ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Morphological and quantitative study of collagen fibers in healthy and diseased human gingival tissues

TATIANE ALMEIDA[1)], THALITA VALVERDE[1)], PAULO MARTINS-JÚNIOR[1)], HEDER RIBEIRO[2)], GREGORY KITTEN[1)], LORENZA CARVALHAES[1)]

[1)]*Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil*
[2)]*Department of Basic Science – Health Area, Federal University of Juiz de Fora, Governador Valadares, MG, Brazil*

Abstract

Objectives: This study aimed to evaluate types I, III and IV collagen in healthy gingival tissue and to compare them to gingival tissues suffering from chronic gingivitis and chronic periodontitis. *Materials and Methods*: Thirty-two man patients were selected. The patients belonged to three diagnostic categories: healthy gingiva (HG), chronic gingivitis (CG) and chronic periodontitis (CP), based on clinical and radiographical criteria. Gingival tissue samples were obtained from patients who underwent periodontal surgery procedures. Hematoxylin and Eosin (HE), Picrosirius red, indirect immunofluorescence by confocal microscopy and quantitative analyses were performed to identify the presence and location of types I, III and IV collagen. Statistical significance was verified using the Kruskal–Wallis test. *Results*: Samples from HG group showed thick collagen fibers arranged in a parallel pattern. Samples from CG group showed dilated blood vessels; collagen fibers and inflammatory cells were found dispersed throughout the tissue. Samples from CP group showed the extracellular matrix severely damaged, disorganized collagen fibers and large amount of inflammatory cells. The HG group showed an apparent higher expression of type I collagen, when compared to tissues with CG and CP, however no statistical differences were detected (p=0.064). The types III and IV collagen fibers showed no difference in expression in tissues with gingivitis and periodontitis. *Conclusions*: Following the periodontal disease there was a morphological destruction of the extracellular matrix with lower expression of collagen, which led to a change in tissue architecture that might compromise its functional capacity. There were differences in type I collagen expression among healthy, chronic gingivitis and chronic periodontitis tissue samples.

Keywords: periodontal disease, collagen, histopathology, indirect immunofluorescence.

Introduction

Periodontal disease is a general term to describe an inflammatory disorder initiated and maintained by plaque bacteria and their metabolic products, characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction [1–4]. It is recognized as one of the most prevalent chronic disease in humans, affecting a high proportion of adult population in Brazil [4, 5].

Because of its high prevalence and implications for dentistry, periodontal disease has been indicated as a worldwide public health problem [6]. In this context, several researches have tried to comprehend the biological process resulting in loss of the supporting tissues [4, 7, 8]. Bacteria secret products, such as metalloproteinases, that led to a direct destruction of the periodontal tissues. In response, various types of gingival cells that function as resident or inflammatory cells release several molecules, such as cytokines and proteases, destroying the supporting structures [1, 4, 9–12]. These unbalanced release of bacterial and host substances results in degradation of extracellular matrix [4].

The collagen is the major extracellular matrix component of gingiva [11, 13] and plays a key role in its architecture [1]. It acquires a significant role in disease progression [1, 4, 14], as collagen degradation is pointed as the main marker of periodontal disease progression [4]. The presence of some collagen fibers such as types I, IV and VI has already been observed in healthy and diseased gingival tissue by western blotting [15] and by immunohistochemistry in hyperplasic gingival tissues [16]. However, to our knowledge, there is a lack of quantitative study relating location and organization of those molecules within healthy and diseased human gingival tissues. The better understanding of the molecular bonds and mechanisms of action of these substances can help and guide dentists in building strategies for early diagnosis and more effective treatment of periodontal disease in the future.

Therefore, the aim of the present study was to evaluate types I, III and IV collagen in healthy gingival tissue and to compare them to gingival tissue suffering from chronic gingivitis and chronic periodontitis.

Materials and Methods

Ethical issues

This study was carried out in compliance with international statutes and national legislation on ethics in research involving human subjects. All subjects consented to participation and signed a term of informed consent. The study received approval from the Ethics Committee of the Federal University of Minas Gerais (UFMG) (process number: ETIC 046/01) and from the Ethics Committee of the Itaúna University (UIT) (process number: ETIC 021/06), Brazil. The study was conducted

in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Study population

Gingival tissue samples were obtained from 32 patients who underwent periodontal surgery procedures in dental clinics of the Itaúna University. The samples were obtained from buccal marginal tissues with the aid of a bistoury. During surgery, local anesthesia was done avoiding anesthetic infiltration into the tissue and deformation or compression of the samples. All patients were males, white-skinned, and aged between 35–50-year-old. The patients had no history of systemic disease, smoking, were not undergoing a treatment with antibiotic or any other medicine or a specific periodontal treatment within six months before the study (Table 1).

Table 1 – ***Distribution of the patients according to their periodontal status***

Groups	No. of samples
Healthy gingiva (HG)	12
Chronic gingivitis (CG)	10
Chronic periodontitis (CP)	10

The patients under investigation belonged to three diagnostic categories: healthy gingiva (HG) (n=12), chronic gingivitis (CG) (n=10) and chronic periodontitis (CP) (n=10). These diagnoses were based on clinical and radiographic criteria, which included color, bleeding, probing depth and radiographic evidence of bone resorption, as previously described [17]. HG involved samples with healthy gingiva without bleeding and no evidence of bone resorption or periodontal pocketing. CG involved samples with red color, swelling of the gingival margin, and bleeding without bone resorption or periodontal pocketing. CP involved samples of patients with more than one periodontal pocketing ≥5 mm and at least one pocket with ≥4 mm attachment loss.

Tissue preparation

Immediately after surgery, tissues were fixed at -20^{0}C in methanol 80%/DMSO for seven days. Afterward, the samples were dehydrated with gradually increasing alcohol concentrations, diaphonized in xylene, and embedded in paraplast.

Histopathology

Serial 5-µm sections were obtained and stained with Hematoxylin and Eosin (HE) for descriptive analysis. Collagen fibers were stained with Picrosirius red [18, 19] to perform a qualitative analysis of the disposition and composition of collagen fibers that constitute the basal membrane and extracellular matrix of the tissues. The histological sections were observed in an Olympus BX51 (Tokyo, Japan) with a filter to polarized light.

Indirect immunofluorescence staining and microscopy

An indirect immunofluorescence of the sections was performed to evaluate qualitatively the collagen fibers. Sections (5 µm thick) of all samples were mounted on gelatin-coated slides. Sections were incubated with each primary antibodies overnight in a humidity chamber at 4^{0}C. Cy3-conjugated goat anti-rat immunoglobulin (IgG) absorbed with human Ig (The Jackson Laboratory, Bar Harbor, Maine, USA) was used as a secondary antibody (Table 2).

Table 2 – ***Specification of the antibodies used in indirect immunofluorescence***

Antibody	Specification	Dilution	Manufacturer
Primary	COLI polyclonal rabbit anti-human collagen I	1:300	Rockland
	COLIIII polyclonal rabbit anti-human collagen III	1:300	Rockland
	COLIV polyclonal rabbit anti-human collagen IV	1:300	Rockland
Secondary	Polyclonal, goat anti (IgG rat) conjugated Cy3	1:400	Jackson Laboratory
	Polyclonal, goat anti (IgG rat) conjugated Cy5	1:400	Jackson Laboratory

The antibodies were diluted in phosphate-buffered saline (PBS), pH 7.2, with 0.1% bovine serum albumin (BSA). After 4×5 minutes washes in PBS, the conjugate was applied for one hour at 4^{0}C. The sections were again washed in PBS (4×5 minutes) and mounted in glycerol containing *p*-phenylene-diamine. An indirect immuno-fluorescence of the section was performed in a confocal microscopy (Zeiss LSM 510 Meta linked to Zeiss Axiovert 200M) (Carl Zeiss, Germany). The staining pattern with each polyclonal antibody was recorded as grades from negative (-) to strongly positive (+++) in each section. A control reaction substituting the primary antibody with 0.1% BSA in PBS was done. Fibronectin was used as a positive control (data not shown).

The images obtained through this technique were processed using the tool Thermo from the software Confocal Assistant® (Todd Clark Brelje, University of Minnesota) for obtaining Pseudo Color. This tool blushed in shades of red to black the higher concentration to lower expression of the fiber marked in accordance with the thermal bar that will be shown on the right side of the figures in results section.

Quantitative analyses of types I, III and IV collagen

To assess if there were statistical differences between the above fibers in evaluated degrees of periodontal disease, their intensity of fluorescence was measured with the aid of the software UTHSCSA ImageTool 2.0. Upon completion of the immunofluorescence for each fibber, six microscope fields were collected from each sample in confocal microscope utilizing 40× objective. For quantitative analysis of types I and III collagen, random regions (under epithelial-papillae *versus* in-depth tissue) were selected. The fluorescence intensity of the connective tissue in those regions was measured, comparing the tissues with different degrees of periodontal disease and healthy tissues (Figure 1, a–c).

For quantitative analyses of type IV collagen, regions of basal membrane were selected. In order to avoid incorrect analyses of the background of the images, the regions without molecular expression of type IV collagen were also selected and quantified. The images were analyzed in grayscale using the software UTHSCSA

ImageTool 2.0. The fluorescence intensity is supplied through the unit "gray level", which ranges from zero (black) to 255 (white) values. The closer to the value to 255, the greater the fluorescence intensity is (Figure 1c).

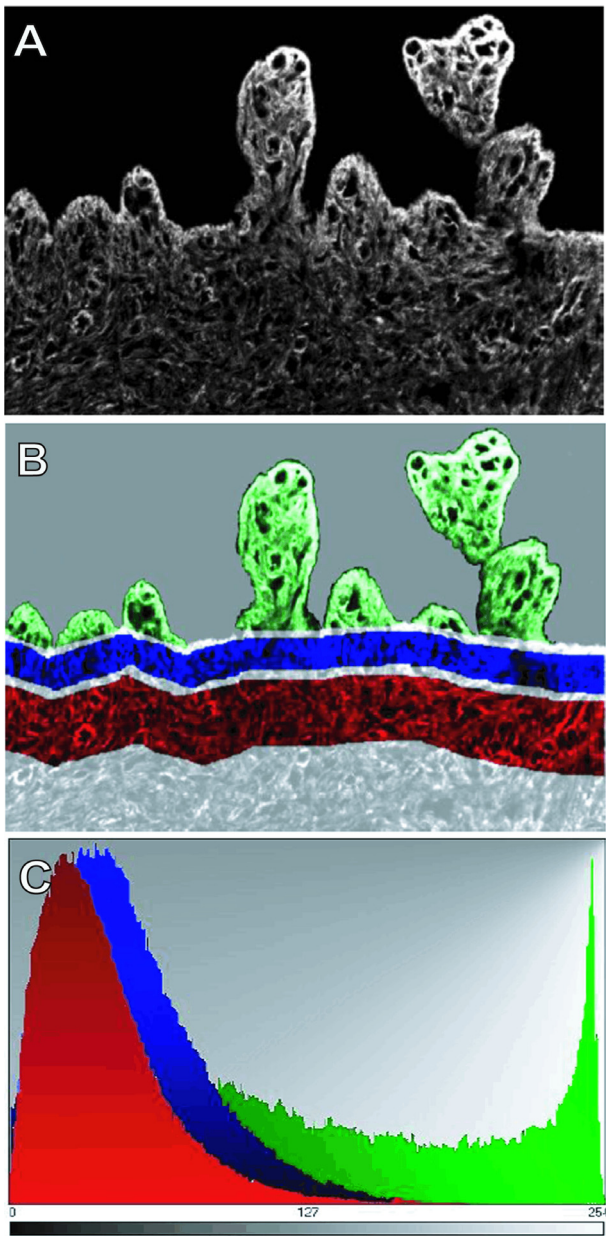

Figure 1 – ***Quantitative analysis using the Image Tool software. The photographs of the samples were obtained from confocal microscopy (A). In the Image Tool, was made a randomly selection from various parts of each photo (B). The program provides a chart with values and number of pixels. Negative values of total protein expression (black, value 0) or high protein expression (white total value 255) (C).***

Statistical analysis

Three researchers in a blind method did the recording and the analysis of all images, and they were unaware of which experimental group each image belonged. Given the reduced number of patients in our study, statistical significance was verified with a non-parametric Kruskal–Wallis test, as implemented by the software GraphPad Prism® 5. In particular, results with $p<0.05$ were considered statistically significant.

Results

Histopathology

HE staining

HE staining was used to classify the epithelium and connective tissue in samples. The epithelium was stratified, squamous and non-keratinized. Connective tissue was classified as loose in regions placed adjacent to epithelium, containing connective papillae. In deeper areas, the tissue was classified as disorganized dense connective presenting thick collagen fibers. The papillae were showed up cut transverse, oblique or longitudinal (Figure 2a).

Tissue samples from HG group showed thick collagen fibers arranged in a parallel pattern, containing fibroblasts. Inflammatory cells in a diffuse form were seen between collagen fibers (Figure 2b). However, these cells were located more frequently near the blood vessels. Papillae could be visualized containing small-caliber blood vessels or even capillaries, proving to be quite vascularized.

Tissue samples from CG group (Figures 2c and 1d) showed dilated blood vessels; collagen fibers and inflammatory cells were found dispersed throughout the tissue. On the other hand, tissue samples from CP group (Figures 2e and 1f) showed a disruption of the extracellular matrix organization, with modified patterns of collagen fibers disposition and large amount of inflammatory cells scattered throughout the tissue, characterizing a chronic inflammation of the tissue.

Picrosirius red

This technique allowed an optimal visualization of collagen fibers and their organization within the extracellular matrix. The HG group showed thick collagen fibers, parallel with some inflammatory cells arranged between these fibers in connective tissue (Figure 3, a and b).

Tissue samples with chronic gingivitis (CG) showed bundles of fibers surrounding inflammatory cells (Figure 3c) tending to restrict such cells in specific areas of connective tissue. At polarization microscopy (Olympus B513, Olympus, Tokyo, Japan), it was observed that the extracellular matrix also appeared similar to that seen in healthy gingiva (HG) (Figure 3d), and the number of inflammatory cells tended to be higher. However, no quantification of these cells was performed in these samples.

The tissue samples from CP group had high destruction of the extracellular matrix together with the presence of inflammatory cells scattered throughout the tissue (Figure 3e). At polarization microscopy, it was observed a small amount of bundles of collagen in the deep region of the tissue (Figure 3f). This result demonstrates the high degradation of collagen fibers during the course of periodontal disease.

Immunofluorescence

Type I collagen

The type I collagen fibers in HG samples exhibited the following pattern: the fibers were thick and arranged in a parallel fascicular pattern showing intense staining. At CG samples, those fibers were still distributed in a similar arrangement of HG samples, but there was less intensity of immunofluorescence staining and the fibers were thinner. At the CP samples, the type I collagen fibers were disperse, evidencing the extracellular matrix des-

truction. Those patterns were observed at under epithelial-papillae and in-depth connective tissue (Figure 4, a–c).

The HG group showed an apparent higher expression of type I collagen, when compared to tissues from CG and CP groups, however no statistical differences were detected (p=0.064). Tissues with chronic gingivitis (CG) showed no significant differences in expression of type I collagen when compared to tissues with chronic periodontitis (CP) (p>0.050) (Figure 4d).

Type III collagen

The type III collagen fibers in HG samples followed a reticular pattern and were well distributed around all regions of connective tissue, with integrity and normal intensity. The type III collagen fibers in CG and CP samples demonstrated a dispersed pattern, without continuity and less intensity at both epithelial-papillae and depth connective tissue (Figure 5, a–c).

No significant differences were found between groups to type III collagen (p=0.217). However, tissue samples from HG group showed an apparent higher concentration of type III collagen in connective tissue when compared to diseased tissue samples (Figure 5d).

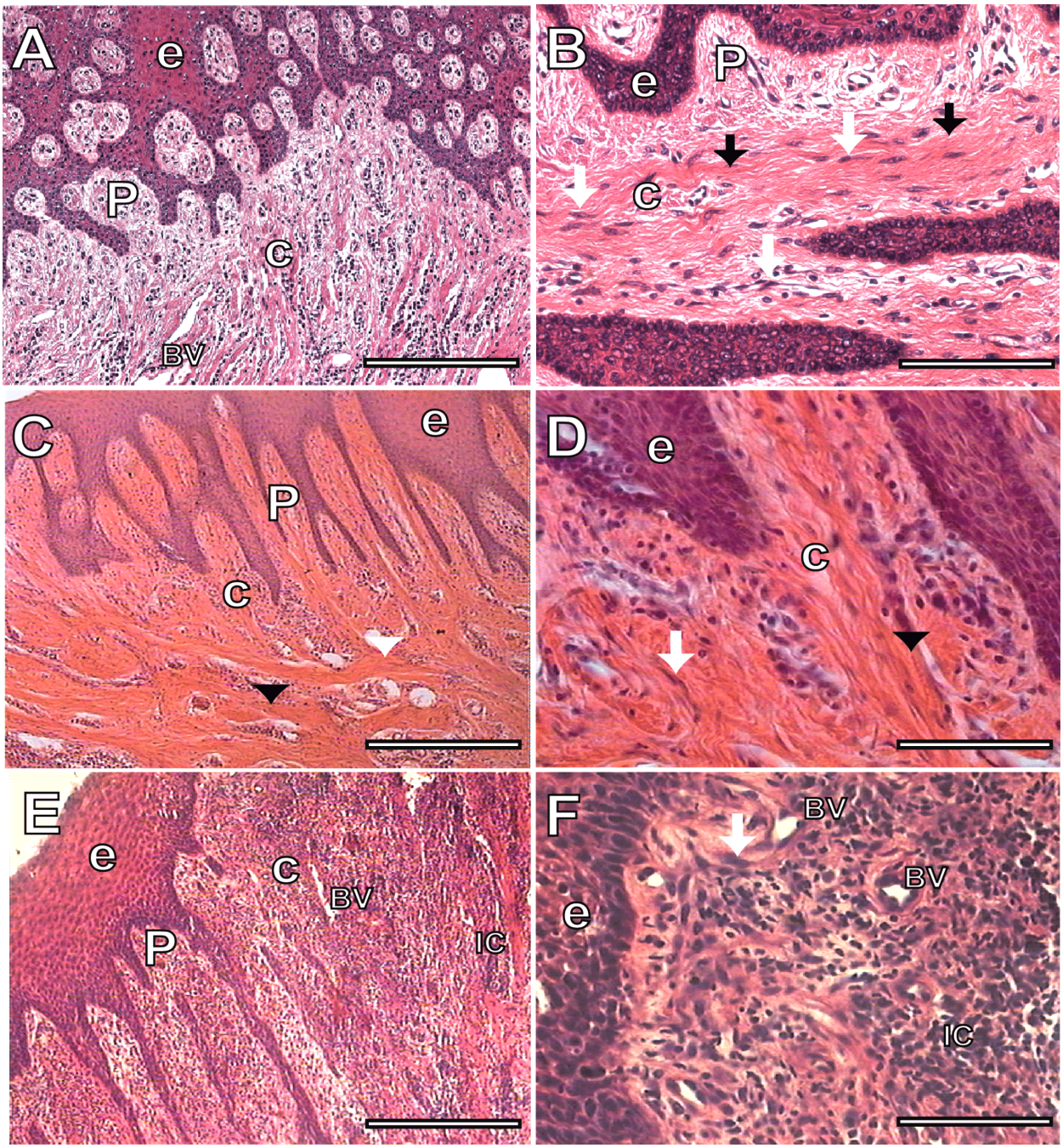

Figure 2 – ***Sections of human gingiva without gingivitis (A and B), with gingivitis (C and D) and chronic periodontitis (E and F). The samples from individuals without gingivitis, with gingivitis and chronic periodontitis showed stratified squamous epithelium non-keratinized (e) with tissue papillae (p). The papillae are formed from connective tissue (c) associated with epithelial projections and blood vessels (BV). Note in (A), disorganized dense connective presenting thick collagen fibers. In (B) observe fibroblasts nuclei (white arrows) and collagen fibers (black arrows). Observe in (C and D), thicker collagen fibers (arrowheads). In (E) more nuclei of inflammatory cells (IC) are seen. HE staining. Bars: (A, C and E) 120 µm; (B, D and F) 48 µm.***

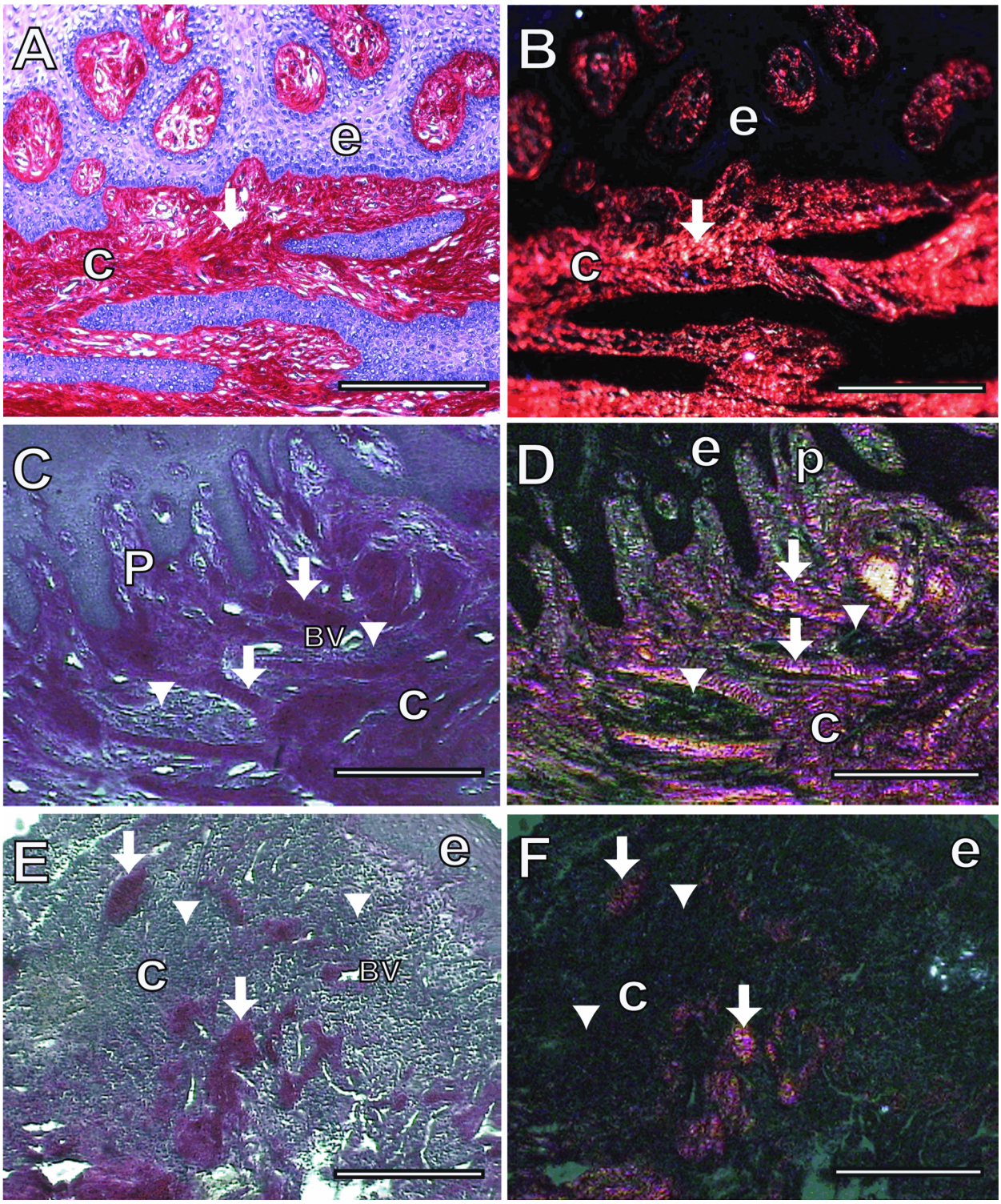

Figure 3 – ***Sections of fragments of human gingival stained with Picrosirius red (A, C and E light microscopy; B, D and F polarized light). Note epithelial tissue (e) and connective tissue (c). See in (A and B) control, collagen fibers (arrows) with normal morphology. Note in (C and D) samples with chronic gingivitis showing bundles of collagen fibers (arrows) compressing groups of inflammatory cells (arrowheads) associated with blood vessels (BV). In (E and F) fragments with chronic periodontitis showing destruction of collagen fibers (arrows) characterizing loss of normal morphology. Note also large amount of inflammatory cells (arrowheads). Bars: (C, D, E and F) 120 µm; (A and B) 48 µm.***

Type IV collagen

The type IV collagen fibers were found distributed at the epithelial and blood vessels basement membrane of all the samples, regardless of groups. Staining for type IV collagen at the CP samples exhibited an apparent thicker aspect of basement membrane around the dilated blood vessels (Figure 6, a–c).

No significant differences were found between groups to type IV collagen (p=0.355) (Figure 6d).

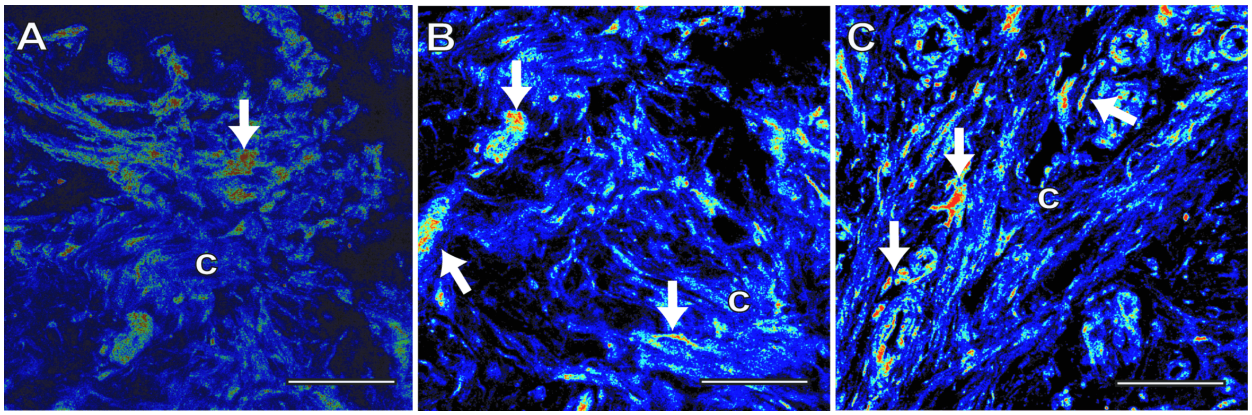

Figure 4 – ***Micrographs of human gingival marked by immunofluorescence to type I collagen. Note marking to type I collagen (arrows) in deep connective tissue (c) of control samples (A), chronic gingivitis (B) and chronic periodontitis (C), respectively. Bars = 100 µm.***

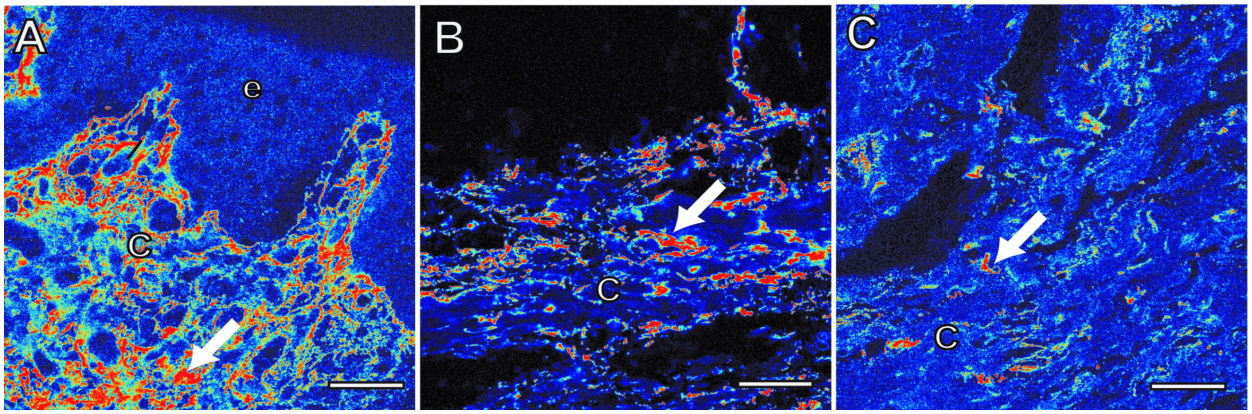

Figure 5 – ***Micrographs of human gingival marked by immunofluorescence to type III collagen. Note epithelial tissue (e) and connective tissue (c). See type III collagen marking (arrows) in control sample (A), samples with chronic gingivitis (B) and chronic periodontitis (C), respectively. Bars = 100 µm.***

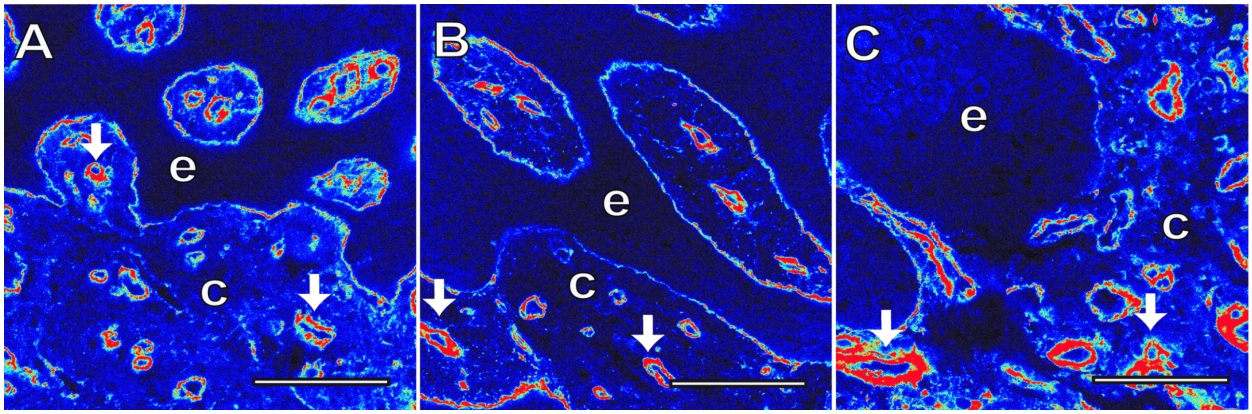

Figure 6 – ***Micrographs of human gingival marked by immunofluorescence to type IV collagen. Note epithelial tissue (e) and connective tissue (c). Observe type IV collagen marking (arrows) in control sample (A), samples with chronic gingivitis (B) and chronic periodontitis (C), respectively. Bars = 100 µm.***

Discussion

The present study evaluated morphologically and quantitatively collagen fibers in healthy and diseased human gingival tissues. As the prevalence and incidence of periodontal disease is high worldwide, several studies have already been conducted looking to elucidate aspects of this disease [7, 8, 11, 20, 21]. However, there was a lack of studies evaluating the fibers of types I, III and IV collagen and relating such fibers to its organization, localization and expression in healthy gingival tissue and varying degrees of periodontal disease. A detailed study of the histological aspects during the development of this disease therefore became necessary in order to establish new techniques that may assist in disease treatment planning and prognosis.

In the present study, thick collagen fibers were in a parallel arrangement in HG group, relatively organized in CG group, but were disorganized in CP group. These results are in agreement with those found by several authors [11, 20, 21]. Ejeil *et al.* [11] also stated that there was a 20% reduction collagen area in the gingiva when comparing normal and severely inflamed gingival tissues.

The results obtained by immunofluorescence technique revealed an apparent higher presence, distribution, and expression of the type I collagen in healthy gingiva, when compared to diseased gingival tissues. Individual factors of the participants may explain the absence of significant differences between groups. Other authors also found a reduction in the amount of soluble collagen in inflamed gingiva [4, 11, 20, 21]. The apparent lower amount of type I collagen fibers in diseased gingiva may be explained by the fact that due to inflammatory process, inflammatory cells (monocytes, macrophages, lymphocytes, polymorphonuclear cells) and resident cells (fibroblasts, epithelial cells and endothelial cells) express metalloproteinases (MMPs), leading to extracellular matrix disorganization and collagen damage [1, 11, 22]. Moreover, according Häkkinen *et al.* [23], with the inflammatory response, the organization of the local extracellular matrix is considerably modified. In accordance with our results, Séguier *et al.* [1] demonstrated that collagen loss was correlated with gingival inflammatory cells during active periodontitis.

There were no statistical differences to the expression of types III and IV collagen when comparing HG group to diseased gingiva groups. These results may be explained, in part, by individual response to chronic gingival inflammation [8, 24]. Some individuals are more susceptible to rapidly destructive disease and others, who have a same pattern of periodontal inflammation, are more resistant to destructive disease [25]. Other studies showed that the degradation of type IV collagen is made by MMP-9 and modulated by MMP-2 [26, 27]. Nevertheless, further studies may be conducted to clarify these findings.

Analysis of data obtained from Picrosirius red and immunofluorescence revealed that despite the increased amount of thicker fibers, there was a decrease in the homogeneous distribution of collagen in the gingival tissue as a whole. In accordance to our results, Lorencini *et al.* [4] observed an increase in the amount of thicker fibers, which showed no preferred orientation, despite the reduction in the area occupied by these fibers. They also found that collagen fibrils are more compact within the fibers in inflamed tissue.

The results of this study suggest a hypothesis based on the rearrangement of collagen fibers in the extracellular matrix of the periodontium following periodontal disease. This process is characterized by the formation of thicker and spaced fibers together with a significant reduction in the area occupied by fibrillar collagen in the gingival connective tissue. Lorencini *et al.* [4] also hypothesized that a "frustrated repair" of the extracellular matrix can occur at the same time as collagen degradation. They also stated that a new configuration of the fibers in the connective tissue would contribute to increase the progression of the periodontal destruction, leading to loss of gingival protection allowing pathogenic microorganisms to invade and reach deeper areas of periodontal tissue.

Although the present study offers originality and provides evidence regarding morphological and quantitative aspects of collagen fibers in healthy and diseased human gingival tissues, some methodological aspects, such as sample size and selection, might limited the extrapolation of the results. Thus, further studies may have conducted with larger and more homogeneous samples to clarify the differences between presence and distribution of more types of collagen in healthy and diseased human gingival tissues.

Conclusions

Following the periodontal disease, there was a morphological destruction of the extracellular matrix with lower expression of collagen, which led to a change in tissue architecture that might compromise its functional capacity. There were differences in type I collagen expression among healthy, chronic gingivitis and chronic periodontitis tissue samples. While based on a relatively small number of patients, the observed tendencies could inform more ample analyses in the future.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was funded by the Brazilian fostering agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). Providers of funding had no role in study design, data collection and analysis, publishing decisions, or preparation of the manuscript.

References

[1] Séguier S, Godeau G, Brousse N. Collagen fibers and inflammatory cells in healthy and diseased human gingival tissues: a comparative and quantitative study by immunohistochemistry and automated image analysis. J Periodontol, 2000, 71(7):1079–1085.

[2] De-Gennaro LA, Lopes JD, Mariano M. Autoantibodies directed to extracellular matrix components in patients with different clinical forms of periodontitis. J Periodontol, 2006, 77(12):2025–2030.

[3] Miller CS, King CP Jr, Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: a cross-sectional study. J Am Dent Assoc, 2006, 137(3):322–329.

[4] Lorencini M, Silva JA, Almeida CA, Bruni-Cardoso A, Carvalho HF, Stach-Machado DR. A new paradigm in the periodontal disease progression: gingival connective tissue remodeling with simultaneous collagen degradation and fibers thickening. Tissue Cell, 2009, 41(1):43–50.

[5] Ministry of Health Project SB Brazil 2010. National survey of oral health: main results. Ministry of Health, Office of Health Care, Department of Primary Care, 2011.

[6] Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet, 2005, 366(9499):1809–1820.

[7] Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol, 1998, 25(2):134–144.

[8] Gürses N, Thorup AK, Reibel J, Carter WG, Holmstrup P. Expression of VLA-integrins and their related basement membrane ligands in gingiva from patients of various periodontitis categories. J Clin Periodontol, 1999, 26(4):217–224.

[9] Meikle MC, Hembry RM, Holley J, Horton C, McFarlane CG, Reynolds JJ. Immunolocalization of matrix metalloproteinases and TIMP-1 (tissue inhibitor of metalloproteinases) in human gingival tissues from periodontitis patients. J Periodontal Res, 1994, 29(2):118–126.

[10] Okada H, Murakami S, Kitamura M, Nozaki T, Kusumoto Y, Hirano H, Shimauchi H, Shimabukuro Y, Saho T. Diagnostic strategies of periodontitis based on the molecular mechanisms of periodontal tissue destruction. Oral Dis, 1996, 2(1):87–95.

[11] Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. J Periodontol, 2003, 74(2): 188–195.
[12] Emingil G, Tervahartiala T, Mäntylä P, Määttä M, Sorsa T, Atilla G. Gingival crevicular fluid matrix metalloproteinase (MMP)-7, extracellular MMP inducer, and tissue inhibitor of MMP-1 levels in periodontal disease. J Periodontol, 2006, 77(12):2040–2050.
[13] Chavrier C, Couble ML, Magloire H, Grimaud JA. Connective tissue organization of healthy human gingiva. Ultrastructural localization of collagen types I–III–IV. J Periodontal Res, 1984, 19(3):221–229.
[14] Lee W, Aitken S, Sodek J, McCulloch CA. Evidence of a direct relationship between neutrophil collagenase activity and periodontal tissue destruction *in vivo*: role of active enzyme in human periodontitis. J Periodontal Res, 1995, 30(1):23–33.
[15] Buduneli N, Atilla G, Güner G, Oktay G. Biochemical analysis of total collagen content and collagen types I, III, IV, V and VI in gingiva of various periodontitis categories. J Int Acad Periodontol, 2001, 3(1):1–6.
[16] Becker J, Schuppan D, Müller S. Immunohistochemical distribution of collagens type I, III, IV and VI, of undulin and of tenascin in oral fibrous hyperplasia. J Oral Pathol Med, 1993, 22(10):463–467.
[17] Kjeldsen M, Holmstrup P, Lindemann RA, Bendtzen K. Bacterial-stimulated cytokine production of peripheral mononuclear cells from patients of various periodontitis categories. J Periodontol, 1995, 66(2):139–144.
[18] Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J, 1979, 11(4):447–455.
[19] Montes GS, Junqueira LCU. The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Mem Inst Oswaldo Cruz, 1991, 86(Suppl 3):1–11.
[20] Gogly B, Godeau G, Gilbert S, Legrand JM, Kut C, Pellat B, Goldberg M. Morphometric analysis of collagen and elastic fibers in normal skin and gingiva in relation to age. Clin Oral Investig, 1997, 1(3):147–152.
[21] Silva JA, Lorencini M, Reis JR, Carvalho HF, Cagnon VH, Stach-Machado DR. The influence of type I diabetes mellitus in periodontal disease induced changes of the gingival epithelium and connective tissue. Tissue Cell, 2008, 40(4): 283–292.
[22] Coleman JW. Nitric oxide in immunity and inflammation. Int Immunopharmacol, 2001, 1(8):1397–1406.
[23] Häkkinen L, Uitto VJ, Larjava H. Cell biology of gingival wound healing. Periodontol 2000, 2000, 24:127–152.
[24] Booth V, Young S, Cruchley A, Taichman NS, Paleolog E. Vascular endothelial growth factor in human periodontal disease. J Periodontal Res, 1998, 33(8):491–499.
[25] Löe H, Anerud A, Boysen H, Morrison E. Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. J Clin Periodontol, 1986, 13(5):431–445.
[26] Gioia M, Monaco S, Van Den Steen PE, Sbardella D, Grasso G, Marini S, Overall CM, Opdenakker G, Coletta M. The collagen binding domain of gelatinase A modulates degradation of collagen IV by gelatinase B. J Mol Biol, 2009, 386(2):419–434.
[27] Rosell A, Ortega-Aznar A, Alvarez-Sabín J, Fernández-Cadenas I, Ribó M, Molina CA, Lo EH, Montaner J. Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke, 2006, 37(6):1399–1406.

Corresponding author
Thalita Valverde, PhD student, MS, Department of Morphology, Federal University of Minas Gerais, Av. Antônio Carlos 6627, ICB, UFMG, 31.270–901 Belo Horizonte, Minas Gerais, Brazil; Phone +55 31 3409 2791, e-mail: thalitamarcolan@yahoo.com.br

Received: May 17, 2014

Accepted: February 6, 2015