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1. Title page

Effect of 2.94 µm Er: YAG laser on the chemical composition of hard tissues

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2. Abstract

The aim was to investigate the effect of the Er-YAG laser radiation on morphology and chemical composition of enamel, dentine and bone. The specimens of the three groups were irradiated with a very long pulse mode (VLP) of 2.94 µm Er-YAG laser with 100 mJ pulse energy and energy density of 8.42 J/cm² for 30s, at a repetition rate of 15 Hz. The organic and inorganic content of the samples were investigated by Fourier Transforms Infrared spectroscopy (FTIR). The morphological characteristics were investigated with Scanning Electron Microscopy (SEM) and elemental analysis (calcium and phosphorus) with Energy-Dispersive X-ray spectroscopy (EDX). FTIR data were analysed with a One-Way ANCOVA test and EDX data with the independent sample t-test. Following the laser radiation, FTIR showed a significant decrease in the organic content of all tissues. The weight percentage (wt. %) calcium content of dentine and bone increased significantly following irradiation with a P-value of 0.002 for both tissues, but the wt. % of phosphorus content was not influenced significantly. The morphological alterations expressed signs of fusion in all the samples.

**Keywords:** Enamel, Dentine, Bone, FTIR, SEM-EDX
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3. Introduction:

Infrared laser irradiation of dental hard tissues produces chemical and morphological changes. The degree of these modifications is influenced by the absorption characteristics of the dental tissues, and thus, the alterations are likely to be varied according to the kind of the dental tissue and laser (Rohanizadeh, LeGeros et al. 1999). The responses of the dental tissues to the laser radiation are affected by the parameters of the laser (i.e., laser wavelength, pulse energy, repetition rate, irradiation time, and the optical properties of dental tissues).

Considering the recent widespread utilisation of the adhesive systems, the laser treatment is expected to promote the suitable compositional changes of dentine and surface characteristics for the adhesive restorative materials, by roughening the surface and changing the mineral content. Alteration in the mineral content of dentine affects the solubility and permeability characteristics of the dentine and also influences the adhesion of restorative materials to the hard tissue. For instance, the variations in the levels of calcium ions or calcium/ phosphorus (Ca/P) ratio could affect the adhesion pattern of the restorative materials, as the adhesive material bonds to the dentine hydroxyapatite by phosphate-calcium bond. Thus, the mineral changes of dentine are important in the restorative dentistry by affecting the bonding technique and microleakage (Ari and Erdemir 2005). The laser treatment of a tooth does not yield a smear layer, with open dentinal tubules facilitating the penetration of adhesive composite and formation of resin tags. In this sense, the knowledge about the morphology and chemical composition of radiated tissue is of paramount importance for the development of studies in the restorative dentistry using the laser.
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The selection of the proper laser parameters is important during dental hard tissue etching; otherwise undesirable modifications of dentine collagen can occur, which would produce a negative effect on the bond strength between the tooth and restorative materials (Bakry, Sadr et al. 2007). Attril (Attrill 1997) has been reported that the irradiation of human enamel with fluence greater than 25 J/cm² of the Er: YAG laser expressed a crater instead of etched surface. The use of a high pulse repetition rate and low energy Er: YAG laser was recommended for improving the performance of the Er: YAG laser (Attrill 1997).

There have been many studies in the literature regarding the chemical composition of hard tissues. However, at the present time, data concerning the application and the analysis of specific parameters of the Er: YAG laser in hard tissues, particularly those with energy density below the ablation threshold are still incomplete. Therefore, the aim of this paper was to measure the effect of the Er:YAG laser radiation on the chemical composition and morphological changes of hard tissues, using Er: YAG laser parameters that have not yet been studied (low energy and high repetition rate). The null hypothesis is that there would be no significant difference in the organic and inorganic content of enamel, dentine and bone before and after the Er: YAG laser radiation.
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4. Materials and Methods

Sample preparation:

Twenty sound human permanent premolar teeth extracted for orthodontic reasons were used in this study. Tooth sections with 0.6 mm thickness were obtained from the teeth using an IsoMet® 1000 Precision Saw (Isomet 1000, Buehler, USA). The teeth were stored in a 10% buffered formalin solution before the experiment under the licence from the Human Tissue Act, 2004 at University of Manchester in the UK. Rat bone slices with 0.6 mm thickness were also used in this experiment. The specimens that were used in the FTIR analysis (7 in each group) were polished with a silicon carbide (SiC) paper of #2100-grit (Buehler Ltd, Lake Bluff, IL, USA) under water cooling for 30 s. In SEM-EDX analysis 14 samples in each group were used with two samples for SEM analysis.

Laser radiation:

Specimens were allocated into three groups: enamel, dentine and bone. The samples were irradiated with an Er-YAG laser (Fidelis Surgical Laser Model 320A Er-YAG Fotona Medical Lasers, Ljubljana, Slovenia) emitting at a wavelength of 29.4 µm, with VLP mode (750-950 msec pulse duration) in a moist environment. The device was adjusted to emit pulses of energy 100 mJ at a pulse repetition rate of 15 Hz and energy density of 8.92 J/cm². The laser beam was delivered in focused and non-contact mode at a 2 mm distance from the specimen. The irradiation distance was standardised. The laser light was delivered to the sample surface perpendicularly and at a constant working distance from the target surface. The laser parameters were chosen based on a pilot study in which many Er: YAG laser parameters were applied on the hard tissue sections. Then, the samples were investigated
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under the scanning electron microscope. We chose the parameters that did not cause a visible ablation with some signs of fusion.

Spectroscopic analysis

The FTIR absorption spectra were recorded on a FTIR spectrometer (Avatar 360, Nicolet Analytical instruments, UK) that equipped with a single reflective horizontal ATR accessory with diameter of crystal is 1.7 mm (MIRacle ATR, PIKE Technologies, 6125 Cottonwood Drive, Madison). Each spectrum was obtained with 80 scans and 4 cm⁻¹ resolutions in the range of 4000 to 500 cm⁻¹. The OMNIC Spectra Software was used for acquisition and storage of data. For each group, the peak absorption of organic matters (Amide I, II and III) and the absorption peaks of inorganic components (carbonate and phosphate) were investigated to identify possible alterations that might take place in the chemical structure of hard tissues following Er-YAG laser irradiation. FTIR analysis was carried out before and after irradiation of the same samples. Table 1 shows the main characteristic bands and the related absorption peaks.

Scanning electron microscope (SEM) and SEM-EDX examination

Because of the coating, it was not possible to analyse the specimens elementally prior and post irradiation. Thus, the samples of each hard tissue were divided into two groups: non-irradiated (control) and irradiated (experimental) groups, with seven specimens in each group. The samples were dehydrated in ascending concentration of ethanol series for 20 minutes for each concentration; 50%, 75%, 90% and 100% respectively. The samples were coated with carbon at approximately 7 nm thickness using a Leica Microsystems Sputter-Coater (Quorum technologies Ltd, Laughton, East Sussex, England) to avoid excessive charge build up on the electrically insulating specimens. The elemental analysis was
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performed using SEM-EDX (FEI, Tokyo, Japan) at 10 KV accelerating voltage and 100x magnification. Three spectrums were taken for each sample and then the average was calculated. Representative samples of each group were selected randomly to scan using the Philips XL30 FEG SEM (field emission gun scanning electron microscope) (FEI, Tokyo, Japan).

The methodology of the experiment was standardised for all the samples, which chosen to be young premolar teeth. The slices were taken from the coronal portion and checked under the microscope to exclude any sections with cracks or hyper-calcified areas. All the experiment analysis was carried out by one operator to avoid bias.

Statistical analysis

The effect of Er-YAG laser radiation on the organic and inorganic content of hard tissue and the differences between irradiated and control samples were assessed by the Analysis of Covariance (ANCOVA) using SPSS Version 23 (IBM Corp., Armonk, US) at a level of significance of p ≤ 0.05. The measurement data of examined elements in the control and lased areas of each sample were analysed statistically using the independent sample Mann-Whitney U-test.
5. Results

(1) FTIR results:

Means and standard deviations of absorption bands for each group are shown in (Table 2). The ANCOVA test and its associated P-value for all absorption bands (Table 3) showed a non-significant reduction for all of the inorganic contents, while the organic matters experienced a significant decrease in the irradiated group of enamel (P-value = 0.004). Dentine expressed a significant reduction in all the examined organic and inorganic contents (P-value < 0.05) except the phosphate, which decreased non-significantly (P-value = 0.28). The organic content of rat bone was decreased significantly following the Er-YAG laser radiation with P-value of 0.001 for amide I, P-value of 0.011 for both amide II and amide III. FTIR spectra for healthy and irradiated hard tissues are seen in Figures 7-9.

(2) EDX Evaluation:

The experimental data (wt. %) obtained by EDX for all groups are displayed in Table 4 and Figures 1-3. Calcium and phosphorus were increased following the Er-YAG laser irradiation for all hard tissues. For enamel tissue, the increase was non-significant. For instance, the mean wt% of enamel calcium was 39.06 in control sample and 39.72 in the lased one. The wt. % of Ca and Ca/P ratio in the irradiated dentine were increased significantly compared to the non-irradiated group, with F-test of 0.0001 and 4.00, and P-values of 0.002 and 0.026 respectively. In the bone tissue, only, the relative calcium showed a significant increase (F-test = 0.0001 and p-value = 0.002). The relative Ca/P ratio of both the enamel and bone decreased non-significantly following the Er: YAG laser radiation (Table 5).
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(3) *Scanning Electron Microscope*:

According to the SEM images, no clear ablation effect of the Er-YAG laser radiation with the used parameters was seen in all samples. However, confocal microscopy expressed some ablation with the amount of removed material was more in dentine than enamel. In the enamel tissue, the prismatic structure was more prominent following the laser irradiation leading to a rough appearance. Also, vitrification (small areas of glazed enamel) was observed in the irradiated enamel (Figure 4). Laser ablation of dentine yielded an etched surface with open dentinal tubules. Areas with signs of probable fusion occurrence were also seen in the irradiated dentine (Figure 5). A rough surface with clear Haversian system, porous areas and some glazed areas were seen in the irradiated surface (Figure 6).
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6. Discussion

The main components of the biological hard tissues that can be analysed by FTIR spectroscopy are the content of amides I, II and III that are related to the organic matrix, mainly collagen type I, and the inorganic matrix represented in phosphate and carbonate. Considering that high intensity infrared lasers including the Er: YAG laser can elevate the temperature of these tissue surfaces; alterations in both organic and inorganic matter were expected. The literature demonstrates that the laser radiation can induce collagen denaturation and water evaporation, which can be observed as a decrease in the infrared bands of amide I, II, and III in the analysis under FTIR spectroscopy. Related to the inorganic matter, the literature shows that the evaporation of carbonate can occur due to laser radiation, which shown as a decrease in the carbonate bands proportional to the phosphate in the irradiated sample when analysed by the FTIR spectroscopy (Zezell, Benetti et al. 2015). Furthermore, the carbonisation effect of the laser energy on the biological hard tissues may be seen as changes in the band positions, disappearance or appearance of new infrared bands (Zezell, Benetti et al. 2015). With a temperature rise, the elimination of carbonate happens progressively and was a maximum value between 400-800°C (Corrêa-Afonso, Bachmann et al. 2012). Thermal elevation higher than 200°C can produce a significant loss of the mineral matrix carbonate (Holcomb and Young 1980, Bachmann, Craievich et al. 2004).

In the spectra in Figures (7, 8 and 9) it is possible to see that all the main infrared bands of hard tissues are maintained, which suggests that the Er-YAG laser irradiation used did not induce the complete degradation of any component of the hard tissues. Therefore, it is possible to infer that the Er-YAG laser radiation did not raise the surface temperature more
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than 700°C, which is the temperature that causes degradation of organic matter (Zezell, Benetti et al. 2015). The low temperature rise might be attributed to the moist environment. Attril et al. (Attrill, Davies et al. 2004) showed that a quantifiable estimate of temperature increase can be calculated by following the linear regression equation:

\[ \Delta T_{\text{max}} = 0.032E - 0.57 \] (with water)

where \( \Delta T_{\text{max}} \) is the peak temperature increment (°C) and \( E \) is the cumulative energy input (J). According to this equation, the temperature rise in our study is 0.87° C, but this describes the overall temperature increase of the tissue.

The changes in the width of the absorption bands are related to a modification of the inorganic crystallinity. In this study, all the tested groups did not show any effect of the Er-YAG laser radiation on the width of the bands. Benetti et al., (Benetti, Santos et al. 2011) found a significant alteration in the width of Amide I + carbonate bands (1500-1600 cm⁻¹) at irradiation of rabbit bone with 6.06 J/cm² of Er-Cr-YSGG laser radiation. The variations might be related to the different laser and samples used. Also, the repetition rate was higher (20 Hz), with more close distance to the sample’s surface than our study.

The present study demonstrated that the Er-YAG laser radiation with an energy density of 8.42 J/cm² did not promote significant chemical changes in the inorganic content of enamel, whereas the dentine expressed significant alterations in both organic and inorganic components. The rationale for this is that the temperature changes of the tissue surfaces during the laser radiation are mainly responsible for the chemical modifications of the hard tissues (Sasaki, Aoki et al. 2002). A previous study has specified that at similar energy densities of laser radiation, dentine showed a markedly higher surface temperature than that measured on the enamel because of the lower thermal diffusivity and reflectance losses of...
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dentine at the respective wavelength (Fried, Zuerlein et al. 2002). The FTIR spectrum of
enamel and dentine were different from each other, with less intensity of organic compounds
in the enamel, which agrees with the known chemical composition of hard tissues. Enamel
has just 1.5% of organic constituents (Antunes, de Rossi et al. 2006, Iijima, Fan et al. 2010).
Our results relating to dentine agree with previous studies (Soares, Resende et al. 2007,
Choeysuppaket, Pokaipisit et al. 2010), and disagree with some previous findings (Antunes,
de Rossi et al. 2006, Bakry, Sadr et al. 2007, Soares, Resende et al. 2007, Zezell, Benetti et
al. 2015). The difference might be due to different methodologies (Sasaki, Aoki et al. 2002).
Also, most of the literature did give a detailed prescription of laser parameters used, the
condition that leads to a difficulty to conduct the right comparison. Rohanizadeh et al.
(Rohanizadeh, LeGeros et al. 1999) reported that the difference could be also, because of the
heterogeneity in dentine composition as a result of the laser irradiation and heterogeneity of
SEM experimental analysis.
Zezell et al. (Zezell, Benetti et al.) demonstrated that the laser effect on the organic matrix of
bone and dentine are more pronounced, since these tissues have a higher organic matrix
content than enamel. This appears clear in our study, whereas only amide II of enamel
organic matrix showed a significant decrease after the laser radiation.
Since a closure adaptation between the sample and FTIR crystal is required to get infrared
spectra, which might be difficult in this experiment because the rough surface gained from
the Er-YAG laser irradiation. Thus, a relative comparison between peak ratios was also
performed. Carbonate/phosphate ratio was decreased significantly following the Er-YAG
laser radiation in dentine (P-value = 0.001), while non-significantly in enamel and bone.
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The calcium increment results in a chalky appearance of the dentine. Thus, the alterations in the Ca/P ratio can be positively effective in the quality of the dental filling materials, because it improves the chemical bond strength between the restorative material and dentine. Increases in the bond strength occur by inducing higher dentine surface energy (Arbabzadeh, Birang et al. 2013). In this study, the SEM-EDX examination revealed that in both dentine and bone groups, calcium levels (Ca wt %) in the irradiated areas increased significantly (p < 0.05) compared with control samples. During the laser radiation, it is likely that the elevation in temperature in the lased area caused an increase in Ca or P elements wt % due to the reduction of the organic components (Hossain, Nakamura et al. 2003, Nagaia, Kinoshitab et al. 2006). This is clear in our FTIR and EDX analysis of irradiated hard tissues, whereas the reduction of organic content accompanied by increase in the composition of Ca and P relative content. However, in the enamel tissue the relative content of Ca increased non-significantly.

Our results for the lased enamel disagree with the previous studies in which there were significant increases of Ca following the laser irradiation (Rodríguez-Vilchis, Contreras-Bulnes et al. 2011), but agree with the study by Nagaia et al. (Nagaia, Kinoshitab et al. 2006). In this experiment, the amount of phosphorus increased following the laser radiation. No significant changes were seen in the Ca/P wt. ratio in the irradiated areas of both enamel and bone tissues. Therefore, it was suggested that no chemical alterations occurred in the lased tissues at the molecular structure level (YU, KIMURA et al. 2000). Dentine showed a significant increase in the Ca/P ratio. Lin et al. (Lin, Lee et al. 2001) stated that the evaporation of phosphorous is considered a major reason for increasing the Ca/P ratio of dentine following the Nd: YAG laser irradiation. In our study, FTIR expressed a decrease in the phosphorous following the Er: YAG laser irradiation. Lizeralli et al. (Lizarelli, Costa et
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al. 2007) also, reported that the USPL removed phosphorous as a result of the light interaction with water as the phosphate is strongly bonded to the water molecules.

However, it should be taken into consideration the difficulty in measuring an accurate Ca or P wt. ratio, even under ideal circumstances, because the interaction volume of the electron beam greatly define the levels of energy recorded at the detector (YU, KIMURA et al. 2000).

No study in the literature has established the analysis of different elements on the same area of hard tissues during experimental phases (prior and post irradiation) (Díaz-Monroy, Contreras-Bulnes et al. 2014).

During the laser radiation, temperature rises in the residual tissues and subsequently, the laser energy produces compositional and ultra-structural changes in the tissue surrounding the target point. These changes may promote the adhesion mechanisms of dental filling materials particularly composite resin in comparison to the conventionally prepared teeth (Ceballos, Osorio et al. 2001, Bertrand, Hessleyer et al. 2004). Under the present study conditions, SEM observations revealed that the irradiated enamel and dentine experienced signs of vitrification. Delmé et al. (Delmé and De Moor 2007) pointed out that the associated vitrification of the Er-YAG irradiated enamel and dentine was related to the laser energy and inevitably any generated heat, without effect of repetition rate. In the present data, the signs of fusion were clearer in dentine than enamel (Figure 4 & 5). The vitrification phenomenon recognised on the SEM images as an area of melted tooth materials revealed flattened shiny or bubble-like changes (Delmé and De Moor 2007). Regarding the bone tissue, previous literature has reported different findings about the impacts of the Er-YAG laser radiation on bone tissues (Hibst 1992, Sasaki, Aoki et al. 2002). The SEM images from this study revealed that the Er-YAG laser conditioning of hard tissues surfaces resulted in a rough surface with varied degrees of irregularities.
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However, the samples that were used in this experiment were stored in formalin. In this condition, they do not resemble the natural hard tissues and bone. Although, the formalin storage provides both fixation and a sterilisation environment, its action occurs through the formation of inter and intra-molecular cross linking and produces protein denaturation (Howat and Wilson 2014). Subsequently, it can influence on the chemical interaction of dental hard tissues with a laser. The storage media is variable and it might be responsible for the variation in the literature. Also, the variation in the crystallinity of the specimens may be important.

Since, the structures studied in hard tissues have heterogeneity in nanometre and resolution of FTIR is ~ 4 cm$^{-1}$, the results are averaged over an area that may be more than the heterogeneity. This might be a reason for the variations in result (Gower 2014). EDX provide the elemental analysis with sampling depth of 1-2 microns, at a specific area of microns and accuracy of about 1% (Celik, Ergücü et al. 2008).

The effect of the Er-YAG laser irradiation on hard tissues varied depending on the type of target tissues and the employed laser parameters. According to the findings observed in the present study, it can be concluded that the Er-YAG infrared lasers could morphologically and chemically change the microstructure of enamel, dentine and bone. However, the addition of EDX provides valuable data about the chemical alterations on the irradiated hard tissues.
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7. Acknowledgments

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Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Role of funding source

There are no any financial relationships with other people or organizations in this study.

Ethical Approval

Ethical approval was given for the experiments. Patient consent was obtained for the teeth to be retained following extraction for unrelated purposes. The teeth were stored under the Human Tissue Authorisation at the University of Manchester in United Kingdom.

Informed Consent

Informed consent was obtained from all individual participants included in the study.
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8. References


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9. Table legends

Table 1: Infrared characteristic bands and peaks associated

Table 2: Mean and standard deviation of absorption bands for each group

Table 3: ANCOVA test comparing peak absorption bands of control and lased groups

Table 4: Weight percent of calcium (Ca), phosphorus (P) and Ca/P ratio for enamel, dentine and bone.

Table 5: Independent samples Mann-Whitney U test and its associated $p$-value of examined elements for the three different hard tissues
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10. Figures legends

Figure 1: A bar chart illustrating mean and standard deviation of elemental analysis of control and irradiated enamel.

Figure 2: A bar chart illustrating mean and standard deviation of elemental analysis of control and irradiated dentine.

Figure 3: A bar chart illustrating mean and standard deviation of elemental analysis of control and irradiated bone.

Figure 4: Representative samples of control (A) and irradiated (B) enamel. A smooth prismatic structure was seen in control specimen. More clear prismatic structure with some glazed areas in the lased enamel.

Figure 5: SEM images of an intact (A) and irradiate (B) dentine samples. A flatter surface topography with smear layer covering dentinal tubules is shown. Laser etched dentine, opening dentinal tubules and removing smear layer. Some glazed areas (circle) are seen.

Figure 6: SEM images of an untreated (A) and irradiate (B) bone samples. The control sample showed a smooth prismatic bony structure. Irradiated bone showed rough surface with some porous and glazed areas.

Figure 7: FTIR spectra for healthy and irradiated human enamel.

Figure 8: FTIR spectra for healthy and irradiated human dentine.

Figure 9: FTIR spectrum for healthy and irradiated human bone.
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