

Comparative methodology for histological analysis of peri-implant soft tissue samples.

Metodología comparativa para el análisis histológico de muestras de tejidos blandos periimplantarios.

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INTRODUCTION.

Conventional histopathology analysis is still considered to be a helpful tool for diagnosis and studies in biomedical research, although modern methods like gene cloning and sequencing or expression analysis are also strongly being used. In the diagnosis field of healthcare, every biopsy taken from a patient requires as first analysis, a conventional histological examination which, in approximately 80% of the cases is the only procedure performed to obtain a proper diagnosis and guide posterior treatment procedures. In the research field, histopathology is also being used to analyze pathological processes like neoplasms and inflammatory diseases.

This article aims to present and discuss a comparative methodology for the histopathological analysis used to investigate cellular and tissue characteristics of soft tissue samples obtained from the gingiva of patients diagnosed with peri-implantitis, a frequent disease associated to an impaired evolution of dental implants.

METHODOLOGY

A histological comparative analysis was applied on six gingival samples from patients with a diagnosis of severe peri-implantitis, processed with conventional histological techniques. Samples were fixed with buffered 10% formalin, paraffin embedded and stained with hematoxylin-eosin (HE), Giemsa, picrosirius and AgNOR histochemical stains. Also, immunohistochemical analysis with anti-smooth muscle actin antibody (α SMA) (Novocastra™ Liquid Mouse Monoclonal Antibody Alpha Smooth Muscle Actin, Product Code: NCL-L-SMA) was implemented to analyze blood vessels. Microscopic analysis was performed with a Leica DM 500 microscope, equipped with a Leica ICC50 W camera. Five microscopic fields were randomly selected from the samples and photomicrographs were analyzed using Image J 1.52 software. Four cellular and tissue characteristics were chosen for analysis:

- 1) Presence of blood vessels, considering absolute number and percentage of vascularization,
 - 2) Presence of inflammatory infiltrate, registering the absolute number of cells and the percentage of tissue occupied by these cells,
 - 3) Connective tissue, considering the percentage of fibrous versus cellular tissue and proportion between collagen type I-III,
 - 4) Number of AgNORs per cell present in each microscopic field.
- Histological structures used for the analysis are shown in Figure 1. Number

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and percentages of histologic structures were counted with Image J 1.52 software.

Percentages were evaluated considering the number of small squares of the grid provided by the Image J software. Seventy small squares were considered as 100% in each microscopic field. Data were registered in Microsoft Excel spreadsheets, considering absolute numbers and percentages for each microscopic field. Mean values were calculated for each sample. Two stains or methods were used for the analysis of each of the histological characteristic and a comparative qualitative analysis was performed using trend lines from Microsoft Excel generated graphs.

RESULTS .

Blood Vessels

Absolute number of blood vessels (MVD) and percentages of vascularization (PV) were counted from slides stained with HE and immunohistochemistry for α SMA. Variable results in mean values were found when count was performed on HE slides, ranging from 5.2 to 32 blood vessels in the six samples analyzed.

The same count performed on α SMA stained immunohistochemistry slides ranged from 2.6 to 11.4. PV showed ranges from 7 to 16.8, and 18.2 to 2.6 % when counted on HE and α SMA slides respectively. Trend lines in Microsoft Excel graphs showed a clear absence of parallelism, which was interpreted as a dissimilarity of most counts. A less dissimilarity trend

was found in the comparative analysis between PV results from HE and α SMA slides.

Inflammatory infiltrate

Number of lymphocytes and plasma cells and percentages of the microscopic field occupied by these cells was counted on HE and Giemsa stained slides. Mean values for number of inflammatory cells counted on HE stained samples ranged from 7.8 to 95.8, and ranged from 19.8 to 232 on Giemsa slides.

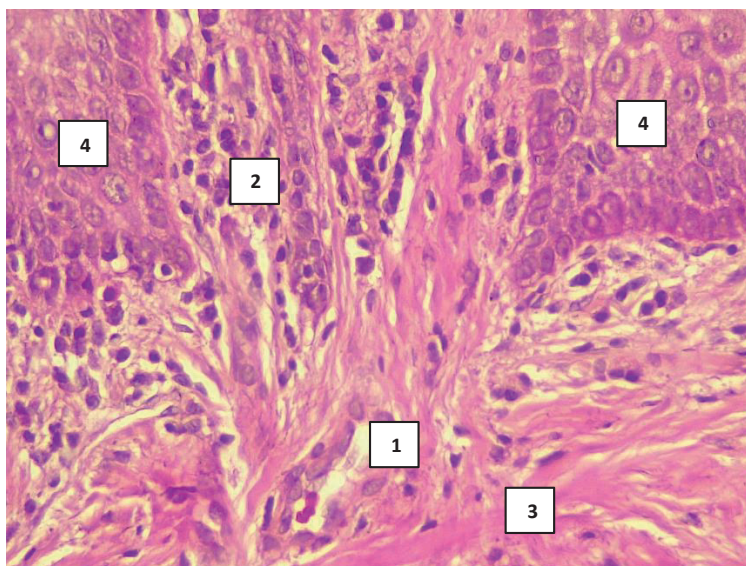
Percentages for HE slides ranged from 4.2 to 52.6 %. A gross dissimilarity was found between the number of inflammatory cells counted with HE and Giemsa, but a less dissimilarity trend was found in comparative analysis between number and percentage of cells counted with HE.

Connective tissue

The percentage of fibrous and cellular connective tissue was analyzed with HE stain. Also, the percentage of collagen type I and III was calculated with a method for picrosirius red-polarization detection of collagen fibers in tissue sections and color deconvolution method for automatic count of pixels.

Mean values for fibrous and cellular connective tissue ranged from 55.8 to 87.4 and 12.6 to 46.4% respectively. Mean values for collagen type I and III ranged from 74.8 to 86.6 and 13.4 to 25.2%, respectively. Proportion of collagen I-III ranged from 3.43 to 10.62. Trend lines from Microsoft Excel graphs also showed absence of parallelism but clear mirror-like curves.

Figure 1. Image showing histopathological structures used for comparative histological analysis.



1: Blood Vessel. 2: Inflammatory cells. 3: Connective tissue. 4: Squamous epithelia. (HE, 40x magnification.)

AgNORs

The proliferation state of squamous cell epithelia was analyzed through the number of AgNORs present in cellular nuclei and its proportion by cell.

AgNOR count in 20 cells showed mean values from 34.8 to 134 and proportion of AgNORs per cell from 1.74 to 6.72. No tendency analysis was performed.

DISCUSSION.

A comparative qualitative analysis of counts of histological structures performed with different stains and methods was conducted. Number of blood vessels analysis is widely used to establish the microvessel density (MVD), which is useful as a prognostic factor in neoplasms.¹ Angiogenesis is also a prominent feature in inflammatory diseases.

In this article, number of blood vessels counted with HE and α SMA immunohistochemistry were compared, finding little similarity between both counts, which implies that use of both methods to analyze a soft tissue sample is not recommended. More similarity in data was observed when the number of blood vessels was compared with percentage of vascularization as analyzed with α SMA immunohistochemistry.

Percentage of vascularization was tested as a method due to the different size of blood vessels counted in MVD analysis, which could imply more tissue irrigated by that blood vessel, not being considered as a biological fact in the absolute number count. This result could be of interest when future analyses are performed to investigate prognosis of disease, although more studies are needed to support this statement.

A similar analysis was done between HE and Giemsa inflammatory cell counts. Giemsa stain was used in this analysis due to the easy observation of inflammatory cells. Nevertheless, a great difference was found when HE analysis was considered. New studies need to be carried out in order to establish a clear conclusion in this regard.

Connective tissue analysis showed clear differences between fibrous *versus* cellular tissue and between collagen type I and III. This last method is described in the literature² and has been used to analyze these structures as markers of the degree of collagen sclerosis in cardiac disease.³ In this work, the proportion between collagen type I and III and between fibrous over cellular connective tissue are in accordance with

what is expected and might be associated with a healing reaction of the connective tissue in the presence of a chronic inflammatory process.

Regarding the number of AgNORs per cell, which has also been analyzed as a probable prognostic factor in neoplasias,⁴ in the present work we found counts similar to those observed in dysplastic epithelia.⁵

CONCLUSION.

Comparative methodologies for histopathological analysis to investigate cellular and tissue characteristics of soft tissue samples is useful to discriminate the best methods of analysis with the aim of providing relevant clinical information.

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