Biodentine, MTA, and the Fuji IX GP on flow cytometry and human gingival fibroblasts. They observed that Biodentine and MTA produced similar responses in cells. They also found that these two materials were more biocompatible than the Fuji IX GP in the test conditions they applied [17]. In a study, Michel et al. investigated the cytotoxicity of different dental materials on HGF (Human Gingival Fibroblast) and hFOB (human Fetal Osteoblasts) cultures with the MTT assay. They found that the Fuji II LC and Glass Fill are more cytotoxic than Biodentine. In addition, while Biodentine showed a similar cytotoxic effect to human gingival fibroblasts than other tested calcium silicate-based cement (ProRoot MTA, Harvard MTA, EndoSequence putty), it showed a more cytotoxic effect against hFOB cells than other tested calcium silicate-based cement [18].

Glass Fill is a glass carbomer-based material. Glass carbomers are separated from glass ionomers by nano-sized powder particles, including fluorapatite and hydroxyapatite fillers. It is also recommended to use glass carbomers with a light device that can generate sufficient heat for clinical use [19]. We can describe Glass Fill as a moderate cytotoxic restorative material compared to other tested materials. Similar to our study,Michel et al. found Glass Fill more cytotoxic than Biodentine and the Fuji II LC on HGF and hFOB cells [18]. In addition, Ülker et al. compared the self-adhesive materials in cytotoxicity with MTT tests on bovine pulp cells. Still, they did not find a statistically significant difference between Glass Fill and Fuji II LC[20]. A slightly proliferative effect was observed at a lower concentration from the 4th day of the RTCA experiment (see Figure3c). We have seen similar effects in the Fuji IX GP Capsule, a high-viscosity glass ionomer, and EQUIA, a glass hybrid. Therefore, we can say that glass ionomer or glass ionomer-like materials have proliferative effects on L929 cells at low concentrations.

ActivaBioActive Restorative describes it as a "bioactive composite" and suggests that it releases calcium, phosphate, and fluoride and can recharge it. In addition, although it is in the class of composites, it does not contain bisphenol A, Bis-GMA, and Bis-GMA derivatives. This material elicits a response that stimulates mineral apatite formation and remineralization, the defining requirement of bioactive materials. The manufacturer claims this processknits the restoration and the tooth together, penetrates and fills micro-gaps, reduces sensitivity, guards against secondary caries, and seals margins against microleakage and failure [21]. It is stated that when it is first released,ActivaBioActive Restorative is chemically bonded to the tooth by the manufacturer and can be used without

any adhesives when retention is not required [22]. However, in the last instruction, it is suggested to be used with a suitable adhesive agent [23]. The most salient result we have observed with ActivaBioActive Restorative. In the RTCA experiment, this bioactive composite was the only material that could maintain the viability of all cell culture groups at the end of the 144th hour. ActivaBioActive Restorative was launched in 2013, and there are very few studies that we can compare our results about this material. ActivaBioActive Restorative was found to be less cytotoxic than other materials tested in our study, even Biodentine, which is indicated for vital pulp therapies. Similarly, ElRash et al. found that ActivaBioActive Restorative's biocompatibility was better than other materials in their studies where they implanted ActivaBioActive Restorative, MTA-HP, and iRoot BP Plus Root Repair Material into mouse subcutaneous tissues and evaluated implantation sites for up to one month [24].

It should be kept in mind that the results obtained from these studies may not apply to in vivo conditions considering the limits of an in vitro study. Considering that a biomaterial can remain in the human body for life, it can be concluded that the relevant materials are tested for a very limited time in laboratory studies. Also, biomaterials may have systemic toxicity, genotoxicity, allergy or teratological effects,besides regional toxicity.

Conclusions

All tested materials have cytotoxic effects on L929 fibroblast cells at undiluted concentrations. If we need to sort the toxicity of these materials for the XTT experiment: ActivaBioActive Restorative <Biodentine<EQUIA Forte <Fuji IX GP Capsule <Glass Fill; for the RTCA experiment: ActivaBioActive Restorative <Biodentine<Glass Fill <EQUIA Forte <Fuji IX GP Capsule. The RTCA method in evaluating the cytotoxicity of dental materials has a higher potential to provide more useful information than the XTT method. Also, Glass ionomers or glass ionomer-like materials have a proliferative effect on L929 cells at low concentrations.

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