The Soft Tissue Immunologic Response to Hydroxyapatite-Coated Transmucosal Implant Surfaces: A Study in Humans

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ABSTRACT

Objective: To evaluate the soft tissue response in humans immunologically and histologically after placement of mini-implants coated with or without nano-size hydroxyapatite coatings.

Material and Methods: Commercially pure (cp) titanium mini-implants ($n = 13$) or nano-hydroxyapatite-coated ones ($n = 12$) were randomly placed into partially edentulous jaws. Crevicular fluid was sampled 1 week after placement and subjected to quantitative polymerase chain reaction analysis to explore the inflammatory markers. After 8 weeks, implants and surrounding soft and hard tissue were trephined, and undecalcified ground sections were prepared. Inflammatory cell accumulation within a defined region of interest in the soft tissue was quantified histomorphometrically.

Results: No statistically significant differences in immunological response to the different implant surfaces were found for IL-6 ($p = .438$), TGF-β2 ($p = .467$), MMP-8 ($p = .758$), CCL-3 ($p = .758$), IL-8 ($p = .771$), and IL-1β (0.771). Histomorphometric evaluation presented no statistically significant difference between the two mini-implant surfaces with regards to number of inflammatory cells ($p = .669$).

Conclusion: Nano-hydroxyapatite-coated surfaces in the transmucosal region yielded similar inflammatory response and is suggested to be as biocompatible as commercially pure titanium surfaces.

KEY WORDS: dental implant, gene expression, human, hydroxyapatite, nanotopography, osseointegration

INTRODUCTION

Implant treatment has become one of the clinically predictable and reliable options in restoring partial or full edentulism. In fact, the long-term survival of implants placed in the jaw bone has been reported to be around 95%.[1,2] While the success of osseointegration has improved, there still exists a demand to shorten the treatment time by enhancing osseointegration. Research on implant surface modification is now starting to shift its focus from pure osseointegration to long-term maintenance of marginal bone and soft tissue levels. As it has been previously reported, bone and soft tissue around

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implants relate to one another, and always maintaining their biologic width,[3,4] this suggests that for successful treatment, both the hard and the soft tissue around implants must remain healthy. Much attention has been given to the bone-to-implant interaction, that is, osseointegration. It is well known that implants with a moderately roughened surface osseointegrate better than smoother or rougher surfaces.[5] However, little attention has been paid to the soft tissue interaction between the transmucosal portion of the implant especially in vitro animal studies.

Recently, there has been additional interest with regards to abutment surface or macrogeometric modifications in order to improve soft tissue sealing or even gain soft tissue attachment. These modifications could improve peri-implant mucosal sealing and contribute to better long-term treatment outcome. One possible way to modify the abutment topography is by introducing the biologically inspired structures, which have been reported to create different functional elements.[6] It has been proven in implant surface modification studies that there is an hierarchical biomimetic architecture that is more advantageous to osseointegration.[7] Thus, it is hypothesized that by introducing biomimetics to the abutment surface modification, there would be alterations in the soft tissue around the abutment, which may improve the sealing.

Modification of both the topography and the surface chemistry are required to obtain a bioactive surface. In a study by Wennerberg and colleagues,[8] a nano-sized porous titanium dioxide (TiO_2) film was coated to abutments, and these were placed in humans to observe the effect of soft tissue contact to the abutment surface. Histologic evaluation revealed that no definite differences between the uncoated abutments and the nano-coated were observed; however, the corresponding clinical observation showed that many of the coated test abutments were completely covered with soft tissue, whereas the noncoated controls did not show this tendency.

Several studies have demonstrated the capability of the nanostructured hydroxyapatite (HA) to accelerate the osseointegration process,[9–13] when the coating thickness is approximately 10–20 nm. Intriguingly, the HA crystals are of similar size and shape as those found in the human bone tissue. Although no studies have yet observed the in vivo or clinical soft tissue responses to nanostructured HA, in vitro studies suggest that fibroblasts cultivated on theses surfaces present enhanced cell attachment and proliferation.[14,15] There is great interest to document the immunological response of nano-HA-coated abutments with regards to the more commonly used turned titanium surface abutments placed in humans. Thus, the objective of the study was to investigate the gene expression of the cell population excreted into the crevicular fluid and to confirm histologically the inflammatory responses in the transmucosal region of both commercially pure titanium mini/implants with or without nano-size HA coatings.

MATERIAL AND METHODS

Study Population

Patients ($n = 13$) lacking at least one tooth that could be replaced by a wide body implant (diameter of 7 mm and length between 7–9 mm) were selected from the population attending the Department Periodontology and Oral Implantology of the University Hospital in Ghent (seven males and six females, mean 58.8 years, range 45–72 years). They were all periodontally healthy and maintained a good oral hygiene, brushed two times a day, and used interdental brushes. The patients received in total 25 mini-implants. Subjects were all informed of the nature and objectives of the study and their full-signed consent was obtained prior to entry into the study. Approval of the ethical committee of the University Hospital in Ghent (EC 2010/536) was obtained.

Study Design

Experimental, commercially pure titanium one-piece mini screws with a transmucosal portion equivalent to an abutment with a diameter of 1.5 mm and length of 8 mm were placed in the jaw bone. Part of the mini screws were coated with a nano-HA coating (Promimic AB, Göteborg, Sweden) as described by Jimbo and colleagues[11] and compared to a turned commercially pure titanium implant. Although in the microlevel, the surface topography for both surfaces are comparable, it has been reported that the nano-HA coating has a homogenous whisker-like morphology, and the topography in the nanolevel has confirmed the diameter of the coated particles to be around 20–30 nm.[9,10] On the other hand, the turned surface did not have a homogenous structure in the nanolevel and the morphology confirmed by the structural equation modeling presented a typical turned surface.[11]

TABLE 1 Distribution of the Mini Screws and Patients (Hydroxyapatite Coated, HA, or CP Titanium, cpTi) 1 Week after Placement

Patient Number	Age	Gender	HA	cpTi	Sample Number
1	72	M	45 HA		1
				46 cpTi	2
2	48	F		17 cpTi	*
3	62	F	16 HA		3
				17 cpTi	4
4	59	M	26 HA		5
				27 cpTi	6
5	61	M		47 cpTi	7
			46 HA		8
6	45	M	24 HA		9
				25 cpTi	10
			14 HA		11
				17 cpTi	12
7	51	F	16 HA		13
8	72	M	16 HA		*
9	53	M	†		†
10	60	F	17 HA		14
				27 cpTi	15
11	62	F		27 cpTi	16
12	67	M		16 cpTi	17
			26 HA		18
			27 HA		19
13	46	F		27 cpTi	20
				26 cpTi	21
14	60	M		25 cpTi	22
			15 HA		23

*Sample lost due to not attending to the visits.
†Exit patient due to cyst.

All surgical procedures were performed by one surgeon (H.D.B.) and implant placements were selected according to a randomization scheme (Table 1). Minimal flap was raised to explore the bone and only cortical bone perforation was performed, basically placing the mini screw in a self-threaded manner. A week after surgery, sampling of the peri-implant crevicular fluid using two paper points, ISO 30 (taper .02, Dentsply Maillefer, Tulsa, OK, USA) was performed at both buccal and lingual points around the mini screws and mRNA samples were taken 1 week after placement of the mini screws.

Subsequently after sampling, the paper points were placed in RNA*later* (miRNeasy Mini Kit (50), Qiagen®, Gmbh, Hilden, Germany) to preserve the mRNA and were stored at −80°C until the extraction procedure. Eight weeks after placement, the mini screws were removed after punching the soft tissue collar. The surrounding bone and soft tissue were obtained with a trephine bur. After the implant retrieval, osteotomy was further prepared to place a wide body implant for subsequent superstructure construction.

Histological Evaluation

The trephined implant-tissue *bloc* samples (Figure 1) were placed in 4% formaldehyde for 48 hours and thereafter underwent dehydration in series of ethanol (70–100%) and gradual infiltration in light polymerizing resin (30–100%, Technovit 7200®, EXAKT®, Heraeus Kulzer GmbH & Co., Wehrheim, Germany). After embedding the samples, the histologic blocks were subjected to undecalcified cut and ground sectioning.

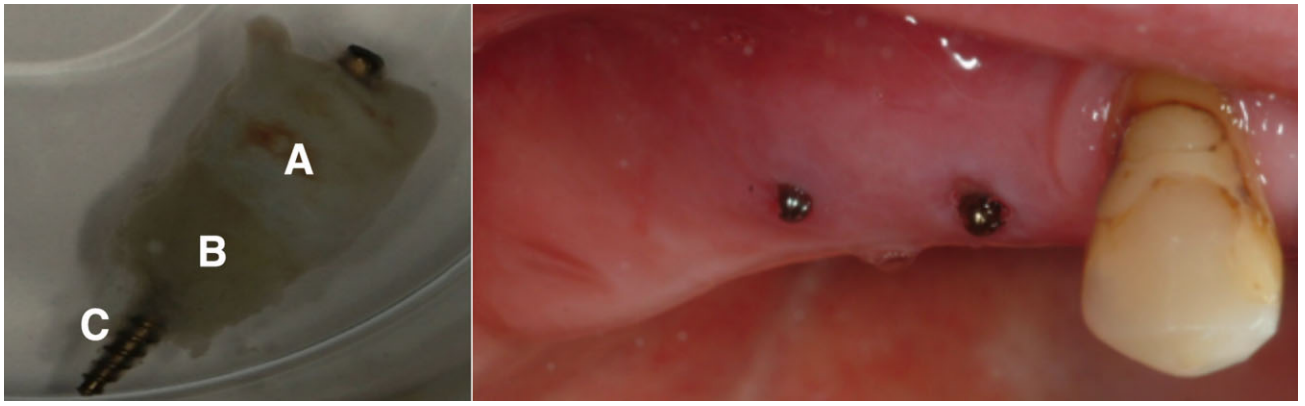

Figure 1 Trephined block biopsy of a mini-implant (C) with surrounding peri-implant tissue (A) and bone (B), and both group of implants placed in the oral cavity. No clinical signs of failure were evident.

The sections were ground to a final thickness of 15 µm and stained with toluidine blue and pyronin G. Histological evaluations were performed with a light microscope (Eclipse ME600; Nikon, Tokyo, Japan) and histomorphometrical analysis by an image analysis software (Image J v. 1.43u; Image J, Bethesda, MD, USA). The presence of inflammatory cells around an implant was quantified at two areas as presented in Figure 2 at

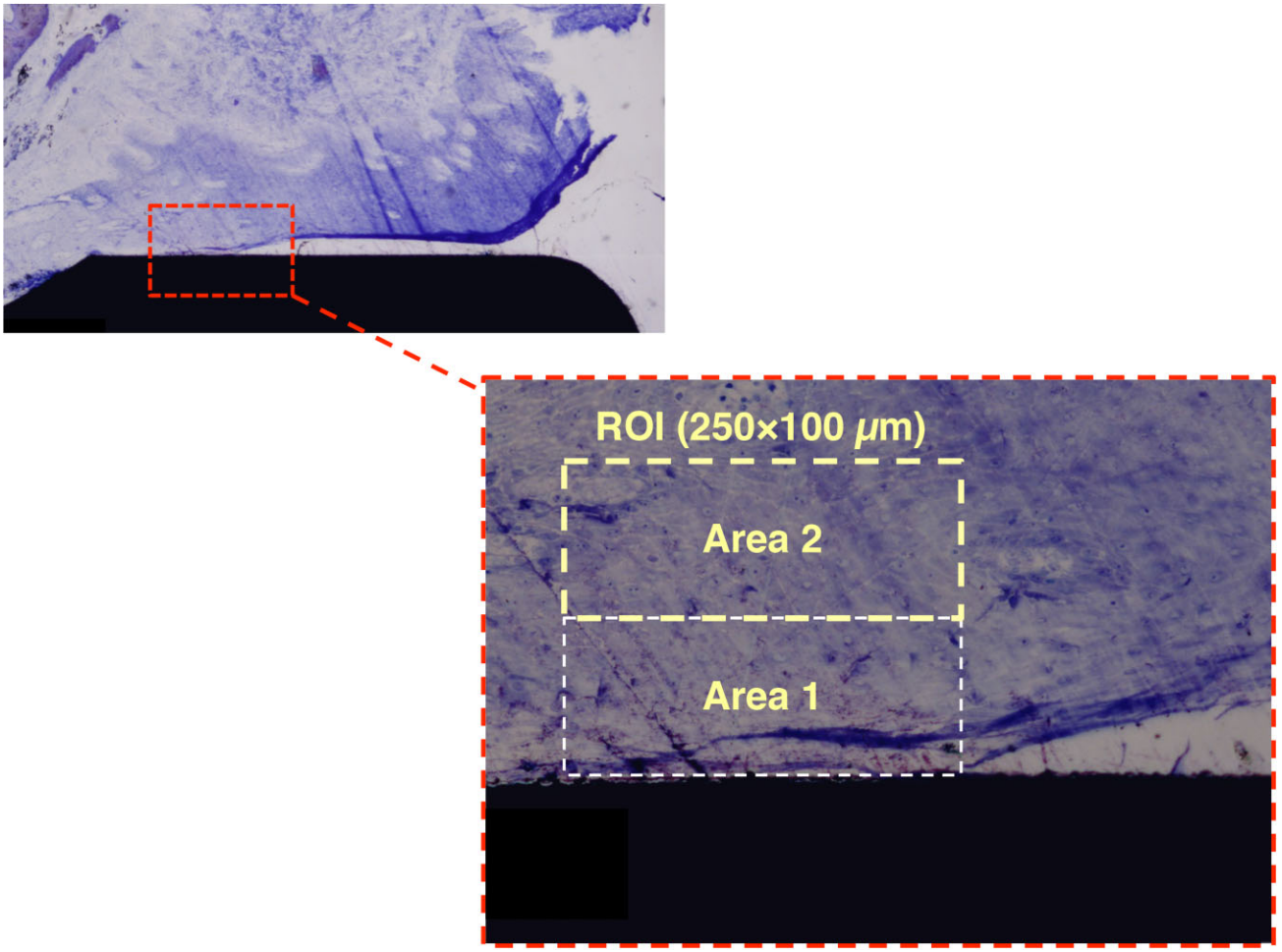

Figure 2 The area of interest (area 1 and area 2) quantified histomorphometrically within a region of interest of 250 × 100 µm.

×10 magnification in a defined region of interest (ROI) of 250 × 100 µm for each area. Area 1 represents the zone of the connective tissue lateral to the neck at the implant-soft tissue interface. Area 2 represents the zone located next to area 1, away from the implant interface. Statistical analysis, using one-way analysis of variance in IBM SPSS statistics version 20 (SPSS Statistics and IBM Company, Chicago, IL, USA) was used to detect differences in the number of inflammatory cells between the HA-coated and titanium surfaces.

Gene Expression

The peri-implant crevicular fluid was submitted for mRNA extraction following the Bench Protocol from Qiagen (miRNeasy Mini Kit (50) and RNAse-Free Dnase set). The quality of the obtained mRNA was examined with the NanoDrop Spectrophotometer (Thermo Scientific®, Wilmington, DE, USA). Reverse transcription was performed on 10 ng/µL RNA using the IScriptcDNASynthesis Kit (Bio-Rad®, Berkeley, CA, USA). Quantitative reverse transcription was done per cDNA sample in a total volume of 5 µL, 3 µL mix + 2 µL cDNA (with a concentration of 2.5 ng/µL). Reactions were performed using the iQ™ SYBR® Green supermix (Bio-Rad) according to manufacturer's recommendations. Selection of the genes of interest was done after a literature research, using the PubMed search engine.[16–18] As a result, the following markers supposedly involved in inflammatory responses were selected: transforming growth factor beta-2 (TGF-β2), collagenase-2 (MMP-8), chemokine ligand-3 (CCL-3), interleukin-8 (IL-8), IL-1β and IL-6. The sequence and detailed explanations are presented in Tables 2 and 3. As control, housekeeping genes were included in the analysis of the genes of interest (Table 4).

The statistical analysis was examined at the implant level. The Qbase^{PLUS} program (Biogazelle, Ghent, Belgium) with the Mann-Whitney test for independent variables (expression levels of commercially pure titanium surfaces versus HA-coated surfaces) was used on a 5% significance level. Qbase^{PLUS} Array Data Analysis software was utilized[19] to analyze polymerase chain reaction (PCR) data and generate results are reported using the MIQE guidelines.[20] Assay specificity was verified by size analysis, a single band was seen for all assays. Amplification efficiency was evaluated using a 7-point 10-fold dilution series. All efficiencies fell in the 90–110% efficiency range.

RESULTS

Histologic and Histomorphometric Analysis

Ten paired samples (HA = 5, commercially pure titanium [cpTi] = 5) that were sequentially selected based on the soft tissue attachment to the implant in the defined ROI were included in the histomorphometric

TABLE 2 Genes Utilized in the Study

Gene	Primer Sequence*	Amplification Efficiency Value (E)†	Correlation Coefficient (r^2)
IL-1β	IL-1β F **5'-CCTGTACGATCACTGAACTG-3'**	1.925	0.99
	IL-1β R **5'-ACCACTTGTTGCTCCATATC-3'**		
TGF-β2	TGF-β2 F **5'-GCCCTACTTGTGCTTTGTGTTTCT-3'**	1.934	0.997
	TGF-β2 R **5'-AGACCACTGAACTCGAACCCATCT-3'**		
CCL-3	CCL-3 F **5'-GACAGCCACTCGGTTGTCAC-3'**	1.937	0.925
	CCL-3 R **5'-TTGTGATTGTTTGCTCTGAGAGTTCCC-3'**		
IL-8	IL-8 F **5'-CAATGCGCCAACACAGAAAT-3'**	1.828	0.996
	IL-8 R **5'-TCTCCACAACCCTCTGCACC-3'**		
IL-6	IL-6 F **5'-TGTTGTTAATGGGCATTCCT-3'**	1.999	0.985
	IL-6 R **5'-AGTGTCCTAACGCTCATACT-3'**		
MMP-8	MMP-8 F **5'-CCTTGGAATTCCTTGGCTTGGAGATG-3'**	1.879	0.991
	MMP-8 R **5'-AGCTTACCAGGGTCTTTGCAGATG-3'**		

*Primer source *Homo sapiens* (IDT, Coralville, IA, USA).
†A good amplification efficiency (E, target value 2) and high correlation coefficient (r^2, target value 1) indicate a high quality from which reliable efficiency estimations can be drawn.

TABLE 3 Genes Utilized in the Study: Type and Function

Gene	Gene Type	Gene Function
IL-1β	Protein coding	Mediator of the inflammatory response Involved in cell proliferation, differentiation, and apoptosis Associated with periodontal disease (matrix breakdown)
TGF-β	Protein coding	Peptide involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis in cells Decreased expression in periodontal and peri-implant disease
CCL-3	Protein coding	Role in inflammatory responses Bone remodeling associated
IL-8	Protein coding	Major mediator of the inflammatory response Chemoattractant Involvement in peri-implantitis
IL-6	Protein coding	Functions in inflammation and the maturation of B cells Produced at sites of acute and chronic inflammation Associated with periodontal and peri-implant disease
MMP-8	Protein coding	Involved in the breakdown of extracellular matrix (tissue remodeling) as well as in disease processes Early signal of peri-implant disease

evaluation. Selection of the readable sections was performed independently by two examiners (F.Y. and E.D.W.) who reached an agreement.

The morphology of the contact zone varied considerably between patients: in some patients the contact zone was occupied by connective tissue, in others by epithelium. The histologic observation presented localization of inflammatory cells in vicinity of the implant transgingival area for both control and test groups (Figure 3) and the histomorphometric evaluation presented no statistically significant differences between the two tested groups for both areas 1 and 2 ($p = .669$, $p = .165$, respectively, Table 5).

Gene Expression

An average yield of 245 ng RNA per sample was obtained after RNA extraction. cDNA with a concentration of 2,5 ng/µL was synthesized from this RNA (IScript cDNA Synthesis kit 100 x 20 µL reactions, Qiagen).

Three tested housekeeping genes; HPRT1, GAPDH, and YWHAZ were used to program a quantitative PCR (qPCR) array for DNA stability and standardization.

Analysis(Qbase^{PLUS} program, Biogazelle)[19] with the housekeeping genes and the genes of interest together, revealed that samples 14, 17, 20, and 23 showed no reaction, as we could not obtain usable quantities of cDNA and were therefore excluded from further

TABLE 4 Housekeeping Genes Utilized in the Study

Gene	Primer Sequence*	Amplification Efficiency Value (E)†	Correlation Coefficient (r^2)†
HPRT-1	HPRT-1 F **5'-TGACACTGGCAAAACAATGCA-3'** HPRT-1 R **5'-GGTCCTTTTCACCAGCAAGCT-3'**	2.006	0.999
GAPDH	GAPDH F **5'-TGCACCACCAACTGCTTAGC-3'** GAPDH R **5'-GGCATGGACTGTGGTCATGAG-3'**	2.023	1
YWHAZ	YWHAZ F **5'-ACTTTTGGTACATTGTGGCTTCAA-3'** YWHAZ R **5'-CCGCCAGGACAAACCAGTAT-3'**	1.995	1

*Primer source *Homo sapiens* (IDT, Coralville, IA, USA).
†A good amplification efficiency (E, target value 2) and high correlation coefficient (r^2, target value 1) indicate a high quality from which reliable efficiency estimations can be drawn.

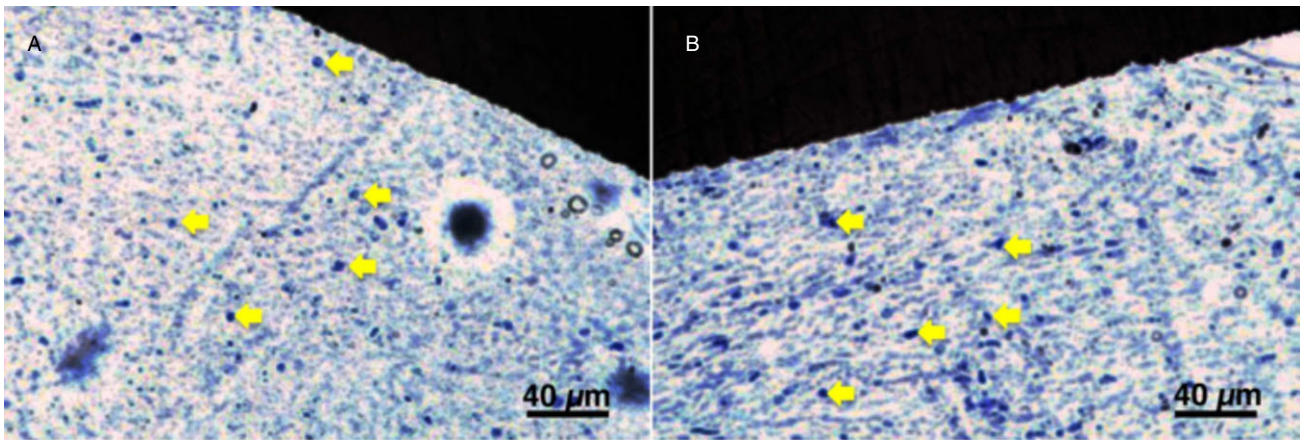

Figure 3 Histological micrographs at the interface of the soft tissue attachment to (A) cp titanium surface and (B) hydroxyapatite-coated surface. Arrows indicate inflammatory cells, which presented no qualitative and quantitative differences between the two groups. Bars indicate 40 µm, magnification ×10.

analysis. Hence, in total, 19 samples were available for the subsequent qPCR analysis, and six genes of interest were selected, namely, TGF-β2, MMP-8, CCL-3, IL-8, IL-1β, and IL-6. The statistical analysis was done on implant level ($n = 19$), using a Mann-Whitney test. Results viewed in Table 6 show no significant differences in the immunological response to the different implant coating for IL-6 ($p = .438$), TGF-β2 ($p = .467$), MMP-8 ($p = .758$), CCL-3 ($p =$ MIP-1α) ($p = .758$), IL-8 ($p = .771$), and IL-1β ($p = .771$).

DISCUSSION

This study investigated the genetic and histologic response of two different implant transmucosal surface topographies in humans with regards to soft tissue immune response. The gene expression analysis of the crevicular fluid cells presented no statistically significant difference concerning the surface treatment. Although some samples could not be utilized due to follow-up problems and damage of the mRNA, the samples we obtained ($n = 19$) were enough to obtain a statistical conclusion. Furthermore, the histomorphometric investigation revealed no qualitative or statistically significant difference in the number of inflammatory cells around

TABLE 5 Number of Inflammatory Cells in the Connective Tissue Lateral to the Neck at the Soft Tissue Attachment to the Mini-Implant

Patient Number	HA (1) or cpTi (0)	Sample Number	Amount of Cells	
			I*	II†
3	1 HA	3	113	121
	0 cpTi	4	85	82
4	1 HA	5	66	50
	0 cpTi	6	89	48
6	1 HA	9	71	120
	0 cpTi	12	100	59
10	1 HA	14	91	80
	0 cpTi	15	53	75
12	1 HA	19	61	70
	0 cpTi	17	44	60

*I = Area 1.
†II = Area 2.

TABLE 6 Fold Induction and Difference of Markers ($n = 23$) Real-Time PCR Measurement of mRNA Levels of Cells Adhering to CP Titanium (S) or Hydroxyapatite-Coated Surfaces (HA)

Target	*p*-Value	Fold Induction	Fold Difference* (HA/S)	95% CI Low	95% CI High
IL6	0.438	HA 0.544 S 1.837	0.296	0.078	1.122
CCL3	0.467	HA 0.825 S 1.212	0.681	0.233	1.989
TGFB2	0.467	HA 1.222 S 0.740	1.652	1.020	2.677
IL-1B	0.758	HA 1.338 S 0.769	1.739	0.293	10.328
MMP8	0.758	HA 0.870 S 1.169	0.744	0.379	1.459
IL8	0.771	HA 1.609 S 0.652	2.470	0.379	16.087

*Fold differences are the ratio between hydroxyapatite (HA) and commercially pure titanium (S) surfaces.
HPRT1, GAPDH, and YWHAZ were used to standardize the samples.

cpTi and nano-HA-coated surfaces. Here, one must acknowledge that only five paired samples were intact between the implant and the soft tissue, and this must also be acknowledged as a drawback of the current study. The difficulties of extracting the biopsies in an intact manner must be recognized and admittedly, the sampling technique must be improved. However, this is the first study to obtain a pair-wise histological human comparison on this topic and the outcomes depicted some evocative information.

In terms of abutment surface topography, it has long been believed that the abutment surface should be smooth, preferably polished to promote positive soft tissue responses. However, recent publications, have shown, for example, that a laser-etched microtextured surface provides firm soft tissue healing around the abutment with collagen fibrils running perpendicular to the implant-abutment, which has not been the case for traditional abutment materials (which the collagen fibrils align parallel to the abutment).[21,22] This is an indication that there may exist a topographical feature that may optimize soft tissue healing and provide for better sealing around the abutment; however, it must be noted that more evidence is necessary to sustain theses conclusions.

It has been reported that abutment material differences influence the soft tissue status around the abutment, showing that gold alloy materials present more inflammatory cells localization as compared to titanium, or zirconium abutments.[23,24] Furthermore, it has also been suggested that there exists different biologic responses between milled and casted abutments.[25] Based on these outcomes, the chemistry of the material may be responsible for the soft tissue inflammatory responses, and interestingly, the materials considered to be biocompatible or bio-inert as an implant material, also apply as an abutment material. Thus, the alteration of topography and chemistry in the nanolevel as conducted in the current study was of great interest.

The sealing between the soft tissue and the implant abutment is a topic studied by many groups however, without a definite answer to what is optimal.[26–28] The definite differences between the implant and the tooth in the soft tissue region, is that the tooth has collagen fibrils in the soft tissue running perpendicular to it, which creates an attachment of the fibers and the tooth via hemi desmosomes. The lack of soft tissue sealing has been demonstrated by Ikeda and colleagues, where utilizing electron microscopy, showed that horseradish peroxidase infiltrated into the soft tissue easily reaches the bone of the implant.[29] This could be one of the reasons for peri-implant bone loss, which causes serious damage to the oral health status, although there are other mechanical reasons that have been shown to contribute to bone loss.[30,31]

Since the nano-HA coating used in the current study presented no statistical difference in terms of inflammatory responses, it can be suggested that the nano-coating commonly used to enhance osseointegration can also be applied as an abutment coating. It has been reported that HAs support gingival fibroblast attachment, proliferation and metabolism such as collagen production.[14,32] The strategically applied nanostructure may guide the soft tissue to a desired form while at the same time the chemistry of the HA may warrant low levels of inflammatory responses. In earlier studies, HA plasma sprayed implants caused inflammation. This is in contrast to the earlier belief that plasma spray hydroxyapatite exposition in the oral cavity, and causes inflammation.[33] In the present study, no differences between the cpTI and HA-coated implants were found, which shows that immunologically, a nano-roughened HA surface is better tolerated. The histologic and immunologic outcomes of the current study suggests that the effect of nano-HA when compared to turned surfaces did not present any differences, thus it is speculated that the nanostructure itself did not play a role in the enhancement of tissue integration. One reason could be that the coated nano-HA dissolved rapidly that at the time of evaluation, no differences could be seen. This is in accordance with previous a report by Ong and colleagues where they suggested that the HA will degrade or dissolve irrespective of their surface crystallinity.[34] Another reason could be that the adsorbed plasma proteins such as albumin or fibronectin presented a masking effect, which may have cached the effect of the surface nanotopography. It has been reported that proteins adsorbed onto a material surface will constantly be replaced by different proteins depending on their molecular weight,[35] and the constant biofilm formation of different proteins may very well hinder the underlying surface topography, chemistry, and their physical properties.

CONCLUSION

The results of the current study indicated that HA-coated nano-surfaces do not provoke greater

inflammation as compared to the turned cpTi surfaces. This suggests that the surface modification in the nanolevel has not changed the biocompatibility of the abutment. Although no significant differences were seen in the current study, it would be of great interest to further conduct observations in the electron microscopy level to identify whether there exist a nano-HA surface unique interaction to the surrounding soft tissue.

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