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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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THE INFLAMMATORY RESPONSE OF EPITHELIAL CELL TO CORROSION PRODUCTS FROM RECAST PALLADIUM –SILVER ALLOY

Mr. Verapol Singkarlsiri

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A Thesis Submitted in Partial Fulfillment of the Requirements
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มีการใช้โลหะเช่นแพลเลเดียมกับเงินมาผสมในโลหะเจือที่ใช้ในทางทันตกรรมมากขึ้นเพื่อทดแทนการใช้ทองและแพลทินัมทำให้ต้นทุนลดลงแต่ยังคงหรือเพิ่มคุณสมบัติให้ดีขึ้นเทียบเท่าเดิม อีกวิธีของการประหยัดต้นทุนคือการนำโลหะเจือที่ผ่านการขึ้นชิ้นงานกลับมาหล่อซ้ำ ได้มีการศึกษาเกี่ยวกับคุณสมบัติทางกลศาสตร์ของโลหะที่นำมาหล่อซ้ำและปริมาณที่เหมาะสมในการใส่โลหะใหม่ในกระบวนการหล่อซ้ำ แต่มีการศึกษาไม่มากเกี่ยวกับความเป็นพิษของโลหะเจือที่มาจากการหล่อซ้ำ มีรายงานพบว่าการปลดปล่อยประจุจากโลหะที่นำมาหล่อซ้ำมีผลต่อความเป็นพิษของโลหะเจือนั้นๆ การวางโลหะเจือเหล่านี้ใกล้กับเหงือกอาจทำให้เกิดการตอบสนองอย่างใดอย่างหนึ่งระหว่างเหงือกต่อประจุจากโลหะนั้นๆ การศึกษานี้มีวัตถุประสงค์ในการศึกษาการตอบสนองการอักเสบของเซลล์เยื่อบุต่อผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินที่ผ่านการหล่อซ้ำ การทดลองเริ่มโดยเตรียมชิ้นงานโลหะเจือแพลเลเดียมและเงิน 3 กลุ่มๆละ 3 ชิ้นคือกลุ่มที่หล่อครั้งแรก กลุ่มที่หล่อครั้งที่สอง และ กลุ่มที่หล่อครั้งที่สี่ ขนาด 10*5*1 ม.ม.^{3} นำชิ้นงานโลหะที่ขัดและทำความสะอาดแล้วแช่ในน้ำลายเทียมเป็นเวลา 15 วัน จากนั้นนำชิ้นงานอย่างละ 1 ชิ้น ในแต่ละกลุ่มมาศึกษาโครงสร้างจุลภาคโดยใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) น้ำลายเทียมที่ผ่านการแช่จากโลหะในแต่ละกลุ่มตัวอย่างจะถูกนำมาตรวจสอบการปลดปล่อยประจุที่ออกมาจากโลหะ โดยใช้เครื่องอินดักทีฟลี คับเปิล พลาสมา แมสสเปกโทรมิเตอร์ (ICP-MS) นำน้ำลายเทียมส่วนที่เหลือมาทดสอบกับเซลล์เยื่อบุและทำการวัดค่าการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนอินเตอร์ลิวคิน-1เบต้า (IL-1β) และทูเมอร์เนโครซิสแฟคเตอร์อัลฟา (TNF-α) โดยใช้วิธีการรีเวอร์ทรานสคริปชั่น-พีซีอาร์ (Reverse Transcription PCR) ผลจากการทดลองพบโครงสร้างจุลภาคของโลหะเจือแพลเลเดียมกับเงินมีการเปลี่ยนแปลงหลังถูกเหวี่ยงซ้ำและมีการปลดปล่อยประจุแพลเลเดียมออกมามากขึ้นในกลุ่มที่ถูกหล่อซ้ำ การตอบสนองต่อการอักเสบเพิ่มขึ้นอย่างชัดเจนของเซลล์เยื่อบุต่อผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินในกลุ่มหล่อซ้ำโดยวิเคราะห์จากการเพิ่มขึ้นของการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนอินเตอร์ลิวคิน-1เบต้า (IL-1β) กล่าวโดยสรุปคือผลิตภัณฑ์การกัดกร่อนของโลหะเจือแพลเลเดียมกับเงินในกลุ่มหล่อซ้ำมีผลทำให้เกิดการกระตุ้นการแสดงออกระดับเอ็มอาร์เอ็นเอของยีนอินเตอร์ลิวคิน-1เบต้า (IL-1β) เพิ่มขึ้น ผลจากการวิจัยทำให้ทราบมากขึ้นถึงความเข้ากันได้กับเนื้อเยื่อในร่างกายของโลหะเจือแพลเลเดียมกับเงินที่ถูกหล่อซ้ำโดยดูจากพฤติกรรมการกัดกร่อนและการแสดงออกของยีนที่มีผลต่อการะบวนการอักเสบ

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ภาควิชา ทันตกรรมประดิษฐ์	ลายมือชื่อนิสิต .....
สาขาวิชา ทันตกรรมประดิษฐ์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5775822032 : MAJOR PROSTHODONTICS
KEYWORDS: PALLADIUM-SILVER ALLOY / , INFLAMMATORY RESPONSE / RECAST / EPITHELIAL CELLS

VERAPOL SINGKARLSIRI: THE INFLAMMATORY RESPONSE OF EPITHELIAL CELL TO CORROSION PRODUCTS FROM RECAST PALLADIUM –SILVER ALLOY. ADVISOR: ASST. PROF. VIRITPON SRIMANEEPONG, Ph.D., CO-ADVISOR: ASST. PROF. ANJALEE VACHARAKSA, Ph.D., 40 pp.

In dentistry, there is an increase in alternative metals such as palladium or silver to add into noble metal alloys in order to improve properties and reduce cost. Apart from using other metals to replace gold or platinum, reuse is another way to save the cost. There are many studies on the recast dental alloys on mechanical properties and how many percentages of new alloys needed to add in the recasting process. It has been well accepted that amount of new and used alloys would affect the mechanical and physical properties. The biocompatibility of the alloys, however, has not been extensively investigated. It is reported that metal ions releasing from recasting alloys, might influence on the biocompatibility of the alloy, but the mechanisms is not yet clear. In the gingival sulci, 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. To investigate the epithelial inflammatory responses to corrosion products from recast palladium-silver alloy. Three specimens of each group from first cast, second cast and fourth cast Pd-Ag alloy ($10*5*1mm^3$) were submerged in artificial saliva for 15 days. Microstructure of alloys were observed by a scanning electron microscope (SEM). The corrosion products from alloys were measured by an inductively coupled plasma mass spectrometry (ICP). To study the biocompatibility property, the oral epithelial cells cultured in monolayer were challenged by corrosion product from recast alloys. Quantitative RT-PCR was used to examine the expression of proinflammatory cytokines-specific mRNA, including IL-1β and TNF-α. Microstructure were changed after recasting. Palladium ion was more found after recast. In a reverse transcriptase PCR, IL-1β mRNA levels in response to corrosion product of second and fourth recast group were significantly higher than first cast group. We suggest that the corrosion product from recast Pd-Ag alloy induce epithelial cells to secrete IL-1β which is a proinflammatory that is also found in inflammed gingival tissue. This research give more information and understanding on biocompatibility of recast palladium-silver alloy by observing their corrosion behavior and potential cytotoxic effect i.e. presence of inflammatory response.

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Department:	Prosthodontics	Student's Signature	.....
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Chulalongkorn University

CONTENTS

Page
THAI ABSTRACT iv
ENGLISH ABSTRACT v
ACKNOWLEDGEMENTS vi
CONTENTS vii
LIST OF TABLES 1
LIST OF FIGURES 1
CHAPTER I 2
INTRODUCTION 2
Background and Rationale 2
Research Question 6
Research Hypothesis 6
Research objective 6
Prospective outcomes 6
Conceptual framework 7
Keywords 8
Research design 8
CHAPTER II 9
REVIEW OF RELATED LITERATURE 9
Use of Alloys in dentistry 9
Corrosion of Dental Alloys 11
Biocompatibility of Dental Alloys 13
Reuse of dental alloys and its properties 14
CHAPTER III 17
RESEARCH METHODOLOGY 17
Specimen preparation 17
Observation of microstructure 18
Immersion test 18
Cell culture test 19

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

Quantitative RT-PCR....19
Statistical analysis....20
CHAPTER IV....22
Results....22
Metallographic observations....22
Observation of metallic ion release....22
Gene expression....23
CHAPTER V....29
DISCUSSION AND CONCLUSION....29
Discussion....29
Conclusion....32
REFERENCES....33
VITA....40

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Table 1. Composition of alloy in wt% 17

Table 2. Sequences of PCR primers.....21

Table 3. EDS analysis wt% of gray and white matrix in dendritic structure. 25

Table 4. Immersion test results from first and recast of Palladium-silver alloys in artificial saliva.....26

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Figure 1. Typical SEM micrographs of the Pd-Ag alloy at 45× magnification.....27

Figure 2. TNF-α, IL-1β mRNA transcription from NOK-SI cell incubated with the different corrosion product from first cast and recast group relative to non-stimulated cells.....28

จุฬาลงกรณ์มหาวิทยาลัย
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CHAPTER I

INTRODUCTION

Background and Rationale

Dental alloys have been used in dentistry for variety of applications. Noble or based-metal alloys may be selected to use for full-metal or porcelain-fused-to-metal crown fabrication. Not only the mechanical properties, but also the biocompatibility and corrosion resistance should be carefully taken in consideration for dental alloy selection. Since the cost of using gold alloys has been substantially increased, other types of alloys such as palladium-based or silver-palladium alloys become more popular. High-palladium alloys, containing more than 75% of palladium, have been used as alternatives to gold-based alloys (1). However, the cost of palladium continues to increase. Therefore, silver is used for subsidize some palladium and reduce the cost of palladium-silver or silver-palladium alloys. Silver-palladium alloys may not contain gold or only a small amount of gold, but at least 25% of the alloys must be palladium for tarnish resistance and nobility (2).

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In the review of corrosion of alloys which consist of Pd, Ag and base metal. The deposition of the metal elements in gingiva from dental casting alloys was reported in an in vivo study, and silver remnants in metallic pigmented gingiva (3) and in biopsies of pigmented gingiva adjacent to dental casting alloys (4) appeared to be prominent. However, many in vitro studies show that gold or palladium release is rarely detected from any solutions (5-8).This finding suggested that gold or palladium has high corrosion resistance due to its nobility (9), while the most common detectable metal

ions in any solutions in laboratory are silver, copper and zinc (5, 8, 10). For binary palladium-silver alloys, some study suggested that the highest corrosion resistance of binary palladium-silver alloys was occurred when it contained 50% and 75% of silver (11). In contrast, one study reported that corrosion resistance of palladium-silver alloys was deceased when there are more than 50 wt.% of silver (12). When more than 40% of the alloys consisted of palladium, the corrosion behavior in electrochemical test will be become passive (13).The corrosion behavior of binary alloys, especially in binary and ternary palladium-silver, is thus difficult to be evaluated by using individual alloying elements.

The corrosion behavior of dental alloys can also indicate their biocompatibility. In a review of the biocompatibility of palladium, its alloys and compounds. Pd has a potential adverse biological effects (7) while that pure silver can be corroded in saliva and causes localized tissue response or systemic side effects (14). According to few studies, silver is also higher detected in their experiment (5, 8, 10) and also found in metallic pigmented gingiva and biopsies of pigmented gingiva adjacent to dental casting alloys (3, 4). Nevertheless, the cytotoxicity of silver ions is low when compared to other base metal alloys such as zinc, copper or nickel (15). The release of silver from noble metal alloys is detectable, and it is suspected of having an effect on the cytotoxicity because of the decreasing of succinic dehydrogenase activity which is representative of the mitochondrial function of the cells of Balb/c 3T3 mouse fibroblasts within 96 hours (16). The formation of insoluble silver chloride depositing on alloy surface can reduce the toxicity of silver release from silver-palladium alloys (17, 18). However, the silver chloride film is not considered to be an efficient barrier to prevent dissolution (19).

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Most of biocompatibility tests refer to the cellular responses including cell viability, metabolic activity, toxicity and the inflammatory responses. Metal salt solution, pure metal or dental alloys have been investigated for their cytotoxicity in variety of cell types such as L-929 mouse fibroblasts, 3T3 mouse fibroblast or TR 146 epithelial cells. There are a studies that test the toxic effect of metal ion by using normal oral epithelial cells which consists of TR 146 cell to reconstituted human epithelium model (20-22).Cell viability measurements in epithelial cells are in agreement with results on fibroblast cell (23).Moreover, under non-toxic or only mild toxic experimental conditions on epithelial cells can induce the release of inflammatory parameters such as Interleukins (IL) and tumor necrosis factor alpha (TNF) which is important in vivo in the course of inflammation or responses of immune modulation and also play a central role in gingival and periodontal inflammation (24).

For the economical reason, based metal alloys are commonly used for casting dental prostheses (25, 26).It is well known that the base metal ions such as zinc ,copper, nickel or chromium share the high percentage of metal ion release (3-6, 8, 10). Therefore, the use of high-noble or noble alloys for dental prostheses remains beneficial because of the high corrosion resistance and biocompatibility (25). Recast 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 (27), increasing of corrosion (28, 29) leading to toxic metal ions release and the toxicity of alloys (30, 31) and lower elastic modulus (32).The elemental composition, hardness, and corrosion behavior of silver-palladium alloys can be affected from recasting, especially in fifth recasting of palladium alloys. In bulk elemental composition, there is no obvious change of the

major elements which were palladium, gold and silver, but the concentration of zinc and copper slightly decreased after recasting. The hardness in different groups of recasting generally increased. While the casting shrinkage was found in the third, fourth and fifth casting generations. The grain size has become steadily after recasting, and the change of subgrain structure was distinguishable after the fifth cast. In Corrosion test, recasting has effect on the corrosion by observing the polarization resistance and the corrosion current density. Polarization resistance significantly decreased while the corrosion current density significantly increased, after the fifth casting (33).

There was a few investigation of the cellular responses and the metal alloys biocompatibility, especially to the recasting palladium base of alloys. Some dental laboratories follow the recommendation for recasting by adding new metals to 50% new metal with previously melted buttons or sprues removed from castings to saving the cost of restoration (34, 35). Thus, this procedure may adversely effect on the soft tissue adjacent to a restoration in the oral environment which has the electrochemical activity that is cause of corrosion in dental materials (36). Therefore, we hypothesize that corrosion product released from recasting specimen will more leads to the release of proinflammatory cytokines including IL-1β and TNF-α from the epithelial cells.

Research Question

Does recast cycle of palladium-silver alloy affect to epithelial cell inflammatory cytokine production?

Research Hypothesis

H_0 : There is not a statistical significant difference in epithelial cell inflammatory response between first casting and recasting of palladium-based alloys containing silver.

H_1: There is a statistical significant difference in epithelial cell inflammatory response between first casting and recasting of palladium-based alloys containing silver.

Research objective

To investigate the epithelial inflammatory cytokine production to the recast palladium-silver alloys.

Prospective outcomes

To have a better understanding of the epithelial responses to recast palladium-silver alloy.

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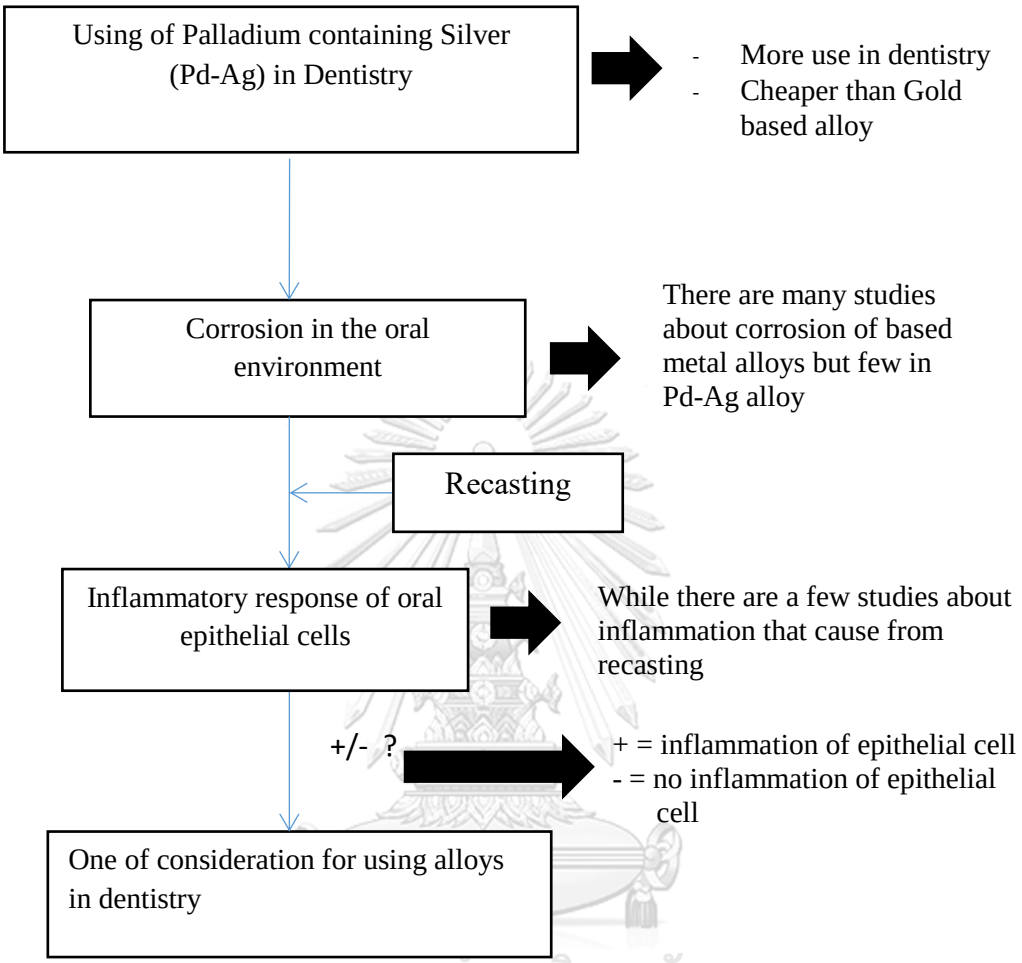
Conceptual framework



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Keywords

- epithelial cell
- Palladium-silver alloy
- inflammatory response
- recast

Research design

Laboratory experiment research

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CHAPTER II

REVIEW OF RELATED LITERATURE

Use of Alloys in dentistry

Alloys are mixtures of two or more metals or nonmetals (elements). They have been used for a long time as dental materials for fixed prosthetic restorations and are classified into three types according to the proportions and types of noble metals they contain: high-noble alloy, noble alloy and base metal alloy. High-noble alloys have a minimum of 60% noble metals (any combination of gold, palladium and silver) with a minimum of 40% by weight of gold. Noble alloys contain at least 25% by weight of noble metal. Any combination of gold, palladium or silver totaling at least 25% places the alloy in this category. Base metal alloys contain less than 25% noble metal, but in actuality, most contain no noble metal at all (37). As a group, they are much harder, stronger and have twice the elasticity of the high-noble and noble metal alloys, but base metal alloys also have disadvantages for the lab technicians and dentists that work with them. Their melting temperature and casting shrinkage (about 2.3%) are higher than that of the other alloy types, which make them more difficult to cast and must be compensated for. Their hardness makes them difficult to burnish and polish and their high melting temperature makes them difficult to solder. They are also more prone to corrosion under acidic conditions.

For crowns and bridges, there are five types of alloys, which are based on gold, silver, palladium, cobalt and nickel (38). If the alloys are classified by their application, there are three categories of prostheses, including all-metal prostheses, metal ceramic

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prostheses, or removable partial dentures. There are differences between the noble and high noble alloys, which are used for all-metal prostheses and metal ceramic prostheses. The alloys for metal ceramic prostheses must have the potential to bond to dental porcelain and have a thermal expansion coefficient compatible with dental porcelain. In addition, their solidus temperature should be significantly higher than the sintering temperature of porcelain to minimize creep deformation. High-noble and noble alloys used for all-metal prostheses are rarely used for metal ceramic prostheses, whereas alloys used for metal ceramic prostheses can be used for all-metal prostheses as well (2). Common components in high-noble and noble alloys for fixed prostheses (i.e. crowns and bridges) are gold, palladium, platinum and silver. The other minor components are copper, zinc, indium and iridium. Every component has their own properties to produce alloys with suitable characteristics. Some noble alloys rely on palladium without the use of gold for corrosion resistance. Therefore, noble alloys are categorized into two groups, i.e. gold-based and high palladium-based alloys. Gold-based alloys have four types (i.e. Type 1 to Type 4). Type 3 and 4 are usually used for constructing crowns and bridges, respectively, in high-stress areas (2). High-palladium alloys, containing more than 75% of palladium, have been used as alternatives to gold-based alloys. The first commercially successful high-palladium dental alloy was a palladium-copper-gallium alloy. Afterwards, palladium-gallium alloys were developed due to their easy casting, finishing and polishing (1).

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The information from Kito Metal Inc. shows that the cumulative average price of palladium significantly increased from 320.27$ per ounce in 2006 to 525.57$ per ounce and 725.43$ per ounce in 2011 and 2013, respectively. In the same period, the price of gold was twice as high as the price of palladium. Because of the continued

increase of palladium prices in the global precious metal market in recent years, silver-palladium alloys are gaining popularity in dental laboratories, and the selection of high palladium alloys has diminished. Silver-palladium is free of gold or may contain a small amount of gold but they must contain at least 25% of palladium to increase the tarnish resistance and provide nobility. If they are free of copper, their physical properties are similarly to Type 3 gold alloys. On the other hand, they may have properties more similar to Type 4 gold alloys if they contain 15% or more copper (2).

Corrosion of Dental Alloys

There are many different types of alloys in the market for selection in dental prostheses. Therefore, the properties of materials must be considered. Mechanical properties are not only important in dental materials which are exposed in oral environment, but biocompatibility and corrosion resistance are also two important factors for manufacturers, patients and dentists. Corrosion is a chemical property of an alloy that influences other properties, including esthetics and strength (38). After corrosion of the alloys, elements will be released to the environment as ions that may or may not cause damage to the surrounding (39).There are many ways to measure corrosion. First of all, you can observe the discoloration of the alloy's surface (i.e., tarnish). Secondly, you can test the alloy for altered current flow, which is known as electrochemical testing. Finally, you can directly measure the released elements (e.g., atomic absorption spectroscopy, atomic emission spectroscopy) (40). All alloys have corrosion and elemental release, but its extent will is influenced by several factors: alloy composition (38), phase structure, surface structure, crevices and pits, thermal treatment/history, combination of alloys and time in service (41). In addition, a reduced

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pH in the oral environment can increase the corrosion of alloys, particularly those based on nickel (42). Studies reported on the influence of pH on the elemental release from dental alloys (42) and on the strength of denture bases (43).

There are many studies that evaluated the corrosion of high palladium alloys, silver-palladium alloys and alloys that consist of palladium and/or silver in their composition. Gold or palladium are rarely detected for any alloy, in any solution (5-8).This is due to their high nobility which increases their corrosion resistance (9). On the other hand, the most common detectable elements ion in any solutions are silver, copper and zinc (5, 8, 10). The metallic deposition of silver is predominantly found in biopsies adjacent to dental alloys (4) and oral pigmentation in gingiva (3). For binary palladium-silver alloys, It has been suggested that the highest corrosion resistance of binary alloys occurs when it contains 50% and 75% of silver (11). In contrast, it has been reported that corrosion resistance of palladium-silver alloys was decreased when there is more than 50 wt.% of silver (12). If the alloys consist of palladium above 40%, the electrochemical behavior changes from active to passive corrosion (13). The corrosion behavior of binary alloys, especially binary and ternary palladium-silver, is difficult to evaluate by using individual alloying elements. For example, the electrochemical behavior of binary palladium-silver alloys differs from that of base metal due to the formation of insoluble salt layers (17) that cover the palladium-rich surface on palladium-silver alloys to prevent the exposure of palladium-rich surface to the surrounding environment (18).

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Biocompatibility of Dental Alloys

Biocompatibility is one of the most important properties of dental materials. Most biocompatibility tests focus on cellular viability and morphology, metabolic activity, cellular toxicity or the presence of inflammatory cytokine markers. For these tests, metal salt solutions, pure metals or dental alloys are prepared to measure the cytotoxicity in a variety of cell culture medium such as L-929 mouse fibroblast, 3T3 mouse fibroblast or TR 146 cell cultures. Epithelial cell cultures are suitable to test toxicity of dental materials because under non-toxic or mildly toxic experimental conditions, epithelial cells can induce the release of inflammatory parameters (20) and other biocompatibility parameters (21, 44). Moreover, some the results of biocompatibility tests are also in agreement with results on fibroblast cells (23).Inflammation of the human gingiva has been observed in the vicinity of dental alloys (45), and disregulated proinflammatory mediators at local sites have been considered to be major contributors to the development of gingival inflammation (46, 47). While the release of metal ions may induce DNA damage in the cells (48), and the silver nanoparticle was shown to induce pulmonary inflammation and injury (49). In order to simulate the in vivo situation, measurement of acute toxicity, by focusing on proinflammatory mediators, may be proper for in vitro testing of dental materials (20). To relate with periodontal inflammation, proinflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) are the first to appear in the periodontal disease pathogenesis pathways(50). This cytokines are produced by resident cells, such as epithelial cells and fibroblasts, and by phagocytes (neutrophils and macrophages) in the acute and early chronic phases of inflammation, and by immune cells (lymphocytes) in established and advanced lesions (51). The secretion of

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proinflammatory cytokines are regulated by genes which generally depend on the activation of nuclear factor kappa-B transcription. The nuclear factor kappa-B regulated pathways are activated by pathogen-associated molecular patterns, such as lipopolysaccharide, through the toll-like receptor pathway (52). Interleukin-1beta is signature innate cytokines and have been characteristically associated with inflammatory cell migration and osteoclastogenesis. Tumor necrosis factor alpha is a multi-effect cytokine that has many functions, from cell migration to tissue destruction. Tumor necrosis factor alpha impacts cell migration by inducing the up-regulation of adhesion molecules to promote rolling and adhesion of neutrophils to the vessel wall, leading to extravasation (24).

Reuse of dental alloys and its properties

Base-metal alloys are more commonly used for dental casting prostheses (25, 26), although they are more difficult to cast and more sensitive during casting than noble alloys. From previous studies about the corrosion of dental alloys, metal ions released in different solutions are those of non-precious metals such as Zn, Cu, Ni and Cr etc. (3-6, 8, 10).Therefore, high noble and noble alloys are interesting for dental prostheses because of their high corrosion resistance and biocompatibility (25). But high noble and noble alloys are often reused by recasting, due to the high price of noble metals. On the other hand, base-metal alloys are sometimes reused because of their low price. Therefore, there are many studies that evaluated the properties of alloys after recasting.In conclusion, from previous studies about the properties of alloys after recasting, there are statistically significant differences for some properties of based-metal alloys after recasting, i.e. the decreasing of bond strength between porcelain(53), high toxicity of alloys(30, 31) due to the increasing of tendency to corrosion of toxic

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metal ions (28, 29) and decrease of elastic modulus (32). On the other hand, some physical properties showed no statistically significant difference (53).Yield strength and percentage elongation after recasting of Type III gold (46%) alloys without adding of new alloys was significant decreased ($P < .01$), and percentage elongation was also decreased but with no statistical difference (54). For silver-palladium alloys, which are an alternative to gold-based alloys, the elemental composition, hardness, and corrosion behavior are affected by recasting, especially for fifth recasting of palladium alloys (33).It can be concluded that corrosion products of dental casting alloys can be toxic to different cell cultures (15, 16, 55). Some studies showed that corrosion products of dental alloys are found in adjacent gingiva (3, 4). Moreover, the recasting affects some properties of alloys, especially the corrosion resistance of dental casting alloys (28, 29). For silver-palladium alloys, there are a few studies about the effect of recasting on corrosion properties. Furthermore, there are a few studies about the biocompatibility of recasting silver-palladium alloys, which are widely used as an alternative choice of gold-based alloys to reduce the cost of restoration (54). Some dental laboratories follow recommendation for recasting by adding up to 50% of new metal with previously melted buttons or sprues removed from castings to save costs of restoration (34, 35). If recasting of silver-palladium alloys is expected to increase their corrosion behavior or potential cytotoxic effect, this procedure may adversely affect the soft tissue adjacent to a restoration in the oral environment, which has an electrochemical activity that causes corrosion in dental materials (36). Therefore, restorations made from recasting of silver-palladium alloys must not be closely placed to soft tissue in oral cavity, e.g. subgingival margin of crown, or other materials should be used instead. Therefore, we

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hypothesize the corrosion product from recasting Pd-Ag alloy leads to more release of proinflammatory cytokines including, IL-1β and TNF-α from the epithelial cells.

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CHAPTER III

RESEARCH METHODOLOGY

Specimen preparation

Three square-shaped specimens (10 mm x 5 mm and 1.0 mm. thickness) of a palladium base alloys (Aurolite 1C, Aurium research, USA) which consist of 38.2 % of silver and 53% of palladium (in wt%) (table 1) 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. All alloy specimens were polished with sandpaper up to No 1200. For any recast alloy group, three specimens were prepared by using 100% once-cast of pervious specimens including sprues and buttons which were cleaned in dilute sulfuric acid to remove the oxidation film for 5 seconds before reusing.

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Table 1. Composition of alloy in wt%

Alloys	Element					
	Pd	Ag	Sn	Cu	Zn	In
Aurolite 1C	53.0	38.2	7.0	< 1.0	< 1.0	1.0

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 scanning electron (SEM) (JSM-6400, JEOL, USA). Grain size was calculated in microns and casting defects are examined. Energy dispersive spectroscopy (EDS) were used to analyze each characteristic type of area.

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 15 days. After 15 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, Thermo scientific ICAP QC, 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 and IL-β gene expression.

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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 and IL-β. 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^{o}C for 1 min followed by 40 amplification cycles consisting of 95^{o}C for 45 s, 60^{o}C for 60 s, 72^{o}C for 90 s, and one extension cycle at 72^{o}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. 18S 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.

Statistical analysis

Statistical evaluation was undertaken with the Statistical Package for Social Sciences (SPSS®, Chicago, Illinois, USA, version 17.0 for Windows). One-way ANOVA follow by Tukey's HSD was used to determine the expression of TNF-α, IL-β and concentration of existing ions in corrosion for the different group of specimens. The statistical significance was considered at $p \leq 0.05$.

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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	NM 030968	186
qIL-β	F : GTC ATT CGC TCC CAC ATT CT R : ACT TCT TGC CCC CTT TGA AT	NM 000576.2	105
18S	F : CTT AGA GGG ACA AGT GGC G R : ACG CTG AGC CAG TCA GTG TA	NM 002046.4	121

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CHAPTER IV

Results

Metallographic observations

Scanning electron microscopy revealed the microstructure of the cast Pd-Ag alloy. The first cast alloy demonstrates fine dendritic structure with a light gray grains surrounded by large dark areas (Figure 1A). Many small and bright particles can be seen scattering inside the dark area. From EDS (Table 3), localized trace element of bright and grey matrix revealed that the bright matrix was rich of Pd while the dark area was richer in Ag. Both area showed low, but in traceable, amount of Sn, but Zinc, Copper and Indium were not detectable. After recasting, trace element detected in bright and grey matrix remains similar between each casting, however, the light grey grains of the second cast (Figure 1B) and the fourth cast alloys (Figure 1C) appeared more condensed than the first cast alloys. The area of bright matrix was obviously displayed larger in recast groups while the dark area was smaller. Then, surface area of grey and dark matrix was measured by Image J® software and compared. The area percentage of dark area in first cast alloy was 27.82% while it decreased to 8.96% and 6.02% in second cast and fourth cast groups, respectively.

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Observation of metallic ion release

To measure the metal ions released in the supernatants, the Pd-Ag alloys were submerged in artificial saliva (0.07 g/L MgCl2, 0.75 g/L KCl, 0.439 g/L KH2PO4,0.005g/L NaF0,0.965 g/L K2HPO4), pH 6.7, at 37 C for 15 days. Released

ions was detected by ICP analysis (Table 4). Consistently with EDS analysis of the alloys, Zinc, Copper, Tin and Indium were not detected in artificial saliva supernatants. Ag released by the recasting was significantly decreased (p <0.05) while Pd released by the second-cast and fourth-cast alloys was significantly increased when compared to the first cast groups (p <0.05). This finding corresponds to the metallographic microstructures in Figure 1.

Gene expression

To investigate the inflammatory response of epithelial cells to corrosion products of Pd-Ag alloys, the normal oral keratinocytes were incubated with immersion from first-cast, second-cast and forth-cast alloys for 24 hrs. Cells incubated with artificial saliva were included as a baseline control and cells incubated with 2.5 μg/mL P.gingivalis LPS (InvivoGen,San Diego,CA,USA) represented inflammatory response as a positive control. The level of IL-1β mRNA was upregulated in response to corrosion product of the alloys. The first-cast alloy stimulates IL-1β mRNA similar to the level stimulated by 2.5 μg/mL P.gingivalis LPS, but not significantly higher than baseline (p =0.071) (figure. 2A). While IL-1β mRNA responding to the second- and fourth-cast group were significantly higher than first-cast group (p =0.022, 0.001), and the baseline control (p =0.01, 0.00). There was not significantly difference of IL-1β between second-cast and fourth-cast group (p =0.929). The level of TNFα was downregulated in response to corrosion product of the alloys. The first-cast alloy stimulates TNFα mRNA similar to the level stimulated by 2.5 μg/mL P.gingivalis LPS, but significantly lower than baseline (p =0.004) (figure. 2B). While TNFα mRNA responding to the second- and fourth-cast group were not significantly different

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between first-cast group (p =0.177, 0.300), and the baseline control (p =0.377, 0.214). There was not significantly difference of TNFα between second-cast and fourth-cast group (*p* =0.961).

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Table 3. EDS analysis wt% of gray and white matrix in dendritic structure.

Alloy	Element					
	Pd	Ag	Sn	Zn	In	Cu
1st cast group						
Dark area						
Average	**50.89 ±1.11[a]**	**42.98 ±1.32[a]**	**6.13 ±0.36[a]**	-	-	-
Gray area						
Average	**57.59 ±1.85[b]**	**35.75 ±1.54[b]**	**6.66 ±0.32[b]**	-	-	-
2nd cast group						
Dark area						
Average	**50.17 ±2.58[a]**	**44.38 ±3.05[a]**	**5.45 ±0.85[a]**	-	-	-
Gray area						
Average	**62.77 ±1.33[b]**	**29.54 ±0.96[b]**	**7.69 ±0.42[b]**	-	-	-
4th cast group						
Dark area						
Average	**51.26 ±1.16[a]**	**42.60 ±1.13[a]**	**6.14 ±0.38[a]**	-	-	-
Gray area						
Average	**61.00 ±3.61[b]**	**31.88 ±3.72[b]**	**7.12 ±0.33[b]**	-	-	-

* Values with different lower case letters in same column of each alloy are significantly different at P<.05

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Table 4. Immersion test results from first and recast of Palladium-silver alloys in artificial saliva* Released ion in ppb 15 days pH 6.7 at 37° C.

Alloy	Element					
	Pd	Ag	Cu	Zn	In	Sn
Aurolite 1C						
1st cast group	**1.307± 0.054[a]**	**0.099 ±0.009[a]**	-	-	-	-
2nd cast group	**2.707 ±0.049[b]**	**0.029 ±0.004[b]**	-	-	-	-
4th cast group	**1.701 ±0.083[b]**	**0.024 ±0.003[b]**	-	-	-	-

*(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).

**Values with different lower case letters in same column for each alloy are significantly different at P<.05

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Figure 1. Typical SEM micrographs of the Pd-Ag alloy at 45× magnification (A) the first cast group (B) 2th cast group (C) 4th cast group

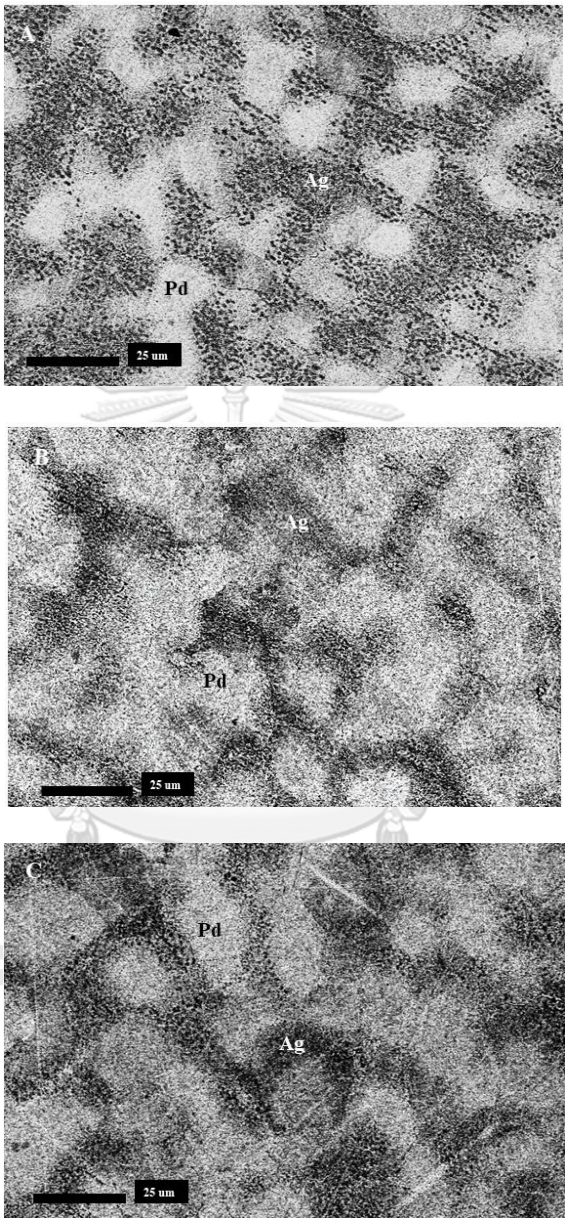
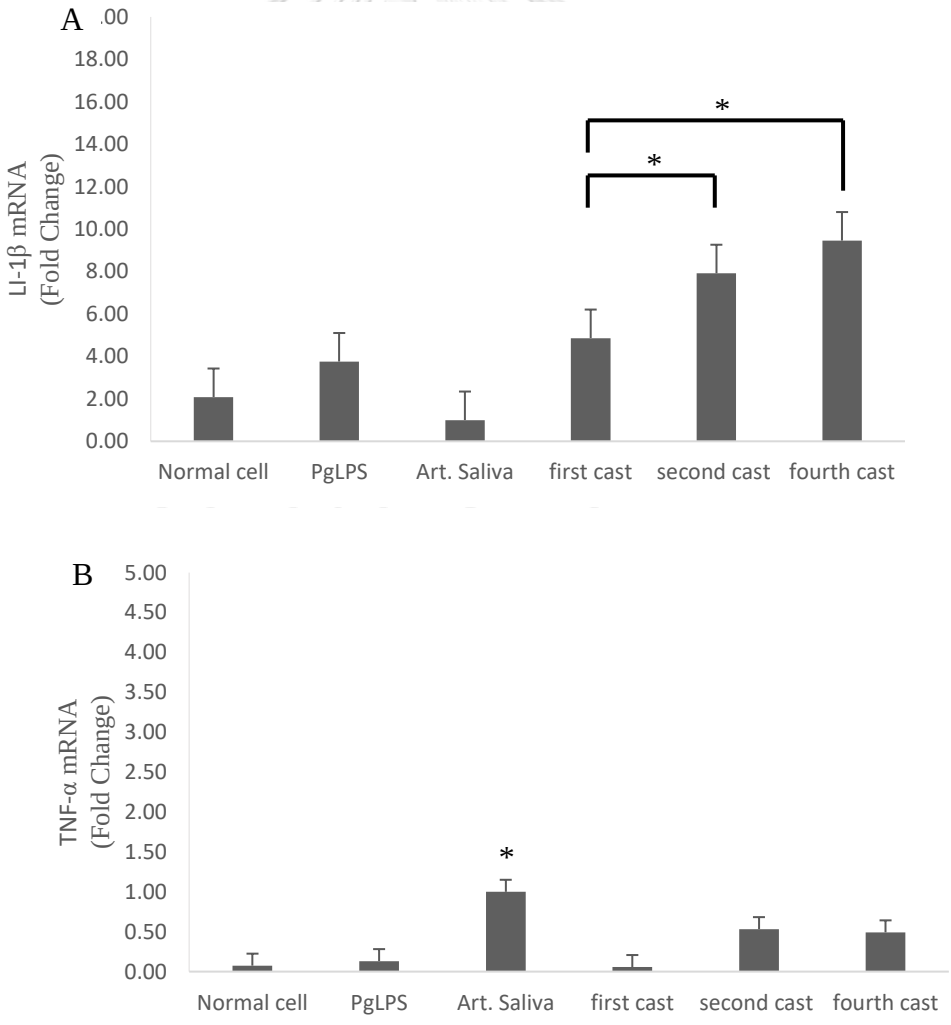


Figure 2. TNF-α, IL-1β mRNA transcription from NOK-SI cell incubated with the different corrosion product from first cast and recast group relative to non-stimulated cells. (A) IL-1β expression in NOK-SI cells treated with corrosion product from first cast and recast group of Pd-Ag alloy. (B) TNF α expression in NOK-SI cells treated with corrosion product from first cast and recast group of Pd-Ag alloy. All experiments were performed in duplicate. * indicates a significant difference using One-Way ANOVA test follow by Tukey's HSD ($p < 0.05$) when comparing by using the Comparative CT Method.



CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

The present study investigated the properties of alloys after recasting such surface morphology, mechanical properties, corrosion resistance and its biocompatibility. The effect of recasting to the properties of alloys is becoming more interest. The results of microstructural changes after recasting due to the increasing of grain dimensions, impurities and porosities is that affect the mechanical properties of alloys (54).In our microstructure analysis, we can see more porosity in recast group of Pd-Ag alloy. The porosity is displayed as a large dark spot and randomly distributed within the matrix. Furthermore, porosities are effect on the corrosion property by reducing the corrosion resistance. From SEM analysis, we found gray area and dark area in the microstructure of Pd-Ag alloy which show a complete miscibility at all compositions because of solid solution in phase diagram of Pd-Ag alloy (56). Due to a complete miscibility of microstructure, the results from EDS analysis can only inform the enrich area of metals. We found that gray area was Pd rich when compare to dark area.

The corrosion resistance of dental alloys in oral environment can be related to its biocompatibility. Many solutions were used in corrosion test such as 0.9% sodium chloride (NaCl), 1% lactic acid and artificial saliva etc. The different of solutions are represented to different of environment in the mouth. In this study, we used artificial saliva as a solution to represent a closely environment in the mouth. Palladium is the

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main component in high palladium-based alloys. Although palladium has a potential adverse biological effects (7), dissolution of palladium from these alloys is low. Thus, the palladium-based alloys shows less cytotoxicity compared to silver, copper and zinc (16, 55). Some studies reported that pure silver can be corroded in saliva and causes localized tissue response or systemic side effects (14). According to few studies, silver is also higher detected in their experiment (5, 8, 10) and also found in metallic pigmented gingiva and biopsies of pigmented gingiva adjacent to dental casting alloys (3, 4). Nevertheless, the cytotoxicity of silver ions is low when compared to other base metal alloys such as zinc, copper or nickel (15). The formation of insoluble silver chloride depositing on alloy surface can reduce the toxicity of silver release from silver-palladium alloys (17, 18). We found that Pd and Ag were detectable while Cu, Sn, In and Zn were not found in all groups. The previous studies support our results that Ag is usually found in elemental releasing (5, 8, 10). But we can also detected Pd in our solution in all groups after immersion test for 15 days. While some study reported that Pd was not detectable (7) .This disagreement may be due to the limitation of measurement of elemental ion releasing. From our results, we found the increasing of Pd in corrosion product from recast group. From EDS analysis also showed the increase of Pd rich areas that may related to the increasing of Pd ion from recast alloy in the corrosion product. While the detection of Ag was low due to the formation of insoluble silver chloride depositing on the alloy.

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For the biocompatibility test, researchers always focus on cellular viability and morphology, metabolic activity, cellular toxicity or the presence of inflammatory cytokine markers(2). In this study, we focus on the presence of inflammatory cytokine markers from the epithelial cell culture when they were challenged by the corrosion

product of first cast, second cast and fourth cast group of Pd-Ag alloy. In order to simulate the in vivo situation, measurement of acute toxicity, by focusing on proinflammatory mediators, may be proper for in vitro testing of dental materials (20). In this study, we select the epithelial cell cultures because epithelial cells which are closely to the metal restoration margin can be induced the release of inflammatory parameters under non-toxic or mildly toxic experimental conditions (20). IL-1β and TNF-α which play a central role in gingival and periodontal inflammation were selected to investigate the inflammatory response of epithelial cell to the corrosion product because we want to relate this response to periodontitis. From the results, we found that IL-1β was significantly unregulated by the corrosion product from recast group when compare to the first cast group and non- stimulated cells. While TNF-α was more upregulated by the artificial saliva when compared to the other groups. This result showed that this artificial saliva more upregulate TNF-α expression than IL-1β in NOK-SI cells. Therefore, this artificial saliva must be more investigated about the upregulation of TNF-α expression. While Muller et al. also reported that the artificial saliva can stimulate the chemokine expression via NF-κB pathway (57). We conclude that the corrosion product effect to the IL-1β mRNA gene expression. Now, we know that the corrosion product from recast Pd-Ag alloy can increase the IL-1β mRNA gene expression in epithelial cell but it is not still proved that which element in corrosion product play the important role to increase IL-1β. Therefore, this question should be investigated for next subject.

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Conclusion

The recasting process increased some elemental releasing of Pd-Ag alloy. Furthermore, the more corrosion product from recast Pd-Ag alloy induce epithelial cell to secrete IL-1β which is the proinflammatory that is also found in periodontitis patients. We suggest that the recasting metal restoration from Pd-Ag alloy should not be placed closely to the gingival margin.

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Chulalongkorn University

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Chulalongkorn University

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

VITA

Mr. Verapol Singkarlsiri was born on March 15, 1988 in Bangkok, Thailand. He graduated the degree of Doctor of Dental Surgery (D.D.S) from Faculty of Dentistry, Mahidol University, Bangkok, Thailand in 2012. After graduation, he worked for Bandan Lanhoi hospital, Sukhothai, Thailand from May 2012- May 2014.He started his study for the Master degree in Prosthodontic at Faculty of Dentistry, Chulalongkorn University. The research component of this degree was performed at the Department of Oral biology, Scientific and Technological Research Equipment center, Chulalongkorn University and oral biology research center, Faculty of dentistry, Chulalongkorn University. At the present, he work for a several famous private dental clinics in Bangkok.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University