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General discussion

In this thesis various aspects of root canal irrigation have been studied and passive ultrasonic irrigation in particular using various ex vivo models. Various aspects of the models and techniques used will be discussed in this chapter.

Groove/depression model

All the ultrasonically activated irrigation studies in this thesis used the groove/depression model to evaluate the effectiveness of different irrigation regimens to remove dentin debris from the apical root canal. This *ex vivo* root canal model, first introduced by Lee (1), has a standard groove or standardized depressions in the apical portion of one canal wall filled with dentin debris, allowing the evaluation of different irrigation procedures on dentin debris removal from an oval extension or irregularities on the apical root canal wall. The dentin debris has been soaked in NaOCl for 5 min before using it, in order to extract its organic component. Since NaOCl has no chemical effect on inorganic matter, dentin debris removal in this setup evaluates the mechanical effect of the irrigation procedure.

The advantage of this model is that the amount of debris present both before and after irrigation can be compared, whereas the methodology normally used did not allow quantification of debris before the irrigation procedure (2-4). Consequently, ranking of the different irrigation procedures may therefore not have been reliable. In this model, the amount of debris before irrigation was standardized, as assessed by the initial scoring. The high kappa value indicates that the inter- and intra-observer agreement was very high, which support the reliability of this model. Another advantage is that the model is sensitive enough to detect differences of the effectiveness between irrigation procedures, and that the operator sensitivity is very low. The operators do not influence the outcome of the results (1, 5-11).

De Groot et al. modified the groove/depression model to improve its standardization by minimizing the original root canal lumen and ensure that the root canal model is closed by embedding the root in resin (5). The modification of the depression model used in Chapter 5 and 6 introduced an extra depression located at 0.5 mm from WL which allowed the evaluation in front of the ultrasonically oscillating file tip (6). Because the natural root canal varies considerably, the removal of the original root canal space and the subsequent preparation of a 'new' root canal resulted in a really standardized root canal. By embedding the root in the resin and using the four screws, it is easy to assemble or disassemble the model, facilitating the root canal treatment procedure *in vitro* within an enclosed system and the evaluation of the interested area before and after the irrigation. Lately, researchers noticed the difference between an open and closed root canal system (12). Basically, the open system overestimated the efficacy of the irrigation procedure. When the roots are embedded in resin, they can be tightly closed which can be controlled before and after the irrigation procedure. Besides, these models can be repeatedly used at least up to 8 times when the root canal walls are not damaged during the irrigation procedure.

The potential drawback of the groove/depression model is the inability to evaluate the volume of the debris present in the groove/depression. The groove/depression is a three-dimensional structure, while dentin debris is scored by evaluating the occupied area in 2D. The question whether a 3D volumetric analysis of the debris in the groove would give us more information and would improve the information we obtain from the model is still open. For the 4 mm-long groove, the 4-scale scoring system gives an indication on the cleaning efficacy of different irrigation protocols. For the depression, with only 0.3 mm in diameter, it was not possible to use the 4-scale scoring system. Therefore, we scored the samples as 'clean' and 'not clean', which might underestimate the differences between the irrigation procedures. OCT or MicroCT could measure the volume of the debris present and therefore could improve the accuracy of the evaluation of the depressions.

Evaluation and improvements of passive ultrasonic irrigation (PUI)

Intermittent flush method

Intermittent flush technique has been applied when performing PUI in all the related studies in this thesis (except chapter 7). We can discern three steps: irrigant delivery, irrigant activation followed by again irrigant delivery (refreshment). Firstly, 2 mL of 2% NaOCl was delivered using a 10 mL syringe with 30-gauge needle (Navitip) placed at 1mm from WL with a flow rate of approximately 5 mL min⁻¹. Thereafter, a stainless steel, noncutting wire (Irrisafe, Acteon, Merignac, France) was inserted into the root canal and driven ultrasonically (frequency approximately 30 kHz, file tip displacement amplitude within 100 µm) by a piezoelectronic unit (Suprasson PMax, Satelec Acteon) to activate the irrigant. After each ultrasonic activation, 2 mL irrigant was delivered again similar to the first time. There were some small variations in this schedule (Table 10-1) needed for the different protocols.

Syringe irrigation was used as control groups in chapter 2-6, and did not remove debris from the groove or depressions. Therefore it can be concluded that irrigant delivery by a syringe with a needle, does not change the debris score in the groove or depression before ultrasonic activation.

Streaming

Since the tip of the instrument was normally located at 1 mm from WL and the instrument could not enter the groove or depressions, the instrument per se could not physically disturb the dentin debris and the activated irrigant should be responsible for the removal of the debris from the groove and the depressions on the apical root canal wall. Therefore, it can be concluded that the streaming of the irrigant induced by the irrigation systems produce shear stress along the root canal wall and in the groove and depressions, resulting in the removal of debris from these areas. The cleaning efficacy is consequently influenced directly by the streaming.

$$v = \frac{\omega \varepsilon_0^2}{a}$$

According to the equation (13) above (where v is the flow velocity (in m/s), ω is 2π times the driving frequency (in Hz), ε_0 is the oscillation amplitude (in m) and a the radius of the tip (in m)), higher frequency, larger displacement amplitude and smaller instrument will result in higher streaming velocity. This could explain the result in chapter 5, where, a high intensity results in a higher cleaning efficacy. However, high intensity brings the risk of instrument breakage and an increased chance of wall damage in case of wall contact. Therefore, a new instrument, in respect of materials and profiles, is needed to solve these problems.

Although a higher amplitude is associated with better cleaning efficacy, other parameters should also be taken into account. With the sonic device, the oscillation amplitude of the sonic tip was so high that it caused a lot of wall contact limiting significantly its free movement and efficacy. The frequency of the sonic device was significantly lower than that of the ultrasonic device, resulting in a lower flow velocity and cleaning efficacy (chapter 2).

Apart from the flow velocity, the direction of the flow also plays an important role. A high-velocity jet observed in the direction of the oscillating file (equal to the axis of the handpiece) resulted in an improved cleaning efficacy. It is therefore advised to direct the handpiece towards the oval extension and/or isthmus during PUI in order to maximize its effect. On the other hand, it is worthwhile to develop a handpiece which could change the oscillating direction automatically, or a device that could activate the flow in all directions equally.

Dimension and taper

It has been shown that dimension and taper of the root canal have an influence on the cleaning efficacy of syringe irrigation (14, 15) and PUI (10, 16).

Using a similar research model, we tested the effect of the insertion depth of an ultrasonically activated instrument on dentin debris removal from depressions in the root canal wall (Chapter 6). If we only evaluate the individual depressions, it is interesting to notice that the cleaning efficacy from these depressions is very similar, but the dimension of the root canal actually changed from apical to the coronal, from 0.39 mm (1 mm from WL) to 0.60 mm (5 mm from WL). According to this result, we could conclude that within this range the dimension of the root canal did not influence the cleaning efficacy of the ultrasonic activation of the irrigant.

This finding seems not to be in line with the previous studies. However, both Lee and van der Sluis used a continuous flush technique with the irrigation duration of 3 min, while the intermittent flush technique with only 10 seconds of ultrasonic activation was performed in our testing. During the continuous flush technique, the irrigant flows continuously in the pulp chamber and the streaming induced by the ultrasonically activated instrument should direct the flow apically. It seems that it takes more or less 2 to 3 minutes before the flow has reached the apical root canal, because the laterally directed flow is more important than the apically directed flow. During the intermittent flush technique, the irrigant is directly delivered in and removed from the apical root canal.

Ultrasonic activation phases

In chapter 4 we described the three phases (start-up, steady and stopping) of the ultrasonic activation period, in combination with a rest phase during pulsed ultrasonic activation of the irrigant. It was the first time to evaluate the effect of pulsed ultrasound on the performance of PUI. Results indicated that the 50% duty cycle produced the best cleaning efficacy, although the total activation time was actually less than continuous activation. More start-ups could explain the results, but other groups with the same amount of start-ups did not result in a better cleaning efficacy, indicating both the duty cycle and the duration of each phase play a role. An explanation could be that the removal of debris from the groove theoretically follows two steps, namely, loosening of the debris in the groove by the shear stress of the activated flow and the removal of the dentine out of the groove and the root canal. Both steps need a certain time, which could explain the importance of the rest phase. Besides, from the chemical point of

view, an increase in the reaction rate was also found in the rest phase of the activated NaOCl solutions (17). In addition, the instrument used in the studies was recommended by the manufacturer for continuous ultrasonic activation. It could be prone to separation under pulsed ultrasound. This needs to be studied.

Other aspects related to PUI

Temperature

Heat generation is inevitable during the use of ultrasound in endodontic treatment because the energy transmission from the ultrasonically oscillating instrument to the irrigant or dentin will result in heat generation or exchange. This effect can be beneficial as well as harmful. Temperature rise can generally enhance the chemical efficacy and disinfectant potential of the irrigant (18, 19); on the other hand, 47 °C seems to be a critical threshold temperature for the occurrence of morphologically evident bone damage in the rabbit (20). Fortunately, irrigant temperature can exceed this critical value without causing any damage to the periodontal ligament, due to the fact that the dentin is a poor conductor of heat, insulating the external root surface from the temperature changes within the root canal (21).

Moorer and Wesselink (28) found that the temperature of the 5-mL NaOCl rose from 22 to 45 °C after ultrasonic activation under the intensity of 100 W per cm², and speculated that the temperature may reach 70 °C within minutes due to the much smaller volume of liquid in the root canal (22). This speculation did not correlate with data from later publications where the temperature rise in root canals was measured.

It is not advised during PUI, to force the oscillating instrument against the root canal wall. Therefore, the effect of heat generation by direct contact of the instrument to the root canal wall is minimal and mainly due to temperature changes in the irrigant.

Ahmad et al reported a minor temperature rise of the irrigant (0.4–0.8 °C) (23) and speculated that the temperature rise originated from heat loss from the file, conversion of sound energy into heat in the irrigant, and frictional contact of the file against the walls of the root canal. In that study, the root canal was instrumented to size 80# (indicating a large volume of the irrigant inside the root canal with the higher thermal buffer capacity). Furthermore, PUI was performed with continuous flow of fresh irrigant in the coronal root canal which cooled down the irrigant temperature in the root canal. Both factors may explain the small temperature changes. Zeltner (31) reported that a continuous flow in the coronal root canal resulted in a consistent temperature drop in the coronal third and a more variable pattern in the middle and apical thirds, indirectly indicating that the irrigant refreshment may be poor in the apical portion. This is consistent with the dentin debris removal capacity, which is limited within a short timeframe for ultrasonic activation of the irrigant combined with a continuous flow in the pulp chamber (24). Both Cameron (21) and Zeltner (25) found a similar pattern of a transient drop in temperature and then an increase when the intermittent flush was performed. Cameron recorded an increase of about 8 °C maximum after 30-second ultrasonic activation, and 1 °C after 10-second activation; Zeltner (31) detected 7.7 °C as the mean maximum in the coronal and middle root canal after 180-second activation. All the studies in this thesis applied the ultrasonic activation for no longer than 20 seconds; we therefore assumed that the minor temperature change should not have influenced the irrigation efficacy on dentin debris removal. During PUI, both the irrigant and the root canal wall buffers the temperature change, it is therefore unlikely to result in heat-induced injury. Unexpectedly, the drop of the temperature during irrigation leading to the hypothermia may have some effect on the surrounding tissue, but this needs further research work.

The following factors may influence the temperature changes in PUI: canal diameter, irrigant delivery method, activation duration, volume of the irrigant, design of the ultrasonic insert, method of generating ultrasonic energy (the magnetostrictive stack generator generates much more heat than its piezo crystal counterpart (21)), and power of the ultrasonic device.

Cavitation

There are two types of cavitation detected during PUI (26), stable and transient. The former could be defined as oscillation of bubbles under low-intensity ultrasound. In the latter situation, the bubbles implode resulting from the high acoustic pressures in an ultrasound field. Though we observed both types of cavitation by high speed imaging technique, it is still not clear what role they play in the cleaning efficacy regarding dentin debris removal.

Curvature

Debridement of curved root canal systems is more challenging than its straight counterpart regardless of instrumentation techniques (4, 27, 28). Innovative instruments and instrumentation techniques

therefore have been developed and evaluated specifically for curved root canal systems (29-33). However, similar irrigation protocols are adopted for both straight and curved root canals. The effectiveness of various irrigation techniques with irrigant activation in the curved canal still needs to be clarified.

Three approaches of ultrasonic activation of the irrigant can be suggested. 1) The oscillating file follows the curvature and reaches close to the working length; 2) the file is located above the curvature in order to perform PUI, minimizing the chance to contact the root canal wall; or 3) the file is pre-bent and just follows the beginning of the curvature.

There are few studies on the evaluation of PUI efficacy in the curved canal. Though some studies (34, 35) claimed that PUI was performed, they did not (or could not in case of a Ni-Ti instrument) pre-bend the file and inserted the file into the apical portion resulting in at least three contact points with the wall (Amato 2011, fig 1B), consequently the file oscillation would be restricted, if not eliminated, and the effectiveness could be hindered. Besides, Rödiger (41) used a cutting file as oscillating instrument. It is likely that the instrument would produce smear layer and unfavorable cutting in the root canal wall, which could confound the results. Furthermore, the power setting plays an important role in the flow penetration and cleaning efficacy of PUI (Chapter 6) (6) for both straight and curved canal. Both studies by Amato and Rödiger used low ultrasonic intensity.

Although pre-bending a file will change the oscillation pattern to lower amplitude and smaller wavelength (36) (unpublished data by Bram Verhaagen), it is unlikely to affect the cleaning efficacy of PUI. If the file is located at the beginning of the curvature (Chapter 6), the flow penetration of the irrigant typically doesn't go much further than halfway the distance to the apex, regardless of the curvature (as long as not a severely curved canal, less than 40° is concerned), but this can be enhanced with increasing power setting. Al-Jadaa reported the effectiveness of PUI on tissue dissolution in simulated accessory canals by using a curved canal model (37), indicating the lateral irrigant penetration as well. We can therefore assume there is an effective field distance around the oscillating file (in front of the tip and laterally). Curvature per se may not influence the effectiveness of the flow, but the difficulty in reaching the working length determined by the location and length of the curvature in a curved canal. In addition, although the penetration depth of the flow observed by visual method was in agreement with the cleaned areas in the *ex vivo* experiments (chapter 6) in which the cleaning effect decreases with the distance in front of the file tip, it needs to further elucidate how the shear stress of the flow changes exactly regarding both in front of and around the oscillating file.

Endodontic biofilm studies

The conditions under which biofilms exist *in vivo* are not fully understood (38). The stages of structural organization of biofilm, the composition and activities of the colonizing microorganisms in various environments may be different, although the establishment of a micro-community on a surface seems to follow essentially the same series of developmental stages (38). As far as endodontic disinfection studies by using a biofilm model are concerned, the following aspects should be considered.

- √ Bacteria colonization condition (substratum, nutrient environment, biofilm formation time)
- √ Selected bacteria (oral species, root canal origins, mono-, dual- or multi-species)
- √ evaluation method (sampling, traditional culturing, SEM, Confocal laser spectrum microscopy [CLSM])

Bacteria colonization condition

Wells (39), membrane filters (40), pegs (41, 42) and dentin or bovine dentin samples (43-47) are commonly used in *in vitro* biofilms studies to test the efficacy of selected disinfectants. The former facilitate optimal standardization conditions while missing dentin characteristics, such as the roughness, the presence and density of the dentin tubules (48). Dentinal tubules contain a considerable amount of unmineralized collagen (49), which might serve as an adhesion substrate to certain bacteria (50).

Collagen-coated hydroxyapatite (HA) and uncoated HA discs were chosen as the biofilm substrate (51). It resembles dentin (HA and collagen) and the standard shape of the discs makes it possible to grow biofilms with consistent characteristics which has proven to be difficult when using dentin as the biofilm substrate due to the variations of the dentin surfaces with tubules (52). HA however, does not possess the fine details of the dentin microanatomy.

The substratum not only influences the initial adhesion of colonizing cells, but also influences the production of signaling molecules that control cell physiology and virulence (53, 54). It has been shown that the biofilm development in the root canal and its penetration into the dentinal tubules have been associated with the environmental conditions (43).

Rich medium, optimal conditions are always provided when biofilms are cultured in the laboratory, which is very unlikely to happen in the real root canal (55). The availability of nutrients determines the bacterial composition of a biofilm (56). There is still no consensus in the literature regarding the time of biofilm formation, ranging from one day to 50 days (53, 57-60), although it is believed that a longer incubation time might produce a better structured and more aggregated biofilm, that is consequently more clinical relevant. It is therefore sometimes difficult to compare different studies testing similar disinfection approaches.

Selected bacteria

Single, dual or multiple species biofilms, found in oral or root canal infections or laboratory strains were used to establish a biofilm model *in vitro*.

Enterococcus faecalis is the most commonly used bacteria in endodontic research. This seems to be logical, because it is commonly isolated in failed root canal treatments, and has biological features that have been revealed to support the assumption of its role as an endodontic pathogen, as mentioned in the introduction. However, *E. faecalis* is not necessarily the dominant pathogen in endodontic infection (61). Easiness of being isolated from the root canal and cultured in the lab might overestimate its role in the root canal infections. Other bacterial phylotypes which are more difficult or impossible to detect or culture might also play an important role.

In recent years, researchers started to validate (new) endodontic disinfectants on single-species biofilm models, in place of tests on planktonic micro-organisms (62-66), as well as on dual or multi species biofilms (51, 67-69). It is well established that endodontic infections have a polymicrobial nature (70). Microbe-microbe interactions may increase total biofilm formation and change the characteristics of a biofilm, which may alter the resistance pattern to antimicrobials (71-73). How different species co-exist in a multispecies ecosystem is a particularly important aspect of polymicrobial disease etiology (74-77). Bacteria can respond differentially to their friend and foe (78). Taken the polymicrobial nature of endodontic infections into consideration, one may suggest a biofilm model needs to be multiple species or mixed species.

We studied dual species biofilms of *E. faecalis* and *S. mutants in vitro*, the two species showed an enhanced resistance to the disinfectant (69). Bacteria never have the same biological behavior under different circumstances. They will propagate when growth factors and other favorable conditions are fully available. On the other hand, when the surroundings or the conditions change, the microorganisms will adapt and behave completely different from the former condition. The role as friend or foe could consequently also change in the dynamic ecosystem. Future studies can shed light on how the adaptation of the microorganisms under different physical, chemical and biological stresses takes place.

Evaluation method

Often, sampling by paper points from the root canal is used to quantify the microorganisms in the root canal, but sampling only shows the planktonic microorganisms which can be removed from the root canal system (basically only the main canal), not the ones which are present as a biofilm on the root canal wall. However, this could partially be solved by using a file larger than the original apical dimension of the root canal to collect dentin shavings from the root canal wall. The oval shape of root canals could still be the cause of an underestimation of the biofilm present. Furthermore, evaluation before and after the treatment is not possible, although this can partially be resolved by control groups.

Another confounding factor is how to evaluate the biofilms. For example, the presence of cells in metabolically inactive states which are incapable of cellular division preventing them from forming colonies on a plate (79, 80) may lead to overestimation of the efficacy of the tested antimicrobials. SEM has been used to visualize the amount and distribution of bacteria on the surface of the biofilm (57, 58, 81-84). However, it will not give information on the viability of the micro-organisms.

Infected tooth models

The generation of biofilms in root canals of extracted teeth could provide a more realistic scenario (85, 86) by using the dentin as the substratum. Attempts to establish such biofilm root canal models have, however, suffered from several drawbacks (87), mainly due to the wide variation and anatomic complexity of a human root canal. Normally the following protocol was used. Teeth were decoronated, and autoclaved after conventional chemomechanical debridement of the root canals. Each root canal was then dispensed with an inoculum of microorganisms. After a certain incubation time, the root canals with biofilms were challenged by different treatment strategies.

Though using dentin as a substratum mimics the clinical situation, the invasion of root dentinal tubules by bacteria is a multi-factorial event which is only possible for a limited number of oral bacterial (88). The penetration of microorganisms in infected root dentin has shown variations in

the experimental model due to the bacteria selected for biofilm formation and the time of incubation employed in the studies (43, 67, 89). Furthermore, the dentin of an extracted root is different because the tubules are 'empty'. In the clinical situation they will be filled with tissue fluid which will influence the adaptation of the biofilm and the migration into the tubules.

In an adaptation of the above mentioned model, the intracanal bacteria/biofilm was established by contamination of the root canals of extracted teeth *in situ* with oral bacteria (90-92) in order to mimic the nutrient environment is the clinical situation. Briefly, volunteers carried appliances in their mouth with roots with prepared root canals which consequently were accessible to the oral microorganisms. In this way, a more clinical relevant biofilm, was established in standardized root canals. However, the composition of the biofilm will vary with the oral microbiota of the different volunteers participated. Once again, if the paper-point sampling was used, no information was obtained about the status of the biofilms in the root canal. Although one of the studies performed a histological evaluation of the biofilm (91), it was not clear whether it was done with a series of sections.

Since the groove model is a standardized model used for testing debris removal, we tried to establish a biofilm groove model to test biofilm removal of an irrigation protocol (93). The fact that bacteria not only attached in the groove but also outside the groove indicated wide variations among the models in the respect of biofilm formation. However, the overload of biofilm formation could be removed. Another problem was that the original groove was too deep to apply the culture medium without air inclusion resulting in non standardized amounts of biofilm in the different models. Shallower grooves could solve this problem. More difficult was the quantification of the biofilm formed in the groove model. Several approaches were attempted, two general staining methods (crystal violet and plaque indicator), two metabolic staining methods [resazurin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] and a nucleic acid staining method (syto9) (Table 2). The syto9 seemed to be the most promising staining and will be evaluated in future studies.

The following points are therefore the potential problems in *ex vivo* biofilm study models: unpredictable even after weeks of culture; culturing on yes or no level; much variation; often poorly documented; mono or mixed species biofilm. These confounding factors should be considered when drawing conclusion from these studies. Furthermore, additional local factors in the root canal environment, complex root canal anatomy, and the polymicrobial nature of root canal infections may affect the function of the various irrigating solutions or techniques. Growing biofilms on standardized readily available surfaces, on the other hand, eliminates these problems and allows a more accurate assessment of antimicrobial efficacy.

Table 10-1. Variations of PUI applied in each chapter.

Chapter	Model	Duration PUI	Orientation of file oscillation	Instrument size/taper	Intensity	Pulse intervals
2	Groove	20 sec * 3	Toward the groove	#20/.00	Blue 4	N/A
3	Groove	10 sec	Perpendicular to the groove Toward the groove	#20/.00	Blue 4	N/A
4	Groove	10 sec	Toward the groove	#20/.00	Yellow 4	None 730msON/ 100msOFF 400msON/ 400msOFF 100msON/ 670msOFF
5	Depression	10 sec	Toward the depression	#25/.00	Blue 4 Yellow 4 Green 4	N/A
6	Depression	10 sec	Toward the depression	#20/.00	Blue 5	N/A

Table 10-2. Overview of the tried quantification techniques

Category	Substance	Result summary
General stain	Crystal violet	Stains biofilm and dentin
	Plaque indicator	Stains biofilm and dentin
Metabolic stain	Resazurin	Stains fluid not cells
	MTT	Stains biofilm to local
Other stain	Syto9	Autofluorescence of dentin

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