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Tissue Reaction to Sealing Materials: Different View at Biocompatibility

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Abstract
The biodegradability of root canal sealers in areas other than the root canal system is crucial to the overall success rate of endodontic treatment. The aim of the present study was to investigate, the cell and tissue reaction to GuttaFlow and AHPlus, both in vitro and in vivo. For the in vitro experiments the materials were incubated with Human Periodontal Ligament Fibroblasts and cell proliferation and cytotoxicity analyses were performed. Additional fluorescence-microscope stainings were carried out in order to visualize cell growth and morphology. For assessment of the tissue reaction to the materials a subcutaneous implantation model in Wistar rats was employed and the inflammatory response to the materials was visualized by means of general and specific histology after 6 weeks. Human gingival fibroblasts proliferation seemed to be dependent upon dental material and cultivation time. After an incubation period of 96 hrs AHPlus proved to be significantly ($p < 0.002$) more cytotoxic than GuttaFlow, as only a small number of fibroblasts survived on AHPlus. In vivo, GuttaFlow was surrounded by a fibrous capsule and no degradation took place, while AHPlus induced a well-vascularized granulation tissue in which the material was phagocyted by macrophages. The results of this study demonstrate that a potential cytotoxic effect of a sealing material may beneficial in order to have antibacterial properties and induce self degradation when accidentally extruded over the apical foramen.
Key words: AHPlus, Guttaflow, sealing material, biocompatibility, biomaterial

Introduction

Increased technical knowledge and scientific improvements have resulted in success rates of endodontic therapy ranging from 92% to 98% [1, 2, 3]. As a consequence, endodontic treatment is considered superior to implantation by some authors [4]. For this reason, both patients and dental professionals favor tooth preservation rather than extraction of teeth. The extrusion of sealing agents over the apical constriction is, however, a phenomenon, frequently observed in clinical routine [5]. Sealing materials should not exert an irritating effect on the peri-radicular tissue [6] and complications relating to persistence of sealing agents in the peri-apical tissue could result in endodontic failure. The knowledge of the inflammatory response pattern to the employed material is crucial in order to assess the clinical consequence of a potential material over extrusion.

A variety of sealing materials with different specifications is currently available, such as, calcium hydoroxide-, zinx-oxide-eugenole-, zirconium oxide-, and resin-based. Among the great variety of sealing agents AHPlus (Dentsply, Konstanz, Germany), an epoxy resin-based root canal sealer, is considered to be the golden standard sealing agent [7]. Nevertheless, several in vitro studies have illustrated the proinflammatory activity of AHPlus. A significant increase of prostaglandine E2 (PGE2) release in human gingival fibroblasts was observed after their incubation on this material [8]. The observed cell toxicity appeared to be independent of the origin of the cells since cytotoxicity was observed after the incubation of mouse fibroblasts with AHPlus [9]. Similar observations such as induction of oxidative stress were reported for human mammalian cells [10]. In vivo, the subcutaneous implantation of the epoxy resign based sealer resulted in an inflammatory response combined with a granulation tissue composed of macrophages and multinucleated giant cells [11]. An inflammatory response characterized by the presence of lymphocytes, macrophages, giant foreign body cells and necrotic bone fragments was also found to occur after AHPlus implantation into the lower jaw of guinea pigs [12]. The observed inflammatory response in vitro and in vivo may reflect the ability of a living tissue such as the periradicular tissue to induce the degradation process of AHPlus. Ac-

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cordingly, materials, which are successfully employed in endodontic treatment, should be able to induce an inflammatory cascade that will contribute to their degradation in case of overextrusion. This knowledge should be considered when sealing agents are analyzed with respect to their suitability for clinical application.

GuttaFlow is a relatively new sealing material for endodontic treatment, which combines gutta-percha and sealer into one injectable system. Due to its thixotropicity, which enables the material to diminish its viscosity when put under stress, it is more likely to be extruded into the periapical tissue after application [13]. When compared to AHPlus, the gutta percha based sealer is significantly less cytotoxic, when assessing the viability and the proliferation of cells by means of the MTT-test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) [14]. The observed low cytotoxicity appears to be independent of the cell origin since very low cytoxicity was reported after incubation of mouse fibroblasts on GuttaFlow [15]. In a further in vitro study GuttaFlow was tested on human peripheral blood lymphocytes using the comet assay and chromosomal aberration analysis. The results of this study displayed a good biocompatibility and genotoxicity of GuttaFlow [16].

In vivo the subcutaneous implantation model in Wistar rats was employed to examine malondialdehyde, glutathione levels in tissue samples and different serum parameters were measured. In parallel, tissues were also examined histologically after implantation of GuttaFlow. A good biocompatibility and acceptable tissue toxicity was also observed [17].

The present combined in vitro and in vivo study was aimed at analyzing the cell and tissue reaction to GuttaFlow in comparison to AHPlus, in order to evaluate the early and the late inflammatory response as a predictor of its potential clinical success. In this study the early inflammatory response was analyzed on a cellular basis for up to 96 hours in vitro, while the late tissue response was assessed with the subcutaneous implantation model by means of histological evaluation at day 60.

Material and Methods

Sealing Materials

AHPlus (Dentsply, Konstanz, Germany) is an epoxy resin-based root canal sealer. It consists of a paste-paste system, with paste A containing epoxy resin and iron oxide and paste B containing amines and silicone oil. It is described as having a favorable mechanical properties, a good adhesion to dentine and little polymerisation shrinkage when inserted into the root canal. The detailed description of the materials preparation for the in vitro experiments is explained in the respective section.

GuttaFlow (Roeko, Coltene, Langenau Germany) is composed of a polydimethylsiloxane matrix filled with very finely ground gutta-percha with a diameter less than 30 μm. It is a cold flowable, self-curing filling system, which combines sealer and gutta-percha in one product. Due to its thixotropicity, the material is able to diminish its viscosity when put under stress. The detailed description of the materials preparation for the in vitro experiments is explained in the respective section.

Clonetics® HPdLF-Human Periodontal Ligament Fibroblasts (Lonza, Switzerland) were cultured in Dulbecco´s Modified Eagle Medium, supplemented with 15% fetal bovine serum, 2mM L-Glutamine and 100U/100μg/ml Penicillin/Streptomycin (Invitrogen, Germany), incubated at 37 0C and 5% CO_2. Different in vitro assays were carried out in order to assess the interaction of the sealing agents with the Human Periodontal Ligament Fibroblasts i.e. cell visualization, cell proliferation, materials cytotoxiticy, and induction of inflammatory agents.

Cell Visualization

In order to visualize the cell/sealing material-interface fluorescence-microscope stainings of cell components (30.000 cells / well), i.e. core and cytoplasm were carried out. The stained cells were visualized by means of an Inverted Microscope, Axiovert 40C (Carl Zeiss, Germany). Magnifications between 25 to 400 fold were used. Pallotoxins were used to stain the cytoskeleton of the cells and DAPI was applied to counterstain the nucleus. In addition, a live/dead assay was applied to visualize the vitality of the cells in close contact to the materials surface. The applied stains are described below.

Phallotoxins (BODIPY® FL phallacidin; Invitrogen,Oregon, USA) stain F-actin at nanomolar concentrations and are used for labeling, identifying, and quantitating F-actin in tissue sections and cell cultures. Unlike antibodies, the binding affinity does not change appreciably with actin from different species or sources. Nonspecific staining is negligible, and the contrast between stained and unstained areas is extremely large.

The blue-fluorescent DAPI (4´,6-Diamidine-2´-phenylindole dihydrochloride; Roche Diagnostics GmbH, Mannheim, Germany) nucleic acid stain preferentially stains DNA (Deoxyribonucleic acid). It can be rapidly taken up into cellular DNA by tissue culture cells, yielding highly fluorescent nuclei and no detectable cytoplasmic fluorescence. Blue fluorescence stands out in vivid contrast to the green Phallotoxin staining.

Calcein /Ethidium homodimer stainig (LIVE/DEAD® Viability/Cytotoxicity Kit; Invitrogen, Eugene, Oregon) is a two-color fluorescence-based method for determining viability / cytotoxicity of cultured cells. Calcein is a fluorogenic esterase substrate that is hydrolyzed intracellularly to a green fluorescent product, which is an indicator of live cells. Ethidium homodimer enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. Ethidium homodimer is excluded by the intact plasma membrane of live cells.

Cell Proliferation Assay/ Cytotoxicity Analysis

Selected root canal sealers GuttaFlow (Coltène/Whaledent, Langenau, Germany) and AHPlus

(Dentsply DeTrey GmbH, Konstanz, Germany) were used to test for possible effects on cell proliferation and metabolic activity of Human Periodontal Ligament Fibroblasts. Manufacturers recommendations were strictly adhered to in the aseptic preparation of the root canal sealers. The sealers were placed at the junction between the base and wall of each multiwell cylinder (16 mm diameter, Greiner Bio-One, Frickenhausen). In this manner, the sealers covered only a small surface area of the well. The materials were allowed to set for 24 h. The sealer amounts were determined according to preliminary experiments and calculated by weighing the sealers with an analytical balance (+ 0.01 g, Pionieer, Ohaus, Pine Brook, USA).

Cell proliferation was assessed by means of the AlamarBlue assay (AlamarBlue® Cell Viability Reagent (Invitrogen, Oregon, USA). This assay is a fluorometric growth indicator based on detection of metabolic cell activity. Human Periodontal Ligament Fibroblasts were incubated in a 96-well plate (10.000 cells /well) under standard cultivation conditions with 10% AlamarBlue® at 0, 2, 6, 24, 48, 72, 96 h the plate was measured at a wavelength of 560/20 and 620/40 nm with a fluorescence reader (Synergy HT-Reader, Biotek). Cells in medium without material served as a control. Logarithmic signals were converted to a linear scale and expressed as relative fluorescence units (RFU). All samples were tested in triplicate.

Cytotoxic potential of the employed sealing agent was investigated by means of the ToxiLight® BioAssay Kit (Lonza Rockland, Inc Rockland, USA). The ToxiLight® BioAssay is a non-destructive, bioluminescent cytotoxicity assay which quantitatively measures the release of Adenylate Kinase (AK) from damaged cells. Human Periodontal Ligament Fibroblasts are incubated under standard cultivation conditions in a 96-well plate (30.000 cells / well). After incubating the cells with the sealing agent for 24 h, the supernatant is mixed with ToxiLight® agent. After a short incubation time the emitted light intensity, is measured using a luminometer. Logarithmic signals were converted to a linear scale and expressed as relative luminescence units (RLU). Cells in medium without material served as a control.

Prostaglandine E-Assay

Prostaglandine E2 (PGE2) is a cytokine released by the cells under inflammatory conditions. The PGE2 Assay (R&D Systems, Inc; Minneapolis, USA) was applied in order to assess the inflammatory potential of the sealing materials. The release of PGE2 was determined in 96-well plates seeding the cells over the root canal filling materials. The cells (30.000 cells /well) were incubated for 30 min in contact with the materials. The supernatant was collected and PGE2 was determined using a highly sensitive and specific competitive enzyme immunoassay based on monoclonal antibodies for PGE2. The color development was stopped, and the absorbance was read at 450 nm. The intensity of the color is inversely proportional to the concentration of PGE2 in the sample.

Statistical Analysis

The statistical analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL). A p-value < 0.05 was considered to indicate statistical significance.

Animals

All experiments were performed with approval of the Committee on the Use of Live Animals in Teaching and Research, Rhineland-Palatinate, Germany. For these studies, female Wistar rats (6-to 8-week old, 90-120 g body weight, Charles River Laboratories, Sulzfeld, Germany) were used and housed one per cage, kept with water ad libitum, an artificial light-dark regime, and fed with regular mouse pellets (Laboratory Rodent Chow, Altromin, Germany) at the Laboratory Animal Unit of the Institute of Pathology, Johannes Gutenberg University, Mainz, Germany.

For assessment of the tissue reaction to GuttaFlow (Coltène/Whaledent, Langenau, Germany) and AHPlus (Dentsply DeTrey GmbH, Konstanz, Germany) 16 animals were randomly assigned to three groups of GuttaFlow (n = 6), AHPlus (n = 6) and physiological saline (n = 4) respectively. For the two experimental groups, animals were prepared for the 'late response' time point of 60 days. Anaesthesia (10 ml Ketamine [50 mg/ml] with 1.6 ml Xylazine [2%] were administered by intraperitoneal injection. The skin of the rostral portion of the interscapular region was shaved. The materials were activated according to the manufacturer's description. For each substance a 0.5 ml was injected pericutaneously into the subcutaneous tissue of the animal under sterile conditions using a 20-gauge needle as previously described [18]. In control animals, physiological saline (0.5 ml) was injected. All animals survived the indicated time periods without any complications.

Histological Preparation of the Implantation Bed

The animals were euthanized by an overdose of Ketamine and Xylazine at the indicated time point. Immediately after sacrifice, the implantation bed was explanted together with the surrounding peri-implantation tissue and fixed in 4% buffered formalin for 24 h prior to histological and histochemical analysis. The fixed tissue was cut into seven segments of identical dimensions covering the margins and center of the implantation bed. The segements were then dehydrated in a series of alcohol, transferred to xylene, and embedded in paraffin. From each segment, three consecutive 3-4 µm thick slices were deparaffinized and rehydrated. These slices were stained with Mayer's hematoxylin and eosin (H & E). The segments that contained the most representative view of the materials were selected for qualitative histopathological evaluation. From the corresponding histological block, two consecutive slices were prepared for further histochemical analysis of connective tissue ingrowth.

Histochemistry for Connective Tissue Ingrowth

The remaining three slides were prepared to visualize connective tissue ingrowth within the implantation

bed using additional histochemical staining procedures. Azan and Movat´s pentachrome [19, 20, 21] stains are commonly used for the detection of reticular and collagen fibers. Azan [19] stains collagen and reticular fibers blue, while Movat's [20] pentachrome stains collagen green. All chemicals were purchased from (Sigma-Aldrich, Germany) and used without further purification.

Morphological Evaluation of the Inflammatory Response

Histopathological evaluation was conducted using a Nikon ECLIPSE 80i microscope (Nikon, Japan) by two independent examiners experienced in histomorphological analysis who were blinded to the experimental protocol. The histological slides of the implantation bed as well of the organs were assessed qualitatively for the following characteristic features: Fibrotic capsules around the biomaterials, fibrosis, hemorrhage, necrosis, vascularization, neutrophils, lymphocytes, plasma cells, macrophages, giant cells and degree of biomaterial degradation. Microphotographs were taken using a Nikon DS-Fi1/Digital camera and a digital sight control unit (Nikon, Japan).

Results

In vitro Cell Visualization

AHPlus-group

The application of Phallacidin / DAPI which were utilized to visualize cell nucleus and cytoskeleton structures revealed that Human Periodontal Ligament Fibroblasts were altered in shape, appearing round with no visible cytoplasm branches (Fig. 1a). Almost no cells were visible in close proximity to the epoxy resin based sealing agent (Fig. 1a).

A similar condition was observed when the cells were stained with Calcein/Ethidium homodimer. Ethidium homodimer enters cells with damaged membranes, binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. The intact cell cytoplasma membrane of live cells is not permeable for Ethidium homodimer. Taking into account this information it is clearly visible that nearly all of the cells in close proximity to AHPlus are damaged, as the red color is visible in nearly all cells close to the biomaterial (Fig. 1b).

GuttaFlow-group

The use of Phallacidin / DAPI staining demonstrated that cells incubated with GuttaFlow proliferated well and showed a great affinity to the materials surface (Fig. 1c). A three dimensional cellular network was observed on the materials surface with cell branches spreading over the surface of the material (Fig. 1c).

A similar condition was observed when Calcein/Ethidium homodimer stain for cell visualization was carried out (Fig. 1d). Hardly any cells were damaged and the cells displayed a brightly luminating green color as a sign of an intact metabolism and intact cell cytoplasm membrane (Fig. 1d).

AlamarBlue Proliferation Assay on GuttaFlow vs AHPlus

The AlamarBlue proliferation assay gives information about the proliferating rate of cells incubated with an agent such as the used sealers over a time period of 96 h. Cells in medium without material served as control. In this assay high cellular proliferation was expressed as high relative fluorescence unit (RFU). After 24 hours AHPlus significantly inhibited cell growth as opposed to GuttaFlow that exhibited similar results as the control group. Surprisingly, in the GuttaFlow group an enhanced cell growth rate was observed at 72 h when compared to the control (Fig. 2a). The data showed that the cells in the GuttaFlow group had a significant higher proliferation rate starting at 24h and at time points 48h, 72h, 96h ($p < 0.002$) after exposure to the material (Fig. 2a).

ToxiLight® BioAssay on GuttaFlow vs AHPlus

The ToxiLight® BioAssay, is a bioluminescent cytotoxicity assay, which quantitatively measures the release of Adenylate Kinase (AK) from damaged cells. A high relative luminescence (RLU) indicates a great release of Adenylate Kinase, which again is an indicator for damaged cells. The RLU is measured once, after the cells have been incubated with the respective sealing agents for a time period of 24 hours. Cells in medium without the material served as a control. Cells incubated with AHPlus released significantly higher amounts of Adenylate Kinase than those incubated with GuttaFlow or the control group, indicating cytotoxicity of on cells exposed to AHPlus ($p < 0.002$) (Fig 2b).

Prostaglandin E2 Release on GuttaFlow vs AHPlus

The release of Prostaglandin E2 (PGE-2) as an inflammatory marker was evaluated. Prostaglandins, thromboxanes, and leukotrienes belong to the class of prostanoid fatty acid derivatives of arachidonic acid. Increased amounts of PGE2 are produced in several pathologic conditions, including inflammation and arthritis, fever and tissue injury among others. The application of the PGE2 assay revealed a significantly higher prostaglandin release in the supernatant of Human Periodontal Ligament Fibroblasts incubated with AHPlus ($p < 0.002$) (Fig. 2c). The results of GuttaFlow were comparable to the control group. This can be interpreted as a sign that GuttaFlow does not exert a negative influence on cell viability.

Histological Results

Subcutaneous Implantation of AHPlus

Sixty days following subcutaneous implantation AHPlus showed excellent integration of the material into the subcutaneous tissue (Fig. 3a). The epoxy resin-based sealer resulted in a cement-like aggregate, which did not allow any connective tissue growth into the material (Fig. 3a). The ultrastructural analysis of the material surface demonstrated a crystalline-like ap-

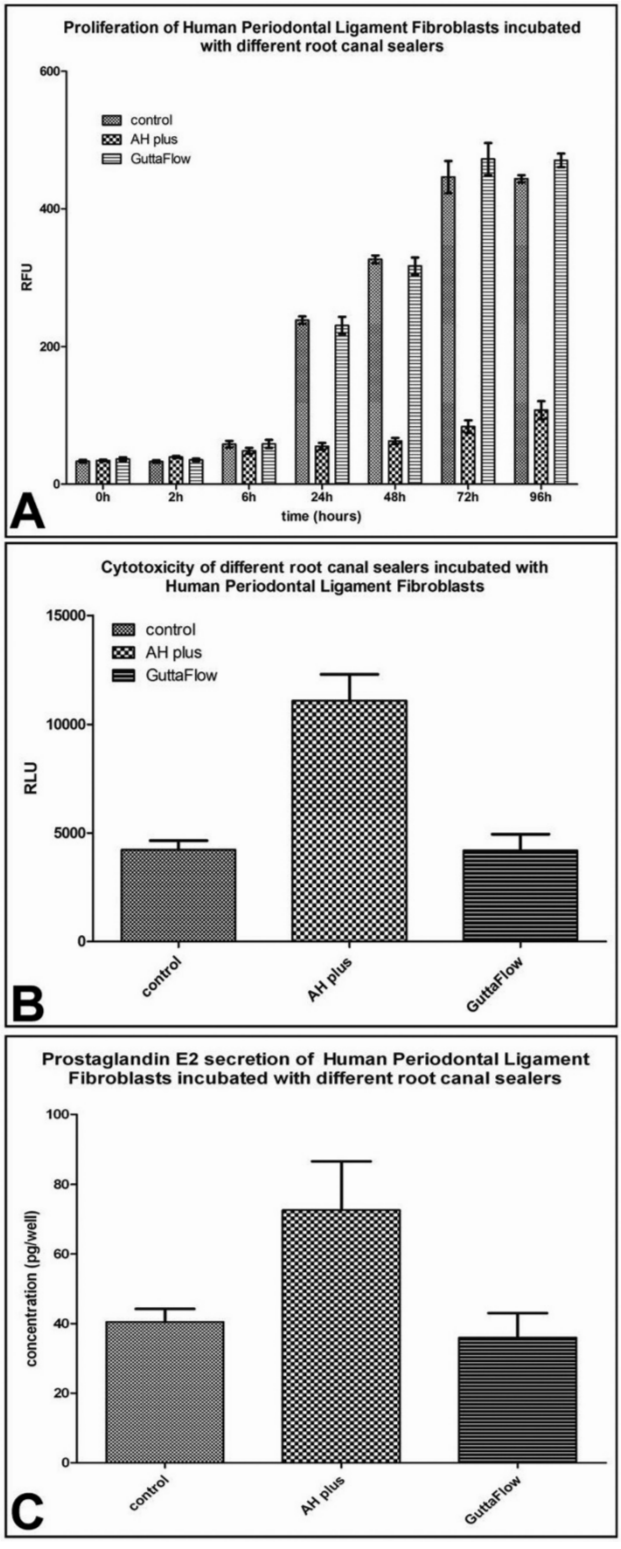


Fig. 2 shows the proliferation rate as well as the inflammatory response of Human Periodontal Ligament Fibroblasts to GuttaFlow, AHPlus and a control group. A) displays the results of the AlamarBlue proliferation assay with Human Periodontal Ligament Fibroblasts. AHPlus significantly inhibited cell growth within the first 24 while GuttaFlow and the control group seemed to have comparable cell growth characteristics. B) shows the results of the ToxiLight® BioAssay after a time period of 24 h. A significantly higher release of Adenylate Kinase was observed in cells, incubated with AHPlus. C) shows the significantly higher prostaglandin release in the supernatant of Human Periodontal Ligament Fibroblasts incubated with AHPlus.

pearance which reflected its composition of epoxy resins (paste A) and amines (paste B) (Fig. 3b). The microscopic analysis the peri-implantary tissue around AHPlus revealed that no fibrous capsule but an active granulation tissue was found within the implantation bed (Fig. 4a). This granulation tissue contained a considerable number of activated macrophages, fibroblasts and microvessels (Fig. 4a). Macrophages at the biomaterial interface and in deeper regions of this granulation tissue were filled with crystalline-like structures indicating a cellular absorption of the epoxy resin-based sealer by these cells (Fig. 4b-c). The cellular absorption of AHPlus, however, appeared to take place from its periphery and no macrophages or connective tissue was found within the core of the implanted material. No multi-nucleated giant cells, lymphocytes, plasma cells or areas of necrosis were detectable surrounding the material.

Subcutaneous Implantation of GuttaFlow

Sixty days following subcutaneous implantation, GuttaFlow demonstrated good integration within the subcutaneous tissue (Fig. 3c). Macroscopically, the gutta-percha based sealing agent appeared as a bulk material. No indication of connective tissue ingrowth was detectable, defined as no inhomogeneity and porosity of the material after its application (Fig. 3c). The analysis of the material's ultrastructure, however, revealed a rough surface (Fig. 3d). The microscopic analysis of the tissue-biomaterial-interface showed that GuttaFlow was surrounded by a thin fibrous capsule (Fig. 4d). Few granulocytes and macrophages were observed, however, a considerable number of fibroblasts were detectable (Fig. 4e). Almost no vessels were present in this fibrous capsule (Fig. 4 d-f). No gutta-percha particles were found within the core cytoplasm of macrophages (Fig. 4 f). No multi-nucleated giant cells, no lymphocytes, no plasma cells or areas of necrosis were found within the capsule and at the interface of GuttaFlow. No sign of cellular degradation or detectable active inflammatory response was present. Thus, the tissue reaction to the gutta-percha-based sealing agent was completely different than that for AHPlus.

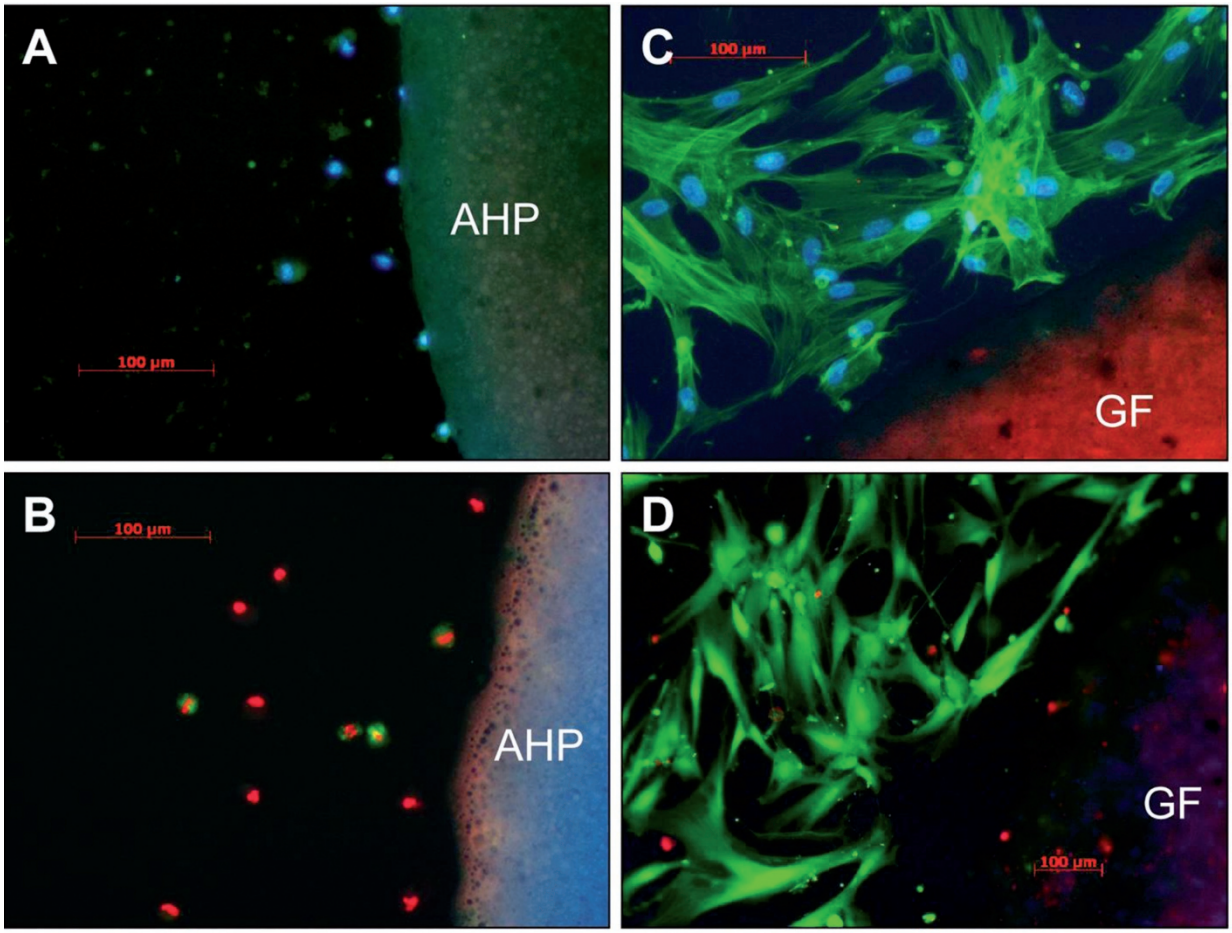
Subcutaneous Implantation of Saline

Sixty days following subcutaneous implantation in the saline group no physiological inflammatory tissue reaction was observed. Sporadic macrophages and fibroblasts were detected embedded in a connective tissue similar to physiological skin tissue (data not shown).

Discussion

The understanding of the inflammatory response to sealing materials is essential for their clinical success. Knowledge about this response might be beneficial in predicting potential complications associated with overextrusion of the material into the periapical tissue.

The experiments with AHPlus showed that cells incubated on this material were altered in size and mor-



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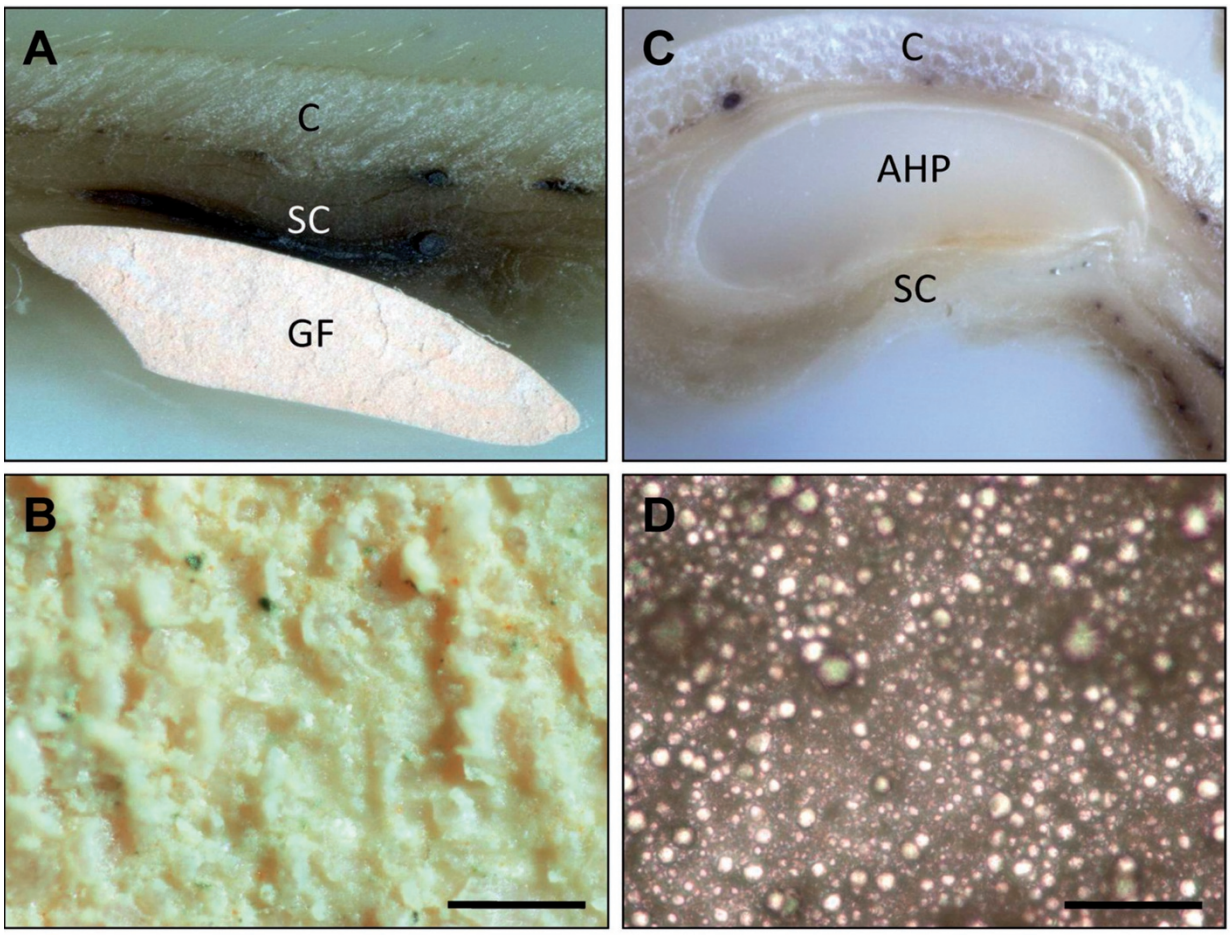

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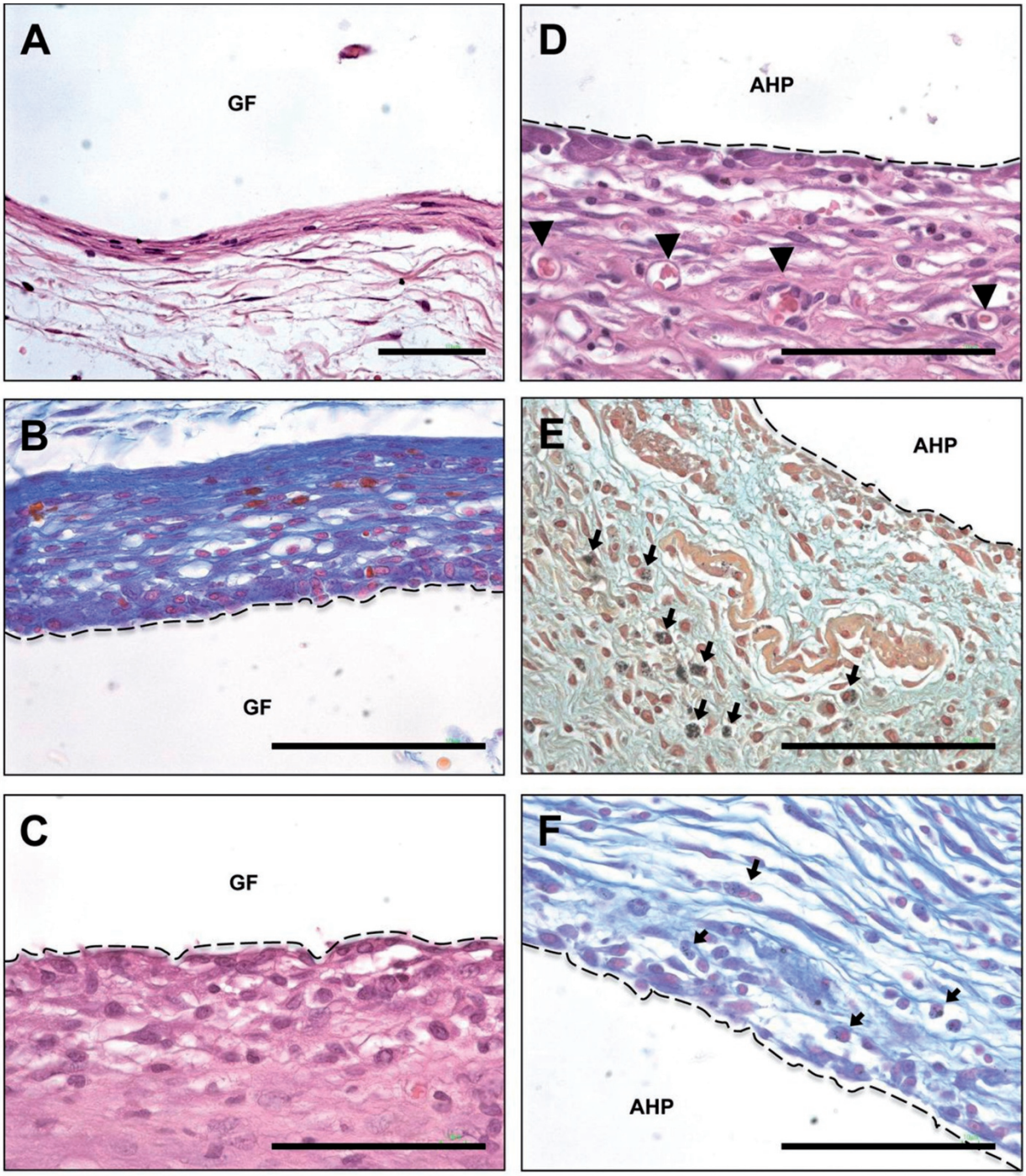

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Fig. 1 shows the reaction of Human Periodontal Ligament Fibroblasts to AHPlus and GuttaFlow stained with Phallacidin / DAPI and Calcein-homodimer. DAPI-stains the cell core blue and Phallacidin counterstains the cytoplasm green. The intact membrane of live cells is not permeable for Ethidium homodimer. a) In the AHPlus group cells were altered in shape and appeared round without cytoplasm brances, Phallacidin / DAPI-staininig 100x, magnification, scale bar =100 µm. b) shows damaged cells in close proximity to AHPlus indicated by the presence of red color in cell cytoplasm, Calcein-homodimer-staininig, 100x magnification, scale bar =100µm. c) cells in the GuttalFlow group were found to built a three dimensional network, reaching to deeper regions of the material. Phallacidin / DAPI-staininig 200 x magnification, scale bar =100 µm. d) shows cells on the surface of GuttaFlow displaying a brightly luminating green color as a sign of an intact metabolism and intact cell cytoplasm Calcein-homodimer-staininig, 200 x magnification, scale bar =100µm.

Fig. 3 shows the tissue integration of GuttaFlow and AHPlus. A+B shows the integration of AHPlus (AHP) within the subcutaneous tissue (SC) of the mouse macroscopically (a) and on an ultra structural level (b). Accordingly, an absence of tissue penetration within AHPlus was observed (a = 40 x magnification), however the ultra structure of this sealing agent appeared more crystalline-like (b = 100 x magnification.); c = Cutis. c+d show the integration of GuttaFlow (GF) within the subcutaneous tissue (SC) of the mouse macroscopically (c) and on an ultra structural level (d). Note the absence of tissue penetration within GuttaFlow (c= 40 x magnification) and the rough ultra structural of this sealing agent (d = 100 x magnification.); c = Cutis.

Fig. 4 shows the reaction to GuttaFlow and AHPlus. A-C shows the tissue reaction to AHPlus; A) shows a well vascularised (double head arrows) granulation tissue. HE, 200 x magnification. B) and C) emphasize the collagen matrix component within this granulation tissue and the active absorption of the biomaterial by macrophages (arrows). (B = Movat´s Pentachrome-staining, 400 x magnification); (C = Azan-staining, 400 x magnification). D-F shows the tissue reaction to GuttaFlow. D) shows GuttaFlow which was surrounded by a thin cell rich and poorly vascularised capsule, HE-staining, 40x magnification. E) and F) demonstrate that no GuttaFlow particle was incorporated by the tissue which surrounded the biomaterial. (E = Azan-staining, 200 x magnification); (F = HE-staining, 400x magnification).

phology. The use of Phallacidin / DAPI and Calcein / Ethidiumhomodimer staining revealed that cells were round-shaped and without visible cell cytoplasm branches. The use of Calcein / Ehtidiumhomodimer, which allows the detection of a bright red color within the cytoplasm of the respective cells, showed that the membranes of cells in close proximity to the material were damaged. An intact cell usually does not show any sign of Calcein uptake. The detection and quantification of PGE-2 and Adenlylat-kinase revealed that this material further induces the release of the pro-inflammatory agents. The results of the cell proliferation assay proved that cells incubated on this material had a significantly lower proliferation rate when compared to GuttaFlow and the control group. In vivo, AHPlus remained as a solid structure through which no connective tissue can penetrate. Its implantation resulted in an active granulation tissue, consisting of macrophages, fibroblasts and microvessels 60 days after implantation. This granulation tissue was induced by the biomaterial as a pathway for macrophages to the implantation bed in order to perform the materials cellular resorption. The presence of crystalline structures within the macrophages verified this resorptive activity. The results of our in vivo study are in accordance with the study of Gomes-Filho et al. [12], in which a histological analysis of AHPlus was performed in the same animal model up to 30 days that demonstrated an active granulation tissue. We assume that in Wistar rats, the cellular resorption of the epoxy resin sealer takes place between day 30 and 60 after implantation. Based on these data we refrained from analyzing the materials at earlier time points.

The present study has shown that in vitro GuttaFlow induces a mild cell toxicity. Cell visualization by means of Phallacidin / DAPI and Calcain / Ehtidiumhomodimer stainings illustrate that HPdLF incubated with GuttaFlow were able to deploy their branches and detach on the materials surface. The PGE-2 and Adenylate-kinase assays underlined the very low pro-inflammatory potential of this material with values comparable to the control group. The results of the proliferation assay revealed that cells cultivated on GuttaFlow had a significantly higher proliferation rate when compared to AHPlus with results comparable to the control group. In vivo, GuttaFlow was surrounded by a fibrous capsule, which isolated the material from its peripheral surrounding tissue. No cellular absorption of the material was seen in any of the analyzed animals. At the same time no connective tissue ingrowth was observed within the material, which again is a sign for its hermetic structure. These data demonstrate that this material does induce an inflammatory response pattern which leads to its isolation by a fibrous capsule within the living organism as it can not be degraded by hosts inflammatory cells.

The extrusions of sealing material into the periapical region, i.e. apical puffs, are well-described complications in endontontic treatment. Apical puffs are the result of over-extension of filling materials, and can be observed in the presence of severe apical lesions. This calls for materials, which are able to hermetically fill the root canals while being resorbable when accidentally overextruded into tissues other than the root canal system. It is known that when certain non- resorbable materials are over-extruded into soft tissue, i.e. human sinus, these materials are capable of triggering chronic infections such as aspergillosis [22, 23]. This would necessitate a surgical intervention leading to a potential loss of the tooth. These cases would have to be declared as endodontic failures endangering the potential of endodontic treatment as a reasonable alternative to implantation.

The findings of this study revealed that the inflammatory response patterns induced by the sealing agents were different in vitro and in vivo. AHPlus obviously has a cell toxic effect on human periodontal ligaments in vitro. In vivo, these findings are reflected by the presence of an active granulation tissue and macrophages, which are involved in materials degradation. AHPlus, obviously has the ability to stimulate a proinflammatory milieu, resulting in its degradation. GuttaFlow, on the other hand evoked almost no inflammatory response in vitro.

In vivo, GuttaFlow did not undergo an adequate degradation but remained encapsulated within the subcutaneous tissue as a foreign body. As a consequence, GuttaFlow might persist in case of an overextrusion into the periapical tissue as a foreign body. The persistence of GuttaFlow as a non-resorbable sealing agent may lead to endodontic complications such as infections of the peri-apical tissue. Accordingly, special caution should be taken when GuttaFlow is applied, as it has been described being easily over-extruded into the periapical tissue [13].

Sealing agents are solely produced for the hermetic closure of the root canal system. This feature is desirable to inhibit the growth of any microorganisms i.e. mainly bacteria within the newly cleaned root canal system [6]. Sealing materials are not designed to be applied in other tissues such as bone and/or soft tissue. Complications may arise only in case of the materials accidental overextrusion into the periapical tissue and its persistence. Thus, the introduction of sealing materials into an undesired tissue, i.e. soft and bone tissue should favorably result in its degradation. A cellular degradation can be only performed by means of a granulation tissue, which serves as a pathway for the inflammatory cells to the implantation site.

Our present data and other previously described data demonstrated the proinflammatory characterists of AHPlus in vitro and in vivo [9, 11, 12, 24] and could therefore be interpreted as an effort of the organism to induce its degradation.

Biomaterials, such as polymer-based membranes and ceramic-based bone substitutes which are designed to find application in bone or soft tissue regeneration, i.e. for Guided Bone regeneration and/or Guided Tissue regeneration induce a inflammatory response which can be related to their structural differences. We have previously shown that changes in size, porosity, shape and chemical composition induce different tissue inflammatory tissue reactions [25-28]. Additionally, changes in different treatment of the same materials resulted in different tissue responses [29]. Bone substitutes or biomembranes which ideally mimick spongious bone particles or extracelluar matrices, enable residual cells and tissue growth along their

three-dimensional architecture. Accordingly, the granulation tissues as well as necrosis in bone, which has been described after AHPlus implantation [11] have to be evaluated as the effort of the tissue to eliminate AHPlus in tissues in which the application of this sealing agent has no benefit. On the other hand, these data support the paradoxical postulation of Grossmann [6], that sealing agents should inhibit the growth of any microorganisms but at the same time not irritate the peri-radicular tissue.

The results of the present study as well as the in vitro and in vivo studies described above indicate that one may use of the term of "biocompatibility" for describing the tissue reaction to the sealing materials. Biocompatibility is the ability of a material to perform an appropriate host response within a specific application [30]. Accordingly, there is no specific application for sealing materials in other tissues than the root canal system. As a consequence, the application of a sealing material in any other tissue but the root canal has to result in its degradation to be considered the golden standard.

Conclusion

In this combined in vitro and in vivo study the cell and tissue reaction to GuttaFlow and AHPlus were compared. AHPlus induced a proinflammatory milieu in vitro and in vivo that resulted in well-vascularized granulation tissue and the sealing agents biodegradation. No proinflammatory activity was reported for GuttaFlow in vitro. In vivo, GuttaFlow remained as a foreign body, which surrounded by a fibrous capsule. These findings demonstrate that the use of sealing materials with a good biodegradation, such as AHPlus, reduce the risk of infections and promote apical repair when accidentally extruded over the apical foramen.

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