INTRODUCTION

Dental enamel, the most highly mineralized tissue in the body, consists of 95% minerals, 4% water, and 1% organic materials\(^1\). With the time of exposure in the oral cavity, enamel maturation occurs presumably through the incorporation of calcium, phosphate, and fluoride from saliva or external origins into the enamel\(^2\). The acids produced by bacteria in the oral environment cause the demineralization of the enamel. The enamel of an immature tooth and around an orthodontic bracket is at extremely high risk of demineralization\(^3,4\). Because fluoride plays an important role in preventing demineralization, fluoride-containing products, such as toothpastes, mouth rinses, pit-and-fissure sealants, and fluoride-releasing adhesives, have been used widely clinically\(^4-6\).

Previous studies have demonstrated the inhibitory potential of CO\(_2\) laser irradiation on enamel demineralization\(^7-12\) and irradiation conditions, such as wavelength, pulse width and pulse energy\(^9,12\), the temperature change of the enamel\(^19\), and changes in the composition and structure of the enamel have been investigated\(^10,11\). Featherstone et al.\(^9\) reported that if sufficiently high temperatures (~900°C) were achieved in the enamel mineral by laser irradiation, carbonate was lost from the laser-treated mineral, and surface melting and recrystallization occurred, which resulted in a reduction in its reactivity. However, Hsu et al.\(^11\) reported that the partial decomposition of the organic matrix of the enamel caused by heating to less than 400°C with CO\(_2\) laser irradiation resulted in blockage of the inter- and intraprismatic spaces; ion diffusion in the enamel was compromised, resulting in a reduction of enamel demineralization. It is known that F improve the acid resistance of teeth, acting on hydroxyapatite and converting it to fluorapatite\(^13-15\). Additionally, combined effects of CO\(_2\) laser irradiation and fluoride application on enamel demineralization have been reported\(^16-18\). However, the mechanism(s) of these effects remain(s) unclear.

It has been demonstrated that the organic components, such as proteins and free water, play an important role in the mechanical properties of enamel\(^19\). Thus, heating due to CO\(_2\) laser irradiation may degrade the mechanical properties of enamel. The embrittlement of enamel may cause iatrogenic damage, such as fracture and the creation of surface defects. No reported study has evaluated the relationship between demineralization effects, compositional and structural changes, and mechanical property changes with laser irradiation.

The aim of the present study was to investigate the effects of CO\(_2\) laser irradiation combined with fluoride application on the inhibition of demineralization, and the mechanical properties, structure, and composition of enamel.
MATERIALS AND METHODS

Sample preparation, fluoride application, laser irradiation and immersion in demineralization solution

Human non-carious premolars, extracted from patients aged between 18 and 29 years, undergoing orthodontic treatment, were used in the present study. Selection criteria included the absence of any visible decalcification or cracking of the enamel surface under a stereoscopic microscope (SMZ 1500, Nikon, Tokyo, Japan) at a magnification of ×10. The buccal surfaces of all teeth were cleaned using non-fluoridated pumice and a rubber cup, and washed thoroughly and then dried using a moisture-free air source. The experimental protocol was approved by the Ethics Committee of the Health Sciences University of Hokkaido (No. 64).

The teeth were divided randomly into five groups: (1) laser irradiation group (L group), (2) fluoride application group (F group), (3) laser irradiation plus fluoride application group (FL group), (4) control group (C group), and (5) native group (N group).

Acidulated phosphate fluoride (APF) gel (Fluor jelly, Oriental Pharmaceutical and Synthetic Chemical, Osaka, Japan) containing 1.23% F (NaF) at pH 3.5 was applied to the buccal enamel of specimens (F group and FL group before laser irradiation) for 1 min. In the present study, a CO₂ laser (10.6-µm wavelength, 10-µs pulse duration; Nanolaser GL-III, GC, Tokyo, Japan) was used and irradiation was performed at a distance of 5 mm for 5 s; the beam diameter at the sample surface was 1.05 mm. The output power used was 0.5, 1.0, 2.0, or 4.0 W (L and FL groups).

Acid-resistant nail varnish was next applied to each tooth, leaving a 5×5-mm² area of exposed, sound enamel. The specimens were then immersed in a demineralization solution (4% Methocel MC gel (Fluka, Buchs, Switzerland) +0.1% lactic acid) as reported previously. The solution was adjusted to pH 4.6. All specimens were immersed in a polystyrene vial with 10 mL of this solution for 72 h at 37°C, with the solution changed every 24 h, and then washed gently with deionized water. The samples of the native group were immersed in distilled water for 72 h.

Depth-dependent mineral density (MD) of the enamel, analyzed by micro-CT

Specimens from each condition were selected randomly after immersion. The enamel block with an approximate size of 1.8×3.0×5.0 mm was sectioned using a slow-speed water-cooled diamond saw (Isomet 11-1280, Buehler, Illinois, USA; Fig. 1a). A scan of the specimens with a cone-beam micro-CT scanner was performed at room temperature (tube voltage=60 kV, tube current=60 µA; TDM1000, Yamato Kagaku, Tokyo, Japan). The specimens were placed in a cylindrical sample holder with a small amount of cotton soaked in distilled water during scanning to avoid dehydration. Seven grades of hydroxyapatite (200–800 mg/cm³) and aluminum (1,550 mg/cm³) were used as phantom materials for the quantitative analysis of mineral density. The resulting two-dimensional images were reconstructed to a three-dimensional (3D) image with a resolution of 1,024×1,024 pixels and an isotropic voxel side of 3.6 µm (TRI-BONE System software, Ratoc System Engineering). For F, C, and N groups, the measurement locations were selected randomly and measurements were performed at 0–100-µm depth from the enamel surface at 3.6-µm intervals (Fig. 1b; n=12). For color mapping, higher mineralized regions were expressed as a red color, whereas relatively less mineralized areas were blue (Fig. 2). For the L and FL groups, locations at 1.0 mm in width from the irradiation center of the laser on the enamel surfaces were selected and measurements were performed at 0–100-µm depth with 3.6-µm intervals (n=12).
**Fig. 2** Representative micro-CT scanning 3D images after color mapping. The arrows in the cross-sectional view indicate the laser irradiation sites.

**Depth-dependent mechanical properties of the enamels, measured using a nanoindentation test**

Specimens from each condition were selected randomly after immersion. All teeth were cut using a slow-speed, water-cooled diamond saw (Isomet, Buehler, Lake Bluff, Illinois, USA) so that they were divided into occlusal and cervical halves; one of the sectioned specimens (transverse planes) was then encapsulated in epoxy resin (Epofix, Struers, Copenhagen, Denmark) for the nanoindentation test \(n=5\). All samples were ground (600-grit sandpaper) and polished using diamond suspensions (particle sizes of 3, 1 and 0.25 µm) to obtain a surface suitable for nanoindentation. The specimens were then washed with distilled water and lightly cleaned ultrasonically. Nanoindentation testing (ENT-1100a, Elionix, Tokyo, Japan) was carried out at constant temperature (28°C) with a peak load of 5 mN using a Berkovich indenter \(n=5\). The same analytical locations at 1.0 mm in width from the irradiation center of the laser on the enamel surfaces were selected and the indentations were made at depths of 1–101 µm (20 locations, spaced 5 µm apart; Fig. 1c). Linear extrapolation methods (ISO Standard 14577) were used for the unloading curve between 95% and 70% of the maximum test force to calculate the elastic modulus. The hardness and elastic modulus were calculated using the software provided with the nanoindentation apparatus.

**Micro-X-ray Diffraction (Micro-XRD)**

The crystal structures of the enamel with and without CO\(_2\) laser irradiation were analyzed by micro-XRD (Micro-XRD; Rint-2500, Rigaku Corp., Tokyo, Japan) with Cu K\(\alpha\) radiation at 40 kV and a tube current of 300 mA \(n=2\). The specimens were oscillated from 0° to +15° about the \(\chi\)-axis and rotated from −120° to +120° about the \(\varphi\)-axis to minimize any effect of a preferred orientation. The locations of these axes are shown in previous publications that describe the micro-XRD technique\(^{21}\). A collimator, 100 µm in diameter, was used to establish the dimensions of the analysis area. The diffracted X-rays were detected by a position-sensitive proportional counter (PSPC), and the measurement time for scanning over the diffraction angle (2\(\theta\)) from 10° to 90° was 10 min.

**Depth-dependent surface compositional analysis of the enamel by X-ray photoelectron spectroscopy (XPS)**

Polished enamel blocks, 2.0×2.0 mm\(^2\) and 1.0 mm in thickness, were used \(n=1\). The surface compositions of the enamel samples (N group, F group, and FL group with 1.0 W laser irradiation) were evaluated by XPS (ESCA-850, Shimadzu, Kyoto, Japan). The specimens were analyzed with Al K\(\alpha\) radiation at 7 kV and 30 mA and the pressure of the main chamber was maintained at <1×10\(^{-6}\) Pa. Argon ion sputtering was used for depth profiling measurements. The relative atomic concentrations of the elements at each depth...
were calculated from the ratios of the element area intensities. For the chemical analysis of the enamel surface, standard reference materials of CaF$_2$ and fluorapatite were also analyzed.

Statistical analyses
The experimental results were analyzed using the PASW software (ver. 18.0 J for Windows; IBM, Armonk, NY). Mean MD, hardness, and elastic modulus, and the standard deviations, for the five groups were compared by one-way ANOVA, followed by Tukey’s test. For all statistical tests, significance was set at $p<0.05$.

RESULTS

Micro-CT scanning 3D images after color mapping are shown in Fig. 2. More highly mineralized regions are indicated by the red color, whereas relatively less mineralized areas are show in blue. The arrows in the cross-sectional view show the areas to which the CO$_2$ laser was applied. The enamel surface of Group C showed lower MD values than Group N by demineralization, although Group F showed similar MD values versus Group N. For Groups F and FL, the enamel surface at the irradiation center of the laser at 4.0 W showed lower MD values.

Figure 3 shows 2D mapping images for values of MD, hardness, and elastic modulus. The measurement locations on the buccal enamel surface ($y$-axis in this figure) for groups N, C, and F were selected randomly. The $x$-axis (depth) shows the distance from the enamel surface. The values of MD, hardness, and elastic modulus for Group C were significantly lower than those in Group N at depths of 0–50 µm. The values of MD, hardness, and elastic modulus for Group F were significantly lower than those in Group N in the shallow depth regions. The $y$-axis in the figure (perimeter) shows distance (mm) from the irradiation center of the laser on the enamel surfaces. In comparing Groups C, L, and FL, limited regions on the enamel surfaces of Groups L showed significantly lower values of MD, hardness, and elastic modulus than Group C, and the decreased values reached deeper regions for specimens irradiated at 4 W. However, most surface regions for Groups FL, except that irradiated at 4 W, showed significantly greater values of MD, hardness, and elastic modulus than Group C. Although most surface regions for Group L showed significantly lower values of MD, hardness, and elastic modulus than Group F, limited surface regions for Groups FL showed significantly higher values of MD and hardness than Group F. There were no significant differences among the values of elastic modulus.

Figure 4 contains representative micro-XRD patterns at 2θ diffraction angles between 20° and 60° for Groups N, L, F, and FL. All spectra in Figs. 4a and b showed peaks for hydroxyapatite or fluorapatite. The specimens irradiated with the CO$_2$ laser at 2 and 4 W showed decreased peak intensities and peaks for
β-tricalcium phosphate (β-TCP) were observed.

Figure 5 shows fluoride depth profiles, and F 1s and Ca 2p spectra obtained by XPS. The fluoride concentration for Group FL was higher than that for Group F; the fluoride uptake for Group FL (280 nm) was also higher than that for Group F (180 nm). There was no significant difference in the concentrations of other elements (calcium, phosphate, sodium; data not shown). The XPS spectra (F 1s and Ca 2p) for Groups F and FL were consistent with peaks for a standard fluorapatite specimen.

**DISCUSSION**

In the present study, laser irradiation alone produced a slight improvement in the acid resistance of the enamel and the effect was inferior versus fluoride application alone. This differs from previous reports that laser treatment alone produced inhibition of enamel demineralization comparable with that found by three-times-per-day use of fluoride dentifrice\(^ {22} \). Rodrigues et al.\(^ {16} \) reported that laser treatment did not enhance remineralization in the absence of fluoride, but inhibited only demineralization. The beam diameter at the sample surface used in this study was 1.05 mm. The enamel region surrounding laser irradiation showed lower MD values compared with the region directly laser-irradiated with 0.5, 1.0 and 2.0 W; the values were lower than that obtained for fluoride application alone. Although detailed investigation of the temperature distribution in laser-irradiated and its peripheral regions is necessity, the improvement of the enamel acid resistance by laser irradiation may be greatly influenced by the temperature difference in different enamel regions.

CO\(_2\) laser irradiation plus fluoride application in the present study produced improved the acid resistance of the enamel, versus fluoride application alone. The specimens laser-irradiated with 0.5 and 1.0 W showed significantly higher MD values at the irradiation center of the laser, although the specimens laser-irradiated with 4.0 W showed significantly lower MD values at the irradiation center of the laser compared with specimen that was not laser-irradiated. The reason for this is that superfluous energy from the laser irradiation at 4.0 W might have damaged the enamel directly under
the irradiated region, although the surrounding enamel received an energy level suitable for improving its structure. Laser irradiation with low output (0.5 or 1.0 W) is preferable for modifying the enamel surface to improve acid resistance.

The effects of CO₂ laser irradiation plus fluoride application on the values of hardness and elastic modulus were also investigated because their relationships are important for understanding the degradation of physical properties during the demineralization of enamel. The use of cross-sectional microhardness measurements with a Knoop indenter is a popular method of investigating the demineralization of enamel quantitatively, and a strong correlation (R² = 0.84–0.92) between enamel microhardness and mineral content determined by transversal microradiography in carious lesions was reported previously. The traditional Knoop microhardness test generally produces a large indentation (indentation length equivalent to a width of 5–20 enamel prisms) and is also influenced by the substrate. However, recent advances in nanoindentation tests now allow the measurement of mechanical properties of extremely small volumes of material, and the hardness and elastic modulus can be determined simultaneously. In the present study, a depth-dependent analysis of the enamel surface region was performed with an isotropic voxel size of 3.6 μm in µCT scanning images and with a 5-μm interval for indentations in nanoindentation testing. The research findings in the present study showed a correlation tendency between MD, hardness, and elastic modulus; this is consistent with previous findings.

The results of the depth-dependent MD, hardness, and elastic modulus for Groups FL were similar to those of Group F. However, Group FL, which was laser irradiated with 1.0 W, showed significantly higher values of MD and hardness than those of Group F. In the present study, although APF gel was applied to the teeth and wiped off before laser irradiation, APF gel remaining on the enamel might have contributed to modification of the tooth crystalline structure in the form of firmly bound fluoride, due to suitable heating by the laser irradiation. This is partially supported by the XPS results; i.e., the fluoride concentration of the enamel surface and the fluoride uptake for Group FL was higher than in Group F.

All XRD spectra obtained in this study contained hydroxyapatite or fluorapatite. Peaks for β-TCP were also observed in the specimens laser-irradiated with 2.0 and 4.0 W. After laser irradiation with 4 W, all peak intensities decreased, indicating that the surface of the enamel melted partially and the structure changed, becoming amorphous-like, reducing its acid resistance. Because the XRD spectra for hydroxyapatite and fluorapatite are similar, it is difficult to identify the spectra obtained from Groups L and F. Previous studies reported that calcium fluoride formed on the enamel surface after fluoride application. The relative atomic concentration of Group FL (11%) obtained from the XPS analysis was similar to that of fluorapatite, and the F 1s and Ca 2p spectra obtained for Groups L and FL were similar to those obtained for standard fluorapatite materials (Fig. 5). These results suggest that the crystal structure of the enamel surface can be changed from hydroxyapatite to fluorapatite by suitable heating with laser irradiation and fluoride application.

CONCLUSIONS

Under the conditions of this study, the following conclusions can be drawn:

1. CO₂ laser irradiation with low output (0.5 or 1.0 W) is preferable for modification of the enamel surface to improve acid resistance.
2. Higher fluoride concentration and superior fluoride uptake of the enamel surface were observed with CO₂ laser irradiation plus APF gel application.
3. The crystal structure of the enamel surface may change from hydroxyapatite to fluorapatite with laser irradiation and fluoride application.
4. The values of MD, hardness, and elastic modulus of the enamel surface after immersion in demineralization solution were correlated.

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