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Radiological, histological and immunohistochemical evaluation of periapical inflammatory lesions

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Abstract

The loss of teeth is largely caused by supporting tissue damage, because of bacterial invasion from the infected root canals. Sixty patients with periapical lesions (PLs) of endodontic origin were included in the study. Clinical and radiological examination was performed. Periapical radiographs were analyzed by two independent observers to determine the size and severity of PLs, using Periapical Index (PAI) scores. The tissue samples collected by periapical curettage during apicoectomy or after dental extractions by alveolar curettage were histologically and immunohistochemically analyzed. The PLs were histologically diagnosed as: periapical granulomas (PGs), granulomas with cystic potential and radicular cysts (RCs) with various degrees of inflammation. Capillary density was evaluated using the angiogenic index after immunohistochemical staining with CD34 monoclonal antibody. A statistically significant correlation was observed between PAI scores and the size of the lesions. 68.33% of cases were PGs, 18.33% PGs with cystic potential and 18.33% RCs with different degrees of inflammation. Seventy-five percent PLs had an angiogenic index 1 and 25% had an angiogenic index 2. Statistically significant differences were obtained between the angiogenic index and lesion size (p<0.05). Capillary density within PLs did not influence the severity scores of lesions detected on radiographs. The angiogenic index appeared not to be associated with the histological lesion type and the intensity of inflammation, but was more likely correlated with the degree of granulation tissue maturation and the size of PLs.

Keywords: periapical lesions, inflammatory infiltrate, capillary density, CD34 marker.

Introduction

Periapical lesion (PL) is an inflammatory process around the apex of the tooth caused by the bacterial infection of the root canal system. Bacteria and their toxic products within root canals progress through the apical foramen into periapical tissues, leading to inflammation, destruction of periapical tissues and development of various types of PL [1, 2]. PL is one of the most frequent pathological conditions within the alveolar bone [3]. Most of the periapical inflammatory lesions of endodontic origin are periapical granulomas (PGs) and radicular cysts (RCs), both being transitional entities of the same pathological lesions, as one type of lesion may turn into another [4, 5]. Having a common etiology, PG and RC are commonly found at the apex of the teeth with necrotic pulp [6]. Epithelial cell rests of Malassez from the apical region of teeth with pulp necrosis are stimulated to divide and proliferate by inflammatory mediators, pro-inflammatory cytokines, and growth factors released from host cells. This process leads to radicular cyst development [7, 8]. Chronic periapical inflammation also leads to periodontal ligament destruction, alveolar bone resorption and development of granulation tissue. Histopathological analysis is an accurate method for the detection of periapical tissue changes [9]. During inflammation, the angiogenic factors involved in angiogenesis are released by the activation of different types of cells (endothelial cells, macrophages, fibroblasts). Macrophages have a pro-angiogenic effect on the site of inflammation; they induce angiogenesis by stimulating the proliferation of blood vessels, which causes secondary macrophage infiltration of the soft tissues.

Angiogenesis is a complex process that occurs as a normal physiological condition during embryogenesis, but also in pathological conditions, such as inflammation and tissue repair. This process consists of the formation of new blood vessels from preexisting ones. Being involved in the pathogenesis of chronic inflammatory diseases, angiogenesis contributes to the formation of granulation tissue. This dynamic process involves endothelial cell division, selective degradation of vascular basement membranes and surrounding extracellular matrix, and endothelial cell migration [10]. An inflammatory state can promote angiogenesis, and angiogenesis can facilitate chronic inflammation [11]. CD34 monoclonal antibody may be used to highlight microvessels in inflammatory disorders due to its capability of staining vascular endothelial cells [12]. During inflammation, angiogenesis plays a role in the development and progression of pathological lesions. Inflammatory cell infiltrate and neoangiogenesis influence the development and the growth of RCs [13]. Inflammation and angiogenesis are actively involved in the progression of lesions, being co-dependent processes [11]. The provisional diagnosis of periapical pathology of endodontic origin includes clinical and radiographic examination, whereas definitive diagnosis is established by histo-

pathological analysis in conjunction with immunohistochemical analysis. The absence or presence of periapical inflammation in teeth affected by pulp necrosis can be evaluated by clinical signs or symptoms, and also by radiological evaluation of the periapical status [14]. Periapical radiolucency on radiographic images is suggestive of alveolar bone resorption surrounding the affected teeth confirming the presence of PL, but sometimes RCs has a radiographic appearance similar to that of PGs [15].

Therefore, the objective of this study was to investigate inflammatory infiltrate and angiogenesis through CD34 staining in periapical lesions of endodontic origin, and to evaluate the relationships between the histological degree of inflammation, capillary density and radiological parameters.

Materials and Methods

The study included 60 patients, with no systemic diseases, having at least one tooth with PL, who did not undergo any treatment with anti-inflammatory or antibiotic medication during the last six months. Patients with immunosuppression status and those with combined periodontal/endodontic lesions or longitudinal fracture of the dental root were excluded from the study. The study protocol was approved by the Medical Ethical Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania (No. 321/02.06.2015). Each patient was informed about the study protocol and signed a consent form.

Clinical and radiographic examination was performed in each patient. Following initial oral examination, pulp vitality tests and intraoral radiographs were carried out for each affected tooth in order to establish the endodontic origin of the lesions. The following data were recorded for each patient: age (years), gender, medical history, type and location of affected teeth, endodontic status, clinical signs and symptoms, response to palpation and percussion. The digital radiographic images were analyzed by two observers for evaluation of the periapical status of the affected teeth. Each observer measured the size (area in mm^2) of the lesion using Digimizer® 4.3 software (MedCalc Image Analysis) and determined the severity with the Periapical Index (PAI) scoring system described by Ørstavik *et al.* [16].

The tissue samples were obtained by alveolar curettage after tooth extraction or periapical curettage during apicoectomy. Immediately after the surgical procedures, the pathological tissues were fixed in 10% neutral buffered formalin and analyzed histopathologically and immunohistochemically.

For microscopic evaluation, the histological sections were stained with Hematoxylin–Eosin (HE). Based on the method proposed by Tsai *et al.* [17], the PLs were divided into three degrees of inflammation: grade I, inflammatory cells less than one-third per field; grade II, inflammatory cells between one-third and two-thirds per field, and grade III, inflammatory cells more than two-thirds per field. Taking into consideration the age of granulation tissue, the PLs were divided into: young lesions, up to two weeks of age, moderately old lesions, between two and four weeks of age, and old lesions, over four weeks of age, with mature granulation tissue. Periapical inflammatory lesions were also classified according to histological findings into PG, PG with cystic potential and RC. Lesions consisting of granulation tissue with various degrees of inflammatory infiltrate, surrounded by a fibrous connective capsule, were diagnosed as PGs. Lesions consisting of granulation tissue with epithelial cell rests of Malassez starting the proliferation process with the formation of fissures were diagnosed as PGs with cystic potential. Lesions consisting of a cavity lined by stratified squamous epithelium resulting from epithelial rests of Malassez proliferation and a connective tissue wall with variable inflammatory infiltrate were considered as RCs.

The study of angiogenesis in PLs was performed by immunohistochemistry using CD34 monoclonal antibody in 4 μm thick histological sections cut with the rotary microtome (MicroTec CUT 4055). The protocol was conducted using the Novolink™ Polymer Detection System Kit (Novocastra, Leica, UK).

Histopathological and immunohistochemical analysis of the samples was performed using the Leica DM750 microscope (Leica Microsystem GmbH, Germany). The angiogenic index of PLs was determined after evaluation of the density of microvessels immunoreactive for CD34 antibody. The angiogenic index consisted of a semi-quantitative score ranging from 1 to 3 according to microvessel density: (1) low capillary density <10 vessels/field; (2) medium capillary density between 20–40 vessels/field; (3) high capillary density >40 vessels/field.

Statistical data analysis was performed using the SPSS software (SPSS Statistics 19, IBM, Chicago, IL, USA). The distribution of data was tested using the Kolmogorov–Smirnov test. Normally distributed data were expressed as arithmetic mean ± standard deviation, whereas non-normally distributed data were expressed as median (25^{th}–75^{th} percentile). The correlation between quantitative variables was analyzed using Pearson's or Spearman's correlation coefficient.

Results

The periapical inflammatory lesions were collected from 60 patients, including 26 (43.3%) females and 34 (56.7%) males, with an average age of 42.33 years.

Radiological evaluation revealed the presence of radiolucent areas of different sizes in the periapical region, in close relation with the roots. The PAI scores allocated by observer 2 were correlated with those given by observer 1 (r=0.76, p=0.001), with Cohen's kappa coefficient κ=0.62, p=0.0001. PAI scores were significantly correlated with the radiological area of the lesions (r=0.31, p=0.03). When comparing the radiological area of the lesions measured in mm^2 with the degree of inflammatory infiltrate, the highest value [20.75 (8.75–32.83)] was detected in lesions with inflammatory infiltrate grade I. The mean PAI scores assigned by two observers were higher in the cases of grade I of inflammation [4 (3.5–4)]. Based on the initial clinical and radiological evaluation of the lesions, 27 (45%) were assessed as PGs, and 33 (55%) were RCs. The histopathological study identified the type of PL and the degrees of inflammatory infiltrate. Thus, 38 (63.3%) cases were histologically diagnosed as PGs, 11 (18.3%) cases as PGs with cystic potential and 11 (18.3%) cases as RCs.

The microscopic analysis of tissue samples showed a variable proportion of inflammatory cells, mainly lymphocytes, macrophages, plasma cells and neutrophils, grouped as inflammatory infiltrates around newly formed vessels. In all samples, the presence of a mixed, predominantly chronic inflammatory infiltrate formed by lymphocytes, macrophages, plasma cells and rare neutrophils was noticed (Figure 1, A–C).

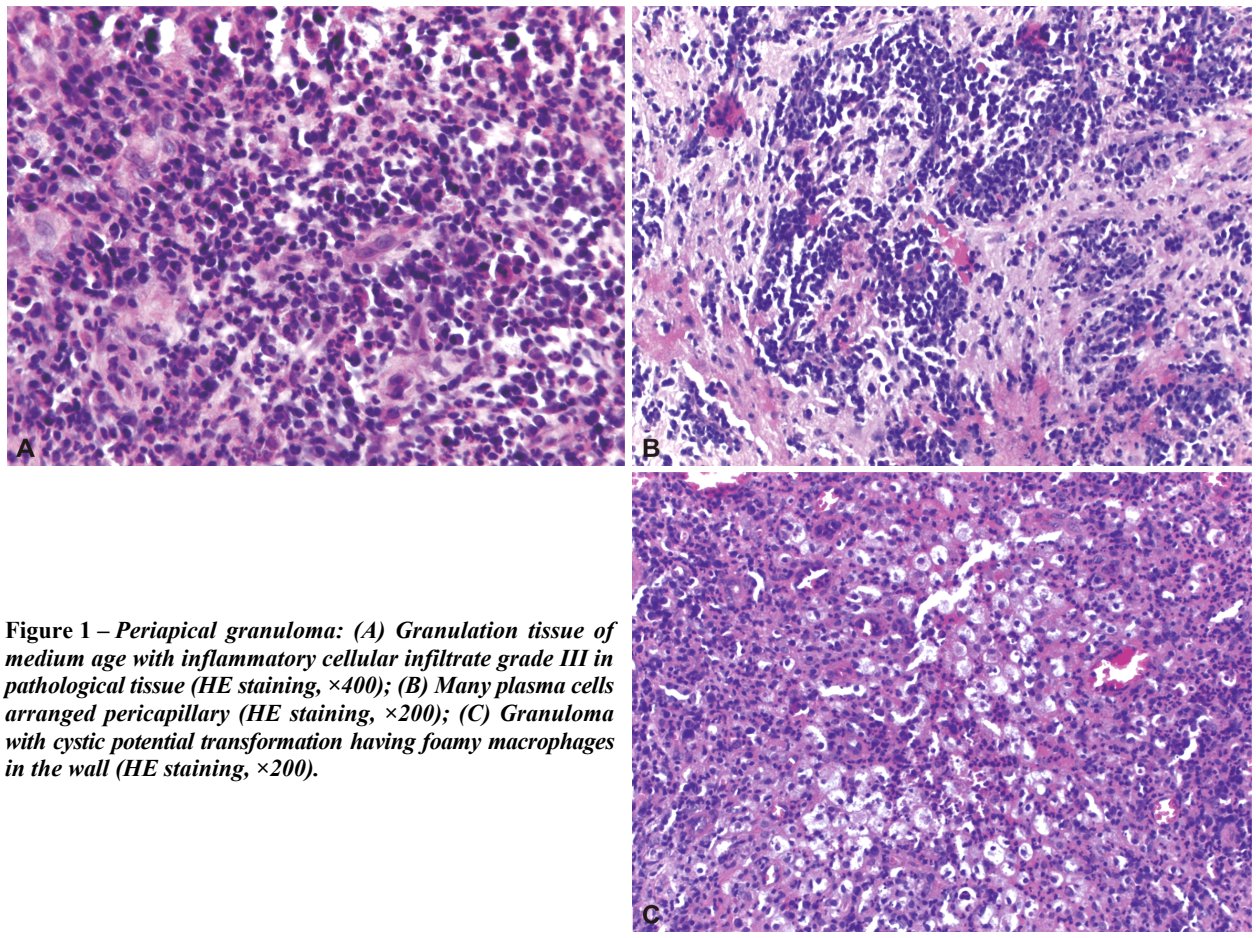

Figure 1 – ***Periapical granuloma: (A) Granulation tissue of medium age with inflammatory cellular infiltrate grade III in pathological tissue (HE staining, ×400); (B) Many plasma cells arranged pericapillary (HE staining, ×200); (C) Granuloma with cystic potential transformation having foamy macrophages in the wall (HE staining, ×200).***

The intensity of inflammatory infiltrate ranged from low (grade I), in seven (11.6%) cases, to moderate (grade II), in 37 (61.66%) cases, and severe (grade III), in 16 (26.66%) cases. No statistically significant differences were found between the intensity of the inflammatory infiltrate and the histological type of the lesion (p=0.107) (Table 1).

Table 1 – ***Intensity of inflammatory infiltrate in relation with age, radiological features and histological types of PL at investigated patients (n=60)***

	Grade I (*n*=7)	Grade II (*n*=37)	Grade III (*n*=16)	*p*
Age* [years]	37.57±14.41	41.97±12.57	45.25±12.54	0.411
Lesion area^{+} [mm^2]	20.75 (8.75–32.83)	15.78 (8.83–19.82)	18.67 (10.85–25.52)	0.233
Mean PAI scores^{+}	4 (3.5–4)	3 (3–4)	3.75 (3.12–4)	0.116
Radiological diagnosis				
PG *n* (%)	2 (28.6)	18 (48.6)	7 (43.8)	0.615
RC *n* (%)	5 (71.4)	19 (51.4)	9 (56.3)	
Histological diagnosis				
PG *n* (%)	4 (57.1)	28 (75.7)	6 (37.5)	0.107
RC *n* (%)	2 (28.6)	4 (10.8)	5 (31.3)	
PG with cystic potential *n* (%)	1 (14.3)	5 (13.5)	5 (31.3)	

*Arithmetic mean ± standard deviation; $^{+}$Median (25^{th}–75^{th} percentile); PAI: Periapical Index; mean PAI: Arithmetic mean of observer 1 and observer 2; PG: Periapical granuloma; RC: Radicular cyst.

The immunohistochemistry technique was used to evaluate newly capillary vessels and interstitial dendritic cells within the PL. The CD34 marker stained the endothelial cells of the blood capillaries, as well as isolated dendritic cells (Figure 2, A–D).

Regarding gender distribution, males were more affected than females, but no statistically significant differences were found (p=0.206). PLs were frequently located in the maxilla [35 cases including four (57.1%) lesions with grade I, 23 (62.2%) lesions with grade II and eight (50%) lesions with grade III, p=0.71], and the most affected tooth was the lateral incisor [20 cases including two (28.57%) lesions with grade I, 13 (35.13%) lesions with grade II, and five (31.25%) lesions with grade III, p=0.726]. The teeth associated with PL and previous endodontic treatment had inflammatory infiltrate grade II and III (62.16% and 62.5% teeth, respectively). Forty-five (75%) cases were treated by root-end resection including 85.71% with grade I, 72.97% with grade II, and 75% with grade III, while 15 (25%) cases were treated by extraction: 14.28% with grade I, 27.02% with grade II, and 25% with grade III. 83.33% PLs had mature granulation tissue with different degrees of inflammation, 13.33% had medium aged granulation tissue with variable inflammatory infiltrate, and 3.33% had young granulation tissue with moderate inflammatory infiltrate. Regarding the intensity of inflammatory infiltrate in old PLs, 71.42% were grade I, 83.78% were grade II, and 87.5% were grade III (Table 2).

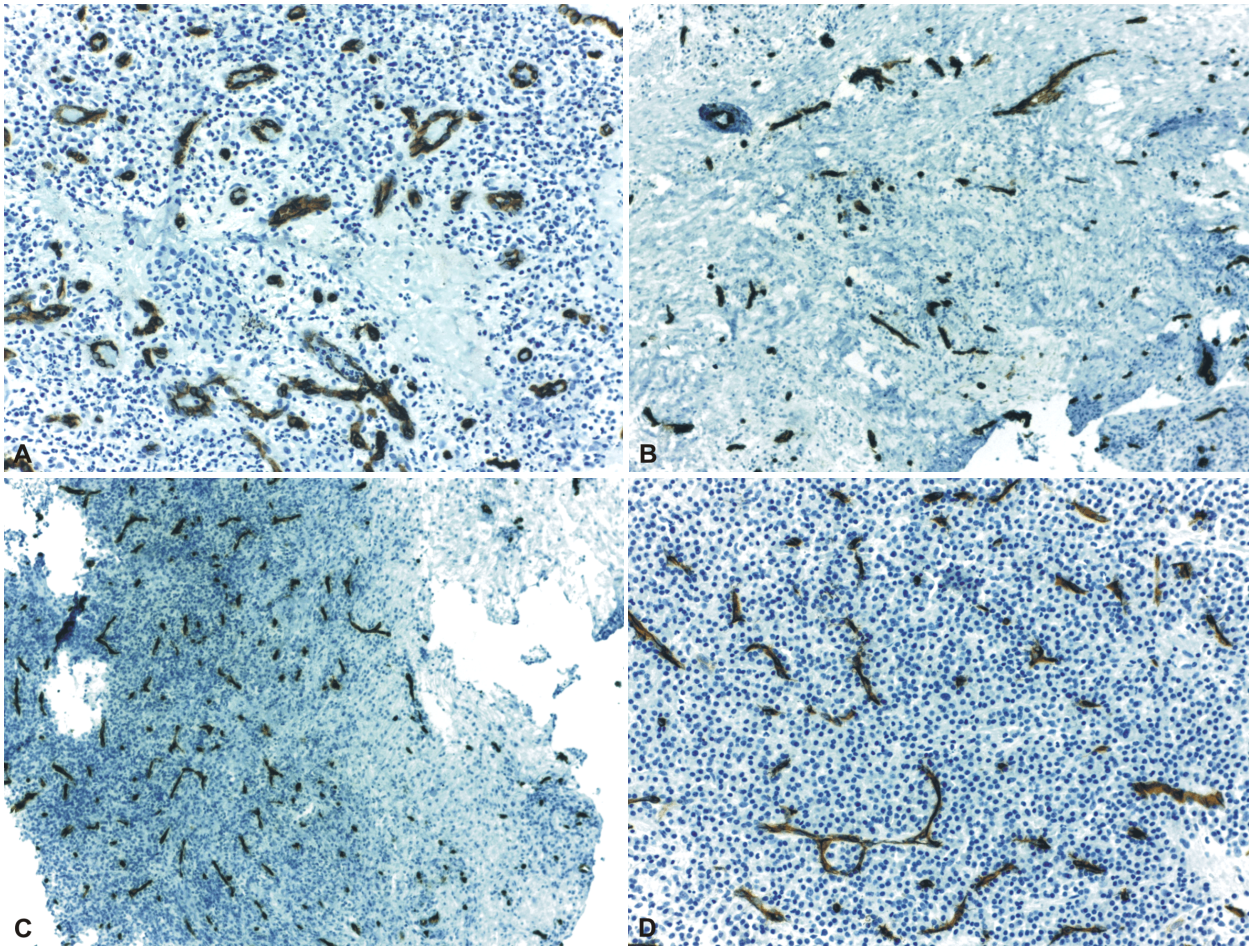

Figure 2 – ***Capillaries and interstitial dendritic cells positive for CD34 monoclonal antibody: (A) Periapical granuloma. Granulation tissue with medium density of capillary vessels and interstitial dendritic cells; (B) Periapical granuloma with cystic potential. Low capillary density and medium density of dendritic interstitial cells in fibrous capsule; (C) Periapical granuloma with cystic potential. Intense inflammation and higher density of capillary blood vessels; (D) Radicular cyst. Intense inflammatory cell infiltrate, but relatively poor in capillaries. Anti-CD34 antibody immunostaining: (A, B and D) ×200; (C) ×40.***

Table 2 – ***Association between inflammatory infiltrate and gender, tooth type, tooth location, endodontic, surgical treatment and age of granulation tissue in PL at investigated patients (n=60)***

	Grade I (*n*=7)	Grade II (*n*=37)	Grade III (*n*=16)	*P*
Gender				
Females *n* (%)	3 (42.9)	19 (51.4)	12 (75)	0.206
Males *n* (%)	4 (57.1)	18 (48.6)	4 (25)	
Tooth type				
Central incisor *n* (%)	0 (0)	4 (10.81)	2 (12.5)	0.726
Lateral incisor *n* (%)	2 (28.6)	13 (35.1)	5 (31.3)	
Canine *n* (%)	1 (14.2)	4 (10.8)	3 (18.8)	
Premolar *n* (%)	4 (57.1)	10 (27)	3 (18.8)	
Molar *n* (%)	0 (0)	6 (16.2)	3 (18.8)	
Tooth location				
Mandible *n* (%)	3 (42.9)	14 (37.8)	8 (50)	0.621
Maxilla *n* (%)	4 (57.1)	23 (62.2)	8 (50)	
Endodontic treatment				
Yes *n* (%)	3 (42.9)	23 (62.1)	10 (62.5)	0.615
No *n* (%)	4 (57.1)	14 (37.8)	6 (37.5)	
Surgical treatment				
Apical resection *n* (%)	6 (85.7)	27 (73)	12 (75)	0.775
Dental extraction *n* (%)	1 (14.3)	10 (27)	4 (25)	
Age of the granulation tissue				
Young *n* (%)	0 (0)	2 (5.4)	0 (0)	0.592
Medium aged *n* (%)	2 (28.6)	4 (10.8)	2 (12.5)	
Mature *n* (%)	5 (71.4)	31 (83.8)	14 (87.5)	

Forty-five lesions had a low capillary density, while 15 had a medium capillary density. Statistically significant differences were found between the radiological area of lesions and the angiogenic index (p=0.044). The radiological area of the lesions had a high value [17.83 (10.29–22.33)] in the case of the angiogenic index 1, compared to the angiogenic index 2 [10.45 (8.09–19.46)]. Regarding the mean scores of the two observers, values were identical for lesions with low and medium capillary density [3.5 (3–4)]. There were no statistically significant differences between the angiogenic index and the histological type of lesions (p=0.65). In the case of low capillary density, 71% of lesions were PGs, 82% RCs, and 82% PGs with cystic potential, while in cases with medium capillary density, 29% were PGs, 18.1% RCs and 18.1% PGs with cystic potential (Table 3).

The inflammatory lesions were frequently located in the maxilla [35 cases including 28 (62.2%) with low

capillary density and seven (46.6%) with medium capillary density]. The most affected tooth was the lateral incisor (33.3%), followed by premolars (26.6%) and molars (17.8%). Regarding the age of the lesion, 50 lesions were old, of which 38 (84.4%) had a low capillary density and 12 (80%) a medium capillary density (Table 4).

Table 3 – ***Angiogenic indices in relation with age, radiological features and histological types of PL at investigated patients (n=60)***

	Angiogenic index 1 (*n*=45)	Angiogenic index 2 (*n*=15)	*P*
Age [years]*	42.38±12.97	42.2±12.5	0.932
Lesion area^{+} [mm^2]	17.83 (10.29–22.33)	10.45 (8.09–19.46)	0.044
Mean PAI scores^{+}	3.5 (3–4)	3.5 (3–4)	0.55
Radiological diagnosis			
PG *n* (%)	19 (30.4)	8 (13.9)	0.454
RC *n* (%)	26 (42.6)	7 (11.5)	
Histological type of the lesion			
PG *n* (%)	27 (71)	11 (29)	0.65
RC *n* (%)	9 (82)	2 (18.1)	
PG with cystic potential *n* (%)	9 (82)	2 (18.1)	
Grade of inflammatory infiltrate			
I *n* (%)	7 (100)	0 (0)	0.253
II *n* (%)	27 (73)	10 (27)	
III *n* (%)	11 (68.8)	5 (31.2)	

*Arithmetic mean ± standard deviation; $^{+}$Median (25^{th}–75^{th} percentile); PAI: Periapical Index; mean PAI: Arithmetic mean of observer 1 and observer 2; PG: Periapical granuloma; RC: Radicular cyst.

Table 4 – ***Association between angiogenic index and gender, tooth type, tooth location, endodontic, surgical treatment and age of granulation tissue in PL at investigated patients (n=60)***

	Angiogenic index 1 (*n*=45)	Angiogenic index 2 (*n*=15)	*P*
Gender			
Females *n* (%)	22 (48.9)	4 (26.6)	0.133
Males *n* (%)	23 (51.1)	11 (73.3)	
Tooth type			
Central incisor *n* (%)	5 (11.1)	1 (66.6)	0.737
Lateral incisor *n* (%)	15 (33.3)	5 (33.3)	
Canine *n* (%)	5 (11.1)	3 (20)	
Premolar *n* (%)	12 (26.6)	5 (33.3)	
Molar *n* (%)	8 (17.8)	1 (6.6)	
Tooth location			
Mandible *n* (%)	17 (37.8)	8 (53.3)	0.29
Maxilla *n* (%)	28 (62.2)	7 (46.6)	
Endodontic treatment			
Yes *n* (%)	25 (55.5)	11 (73.3)	0.224
No *n* (%)	20 (44.4)	4 (26.6)	
Surgical treatment			
Apical resection *n* (%)	33 (73.3)	12 (80)	0.74
Dental extraction *n* (%)	12 (26.6)	3 (20)	
Age of the granulation tissue			
Young *n* (%)	1 (2.2)	1 (6.6)	0.707
Medium aged *n* (%)	6 (13.3)	2 (13.3)	
Mature *n* (%)	38 (84.4)	12 (80)	

Discussion

In the present study, we evaluated the correlation between angiogenesis, severity scores observed on periapical radiographs, and the intensity of inflammatory infiltrate in PL. Histopathological evaluation of PLs was necessary to establish definitive and differential diagnosis. All the samples were collected from PLs without clinical symptoms associated with non-vital teeth. The histopathological analysis of PLs identified the type of lesion, with various degrees of inflammatory cell infiltrate. Although newly formed vessels can be identified by classical histological staining (HE), limited information on the angiogenesis process can be obtained by histological examination alone [18]. Thus, using the immunohistochemistry technique, angiogenesis in the histological type of PL can be better investigated. Given that the proliferation of new blood vessels within PLs is an important factor for the progression, in this study angiogenesis was assessed in relation to the intensity of inflammatory infiltrate and the severity of lesions detected on digital radiographs. Angiogenic activity in PLs was quantified indirectly by evaluating capillary density in pathological periapical samples. Immunohistochemical analysis using the CD34 marker detected all capillary vessels in PLs. The radiological area of the lesions was associated with capillary density; PL with a larger radiological area had a low capillary density, while PL with a smaller radiological area had a medium capillary density.

In PGs with young granulation tissue, many widely patent capillaries with edematous stroma and abundant round nuclear inflammatory infiltrate grade III were observed. In the newly formed capillaries, there were leukocyte margination phenomena, with the migration of neutrophils attracted by microbial pathogens through the capillary wall, towards the granulation tissue. Old lesions had mature granulation tissue rich in fibroblasts and poor in capillaries. Newly formed CD34-positive capillaries with a closed lumen were found in PGs with mature granulation tissue. Towards the center of the granulation tissue, inflammation was more intense, the capillaries having an open lumen. CD34 stained the newly formed capillaries, as well as interstitial dendritic cells in PGs with cystic potential. A capsule rich in fibroblasts and poor in capillaries, separating the granulation tissue from the surrounding alveolar bone, was observed at the periphery of the lesions. The maturation of granulation tissue evolves centripetally, from the periphery to the center of the lesions. As the maturation of granulation tissue took place, blood vessels progressively diminished and the inflammatory infiltrate was reduced.

The prevalence of RC varies between 6% and 55%, and that of PG ranges between 46% and 94% in PL cases. This variation is due to the different biopsy sampling methods and histopathological criteria used for the diagnosis of PL [19]. In our study, the frequency of PG and RC was 68.33% and 18.33%, respectively. Compared to other studies (Diegues *et al.* [3], who analyzed 255 cases, reporting a frequency of 53% for RC and 42% for PG), the frequency of RC was higher, and that of PG was lower. The study of Koivisto *et al.* [20] indicates a frequency of 40.4% for PG and 33.1% for RC. These differences are due to the higher number of patients

included in these studies. Our observations showed that the PLs were most frequently diagnosed in the maxilla, 35 cases (58.3%), in accordance with the results of other studies [20, 21]. Twenty-three (60.52%) cases of PGs and seven (63.63%) cases of RCs were located in the maxilla, while in six (54.54%) cases of PGs with cystic potential, the most frequent location was the mandible. The most frequently involved tooth was the lateral incisor, in 20 (33.3%) cases, with a moderate degree of inflammation and low capillary density. This is similar to the observations of other studies [22].

The intensity of inflammatory infiltrate in PLs was variable: 37 cases had grade II inflammation and 16 cases had grade III inflammation, which suggests a moderate and severe inflammatory response in most of the PL PGs. Martín-González *et al.* [23] investigated the inflammatory infiltrate and leptin expression in 15 human periapical granuloma samples. All PG had inflammatory infiltrate grade III and showed expression of the adipokine leptin. In our study, out of 38 PG, four had inflammatory infiltrate grade I, 28 grade II, and six grade III.

The limitations of the study include the small size of the studied sample and the old age of most periapical lesions. In this study, no significant correlations were established in PLs between capillary density and the histological degree of inflammatory infiltrate. This is mainly due to the advanced age of the lesions. Based on our observations, the number of capillaries in PL decreased along with the age of the lesions. As most of the lesions were old, a high capillary density with an angiogenic index 3 was detected only in two cases. Lima *et al.* [24] studied angiogenesis and mast cell infiltration in PLs by immunohistochemistry, using CD34 and CD105 markers. Angiogenesis was correlated with mast cell density in radicular cysts and periapical granulomas, the results showing no differences between RCs and PGs regarding angiogenesis and mast cell density. Under local hypoxia conditions, macrophages and T-lymphocytes secrete pro-angiogenic factors (VEGF, angiopoietin 1/2, MMP-2, -7, -9), together triggering angiogenesis and exacerbating inflammatory response [25]. Inflammation is also associated with immune response, which is demonstrated by the lymphocytes present in the inflammatory response. The interstitial dendritic cells detected by CD34 immunostaining in the present study are cells that initiate the immune response involved in the pathogenesis of PLs.

Conclusions

PG with and without epithelium and RC represent different stages in the development of the periapical inflammatory process associated with the proliferation of new capillary vessels. Capillary density within PL does not influence the severity scores of lesions detected on radiographs. The angiogenic index appears not to be associated with the histological type of lesion and the intensity of inflammatory infiltrate, but was more likely correlated with the degree of granulation tissue maturation and the size of PLs.

Conflict of interests

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

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