University Ss Cyril and Methodius

Faculty of Dental Medicine

DOCTORAL THESIS

ANTIMICROBIAL EFFICACY OF ERBIUM LASER IN ENDODONTIC TREATMENT OF INFECTED ROOT CANALS

In vitro study

Agime Dragidella

Mentor: Prof. D-r Mira Jankulovska

Skopje, 2017

CONTENT

FIGURE LEGENDS

1 ABSTRACT

Background: This study was conducted to estimate efficacy of Er:YAG laser compared to different antibacterial methods in therapy of infected root canals. Chemo-mechanical irrigation or only instrumentation techniques for cleaning the infected root canals does not ensure complete removal of microorganisms due to anatomy of root dentin, where microorganisms can form complex biofilm or can penetrate into dentin tubules. Repeated infections are possible. Our research determined whether there are differences in disinfecting efficacy between the testing methods, and if laser application with Er:YAG laser could be used in clinical terms as sufficient for therapy.

Methods: In the study 200 freshly extracted premolar single-rooted teeth were included. The crown of each tooth was cut off obtaining 15 mm long root canal. The apical part was drilled through. Teeth were sterilized with absolute alcohol. 200 teeth were equally divided into four groups depending on the strain which were they inoculated with (*Enterococcus faecalis, Candida albicans, Streptococcus sanguinis, Fusobacterium nucleatum*). The growth of the biofilm was confirmed microbiologically on agar plates. Three different treatments were applied; laser radiation with Er:YAG laser, irrigation with 5.20% NaOCl or Qmix. Viability of microorganisms in the samples after treatments and compared to samples without treatment, were analyzed with a fluorescence method by flow cytometry.

Results: Results indicated that all treatment methods effectively eliminated major percent of microbial cells. Statistically significant differences in mean percentage of dead cells between tested organisms ($p<0.05$) for radiation with Er:YAG laser 30 and 90 seconds, irrigation with 5.20% NaOCl and almost ($p=0.052$) for irrigation with Qmix were observed. There were statistically significant differences between treatment methods ($p<0.001$) for all microorganisms, as well as in interaction between tested organisms and treatment methods ($p=0.040$). The percent of dead cells was significantly higher for *C. albicans* and *S. sanguinis* compared to *E. faecalis* or *F. nucleatum* in all treatment groups. Longer duration of irradiation showed was statistically significant compared to 30-seconds irradiation ($p<0.001$) and

achieved over 80% of dead cells. Disinfecting activity in combination with irrigant was over 90% for addition of NaOCl and over 95% for addition of Qmix.

Conclusions: According to the results we can conclude that Er:YAG irradiation and its disinfecting capabilities could be used sufficiently as standard disinfection method in endodontics, or can serve as adjuvant therapy to standard mechanical and irrigational treatments. However, this study included only 200 samples, so we need to expand study to more samples of root canals or even in *in vivo* conditions.

2 INTRODUCTION

Pathogenic bacteria are a major problem in dentistry, so the goal of endodontic treatment is to eliminate bacteria which cause problems in therapy treatment of infected root canals and periapical healing.[1,2] Because of the complex structure of root canal system, the complete elimination of microorganisms still presents a major challenge and enables resistance to irrigation and mechanical cleaning of root canals. Bacteria also produce biofilm that represents the only resistance to antibiotic therapy or to washing and mechanical cleaning of teeth.[3] Periodontal pathogenic bacteria cause various diseases; periodontitis, endodontitis, caries, alveolar ostitis, necrosis. Therefore, the control of microorganisms is extremely important in preventing infection inside the tooth and the tooth root. For prevention of such infections in particular treatment an increasingly important role is played by chemo-mechanical processes. The effectiveness of these processes depends largely on the individual anatomy of the tooth (round, oblong, irregular shapes...). Existing treatment procedures include mechanical treatment with rotary files accompanied by chemical cleaning and irrigation with irrigants such as sodium hypochlorite (NaOCl) or chlorhexidine (CHX) following application of medications and sealing of the root canal.[4]

Although during endodontic treatment, irrigation with NaOCl removes the majority of infecting microorganisms, it is still possible to regain infection of the root canal because of a smear layer that reduces effectiveness of disinfecting agents.[2] On the other hand, according to the small diameter of root canal it is difficult to irrigate the whole surface. The use of chelator substances such as ethylenediaminetetraacetic acid (EDTA) or Qmix preparation that remove smear layer have been suggested as enhanced methods of irrigation.[5] Numerous studies have shown that, in particular in the oval-shaped dental channels, persistent infections occur due to the ineffective treatment. This is due to the use of dental drills and insufficient cleaning of the channel, in which necrotic dental pulp and unremoved bacterial biofilm remain. Until now, the problem was addressed by ultra sonification or by use of a combination of tools for cleaning (instruments with flexible head made of nickel and titanium) and traditional dental devices. For the eradication of infectious agent such as *Enterococcus faecalis*, such procedures are not

effective because of the difficulty of access to dental channel. This requires new methods and approaches to solve these problems.

After using the instrument as a supplement to effective disinfection, appeared approach leaching with NaOCl and CHX. In addition, irrigants to disinfect teeth are increasingly using modern methods such as lasers. At the moment there are various equipment laser available with radiation (Er:YAG, Er, Cr:YSGG, Nd:YAG, diode, CO2), which are appropriate in periodontology and endodontics, each with special and different features. Some of these lasers are effective in removing residuals and detoxifying radicular cement (Er:YAG).[6-8] On the contrary other can eliminate scale, but can act on the soft tissues, reduce inflammation, as well as modify the oxidation of the tissue system and cytokines that mediate inflammation (Nd:YAG, diode).[9]

2.1 CLEANING AND SHAPING THE ROOT CANAL SYSTEM

Periradicular periodontitis following pulp necrosis is caused by microorganisms and their products emanating from the root canal system.[10-12] Successful endodontic therapy, which mainly depends on the elimination of microorganisms from the root canal system, is accomplished by means of biomechanical instrumentation of the root canal. Studies have shown, however, that complete removal of microorganisms from the root canal system is virtually impossible[13,14] and a smear layer covering the instrumented walls of the root canal is formed.[15-17] The smear layer consists of a superficial layer on the surface of the root canal wall approximately 1 to 2 µm thick and a deeper layer packed into the dentinal tubules to a depth of up to 40 µm.[17] It contains inorganic and organic substances that also include microorganisms and necrotic debris.[18] In addition to the possibility that the smear layer itself may be infected, it also can protect the bacteria already present in the dentinal tubules by preventing the application of successful intracanal disinfection agents.[19] Pashley et al.[20] considered that a smear layer containing bacteria or bacterial products might provide a reservoir of irritants. Thus, complete removal of the smear layer would be consistent with the elimination of irritants from the root canal system.[21]

According to Oguntebi BR.[22], the most currently used intracanal medicaments have a limited antibacterial spectrum and some of them have a limited ability to diffuse into the dentinal tubules. In his review, he suggested that newer treatment strategies designed to eliminate microorganisms from the root canal system must include agents that can penetrate the dentinal tubules and destroy the microorganisms because they are located in an area beyond the host defense mechanisms where they cannot be reached by systemically administered antibacterial agents. It also was clearly demonstrated that more than 35% of the canals' surface area remained unchanged following instrumentation of the root canal using four nickel-titanium preparation techniques.[23]

In various laser systems used in dentistry, the emitted energy can be delivered into the root canal system by a thin optical fiber (Nd:YAG, erbium,chromium:yttrium-scandium-gallium-garnet [Er,Cr:YSGG], argon, and diode) or by a hollow tube (CO_2 and Er:YAG). Thus, the potential bactericidal effect of laser irradiation can be used effectively for additional cleansing of the root canal system following biomechanical instrumentation. This effect was studied extensively using lasers such as CO_2 (24, 25), Nd:YAG[26-29], excimer[30,31], diode[32], and Er:YAG.[33-35] The apparent consensus is that laser irradiation emitted from laser systems used in dentistry has the potential to kill microorganisms. In most cases, the effect is directly related to the amount of irradiation and to its energy level. It has also been documented in numerous studies that CO_2[36], Nd:YAG[36-38], argon[36,39], Er,Cr:YSGG[40], and Er:YAG[41,42] laser irradiations have the ability to remove debris and the smear layer from the root canal walls following biomechanical instrumentation.

There are several limitations that may be associated with the intracanal use of lasers that cannot be overlooked.[43] The emission of laser energy from the tip of the optical fiber or the laser guide is directed along the root canal and not necessary laterally to the root canal walls.[44] Thus, it is almost impossible to obtain uniform coverage of the canal surface using a laser.[43,44] Another limitation is the safety of such a procedure because thermal damage to the periapical tissues potentially is possible.[43,45] Direct emission of laser irradiation from the tip of the optical fiber in the vicinity of the apical foramen of a tooth may result in transmission of the irradiation beyond the foramen. This transmission of irradiation, in turn, may affect the

supporting tissues of the tooth adversely and can be hazardous in teeth with close proximity to the mental foramen or to the mandibular nerve.[44] In the review, Kimura Y and colleagues[45] also emphasized the possible limitations of the use of lasers in the root canal system. They suggested that removal of smear layer and debris by laser is possible, however it is difficult to clean all root canal walls, because the laser is emitted straight ahead, making it almost impossible to irradiate the lateral canal walls. These investigators strongly recommended improving the endodontic tip to enable irradiation of all areas of the root canal walls.

Stabholz and colleagues[44,46] reported the developed endodontic tip that can be used with an Er:YAG laser system. The Er:YAG laser has gained increasing popularity among clinicians following its approval by the Food and Drug Administration for use on hard dental tissues.[47] The beam of the Er:YAG laser is delivered through a hollow tube, making it possible to develop an endodontic tip that allows lateral emission of the irradiation (side-firing), rather than direct emission through a single opening at its far end.

This new endodontic side-firing spiral tip (RCLase; Lumenis, Opus Dent, Israel) was designed to fit the shape and the volume of root canals prepared by nickel-titanium rotary instrumentation. It emits the Er:YAG laser irradiation laterally to the walls of the root canal through a spiral slit located all along the tip. The tip is sealed at its far end, preventing the transmission of irradiation to and through the apical foramen of the tooth. The dentinal tubules in the root run a relatively straight course between the pulp and the periphery, in contrast to the typical S-shaped contours of the tubules in the tooth crown.[18] Studies have shown that bacteria and their by-products, present in infected root canals, may invade the dentinal tubules. The presence of bacteria in the dentinal tubules of infected teeth at approximately half the distance between the root canal walls and the cementodentinal junction also was reported.[48,49] These findings justify the rationale and need for developing effective means of removing the smear layer from root canal walls following biomechanical instrumentation. This removal would allow disinfectants and laser irradiation to reach and destroy microorganisms in the dentinal tubules.

A pilot study[44] examined the efficacy of the endodontic side-firing spiral tip in removing debris and smear layer from distal and palatal root canals of freshly extracted human molars that were instrumented using nickel-titanium (ProTaper; Dentsply, Tulsa Dental, Tulsa Oklahoma) files to size F3. Following root canal preparation, the pulp chamber and the root canals of the prepared teeth were filled with 17% EDTA and irradiated with Er:YAG laser (Opus 20, Lumenis, Opus Dent, Israel), using 500 mJ per pulse at a frequency of 12 Hz for four cycles of 15 seconds each. The RCLase Side-Firing Spiral Tip was used for the irradiation. The lased roots were removed, split longitudinally, and submitted for SEM evaluation.

Distal and palatal roots of freshly extracted human molars that had undergone similar preparation but were not lased served as control. Scanning Electron Microscopy (SEM) of the lased root canal walls revealed clean surfaces, free of smear layer and debris. Open dentinal tubules were clearly distinguishable. In contrast, SEM of the nonlased root canals showed the presence of smear. It appears that an efficient cleansing of the root canal system can be achieved by using the Er:YAG laser with the RCLase Side-firing Spiral Tip after biomechanical preparation of the root canal with nickel-titanium (ProTaper) files.[50]

2.2 LASERS AS THERAPY

It is well known that biofilms and dental plaques are responsible for the development of periodontal disease. Physical or chemical methods of elimination may differ, depending on the nature of the disease, supra or subgingive position, and can produce different results in both sites.[51] Using a laser confocal microscope and studying biofilm in its natural state, it has been observed that bacterial behavior is quite different as viewed in traditional cultures. In their natural state, bacterial colonies formed several microcolumns involved in the matrix, which had channels through which fluid flow, nutrient transport, metabolic waste, enzymes, oxygen and other products.[52]

Biofilm, which adheres to the inner and outer walls of the periodontal pocket, allows bacteria to penetrate the epithelium of connective tissue which can cause gingival inflammatory reactions. This inflammation can progress from vasodilation, cell migration, and

mediator release, thereby increasing the inflammatory response and the perennial disease. This situation makes the microorganisms more resistant to drugs, which are often unable to reach colonies protected by a matrix and that makes bacteria become resistant.[53] Inflammatroy processe induced by bacteria and their waste products attract macrophages that produce, among others, interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), which have the ability to activate osteoclasts and production of bone resorption. TNF-α activates adhesion molecules on endothelial blood vessel cells, which favors monocyte adhesion. They also stimulate the arrival of T lymphocytes, which contribute to the activation of the nucleus factor capa B ligand (Rankla) receptor on the bones, thereby favoring bone loss.[54] This process affects more complexes, such as proteins Nuclear Factor Kappa B (NF-κB), reactor activator of nuclear factor Kappa B (RANK), RANKL and osteoprotegerin (OPG), among other it may change the response from osteoclastic predecessors, and thereby alter bone destruction. NF-κB plays the basic role as activator of immunoglobulin during the contagious process. Thus, as IL-1 and TNF-α are used for the Rank-L synthesis and thus the activation of the rank, which makes it possible to differentiate the preosteoclasts into osteoclasts. However, this procedure can be concealed when OPG (soluble protein expressed in many tissues and osteoblasts) appears, blocking RANKL, and stops the process of bone destruction. OPG is a receptor for tumor necrosis factor such as molecules that produce gingival fibroblasts, ligaments and epithelial cells, which can be modulated by several inflammatory cytokines. These proteins can be detected in the periodontal pocket, referring to the degree of periodontal disease evolution.[55] His control gives us much more precision than the clinics to detect possible biological effects during periodontal status when we apply the treatment.

Looking for an efficient biofilm removal and reducing inflammation, mechanical processing is still considered a golden standard. Using root scaling and polishing (SRP), ultrasonic chisel and adequate hygienic techniques, acceptable results can be achieved, but treatments themselves are unable to completely eliminate all bacteria due to radicular morphologic factors, deep pockets with reduced access, bacterial attacks on adjacent gingival tissues and fast variants of bacterial colonies.[56] Antibiotics are now being used as an integral element, but their use must be limited to a minimum due to development of frequent resistance

and difficulties in maintaining a stable and effective level over a long period of time.[57] For this reason, additional research into substances or techniques that may alter pH, oxygen concentration or nutrition, dental plaque layout, become necessary to alter the biofilm microflora. We also need to find systems capable of hindering the bacterial genetic signal and altering the inflammatory response in the periodontal tissues. An alternative is to consider the use of laser technology. Several studies guarantee a beneficial effect such as sulcular and/or pocket debridement, reduction of subgingival bacterial load, and reduction of inflammation.[58,59] Photodynamic therapy has also been introduced in the periodontal field. Today, the use of lasers within the periodontal pocket has become a promising field in periodontal treatment.

2.2.1 Lasers in periodontology

Currently, various laser radiation equipment (Er:YAG, Er, Cr:YSGG, Nd:YAG, Diode, CO2) are available in periodontics, each with special features and different effects, what is needed to select the most suitable type of application (Table 1).

Table 1: Wavelength of the laser and their possible application in endodontics

Laser	Procedure
Diode (810-980 nm)	Desensitization, pulp pulping, disinfection of root canals
Nd:YAG (1064 nm)	Desensitization, pulp pulping, pulpectomy, cleaning and disinfection of root canals
Er, Cr:YSGG (2780 nm)	Cavity access preparation, shaping, cleaning and disinfection of the root canals
Er:YAG (2940 nm)	Cavity access preparation, pulpectomy, shaping, cleaning and disinfection of the root canals
CO2 (10600 nm)	Desensitization, pulp pulping, pulpectomy

Some of these lasers are effective in removing residual plaque and detoxification of radical cements (Er:YAG).[59,60,61] On the contrary, others cannot eliminate plaque, but may act on soft tissues, reduce inflammation, such as altering tissue oxidation and cytokines mediating inflammation (Nd:YAG, diode). Although these effects over tissue are difficult to evaluate clinically, they are guaranteed molecular biology techniques. The results are variable, but the investigation should help us to select wavelengths of radiation, duration of impulse, energy/power, impulse, frequency, exposure time, sequence, wave type, continuous (HV) or pulse, type of applicator (cut or solid fiberglass), and other factors that can provide the desired goals.

The therapeutic application of laser radiation may be clinically useful only if appropriate instrumentation is available. Since laser is introduced into medicine and dental scineces, the number of different applicators was developed for clinical use.

In periodontics, we need treatments for the removal of plaque and calculus, to eliminate and/or reduce the sulcular inflammation. That is why we have to work on soft and hard dental tissues. For this reason, the basic effects of periodontal lasers for soft and hard tissue have been demonstrated to demonstrate the capabilities of this technology, mainly in combination with SRP.

2.2.2 Lasers on hard tooth tissues

By the early 1990s, the use of laser systems for periodontal therapy was limited for soft tissue procedures, such as gingivectomy and frenectomy, but when used on periodontal hard tissues it has been previously shown to be clinically non-effective.[62] In recent decades, laser therapy has been suggested as an alternative or addition to conventional non-surgical therapy due to its ability, ablation of tissue and hemostasis, bactericidal action against periodontal pathogens and root detoxification.[58,59,60,61]

In 1989, Hibst R, Keller U[63] reported the possibility of ablation of hard tooth tissues with irradiation of Er:YAG laser, which was absorbed by water. Since then, numerous researches on hard tissue have been using Er:YAG laser, that points to the ability of this laser

for ablation of hard dental tissues and carious lesions without producing large thermal side effects.

Absorption of the Er:YAG laser in water is highest due to emission of its 2940 nm wave length that coincides with high water absorption. In addition, the OH group exhibits relatively high absorption at 2940 nm. As Er:YAG laser is well absorbed by numerous biological tissues that contain water molecules, it is pleasant to use it not only for the treatment of soft tissue, but also because of the ablation for hard tissue. *In vitro* studies have shown that scattering of Er:YAG laser effectively eliminates subgingival scaling, with similar results compared to ultrasound instrumentation.[60, 64, 65] However, some authors have shown a higher amount of residual calculus in areas treated with Er:YAG laser.[66] Factors such as quantity and quality of initial calculus (texture, thickness and water content) and root anatomy, together with individualized instrumentation techniques, can affect results regardless of the way they are implemented.[64] Due to a similar composition of dental calculus and cement it would be impossible to selectively and efficiently remove the scale using Er:YAG laser without root damaging.[60]

They reported that different energy settings were more efficient for removing the plaques without damaging the root cement. Most of these researches suggest the use of energy between 100-160 mJ. Higher energy can damage the radicular surface, and lower energy is unable to efficiently eliminate the calculus.[9] Folwaczny M et al. reported that the inclination of the laser on the root surfaces has a strong influence on the amount of substance removed during the irradiation of Er:YAG laser.[65] In addition, the use of water as a cooler reduces heat generation by cooling the irradiated space and absorbs excess laser energy. The secondary effect of calculus removal is the elimination of cement and the subsequent exposure of dentin tubules. Although the amount of cement on the root surface is very variable, and depends on factors such as the age of the patient, prior periodontal treatment, *in vitro* studies have shown that the number and diameter of exposed dentin tubules was significantly higher in areas treated with lasers than with ultrasonic chasms.[64,67,68] The latest generation of Er:YAG lasers contains a lithium-ray detector based on the mineralized fluorescence signal (feedback system). Preliminary *in vitro* and clinical studies show that Er:YAG laser debridman, when it

comes to automatic detection of plaque, provides effective removal of plaques similar to ultrasound scaling without ablation resulting in virtually no exposure to dentinal tubes[64,69,70]

Er: YAG laser does not cause coagulation or dissolution of the irradiated root surface, but it has been shown that the surface has micro-irregularities, rough and chalky, probably due to the effect of mechanical engraving.[61,64,65,71] Some craters can be shaped by the result of perpendicular directing the laser beam to the root surface or using a nonconforming extension, hindering the unique radiation.[58,65] There is no clear indicator in the literature which is the ideal root area for the treatment. It is well accepted that the rough surface does not adversely affect the treatment of periodontal disease. According to several authors[65], the surface treated with Er:YAG laser is similar to that observed after EDTA or treated citric acid, which has been used for many years as a device for improving the outcome of periodontal treatment. On the contrary, the presence of a rough surface of the root can favor plaque retention and, therefore, limit the results associated with periodontal therapy.[56]

2.2.3 Laser for the treatment of peri-implantitis

Bacterial colonization on the surface of the implant is considered the major etiological factor of implant failure. Bacterial presence on the surface of the implant can cause inflammation of the peri-implant mucous membrane and, if not treated, can progress apically, resulting in periimplantitis and bone resorption. Therefore, removal of bacterial plaque is the main goal in the treatment of peri-implant infections. However, debridement of the implant surface is difficult to achieve, especially in rough ones. Various mechanical and chemical methods have been proposed to achieve this goal. *In vitro* studies suggest the use of certain devices, made from lower titanium hardness (plastic gauges) for mechanical debridement. Because mechanical methods are ineffective when used alone, chemical agents, such as local or systemic antimicrobial agents, also have a beneficial role. Various laser systems are also proposed for debridement of the cross-sectional area. *In vitro* studies show also that, due to radiation, only CO_2, diode and Er:YAG lasers are adequate for the debridement of the implant surface. This is because titanium barely absorbs their wavelength, which only slightly increases the temperature of the implant during irradiation. However, Nd:YAG laser creates

important permanent damage to the surface of titanium. In addition, only CO2 and Er:YAG have shown a bactericidal effect in *in vitro* studies, so both systems can be used for decontamination and detoxification of implant surfaces. CO2 as well as diode lasers are not effective for removing plaques in radicular surfaces or in titanium implants. They should only be used as auxiliary agents of mechanical procedures[9]. However, studies have shown that non-surgical instrumentation of implants with Er:YAG laser, and using specific applicators, effectively removes lipid and subgingival plaque without thermal damage to the surface of the implant. The research results show that Er:YAG laser radiation does not damage the titanium surface and does not affect the rate of attachment of human cells to osteoblasts[72].

Recently, 940 and 980 nm diode lasers have created great expectations in view of their excellent cut, removal and coagulation properties of soft tissue, enabling low energy applications, while reducing the inflammatory process and achieving faster tissue healing. The use of lasers for the treatment of peri-implantitis is promising, but more studies are needed to evaluate its actual efficacy[9].

We need to consider that laser treatments are in constant development; maybe in the next few years we will have a combination of equipment of different photonic properties, allowing us to choose the most appropriate system for every need. Although great progress has been made over the last few years, most studies are difficult to evaluate clinically due to their short duration (2 to 3 months). More long-term systematic studies are required to evaluate the clinical and biological effects of each type of laser, time and mode of administration, single/multiple dose and frequency usage. It will also be important to know the appropriate energy of each type of laser, deepen its knowledge, as well as its application of comfort, silence, reduction of anesthesia and other benefits that make them attractive to society and professionals.

2.2.4 Lasers in endodontics

All present wavelengths of dental lasers were used in a wide range of endodontic treatment, whether it was to aid preparation phases, or root canal therapy techniques, or alleviate the damage of poor quality pulp[73].

The areas of endodontics where the laser was investigated as follows:

- Directly covering the pulp
- Removing pulp tissue
- Access/design of channel walls and morphological changes in structure
- Bacterial decontamination
- Closing or removing gutta percha material
- Reducing the sensitivity of root dentin

As with the laser use of debridement in the periodontal pocket, it should be in mind that non-visual access points are potentially limiting controls that are carried out by the operator using laser energy in the canal. Additionally, laser use must be apt to good clinical practice if we are to maximize utilization. Wherever the laser is used, it is recommended that this should be based on evidence and, if necessary, complemented by all other treatment measures.

2.2.5 Pulp capping and pulpotomy

Consideration for capping the pulp and/or pulpotomy by laser we should complement the current protocol for such actions. Exposure to vital pulp (resulting from caries or trauma) and subsequent local action, leading to the preservation of vital tissues, is controversial in permanent dentition and the rate of success is very low. It was suggested that permanent teeth with open tips or dairy teeth offer a better chance of replenishing pulp. However, the use of laser energy to assist hemostasis and elimination of bacterial contamination in order to create a repairing dentine bridge can offer increased chances for a successful solution[74].

Laser technique, including exposed tissue of pulp, should be controlled under the control of the contamination of bacteria in saliva. The minimum energy level (1-2 W of average power) per wavelength should be sufficient to ensure hemostasis and sterilize the

cutting surface. Calcium hydroxide as a dressing should be applied directly, so that the cavity is totally resected.

Access and design of canal wall and morphological changes in structure

Accepted interaction of Er:YAG and Er, Cr:YSGG lasers with hard dental tissue makes these wavelengths ideal for removing covering dentin at the top of the pulp. Within the confidence of the root canals, the use of a laser wavelength without water cooling can lead to a potentially high rise in temperature. Risks are associated with melting or cracking dentine walls and trans-apical dental radiation[75]. With short infrared and CO_2 lasers, the power level of 0.75 to 1.5 W should be considered as the required maximum. With the help of erbium laser, the power values of 150-250 mJ/4-8 pps are considered appropriate but important to allow the water to reach the ablation site to prevent overheating and cavitation of the canal walls[76,77].

In order to solve the emission of laser light from the system, scientists developed intracanal instruments[78] and revised experimental devices for the production of non-linear spread of laser radiation with optical fibers. With the mechanical preparation of the canal, a layer of impurities, which bind bacteria, is produced. Most laser wavelengths will remove the residual layer and can be used in combination with irigants and chelators such as NaOCl or EDTA. Nd:YAG laser has been extensively investigated, but many reports are made about melting and carbonization. It is believed that a group of erbium laser wavelengths is best placed to achieve this and does not cause damage due to temperature increase[79,80].

2.2.6 Application of Er:YAG laser in endodontics

Erbium laser is built from erbium ions (Er^{3+}) in solid state of yttrium aluminium grenade (YAG) materials. Its pulsed infrared radiation (2940 nm) is characterized by water absorption, so it is particularly suitable and precise for ablation of biological tissues with high water content. Theoretically, the water absorption coefficient for Er:YAG laser is 10000 cm-1, so 15 and 2000 times higher than for CO2 or Nd:YAG laser. This high absorption coefficient results in an extremely small optical penetration in depth and thus the ablation of tissue with minimal damage. Furthermore, as the OH component of hydroxyapatite, showing maximum

absorption around 2800 nm, thus explaining its ablation volume through the enamel, dentin and bone, so that the above indicated types of lasers used for the soft and hard tissues[7,81]. The transfer of energy with the laser system is done by a hinge or flexible waveguide made of zirconium fluoride or crystal sapphire. A number of new applicators are continually expanding for potential dental benefits[81]. Based on results *in vitro,* Watanabe performed the first clinical use of Er:YAG laser for the debridement in 1996[82]. Today, following clinical research, for this type of laser radiation, Er:YAG laser appears to be the most suitable laser system, an alternative or auxiliary SRP tool[59]. Although the *in vitro* ability to remove calculus and plaque with Er:YAG laser has been proven, different clinical studies have shown different clinical outcomes in the initial treatment of chronic periodontitis. Crespi[71] reported a significant reduction in clinical parameters after 6 months in the Er:YAG group compared to a group of treated SRPs with ultrasound scales. A study found that the auxiliary use of Er:YAG lasers for conventional SRP did not reveal a more efficient outcome than SRP itself in a short period of six months.

New Er:YAG laser equipment, introduced to improve results, is a device that allows control of Er:YAG laser radiation with a feedback system that selectively detects subgingival calculus or plaque. Several studies have assessed the outcomes of treatment after laser debridement by means of fluorescence of Er:YAG radiation. In the study by Sculean A, it was observed that fluorescence-controlled Er:YAG radiation led to clinical improvements after 3 and 6 months, similar to ultrasonic debridement[83]. Tomasi C et al. evaluated clinical and microbiological outcomes after feedback under the control of Er:YAG laser and ultrasonic debridmann device during periodontal support therapy[84]. They noted that the mean reduction in PPD and gain of CAL (clinical attachement level) were significantly higher in the laser group after 1 month of treatment. However, both treatments resulted in significant reduction in subgingive microflora, although no observed significant differences were observed between the groups at each time point. Derdilopoulou[85] compared the microbiological effect of SRP, Er:YAG laser with feedback, sonic and ultrasound sonus in pacients with chronic periodontitis for a period of 6 months. Methods of treatment resulted in a comparable reduction in the evaluation of periodontal pathogens, where Er:YAG laser did not show much better. Finally,

better results were obtained by Dominguez A when using Er:YAG laser as an SRP supplement[86]. Although there were no statistically significant differences between the two groups in any of the investigated clinical parameters, the cytokine levels in GCF have been reduced with the feedback control of Er:YAG laser radiation. Outcome in SRP + Er:YAG group was only slightly better than in the SRP group, so the mechanical subgingival debridement is still needed. Despite the advantages described above in using this laser prototype, they did not find any new additional effects of local Er:YAG laser therapy.

Finally, Schwarz F et al. described the immunohistochemical characterization of the wound healing after non-surgical periodontal treatments, where fluorescence under control of Er:YAG laser radiation was effective in controlling disease progression, and could support forming of new bonding of connective tissue[69].

De Meyer S et al[87] evaluated the antimicrobial effect of laser-activated irrigation with Er:YAG on biofilms formed in simulated root canals. They grew a mixed biofilm of *Enterococcus faecalis* and *Streptococcus mutans* in a root canal model. Biofilms were subjected to the following treatments, all executed for 20 seconds: syringe irrigation with a needle, ultrasonically activated irrigation, and laser-activated irrigation with a 2940nm Er:YAG laser (20 Hz, 50 μs, 20 or 40 mJ, conical fibre tip). Performance of sterile saline as well as NaOCl (2.5%) as irrigants was tested. They proved that when using saline as the irrigant, significant reductions in viable counts were observed for ultrasonically activated irrigation and for laser-activated irrigation groups, but not for syringe irrigation. The reductions in the laser-activated irrigation groups were significantly greater than those of ultrasonically activated irrigation. With NaOCl as the irrigant, significant reductions in the number of attached bacteria were observed for all treatment groups and there was no significant difference between laser-activated and ultrasonically activated irrigation. Laser-activated irrigation removed more biofilm than ultrasonically activated irrigation when using saline as the irrigant, indicating greater physical biofilm removal. The use of NaOCl resulted in greater biofilm reduction with no significant differences between treatment groups[87].

Similar was confirmed by Cheng X et al[88]. who also evaluated the bactericidal effect of Er:YAG laser radiation combined with NaOCl irrigation in the treatment of *Enterococcus faecalis* deep inside dentinal tubules. The Er:YAG laser was activated at 0.3, 0.5 and 1.0 W for either 20 or 30 seconds; NaOCl and saline suspension were used for the control groups. Scanning electronic microscopy results showed that the Er:YAG laser combined with NaOCl disinfected the dentinal tubules as irradiation power and time increased. It reached 100 % in all experimental groups, both on the root canal walls and at 100 and 200 μm inside the dentinal tubules. However, at 300, 400 and 500 μm inside the dentinal tubules, only the groups treated with 0.5 and 1 W for 30s exhibited no bacterial growth. Of the two groups in which no bacteria were detected at all tested depths, Er:YAG laser irradiation at 0.5 W for 30 seconds combined with NaOCl irrigation was preferable because of the lower emission power and shorter irradiation time. They concluded that laser irradiation may serve as a new option for effective root canal disinfection.

In 2010 it was proposed that lasers could be used to enhance the decontaminating action of NaOCl. Olivi G et al. conducted a study where they compared the disinfection efficacy of laser-activated irrigation by using a photon-induced photoacoustic streaming tip with conventional irrigation and specifically laser-activated irrigation's ability to remove bacterial biofilm formed on walls of root canal. They used 26 human anterior teeth, infected with *Enterococcus faecalis* for four weeks, and conducted two irrigation protocols. Group A received two cycles of 30 seconds of 5% NaOCl laser activation and one cycle of 30 seconds with laser activation only, involving the use of 17% EDTA. The Er:YAG laser's settings were 20 millijoules, 15 Hz, 50-microsecond pulse duration, and it had a 600 micrometer photon-induced photoacoustic streaming tip. Group B received two cycles of 30 seconds of 5% NaOCl and 17% EDTA irrigation alone, delivered via a syringe needle. The authors found that group A had significantly better disinfection compared to group B. Scanning electron microscopic images showed absence of bacterial biofilm remaining after laser-activated irrigation using photon-induced photoacoustic streaming. Er:YAG laser activation of 5%

NaOCl and 17% EDTA was more effective than conventional irrigation for eradicating *E. faecalis* and preventing new bacterial growth *ex vivo*[89].

Furthermore, Neelakantan P et al[90] in 2015 investigated the impact of three irrigation protocols, activated by three different methods, on mature biofilms of *Enterococcus faecalis in vitro*. Samples were divided into three experimental (n=80) and one control (n=40) group based on the irrigation protocol employed: group 1 (NaOCl + Etidronic acid), 1:1 mixture of 6% NaOCl and 18% etidronic acid; group 2 (NaOCl-EDTA), 3% NaOCl followed by 17% EDTA; group 3 (NaOCl-EDTA-NaOCl), 3% NaOCl followed by 17% EDTA and a final flush of 3% NaOCl. Saline served as the control. Samples were further divided into four subgroups (n=20) based on the activation method: subgroup A, no activation; subgroup B, ultrasonic activation; group C, diode laser; group D, Er:YAG laser. Disinfection rates were determined with confocal laser scanning microscopy and root dentine powder was obtained for determining the colony-forming units (CFU /mL). Interestingly they found out that all experimental irrigation protocols caused complete destruction of the biofilm in the root canal. Within the dentinal tubules, all groups had a significantly higher percentage of dead bacteria than the saline control. There was no significant difference between NaOCl + etidronic acid and NaOCl-EDTA-NaOCl, whereas both groups brought about more bacterial reduction than NaOCl-EDTA. There was also no significant difference between diode laser and Er:YAG laser in any of the groups . Both diode and Er:YAG laser were more effective than ultrasonic activation and conventional syringe irrigation in reducing *E. fecalis* biofilms. The use of NaOCl after or in combination with a chelator caused the greatest reduction of E. *faecalis*. So at the end they concluded that using lasers, diode laser and Er:YAG laser activation, were superior to ultrasonics in dentinal tubule disinfection[90].

The purpose of the study from Zan R et al. 2013, was to investigate the antibacterial effects of two different types of laser and aqueous ozone in human root canals infected by *Enterococcus faecalis*. Many techniques have been developed to find an alternative to NaOCl as a disinfection agent for infected root canals. Eighty mandibular premolar teeth with single roots

and canals were selected. Following root canal preparation and irrigation, sterilization was performed in an autoclave. *E. faecalis* was incubated in the root canals and kept at 37°C for 24 h. The teeth contaminated with *E. faecalis* were divided into one negative control group NaOCl and three experimental groups; (Er:YAG laser, KTP laser, and aqueous ozone groups) (n=20). A disinfection procedure was performed for 3 min in order to standardize all groups. After this procedure, the microbial colonies were counted. The results indicated that whereas the NaOCl group exhibited the highest antibacterial effect among all groups, the aqueous ozone showed the highest antibacterial effect among the experimental groups. Whereas a statistically significant difference was noted between the aqueous ozone and laser groups ($p<0.05$), the difference between the Er:YAG and KTP lasers was not statistically significant ($p>0.05$). The end results showed that when aqueous ozone was applied with the aim of disinfecting the root canals, it exhibited a higher antibacterial effect than the KTP and Er:YAG lasers. However, the antibacterial effect of the aqueous ozone was insufficient when compared with NaOCl[91].

In recent years, various laser systems have been introduced into the field of laser-assisted endodontic therapy. The performance of such systems was investigated by Cheng et al. 2012, who evaluated the bactericidal effect of Nd:YAG, Er:YAG, Er,Cr:YSGG laser radiation, and antimicrobial photodynamic therapy (aPDT) in experimentally infected root canals compared with standard endodontic treatment of 5.25% NaClO irrigation. Two hundred and twenty infected root canals from extracted human teeth (contaminated with *Enterococcus faecalis* ATCC 4083 for 4 weeks) were randomly divided into five experimental groups (Nd:YAG, Er:YAG + 5.25% NaClO + 0.9% normal saline + distilled water (Er:YAG/NaClO/NS/DW), Er:YAG + 0.9% normal saline + distilled water (Er:YAG/NS/DW), Er,Cr:YSGG, and aPDT) and two control groups (5.25% NaClO as positive control and 0.9% normal saline (NS) as negative control). The numbers of bacteria on the surface of root canal walls and at different depths inside dentinal tubules before and after treatment were analysed. The morphology of bacterial cells before and after treatment was examined by scanning electron microscopy. After treatment, the bacterial reductions in the experimental groups and the positive control

group were significantly greater than that of the negative control group ($P < 0.001$). However, only Er:YAG/NaClO/NS/DW group showed no bacterial growth (the bacterial reduction reached up to 100%) on the surface of root canal walls or at 100/200 μm inside the dentinal tubules. All the laser radiation protocols tested, especially Er:YAG/NaClO/NS/DW, had effective bactericidal effect in experimentally infected root canals. Regarding all only Er:YAG/NaClO/NS/DW seemed to be an ideal protocol for root canal disinfection during endodontic therapy[92].

After many studies both Nd:YAG and Er:YAG lasers have been suggested as root canal disinfection aids. Nd:YAG of more as disinfecting agent and Er:YAG more of a smear layer removal. In recent years, various laser systems have gained importance in the field of laser-assisted endodontics, namely the Nd:YAG, the diode, the Er:YAG, and the Er,Cr:YSGG laser. Individual studies have been carried out so far, focusing on the respective wavelength, its specific bactericidal capabilities, and potential usefulness is root canal disinfection. The *in vitro* investigation by Schoop U et al. 2004, however, was performed to compare the microbicidal effect of these laser systems under standardized conditions and to draw a conclusion upon their relative effectiveness in the deep layers of dentin. In total, 360 slices of root dentin with a thickness of 1 mm were obtained by longitudinal cuts of freshly extracted human premolars. The samples were inoculated with a suspension of either *Escherichia coli* or *Enterococcus faecalis*. After the incubation, the samples were randomly assigned to the four different laser systems tested. Each laser group consisted of two different operational settings and a control. The dentinal samples underwent "indirect" laser irradiation through the dentin from the bacteria free side and were then subjected to a classical quantitative microbiologic evaluation. To assess the temperature increase during the irradiation procedure, additional measurements were carried out using a thermocouple. Microbiology indicated that all laser systems were capable of significant reductions in both test strains. At an effective output power of 1W, *E. coli* was reduced with the best results for the Er:YAG laser showing complete eradication of *E. coli* in 75% of the samples. *E. faecalis*, a stubborn invader of the root canal, showed minor changes in bacterial count at 1 W. Using the higher setting of 1.5 W,

significant reductions of *E. coli* were again observed with all laser systems, where only the diode and the Er:YAG laser were capable of complete eradication of *E. faecalis* to a significant extent. There was no significant relation between the temperature increase and the bactericidal effect[93].

There are lot of studies which deal with the performance and the disinfecting effects of Er:YAG laser: Results of Dostalova T et al 2002, study showed that Er:YAG laser (100 mJ energy, 30 pulses and 4 Hz) is effective in disinfecting canals[94] . Perin FM et al 2004, assessed the antimicrobial effect of Er:YAG laser and 1% NaOCl in eliminating 4 types of bacteria and one type of fungus. Results demonstrated that Er:YAG laser (7 Hz, 100 mJ, 80 pulses/canal, 11 seconds) and 1% NaOCl if used throughout the canal length are effective against 5 types of microorganisms, and if laser and irrigating solutions are used 3mm shorter than the canal apex, 70% of the samples would remain contaminated[95].

In a study, disinfecting effect of Er:YAG laser with different frequencies in roots of extracted teeth, which were voluntarily contaminated, was evaluated. Frequencies used were 7, 10 and 16 Hz with 1%, 2.5% NaOCl irrigating substances. Results showed that all frequencies were effective in disinfecting canals but none completely eliminated microorganisms. 2.5% NaOCl was a little more effective but this difference wasn't statistically significant[96]. Gordon W et al 2007, studied antimicrobial effect of Er,Cr:YSGG laser on dentinal walls infected by *E. faecalis* and reached the conclusion that Er,Cr:YSGG laser under the study conditions led to a 99.7% reduction in microbial count[97]. A comparative study on the effectiveness of Er,Cr:YSGG laser with 3% NaOCl indicated that Er,Cr:YSGG laser (0.5 W power, 20% water and air) resulted in reduction of bacterial count, but bacteria were not completely eliminated. Hypochlorite solution was able to prevent *E. faecalis* growth and efficiently sterilize canals[98] . In a study Schoop et al 2007, assessed the effects of Er,Cr:YSGG on two types of microorganism cultures in root canals. They stated that this laser can eliminate intra canal bacteria. Also SEM evaluation showed that this laser can remove intra canal debris and open dentinal tubules entries[99].

Bacteria and their products are a major cause of the development of periapical lesion[5]. The main objective of microbiological chemo-mechanical preparation of infected root canal is to completely eliminate intra-canal bacterial population or at least that they are reduced to the amount that is compatible to treat[100]. The goal of endodontic treatment is prevention and sometimes treatment of endodontic diseases of apical periodontitis. Despite the fact that most infectious microorganism is removed in endodontic instrumentation from infected channel, the rest of the bacteria can be identified in more than half cases of teeth before putting the seal[2] . The bacteria that have persistence on chemo-mechanical procedures have a negative impact on recurring treatment[100].

Successful endodontic therapy has increased dramatically with the development and adaptation of new technologies. Despite that most defects are associated with inadequate cleaning and disinfection channel system[101]. The rest of the bacteria can proliferate in the root canal, the dentin or periapical region. Chemo-mechanical irrigation or only instrumentation techniques for cleaning the infected root canals does not ensure complete removal of microorganisms due to anatomy of root dentin, where microorganisms can form complex biofilm or can penetrate into dentin tubules. Repeated infections are possible[101,102]. It is assumed that currently used hand instrumentation methods and substances in endodontics could not be able to destroy the remaining bacteria after primary therapy or could only damage cells on the surface of formed biofilm[100,103].

3 SUBJECT OF THE RESEARCH

This study was established to check the efficiency and effectiveness of antibacterial method of treatment of infected root canal. Chemo-mechanical irrigation or the instrumentation techniques for cleaning infected root canal does not foresee a complete removal of microorganisms due to the anatomy of the tooth dentin, because for him microorganisms form a complex biofilm or penetrate into the dentin tubules. Therefore, recurrent infections are possible. It is assumed that now used methods and substances in endodontics do not have possibility of eliminating residual bacteria after primary therapy or can destroy only the cells on the surface of the formed biofilm.

The subject of our research is to:

- Check disinfecting efficiency of 5.20% NaOCl *ex vivo* on *Enterococcus faecalis* viability.
- Check disinfecting efficiency of 5.20% NaOCl *ex vivo* on *Candida albicans* viability.
- Check disinfecting efficiency of 5.20% NaOCl *ex vivo* on *Streptococcus sanguinis* viability.
- Check disinfecting efficiency of 5.20% NaOCl *ex vivo* on *Fusobacterium nucleatum* viability.
- Check disinfecting efficiency of Erbium laser (Er:YAG) *ex vivo* on *Enterococcus faecalis* viability.
- Check disinfecting efficiency of Erbium laser (Er:YAG) *ex vivo* on *Candida albicans* viability.
- Check disinfecting efficiency of Erbium laser (Er:YAG) *ex vivo* on *Streptococcus sanguinis* viability.
- Check disinfecting efficiency of Erbium laser (Er:YAG) *ex vivo* on *Fusobacterium nucleatum* viability.
- Check disinfecting efficiency of Qmix *ex vivo* on *Enterococcus faecalis* viability.

- Check disinfecting efficiency of Qmix *ex vivo* on *Candida albicans* viability.
- Check disinfecting efficiency of Qmix *ex vivo* on *Streptococcus sanguinis* viability.
- Check disinfecting efficiency of Qmix *ex vivo* on *Fusobacterium nucleatum* viability.
- Check the effectiveness of combination of Er:YAG and 5.20% NaOCl *ex vivo* on *Enterococcus faecalis* viability.
- Check the effectiveness of combination of Er:YAG and 5.20% NaOCl *ex vivo* on *Candida albicans* viability.
- Check the effectiveness of combination of Er:YAG and 5.20% NaOCl *ex vivo* on *Streptococcus sanguinis* viability.
- Check the effectiveness of combination of Er:YAG and 5.20% NaOCl *ex vivo* on *Fusobacterium nucleatum* viability.
- Check the effectiveness of combination of Er:YAG and Qmix *ex vivo* on *Enterococcus faecalis* viability.
- Check the effectiveness of combination of Er:YAG and Qmix *ex vivo* on *Candida albicans* viability.
- Check the effectiveness of combination of Er:YAG and Qmix *ex vivo* on *Streptococcus sanguinis* viability.
- Check the effectiveness of combination of Er:YAG and Qmix *ex vivo* on *Fusobacterium nucleatum* viability.
- To compare the efficiency of all methods.

3.1 HYPOTHESIS OF THE RESEARCH

Working Hypothesis 1: The first application of laser Er:YAG disinfects infected root canals and destroys the majority of bacterial biofilm.

Working Hypothesis 2: For effective disinfection with Er:YAG system is not necessary to use supplement material such as reactive dyes for disinfection, or irrigation agents, because the lasers irradiation destroys microorganisms.

Working Hypothesis 3: Laser disinfecting system will overcome other methods of disinfection.

Zero Hypothesis: According to our research laser will have no influence on the disinfection of root canals.

4 MATERIALS AND METHODS

This research was realized in:

- Faculty of Medicine, Departament of Stomatology, University of Prishtina, Prishtina, Kosovo
- Medical faculty of Ljubljana, Institute of Microbiology and Immunology, Ljubljana, Slovenia

4.1 SAMPLES

In the study we included 200 freshly extracted premolar single-rooted teeth (Figure 1). The crown of each tooth was cut off using a water cooled diamond blade in a low speed saw Isomet 1000 (Buehler GmbH, Germany) obtaining 15 mm long root specimens. Only teeth with round canals were included in the study (Figure 2). For determination of the working length we used #10 Kerr file (Maillefer Instruments SA, Switzerland). The canals were then enlarged to an apical size of #35 (F3) using Protaper files (Maillefer Instruments, Switzerland). Between each file copious, irrigation with 2.5% NaOCl was performed. After root canal instrumentation, teeth were rinsed with 17% EDTA and then sterilized with absolute alcohol. The canals were dried with paper points (Dentsply Maillefer) and finally root apex was closed with composite material. Confirmation of sterilization was microbiologically tested on blood agar for 24 h at 37 ºC. When the growth of any microorganism was observed, the sample followed above mentioned procedure of sterilization once again, until no bacterial of fungal contamination was observed on blood agar plates.

Figure 1: Preparation of freshly extracted premolar single-rooted teeth with drilled root canals for further procedures.

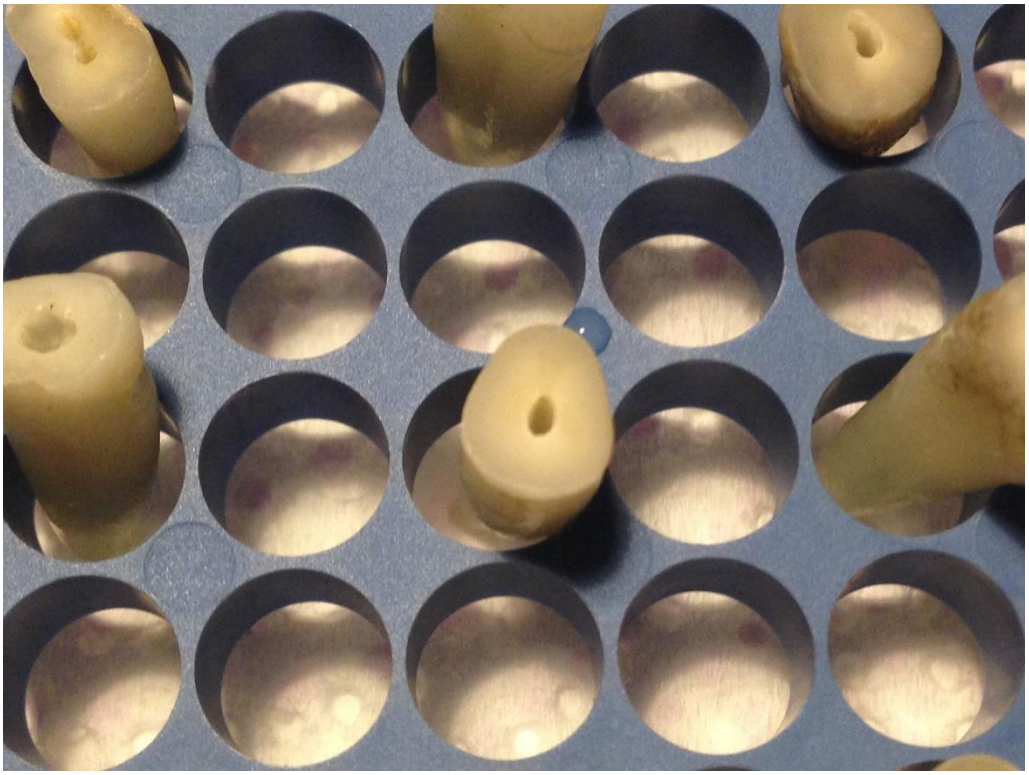

Figure 2: For good standardization of furtherly tested instrumentations only teeth with round canal were appropriate for inoculation of bacterial culture.

4.2 PREPARATION OF BACTERIAL SUSPENSION AND BIOFILM GROWTH

200 teeth were equally divided into four groups depending on the strain which were they inoculated with (*Enterococcus faecalis, Candida albicans, Streptococcus sanguinis, Fusobacterium nucleatum*). These bactraial and fungal strains are one of the most probable cause for oral infections and reinfections after failure of endodontic therapies. They cause periodontal diseases, and are commonly found in the dental plaque of humans and are frequently associated with gum disease. Due to their ability to adhere to different surfaces and develop microbial biofilms are very difficult to eliminate.

The four groups were further divided into 7 groups, depending on the used method of disinfection (Er:YAG laser, NaOCl, Qmix, combination of laser and NaOCl, combination of laser and Qmix, positive, negative control) (Table 2).

Table 2: Distribution of samples into testing groups.

	Enterococcus faecalis	***Candida albicans***	***Streptococcus sanguinis***	***Fusobacterium nucleatum***
Er:YAG				
30 sec	5	5	5	5
90 sec	5	5	5	5
5,20% NaOCl	5	5	5	5
Qmix	5	5	5	5
Er:YAG+NaOCl				
30 sec	5	5	5	5
90 sec	5	5	5	5
Er:YAG+Qmix				
30 sec	5	5	5	5
90 sec	5	5	5	5
Positive control	5	5	5	5
Negative control	5	5	5	5
Total	50	50	50	50

4.2.1 Microbiological cultures

E. faecalis is a Gram-positive bacterium, which under the microscope is seen as diplococci. As facultative anaerobic bacteria it can persist and lice without nutrients. *E. faecalis* forms bacterial biofilms on almost all kinds of surfaces, so we can often find it in anaerobic conditions in mouth cavity.

S. sanguinis is similar to *Enterococcus*, which are commensal organisms in gastrointestinal tract, specifically in the mouth. Sometimes they cause opportunistic infections in the mouth or in other systems of the body. In worst case scenario they may cause sepsis and endocarditis.

Mitis salviarius agar is frequently used to distinguish between types of *Streptococcus*, and very close to the genus *Enterococcus* (formerly the *Streptococcus* genus) that represent ordinary flora in the mouth. These organisms are often in the mouth, and are associated with caries, and caused endocarditis. The sugars in this medium are sucrose and glucose, as well as color trypan blue and crystal violet. Trypan blue is absorbed by the bacterial colonies, causing them to turn blue. Crystal violet in combination with 1% added telluride added to this medium, can inhibit gram negative bacteria, and many other Gram-positive bacteria. To prepare the culture we used plates with blood agar and *mitis salivarius* agar as medium, microbiological chamber for aseptic operation and incubator.

The method is based on the cultivation of bacteria and fungi on the blood agar plates and then their identification with the microscope. We used frozen strains ATCC, blood agar, reagents for preparing microscopic Gram dying.

Frozen ATCC strains of microorganisms were taken and then inoculated on the blood agar plates. Medium was incubated for 24 hours in an anaerobic atmosphere at 37 °C. After successful microbial growth on blood agar plates, microorganisms were identified using phenotypical features of each microorganism and microscopic examination and dying cells according to Gram.

4.2.1.1 *Ingredients for preparation of blood-agar plates*

Blood agar was prepared according to ingredients mentioned below.

Components	Amount g/L
Blood Agar Base:	
Heart extract 20g, NaCl 5g, Agar-agar 15 g	40 g
Destilated water	1000 mL
Citrated bovine blood	50 mL

4.2.1.2. Identification of microbes

Typical colonies of *Enterococcus faecalis* and *Streptococcus sanguinus* were small and white. For the identification of isolated colonies, we made microscopic examination of Gram-staining.

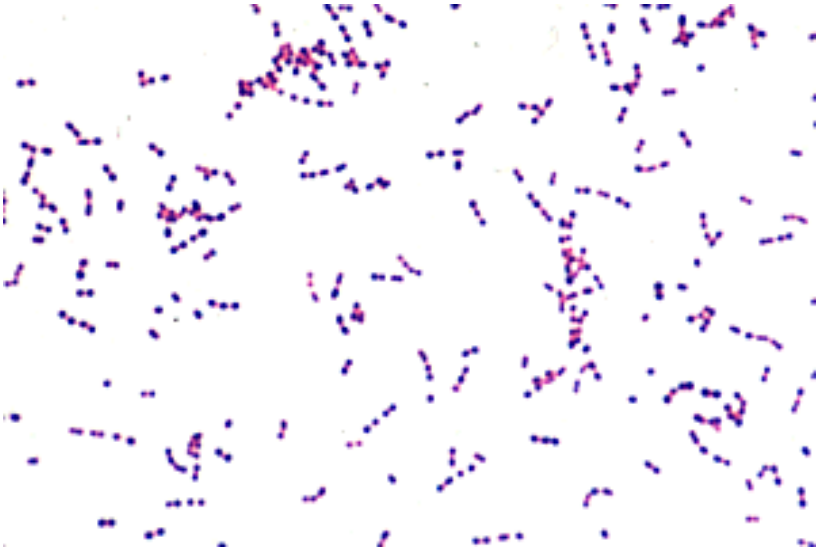

Figure 3: *Streptococcus sanguinis* stained by Gram (Gram positive cocci in chains).

4.2.1.2 *The growth of bacteria and bacterial biofilms in the root canal*

The method is based on the incubation of a very high concentration of bacteria in suspension in sterilized root canals. For inoculation of root canals, we used the bacterial strain *Enterococcus faecalis, Streptococcus sanguinis, Candida albicans and Fusebacterium nucleatum,* that successfully grown on blood agar plates and were identified. 200 samples of teeth were divided into groups, depending on the microorganism which were inoculated into the root canal. For bacterial inoculation, we have prepared a suspension culture of 5 McFarlands ($1.5x10^9$ cells/mL) in the TIO (thioglycolate) broth. The bacterial concentrations were measured by spectrophotometer. Teeth were inoculated with the suspension and incubated for seven days at 37 °C in an anaerobic atmosphere.

4.2.1.3 *Inoculation protocol*

Day 1: 30 mL of suspension 5 McFarlands into the root canal, incubating for 24 h at 37 °C.
Day 2: 30 ml of suspension 5 McFarlands into the root canal, 24 h incubation at 37 °C.
Day 3: 30 ml of suspension 5 McFarlands into the root canal, 24 h incubation at 37 °C.
Day 4: 30 uL thioglycolate broth into the root canal, 24 h incubation at 37 °C.
Day 5: 30 uL thioglycolate broth into the root canal, 24 h incubation at 37 °C.
Day 6: 30 uL thioglycolate broth into the root canal, 24 h incubation at 37 °C.
Day 7: 30 uL thioglycolate broth into the root canal, 24 h incubation at 37 °C.

The growth of biofilm was examined with microbiological methods of cultivation of bacteria on agar plates. Root canals were washed with 1X phosphate buffer (PBS) with a pH of 8.3. The washed cell suspension was inoculated and smeared on blood agar plates, which were incubated 24 hours at 37 °C. The following day we checked growth of bacteria on the plates and assessed or confirmed growth of biofilm, if more than 300 white bacterial colonies were grown on a single plate.
Preparation of 1X PBS diluted 10X PBS 1:10.

Ingredients for 10X PBS buffer

Components	**Amount**
Sodium chloride – NaCl	788.5 g
Dipotassium hydrogen phosphate- K_2HPO_4	261.04 g
Potassium dihydrogen phosphate- KH_2PO_4	39 g
Distilated water	10 L

Figure 4: The appearance of colonies of *E. faecalis* on blood agar plate.

4.3 IRRIGATION WITH 5.2% NaOCl

The procedure for application of 5.2% NaOCl was the same for all microorganisms.

Inoculated teeth were rinsed with 3 mL of 5.2% NaOCl. After flushing of the root canal with NaOCl, we washed the dental root canal with 2 ml of 10X PBS with fetal bovine serum (FBS) for the neutralization of the toxic effect on the persistent live bacterial cells. FBS includes a protein albumin that neutralizes hypochlorite by binding to the toxin radicals of hypochlorite and denaturing it. At the end we had 5 ml of suspension, of which 500 μL was pipetted to prepare a sample for measuring cell viability on the flow cytometer.

Components of 10X PBS

Components	**Amount**
Sodium chloride – NaCl	788.5 g
Dipotassium hydrogen phospate - K_2HPO_4	261.04 g
Potassiom dihydrogen phospate - KH_2PO_4	39 g
Destilled water	10 L

For prepartaion of 1X PBS we diluted 10X PBS 1:10.

4.4 IRRIGATION WITH QMIX

The procedure for application of Qmix was the same for all microorganisms in all root canals.

Inoculated teeth were rinsed with 3 mL of Qmix (Dentsply Tulsa, Tulsa, OK). After flushing of the root canal with Qmix, we collected 3 mL of suspension of which 500 µl was pipetted to prepare a sample for measuring cell viability on the flow cytometer.

4.5 Er:YAG LASER APPLICATION

Laser application was performed according to publication by De Meyer et al. (87) using a Er:YAG laser as disinfecting agent and in combination with NaOCl as irrigant. We used Fotona's Er:YAG laser TwinLight® Endodontic Treatment (TET) (Fotona, Ljubljana, Slovenia) which successfully addresses two major disadvantages of classical chemo-mechanical treatment procedures: the inability to clean and debride anatomically complex root-canal systems and to deeply disinfect dentinal walls and tubules, but the sample were only irradiated with Er:YAG laser.

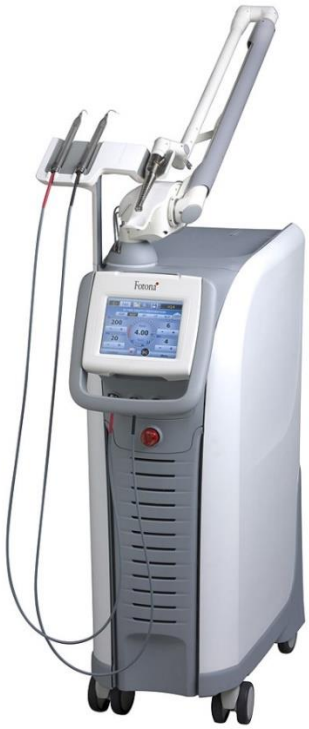

Figure 5: Er:YAG laser TwinLight® Endodontic Treatment (TET) (Fotona, Ljubljana, Slovenia).

The Fotona TwinLight® Endodontic Treatment successfully addresses two major disadvantages of classical chemo-mechanical procedures: the inability to clean and debride anatomically complex root canal systems and to deeply disinfect dentinal walls.

Figure 6: Dental, contact 200 µm fiber-optic handpiece.

Fotona's TwinLight® Endodontic Treatment (TET) successfully addresses the two major disadvantages of classical chemo-mechanical treatment procedures: the inability to clean and debride anatomically complex root-canal systems and to deeply disinfect dentinal walls and tubules.

- In the first step of the TwinLight® treatment process, a revolutionary photon-induced photoacoustic streaming method is employed, using the power of the Er:YAG laser to create non-thermal photoacoustic shock waves within the cleaning and debriding solutions present in the canal. Following this photoacoustic treatment, the canals and subcanals are left clean and the dentinal tubules are free of a smear layer. The process is also highly effective for final water rinsing prior to obturation.

- In the second step of the TwinLight® endodontic treatment, a deeply penetrating Nd:YAG laser wavelength is utilized to thoroughly decontaminate the dentinal walls up to 1000 μm deep. In this step, the high peak-pulse power of the Nd:YAG laser plays an important role as it induces maximum temperature pulsing for elimination of bacteria.

Optimal Wavelength Combination

Utilizing a combination of Er:YAG and Nd:YAG laser wavelengths in endodontic treatments makes optimum use of the unique laser-tissue interaction characteristics of each wavelength. The combined power of the Er:YAG-induced shock waves in the cleaning solutions and the Nd:YAG laser's superior bactericidal effect can dramatically improve the outcome of laser-assisted endodontic treatments, guaranteeing maximum efficacy and long-term success. However, we only used the application of Er:YAG laser to estimated it's disinfecting ability.

Method procedure

The inoculated teeth were subjected to the following treatments, all executed for 20 seconds: irrigation with a needle for 5.2% NaOCl and Qmix irrigation, and laser-activated irrigation with 2940 nm Er:YAG laser with pulse activated irradiation (power output 15 W, 20 Hz, pulsing rate of 50 μs, 1500 mJ, conical fibre tip). The laser beam was completely inserted through root canal with optical fibres of 200 μm and then laser irradiation for 20 or 90 seconds was performed.

When testing the effectiveness of laser irradiation with NaOCl or Qmix irrigation the procedure was as follows: in the first step of the treatment process, a revolutionary photon-induced photoacoustic streaming method was employed, using the power of the Er:YAG laser to create non-thermal photoacoustic shock waves in the canal for 10 seconds. Following this photoacoustic treatment the canals and subcanals were left for irrigation with NaOCl or Qmix for 10 seconds.

After laser irradiation, rinsing of damaged biofilm from the root canals with 2 mL of 1x PBS with 1mM EDTA, pH 8.3 was followed. Between the rinses we lightly drew the walls of root canal to wash put as many cells as possible. We collected the rinsed solution and at the end, we had 2 mL of washed liquid with the cells, which we used for further analysis on the flow cytometer.

4.6 MEASUREMENT OF BACTERIAL VIABILITY BY FLOW CYTOMETRY

Flow Cytometry has multiple uses in biological and medical sciences and is used in diagnostics and research. The method is increasingly being used for identification, characterization, monitoring and control of cell organisms in bioprocesses. With the help of flow cytometry, we can get more information on these organisms, which then makes it easier to optimize and run bioprocesses. Flow cytometry with its techniques among others enables measurement and determination of apoptotic cells, measurement and detection of dead cells, cell cycle analysis... The methods of flow cytometry utilize morphological and biochemical cell changes that are typical for apoptosis, necrosis or a particular cell cycle phase. With such techniques, we get more information about the condition of the cell in the exploratory sample.

Figure 7: BD FACSCanto II flow cytometer

Method principles

To determine the viability of the cell with flow cytometer, we use a simple method, which includes the use of a pair of fluorescent dyes TO and PI. Both dyes bind to nuclear DNA. The cell membrane is permeable for the dye TO, and therefore it enters the living as well as dead cells, but to varying degrees. The living cell membrane is impermeable to PI, which can enter only cells with damaged membrane (necrotic cells). The combination of these dyes allows the flow cytometer to differ between live and dead cells and thus establish their viability (BD Cell Viability Kit, Becton Dickinson Biosciences, USA).

Kit contains as metioned two dyes; thiazole orange (TO) for dyeing all cells, propidium iodide (PI) for dyeing dead cells, and liquid suspension of fluorescent beads for determination of absolute cell concentration. The living cells have untouched membranes so they are not permeable to dyes such as PI, which enter only in damaged membrane cells. This dye is permeable to all cells, alive and dead. Then the fluorescence signal is detected. The combination of these dyes represents a fast and definitive method for the discrimination of

living or dead cells. The advantage of flow cytometry by growing on agar plates is in the speed of results. Results are obtained less than a minute on 24 hours for agar plates cultivation.

Method procedure

After laser irradiation or irrigations damaged biofilm cells were rinsed out. After obtaining cell suspensions after different treatments in each root canal we have subtracted 500 µL of suspension for analyses on the flow cytometer.

To assure viability, we used "The Cell Viability Kit with Liquid Countailing Beads" (BD Biosciences). We proceeded according to the manufacturer's instructions. 500 µL of suspension was placed in a test tube for a cytometer. To the pipetted suspension 5 µL of TO dye and 5 µL of PI dye were added, and addtionly 50 µL of BD Liquid Counting Beads. The test tube was well stirred and followed incubation for 10 minutes at room temperature and in darkness. After incubation, the sample was analyzed on the flow cytometer BD FACSCanto II and we obtained results of viability (% of live organisms) of microorganisms after treatment.

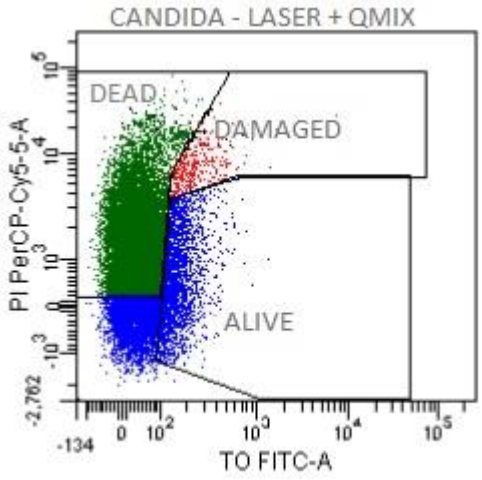

Figure 8: Example of flow cytometry analysis of dead *Candida albicans* cells after combined Er:YAG laser radiation and Qmix irrigation methodology.

Fluorescent dyes on flow cytometer enable to detect live, dead and damaged cells.

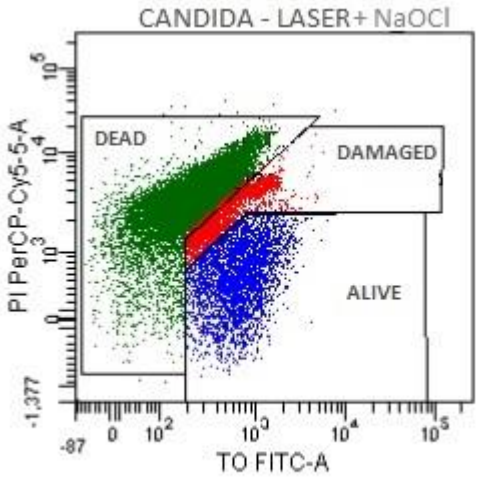

Figure 9: Example of flow cytometry analysis of dead *Candida albicans* cells after combined Er:YAG laser radiation and 5.20% NaOCl irrigation methodology

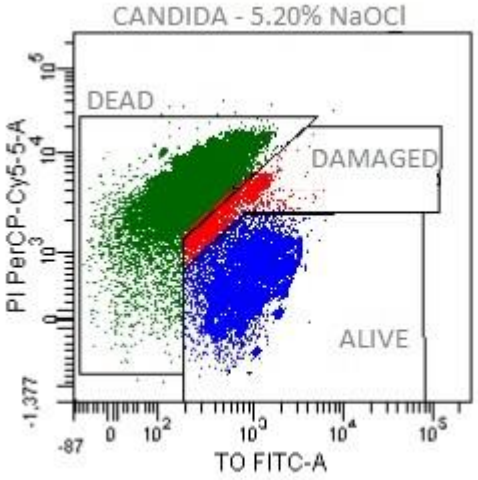

Figure 10: Another example of flow cytometry analysis of dead *Candida albicans* cells after irrigation with 5.20% NaOCl.

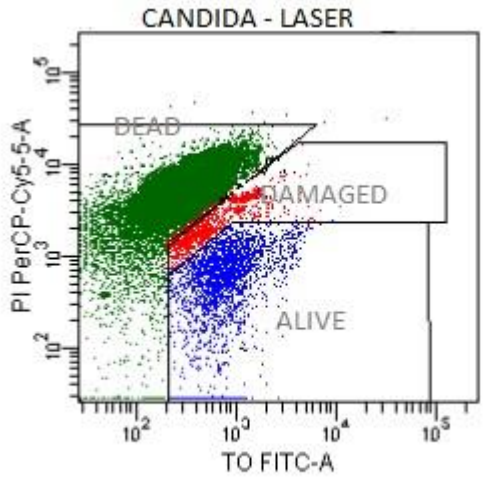

Figure 11: Example of flow cytometry analysis of dead *Candida albicans* cells after Er:YAG laser radiation.

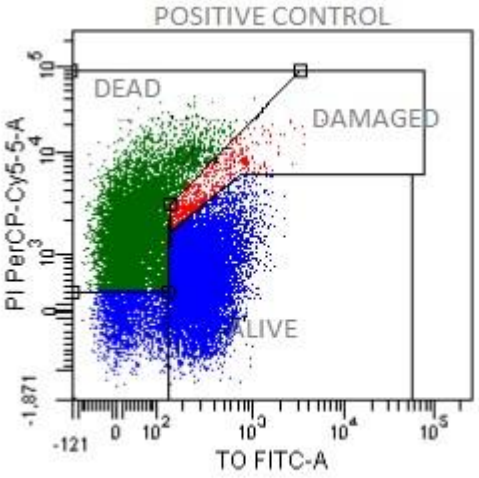

Figure 12: Example of *Candida albicans* positive control on flow cytometry analysis.

4.7 CONTROL PROCEDURES

Positive controls represented successful microbial biofim growth and were without the application of Er:YAG laser or irrgation. Inoculated teeth were washed with 5 mL of 1X PBS with 1 mM 17% EDTA and treated slightly on the walls of root canal. From the 5 mL suspension we took 500 μL for the cytometry viability test and 100 μL for inoculation on the blood agar.

For confirmation of positive growth of microorganisms on the agar plates we took washed suspension from the root canals and inoculated onto the blood agar. We pipetted 100 μL of suspensions of the washed cells and placed on the blood agar. The rinsed solution was mixed for a better cell extension in the liquid - resuspension. Confluent smear of suspension followed (across the board) the procedure. The plates were incubated for 24 hours at 37 ºC in an anaerobic atmoshere, and the next day the number of grown microbial colonies were counted (CFU – colony forming units). For a successful growth, we estimated the number of more than 300 of white colonies on one plate.

4.8 STATISTICAL ANALYSIS

Statistical analysis was performed using the statistical software SPSS 20 (IBM, New York, USA). Two-Way ANOVA statistical test with post-hoc Tukey test was used with the percentage of dead microbial cells as a dependent variable, and the type of microorganism, and disinfecting method as factor variables. Statistical significance was set at $p<0.05$.

5 RESULTS

The aim of our study was to check the efficiency and effectiveness of different antibacterial methods of treatment of infected root canal. It is assumed that now used methods and substances in endodontics do not have possibility of eliminating residual bacteria after primary therapy or can destroy only the cells on the surface of the formed biofilm. Therefore we checked the efficiency of standard irrigants 5.2% NaOCl and Qmix in comparison to laser radiation with Er:YAG laser system. Furthermore, we also combined the laser therapy with irrigation by irrigants and evaluated elimination of infectious microorganism with percent of dead cells in each sample by most accurate method of flow cytometry.

In the analysis we included 200 samples of root canals. In the Table 11 are presented results for Er:YAG laser application and irrigation of infected root canals with 5.20% NaOCl and Qmix. Results are presented as average percentage of dead cells, that were detected by flow cytometer in each sample. We can see that in total combination of two treatments (irrigation with NaOCl or Qmix and laser application) showed the highest percent of dead microbial cells from infected root canals and consequently showed the best disinfection efficacy.

Positive controls were infected root canals that were not treated with any method. However, we still analysed the percent of dead microbial cells in these samples. We have observed that despite no treatment in all samples more than 30% of all microbial cells were dead. Meanwhile, negative controls presented root canals that had no inoculation of microorganisms. When these samples were rinsed no cells were detected.

Table 3 represents the percent of dead microbial cells for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after treatment with Er:YAG radiation for 30 seconds. Each microorganism was inoculated into 5 samples of root canals, and totally radiation was performed in 20 root canals. On average, the highest rate of dead cells was observed for *S. sanguinis* and the lowest for *F. nucleatum*.

Table 3: Percent of dead microbial cells from infected root canals after radiation with Er:YAG laser for 30 seconds.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 30 sec				
Sample 1	71,7	74,2	85,5	68,0
Sample 2	74,5	81,2	84,6	69,8
Sample 3	76,7	81,0	83,8	74,5
Sample 4	76,2	80,5	77,9	73,1
Sample 5	73,5	78,2	79,1	76,9
Mean %	74,5	79,0	82,2	72,5
±SD	2,0	2,9	3,4	3,6

*SD-standard deviation

Table 4 represents the percent of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after treatment with Er:YAG radiation for 90 seconds. Each microorganism was once again inoculated into 5 different samples of root canals, and totally radiation was performed in 20 root canals. On average, the highest rate of dead cells was observed for *C. albicans* and the lowest for *F. nucleatum*.

Table 4: Percent of dead microbial cells from infected root canals after radiation with Er:YAG laser for 90 seconds.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 90 sec				
Sample 1	80,6	89,1	84,8	67,9
Sample 2	84,2	84,6	90,5	80,5
Sample 3	80,2	93,7	89,0	82,6
Sample 4	78,2	89,3	89,2	83,1
Sample 5	79,0	88,4	88,4	76,8
Mean %	80,4	89,0	88,4	78,2
±SD	2,3	3,2	2,1	6,3

*SD-standard deviation

Moreover, table 5 represents the percent of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis*, *C. albicans*, *S. sanguinis*, *F. nucleatum*) after treatment with irrigation with 5.20% NaOCl. Each microorganism was inoculated into 5 different samples of root canals, and totally in 20 root canals. On average, the highest rate of dead cells was observed for *F. nucleatum* and *S. sanguinis*, and the lowest for *E. faecalis*.

Table 5: Percent of dead microbial cells from infected root canals after irrigation with 5.20% NaOCl.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
5,20% NaOCl				
Sample 1	59,2	68,6	70,7	59,0
Sample 2	60,7	61,5	74,3	69,6
Sample 3	55,7	74,6	77,2	78,9
Sample 4	58,6	62,8	69,0	75,1
Sample 5	61,8	67,3	69,9	80,4
Mean %	59,2	67,0	72,2	72,6
±SD	2,3	5,2	3,4	8,7

*SD-standard deviation

Table 6 represents the percentage of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after treatment with Qmix irrigation. Each microorganism was inoculated into 5 different samples of root canals, and totally irrigation was performed in 20 root canals. The highest mean rate of dead cells was observed for *S. sanguinis* and the lowest rate for *F. nucleatum*.

Table 6: Percent of dead microbial cells from infected root canals after irrigation with Qmix.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Qmix				
Sample 1	70,5	76,5	79,8	55,1
Sample 2	69,3	78,5	82,2	79,4
Sample 3	76,1	79,4	84,9	75,9
Sample 4	78,7	82,6	88,0	70,6
Sample 5	82,7	80,0	85,7	69,7
Mean %	75,5	79,4	84,1	70,1
±SD	5,6	2,2	3,2	9,3

*SD-standard deviation

Table 7 represents the percent of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after combined treatment with Er:YAG radiation for 30 seconds and in addition of irrigation with 5.20% NaOCl. Each microorganism was inoculated into 5 separate samples of root canals, and totally in 20 root canals. On average, the highest rate of dead cells was observed for *S. sanguinis* and the lowest for *F. nucleatum*.

Table 7: Percent of dead microbial cells from infected root canals after application of combined treatment methods Er:YAG radiation for 30 seconds and irrigation with 5.20% NaOCl.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 30 sec + 5.20% NaOCl				
Sample 1	87,7	90,3	93,8	80,4
Sample 2	88,1	87,4	92,8	83,2
Sample 3	71,9	75,8	94,8	80,2
Sample 4	88,3	85,5	94,8	77,2
Sample 5	69,8	84,2	97,0	79,2
Mean %	81,2	84,6	94,6	80,0
±SD	9,4	5,4	1,6	2,2

*SD-standard deviation

Table 8 represents the percent of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after combined treatment with Er:YAG radiation for 90 seconds and in addition of irrigation with 5.20% NaOCl. Each microorganism was inoculated into 5 separate samples of root canals, and totally in 20 root canals. On average, the highest rate of dead cells was observed for *S. sanguinis* and *C. albicans* and the lowest for *F. nucleatum* and *E. faecalis.*

Table 8: Percent of dead microbial cells from infected root canals after application of combined treatment methods Er:YAG radiation for 90 seconds and irrigation with 5.20% NaOCl.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 90 sec + 5.20% NaOCl				
Sample 1	89,7	93,9	92,5	88,1
Sample 2	90,3	93,5	92,8	85,7
Sample 3	89,0	90,8	92,3	94,7
Sample 4	89,7	90,3	93,0	90,0
Sample 5	93,1	93,3	93,5	88,2
Mean %	90,4	92,4	92,8	89,3
±SD	1,6	1,7	0,5	3,4

*SD-standard deviation

Table 9 represents the percent of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after combined treatment with Er:YAG radiation for 30 seconds and in addition of irrigation with Qmix. Each microorganism was inoculated into 5 separate samples of root canals, and totally in 20 root canals. On average, the highest rate of dead cells was observed for *S. sanguinis* and the lowest for *F. nucleatum*.

Table 9: Percent of dead microbial cells from infected root canals after application of combined treatment methods Er:YAG radiation for 30 seconds and irrigation with Qmix.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 30 sec + Qmix				
Sample 1	90,7	91,6	91,9	86,2
Sample 2	94,9	91,9	94,5	91,5
Sample 3	85,9	90,4	91,0	90,4
Sample 4	86,1	90,0	89,9	92,6
Sample 5	94,9	90,7	93,4	83,6
Mean %	90,5	90,9	92,1	88,9
±SD	4,5	0,8	1,8	3,8

*SD-standard deviation

In table 10 are presented the percents of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) after combined treatment with Er:YAG radiation for 90 seconds and in addition of irrigation with 5.20% Qmix. Each microorganism was inoculated into 5 separate samples of root canals, and totally in 20 root canals. On average, the highest rate of dead cells was observed for *C. albicans* and the lowest for *F. nucleatum*.

Table 10: Percent of dead microbial cells from infected root canals after application of combined treatment methods Er:YAG radiation for 90 seconds and irrigation with Qmix.

	% of dead microbial cells detected by flow cytometry			
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*
Er:YAG 90 sec + Qmix				
Sample 1	94,8	94,8	92,4	92,1
Sample 2	90,5	96,6	95,5	93,5
Sample 3	98,7	96,2	97,0	89,1
Sample 4	96,8	97,8	96,3	90,0
Sample 5	95,3	97,1	97,7	92,7
Mean %	95,2	96,5	95,8	91,5
±SD	3,0	1,1	2,1	1,9

*SD-standard deviation

Table 11 represents the average percents of dead microbial cells by flow cytometry for each tested microorganism (*E. faecalis, C. albicans, S. sanguinis, F. nucleatum*) and after treatment methods (Er:YAG radiation for 30 and 90 seconds, irrigation with 5.20% NaOCl or Qmix). The mean rates of dead microbial cells are also presented in the following figures, just for better overview of dead cells according to the type of microorganism and type of treatment method (Figure 13-17).

Table 11: Average percent of dead microbial cells from 200 treated root canals with Er:YAG laser application and irrigation with 5.20% NaOCl or Qmix measured by flow cytometry.

	% of dead microbial cells detected by flow cytometry				
	Enterococcus faecalis	*Candida albicans*	*Streptococcus sanguinis*	*Fusebacterium nucleatum*	***p-value***
Er:YAG					
30 sec	74,48	79,02	82,19	72,46	0,049
90 sec	80,44	89,04	88,38	78,18	0,034
5,20% NaOCl	59,24	66,97	72,22	72,61	0,022
Qmix	75,51	79,45	84,13	70,15	0,052
Er:YAG+NaOCl					
30 sec	81,16	84,63	94,64	80,04	0,041
90 sec	90,36	92,42	92,81	89,35	0,152
Er:YAG+Qmix					
30 sec	90,50	90,88	92,14	88,86	0,112
90 sec	95,21	96,49	96,79	91,48	0,687
p-value	<0,001	<0,001	<0,001	<0,001	
CFU/mL					
Positive control	>300	>300	>300	>300	/
Negative control	0	0	0	0	/

*CFU-colony forming units

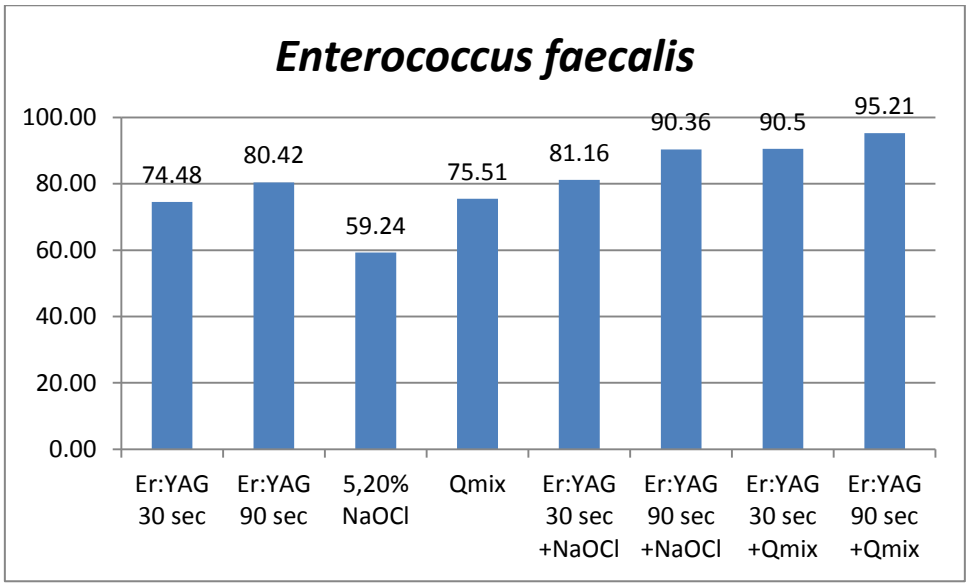

Figure 13: Distribution of dead *E. faecalis* cells according to treatment mode.

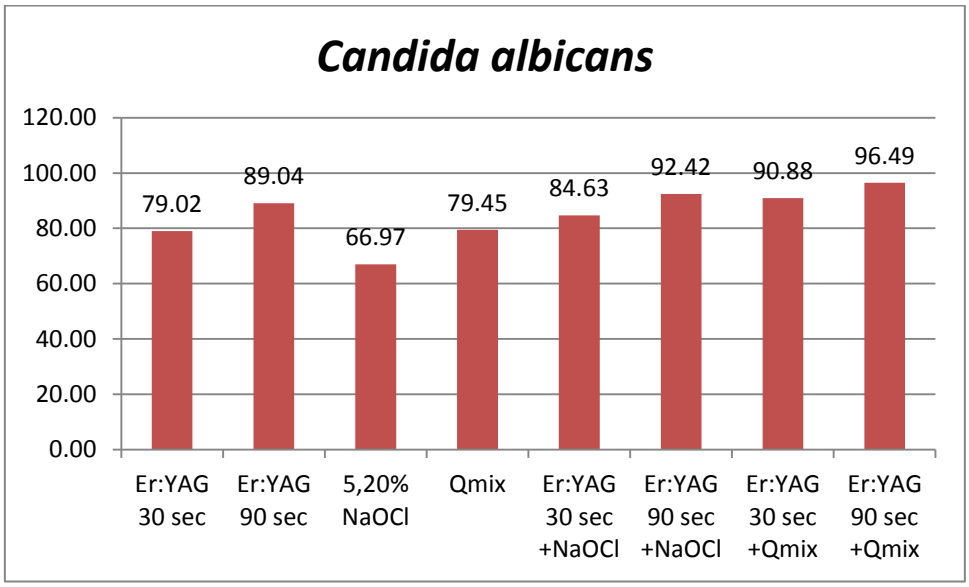

Figure 14: Distribution of dead *C. albicans* cells according to treatment mode.

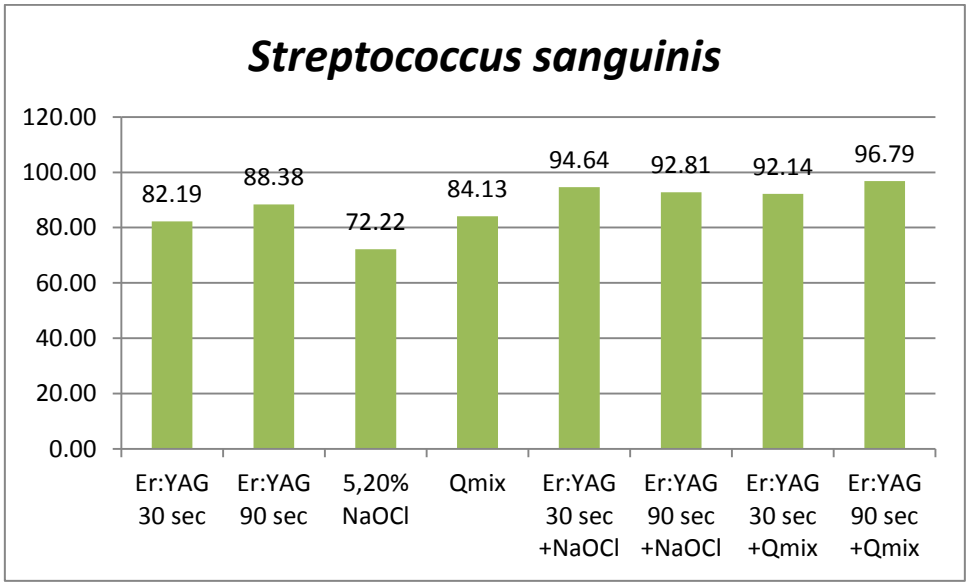

Figure 15: Distribution of dead *S.sanguinis* cells according to treatment mode.

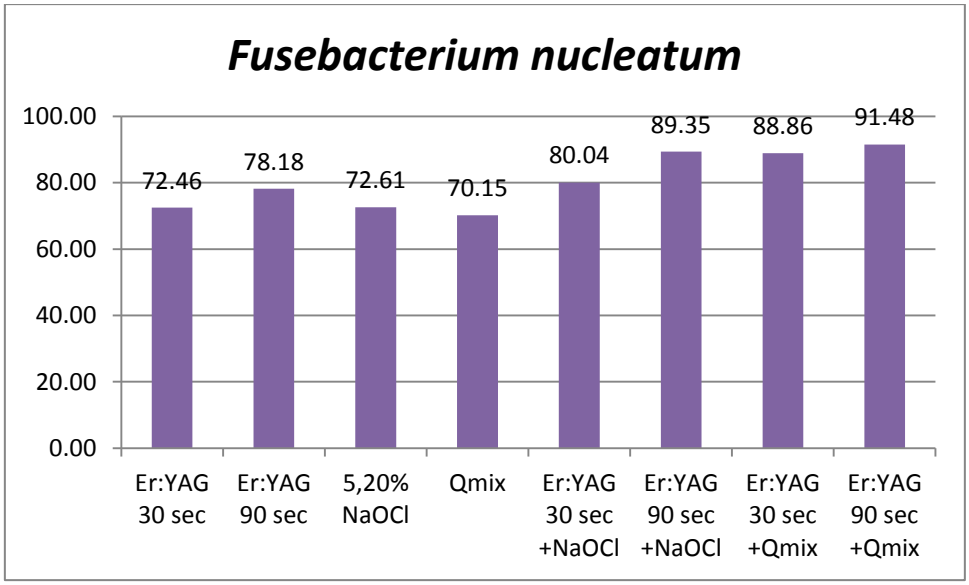

Figure 16: Distribution of dead *F. nucleatum* cells according to treatment mode.

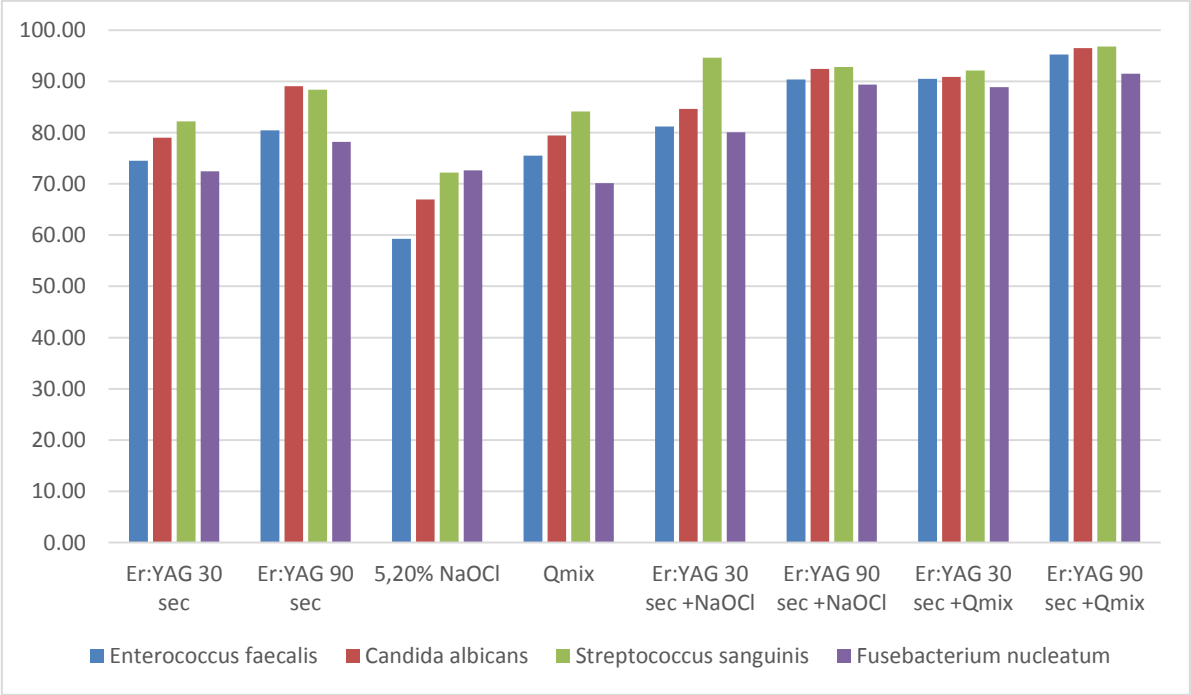

Figure 17: Comparison of treatment methods antibacterial efficacy among microorganisms, that were inoculated into root canal samples.

Two factor ANOVA showed statistically significant differences in mean percentage of dead cells between tested organisms ($p<0.05$) for radiation with Er:YAG laser 30 and 90 seconds, irrigation with 5.20% NaOCl and almost ($p=0.052$) for irrigation with Qmix. There were also statistically significant differences between treatment methods ($p<0.001$) for all microorganisms, as well as in interaction between tested organisms and treatment methods ($p=0.040$).

The percent of dead cells was significantly higher for *C. albicans* and *S. sanguinis* compared to *E. faecalis* or *F. nucleatum* in all treatment groups. However, when comparing disinfecting methods, combination of laser radiation and irrigation was significantly more effective ($p<0.001$) compared to other groups. While the lowest percent of dead cells was detected in the group with 5.20% NaOCl irrigation, more accurate analysis showed statistically significant higher percent of dead microbial cells in group Er:YAG for 90 seconds

+ Qmix irrigation. There were also no statistical differences between microorganism, what indicates on good disinfecting activity that can hande most infections.

Longer duration of irradiation showed better disinfecting action. 90-seconds irradiation was statistically significant compared to 30-seconds irradiation ($p<0.001$) and achieved over 80% of dead cells. Disinfecting activity in combination with irrigant was over 90% for addition of NaOCl and over 95% for addition of Qmix.

Post-hoc analysis showed significant differences in the percentages of dead cells between different treatments. Values were comparable for *E. faecalis* and *F. nucleatum* and for *C. albicans* with *S. sangunis,* and further proved that laser irradiation had greater disinfecting effect ($p<0.001$, respectively). Efficiency of disinfection also showed differences between time duration of irradiation with laser ($p<0.001$) for all microorganisms.

The amount of dead cells in dentin increased with increased NaOCl concentration and time of exposure to laser radiation ($p<0.05$). Qmix was equally effective in killing bacteria in root canals as laser and even better when compared to 5.20% NaOCl. In the control group, which was treated with PBS buffer, no bacteria was observed and consequently no dead cells.

Despite good study design all treatments and several chelating agents containing antimicrobials could not remove biofilms nor kill all microbial cells significantly. Combination of laser irradiation and irrigation with Qmix came the closest to total elimination of cells. Dissolution ability is mandatory for an appropriate eradication of biofilms attached to dentin. Significantly fewer bacteria were killed in the standard method of irrigation with NaOCl. We believe that longer time of exposure could result to more dead bacteria than only 1 minute of exposure. However, we confirmed that all the disinfecting agents killed significantly more bacteria than the PBS alone used as a negative control ($P<0.001$).

In addition to identification with flow cytometry, positive culturing of bacteria on blood agar plates indicated that bacterial biofilm was present in all of the positive control specimens (Table 11). Culturing of bacteria on agar plates was also used as suggestive of a qualitative reduction of bacteria in tested groups compared with the positive control specimens. To provide a quantitative assessment, we enumerated CFUs of bacterial suspensions. As presented irrigation regimens (NaOCl, Qmix and NaOCl or Qmix in combination with laser) significantly reduced bacterial growth ($p<0.001$). Immediately after the treatment, all groups resulted into elevated percent of dead bacterial cells, whereas there was no detectable growth in negative control group. When we compared bacterial counts on agar plates (results below), this difference was statistically significant ($p<0.001$). After 24 hours, we detected no bacterial growth in any of the incubated samples in negative control group compared to other groups which had bacterial growth.

- *Enterococcus faecalis*:

  Positive control 1: > 300 CFU

  Positive control 2: > 300 CFU

  Positive control 3: > 300 CFU

  Positive control 4: > 300 CFU

  Positive control 5: > 300 CFU

- *Candida albicans:*

  Positive control 1: > 300 CFU

  Positive control 2: > 300 CFU

  Positive control 3: > 300 CFU

  Positive control 4: > 300 CFU

  Positive control 5: > 300 CFU

- *Streptococcus sangunis*:

  Positive control 1: > 300 CFU

  Positive control 2: > 300 CFU

  Positive control 3: > 300 CFU

  Positive control 4: > 300 CFU

  Positive control 5: > 300 CFU

- *Fusebacterium nucleatum:*

  Positive control 1: > 300 CFU

  Positive control 2: > 300 CFU

  Positive control 3: > 300 CFU

  Positive control 4: > 300 CFU

  Positive control 5: > 300 CFU

6 DISCUSSION

The aim of this study was to established the efficiency of antibacterial methods for treatment of infected root canals. According to the knowledge published in the literature chemo-mechanical irrigation and instrumentation techniques for cleaning infected root canal do not completely remove causative microorganisms due to various reasons; the anatomy of the tooth dentin, microorganisms can form complex biofilms or penetrate into the dentin tubules and hide from the influence of antimicrobial agents, or specific method of disinfection does not harm microorganisms enough. Therefore, recurrent infections are possible and very common. It is assumed that commonly used methods and substances in endodontics do not have possibility of eliminating residual bacteria after primary therapy or can destroy only the cells on the surface of the formed biofilm.

In this study, we compared the effectiveness of laser-activated root canal disinfection by using Er;YAG laser compared to the conventional hand irrigation methods using irrigants as 5.20% NaOCl or Qmix. Furthermore, we also combined the laser therapy with irrigation by irrigants and evaluated elimination of infectious microorganism with percent of dead cells in each sample by most accurate method of flow cytometry. In the analysis we included 200 extracted single rooted premolar teeth with removed crown. The root canals were standardized to 15 mm of length for better evaluation and representation of results. The apex of the root canals was drilled through for better rinsing of root canal and to allow for the laser light or irrigant to reach the apical part of the tooth. Before microbial inoculation the teeth were sterilized and then infected *in vitro* with four different microorganisms, which are most commonly found in oral cavity (*E. faecalis, S. sanguinis, F. nucleatum* and *C. albicans*). These strains also form microbial biofilm, which is hard to eliminate. The growth of bacterial biofilm was microbiologically confirmed with the growth of rinsed microorganism from positive control samples. After incubation we counted colony forming units (CFU/mL) and evaluated biofilm as positive with number colonies more than 300 CFU/mL.

As mentioned, we used strains of bacteria and fungus that most frequently cause complicated infections; *E. faecalis, S. sanguiis, F. nucleatum,* and *C. albicans.* These microorganisms are commonly isolated and found in teeth in which treatments of root canals have failed, and moreover they are difficult to eradicate from the canals. We chose *E. faecalis* as the reference microorganism due its high frequency of isolation from cases of failed endodontic treatment, its resistance to calcium hydroxide treatment and its relative insensitivity to laser irradiation[93,104,105]. We believed that if laser treatment was effective in eliminating this organism from infected dentinal tubules, we could then conclude that it would be effective against other organism found in endodontic infections, and that this technology might have clinical application in disinfecting of root canals during endodontic therapy.

C. albicans was chosen on its various pathogenic characteristics. Not only *C. albicans* has the ability to bind to dentin collagen, invade deep dentin tubules and form biofilms, it is also known to activate host defences and to show resistance to different antimicrobial agents used in endodontics. *C. albicans* cells have also been found in the resorption of periapical root surfaces and in periapical granuloma. Moreover, oral candidiasis – a common infection of the oral mucous membranes in which *C. albicans* is frequently implicated – is highly prevalent in immunocompromised patients, whose compromised immune systems might increase the risk of fungi colonization of the root canal system[106]. For these reasons, an optimal solution for irrigation during cleaning and shaping of root canals should possess antifungal properties.

In the experimental part we used an incubation period of one week to enhance bacterial penetration into dentin tubules and promote bacterial biofilm formation. After irradiation and irrigation or combination of both, we sampled the specimens of any remaining bacteria from the main root canal, and counted them using flow cytometry. The viability of the microorganisms in the samples after laser treatment or irrigations was analyzed on the flow cytometer compared to non-treated samples and then we made statistical analysis with ANOVA test. Flow cytometry analyses according to detected dyes gave us results as percentage of dead cells, so viability was easily evaluated.

Here presented results are analyses from 200 of teeth samples that underwent specific procedure of disinfection. Qmix has been employed after the root canal preparation as a rinse to improve root canal cleaning and disinfection already in the past[107]. It comprises an aqueous solution of EDTA, chlorhexidine and N-acetyl-N,N,Ntrimethylammonium bromide[108]. A previous study[107] employed a 3-minute period to evaluate the antimicrobial effect and it was demonstrated that Qmix promoted additional antimicrobial action, especially in longer periods (>1 minute)[109]. According to these results we also expected and showed that Qmix performed as a good disinfecting irrigant, and obtained even better results compared to irrigation with 5.20% NaOCl, as was also observed by Gründling GL et al[110]. On the other hand, in total, combination of two treatments (irrigation with NaOCl or Qmix and laser application) showed the highest percent of dead microbial cells from infected root canals and consequently showed the best disinfection efficacy. Positive controls were infected root canals that were not treated with any method and were used as confirmation of biofilm. We have observed that despite no treatment in all samples more than 30% of all microbial cells were dead. All treatment methods successfully eliminated majority of microbial cells when compared to control samples. Meanwhile, therapy with Er:YAG laser statistically proved higher percent of dead microbial cells compared to other disinfecting methods ($p<0.001$). Furthermore, with prolonged irradiation (=90 seconds) we have observed even better disinfection rate for laser radiation (Table 11). With irradiation of 90 seconds in average more than additional 5% of microbial cells were killed. Moreover, we have also observed that lowest percentage of dead cells was recorded for *F. nucelatum* and than *E. faecalis*. *C. albicans* and *S. sanguinis* showed higher percent of dead cells. *E. faecalis* showed 80,44% of dead cells, *C. albicans* 89,02%, *S. sanguinis* 88,38%, and *F. nucleatum* 78,18% of dead microbial cells. Very similar results were also obtained for irrigation with 5.20% NaOCl or Qmix. It seems that *F. nucleatum* and *E. faecalis* are very persistant bacteria in root canal infections, but *C. albicans* and *S. sanguinis* not so.

According to the results in our study, laser irradiation showed better disinfection compared to irrigation with 5.20% NaOCl for all tested microorganisms. When we activated 5.20% NaOCl via the Er:YAG laser, we have found in every testing microbial group even greater reduction in bacterial infection compared to using conventional NaOCl irrigation or irradiation with laser alone. When the irrigation with 5.20% NaOCl was accompanied with Er:YAG radiation the percent of dead microbial cells elevated over 80% for all microorganism. When Qmix was used these percents were even higher than 88%. Moreover, there were also no statistical differences between microorganisms ($p=0.687$), what accordingly means that the number of dead microorganisms were so high, that this type of method could represent satisfactory procedure that eliminates polymicrobial infections and is not bacteria-dependent. Combination protocol introduced several modifications to currently used laser techniques and protocols that involve higher laser energy and longer laser pulse duration. The 2940 nm wavelength of the Er:YAG laser was chosen for its high absorption in water. We used conditions and specifications of laser irradiation as were described in the study by Olivi et al[111],a pulsed energy (20 mJ at 15 Hz, average power 0.3 W) to produce an effective activation and streaming of fluids within the canal while reducing the thermal side effects of laser irradiation on the dentin walls. The use of a short pulse duration of 50 μs produced a high peak power of 400 W at only 20 mJ, generating shock wave phenomenon (photoacoustic/mechanical effect) and secondary and tertiary cavitation in the fluids[112,113] . Longer duration of irradiation showed better disinfecting action. 90-seconds irradiation was statistically significant compared to 30-seconds irradiation ($p<0.001$) and achieved over 80% of dead cells. Disinfecting activity in combination with irrigant was over 90% for addition of NaOCl and over 95% for addition of Qmix. We believe since radiation of Er:YAG laser was already established as good smear layer remover, that laser effectively removes microbial biofilms and causes death of microbial cells. After that application or irrigant kills the remaning microbial cells. That is why the combination of two treatment methods acieved more than 90-95% reduction of viable bacterial cells.

Post-hoc analysis showed significant differences in the percentages of dead cells between different treatments. Values were comparable for *E. faecalis* and *F. nucleatum* and for *C. albicans* with *S. sangunis,* and further proved that laser irradiation had greater disinfecting effect ($p<0.001$, respectively). Efficiency of disinfection also showed differences between time duration of irradiation with diode laser ($p<0.001$) for all microorganisms. The amount of dead cells in dentin increased with increased NaOCl concentration and time of exposure to laser radiation ($p<0.05$). Qmix was equally effective in killing bacteria in root canals as laser and even better than 5.2% NaOCl. In the control group, which was treated with PBS buffer, no bacteria was observed and consequently no dead cells.

Despite good study design all treatments and several chelating agents containing antimicrobials could not remove biofilms nor kill all microbial cells significantly. Combination of laser irradiation and irrigation with Qmix came the closest to total elimination of cells. Dissolution ability is mandatory for an appropriate eradication of biofilms attached to dentin. Significantly fewer bacteria were killed in the standard method of irrigation with NaOCl. We believe that longer time of exposure could result to more dead bacteria than only 30-60 seconds of exposure. However, we confirmed that all the disinfecting agents killed significantly more bacteria than the PBS alone used as a negative control ($P<0.001$).

In the presence of a smear layer, combination of exposure to QMiX or 5.20% NaOCl + laser radiation resulted in significantly more dead bacteria than of exposure to these same disinfecting solutions alone ($p<0.001$). Er:YAG effectively eluted smear layer and then killed bacteria and destroyed attached biofilm, moreover irrigation with NaOCl or Qmix eliminated the rest of viable cells, that were not harmed by laser irradiation. The smear layer reduces the effectiveness of disinfecting agents against *E. faecalis* in infected dentin, as this bacteria form very tough biofilms. Solutions containing NaOCl and/or Qmix could not alone show high antibacterial activity.

In addition to identification with flow cytometry, positive culturing of bacteria on blood agar plates indicated that bacterial biofilm was present in all of the positive control specimens (Table 11). Culturing of bacteria on agar plates was also used as suggestive of a qualitative reduction of bacteria in tested groups compared with the positive control specimens. To provide a quantitative assessment, we enumerated CFUs of bacterial suspensions. As presented irrigation regimens (NaOCl, Qmix and NaOCl or Qmix in combination with laser) significantly reduced bacterial growth ($p<0.001$). Immediately after the treatment, all groups resulted into elevated percent of dead bacterial cells, whereas there was no detectable growth in negative control group. When we compared bacterial counts on agar plates (results below), this difference was statistically significant ($p<0.001$). After 24 hours, we detected no bacterial growth in any of the incubated samples in negative control group compared to other groups which had bacterial growth.

The results of our study were similar as results in other *ex vivo* studies[114-122]. The use of 5.20% NaOCl alone could not eliminate most of bacteria and therefore prevent bacterial growth completely. However, after we used combination of laser–activated NaOCl for a total of 90 seconds, we observed almost complete eradication of bacteria and biofilm. The results of subsequent testing showed that there were still present life bacteria from the rinsed root canal so growth on incubated agar plates and reinfection in the oral cavity could be expected. However, mainly the rate of dead microorganisms was above 90%. The effectiveness of this laser technique could be explained by the increased consumption of available chlorine ions that occurred after the activation of the irrigant by an Er:YAG laser[117] . Another explanation might be related to the lysing and mechanical breaking up of the bacterial biofilm due to the laser application, after which irrigation with NaOCl killed most of free bacteria. Because the volume of the liquid in the root canal is small, this effect amplifies and improves the removal of bacteria, which has also been confirmed before[112,113] .

Results of Dostalova T et al. using Er:YAG laser (100mJ energy, 30 pulses and 4 Hz) showed that irradiation was effective in disinfecting canals[123] . Perin FM et al. assessed the

antimicrobial effect of Er:YAG laser and 1% NaOCl in eliminating 4 types of bacteria and one type of fungus. Results demonstrated that Er:YAG laser (7 Hz, 100mJ, 80 pulses/canal, 11sec) and 1% NaOCl if used throughout the canal length are effective against 5 types of microorganisms, and if laser and irrigating solutions are used 3 mm shorter than the canal apex, 70% of the samples would remain contaminated[124]. In another study, disinfecting effect of Er:YAG laser with different frequencies in roots of extracted teeth, which were voluntarily contaminated, was evaluated. Frequencies that were used were 7, 10 and 16 Hz with 1%, 2.5% NaOCl irrigating substances. Results showed that all frequencies were effective in disinfecting canals but none completely eliminated microorganisms. These results are concordant with our findings here. 2.5% NaOCl was a little more effective but this difference was not significant[125]. Gordon W et al. studied antimicrobial effect of Er:YAG laser on dentinal walls infected by *E. faecalis* and reached the conclusion that laser under the study conditions led to a 99.7% reduction in microbial count[126] . A comparative study on the effectiveness of laser with 3% NaOCl indicated that laser (0.5W power, 20% water and air) resulted in reduction of bacterial count, but bacteria were not completely eliminated. NaOCl solution was able to prevent *E. faecalis* growth and efficiently sterilize canals[127]. In a study by Schoop U et al., they assessed the effects of Er:YAG on two types of microorganism cultures in root canals. They stated that this laser can eliminate intra-canal bacteria. SEM evaluation showed that this laser can remove intra-canal debris and open dentinal tubules entries[99].

Untill now many lasers such as CO2, Nd:YAG, Er:YAG and Er,Cr:YSGG have been used to remove debris and smear layer from infected canals. Several studies showed that Er:YAG is the most appropriate laser for intra-canal removal of debris and smear layers (127). It appears that Erbium lasers, because of their effect on minerals existing in debris and smear layer, can be more effective in removing these two components from the canals. In this respect Erbium laser competes with canal irrigating solutions in debris and smear layer removal. Laser can, directly or as an adjunctive device, be effective in disinfecting canals. Laser treatment permits the delivery of a non-contact, homogenous, heating effect; independent of the distance of the target tissue from the heat source. This is a major advantage when you consider the irregular

surface of the target tissues. Laser light can penetrate area of canals where rinsing solutions have no access like secondary canals and depth of dentin tubules, and eliminates microorganisms. Studies to disinfection by Erbium laser family show that use of this laser alone can be effective in canal disinfection[99,123,124,126.]. However, not always have been obtained the same results. Studies which evaluated the disinfecting effect of this laser with NaOCl in different concentrations demonstrated that NaOCl is more effective than Erbium laser alone in disinfecting canals[125]. But when Erbium laser is used in combination with NaOCl in canals, better results are obtained[124]. The results of the aforementioned studies clear that this laser in combination with a standard root treatment and an appropriate rinsing solution is effective. Results of studies in which many types of lasers were used in disinfection of canals showed that all wavelengths used for disinfection in different thicknesses of dentin were effective without inappropriate heat effect[93,128,129.].

NaOCl is the most widely used root canal irrigant, yet there is no consensus about its optimal concentration[129]. A past study has indicated that exposure to high concentrations of NaOCl is the most predictable method for eliminating intra-canal bacteria and removing intra-canal biofilm[130]. According to the literature and to our knowledge, in the present study, 5.20% NaOCl was one of the most effective irrigants, with lowest amount of bacteria counted in the NaOCl group. Besides, its unpleasant taste, NaOCl is also highly toxic, may cause severe irritation if inadvertently extruded into the periapical area, and unable to completely remove the smear layer. For these reasons, the use of Qmix as an irrigant may be a good alternative to NaOCl. Considering that endodontic infections are polymicrobial biofilm‐based diseases, evaluating against only one organism represents a limitation to the present study, since the presence of multiple microorganisms might have altered the dynamics demonstrated by the present study.

The difference in the results may be attributed to differences in the methodology used in different studies. In the study by Jha D and colleagues[131] , the researchers recovered residual viable bacteria after laser treatment of infected root dentin by collecting dentin shavings from

the root canal wall. In our model, the surviving bacteria were quantified by immediately rinsing the material recovered from the lased infected dentin and counting by flow cytometer. This allowed us to detect and measure the degree of disinfection achieved by the laser treatment. Consequently, although we did not find total elimination of viable organisms (that is, sterilization), we did achieve a significant reduction in the viable bacterial load, approaching sterility. Our results suggest that the Er:YAG laser may be a valuable tool for root canal disinfection when one uses radially emitting laser tips. One benefit of the laser over conventional treatment is that it has the ability to achieve significant disinfection of canals infected with bacteria for which there is evidence that conventional calcium hydroxide is not as effective, owing to the resistance of this type of bacterium. Should modification of the lasing procedure permit predictable, total elimination of viable bacteria in the dentin, this could justify a one-visit endodontic treatment for infected root canals. Other potential benefits of using the laser include conservation of root structure and less emphasis on mechanical instrumentation, especially in curved roots. This benefit is promising, as the laser tips are flexible and come in sizes as small as 200 μm in diameter (equal to the diameter of the tip of a no. 20 file), both of which would minimize length of procedure and dependence on mechanical instrumentation. These tips are capable of penetrating narrow, long and curved canals more efficiently, in areas that NaOCl irrigation may not be able to reach. One of our goals was to determine if either the irrigation disinfection or the laser treatments under specified conditions are capable of a 100 percent reduction in infection. None of the treatment conditions was able to demonstrate such effects. Further studies to evaluate new treatment protocols that could count for completed bacterial eradication need to be considered in the future. From all that we know of pulpal and periapical disease, the elimination of infection (that is, sterilization) and prevention of subsequent infection is at the heart of endodontic therapy. To date, no existing procedure allows the clinician to sterilize an infected root canal system quickly and easily and with absolute surety. Therefore, the goal of our work was to learn whether the use of this particular laser system could reliably accomplish this goal of root canal sterilization.

While our results did not demonstrate complete elimination of infection from our root canals, our test system did allow us to quantify the degree of change in bacterial load after laser treatment. We found that we could achieve over 90% reduction in viable bacteria using the combination of laser and NaOCl[132]. Due to the difficulty in removing microbial biofilm after common disinfecting procedures, the use of auxiliary techniques, such as the use of lasers, may be useful during endodontic treatment. Perhaps this is another economical tool that can be added to the dentist's armamentarium for future applications. However, future studies should be conducted to evaluate the effectiveness of this procedure in clinical trials.

7 CONCLUSIONS

In our study we wanted to investigate the antimicrobial activity of Er:YAG laser microorganism that commonly cause root canal infections and are hard to eliminate, as *E. faecalis, S. sanguinis, C. albicans, F. nucleatum*. According to the results we can concluded that:

- Under the *ex vivo* conditions in our study, Er:YAG laser irradiation appeared to be effective in enhancing the efficacy of irrigation solutions that are used commonly in endodontics.
- All methods (Er:YAG radiation, irrigation with 5.20% NaOCl or Qmix) successfully killed microbial cells in the infected root canals, but none treatment achieved 100% elimination.
- Longer duration of laser radiation permits better disinfection. The 90-second radiation showed an average above 80% removal of the microorganism, 30-second on average only above 70%.
- The use of a combination of two methods; irrigation with 5.20% NaOCl or conventional Qmix and radiation with Er:YAG for 90 seconds was effective in eradicating 90-95% of causative bacteria and in inhibiting new bacterial growth. The

combined use of different methods is therefore necessary to enhance antimicrobial effectiveness.

- Additional clinical studies are needed to clarify the effect on endodontic treatment outcomes *in vivo*.

8 REFERENCES

1. Saleh IM, Ruyter IE, Haapasalo M, Orstavik D. Survival of Enterococcus faecalis in infected dentinal tubules after root canal filling with different root canal sealers in vitro. Int Endod J 2004; 37(3): 193-8.
2. Soukos NS, Chen PSY, Morris JT, Ruggiero K, Abernethy AD, Som S, et al. Photodynamic therapy for endodontic disinfection. J Endod 2006; 32(10): 979-84.
3. Ng R, Singh F, Papamanou DA, Song X, Patel C, Holewa C, et al. Endodontic photodynamic therapy ex vivo. J Endod 2011; 37(2): 217-22.
4. Garcez AS, Ribeiro MS, Tegos GP, Nunez SC, Jorge AOC, Hamblin MR. Antimicrobial photodynamic endodontic treatment to eliminate root canal biofilm infection. Laser Sug Med 2007; 39(1): 59-66.
5. Gründling GL, Zechin JG, Jardim WM, Dias de Oliveira S, Poli de Figueiredo JA. Effect of ultrasonics on Enterococcus faecalis biofilm in a bovine tooth model. J Endod 2011; 37(8): 1128-33.
6. Schwarz F, Aoki A, Becker J, Sculean A. Laser application in non-surgical periodontal therapy: a systematic review. J Clin Periodontol 2008;35(8 Suppl):29-44.
7. Aoki A, Sasaki KM, Watanabe H, Ishikawa I. Lasers in nonsurgical periodontal therapy. Periodontol 2000;36(1):59-97.
8. Ishikawa I, Aoki A, Takasaki AA. Potential applications of Erbium:YAG laser in periodontics. J Periodontal Res 2004;39(4):275-85.
9. Juan Antonio García Núñez, Alicia Herrero Sánchez, Ana Isabel García Kass and Clara Gómez Hernández (2012). Laser Radiation as an Adjunct to Nonsurgical Treatment of Periodontal Disease, Periodontal Diseases - A Clinician's Guide, Dr. Jane Manakil (Ed.), ISBN: 978-953-307-818-2, InTech, Available from: http://www.intechopen.com/books/periodontal-diseases-a-clinician-s-guide/laser-radiation-as-an-adjunctivetool-to-non-surgical-treatment-of-periodontal-disease

10. Kakehashi S, Stanley HR, Fitzgerald RJ. The effect of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965;20:340–9.
11. Bergenholz G. Microorganisms from necrotic pulps of traumatized teeth. Odontol Revy 1974;25:347–58.
12. Moller AJ, Fabricius L, Dahlen G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. Scand J Dent Res 1981;89:1475–84.
13. Bystrom A, Sundquist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 1981;89:321–8.
14. Sjogren U, Hagglund B, Sundquist G, Wing K. Factors affecting the long-term results of endodontic treatment. J Endod 1990;16:498–504.
15. McComb D, Smith DC. A preliminary scanning electron microscope study of root canals after endodontic procedures. J Endod 1975;1:238–42.
16. Moodnik RM, Dorn SO, Feldman MJ, Levey M, Borden BG. Efficacy of biomechanical instrumentation: a scanning electron microscopy study. J Endod 1976;2:261–6.
17. Mader CL, Baumgartner JC, Peters DD. Scanning electron microscopic investigation of the smeared layer on root canal walls. J Endod 1984;10:477–83.
18. Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in endodontics: a review. Oral Surg Oral Med Oral Pathol 2002;94:658–66.
19. HaapasaloM, Orstavik D. In vitro infection and sisinfection of dentinal tubules. J Dent Res 1986;66:1375–9.
20. Pashley DH. Smear layer: physiological considerations. Oper Dent Suppl 1984;3:13–29.
21. Drake DR, Wiemann AH, Rivera EM, Walton RE. Bacterial retention in canal walls in vitro: effect of smear layer. J Endod 1994;20:78–82.
22. Oguntebi BR. Dentin tubule infection and endodontic therapy implications. Int Endod J 1994;27:218–22.
23. Peters OA, Schonenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001;34:221–30.

24. Zakariasen KL, Dederich DN, Tulip J, DeCoste S, Jensen SE, Pickard MA. Bactericidal action of carbon dioxide laser radiation in experimental root canals. Can J Microbiol 1986;32:942–6.
25. Le Goff A, Morazin-Dautel A, Guigand M, Vulcain JM, Bonnaure-Mallet M. An evaluation of the CO2 laser for endodontic disinfection. J Endod 1999;25:105–8.
26. Moshonov J, Orstavik D, Yamauchi S, Pattiette M, Trope M. Nd:YAG laser irradiation in root canal disinfection. Endod Dent Traumatol 1995;11:220–4.
27. Fegan SE, Steiman HR. Comparative evaluation of the antibacterial effects of intracanal Nd:YAG laser irradiation: An in vitro study. J Endod 1995;21:415–7.
28. Rooney J, Midda M, Leeming J. A laboratory investigation of the bactericidal effect of Nd:YAG laser. Br Dent J 1994;176:61–4.
29. Gutknecht N, Moritz A, Conrads G. Bactericidal effect of the Nd:YAG laser in in vitro root canals. J Clin Laser Med Surg 1996;14:77–80.
30. Stabholz A, Kettering J, Neev J, Torabinejad M. Effects of XeCl excimer laser on Streptococcus mutans. J Endod 1993;19:232–5.
31. Folwaczny M, Liesenhoff T, Lehn N, Horch HH. Bactericidal action of 308nm excimerlaser radiation: an in vitro investigation. J Endod 1998;24:781–5.
32. Moritz A, Gutknecht N, Goharkhay K, Schoop U, Wernisch J, Sperr W. In vitro irradiation of infected root canals with diode laser: results of microbiologic, infrared spectrometric and stain penetration examination. Quintessence Int 1997;28:205–9.
33. Mehl A, Folwaczny M, Haffner C, Hickel R. Bactericidal effects of 2.94 l Er:YAG laser irradiation in dental root canals. J Endod 1999;25:490–3.
34. Dostalova T, Jelinkova H, Housova D, Sulc J, Nemec M, Duskova J, et al. Endodontic treatment with application of Er:YAG laser waveguide radiation disinfection. J Clin Laser Med Surg 2002;20:135–9.
35. Schoop U, Moritz A, Kluger W, Patruta S, Goharkhay K, Sperr W, et al. The Er;YAG laser in endodontics: results of an in vitro study. Lasers Surg Med 2002;30:360–4.

36. Anic I, Tachibana H, Matsumoto K, Qi P. Permeability, morphologic and temperature changes of canal dentin walls induced by Nd:YAG, CO2 and argon lasers. Int Endod J 1996;29:13–22.
37. Harashima T, Takeda FH, Kimura Y, Matsumoto K. Effect of Nd:YAG laser irradiation for removal of intracanal debris and smear layer in extracted human teeth. J Clin Laser Med Surg 1997;15:131–5.
38. Saunders WP, Whitters CJ, Strang R, Moseley H, Payne AP, McGadey J. The effect of an Nd:YAG pulsed laser on the cleaning of the root canal and the formation of a fused apical plug. Int Endod J 1995;28:213–20.
39. Moshonov J, Sion A, Kasirer J, Rotstein I, Stabholz A. Efficacy of argon laser irradiation in removing intracanal debris. Oral Surg Oral Med Oral Pathol 1995;79:221–5.
40. Yamazaki R, Goya C, Yu DG, Kimura Y, Matsumoto K. Effect of Erbium, Chromium:YSGG laser irradiation on root canal walls: a scanning electron microscopic and thermographic study. J Endod 2001;27:9–12.
41. Takeda FH, Harashima T, Kimura Y, Matsumoto K. Efficacy of Er:YAG laser irradiation in removing debris and smear layer on root canal walls. J Endod 1998;24:548–51.
42. Kimura Y, Yonaga K, Yokoyama K, Kinoshita J, Ogata Y, Matsumoto K. Root surface temperature increase during Er:YAG laser irradiation of root canals. J Endod 2002;28: 76–8.
43. Goodis HE, Pashley D, Stabholz A. Pulpal effects of thermal and mechanical irritants. In: Hargreaves KM, Goodis HE, editors. Seltzer and Bender's dental pulp. Quintessence Publishing, Inc; 2002. p. 371–410.
44. Stabholz A, Zeltzser R, Sela M, Peretz B, Moshonov J, Ziskind D. The use of lasers in dentistry: principles of operation and clinical applications. Compendium 2003;24:811–24.
45. Kimura Y, Wilder-Smith P, Matsumoto K. Lasers in endodontics: a review. Int Endod J 2000;33:173–85
46. Stabholz A. The role of laser technology in modern endodontics. In: Ishikawa I, Frame JW, Aoki A, editors. Lasers in dentistry, revolution of dental treatment in the new millennium. Elsevier Science BV Int Congr Series 2003;1248:21–7.

47. Cozean C, Arcoria CJ, Pelagalli J, Powell GL. Dentistry for the 21st century? Erbium:YAG laser for teeth. J Am Dent Assoc 1997;128:1080–7.
48. Ando N, Hoshino E. Predominant obligate anaerobes invading the deep layers of root canal dentine. Int Endod J 1990;23:20–7.
49. Armitage GC, Ryder MI, Wilcox SE. Cemental changes in teeth with heavily infected root canals. J Endod 1983;9:127–30.
50. Stabholz A, Sahar-Helft S, Moshonov J. Lasers in endodontics. Dent Clin North Am 2004;48(4):809-32.
51. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to cell in the development of bacterial biofilm. Science 1998;280(5361):295-8.
52. Costerton JW; Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marcie TJ. Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987;41:435-64.
53. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinical relevant microorganisms. Clin Microbiol Rev 2002;15(2):167-93.
54. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 1999;397(6717):315-23.
55. Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Töz H, Atilla G, Hughes FJ, Belibasakis GN. Gingival crevicular levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. J Clin Periodontol 2007;34(5):370-6.
56. Adriaens PA, Adriaens LM. Effects of nonsurgical periodontal therapy on hard and soft tissues. Periodontol 2000;36(1):121–45.
57. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000;28(1):12–55.
58. Folwaczny M, Mehl A, Aggstaller H, Kickel R. Antimicrobial effects of 2.94 micron Er:YAG laser radiation on root surfaces: an in vitro study. J Clin Periodontol 2002;29(1):73-8.

59. Schwarz F, Aoki A, Becker J, Sculean A. Laser application in non-surgical periodontal therapy: a systematic review. J Clin Periodontol 2008;35(8 Suppl):29-44.
60. Aoki A, Sasaki KM, Watanabe H, Ishikawa I. Lasers in nonsurgical periodontal therapy. Periodontol 2000;36(1):59-97.
61. Ishikawa I, Aoki A, Takasaki AA. Potential applications of Erbium:YAG laser in periodontics. J Periodontal Res 2004;39(4):275-85.
62. Cohen RE, Ammons WF. Lasers in periodontics (Academy report). AAP (The American Academy of Periodontology). The Research, Science and Therapy Committee of the American Academy of Periodontology. J Periodontol 2002;73(10):1231-9.
63. Hibst R, Keller U. Experimental studies of the application of the Er:YAG laser on dental hard substances. I. Measurement of the ablation rate. Lasers Surg Med 1989;9(4):338-44.
64. Herrero A, García-Kass AI, Gómez C, Sanz M, García JA. Effects of two kinds of Er:YAG laser system on root surface in comparison to ultrasonic scaling: an in vitro study. Photomed Laser Surg 2010;28(4):497-504.
65. Folwaczny M, Mehl A, Haffner C, Benz C, Hickel R. Root substance removal with Er:YAG laser radiation at different parameters using a new delivery system. J Periodontol 2000;71(2):147-55.
66. Eberhard, J, Ehlers H, Falk W, Açil Y, Albers HK, Jepsen S. Efficacy of subgingival calculus removal with Er:YAG laser compared to mechanicaldebridement: an in situ study. J Clin Periodontol 2003;30(6):511-8.
67. Theodoro LH, Haypek P, Bachmann LM, Garcia VG, Sampaio JE, Zezell DM, Eduardo Cde P. Effect of Er:YAG and Diode laser irradiation on the root surface: morphological and thermal analysis. J Periodontol 2003;74(6):838-43.
68. Gómez C, Bisheimer M, Costela A, García-Moreno I, García A, García JA. Evaluation of the effects of Er:YAG and Nd:YAG lasers and ultrasonic instrumentation on root surfaces. Photomed Laser Surg 2009;27(1):43-8.
69. Schwarz F, Jepsen S, Herten M, Aoki A, Sculean A, Becker J. Immunohistochemical characterization of periodontal wound healing following nonsurgical treatment with

fluorescence controlled Er:YAG laser radiation in dogs. Lasers Surg Med 2007;39(5):428-40.

70. Byrnes KR, Wu X, Waynant RW, Ilev IK, Anders JJ. Low power laser irradiation alters gene expression of olfactory ensheathing cells in vitro. Lasers Surg Med 2005;37(2):161-71.
71. Crespi R, Cappare P, Toscanelli I, Gherlone E, Romanos GE. Effects of Er:YAG laser compared to ultrasonic scaler in periodontal treatment: a 2-year follow-up split-mouth clinical study. J Periodontol 2007;78(7):1195-1200.
72. Schwarz F, Sculean A, Rothamel D, Schwenzer K, Georg T, Becker J. Clinical evaluation of an Er:YAG laser for non-surgical treatment of peri-implantitis: a pilot study. Clin Oral Implants Res, 2005;16(1):44-52.
73. Kimura Y, Wilder-Smith P, Matsumoto K. Lasers in endodontics:a review. Int Endod J 2000;33:173-85.
74. Jayawardena JA, Kato J, Moriya K, Takagi Y. Pulpal response to exposure with Er:YAG laser. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91:222-9.
75. Chen WH. Laser root canal therapy. J Indiana Dent Assoc 2002-2003;81:20-23.
76. Ebihara A, Majaron B, Liaw L H, Krasieva T B, Wilder-Smith P. Er:YAG laser modifi cation of root canal dentine: infl uence of pulse duration, repetitive irradiation and water spray. Lasers Med Sci 2002;17:198-207.
77. Kimura Y, Yonaga K, Yokoyama K, Kinoshita J, Ogata Y, Matsumoto K. Root surface temperature increase during Er:YAG laser irradiation of root canals. J Endod 2002;28:76-8.
78. Shoji S, Hariu H, Horiuchi H. Canal enlargement by Er:YAG laser using a cone-shaped irradiation tip. J Endod 2000;26:454-8.
79. Kesler G, Gal R, Kesler A, Koren R. Histological and scanning electron microscope examination of root canal after preparation with Er:YAG laser microprobe: a preliminary in vitro study. J Clin Laser Med Surg 2002;20:269-77.

80. Kaitsas V, Signore A, Fonzi L, Benedicenti S, Barone M. Effects of Nd: YAG laser irradiation on the root canal wall dentin of human teeth: a SEM study. Bull Group Int Rech Sci Stomatol Odontol 2001;43:87-92.
81. Nandakumar K, Obika H, Shinozaki T, Ooie T, Utsumi A, Yano T. Inhibition of bacterial attachment by pulsed Nd:YAG laser irradiations: an in vitro study using marine biofilm-forming bacterium Pseudoalteromonas carrageenovora. Biotechnol Bioeng 2002;80(5):552-8.
82. Watanabe H, Ishikawa I, Suzuki M, Hasegawa K. Clinical assessments of the Erbium:YAG laser for soft tissue surgery and scaling. J Clin Laser Med Surg 1996;14(2)67-75.
83. Sculean A, Schwarz F, Berakdar M, Romanos GE, Arweiler NB, Becker J. Periodontal treatment with an Er:YAG laser compared to ultrasonic instrumentation: a pilot study. J Periodontol 2004;75(7):966-73.
84. Tomasi C, Schander K, Dahlén G, Wennström JL. Short-term clinical and microbiologic effects of pockets debridement with an Er:YAG laser during periodontal maintenance. J Periodontol 2006;77(1):111-8.
85. Derdilopoulou FV, Nonhoff J, Neumann K, Kielbassa AM. Microbiological findings after periodontal therapy using curetters, Er:YAG, laser, sonic, and ultrasonic scalers. J Clin Periodontol 2007;34(7):588-98.
86. Domínguez A, Gómez C, García-Kass AI, García-Núńez JA. IL-1beta, TNF-alpha, total antioxidative status and microbiological findings in chronic periodontitis treated with fluorescence-controlled Er:YAG laser radiation. Lasers Surg Med 2010;42(7):24-31.
87. De Meyer S, Meire MA, Coenye T, De Moor R. Effect of laser-activated irrigation on biofilms in artificial root canals. J Int Endod J 2016; doi: 10.1111/iej.12643.
88. Cheng X, Chen B, Qiu J, He W, Lv H, Qu T, Yu Q, Tian Y. Bactericidal effect of Er:YAG laser combined with sodium hypochlorite irrigation against Enterococcus faecalis deep inside dentinal tubules in experimentally infected root canals. J Med Microbiol 2016; 65(2):176-87.

89. Olivi G, DiVito E, Peters O, Kaitsas V, Angiero F, Signore A, Benedicenti S. Disinfection efficacy of photon-induced photoacoustic streaming on root canals infected with Enterococcus faecalis: an ex vivo study. J Am Dent Assoc 2014; 145(8):843-8.
90. Neelakantan P, Cheng CQ, Mohanraj R, Sriraman P, Subbarao C, Sharma S. Antibiofilm activity of three irrigation protocols activated by ultrasonic, diode laser or Er:YAG laser *in vitro*. Int Endod J 2015; 48(6):602-10.
91. Zan R, Hubbezoglu I, Sümer Z, Tunç T, Tanalp J. Antibacterial effects of two different types of laser and aqueous ozone against Enterococcus faecalis in root canals. Photomed Laser Surg 2013; 31(4):150-4.
92. Cheng X, Guan S, Lu H, Zhao C, Chen X, Li N, Bai Q, Tian Y, Yu Q. Evaluation of the bactericidal effect of Nd:YAG, Er:YAG, Er,Cr:YSGG laser radiation, and antimicrobial photodynamic therapy (aPDT) in experimentally infected root canals. Lasers Surg Med 2012; 44(10):824-31.
93. Schoop U, Kluger W, Moritz A, Nedjelik N, Georgopoulos A, Sperr W. Bactericidal effect of different laser systems in the deep layers of dentin. Lasers Surg Med 2004; 35(2):111-6.
94. Dostalova T, Jelinkova H, Housova D, Sulc J, Nemec M, Duskova J, et al. Endodontic treatment with application of Er: YAG laser waveguide radiation disinfection. J Clin Laser Med Surg 2002; 20(3):135-9.
95. Perin FM, França SC, Silva-Sousa YT, Alfredo E, Saquy PC, Estrela C, et al. Evaluation of the antimicrobial effect of Er:YAG laser irradiation versus 1% sodium hypochlorite irrigation for root canal disinfection. Aust Endod J 2004;30(1):20-2.
96. Vezzani MS, Pietro R, Silva-Sousa YT, Brugnera-Junior A, Sousa-Neto MD. Disinfection of root canals using Er: YAG laser at different frequencies. Photomed Laser Surg 2006; 24(4):499-502.
97. Godon W, Atabakhsh VA, Meza F, Doms A, Nissan R, Rizoiu I, et al. The antimicrobial efficacy of the erbium, chromium:yttrium-scandium-gallium-garnet laser with radial emitting tips on root canal dentin walls infected with Enterococcus faecalis. J Am Dent Assoc; 138(7):992-1002.

98. Eldeniz AU, Ozer F, Hadimli HH, Erganis O. Bactericidal efficacy of Er,Cr: YSGG laser irradiation against Enterococcus faecalis compared with NaOCI irrigation: an ex vivo pilot study. Int Endod J;40(2):112-9.
99. Schoop U, Goharkhay K, Klimscha J, Zagler M, Wernisch J, Georgopoulos A, et al. The use of the Erbium, Chromium:Yttrium-Scandium-Gallium-Garnet laser in endodontic treatment: the results of an in vitro study. J Am Dent Assoc 2007;138(7):949-55.
100. Siqueira JF Jr, Batista MM, Fraga RC, de Uzeda M. Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. J Endod 1998; 102: 414-6.
101. Fonseca MB, Junior POT, Pallota RC, Filho HF, Denardin OVP, Rapoport A, et al. Photodynamic therapy for root canals infected with Enterococcus faecalis. Photomed Laser Surg 2008; 26(3): 209-13.
102. Garcez AS, Nunez SC, Lage-Marques JL, Cardoso Jorge AO, Ribeiro MS. Efficiency of NaOCl and laser-assisted photosensitization on the reduction of Enterococcus faecalis in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006; 102: 93-8.
103. Brito PRR, Souza LC, Marchado de Oliveira JC, Alves FRF, De-Deus G, Lopes HP, et al. Comparison of the effectiveness of three irrigation techniques in reducing intracanal Enterococcus faecalis population: An in vitro study. J Endod 2009; 35(10): 1422-7.
104. Silbert T, Bird PS, Milburn GJ, Walsh LJ. Disinfection of root canals by laser dye photosensitization (abstract 3408). J Dent Res 2000;79(special issue):569.
105. Moritz A, Jakolitsch S, Goharkhay K, et al. Morphologic changes correlating to different sensitivities of Escherichia coli and Enterococcus faecalis to Nd:YAG laser irradiation through dentin. Lasers Surg Med 2000;26(3):250-61.
106. Ruff ML, McClanahan SB, Babel BS. In vitro antifungal efficacy of four irrigants as a final rinse. J Endod 2006;32:331‐3.
107. Stojicic S, Shen Y, Qian W, Johnson B, Haapasalo M. Antibacterial and smear layer removal ability of a novel irrigant, QMix. Int Endod J 2011;45:363-71.
108. Haapasalo M, inventor. The University of British Columbia. Composition and method for irrigation of a prepared dental root canal. US Patent US 2012/0101166 A1, Apr. 26 2012.

109. Morgental RD, Singh A, Sappal H, Kopper PMP, Vier-Pelisser FV, Peters OA. Dentin inhibits the antibacterial effect of new and conventional endodontic irrigants. J Endod 2013; 39:406-10.
110. Gründling GL, Melo TA, Montagner F, Scarparo RK, Vier-Pelisser FV. QMix® irrigant reduces lipopolysaccharide (LPS) levels in an in vitro model. J Appl Oral Sci 2015; 23(4):431-5.
111. Olivi G, DiVito E, Peters O, Kaitsas V, Angiero F, Signore A, Benedicenti S. Disinfection efficacy of photon-induced photoacoustic streaming on root canals infected with Enterococcus faecalis: an ex vivo study. J Am Dent Assoc 2014;145(8):843-8.
112. DiVito E, Peters OA, Olivi G. Effectiveness of the erbium:YAG laser and new design radial and stripped tips in removing the smear layer after root canal instrumentation. Lasers Med Sci 2012;27(2):273-280.
113. Peters OA, Bardsley S, Fong J, Pandher G, DiVito E. Disinfection of root canals with photon-initiated photoacoustic streaming. J Endod 2011;37(7):1008-1012.
114. Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J 1998;31(1):1-7.
115. Matsumoto H, Yoshimine Y, Akamine A. Visualization of irrigant flow and cavitation induced by Er:YAG laser within a root canal model. J Endod 2011;37(6):839-843.
116. Blanken J, De Moor RJ, Meire M, Verdaasdonk R. Laser induced explosive vapor and cavitation resulting in effective irrigation of the root canal, part 1: a visualization study. Lasers Surg Med 2009;41(7):514-519.
117. Macedo RG, Wesselink PR, Zaccheo F, Fanali D, Van Der Sluis LW. Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. Int Endod J 2010;43(12): 1108-1115.
118. De Moor RJ, Meire M, Goharkhay K, Moritz A, Vanobbergen J. Efficacy of ultrasonic versus laser-activated irrigation to remove artificially placed dentin debris plugs. J Endod 2010;36(9):1580-1583.
119. George R, Walsh LJ. Thermal effects from modified endodontic laser tips used in the apical third of root canals with erbium-doped yttrium aluminium garnet and erbium,

chromium-doped yttrium scandium gallium garnet lasers. Photomed Laser Surg 2010;28(2):161-165.

120. Benedicenti S, Cassanelli C, Signore A, Ravera G, Angiero F. Decontamination of root canals with the gallium-aluminum-arsenide laser: an in vitro study. Photomed Laser Surg 2008;26(4):367-370.
121. Jaramillo DE, Aprecio RM, Angelov N, DiVito E, McClammy TV. Efficacy of photon induced photoacoustic streaming (PIPS) on root canals infected with Enterococcus faecalis: a pilot study. Endod Pract 2012;5(3):28-32.
122. Ordinola-Zapata R, Bramante CM, Aprecio RM, Handysides R, Jaramillo DE. Biofilm removal by 6% sodium hypochlorite activated by different irrigation techniques (published online ahead of print Nov. 13, 2013). Int Endod J 2014;47(7):659-66.
123. Dostalova T, Jelinkova H, Housova D, Sulc J, Nemec M, Duskova J, et al. Endodontic treatment with application of Er: YAG laser waveguide radiation disinfection. J Clin Laser Med Surg 2002; 20(3):135-9.
124. Perin FM, França SC, Silva-Sousa YT, Alfredo E, Saquy PC, Estrela C, et al. Evaluation of the antimicrobial effect of Er:YAG laser irradiation versus 1% sodium hypochlorite irrigation for root canal disinfection. Aust Endod J 2004;30(1):20-2.
125. Vezzani MS, Pietro R, Silva-Sousa YT, Brugnera-Junior A, Sousa-Neto MD. Disinfection of root canals using Er: YAG laser at different frequencies. Photomed Laser Surg 2006; 24(4):499-502. 59.
126. Gordon W, Atabakhsh VA, Meza F, Doms A, Nissan R, Rizoiu I, et al. The antimicrobial efficacy of the erbium, chromium:yttrium-scandium-gallium-garnet laser with radial emitting tips on root canal dentin walls infected with Enterococcus faecalis. J Am Dent Assoc; 138(7):992-1002.
127. Eldeniz AU, Ozer F, Hadimli HH, Erganis O. Bactericidal efficacy of Er,Cr: YSGG laser irradiation against Enterococcus faecalis compared with NaOCI irrigation: an ex vivo pilot study. Int Endod J;40(2):112-9.
128. Shahabi S, Zendedel S. Atomic analysis and hardness measurement of the cavity prepared by laser. Lasers Med Sci. 2010 May;25(3):379-83.

129. Zehnder M. Root canal irrigants. J Endod 2006;32:389‐98.
130. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod 2006; 32:434‐7.
131. Jha D, Guerrero A, Ngo T, Helfer A, Hasselgren G. Inability of laser and rotary instrumentation to eliminate root canal infection. JADA 2006;137(1):67-70.
132. Gordon W, Atabakhsh VA, Meza F, Doms A, Nissan R, Rizoiu I, Stevens RH. The antimicrobial efficacy of the erbium, chromium: yttrium-scandium-gallium-garnet laser with radialemitting tips on root canal dentin walls infected with Enterococcus faecalis. J Am Dent Assoc 2007;138(7):992-1002.