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Effect of Coating Titanium Implants with Silk on Osseointegration

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Introduction

1. Dental Implants:

According to the World Health Organization (WHO), by the year 2020, 37.9 million of the U.S. population will suffer from one form or another of edentulism [1]. Dental implants (Figure 1) are considered to be the gold standard in modern dentistry in terms of replacing missing teeth [2]. The other option available to edentulous patients is dentures, whether fixed or removable, neither of which is as favorable as implants [3]. The possibility of restoring missing teeth with fixed restorations supported by dental implants is the treatment of choice to many or majority of clinicians [4].

The ultimate goal of dental implant therapy is to satisfy the patient's desire to replace one or more missing teeth in an esthetic, secure, functional and long lasting manner [5]. To achieve this goal, clinicians must accurately diagnose the current dentoalveolar condition as well as the overall mental and physical well being of the patient to determine whether implant therapy is possible or practical, and most importantly, whether it is indicated for a particular patient. Therefore, the unequivocal success of endosseous dental implants is driving the need for continuing refinements in implant design and optimization of the biological healing response following implant placement [5].

Understanding the biological cascade of early peri-implant bone healing is very important because by the time that bone is formed on the implant surface, the most important healing events have already occurred [6].

Implant fixtures are made mainly of titanium due to its superior properties, such as durability, high strength, and the ability to be shaped in different forms and textures [7]. Titanium has also proven to be an ideal material to be placed into the human body due to its resistance to corrosion. Titanium has superior mechanical properties as well and it is light in weight, which makes it favorable for bone implantation [8].

2. Osseointegration

Osseointegration is defined as a direct structural and functional connection between ordered, living bone and the surface of a load-bearing implant (Figure 2) [9]. Osseointegration proceeds through 3 phases:

- ***Osseoinduction***

Osteogenic mesenchymal cells migrate to the implant surface and form a connective tissue matrix that would work as a scaffold for the bone to deposit its cells. Implant surface would determine the success of this scaffold anchorage [10, 11]. Dental implant surfaces interact with blood components from ruptured blood vessels. Within a short period of time, various plasma proteins such as fibrin (Figure 3) get adsorbed on the material surface. Fibrinogen is converted to fibrin and the complement and kinin systems become activated. As in fracture healing (Figure 4), the migration of bone cells in peri-implant healing will occur through the fibrin of a blood clot. Since fibrin has the

potential to adhere to almost all surfaces, it can be anticipated that the migration of osteogenic cell populations towards the implant surface will occur. However, as the migration of cells through fibrin will cause retraction of the fibrin scaffold, the ability of an implant surface to retain this fibrin scaffold during the phase of wound contraction is critical in determining whether the migrating cells will reach the implant surface [12].

- ***New bone formation***

Mineralization of the previously laid collagen matrix takes place on the implant surface [13]. This stage is tightly regulated by numerous biological factors, including extracellular matrix proteins, cytoskeletal proteins, chemical characteristics and topographies at the implant surface and by the released ions/products from the material [14].

- ***Bone remodeling***

Bone remodeling refers to the phase of maturation of previously, *de novo,* laid bone at and around the implant surface, which can last up to several decades [15, 16]. However, adverse responses such as pathological inflammation, fibrous encapsulation and implant failure, can also occur during this stage [17-19].

Osseointegration is critical for implant stability and is considered a prerequisite for implant loading and long-term clinical success of endosseous dental implants. The alternative would have been fibrous encapsulation of dental implant, which is called

(fibrointegration) which results in mobility of the implant after loading [20]. Osseointegration requires a precise fitting into the bone and primary stability [21], along with a bioactive surface that would attract bone formation around a bioinert material such as titanium.

Cochran et al. concluded that the influence of physical properties such as surface topography and roughness on osseointegration have translated to shorter healing times from implant placement to restoration [22]. The ultimate success of dental implant therapy, depends on the collaboration of different factors that Albrektsson et al. suggested as being six factors that are particularly important for the establishment of reliable osseointegration: implant material, implant design, surface conditions, status of the bone, surgical technique, and implant loading conditions [9].

Sul et al. described osseointegration of titanium implant surfaces as being dependent upon both physical and chemical properties. Surface properties of the oxidized implants, especially surface chemistry, influenced bone responses. Structural and functional union of the implant with living bone is strongly influenced by the surface properties of the titanium implant. He pointed out that titanium and its alloys cannot directly bond with living bone, and that modification of the implant surface has been proposed as a method for enhancing osseointegration [23].

3. Surface Modification

Optimizing the surface topography of the inert implant will directly influence the efficacy of all osseointegration phases, specially the first two phases. Surface modeling has been the quest of researchers for a long time in order to improve the osseointegration concept. Modifying the surface of inert titanium changes it to a bioactive surface that would enhance its interaction with the biological environment around the implant ultimately leading to better osseointegration.

The methods employed for surface modifications of implants can be broadly classified into 3 types: mechanical, chemical, and physical. These different methods can be employed to change the implant surface chemistry, morphology, and structure. The main objective of these techniques is to improve the biomechanical properties of the implant such as stimulation of bone formation to enhance osseointegration, removal of surface contaminants and improvement of wear and corrosion resistance.

Singhatanadgit et al. summarized the current advances made in the field of implant surface modification [24]. He broke it down to three main categories:

1. Nano-scale surface roughening (chemical modification, physical compaction, nanoparticle deposition and molecular self-assembly method)
2. Calcium phosphate coatings (done either with immersion or electrochemical deposition)
3. Biological active agents incorporation of:

- Osteoinductive agents such as: Bone morphogenic proteins (BMP), Transforming growth factors (TGF), Vascular endothelial growth factors (VEGF), Platelet-derived growth factors (PDGF)s, Insulin-like growth factors (IGF) and Bone remodeling related agents, such as bisphosphonates
- Synthetic arginine-glycine-aspartic acid (RGD) peptides
- Antibiotics

Surface modification of an implant can be done in various ways one of which is utilizing silk as a scaffold to carry different types of proteins.

Silk in biomedical field

Bombyx mori (silkworm) silk (Figure 5) is a unique material, which has historically been highly used for its strength. Physicians have used silk as a suture material for centuries (Figure 6), and it has recently gained attention as a biomaterial because of several desirable properties. These properties include biocompatibility, the ease with which it can be chemically modified, slow rate of degradation *in vivo* and ability to be processed into multiple material formats from either aqueous solution or an organic solvent [25-28].

Arginine-glycine-aspartic acid (RGD) peptides serve as receptors for integrin and mediate adhesion of cells to the extracellular matrix (Figure 7). They address selectively certain cell lines and subsequently, elicit a specific cell response. Those peptides have low space requirements that allow them to compact on surfaces with high density, which allows more cellular adhesion activity. Furthermore, ECM contains many cell recognition

motives. Therefore, placing a peptide that represents only one single motif will selectively activate one type of cellular adhesion receptor [29]. RGD peptides can be incorporated into the silk that is used in coating implants [29]. It is expected that the RGD motif will stimulate the adhesion of fibroblasts from the blood clot leading to a more predictable and accelerated osseointegration. Schliephake et al. reported that synthetic RGD peptides coated onto the surface of implant materials increase bone-to-implant contact (BIC) and newly formed peri-implant bone, presumably by enhancing early cellular attachment to the implant surface [30] (Figure 8). An in-vitro study done by Vidal et al. showed that the adherence of fibroblasts on the titanium surface modified with the multifunctional silk coating demonstrated an increase in the number of adhering cells by 60% after they have grafted silk proteins with TiBP (titanium binding proteins) and fibronectin-derived RGD peptide [31]. Eriksson et al. showed that the adhesion of plasma proteins on the surface of titanium implants has been reported to play an essential role in the process of Osseointegration [32]. Walivaara et al. further showed that the specific pattern of adsorbed proteins determines the type of tissue that will develop at the interface between the implanted material and the host [33].

Utilizing silk, as a scaffold, has been recently the center of attention in research. Gosline et al} reported that characteristic features of silk rivals the mechanical properties of synthetic high performance fibers offering new options for the design of biomaterials and tissue engineering scaffolds with a wide range of mechanical properties. [34]. Meinel L et al. used it successfully as carrier of stem cells for the in vitro engineering of bone-like

tissues [35]. Jang E et al. showed in an animal study, that silk fibroin powder was successful in the restoration of peri-implant defects with Choukroun PRF (Platelet rich fibrin Growth Factor). Thus, biocompatibility, the ability to engineer the materials with specific mechanical properties and a diverse range of surface chemistries for modification or decoration suggests that silk may provide an important class of biomaterial [36].

I. Specific Aims

AIM I: To compare the amount of bone-implant contact (BIC) in silk coated titanium implants (test) versus uncoated implants (control), through histological investigation; we will calculate the percentage of bone contact to the implant surface. We expect that test group will have more BIC in quantity than control group.

AIM II: To compare early wound healing events at 2, 4 and 6 weeks between test and control groups in terms of presence of inflammatory cells. We expect the test group to show less inflammation compared to control since it will be replaced with early signs of new bone formation in terms of wound healing process.

II. Hypothesis

The hypothesis being tested is that coating titanium implants with RGD-decorated silk proteins will result in improved osseointegration. The sooner the newly formed bone is observed, the better the chances that osseointegration would be stable since infection and fibrous encapsulation would not be given the chance to reach the implant surface. The osseous cells would populate the surface before epithelial downgrade gets the chance to reach the implant surfaces.

III. Outcomes

Primary outcome: Bone to implant contact (BIC) visualized (Figure 9) in each implant and quantified histologically.

Secondary outcome: Wound healing events at 2, 4 and 6 weeks and comparison of the histological findings between test and control groups.

Based on published reports, the following histological events should be observed in regular titanium implants placed into rabbit tibiae as described by Sennerby et al. and are expected to be observed in our control group of titanium implants [37] (Figure 10):

- **2 Weeks:** Newly formed bone filling the threads can be observed as well as a compaction of this bone against the threads of the implant.

- **4 Weeks:** Bone fills larger sites of implant surface though still immature and osteocytes lacunae are still evident suggesting continuous remodeling of newly formed bone.
- **6 Weeks:** Newly formed bone is rather dense and more compact against the surface of the implant and it is still possible to distinguish between old bone and new bone in the area surrounding the implant.

In the test group we expect to observe a faster healing process whereas that of the control group will be one step behind when it comes to new bone formation or tissue maturation around implants. These findings would prove that modifying the surface of implants will accelerate the wound healing process around implants leading to a more stable and rapid osseointegration, leaving no chance for epithelial down growth to take place which usually causes fibrous encapsulation and implant loss. Indeed, it is well accepted that bone formation does not initiate at the implant surface, instead, newly formed bone grows towards rather than from the implant surface [38]. Thus, the more this process can be accelerated, the better the outcome of implant stability will be.

IV. Sample Size Calculation

For sample size calculation, we used the percent of BIC as the primary outcome of the study. Based on data from published studies, we anticipate that the mean (SD) BIC in the control group to be 40% (4.0%) [39, 40]. For the coated group to be considered to result in a greater BIC, there must be at least 15% more BIC. Thus, the sample size calculation was based on comparing a mean BIC of 40% in the control group to 46% in the experimental group, and assuming a common SD of 4.0. Setting alpha equal to 0.05, we will have 94% power to detect a difference between the groups with 10 implants placed per group (nQuery Advisor, Version 7.0).

V. Materials and methods

1. Animals

Male New Zealand white rabbits, aged 1 to 2 years and weighing 2.6 to 3 Kg were used in this study. The Tufts Medical Center's Institutional Animal Care and Use Committee (IACUC) approved the study design. Rabbits were chosen for this study for several reasons. Approximately 35% of published musculoskeletal research studies use the rabbit as a first choice of animal model [41] due to its size and ease of handling. Rabbits reach their skeletal maturity at around 6 months of age, making them a good choice to assess bone formation around implants. Rabbit tibiae have a primary vascular longitudinal tissue structure, comprising vascular canals of osteons running parallel with the long axis of the bone, making it a suitable environment for implant placement and osteogenesis would be expected due to the high vascularity. Accelerated healing, when compared to humans, makes it a little difficult to extrapolate results to human use. Nevertheless, rabbits are commonly used for screening implant materials prior to testing in a larger animal model [42].

2. Materials

We have used titanium screws since they mimic dental implants in composition and design. Twenty screws were purchased from ACE surgical supply company Inc. (Brockton, MA). Ten of those screws were coated with silk and the other ten were left

untreated and used as controls. Special screwdrivers for placing implants, suture materials and surgical blades were provided from the dispensary of the Department of Periodontology at Tufts University School of Dental Medicine (TUSDM). The Surgical Interventional Research Laboratories (SIRL) at Tufts Medical Center provided all other surgical supplies.

3. Experimental Design

Two groups of implants were used: pure titanium (uncoated) that represented the control group and silk-coated implants decorated with RGD peptides representing the test group. Coating of implants was performed in Dr. David Kaplan's laboratory in Medford, MA.

1. *Process of coating titanium implants:*

Natural silkworm fibers are encased in a coat of Sericin, a family of glue-like proteins, which must be purified, degummed, from fibroin for use in tissue engineering. Even after degumming, silk fibers maintain high mechanical integrity. Silk preparation was performed as described by Dr. Kaplan's protocol (Figure 11): Silk was boiled in water with sodium carbonate for 10 min to degum the Sericin component and isolate fibroin protein, then washed three times for 20 minutes in deionized water and allowed to dry for 24 h. Silk fibroin was then removed and spread over clean aluminum foil and let dry overnight in a fume hood. Lithium Bromide (LiBr) was added to the silk fibroin and

incubated at 60°C for 3 hours, producing a 20% solution. Silk solution was placed into dialysis cassettes and dialyzed against deionized water for 3 days to remove excess ions. Centrifugation was used to remove impurities at 10,000 rpm at 4°C for 20 min. The negative terminal was connected to a platinum wire, while the positive terminal was connected to the titanium implants (Figure 12). Implants were submerged 2 mm into the silk solution; the power supply was adjusted to 20 volts and turned on (Figure12). Gelation (collection of silk gel on titanium surface) was allowed to proceed for 30 seconds and silk-coated implants were allowed to dry overnight. Implants were then sterilized in an autoclave.

- RGD Functionalization:

In order to increase cell adhesion to the titanium screws RDG cell binding sites were chemically added to the silk coatings. The 30% tyrosine residues of silk fibroin were modified allowing for the installation of carboxylic acids at a concentration of 214.86 mL diazonium salt solution per 1 g dried silk. Silk coated screws were soaked on ice in borate buffer for at least 30 min. For each gram of dry silk, 107 mL of cooled 0.01 M acetonitrile solution of 4-aminobenzoic acid and 53.7 mL of 1.6 M aqueous p-toluene sulfonic acid was combined with 50.9 mL of a cooled 0.8 M sodium nitrite.

This mixture was vortexed and placed in an ice bath for 30 min. After combining the screws and the diazonium salt solution, the reaction was placed in an ice bath and

allowed to proceed for 30 min. The diazo-screws were rinsed in ultrapure distilled water three times.

Briefly, electro spun mats were soaked in MES buffer for 15 min. The –COOH groups in the silk fibroin were activated by reaction with EDC and NHS solution (0.5 mg/mL of EDC and 0.7 mg/mL of NHS in MES buffer) for 15 minutes at room temperature. The activated silk coatings were rinsed once with MES buffer and reacted with 0.1 mg/mL GRGDS peptide in MES buffer at room temperature for 4 hours. After the coupling reaction, the coated screws were rinse once with MES buffer and twice in ultrapure distilled water, and then sealed in sterile bags.

2. *Surgical intervention*

Three male New Zealand rabbits were purchased from Millbrook breeding laboratories and delivered to Tufts animal facility in the Boston campus. Each animal was placed into a separate cage. Food, water and toys were provided. A veterinarian examined the animals and approved scheduling a date for the surgery. All surgeries were carried out at the Surgical Interventional Research Laboratories (SIRL) at Tufts Medical Center, and were supervised by the appointed Veterinarian and two technicians.

On the day of surgery, rabbits were injected IM with a muscle relaxant. Preparation of animals took place by shaving both tibiae with an electrical shaving machine. The first animal was brought to the operating room (OR) and placed supine on his back on the operating table. Inhalation anesthesia was administered and when the animal lost his

reflexes, procedure took place. All surgical steps were performed under sterile conditions. The whole animal was covered with sterile drapes except for the surgical area that was exposed.

A horizontal incision on the medial side of the tibia was performed using size 15-blade. Incision line was approximately 35 mm in length and did not go beyond the skin. Blunt dissection took place through the muscle layers until the bone was exposed. Predrilling, to facilitate insertion without applying strain on the coated implants, was performed using a 2 mm round bur at low speed with saline irrigation. The bur was inserted to 5 mm in depth. Three holes were predrilled leaving 5 mm distance between each (Figure 15). The implants were then threaded and screwed into the tibia. Trying to dislodge implant after insertion tested the presence of primary stability [43].

Implants were inserted passively into predrilled holes and implants heads were flushed with tibial bone surface (Figure 16). Table 1 shows the distribution of test and control on each tibia. To prevent total loss of test or control samples in case infection occurred in one of the wounds, tests and controls were mixed within one tibia. Muscular layer was sutured first using internal continuous suture technique with 5.0-Vicryl-suture material. Skin layer was sutured the same way as well and skin-glue was used to ensure total closure of wounds. Subcutaneous topical anesthesia was administered around the wound to ensure total comfort of rabbits and facilitate rapid recovery. Rabbits were placed on top of body warmers until they regained consciousness and then were placed back into their cages. Analgesics were given BID for the first 3 days IM, and antibiotics (0.6 ml of Butryl) was administered BID for the first 7 days after surgery (including

weekends). Rabbits were monitored daily for 7 days after surgery and every other day until euthanasia. The following was noted and recorded: movement, metabolic waste, appetite, water consumption, wound texture, absence/presence of pus discharge and fever.

The second and third animals underwent the same procedure. The same operator performed all procedures (M.Q.)

Two weeks later, the first animal was euthanized using IV injection of pentobarbital of the barbiturates family of medicine, which causes cerebral death and respiratory arrest [44]. The second animal was euthanized 2 weeks after the first. First and second animals marked the 2nd and 4th week's samples, respectively. The third animal was euthanized 2 weeks after, marking the 6th week of surgery. Tibiae were collected, properly labeled (Table 2) and placed into separate jars containing 10% formalin made in phosphate buffered saline (PBS). Jars were transported to CBSET laboratories, Lexington, MA to be processed.

3. Sample processing

Upon receipt, samples were sectioned individually with a band saw into three separate cylinders, each containing one implant (Figure 17) and placed into 10% neutral buffered formalin for additional fixation. Samples were then dehydrated in graded alcohols, cleared with xylene, and processed in methyl-butyl methacrylate resin. Once

polymerized, samples were glued to their final slide with Technovit 7210 and ground down to ~30-50 μm using a precision micro-grinder and graded grit papers (320, 800, 1000, 1200, and 4000 grit). Final polished slides were etched with acetic acid alcohol and stained with Gills hematoxylin and eosin (H&E). Samples were analyzed using a microscope under 40X magnification and histomorphomitric analyses were conducted independently by two analysts (J.K. & G.W.) from the laboratory and supervised by two of the investigators (D.Z. & M.Q.). Results were extrapolated upon agreement of all parties.

VI. Data presentation and Statistical analyses

Data were analyzed from images taken from histology slides using Olympus Microsuite™ biological suite imaging software (version 2.6). Percent total BIC (Zone C) was obtained from the sum of percent BIC in the cortical part of the bone (Zone A) and the percent BIC from the marrow space area (Zone, B), as schematically shown in Figure 18. According to Sennerby et al. [45], newly bone formation tends to flow from the cortical area to marrow space along the surface of the implant and that was the reason why both BIC percentages were quantified and summed up to evaluate the total implant length BIC (Figure 18).

Mann-Whitney non-parametric test was performed to compare test and control groups at 2, 4 and 6 weeks. An independent samples t-test was performed to determine statistical significance of the given data. All tests were performed using SPSS analytical software.

VII. Results

A total of three animals were used. Each animal resulted in three test-group samples and three control-group samples at three different time points: 2, 4 and 6 weeks. A total of 9 silk-coated implants and 9 plain titanium implants were placed in rabbits' tibiae. Two samples, both from the control groups, one from the 2nd week time point and the other from the 6th were not included in the reported results due to damages that occurred during grinding down the specimens (Table 3). Data will be presented as % BIC mean value (SD) and inflammatory cells as median value (Range) (Table 4).

1. *Bone-implant contact (BIC)*

At 2 weeks, the test group showed higher BIC over control group in general, though the difference did not reach statistical significance. Total BIC mean (SD) was 82% (0.83) in test group vs. 62% (0.13) in control group at 2 weeks (p=0.083). Marrow space BIC mean (SD) was higher in test group with 55% (0.08) vs. 41% (0.02) in control group (p=0.564) with no statistical significance in mean difference. Cortical BIC mean (SD) in both test and control groups was lower than marrow space BIC since the test group showed 27% (0.02) vs. 21% (0.03) in control group, p=0.083. (Figure 19)

At 4 weeks, the control group showed higher total BIC with the cortical bone showing the higher increase. Mean values of total BIC in test group were 57% (0.14) vs. control group 39% (0.04) in the same timeline but did not reach statistical significance (p=0.275). Higher BIC mean value in test group was at the cortical part of bone 26% (0.03) vs. 24% (0.02) in control group, p=0.513. Marrow space part showed BIC of 13%

(0.03) in test group vs. 33% (0.16) in control group with the difference being statistically significant, p=0.05.

At 6 weeks, total BIC mean values of control group was slightly higher 34% (0.06) than test group 32% (0.07) with no statistical significance in difference, p=1.000. However, marrow space BIC% in test group 14% (0.09) was higher than control group 5% (0.07) p=0.248 and cortical space of test group showed 18% (0.03) vs. 29% (0.01), p=0.083.

2. *Inflammatory response*

The inflammatory response (Table 5) was generally equally distributed with higher values in the test group. Inflammatory response was given the value of 0 when inflammatory cells were absent. A value of 1 was given when cells were minimally observed in tissues. The value of 2 was given when cells were notable (mild to moderate) and value of 3 was given when there was a prominent observation of cells in tissues (marked/severe).

The following was evaluated according to the criteria mentioned earlier. ***Medullary inflammation***, which represented the overall accumulation of different inflammatory cell types. ***Implant associated inflammation***, which evaluated the accumulation of inflammatory cells in close proximity to dental implants. ***Macrophages***, which play an important role in phagocytosis of debris and old bone that is necessary in regeneration process [46]. ***Multinuclear giant cells (MNG),*** which appear in close proximity with osteoclasts and they are the result of fusion of several macrophages together [47].

Lymphocytes, which are inflammatory cells that are considered to be the first line of defense in the presence of an antigen or a foreign body [48]. ***Neutrophils***, which are cells that represent the hallmark of acute inflammation status following an injury [49]. Other cells that were evaluated were ***fibrous tissue*** and ***newly formed osteocytes.***

At 2 weeks, Lymphocytes had the upper hand in presence with a higher median, 1 (0) in test group vs. 0 (1) in control group p=0.025 and implant associated inflammatory cells were higher in test group 2 (0) than control group 1 (1) p=0.034. Both cells were significantly higher in test group with a *p value* <0.05. Both cells increased in number as healing time increased. Medullary inflammation, neutrophils, MNG and fibrous tissues were equally present and not statistically significant in difference when test and control groups were compared. The presence of newly formed osteocytes was higher in test group when compared to control without statistical significance in difference.

At 4 weeks, all inflammatory cells increased in quantity in test group when compared to control group, although the increase did not reach statistical significance. The median values were higher when it came to medullary inflammation and implant-associated inflammation in test group. Whereas, the median values of neutrophils, MGC, macrophages and fibrous tissue were all equal to the control group. The prominence of lymphocytes in test group 2 (1) p=0.034, which was statistically significant, was the hallmark of this timeline in terms of inflammatory response.

At 6 weeks, higher median values in test group was seen at this timeline with the prominence of lymphocytes 2 (0) vs. 0 (0) in control group, p=0.025. Inflammation around implant was higher in test group 3 (1) vs. 1 (0) in control group (p=0.034) and prominence of macrophages can also be seen in test group 2 (0) vs. 0 (1) in control group (p=0.034). All previous values were statistically significant in difference. Medullary inflammation, MNG, neutrophils and fibrous tissues were equal to control group.

VIII. Discussion

Our study evaluated the effect of silk-coated titanium implant on osseointegration using an animal model. To the best of our knowledge, this is the first in-vivo study that addressed such question using rabbit as an animal model. We have compared silk coating of titanium with RGD peptides to pure titanium at three different timelines. Our hypothesis was that silk-coated implants would show better osseointegration in terms of quantity of bone in contact with implant surface (BIC). Our primary outcome was to quantify BIC around each silk-coated implant and compare it with pure titanium implant. We have found that at 2 weeks, the mean of BIC covering the total length of the implant in the test group was higher than the control group with 82% of BIC vs. 62% in control group but it was not statistically significant (p=0.083). Figure 20A and B show 2 weeks histology and there is evidence of newly formed bone around both groups and immature bone is on its way to condense around implant surface in the cortical area. These events are with agreement with what Sennerby et al. [50] published in regards of

bone formation around pure titanium in rabbit tibiae. The quantity in the test group showed higher mean values but the quality of newly deposited bone seemed similar. The silk layer was still intact around the whole implant and it showed that it attracted newly formed bone at the marrow space more than control group possibly because of the RGD peptides. This is in agreement with Schliephake et al. [30] who confirmed early cellular adhesion to implant surfaces coated with RGD as early wound healing event. Signs of early inflammatory reaction were evident in test group around implant's apical part in the marrow space at this time point with evident presence of lymphocytes. Although lymphocytes and implant-associated inflammation in test group are statistically higher than control group, the median was as low as 1 in 2 weeks. Figure 20C and D show 4 weeks histology results that indicate more maturation of bone in contact with implants in both test and control groups with evidence of few empty lacunae, which represents evidence of immaturity [51]. BIC% of total length of implant was higher in control group (57%) vs. test group (39%) but the difference did not reach statistical significance (p=0.275). However, it was statistically significant that BIC% at the marrow space level was higher in control group when compared with test at 4 weeks (Figure 19, p=0.05). Silk is showing signs of dislodgement from the surface of implant in the test group at this point of time. Median value of lymphocytes as an inflammatory cell increased to 2 in test group with statistical significance difference (p=0.034). Level of maturation of newly formed bone was equal as an observation between both groups at this point of time. Figure 20E and F shows histology of control and test groups, respectively, at 6 weeks with full maturation of newly formed bone and condensation of osteocytes around

implants in both groups with slightly increased BIC % in control group for the total length of the implant 34% vs. 32 % in test group. At this time point, inflammatory cells increased in quantity around the implant. The silk is defragmented from around the implant at this time point and it was evident that the inflammatory cells engulfed parts of it (Figure 20). Defragmentation of silk; yield segments of peptides-chains, which are recognized by the inflammatory cells as foreign antigens, which would explain the increase in inflammatory response at this stage. A follow up study that looks into longer time points is needed to explain the extent of this inflammatory response and whether it would continue even after total degradation of the silk and whether it would affect the osseointegration that have occurred on the surface of the implant. Displacement of bone to implant contact by fibrous tissue due to the presence of prolonged inflammation should also be investigated in further studies.

IX. Limitations

Our study was underpowered by 2 samples dropout due to grinding process accidents. A better-powered study would give a better statistical significance extrapolation. The study was also short in follow up and further studies should look into results as far as 3-4 months to further investigate the effect of silk degradation on the inflammatory response and osseointegration. Increasing the number of animals in each group would also be another suggestion to improve the study by testing variability in host response and its effect on osseointegration. The effect of the addition of growth factors and morphogenic proteins could be also studied in the same manner as the RGD peptide to improve the anti-inflammatory effects of silk. Due to financial limitations, titanium screws were used instead of dental implants. Using actual dental implants, could give a better picture on how these results relate to dental practice. The thickness of the silk coating could be evaluated in terms of proximity of newly formed bone to implant surface. Furthermore, controlling the rate of biodegradability of the silk coating is another parameter that should be investigated. If degradation of the silk biomaterial occurs faster, then maybe the inflammatory response would subside sooner. Lastly, a study could be designed using pure titanium implant as a negative control, RGD-coated implant (without silk) as positive control, and silk coated implant with RGD peptides as a test to investigate the actual role of the silk as a viable scaffold option for coating implant surfaces with various other peptides.

X. Conclusion

Within the limitation of our study, we can conclude that, coating implants with RGD decorated silk was not superior to pure titanium implants in terms of osseointegration in an animal model. Some improvement was seen at early stages of wound healing but quickly evened out with time. Silk also initiated more inflammatory response that escalated with time. Further studies are needed to follow up on the extent of this inflammatory response and its impact on osseointegration of titanium implants.

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