Vasileios Kapralos[1]
Jannis Böcker[2]
Kirstin Vach[3]
Markus Altenburger[2]
Susanne Proksch[2]
Lamprini Karygianni[4]

[1] Department of Endodontics, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, Oslo, Norway
[2] Department of Operative Dentistry and Periodontology, Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[3] Institute for Medical Biometry and Statistics, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[4] Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

CORRESPONDENCE
Vasileios Kapralos
Department of Endodontics, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo
Geitmyrsveien 71
N-0455 Oslo
E-mail:
vasilis.kapralos@gmail.com, vasileios.kapralos@odont.uio.no

SWISS DENTAL JOURNAL SSO 132: 586–597 (2022)
Accepted for publication: 9 May 2022

On the biocompatibility of endodontic sealers

KEYWORDS
Osteoblasts (MeSH-ID D010006)
Endodontics (MeSH-ID D004708)
Root Canal Therapy (MeSH-ID D012390)
Biocompatibility Testing (MeSH-ID D008422)

SUMMARY

Periapical tissue may be exposed to root canal filling materials in consequence of root canal therapy. There is scant scientific data about the biocompatibility of root canal filling materials of various chemistry on the periapical area. This study aimed to investigate the effects of different root canal sealers and their eluates on human alveolar osteoblasts in terms of cell proliferation, adhesion, morphology and gene expression *in vitro*. Five endodontic sealers (AH Plus®, Apexit® Plus, Tubli-Seal®, RealSeal SE®, EndoREZ®) and one gutta-percha obturation material (BeeFill®) were tested. Human alveolar osteoblasts derived from three different donors following incubation with sealer eluates after 24 and 72 hours were investigated by means of qPCR (gene expression). Morphological reactions of the alveolar osteoblasts were measured by culturing the cells for 3, 7 and 14 days, respectively, followed by scanning electron microscopy (morphology, adhesion) and fluorescence imaging of the actin cytoskeleton (morphology, proliferation). A repeated measures analysis was performed and P values were adjusted by Tukey. While all sealers influenced the cell morphology and the expression of genes associated with apoptosis (Casp3), proliferation (histone H3) and inflammation (interleukin-6 and matrix metalloproteinases 1 and 3), mainly AH Plus® and Apexit® yielded a regular actin cytoskeleton and beneficial gene expression patterns. Regarding cell adhesion, only AH Plus® supported proper anchorage for alveolar osteoblasts. Our results provide evidence for the biocompatibility of epoxy resin-based endodontic sealers, i.e. AH Plus®, while other sealers proved cytotoxic for alveolar osteoblasts. Further studies are needed for understanding the bone cell reactions after endodontic treatment and the clinical decision-making regarding the sealer of choice for root canal fillings.

Introduction

The root canal treatment aims to eliminate bacteria from the infected root canal, prevent reinfection of the tooth and promote healing of periapical tissues (Möller et al. 1981; Cook et al. 2007). The success of endodontic treatment is multifactorial, with each distinct procedural step (mechanical: instrumentation; chemical: irrigation and obturation) playing a significant role and contributing to the overall therapeutic result (Friedman et al. 2003; de Chevigny et al. 2008). Following chemo-mechanical preparation of the dentinal walls, a root canal treatment is completed by creating a bacteria- and fluid-tight seal into the root canal system with the use of filling materials (Möller et al. 1981; Guldener 1993; Wesselink 1995; Cook et al. 2007).

The most common and well-known root canal filling material is gutta-percha. Despite its high degree of volumetric stability, gutta-percha cannot adequately seal the root canal space by itself and does not adhere to the dentinal walls (Skinner & Himel 1987; Tagger et al. 1994; Li et al. 2014). Therefore, it is used as a core root canal filling material in combination with adjacent root canal filling materials, the endodontic sealers, which aim to create a homogenous interface between the dentinal walls and gutta-percha.

Endodontic sealers play an important role in hermetic obturation of the root canal system especially at the sealer-dentin interface, sealing accessory root canals and dentinal tubules (Heidemann 1989). Sealers entomb residual bacteria, prevent leakage of nutrients and ideally possess antimicrobial properties (Ørstavik 1988; Donnermeyer et al. 2019). Different types of root canal sealers, such as zinc oxide eugenol-, resin-, silicone-, calcium silicate-based materials with different setting formations are used in endodontics (Ørstavik 2014). These materials should ideally offer many biological and physiochemical properties such as antimicrobial activity, remain unaffected by the irrigating solutions, keep a long-term dimensional and physicochemical stability inside the root canal space (Ørstavik et al. 2001; Williamson et al. 2005; Camilleri & Mallia 2011; Gopikrishna 2014), remain insoluble and not induce cytotoxic effects to surrounding periapical tissues (Dahl 2005).

Low cytotoxicity of sealers is important since extrusion of these materials to the periapical area may occur and lead to an inflammatory reaction, which may retard the healing process and even degenerate the periapical tissues (Nair 2004; Huang et al. 2005). Moreover, contact between tissue fluids or irrigation liquids and sealers may cause leaching of constituents from the sealer. Leachates could potentially migrate to exposed dentinal tubules, lateral canals or to periapical tissues through the bulk of filling materials or the dentine-sealer interface (Kazemi et al. 1993; Geurtsen & Leyhausen 1997; Ørstavik 2005; Elyassi et al. 2019). Eluates and further constituents from sealers' degradation processes may be in long-term contact with periapical tissues and thereby induce cytotoxic effects.

Leachates of endodontic materials have attracted the attention with regard to antimicrobial properties and cytotoxicity (Arias-Moliz et al. 2017). The leachable compounds ideally should be biocompatible and hence prevent cytotoxic effects to the periapical tissues as this may jeopardize the clinical success of root canal therapies (Barbosa et al. 1993; Dahl 2005). A recent literature review on standardization of antimicrobial testing of dental materials highlights the importance of biocompatibility testing along with characterization of elution/degraded materials (Camilleri et al. 2020).

Both the biological and the physicomechanical properties of sealers depend on the chemistry of the root canal sealers. Hence, root canal sealers need to possess good physicochemical properties along with low cytotoxicity (Huang et al. 2002). Most of the endodontic root canal sealers may have some toxic properties or exhibit different levels of cytotoxicity on tissues, which would result in prolonged wound healing, inflammation and bone resorption (Kaur et al. 2015; Silva et al. 2021). To date, there is scant scientific data about the biocompatibility of root canal filling materials of various chemistry on the periapical area. The present study aimed to investigate the effects of five different root canal sealers and their eluates on human alveolar osteoblasts as representatives of periapical bone tissue in terms of cell proliferation, adhesion, morphology and gene expression *in vitro.*

Materials and methods

Root canal filling materials

An epoxy resin-based sealer, AH Plus® (Dentsply International Inc, York, PA, USA), a zinc oxide eugenol-based sealer, Tubli-Seal® (SybronEndo, Kerr, Romulus, MI, USA), a methacrylate resin-based sealer, RealSeal SE® (SybronEndo, Kerr, Romulus, MI, USA), EndoREZ® (Ultradent Products, South Jordan, UT, USA), a methacrylate resin-based sealer, Apexit® Plus (Ivoclar Vivadent, Schaan, Liechtenstein), and BeeFill® (VDW, Munich, Germany), a gutta-percha, were tested. The materials were mixed according to the manufacturers' instructions.

Preparation of eluates and pellets from root canal filling materials

The root canal filling materials were transferred into the plastic lids of sterile Eppendorf microcentrifuge tubes and incubated for 24 hours (h) in a humidified atmosphere of 5% CO_2 at 37 °C, yielding solid sealer discs (diameter 4.5 mm, 15.89 mm^2 surface area, height 2.5 mm) that were subsequently rinsed with 70% ethanol, washed with phosphate buffered saline (PBS, Life Technologies, Darmstadt, Germany) and exposed to UV light (60 min, VL-6.L., LTF-Labortechnik GmbH & Co. KG, Wasserburg, Germany) for sterilization purposes. For eluate preparation, 1 disc/well was placed in 24-well plates containing 1 ml growth medium each, and incubated for 24 h or 72 h, respectively, at 37 °C with 5% CO_2. For quality assessment, the surface morphology of the remaining discs was analyzed by scanning electron microscopy (SEM).

Cell culture and exposure to root canal filling materials

All experiments were carried out in accordance to the guidelines of the World Medical Association Declaration of Helsinki and were approved by the Committee of Ethics of the Medical Faculty of the Albert Ludwigs University Freiburg, Germany (vote number 516/12). All primary human alveolar osteoblasts were derived from operative biopsies of n = 3 donors with informed and written consent and kindly provided by Prof. Thorsten Steinberg, Center for Dental Medicine, University Medical Center Freiburg, Dept of Oral Biotechnology. Osteoblasts were cultured in MEMalpha (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 1% glutamax (Life Technologies), 1% amphotericin and 1% antibiotics. Upon confluency, cells were trypsinized and passaged up to 6–9 times. For gene expression analysis, 5×10^3 cells/well were incubated in

eluate medium for 24 h and 72 h, respectively. For morphology analysis, 1×10^3 cells/cm^2 were cultured in microtiter plates for 3 days (d), 7 d and 14 d. For morphology testing, 2×10^3 cells/well were cultured in microtiter plates for 3 d, 7 d and 14 d at the surface of root canal filling material pellets or incubated with eluates, respectively.

Scanning electron microscopy (SEM)
The specimens were fixed in 8% formaldehyde and dehydrated in an ascending ethanol series (50%, 70%, 80%, 90% once each and twice in 100% for 1 h). Afterwards, critical point drying (Critical Point Dryer CPD 030; Bal-Tec, Wallruf, Germany) was performed, and the samples were sputter-coated with a gold palladium for 60 sec at 60 mA (SCD 050; Bal-Tec) and scanned by a Zeiss Leo 435 VP scanning electron microscope at 10 kV (Zeiss, Oberkochen, Germany).

Fluorescence microscopy (FM)
For actin staining after 3 d, 7 d and 14 d, respectively, the cells were washed with phosphate buffered saline (PBS, Biochrom, Berlin, Deutschland), fixed in 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany) and permeabilized with 1% Triton X-100 (Life Technologies, Darmstadt, Germany) for 3–5 min. Non-specific binding sites were blocked with ImageIt FX® signal enhancer (Life Technologies, Darmstadt, Germany) for 30 min, and the cells were incubated with Alexa Fluor 488-conjugated phalloidine (1:40; Abcam, Cambridge, UK) at room temperature for 30 min in the dark. After intense rinsing, cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI, Merck, Darmstadt, Germany), and cells were mounted with an aqueous permanent mounting medium (Fluoromount, Southern Biotech, Birmingham, UK). Fluorescent images were acquired by a fluorescence microscope equipped with a CCD camera (BZ-9000, Keyence, Neu-Isenburg, Germany) and analyzed with BZ image analysis application software (Keyence).

qPCR assays
Total cellular RNA was purified using a guanidium-thiocyanate method (RNeasy Mini kit; Qiagen, Hilden, Germany) and stored at –80 °C. Genomic DNA contamination was eliminated by DNase digestion according to the manufacturer's instructions (RNase free DNase kit; Qiagen, Hilden, Germany). The RNA integrity and quantity were verified using the Experion RNA StdSens chip microfluidic technology according to the manufacturer's instructions (Bio-Rad, Munich, Germany), and cDNA was synthesized from 200 ng of total RNA each by using the RT2 PreAmp cDNA synthesis kit (Qiagen, Hilden, Germany), preceded by a genomic DNA elimination step at 42 °C for 5 min, in a C1000 Thermal Cycler (Bio-Rad) at 42 °C for 30 min followed by 95 °C for 5 min. Signal intensity and specificity were enhanced pre-amplifying cDNA templates by multiplex PCR by using the RT2 Pathway Primer Mix (Qiagen) in a 25 µl volume, and cycling conditions were 95 °C for 10 min followed by 12 cycles of 95 °C for 15 sec and 60 °C for 120 sec for all specific array primer sets. For real-time qPCR array, pre-amplified templates were amplified in 25 µl reaction mixture using RT2 SYBR Green qPCR mastermix (Qiagen) in a CFX96 cycler (Bio-Rad), according to the manufacturer's instructions. The products' specificity of each amplicon was checked by examining the melting temperatures (heating at 0.05 °C/s to 95 °C). Negative reverse transcription, negative genomic DNA and negative template controls were included in all PCR runs. Data were collected with CFX96 Manager Software version 1.0 (Bio-Rad), and relative quantities of the respective genes of interest were normalized to the relative quantity of hydroxymethylbilane synthase, ribosomal protein L13a and ubiquitin as references, which were validated for Ct value consistency.

Statistical analysis
To evaluate the effect of groups (control, root filling materials), alveolar osteoblast donors (1, 2, 3) and time, a repeated measures analysis was performed for the outcomes of interest. Therefore, a linear model was fitted within which the structure of data, i.e. repeated measurements, is accounted for (repeated measurement per example, several examples per 'donor' and several materials per donor). An interaction term between material and donor is included in the model to detect inter-individual differences between the materials. The group effects and differences of least-square (ls) means are calculated with their 95% confidence intervals. Several multiple comparisons of ls means in relevant donor and material combinations are done. P values are adjusted by the method of Tukey. All calculations were performed with the statistical software SAS 9.1.2. (SAS Institute S.A., Cary, NC, USA) using PROC MIXED.

Results
Microscopy analysis
Regarding control, the actin filaments of osteoblasts (Fig. 1Ai) were evenly distributed in the cytoplasm and showed an intact, characteristic actin cytoskeleton. The fibers appeared linear and were arranged in parallel bundles longitudinal to the cell axis. The cells displayed star- to spindle-like shapes. The blue stained DNA of the round-oval cell nuclei was abundant and evenly stained. In Figures 1Aii and iii, a clear increase in the number of cells is recognizable. The spindle- to dumbbell-shaped cells were evenly distributed and had long filaments.

For AH Plus® (Fig. 1B), an increase in cell number was evident in FM images (i), (ii), and (iii). In image (i), compared to the control (Fig. 1Ai), the cell number seemed to be lower after 3 d, and the cells showed spacing from each other. In images (ii) and (iii) there was a clear increase in cell number, and the cells were densely packed next to each other. The cellular actin filaments stained in green by phalloidin were even less directed in (i) than in (ii) and (iii) images. In the latter, the actin filaments were arranged in parallel to each other, indicating a flat shape of the cells. Initially, the cells were rather star-shaped (Fig. 1Bi), while they exhibited spindle- after 7 d (Fig. 1Bii) and dumbbell-like shapes after 14 d of culture (Fig. 1Biii). The cell nuclei, stained blue by DAPI nuclei were clearly visible in cells attached to AH Plus® at each time point of examination. In SEM, cell proliferation was also evident with prolonged culture time. A dense cell layer could be detected on the surface of the specimens. The osteoblasts exhibited typical characteristics of polymorphic cells with their flattened and spindle-shaped shape, a fact which indicates good adherence to the sealer surface. The cell layer had already been very dense after 3 d (iv) and increased over time at 7 d (v) and 14 d (vi). The cells did not show any damage such as membrane defects or rounding.

The FM images (Fig. 1C) showed a lower number of osteoblasts growing close to Apexit® Plus compared to matched

controls. The green-stained actin filaments of the osteoblasts showed star-shaped cells with short cell filaments after 3 d of culture (i). The cytoskeleton was less structured compared to the control group, and fragmented filamentous actin could be detected. After 7 d (ii), only a slight increase in the number of cells was observed, and the cytoskeleton was also not well developed. The cell nuclei were partially detached from the cell bodies. Further, after 7 d (ii), cell variations could be identified. The actin filaments of the spindle- to dumbbell-shaped cells were arranged in parallel. The cell nuclei were abundant and clearly stained. In the SEM micrographs (iv)–(vi), no intact cells are present on the surface of the specimens even after only 3 d of incubation. The osteoblasts, which could be found in isolated cases, had a round, apoptotic shape. This observation could be indicative of a cytotoxic effect of Apexit® Plus. The differing results in immunofluorescent stainings compared to SEM images could either be attributed to the fact that cell shapes differed in various microscopic regions, meaning that both living and dead cells were detectable, or merely be an artefact resulting from different experimental procedures to fix the cells.

When osteoblasts were grown on Tubli-Seal® pellets (Fig. 1Di–iii), a lower increase in cell number was observed over time compared to the control group. After 3 d, only few cells with short projections were detectable (i), and the cellular actin filaments were fragmented. After 7 d of culture (ii), the cells had a flattened and wide-spread cell body with a structured cytoskeleton. However, the cytoskeleton of the cells was poorly organized and appeared partially disrupted after 14 d (iii), while the morphology varied from star-shaped to dumbbell-like shapes. In SEM images (iv) and (v), only round cells were present on the sample surfaces, indicative of cell apoptosis. The osteoblasts were dislocated and lacked extensions. After 7 d (v), the typical apoptotic spiny shape of the cell membrane was obvious, while only cell remnants were visible on the sample surfaces at 14 d (vi).

Alveolar osteoblasts grown on the surface of RealSeal SE® pellets (Fig. 1E) showed elongating filaments after 3 d (i). After 7 d of incubation (ii), the cell number was increased, and the cell bodies were flat and displayed a dumbbell-like morphology. Compared to the control group, the actin fibers did not run in rectified bundles and were less evenly distributed over the cytoplasm. The nuclei were numerous and clearly stained. Fewer osteoblasts were observed after 14 d (iii) than after 7 d (ii). The cytoskeleton of the cells was less elongated than in image (ii), suggesting small, compact cells. In the SEM images at 3 d (iv), only few osteoblasts with single long filaments were visible on the sample surface. Cells with spindle-shaped morphology could not be found in direct contact with the material, while inactive, rounded osteoblasts were shown. After 7 (v) and 14 d (vi), only apoptotic cells were visible, clearly indicating a cytotoxic effect of RealSeal SE®.

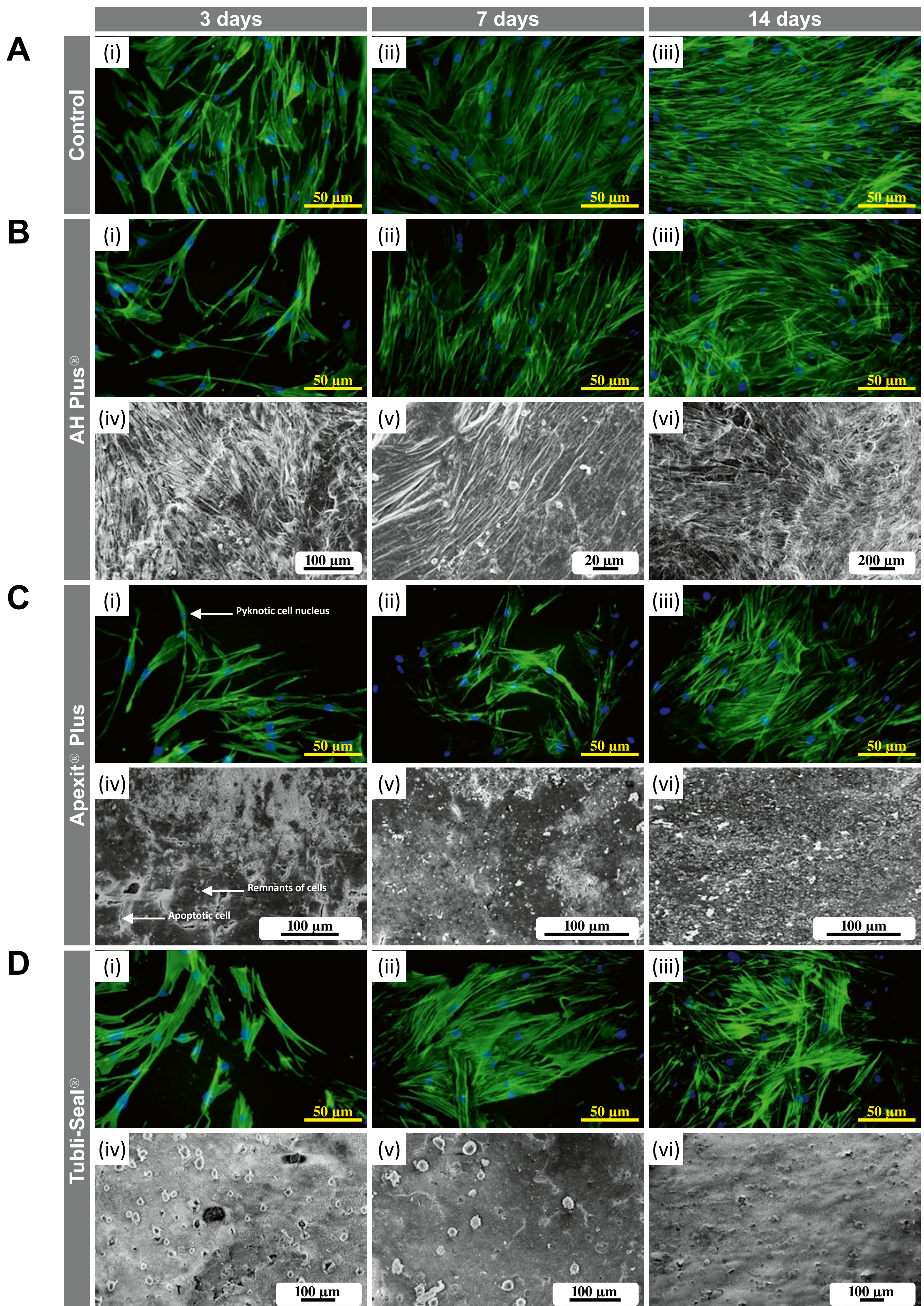
Regarding EndoREZ® (Fig. 1F), the FM image (i) after 3 d of incubation, showed small cells in size. After 7 d (ii), a higher number of cells could be detected. The actin filaments were, however, less aligned compared to the control group in Figure 1A. In image (iii) after 14 d, fewer osteoblasts could be identified, and the actin filaments were also superimposed and fragmented. The osteoblasts appeared more rounded and did not show extensions. The SEM image (iv) showed a few round osteoblasts on the sample surface after 3 d of incubation. After 7 d (v) and 14 d (vi), only cell debris was present on the sample surface.

Based on the FM images (Fig. 1G), BeeFill® had a stimulative effect on cell number with increasing culture. After 3 d (i), short, spindle-shaped osteoblasts with extending filaments were visible. After 7 d (ii) and 14 d (iii), only in the BeeFill® group of all sealers under study was the cell number comparable to that of the control group (Fig. 1A). There were evenly distributed, spindle-shaped cells with oval nuclei. The actin filaments seemed to overlap partially and showed a parallel arrangement. In SEM image (iv) a large number of round cells could be detected on the surface of the sample.

Gene expression analysis

Next, the expression of selected genes for inflammation (IL-6, MMP1, MMP3; Fig. 2), proliferation (Hist3H3; Fig. 3A) and apoptosis (Casp3; Fig. 3B) was measured, all normalized to a pool of three house-keeping genes. Due to insufficient RNA amounts, the analysis of the EndoREZ® and Tubli-Seal® samples was not feasible.

With regard to inflammation markers, no statistically significant difference was found in the expression of IL-6 in the 24-hour, nor in the 72-hour samples (Fig. 2A). However, the expression of IL-6 was increased over time for Apexit® Plus. No statistically significant differences could be detected in the expression of MMP1 after 24 h, both between different sealer groups and compared to controls. However, after 72 h, osteoblasts expressed significantly more MMP1 RNA in the AH Plus® group compared to controls ($p = 0.009$, Fig. 2B). An increasing trend in expression of the inflammation marker MMP1 was observed over time with Apexit® Plus and RealSeal SE®. At 24 h, MMP3 was expressed to a significantly higher degree compared to controls in cells cultured with eluates from Apexit® Plus ($p = 0.001$) and BeeFill® ($p = 0.001$). In addition, cells in the BeeFill® group expressed significantly more MMP3 RNA than cells in the Apexit® Plus group ($p = 0.004$). Interestingly, there were no significant differences for the 72-hour samples. On the other hand, over time, there was a decreasing trend in the expressed inflammation markers in all samples. With regard to gene expression of the proliferation marker Hist3H3 (Fig. 3A), osteoblasts cultured in the presence of RealSeal SE® showed a significantly lower expression compared to controls ($p = 0.017$). Interestingly, after 72 h, the opposite effect was noticed, because cells in the RealSeal SE® group expressed significantly higher HistH3 RNA levels than controls ($p = 0.0001$). Similarly, cells incubated with eluates from Apexit® Plus expressed more HistH3 than cells in the control group ($p = 0.063$; Fig. 3A). Over time, an increase in the expression of Hist3H3 was observed for Apexit® Plus and BeeFill®, whereas for AH Plus® and the control group the expression of Hist3H3 tended to decrease. As for apoptosis (Fig. 3B) in the 24-hour samples, no significant difference was found between the material groups. After 72 h, osteoblasts in the AH Plus® ($p = 0.0003$) and in the Apexit® Plus ($p < 0.0001$) groups expressed significantly less Casp3 RNA than cells in the RealSeal SE® group. Further, cells in the Apexit® Plus group expressed significantly less Casp3 RNA than cells in the AH Plus® group ($p = 0.026$). There was not significant difference in gene expression between the sealer groups and controls, respectively, which presumably results from a high variation of Casp3 expression in control. RealSeal SE® and BeeFill® showed an increasing trend in gene expression of Casp3 over time. In contrast, there was a decreasing trend in Casp3 expression for AH Plus®.



3 days
7 days
14 days
A
Control
(i)
(ii)
(iii)
50 µm
B
AH Plus®
(iv)
(v)
(vi)
100 µm
20 µm
200 µm
C
Apexit® Plus
Pyknotic cell nucleus
Remnants of cells
Apoptotic cell
100 µm
D
Tubli-Seal®

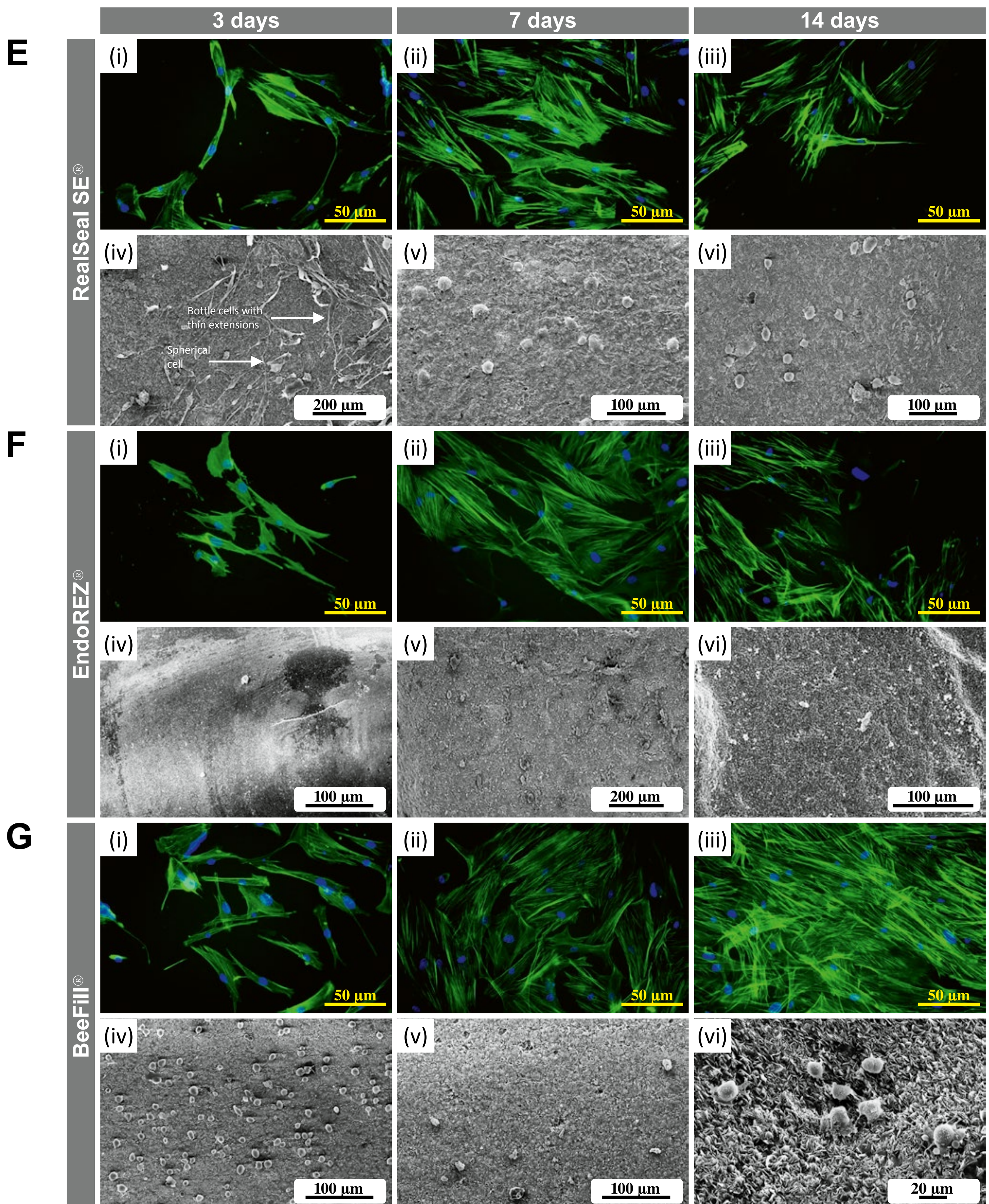


Fig. 1 Indicative FM (i, ii and iii) and SEM (iv, v and vi) micrographs of the human osteoblasts seeded on the surface of the tested materials for 3, 7 and 14 d. A: control; B: AH Plus®; C: Apexit® Plus; D: Tubli-Seal®; E: RealSeal SE®; F: EndoREZ®; G: BeeFill®

Fig. 2 Gene expression of IL-6 (A), MMP1 (B) and MMP3 (C) after 24 and 72 hours, indicative of inflammatory response.

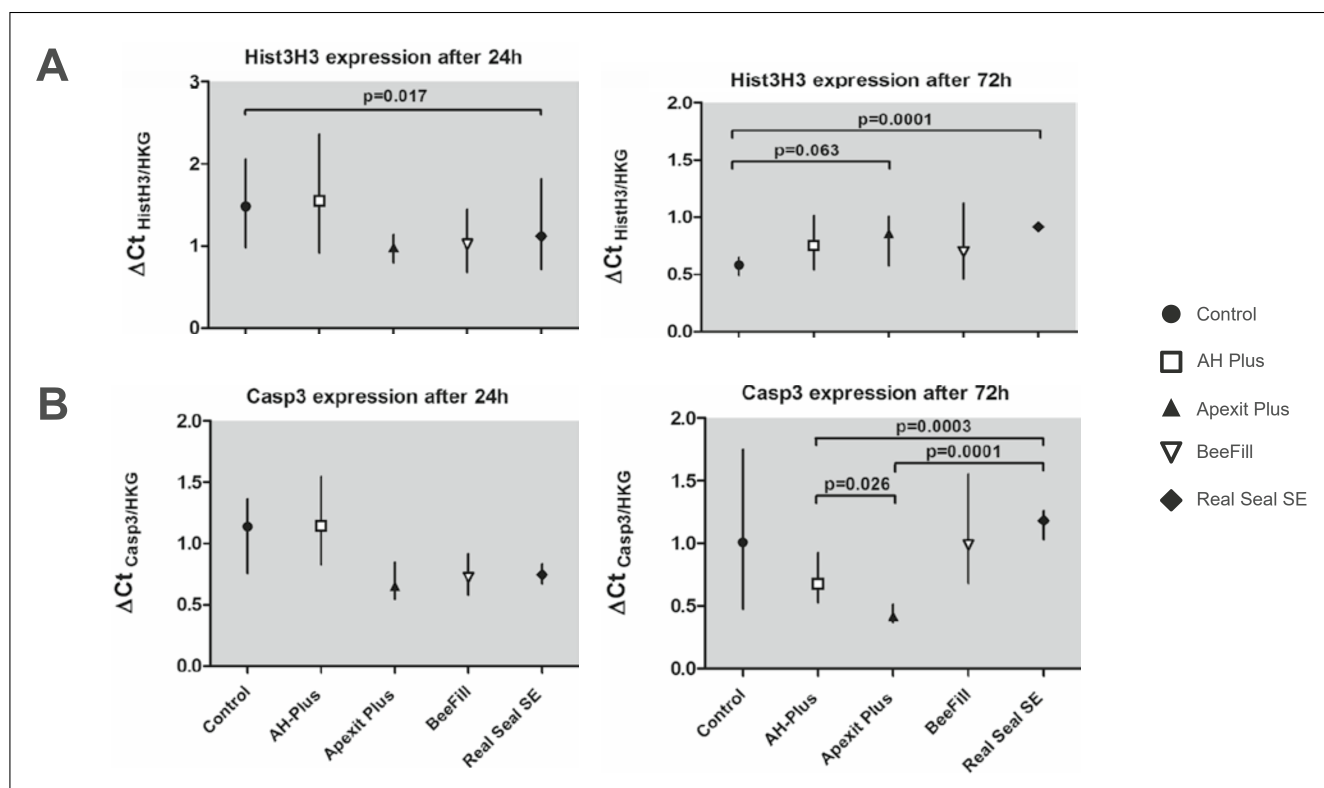


Fig. 3 Gene expression of Hist3H3 (A) and Casp3 (B) after 24 and 72 hours, indicative of the cell apoptosis.

Discussion

In root canal treatments, the use of sealers in combination with gutta-percha is the standard procedure. Based on the established ISO 10993 series, a medical device should aim at the protection of humans from potential biological risks. The root canal filling materials (endodontic sealers, gutta-percha) can be categorized to externally communicating dental medical devices (non-implantable) as they are in contact with tissue, bone or pulp/dentin systems. Thus, the biological evaluation of endodontic sealers is crucial to assess the safety of these materials for clinical use. A series of tests for genotoxicity, in vitro cytotoxicity, systemic toxicity, potential degradation and leachables is suggested (International Organization for Standardization 2018). The physical and biological properties of dental materials have been and are being investigated *in vitro, in vivo* and in clinical studies. The results of our study render valuable insights on the cytotoxicity of endodontic sealers, because we investigated primary human cells isolated from the target tissue, i.e. alveolar bone, which reflect the clinical situation much more realistically than animal tissue-unspecific or immortalized cells (Huang & Chang 2002).

Five different sealers as well as BeeFill® as a representative of the gutta-percha material group were, after having set, tested for their cytotoxicity with primary human osteoblasts. It is well documented that set materials are less cytotoxic than freshly mixed as they release less components (Camargo et al. 2014). Since the filling material affects the periapical tissues by releasing components (Tronstad et al. 1988), but also by direct contact if extrusion occurs, both material surfaces and eluates were investigated. Scanning electron micrographs were used to investigate the cell morphology of osteoblasts seeded on the surface of sealer samples. Through surface receptors and adhesion links, osteoblasts attach to materials (Hong et al. 2003). As a result, cell adhesion, as a prerequisite for proliferation and growth of the cells, is dependent on the chemical and physical properties of the material surfaces (Wang et al. 2013).

In direct contact with the sample surface, alveolar osteoblasts in the AH Plus® group showed good adhesion after 3 d and an increase in cell number over time. Together with the gene expression data in the AH Plus® group, our results do not reflect a strong initial inflammatory reaction, as observed in an implantation study in porcine bone (Sousa et al. 2006).

On the surface of the RealSeal SE® samples after 3 d, a few spindle-shaped cells could be detected next to dislocated, apoptotic cells. After 7 d and 14 d, few living cells were detectable. The cytotoxic effect of RealSeal SE® on osteoblasts has already been shown in a previous study (Scelza et al. 2012) and can be presumably attributed to the release of toxic monomers of incompletely polymerized metacrylate (Bouillaguet et al. 2006). With regard to Apexit® Plus, EndoREZ® and Tubli-Seal® samples, only inactive round cells were observed. The initial toxic effect of these materials has already been reported in various studies (Zmener et al. 2005; Eldeniz et al. 2007; Kangarloo et al. 2009); however, our study is the first to show that the cells do not recover from this initial cytotoxic effect. On the surface of the BeeFill® samples, apoptotic cells were also visible after 3 d. A toxic effect of gutta-percha caused by zinc oxide and zinc ions has already been demonstrated in 1990 by means of a chromium release assay with mouse fibroblasts (Pascon & Spangberg 1990). Nevertheless, osteoblasts nicely recovered from the initial BeeFill® cytotoxicity, as shown by FM, SEM and qPCR results.

For the morphological examination of the osteoblasts by FM and for cell analysis by quantitative qPCR, sample eluates were prepared. Sealers are supposed to have a certain solubility, thus release of components may have a biological effect on the surrounding tissue (Geurtsen & Leyhausen 1997; Schafer & Zandbiglari 2003).

In FM, cells are fixed in their vital state, which allows the morphological examination of cells in their natural behavior and their protein metabolism. Cell functions, such as adhesion strength, differentiation and proliferation, are closely related to cell shape (Chen et al. 1997). It is a well-established concept that human osteoblasts proliferate rapidly in vitro within the first few days (Auf'mkolk et al. 1985; Robey & Termine 1985). After incubation of the cells in the material eluates for 3 d, 7 d and 14 d, a reduced cell number and a change in the morphology could be detected, which varied among the different materials. The AH Plus® samples affected the cell morphology after 3 d of incubation while the cells appeared to proliferate over time. The cell density and the morphology of the cells were similar to the control group after 7 d and 14 d. An initial toxic effect for AH Plus® has already been demonstrated by Sousa et al. (Sousa et al. 2006), in an implantation study and is probably due to the release of formaldehyde (Leonardo et al. 1999; Cohen et al. 2000). Low cytotoxicity of AH Plus® was found in 2007 in a study with human gingival fibroblasts (Eldeniz et al. 2007). The same study showed a significantly more toxic effect of Apexit® Plus and EndoREZ® compared to AH Plus®, a result that is consistent with the morphological examination of cells in the present study exhibiting a reduced cell density at all three time points. Similar results were obtained with Tubli-Seal® and RealSeal SE®, and showed an even more pronounced effect. Different studies on the biocompatibility of RealSeal SE® have produced conflicting results so far (Kim et al. 2010). On the one hand, low cytotoxicity was demonstrated in an indirect test with human gingival fibroblasts (Scotti et al. 2008), whereas other studies showed a strong toxic effect of the materials (Scelza et al. 2012). Morphological examination also indicated a toxic effect, which is demonstrated by a reduced cell number of the osteoblasts and an effect on the actin cytoskeleton. A possible cause for this may be the release of toxic monomers, which occurs due to a non-complete polymerization of the methacrylate (Bouillaguet et al. 2006). A study by Chang et al. showed a toxic effect of Tubli-Seal® on periodontal ligament fibroblasts due to the eugenol contained in the material (Chang et al. 2010). Contrary to the results of Pascon and Spangberg (Pascon & Spangberg 1990), the morphological examination of BeeFill® samples revealed an initial interaction with the osteoblasts after 3 d. However, after 7 d and 14 d, the cell density and the morphology of the cells were comparable to those of the control group.

In this study, the behavior of human osteoblasts after cultivation with different sealer eluates was analyzed on a molecular level using the gene expression of specific genes. The most impressive result was that no RNA at all could be retrieved from cells cultured in eluates from EndoREZ® and Tubli-Seal®. Thus, both materials seem to exhibit a massive toxicity on osteoblasts so that the amount of RNA extracted after 24 h and 72 h was either too low or non-existent, indicating the absence of any living cells. This interpretation is substantiated by our SEM findings of round and apoptotic-like cells in both groups after prolonged culture periods. In line with our results, this toxicity has been documented in several studies (Eldeniz et al. 2007; Chang et al. 2010; Diomede et al. 2014).

In line with scientific evidence, our data reveal a clear cytotoxic effect of Tubli-Seal®, which is supposed to result from its component eugenol (Briseno & Willershausen 1990; Gulati et al. 1991). Al-Awadhi et al. documented increased apoptosis activity in primary osteoblasts in the presence of an eugenol-containing sealer (Al-Awadhi et al. 2004). Futhermore, human PDL fibroblasts in contact with the eugenol-containing sealer endomethasone and N2 showed decreased metabolic activity (Schwarze et al. 2002). The other sealer that proved highly cell damaging in our study was EndoREZ®. Its cytotoxicity may presumably be attributed to the release of urethane dimethacrylate (UDMA), which was already recognized as a toxic substance by Hikage et al. (Hikage et al. 1999). The results of several *in vivo* and *in vitro* studies substantiate clear toxic properties of the material (Zmener 2004; Bouillaguet et al. 2006).

Besides apoptosis, we aimed at measuring the inflammatory reaction of alveolar osteoblasts exposed to the different endodontic sealers. Interleukins are peptide hormones from the cytokine group and are well-known immunomodulators. The cytokine IL-6 is a marker for tissue injuries and infections (Hirano et al. 1990; Kishimoto et al. 1992) and plays a significant role in the activation of the immune system through the differentiation of B cells, as well as being an initiator of bone resorption (Ishimi et al. 1990). In addition, various studies have demonstrated a link between the progression of inflammatory reactions in the periapical tissue and the expression of IL-6 (Bando et al. 1993; Euler et al. 1998; Honma et al. 1998). In a study by Huang et al., the effect of AH Plus® on the expression of IL-6 and IL-8 genes of human osteoblasts was investigated. There is evidence that AH Plus® triggers the gene expression of IL-6 and even further of IL-8, which was interpreted as a result of the sealer release of formaldehyde (Huang et al. 2005). In the present study, no sealer significantly affected the expression of IL-6 in alveoar osteoblasts. Matrix metalloproteinases (MMPs) are members of the zinc-dependent endopeptidase family, and regulate tissue homeostasis, bone remodeling and wound healing (Chen 1992). Nevertheless, they are also involved in pathological processes such as tumor growth and metastasis, rheumatoid arthritis or oral diseases (Stetler-Stevenson et al. 1996; Basset et al. 1997). The interstitial collagenase MMP1 degrades fibrillar and non-fibrillar collagen (types I, II, III, VI and IX) and gelatin (Bhuvarahamurthy et al. 2006). Stromelysin (MMP3) is involved in the degradation of proteoglycans, laminin, fibronectin, collagen (types III, IV, V and IX), gelatin and pro-MMP1 and plays an important role in the physiology of inflammatory processes and wound healing (Chen 1992). A dysregulation of MMP gene expression or activation of MMPs can lead to various clinical conditions such as chronic inflammation (Rodriguez et al. 2010). A study by Paula-Silva et al. found significantly increased MMP expression and degeneration of the periapical tissue as a result of bacterial invasion in untreated apical periodontitis (Paula-Silva et al. 2010). In our hands, the significantly increased expression of the MMP3 gene in the Apexit® Plus, BeeFill® and RealSeal SE® samples after 24 h, suggests a similar inflammatory reaction. After 72 h, increased expression of the MMP1 gene was observed in cells incubated with Apexit® Plus eluates and slightly in the AH Plus® group. From these results, it can be concluded that exposure of the periapical tissues to endodontic materials after the root canal treatment could induce an inflammatory reaction, which may undermine the therapy success.

Histone 3H3 (Hist3H3) from the histone family is involved in chromosome organization in the cell nucleus. The expression for these proteins takes place primarily during the replication of DNA and is thus a sign of cell proliferation (Peterson & Laniel 2004). The significantly lower expression of the Hist3H3 gene in the RealSeal SE® group compared to the control group after 24 h indicates a low proliferation of osteoblasts, which increased slightly at the 72-hours mark. The same tendency was also observed for Apexit® Plus. The low proliferation in RealSeal SE® group can be explained by the time-dependent cytotoxic effect of unbound monomers as already described by Scelza et al. (Scelza et al. 2012).

In this study, the effect of RealSeal SE® on the cellular behavior of human osteoblasts at two time points (1 d and 3 d) was tested. It was shown that the freshly prepared samples had a high toxic effect on the cells 24 h after mixing. After 72 h, this effect was also detectable, albeit to a lesser extent (Scelza et al. 2012). The reduced proliferation of the RealSeal SE® samples, which is presumably caused by monomer release, also appears to be time-dependent, since a slight increase in cell proliferation was observed after 72 h.

Caspase 3 (Casp3) is a cysteine protease that, as a component of an enzyme cascade, is involved in the initiation of cell apoptosis (Kurokawa & Kornbluth 2009). As a result of the activated cascade, there is dissolution of the nuclear envelope, disruption of transcription and translation, chromatin condensation and DNA cleavage through increased nuclease activity (Scaffidi et al. 1998). In this study, an increased expression of the Casp3 gene in Apexit® Plus, AH Plus® and RealSeal SE® samples after 72 h was observed. Interestingly, the toxic effect of RealSeal SE® seemed to be the most pronounced one, while the apoptosis stimulus was highest among the materials tested. RealSeal SE® contains calcium hydroxide, barium sulphate, barium glass and silicates as fillers. There are reports in which the filler content of the material is up to 70% (Versiani et al. 2006). One possible explanation for the cytotoxic effect of this sealer is the release of filler particles or monomers as mentioned above. The toxic effect of the calcium hydroxide-based sealer Apexit® Plus was not confirmed in previous studies (Beltes et al. 1995; Miletic et al. 2000). The reason for our contradictory results could be the use of different cell lines (mouse fibroblasts, PDL fibroblasts). However, a study by Eldeniz et al. showed similar results showing a high cytotoxicity of Apexit® Plus on mouse and human gingival fibroblasts (Eldeniz et al. 2007).

Conclusions

In the present study, it was shown that the sealers investigated from different material groups as well as BeeFill® as a representative of the gutta-percha material group have varying degrees of cytotoxic effect on human osteoblasts. The toxic substances released into the periapical area cause reduced density of the cells, have an inflammatory effect and are able to induce apoptosis. In inter-group comparison, EndoREZ® and Tubli-Seal® performed the worst, followed by Apexit® Plus and RealSeal SE®. BeeFill® and AH Plus® proved to have low cytotoxicity.

The *in vitro* tests carried out are very helpful in investigating the biological properties of sealers. However, they cannot directly simulate the clinical scenario. Nevertheless, such investigations are necessary in the search for sealers with all required chemical, physical and biological properties. In everyday dental practice, the human immune system may restore healthy tissues and inflammatory reactions can be compensated for a long

period of time. However, practitioners should take special care to avoid iatrogenic periapical inflammation caused by root canal filling materials, to ensure complete healing of the apical and periapical tissues.

Acknowledgments and conflicts of interest

All authors declare that they do not have any conflict of interest.

Zusammenfassung

Einleitung

Das periapikale Gewebe kann infolge einer Wurzelkanalbehandlung mit Wurzelkanalfüllungsmaterialien in Berührung kommen. Eine geringe Zytotoxizität von Wurzelkanalsealer ist wichtig, da es zu einer Extrusion dieser Materialien in den periapikalen Bereich und zu einer Fremdkörperreaktion kommen kann, die den Heilungsprozess verzögern und sogar zu einer Degeneration des periapikalen Gewebes führen kann. Es gibt nur wenige wissenschaftliche Daten über die Biokompatibilität von Wurzelkanalfüllungsmaterialien unterschiedlicher Chemie im periapikalen Bereich. Ziel dieser Studie war es, die Auswirkungen verschiedener Wurzelkanalsealer und ihrer Eluate auf menschliche Alveolar-Osteoblasten in Bezug auf Zellproliferation, Adhäsion, Morphologie und Genexpression in vitro zu untersuchen.

Material und Methoden

Fünf Wurzelkanalsealer (AH Plus®, Apexit® Plus, Tubli-Seal®, RealSeal SE®, EndoREZ®) und ein Guttapercha-Obturationsmaterial (BeeFill®) wurden getestet. Humane Alveolar-Osteoblasten von drei verschiedenen Spendern wurden nach Inkubation mit 24- und 72-stündigen Sealer-Eluaten mittels qPCR (Genexpression) untersucht. Die morphologischen Reaktionen der alveolären Osteoblasten wurden visualisiert, nachdem die Zellen für 3 Tage, 7 Tage und 14 Tage kultiviert wurden, gefolgt von Rasterelektronenmikroskopie (Morphologie, Adhäsion) und Fluoreszenzmikroskopie (Morphologie des Aktinzytoskeletts, Proliferation). Es wurde eine Analyse mit wiederholten Messungen durchgeführt und die p-Werte wurden mit Tukey angepasst.

Resultate

Während alle Wurzelkanalsealer die Zellmorphologie und die Genexpression beeinflussten, die mit Apoptose (Casp3), Proliferation (Histon H3) und Entzündung (Interleukin-6 und Matrix-Metalloproteinasen 1 und 3) in Verbindung gebracht werden, sorgten vor allem AH Plus® und Apexit® Plus für ein regelmässiges Aktinzytoskelett und gutes Genexpressionsmuster. Was die Zelladhäsion betrifft, unterstützte nur AH Plus® die ordnungsgemässe Verankerung der alveolären Osteoblasten.

Diskussion

In der vorliegenden Studie konnte gezeigt werden, dass die untersuchten Sealer aus verschiedenen Materialgruppen sowie BeeFill® als Vertreter der Materialgruppe Guttapercha Toxizität auf die Zellen aufweisen. Im Zytotoxizitätsvergleich schnitten EndoREZ® und Tubli-Seal® am schlechtesten ab, gefolgt von Apexit® Plus und RealSeal SE®. Am besten schnitten BeeFill® und AH Plus® ab, die eine vergleichsweise geringe Zytotoxizität aufweisen. Unsere Ergebnisse belegen die Biokompatibilität von Wurzelkanalsealer auf Epoxidharzbasis, d.h. AH Plus®, während sich andere Wurzelkanalsealer als zytotoxisch für alveoläre Osteoblasten erwiesen. Weitere Studien sind erforderlich, um die Reaktionen der Knochenzellen nach einer Wurzelkanalbehandlung zu verstehen und die klinische Entscheidung über den Wurzelkanalsealer der Wahl zu treffen.

Résumé

Objectif

Les tissus périapicaux peuvent être exposés aux matériaux d'obturation canalaire à la suite d'un traitement canalaire. La faible cytotoxicité des produits d'obturation est importante car l'extrusion de ces matériaux dans la zone périapicale peut se produire et entraîner une réaction à corps étranger qui peut retarder le processus de guérison et même dégénérer les tissus périapicaux. Il existe peu de données scientifiques sur la biocompatibilité des matériaux d'obturation des canaux radiculaires de différentes compositions chimiques sur la zone périapicale. Cette étude visait à étudier les effets de différents matériaux d'obturation canalaire et de leurs éluats sur les ostéoblastes alvéolaires humains en termes de prolifération cellulaire, d'adhésion, de morphologie et d'expression génétique in vitro.

Matériels et méthodes

Cinq scellants endodontiques (AH Plus®, Apexit® Plus, Tubli-Seal®, RealSeal SE®, EndoREZ®) et un matériau d'obturation à base de gutta-percha (BeeFill®) ont été testés. Les ostéoblastes alvéolaires humains dérivés de trois donneurs différents après incubation avec des éluats de scellement de 24 et 72 heures ont été étudiés au moyen de la qPCR (expression génétique). Les réactions morphologiques des ostéoblastes alvéolaires ont été mesurées en cultivant les cellules pendant 72 h, puis 7 et 14 jours, respectivement, et en procédant à une microscopie électronique à balayage (morphologie, adhésion) et à une imagerie par fluorescence du cytosquelette d'actine (morphologie, prolifération). Une analyse de mesures répétées a été réalisée et les valeurs p ont été ajustées par Tukey.

Résultats

Alors que tous les scellants endodontiques ont influencé la morphologie cellulaire et l'expression des gènes associés à l'apoptose (Casp3), à la prolifération (histone H3) et à l'inflammation (interleukine-6 et métalloprotéinases matricielles 1 et 3), AH Plus® et Apexit® Plus ont principalement produit un cytosquelette d'actine régulier et des modèles d'expression génétique bénéfiques. En ce qui concerne l'adhésion cellulaire, seul AH Plus® a permis un ancrage correct des ostéoblastes alvéolaires.

Discussion

Dans la présente étude, il a été démontré que les scellants étudiés provenant de différents groupes de matériaux, ainsi que BeeFill® en tant que représentant du groupe de matériaux gutta-percha, ont des degrés variables d'effet cytotoxique sur les cellules. Dans la comparaison de la cytotoxicité, EndoREZ® et Tubli-Seal® ont obtenu les pires résultats, suivis par Apexit® Plus et RealSeal SE®. BeeFill® et AH Plus® se sont avérés être les meilleurs, et ont donc comparativement une faible cytotoxicité. Nos résultats fournissent des preuves de la biocompatibilité des scellants endodontiques à base de résine époxy, c'est-à-dire AH Plus®. D'autres études sont nécessaires pour comprendre les réactions des cellules osseuses après un traitement endodontique et la prise de décision clinique concernant le scellant de choix pour les obturations canalaires.

References

Al-Awadhi S, Spears R, Gutmann J L, Opperman L A: Cultured primary osteoblast viability and apoptosis in the presence of root canal sealers. J Endod 30: 527–533 (2004)

Arias-Moliz M T, Farrugia C, Lung C Y K, Wismayer P S, Camilleri J: Antimicrobial and biological activity of leachate from light curable pulp capping materials. J Dent 64: 45–51 (2017)

Auf'mkolk B, Hauschka P V, Schwartz E R: Characterization of human bone cells in culture. Calcif Tissue Int 37: 228–235 (1985)

Bando Y, Henderson B, Meghji S, Poole S, Harris M: Immunocytochemical localization of inflammatory cytokines and vascular adhesion receptors in radicular cysts. J Oral Pathol Med 22: 221–227 (1993)

Barbosa S V, Araki K, Spångberg L S: Cytotoxicity of some modified root canal sealers and their leachable components. Oral Surg Oral Med Oral Pathol 75: 357–361 (1993)

Basset P, Okada A, Chenard M P, Kannan R, Stoll I, Anglard P, Bellocq J P, Rio M C: Matrix metalloproteinases as stromal effectors of human carcinoma progression: therapeutic implications. Matrix Biol 15: 535–541 (1997)

Beltes P, Koulaouzidou E, Kotoula V, Kortsaris A H: In vitro evaluation of the cytotoxicity of calcium hydroxide-based root canal sealers. Endod Dent Traumatol 11: 245–249 (1995)

Bhuvarahamurthy V, Kristiansen G O, Johannsen M, Loening S A, Schnorr D, Jung K, Staack A: In situ gene expression and localization of metalloproteinases MMP1, MMP2, MMP3, MMP9, and their inhibitors TIMP1 and TIMP2 in human renal cell carcinoma. Oncol Rep 15: 1379–1384 (2006)

Bouillaguet S, Wataha J C, Tay F R, Brackett M G, Lockwood P E: Initial in vitro biological response to contemporary endodontic sealers. J Endod 32: 989–992 (2006)

Briseno B M, Willershausen B: Root canal sealer cytotoxicity on human gingival fibroblasts. 1. Zinc oxide-eugenol-based sealers. J Endod 16: 383–386 (1990)

Camargo C H R, Oliveira T R, Silva G O, Rabelo S B, Valera M C, Cavalcanti B N: Setting time affects in vitro biological properties of root canal sealers. J Endod 40: 530–533 (2014)

Camilleri J, Arias Moliz T, Bettencourt A, Costa J, Martins F, Rabadijeva D, Rodriguez D, Visai L, Combes C, Farrugia C, Koidis P, Neves C: Standardization of antimicrobial testing of dental devices. Dent Mater 36: e59–e73 (2020)

Camilleri J, Mallia B: Evaluation of the dimensional changes of mineral trioxide aggregate sealer. Int Endod J 44: 416–424 (2011)

Chang M C, Lin L D, Chen Y J, Tsai Y L, Cheng Y A, Kuo C S, Chang H H, Tai T F, Lin H J, Jeng J H: Comparative cytotoxicity of five root canal sealers on cultured human periodontal ligament fibroblasts. Int Endod J 43: 251–257 (2010)

Chen C S, Mrksich M, Huang S, Whitesides G M, Ingber D E: Geometric control of cell life and death. Science 276: 1425–1428 (1997)

Chen W T: Membrane proteases: roles in tissue remodeling and tumour invasion. Curr Opin Cell Biol 4: 802–809 (1992)

Cohen B I, Pagnillo M K, Musikant B L, Deutsch A S: An in vitro study of the cytotoxicity of two root canal sealers. J Endod 26: 228–229 (2000)

Cook J, Nandakumar R, Fouad A F: Molecular- and culture-based comparison of the effects of antimicrobial agents on bacterial survival in infected dentinal tubules. J Endod 33: 690–692 (2007)

Dahl J E: Toxicity of endodontic filling materials. Endod Topics 12: 39–43 (2005)

de Chevigny C, Dao T T, Basrani B R, Marquis V, Farzaneh M, Abitbol S, Friedman S: Treatment outcome in endodontics: the Toronto study – phase 4: initial treatment. J Endod 34: 258–263 (2008)

Diomede F, Caputi S, Merciaro I, Frisone S, D'Arcangelo C, Piattelli A, Trubiani O: Pro-inflammatory cytokine release and cell growth inhibition in primary human oral cells after exposure to endodontic sealer. Int Endod J 47: 864–872 (2014)

Donnermeyer D, Vahdat-Pajouh N, Schafer E, Dammaschke T: Influence of the final irrigation solution on the push-out bond strength of calcium silicate-based, epoxy resin-based and silicone-based endodontic sealers. Odontology 107: 231–236 (2019)

Eldeniz A U, Mustafa K, Ørstavik D, Dahl J E: Cytotoxicity of new resin-, calcium hydroxide- and silicone-based root canal sealers on fibroblasts derived from human gingiva and L929 cell lines. Int Endod J 40: 329–337 (2007)

Elyassi Y, Moinzadeh A T, Kleverlaan C J: Characterization of Leachates from 6 Root Canal Sealers. J Endod 45: 623–627 (2019)

Euler G J, Miller G A, Hutter J W, D'Alesandro M M: Interleukin-6 in neutrophils from peripheral blood and inflammatory periradicular tissues. J Endod 24: 480–484 (1998)

Friedman S, Abitbol S, Lawrence H P: Treatment outcome in endodontics: the Toronto Study. Phase 1: initial treatment. J Endod 29: 787–793 (2003)

Gopikrishna V: Grossman's Endodontic Practice. 14th edn, Wolters Kluwer, Gurgaon (2020)

Geurtsen W, Leyhausen G: Biological aspects of root canal filling materials – histocompatibility, cytotoxicity, and mutagenicity. Clin Oral Investig 1: 5–11 (1997)

Gulati N, Chandra S, Aggarwal P K, Jaiswal J N, Singh M: Cytotoxicity of eugenol in sealer containing zinc-oxide. Endod Dent Traumatol 7: 181–185 (1991)

Guldener P H, Lungeland K: Endodontologie: Diagnostik und Therapie. Thieme, Stuttgart (1993)

Heidemann D: Die Wurzelfüllung – manuell – maschinell. Dtsch Zahnärztl Z 44: 414–416 (1989)

Hikage S, Sato A, Suzuki S, Cox C F, Sakaguchi K: Cytotoxicity of dental resin monomers in the presence of S9 mix enzymes. Dent Mater J 18: 76–86 (1999)

Hirano T, Akira S, Taga T, Kishimoto T: Biological and clinical aspects of interleukin 6. Immunol Today 11: 443–449 (1990)

Hong J Y, Kim Y J, Lee H W, Lee W K, Ko J S, Kim H M: Osteoblastic cell response to thin film of poorly crystalline calcium phosphate apatite formed at low temperatures. Biomaterials 24: 2977–2984 (2003)

Honma M, Hayakawa Y, Kosugi H, Koizumi F: Localization of mRNA for inflammatory cytokines in radicular cyst tissue by in situ hybridization, and induction of inflammatory cytokines by human gingival fibroblasts in response to radicular cyst contents. J Oral Pathol Med 27: 399–404 (1998)

Huang F M, Chang Y C: Cytotoxicity of resin-based restorative materials on human pulp cell cultures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 94: 361–365 (2002)

Huang F M, Tai K W, Chou M Y, Chang Y C: Cytotoxicity of resin-, zinc oxide-eugenol-, and calcium hydroxide-based root canal sealers on human periodontal ligament cells and permanent V79 cells. Int Endod J 35: 153–158 (2002)

Huang F M, Tsai C H, Yang S F, Chang Y C: Induction of interleukin-6 and interleukin-8 gene expression by root canal sealers in human osteoblastic cells. J Endod 31: 679–683 (2005)

Ishimi Y, Miyaura C, Jin C H, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T, Hirano T: IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 145: 3297–3303 (1990)

International Organization for Standardization: Biological evaluation of medical devices – part. 1: Evaluation and testing within a risk management process. ISO 10993-1 (2018)

Kangarloo A, Sattari M, Rabiee F, Dianat S O: Evaluation of cytotoxicity of different root canal sealers and their effect on cytokine production. Iran Endod J 4: 31–34 (2009)

Kaur A, Shah N, Logani A, Mishra N: Biotoxicity of commonly used root canal sealers: A meta-analysis. J Conserv Dent 18: 83–88 (2015)

Kazemi R B, Safavi K E, Spångberg L S: Dimensional changes of endodontic sealers. Oral Surg Oral Med Oral Pathol 76: 766–771 (1993)

Kim Y K, Grandini S, Ames J M, Gu L S, Kim S K, Pashley D H, Gutmann J L, Tay F R: Critical review on methacrylate resin-based root canal sealers. J Endod 36: 383–399 (2010)

Kishimoto T, Hibi M, Murakami M, Narazaki M, Saito M, Taga T: The molecular biology of interleukin 6 and its receptor. Ciba Found Symp 167: 5–16; discussion 16–23 (1992)

Kurokawa M, Kornbluth S: Caspases and kinases in a death grip. Cell 138: 838–854 (2009)

Leonardo M R, Bezerra da Silva L A, Filho M T, Santana da Silva R: Release of formaldehyde by 4 endodontic sealers. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 88: 221–225 (1999)

Li G H, Niu L N, Zhang W, Olsen M, De-Deus G, Eid A A, Chen J H, Pashley D H, Tay F R: Ability of new obturation materials to improve the seal of the root canal system: a review. Acta Biomater 10: 1050–1063 (2014)

Miletić I, Anić I, Karlović Z, Marsan T, Pezelj-Ribarić S, Osmak M: Cytotoxic effect of four root filling materials. Endod Dent Traumatol 16: 287–290 (2000)

Möller A J, Fabricius L, Dahlén G, Öhman A E, Heyden G: Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. Scand J Dent Res 89: 475–484 (1981)

Nair P N R: Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med 15: 348–381 (2004)

Ørstavik D: Antibacterial properties of endodontic materials. Int Endod J 21: 161–169 (1988)

Ørstavik D, Nordahl I, Tibballs J E: Dimensional change following setting of root canal sealer materials. Dent Mater 17: 512–519 (2001)

Ørstavik D: Materials used for root canal obturation: technical, biological and clinical testing. Endod Topics 12: 25–38 (2005)

Ørstavik D: Endodontic filling materials. Endod Topics 31: 53–67 (2014)

Pascon E A, Spångberg L S: In vitro cytotoxicity of root canal filling materials: 1. Gutta-percha. J Endod 16: 429–433 (1990)

Paula-Silva F W G, da Silva Bezerra L A, Kapila Y L: Matrix metalloproteinase expression in teeth with apical periodontitis is differentially modulated by the modality of root canal treatment. J Endod 36: 231–237 (2010)

Peterson C L, Laniel M A: Histones and histone modifications. Curr Biol 14: R546–551 (2004)

Robey P G, Termine J D: Human bone cellsin vitro. Calcif Tissue Int 37: 453–460 (1985)

Rodriguez D, Morrison C J, Overall C M: Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta 1803: 39–54 (2010)

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli K J, Debatin K M, Krammer P H, Peter M E: Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17: 1675–1687 (1998)

Scelza M Z, Linhares A B, da Silva L E, Granjeiro J M, Alves G G: A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. Int Endod J 45: 12–18 (2012)

Schäfer E, Zandbiglari T: Solubility of root-canal sealers in water and artificial saliva. Int Endod J 36: 660–669 (2003)

Schwarze T, Fiedler I, Leyhausen G, Geurtsen W: The cellular compatibility of five endodontic sealers during the setting period. J Endod 28: 784–786 (2002)

Scotti R, Tiozzo R, Parisi C, Croce M A, Baldissara P: Biocompatibility of various root canal filling materials ex vivo. Int Endod J 41: 651–657 (2008)

Silva E, Cardoso M L, Rodrigues J P, De-Deus G, Fidalgo T: Solubility of bioceramic- and epoxy resin-based root canal sealers: A systematic review and meta-analysis. Aust Endod J 47: 690–702 (2021)

Skinner R L, Himel V T: The sealing ability of injection-molded thermoplasticized gutta-percha with and without the use of sealers. J Endod 13: 315–317 (1987)

Sousa C J A, Montes C R M, Pascon E A, Loyola A M, Versiani M A: Comparison of the intraosseous biocompatibility of AH Plus, EndoREZ, and Epiphany root canal sealers. J Endod 32: 656–662 (2006)

Stetler-Stevenson W G, Hewitt R, Corcoran M: Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. Semin Cancer Biol 7: 147–154 (1996)

Tagger M, Katz A, Tamse A: Apical seal using the GPII method in straight canals compared with lateral condensation, with or without sealer. Oral Surg Oral Med Oral Pathol 78: 225–231 (1994)

Tronstad L, Barnett F, Flax M: Solubility and biocompatibility of calcium hydroxide-containing root canal sealers. Endod Dent Traumatol 4: 152–159 (1988)

Versiani M A, Carvalho-Junior J R, Padilha M I A F, Lacey S, Pascon E A, Sousa-Neto M D: A comparative study of physicochemical properties of AH Plus and Epiphany root canal sealants. Int Endod J 39: 464–471 (2006)

Wang Y, Jiang X L, Peng S W, Guo X Y, Shang G G, Chen J C, Wu Q, Chen G Q: Induced apoptosis of osteoblasts proliferating on polyhydroxyalkanoates. Biomaterials 34: 3737–3746 (2013)

Wesselink P: Die Wurzelkanalfüllung (I). Endodontie 4: 181–192 (1995)

Williamson A E, Dawson D V, Drake D R, Walton R E, Rivera E M: Effect of root canal filling/sealer systems on apical endotoxin penetration: a coronal leakage evaluation. J Endod 31: 599–604 (2005)

Zmener O: Tissue response to a new methacrylate-based root canal sealer: preliminary observations in the subcutaneous connective tissue of rats. J Endod 30: 348–351 (2004)

Zmener O, Banegas G, Pameijer C H: Bone tissue response to a methacrylate-based endodontic sealer: a histological and histometric study. J Endod 31: 457–459 (2005)