The Increase Of IL-1β and IL-6 In Oral Epithelial Cells Induced by Corrosion Products of Multiple-Recast Palladium-Silver Dental Alloy

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Abstract

Reuse of dental alloys is common in dental laboratory but the effect of reused alloys on pro-inflammatory cytokines in oral epithelial cells is scarce. The aim was to investigate the epithelial inflammatory responses stimulated by corrosion products of multiple-recast palladium-silver alloy. A commercial palladium-silver (Pd-Ag) dental alloy was cast and recast up to four times. They were immersed in artificial saliva for 15 days to determine the corrosion process. The corrosion products in artificial saliva were investigated by using inductively coupled plasma mass spectrometry (ICP-MS). Oral epithelial cells were cultured with corrosion product from single- or multiple-recast alloys. IL-1β, IL-6, TNF-α mRNA and protein expression were determined by quantitative RT-PCR and ELISA, respectively. The results were analyzed by one-way ANOVA, and post hoc Tukey's test at a significance level of 0.05. Corrosion products released was increased in the second and fourth-recast groups. The change in alloy microstructure demonstrated different amount of light- and dark-gray matrix after recasting. The expression of the inflammatory cytokines IL-1β and IL-6 were significantly unregulated in the recast groups. Recast Pd-Ag alloy resulted in increased corrosion products and this would induce an inflammatory reaction in oral epithelial cells.

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Introduction

The mechanical, physical properties and biocompatibility of dental alloys must be carefully considered when selecting which alloy to use. Most studies show that release of gold or Pd ions is rarely detected in vitro in any solutions used as supernatant, while the most common detectable metal ions are Ag, copper and zinc.[1-3] One study has demonstrated that gold-based or Pd-based alloys may also induced inflammatory reactions and were reported as causative agents in cases of stomatitis, oral lichenoid reactions and disseminated urticarial.[4] Pd-based alloys are a commonly used alternative to gold alloys. The corrosion behavior of dental alloys suggests their biocompatibility. One study showed that Pd-Ag alloys containing 50% or 75% Ag demonstrated the highest corrosion resistance[5], while the other study claimed that the corrosion is not detectable in alloys consist of more than 40% Pd.[6]

Reusing of alloy is a common dental laboratory procedure. However, it is unknown if the multiple-recast alloys adversely affect the oral soft tissue. Many studies have reported the significant changes in various based-metal alloy properties after recasting either with or without adding new alloys. These changes include compromised bond strength to porcelain[7] or decreased elastic modulus.[8] The increased corrosion products after recasting potentially increase the toxicity of alloys.[9,10] Several studies reported the toxic effect of metal ions on TR146 human oral epithelial cells which represent the oral epithelium[11], and fibroblast model.[12] The epithelial cells and fibroblasts may be induced to release inflammatory mediators such as tumor necrosis factor (TNF-α) and interleukins (IL) such as IL-1β or IL-6.[13] These inflammatory cytokines are important for immune modulation. IL-1β is a

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signature innate cytokine that is associated with inflammatory cell migration and osteoclastogenesis. In addition, TNF-α is a potent inflammatory cytokine that upregulates the production of collagenases, prostaglandin (PG) E_2, chemokines and cytokines, cell adhesion molecules, and bone resorption related factors.[14] These mediators also play an important role on periodontal inflammation. Little is known of the cellular responses to multiple-recast alloys, especially recast Pd-based alloys. Therefore, the aim of this study was to investigate the effect of the corrosion products released from multiple-recasting Pd-Ag alloys on the upregulation of inflammatory genes in normal oral epithelial cells. The characteristics including alloy composition and its microstructure were examined.

Materials and methods

Specimen preparation

Pd-based alloy specimens (10 mm x 5 mm and 1.0 mm) consisting of 53% Pd and 38.2 % Ag and other elements (7% Sn, <1% Cu, <1% Zn and 1% In) (Aurolite 1C, Aurium research, USA) were cast following the manufacturer's instruction using conventional casting machine (Argoncaster-AE, Shofu, Japan) with phosphate-bonded investment (Bellavest® SH, BEGO, USA). For the first-cast group, the specimens were prepared by being cast only once and cut into required specimen size. Remaining alloy including sprues and buttons were kept to reuse for the next recasting groups. These methods were repeated for multiple-recasting up to 4 times so the recast specimen groups were recast without adding new alloy. The residual investment material on the specimen surface was removed by alumina particle air abrasion. The specimens were ultrasonically cleaned and polished up to 1200 grit sandpapers before testing.

Immersion test

The specimens of the first-cast and recast groups (second- and fourth-cast groups) were immersed in 6 mL of artificial saliva containing 0.07 g/L $MgCl_2$, 0.75 g/L KCl, 0.439 g/L KH_2PO_4, 0.005g/L NaF, and 0.965 g/L K_2HPO_4 buffered to pH 6.7. The specimens were then incubated at 37^o C for 15 d. After immersion, the concentration of the metallic elements in artificial saliva were measured using Inductively Coupled Plasma Mass Spectrometry (ICP, Thermo scientific ICAP QC, USA) in parts per billion (ppb) and analyzed. But any dissolved elements with a value lower than the detection limit was considered to be 0 ppb.

Surface characteristics and chemical characterization

After the immersion test, one sample from each group was randomly chosen for microstructure observation. The specimens were polished until achieving a mirror surface and were etched in 45 ml glycerol with 15 ml nitric acid and 30 ml hydrochloric acid for 1 sec. Microstructures of all specimens were observed using scanning electron microscope (JSM-6400, JEOL, USA). The elemental analysis or chemical characterization of the specimens was then analyzed by energy dispersive X-ray spectroscopy (EDS).

Co-culture of normal human oral keratinocytes

The inflammatory response of oral epithelial cells was examined. A normal human oral keratinocytes spontaneous immortalized (NOK-SI) cell line was established from oral mucosal epithelium as previously described and grown in keratinocyte serum-free medium (KSFM; Invitrogen, Carlsbad, CA) supplemented with 0.1mM $CaCl_2$ (keratinocyte medium) at 37°C in a humidified 5% CO_2 atmosphere.[15] The cells were cultured (2.5×10^5 cells/well) in keratinocyte medium to form a monolayer in 6-well tissue culture plate (Corning® Life Sciences, Tewksbury, MA) overnight. Cells in media and artificial saliva groups were served as a negative control. Cells incubated with 2.5 μg/mL *P.gingivalis* LPS (InvivoGen, San Diego, CA, USA) represented an inflammatory response as a positive control. The corrosion product in artificial saliva from each group was added into the culture, and incubated for 24 h. The NOK-SI cells were then collected for quantitative RT-PCR to detect the mRNA expression of TNF-α and IL-1β, and the NOK-SI culture supernatant was collected for ELISA.

Quantitative RT-PCR

Total RNA of NOK-SI cells from each group was isolated using TRIzol Reagent (Invitrogen, Milan, Italy) and 1 μg was reverse transcribed using the RevertAid H Minus First

Strand cDNA Synthesis Kit (Fermentas, Hanover, MD) and random primers System (Invitrogen, Milan, Italy). Quantitative SYBR Green PCR analysis on CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA) was performed to evaluate the mRNA expression. The expression of TNF-α, IL-1β, and IL-6 induced by the corrosion products was investigated. The qPCR reactions were performed in a total volume of 25 µL, containing 250 – 500 ng cDNA at 95°C for 1 min followed by 40 amplification cycles consisting of 95°C for 45 sec, 60°C for 60 sec, 72°C for 90 sec, and one extension cycle at 72°C for 10 min. The reactions were performed in duplicate, and the average values were used for gene expression analysis. The data for comparative analysis of gene expression was obtained using the Ct method. The 18S mRNA expression was used as an internal control. The qPCR products were stained with ethidium bromide on a 1.8% agarose gel to confirm the specific product size.

Enzyme-Linked Immunosorbent Assay (ELISA)

The expression of TNF-α, IL-β, and IL-6 induced by the corrosion products from all groups of specimens were analyzed. ELISA for TNF-α, IL-1β and IL-6 proteins in the epithelial cell supernatant was performed. ELISA microplates were coated overnight with their respective capture antibody. Epithelial supernatant was incubated at room temperature for 2 h. The wells were rinsed with ELISA wash buffer, and incubated with the substrate solution for 20 min. The absorbance was measured at 450 nm using a microplate reader. A standard curve was generated using standards, and the concentration of each protein was determined.

Statistical analysis

The data were analyzed using SPSS 18 program (SPSS, Chicago, IL, USA). The normal distribution and variance of the data were analyzed by one-way ANOVA, and post hoc Tukey's test at a significance level of 0.05.

Results

Surface characteristics and chemical characterization

Scanning electron microscopy revealed the microstructure of the Pd-Ag alloys after casting. The first-cast alloy demonstrated as fine dendritic structure with a light gray grains surrounded by large dark-gray areas (Figure. 1A). Many small and bright particles can be seen scattering inside the dark-gray area. The EDS analysis (Table 1) of the localized trace elements in the light- and dark-gray matrix revealed that the light-gray matrix was rich in Pd while the dark-gray area was enriched with Ag. Both areas showed trace amounts of Sn while Zn, Cu and In were undetectable.

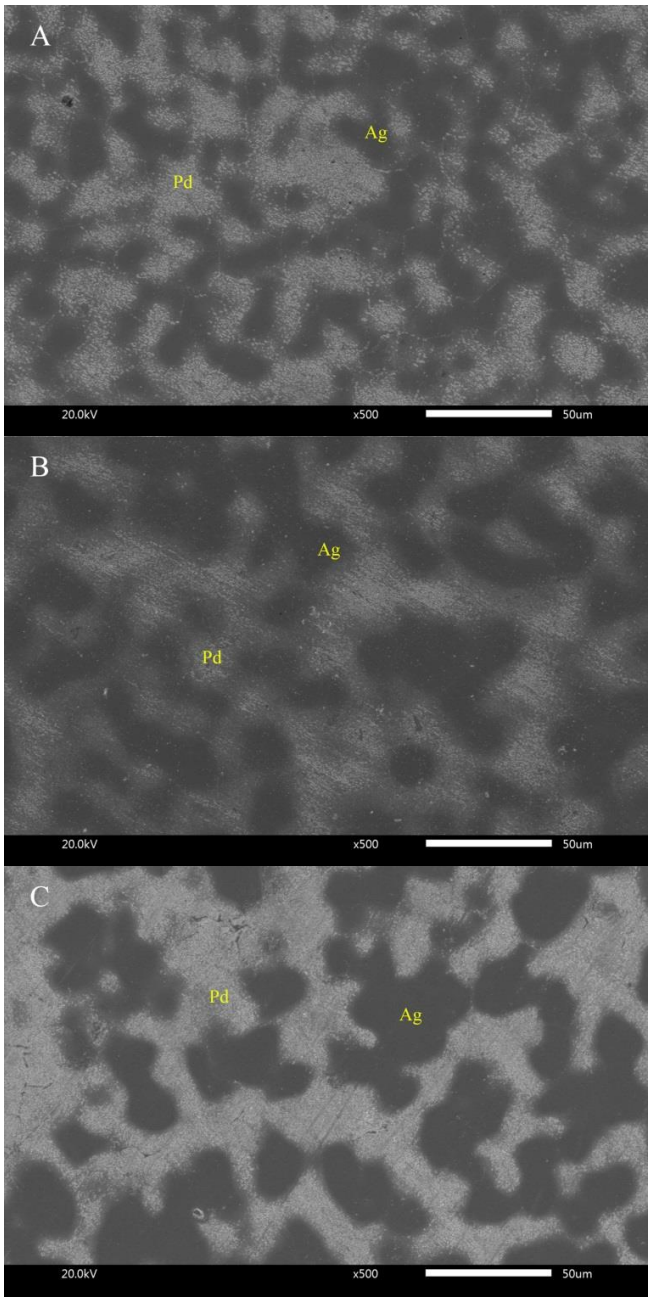


Figure 1. SEM micrographs of the Pd-Ag alloy at 45× magnification (**A**) First-cast group (**B**) Second-cast group (**C**) Fourth-cast group.

The trace elements detected in the light and dark-gray matrices remained similar after multiple castings. However, the dark-grey matrix areas obviously displayed smaller, while the light-grey matrix areas enlarged in the recast groups. The surface area of the light-gray matrix estimated by Image J® software was 72% of the total surface area in the first-cast alloy, and light-gray matrix area increased to 91% and 94% in the second-cast and the fourth-cast alloy, respectively.

Alloy	Elements					
	Pd	Ag	Sn	Zn	In	Cu
First-cast group						
Dark-gray area	50.89 ±1.11[a]	42.98 ±1.32[a]	6.13 ±0.36[a]	N/A	N/A	N/A
Light-gray area	57.59 ±1.85[b]	35.75 ±1.54[b]	6.66 ±0.32[b]	N/A	N/A	N/A
Second-cast group						
Dark-gray area	50.17 ±2.58[a]	44.38 ±3.05[a]	5.45 ±0.85[a]	N/A	N/A	N/A
Light-gray area	62.77 ±1.33[b]	29.54 ±0.96[b]	7.69 ±0.42[b]	N/A	N/A	N/A
Fourth-cast group						
Dark-gray area	51.26 ±1.16[a]	42.60 ±1.13[a]	6.14 ±0.38[a]	N/A	N/A	N/A
Light-gray area	61.00 ±3.61[b]	31.88 ±3.72[b]	7.12 ±0.33[b]	N/A	N/A	N/A

Table 1. Average of trace elements (wt%) in dark-gray and light-gray matrix of dendritic structure examined by EDS analysis.
Values with different lower case letters in same column of each alloy are significantly different at P<.05.

Ion release

The metal ions released from the cast Pd-Ag alloys after immersion in artificial saliva for 15 d were detected by ICP analysis (Table 2). Consistent with EDS analysis of the alloys, Zn, Cu, Sn and In were not detected in the artificial saliva supernatants. Ag released from the multiple-recast alloys significantly decreased (*P*<.05) while the Pd released from the second-cast and fourth-cast alloys significantly increased compared to the first-cast groups (*P*<.05).

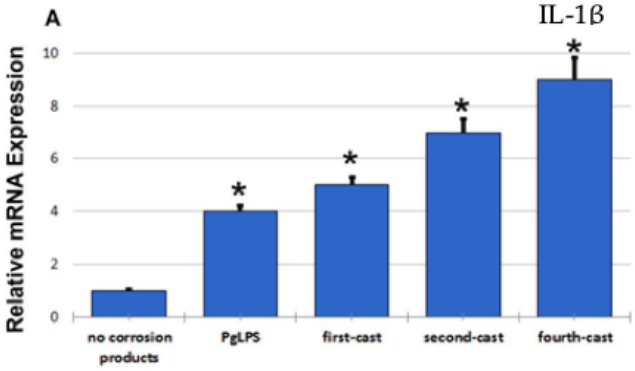
Alloy (Aurolite 1C)	Elements (Released ion in ppb)					
	Pd	Ag	Sn	Zn	In	Cu
First-cast group	1.307 ±0.054[a]	0.099 ±0.009[a]	–	–	–	–
Second-cast group	2.707 ±0.049[b]	0.029 ±0.004[b]	–	–	–	–
Fourth-cast group	1.701 ±0.083[b]	0.024 ±0.003[b]	–	–	–	–

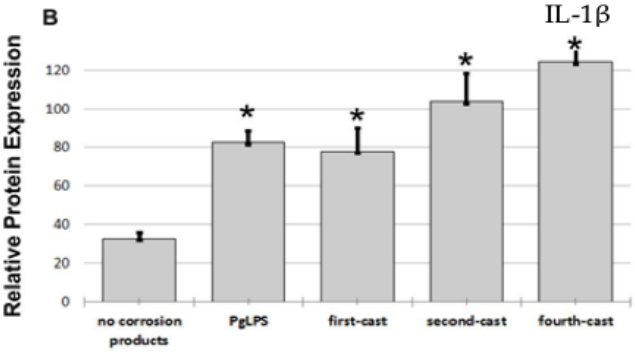
Table 2. Immersion test results from first and recast of Palladium-silver alloys in artificial saliva after 15 days pH 6.7 at 37° C
Values with different lower case letters in same column for each alloy are significantly different at P<.05.

Inflammatory responses

To investigate the inflammatory response of epithelial cells to the corrosion products of Pd-Ag alloys, the normal oral keratinocytes were incubated with immersion media from first-cast, second-cast and fourth-cast alloys for 24 hrs. The cytotoxic test using the MTT assay was performed at 24 h, and none of the treatment markedly affected cell viability (data not shown). Cells incubated with artificial saliva and cells incubated with 2.5 μg/mL *P.gingivalis* LPS were included as a positive control to represent an inflammatory response. The level of IL-1β mRNA (Figure. 2A) and protein (Figure. 2B) was significantly upregulated in response to *P.gingivalis* LPS (*P*<.01). While the level of TNFα mRNA remained unchanged (Figure. 2C), TNF-α protein level is upregulated after multiple casting (Figure. 2D). In addition, the IL-1β released in the culture supernatant (Figure. 2B) significantly increased after incubating with immersion media from first-cast, second-cast and fourth-cast alloys (*P*<.01). There was no significant difference in IL-1β release between the second-cast and fourth-cast groups (*P*>.05).

The levels of IL-6 mRNA (Figure. 2E) and protein (Figure. 2F) were significantly upregulated in the second- and the fourth-cast groups (*P*<.01), however, the levels of those in the first-cast group were unchanged compared with baseline. Although there was no significant difference in IL-6 between the second-cast and fourth-cast group (*P*>.05), IL-6 expression in the first-cast was significantly lower compared with the multiple-recast groups.





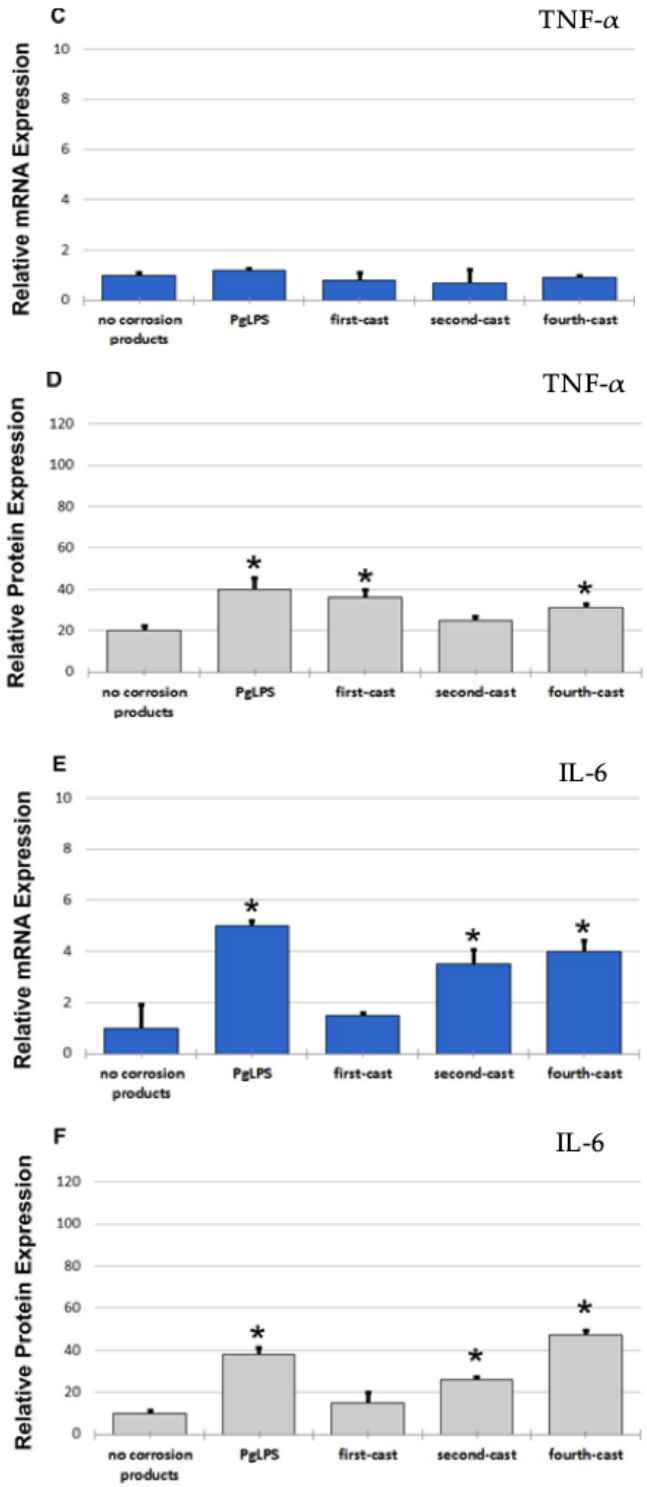


Figure 2. Corrosion products from recast alloys upregulate inflammatory cytokine mRNA expression. Relative expression of (A) IL-1β (C) TNF-α and (E) IL-6, mRNA in normal oral keratinocytes after incubated with the different corrosion products from first-cast and recast groups compared to non-stimulated cells (no corrosion product). All experiments were performed in duplicates.

ELISA detection of TNF-α, IL-1β and IL-6 released in the culture supernatant of normal oral keratinocytes after co-cultured with corrosion products from first-cast and recast group for 24h as described in materials and methods. (B) IL-1β (D) TNF-α and (F) IL-6 downregulation in NOK-SI cells treated with corrosion product from first-cast and recast groups of Pd-Ag alloy. All experiments were performed in triplicates.

*indicates a significant difference using One-Way ANOVA test followed by Turkey's HSD (P<.05)

Discussion

This study investigated the release of corrosion products after recasting and the inflammatory responses by epithelial cells to the corrosion products. It has been reported that microstructural changes occurred in alloys after recasting due to increased grain dimensions, impurities and porosities.[16] These changes could affect the mechanical and physical properties of alloys. In this study, the microstructure analysis revealed the light- and dark-gray area in the microstructure suggesting a complete miscibility, forming a homogeneous solution, of all compositions in solid solution of Pd-Ag alloy. Moreover, the light-gray area was Pd-rich compared with dark-gray area as demonstrated by EDS analysis. The large dark spots randomly distributed within the matrix represent a porous surface that increased in the fourth-recast group and may affect the corrosion behavior of the alloy.

The release of elements from the alloy can induce adverse biologic reactions such as toxicity, allergy, or mutagenicity. Corrosion can be observed by visualizing the change in the alloy surface, by estimating the elemental release indirectly through the flow of the released electrons, or by measuring the release of the elements. The present study demonstrated that Pd-Ag alloy corroded in artificial saliva. Release of Pd and Ag were detected while other elements could not be found in any groups after immersion test. This finding is consistent with previous studies where Ag release was commonly found[2,17], however, the study reported that Pd was not detectable.[3] These different results may be due to the limitation of measurement of elemental ion release. This study found that Pd release was detected after 15-day immersion test and the Pd level increased in multiple-recast groups. Because Pd is the main component

found in the alloy microstructure after multiple casting, dissolution of Pd from these alloys is therefore in relative proportion to their Pd composition. Alloy corrosion can result in high amounts of Ag ions in saliva and caused localized tissue response or systemic side effects.[18] However, the cytotoxicity of Ag ions remains relatively low compared with other base metal alloys such as zinc, copper or nickel.[19] Other studies suggested that the deposition of a layer of newly-formed silver chloride on an alloy surface may also reduce the toxicity of Ag released from Pd-Ag alloys.[20,21]

The biologic response to released elements depends on the type of elements, the quantity released and the duration of tissue exposure. We could detect only two metal elements released from the recast dental alloy. However, we did not investigate the effect of individual metallic ion which will have the most unfavorable effect on cytokine of inflammatory response. Therefore, the term of corrosion product was used in this study to represent the released metal ions. Because epithelial cells are positioned close to the dental restorations, the expression of inflammatory cytokines in epithelial cells is another key marker to examine the host response to external stimuli such as corrosion products. We found that the key inflammatory-related cytokines were expressed in normal oral epithelial cells when they were challenged by the corrosion products of the first-cast, second-cast and fourth-cast Pd-Ag alloy groups. The pro-inflammatory cytokines, IL-1β and IL-6, were upregulated in response to the corrosion products from the recast group compared with the first-cast group and non-stimulated cells. In contrast, TNF-α expression was not stimulated by the corrosion products compared with the non-stimulated cells. The significant increase in IL-1β and IL-6 expression suggests that the nuclear factor-κB (NF-κB) pathway[22] may be activated by the corrosion products from the recast group, and this may trigger inflammation in the absence of infection.[23] IL-1β[24] and TNFα represent the pro-inflammatory response cytokines that are rapidly released on tissue injury or infection and are involved in periodontal tissue inflammation, loss of attachment and bone resorption.[14] However, IL-6 is reported as a pro-inflammatory response cytokine with pleiotropic function of an epithelial regeneration-promoting factor.[25] A previous study demonstrated that levels of IL-1β and IL-6 in gingival tissues are closely related to the severity of periodontal disease compared with healthy gingival tissue.[26] In addition, the concentration of IL-1β and IL-6 in gingival crevicular fluid in chronic periodontitis patients is higher than that of healthy patients.[27] The regulatory effect of IL-1β is concentration-dependent. Low doses of IL-1β activate the BMP/Smad signaling pathway and promote osteogenesis whereas higher doses of IL-1β activates NF-κB and mitogen-activated protein kinase (MAPK) signaling to that inhibits osteogenesis.[28] Whether the corrosion products from dental alloys contributed to inflammatory responses remains to be elucidated in an in vivo model. The release of theses cytokines may lead to sequential release of lipid mediators, cytokines, and chemokine that drive recruitment and activation of other inflammatory cells.

Conclusions

Recasting altered the physical properties and increased ion release of Pd-Ag dental alloys. The increased corrosion product (released metallic ions) from multiple-recast Pd-Ag alloys induces oral epithelial cells to secrete IL-1β and IL-6 in vitro. These pro-inflammatory response cytokines are also found in periodontitis patients. Therefore, the corrosion products from recast Pd-Ag alloy could increase the severity of periodontal tissue inflammation.

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Declaration of Interest

The authors declare no conflict of interest.

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