Gene Expression of Epithelial Cells Induced by Corrosion Product of Recast Palladium - Silver Alloy

การแสดงออกของยีนในเซลล์เยื่อบุที่ถูกกระตุ้นโดยผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินที่ผ่านการหล่อซ้ำ

Verapol Singkarlsiri (วีรพล ศฤงคารศิริ)* Dr.Viritpon Srimaneepong (ดร.วิริทธิ์พล ศรีมณีพงศ์)**
Dr.Anjalee Vacharaksa (ดร.อัญชลี วัชรักษะ)***

ABSTRACT

This study aimed to investigate the expression of TNF-α from epithelial actuated by the corrosion products from recast palladium-silver alloy. Three specimens of each group from first cast and recast Pd-Ag alloy ($10*15*1mm^3$) were submerged in artificial saliva for 7 days. The oral epithelial cells cultured in monolayer were challenged by corrosion product from different groups of alloys. Quantitative RT-PCR was used to examine the mRNA gene expression of TNF-α. The results showed that the response on TNF-α mRNA from corrosion product in the recast group was significantly higher than that from the first cast group. This implies that the more corrosion product from recast group could induce more gene expression of TNF-α in the epithelial cell which could relate to more periodontal inflammation.

บทคัดย่อ

การศึกษามีวัตถุประสงค์ในการศึกษาการตอบสนองการอักเสบของเซลล์เยื่อบุต่อผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินที่ผ่านการหล่อซ้ำโดยดูจากการแสดงออกของยีนทูเมอร์เนโครซิสแฟคเตอร์อัลฟา การทดลองเริ่มโดยเตรียมชิ้นงานโลหะเจือแพลเลเดียมและเงิน 2 กลุ่มๆละ 3 ชิ้นคือกลุ่มที่หล่อครั้งแรก และ กลุ่มที่หล่อซ้ำ ขนาด 10*15*1 ม.ม.^{3} นำชิ้นงานโลหะที่ขัดและทำความสะอาดแล้วแช่ในน้ำลายเทียมเป็นเวลา 7 วัน นำสารละลายที่ได้ไปทดสอบกับเซลล์เยื่อบุและทำการวัดค่าการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนทูเมอร์เนโครซิสแฟคเตอร์อัลฟา โดยใช้วิธีการรีเวอร์ทรานสคริปชั่น-พีซีอาร์ (RT-PCR) ผลจากการทดลองพบการตอบสนองต่อการอักเสบเพิ่มขึ้นอย่างชัดเจนของเซลล์เยื่อบุต่อผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินในกลุ่มหล่อซ้ำจากการเพิ่มขึ้นของการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนทูเมอร์เนโครซิสแฟคเตอร์อัลฟา กล่าวโดยสรุปคือผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินในกลุ่มหล่อซ้ำมีผลทำให้เกิดการกระตุ้นการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนทูเมอร์เนโครซิสแฟคเตอร์อัลฟา เพิ่มขึ้นซึ่งอาจมีความเชื่อมโยงกับการเกิดการอักเสบในโรคปริทันต์ได้

Keywords: Palladium-silver Alloy, Gene expression, Recast
คำสำคัญ: โลหะเจือแพลเลเดียมกับเงิน การแสดงออกของยีน การหล่อซ้ำ

* *Student, Master of Science Program in Prosthodontics, Faculty of Dentistry, Chulalongkorn University*
** *Assistant Professor, Department of Prosthodontics, Faculty of Dentistry, Chulalongkorn University*
*** *Lecturer, Department of Microbiology, Faculty of Dentistry, Chulalongkorn University*

Introduction

Palladium-based alloy which containing more than 75% of palladium become more popular and are used as alternatives to gold-based alloys (Carr, Brantley, 1991). To reduce the cost of alloys, silver is added to subsidize amount of palladium. Palladium-silver alloys may not contain gold or only a small amount of gold, but at least 25% of the alloys must be palladium for properties of tarnish resistance and nobility. In dental practice, noble and semi-noble alloys are commonly reused. While recasting of base metal alloy is also performed in some laboratories. There are significant changes in some properties of based-metal alloys after recasting, including compromising bond strength to porcelain in porcelain-fused to metal restoration (Madani et al., 2011), decreasing in corrosion resistance (Jabbar, 2008; Oyar et al., 2014) and lower elastic modulus (Mirkovic, 2007). One study shows changing in the elemental composition, hardness, and corrosion behavior of recast palladium-silver alloy, especially in fifth recasting, (Horasawa, Marek , 2004). In oral environment which is highly conductive to electrochemical action (i.e. warm, moist and subjected to wide fluctuation in temperature and pH), metal restoration can release metal ions as a result of chemical reactions, electrochemical forces or mechanical forces. Moreover, the metal restoration margin is in close proximity to the sulcular epithelium. Therefore the epithelial cells may encounter the released metal ions as an external stimulus. In biocompatibility test, corrosion resistance should be carefully taken in consideration for dental alloy selection. The deposition of the metal elements in gingiva from dental casting alloys was reported in a clinical study. Silver remnants in metallic pigmented gingiva (Venclikova et al., 2007) and in biopsies of pigmented gingiva adjacent to dental casting alloys (Garhammer et al., 2003) appeared to be prominent. Some study reported that not only the base metal alloys caused the allergy, but gold based or palladium based alloys can also induced the allergy and were reported as a causative agents in cases of stomatitis, oral lichenoid reactions and disseminated urticarial (Hensten-Pettersen, 1992).

Little is known of the inflammatory responses of epithelial cells and the metal alloys biocompatibility to the recasting palladium base of alloys. Some study reported that the release of inflammatory parameters can be detected under non-toxic or only mild toxic experimental conditions after exposure compounds of dental materials (Schmalz et al., 2000). Under pathologic condition, balance between pro- and anti-inflammation is tipped toward proinflammatory activity. Many proinflammatory cytokines including TNF-α are released during periodontal destruction. TNF-α is a potent inflammatory cytokine that upregulates the production of collagenases, prostaglandin (PG) E2, chemokines and cytokines, cell adhesion molecules and bone resorption related factors. Moreover, TNF-α are also play a major role in bone loss gingival and periodontal inflammation (Graves, Cochran, 2003). The excessive production of TNF-α is cause of periodontal tissue destruction by overreaction of host response to periodontal pathogens in periodontitis.

Objective of the study

This study aimed to investigate the gene expression of TNF-α from epithelial cell induced by the corrosion product from recast palladium-silver alloy in order to understand the relation with progression of periodontal inflammation.

Materials and methods

Specimen preparation

A palladium base alloys (Aurolite 1C, Aurium research, USA) which consist of 38.2 % of silver and 53% of palladium (in mass %) as a major components, were employed (table 1). Three square-shaped specimens (10 mm x 15 mm and 1.0 mm. thickness) of alloy were prepared for first cast group (control group) from wax pattern and connected by 3 mm diameter of the sprue to the sprue former and positioned in the center of casting ring using phosphate bonded investment. Alumina particle air abraded was used to remove the residual investment material from each casting and all specimens were ultrasonic cleaned. The all alloy specimens were polished with sandpaper up to No 1200. For recast alloy group, three specimens were prepared by using 100% once-cast from first cast group specimens including sprues and buttons which were cleaned in dilute sulfuric acid to remove the oxidation film for 5 seconds before reusing.

Table1 Composition of Palladium based alloy (Aurolite 1C)

Palladium	53.0 %
Silver	38.2 %
Tin	7.0 %
Indium	1.0 %
Copper	< 1 %
Zinc	< 1%

Observation of microstructure

One sample of each group was randomly chosen for microstructure observation. The specimens were polished on the polishing discs which were covered with soft cloth impregnated with 6 microns and 1 micron in diameter abrasive diamond particles and an oily lubricant until becoming mirror surface. After polishing, the specimens were etched using reagent which consisted of 45ml Glycerol, 15ml Nitric Acid and 30ml Hydrochloric Acid for 1 second. Microstructure was examined with a stereo microscope (SZ 61, OLYMPUS, USA). Grain size was calculated in microns and casting defects are examined.

Immersion test

Specimens of first cast and recasting groups were immersed in 6 mL of artificial saliva (0.07 g/L $MgCl_2$, 0.75 g/L KCl, 0.439 g/L KH_2PO_4,0.005g/L NaF0,0.965 g/L K_2HPO_4) which is buffered to pH = 6.7 and incubated at 37^{o}C for 7 days. After 7 day of immersion, specimens were removed from the artificial saliva. The metallic elements in artificial saliva were measured by an inductively coupled plasma mass spectrometry (ICP, Agilent 7900 ICP-MS, USA) in ppb (parts per billion) . The determination of each test solution was performed in duplicate. The concentrations of any dissolved elements whose values were lower than the detection limit were considered to be 0 ppb.

Cell culture test

A normal human oral keratinocytes spontaneous immortalized (NOK-SI) cell line were 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 atmosphere of 5% CO_2. For the experiments, cells were cultured ($2.5x10^5$ cells/well) in keratinocyte medium to form a monolayer on the transwell inserts 6 well with membrane pore size 0.04 μ (Corning® Life Sciences, Tewksbury, MA) overnight. When approximately 70 % confluent, the cells were subcultured by using 0.25 % trysin-EDTA (Gibco) and plated at 1:4 ratio. Cells from passages 70-73 were used in the experiments. Then, corrosion product from each group was added to the top of the monolayer, and incubated for 24 h. Cells in media and artificial saliva groups were used as a control. The NOK-SI cells were then collected for quantitative RT-PCR to detect TNF-α gene expression.

Quantitative RT-PCR

Total RNA of NOKs cell from each group was isolated using TRIzol Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions 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), according to the manufacturer's instructions. Quantitative SYBR Green PCR analysis on CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA) was performed to evaluate the mRNA expression of TNF-α. The sequences of the primers of the genes analyzed in PCR are shown in Table 2. Reactions of qPCR were performed in a total volume of 25 μL, containing 250 to 500 ng of cDNA. The qPCR was performed at 95°C for 1 min followed by 40 amplification cycles consisting of 95°C for 45 s, 60°C for 60 s, 72°C for 90 s, 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. Analysis of genes expression was performed using CFX Manager™ Software (Bio-Rad, Hercules,CA). Data for comparative analysis of gene expression were obtained using the Ct method. GAPDH mRNA expression was used as an internal control. The PCR products were stained with ethidium bromide on a 1.8 % agarose gel to confirm the specific product size.

Table 2 Sequences of PCR primers (following the reported sequences from GenBank)

Gene	Primer Sequence 5' ⟶3'	Accession No.	bp
qTNF-α	F : CCG CTG TCT GCT TCA CGC T R : CTG GTC CTG GTT CAC TCT C	EU047718	186
qGAPDH	F : CAC TGC CCA CGT GTC AGT GGT G R : GTA GCC CAG GAT GCC CTT GAG	NM 002046.4	121

Data analysis

Descriptive analysis was used to explain the result between the expression of TNF-α and concentration of existing ions in corrosion for the different group of specimens.

Results

The results from ICP analysis showed that Zinc, Copper and Indium were not detected. Ag was more higher in recast while Pd was lower in recast group (p <0.05) (Table 3).

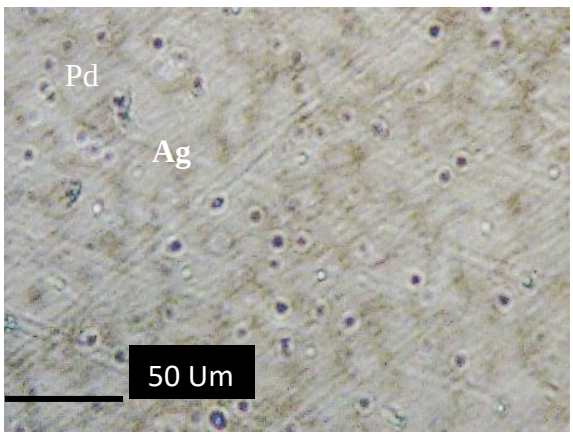
Table 3 Average concentration (ug/L) of elements from first and recast of Palladium-silver alloys.

Alloy	Element				
	Pd	Ag	Cu	Zn	In
First cast group					
Average	3.39	0.54	-	-	-
St.dev	0.05	0.03	-	-	-
Recast group					
Average	0.64	0.79	-	-	-
St.dev	0.02	0.05	-	-	-

Metallographic observation

The microstructure of Pd-Ag alloy was fine dendritic structure and displayed a bright and dark matrix. The bright matrix was rich of Pd while the dark was Ag. There were dark spots which randomly distributed within the matrix. Those dark spots were base metal in Pd based alloy (figure 1A). After recasting, grain size were a larger than first cast group (figure 1B).

A



B

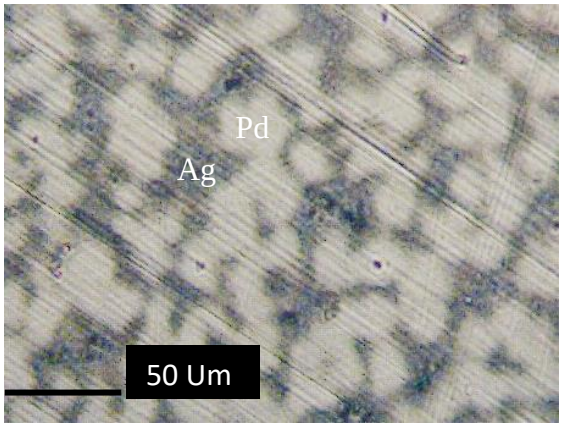


Figure 1 Typical micrographs of the Pd-Ag alloy at 10X magnification (A) the first cast group showing the fine dendritic structure with randomly distributed regular dark spots. (B) The recast group displaying a larger of

Gene expression

In a reverse transcriptase PCR, TNF- α mRNA levels in response to corrosion product of recast group were 7- and 70 - fold higher than first cast group and cell in artificial saliva (control), respectively (Figure 2).

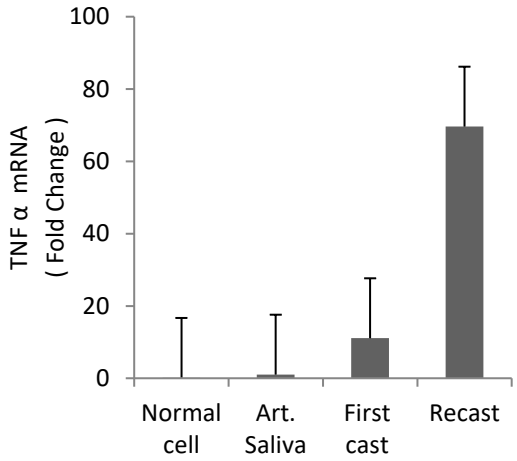


Figure 2 TNF-α mRNA transcription from NOK-SI cell incubated with the different corrosion product from first cast and recast group relative to non-stimulated cells .TNF α expression in NOK-SI cells treated with corrosion product from first cast and recast group of Pd-Ag alloy. All experiments were performed in duplicate.

Discussion

By observation of the microstructure of recast alloy, the increasing in grain dimensions, impurities and porosities could affect the mechanical properties of alloys (Reisbick, Brantley, 1995). In our microstructure analysis, we can see a lager grain size in recast group of Pd-Ag alloy. The new grain size may be different from the original one by remelting and re-solidification during recasting. If the melting temperature and casting conditions are the same, the new grain size depends mainly on the concentration of nuclei for grain formation. Furthermore, Although impurities may facilitate grain nucleation but increasing of impurities, such as oxides, nitrides and carbides, which impede the motion of dislocations and may be one of the causes of the reduction in the corrosion resistance with repeated recasting (Horasawa, Marek, 2004).

The corrosion resistance of dental alloys in oral environment can be related to its biocompatibility. We found that Ag was detected while In, Cu and Zn were not found in both first cast and recast group of the Pd-Ag alloy. This could be due to small amount of element and limitation of measurement. Pd was also detected in solution in both groups after immersion test for 7 days. While previous study reported that Pd was not detectable using atomic absorption spectroscopy (AAS) (Wataha, Hanks, 1996). This disagreement may be due to different method of measurement. Interestingly, we found the decrease of Pd in corrosion product from the recast group.

Many solutions were used in corrosion test such as 0.9% sodium chloride (NaCl), 1% lactic acid and artificial saliva etc. In this study, artificial saliva was used to mimic the oral environment. Palladium is the main

component in high palladium-based alloys. Although palladium was claimed to have a potential adverse biological effects (Wataha, Hanks, 1996), dissolution of palladium from these alloys is low.

For the biocompatibility test, the expression of TNF-α from the epithelial cell culture was observed because this proinflammatory maker is usually found and release under non-toxic or mildly toxic experimental conditions. Epithelial cell cultures are selected because epithelial cells which are usually close to the metal restoration in oral cavity. TNF-α which plays a major role in gingival and periodontal inflammation, was also selected to investigate the inflammatory response of epithelial cell to the corrosion product. In this study, we want to relate this response to periodontitis which may contribute to periodontal disease severity. From the results, we found that TNF-α was unregulated by the corrosion product from recast group when compare to the first cast group, control group and non- stimulated cells. TNF-α was regulated by the transcription factor nuclear factor kappa B (NF-□ B) (Gupta et al., 2005) and metal nanoparticles (NP) can activates the NF-□ B by mediating Reactive oxygen species (ROS) (Persichini et al., 2006). Therefore, this could be the reason of our finding that the more corrosion product from recast group induced more gene expression of TNF-α in epithelial cells by using NF-□ B pathway. The corrosion product could affect the TNF-α mRNA gene expression. Now, we know that the corrosion product from recast Pd-Ag alloy can increase the TNF-α mRNA gene expression in epithelial cell. However it is still not proved that Ag is element in corrosion product that play the important role to increase TNF-α. It is known that Ag cloud be the primary cause of cytotoxicity. Therefore, this question should be further responded.

Conclusion

Within the limitation of the study, it could be concluded that the recasting process increased some elemental releasing of Pd-Ag alloy. The corrosion product from recast Pd-Ag alloy induce epithelial cell to express TNF-α which is the proinflammatory marker found in periodontal inflammation. We suggest that the 100% recasting metal restoration of Pd-Ag alloy without adding new alloy is harmful.

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