Bioactivity potential of Portland cement in regenerative endodontic procedures

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Manuscript Details

Manuscript number	DEMA_2019_169_R1
Title	Bioactivity potential of Portland cement in regenerative endodontic procedures: from clinic to lab
Article type	Full Length Article

Abstract

Objectives The aim of this study was to evaluate the bioactivity potential of an hydraulic calcium-silicate cement, Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland: 'MPC'), applied in a tooth extracted because of failed regenerative endodontic procedures (REP) and by means of ex vivo (EV) specimens. **Methods** Ten EV cylindrical dentin cavities were prepared and filled with MPC and stored for 1 month in distilled water (DW), Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), simulated body fluid (SBF), versus no media (NM) serving as control. Six additional EV specimens were filled with MPC and exposed for 2 weeks to leucocyte-and-platelet-rich fibrin (LPRF)-clot (C), LPRF-membrane (M) and LPRF-exudate (E). MPC in the EV specimens and in the coronal part of the REP tooth was analyzed by means of micro-Raman spectroscopy (MR), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). **Results** SEM showed rough crystallite surfaces for the EV samples and a porous surface for the REP tooth. EDS of the EV samples revealed prominent peaks for Ca, Si and O. Storage in HBSS, DPBS, SBF, exposure to LPRF and the REP tooth showed considerable amounts of P as well. MR exhibited vibrations of phosphate (DPBS, SBF), carbonated hydroxyapatite (DPBS, SBF), calcium carbonate (DW, HBSS, NM, REP-tooth, LPRF-E), oxidized (ferric) proteins (LPRF-E/C/M) and the amide III band (all samples). Hence, only storage of MPC in DPBS and SBF for 1 month revealed bioactivity. **Significance** The environmental conditions, namely the laboratory and clinical settings, affect the bioactivity potential of MPC.

Keywords	microscopy; Raman; root canal; spectroscopy; stem cells; tricalcium silicate
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Prof. Dr. D.C. Watts
 Editor-in-chief
Dental Materials

09/06/2019

Dear editor, dear Professor D.C. Watts,

We would like to thank you and the reviewer for the comments concerning our manuscript ID DEMA_2019_169 entitled “Bioactivity potential of Portland cement in regenerative endodontic procedures: from clinic to lab”. Please find the accompanying revised manuscript and a point-by-point rebuttal to the reviewer’s concerns. All the modifications are highlighted in yellow in the manuscript and we sincerely hope to have responded to the reviewer’s questions in an appropriate way, so that this manuscript is now according to the standards of *Dental Materials*.

We are looking forward to your response and thank you in advance.

Yours sincerely,

Drs. Nastaran Meschi

Xin Li

Dr. Xin Li

Drs. Gertrude Van Gorp

Prof. Dr. Josette Camilleri

Prof. Dr. Bart Van Meerbeek

Prof. Dr. Paul Lambrechts

Author's Point-by-Point Response to Reviewer

Dear reviewer, please find below a point-by-point rebuttal on the concerns you have mentioned. All the modifications are highlighted in yellow in the manuscript and we sincerely hope to have responded to your questions in an appropriate way.

*** Concern 1:**

INTRODUCTION:

This section should be widely modified and implemented; many references must be deleted and replaced with adequate articles.

The role of tricalcium silicate based materials as filler materials in tissue engineering procedures should be clarified in the introduction section. Several scaffolds have been proposed with the incorporation of TCS for tissue engineering (bone regeneration and pulp revascularization procedures). The reported articles must be discussed and cited in this section

- Page 1: "many scaffolds have been proposed and are being developed". Replace the reference 3 with references concerning scaffolds for tissue regeneration.

Chiu YC, Fang HY, Hsu TT, Lin CY, Shie MY. The Characteristics of Mineral Trioxide Aggregate/Polycaprolactone 3-dimensional Scaffold with Osteogenesis Properties for Tissue Regeneration. J Endod 2017;43:923-929.

Cho YS, Hong MW, Quan M, Kim SY, Lee SH, Lee SJ, Kim YY, Cho YS. Assessments for bone regeneration using the polycaprolactone SLUP (salt-leaching using powder) scaffold. J Biomed Mater Res A 2017;12:3432-3444.

Gandolfi MG, Zamparini F, Degli Esposti M, Chiellini F, Aparicio C, Fava F, Prati C. Polylactic acid-based porous scaffolds doped with calcium silicate and dicalcium phosphate dihydrate designed for biomedical application. Mater Sci Eng C 2018;82:163-181.

Gandolfi MG, Zamparini F, Degli Esposti M, Chiellini F, Fava F, Fabbri P, Taddei P, Prati C. PCL Highly porous polycaprolactone scaffolds doped with calcium silicate and dicalcium phosphate dihydrate designed for bone regeneration, Mat Sci Eng C 2019;102:341-361.

Moussa DG, Aparicio C. Present and future of tissue engineering scaffolds for dentin-pulp complex regeneration. J Tissue Eng Regen Med 2019;13:58-75.

Response: Dear reviewer, thank you for your comments. We have tried to implement them in the introduction, as long as the reasoning of the suggestions and suggested references

were in line with the scope of the manuscript. After all, REP is a novel, biologically based endodontic treatment modality that aims to restore the function of the damaged/inflamed pulp of immature permanent teeth. Hence, further root development (by restoring/regeneration of the pulp-dentincomplex) is one of REP's aims. The scaffolds applied in REP should serve as a skeleton in which the mesenchymal stem cells could nestle in order to enhance further pulp healing and root dentin development. However, many of the articles you suggest rely on scaffolds inducing bone and bone regeneration, which are not wanted inside the root canal. The TCS (MTA, Portland cement, ...) applied in REP are necessary to seal tightly the intracanal blood clot induced during REP. As the TCS in REP are in close contact with blood, synthetic/natural scaffolds and even dental pulp in some cases, it should reveal a biological response. Even if the bioactivity potential of Portland cement has been shown in previous *in vitro*, animal and human studies, the clinical situation is not comparable to the lab situation and depends on the (endodontic) treatment modality. The Portland cement investigated in dentin slices of extracted teeth (where cement is put into the dentin slices in the lab) undergoes other manipulations and environmental conditions than the Portland cement put into a patient's root canal during REP. Furthermore, TCS put as a root-end filling in apexification or apex-resection is much nearer to periapical medullary bone than the TCS put directly below the CEJ in REP. Hence, to our knowledge no previous study has reported a chemical and micro-structural analysis of Portland cement applied in a patient's REP-treated tooth until now, which is the novelty of this manuscript.

Apparently, this was not clear enough and we have tried to clarify this in the manuscript.

Text: INTRODUCTION:

page 1:

- This novel endodontic treatment modality is a biologically based treatment that aims to restore the function and further development of the damaged pulp-dentincomplex [3].

+ the following reference is added: Wigler R, Kaufman AY, Lin S, Steinbock N, Hazan-Molina H, Torneck CD. Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. *J Endod.* 2013;39:319-26.

- The former reference 3 has been replaced by suggested reference: Moussa DG, Aparicio C. Present and future of tissue engineering scaffolds for dentin-pulp complex regeneration. *J Tissue Eng Regen Med* 2019;13:58-75.

+ text altered:

Many scaffolds have been proposed and are being developed [4]. The ideal scaffold for biomedical application should be biocompatible, biodegradable and mimic the extracellular matrix (ECM). On one hand, synthetically-engineered polymeric and ceramic scaffolds seem

to give promising results in terms of bone regeneration. On the other hand, the degradation of these synthetic scaffolds might be a concern and they lack the physiological information that is available in the ECM [4]. However, post REP, rather pulp and dentin are appreciated inside the root canal than bone. Natural scaffolds such as an autologous platelet concentrate have been applied in the medical and dental field for decades [5, 6], among which leucocyte- and-platelet-rich fibrin, commonly referred to as 'LPRF', seemed to reveal one of the most favorable outcomes in terms of regenerative wound healing [7]. Nevertheless, An autologous scaffold loaded with growth factors is most ethical and cost-effective. Also-In the field of ...

- The following references were not added due to emphasis on bone regeneration. Furthermore, polycaprolactone is not suitable for use in infected areas (for example inside a root canal) as it will be digested by bacteria (cfr. Resilon).

Chiu YC, Fang HY, Hsu TT, Lin CY, Shie MY. The Characteristics of Mineral Trioxide Aggregate/Polycaprolactone 3-dimensional Scaffold with Osteogenesis Properties for Tissue Regeneration. *J Endod* 2017;43:923-929.

Cho YS, Hong MW, Quan M, Kim SY, Lee SH, Lee SJ, Kim YY, Cho YS. Assessments for bone regeneration using the polycaprolactone SLUP (salt-leaching using powder) scaffold. *J Biomed Mater Res A* 2017;12:3432-3444.

Gandolfi MG, Zamparini F, Degli Esposti M, Chiellini F, Fava F, Fabbri P, Taddei P, Prati C. PCL Highly porous polycaprolactone scaffolds doped with calcium silicate and dicalcium phosphate dihydrate designed for bone regeneration, *Mat Sci Eng C* 2019;102:341-361.

Gandolfi MG, Zamparini F, Degli Esposti M, Chiellini F, Aparicio C, Fava F, Prati C. Polylactic acid-based porous scaffolds doped with calcium silicate and dicalcium phosphate dihydrate designed for biomedical application. *Mater Sci Eng C* 2018;82:163-181.

*** Concern 2:**

INTRODUCTION:

- Page 2: The aim of the study is no clear, in particular considering the title. The bioactivity potential (i.e induction of biological response) of Portland Cement has been already investigated. In this context, apatite forming ability has been tested following ISO 23317, but many articles have been already published. Please expand and clarify the introduction.

M. Mattioli Belmonte, G. Biagini, R.A.A. Muzzarelli, C. Castaldini, A. Krajewski, A. Ravaglioli, et al. Osteoinduction in the presence of chitosan-coated porous hydroxyapatite *J Bioact Compat Polym*, 10 (1995), pp. 249-257

Gandolfi MG, Iezzi G, Piattelli A, Prati C, Scarano A. Osteoinductive potential and bone-bonding ability of ProRoot MTA, MTA Plus and Biodentine in rabbit intramedullary model: Microchemical characterization and histological analysis. *Dent Mater* 2017;33(5):e221-e238.

Response: cfr response to concern 1 and modifications in text.

The following references were added:

Gandolfi MG, Iezzi G, Piattelli A, Prati C, Scarano A. Osteoinductive potential and bone-bonding ability of ProRoot MTA, MTA Plus and Biodentine in rabbit intramedullary model: Microchemical characterization and histological analysis. *Dent Mater* 2017;33(5):e221-e238.

(cfr concern 14) Zadpoor AA. Relationship between *in vitro* apatite-forming ability measured using simulated body fluid and *in vivo* bioactivity of biomaterials. *Mater Sci Eng C Mater Biol Appl* 2014;35:134-143.

Text:

Pages 2 and 3:

A particular property of TCS is its bioactivity potential [15, 16]. As the TCS in REP are in close contact with blood, synthetic/natural scaffolds and even dental pulp in some cases, it should reveal a biological response. After all, a bioactive material has the ability to induce a specific biologic activity [17]. In the field of tissue engineering, this definition has undergone a slight modification, namely that a bioactive material will form carbonated apatite after being immersed in a serum-like solution (such as simulated body fluid (SBF)) [18]. During and after setting in REP, TCS remains in contact with blood and tissue fluids. Ageing of TCS for endodontic applications in SBF have already been tested *in vitro* [19] and even if an ISO standard (ISO 23317(2012)) has been prescribed to test the apatite forming ability of Portland cement *in vitro* [18], the clinical situation is not comparable to the lab setting. The TCS investigated in human dentin slices of extracted teeth (where cement is put into the dentin slices in the lab) undergoes other manipulations and environmental conditions than when it has been put into a patient's root canal during treatment [20]. Furthermore, the type of (endodontic) treatment modality is another factor. TCS put as a root-end filling during apexification or apex-resection [21] is much nearer to periapical medullary bone [22] than when the TCS is put directly below the cemento-enamel junction in REP, which directly influences the apatite-forming ability of the TCS. Thus, specific environmental conditions may modify the material setting [23-25]. Very little information is available on the environment TCS is placed in during REP and almost nothing is known on the material chemistry of failed clinical cases [21]. Hence, the purpose of this study was to investigate the effect of environmental conditions on MPC bioactivity by analyzing an extracted tooth after REP failure posttrauma and comparing it with *ex vivo* (EV) specimens. To our knowledge no

previous study has reported the chemical and micro-structural analysis of Portland cement applied in a patient's REP-treated tooth.

*** Concern 3:**

INTRODUCTION:

- Page 2: ESEM-EDX evaluation of CaSi based materials placed in root canal sections have been already used to study bioactivity potential. What innovations does the present paper provide?

Check the present reference: Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. Dent Mater 2011;27(7):e134-e157.

Response:

- cfr response to concern 2

- Concerning the proposed reference: Is not applicable to our study, as we have analyzed a non-light curable TCS without resin in the coronal part of the root canal. The setting time of the TCS was also not analyzed in our study.

Text: /

*** Concern 4:**

INTRODUCTION:

-Page 2 Replace the name Med-Pz with MedCem MTA, if this is the material used in the study.

Response: Dear reviewer, we used portland cement (Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland)) and not mineral trioxide aggregate (such as MedCem MTA). Hence, it would be inappropriate to change the name.

<https://www.medcem.eu/en/products.html>

Text: /

*** Concern 5:**

INTRODUCTION:

- Page 2 Delete reference 20 reported in the purpose sentence.

Response and Text: The former reference 20 has been removed.

*** Concern 6:**

MATERIALS AND METHODS:

Page 2:

-Replace reference 13 (first appear) with one reporting the absence of bismuth oxide in MedCem MTA.

-Replace reference 13 with articles demonstrating the bioactivity of Calcium silicates cements:

Response: Dear reviewer, the first appear of (former) reference 13 was in the introduction section and not in “Materials and Methods”. Reference 13 reports the absence of bismuth oxide in Portland cement in comparison with mineral trioxide aggregate. As we were using Pure Portland Cement Med-PZ and not MedCem MTA, we did not replace the first appear of reference 13. However, the second appear of reference 13 is replaced with articles demonstrating the bioactivity of calcium silicate cements, suggested by yourself. Also, all other appears of reference 13 have been deleted/replaced throughout the manuscript (see further).

Text:

Page 2:

A particular property of TCS is its bioactivity potential [16, 17].

References added:

- Niu LN, Jiao K, Wang TD, Zhang W, Camilleri J, Bergeron BE, et al. A review of the bioactivity of hydraulic calcium silicate cements. *J Dent.* 2014;42:517-33.

- Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. *Dent Mater* 2015;31:351-370

*** Concern 7:**

MATERIALS AND METHODS:

-Replace 14 with PAS specification 2012.

-Replace reference 15 with ISO 23317 and PAS specification 2012.

Response: Dear reviewer, the first appear of (former) references 14 and 15 were in the introduction section and not “Materials and Methods”.

The PAS specification 2012 is unclear to us, but reference 15 has been replaced by ISO 23317(2012).

Text:

Page 2:

In the field of tissue engineering, this definition has undergone a slight modification, namely that a bioactive material will form carbonated apatite after being immersed in a serum-like solution (such as simulated body fluid (SBF)) [18].

*** Concern 8:**

MATERIALS AND METHODS:

Page 2 Remove the sentence “MR analysis of a patient’s sample has not been performed for this specific topic until now” as vibrational investigations have already been used to analyse living specimens from a patient (e.g. dentine slices). Some references have been provided below. How the analyses reported in the present paper differ from the literature methodology?

Almahdy A, Downey FC, Sauro S, Cook RJ, Sherriff M, Richards D, Watson TF, Banerjee A, Festy F. Microbiochemical analysis of carious dentine using Raman and fluorescence spectroscopy. Caries Res. 2012;46(5):432-40.

Di Foggia M, Prati C, Gandolfi MG, Taddei P. An in vitro study on dentin demineralization and remineralization: Collagen rearrangements and influence on the enucleated phase. J Inorg Biochem 2019;193:84-93.

Gandolfi MG, Taddei P, Pondrelli A, Zamparini F, Prati C, Spagnuolo G. Demineralization, collagen modification and remineralization degree of human dentin after EDTA and citric acid treatments. Mater 2018;12(1).

Eliades G, Mantzourani M, Labella R, Mutti B, Sharma D. Interactions of dentine desensitisers with human dentine: Morphology and composition. J Dent 2013;41(SUPPL. 4):S28-S39.

Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. Dent Mater 2011;27(7):e134-e157

P. Taddei, A. Tinti, M.G. Gandolfi, P.L. Rossi, C. Prati Ageing of calcium silicate cements for endodontic use in simulated body fluids: a micro-Raman studyJ. Raman Spectrosc., 40; 1858-1866

Response: The sentence has been removed in the “Methods” section. However, as mentioned in the “Introduction”: to our knowledge no previous study has reported the

chemical and micro-structural analysis of Portland cement applied in a patient's REP-treated tooth. Dentin slices filled with TCS in the lab cannot be compared to TCS put into a patient's tooth during an endodontic treatment. We have addressed this concern in our response and in the text, as a response to concerns 1, 2 and 14. Also the reference (Zadpoor AA, 2014) you have suggested, supports our response.

Text:

Page 3:

- Methods: **MR analysis of a patient's sample has not been performed for this specific topic until now.**

- Concerning the suggested references: none of them involved patient's samples.

* The following suggested references have been added:

Di Foggia M, Prati C, Gandolfi MG, Taddei P. An in vitro study on dentin demineralization and remineralization: Collagen rearrangements and influence on the enucleated phase. J Inorg Biochem 2019;193:84-93.

=> in the discussion section, cfr concern 13

P. Taddei, A. Tinti, M.G. Gandolfi, P.L. Rossi, C. Prati Ageing of calcium silicate cements for endodontic use in simulated body fluids: a micro-Raman studyJ. Raman Spectrosc., 40; 1858-1866

=> in the discussion section (cfr concern 17) and in the introduction section (cfr concern 2)

* The following suggested references were not added, due to the fact that we are not studying the Raman spectra of dentin but that of portland cement.

Almahdy A, Downey FC, Sauro S, Cook RJ, Sherriff M, Richards D, Watson TF, Banerjee A, Festy F. Microbiochemical analysis of carious dentine using Raman and fluorescence spectroscopy. Caries Res. 2012;46(5):432-40.

Gandolfi MG, Taddei P, Pondrelli A, Zamparini F, Prati C, Spagnuolo G. Demineralization, collagen modification and remineralization degree of human dentin after EDTA and citric acid treatments. Mater 2018;12(1).

Eliades G, Mantzourani M, Labella R, Mutti B, Sharma D. Interactions of dentine desensitisers with human dentine: Morphology and composition. J Dent 2013;41(SUPPL. 4):S28-S39.

* The following reference is not applicable to our study, as we have analyzed a non-light curable TCS without resin in the coronal part of the root canal. The setting time of the TCS was also not analyzed in our study.

Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. *Dent Mater* 2011;27(7):e134-e157

- Please see also responses to concerns 1, 2 and 14.

*** Concern 9:**

MATERIALS AND METHODS:

- Page 3 This section should be more logically organized. I suggest to divide in subsections Start a new paragraph after “ Directly after extraction...”

Response: In the “Materials and Methods” section: points 2.1 and 2.2. have been divided into 2 sections in order to obtain a more logical structure.

Text: Pages 3-5:

2.1 Patient's sample

2.1.1 Clinical part

2.1.2 Tissue processing

Directly after extraction, ...

2.2 Ex vivo samples in different media

2.2.1 Storage media

2.2.2 LPRF

*** Concern 10:**

RESULTS:

-Troughout Micro-Raman analyses: According to Awonusi, Peaks ascribable to B type carbonated apatite may be found also at 715-689 cm⁻¹, did authors found those peaks? Awonusi A, Morris MD, Tecklenburg MM. Carbonate assignment and calibration in the Raman spectrum of apatite. *Calcif Tissue Int.* 2007 Jul;81(1):46-52. Epub 2007 Jun 6. PubMed PMID: 17551767.

Response: Dear reviewer, thank you for this remark. We did not find any peak for B-type carbonated apaptite at 715-689 cm⁻¹, but we have mentioned this in the discussion section and have added the reference.

Text:

Page 9:

Noticeable is that another study has reported a peak for β -type of carbonated apatite at 715-689 cm^{-1} that was not found throughout the current study [40].

***Concern 11:**

DISCUSSION:

-Page 7 "This clinker is ground to a fine powder with gypsum, resulting in Portland cement. MPC consists mainly of tricalcium silicate and dicalcium silicate, and is classified as a hydraulic cement as it sets in the presence of moisture"

-Page 7: Replace 13 with the following references:

Niu LN, Jiao K, Wang TD, Zhang W, Camilleri J, Bergeron BE, et al. A review of the bioactivity of hydraulic calcium silicate cements. *J Dent.* 2014;42:517-33.

Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. *Dent Mater* 2015;31:351-370.

Response and text: Reference 13 has been replaced by the suggested references on page 8.

*** Concern 12:**

DISCUSSION:

- Page 7: Delete reference 30 not on animal model, but on in vitro cells.

Response: Indeed, we apologize for this.

Text:

Page 8:

Research performed on ~~animal models and~~ human specimens retrieved from the root end [21] revealed only crystalline calcium carbonate in the presence of blood.

*** Concern 13:**

DISCUSSION:

- Page 7: Modify the sentence (This is the first paper reporting....) as not true and discuss previous papers on this topic.

Almahdy A, Downey FC, Sauro S, Cook RJ, Sherriff M, Richards D, Watson TF, Banerjee A, Festy F. Microbiochemical analysis of carious dentine using Raman and fluorescence spectroscopy. *Caries Res.* 2012;46(5):432-40.

Di Foggia M, Prati C, Gandolfi MG, Taddei P. An in vitro study on dentin demineralization and remineralization: Collagen rearrangements and influence on the enucleated phase. *J Inorg Biochem* 2019;193:84-93.

Gandolfi MG, Taddei P, Pondrelli A, Zamparini F, Prati C, Spagnuolo G. Demineralization, collagen modification and remineralization degree of human dentin after EDTA and citric acid treatments. *Mater* 2018;12(1).

Eliades G, Mantzourani M, Labella R, Mutti B, Sharma D. Interactions of dentine desensitisers with human dentine: Morphology and composition. *J Dent* 2013;41(SUPPL. 4):S28-S39.

Gandolfi MG, Taddei P, Siboni F, Modena E, Ciapetti G, Prati C. Development of the foremost light-curable calcium-silicate MTA cement as root-end in oral surgery. Chemical-physical properties, bioactivity and biological behavior. *Dent Mater* 2011;27(7):e134-e157

P. Taddei, A. Tinti, M.G. Gandolfi, P.L. Rossi, C. Prati Ageing of calcium silicate cements for endodontic use in simulated body fluids: a micro-Raman study. *J. Raman Spectrosc.*, 40; 1858-1866

Response:

- The sentence has been altered in the discussion section.
- Concerning the suggested references: see response to concern 8. The following reference has been added to the discussion section and discussed: Di Foggia M, Prati C, Gandolfi MG, Taddei P. An in vitro study on dentin demineralization and remineralization: Collagen rearrangements and influence on the enucleated phase. *J Inorg Biochem* 2019;193:84-93. However, we have to mention that the scope of our study was not to analyze the remineralization of demineralized dentin.

Text:

Page 8:

MR has already been applied to study *in vitro* the ageing of TCSs in SBF [19] and remineralization of demineralized human dentin slices when put in close contact to calcium silicate/hydroxide cements in physiological-like solutions [36]. However, until now, no MR-study has investigated TCS put for several months in the coronal part of the root of a patient's tooth. This is the first paper reporting the use of MR, SEM and EDS to analyze MPC retrieved from a patient's tooth due to failed REP posttrauma.

*** Concern 14:**

DISCUSSION:

- Page 7: Numerous bioactivity tests exist to predict the role of bioactive materials in-vivo, the following reference may be useful to discuss this topic.

Zadpoor AA. Relationship between in vitro apatite-forming ability measured using simulated body fluid and in vivo bioactivity of biomaterials. *Mater Sci Eng C Mater Biol Appl* 2014;35:134-143.

Response: Dear reviewer, thank you for this suggestion. We have tried to implement it in the introduction and discussion and have added the reference. More specifically, the final part of the discussion has been re-written and re-organized.

Text:

Pages 10-11:

Before a biomaterial is applied in a clinical trial in order to predict its bioactivity, *in vitro* SBF-tests, *in vitro* cell culture tests and an *in vivo* animal study are performed. However, regardless the use of these pre-clinical tests, certain physiological and environmental conditions cannot be (easily) reproduced [20]. The lab protocol followed in this study might have influenced the material properties. Potential resin contamination due to embedding of the dentin slices, fixation and dehydration of MPC (= a non-living material), carbon-dioxide contamination during analysis and potential bacterial growth during storage in the incubator, may have occurred. Additionally, concerning the patient's REP-tooth in the current study, the restorative materials placed upon the MPC, the residual microbial load of the root canal post disinfection and the mechanical load of the tooth during function, are limitations to be aware of when interpreting the results. Additionally, patient-dependent factors such as general health, mouth hygiene and compliance during treatment, are not reproducible in lab conditions. Further research is necessary to distinguish the impact of these factors. Hence, caution is needed while interpreting *in vitro* results and extrapolating them to the clinical situation.

*** Concern 15:**

DISCUSSION:

-Page 8: Remove Reference 31 and replace with ISO 23317 2012.

Response and text:

page 8:

SBF is an acellular storage medium with ion concentrations equal to those of human blood plasma [18] ...

*** Concern 16:**

DISCUSSION:

-Page 8: Remove the sentence “Kokubo et al. (2006) have introduced an in vitro method to examine the bioactivity potential of a material by immersion in SBF”

Response: Two sentences have been removed.

Text: Page 8:

SBF is an acellular storage medium with ion concentrations equal to those of human blood plasma [18] ~~Kokubo et al. (2006) have introduced an in vitro method to examine the bioactivity potential of a material by immersion in SBF [32]. They concluded that apatite formation within 1 month is an indication of bioactivity potential.~~ and demands preparation,

...

*** Concern 17:**

DISCUSSION:

-Page 8: cite the first article that used HBSS to test the apatite forming ability of PC:
Gandolfi MG, Taddei P, Tinti A, De Stefano Dorigo E, Prati C. Alpha-TCP improves the apatite-formation ability of calcium-silicate hydraulic cement soaked in phosphate solutions. *Mater Sci Eng C* 2011;31:1412-1422.

Response: Dear reviewer, your request is a bit unclear to us, as in studies published earlier than 2011 HBSS has already been used to test the apatite forming ability of PC:

- Kinetics of apatite formation on a calcium-silicate cement for root-end filling during ageing in physiological-like phosphate solutions.

Gandolfi MG, Taddei P, Tinti A, De Stefano Dorigo E, Rossi PL, Prati C.

Clin Oral Investig. 2010 Dec;14(6):659-68. doi: 10.1007/s00784-009-0356-3. Epub 2009 Nov 27.

- P. Taddei, A. Tinti, M.G. Gandolfi, P.L. Rossi, C. Prati Ageing of calcium silicate cements for endodontic use in simulated body fluids: a micro-Raman study *J. Raman Spectrosc.*, 2009, 40; 1858-1866.

Hence, we have added P. Taddei et al., 2009 as reference.

Text: Page 9:

Regarding HBSS, as mentioned in previous studies, a storage time longer than one month is necessary to detect a higher bioactivity of TCS immersed in HBSS, due to the lower phosphate concentration of HBSS in comparison with DPBS [19, 33].

* **Concern 18:**

DISCUSSION:

-Page 8: The reference Gandolfi 2010 used DPBS and not HBSS. Please correct.

Response: Dear reviewer, Gandolfi 2010 mentioned DPBS as well as HBSS. Find below a printscreen of the abstract:

Clin Oral Invest (2010) 14:659–668
DOI 10.1007/s00784-009-0356-3

ORIGINAL ARTICLE

Kinetics of apatite formation on a calcium-silicate cement for root-end filling during ageing in physiological-like phosphate solutions

Maria Giovanna Gandolfi · Paola Taddei · Anna Tinti · Elettra De Stefano Dorigo · Piermaria Luigi Rossi · Carlo Prati

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Abstract The bioactivity of calcium silicate mineral trioxide aggregate (MTA) cements has been attributed to their ability to produce apatite in presence of phosphate-containing fluids. This study evaluated surface morphology and chemical transformations of an experimental accelerated calcium-silicate cement as a function of soaking time in different phosphate-containing solutions. Cement discs were immersed in Dulbecco's phosphate-buffered saline (DPBS) or Hank's balanced salt solution (HBSS) for different times (1–180 days) and analysed by scanning electron microscopy connected with an energy dispersive X-ray analysis (SEM-EDX) and micro-Raman spectroscopy. SEM-EDX revealed Ca and P peaks after 14 days in DPBS. A thin Ca- and P-rich crystalline coating layer was detected after 60 days. A thicker multilayered coating was observed after 180 days. Micro-Raman disclosed the 965-cm^{-1} phosphate band at 7 days

only on samples stored in DPBS and later the 590- and 435-cm^{-1} phosphate bands. After 60–180 days, a layer $\sim 200\text{--}900\text{ }\mu\text{m}$ thick formed displaying the bands of carbonated apatite (at $1,077$, 965 , 590 , 435 cm^{-1}) and calcite (at $1,088$, 713 , 280 cm^{-1}). On HBSS-soaked, only calcite bands were observed until 90 days, and just after 180 days, a thin apatite-calcite layer appeared. Micro-Raman and SEM-EDX demonstrated the mineralization induction capacity of calcium-silicate cements (MTAs and Portland cements) with the formation of apatite after 7 days in DPBS. Longer time is necessary to observe bioactivity when cements are immersed in HBSS.

Keywords Calcium-silicate cements · MTA cements · Portland cement · Ageing · Phosphate solutions · Apatite · Calcite

Text: /

* **Concern 19:**

REFERENCES:

Delete the references 3,13,14,15,31 and introduce the suggested references.

Response and text: References 3, 14, 15 and 31 have been deleted and the following suggested references were introduced throughout the manuscript:

- Moussa DG, Aparicio C. Present and future of tissue engineering scaffolds for dentin-pulp complex regeneration. J Tissue Eng Regen Med 2019;13:58-75.
- Prati C, Gandolfi MG. Calcium silicate bioactive cements: Biological perspectives and clinical applications. Dent Mater 2015;31:351-370.
- P. Taddei, A. Tinti, M.G. Gandolfi, P.L. Rossi, C. Prati Ageing of calcium silicate cements for endodontic use in simulated body fluids: a micro-Raman studyJ. Raman Spectrosc., 2009, 40; 1858-1866.
- Gandolfi MG, Zamparini F, Degli Esposti M, Chiellini F, Aparicio C, Fava F, Prati C. Polylactic acid-based porous scaffolds doped with calcium silicate and dicalcium phosphate dihydrate designed for biomedical application. Mater Sci Eng C 2018;82:163-181.
- ISO 23317-2. Implants for surgery- In vitro evaluation for apatite-forming ability of implant materials. Switzerland. 2012.
- Gandolfi MG, Iezzi G, Piattelli A, Prati C, Scarano A. Osteoinductive potential and bone-bonding ability of ProRoot MTA, MTA Plus and Biodentine in rabbit intramedullary model: Microchemical characterization and histological analysis. Dent Mater 2017;33(5):e221-e238.
- Zadpoor AA. Relationship between in vitro apatite-forming ability measured using simulated body fluid and in vivo bioactivity of biomaterials. Mater Sci Eng C Mater Biol Appl 2014;35:134-143.
- Di Foggia M, Prati C, Gandolfi MG, Taddei P. An in vitro study on dentin demineralization and remineralization: Collagen rearrangements and influence on the enucleated phase. J Inorg Biochem 2019;193:84-93.

However, as we were using Pure Portland Cement Med-PZ and not MedCem MTA, we did not delete the first appear of reference 13 on page 2.

***Concern 20:**

FIGURES

- Fig3: *Why did you include the Au peak in the EDX of sample stored in DW? This could be removed as not relevant. In addition, I suggest to size all the EDS spectra at the same scale level for better comparison of the peaks.*
- Fig 4: *similarly to Fig 3, I suggest to size all the EDS spectra at same scale level.*

Response and Text:

- Au in Fig. 3c has been removed. This was due to the SEM gold sputtering and should have been removed, our apologies.
- All the EDS-spectra in Figs. 3 and 4 have been sized until 3.9 Energy keV.

* **Concern 21:**

TABLES

Table 1: Au in DW should be removed as in the other stored samples.

Response and text: Table 1 has been revised: the row "AuM" has been removed.

- Storage of Portland cement in DPBS and SBF for 1 month revealed apatite formation.
- The observed CaCO_3 was due to the presence of blood and/or CO_2 .
- Discrepancies between laboratory and clinical set-up directly influence the outcome.
- Environmental conditions affect the bioactivity potential of Portland cement.

Bioactivity potential of Portland cement in regenerative endodontic procedures: from clinic to lab

Abstract

Objectives

The aim of this study was to evaluate the bioactivity potential of an hydraulic calcium-silicate cement, Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland: 'MPC'), applied in a tooth extracted because of failed regenerative endodontic procedures (REP) and by means of *ex vivo* (EV) specimens.

Methods

Ten EV cylindrical dentin cavities were prepared and filled with MPC and stored for 1 month in distilled water (DW), Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), simulated body fluid (SBF), versus no media (NM) serving as control. Six additional EV specimens were filled with MPC and exposed for 2 weeks to leucocyte-and-platelet-rich fibrin (LPRF)-clot (C), LPRF-membrane (M) and LPRF-exudate (E). MPC in the EV specimens and in the coronal part of the REP tooth was analyzed by means of micro-Raman spectroscopy (MR), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS).

Results

SEM showed rough crystallite surfaces for the EV samples and a porous surface for the REP tooth. EDS of the EV samples revealed prominent peaks for Ca, Si and O. Storage in HBSS, DPBS, SBF, exposure to LPRF and the REP tooth showed considerable amounts of P as well. MR exhibited vibrations of phosphate (DPBS, SBF), carbonated hydroxyapatite (DPBS, SBF), calcium carbonate (DW, HBSS, NM, REP-tooth, LPRF-E), oxidized (ferric) proteins (LPRF-E/C/M) and the amide III band (all samples). Hence, only storage of MPC in DPBS and SBF for 1 month revealed bioactivity.

Significance

The environmental conditions, namely the laboratory and clinical settings, affect the bioactivity potential of MPC.

Key words

microscopy; Raman; root canal; spectroscopy; stem cells; tricalcium silicate

Bioactivity potential of Portland cement in regenerative endodontic procedures: from clinic to lab

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Bioactivity potential of Portland cement in regenerative endodontic procedures: from clinic to lab

1. Introduction

Infected immature permanent teeth with pulp necrosis are more recently treated with regenerative endodontic procedures (REP) [1, 2]. This novel endodontic treatment modality is a biologically based treatment that aims to restore the function and further development of the damaged pulp-dentin complex [3]. After disinfection of the root canal during the first REP session, bleeding is provoked periapically at the second REP session to induce the formation of a blood clot inside the root canal and to attract stem cells that might aid further root development. Additionally, 2 other pillars of tissue engineering are introduced during REP, namely the placement of a scaffold (in which the stem cells might nestle) and the administration of growth factors (aiding the healing process) [2]. Many scaffolds have been proposed and are being developed [4]. The ideal scaffold for biomedical application should be biocompatible, biodegradable and mimic the extracellular matrix (ECM). On one hand, synthetically-engineered polymeric and ceramic scaffolds seem to give promising results in terms of bone regeneration. On the other hand, the degradation of these synthetic scaffolds might be a concern and they lack the physiological information that is available in the ECM [4]. However, post REP, rather pulp and dentin are appreciated inside the root canal than bone. Natural scaffolds such as an autologous platelet concentrate have been applied in the medical and dental field for decades [5, 6], among which leucocyte-and-platelet-rich fibrin, commonly referred to as 'LPRF', seemed to reveal one of the most favorable outcomes in terms of regenerative wound healing [7]. Nevertheless, An autologous scaffold loaded with growth factors is most ethical and cost-effective. Also In the field of REP, LPRF has been implemented with promising results in terms of periapical bone healing, further root development [8] and regaining pulp sensibility [9]. The LPRF-clot has a specific anatomy (Fig. 1) with underneath the red thrombus a buffy coat, in which most of the platelets, leukocytes and growth

factors are entrapped [7]. This LPRF-clot is also very plastic and can be adapted to the operation site (e.g. root canal). More specifically, it can be transformed into a membrane by squeezing the exudate out by means of a sterile glass plate (Fig. 1) [10].

During the second REP session, a hydraulic tricalcium silicate-based cement (TCS) is placed upon the blood clot and/or scaffold to seal the root canal [1, 2]. So-called mineral trioxide aggregate (MTA) has been one of the most investigated TCSs [11]. However, MTA may discolor the tooth due to bismuth oxide [12], by which it is not favorable to be applied coronally [13]. Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland: 'MPC') has similar chemo-mechanical properties as MTA and does not contain bismuth oxide [14]. A particular property of TCS is its bioactivity potential [15, 16]. As the TCS in REP are in close contact with blood, synthetic/natural scaffolds and even dental pulp in some cases, it should reveal a biological response. After all, a bioactive material has the ability to induce a specific biologic activity [17]. In the field of tissue engineering, this definition has undergone a slight modification, namely that a bioactive material will form carbonated apatite after being immersed in a serum-like solution (such as simulated body fluid (SBF)) [18]. During and after setting in REP, TCS remains in contact with blood and tissue fluids. Ageing of TCS for endodontic applications in SBF have already been tested *in vitro* [19] and even if an ISO standard (ISO 23317(2012)) has been prescribed to test the apatite forming ability of Portland cement *in vitro* [18], the clinical situation is not comparable to the lab setting. The TCS investigated in human dentin slices of extracted teeth (where cement is put into the dentin slices in the lab) undergoes other manipulations and environmental conditions than when it has been put into a patient's root canal during treatment [20]. Furthermore, the type of (endodontic) treatment modality is another factor. TCS put as a root-end filling during apexification or apex-resection [21] is much nearer to periapical medullary bone [22] than when the TCS is put directly below the cemento-enamel junction in REP, which directly influences the apatite-forming ability of the TCS. Thus, specific environmental conditions may modify the material setting [23-25]. Very little information is available on the environment TCS is placed in during REP and almost nothing is known on the material chemistry of failed clinical cases [21]. Hence, the purpose of this study was to

investigate the effect of environmental conditions on MPC bioactivity by analyzing an extracted tooth after REP failure **posttrauma** and comparing it with *ex vivo* (EV) specimens. **To our knowledge no previous study has reported the chemical and micro-structural analysis of Portland cement applied in a patient's REP-treated tooth.**

2. Methods

This study was designed as an *in vitro* (IV) study and was conducted according to the Good Clinical Practice standards (International Council on Harmonization, 1996), which rely on the ethical principles of the Declaration of Helsinki (World Medical Association, 1964). Ethical approval was obtained by the Medical Ethics Committee of UZ/KU Leuven under the file number S60773. One patient's sample and 16 EV samples placed in different media were investigated by means of micro-Raman spectroscopy (MR), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). MR is a spectroscopic technique commonly used in chemistry to provide the structural fingerprint of a material in a non-destructive manner [26]. **MR analysis of a patient's sample has not been performed for this specific topic until now.**

2.1 Patient's sample

2.1.1 Clinical part

The right and left central maxillary permanent incisors of an eight-year-old girl suffered an uncomplicated crown fracture. Both teeth reacted positively to carbon dioxide snow (CDS) and were restored with a calcium-hydroxide lining (Life, Kerr, Scafati, Italy), glass-ionomer cement (Fuji 2 LC, GC, Tokyo, Japan; 'GIC') and composite (Filtek Supreme XTE, 3M Oral Care, Seefeld, Germany). Nevertheless, 8 months later, the left central maxillary permanent incisor reacted negatively to CDS and presented a vestibular abscess. The tooth received REP following the procedure detailed by the European Society of Endodontology [1], with the exception that no CollaPlug (Zimmer Biomet, Berlin, Germany) was used underneath the MPC and a zinc phosphate cement (De Trey Zinc, Densply Sirona, Konstanz, Germany) was placed instead of GIC underneath the composite restoration. At the recall 20

months post REP, the periapical lesion was healed, but not enough root-canal wall thickening was obtained (Fig. 2b). After multi-disciplinary consultation at 22 months post REP, it was decided to remove this tooth rather than extracting a sound premolar to create space for orthodontic reasons. Written informed consent was obtained by the patient and the parents to use the tooth for further research.

2.1.2 Tissue processing

Directly after extraction, the REP tooth was sectioned using a diamond saw (Isomet 1000, Lake Bluff, IL, USA) (Fig. 2b). The coronal part was kept in a carbon dioxide-free environment (= desiccator with soda lime and silica gel) for 24 h. The apical part of the tooth was fixed using 6% formaldehyde and processed for immunohistology (data not in the scope of this study). Afterwards, the coronal part of the REP tooth was imbedded in resin (Unifast III, GC) and segmented with a microtome (Micracut 151, Metkon, Bursa, Turkey) into a cylindrical dentin slice with a 2-mm thickness, containing MPC in the central root-canal space (Fig. 2b). Subsequently, the specimen was processed for SEM by fixation in 2.5% glutaraldehyde for 24 h, dehydration in ascending concentrations of ethanol (25, 50, 75, and 95% for 30 min each, and 100% for 1 h), and finally drying by immersion in hexamethyldisilazane for 10 min. Prior to further SEM processing, MR was performed using a Senterra device (Bruker, Optik, Ettlingen, Germany). MPC was scanned on 9 randomly selected spots using a near-infrared (785 nm) laser with 50 mV power, a ×50 microscope objective and 50×1000 aperture, and an integration time of 10 s with 3 co-additions. The CCD detector possessed a 1024 × 256 pixel resolution and was cooled down thermoelectrically to -65°C. MR peaks characteristic for MPC were recorded. Post-processing of the spectra was performed using the Opus Spectroscopy Software version 7.0 (Bruker, Ettlingen, Germany). Subsequently, the specimen was mounted by means of a double-sided carbon tape on an aluminum stub, carbon coated and gold-sputtered by means of a JEOL JFC-1300 auto-fine coater (Jeol, Tokyo, Japan). SEMs were taken using a FEI XL30 field-emission-gun (Feg)-SEM (FEI, Eindhoven, The Netherlands) employed in the secondary electron mode at different magnifications, a voltage of 15 kV and a 10-mm working distance. At a magnification of 500x and a voltage of 20 kV, the EDS detector

(EDAX, Tilburg, The Netherlands) of the FEI XL30 Feg-SEM (FEI) was used to chemically map the surface composition of the selected area.

2.2 Ex vivo samples in different media

2.2.1 Storage media

Eight human central incisors (gathered as approved by the Medical Ethics Committee of UZ/KU Leuven under the file number S60773) were stored in 0.5% chloramine solution at 4°C and were used within 3 months after extraction. The teeth were embedded in resin (Unifast III, GC) and segmented with a microtome (Micracut 151, Metkon) into 16 cylindrical dentin slices (2 per tooth) with a 2-mm thickness. At the root-canal region of the dentin slices, a cavity with a diameter of 2 mm was prepared using a diamond bur (Komet, Lemgo, Germany). MPC (Medcem; lot-number: MWZ170215) was prepared strictly following the manufacturer's instructions and placed into the central cavity of the EV specimens by means of an amalgam gun. The resin border of each specimen was marked with a bur for upward orientation. Prior to setting, the EV samples were immersed in 25 mL of the following media (2 samples per media) with the resin mark pointing upwards: distilled water (DW), Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), simulated body fluid (SBF), versus no media (NM) serving as control. The EV samples were stored in an incubator at 37°C for 1 month with the media changed every 2 weeks. SBF was made by the main investigator (MN) and consisted of 136.8 mM NaCl, 4.2 mM NaHCO₃, 3.0 mM KCl, 1.0 mM K₂HPO₄·3H₂O, 1.5 mM MgCl₂·6H₂O, 40 mM HCl, 2.5 mM CaCl₂, 0.5 mM Na₂SO₄, 50 mM (CH₂OH)₃CNH₂; its pH was adjusted to 7.4 using a pH 3110 Set VWR pH meter (WTW, Weilheim, Germany).

2.2.2 LPRF

Furthermore, a venipuncture on the median cubital vein of MN was performed by a nurse (UZ Leuven, Leuven, Belgium) and blood was drawn into 2 sterile, 10 mL plastic tubes without anticoagulant. The tubes were immediately centrifuged at 702 RCF in an IntraSpin centrifuge (Intra-Lock International, Boca Raton, FL, USA) to obtain LPRF-clots (Fig. 1). One clot was pressed for 5 min

under a sterile glass plate, in order to obtain LPRF-membranes and -exudate (Fig. 1). After mixing, but prior to MPC setting, 4 separate EV specimens were placed in contact with LPRF-clots (n=2) and LPRF-membranes (n=2), while 2 additional EV specimens were immersed in LPRF-exudate. All LPRF-specimens were placed for 2 weeks in the incubator at 37°C, as LPRF loses its functionality after 7-14 days [7]. Prior to analysis, the specimens were retrieved and placed in a carbon dioxide-free environment (= desiccator with soda lime and silica gel) for 24 h. Sample preparation prior to analysis and the analyses themselves were performed as described for the patient's sample. Per medium 1 EV sample was used for MR and another was further processed for Feg-SEM and EDS.

3. Results

3.1 Patient's sample

SEM revealed a rough porous surface (Fig. 2d). EDS showed high concentrations of zinc, phosphorus and oxygen, and lower amounts of magnesium, aluminum, silicon and calcium (Fig. 2e). The relative weight percentage of the elements is presented in Table 1. MR exhibited vibrations of the amide III band (N-H) at 1242 cm^{-1} [27] and calcium carbonate at 1082 cm^{-1} [26, 28] (Fig. 2c).

3.2 Ex vivo samples

3.2.1 Five different media

SEM overall presented rough surfaces (DPBS, SBF; Fig. 3h,k) and some samples presented crystals (DW, HBSS, NM; Fig. 3b,e,n). This difference in microstructure depending on the storage media is related to the discrepancies found in the chemical surface analysis. On the one hand, EDS for DW, DPBS and NM (Fig. 3c,i,o) showed high calcium and silicon peaks ($>7,6\text{ K}$) and intermediate-to-small peaks representing oxygen, sodium, aluminum and magnesium ($<4,75\text{ K}$). On the other hand, HBSS and SBF presented similar chemical compositions, being more specifically rich in phosphorus, oxygen and calcium (Fig. 3f,l). Phosphorus was also present when stored in DPBS (Fig. 3i). Hence, the phosphate

peak at 960 cm^{-1} was also detected by MR when the EV samples had been stored in DPBS and SBF (Fig. 3p). MPC in all groups presented vibrations for the amide III band (N-H) at 1242 cm^{-1} (γ -peak in Fig. 3p) [27]. Furthermore, DPBS and SBF storage resulted in a vibration of the carbonate group (β -type of carbonate) in hydroxyapatite at 1070 cm^{-1} [26, 28, 29]. HBSS, DPBS and NM revealed a calcium-carbonate peak with high Raman intensity at 1082 cm^{-1} [26, 28].

3.2.2 LPRF

MPC exposed to LPRF-clot was greyish discolored (Fig. 4a) versus yellowish discolored when having been exposed to LPRF-membrane (Fig. 4e) and -exudate (Fig. 4i). SEM of the sample exposed to LPRF-clot presented a rough and granular surface with deposits (Fig. 4b,c), while the sample exposed to LPRF-membrane showed a rough structure with round crystals (Fig. 4f,g). These granular desposits and crystals were rich in calcium, but they also revealed a phosphorus peak (Fig. 4d,h,i) implicating the presence of phosphate.

MR of MPC exposed to LPRF-clot and -membrane presented similar vibrations for the amide III band (N-H) at 1242 cm^{-1} [27] and a peak at $1370\text{-}1378\text{ cm}^{-1}$ representing oxidized (ferric) proteins [30] (Fig. 4m). Nevertheless, EDS of MPC exposed to LPRF-clot presented 2.40 wt% of iron (Table 1). The other EDS peaks for LPRF-clot and -membrane were similar, namely high carbon, oxygen, calcium and silicon peaks and moderate aluminum, magnesium, sodium and phosphorus peaks (Fig. 4d,h).

SEM of MPC exposed to LPRF-exudate showed a rough surface with clusters of acicular (needle-like) and round crystals (Fig. 4j,k). No iron was detected by EDS, but the spectra revealed high carbon, oxygen and calcium peaks and moderate aluminum, magnesium, phosphorus, silicon and sodium peaks (Fig. 4l, Table 1). MR of MPC exposed to LPRF-exudate was similar to that exposed to LPRF-clot and -membrane except for the calcium carbonate peak at 1082 cm^{-1} [26, 28]. This mixed presence of phosphate and calcium carbonate explains the mixed crystal structure imaged by SEM.

4. Discussion

Portland cement is produced by burning limestone and clay in a kiln, which results in a cement clinker. This clinker is ground to a fine powder with gypsum, resulting in Portland cement. MPC consists mainly of tricalcium silicate and dicalcium silicate, and is classified as a hydraulic cement as it sets in the presence of moisture [15, 16]. Hydrated MPC's end products are mainly calcium-silicate hydrate, calcium hydroxide and ettringite. About one third of the volume of hydrated MPC consists of calcium hydroxide that can be released [31]. Calcium hydroxide leads to an alkaline pH and induces apatite formation when MPC is immersed in physiological-like phosphate solutions [32-35]. A bioactive material has the potential to elicit a response from a living tissue or cells [17]. Hydraulic TCSs are regarded as bioactive materials because of their ability to induce hydroxyapatite formation [15]. Hydroxyapatite formation has to date only been observed in *in vitro* samples [34, 35]. Research performed on animal models and human specimens retrieved from the root end [21] revealed only crystalline calcium carbonate in the presence of blood. In previous studies, only X-ray diffraction and EDS were used to analyze the retrieved materials. MR has already been applied to study *in vitro* the ageing of TCSs in SBF [19] and remineralization of demineralized human dentin slices when put in close contact to calcium silicate/hydroxide cements in physiological-like solutions [36]. However, until now, no MR-study has investigated TCS put for several months in the coronal part of the root of a patient's tooth. This is the first paper reporting the use of MR, SEM and EDS to analyze MPC retrieved from a patient's tooth due to failed REP posttrauma.

Physiological-like phosphate solutions such as SBF, HBSS and DSPS were applied in the current study as ageing media for MPC in order to mimic the clinical situation (patient's sample and LPRF-specimens) [32-35]. SBF is an acellular storage medium with ion concentrations equal to those of human blood plasma [18]. Kokubo *et al.* (2006) have introduced an *in vitro* method to examine the bioactivity potential of a material by immersion in SBF [32]. They concluded that apatite formation within 1 month is an indication of bioactivity potential, and demands preparation, as performed in the current study

by the principal investigator (MN). However, there are also commercially available media that mimic the ion composition of human blood plasma, such as HBSS and DPBS.

EDS was applied to chemically analyze the elemental composition of the specimens. Nevertheless, quantitative data obtained by EDS should be interpreted with caution as they are less accurate in comparison with wavelength spectroscopy [37]. The high EDS-peaks for calcium, oxygen and silicon for the MPC samples exposed to DW, HBSS, DPBS, NM and LPRF were characteristic for MPC, as MPC releases calcium-silicate hydrate and calcium hydroxide during setting [31]. Moreover, calcium was present in all specimens. Furthermore, MPC exposed to HBSS, DPBS, SBF and LPRF showed a considerable amount of crystals rich in phosphorus (Table 1), indicating the occurrence of phosphates. Phosphorus was also detected by MR; more specifically DPBS and SBF presented a phosphate peak at 960 cm^{-1} and a vibration of the carbonate group (β -type of carbonate) in hydroxyapatite at 1070 cm^{-1} , which is in accordance with previous studies [32, 33]. Such MR vibration was missing for MPC exposed to DW and NM, due to the lack of phosphorus. Even if phosphorus was present in MPC exposed to LPRF and the patient's sample, rather calcium carbonate than phosphate and/or apatite was detected. This must be attributed to contamination with blood [21, 33, 38]. Regarding HBSS, as mentioned in previous studies, a storage time longer than one month is necessary to detect a higher bioactivity of TCS immersed in HBSS, due to the lower phosphate concentration of HBSS in comparison with DPBS [19, 33]. Gandolfi *et al.* (2010) observed apatite formation only after 180-day storage of TCS in HBSS [33]. In the current study, MPC exposed to HBSS presented a calcium-carbonate peak due to the reaction of calcium hydroxide with environmental carbon dioxide [39]. Noticeable is that another study has reported a peak for β -type of carbonated apatite at $715\text{-}689\text{ cm}^{-1}$ that was not found throughout the current study [40].

Concerning the other MR vibrations recorded, the peak representing the amide III band (N-H) at 1242 cm^{-1} [27] was detected for the EV specimens in 5 different media (Fig. 3), indicating the presence of organic components (e.g. collagen) or bacterial contamination, even if SEM did not reveal any signs of bacterial growth. However, for the patient's sample and the LPRF specimens (Fig. 4), this amide III band

was more obvious due to the direct contact with blood. The MR vibration for oxidized (ferric) proteins (Fig. 4m) and the detection of iron by EDS (Table 1) refer to the red blood cells in the thrombus of LPRF (Fig. 1).

Regarding the surface characteristics of MPC, except for the LPRF samples, none of the other samples were discolored. As the red thrombus of the LPRF-clot was in contact with MPC, a greyish discoloration occurred (Fig. 4a). The MPC-samples exposed to LPRF-membrane and -exudate were still yellowish discolored (Fig. 4e, i), even if there was no contact with the thrombus.

As the blood clot triggered during REP should reach approximately 3 mm below the cemento-enamel junction, a biocompatible material that is capable to induce mineralization, is required [1]. Nevertheless, this blood clot should be stable or a sterile and auto-resorbable collagen plug should be applied as a scaffold for TCS. If not, TCS might be pushed deeper into the root canal, as was the case in the present study. Hence, no further root development took place (Fig. 2a). Furthermore, the specimen retrieved from the patient (Fig. 2b) did not show the presence of calcium hydroxide but was rather rich in calcium carbonate and zinc phosphate due to contamination during REP with blood and zinc phosphate cement, respectively. Zinc phosphate was placed upon MPC as an intermediate layer between MPC and composite. However, the prescribed treatment protocols concerning REP rather recommend the application of GIC [1, 41], even if research in materials have shown that the placement of GIC over fresh TCS cement interferes with cement setting [42]. The use of zinc phosphate was also representative in the weight percentage of the elements in MPC (Table 1): for the patient's sample the amount of zinc was prominent.

Even if *in vitro* settings strive to mimic as much as possible the clinical situation, it is questionable whether *in vitro* study results could be compared to clinical outcomes and vice versa. Before a biomaterial is applied in a clinical trial in order to predict its bioactivity, *in vitro* SBF-tests, *in vitro* cell culture tests and an *in vivo* animal study are performed. However, regardless the use of these pre-clinical tests, certain physiological and environmental conditions cannot be (easily) reproduced [20]. The lab protocol followed in this study might have influenced the material properties. Potential resin

contamination due to embedding of the dentin slices, fixation and dehydration of MPC (= a non-living material), carbon-dioxide contamination during analysis and potential bacterial growth during storage in the incubator, may have occurred. Additionally, concerning the patient's REP-tooth in the current study, the restorative materials placed upon the MPC, the residual microbial load of the root canal post disinfection and the mechanical load of the tooth during function, are limitations to be aware of when interpreting the results. Additionally, patient-dependent factors such as general health, mouth hygiene and compliance during treatment, are not reproducible in lab conditions. Further research is necessary to distinguish the impact of these factors. Hence, caution is needed while interpreting *in vitro* results and extrapolating them to the clinical situation.

5. Conclusion

In the present study, only storage of MPC in DPBS and SBF for 1 month revealed apatite formation. Discrepancies between *in vitro* and clinical study protocols directly influence the outcome. Hence, the observed calcium carbonate was due to the presence of blood and/or environmental carbon dioxide. Furthermore, the detected amide III band indicated the presence of organic (collagen/blood) or bacterial components. In general, we can conclude that environmental conditions affect the bioactivity potential of MPC.

6. Acknowledgements

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8. Figure Legends

Fig. 1: Leucocyte-and-platelet-rich fibrin (LPRF): (a) LPRF in a sterile tube post centrifugation; (b) LPRF-clot; (c) LPRF-membrane.

Fig. 2: Patient's case: (a) Conebeam CT 22 months post regenerative endodontic procedures of the left central maxillary permanent incisor; (b) Cylindrical dentin slice with a 2-mm thickness, containing a Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) root-canal filling in the center; (c) micro-Raman analysis of the filling material in (b), revealing a β -calcium carbonate peak at 1082 cm^{-1} and a γ -peak for the amide III band (N-H) at 1242 cm^{-1} ; (d) SEM micrograph of the filling material in (b); (e) EDS of the filling material in (b).

Fig. 3: Analyses performed for Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) stored in 5 different media. (a) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center and stored for 1 month in distilled water; (b) SEM of MPC in (a) at 500x; (c) EDS of the MPC in (a); (d) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, stored for 1 month in Hank's balanced salt solution (HBSS); (e) SEM of the MPC in (d) 500x; (f) EDS of the MPC (d); (g) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center and stored for 1 month in Dulbecco's phosphate-buffered saline (DPBS); (h) SEM of the MPC in (g) at 500x; (i) EDS of the MPC in (g); (j) Cylindrical dentin slice with a 2mm thickness containing MPC in the center and stored for 1 month in simulated body fluid (SBF); (k) SEM of the MPC in (j) at 500x; (l) EDS of the MPC in (j); (m) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center with no additional media; (n) SEM of the MPC in (m) at 500x; (o) EDS of the MPC in (m); (p) micro-Raman analysis for 5 different

media with the following peaks: α : phosphate peak at 960 cm^{-1} , β : calcium-carbonate peak at 1082 cm^{-1} , γ : amide III band (N-H) at 1242 cm^{-1} , μ : peak for the carbonate group (β -type of carbonate) in hydroxyapatite at 1070 cm^{-1} .

Fig. 4: Analyses performed for Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland; MPC) in contact with leucocyte-and-platelet-rich fibrin (LPRF). (a) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, upon which a LPRF-clot (LPRF-C) was placed for 2 weeks; (b) SEM of the MPC in (a) at 33x (b) and at 500x (c); (d) EDS of the MPC in (a); (e) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center, upon which a LPRF-membrane (LPRF-M) was placed for 2 weeks; (f) SEM of the MPC in (e) at 33x (f) and at 500x (g); (h) EDS of the MPC in (e); (i) Cylindrical dentin slice with a 2-mm thickness containing MPC in the center that was immersed in LPRF-exudate (LPRF-E) for 2 weeks; (j) SEM of the MPC in (i) at 500x (j) and at 2200x (k); (l) EDS of the MPC in (i); (m) micro-Raman analysis for LPRF-E/M/C with the following peaks: β : calcium-carbonate peak at 1082 cm^{-1} , γ : amide III band (N-H) at 1242 cm^{-1} , λ : oxidized (ferric) proteins at $1378\text{-}1370\text{ cm}^{-1}$.

Table 1. Elemental composition (wt%) of Pure Portland Cement (Med-PZ, Medcem, Weinfelden, Switzerland) stored following the different experimental conditions.

Chemical element	Patient's sample	Distilled water	HBSS	DPBS	SBF	No media	LPRF-C	LPRF-M	LPRF-E
OK	28.95	43.94	46.52	46.49	41.68	37.94	38.99	39.71	35.67
ZnL	40.68	-	-	-	-	-	-	-	-
MgK	6.63	1.68	-	2.67	-	0.21	0.89	0.80	1.03
AlK	4.34	5.74	-	5.17	-	0.25	3.33	2.82	1.21
SiK	2.23	17.18	-	24.51	0.59	10.68	20.03	11.92	2.78
PK	14.18	-	4.05	2.99	14.50	-	4.56	2.94	2.27
CaK	2.97	11.88	49.43	15.47	42.17	28.63	13.05	21.18	28.21
CK	-	9.69	-	-	-	21.99	15.20	18.50	27.97
NaK	-	2.46	-	2.70	1.07	0.30	1.55	2.13	0.86
FeK	-	1.49	-	-	-	-	2.40	-	-

C = clot; DPBS = Dulbecco's phosphate-buffered saline; E = exudate; HBSS = Hank's balanced salt solution; LPRF= leukocyte and platelet rich fibrin; M = membrane; SBF = simulated body fluid.