IMMUNOHISTOCHEMICAL ASSESSMENT OF THE PERI-IMPLANT SOFT TISSUES AROUND DIFFERENT ABUTMENT MATERIALS: AN EXPERIMENTAL STUDY IN HUMAN

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Thesis Title	IMMUNOHISTOCHEMICAL ASSESSMENT OF THE PERI-IMPLANT SOFT TISSUES AROUND DIFFERENT ABUTMENT MATERIALS: AN EXPERIMENTAL STUDY IN HUMAN
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Field of Study	Esthetic Restorative and Implant Dentistry
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ศิริกาญจน์ ทองมีอาคม : การประเมินลักษณะทางเคมีจุลกายวิภาคภูมิคุ้มกันของเนื้อเยื่ออ่อนรอบวัสดุหลักยึดต่างชนิดในมนุษย์ (IMMUNOHISTOCHEMICAL ASSESSMENT OF THE PERI-IMPLANT SOFT TISSUES AROUND DIFFERENT ABUTMENT MATERIALS: AN EXPERIMENTAL STUDY IN HUMAN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพ. ประเวศ เสรีเชษฐพงษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ทพ. ดร. อาทิ พันธุ์ พิมพ์ขาวขำ, 84 หน้า.

วัตถุประสงค์ เพื่อประเมินผลของหลักยึดที่ทำจากวัสดุชนิดต่างๆ คือ ไทเทเนียม เซอร์โคเนีย โลหะผสมทอง และไทเทเนียมส่วนฐานยึดกับเซอร์โคเนียส่วนคลุม ต่อเนื้อเยื่อรอบหลักยึด

วิธีการศึกษาวิจัย รากเทียมในบริเวณฟันหลังทั้งสิ้น 20 ตัว สุ่มแบ่งออกเป็น 4 กลุ่ม และใส่หลักยึดที่ทำจากวัสดุ 4 ชนิดคือ ไทเทเนียม เซอร์โคเนีย โลหะผสมทอง และไทเทเนียมส่วนฐานยึดกับเซอร์โคเนียส่วนคลุม ในวันเดียวกันกับการผ่าตัดฝังรากเทียม เมื่อครบ 8 สัปดาห์ เนื้อเยื่อรอบหลักยึดถูกตัดและแบ่งตามด้านของรากเทียม คือ ใกล้แก้ม ใกล้ลิ้น ใกล้กลาง และ ไกลกลาง เนื้อเยื่อดังกล่างถูกนำไปผ่านกระบวนการทางเคมีจุลการวิภาคภูมิคุ้มกัน และย้อมด้วย ซีดี3 (CD3) ซีดี20 (CD20) ซีดี68 (CD68) ซีดี138 (CD138) และ แฟคเตอร์เอท (FactorVIII) เพื่อระบุ ทีเซลล์ (T-cell) บีเซลล์ (B-cell) มาโครฟาจ (Macropahge) และ หลอดเลือดขนาดเล็ก (Microvessel) ตามลำดับ พยาธิแพทย์เพียงคนเดียวกระทำการประเมินเชิงปริมาณของเครื่องหมายเซลล์ เซลล์ที่ให้ผลบวกในชิ้นเนื้อถูกแสดงเป็นจำนวนเซลล์บวกต่อตารางมิลลิเมตรของพื้นที่ชิ้นเนื้อ ผลลัพธ์ได้รับการประเมินลักษณะทางเคมีจุลกายวิภาครภูมิคุ้มกัน และ ความหนาแน่นของหลอดเลือดขนาดเล็ก (Microvessel density)

ผลการวิจัย คนไข้สุขภาพแข็งแรงทั้งสิ้น 16 คน เพศชาย 6 คน และ เพศหญิง 10 คน ได้เข้าร่วมในงานวิจัย จำนวนเซลล์ที่ให้ผลบวกของกลุ่ม ไทเทเนียม เซอร์โคเนีย โลหะผสมทอง และไทเทเนียมส่วนฐาน คือ 119.28 117.06 445.18 และ 109 ตามลำดับ การวิเคราะห์ความแปรปรวนของการทดลองแบบแฟกทอเรียลได้ถูกใช้ในการวิเคราะห์ข้อมูล กลุ่มโลหะผสมทองมีจำนวนเซลล์ที่ให้ผลบวกมากกว่าอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มไทเทเนียม (p-value=0.009) และ กลุ่มเซอร์โคเนีย (p-value=0.042) ด้านของรากเทียมส่งผลที่ไม่แตกต่างกันต่อจำนวนเซลล์ที่ให้ผลบวก ความหนาแน่นของหลอดเลือดขนาดเล็กระหว่างกลุ่มไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ จากการวิเคราะห์ความแปรปรวนของการทดลองแบบแฟกทอเรียล พบว่า ไม่มีผลของวัสดุหลักยึด (p-value=0.501) และด้านของรากเทียม (p-value=0.910) ต่อความหนาแน่นของหลอดเลือดขนาดเล็ก

สรุปผลการวิจัย วัสดุหลัดยึดต่างชนิดส่งผลต่อลักษณะทางเคมีจุลกายวิภาคภูมิคุ้มกันของเนื้อเยื่ออ่อนรอบรากเทียม โดยหลักยึดที่ทำจากโลหะผสมทองแสดงผลของจำนวนเซลล์อักเสบในเนื้อเยื่อโดยรอบมากกว่าหลักยึดที่ทำจากไทเทเนียมและเซอร์โคเนีย เนื้อเยื่อรอบรากเทียมจากด้านของรากเทียมที่แตกต่างกันไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ทั้งในแง่มุมเคมีจุลกายวิภาคภูมิคุ้มกันและความหนาแน่นของหลอดเลือดขนาดเล็ก เนื้อเยื่อรอบหลักยึดที่ทำจากโลหะผสมทองมีแนวโน้มที่จะพบอัตราการอักเสบที่สูงกว่า เมื่อเปรียบเทียบกับหลักยึดที่ทำจากไทเทเนียมและเซอร์โคเนีย

สาขาวิชา ทันตกรรมบูรณะเพื่อความสวยงามและทันตกรรมรากเทียม ลายมือชื่อนิสิต .....

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5775823632 : MAJOR ESTHETIC RESTORATIVE AND IMPLANT DENTISTRY
KEYWORDS: IMMUNOHISTOCHEMISTRY / DENTAL IMPLANT / ABUTMENT / GOLD ALLOY / ZIRCONIA / TITANIUM / TITANIUM-BASE / PERI-IMPLANT SOFT TISSUE

SIRIKARN THONGMEEARKOM: IMMUNOHISTOCHEMICAL ASSESSMENT OF THE PERI-IMPLANT SOFT TISSUES AROUND DIFFERENT ABUTMENT MATERIALS: AN EXPERIMENTAL STUDY IN HUMAN. ADVISOR: ASSOC. PROF. PRAVEJ SERICHETAPHONGSA, CO-ADVISOR: ASSOC. PROF. ATIPHAN PIMKHAOKHAM, Ph.D., 84 pp.

Objective To evaluate the effect of 4 different types of abutment material, which are titanium, zirconium oxide, gold alloy, and zirconia-coping cemented on titanium-base, on the surrounding soft tissues.

Material and Methods Twenty dental implants in posterior edentulous area were randomly divided into 4 groups and inserted 4 types of abutment materials; Titanium, zirconia, gold-alloy, and titanium-base, on the implant installation surgery day. Eight weeks after implant surgery, peri-implant soft tissues around experimental abutments were harvested and split according to implant side; buccal, lingual, mesial, and distal. The specimens were processed through immunohistochemical preparation and stained with CD3, CD20, CD68, CD138, and factorVIII to identify T-cells, B-cells, macrophages, plasma cells, and microvessels, respectively. The quantitative assessment of cell markers was performed by one pathologist. The total counts of positive cell for one compartment were expressed as numbers of positive cells per square millimetre of soft tissues. The outcome was assessed immunohistochemical characteristic and microvessel density (MVD).

Results Sixteen healthy patients, 6 males and 10 females, were included in this study. Total positive cells for titanium, zirconia, gold-alloy, and titanium-base, were 119.28, 177.06, 445.18, and 109, respectively. Factorial analysis of variance (factorial ANOVA) was used to analyse the data. Gold alloy group showed statistical significance higher number of positive cells, compared to titanium (p-value=0.009) and zirconia (p-value=0.042). Implant side exhibited no influence on positive cell number (p-value=0.825). Microvessel density was found no statistical difference between groups. Factorial AVOVA was performed and reported that no main effect was found in both abutment material (p-value=0.501) and implant side (p-value=0.910) to have influence on microvessel density.

Conclusions Different types of abutment material had an influence on peri-implant soft tissues in immunohistochemical features. Gold alloy abutments exhibited more inflammatory cells in surrounding tissues than titanium and zirconia abutments. Different sides of implant showed no statistical difference in peri-implant tissues response in immunohistochemical aspect and microvessel density. The tissues around gold alloy abutment tended to experience a higher rate of inflammation-associated processes when compared to titanium and zirconia abutments.

Field of Study:	Esthetic Restorative and Implant Dentistry	Student's Signature	.....
		Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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Background and rationale

Dental implant has become a treatment of choice for dental substitution and has been widely used as an anchorage of prosthesis. Many previous studies supported that dental implant survival rate and success rate were both high [1]. However, to restore a missing tooth in the anterior region is considered to be very challenging. Patient's aesthetic satisfaction has to be met in order to achieve success in restoration [2]. Dental implant treatment comprises of not only an implant fixture, but also the abutment part which establishes a transmucosal connection between the intraoral environment and the implant body [3].

Peri-implant tissues have a significant difference from periodontal tissues with lack of cementum and periodontal ligament, less blood vessels and fibroblasts in connective tissue and absence of an attached supra-crestal connective tissue [3, 4]. The establishment of a stable and healthy perimucosal seal that protects the underlying tissues from the intraoral environment is mainly determined by the adhesion, proliferation and colonisation of fibroblastic cells and microorganisms. The biocompatibility of

abutment materials to transmucosal areas is one of key influencing factors for soft tissue stability [5].

Because the peri-implant soft tissues morphology in anterior areas is not flat, the prosthetic emergence profile should reproduce the natural soft tissues scalloping [2]. The use of stock abutment can result in a round shape of the mucosa, with an emergence profile not showing a natural appearance. Anatomical shape of the customised abutment is able to help supporting the surrounding soft tissues and locating a proper cementation margin for cleaning cement excess [2, 6]. Currently, various materials are used to fabricate customised prosthetic abutments.

Gold alloy has been used to cast a customised UCLA abutment. The yellow colour of gold can favourably enhance the pink colour of the soft tissue, resulting in better aesthetic appearance [7]. It has been reported that the peri-implant soft tissue dimensions were not different between gold and titanium materials in previous study [8].

Zirconium abutment is one of materials used for fabrication of individually customised abutment. Zirconia offers a much better aesthetic outcome compared with titanium [6, 9] and some studies even claimed that it was the most biocompatible material with lower adhesion of bacteria [10]. However, its brittleness is considered to be a shortcoming of zirconium abutment [11].

On the contrary, zirconia abutments with an external connection or an internal hexagon two-piece construction, where zirconium abutment-like coping is cemented on a titanium base, showed excellent outcome in previous studies [12-14]. Titanium-base abutment with zirconia coping is another option to overcome the shortcoming of one-piece zirconia abutment.

The matter of biocompatibility of the material used in the transmucosal part can cause a chronic inflammation of soft tissue and result in a persistent inflammation process, tissue recession and demolition of the bone under the area [3, 5, 8, 15]. The soft tissue response can be evaluated directly by the biopsy specimens of the soft tissue around

implant abutments. However, the immunohistological analysis comparison of soft tissue response to different materials is very limited.

Research question

1. Do the peri-implant soft tissues respond similarly to 4 different experimental abutments: titanium, zirconia, gold alloy, and titanium-base with zirconia coping, in immunohistochemical features?

2. Does each side of the peri-implant soft tissues respond differently from others?

Research objectives

To evaluate the effect of 4 different types of abutment material, which are titanium, zirconium oxide, gold alloy, and zirconia-coping cemented on titanium-base, on each side of the surrounding soft tissues.

Hypothesis

Soft tissues response to the 4 different abutment materials demonstrates similar immunohistochemical characteristics.

Conceptual framework

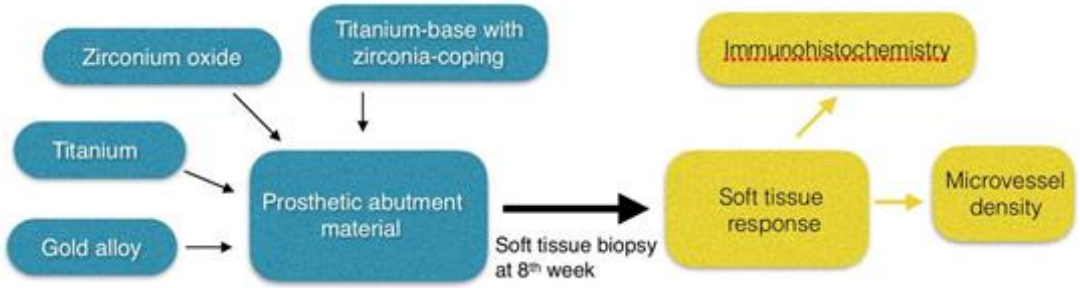


Figure 1

Conceptual framework of the study

Limitations

Even though the oral hygiene instruction will be provided to every participant, the plaque control ability of individuals is still not equal, and plaque accumulation can directly affect the inflammatory response in soft tissues.

Expected benefits of the study

The results achieved from this study will be beneficial for dentists to select the abutment, especially in a case that requires individually customised abutment in order that the best outcome would be achieved. Moreover, if the peri-implant soft tissues around zirconia abutment, gold alloy abutment, and zirconia coping with titanium-base abutment,

showed similar or less inflammatory response than ones around titanium abutment, the zirconia abutment, gold alloy abutment, and zirconia coping with titanium-base abutment would become restorative choices instead. The immunohistochemical characteristics of peri-implant soft tissues around 4 experimental abutments could be used as baseline evidence in future study.

Review of literatures

1. Abutment materials

Prosthetic abutment is a critical part of implant treatment due to the ability to establish a transmucosal connection and to maintain soft tissue around dental implant. In other words, this part plays an important role in protecting the peri-implant structures. The material used in abutment fabrication is one of the factors that determine the quality of the attachment presenting between the mucosa and the implant [3, 15].

Stock abutments were the only option available provided from implant manufacturers for many years. The shortcomings of these abutments are predetermined cement line position, which impedes the removal of cement remnants causing peri-

implant disease [16], and lack of emergence profile. On the contrary, customised implant abutments overcome the shortcomings above with their advantages, including soft tissue support and a favourable location of the cementation margin for cleaning cement excess [2, 6]. Various materials are used to fabricate individually customised abutment, currently.

Titanium was the preferred implant abutment material for decades because of its strength, resistance to distortion, and possibility to produce the abutment as one-piece, as which zirconium cannot be used to produce [6]. Nevertheless, titanium abutment has such a crucial drawback which is that its dark colour may shine through peri-implant soft tissue, resulting in a greyish appearance of the gingiva. This drawback is considered to be aesthetically unacceptable [5].

Gold has been used to cast the customised abutment for a long time, but the use has decreased recently because of low biocompatibility and higher pricing [6]. The yellow colour of gold can enhance the pink colour of gingiva, which results in favourable aesthetic outcome [7]. There is still a controversial issue that whether the titanium abutments have superiority over the gold ones [8].

An experimental study in dog, by Abrahamsson et al in 1998, has shown that peri-implant soft tissues do not form a proper attachment with gold abutments, in contrast to titanium abutments, and soft tissue recession and bone resorption can be expected [3]. Linkevicius and Aspe, from a systematic review in 2008, concluded that it is still unclear if titanium is superior to gold as an abutment material. Almost similar peri-implant tissue dimensions around implant abutments of both materials can be observed in animal histologic studies. There is also the evidence from clinical control trials showing that no difference can be found between gold alloy abutments and titanium abutments in terms of peri-implant bone stability [7, 8].

Zirconia is a widely used material for individually customised abutment fabrication. Zirconia abutments offer a much better aesthetic outcome than titanium ones and eliminate greyish appearance of the peri-implant mucosa, which is aesthetically unacceptable, especially in the case of thin gingival biotype [4, 6]. Number of studies even claimed that zirconia is the most biocompatible material [10]. Nevertheless, the

drawback of this material is its brittleness. Fractures of one-piece zirconia abutment with internal connection were found in both short- and long-term observation [6].

Degidi et al, 2006, conducted a comparative immunohistochemical evaluation in peri- implant soft tissues of titanium and zirconium healing caps. They found higher inflammatory infiltration and micro vessel density in the titanium specimens. From the level of expression of VEGF, Ki-67, NOS1 and NOS3, higher inflammation processes and higher amount of bacteria present around the titanium samples can be correlated [10]. According to a systematic review by Linkevicius and Aspe in 2008, peri-implant soft and hard tissues reacted very similarly to titanium and zirconium in animal histologic studies. In addition, even better reaction of human mucosa to zirconium were indicated in human histologic material [8]. Van Brakel R et al 2012, compared zirconia and titanium abutments in man in regard to the soft tissues health and reported that no differences were seen in peri-implant mucosa [5]. The 4th EAO consensus conference 2015 stated no significant differences in clinical outcomes, when comparing zirconia and titanium as abutment materials, in terms of probing pocket depth, bleeding on probing, marginal bone level,

and mucosal recessions. Zirconia abutments showed superiority in achieving natural soft tissue colour, although they may be associated with more biological complications [9].

Because the internal connection between a customised zirconia abutment and the implant continues to be a technical challenge, a zirconia abutment-like coping cemented to an antirational titanium component is introduced [14]. In contrast to one-piece zirconia abutment, in which internal connection fracture could be found, this customised bicomponent abutment showed the excellent outcome in previous studies [12-14].

Canullo, in 2007, evaluated the clinical performance of metallic-zirconia abutments in 25 patients with 30 implant-supported single-tooth restorations and found that these abutments may be comparable to other aesthetic implant abutments. The result from this study showed no abutment fractures and screw loosening throughout the clinical observation period [12]. Rosentritt et al 2015 investigated the influence of the combination of individually customized zirconia abutments and adhesive bases on the long-term in vitro performance of anterior crowns. They concluded that titanium adhesive bases and bonded patient-specific zirconia abutments provided good in vitro performance and high

fracture resistance for anterior implant-supported zirconia crowns. Moreover, sufficient high torque moments and early re-screwing would be recommended [14]. The influence of this type of abutment and cementation, between titanium-base and zirconia coping, on the peri-implant mucosa still remains unclear.

2. **Soft tissue interface**

The establishment of an early and long-standing effective barrier able to protect the peri-implant structures is crucial for initial healing or long-term behaviour of implants. The formation of this soft tissue barrier prevents the penetration of oral bacteria and their products into the implant body [3, 17]. The soft tissue interface was assessed and found to be 3-4 mm in the apico-coronal direction. This interface is called biological width, which consists of 2 zones: junctional epithelium and connective tissue attachment [3, 15].

Junctional epithelium is approximately 2 mm long [18]. The epithelium attaches to the implant surface via a basal lamina and hemidesmosomes, which can be formed at 2-3 days of healing. The presence of granulation tissue on the transmucosal part of implant prevents the down growth of epithelium [15].

Connective tissue attachment is located between the barrier epithelium and the marginal bone. It has found to be rich in collagen fibers, but poor in cells and vascular structure. This connective tissue consists of 2 zones, the inner zone contacting directly on the implant abutment surface and the outer zone that is richer in cells and blood vessels. The collagen fibers run parallel to the implant surface and are separated from the surface by a proteoglycan layer [15, 18].

3. **Inflammatory process in peri-implant soft tissue**

Healthy soft tissue around implant abutment is considered to be important for the long- term success of dental implant [17]. Peri-implantitis is an inflammatory lesion of bacterial etiology leading to mucosal inflammation and bone loss. The chronic inflammation can be identified from neovascularization and neoformation of collagen [19].

Angiogenesis is the formation of new capillaries due to the budding of endothelial cells. It is considered to play an important role in developing organs, inflammation, and wound healing. In periodontal tissues, the angiogenesis may be important both in maintenance of tissue health and in chronic inflammation of periodontal diseases.

Inflamed tissues tend to increase the expression of inflammatory mediators, which in turn may enhance angiogenesis. Vascular endothelial growth factor (VEGF), which is one of the major angiogenic activator, can be detected in vascular endothelial cells, inflammatory cells, and junctional, sulcular, and gingival epithelium. It is also involved in inflammation-associated process [10, 20, 21].

4. Immunohistochemical staining method

Immunohistochemistry is an investigative tool for providing information to the routine biological assessment of tissues. It has been used to define specific phenotypes from cellular markers. Moreover, it can also offer important diagnostic, prognostic, and predictive information relative to disease status and biology. Formalin-fixed paraffin-embedded tissues, has become the medium of choice for most clinical and research studies, since the superior morphology could be provided. In 1968, the first practical application of antibodies to paraffin-embedded tissues was introduced, called the peroxidase-labeled antibody method (figure 2). It was considered to be able to overcome some of the limitations of earlier fluorescence antibody methods [22].

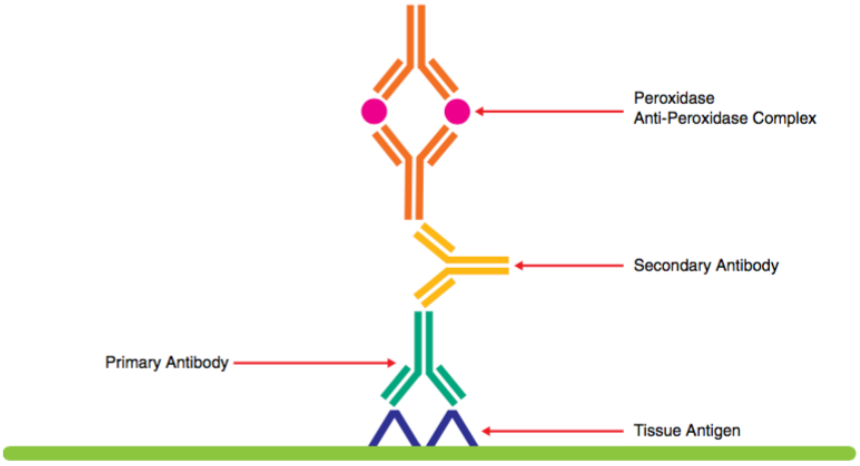


Figure 2

Peroxidase Anti-Peroxidase (PAP) Complex Method

Avidin-biotin complex (ABC) method (figure 3) came up in 1981 as a new generation of immunohistochemical method and remains widely used until today. This method relies on the strong affinity of avidin or streptavidin for the vitamin biotin. The biotin molecule, which is easily conjugated to antibodies and enzymes, can be bind to both streptavidin from Streptomycin avidinii and avidin from chicken egg via four binding sites. This method has secondary antibodies that are conjugated to biotin and function as links between avidin-biotin-peroxidase complex and tissue-bound primary antibodies [23].

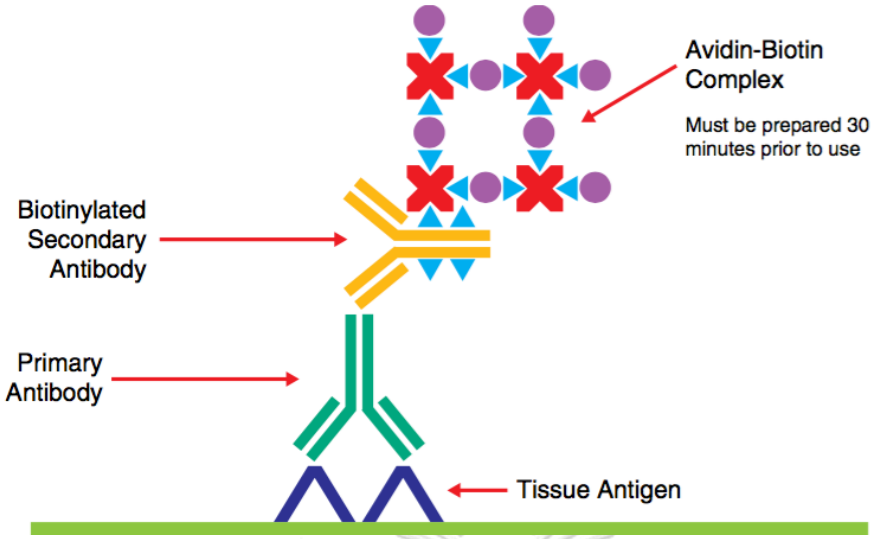


Figure 3

Avidin-Biotin Complex (ABC) Method

Labeled streptavidin-biotin (LSAB) method (figure 4) also uses a biotinylated secondary antibody that links primary antibodies and a streptavidin-peroxidase conjugate together, in a similar method to ABC method [24]. In both ABC and LSAB methods, a single primary antibody is connected with multiple peroxidase molecules, and due to the large enzyme-to-antibody ratio, an important increase in sensitivity is accomplished compared to direct peroxidase-conjugate methods.

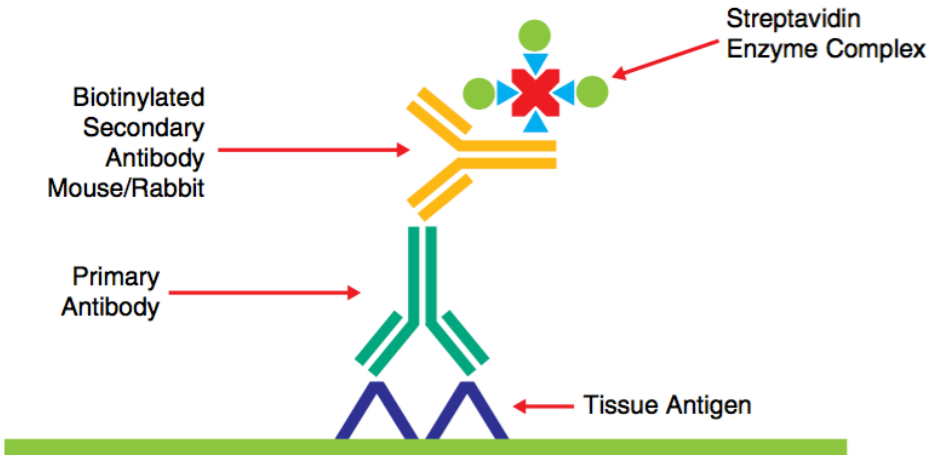


Figure 4

Labeled Streptavidin-Biotin (LSAB) Method

5. **Animal immunohistochemical study**

A study, comparing the soft tissues around implants with screwed and cemented abutments, was conducted in 6 beagle dogs by Assenza et al in 2006. They aimed to assess the differences in the expression of VEGF, MVD, proliferative activity (MIB-1), and inflammatory infiltrate in peri-implant soft tissues between 2 types of abutment connection. A total of 8 loosened screws, from 30 screwed abutments, were recorded while on the contrary no screw loosening was found among 30 cemented abutments. Gingival biopsies were retrieved from 8 implants of each cemented, screwed, and unscrewed abutment group. The results showed a statistically significant difference in MVD between screwed

and unscrewed abutments, and between cemented and unscrewed abutments. Plus, a high intensity of VEGF was prevalent in unscrewed abutment. Therefore, the assumption was made that the presence of bacteria inside the hollow portion of the implants or enhanced reparative processes could induce the results [20].

6. **Human immunohistochemical study**

Few immunohistochemical studies in human have been conducted and compared the peri-implant soft tissues response to different abutment materials. Some of them compared the soft tissue health between peri-implant mucositis and peri-impalntitis and/or some compared between periodontitis and peri-implantitis. Many antibodies have been used to detect different cellular markers in previous studies.

In 1997, Esposito M. et al investigated the cellular composition of the soft tissues surrounding late failed Branemark implants. The immunohistochemical assessment found that the peri-implant soft tissues contained a large number of macrophages, HLA-DR positive cells, lymphocytes and plasma cells. They concluded that a chronic inflammatory

response of the soft tissues surrounding late failures of implant displayed macrophages as the predominant cell type. To identify macrophages, CD68 was used in this study [25].

Gualini and Berglundh, 2003, assessed immunohistochemical characteristics of peri- implant mucositis and per-implantitis lesions. The avidin-biotin method (ABC) was used for immunohistochemical preparation. The size of the infiltrated connective tissue (ICT) and the proportions of different cell markers within the ICT were also assessed. In peri-implantitis, the proportion of B cells (CD19 positive) was found to be three times larger than in mucositis sites. This finding was consistent with studies reported the presence of large numbers of B cells on periodontitis lesions at natural teeth (Berglundh et al. 2001, Seymour & Greenspan 1979, Mackler et al. 1977). Moreover, the results showed that peri-implantitis lesions were larger. This was in agreement with Sanz et al, 1991, who reported that an inflammatory infiltrate occupied 65% of the connective tissue portion [26].

A comparative immunohistochemical evaluation in soft tissues surrounding titanium and zirconium oxide healing caps was conducted by Degidi et al, 2006. A gingival biopsy, with the dimension of 1.7 mm in thickness and 3 mm in height, was

retrieved from around the healing caps of both groups, without the healing caps removal. The immunohistochemical staining of VEGF, factor VIII, Ki-67, leukocyte common antigen (LCA), CD3, CD20, NOS1, NOS3 was done with streptavidine-biotine-peroxidase method (LSAB). The results demonstrated no significant differences in the number of B-lymphocytes and T-lymphocytes between groups, and higher inflammatory infiltrate and MVD in the titanium specimens. In addition, because of the level of expression of VEGF, Ki-67, NOS1 and NOS3, higher inflammation processes and higher amount of bacteria present in titanium specimens could be concluded [10].

Degeidi et al also conducted a prospective randomised study in 2012 to compare immunohistochemical features in the peri-implant soft tissues around machined and acid-etched titanium healing abutments. All healing caps were inserted on the same day as implant surgery, and the soft tissues were sutured around the caps. After the retrieval of gingival biopsy around healing caps, the LSAB method was used to perform the immunohistochemical staining of VEGF, factor VIII, Ki-67, CD3, CD20, CD68, NOS1, and NOS3. In the acid-etched titanium specimens, the inflammatory infiltrate, higher values of

MVD, a higher expression of VEGF intensity and Ki-67, and a higher number of T lymphocytes and B lymphocytes were obsereved. All these findings indicated that the tissues around acid-etched titanium healing abutments experienced a higher rate of restorative processes, most presumably correlated to the higher inflammation processes [21].

The human tissue response to titanium cover screws was studied by Olmedo et al 2012. Langerhans cells, macrophages, and T lymphocytes were identified using immunohistochemical techniques. The results confirmed the presence of macrophages and T lymphocytes and their association with the metal particles. In agreement with previous studies, the T lymphocytes infiltrate may suggest the presence of a cell-mediated immune response [27].

Carcuac and Berglundh, 2014, examined differences in cellular composition of human peri-implantitis and periodontitis lesions. To identify T cells, B cells, plasma cells, macrophages, and endothelial cells, the following antibodies were used in immunohistochemical preparation: CD3, CD20, CD138, CD68, and CD34, respectively.

The results demonstrated that the numbers and densities of plasma cells, macrophages, and PMN cells were higher in peri-implantitis than periodontitis sites. According to the results, they indicated that the inflammatory response in peri-implantitis sites was more intense by promoting cells of both innate and adaptive immune response [28].

7. Microvascular density

Many histological studies have utilised the microvascular density to evaluate the soft tissue characteristics. The microvessels are counted in the region of interest and the values would be expressed as number of micro vessels per square millimetre of peri-implant soft tissues [10, 20, 21, 29].

Materials and methods

1. Research design

This was a double blinded, randomised controlled clinical trial study, and designed to evaluate the differences in immunohistochemical features of soft tissues around 4 different abutments.

2. Diagram of study design

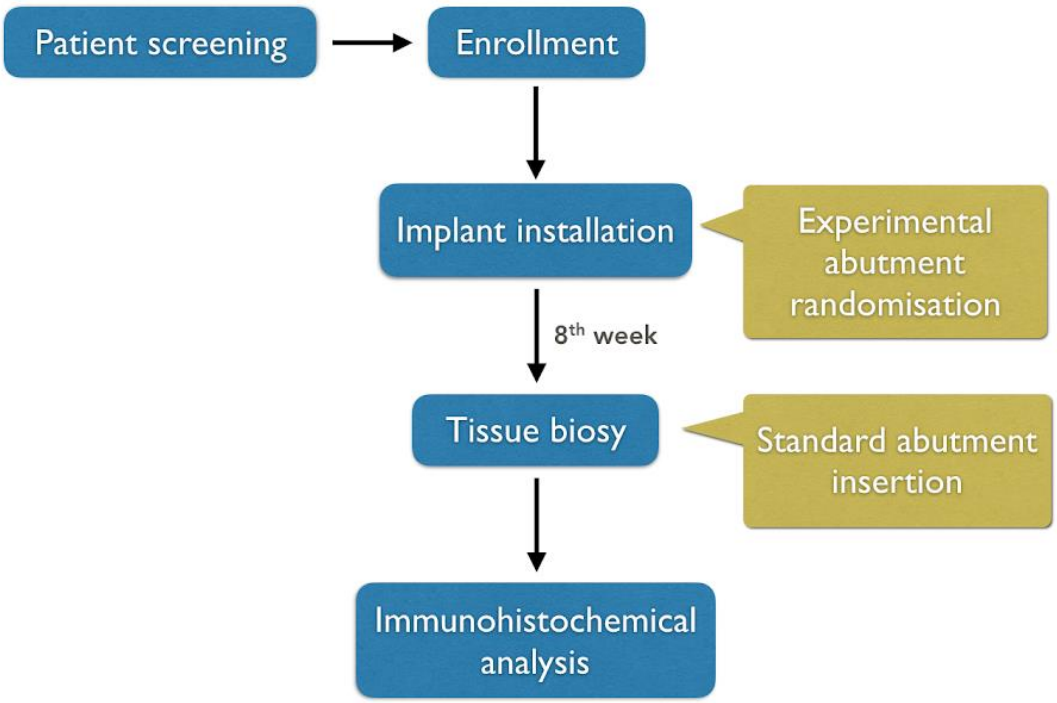


Figure 5

Diagram of the study

3. Ethical considerations

This experimental study in human involves the use of 4 different abutment materials which are currently used in the prosthetic part of implant restoration. The abutment of different materials will be randomly allocated in order to prevent the bias. In this study, the gingival biopsy method will be performed with scalpel blade.

The study protocol had been approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The approval number was HREC-DCU 2016-052.

4. **Population and sample**

Patients who required implant treatment in posterior teeth were asked for agreement to be included in this study on condition that they had tooth extraction at least 4 months with sufficient residual bone volume for the insertion of 4.8 mm-diameter implant and sufficient band of keratinized mucosa (>5mm). The exclusion criterions were smoker, pregnancy, a handicap that would not be able to perform adequate oral hygiene maintenance, and patients with systemic disease that required routine use of antibiotics.

5. **Sample size**

According to previous studies with the closest research design, which evaluated the immunohistochemical features in peri-implant soft tissues and compared between different healing abutments, one study used 5 samples per group in order to compare between 2 groups [21]. In another study, 5 patients participated without the disclosure of

implant and abutment numbers [10]. Both studies could find statistically significant differences with that number of sample size.

Therefore, the number of sample size of this study was planned to be 5 abutments per group, 20 abutments in total. Patients were divided randomly into 4 groups: titanium, zirconia, gold alloy, and zirconia-coping cemented on titanium-base.

6. **Allocation technique**

Each implant was allocated to one of the 4 groups (group1 = titanium, group2 = zirconia, group3 = gold alloy, and group 4 = zirconia-coping cemented on titanium-base). A randomization was performed by draw lots, letting the patient pick one envelope out. The envelope was opened after implant installation in order to blind an operator. An abutment that was made from the type of material written in the envelope will be screwed in the implant on the same day.

7. Experimental abutment

7.1 Group 1: Titanium

The TiDesign™ EV 4.8 triangular shaped abutment with a diameter of 5.5 mm (product code 25340) from Astra Tech Dental, Densply, Mölndal, Sweden (figure 6).

Figure 6

TiDesign™ EV 4.8 abutment diameter 5.5 mm (product code 25340)

7.2 Group 2: Zirconia

The ZirDesign™ EV 4.8 triangular shaped abutment with a diameter of 5.5 mm (product code 25322) from Astra Tech Dental, Densply, Mölndal, Sweden (figure 7).

Figure 7

ZirDesign™ EV 4.8 abutment diameter 5.5 mm (product code 25322)

7.3 Group 3: Gold alloy

The CastDesign™ EV 4.8 abutment with a diameter of 5.1 mm (product code 25328) from Astra Tech Dental, Densply, Mölndal, Sweden (figure 8). The abutment was casted with gold type 4 to the same shape and diameter as the abutment in group 1 and group 2.

Figure 8

CastDesign™ EV 4.8 abutment diameter 5.1 mm (product code 25328)

7.4 Group 4: Titanium-base

The TitaniumBase EV 4.8 abutment with a diameter of 5.0 mm (product code 25930) (figure 9) from Astra Tech Dental, Densply, Mölndal, Sweden. Zirconia-coping will be fabricated by one lab technician using CAD/CAM technique into the same shape and diameter as the abutment in group 1 and group2 and cemented to the titanium-base abutment with resin cement (Multilink® Automix, Ivoclar Vivadent, Liechtenstein)

Figure 9

TitaniumBase EV 4.8 abutment diameter 5.0 mm (product code 25930)

8. Intervention

8.1 Surgical protocol

OsseoSpeed™ EV implants with a diameter of 4.8 mm were used. The length of the implant was selected depending on each individual bone volume available, which could be determined by computed tomography scan. Implant surgical protocol was

performed under local anesthesia with a standard protocol by dentists who were attending the CE course and/or studying at Esthetic Restorative and Implant Dentistry program, Faculty of Dentistry, Chulalongkorn University, during years 2016-2017. All dentists performed the implant surgery under supervision of one experienced surgeon. After flap elevation and osteotomy site preparation, the implant fixture was placed at the crestal bone level in all aspects. Then patient was asked to draw one envelop for abutment randomization.

An abutment, that is made from the type of material in the envelope, was screwed in the implant fixture, instead of routine use of healing abutment, on the same day. Flap was approximated and sutured around the experimental abutment. The occlusal part of abutment will be adjusted to avoid occluding teeth in every direction and jaw movement. The screw access hole will then be covered with esthetic tape, cavit, and resin composite.

An antibiotic for 1 week and a 0.2% Chlorhexidine mouth wash for 2 weeks were prescribed for all patients. Two weeks after the surgery, patients were appointed for stitch-off and wound evaluation. At 8 weeks, patients were appointed for a tissue biopsy visit.

8.2 Gingival biopsy

Eight weeks after implant installation, patients were asked to rinse the mouth with a 0.2% chlorhexidine mouthwash solution prior to soft tissues biopsy. Scalpel blade no.15C was used to cut the peri-implant soft tissue with 1 mm apart from the experimental abutment and the angle of the blade was paralleled to the abutment surface.

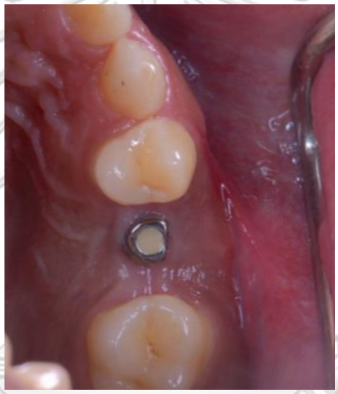


Figure 10

Clinical intraoral photograph demonstrated gingival condition around abutment at 8 weeks after implant placement.

The experimental abutment was unscrewed and removed together with the specimen, then regular titanium healing abutment (HealDesign™ EV 4.8, product code 25919) with a diameter of 7.5 mm was screwed in the implant fixture instead. The

specimen was sutured at mesio-buccal line angle in order to identify the abutment side and immediately fixed in 10% formalin solution.

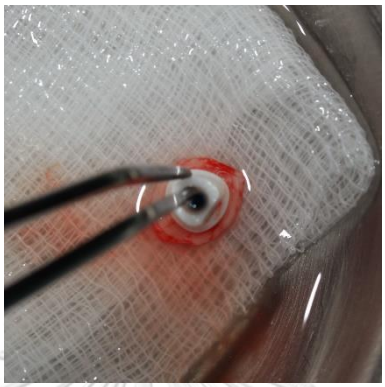


Figure 11

A cuff of soft tissue biopsy (Degidi et al, 2012)

One month later, the patient was appointed for the impression and further prosthetic restoration.

8.3 Immunohistochemical preparation

The immunohistochemical staining of factor VIII, CD3, CD20, CD68, and CD138 was prepared using labeled streptavidin-biotin (LSAB) method.

The peri-implant soft tissue specimen was fixed in 10% formalin solution for 24 hours and embedded in paraffin. Six micrometers tissue sections were obtained by a

microtome and mounted on glass slides coated with 3-aminopropyltriethoxy-silane (Sigma Aldrich, St. Louis, MO, USA). Following deparaffinization by xylene and rehydration, the sections was washed with phosphate buffered saline (PBS), pH 7.4, for 10 minutes. To unmask the antigens, the slides were incubated with a 2.1% content of citric acid for 30 minutes. Moreover, 2% PBS- BSA (Bovine Serum Albuin) was used, in order to block nonspecific bindings, for 30 minutes. Then, the sections were incubated in a solution containing the primary antibodies, in the refrigerator overnight. On the following day, the slides were rinsed in buffer and treated with methanol and 3% hydrogen peroxide (H_2O_2) for 15 minutes to block endogenous peroxidase. Biotinylated link antibody and streptavidin peroxidase antibody (DAKO-LSABkit, Carpinteria, USA) were incubated for 40 min at 22^{o}C. To visualize the specific reaction to each antibody, 3,3'-diaminobenzidin(DAB) was utilized by application and incubation in the dark for 7 minutes. After this, the specimens were counterstained with Mayer's haematoxylin and coverslipped.

9. Outcome measurement

9.1 Immunohistochemical assessment

The surface area of the specimen was evaluated. The quantitative assessments of cell markers were performed using a microscope equipped with an image system. To identify T cells, B cells, plasma cells, and macrophages, the antibodies to CD3, CD20, CD138, and CD68, were used respectively. The positive cell in the compartment was counted twice and related to the surface area and expressed as positive cell number per square millimeter. Every specimen was evaluated by only one pathologist.

9.2 Microvessel density

The antibody to the human factor VIII was used in this study to highlight the blood microvessels. All the morphologic structures with a lumen surrounded by factor VIII-positive endothelial cells were considered as blood microvessels. Counting of the microvessels was performed with a 200-fold magnification, and the individual microvessel profiles were circled for prevention of the duplicates counting. The microvessel density

(MVD) values were expressed as numbers of microvessels per square millimetre of soft tissues.

9.3 Data collection and analysis

The mean value and standard deviation of the data among each experimental group will be analyzed by descriptive statistics, using statistical software SPSS 23.0 (SPSS, Chicago, IL, USA). The data will be categorized by the location of soft tissue which are buccal, lingual, mesial, and distal.

To compare between groups of positive cell number of each marker and microvessel density, factorial analysis of variance (factorial ANOVA) and Tukey HSD post hoc test for multiple comparisons will be performed. Results will be considered to have statistically significant difference at p-value < 0.05.

Results

Seventeen healthy patients, 6 males and 11 females, who fulfilled the inclusion criteria, were included in this study. Mean age was 54 for titanium group, 52.4 for zirconia group, 51.6 for gold alloy group, and 45 for titanium-base group. Tooth number of each inserted experimental abutment was clarified in Table 1.

Table 1 *Demographic data of all cases*

Case	Sex	Age	Tooth number
T1	M	29	15
T2			46
T3	F	50	36
T4	F	71	36
T5	F	66	47
Z1	F	55	36
Z2	M	58	46
Z3	F	47	57
Z4	M	30	46
Z5	F	62	16
G1	M	56	47
G2	M	63	46
G3	F	61	47
G4	M	51	36
G5	F	27	35
TZ1	F	59	36
TZ2			37
TZ3	F	27	26
TZ4	F	50	46
TZ5			47

1. Clinical findings

All implants healed completely on the day of peri-implant soft tissue biopsy, without any complications observed. No clinically plaque accumulation, suppuration, and soft-tissue swelling were presented in all cases.

2. Immunohistochemical findings

In titanium group, mean of total positive cells was 119.28 (±117.92) which was considered to be the lower than zirconia and gold alloy, but higher than titanium-base. Buccal side of the implant showed the highest mean positive cells and distal side showed the lowest which were 161.45 and 70.20, respectively (Table 2).

In zirconia group, mean of total positive cells was 177.06 (±213.76) which was higher than than titanium and titanium-base, but lower than gold alloy. The side that showed the highest mean positive cells was mesial, which was 240.69. And the lowest mean positive cells provided from buccal side which was 105.07 (Table 2).

In gold alloy group, mean of total positive cells was 445.18 (±476.66) which was the highest amount among 4 experimental groups. Distal side was observed the highest mean positive cells which was 622.56, while buccal side was observed the lowest mean positive cells which was 280.15 (Table 2).

In titanium-base group, mean of total positive cells was 109 (±56.32) which was the lowest amount among 4 experimental groups. The highest mean positive cells were presented in distal side and the lowest mean positive cells was presented in mesial side, which were 107.34 and 76.25, respectively (Table 2). Due to the limitation of study time, the specimens in this group was harvested and evaluated in time only 2 specimens.

Table 2 Mean of positive cells

Type of Abutment	Site of collected tissue	Mean	Std. Deviation	N
Titanium	Mesial	142.1660	104.62784	5
	Buccal	161.4480	187.56968	5
	Distal	70.2000	57.70928	5
	Lingual	103.2900	101.92833	5
	Total	119.2760	117.92697	20

Zirconia	Mesial	240.6860	142.18981	5
	Buccal	105.0740	95.91523	5
	Distal	207.4060	376.30824	5
	Lingual	155.0640	180.91321	5
	Total	177.0575	213.76271	20
Gold	Mesial	510.6740	388.04237	5
	Buccal	280.1520	304.60918	5
	Distal	622.5600	642.22461	5
	Lingual	367.3440	580.59101	5
	Total	445.1825	476.65706	20
TiBase	Mesial	76.2500	22.98097	2
	Buccal	107.3350	78.72220	2
	Distal	131.0000	100.40916	2
	Lingual	121.4000	44.40631	2
	Total	108.9963	56.32231	8
Total	Mesial	271.7724	271.35587	17
	Buccal	173.4141	200.72341	17
	Distal	280.1665	441.39075	17
	Lingual	198.3112	329.01172	17
	Total	230.9160	318.95045	68

To analyse the results data, factorial analysis of variance (Factorial ANOVA) was used and 3 statistical facts could be stated. First, main effect was found in abutment material to have the influence on positive cell number (p-value=0.006). Second, main

effect was not found in implant side to have the influence on positive cell number (p-value=0.825). And the last one, there was no interaction effect of abutment material and implant side (p-value=0.963) (Table 3). Moreover, Tukey HSD post hoc test demonstrated that gold alloy showed statistical significance higher number of positive cells, compared to titanium (p-value=0.009) and zirconia (p-value=0.042) (Table 4).

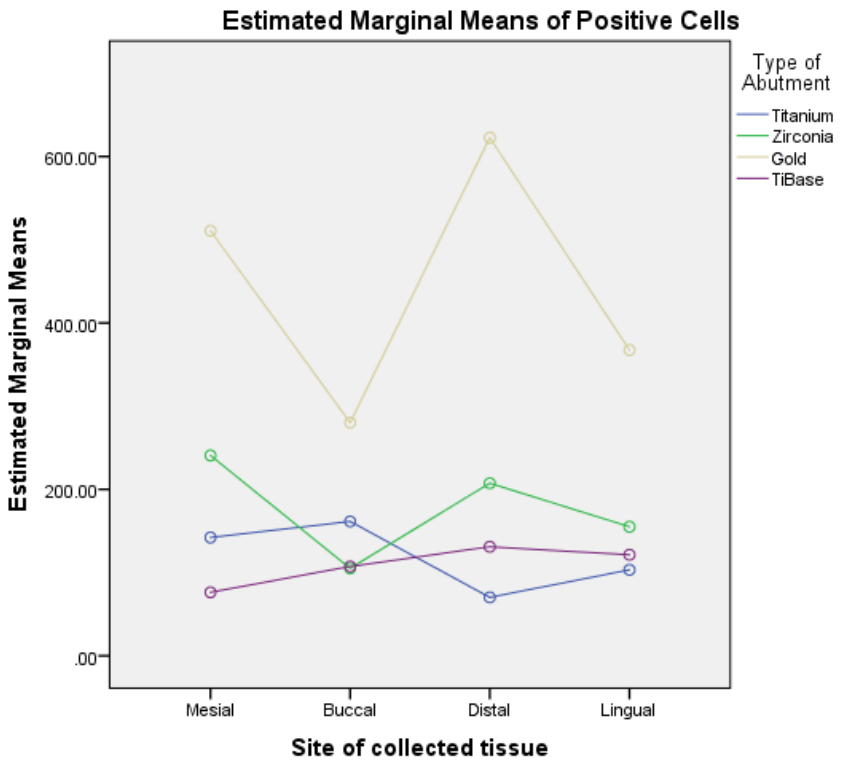


Figure 12

Effect of abutment type and implant side on sum of positive cells

Table 3 *Factorial ANOVA results to test the influence of type of abutment and implant side on positive cells*

Source	SS	df	MS	F	Sig.
Abutment	1344402.616	3	448134.205	4.619	.006
Site	87425.140	3	29141.713	.300	.825
Abutment * Site	282767.553	9	31418.617	.324	.963
Error	5044804.342	52	97015.468		
Total	10441779.625	68			

Table 4 *Tukey HSD post hoc test for multiple comparisons of type of abutment*

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Titanium	Zirconia	98.49643	.936	-319.2008	203.6378
	Gold	98.49643	.009	-587.3258	-64.4872
	TiBase	130.29853	1.000	-335.5454	356.1049
Zirconia	Titanium	98.49643	.936	-203.6378	319.2008
	Gold	98.49643	.042	-529.5443	-6.7057
	TiBase	130.29853	.953	-277.7639	413.8864
Gold	Titanium	98.49643	.009	64.4872	587.3258
	Zirconia	98.49643	.042	6.7057	529.5443
	TiBase	130.29853	.060	-9.6389	682.0114
TiBase	Titanium	130.29853	1.000	-356.1049	335.5454
	Zirconia	130.29853	.953	-413.8864	277.7639
	Gold	130.29853	.060	-682.0114	9.6389

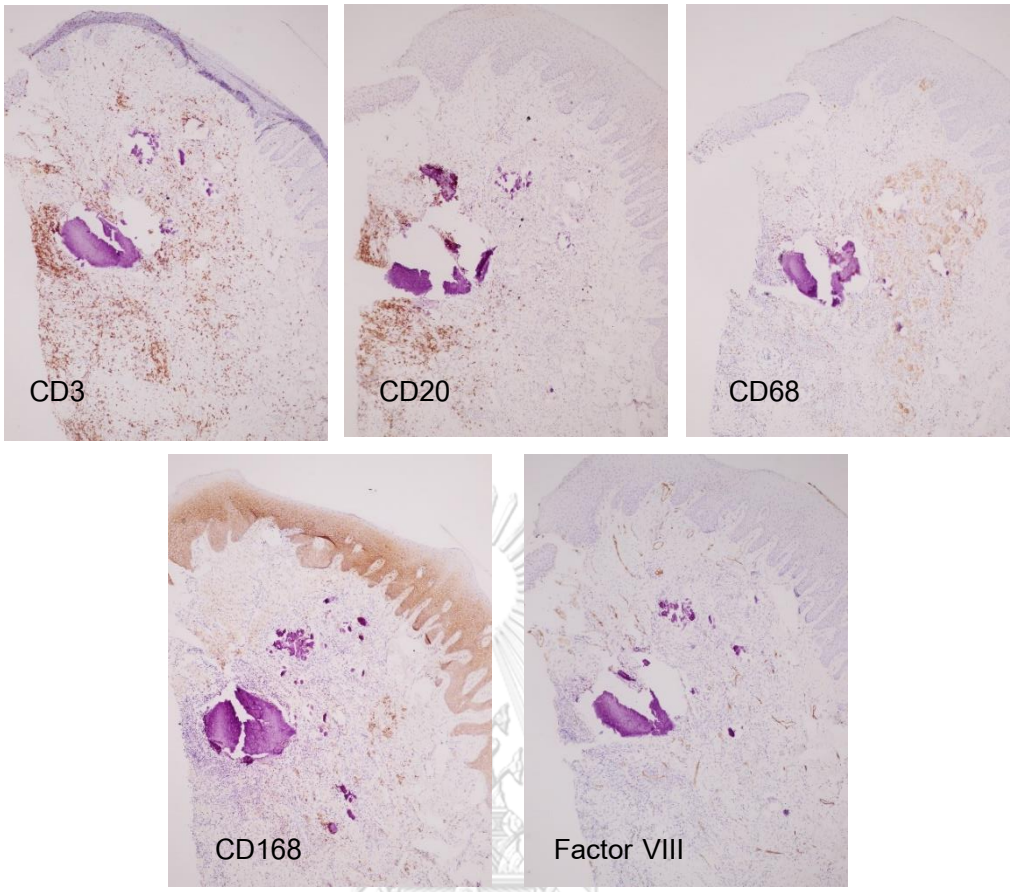


Figure 13

Sections prepared from soft tissues surrounding gold abutment. Positive cells were marked in brown color. From top left: CD3, CD20, CD68, CD138 and Factor VIII markers.

To evaluate thoroughly by each marker, CD3 and CD138 positive cells demonstrated no significant difference between groups (Table5, 6), while CD20 positive cells were found to be significantly greater in gold alloy group than titanium (p-value=0.018) and zirconia (p-value=0.038) (Table7). Moreover, CD68 positive cells

showed significantly higher number in gold alloy group than titanium (p-value=0.017), zirconia (p-value=0.022), and titanium-base (p-value=0.049) (Table8).

Table 5 *The numbers of CD3 positive cells were analysed by Tukey HSD post hoc test for multiple comparision between groups.*

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Titanium	Zirconia	41.71313	.803	-148.4168	73.0048
	Gold	41.71313	.057	-218.9843	2.4373
	TiBase	55.18128	1.000	-141.4883	151.4248
Zirconia	Titanium	41.71313	.803	-73.0048	148.4168
	Gold	41.71313	.338	-181.2783	40.1433
	TiBase	55.18128	.866	-103.7823	189.1308
Gold	Titanium	41.71313	.057	-2.4373	218.9843
	Zirconia	41.71313	.338	-40.1433	181.2783
	TiBase	55.18128	.183	-33.2148	259.6983
TiBase	Titanium	55.18128	1.000	-151.4248	141.4883
	Zirconia	55.18128	.866	-189.1308	103.7823
	Gold	55.18128	.183	-259.6983	33.2148

Table 6 *The numbers of CD138 positive cells were analysed by Tukey HSD post hoc test for multiple comparision between groups.*

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Titanium	Zirconia	4.61448	.990	-13.6603	10.8343
	Gold	4.61448	.324	-20.1798	4.3148
	TiBase	6.10438	.922	-12.3614	20.0419
Zirconia	Titanium	4.61448	.990	-10.8343	13.6603
	Gold	4.61448	.497	-18.7668	5.7278
	TiBase	6.10438	.825	-10.9484	21.4549
Gold	Titanium	4.61448	.324	-4.3148	20.1798
	Zirconia	4.61448	.497	-5.7278	18.7668
	TiBase	6.10438	.229	-4.4289	27.9744
TiBase	Titanium	6.10438	.922	-20.0419	12.3614
	Zirconia	6.10438	.825	-21.4549	10.9484
	Gold	6.10438	.229	-27.9744	4.4289

Table 7 *The numbers of CD20 positive cells were analysed by Tukey HSD post hoc test for multiple comparision between groups.*

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Titanium	Zirconia	60.82703	.991	-179.3819	143.4999
	Gold	60.82703	.018	-347.4624	-24.5806
	TiBase	80.46660	1.000	-215.6073	211.5253
Zirconia	Titanium	60.82703	.991	-143.4999	179.3819
	Gold	60.82703	.038	-329.5214	-6.6396
	TiBase	80.46660	.997	-197.6663	229.4663
Gold	Titanium	60.82703	.018	24.5806	347.4624
	Zirconia	60.82703	.038	6.6396	329.5214
	TiBase	80.46660	.114	-29.5858	397.5468
TiBase	Titanium	80.46660	1.000	-211.5253	215.6073
	Zirconia	80.46660	.997	-229.4663	197.6663
	Gold	80.46660	.114	-397.5468	29.5858

Table 8 *The numbers of CD68 positive cells were analysed by Tukey HSD post hoc test for multiple comparision between groups.*

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Titanium	Zirconia	7.71730	1.000	-21.2000	19.7650
	Gold	7.71730	.017	-44.1605	-3.1955
	TiBase	10.20903	.986	-23.5800	30.6115
Zirconia	Titanium	7.71730	1.000	-19.7650	21.2000
	Gold	7.71730	.022	-43.4430	-2.4780
	TiBase	10.20903	.976	-22.8625	31.3290
Gold	Titanium	7.71730	.017	3.1955	44.1605
	Zirconia	7.71730	.022	2.4780	43.4430
	TiBase	10.20903	.049	.0980	54.2895
TiBase	Titanium	10.20903	.986	-30.6115	23.5800
	Zirconia	10.20903	.976	-31.3290	22.8625
	Gold	10.20903	.049	-54.2895	-.0980

3. Microvessel density

In titanium group, the total mean number of microvessel density was 14.62(±9.16). Lingual side and distal side showed the highest value, which was 18.11, and lowest value which was 10.03, respectively (Table 9).

In zirconia group, the total mean number of microvessel density was 14.97(±20.50). The highest number was showed in distal side (23.33) and the lowest number was showed in lingual side (9.31) (Table 9).

In gold alloy group, the total mean number of microvessel density was 24.36(±32.98). Mesial side exhibited the highest number of 46.92 whereas buccal exhibited the lowest number of 14.23 (Table 9).

In titanium-base group, the total mean number of microvessel density was 19.27(±11.79), which was calculated from only 2 abutments. Because of the limitation of study time, the specimens from 3 remaining abutments were not available. Buccal side showed the highest number and mesial side showed the lowest number, which were 28.34 and 11.34, respectively (Table 9).

Table 9 *Microvessel density (MVD)*

Type of Abutment	Site of collected tissue	Mean	Std. Deviation	N
Titanium	Mesial	14.5920	5.94904	5
	Buccal	15.7780	7.34466	5
	Distal	10.0320	6.05394	5
	Lingual	18.1100	15.13291	5
	Total	14.6280	9.15601	20

Zirconia	Mesial	14.1400	7.40689	5
	Buccal	13.1060	11.02708	5
	Distal	23.3280	40.64347	5
	Lingual	9.3100	5.94906	5
	Total	14.9710	20.50063	20
Gold	Mesial	46.9220	60.19840	5
	Buccal	14.2300	8.82098	5
	Distal	18.3340	13.69773	5
	Lingual	17.9620	20.41488	5
	Total	24.3620	32.98225	20
TiBase	Mesial	11.3350	9.42573	2
	Buccal	28.3350	22.15366	2
	Distal	20.1200	9.26310	2
	Lingual	17.3150	2.99106	2
	Total	19.2762	11.79434	8
Total	Mesial	23.5847	34.29546	17
	Buccal	16.0141	10.80010	17
	Distal	17.5712	22.43921	17
	Lingual	15.3847	13.68373	17
	Total	18.1387	22.00837	68

Factorial analysis of variance (Factorial ANOVA) was performed to analyse the data and compare the number of microvessel density between groups. No main effect

was found in both abutment material (p-value=0.501) and implant side (p-value=0.910) to have influence on microvessel density. There was no interaction effect of abutment material and implant side (p-value=0.613) (Table 10).

Table 10 *Factorial ANOVA results to test the influence of type of abutment and implant side on positive cells*

Source	SS	df	MS	F	Sig.
Abutment	1232.128	3	410.709	.798	.501
Site	277.665	3	92.555	.180	.910
Abutment * Site	3731.687	9	414.632	.805	.613
Error	26773.515	52	514.875		
Total	54825.468	68			

Discussion

In the present study, some immunohistochemical features of peri-implant soft tissues, around titanium, zirconia, gold alloy and titanium-base combined with zirconia abutments, were evaluated. It was demonstrated that gold alloy group contained greater number of inflammatory cells in surrounding soft tissues, comparing to titanium and zirconia groups. While titanium, zirconia, and titanium-base contained considerably similar number.

The finding which greater number of inflammatory cells were presented in soft tissue surrounding gold alloy abutment was in agreement with previous studies. Abrahamsson et al, 1998, examined histological features of peri-implant soft tissue after 3 months healing period in 5 beagle dogs and found significant soft tissue margin recession and bone resorption in gold alloy group, unlike titanium and zirconia groups [3]. Welander et al, 2008, suggested that soft tissue healing around abutment made of gold alloy was different to that at abutments made of titanium and zirconia. In gold alloy group,

it was reported lower amounts of collagen and fibroblasts and larger fractions of leukocytes [30].

In addition, in peri-implantitis, the proportion of B cells was found to be three times larger than in mucositis sites [26], which was consistent with studies reported the presence of large numbers of B cells on periodontits lesions at natural teeth [31-33]. It is known that, in the case of adult chronic periodontitis, the inflammatory infiltrate is mainly composed of B lymphocytes [32, 34]. B-cells were the only cells that produce antibodies. B-cells also expressed membrane antibodies that recognize antigens and effector B-cells secrete the antibodies that neutralize and eliminate the antigen [35]. Likewise, in the present study, the amount of B cells in gold alloy group was significantly higher than titanium and zirconia, even though the amount of T cells was considered to be similar among different groups.

Besides, Esposito M, 1997, concluded that a chronic inflammatory response of the soft tissues surrounding late failures of implant displayed macrophages as the predominant cell type. This present study found significantly greater number of

macrophage in gold alloy group than the others [25]. Taken all immunohistochemical features together, gold alloy abutment as observed to be inferior to titanium, zirconia, and titanium-base abutment.

On contrary, conflicting data were demonstrated in an animal experiment by Abrahamsson and Cardaropoli, 2007. The study on 4 beagle dogs reported that the peri-implant soft tissue dimensions were not influenced by different types of abutment, whether titanium or gold alloy were used [36]. Furthermore, Vigolo et al, 2006, in the study on 20 patients reported no different behaviour of peri-implant soft tissue was observed when titanium abutments or gold alloy abutments were used [7]. In this context, it should be recognised the major difference in experimental design, though. This present study reported the peri-implant soft tissue in human, whereas Abrahamsson and Cardaropoli, 2007, conducted the experiment in dogs. Vigolo et al, 2006, reported periodontal parameter data for peri-implant mucosal response and radiographic assessment for marginal bone response comparison with the fact that the present study assessed the immunohistochemical features of peri-implant soft tissues which could identify

inflammation cells and microvessels in soft tissues. Therefore, the difference found in this study between soft tissues surrounding titanium and gold alloy abutments, might not be able to be observed by intraoral periodontal examination, but the cell-level evaluation might be necessary.

The results presented in this study, which no differences in peri-implant soft tissue adjacent to titanium and zirconia abutment surfaces were observed in immunohistochemical aspect, was in agreement with many previous reports. Welander et al, 2007, in a study on labrador dogs found no statistically significant differences in soft tissue healing to abutments made of titanium and zirconia. The soft tissue dimensions at both abutment materials remained stable between 2 and 5 months of healing [30]. Likewise, van Brakel et al,2012, studied soft tissue response to zirconia and titanium implant abutments in 20 edentulous patients, a total of 40 implants. Three months after implant installation, soft tissue biopsies were prepared for histological evaluation. The results showed that no differences in soft tissue health were seen in peri-implant soft tissue surrounding titanium and zirconia abutments [5]. Furthermore, Ferrari et al, 2015, in an

randomised clinical trial study assessed the effect of different prosthetic abutment on peri-implant soft tissue. They reported that soft tissues around abutment were not influenced by different types of abutments, which were titanium, titanium nitrade, and zirconia, after 2 years of clinical service [4].

Linkevicius and Vaitelis, 2015, conducted the systematic review and meta-analysis about the effect of titanium or zirconia as implant abutment material on peri-implant soft tissues. They concluded no obvious advantage of titanium or zirconia abutment over each other on soft tissue recession, probing depths, bleeding on probing, marginal bone level, and patient-reported outcome. Nevertheless, only a tendency in zirconia abutment giving rise to better colour response of peri-implant mucosa and better esthetic outcome as a consequence [6]. Sicilia et al, 2015, also summarized the effect of titanium and zirconia abutments on peri-implant soft tissues in the 4th EAO consensus conference. It was demonstrated that no significant differences were seen between titanium and zirconia abutments when evaluating probing depth, bleeding on probing, mucosal recession, and marginal bone levels [9].

On the other side, Degidi et al, 2006, conducted a comparative immunohistochemical evaluation in peri-implant soft tissue of titanium and zirconia healing caps. Five patients were participated in the study and after 6 months of healing period, gingival biopsies were performed. The results revealed that the tissues in titanium group experienced a higher rate of inflammation-associated process, most probably correlated to the higher inflammation processes observed in these tissues. Including microvessel density (MVD), higher number was observed in titanium group. Nevertheless, statistically similar MVD values were observed in this present study [10]. The difference may be explained by the finding of Rimondini et al, 2002, which stated that zirconia accumulated significantly fewer bacteria compared to titanium. Titanium surfaces appeared to be uniformly coated with a biofilm, when in fact, zirconia surfaces were only colonised by clusters of bacteria [37]. Nakamura et al, 2010, also reported that zirconia abutment surface was found to be less attractive for early plague retention compared to titanium [38]. However, further investigation should be conducted due to the fact that only 5

patients were included in Degidi et al, 2006, whereas 17 patients were included in this present study.

Few studies were available on titanium-base cemented with zirconia abutment. Martin et al, 2015, investigated the influence of the individually customised, zirconia or polyetherketone (PEEK), and titanium adhesive base on in vitro performance of anterior crowns. The results were able to be concluded that titanium adhesive bases and bonded customised zirconia abutments provided good in vitro performance and high fracture resistance on supporting zirconia crowns [14]. In agreement with the present study, adhesive base and abutment combinations might be appropriate for anterior application with the advantage of esthetic outcome over titanium abutments and endurance over zirconia abutments.

Moreover, Canullo evaluated clinical outcome of cemented customised zirconia abutments on titanium post abutment for single-implant restorations. Twenty-five patients are recruited for 30 implant-supported single-tooth restorations. No abutment fracture or screw loosening was reported during clinical loading, resulting in 100% survival rate at 6

monthly intervals over a 36- to 44- month period [12]. According to the conclusion, metallic-zirconia abutments might be comparable to other available esthetic implant abutment.

As reported in the results, macrophages (CD68 positive cells) were observed less in titanium-base group than gold alloy group. It's acknowledged that the infiltrating cells in inflamed periodontal lesions were constituted of macrophages from 5 to 30% and also shown that macrophages produce cytokines such as IL-1, IL-6, IL-10, IL-12, IL-13, IFN-α and TNF-α [39]. The inflamed gingival tissues demonstrated that IL-1β or IL-1β mRNA-expressing cells were mostly macrophages [40]. Taken together from currently available studies, titanium-base abutment was one of the abutment of choice and suitable for clinical use, which was in accordance with the results of the present study. Comparing titanium-base to gold alloy abutment, the results of this study demonstrated that titanium-base abutment was even better in immunohistochemical features.

Conclusion

In summary, this in vivo study demonstrated that different types of abutment material had an influence on peri-implant soft tissues in immunohistochemical features. Gold alloy abutments exhibited more inflammatory cells in surrounding tissues than titanium and zirconia abutments, while microvessel density exhibited no significant difference among experimental abutment materials. Furthermore, different sides of implant showed no statistical difference in peri-implant tissues response in immunohistochemical aspect and microvessel density. According to the results, it was suggested that the tissues around gold alloy abutment experienced a higher rate of inflammation-associated processes when compared to titanium and zirconia abutments.

REFERENCES

1. Moraschini, V., et al., *Evaluation of survival and success rates of dental implants reported in longitudinal studies with a follow-up period of at least 10 years: a systematic review.* Int J Oral Maxillofac Surg, 2015. **44**(3): p. 377-88.
2. Lops, D., et al., *Soft tissues stability of cad-cam and stock abutments in anterior regions: 2-year prospective multicentric cohort study.* Clin Oral Implants Res, 2015. **26**(12): p. 1436-42.
3. Abrahamsson, I., et al., *The mucosal attachment at different abutments. An experimental study in dogs.* J Clin Periodontol, 1998. **25**(9): p. 721-7.
4. Ferrari, M., et al., *Effect of different prosthetic abutments on peri-implant soft tissue. A randomized controlled clinical trial.* Am J Dent, 2015. **28**(2): p. 85-9.
5. van Brakel, R., et al., *Soft tissue response to zirconia and titanium implant abutments: an in vivo within-subject comparison.* J Clin Periodontol, 2012. **39**(10): p. 995-1001.
6. Linkevicius, T. and J. Vaitelis, *The effect of zirconia or titanium as abutment material on soft peri-implant tissues: a systematic review and meta-analysis.* Clin Oral Implants Res, 2015. **26 Suppl 11**: p. 139-47.
7. Vigolo, P., et al., *A 4-year prospective study to assess peri-implant hard and soft tissues adjacent to titanium versus gold-alloy abutments in cemented single implant crowns.* J Prosthodont, 2006. **15**(4): p. 250-6.
8. Linkevicius, T. and P. Apse, *Influence of abutment material on stability of peri-implant tissues: a systematic review.* Int J Oral Maxillofac Implants, 2008. **23**(3): p. 449-56.
9. Sicilia, A., et al., *Long-term stability of peri-implant tissues after bone or soft tissue augmentation. Effect of zirconia or titanium abutments on peri-implant soft tissues. Summary and consensus statements. The 4th EAO Consensus Conference 2015.* Clin Oral Implants Res, 2015. **26 Suppl 11**: p. 148-52.
10. Degidi, M., et al., *Inflammatory infiltrate, microvessel density, nitric oxide synthase expression, vascular endothelial growth factor expression, and*

proliferative activity in peri-implant soft tissues around titanium and zirconium oxide healing caps. J Periodontol, 2006. **77**(1): p. 73-80.

11. Belser, U.C., et al., *Outcome analysis of implant restorations located in the anterior maxilla: a review of the recent literature.* Int J Oral Maxillofac Implants, 2004. **19 Suppl**: p. 30-42.
12. Canullo, L., *Clinical outcome study of customized zirconia abutments for single-implant restorations.* Int J Prosthodont, 2007. **20**(5): p. 489-93.
13. Glauser, R., et al., *Experimental zirconia abutments for implant-supported single-tooth restorations in esthetically demanding regions: 4-year results of a prospective clinical study.* Int J Prosthodont, 2004. **17**(3): p. 285-90.
14. Rosentritt, M., et al., *In vitro performance of implant-supported monolithic zirconia crowns: Influence of patient-specific tooth-coloured abutments with titanium adhesive bases.* J Dent, 2015. **43**(7): p. 839-45.
15. Rompen, E., et al., *The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review.* Clin Oral Implants Res, 2006. **17 Suppl 2**: p. 55-67.
16. Linkevicius, T., et al., *The influence of margin location on the amount of undetected cement excess after delivery of cement-retained implant restorations.* Clin Oral Implants Res, 2011. **22**(12): p. 1379-84.
17. Berglundh, T., et al., *The soft tissue barrier at implants and teeth.* Clin Oral Implants Res, 1991. **2**(2): p. 81-90.
18. Ericsson, I., et al., *Different types of inflammatory reactions in peri-implant soft tissues.* J Clin Periodontol, 1995. **22**(3): p. 255-61.
19. de Araujo, M.F., et al., *Evaluation of peri-implant mucosa: clinical, histopathological and immunological aspects.* Arch Oral Biol, 2014. **59**(5): p. 470-8.
20. Assenza, B., et al., *Screw vs cement-implant-retained restorations: an experimental study in the beagle. Part 2. Immunohistochemical evaluation of the peri-implant tissues.* J Oral Implantol, 2006. **32**(1): p. 1-7.

21. Degidi, M., et al., *Histological and immunohistochemical evaluation of the peri-implant soft tissues around machined and acid-etched titanium healing abutments: a prospective randomised study.* Clin Oral Investig, 2012. **16**(3): p. 857-66.

22. Nakane, P.K., *Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat.* J Histochem Cytochem, 1968. **16**(9): p. 557-60.

23. Hsu, S.M., L. Raine, and H. Fanger, *Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures.* J Histochem Cytochem, 1981. **29**(4): p. 577-80.

24. Chilosi, M., et al., *A rapid immunostaining method for frozen sections.* Biotech Histochem, 1994. **69**(4): p. 235-9.

25. Esposito, M., et al., *Immunohistochemistry of soft tissues surrounding late failures of Branemark implants.* Clin Oral Implants Res, 1997. **8**(5): p. 352-66.

26. Gualini, F. and T. Berglundh, *Immunohistochemical characteristics of inflammatory lesions at implants.* J Clin Periodontol, 2003. **30**(1): p. 14-8.

27. Olmedo, D.G., et al., *Oral mucosa tissue response to titanium cover screws.* J Periodontol, 2012. **83**(8): p. 973-80.

28. Carcuac, O. and T. Berglundh, *Composition of human peri-implantitis and periodontitis lesions.* J Dent Res, 2014. **93**(11): p. 1083-8.

29. Bullon, P., et al., *Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis.* Clin Oral Implants Res, 2004. **15**(5): p. 553-9.

30. Welander, M., I. Abrahamsson, and T. Berglundh, *The mucosal barrier at implant abutments of different materials.* Clin Oral Implants Res, 2008. **19**(7): p. 635-41.

31. Berglundh, T., et al., *Some local and systemic immunological features of prepubertal periodontitis.* J Clin Periodontol, 2001. **28**(2): p. 113-20.

32. Seymour, G.J. and J.S. Greenspan, *The phenotypic characterization of lymphocyte subpopulations in established human periodontal disease.* J Periodontal Res, 1979. **14**(1): p. 39-46.

33. Mackler, B.F., et al., *Immunoglobulin bearing lymphocytes and plasma cells in human periodontal disease.* J Periodontal Res, 1977. **12**(1): p. 37-45.

34. Okada, H., T. Kida, and H. Yamagami, *Identification and distribution of immunocompetent cells in inflamed gingiva of human chronic periodontitis.* Infect Immun, 1983. **41**(1): p. 365-74.

35. Abul K. Abbas, A.H.L., *Basic Immunology: Functions and Disorders of the Immune System*. Second ed. 2006: Saunders.

36. Abrahamsson, I. and G. Cardaropoli, *Peri-implant hard and soft tissue integration to dental implants made of titanium and gold.* Clin Oral Implants Res, 2007. **18**(3): p. 269-74.

37. Rimondini, L., et al., *Bacterial colonization of zirconia ceramic surfaces: an in vitro and in vivo study.* Int J Oral Maxillofac Implants, 2002. **17**(6): p. 793-8.

38. Nakamura, K., et al., *Zirconia as a dental implant abutment material: a systematic review*. Int J Prosthodont, 2010. **23**(4): p. 299-309.

39. Okada, H. and S. Murakami, *Cytokine expression in periodontal health and disease.* Crit Rev Oral Biol Med, 1998. **9**(3): p. 248-66.

40. Matsuki, Y., T. Yamamoto, and K. Hara, *Detection of inflammatory cytokine messenger RNA (mRNA)-expressing cells in human inflamed gingiva by combined in situ hybridization and immunohistochemistry*. Immunology, 1992. **76**(1): p. 42-7.

APPENDIX

Appendix1 Raw data; titanium group

Sample	Side	CD3	CD20	CD68	CD138	FVIII	Area
T1	B	235	65	20	0	25	1.70
T1	D	307	274	5	10	35	2.50
T1	M	148	75	9	12	24	2.25
T1	L	163	47	7	0	27	0.80
T2	B	30	37	11	4	12	1.50
T2	D	109	54	20	19	39	3.00
T2	M	98	25	16	8	20	1.00
T2	L	22	19	6	2	11	1.25
T3	B	75	55	26	10	57	6.00
T3	D	16	10	4	4	20	2.40
T3	M	35	32	15	12	38	4.50
T3	L	72	65	28	23	53	1.50
T4	B	26	21	10	20	11	0.50
T4	D	12	8	2	1	21	0.75
T4	M	34	20	10	3	25	6.00
T4	L	23	5	11	1	8	2.00
T5	B	104	95	12	18	15	0.80
T5	D	185	196	10	20	14	0.90
T5	M	283	123	75	27	55	8.00
T5	L	109	51	15	7	26	3.00

Raw data; zirconia group

Sample	Side	CD3	CD20	CD68	CD138	FVIII	Area
Z1	B	371	79	10	9	16	1.50
Z1	D	99	12	7	11	15	0.50
Z1	M	132	69	13	6	24	0.25
Z1	L	179	75	2	12	16	1.40
Z2	B	58	25	7	31	22	0.88
Z2	D	155	75	23	15	26	2.25
Z2	M	56	21	5	12	11	1.70
Z2	L	185	164	12	4	15	0.80
Z3	B	235	258	12	15	23	1.25
Z3	D	264	323	27	58	27	6.75
Z3	M	228	64	19	52	44	7.00
Z3	L	115	89	11	12	26	3.75
Z4	B	42	32	6	0	13	1.35
Z4	D	21	12	8	2	22	1.30
Z4	M	48	20	9	1	19	4.60
Z4	L	29	11	2	1	10	2.25
Z5	B	114	121	24	19	7	1.00
Z5	D	31	39	8	4	16	5.25
Z5	M	48	15	11	5	9	2.40
Z5	L	46	47	2	1	10	2.00

Raw data; gold alloy group

Sample	Side	CD3	CD20	CD68	CD138	FVIII	Area
G1	B	47	40	45	0	38	0.25
G1	D	745	311	67	46	32	1.50
G1	M	456	193	43	8	27	0.75
G1	L	252	101	31	5	18	1.50
G2	B	122	47	31	28	18	3.75
G2	D	95	111	52	11	22	2.25
G2	M	194	123	54	21	29	3.00
G2	L	49	65	8	6	8	2.25
G3	B	157	255	53	38	17	0.50
G3	D	195	326	16	8	36	1.50
G3	M	298	182	41	29	8	2.50
G3	L	45	53	65	15	18	2.00
G4	B	123	175	6	1	50	1.50
G4	D	152	123	6	26	52	3.75
G4	M	480	465	62	67	69	5.00
G4	L	76	62	12	0	45	4.00
G5	B	215	548	17	13	11	1.05
G5	D	105	229	5	1	13	6.00
G5	M	532	984	36	62	29	1.00
G5	L	118	541	19	19	27	0.50

Raw data; titanium-base group

Sample	Side	CD3	CD20	CD68	CD138	FVIII	Area
TZ1	B	35	53	0	2	7	1.50
TZ1	D	91	55	5	4	38	3.00
TZ1	M	184	102	17	0	40	1.50
TZ1	L	172	122	14	7	68	3.50
TZ2	B	98	78	7	2	36	2.00
TZ2	D	88	63	9	3	44	1.00
TZ2	M	45	35	2	2	19	1.40
TZ2	L	184	146	12	40	38	2.50
TZ3	B	N/A	N/A	N/A	N/A	N/A	N/A
TZ3	D	N/A	N/A	N/A	N/A	N/A	N/A
TZ3	M	N/A	N/A	N/A	N/A	N/A	N/A
TZ3	L	N/A	N/A	N/A	N/A	N/A	N/A
TZ4	B	N/A	N/A	N/A	N/A	N/A	N/A
TZ4	D	N/A	N/A	N/A	N/A	N/A	N/A
TZ4	M	N/A	N/A	N/A	N/A	N/A	N/A
TZ4	L	N/A	N/A	N/A	N/A	N/A	N/A
TZ5	B	N/A	N/A	N/A	N/A	N/A	N/A
TZ5	D	N/A	N/A	N/A	N/A	N/A	N/A
TZ5	M	N/A	N/A	N/A	N/A	N/A	N/A
TZ5	L	N/A	N/A	N/A	N/A	N/A	N/A

Appendix2 Descriptive statistics, Factorial-ANOVA and Tukey's post hoc test

CD3 positive cells per area

Descriptive Statistics

Dependent Variable: CD3/mm2

Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	70.5480	59.96967	5
	Zirconia	129.2700	88.41723	5
	Gold	164.2580	110.39181	5
	TiBase	36.1650	18.15143	2
	Total	111.3359	90.12569	17
Buccal	Titanium	77.4720	85.06697	5
	Zirconia	65.6100	77.86478	5
	Gold	145.3840	201.01618	5
	TiBase	59.1650	40.77885	2
	Total	91.8035	121.81762	17
Distal	Titanium	42.5220	39.48046	5
	Zirconia	124.7880	225.59832	5
	Gold	283.9740	263.19607	5
	TiBase	77.4050	64.01438	2
	Total	141.8371	201.72507	17
Lingual	Titanium	63.4360	79.77987	5
	Zirconia	85.1340	93.83703	5
	Gold	93.4560	101.96997	5
	TiBase	61.3700	17.29583	2
	Total	78.4041	81.25243	17
Total	Titanium	63.4945	64.25689	20
	Zirconia	101.2005	127.45846	20
	Gold	171.7680	181.57686	20
	TiBase	58.5262	34.05716	8
	Total	105.8451	131.73933	68

Tests of Between-Subjects Effects

Dependent Variable: CD3/mm2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	258009.561[a]	15	17200.637	.989	.480
Intercept	567332.755	1	567332.755	32.606	.000
Site	25941.210	3	8647.070	.497	.686
Abutment	141132.076	3	47044.025	2.704	.055
Site * Abutment	78189.854	9	8687.762	.499	.868
Error	904792.201	52	17399.850		
Total	1924619.033	68			
Corrected Total	1162801.762	67			

a. R Squared = .222 (Adjusted R Squared = -.003)

Multiple Comparisons

Dependent Variable: CD3/mm2

Tukey HSD

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	41.71313	.803
	Gold	41.71313	.057
	TiBase	55.18128	1.000
Zirconia	Titanium	41.71313	.803
	Gold	41.71313	.338
	TiBase	55.18128	.866
Gold	Titanium	41.71313	.057
	Zirconia	41.71313	.338
	TiBase	55.18128	.183
TiBase	Titanium	55.18128	1.000
	Zirconia	55.18128	.866
	Gold	55.18128	.183

Based on observed means.

*. The mean difference is significant at the .05 level.

CD20 positive cells per area

Descriptive Statistics

Dependent Variable: CD20/mm2

Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	46.5660	42.36330	5
	Zirconia	86.4360	77.50645	5
	Gold	264.2200	236.00183	5
	TiBase	37.1650	2.59508	2
	Total	121.2024	158.96010	17
Buccal	Titanium	72.0440	92.11927	5
	Zirconia	24.3680	16.94043	5
	Gold	108.9920	94.58860	5
	TiBase	40.6650	31.58646	2
	Total	65.1971	75.51513	17
Distal	Titanium	16.8300	12.42809	5
	Zirconia	61.6180	119.88136	5
	Gold	289.6260	397.07948	5
	TiBase	46.5000	30.40559	2
	Total	113.7276	239.00576	17
Lingual	Titanium	27.3560	22.98689	5
	Zirconia	62.1380	81.74406	5
	Gold	244.0440	468.84088	5
	TiBase	46.6300	16.64529	2
	Total	103.5853	256.31293	17
Total	Titanium	40.6990	52.65445	20
	Zirconia	58.6400	79.21752	20
	Gold	226.7205	313.39635	20
	TiBase	42.7400	18.26518	8
	Total	100.9281	192.86727	68

Tests of Between-Subjects Effects

Dependent Variable: CD20/mm2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	568289.295[a]	15	37885.953	1.024	.447
Intercept	494592.986	1	494592.986	13.368	.001
Site	19626.403	3	6542.134	.177	.912
Abutment	451877.956	3	150625.985	4.071	.011
Site * Abutment	84814.430	9	9423.826	.255	.984
Error	1923962.320	52	36999.275		
Total	3184932.186	68			
Corrected Total	2492251.614	67			

a. R Squared = .228 (Adjusted R Squared = .005)

Multiple Comparisons

Dependent Variable: CD20/mm2

Tukey HSD

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	60.82703	.991
	Gold	60.82703	.018
	TiBase	80.46660	1.000
Zirconia	Titanium	60.82703	.991
	Gold	60.82703	.038
	TiBase	80.46660	.997
Gold	Titanium	60.82703	.018
	Zirconia	60.82703	.038
	TiBase	80.46660	.114
TiBase	Titanium	80.46660	1.000
	Zirconia	80.46660	.997
	Gold	80.46660	.114

Based on observed means.

*. The mean difference is significant at the .05 level.

CD68 positive cells per area

Descriptive Statistics

Dependent Variable: CD68/mm2

Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	11.6840	6.18862	5
	Zirconia	10.5320	7.76128	5
	Gold	62.8920	77.79057	5
	TiBase	1.7500	2.47487	2
	Total	25.2376	46.63565	17
Buccal	Titanium	4.8240	4.04611	5
	Zirconia	7.1780	4.97400	5
	Gold	16.1760	18.29114	5
	TiBase	5.3350	5.18309	2
	Total	8.9153	10.94756	17
Distal	Titanium	6.8760	5.86228	5
	Zirconia	12.8380	21.91309	5
	Gold	28.0260	18.72275	5
	TiBase	6.3800	7.00036	2
	Total	14.7918	17.42662	17
Lingual	Titanium	8.5440	5.88264	5
	Zirconia	4.2500	6.06443	5
	Gold	19.5460	16.11631	5
	TiBase	4.4000	.56569	2
	Total	10.0294	11.23344	17
Total	Titanium	7.9820	5.71541	20
	Zirconia	8.6995	11.74453	20
	Gold	31.6600	42.83420	20
	TiBase	4.4663	3.88980	8
	Total	14.7435	26.32212	68

Tests of Between-Subjects Effects

Dependent Variable: CD68/mm2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	15451.701[a]	15	1030.113	1.730	.074
Intercept	10140.576	1	10140.576	17.027	.000
Site	1628.729	3	542.910	.912	.442
Abutment	8213.291	3	2737.764	4.597	.006
Site * Abutment	4410.969	9	490.108	.823	.598
Error	30969.500	52	595.567		
Total	61202.474	68			
Corrected Total	46421.201	67			

a. R Squared = .333 (Adjusted R Squared = .140)

Multiple Comparisons

Dependent Variable: CD68/mm2

Tukey HSD

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	7.71730	1.000
	Gold	7.71730	.017
	TiBase	10.20903	.986
Zirconia	Titanium	7.71730	1.000
	Gold	7.71730	.022
	TiBase	10.20903	.976
Gold	Titanium	7.71730	.017
	Zirconia	7.71730	.022
	TiBase	10.20903	.049
TiBase	Titanium	10.20903	.986
	Zirconia	10.20903	.976
	Gold	10.20903	.049

Based on observed means.

*. The mean difference is significant at the .05 level.

CD138 positive cells per area

Descriptive Statistics

Dependent Variable: CD138/mm2

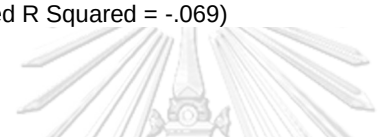
Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	13.3680	17.48321	5
	Zirconia	14.4460	13.58969	5
	Gold	19.3040	32.10254	5
	TiBase	1.1650	.23335	2
	Total	13.9953	20.24358	17
Buccal	Titanium	7.1100	8.68324	5
	Zirconia	7.9120	8.54733	5
	Gold	9.5980	12.04591	5
	TiBase	2.1650	1.18087	2
	Total	7.4959	8.86063	17
Distal	Titanium	3.9760	2.83590	5
	Zirconia	8.1580	9.38960	5
	Gold	20.9340	23.07492	5
	TiBase	.7150	1.01116	2
	Total	9.8100	14.75558	17
Lingual	Titanium	3.9520	6.42566	5
	Zirconia	3.5420	3.40587	5
	Gold	10.3000	15.71638	5
	TiBase	9.0000	9.89949	2
	Total	6.2924	9.54513	17
Total	Titanium	7.1015	10.30165	20
	Zirconia	8.5145	9.54766	20
	Gold	15.0340	20.95804	20
	TiBase	3.2613	5.21669	8
	Total	9.3984	14.11153	68

Tests of Between-Subjects Effects

Dependent Variable: CD138/mm2

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2269.480[a]	15	151.299	.711	.763
Intercept	4181.720	1	4181.720	19.639	.000
Site	280.002	3	93.334	.438	.727
Abutment	1057.657	3	352.552	1.656	.188
Site * Abutment	624.168	9	69.352	.326	.963
Error	11072.579	52	212.934		
Total	19348.471	68			
Corrected Total	13342.059	67			

a. R Squared = .170 (Adjusted R Squared = -.069)



Multiple Comparisons

Dependent Variable: CD138/mm2

Tukey HSD

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	4.61448	.990
	Gold	4.61448	.324
	TiBase	6.10438	.922
Zirconia	Titanium	4.61448	.990
	Gold	4.61448	.497
	TiBase	6.10438	.825
Gold	Titanium	4.61448	.324
	Zirconia	4.61448	.497
	TiBase	6.10438	.229
TiBase	Titanium	6.10438	.922
	Zirconia	6.10438	.825
	Gold	6.10438	.229

Based on observed means.

*. The mean difference is significant at the .05 level.

Sum of positive cells per area

Descriptive Statistics

Dependent Variable: Positive Cells

Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	142.1660	104.62784	5
	Zirconia	240.6860	142.18981	5
	Gold	510.6740	388.04237	5
	TiBase	76.2500	22.98097	2
	Total	271.7724	271.35587	17
Buccal	Titanium	161.4480	187.56968	5
	Zirconia	105.0740	95.91523	5
	Gold	280.1520	304.60918	5
	TiBase	107.3350	78.72220	2
	Total	173.4141	200.72341	17
Distal	Titanium	70.2000	57.70928	5
	Zirconia	207.4060	376.30824	5
	Gold	622.5600	642.22461	5
	TiBase	131.0000	100.40916	2
	Total	280.1665	441.39075	17
Lingual	Titanium	103.2900	101.92833	5
	Zirconia	155.0640	180.91321	5
	Gold	367.3440	580.59101	5
	TiBase	121.4000	44.40631	2
	Total	198.3112	329.01172	17
Total	Titanium	119.2760	117.92697	20
	Zirconia	177.0575	213.76271	20
	Gold	445.1825	476.65706	20
	TiBase	108.9963	56.32231	8
	Total	230.9160	318.95045	68

Tests of Between-Subjects Effects

Dependent Variable: Positive Cells

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1771064.823[a]	15	118070.988	1.217	.290
Intercept	2630440.318	1	2630440.318	27.114	.000
Site	87425.140	3	29141.713	.300	.825
Abutment	1344402.616	3	448134.205	4.619	.006
Site * Abutment	282767.553	9	31418.617	.324	.963
Error	5044804.342	52	97015.468		
Total	10441779.625	68			
Corrected Total	6815869.165	67			

a. R Squared = .260 (Adjusted R Squared = .046)

Multiple Comparisons

Dependent Variable: Positive Cells

Tukey HSD

(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	98.49643	.936
	Gold	98.49643	.009
	TiBase	130.29853	1.000
Zirconia	Titanium	98.49643	.936
	Gold	98.49643	.042
	TiBase	130.29853	.953
Gold	Titanium	98.49643	.009
	Zirconia	98.49643	.042
	TiBase	130.29853	.060
TiBase	Titanium	130.29853	1.000
	Zirconia	130.29853	.953
	Gold	130.29853	.060

Based on observed means.

*. The mean difference is significant at the .05 level.

Microvessel density (MVD)

Descriptive Statistics

Dependent Variable: MVD

Site of collected tissue	Type of Abutment	Mean	Std. Deviation	N
Mesial	Titanium	14.5920	5.94904	5
	Zirconia	14.1400	7.40689	5
	Gold	46.9220	60.19840	5
	TiBase	11.3350	9.42573	2
	Total	23.5847	34.29546	17
Buccal	Titanium	15.7780	7.34466	5
	Zirconia	13.1060	11.02708	5
	Gold	14.2300	8.82098	5
	TiBase	28.3350	22.15366	2
	Total	16.0141	10.80010	17
Distal	Titanium	10.0320	6.05394	5
	Zirconia	23.3280	40.64347	5
	Gold	18.3340	13.69773	5
	TiBase	20.1200	9.26310	2
	Total	17.5712	22.43921	17
Lingual	Titanium	18.1100	15.13291	5
	Zirconia	9.3100	5.94906	5
	Gold	17.9620	20.41488	5
	TiBase	17.3150	2.99106	2
	Total	15.3847	13.68373	17
Total	Titanium	14.6280	9.15601	20
	Zirconia	14.9710	20.50063	20
	Gold	24.3620	32.98225	20
	TiBase	19.2762	11.79434	8
	Total	18.1387	22.00837	68

Tests of Between-Subjects Effects

Dependent Variable: MVD

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5679.165[a]	15	378.611	.735	.738
Intercept	19504.345	1	19504.345	37.882	.000
Site	277.665	3	92.555	.180	.910
Abutment	1232.128	3	410.709	.798	.501
Site * Abutment	3731.687	9	414.632	.805	.613
Error	26773.515	52	514.875		
Total	54825.468	68			
Corrected Total	32452.680	67			

a. R Squared = .175 (Adjusted R Squared = -.063)

Multiple Comparisons

Dependent Variable: MVD

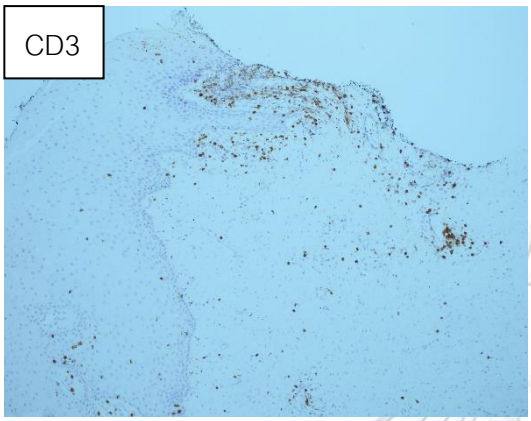
Tukey HSD

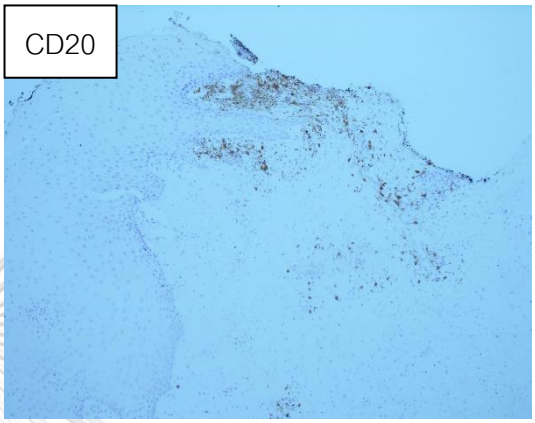
(I) Type of Abutment	(J) Type of Abutment	Std. Error	Sig.
Titanium	Zirconia	7.17548	1.000
	Gold	7.17548	.532
	TiBase	9.49227	.961
Zirconia	Titanium	7.17548	1.000
	Gold	7.17548	.562
	TiBase	9.49227	.969
Gold	Titanium	7.17548	.532
	Zirconia	7.17548	.562
	TiBase	9.49227	.950
TiBase	Titanium	9.49227	.961
	Zirconia	9.49227	.969
	Gold	9.49227	.950

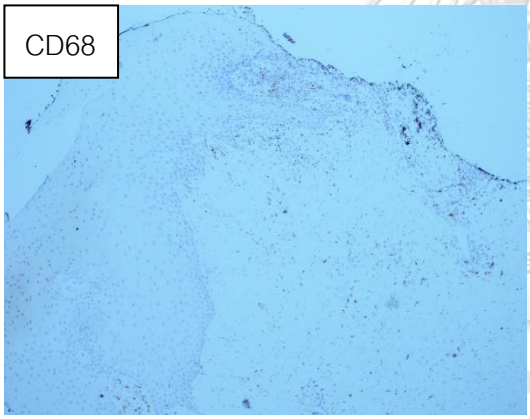
Based on observed means.

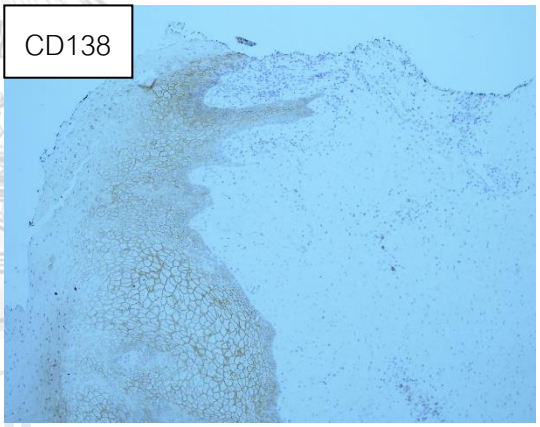
Appendix3 Some immunohistochemical pictures

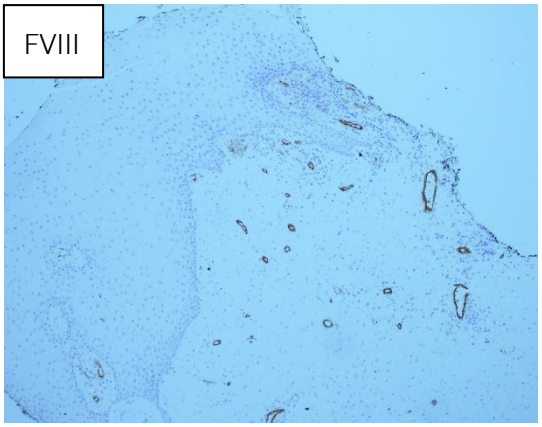
Titanium group:



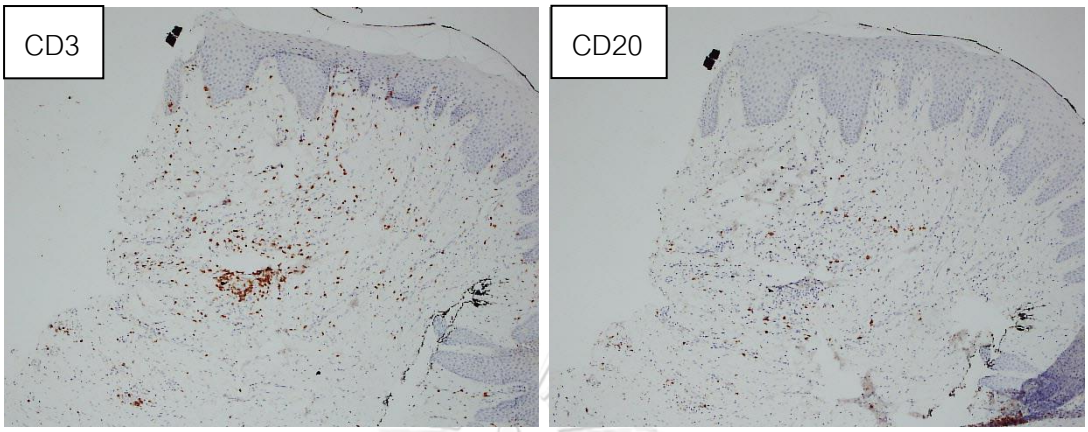


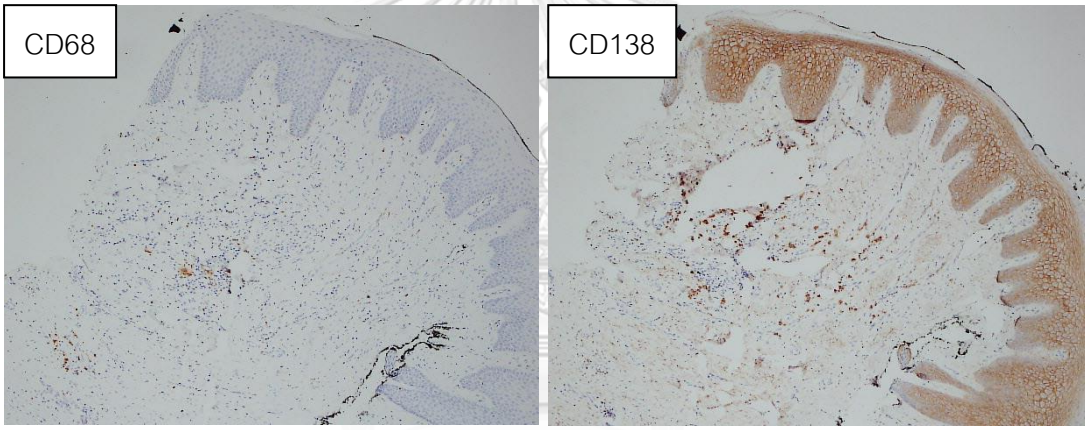


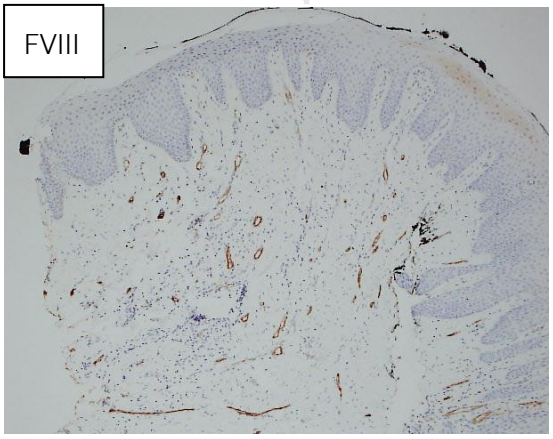




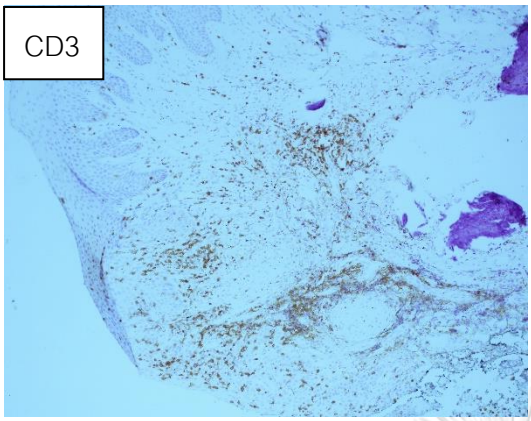
Zirconia group:

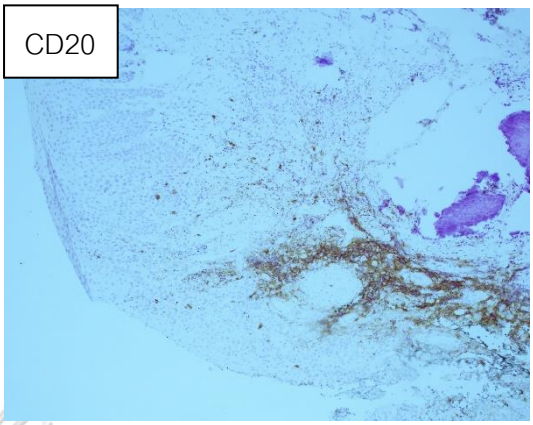


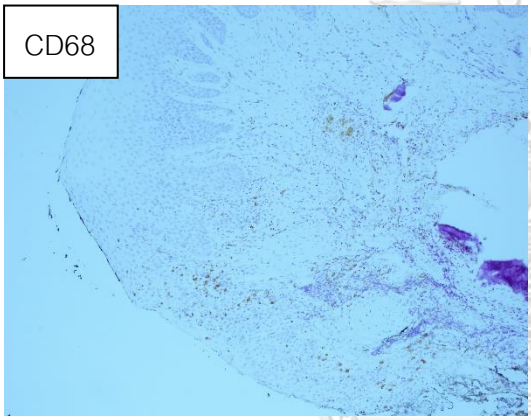


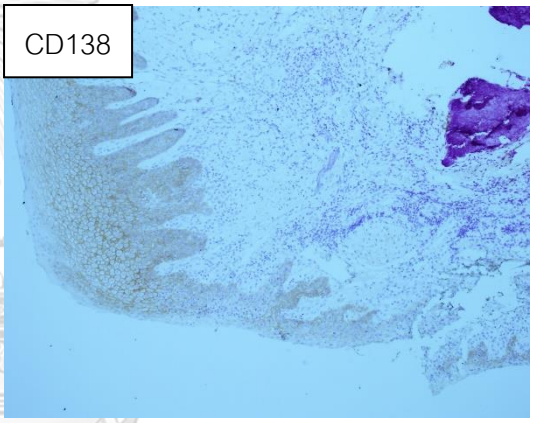


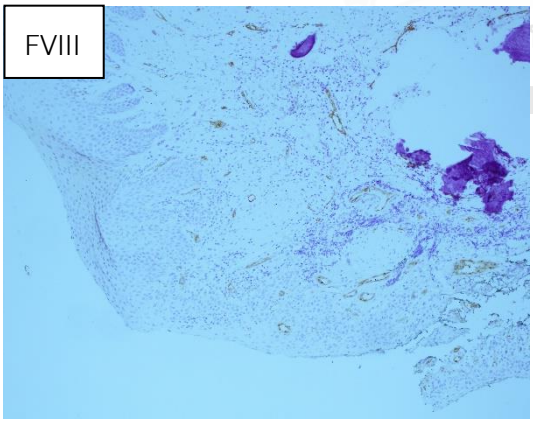
Gold alloy group:



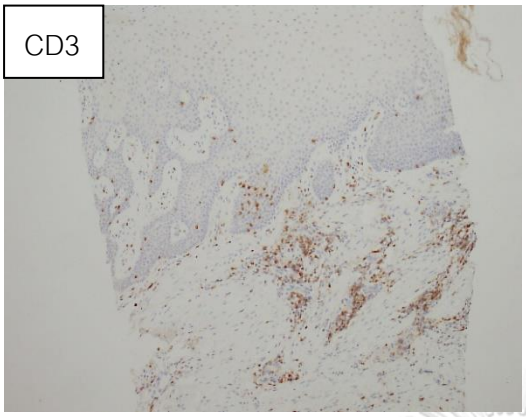


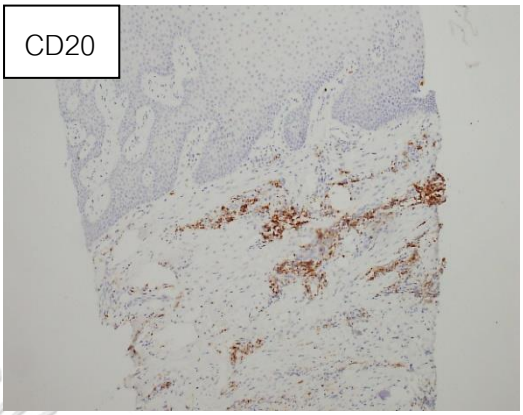


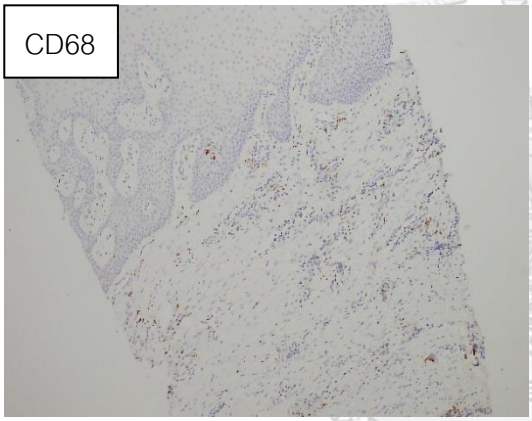


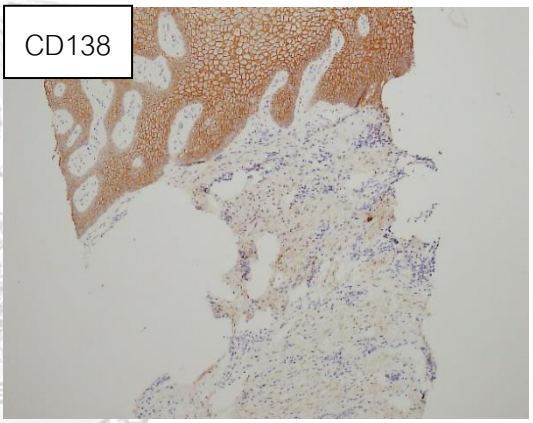


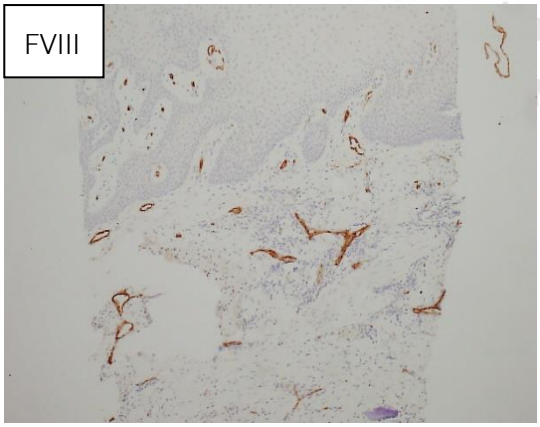
Titanium-base group:











Appendix4 Consent form

เอกสารยินยอมเข้าร่วมการวิจัย (Consent Form)

การวิจัยเรื่อง <u>การศึกษาลักษณะทางจุลกายวิภาคศาสตร์ และ ปฏิกิริยาการอักเสบของเนื้อเยื่อรอบหลักยึดที่ทำจากวัสดุต่างชนิดในมนุษย์</u>

ข้าพเจ้า (นาย, นาง, นางสาว, เด็กชาย,

เด็กหญิง).....

อยู่บ้านเลขที่.....ถนน.....ตำบล/แขวง.....

อำเภอ/เขต.....จังหวัด.....รหัสไปรษณีย์.....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้

1. ข้าพเจ้าได้รับทราบรายละเอียดข้อมูลคำอธิบายสำหรับอาสาสมัครที่เข้าร่วมในการวิจัย รวมทั้งได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการทำวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการทำวิจัยหรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียดและมีความเข้าใจดีแล้ว
2. ผู้วิจัยรับรองว่าจะตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ
3. ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการเท่านั้น และผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่า
4. ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้และการบอกเลิกการเข้าร่วม การวิจัยนี้จะไม่มีผลต่อการรักษาโรคที่ข้าพเจ้าจะพึงได้รับต่อไป

ข้าพเจ้าจึงสมัครใจเข้าร่วมโครงการวิจัยนี้ตามที่ระบุในเอกสารข้อมูลคำอธิบายสำหรับอาสาสมัคร และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ และได้รับสำเนาเอกสารใบยินยอมที่

ข้าพเจ้าลงนามและลงวันที่ และเอกสารยกเลิกการเข้าร่วมวิจัย อย่างละ 1 ฉบับ เป็นที่เรียบร้อยแล้ว

ลงนาม..... ผู้ยินยอม

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม..... พยาน

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม..... ผู้วิจัยหลัก

(.....)

วันที่.....เดือน.....พ.ศ.....

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ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนาม หรือประทับลายนิ้วหัวแม่มือขวาของข้าพเจ้าในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม.....ผู้ยินยอม

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม.....พยาน

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม.....ผู้วิจัยหลัก

(.....)

วันที่.....เดือน.....พ.ศ.....

ในกรณีที่ผู้ถูกทดลองยังไม่บรรลุนิติภาวะ จะต้องได้รับการยินยอมจากผู้ปกครองหรือผู้อุปการะโดยชอบด้วยกฎหมาย

ลงนาม..... ผู้ปกครอง

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม..... พยาน

(.....)

วันที่.....เดือน.....พ.ศ.....

ลงนาม..... ผู้วิจัยหลัก

(.....)

วันที่.....เดือน.....พ.ศ.....

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