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Biocompatibility of Novel Endodontic Sealers

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SUMMARY

RESUMO

Introdução: Um tratamento endodôntico de sucesso, visa o selamento tridimensional do sistema canalar, bem como a erradicação da infeção, a reparação de lesões apicais e a prevenção de uma eventual recontaminação.
Durante a etapa de obturação canalar podem ser utilizados diversos tipos de cimentos endodônticos combinados com a guta-percha. Um cimento endodôntico ideal deverá ser biocompatível, estável dimensionalmente, possuir atividade antimicrobiana e qualidade adequada de selamento marginal. A biocompatibilidade é a capacidade do material, após contacto, não suscitar respostas adversas por parte do hospedeiro.

Objetivo: Este estudo tem como objetivo principal avaliar a biocompatibilidade de um novo cimento endodôntico à base de silicato de cálcio (Bioroot™RCS; Septodont, France) e de dois novos cimentos à base de silicone (Guttaflow® 2 e GuttaFlow® Bioseal; Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland). Neste projeto foi usado como referência um cimento *gold standard*, largamente utilizado na clínica e em estudos prévios, à base de resina epoxi (AH Plus®, Maillefer Dentsply, Ballaigues, Switzerland). O objetivo secundário foi testar a solubilidade destes cimentos de obturação canalar após imersão em meio de cultura durante 24h.

Materiais e Métodos: As células do ligamento periodontal foram incubadas com eluatos dos quatro cimentos à temperatura de 37ºC numa atmosfera humedecida contendo 5% de CO_2 durante 24h, 48h e 72h. Foram testadas diferentes concentrações ao longo do tempo de modo a determinar qual a dose-resposta e a exposição-resposta das células a estes materiais. A citotoxicidade foi determinada através do teste Alamar Blue® e confirmada com microscopia electrónica. A análise estatística foi efetuada recorrendo ao programa Prism (GraphPad Software, CA). Foram aplicados os testes Kolmogorov-Smirnov; Two-way ANOVA com Tukey *post hoc*; One-way ANOVA com Dunnetts *post hoc;* e T-test.

Resultados: Todos os cimentos apresentaram algum grau de citotoxicidade com exceção para o Guttaflow®2. Foram registados valores superiores de biocompatibilidade para cimentos à base de silicone (Guttaflow®2 e Guttaflow® Bioseal) e de silicato de cálcio (BioRoot™RCS), comparativamente ao de resina epoxi AH Plus®. Relativamente ao grau de solubilidade destes materiais, o BioRoot™RCS foi o único cimento que demonstrou dissolução significativa.

Conclusão: Os cimentos endodônticos à base de silicone (Guttaflow®2 and Guttaflow® Bioseal) apresentaram os valores mais elevados de biocompatibilidade, seguidos pelo

cimento à base de silicato de cálcio (BioRoot™RCS). O cimento AH Plus® demonstrou ser o material com mais efeitos citotóxicos.

Palavras-chave: Biocompatibilidade; Citotoxicidade; Cimentos endodônticos; Cimentos de silicato de cálcio; Cimentos de silicone.

ABSTRACT

Introduction: A successful endodontic treatment promotes eradication of bacterial infection, periapical repair, and prevents tooth re-contamination. Endodontic sealers and gutta-percha cones are used as root canal filling materials. The ideal root canal sealer has antimicrobial activity, adequate marginal sealing quality, dimensional stability and biocompatibility. Biocompatibility is the capacity of the sealer to not produce any adverse host response after contact with the living system.

Aim: The primary objective of this study is to evaluate the biocompatibility of a new tricalcium silicate-containing (Bioroot™RCS; Septodont, France) and two silicone-based materials (Guttaflow® 2 and GuttaFlow® Bioseal; Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland) on human periodontal ligament cells. In our project we used a well-known epoxy resin-based sealer (AH Plus®, Maillefer Dentsply, Ballaigues, Switzerland), as a comparison term, since this sealer has been used as the gold standard for the last few years and as a control material in most studies on endodontic sealers. The secondary aim of this project is to test the solubility of these root canal sealers when immersed in culture media for 24h.

Material and Methods: Cells were incubated with fresh eluates from the 4 root canal materials at 37ºC in a humidified air atmosphere containing 5% CO_2 for 24h, 48h and 72h. Different concentrations were tested over time to determine a dose-response and exposure-response effect. The cytoxicity was determined performing Alamar Blue® assay and confirmed with electronic microscopy. Statistical analysis was performed using Prism (GraphPad Software, CA). Kolmogorov-Smirnov test; Two-way ANOVA with Tukey *post hoc* test; One-way ANOVA with Dunnetts *post hoc* test, and one-sample T-test were used.

Results: According to our results all four sealers presented cytotoxicity with exception for Guttaflow®2. Cells of the periodontal ligament showed significantly higher cell viability when exposed to the silicone-based (Guttaflow®2 and Guttaflow® Bioseal) and calcium silicate-based (BioRoot™RCS) sealers eluates in comparison with AH Plus®. Concerning solubility, BioRoot™RCS was the only root canal sealer that presented statistically significant weight loss.

Conclusion: Silicone-based sealers (Guttaflow®2 and Guttaflow® Bioseal), exhibited the highest cell viability values, followed by the calcium silicate-based (BioRoot™RCS). The worst results to the epoxy resin-based sealer (AH Plus®).

Key-words: Biocompatibility; Cytotoxicity; Endodontic Sealers; Calcium-silicate sealers; Silicone sealers.

1.Introduction

INTRODUCTION

The main goal of endodontic treatment is either to prevent or cure apical periodontitis (1,2). In order to achieve that, it is necessary to remove residual pulp along with its breakdown products and microorganisms present inside the root canal system (3,4). Cleaning and shaping, must be followed by a three-dimensional obturation of the endodontic space (5) to prevent further coronal or apical contamination (6,7). The cleaning and shaping process defines both the ability to obturate the root canal and the degree of disinfection. Hence, obturation is a reflection of the previous preparations (8).

During the process of cleaning and shaping, there may occur some procedural errors such as perforations, root fractures, loss of length, canal transportation or loss of coronal seal which can cause a poor obturation and consequently affect adversely the apical seal (8).

In the last few years, there has been a growing emphasis on developing materials and techniques for a proper obturation of the radicular space (8).

Obturation of the root canal usually demands a sealer along with a core material, classically a gutta-percha solid cone (9), whereby the sealer performs as a binding agent between the core material and the root canal dentin (1,10,11).

While endodontic sealers are supposed to be used only inside the root canal system during its filling, sometimes they extrude to the periapical area (**Figure 1**), becoming in intimate contact with the periapical tissues for a long period (7,12–17). Therefore, these materials must be biocompatible (5) and nontoxic to provide good healing and avoid adverse inflammatory reactions (2,18,19).

Ideally, a root canal sealer should be dimensionally stable in order not to create gaps between the root canal walls and the materials, which could lead to bacterial infiltration; radiopaque, so that the clinician can evaluate the obturation, and assess its quality; biocompatible and nontoxic in order to not harm the tissues in contact, especially the periapical area, and have a known solvent, essential in cases of retreatment (19,20).

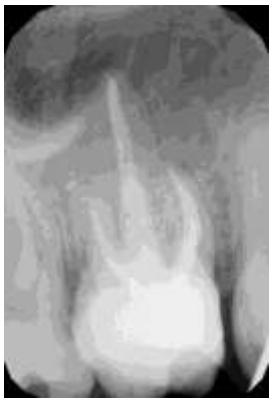

Figure 1. *Representation of sealer extrusion in an endodontic treatment*.

Table I. Requirements for an ideal root filling cement. From Grossman *cit in* (21).

It should be easily introduced into the canal.
It should seal the canal laterally as well as apically.
It should not shrink after being inserted.
It should be impervious to moisture.
It should be bacteriostatic or at least not encourage bacterial growth.
It should be radiopaque.
It should not stain tooth structure.
It should not irritate periapical tissue.
It should be sterile, or quickly and easily sterilized before insertion.
It should be easily removed from the root canal if necessary.

With the necessity of development of an ideal root canal sealer, with most of the requirements present in **Table I**, numerous tests have been developed to evaluate the technological and biological properties of these materials.

The main goal of the technological trials is to guarantee that the sealers have the right workability suitable for a practical clinical usage. There is a range of properties that must be assessed before the commercialization of the sealer such as its radiopacity, working time, setting time, flow, solubility and dimensional stability.

Therefore, these testes must provide a physical characterization of the sealer, previewing how it will perform clinically, with regard to all the limitations of *in vitro* studies.

Before assuring that the material is suitable, there may also be performed the biological testing, mainly to assess the material's biocompatibility and cytotoxicity profile of the sealer. Cytotoxicity defines a material's impact on cell viability (22), for which these tests are of utmost importance.

Other properties that can be tested are the usage of the material and it's antibacterial effects (21).

There are many sealers available to use in clinical practice based on different materials such as zinc oxide-eugenol, calcium hydroxide, epoxy resin, polydimethylsiloxane, glass ionomer, calcium silicate, silicone and methacrylate resin (23,24). However, none of them meet all the appropriate requirements of an ideal sealer.

In this project, we will approach four types of materials: epoxy resin, silicon and tricalcium silicate-based endodontic sealers.

Di and tricalcium silicate-based cements such as mineral trioxide aggregate (MTA) were firstly used in endodontics to repair root perforations and in apical surgery for retrograde root-end fillings (10,13).

Since their high levels of biocompatibility and bioactivity, root canal sealers based on di and tricalcium silicate-based were developed (10). These sealers have proven alkalinity with potential antimicrobial activity and are able to set in a wet field (1). Though there are many calcium silicate-based sealares clinically available none of them is stable enough and interacts properly with the dentin (1).

BioRoot™RCS (Septodont, St. Maure de Fosses, France) (**Figure 2**) is a new bioactive tricalcium silicate-based sealer. It is composed by a powder that mainly consists of tricalcium silicate, povidone as the stickiness agent and zirconium oxide added for radiopacity; and a liquid, which is an aqueous solution of calcium chloride with polycarboxylate, a curing accelerator and a superplasticizer respectively (10). It is resin and eugenol free, which differentiates it from other conventional eugenol and resin based sealers (24).

Since this sealer properties are changed when heated, it is recommended to be used with a single cone obturation technique or lateral conmpaction, rather than warm vertical compactation.

Figure 2. *Ilustration of BioRoot™RCS*.

Silicone-based endodontic sealers are also a good alternative when it comes to biological properties. Guttaflow®2 (Coltène Whaledent, GmBH + Co KG) and Guttaflow®Bioseal (Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland) (**Figure 3**) which is an evolution of its previous, are silicone-based sealers that have showed good biological properties on human ligament periodontal cells (23).

These sealers are composed by a mixture of gutta-percha powder and polydimethylsiloxane with nanometer-sized silver particles added as a preservative. Guttaflow®2 is a cold flowable system combining gutta-percha powder form with a particle size of less than 30 µm and sealer, which differentiates it from its Bioseal (23).

Figure 3. *Ilustration of Guttaflow® 2 and Guttaflow® Bioseal.*

Nowadays one of the most widely used root canal sealers is AH Plus® (Maillefer Dentsply, Ballaigues, Switzerland) (**Figure 4**), mainly due to the fact that it meets most of the requirements of an ideal sealer.

AH Plus® lies on a paste-paste system, and consists of an epoxy resin-based sealer that it's easy to handle, dimensionally stable, radiopaque, presents good sealing, resistance, adhesion to root canal walls, high flow, low solubility (3).

Although some studies state its initial cytotoxicity which might be related to the release of formaldehyde during polymerization or to bisphenol A, it has satisfactory biocompatibility (3).

Figure 4. *Ilustration of AH Plus®.*

The composition of the tested materials is shown in **Table II.**

Table II. Main components of tested sealers.

TESTED MATERIAL	MANUFACTURER	COMPOSITION
AH Plus®	Maillefer Dentsply, Ballaigues, Switzerland	Epoxy paste: diepoxy, calcium tungstate, zirconium oxide, aerosol, and dye Amine paste: 1-adamantane amine, N.N'dibenzyl-5 oxanonandiamine-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosol, and silicone oil
Guttaflow®2	Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland	Gutta-percha powder, polydimethylsiloxane, silicone oil, paraffin oil, platinum catalyst, zirconium dioxide, microsilver (preservative), coloring
Guttaflow®Bioseal	Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland	Gutta-percha powder, polydimethylsiloxane, platinum catalyst, zirconium dioxide, silver (preservative), coloring, bioactive glass ceramic
BioRoot™RCS	Septodont, St. Maure de Fosses, France	Powder based on tricalcium silicate, zirconium oxide and excipients. Aqueous solution of calcium chloride and excipients

Since the biocompatibility of root canal sealers is of primary importance for successful endodontic treatment, it should be accurately assessed before the material contacts with biological tissues.

When assessing the biocompatibility of a root canal sealer, in vitro cytotoxicity tests can be performed. These studies must be suitable for the evaluation of biological compatibility, significant and readily reproducible (3).

Although one of the primary disadvantages of in vitro studies is that it may be difficult to extrapolate its results to an entire organism, these studies have been more and more used nowadays due to their simplicity, specificity and convenience.

The aim of this study was to evaluate the biocompatibility of three main sealers calcium silicate (BioRoot™RCS) and silicone-based (GuttaFlow®2 and Guttaflow®Bioseal), comparing them to and epoxy resin-based sealer (AH Plus®), which has been used as the gold standard for the last few years and has been used as a control material in most studies on endodontic sealers.

To assess that, the cytotoxic effects of eluates of the four mentioned sealers on human periodontal cells were estimated in three different time periods.

2. Materials and Methods

MATERIALS AND METHODS

Sealer Extracts

The materials tested in this project were BioRoot™RCS (Septodont, St Maure de Fosses, France), Guttaflow®Bioseal (Coltène Whaledent, GmBH + Co KG, Langenau, Switzerland), Guttaflow®2 (Coltène Whaledent, GmBH + Co KG), and AH Plus® (Maillefer Dentsply, Ballaigues, Switzerland).

All tested sealers were mixed according to manufacturer instructions. Sample discs (5-mm diameter by 2-mm height) were prepared under aseptic conditions in metallic moulds. The ratio between the surface of the sample and the volume of the medium was 6 cm^2/ml, in accordance with ISO guidelines.

Sample discs were then sterilized by ultraviolet irradiation for 15 minutes, followed by a 48h incubation at 37ºC to achieve complete setting.

In order to mimic the clinical conditions in which the endodontic sealer contacts with the periapical tissues present in the apical region of the root, the cells selected for this experience were human cells from periodontal ligament. Cells were incubated with different eluates according to ISO 10993-5. The vehicle for extraction was prepared with the same composition as culture media, specifically, Dulbecco Modified Eagle Medium (DMEM) (Gibco Invitrogene) supplemented with 10% fetal bovine serum (FBS) (Gibco Invitrogen), 2mM L-glutamine (Gibco Invitrogene) and 100 units/ml penicillin (Gibco Invitrogene) and 100mg/ml streptomycin (Gibco Invitrogene).

Eluate extraction was performed in sterilised recipients according to ISO 10993-12, for a period of 24h at 37ºC in a humid atmosphere containing 5% CO_2. Next, collected eluates were centrifuged at 1200g for 3 minutes to remove insoluble particles. These were then prepared as undiluted (1/1) and 1/2 dilution and 1/4 dilution using culture media.

Cell Viability Assay

Metabolic activity of the periodontal ligament cells and possible effects on cell proliferation were tested for the four sealers.

The test used to evaluate the previous parameters was the Alamar Blue® assay (Alamar Blue Cell Viability Reagent; Biozol, Eching, Germany), which is based on detection of metabolic cell activity.

The Alamar Blue® reagent (resazurin) is a non-toxic cell permeable compound that is blue in colour and virtually non-fluorescent. After contacting with the cells, since viable cells continuously metabolise this reagent, the compound is reduced to resorufin, which is highly fluorescent and red in colour. To assess metabolic activity, the cells were incubated in a 24-

well plate (10,000 cells/well) under standard conditions with 10% Alamar Blue®, following incubation with 1/1, 1/2 and 1/4 eluate dilutions.
Fluorescence was measured at 24h, 48h and 72h at a wavelength of 570nm and 600nm with a fluorescence reader (Synergy HT-Reader, Biotek, Winooski, VT, USA). Negative controls were cells incubated with standard culture media.

Solubility Test

Sealant discs were weighted before and after the extraction procedure. Briefly, following the 24h contact with culture media, discs were dried in an air incubator and final weight was determined.

Statistical Analysis

Number of replicates is indicated in the text and figure legends. Results are expressed and means ± standard error of the mean. Statistical analysis was performed using Prism (GraphPad Software, CA). Preliminary analysis revealed data as normally distributed using Kolmogorov-Smirnov test. Results were then analysed by Repeated measures Two-way ANOVA with Tukey *post hoc* test; Repeated measures One-way ANOVA with Dunnetts *post hoc* test, and one-sample T-test against 100% as the hypothetical value for no change. Statistical significance was set as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

RESULTS

Cell Viability Assay

As mentioned previously, the Alamar Blue® assay gives information on cellular metabolism and viability. In our study, we evaluated these parameters at a 24h, 48h and 72h interval using different concentrations of sealer extracts (undiluted, 1:2 and 1:4 dilutions).

We present the effect of four different sealer eluates on cellular viability over the time when normalized relative to control.

The results presented in **Figure 5** and **Table III** show the biocompatibility of different concentrations of the epoxy resin-based sealer AH Plus® in comparison with the control group. At the 24h period, we observed that the eluates from this sealer were highly cytotoxic regardless of the dilution used. At the 48h and 72h time points, cell viability displays a trend for decreased cytotoxicity with decreased concentrations, consistent with a dose-response effect. At the highest concentration, there is also an exposure effect since viability decreases progressively over time. The overall effect of AH Plus® suggest a very high cytotoxicity across all time points and concentrations tested, ranging from 1,2% to 14,7% of control. Cellular morphology and cellular growth were grossly altered under microscopic examination **Figure 6**, and cellular debri was observed in all conditions where cells were incubated with AH Plus®' eluates.

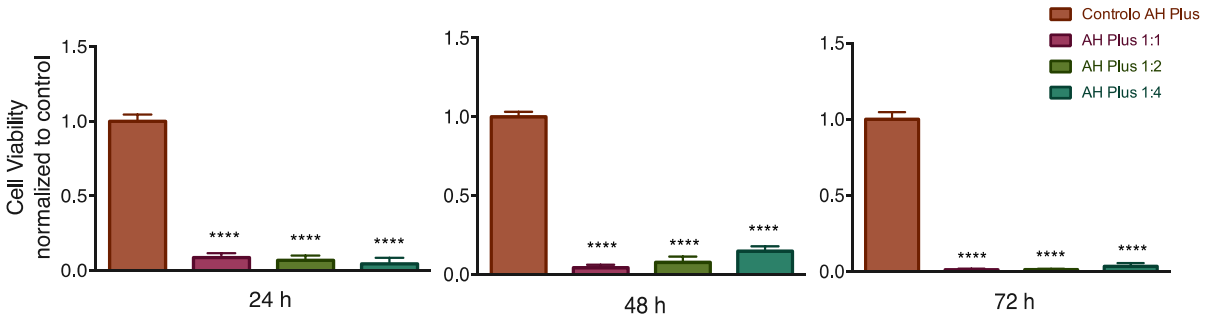

Figure 5. *AH Plus® effect on cell viability over time and at different concentrations.* Cellular viability was assessed at 24h (left), 48h (middle) and 72h (right), at 1:1, 1:2 and 1:4 concentrations. Values are presented as means ± SEM, **** p < 0,0001, One-Way ANOVA with Dunnett post hoc test, n=5 for all conditions.

Table III. AH Plus® effect on cellular viability.

	Cell Viability (%)	SEM	N	Significance Value
	24H			
1:1	8,6	3	5	p<0,0001
1:2	6,9	3,2	5	p<0,0001
1:4	4,5	4	5	p<0,0001
	48H			
1:1	4,2	1,9	5	p<0,0001
1:2	7,6	3,8	5	p<0,0001
1:4	14,7	3,2	5	p<0,0001
	72H			
1:1	1,2	0,7	5	p<0,0001
1:2	1,4	0,5	5	p<0,0001
1:4	3,6	2,2	5	p<0,0001

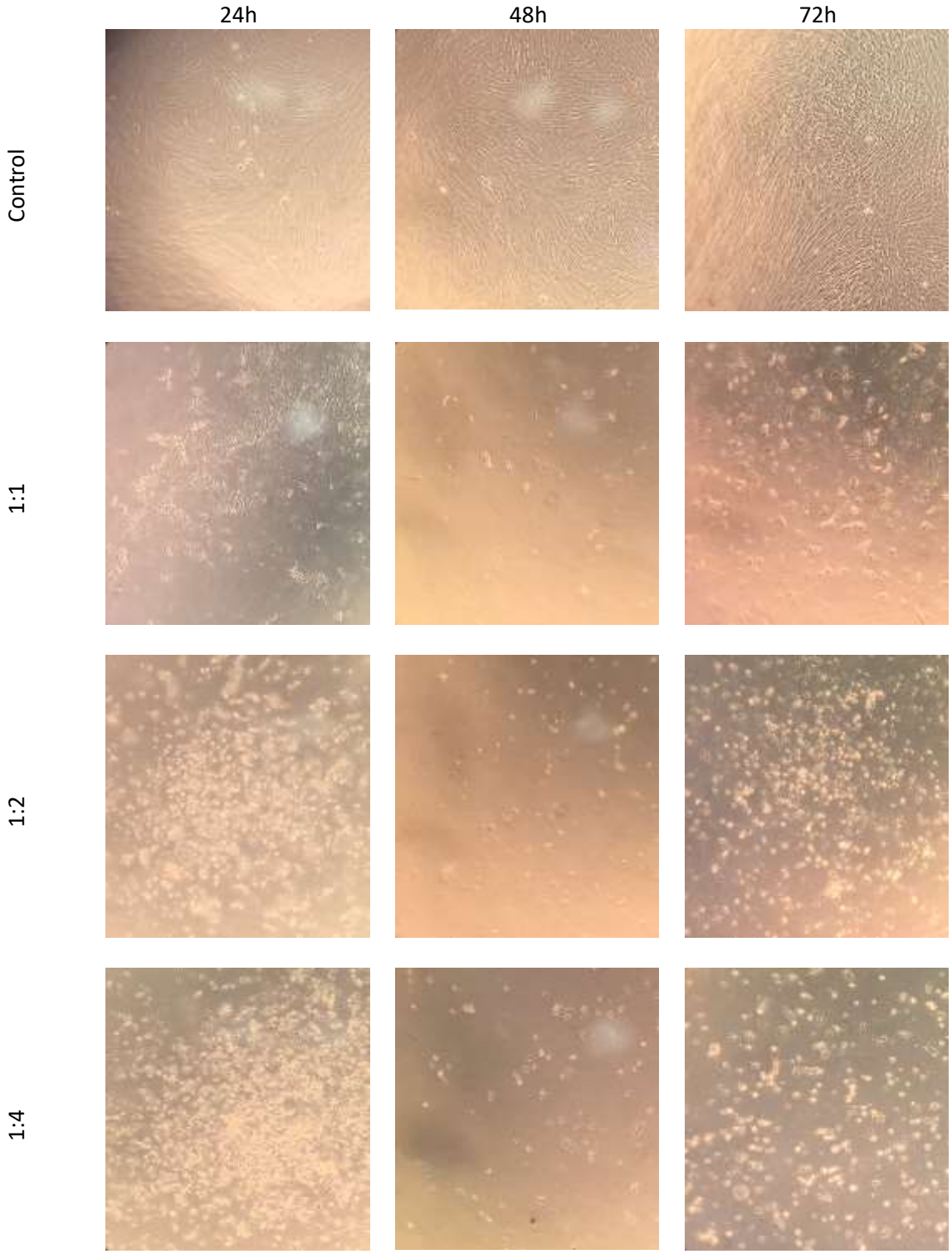

***Figure 6**. Morphology of cells incubated with AH Plus® over time and at different sealer concentrations.*

Regarding silicone-based sealers, one of the materials evaluated in this study was Guttaflow®2. In the 24h and 48h period, as shown in **Figure 7** and **Table IV**, no significant changes in cell viability was observed and mean cellular viability was mostly above 90%. However, in 1:2 concentrations at the 72h time point we observed a small decrease in cellular viability, nevertheless, we did not observe a dose-response effect and cellular viability remained very high (above 80%) in all tested conditions. Additionally, cellular morphology and cellular growth (**Figure 8**) remained unaltered under microscopic examination.

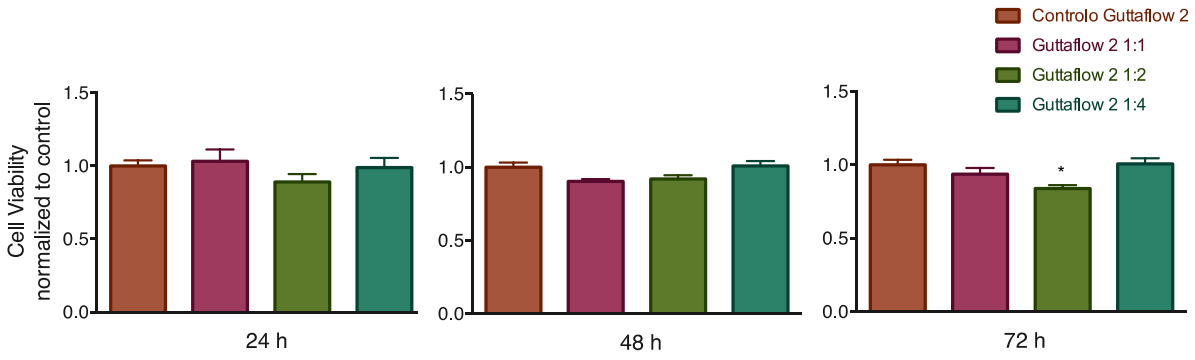

Figure 7 *Guttaflow®2 effect on cell viability over time and at different concentrations.* Cellular viability was assessed at 24h (left), 48h (middle) and 72h (right), at 1:1, 1:2 and 1:4 concentrations. Values are presented as means ± SEM, * p < 0,05, One-Way ANOVA with Dunnett post hoc test, n=5.

Table IV. Guttaflow®2 effect on cellular viability.

	Cell Viability (%)	SEM	N	Significance Value
	24H			
1:1	103,2	8,1	5	NS
1:2	89,1	5,4	5	NS
1:4	98,9	6,5	5	NS
	48H			
1:1	90,3	1,6	5	NS
1:2	92	2,5	5	NS
1:4	100,9	3,3	5	NS
	72H			
1:1	93,7	4,2	5	NS
1:2	83,9	2,2	5	p<0,05
1:4	100,7	3,8	5	NS

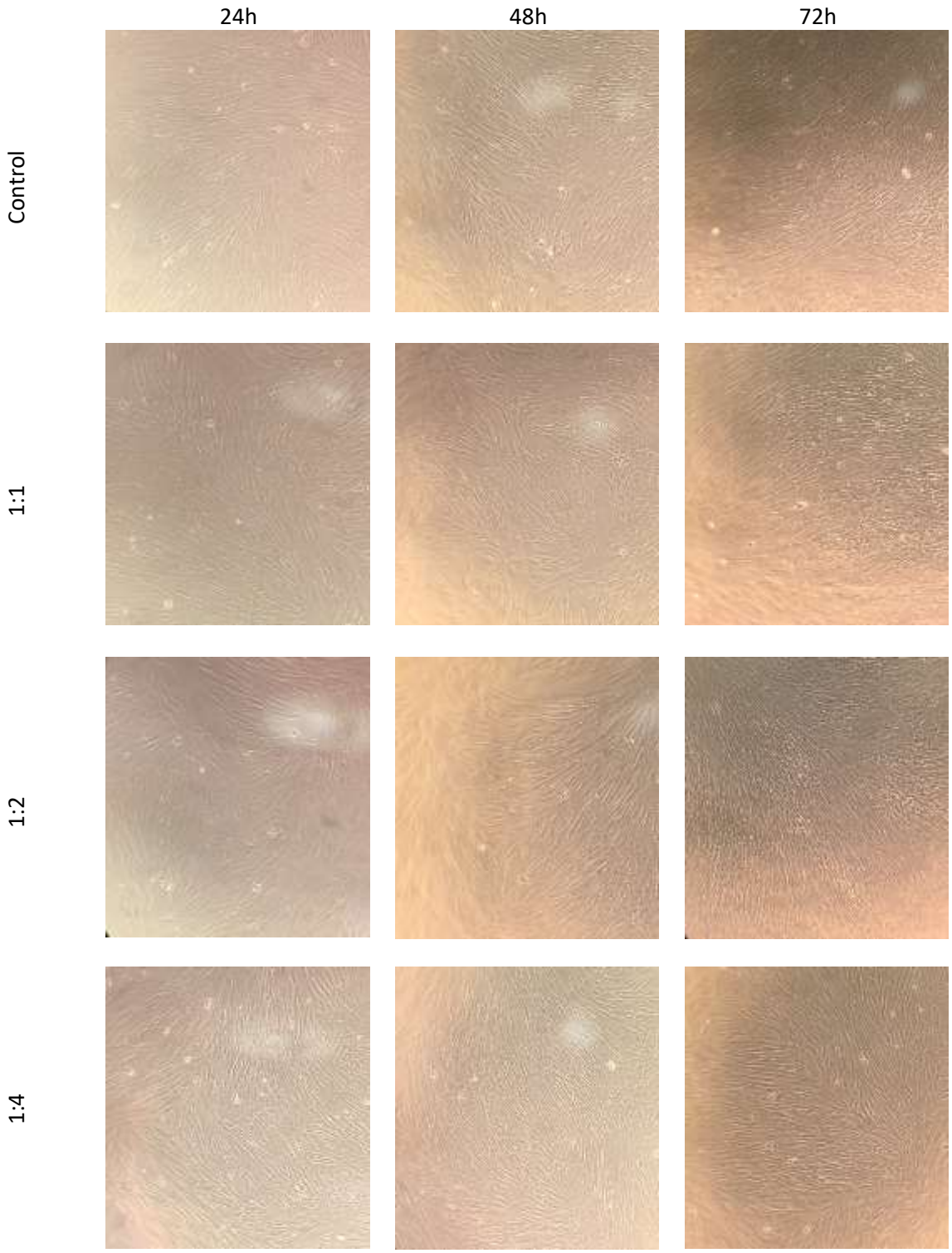

***Figure 8**. Morphology of cells incubated with Guttaflow®2 over time and at different sealer concentrations.*

The second silicone-based sealer tested was Guttaflow®Bioseal, the evolution of Guttaflow®2. The results presented in **Figure 9** and **Table V** show that the viability of cells exposed to Guttaflow®Bioseal decreases with increasing concentrations across all time points.

Additionally, both at 24h, 48h and 72h, we observed an exposure effect as cellular viability decreases over time for the same eluate concentrations. Gross alterations to cellular morphology and cellular debris were not observed under microscopic evaluation (**Figure 10**).

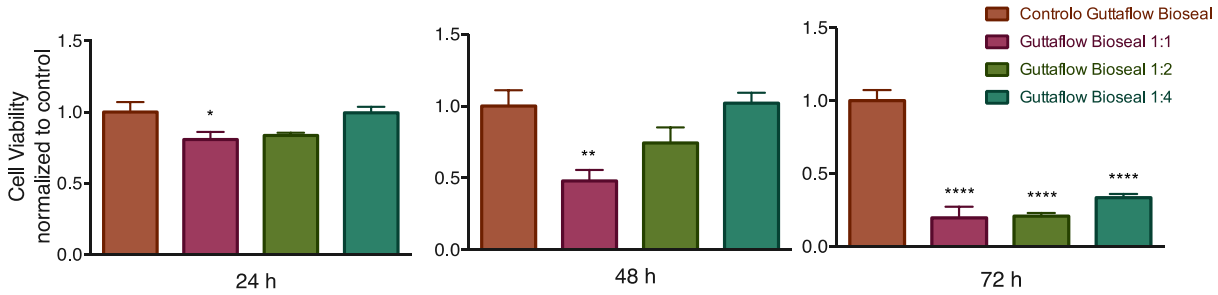

Figure 9. *Guttaflow®Bioseal effect on cell viability over time and at different concentrations.* Cellular viability was assessed at 24h (left), 48h (middle) and 72h (right), at 1:1, 1:2 and 1:4 concentrations. Values are presented as means ± SEM, * p < 0,05; **p < 0,01; **** p < 0,0001, One-Way ANOVA with Dunnett post hoc test, n=5.

Table V. Guttaflow®Bioseal effect on cellular viability.

	Cell Viability (%)	SEM	N	Significance Value
	24H			
1:1	80,8	5,3	5	p<0,05
1:2	83,6	1,9	5	p<0,05
1:4	99,6	4,2	5	NS
	48H			
1:1	47,9	7,7	5	NS
1:2	74,4	11	5	p<0,01
1:4	102,1	7,2	5	NS
	72H			
1:1	19,6	7,6	5	p<0,0001
1:2	20,7	2,2	5	p<0,0001
1:4	33,4	2,6	5	p<0,0001

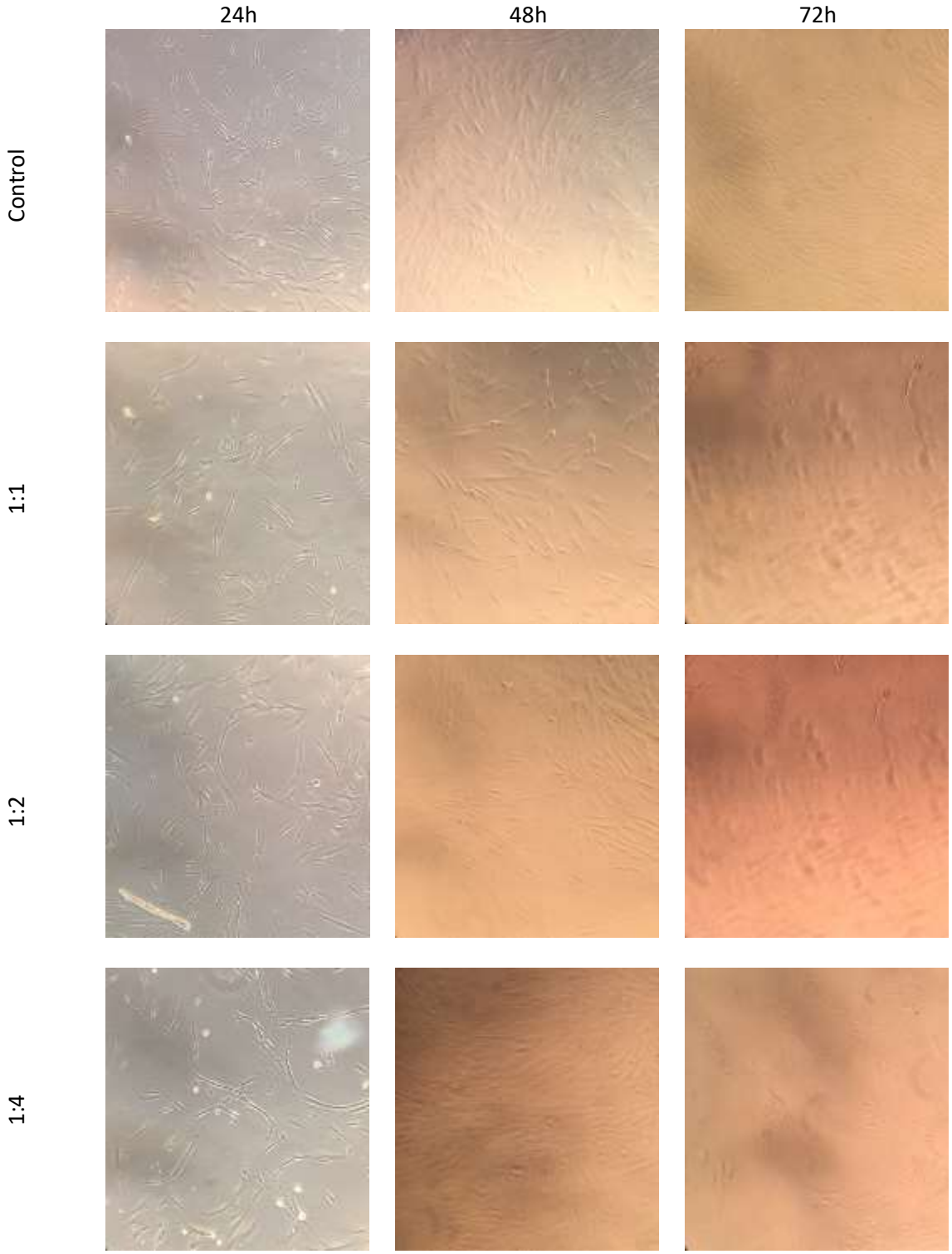

***Figure 10**. Morphology of the cells incubated with Guttaflow®Bioseal over time and at different sealer concentrations*

The fourth and last material tested was BioRoot™RCS which is a new bioactive tricalcium silicate-based sealer as mentioned previously.

As shown in **Figure 11** and **Table VI**, at 24h, 48h and 72h time points, this material presents a decrease in cell viability with the increase of sealer concentration, and an exposure effect, where prolonged presence of the eluates decreases viability over time when the same concentration is maintained. Cellular debri were found in most time points in accordance with decreased cellular viability taken from the Alamar Blue® assay (**Figure 12**).

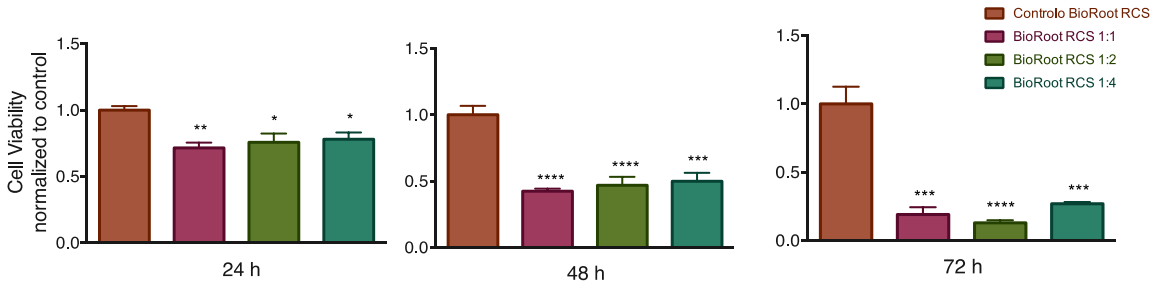

Figure 11 *Bioroot™RCS effect on cell viability over time and at different concentrations.* Cellular viability was assessed at 24h (left), 48h (middle) and 72h (right), at 1:1, 1:2 and 1:4 concentrations. Values are presented as means ± SEM, * p < 0,05; **p < 0,01; *** p < 0,001; ****p < 0,0001 One-Way ANOVA with Dunnett post hoc test, n=4.

Table VI. BioRoot™RCS effect on cell viability over time with different concentrations.

	Cell Viability (%)	SEM	N	Significance Value
	24H			
1:1	71,6	3,9	4	p<0,01
1:2	75,7	6,7	4	p<0,05
1:4	78	5	4	p<0,05
	48H			
1:1	42,5	2	4	p<0,001
1:2	47	6,3	4	p<0,001
1:4	50	6,4	4	p<0,001
	72H			
1:1	19,1	5,4	4	p<0,001
1:2	12,9	1,9	4	p<0,0001
1:4	27,1	1,2	4	p<0,001

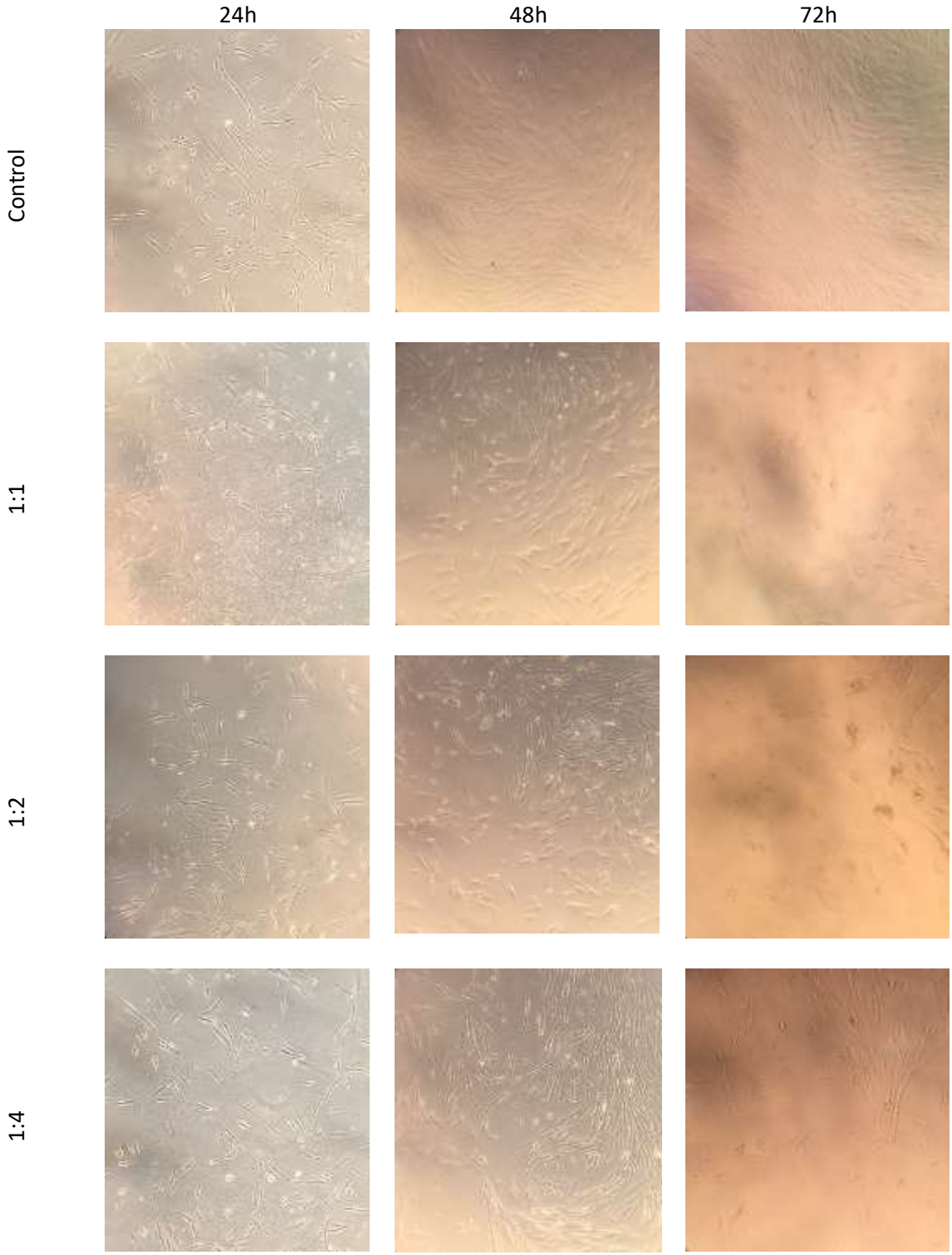

***Figure 12**. Morphology of the cells incubated with BioRoot™RCS over time and at different sealer concentrations.*

In **Figure 13** we plot the profile of cell viability at 24h, 48h and 72h time in 1:1 concentration for each material, normalized to control. It is notable that AH Plus® sealer is the material that shows the lowest biocompatibility.

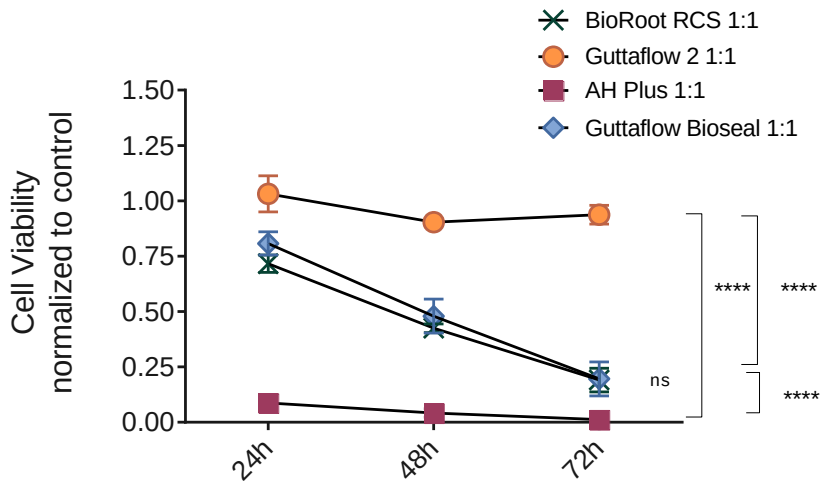

Figure 13 *Sealant effect on cell viability over time and at 1:1 concentration.* Cellular viability was assessed at 24h, 48h and 72h, at 1:1, concentrations. Values are presented as means ± SEM, ****p < 0,0001 Repeated measures Two-Way ANOVA with Tukey post hoc test, n=5-4.

When comparing AH Plus® cell viability results with the silicone-based sealers, Guttaflow®2 and Guttaflow®Bioseal, the first material presents values of 4,7% of cell viability (average value over the 3 time points) for this concentration, whereas the second has a 95,7% and the third 49,4% of cell viability for the same concentration, which suggests that AH Plus® is approximately 20 times more cytotoxic than Guttaflow®2 and 10 times more toxic than Guttaflow®Bioseal.

Comparing AH Plus® with the calcium silicate-based sealer BioRoot™RCS viability, we observed that BioRoot™RCS presents a value of 44,4% (average value over the 3 time points) for the same concentration which means that AH Plus® is around 10 times more cytotoxic than BioRoot™RCS.

The calcium silicate-based sealer BioRoot™RCS was the second most cytotoxic material. Comparing BioRoot™RCS with Guttaflow®2 and Guttaflow®Bioseal, we observed that viability values between BioRoot™RCS and Guttaflow®Bioseal are very similar to each other, whereas Guttaflow®2 is approximately 2 times less cytotoxic than the calcium-silicate based material.

The silicone-based sealers Guttaflow®2 and Guttaflow®Bioseal are the least cytotoxic. However, Guttaflow®2 is around 2 times less cytotoxic than Guttaflow®Bioseal.

Cell viability at 24h, 48h and 72h time in 1:2 concentration is depicted in **Figure 14**, in which we can observe similarities with the no dilution data.

Our results again show that AH Plus® sealer is the material that shows the lowest biocompatibility, presenting an average value of 5,3% over time, Guttaflow®2 88,3% and Guttaflow®Bioseal 59,6% cell viability for the same concentrations, suggesting that AH Plus® is approximalty 17 times more cytotoxic than Guttaflow®2 and 10 times cytotoxic more than Guttaflow®Bioseal.

The calcium silicate-based sealer BioRoot™RCS is the second most cytotoxic, though still less than AH Plus®, presenting average 45,2% cell viability, being around 9 times less cytotoxic than the epoxy resin-based sealer.

In this case, the calcium silicate-based sealer BioRoot™RCS is approximately 2 times more cytotoxic than Guttaflow®2. The silicone-based sealers Guttaflow®2 and Guttaflow®Bioseal are again the least cytotoxic, though there is a significant difference between their effects.

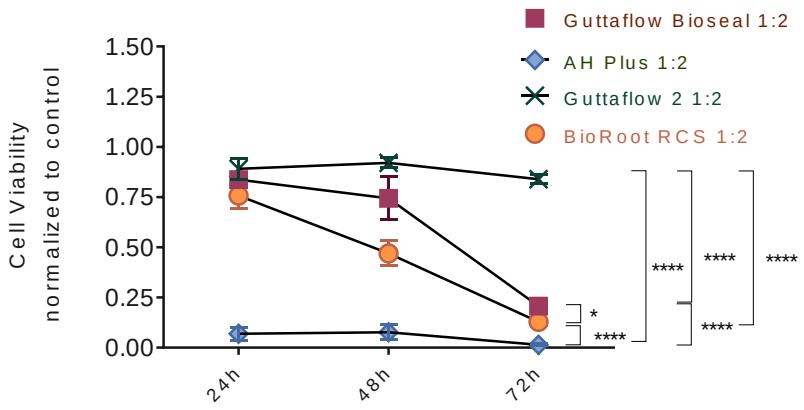

Figure 14 *Sealant effect on cell viability over time and at 1:2 concentration.* Cellular viability was assessed at 24h, 48h and 72h, at 1:1, concentrations. . Values are presented as means ± SEM, *p < 0.05; ****p < 0,0001 Repeated measures Two-Way ANOVA with Tukey post hoc test, n=5-4.

The final and lowest concentration tested in this project was 1:4 presented in **Figure 15**. Again, AH Plus® results are in accordance with the other two concentrations, showing the lowest cell viability. Comparing the epoxy-resin based sealer AH Plus® cell viability results with Guttaflow®2 and Guttaflow®Bioseal viability, we observed that AH Plus® presents a 7,6% average viability where Guttaflow®2 presents a value of 100% and Guttaflow®Bioseal 78,3% for the same concentration suggesting that AH Plus® is around 13 and 10 times more cytotoxic than Guttaflow®2 and Guttaflow®Bioseal, respectively.

AH Plus® is also around 7 times more cytotoxic than BioRoot™RCS, which presents an average cell viability value of 51,7%. Similarly, to the 1:1 and 1:2 concentrations, the calcium silicate-based sealer BioRoot™RCS is the second most cytotoxic and the silicone-based sealers Guttaflow®2 and Guttaflow®Bioseal are again the least cytotoxic.

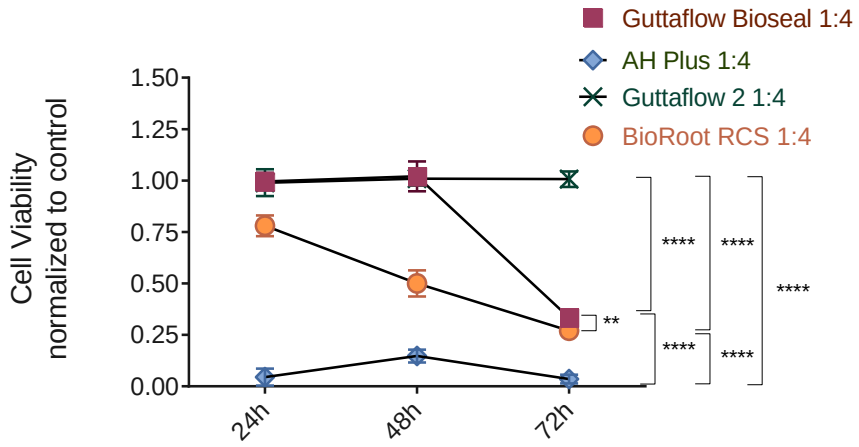

Figure 15 *Sealant effect on cell viability over time and at 1:4 concentration.* Cellular viability was assessed at 24h, 48h and 72h, at 1:1, concentrations. .Values are presented as means ± SEM, *p < 0.05; ****p < 0,0001 Repeated measures Two-Way ANOVA with Tukey post hoc test, n=5-4.

Solubility Test

We also tested solubility index of the four materials, we weighted the dry sealant disks before and after eluate extraction. Concerning solubility, the only material that showed a statistically significant ($p<0,05$) decrease was BioRoot™RCS, which presented a final weight percentage of 94,0%, indicating a weight loss of 6,0%. However, the presence of any insoluble material in the eluate suspension was removed via high speed centrifugation and pelleting of insoluble particles. Therefore, our study mainly addresses the cytotoxic effects of solubilized material.

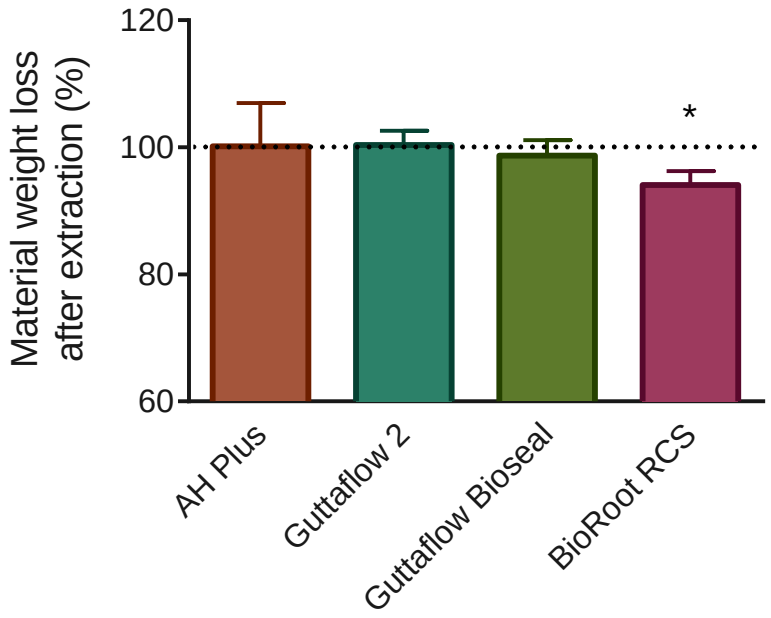

Figure 16 *Material weight loss following eluate extraction in culture media over 24h*. Values are presented as means ± SEM *p < 0.05, one sample t-test against hypothetical value of 100%.

4. Discussion

DISCUSSION

Regarding cell viability and cytotoxicity, it is of extreme significance that root canal sealers have good performance (25). A crucial aspect when developing new materials for clinical application is the evaluation of their cytotoxicity in order to minimize possible adverse effects (2,14). There are several testing methods to analyse possible adverse effects of endodontic sealers, such as cell cultures (2,5,7), animal experiments and clinical trials (2). It is of utmost importance to note that although some materials present cytotoxic effects *in vitro* they may not occur *in vivo* because of the different conditions of the milieu (14). In the present study we used a classic cell model to determine the biocompatibility of four endodontic sealers.

The presence of human periodontal ligament cells in periapical tissues (22) associated with the fact that there may be sealer extrusion during endodontic treatment, leads to the necessity of evaluating a sealer cytocompatibility. Furthermore, we used cells of the periodontal ligament due to the fact that these cells are the predominant cells with potential contact with endodontic sealers and the major elements of connective tissue. Considering that human periodontal ligament stem cells (hPDLSCs) are another well-established *in vitro* model to evaluate cell differentiation and viability (23), it would be interesting to evaluate cytotoxicity in these cells, as well as effects on differentiation and stem cell proliferation.

The biocompatibility test used was the Alamar Blue® Assay which is a reliable test based on the detection of metabolic cell activity, and consequently indicates cell survival rates.

This method has some limitations, such as the fact that it only measures cell metabolic activity. As such, cells can be viable and present only low metabolic activity. Therefore, it is necessary to validate the Alamar Blue® results using other methods, such as electronic microscopy observation (23). In our case we performed microscopic observation and could concluded that decreased metabolic activity correlated well with increased cell death (presence of debris) and decreased cell proliferation (lower cellular confluence).

Another important consideration in our study is that cells were not in direct contact with the sealer but with its eluates. This factor is particularly important since direct contact could present many disadvantages such as result in false high cytotoxic results. Also, when in direct contact, accurate inset and distribution of an equal quantity of root canal sealer is problematic (26).

The materials tested in our project were an epoxy resin-based sealer – AH Plus®, two silicone-based sealers – Guttaflow®2 and Guttaflow®Bioseal and a calcium silicate-based sealer – BioRoot™RCS.

We found that the epoxy resin-based sealer AH Plus® showed the highest level of cytotoxicity independent of the concentration or observed periods comparing with the other tested materials, which is in agreement with other reports in the literature (25). Silicone-based

Guttaflow®2 presented the best cell viability values, and was the most biocompatible root canal sealer in our panel.

Our results also show that eluates extracted from AH Plus®**,** Guttaflow®2, Guttaflow®Bioseal and BioRoot™RCS presented dose and exposure-dependent effects on biological response of cells from the periodontal ligament. **Figures 5, 7, 9** and **11** represent cell viability over time with different concentrations for all tested sealers separately. AH Plus® presented the worst biocompatibility results for all tested concentrations and it was concluded that its cytotoxicity increases with the increase of concentration. This pattern was also valid for Guttaflow® Bioseal and BioRoot™RCS whereas Guttaflow®2 only presented a significant cell viability decrease at 72h for 1:2 concentration. The overall effects of AH Plus® suggest a very high cytotoxicity across all time points and concentrations tested whereas Guttaflow®2 presented high biocompatibility in all conditions.

All four tested materials were also compared concerning cell viability for a specific concentration over time.

As it was concluded in previous studies, such as the one conducted by Collado-González *et al.*, 2017, the silicone based sealers showed the best cytocompatibility results comparing to an epoxy resin-based and a calcium silicate-based sealer (18,23). However, in the study previously mentioned, Guttaflow® Bioseal showed better results than Guttaflow®2. That difference may be due to the fact that the tests were conducted in human periodontal ligament stem cells (hPDLSCs), and that cell viability was tested using MTT Assay. Another study also showing better results concerning cell viability comparing AH Plus® with Guttaflow®2 was conducted by Silva *et al.*, 2015 in which the silicone-based displayed higher levels of cytocompatibility than the epoxy resin-based sealer, as was also seen in our assay (27).

In the present study, and in agreement with other previous reports (9,28,29) such as the one conducted by Bouillaguet *et al.*, 2006, in which Guttaflow® (the predecessor of Guttaflow®2) and AH Plus®' cytotoxicity were first compared, the results indicate that the silicone-based was significantly less cytotoxic than the epoxy-resin based sealer. Nevertheless, it was observed that the Guttaflow® biocompatibility decreased with time, meaning the toxic response increased throughout the studied period as was also shown in other studies (6,29). It is believed that the reason for the cytotoxicity growth is the release of silver particles present in this sealer as a preservative (29). Besides, the existence of small voids in the core of Guttaflow® may allow the releasing of unreacted components such as porosities (6,30,31) which may contribute to the growing cytotoxicity over time.

Regarding calcium silicate compared to epoxy resin-based, Silva et al. 2016, concluded that AH Plus® presented higher cytotoxic effects than EndoSequence®BC Sealer™, which is a bioceramic material calcium silicate-based, comparable to BioRoot™RCS. Our results are in accordance with this study.

Another parameter tested in this project was the solubility of all four root canal sealers, outlined in **Figure 16**. The values obtained lead us to conclude that the only sealer that presented a significant dissolution was BioRoot™RCS, which is in accordance with previous studies (10). Prüllage et al. 2016, previously evaluated solubility for three sealers, two of which are in our project (AH Plus® and BioRoot™RCS). In their study, solubility was determined after the immersion in destilled water and PBS for seven different time periods (1min, 20min, 2h, 24h, 72h, 14d, 28d). BioRoot™RCS presented the highest solubility value for the period evaluated in our study all the tested periods comparing to AH Plus®, which is in accordance with our results, although no silicone-based material was tested by Prüllage et al. 2016. It should be considered that sealer samples' weight differences may be a result of disintegration processes and not necessarily a result of dissolution (32,33) and that water uptake, can increase the material weight, compensating for dissolved material (32,34). To mitigate these two factors, we standardized our materials in disk-shaped moulds with defined volume to area ratio, and we performed weight measurements after drying material extensively.

Although solubility is considered deleterious for a root canal sealer, in some cases such as the calcium silicate-based sealers it may be an advantage. These sealers form calcium during setting, and release OH^- and Ca^{2+} during dissolution (10). This fact promotes bioactivity since calcium ions react with the phosphate present in the PBS buffer or body liquids to precipitate hydroxyapatite (35). Therefore, BioRoot™RCS probably stimulates bioactivity (16,36) and solubility may be necessary for this process. Besides, there is a correlation between a calcium silicate-containing sealer's high solubility and their antimicrobial efficiency (37).

4. Conclusion

CONCLUSION

The main goal of the present study was to evaluate four sealers cytotoxicity and solubility to conclude which ones would be the most advantageous to use in the sealing process of the endodontic treatment.

All of the tested material presented significant cytotoxicity with exception for Guttaflow®2. From these results we can conclude that the best cell viability values correspond to the silicone-based sealers (Guttaflow®2 and Guttaflow® Bioseal), followed by the calcium silicate-based (BioRoot™RCS) and the worst results correspond to the epoxy resin-based sealer (AH Plus®). Concerning solubility, BioRoot™RCS was the only root canal sealer that presented statistically significant weight loss, suggesting it is the most soluble material. However, as mentioned previously, this is not necessarily deleterious since it may induce bioactivity.

4. Bibliography

BIBLIOGRAPHY

1. Khalil I, Naaman A, Camilleri J. Properties of Tricalcium Silicate Sealers. J Endod. 2016;42(10):1529–35.
2. Silva LAB, Azevedo LU, Consolaro A, Barnett F, Xu Y, Battaglino RA, et al. Novel endodontic sealers induce cell cytotoxicity and apoptosis in a dose-dependent behavior and favorable response in mice subcutaneous tissue. Clin Oral Investig [Internet]. 2017; Available from: http://link.springer.com/10.1007/s00784-017-2087-1
3. Bin C V., Valera MC, Camargo SEA, Rabelo SB, Silva GO, Balducci I, et al. Cytotoxicity and genotoxicity of root canal sealers based on mineral trioxide aggregate. J Endod [Internet]. 2012;38(4):495–500. Available from: http://dx.doi.org/10.1016/j.joen.2011.11.003
4. Collado-González M, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Álvarez-Muro T, Lozano A, et al. Cytotoxicity and bioactivity of various pulpotomy materials on stem cells from human exfoliated primary teeth. Int Endod J. 2017;0–2.
5. Yilmaz Z, Dogan AL, Ozdemir O, Serper A. Evaluation of the cytotoxicity of different root canal sealers on L929 cell line by MTT assay. Dent Mater J [Internet]. 2012;31(6):1028–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23207211
6. KonjhodzicPrcic A, Jakupovic S, Brankovic L, Vukovic A. In Vitro Comparison of Cytotoxicity of Four Root Canal Sealers on Human Gingival Fibroblasts. Med Arch [Internet]. 2015;69(1):24. Available from: http://www.scopemed.org/?mno=180717
7. Khashaba RM, Chutkan NB, Borke JL. Comparative study of biocompatibility of newly developed calcium phosphate-based root canal sealers on fibroblasts derived from primary human gingiva and a mouse L929 cell line. Int Endod J. 2009;42(8):711–8.
8. COHEN, S. & HARGREAVES K. Pathways of the Pulp. 10th ed. Mosby, editor. 2011
9. KonjhodzicPrcic A, Jakupovic S, Brankovic L, Vukovic A. Evaluation of Biocompatibility of Root Canal Sealers on L929 Fibroblasts with Multiscan EX Spectrophotometer. Acta Inform Medica [Internet]. 2015;23(3):135. Available from: http://www.scopemed.org/fulltextpdf.php?mno=189736
10. Prüllage RK, Urban K, Schäfer E, Dammaschke T. Material Properties of a Tricalcium Silicate-containing, a Mineral Trioxide Aggregate-containing, and an Epoxy Resin-based Root Canal Sealer. J Endod. 2016;42(12):1784–8.
11. CAMPS J, ABOUT I. Cytotoxicity Testing of Endodontic Sealers: A New Method. J Endod [Internet]. 2003;29(9):583–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0099239905603287

12. Brackett MG, Lewis JB, Kious AR, Messer RLW, Lockwood PE, Brackett WW, et al. Cytotoxicity of endodontic sealers after one year of aging in vitro. J Biomed Mater Res - Part B Appl Biomater. 2012;100 B(7):1729–35.
13. Zhou HM, Du TF, Shen Y, Wang ZJ, Zheng YF, Haapasalo M. In Vitro Cytotoxicity of Calcium Silicate-containing Endodontic Sealers. J Endod [Internet]. 2015;41(1):56–61. Available from: http://dx.doi.org/10.1016/j.joen.2014.09.012
14. Silva EJNL, de Carvalho NK, Ronconi CT, De-Deus G, Zuolo ML, Zaia AA. Cytotoxicity profile of endodontic sealers provided by 3D cell culture experimental model. Braz Dent J. 2016;27(6):652–6.
15. SCHWARZE T, FIEDLER I, LEYHAUSEN G, GEURTSEN W. The Cellular Compatibility of Five Endodontic Sealers during the Setting Period. J Endod [Internet]. 2002;28(11):784–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S009923990560475X
16. Camps J, Jeanneau C, Laurent P. Bioactivity of a Calcium Silicate – based Endodontic Cement (BioRoot RCS): Interactions with Human Periodontal Ligament Cells In Vitro. J Endod [Internet]. 2015;41(9):5–9. Available from: http://dx.doi.org/10.1016/j.joen.2015.04.011
17. Rodríguez-Lozano FJ, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Forner L, Moraleda JM. Evaluation of cytocompatibility of calcium silicate-based endodontic sealers and their effects on the biological responses of mesenchymal dental stem cells. Int Endod J. 2017;50(1):67–76.
18. SILVA EJNL da, SANTOS CC, ZAIA AA. Long-term cytotoxic effects of contemporary root canal sealers. J Appl Oral Sci [Internet]. 2013;21(1):43–7. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1678-77572013000100043&lng=en&nrm=iso&tlng=en
19. Parirokh M, Forghani FR, Paseban H, Asgary S, Askarifard S, Mahani SE. Cytotoxicity of two resin-based sealers and a fluoride varnish on human gingival fibroblasts. Iran Endod J. 2015;10(2):89–92.
20. Vivan RR, Zapata RO, Zeferino MA, Bramante CM, Bernardineli N, Garcia RB, et al. Evaluation of the physical and chemical properties of two commercial and three experimental root-end filling materials. Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology [Internet]. 2010;110(2):250–6. Available from: http://dx.doi.org/10.1016/j.tripleo.2010.04.021
21. Ørstavik D. Materials used for root canal obturation : technical , biological and clinical testing. Endod Top. 2005;12(3):25–38.
22. Collado-González M, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Lozano A, Forner L, et al. Biocompatibility of three new calcium silicate-based

endodontic sealers on human periodontal ligament stem cells. Int Endod J. 2016;

23. Collado-González M, Tomás-Catalá CJ, Oñate-Sánchez RE, Moraleda JM, Rodríguez-Lozano FJ. Cytotoxicity of GuttaFlow Bioseal, GuttaFlow2, MTA Fillapex, and AH Plus on Human Periodontal Ligament Stem Cells. J Endod. 2017;43(5):816–22.
24. Dimitrova-Nakov S, Uzunoglu E, Ardila-Osorio H, Baudry A, Richard G, Kellermann O, et al. In vitro bioactivity of Bioroot™ RCS, via A4 mouse pulpal stem cells. Dent Mater [Internet]. 2015;31(11):1290–7. Available from: http://dx.doi.org/10.1016/j.dental.2015.08.163
25. Silva Almeida LH, Moraes RR, Morgental RD, Pappen FG. Are Premixed Calcium Silicate–based Endodontic Sealers Comparable to Conventional Materials? A Systematic Review of In Vitro Studies. J Endod [Internet]. 2017;43(4):527–35. Available from: http://dx.doi.org/10.1016/j.joen.2016.11.019
26. AZAR N, HEIDARI M, BAHRAMI Z, SHOKRI F. In Vitro Cytotoxicity of a New Epoxy Resin Root Canal Sealer. J Endod [Internet]. 2000;26(8):462–5. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0099239905609193
27. Silva EJNL, Neves AA, De-Deus G, Accorsi-Mendonça T, Moraes AP, Valentim RM, et al. Cytotoxicity and gelatinolytic activity of a new silicon-based endodontic sealer. J Appl Biomater Funct Mater [Internet]. 2015;13(4):0–0. Available from: http://www.jabfm.com/article/cytotoxicity-and-gelatinolytic-activity-of-a-new-silicon-based-endodontic-sealer
28. Willershausen I, Callaway A, Briseño B, Willershausen B. In vitro analysis of the cytotoxicity and the antimicrobial effect of four endodontic sealers. Head Face Med [Internet]. 2011;7(1):15. Available from: http://head-face-med.biomedcentral.com/articles/10.1186/1746-160X-7-15
29. Bouillaguet S, Wataha JC, Tay FR, Brackett MG, Lockwood PE. Initial In Vitro Biological Response to Contemporary Endodontic Sealers. J Endod. 2006;32(10):989–92.
30. Sjögren U, Sundqvist G, Nair PMR. Tissue reaction to gutta-percha particles of various sizes when implanted subcutaneously in guinea pigs. Eur J Oral Sci. 1995;103(5):313–21.
31. ElAyouti A, Achleithner C, Löst C, Weiger R. Homogeneity and adaptation of a new gutta-percha paste to root canal walls. J Endod. 2005;31(9):687–90.
32. ÖRSTAVIK D. Weight loss of endodontic sealers, cements and pastes in water. Eur J Oral Sci. 1983;91(4):316–9.
33. Wilson AD. Specification test for the solubility and disintegration of dental cements: a critical evaluation of its meaning. J Dent Res [Internet]. 1967;55(5):721–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1067288
34. Kazemi RB, Safavi KE, Spångberg LSW. Dimensional changes of endodontic sealers.

Oral Surgery, Oral Med Oral Pathol. 1993;76(6):766–71.

35. Sarkar NK, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I. Physicochemical basis of the biologic properties of mineral trioxide aggregate. J Endod [Internet]. 2005;31(2):97–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15671817

36. Camilleri J. Sealers and warm gutta-percha obturation techniques. J Endod [Internet]. 2015;41(1):72–8. Available from: http://dx.doi.org/10.1016/j.joen.2014.06.007

37. Faria-Júnior NB, Tanomaru-Filho M, Berbert FLC V, Guerreiro-Tanomaru JM. Antibiofilm activity, pH and solubility of endodontic sealers. Int Endod J. 2013;46(8):755–62.

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