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IZVORNI ZNANSTVENI RAD
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Citotoksičnost dvaju bioaktivnih materijala za punjenje korijenskih kanala

Cytotoxicity of Two Bioactive Root Canal Sealers

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Sažetak

Uvod: Svrha istraživanja bila je ispitati citotoksičnost dvaju različitih bioaktivnih materijala za punjenje korijenskih kanala temeljenih na mineral-trioksidnom agregatu MTA Fillapex (Angelus, Solucoes Odontologicas, Londrina, PR, Brazil) i biokeramici, Endosequence BC Sealer (Brasseler, Savannah, Georgia, SAD) u kulturi mišjih fibroblasta L929. **Materijali i postupci:** Mišji fibroblasti L929, dobiveni iz potkožnog veziva miševa linije C3Hf, uzgojeni su u plastičnim posudama za staničnu kulturu površine 75 cm^2 u inkubatoru na temperaturi od 37 ^{0}C, uz 5-posto CO_2 i 90 posto vlažnosti. Svježe zamiješani materijali – (0,1 g) Endosequence BC Sealer i MTA Fillapex – naneseni su na sterilne teflonske diskove promjera šest milimetara. Diskovi s materijalom i prazni teflonski diskovi koji su služili kao kontrola, stavljeni su u bunariće pločica za staničnu kulturu. Nakon inkubacije od jedan sat, šest, 20 i 24 sata, uklonjeni su teflonski diskovi i određen je broj živih stanica tripanskim modrilom u Neubaerovoj komorici. **Rezultati:** Promatranjem razlike između ispitivanih materijala i kontrolne skupine u pojedinim inkubacijskim razdobljima, dokazano je da punilo MTA u svim inkubacijskim razdobljima pokazuje statistički značajan pad broja živih stanica ($p \leq 0,05$), a kod punila BC pojavljuje se statistički značajna razlika od šestog do dvadeset i četvrtog sata inkubacije ($p \leq 0,05$). Punilo MTA u odnosu na punilo BC pokazalo je statistički značajan pad broja živih stanica samo nakon prvog i dvadesetog sata inkubacije ($p \leq 0,05$), a u ostalim inkubacijskim razdobljima ta razlika nije bila statistički značajna ($p \geq 0,05$). **Zaključak:** Punila MTA i Endosequence BC bila su citotoksična u kulturi mišjih fibroblasta.

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Ključne riječi
korijenski kanal, materijali za punjenje; aluminij, spojevi; kalcij, spojevi; toksikološki testovi

Uvod

Osnovna svrha endodontskog liječenja jest očistiti i proširiti korijenske kanale te postići njihovo dobro brtvljenje. Iako se za punjenje korijenskih kanala koristimo različitim materijalima, stalno se razvijaju novi kako bi se poboljšala njihova fizičko-mehanička i biološka svojstva. Nedavno su se u endodonciji počeli upotrebljavati materijali za punjenje korijenskih kanala temeljeni na mineral-trioksidnom agregatu (MTA) i biokeramici.

MTA je materijal za direktno prekrivanje zubne pulpe, apeksifikaciju, zatvaranje perforacija i za retrogradno punjenje korijenskih kanala (1 – 4). Njegove su glavne prednosti biokompatibilnost, dobro brtvljenje te poticanje regeneracije parodontnog ligamenta uz stvaranje kosti i cementa (5, 6). Osim toga, MTA se kemijski veže s tvrdim zubnim tkivima i stvara kristale hidroksilapatita u intersticijalnom sloju (7). MTA Fillapex (Angelus, Solucoes Odontologicas, Londrina, PR, Brazil) je bioaktivni materijal za punjenje korijenskih kanala koji čine dvije paste. Nakon miješanja materijala nasta-

Introduction

The primary goal of endodontic treatment is to clean and shape the root canal system to the greatest possible extent and to achieve a hermetic seal. Although different materials have been used as root canal sealers, new materials are constantly being developed in order to improve their physical-mechanical and biological properties. Recently, mineral trioxide aggregate (MTA) and bioceramic based root canal sealers have been introduced in endodontics.

MTA is an endodontic material currently used for pulp capping, apexification, perforation repair and root-end filling (1-4). The main advantages of this material are biocompatibility, good sealing ability and regeneration of periodontal ligament tissues with formation of bone and cementum (5, 6). Moreover, MTA forms a chemical bond to hard dental tissues through crystals of hydroxylapatite created at the interstitial layer (7). MTA Fillapex (Angelus, Solucoes Odontologicas, Londrina, PR, Brazil) is a bioactive root canal sealer consisting of two pastes. After mixing the material, a semi-

je polupropusna struktura u kojoj je raspršen MTA. Aktivnost MTA temelji se na propusnosti zamiješanog materijala (8), a visoki pH odgovoran je za njegovo antibakterijsko djelovanje (9).

Druga skupina bioaktivnih materijala koji se mogu koristiti za punjenje korijenskih kanala temeljena je na biokeramici. Jedan od njih je Endosequence BC Sealer (Brassler, Savannah, GA, SAD), kalcij-silikatni cement s česticama manjima od dva mikrona. Ovaj materijal ima prednosti poput visokoga pH (12,8) i hidrofilnih svojstava, a osim toga ne skuplja se i ne resorbira, izvrsno brtvi i brzo se stvrdnjava (10). Endosequence BC također može stvoriti hidroksiapatit zahvaljujući kalcij-silikatu koji u reakciji hidracije stvara kalcij-hidrat silikatni gel i kalcijev hidroksid (11). Kalcijev hidroksid ulazi u reakciju s fosfatnim ionima, čime se precipitiraju kristali hidroksiapatita i voda (11).

Materijali za punjenje korijenskih kanala dolaze u bliski doticaj s tkivom parodonta te se postupno otapaju kad su dulje izloženi vlažnom mediju (12). Pojedini spojevi u sastavu tih materijala mogu djelovati kao toksini i oštetiti stanice, što može rezultirati oštećenjem tkiva ili odgodom cijeljenja (13). Ispitivanje biokompatibilnosti materijala za punjenje korijenskih kanala važno je jer odgovor tkiva nakon njihova postavljanja može utjecati na uspjeh endodontskog liječenja (14). Za ispitivanje citotoksičnosti, mogu se upotrijebiti različite kulture životinjskih stanica da bi se dobili podatci o osnovnom biološkom ponašanju materijala (15). Stanične kulture, poput mišjih fibroblasta L929, korisne su kao model za ispitivanje zato što omogućuju velik broj jednakih stanica koje imaju očuvana i ista stanična obilježja pa su tako dobiveni rezultati pouzdani (16).

Svrha istraživanja bila je ispitati citotoksičnost dvaju različitih bioaktivnih materijala za punjenje korijenskih kanala temeljenih na mineral-trioksidnom agregatu i biokeramici u kulturi mišjih fibroblasta L929.

Materijali i postupci

Stanična linija

In vitro ispitivanje citotoksičnosti materijala provedeno je u kulturi mišjih fibroblasta L929 dobivenih iz potkožnog veziva miševa linije C3Hf. Stanice su uzgojene u plastičnim posudama za staničnu kulturu površine 75 cm^2 u inkubatoru na temperaturi od 37 °C, uz 5 posto CO_2 i 90 posto vlažnosti. Redovito su dohranjivane hranjivim medijem koji je sadržavao 10 posto Dulbecco's Modified Eaglea (DMEM – Gibco BRL-Life Technologies) kojemu je dodan 10-postotni fetalni teleći serum (FCS – Fetal Bovine Serum, Gibco Brazil), antibiotici (100 IU/ml penicilin i 50 µl/ml streptomicin) i 200 mM L-glutamina (Gibco Brazil).

Stanice su rasle u plastičnim posudama za staničnu kulturu 20 dana dok nisu prekrile njezinu cijelu površinu. Nakon toga su tripsinizacijom skupljene u sterilnu epruvetu te centrifugirane (1200 okr/4 min.), resuspendirane u novom mediju i tripanskim modrilom prebojene u Neubaerovoj komorici, a početni broj je podešen na 3 x 10^5 stanica po jednom mililitru.

permeable structure is formed with MTA dispersed throughout. Therefore, MTA activity is possible due to permeability of the mixed material (8) while an alkaline pH explains its extended antibacterial action (9).

Other types of bioactive materials proposed for root canal obturation are based on bioceramics. One of the new bioceramic root canal sealers is Endosequence BC Sealer (Brassler, Savannah, GA, USA), consisting of premixed calcium silicate cement with particle size less than 2 microns. This material has advantages including: high pH (12.8), hydrophilic properties, no shrinkage or resorption, excellent sealing ability and fast setting (10). Endosequence BC sealer has the ability to form hydroxyapatite due to calcium silicates in powder, which in a hydration reaction produces a calcium silicate hydrate gel and calcium hydroxide (11). The calcium hydroxide reacts with the phosphate ions to precipitate hydroxyapatite and water (11).

Root canal sealers come in close contact with the periodontal tissues and it has been shown that they constantly dissolve when exposed to an aqueous environment for extended periods (12). The components of these materials can act as toxins causing cellular injury that can lead to tissue damage or delay and impede tissue repair (13). Biocompatibility tests for root canal sealer are important since tissue response after their placement may influence the success of endodontic treatment (14). For cytotoxicity testing, different animal cell cultures can provide information on basic biological behavior of the material (15). Cell cultures, such as mouse L929 fibroblasts, are useful models since they provide large amounts of consistent cells and because of the fact that most cellular characteristics are maintained, providing reliable experimental results (16).

The aim of this study was to investigate the cytotoxicity of two different bioactive root canal sealers, one based on mineral trioxide aggregate and the other based on bioceramics, in culture of mouse L929 fibroblasts.

Materials and methods

Cell lines

Mouse L929 fibroblasts, obtained from subcutaneous connective tissue of mouse line C3Hf, were cultivated in plastic culture flasks, 75 cm^2 in diameter (Sterile Tissue Culture Flask) in an incubator at a temperature of 37°C, with 5% CO_2 and 90% humidity. The cells were constantly enriched with a nourishing medium: 10% Dulbecco's Modified Eagle medium (DMEM – Gibco BRL-Life Technologies) with 10% fetal bovine serum (FCS – Fetal Bovine Serum, Gibco BRL), antibiotics (100 IU/ml penicillin and 50 µl/ml streptomycin) and 200 mM L-glutamine (Gibco BRL). The cells grew in plastic cell culture flasks for 20 days until they covered the entire bottom of the flask. The cells were then trypsinized in sterile tubes and centrifuged (1200 rotary/min for 4 min). The cells were resuspended in a new medium and counted in Neubaer chamber using trypan blue and the number of cells was set at 3 x 10^5 cells/ml.

Ispitivanje citotoksičnosti

Svježe zamiješani materijali (0,1 g) Endosequence BC Sealer (Brasseler, Savannah, SAD), (tablica 1.) i MTA Fillapex (Angelus, Londrina, PR, Brazil) (tablica 1.) naneseni su na sterilne teflonske diskove promjera šest milimetara. Diskovi s materijalom i prazni teflonski diskovi koji su služili kao kontrola, postavljeni su u bunariće pločica za staničnu kulturu (Techno Plastic Products AG, Trasadingen, Švicarska) nakon čega je na njih nanesena suspenzija stanica u ukupnom volumenu od 3000 µl i s početnim brojem stanica od 3 x 10^5/ml. Cijela pločica stavljena je u CO_2 inkubator na 37 °C. Nakon inkubacije od jedan sat, šest, dvadeset i dvadeset i četiri sata, uklonjeni su teflonski diskovi i određen je broj živih stanica tripanskim modrilom u Neubaerovoj komorici. Uzorci su obrađeni u triplikatu, a ispitivanje za svaki materijal i za svako inkubacijsko razdoblje ponovljeno je tri puta.

Cytotoxicity study

Freshly mixed materials (0.1 g), Endosequence BC Sealer (Table 1) and MTA Fillapex (Table 1), were placed on sterile teflon discs, 6 mm in diameter. Teflon discs with tested materials and empty teflon discs, serving as a control group, were placed in wells of 12-well plate (Techno Plastic Products AG, Trasadingen, Switzerland). The teflon discs were covered with cell suspension (3000 µl) with number of cells 3 x 10^5/ml. 12-well plate was placed in a CO_2 incubator at 37°C. After incubation time of one, six, 20 and 24 hours, the teflon discs were removed from the wells and the number of viable cells was determined using trypan blue in Neubaer chamber. All samples were done in triplicate and testing for each material and each incubation period was done three times.

Tablica 1. Sastav punila Endosequence BC Sealer i MTA Fillapex
Table 1 Composition of Endosequence BC Selaer and MTA Fillapex

Materijal • Material	
Endosequence BC Sealer	cirkonijev oksid, kalcijev silikat, monobazični kalcijev fosfat, kalcijev hidroksid, punilo i plastifikatori • Zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents.
MTA Fillapex	*Pasta A* • *Paste A*: salicilatna smola, bizmut trioksid, silikat • salicylate resin, bismut trioxide, silica *Pasta B* • *Paste B*: silikat, titanijev dioksid, MTA (40 %), smola (plastifikator) • silica, titanium dioxide, MTA (40%), resin

Statistička analiza

Za statističku analizu korišten je Mann-Whitneyev U test. Razina značajnosti postavljena je na pet posto.

Statistical analysis

The Mann-Whitney U test was used for statistical analysis of data. The level of significance was set at 5%.

Rezultati

U kontrolnoj skupini nije bilo statistički značajne promjene u broju živih stanica tijekom ispitivanih inkubacijskih razdoblja. Promatranjem razlike između ispitivanih materijala i kontrolne skupine u pojedinim inkubacijskim razdobljima dokazano je da punilo MTA u svim inkubacijskim razdobljima pokazuje statistički značajan pad broja živih stanica ($p \leq 0,05$), a kod punila BC pojavljuje se statistički značajna razlika od šestog do dvadeset i četvrtog sata inkubacije ($p \leq 0,05$) (slika 1.). MTA u odnosu na BC pokazao je statistički značajan pad broja živih stanica samo nakon prvog i dvadesetog sata inkubacije ($p \leq 0,05$), a u ostalim inkubacijskim razdobljima ta razlika nije bila statistički značajna ($p \geq 0,05$).

Results

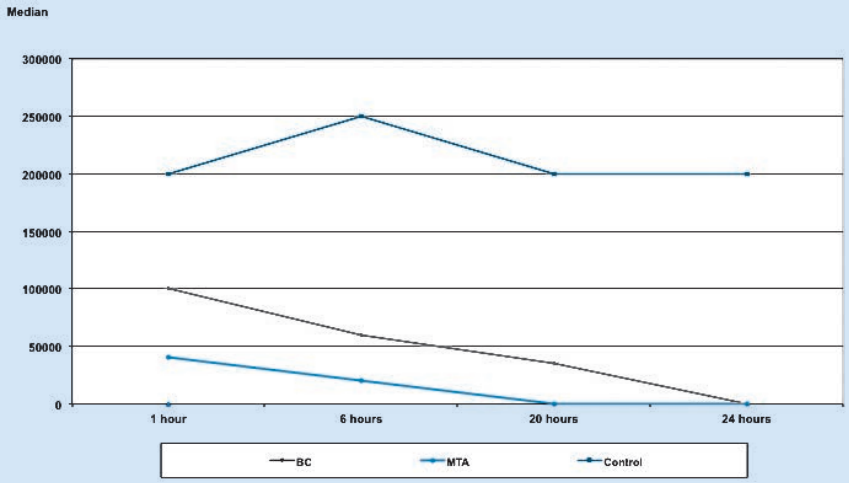
In the control group, there was no statistically significant difference in the number of viable cells during four incubation periods ($p \geq 0.05$). In comparison to the control group, MTA showed less viable cells for all incubation periods ($p \leq 0.05$), while BC sealer showed less viable cells from hour 6 to hour 24 of incubation ($p \leq 0.05$), (Figure 1). The MTA sealer showed less viable cells in comparison to the BC sealer after the first hour and after 20-hour incubation period ($p \leq 0.05$), while for other incubation periods there was no statistically significant difference ($p \geq 0.05$).

Rasprava

U ovom istraživanju ispitana je citotoksičnost dvaju bioaktivnih materijala za punjenje korijenskih kanala temeljenih na MTA-u i biokeramici, u kulturi mišjih fibroblasta L929. Testovi citotoksičnosti obično su inicijalni i njima se procjenjuje biokompatibilnost dentalnih materijala. Rezultati ovog istraživanja pokazali su da su oba ispitana punila za korijenske kanale pokazala citotoksični učinak.

Discussion

This study was designed to determine the cytotoxicity of two bioactive root canal sealers, based on MTA and bioceramics, in a cell line of mouse L929 fibroblasts. Cytotoxicity tests are usually the initial screening tests assessing the biocompatibility of dental materials. The results of the present study showed that both root canal sealers had a cytotoxic effect.



Slika 1. Prosječan broj živih stanica/ml (medijan) tijekom četiriju inkubacijskih razdoblja
Figure 1 Number of viable cells per ml (median) during four incubation periods

MTA Fillapex razvijen je kako bi se iskoristila izvrsna biološka svojstva MTA za materijal kojim se pune korijenski kanali. Prema našim rezultatima, MTA Fillapex bio je citotoksičan u svim razdobljima inkubacije. Spomenuti rezultati slažu se s rezultatima nekoliko istraživanja koja su pokazala da ovaj materijal znatno smanjuje broj živih stanica, iako su korišteni različiti postupci ispitivanja citotoksičnosti (17 – 19). Citotoksični učinak materijala može se objasniti njegovim kemijskim sastavom. MTA Fillapex čine dvije paste – jedna sadržava MTA, a druga salicilatnu smolu. Točna kemijska formula salicilatne smole je 1,2 butilen-glikolni disalicilat, spoj koji bi trebao biti biokompatibilan (20). Salicilatna smola pokazala je manju toksičnost u usporedbi s materijalima koji se prema sastavu temelje na epoksi smoli (20) čija je toksičnost i mutagenost već dokazana (21). Različiti derivati salicilatne smole, s razlikama u molekularnoj strukturi i dužini ugljikova lanca, mogu utjecati na različita fizička svojstva materijala, poput topljivosti (22). Topljivost materijala za punjenje korijenskih kanala nepoželjna je jer se na taj način mogu osloboditi spojevi koji mogu iritirati periapikalna tkiva (23). Iako je topljivost MTA Fillapexa samo 0,1 posto (20), čak i ovako mala topljivost može biti dovoljna za otpuštanje salicilatne smole iz materijala, čime se može objasniti citotoksični učinak na mišje fibroblaste L929. Zanimljivo je da su Vitti i suradnici (24) pokazali da se topljivost MTA Fillapexa povećava tijekom vremena, od -9,31 posto razlike u težini prvoga dana do -25,55 posto razlike u težini nakon dvadeset i osam dana. Drugo moguće objašnjenje za citotoksičnost MTA Fillapexa jest visoki pH koji se pojavljuje zbog spojeva nastalih tijekom stvrdnjavanja materijala (kalcijev hidroksid) koji oslobađaju hidroksilne ione (24). Porast pH može ubiti bakterijske stanice, ali i stanice domaćina oštećujući citoplazmatsku membranu i DNK te denaturiranjem proteina (25). Tijekom vremena manje je hidroksilnih iona koji se otpuštaju iz materijala i stvara se fiziološki pH koji pogoduje aktivnosti stanica (26). Prema istraživanju Vittija i suradnika (24), fiziološki pH nije postignut ni nakon dvadeset i osam dana. Citotoksičnost punila Endosequence BC također se može objasniti alkalnim pH za sva ispitana razdoblja inkubacije, osim prvoga sata. S obzirom na sastav tog punila, ne bi se očekivala toksičnost. No Loushine i suradnici (27) pokazali su da je konačno vri-

MTA Fillapex was developed in an attempt to take advantage of excellent biological properties of MTA for root canal sealers. According to the present results, MTA Fillapex showed cytotoxicity for all tested incubation periods. The findings of this study agree with several previous studies which showed that the material strongly affected cell viability although different methodologies were used (17-19). The observed cytotoxicity can be explained by its chemical composition. MTA Fillapex is composed of two pastes, one containing MTA and the other containing salicylate resin. The exact chemical formula of salicylate resin is 1,2 butylene glycol disalicylate which should be biologically compatible (20). Salicylate resin showed less toxicity in comparison to epoxy resin based materials (20), which have shown well documented toxicity and mutagenicity (21). Different derivates of salicylates resins with differences in molecular structures and size of carbon chains may influence different physical properties of the material such as solubility (22). High solubility of root canal sealer is undesirable because dissolution may cause the release of the components that could irritate periapical tissues (23). Although the solubility of MTA Fillapex material is just 0.1% (20), this might be enough for release of salicylate resin from the material, which explains the toxic effect on L929 fibroblasts. Interestingly, Vitti et al. (24) showed that MTA Fillapex solubility increases over time, from -9.31% weight variation at day one to -25.55% weight variation after 28 days. Another possible explanation for the cytotoxicity of MTA Fillapex is a highly alkaline pH environment, which is associated with setting products (calcium hydroxide) that releases hydroxyl ions (24). The increase of pH value may kill bacteria, and host cells as well, by damaging the cytoplasmatic membrane and DNA and denaturing proteins (25). Over time, hydroxyl ions release from the material decreases creating a physiological pH, which is beneficial for cell activity (26). According to Vitti et al. (24), physiological pH is not obtained even after 28 days. Alkaline pH may also explain the results obtained for Endosequence BC sealer which was also found to be cytotoxic for all incubation time periods except in the first hour of incubation. Based on the composition of Endosequence, no toxicity would be expected. However, Loushine et al. (27) have demonstrated that the final setting time of this

jeme stvrdnjavanja ovog materijala između 160 i 240 sati u vlažnom mediju. Zbog tako dugog stvrdnjavanja mogu se otpustiti neki spojevi iz materijala koji djeluju citotoksično, čime se mogu objasniti rezultati dobiveni u ovom radu. Isti materijal pokazao je citotoksičnost i u kulturi mišjih osteoblasta (27), a Zhang i suradnici (28) dobili su slične rezultate u kulturi mišjih fibroblasta. Ipak treba uzeti u obzir da je citotoksičnost samo jedan dio biokompatibilnosti te se zato rezultati moraju protumačiti s oprezom kako se materijal ne bi proglasio nebiokompatibilnim samo zbog citotoksičnosti.

Zaključak

Punila MTA Fillapex i Endosequence BC pokazala su citotoksični učinak u kulturi mišjih fibroblasta L929.

Sukob interesa

Nije bilo sukoba interesa.

material occurs between 160 and 240 hours in a moist medium. Long setting times may be responsible for some components of the material to leach for extended periods of time and influence adversely cell viability, which may also explain the cytotoxic effect of Endosequence BC sealer in this study. The same sealer was also found to be cytotoxic in a cell culture of mouse osteoblasts (27) while Zhang et al. (28) obtained similar results in a culture of mouse fibroblast which corresponds to the results of the present study even though different cell cultures were used. However, cytotoxicity is just one aspect of biocompatibility and the results should be interpreted with caution in order not to state that a material is not biocompatible just due to its cytotoxicity.

Conclusion

MTA Fillapex and Endosequence BC sealers were both cytotoxic in a culture of mouse L929 fibroblasts.

Conflict of interest

None declared

Abstract

Objective: The aim of this study was to investigate the cytotoxicity of two different bioactive root canal sealers: one based on mineral trioxide aggregate, MTA Fillapex (Angelus, Solucoes Odontologicas, Londrina, PR, Brazil), and the other based on bioceramics, Endosequence BC Sealer (Brasseler, Savannah, Georgia, USA), in culture of mouse L929 fibroblasts. **Materials and methods:** Mouse fibroblasts (L929), obtained from subcutaneous connective tissue of mouse line C3Hf, were cultivated in plastic culture flasks in an incubator at 37ºC, with 5% CO2 and 90% humidity. Freshly mixed Endosequence BC Sealer and MTA Fillapex (0.1 g each) were placed on sterile teflon discs, 6 mm in diameter. Teflon discs with the materials as well as empty discs serving as control were placed in wells of 12-well plate. After incubation times of 1, 6, 20 and 24 hours, the teflon discs were removed from the wells and the number of viable cells was determined using trypan blue in Neubauer chamber. **Results:** In comparison to the control group, MTA Fillapex had significantly less viable cells for all incubation periods ($p \leq 0.05$), while Endosequence BC sealer had significantly less viable cells after 6, 20, and 24 hours of incubation ($p \leq 0.05$). MTA Fillapex exhibited significantly less viable cells in comparison to Endosequence BC sealer after the first hour and after 20 hours of incubation ($p \leq 0.05$), while for the other incubation periods there were no significant differences ($p \geq 0.05$). **Conclusion:** MTA Fillapex and Endosequence BC sealer were both cytotoxic in cultures of mouse L929 fibroblasts.

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