Collagen Quantification in Peri-implant Soft Tissues in Human Peri-Implantitis Lesions

Cuantificación de Colágeno en Tejidos Blandos Periimplantarios en Lesiones de Periimplantitis en Humanos

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SUMMARY: Peri-implantitis is an inflammatory lesion of bacterial etiology characterized by inflammation of the mucosa and bone loss. Chronic inflammation is characterized by neovascularization and collagen neoformation. Mast cells have been shown to participate in the inflammatory process by releasing mediators and proteases such as chymase and tryptase, important in the collagen neoformation process. Although a higher percentage of collagen has been described in periodontal disease, the literature is scarce about the percentage of collagen in peri-implantitis. The aim of this study was to quantify the percentage of collagen fibers present in the peri-implant soft tissue of patients with peri-implantitis lesions. A descriptive observational cross-sectional study was performed. Samples of peri-implant soft tissue were collected from eleven patients with peri-implantitis and then processed by Masson's Trichrome Technique. In microscopic analysis, collagen fibers were observed in all samples, with an average percentage of 39.89 %, standard deviation of 17.02 %, with a minimum value of 8.99 % and a maximum value of 75.65 % density. From these results, it can be concluded that in human peri-implantitis lesions with bone loss greater than 50 %, there is an important percentage of collagen fibers, which is interpreted as connective tissue in a permanent process of reparative response, in the presence of inflammatory infiltrate.

KEY WORDS: Peri-implantitis; Inflammation; Collagen; Masson's Trichrome Technique.

INTRODUCTION

Peri-implantitis (PI) is defined as the pathology associated with bacterial plaque, in the tissues around dental implants, characterized by inflammation in the peri-implant mucosa and the progressive loss of supporting bone (Berglundh *et al*., 2018). Peri-implantitis is one of the leading causes of failure and loss of dental implants. Its prevalence at the subject level is 20 % and at the implant level is 9.25 % (Lee *et al*., 2017).

Peri-implantitis, as a chronic inflammatory lesion, is characterized by neovascularization and neoformation of collagen. Lymphocytes and plasma cells are the most frequent inflammatory cells in peri-implantitis lesions. It has been possible to show through immunohistochemical studies that peri-implantitis lesions contain significantly higher proportions of B cells and elastase positive cells than mucositis lesions, which suggests that they exhibit properties different from mucositis but similar to periodontitis lesions (Gualini & Berglundh, 2003).

Mast cells have been shown to participate in the inflammatory process by releasing mediators and proteases such as chymase and tryptase, which are important in the collagen neoformation process (Zizzi *et al*., 2011). However, although a higher percentage of collagen has been described in periodontal disease, there is scarce literature that describes the percentage of collagen in peri-implantitis.

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Knowing the histopathology of the peri-implant soft tissues in peri-implantitis lesions helps to understand the pathogenesis of the inflammatory process and its consecutive fibrous reaction, in order to contribute to new therapies that prevent the early loss of the dental implant and its associated consequences.

The aim of this study is to quantify the percentage of collagen fibers present in the peri-implant soft tissue in human peri-implantitis lesions.

MATERIAL AND METHOD

A descriptive study was conducted. Eleven peri-implant soft tissue samples were obtained from the same number of patients with peri-implantitis (six men and five women, 33 to 79 years old, function time of implants from five to sixty months). The participants signed an informed consent during the visit to the implant clinic of the Faculty of Dentistry, Universidad de La Frontera, Temuco, Chile and Universidad de Concepción, Chile. The study was conducted in accordance with the principles outlined in the Declaration of Helsinky on experimentation with human participants. Ethical approval for the study was granted by the Ethics Committee of the Universidad de La Frontera, Report N ° 024_2018. After written informed consent, the biopsies were submitted to histopathological analysis at the Oral Pathology Laboratory of Universidad de Talca. The parameters to determine the diagnosis of peri-implantitis were based on the classification proposed by Working Group 4 of the 2017 Global Workshop on the Classification of Peri-implant Diseases and Conditions (Berglundh *et al*., 2018).
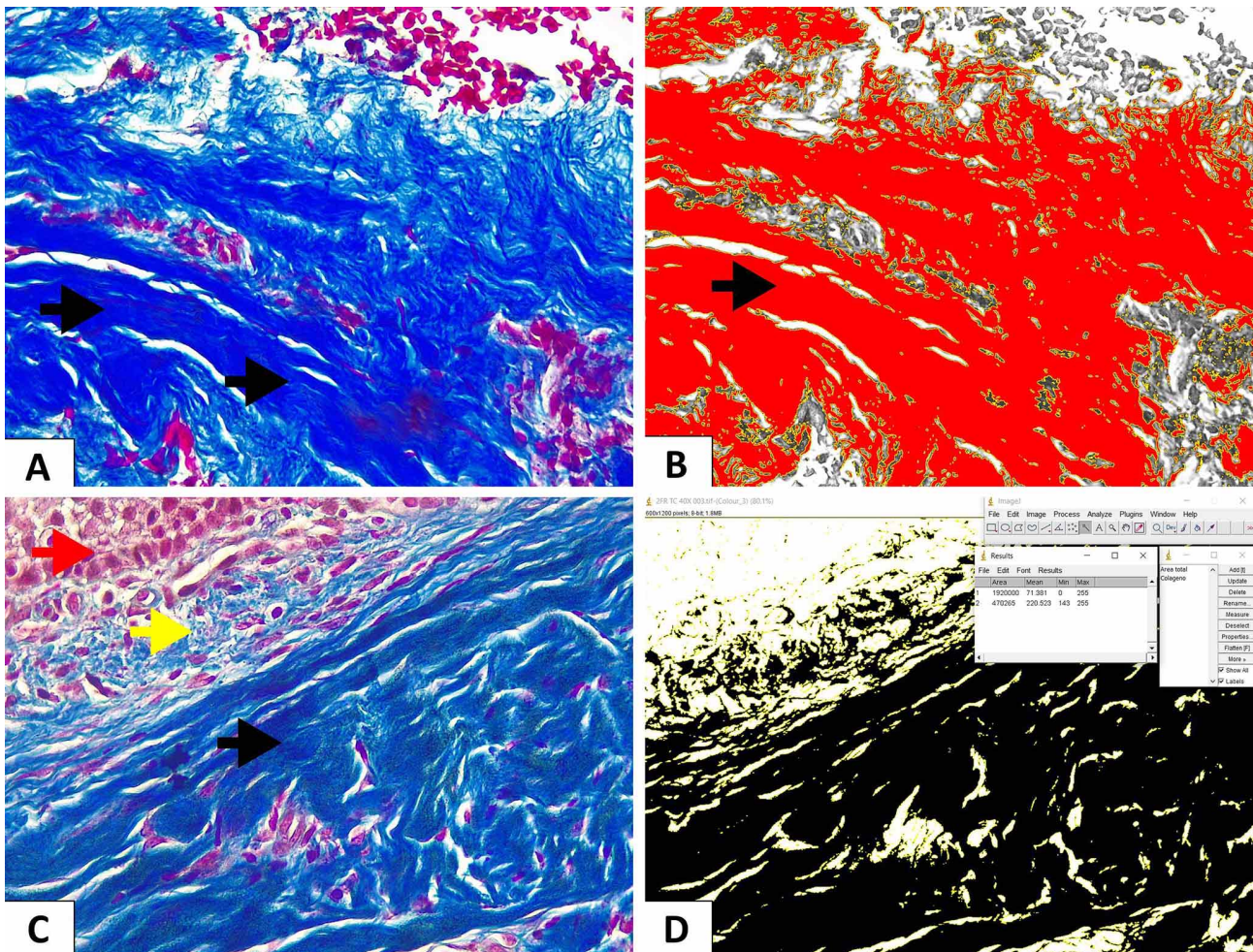

Fig. 1. A. Shows connective tissue in blue (Masson's trichrome technique 40x). Collagen fibers are marked with black arrows. B. Shows the application of color deconvolution in Image J software to visualize selected collagen fibers (Red Option).C. Red arrow: Squamos epithelium; Yellow arrow: scarce inflammatory cells in loose fibrous connective tissue; Black arrow: dense fibrous connective tissue (Masson's trichrome technique 40x). D. Shows the selected areas to be quantified throw application of color deconvolution in Image J software (B&W option) and summary table with the area results in square pixels.

Inclusion criteria considered patients with osseointegrated implants with a function time equal to or greater than five months, with a diagnosis of peri-implantitis with bone loss greater than 50 % and significant aesthetic compromise. The exclusion criteria were as follows: Children, adolescents, patients with autoimmune diseases, and pregnant or lactating women.

Surgical Procedure. The soft tissue biopsies were obtained during the surgical peri-implantitis treatment. The biopsies dimensions were approximately 2x3 mm. Each biopsy was immediately placed in 10 % buffered formalin for histological analysis.

Histological processing and analysis. All samples were processed by Masson's trichromic technique (Fig. 1). From the samples, 4 µm histological sections were obtained which were deparaffinized and hydrated in distilled water. First, the samples were fixed in formaldehyde solution and then an assembly was carried out with Bouin's liquid for 15 minutes at 56-60 ºC, cooled and washed in distilled water to remove the yellow color. Weigert´s Iron hematoxylin staining was performed for 10 minutes and washed in distilled water for 5 minutes. Dyeing with acid scarlet fuchsin solution was carried out for 5 minutes and washed with distilled water. A phosphomolybdic-phosphotungstic acid solution treatment followed for 5 minutes and then staining was carried out with aniline blue solution for 5 minutes. The slides were then treated with 1 % acetic acid for 2 minutes. After that, sections were washed in distilled water, dehydrated, rinsed and mounted on a slide.

The reading, interpretation of the results, and the capture of images were carried out under optical microscopy at 40X magnification with a camera (Canon EOS Rebel XSI, Tokyo, Japan).

Quantification of collagen fibers. Five microscopic fields per each sample were analyzed. The quantification of collagen fibers was carried out using the Image J software (version 1.46j; National Institute of Health, USA) and the color deconvolution application. The image was decomposed into 3 colors and a representative color area was selected to be quantified. Once the imagen values are adjusted, the "Red" option is changed to "B&W" option, and areas of interest areselected. Finally, the system delivers a summary table with the area results in square pixels, which will later allow to calculate the percentage of collagen. Collagen quantification was expressed as a percentage of the total area evaluated. The data were grouped to represent a mean value and standard deviation, determining minimum and maximum values.

RESULTS

Clinical Characteristics. Eleven peri-implant soft tissue samples from eleven patients diagnosed with peri-implantitis were analyzed. The mean age of the patients was 59 years, with a range of 33 to 79 years. Five patients were women (55 %) and six men (45 %). Only two patients were smokers (less than 10 cigarettes a day) and five suffered from related chronic diseases, four were undergoing treatment for arterial hypertension (HA) and one patient for HA and Diabetes Mellitus (MD). Six patients did not report any chronic disease. The functional time of implant (FTI) ranged from five to sixty months.

Histological Results. The lesions that were included in biopsies had similar histopathological characteristics to each other. In all peri-implant soft tissue samples with peri-implantitis lesions, collagen fibers were observed. The composition of the samples was not uniform with respect to collagen quantity. The values were measured in percentage with a minimum value of 8.99 %, and a maximum value of 75.65 % of the total area evaluated, with an average of 39.89 % and a standard deviation of 17.02 % (Tables I and II).

Table I. Percentage value of collagen fibers per sample.

n=	Minimum Value (%)	Maximum Value (%)	Average (%)
1	19,57	49,24	33,53
2	26,96	33,45	32,43
3	11,31	35,99	30,38
4	10,49	65,67	53,82
5	11,67	27,18	23,07
6	33,29	69,91	56,91
7	28,75	63,90	41,78
8	24,62	59,81	41,63
9	31,99	75,65	59,15
10	16,15	51,22	39,27
11	8,99	34,46	26,78

Table II. Percentage value of collagen fibers of the total samples.

Mínimum Value (%)	Maximum Value (%)	Total Average (%)	Standard Deviation (%)
8,99	75,65	39,89	17,02

DISCUSSION

Connective tissue constitutes a fundamental part in every inflammatory disease. It is the place where cellular and vascular structures interact between them and induce the healing process, which is the final purpose of any inflammatory course. Therefore, determination of the amount

of this tissue becomes of great importance in terms of associating the histological structure with the dynamics of the pathogenesis of disease.

In chronic peri-implantitis, few reports on human studies have been found in the literature. Most of the research is performed in animal studies using models with ligation-induced peri-implantitis which represents an acute process (Lang *et al*., 1993; Comut *et al*., 2001; Martins *et al*., 2005; Berglundh *et al*., 2007). Considering that peri-implantitis is a chronic inflammatory disease, these studies could not be comparable due to the differences in the pathogenesis between an acute and a chronic process. Although inflammatory process involves similar mechanisms, type of cells in one or another are not the same, and different molecular interactions occur. Finally, considering a clinical point of view, conclusions obtained in an experimental model cannot always be extrapolated to a human process. Our study was based on the histological analysis of the amount of collagen tissue directly on human soft tissue from peri-implant lesions, which allows a possible application in the clinical field.

There is scarce literature that evidences the percentage of collagen fibers in peri-implant soft tissue in human peri-implantitis. The composition of peri-implant soft tissue in healthy implants has been described, comparing the orientation of the collagen fibers with the natural dentition, analyzing the adaptive process in relation to the implant (Sculean *et al*., 2014; Ivanovski & Lee, 2018). In the present study, it was possible to demonstrate that the average quantity of collagen fibers in peri-implantitis lesions is around 40 % of the total area evaluated, which is considered as an important percentage. When comparing the quantification of collagen fibers in other similar studies, some differences in this figure were found.

In a descriptive observational study, eighteen implants were analyzed histologically; nine patients in the group with peri-implantitis (PP) and nine in the group with healthy peri-implant tissues (PH). This study shows that the PH group had a higher percentage of collagen fibers (28 %) compared to the PP group (20 %), with a significant difference. There was also a significant negative correlation between the density of IL-17 and the percentage of collagen, indicating that a higher secretion of this interleukin decreases the percentage of collagen fibers (de Araújo *et al*., 2017). The amount of collagen tissue described in this study differ from results found in our investigation, in which we registered a mean total percentage of 39,89 %. A possible explanation for this difference could be a greater healing reaction, in which the inflammatory cells are a stimuli for fibroblasts to secrete more extracellular matrix components.

According to a study carried out by Berglundh *et al*. (2004), in which soft tissue biopsies from six patients with peri-implantitis were analyzed, it was shown that the composition of the connective tissue was not uniform with respect to the densities of collagen, vascular and inflammatory structures in different compartments of the injury. The proportions of collagen and vascularization were 3.6 % and 3.5 %, respectively.

In an immunohistochemical study that shows the pattern of collagen distribution in healthy and peri-implant mucosa, an important increase in collagen V was shown in peri-implantitis (Borsani *et al*., 2005). The authors propose that this marked increase in collagen V could influence the homeostasis of gingival stroma and could permit greater bacterial penetration. Some authors refer to other investigation in which it is mentioned that Collagen type V is resistant to collagenase digestion (Liotta *et al*., 1979) and is found localized in inflamed gingival tissues. In our study, the collagen found could be type V collagen, which might be associated to the inflammatory process found, but more investigation needs to be performed.

It has been reported that when there is a permanent association between pathogenic molecules, host tissue destruction, mediators, and receptors in the context of a chronic inflammatory process develops in peri-implant tissues, as well as in periodontal disease. It has been reported that the extent of inflammation in the cellular infiltrate seemed to be more pronounced in peri-implantitis, where it can spread to the bone marrow (Heitz-Mayfield & Lang, 2010), compared to that reported in periodontitis where the disease was well contained within the compartment. This can be attributed to structural differences between the periodontal mucosa and the peri-implant soft tissue.

The stain used in this study was Masson's Trichrome, which is a common technique that distinguishes 3 colors and allows collagen fibers to be clearly visible (Pujari *et al*. 2013). Numerous studies have used Masson's Trichrome stain to identify the collagen fibers in different diseases (Cáceres *et al*., 2017; Ying *et al*. 2017). However, since this procedure could cause a considerable number of errors, it has been complemented with the use of Image J software method to quantify collagen fibers using ImageJ with its color deconvolution plugin for a more exact quantification of collagen fibers.

This investigation is a descriptive design, therefore it only refers to the amount of collagen in peri-implantitis disease as a preliminary study. The main limitation of this research is the number of samples and the lack of a control group. Increasing the number of samples and comparing the

quantification of collagen fibers with a control group would be recommended in order to obtain more significant information about the pathogenesis of peri-implantitis. In the present study, an important percentage of collagen fibers is reported; this is interpreted as connective tissue in a permanent process of reparative response, in the presence of an inflammatory infiltrate. This phenomenon should be investigated in depth in future studies with a larger sample size, however, it serves as a preliminary result for future research.

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RESUMEN: La periimplantitis es una lesión inflamatoria de etiología bacteriana caracterizada por inflamación de la mucosa y pérdida ósea. La inflamación crónica se caracteriza por neovascularización y neoformación de colágeno. Se ha demostrado que los mastocitos participan en el proceso inflamatorio liberando mediadores y proteasas como quimasa y triptasa, importantes en el proceso de neoformación del colágeno. Aunque se ha descrito un mayor porcentaje de colágeno en la enfermedad periodontal, la literatura sobre el porcentaje de colágeno en la periimplantitis es escasa. El objetivo de este estudio fue cuantificar el porcentaje de fibras de colágeno presentes en el tejido blando periimplantario de pacientes con lesiones de periimplantitis. Se realizó un estudio observacional descriptivo transversal. Se recogieron muestras de tejido blando periimplantario de once pacientes con periimplantitis y luego se procesaron mediante la técnica tricrómica de Masson. En el análisis microscópico, se observaron fibras de colágeno en todas las muestras, con un porcentaje promedio de 39,89 %, desviación estándar de 17,02 %, con un valor mínimo de 8,99 % y un valor máximo de 75,65 % de densidad. De estos resultados se puede concluir que en las lesiones de periimplantitis humana con pérdida ósea superior al 50 %, existe un porcentaje importante de fibras de colágeno, que se interpreta como tejido conectivo en un proceso permanente de respuesta reparadora, en presencia de infiltrado inflamatorio.

PALABRAS CLAVE: Periimplantitis; Inflamación; Colágeno; Tricrómico de Masson.

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