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Using autofluorescence to detect bacterial contamination in root fractures

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ABSTRACT

Objectives: Conventional methods for detecting root fractures cannot assess their depth or bacterial contamination. This study was designed to measure the autofluorescence emitted from a root fracture, with the aim of determining whether this is a suitable method for quantifying the depth and bacterial invasion of a fracture.

Methods: This in vitro study investigated 33 mandibular second molars with periapical lesions that had been extracted after finding root fractures in endodontically treated teeth during intentional replantation or diagnostic surgery. The root fractures were scanned using a fluorescence technique, and the association between fluorescence parameters and fracture depth was analyzed. The significance of the association between the red fluorescence among autofluorescence parameters and bacterial contamination within the fracture was examined.

Results: When the depth of the root fractures was evaluated by micro computed tomography, the scattering of light in the fractures increased with depth, and there was a gradual increase in the quantitative fluorescence parameter indicating the deepest point (ΔFmax) in the fractures. In addition, we observed red fluorescence on the outer surface of deeper fractures. The tooth fractures exhibiting red fluorescence were evaluated for bacterial contamination associated with red-fluorescent porphyrin, which revealed bacterial invasion into these fractures. On the other hand, non-red-fluorescing fractures contained necrotic tissue, debris, and irritants.

Conclusions: This viable fluorescent technique can potentially quantify the depth of root fractures and be used as a risk indicator for root fractures with periodontal inflammation.

Clinical significance: The auto-fluorescence technique can be used to detect depth and bacterial contamination of root fractures. It is postulated that the auto-fluorescence can be used as a risk indicator of deep fractures and can replace conventional fracture detection methods.

1. Introduction

The conventional method for diagnosing root fractures is to obtain magnified images using an operating microscope after staining with methylene blue dye. However, a previous study found that methylene blue showed a low sensitivity for identifying dentine cracks [1]. The use of methylene blue in the most common technique only allows the fracture site to be identified, with it not being possible to quantitatively assess the depth of the fracture. There are numerous reports of the importance of assessing the extent of root fractures in addition to simply identifying fracture [2,3], since it is difficult to evaluate periapical pathosis around a root fracture.

The American Association of Endodontics newsletter has described that the treatment plan must vary with the location and extent of cracks extending into the pulp and root portions [4]. A recent study found that autofluorescence emitted from tooth structure could be utilized to quantify cracks without requiring staining with methylene blue [5]. Teeth exhibit autofluorescence—the natural emission of light—when they absorb short-wavelength radiation, with the spontaneous emissions occurring at longer wavelengths. When a crack is detected, its dark appearance is caused by the light scattering in the crack, similar to the principle of transillumination. This scattering of light is caused by the disappearance of autofluorescence, and proprietary software can be used to quantify the loss of autofluorescence according to the depth of a crack [5]. A previous investigation of enamel cracks with disappearance of autofluorescence only assessed the depth of the craze line, whereas enamel cracks are commonly found in adult anterior and posterior teeth and cause no pain and no inflammation [6]. It is therefore necessary to

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assess the depth of fractures extending into the dentin and root portions.

The optical technique gives information not only about the disappearance of autofluorescence in teeth, but also red autofluorescence in tooth lesions associated with bacterial metabolites porphyrin. Oral microorganisms are able to synthesize chromophores of porphyrins that generate the typical autofluorescence in the red spectral region [7–9]. Previous histobacteriological investigations of cracked teeth found that bacteria penetrating into cracks could cause inflammation of pulp or periodontal tissue [10,11]. However, traditional methods for detecting cracks could not assess the microbiological aspects, and the method involving methylene blue dye is limited by its low relationship with bacterial leakage [12]. The depth of fracture and the correlation between autofluorescence and bacterial contamination need to be assessed quantitatively in order to overcome the limitations of using methylene blue dye.

The purpose of this study was to quantify the depth of fractures exhibiting autofluorescence and assess whether bacteria have invaded them. Root fractures with periapical lesions exhibiting autofluorescence elicited by a fluorescence technique were analyzed to determine the depth of the fractures and whether bacterial contamination in fractures is associated with autofluorescence in the red spectral region.

2. Materials and methods

2.1. Sample preparation

The study sample consisted of 33 mandibular second molars with periapical lesions that were extracted from patients who visited Yonsei Dental Hospital. Root fractures in endodontically treated teeth were identified after intentional replantation or diagnostic surgery. This study was approved by the Institutional Review Board of Yonsei Dental Hospital (IRB No. 2-2017-0038). The granulation tissue and calculus were removed from all teeth using a No. 15 scalpel blade (Paragon, Sheffield, UK). The study was conducted by following the procedure shown in Fig. 1.

2.2. Fluorescence evaluation

The autofluorescence of a root fracture was confirmed using a fluorescence camera (QLF-D Biluminator™ 2+, Inspektor Research Systems, Amsterdam, The Netherlands). The root surface fracture and the fluorescence camera were positioned as vertically as possible in order to evaluate the fluorescence aspect in all samples under the same conditions. In this system, the fluorescence camera is mounted on a single-lens-reflex camera with a 60-mm macro lens and is equipped with two light-emitting diodes (LEDs) to allow two images to be taken. One standard white-light image was obtained using 4 white LEDs, and the fluorescence image was obtained during illuminating with 405-nm excitation light from 12 blue-violet LEDs. The parameter values for images obtained using the fluorescence camera were as follows: a shutter speed of 1/10 s, aperture value of 11.0, and ISO speed of 1600 for blue light. A high pass cutoff optical filter ($\lambda > 480$ nm) in combination with a special filter to enhance the porphyrin peak at 630-540 nm was mounted on the lens to remove the excitation light from the autofluorescence image of the root fracture.

2.3. Analysis of fluorescence parameters

Fluorescence camera allow for both acquisition of image and simultaneous quantitative analysis (QA2 version 1.26, Inspektor Research Systems). Quantitative analysis software was used to calculate the disappearance of autofluorescence from the root fracture. An analysis area was designated between 3 mm below the root coronal portion and 3 mm above the root apex in each fluorescence image. It was observed that fractures appear darker than the surrounding sound surfaces. To calculate the maximum fluorescence loss (ΔFmax) at the fracture, an analysis patch was made to fit closely to the fracture with its borderlines positioned on the sound root surface. The ΔFmax represents the darkest area in the fracture.

The red autofluorescence, which is indirectly related to bacterial invasion into the fracture area, was quantified as ΔR [%] and ΔRmax [%] using proprietary software. The threshold for red fluorescence in the root fracture was a ΔR value of > 25. ΔR is a parameter that represents an increased intensity of red fluorescence with respect to the fluorescence of a sound surface, and is quantified as the average value as the percentage of the increase in the ratio of the red and green components compared with that in a sound area. Meanwhile, ΔRmax represents the maximum red fluorescence intensity in the area where the red fluorescence is most intense.

2.4. Micro-CT for depth measurements

All teeth were scanned using the Skyscan 1076 micro-CT device (SkyScan, Aartselaar, Belgium) with the following parameters: accelerating voltage of 100 kV, beam current of 100 μA, 0.5-mm Al filter, resolution of 18 μm, and 360° rotation in 0.5° steps. The scanned images were analyzed using a data viewer program (SkyScan, Aartselaar, Belgium). The fracture depth was measured in the axial plane obtained from a three-dimension view that included 3 mm of the coronal and apex portions of the root region. The deepest point for all samples was analyzed at three points, and their average values were calculated.

2.5. Histological analysis

After obtaining the autofluorescence images, all root fractures were fixed with 10% neutral buffered formalin. Tooth decalcification was performed by washing the fixed tissue sample in running tap water and then immersing each tooth in 5% nitric acid at room temperature. The decalcified samples were dehydrated in ethanol and embedded in paraffin. Each paraffin block was sliced into 4-μm-thick sections and then deparaffinized in xylene.

Bacterial staining was used to visualize bacterial invasion into the fractures. This can also be used to distinguish between Gram-positive and Gram-negative bacteria in tissue using the Modified Brown and Brenn stain [13]. This procedure resulted in Gram-positive bacteria being stained blue or violet, while Gram-negative bacteria were stained red. Bacterial contamination of the root fractures were identified under a light microscope at magnifications of 4×, 20×, and 40×. The histological findings for bacterial contamination were categorized independently by two examiners, who identified the bacteria according to the presence of red fluorescence in the fractures.

2.6. Statistical analysis

All analyses were performed in the study using statistical testing in the R statistical language (R Foundation for Statistical Computing, Vienna, Austria).

The fluorescence parameters as analyzed by proprietary software yielded negative values for ΔFmax and positive values for ΔR; however, all of the data are reported here as absolute values. The association between the depth of a root fracture and the fluorescence parameters was analyzed using Spearman's rank correlation. The correlation between the presence of red fluorescence and the identification of bacterial invasion into a fracture was evaluated using Fisher's exact test.

3. Results

In total, 33 root fractures were confirmed on the root surface as observed with the aid of the operating microscope under high magnification. Under illumination by blue-violet light, root fractures appeared darker than the surrounding sound surface.

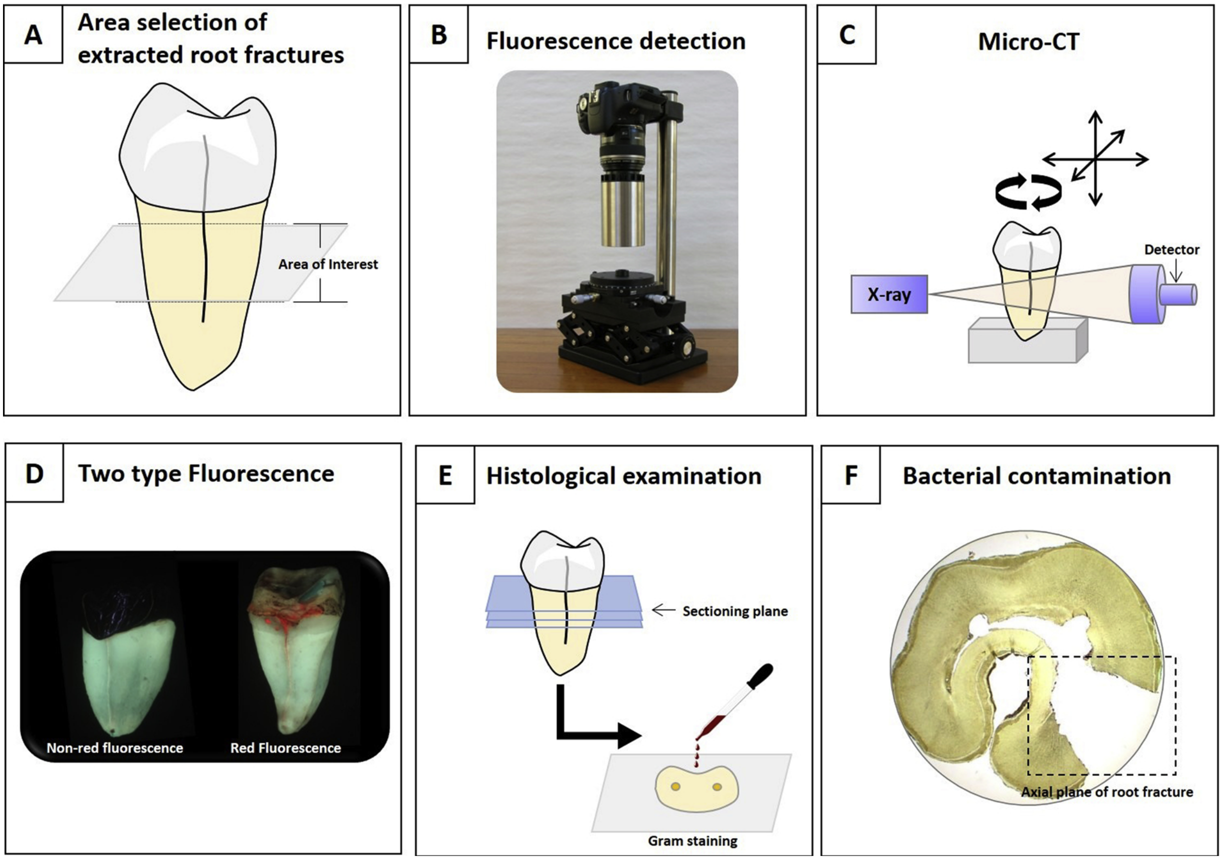

Fig. 1. Schematic representation of the experimental procedure of root fracture evaluation.

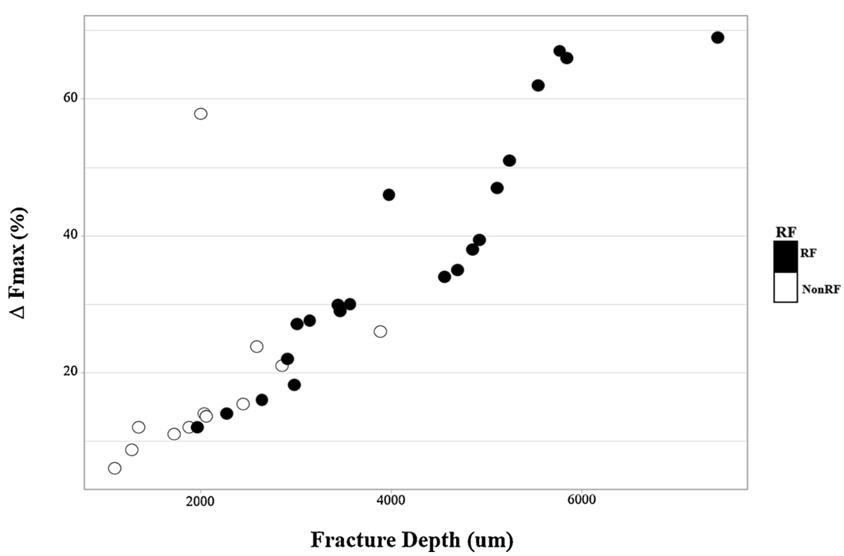

Fig. 2. Relationship between fracture depth from micro-CT and autofluorescence as measured using the fluorescence technique. RF means red fluorescence emitted from the fracture.

Table 1
Correlation of fracture depth and fluorescence parameters obtained by QLF-D.

	Fracture Depth	ΔFmax	ΔR	ΔRmax
Fracture Depth	1			
ΔFmax	0.89*	1		
ΔR	0.65*	0.76*	1	
ΔRmax	0.60*	0.71*	0.94*	1

Spearman's rank correlation analysis, $P < 0.01$*

An increased depth of a root fracture detected using micro-CT was associated with an increased ΔFmax. The proportion of visible red fluorescence (RF) in the fracture also increased with ΔFmax (Fig. 2). There were significant correlations between the fluorescence parameters and the depth of a root fracture. The depth of a fracture showed the strongest correlation with ΔFmax (Table 1, correlation coefficient of 0.89, $p < 0.01$). The intra-examiner reliability was checked and the intra-class correlation coefficient value (ICC) showed excellent reliability for fluorescence parameters (0.94).

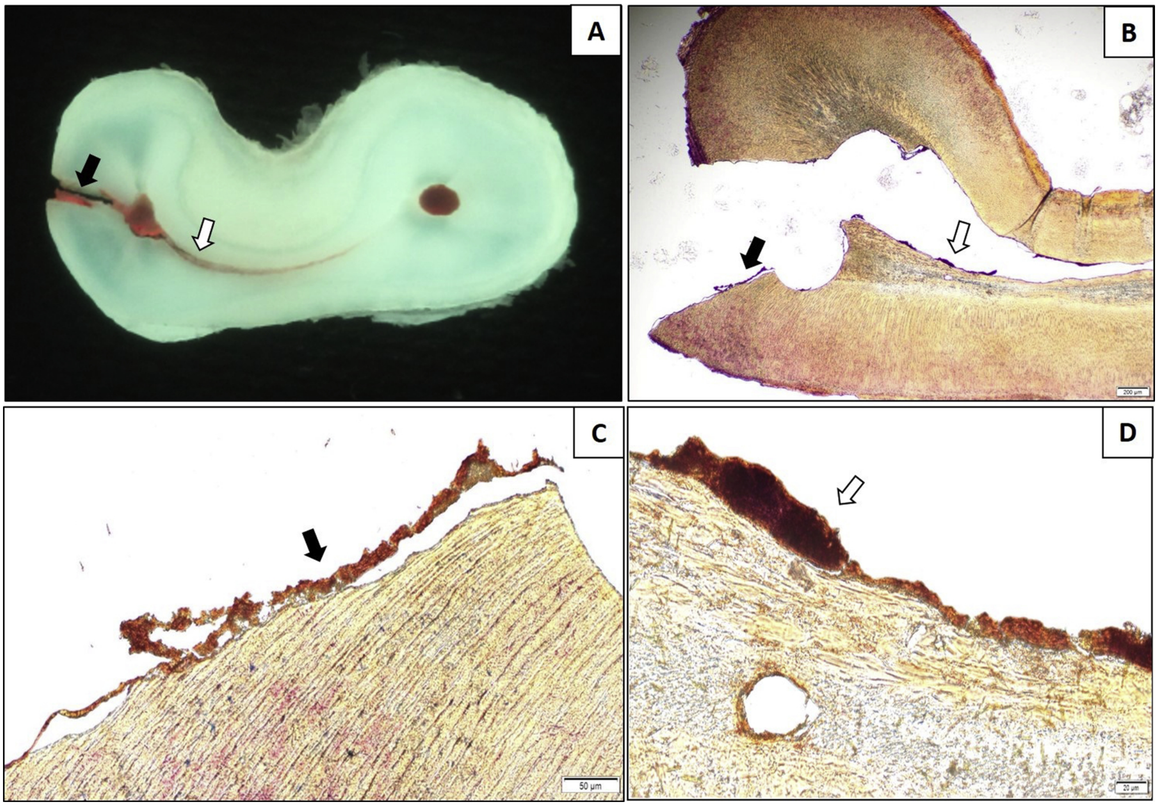

Fig. 3. Representative images of a fracture exhibiting red fluorescence in the axial plane of the root (A), and a histological section of a root fracture (B). High-magnification images of the red-fluorescing fracture (C and D). The black and white arrows indicated each red fluorescing point in high magnification images. Colonization by Gram-positive bacteria is evident inside the fracture.

Table 2
Summary of the presence of bacteria within fracture according to the presence of red fluorescence.

Bacteria within fracture	Absence	Presence	Total	*P-value**
Red Fluorescence (RF)				< 0.001
RF	5.3%	94.7%	100%	
Non RF	71.4%	28.6%	100%	

* *P-value* is from Fisher's exact test for comparison of bacteria and the presence of red fluorescence.

Red autofluorescence associated with bacteria was found in 57% of the root fractures. In all cases where the red fluorescence was detected on the outer surface of the root it was found that red fluorescence extended into the tooth up the end of the fracture (Fig. 3A). Bacterial invasion was detected in fractures with red fluorescence (Fig. 3B–D). Bacteria were detected with fractures in 94% of the 19 teeth exhibiting red fluorescence (Table 2).

On the other hand, 43% of non-red fluorescence fractures on the root surface did not exhibit the penetration of red fluorescence into the fractures (Fig. 4A). As the result of bacterial staining, some irritants such as necrotic tissue and debris were detected within the fractures (Fig. 4B–D). Bacteria had not penetrated into fractures in 71% of 14 teeth with non-red fluorescence (Table 2).

4. Discussion

The conventional methylene blue penetration method for diagnosing root fractures can be used to determine the location of fractures, but it has been difficult to detect the severity of cracks and bacterial colonization in fractures. The present study therefore aimed to develop an optical technique based on autofluorescence that would allow clinicians to objectively detect the depth and bacterial invasion of root fractures. The root fracture appeared dark due to light scattering during light illumination. In the present study, root fractures were detected by the autofluorescence emitted from the surface of the root fracture when exposed to blue-violet light. However, there was a limitation of sample selection. Fractures with severe curvature in the axial plane under micro-CT evaluation were excluded from evaluation with the fluorescence camera because it was difficult to detect the fluorescence aspect of the severe curved fracture inside the root due to the characteristics of light; "light travels only in straight lines." We also confirmed a characteristic behavior of fractures in the red spectral region during excitation at 405 nm due to the optical properties of a fracture differing from those of the adjacent sound surface. This red autofluorescence phenomenon is assumed to originate from fluorescent porphyrins formed by the indigenous microbiota [14]. A particularly interesting observation was that bacterial invasion was clearly observed in root fractures that exhibited red fluorescence.

There was statistically significant correlation between the fracture depth and maximum fluorescence (ΔFmax) related to the darkest area of a fracture during illumination with blue-violet light. Previous studies found that the fluorescence peak indicates the deepest point of a lesion [15,16], and is correlated with the depth of a crack [5]. The present study confirmed the presence of autofluorescence emitted from a fracture on the mesial and distal surfaces of the root, and revealed fractures with red fluorescence on the outer root surface (especially for deep fractures), when a fracture starts superficially on the mesial surface and subsequently propagates to the distal surface. The authors believe that the extent of the fracture into the tooth increases with progressing time. Hence the depth of the fracture represents its age. Superficial fractures may develop into deep fractures causing periapical pathosis leading to diagnosis of extraction [17]. All teeth with superficial fractures that could become deep and wide consistently experienced repetitive

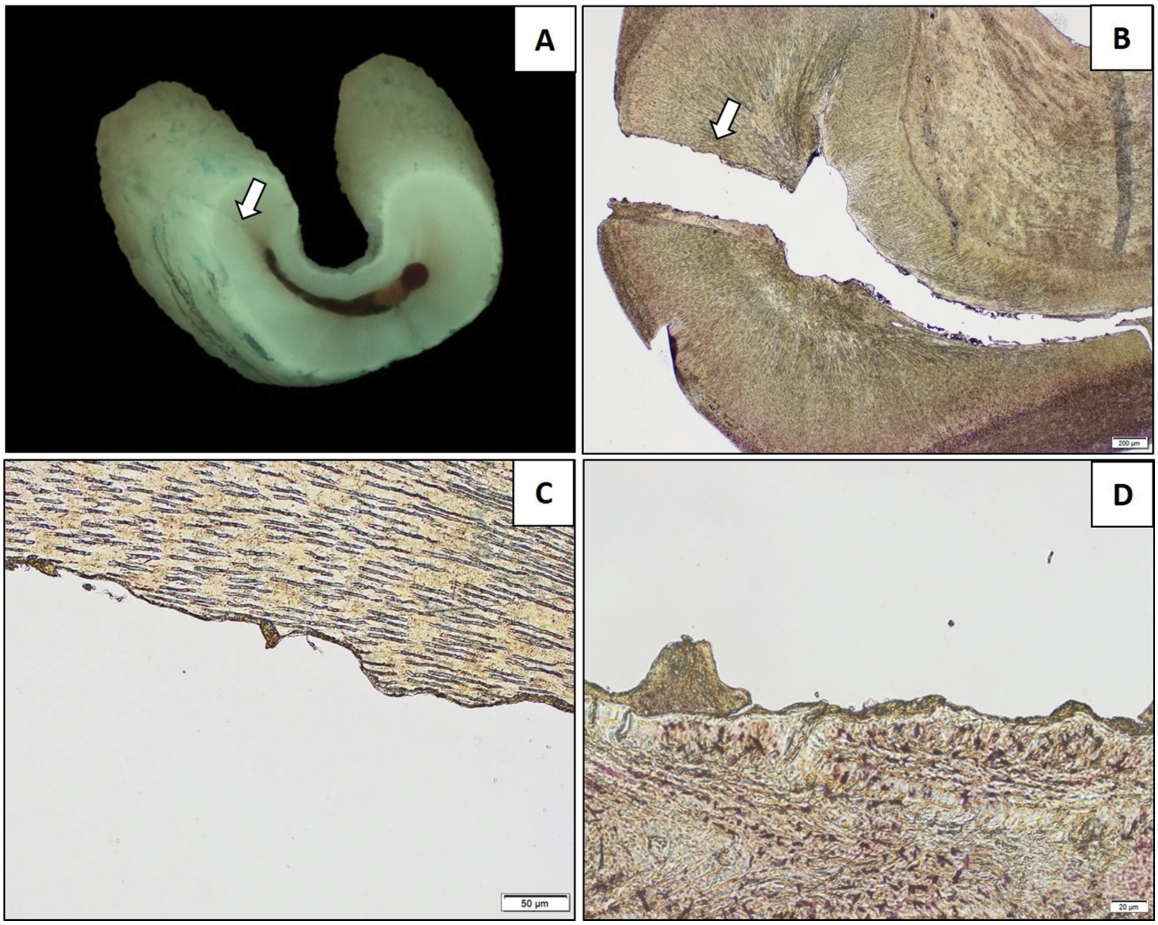

Fig. 4. Representative images of a non-red-fluorescent root fracture (A), a histological section in the axial plane of the root (B) and High-magnification images of the non-red-fluorescing fracture (C, D). The white arrows indicate fractured space.

loading during mastication. This could allow numerous irritants to directly enter through the outer surface penetrating into the extended fracture, leading to inflammation of periodontal tissue adjacent to a fracture [10]. The present study found bacterial metabolites among numerous irritating properties that could be detected as red fluorescence with the naked eye. Previous researches indicated that oral microorganisms are able to synthesize high levels of endogenous metal-free fluorescent porphyrin. The oral microorganisms occurring in dental biofilm exhibit autofluorescence in the red spectral region when excited at 405 nm or in the visible spectrum [18,19]. The molecular fluorescence of bacterial porphyrin absorbs photons in the violet wavelength around 405 nm and result in the subsequent re-emission of radiation at a longer wavelength [14].

Previous research identified that the chromophores of porphyrins were synthesized by late-colonizing bacteria during biofilm formation, but no previous study has demonstrated an association between bacterial contamination and red autofluorescence inside a tooth lesion. In this study we found that the bacterial contamination differed according to the presence or absence of red-fluorescent porphyrin in the fracture. According to the literature, the primary biofilm appears predominantly as green fluorescence, while the secondary colonizer actinomyces and black-pigmented obligate anaerobes—which indicate a mature biofilm—appear predominantly as red fluorescence for excitation at 405 nm. Moreover, the primary biofilm exhibiting green fluorescence may mature into a biofilm exhibiting red fluorescence [8,20]. As the depth of fracture increased, numerous species of bacteria interacted with each other to supply vitamin K or hemin and then produce red-fluorescent porphyrin caused by late-colonizing biofilm. Our results indicate that bacterial contamination was present in root fractures exhibiting red fluorescence, and so the presence of red-fluorescent porphyrin may be an indication of bacterial invasion into a fracture. The anaerobic bacteria that are able to synthesize fluorescent porphyrin penetrated into the fracture, with autofluorescence observed in the red spectral region. These bacteria might be responsible for complications of the periodontal tissue surrounding a root fracture.

This result is valuable it constitutes the first report of an evident difference in autofluorescence in fractured roots extracted due to periodontal inflammation being associated with bacterial contamination. Therefore, the red fluorescence of a root fracture can be used to indicate bacterial penetration, and it can also be used as a risk indicator to inform when a fracture extends to the outer surface and there is invasion by bacteria and bacterial metabolites causing periodontal destruction.

This fluorescence technique could be applied in clinical practice for crack detection during surgical procedures such as diagnostic surgery or apical surgery.

5. Conclusions

We have demonstrated the potential benefits of directly detecting the autofluorescence emitted from a root fracture. This autofluorescence can be used to quantify the fracture depth and to examine bacterial invasion into a fracture, which manifests as red fluorescence caused by bacteria and bacterial metabolites that have penetrated via repetitive mastication. Furthermore, the described simple fluorescence technique could be helpful to diagnosing root fracture and providing objective and quantitative information.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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