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Associated lesions of peri-implant mucosa in immediate *versus* delayed loading of dental implants

ALEXANDRU ANDREI ILIESCU[1)], SABINA ANDRADA ZURAC[2)], VASILE NICOLAE[3)], MIHAELA GEORGIANA ILIESCU[4)], PAULA PERLEA[5)]

[1)]*Department of Oral Rehabilitation, University of Medicine and Pharmacy of Craiova, Romania*

[2)]*Department of Pathological Anatomy, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

[3)]*Department of Dental Medicine, "Victor Papilian" Faculty of Medicine, "Lucian Blaga" University of Sibiu, Romania*

[4)]*Department of Orthodontics and Dento-Facial Orthopedics, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

[5)]*Department of Endodontics, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

Abstract

Currently, immediate loading of dental implants is very attractive as a standard protocol for prosthetic restorations in edentulous patients. The aim of this study is to find out the intimate peri-implant mucosa response depending on timing of implant loading, immediate or delayed. Fifty-one screw implants Alpha Bio (Alpha-Bio Tec, Israel) were inserted in 42 partially edentulous patients according to standardized surgical techniques. At six months of loading, samples of peri-implant mucosa were harvested from 27 immediate loaded, respectively 24 delayed loaded implants, and subjected to microscopic examination. Peri-implant mucosa in both loadings revealed a continuous and stable stratified squamous epithelium with moderate acanthosis and slight hyperkeratosis. Severe fibrosis and tendency to scar-like lesions were present mainly in immediate loading. Slight to moderate density of inflammatory chronic cell populations of non-uniform feature was common to both loading protocols. As compared to lymphocytes, higher scores of plasma cells were encountered in immediate loading. In immediate and delayed loading, the peri-implant mucosa as a new generated structure does not reveal different tissue responses. After six month of prosthetic loading, the healthy peri-implant mucosa is compatible with fibrosis and minor chronic inflammatory reactions.

Keywords: dental implants, peri-implant mucosa, immediate loading, delayed loading.

Introduction

The endosseous dental implants offer a quite predictable treatment opportunity for replacing missing or clinically compromised teeth, regardless they are placed into a more or less healed alveolar ridge, respectively immediately into an extraction socket [1].

The key to higher long-term survival rates of dental implants is to achieving a good osseointegration that depends on surgical step and proper loading as well [2–4]. Albeit in the literature are widely discussed many loading protocols the clinical interest is currently focused only on immediate, early, conventional, and delayed loading [5]. Among these treatment decisions, the immediate loading is the most challenging because reduces the number of appointments and the total rehabilitation time [6–8].

While the primary stability of dental implants is better known and managed, the secondary one, that is based on a good osseointegration development also interferes with the preservation of a healthy peri-implant mucosa [9–14].

The issue of failing dental implants related to peri-implantitis is still a matter of debate. Though a bulk of evidence emphasizes the cumulative role of bacterial infection and inadequate loading of prosthetic reconstruction, a border between them hardly could be traced [15–17]. Moreover, despite the introduction in recent years among dental practitioners of this very attractive standard protocol dedicated to prosthetic restorations in partially and completely edentulous patients simultaneously with dental implant placement, too little is known about the intimate gingival reaction provoked by immediate occlusal loading [18–21].

Since to our knowledge, no previous studies considered peri-implant soft tissue response according to the timing of implant occlusal loading, immediate respective delayed, the purpose of this study was to find out the possible different consequences by comparatively investigating and quantifying the gingival collagen and inflammatory cells infiltrates.

Patients, Materials and Methods

Forty-two partially edentulous patients, 20 females and 22 males, 25–57 years of age, participated in the study. The edentulous alveolar ridges were restored by inserting 51 screw implants Alpha Bio (Alpha-Bio Tec, Israel) with an adjustable wrench torque (Salvin Torque) of at least 30 Ncm, according to standardized surgical techniques. The implant loading, either immediate or delayed, by subsequent prosthetic fixed therapy, followed the conventional recommended protocol. Samples of peri-implant mucosa were harvested from 27 immediate loaded implants, respectively 24 delayed loaded implants at six

months after the prosthetic treatment, in order to perform both histological and immunohistochemical evaluations.

According to the method used by Liljenberg *et al.* [22] and Zitzmann *et al.* [23], the local anesthetic Ubistesin™ (4% articaine, 1:60 000 R-epinephrine, 3M ESPE AG, Germany) was injected at least 10 mm apical of the proposed area for harvesting. For retrieving the biopsies, each peri-implant mucosa sample was excised by a single-use scalpel Aesculap® BA215-C (Aesculap AG, Germany), using two horizontal parallel incisions of 3 mm, with a 2 mm distance connected at extremities, with perpendicular incisions. Three weeks afterwards, adequate oral hygiene measures were followed.

The soft tissue samples were fixed and stored for 24 hours, at room temperature, in a 10% buffered formalin solution (pH 7.4). Afterwards, the samples were washed, dehydrated through graded series of ethanol, cleared in xylene and embedded in paraffin. Subsequently 3-μm thick cross-sections were cut, exposed to Hematoxylin–Eosin (HE) and van Gieson stainings, and assessed by light microscope Olympus BX41 (Olympus America, Inc.). The inflammation and fibrosis were recorded based on single-blind evaluation and scored depending on the degree of lesions on a four-point scale, as none (score 0), mild (score 1), moderate (score 2), and severe (score 3).

The immunohistochemical analysis was used an indirect three stages method. The soft tissue cross-sections were automatically deparaffinized and hydrated through xylene and graded series of ethanol. Afterwards, it were rinsed in distilled water, incubated in 3% H_2O_2, to blocking the tissue peroxidase activity, washed in phosphate-buffered saline (PBS) and finally incubated one hour with a panel of primary monoclonal and polyclonal antibodies having well-defined specificities, such as anti-CD3 and anti-CD138, delivered by Thermo Fisher Scientific (Fremont, CA, USA). The cross-sections were also counter-stained with Hematoxylin–Eosin.

Statistical analysis

Statistics was performed using the GraphPad InStat software. Wilcoxon matched-pairs signed-ranks test was chosen for analysis in each group of loading and Mann–Whitney *U*-test for comparison of lesions between immediate and delayed loading. A level of significance of 5% ($p<0.05$) was adopted for both tests.

Results

The prevalent epithelial lesions of peri-implant mucosa were acanthosis and orthokeratosis or hyperkeratosis (Figures 1 and 2). Both in immediate (Table 1) and in delayed loading (Table 2), acanthosis was assessed as moderate and to a lesser extent slight. As against acanthosis, hyperkeratosis occurred in a lesser degree and frequently was evaluated as slight, either in the immediate loading (Table 1) or in the delayed one (Table 2).

The collagen fibers play a key role in the architecture of peri-implant mucosa. Since they are the major tissue component, can be also involved in structural changes during the aging processes or pathological conditions such as the local inflammation. The main lesion of peri-implant mucosa as recorded underneath the epithelium was connective tissue fibrosis (Figure 3), which was ubiquitously present in both kind of loading, immediate (Table 1) and delayed (Table 2). A tendency to scar-like lesions in chorion (Figure 4), it could also be noted in immediate (Table 1) and, respectively, delayed loading (Table 2). Moreover, in both loadings protocols, the assessed score of fibrosis was severe. Statistical correlation ($r=0.5318$) was found in immediate loading between fibrosis and scar-like lesions.

Table 1 – ***Immediate loading: distribution of lesions in peri-implant mucosa***

Score	Acanthosis [%]	Hyperkeratosis [%]	Fibrosis [%]	Scar [%]
0	14.81	44.44	0	59.26
1	22.22	44.44	7.40	40.74
2	62.96	3.70	22.22	0
3	0	7.40	70.37	0

Table 2 – ***Delayed loading: distribution of lesions in peri-implant mucosa***

Score	Acanthosis [%]	Hyperkeratosis [%]	Fibrosis [%]	Scar [%]
0	16.66	41.66	0	58.33
1	20.83	37.5	4.16	41.66
2	62.5	12.5	29.16	0
3	0	8.33	66.66	0

Frequently, a slight to moderate density of inflammatory infiltrate was recorded in immediate (Table 3) and delayed loading (Table 4). Commonly, it was noted a pretty similar degree of inflammatory cell density in both loading protocols, excepting the immediate loading, where it was also found a severe inflammation in 3.7% of cases. The immunohistochemical evaluation of inflammatory infiltrate showed that lymphocytes, identified as $CD3^+$ cells, were omnipresent (Figure 5).

Table 3 – ***Immediate loading: distribution of inflammatory cells in peri-implant mucosa***

Score	Cell density [%]	Lymphocytes [%]	Plasma cells [%]	PMNs [%]
0	0	0	44.44	81.48
1	33.33	29.62	18.51	14.81
2	62.96	59.26	25.92	3.70
3	3.70	11.11	11.11	0

PMNs: Polymorphonuclear neutrophils.

Table 4 – ***Delayed loading: distribution of inflammatory cells in peri-implant mucosa***

Score	Cell density [%]	Lymphocytes [%]	Plasma cells [%]	PMNs [%]
0	0	0	58.33	83.33
1	33.33	41.66	20.83	12.5
2	66.66	54.16	16.66	4.16
3	0	4.16	4.16	0

PMNs: Polymorphonuclear neutrophils.

The content of inflammatory cells, which infiltrate peri-implant mucosa, has shown different changes depending on implant loading procedure. In our study, the predominant cell type of the chronic gingival inflammation was the lymphocyte, which was proved by immunohistochemical staining of $CD3^+$ cells. Due to the CD3 marker, which is coupled with T-cell receptor (TCR), it was possible to identify and locate T-lymphocytes. Decisively, the $CD3^+$ expression is restricted to T-lymphocytes and

there is a general agreement that CD3 can be considered a specific marker of T-lymphocytes. Purposely, we used in this study the staining for CD3 instead for CD45, since the second marker is expressed on a more extended group of cells including lymphocytes, macrophages, and monocytes.

In our study, significant statistical correlation was found in both loading protocols between the density of chronic inflammatory cells and the lymphocytes score (r=0.8780 in immediate loading, respectively r=0.8149 in delayed loading).

It has to be noted that in case of plasma cells (Figure 6), immunohistochemicaly identified as $CD138^+$ cells (Figure 7), their global occurrence in moderate and severe inflammation was about two times higher in immediate loading than in the delayed one.

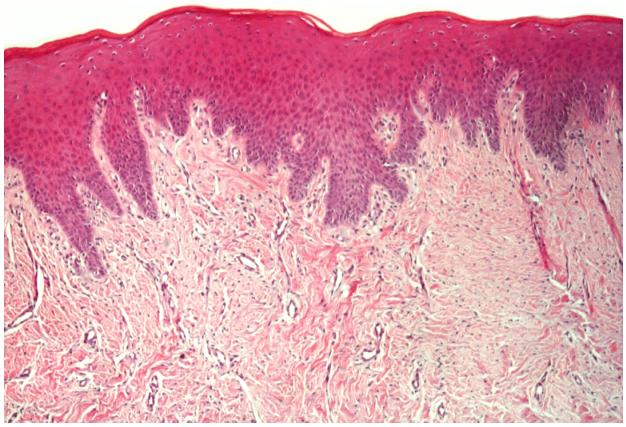

Figure 1 – ***Stratified squamous epithelium. Moderate lengthening of epithelial ridges. Moderate acanthosis and minimal hyperorthokeratosis. Severe fibrosis in chorion, scar-like in some places. Scarce inflammatory cells of lymphoid type in chorion. HE staining, ×100.***

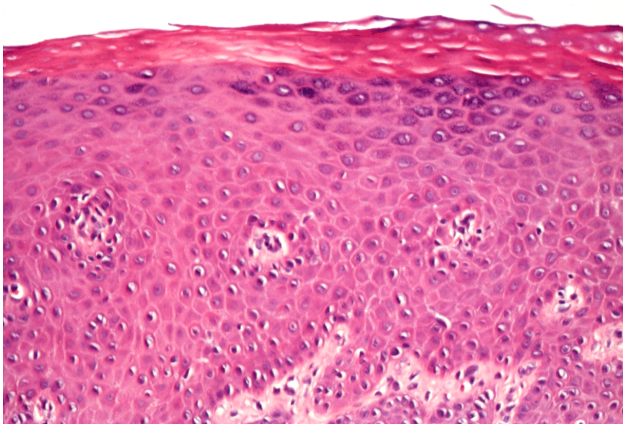

Figure 2 – ***Stratified squamous epithelium. Moderate acanthosis and slight to moderate hyperorthokeratosis. HE staining, ×200.***

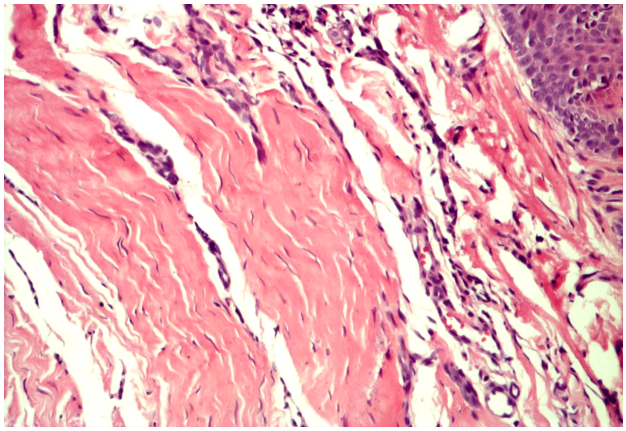

Figure 3 – ***Bulk of coarse collagen bundles separated by elongated blood vessels, bordered with rare fibrocytes and covered with stratified squamous epithelium. HE staining, ×200.***

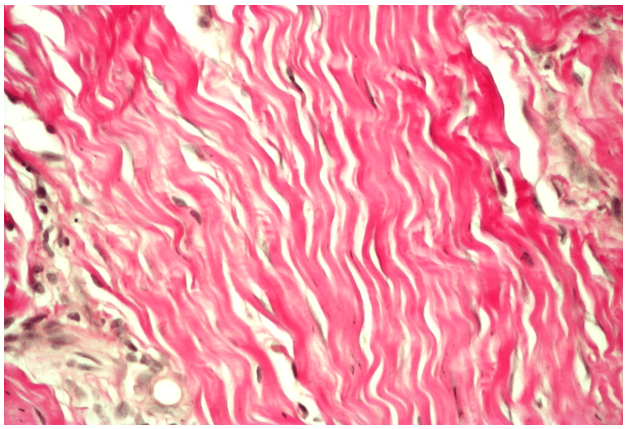

Figure 4 – ***Bulk of coarse collagen fibers with disorganized distribution (scar-like collagen). Van Gieson staining, ×400.***

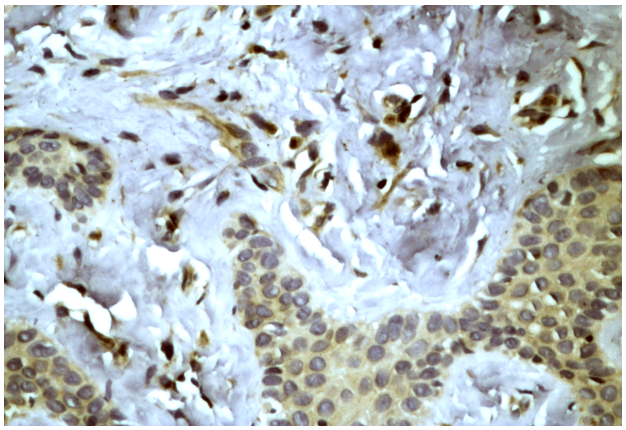

Figure 5 – ***Lymphocytes ($CD3^+$) infiltration in peri-implant mucosa. Anti-CD3 antibody immunostaining, ×200.***

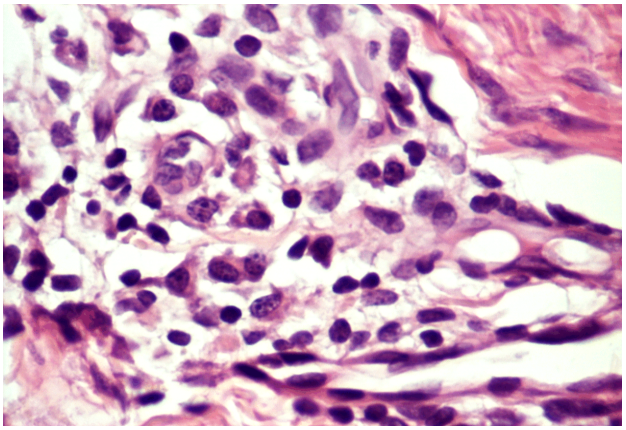

Figure 6 – ***Inflammatory infiltrate with numerous plasma cells. HE staining, ×400.***

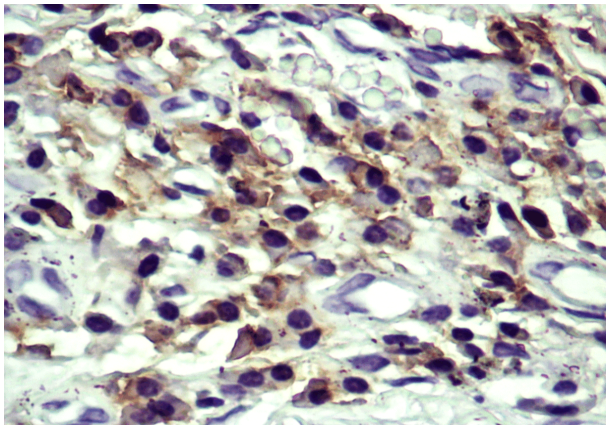

Figure 7 – ***Plasma cells (CD138^{+}) in inflammatory infiltrate with numerous Russell bodies. Anti-CD138 antibody immunostaining, ×400.***

Even though clinically, there were no signs of soft tissue inflammation, microscopically slight to moderate scores of PMNs were described in both immediate (18.52%) and delayed loading (16.67%).

Discussion

The healthy status of the surgically new generated soft tissue anatomical structure of peri-implant mucosa, around the abutments of dental implants, is of paramount importance for their long-term clinical survival [9, 10].

Many previous papers performed by light microscopy found basic resembling features between gingival and peri-implant epithelium and respectively underlying connective tissue. However, too little is known about the characteristics of peri-implant mucosa that depends on loading of dental implants and timing of loading, immediate or delayed. This study was focused on morphological characteristics of the peri-implant mucosa in immediate *versus* delayed loading of dental implants.

Our study confirms that peri-implant mucosa, as component of masticatory mucosa, is morphologically and functionally similar to gingival structures. Though the peri-implant mucosa is a new structure, surgically generated, the interface between the keratinized, stratified, squamous epithelium and chorion is characterized by the same scalloped course of interconnected rete pegs and connective tissue papillae.

We found that the peri-implant mucosa is covered by a stratified squamous epithelium that displays a high frequency of acanthosis, 85.19% in immediate and 83.34% in delayed loading. The acanthosis was mainly of moderate degree and to a lesser extent of a slight one. In our study, acanthosis was commonly observed in the epithelium of peri-implant mucosa, regardless the kind of occlusal loading. Ranging from slight (61%) to moderate (22%) acanthosis might be considered a normal outcome of gingival inflammation or a mucosal adaptation to masticatory stimulation.

Associated with a moderate lengthening of rete pegs, acanthosis may separately occur in peri-implant mucosa during the progress of keratinization that is linked in 50% of cases with a slight to moderate hyperorthokeratosis, in areas exposed to mechanical irritation [24]. In more than one half of loaded implants, this epithelial diffuse hyperplasia of Malpighian layer was associated with hyperkeratosis that was mainly assessed as slight to moderate, 55.56% in immediate and 58.34% in delayed loading, but in some cases the severe score also occurred (7.4% and respectively 8.33%).

Microscopically, in immediate and delayed loading, the interface between the epithelium of peri-implant mucosa and its underlying connective tissue presented epithelial ridges indented with the connective tissue papillae. It seems that resembling the common architecture of the gingiva, the peri-implant mucosa is also capable to play at least the same role as a defensive barrier, because an insufficient keratinized peri-implant mucosa is a risk factor of peri-implantitis [11]. In that respect, a strong argument could be the abovementioned degree of hyperkeratosis, expressed by peri-implant mucosa that was found in our study, in contrast with other papers [11].

The collagen fibers and the extracellular matrix of the underlying chorion of peri-implant mucosa are also involved in getting an efficient seal, around the implant abutment. As compared to the teeth, the new generated collagen fibers around dental implant have different spatial orientation. The majorities are longitudinally parallel or oblique oriented but sometimes show a disorganized feature [9, 10].

In recent years, a bulk of studies supports the participation of molecular growth and differentiation factors in wound healing and regeneration. Fibrosis that was noted in our study in peri-implant mucosa, in all cases suggests that the abovementioned factors are heavily involved in configuration of this new induced mucosal structure. The additional occlusal load of implants, either immediate or delayed, explains the occurrence of fibrosis, mainly assessed as severe.

In our study, could be recognized a well organized connective tissue and disorganized collagen fibers as well, leading in some areas to a scar-like appearance. Fibrosis was noted in all cases. The lesion was mainly severe (70.37% in immediate loading *versus* 66.66% in delayed loading) and to a lesser extent moderate (22.22% in immediate loading *versus* 29.16% delayed loading). Scar-like collagen, assessed only as slight, riches close values in the immediate loading (40.74%) and in the delayed one (41.66%).

Since peri-implant mucosa is a new tissue surgically formed, fibrosis seems to be a ubiquitous phenomenon, highly expressed in 2/3 of cases and to a less intensity in the remaining ones. Changes induced in collagen fibers that become coarse and thicker, is critical in rendering a more fibrous appearance to peri-implant mucosa. It has to be highlighted that this type of scar-like collagen is fully associated with severe tissue fibrosis. The additional chronic inflammation explains the frequency (39%) of the scar-like lesions in chorion, a phenomenon that is developing prior to hyalinization.

In peri-implant mucosa of successfully osseointegrated implants is not uncommon an insignificant inflammatory infiltrate because a new generated immature connective tissue contains fibroblasts and inflammatory cells [9, 10].

It has been revealed that after six months of prosthetic loading, the peri-implant mucosa already showed a well keratinized stratified squamous epithelium and a chorion

architecture similar structured as mucosa covering the edentulous ridge of the same patient. Nevertheless, the inflammatory infiltrate and the blood vessel network had significant higher density as compared to the alveolar ridge mucosa [25].

Chronic inflammation in peri-implant mucosa has been reported in all cases of immediate or delayed loading of our study but its occurrence was uneven, ranging from areas of typical granulation tissue, characterized by a marked inflammatory infiltrate and numerous small caliber vessels, to chorion areas with extremely rare lymphocytes.

However, no differences in qualitative histological features between both groups of immediate and delayed loaded implants were noted. The chronic inflammatory infiltrate was ubiquitous in peri-implant mucosa, having mainly a moderate cell density (62.96% in immediate loading, respectively 66.66% in delayed loading) and decreasing to half values (slight score) in the remaining one-third of samples of both loadings (33%).

In all cases, the chronic inflammatory infiltrate confirmed the presence of lymphocytes, followed in descending order by the plasma cells (55% of cases). The scores for lymphocytes, that were ubiquitous, were in decreasing order mainly moderate (59.26% in immediate loading, respectively 54.16% in delayed loading) and slight (29.62% in immediate loading, respectively 41.66% in delayed loading), but in immediate loading 11.11% out of all cases their occurrence was assessed as severe. An increased local number of $CD3^+$ T-lymphocytes emphasizes their significant role in cell-mediated immunity. However, it is imperative to consider that the lymphocytes could be underscored in peri-implant mucosa because not all T-lymphocytes express $CD3^+$ marker [26].

Plasma cells were recorded to a lesser extent than lymphocytes in immediate loading (55.56%) and in delayed loading as well (41.67%). It has to be noted that in case of plasma cells the occurrence of moderate and severe scores together were about twice in immediate loading than in delayed loading.

PMNs were also reported in both loading protocols. The scores were mainly slight and the rest did not overpass the moderate degree. Even though clinically there were no signs on inflammation, PMNs were recorded in 18.52% cases of immediate loading, respectively in 16.67% cases of delayed loading.

Some papers support the idea that chronic inflammation gradually developed in the chorion of peri-implant mucosa, even at six to 24 months after completion of implant-prosthetic treatment, will get similar quantitative and qualitative parameters as in chronic gingivitis [22, 27].

The peri-implant mucosa is definitely a younger morphofunctional structure than gingiva as it was surgically generated and not formed simultaneously during the tooth eruption. Because the amount of chronic inflammatory cells and the lymphocytes level in peri-implant mucosa reported in our research are between the values recorded in clinical healthy mucosa of alveolar ridge and gingiva, it might be assumed that the immune response to osseointegrated implants has a stable character, regardless the timing of prosthetic loading, as long as the risk of peri-mucositis or peri-implantitis is avoided.

In case of immediate prosthetic loading, further research should explore to what extent the healthy clinical status of peri-implant mucosa is compatible with developing fibrosis, scar-like lesions and minor chronic inflammatory reactions.

Conclusions

After six-month occlusal loading, immediate or delayed, was revealed a continuous and stable stratified squamous epithelium with moderate acanthosis and slight hyperkeratosis. In both loadings, fibrosis score was severe and occurs a tendency to scar-like lesions, mainly in immediate loading. Subacute inflammation with slight to moderate density of identical chronic cell populations was common to both loading protocols. A significant correlation in both cases was found between the lymphocytes score and density of chronic inflammatory cells. However, higher scores of plasma cells were encountered in immediate loading. Even though clinically, there were no signs of inflammation in peri-implant mucosa in few cases neutrophils were also recorded regardless the timing of implants occlusal loading.

Conflict of interests

The authors declare that they do not have any conflict of interests.

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Corresponding author
Vasile Nicolae, Associate Professor, DMD, PhD, Department of Dental Medicine, "Victor Papilian" Faculty of Medicine, "Lucian Blaga" University of Sibiu, 2A Lucian Blaga Street, 550169 Sibiu, Romania; Phone +40269–212 320, e-mail: dento.medica@yahoo.com

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