Infiltration of trace metal ions in the oral mucosa of a rat analyzed using SR-XRF, XAFS, and ICP-MS

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Although the accumulation and distribution of metals from metallic orthodontic appliances in the oral mucosa have been studied extensively, they remain unclear because their concentration is quite low. In this study, metal specimens (Ni, Ni-Ti, and Co-Cr) were sutured in the unilateral oral mucosa of rats, and the distribution of the eluted elements in the mucosal tissue was estimated using inductively coupled plasma mass spectrometry (ICP-MS) and synchrotron radiation X-ray fluorescence analysis (SR-XRF). While the infiltrations of Ni, Co, and Cr into the oral mucosal connective tissue were observed with SR-XRF, significant increases were only found in Ni from the pure Ni group and Cr from the Co-Cr group. Furthermore, Ni and Co were estimated as hydrated ions while Cr was estimated in oxide form through X-ray absorption fine structure (XAFS) analysis.

Keywords: Inductively coupled plasma mass spectrometry (ICP-MS), Synchrotron radiation X-ray fluorescence analysis (SR-XRF), X-ray absorption fine structure analysis (XAFS), Trace elemental analysis, Orthodontic appliances

INTRODUCTION

Metallic materials are widely used in orthodontic devices because of their excellent mechanical properties. For instance, wires composed of cobalt-chromium (Co-Cr) and nickel-titanium (Ni-Ti) alloys are particularly useful for achieving high resilience and excellent elastic properties. However, the oral environment may cause the elution of metal ions, occasionally leading to metal allergy. Although Ni is an essential trace element related to metabolism in the living body, it is also known to cause toxicity, carcinogenesis, and allergy1). Ni is widely contained in various alloys and is the most well-known contact allergen among metals. Similarly, toxicity and allergenicity are caused by Co and Cr, which are also common allergens2). It is believed that the amount of Ni, Co, and Cr elution from dental alloys in the oral cavity is quite low and negligible toxicity is expected, but the prevalence of Ni allergy is reported to be 4.5–28.5% among women3). Therefore, many studies have been conducted to evaluate the biocompatibility of metal alloys in the oral cavity.

Metal allergy is classified as type IV allergy, which is initiated by the release of metal ions and the formation of metal-binding proteins. Metal-binding proteins are recognized as antigens by antigen-presenting cells in the epithelium, such as dendritic cells and macrophages. These cells activate T cells and induce immune response4). According to this theory, metal allergy is triggered by intraoral metal devices when eluted metal ions infiltrate the oral mucosa and then interacting with proteins.

Metal elution from orthodontic devices during treatment is influenced by various factors such as chemical (e.g., salivary pH) and biological (e.g., bacteria and inflammation) environments, morphological changes (e.g., bending stress of devices), and physiological properties (e.g., fluidity of saliva)5-11). Simulating the accurate environment of the oral cavity during orthodontic treatment is difficult because the aforementioned metal elution is influenced by such a variety of factors, and therefore, previous studies primarily focused on the metal-ion release from devices under certain conditions, as described above. It has been also reported that the Ni concentrations in saliva, blood, and urine do not increase significantly after the placement of fixed orthodontic appliances5,12,13). In fact, the increase is even less than the amount of Ni taken from an average daily diet14). Therefore, the metallic elements eroded and accumulated from the orthodontic appliance into the oral mucosa should be directly measured.

Concerning the concentration and distribution of metals in oral mucosa, only the total amount has been analyzed15), and the elemental distribution could not be analyzed, because the concentration is too low to

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apply conventional elemental-distribution analysis methods. In order to examine the metal-ion distribution in the mucous membrane, a micro-area analysis of trace elements in a solid sample is required. Recently, one of the authors applied synchrotron radiation X-ray fluorescence analysis (SR-XRF) and X-ray absorption fine structure analysis (XAFS), which are highly sensitive and non-destructive analysis methods, to visualize the distribution and analyze the chemical state, respectively, of accumulated trace metallic elements, which were suspected to be derived from dental metallic restorations, in the oral mucosa. These methods were also applied for the Ni-doped mouse skin which were suspected to be derived from dental metallic restorations, in the mucous membrane, a micro-area analysis of metals in a solid sample is required. Recently, one of the authors applied synchrotron radiation X-ray fluorescence analysis (SR-XRF) and X-ray absorption fine structure analysis (XAFS), which are highly sensitive and non-destructive analysis methods, to visualize the distribution and analyze the chemical state, respectively, of accumulated trace metallic elements, which were suspected to be derived from dental metallic restorations, in the oral mucosa. These methods were also applied for the Ni-doped mouse skin, and their usefulness was shown. However, metal accumulation into adjacent oral mucosa derived from a metallic orthodontic appliance remains unknown.

The aim of the present study was to investigate the distribution of metal ions infiltrated in the oral mucosa under direct contact with metal alloys by applying SR-XRF, XAFS, and inductively coupled plasma mass spectrometry (ICP-MS). The subjects for research were nickel (Ni), titanium (Ti), cobalt (Co), and chromium (Cr). We used SR-XRF to visualize the distribution of these alloy elements in the oral mucosa. Samples were analyzed using XAFS to determine chemical state of metals, and the samples were subsequently collected for qualitative analysis using ICP-MS.

MATERIALS AND METHODS

Forty female Wistar rats of mass ranging from 200 to 250 g were used in this investigation. All animals were fed a powdered diet (CE-2, Clea Japan, Tokyo, Japan) and had access to tap water ad libitum. All experimental procedures to which they were subjected in this study were approved by the Animal Ethics Review Committee of Tokyo Medical and Dental University (approval number 0150174A).

Experimental model and tissue preparation

All experiments were performed under anesthesia using intraperitoneal injection with chloral hydrate (300 mg/kg). The animals were divided randomly into four groups of 10 rats — (1) sham surgery group, (2) nickel (Ni) group, (3) nickel-titanium (Ni-Ti) group, and (4) cobalt-chromium (Co-Cr) group — and treated using the procedures described below. Three types of alloys — pure Ni, Ni-Ti and Co-Cr (Table 1) — were prepared for the experiment. Wires of each alloy were cut into 10-mm-long test pieces and washed through ultrasonication in 70% ethanol, followed by air seasoning. The animals were anesthetized, and each test piece was sutured using a silk thread on the buccal oral mucosa of the right side (hereafter, sutured side) of the rat of the relevant group so that the alloys came in contact with the mucous membrane. The left side of the oral mucosa of each animal served as the control (hereafter control side). In the animals of the sham surgery group, after anesthetization, the silk threads were sutured 10-mm apart. After two weeks, animals were deeply anesthetized and sacrificed, and bilateral buccal mucosa (2×12 mm) was then extracted for investigation.

ICP-MS analysis

The tissue samples were dried naturally 24 h, weighed to 0.03 g, and dissolved in 10 mL of diluted nitric acid (69%, Ultra-pure grade, Wako, Tokyo, Japan), and the amount of metals in tissues was quantitatively analyzed using ICP-MS (ELAN DRC, Perkin Elmer, OH, USA). Five specimens of each condition were applied for ICP-MS analysis. The statistical significance of the results was analyzed with Dunnet test for multiple comparisons between sham surgery group and each metal sutured groups. Element concentration under the limit of detection was treated as the detection limit.

SR-XRF analysis

Frozen 20-μm-thick sections were made from the bilateral buccal mucosa taken from animals of each group, and they were stuck to 12.5-μm-thick polyimide films (Kapton, Du pont-Toray, Tokyo, Japan). SR-XRF analysis was conducted at BL-4A, Photon Factory, High-Energy Accelerator Research Organization (KEK-PF, Tsukuba, Japan) with 12.9-keV incident X-rays by capillary focusing into a beam diameter of 20 μm and scanning sample points with 40-μm steps in the X and Y directions, analyzing them as pixels of an image. The multiplication time was set to 2 s in the Ni group, 4 s in both the Ni-Ti group and the Co-Cr group, and 1 s in the sham surgery group and control sides of each animal. The obtained XRF data were processed with PyMCA software (Version 4.7.3) (16-17). The elemental distribution images were compared with the structure in the adjoining section dyed with hematoxylin-eosin (HE).

Table 1 Metal materials tested in this study

| Common name | Trade name | Use            | Manufacturer                        | Shape/Size | Composition (%) |
|-------------|------------|----------------|-------------------------------------|------------|----------------|
| Ni          | Pure nickel | General        | Nilaco, Tokyo, Japan                | Round/1 mm diameter | Ni: 99         |
| Ni-Ti alloy | Reflex     | Orthodontics   | TP Orthodontics Japan, Tokyo, Japan | Rectangular/0.45×0.55 mm | Ni: 55 Ti: 45  |
| Co-Cr alloy | Elgiloy yellow | Orthodontics | Rocky Mountain Morita, Tokyo, Japan | Rectangular/0.45×0.55 mm | Co: 40 Cr: 20 Fe: 15.81 Ni: 15 Mo: 7 Mn: 2 |
Table 2  Concentrations of metals in buccal mucosa in four groups analyzed using ICP-MS

|       | Ti      | Cr      | Co      | Ni        |
|-------|---------|---------|---------|-----------|
| Mean  | SD      | Mean    | SD      | Mean      | SD      |
| Sham surgery | 0.12 (0.26) | 0.05 (0.08) | 0.05 (0.08) | 0.00 (0.00) |
| Ni    | 0.01 (0.00) | 0.01 (0.00) | 0.02 (0.03) | 46.92* (48.61) |
| Ni-Ti | 0.01 (0.02) | 0.05 (0.08) | 0.00 (0.00) | 0.00 (0.00) |
| Co-Cr | 0.44 (0.47) | 3.36* (3.10) | 0.12 (0.12) | 0.00 (0.00) |

Numbers are expressed in parts per billion (ppb).
SD: standard deviation.
*: p<0.05

XAFS analysis
The chemical state of the metal was determined by measuring the XAFS spectrum using the fluorescence method (BL-4A, KEK-PF, Tsukuba, Japan) in the concentrated parts of Ni, Co, and Cr that had been obtained from the SR-XRF measurement. The obtained XANES spectra were compared with those of standard specimens that were measured at BL-9A in KEK-PF.

RESULTS
ICP-MS
A significant difference in metal concentration was detected only for Ni in the Ni group and Cr in the Co-Cr group (Table 2). In the Co-Cr group, although the average value of Co increased compared to the sham surgery group, the difference was not significant. No metals were detected in significant quantity in the sham surgery group. The increases in Ni and Ti were not significant in the Ni-Ti group.

SR-XRF
Since both P and S are major elements in a living body and exist in entire organs, it is reasonable to observe their distributed image (Fig. 1). In contrast, Ni, Ti, Co, and Cr were not detected in the sham surgery group.

In the Ni group, remarkable Ni accumulation was observed in the mucosal tissue on the sutured side (Fig. 2A). Ni concentration was high at the surface of the epithelium and gradually decreased with increasing distance from the surface. Although Ni mostly remains in the epithelium, the distribution images indicated that a little amount of Ni penetrated the connective tissue. No Ni accumulation was observed on the control side (Fig. 2B).

In the Ni-Ti group, an accumulation of small amount of excess Ni was detected in the sutured side (Fig. 3A), whereas Ni was not clearly detected on the control side (Fig. 3B). Figure 4 shows a comparison of the Ni distributions in the mucosae in contact with Ni and Ni-Ti with the same contrast. The Ni accumulated in the Ni-Ti group was remarkably less than that of the Ni group, which suggests extremely low Ni erosion from

Fig. 1  Representative hematoxylin-eosin (HE) image and elemental distribution images with synchrotron radiation X-ray fluorescence analysis (SR-XRF) of the sham surgery group.

The color bar indicates relative concentration with an arbitrary unit. In this and the subsequent figures, the scale of Ni pertains to the Ni group, and those of Co and Cr pertain to the Co-Cr group. P: phosphorus, S: sulfur, Fe: iron, Ni: nickel, Ti: titanium, Co: cobalt, Cr: chromium.
Fig. 2 Representative hematoxylin-eosin (HE) image and elemental distribution images with synchrotron radiation X-ray fluorescence analysis (SR-XRF) of the Ni group.
A: sutured side; B: control side. Color bar indicates relative concentration with an arbitrary unit.

Fig. 3 Representative hematoxylin-eosin (HE) image and elemental distribution images with synchrotron radiation X-ray fluorescence analysis (SR-XRF) of the Ni-Ti group.
A: sutured side, B: control side. Color bar indicates relative concentration with an arbitrary unit.

Fig. 4 Representative Ni distribution images of the Ni group (left) and the Ni-Ti group (right). Color bar indicates relative concentration with an arbitrary unit.

The Kα line (6.924 keV) of Co is extremely close to the Kβ line (7.057 keV) of Fe. Fe is abundant in living tissue. In order to analyze the Co distribution, it is necessary to eliminate the influence of Fe Kα. Therefore, we performed spectral deconvolution with all points and obtained the exact distribution image of Co. Figure 5 shows a demonstration of the spectral deconvolution.
spot among Co Kα, Fe Kα, and Fe Kβ at the spot of Co accumulation. The distribution image of Co obtained using this method is different from that of Fe (Fig. 6).

Thus, it is assumed that the Co distribution obtained is correct. Both Co and Cr were detected in higher amounts in the sutured side of the Co-Cr group when compared with the control side.

**XAFS**

Figure 7 shows the XANES spectra of accumulated Ni in the mucosa of the sutured sides of both the Ni and the Ni-Ti groups and those of the standard specimens. The spectra from the Ni-accumulated mucosa were clearly different from that of metallic Ni (Ni foil) but close to that of Ni(OH)₂, which suggests that Ni existed as hydrated ions in the mucosa. It was thus suggested that the eluted Ni accumulated as aqueous ions in the buccal mucosa, rather through the physical falling of metal debris.

Similarly, the XANES spectrum of the Co
accumulated in the mucosa on the sutured side of the Co-Cr group is shown in Fig. 8. In contrast to the standard specimens, it is revealed that Co was permeated into the buccal mucosa as aqueous ions in the Co-Cr group. In contrast, Cr detected in the Co-Cr group was close to that of Cr_2O_3 as a standard sample unlike Ni and Co.

**DISCUSSION**

Ni, Co, and Cr are well-known contact allergens among metals. These metals are contained in various metal alloys including stainless steels, Co-Cr, and Ni-Ti. Thus, they can be released from almost every metal orthodontic device in the oral cavity. Therefore, the most effective approach to inhibit the onset of metal allergy is thought to be blocking the release of metal, and many previous studies have revealed various conditions for metal elution. However, it is impractical to block the release of metal ions completely because the factors that may affect metal elution are numerous, and these metals can be taken from the daily diet, in which the amounts are reported to be larger than those released from orthodontic devices. On the other hand, the onset of metal allergy requires not only metal elution but also metal-binding proteins to be recognized as antigens by the dendritic cells and macrophages in nearby lymphatic tissue or basement membrane under the epithelium. That is, the infiltration of these metal ions into the mucous membrane is necessary for the onset of metal allergy.

SR-XRF was applied in this study to visualize the infiltrated metallic elements. In conventional XRF, all X-rays from the X-ray source are irradiated. Therefore, the background of the spectrum is increased by the scattered incident X-rays, which makes difficult to detect the small peaks of the trace elements obscured by the background noise. In SR-XRF, only monochromatized X-rays with high intensity are irradiated. Therefore, the background becomes negligible and the small peaks of the trace elements are easily identified. By irradiating micro-focused X-rays and scanning the specimen in one or two dimensions, elemental distribution images can be obtained. SR-XRF is highly sensitive and non-destructive, and it is widely used in the trace element analysis of medical and biological specimens. As reported in previous studies, SR-XRF analysis can be performed with thin, sectioned tissue specimens. Then, comparison with histopathological diagnosis can provide relational information between various changes in tissues and the elemental distribution. As the sulfur distribution image obtained using SR-XRF shows the shape of entire specimen, alignment among the elemental distribution images and histopathological images could be easily performed.

The chemical state of the accumulated metallic elements provides additional information concerning erosion from alloys and accumulation into the mucosa. XAFS analysis was performed to determine the chemical state. The X-ray absorption spectrum near the absorption edge of each element was strongly affected by its chemical state. Thus, the chemical state of the target elements could be estimated by comparing the XAFS spectrum to the other standard chemicals.

In our study, the localization of metals was clear in the sutured side in all groups but not detected in the control side. Although previous studies have estimated that the release of metals in the mucosa is in minuscule quantities and that the concentration of metal ions may not directly affect the onset of metal allergy, there is no report verifying the threshold value for metal ions to infiltrate the mucous membrane. Our results indicate that the infiltration of metal ions requires the direct contact of alloys with the surface of the mucous membrane. Moreover, the XAFS analysis (Figs. 7 and 8) indicates that the observed elements were not in the metallic state but in the ionic (Ni, Co) or the oxide (Cr) states. These findings confirm that the trace elements were infiltrated metal ions, rather than debris from the surgical treatment. The aqueous ions of Ni and Co could be derived from the erosion of the applied metal specimens and easily infiltrate the mucosa. Cr was estimated to exist in the oxide state; the solubility of the Cr ion is quite low, and it would be easily precipitated in the oxide state. Thus, Co would also be accumulated in the mucosa with the same process as in Ni and Co.

The HE staining and SR-XRF elemental distribution images suggest that Ni ions were mostly concentrated within the epidermis in the Ni group but slightly penetrated the lamina propria, while only limited Ni ions were scattered in the Ni-Ti group specimens. Because the epithelium is a dense structure and the lamina propria is thought to control the penetration of foreign substances, Ni ions in the Ni group may have had higher concentration and could have infiltrated the lamina propria. In a previous report concerning Ni-impregnated ion penetration into mouse skin, a distribution similar to ours was observed. In the case of the Co-Cr alloy, Co and Cr were distributed in the entire area of the specimen in the Co-Cr group. In the ICP-MS analysis, the Cr concentration was much higher than the Co concentration. The Co-Cr alloy has higher Cr content than Co. Therefore, the Co concentration in the mucosa should be higher than the Cr concentration if both elements eroded simultaneously. We suggest two possible reasons for the different trends in Co and Cr concentrations. One is the selective dissolution of Cr from the Co-Cr alloy. However, Co is usually more soluble in aqueous solutions. The other is the selective accumulation of Cr in mucosal tissues. As shown in Fig. 8, Co is in the ionic state, and Cr is precipitated as an oxide. The Co ion has higher mobility than the precipitated Cr. Therefore, Co might be diffused out through the body fluids, and Cr precipitation might be selectively accumulated locally. Further study is required to confirm these hypotheses.

In comparison with Ni accumulation in the mucosa between the sutured sides of the Ni and Ni-Ti groups, the Ni-Ti group showed extremely low Ni concentration in the SR-XRF result (Fig. 4). Furthermore, in the quantitative ICP-MS analysis of Ni concentration in
the mucosa, Ni increment was detected in the sutured tissue of the Ni group, but no Ni increment was detected in the sutured tissue of the Ni-Ti group compared to sham surgery group. Therefore, the results of SR-XRF and ICP-MS are in good agreement. Furthermore, the Ni-Ti alloy did not show obvious accumulation of Ni in the mucosal tissue with SR-XRF and ICP-MS analyses, which suggests that Ni dissolution from the Ni-Ti alloy is negligible and that the Ni-Ti alloy is relatively safe in terms of metal allergy compared to Co-Cr alloy. The corrosion resistance of stainless steel has been well studied through in vivo implantation\(^\text{20}\) and in vivo estimation with artificial saliva immersion\(^\text{20}\). The mechanisms suggested that the participation of lipopolysaccharide release and filtration into the tissues surrounding the metal alloy accelerates the metal-ion mucosa membrane when the metal alloys are in contact our results may explain the mechanism whereby even of metal allergy requires the antigen-presenting cells to inhibit metal allergy, which causes orthodontic patients to suffer from inflammation of the oral mucosa due to the contact of orthodontic devices.

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