Detection and Assessment of Interleukin 6 in Irreversible Pulp Inflamation

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The study aimed to assess the number, localization and distribution of interleukin 6 (IL-6) positive cells in healthy pulp, acute and chronic pulpitis. The study group included 48 patients aged between 18-72, treated in University of Medicine and Pharmacy Grigore T. Popa Iasi, Romania. The pulpectomy was performed on 42 patients diagnosed with acute and chronic pulpitis. The other 6 patients, without signs of dental caries or periodontal disease, were submitted to extractions of teeth for orthodontic purposes, with pulpectomy performed before extraction. The pulp samples were examined with optic microscope. The detection and assessment of IL-6 were performed using immunohistochemical technique. Data were statistically analysed using non-parametric tests. According to morphopathological criteria, 42.85% were classified as acute pulpitis and 57.14% as chronic pulpitis. The pulp samples in control group were not associated with IL-6 positive cells. The analysis of all samples with acute and chronic pulpitis identified 73.80% samples with IL-6 and 26.20% associated with the absence of IL-6. The highest frequency of IL-6 positive cells was recorded in rich-cell zone of crown dental pulp. The systemic distribution of IL-6 positive cells was mostly diffused without well-defined orientation. IL-6 release in acute and chronic pulpitis is significantly higher comparing with healthy pulp tissue.

Keywords: acute pulpitis, chronic pulpitis, cytokine, ekoscope

Dental pulp reacts against repeated exposures to bacteria due to autoimmune specific and nonspecific mechanisms. The pulp contamination take place through various invasion pathways related with dentinal tubules[1]. The level of pulp damage varies from mild inflammation to pulp necrosis associated with periapical lesions [2]. The researches regarding pulp tissues identified immune cells and cell that have the ability to recognize foreign antigens. The pulp-dentinal tissue is infiltrated initially with chronic inflammatory cells, like macrophages, lymphocytes and plasmatic cells [3]. The analysis of cytokines represents an essential key to understand the etiopathogenic reactions of different dental diseases [4]. The cytokines are soluble proteins that play an important role in the initiation and maintaining of inflammatory immune responses as well as in intercellular communications. These cytokines include interleukins (IL): IL-1α, IL-1 β, IL-6, IL-8 and necrosis tumor factor (TNF-α). Neutrophils and macrophages are major producers of interleukins; other cells that release interleukins are phagocytes, vascular endothelial cells, and keratinocytes [5]. One of the most important interleukin intervening in oromaxillofacial area is interleukin 6 (IL-6); IL-6 influences the evolution of pulpitis, periapical lesions, chronic marginal periodontitis, gingivitis, odontogenic cysts, lichen planus [6-8]. Interleukin-6 is a multifunctional cytokine with both pro-inflammatory (induces acute phase reactants in liver) and anti-inflammatory (regulates neutrophils function and secretion of pro-inflammatory cytokines) features, as a response to trauma and infectious agents [9-12]. IL-6 is able to stimulate a number of biological processes including antibody production, cells T activation, cells B differentiation, proteins growth in acute phase, hemato-poiesis, angiogenesis, vascular permeability, osteoclasts differentiation [13, 14]. IL-6 is produced as a result of interactions between gram negative bacteria, their metabolites (exotoxins, endotoxins) and various inflammation mediators (neuropeptides, kinines, complement system, metabolites), in relation with quantity, time and pathogenicity [9-12, 15].

Within this context, our study aimed to assess the number of IL-6 positive cells, localization and distribution in healthy pulp, acute pulpitis and chronic pulpitis.

Experimental part

The study group included 48 patients, 28 men and 20 women, aged between 18-72 years and the average age was 48 years, treated in Clinical Base of Medical Dental Teaching Mihail Kogalniceanu – University of Medicine and Pharmacy Grigore T. Popa Iasi, Romania. The pulpectomy was performed to 42 patients, diagnosed with acute and chronic pulpitis. The other 6 patients, without signs of dental caries or periodontal disease, were submitted to extractions of teeth for orthodontic purposes, with pulpectomy performed before extraction.

The study has been approved by a research ethics committee of Medicine and Pharmacology University

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Grigore T.Popa Iasi, Romania (approval protocol number 10353). The research was performed accordingly to the Assessment of Helsinki World Medical Association.

Clinical and morphopatological diagnosis

The clinical diagnosis of acute pulpitis was performed in relation to a number of symptoms and signs: (i) symptomatology (spontaneous pain as pulp hypersensitivity), (ii) positive intense response to sensitivity pulp test (prolonged pain, over 8 min , after the removal of cold stimulus, represented by the application of *tetrafluorethane*, Pharmaethyl Spray, Septodont®), (iii) pain provoked by tooth percussion. For chronic pulpitis study group the diagnosis was reported according to the next symptoms and signs: (i) symptomatology (provoked and/ or spontaneous pain as pulp hyposensitivity), (ii) mild positive response to pulp sensitivity testing (after the removal of cold stimulus, represented by the application of tetrafluorethane, Pharmaethyl Spray, Septodont®), (iii) blood and pain after palpation of exposed pulp tissue [16-18].

The testing of pulp sensitivity was performed comparatively with homologous teeth. The criteria of morphopathological diagnosis for acute pulpitis were as follows: vascular congestion, polymorphic inflammatory infiltration, edema, destructive alterations of odontoblastic layer. The criteria of morphopathological diagnosis for chronic pulpitis were as follows: fibrous and sclerotic atrophy, chronic inflammatory infiltrate, hyaline degeneration and cellular detritus [19].

Therapeutic protocole of vital pulp extirpation (pulpectomy)

Irreversibly inflamed dental pulp is removed (by pulpectomy) for curative therapy of this diseases. The pulpectomy was performed accordingly to defined standards: locoregional anesteshia (Ubistesin Forte 4%, 3M ESPE®), operatory field isolation with diga and salivary aspiration system, tooth antiseptisation with NaOCl 2% (Chloraxid 2%, Cerkamed®, PL), access cavity on occlusal surface. Working length was established at 1 mm to radiographic apex and it was compared with working length detected on apex locator Root ZX II (J. Morita®, U.S.A.). Permeabilisation of endodontic space was performed with Kerr file (Sendoline-Poldent®, PL) to create a space to facilitate the release of the conjunctive tissue and its removal with Tire Nerf files (Sendoline-Poldent®, PL). The root canal was further enlarged (files Protaper, Dentsplay®, U.S.A.), irrigated with antiseptic agents (NaOCl 2% - Chloraxid 2%, Cerkamed®, PL, oxygenated water 3% - Tis Farmaceutic®, RO) and root filling was performed (Sealapex, Kerr Corporation®, U.S.A., guta-percha cones Protaper, Dentsplay®, U.S.A.) in the same therapeutic session [16].

Morphopatological examen

The pulp tissues were immersed, immediately after collecting, in neutral buffered formaline 10%. After fixation the samples were processed as follows: introduction in paraffin using the device Leica TP1020 (Leica Microsistem GmbH®, D), longitudinal cut with microtom (5μm) SLEE CUT 6062 (Slee Medical GmbH®, D), the removal of paraffin and standard coloration with Hematoxiline-Eosine (HE) and special coloration with Hematoxiline Mayer (HM). The detection and assessment of IL-6 were performed using immunohistochemical technique (IHC). We applied the protocole specified by producer of kit Novolink Polymer DS RE7140-CE - Leica Novocastra GmbH®. The preparation of samples included the removal of paraffin in two xylene solutions (I + II), 2 times, 30 min, immersion in alcohol solution for 10 min; immersion in modified alcohol solution (90 mL alcohol + 10 mL methyl alcohol); the blocking of peroxidase activity using oxygenated water (4 mL H_2O_2 3% + 1 mL methanol) – 15 min. RT; three alcohol baths 95, 80, 50° – 5 min for each immersion; immersion in doubled distilled H_2O – 5 min.; TBS solution – 5 min; proteine Block – 10 min; incubation with Antibody I – 1 hour – RT – 1:50 (dilution); post-primary incubation – 30 min – RT; washing TBS – 2x5 min; incubation with polymer novolink – 30 min – RT; washing TBS - 2x5 min ; DAB – 5 min (50 μLDAB + 1 mL DAB substrate) (DAB/diaminobenzidine); tap water washing – 3 min+ distilled water washing – 3 min; hematoxylin – 3 min; tap water – 3 min + H_2O dd – 3 min; alcohol 50, 80, 95° – 2 min for each immersion; modified alcohol solution 5 min; alcohol absolute 5 min ; xylene I + II – 10 min for each immersion; lamella assembly; microscope examination. The pulp samples were examined with optic microscope Leica DM 750 (Leica Microsistem GmbH®, D). The acquisition of images was performed with digital foto device Leica ICC50 HD (Leica Microsistem GmbH®, D). The photos were processed using Leica Software Application Suit (LAS) 4.2. All technological stages were performed in Pathological Anatomy Discipline of University of Agricultural Sciences and Veterinary Medicine Ion Ionescu de la Brad Iasi, Romania.

Semi-quantitative assessment

For each pulp sample were assessed 7 microscopic fields randomly selected, using magnification from x200 to x400; the aim was to record the number, coronal/root localization, diffuse/compact distribution of cells expressing positive IL-6 (colored IHC with brown substrate). The samples were classified in relation to number of IL-6 positive cells, we used scores as follows: (i) score 0- absence of IL-6, (ii) score 1- IL-6 poor expression IL-6 (<3 pro-inflammatory cells/ 5 cm^2), (iii) score 2- IL-6 mild expression (<6 pro-inflammatory / 5 cm^2), (iv) score 3- high level of IL-6 expression (>6 pro-inflammatory cells / 5 cm^2). This classification was performed using device Ekoscope (Eon Trading LLC), based on optic microscopy, and used for calculation of somatic cells in complex research applications. The final score for acute and chronic pulpitis samples was obtained by calculation of mean values compared with morphopathological diagnostic. This method allowed the establishment of clear limits between IL-6 values between acute and chronic pulpitis study groups.

Statistic analysis

To compare morphopathological diagnostic with IL-6 values between acute and chronic pulpitis study groups, data were statistically analysed using non-parametric test Mann-Whitney and Kruskal-Wallis, with $p=0.05$.

Results and discussions

Accordingly to morphopathological criteria, 18 cases (42.85%) were classified as acute pulpitis and 24 cases (57.14%) as chronic pulpitis. The pulp samples in control group were not associated with IL-6 expression. In the study group with acute pulpitis, the scores distribution was as follows: three cases (16.66%) with score 0, for cases (22.22%) with score 1, five cases (27.77%) with score 2 and six cases (33.33%) with score 4 (table 1). In the study group with chronic pulpitis, the scores distribution was as follows: eight cases (33.33%) with score 0, six cases (25%) with score 1, seven cases (29.16%) with score 2 and three cases (4.16%) with score 4 (table 1).

Score	IL6 marked				Total
	Absent marking	Showed weak marking	Average mark	Intense mark	
0 Pulpitis chronic	8				8
acute	3				3
Total	11				11
1 Pulpitis chronic		6			6
acute		4			4
Total		10			10
2 Pulpitis chronic			7		7
acute			5		5
Total			12		12
3 Pulpitis chronic				3	3
acute				6	6
Total				9	9

Table 1
PULPITIS * IL6 MARKED * SCORE

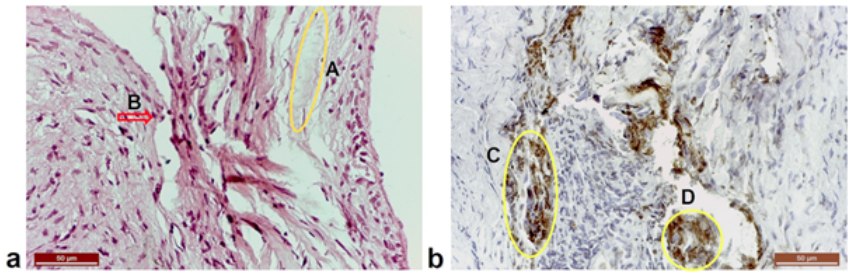

Fig. 1. Acute pulpitis (a) with: edema (A) and cell infiltration (B) among conjunctive structures of pulp, mechanical dilaceration and moderate leukocyte diapedesis (Col. HE, x400), respectively acute pulpitis with high level of IL-6 expression (b) *(brown surface staining)* and cluster arrangement (C) and (D) (IHC, anti IL-6, x400)

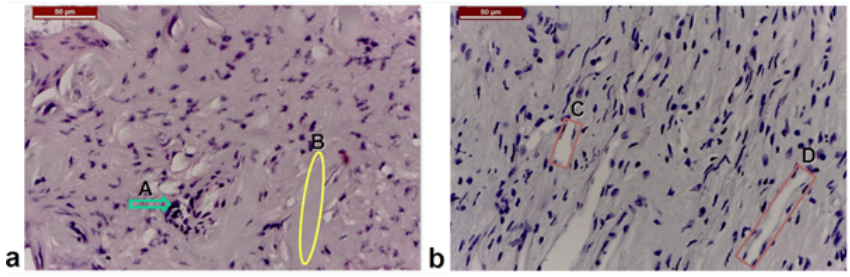

Fig. 2. Chronic pulpitis (a) with fibroblastic proliferation (A) and disordered arrangement of collagen fibers (B) (Col. HE, x400) and respectively chronic pulpitis (b) with proliferative fibrous, sclerosis, neoformation vessels and with IL-6 absence (C) and (D) (IHC, anti IL-6, x400)

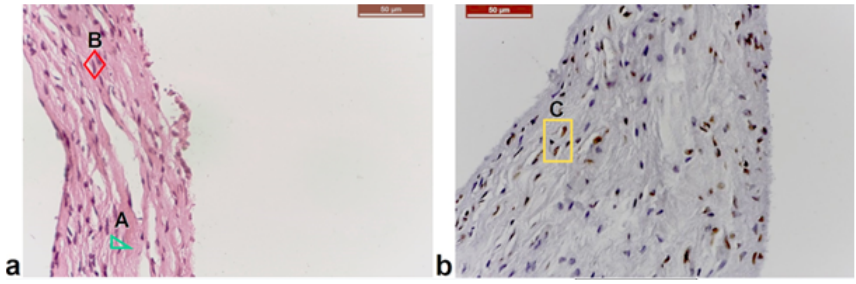

Fig. 3. Chronic pulpitis (a) with collagen fibers (A) and fibroblastic proliferation (B) (Col. HE, x400), respectively (b) chronic pulpitis with IL-6 poor expression *(brown surface staining)* (C) (IHC, anti IL-6, x400)

The analysis of all samples with acute and chronic pulpitis identified 31 pulp samples (73.80%) with cells expressing positive IL-6 and 11 pulp samples (26.20%) associated with the absence of positive IL-6, perhaps due to anti-inflammatory medication.

Regarding location of IL-6 in pulp tissue, the highest frequency was recorded in rich-cell zone of crown dental pulp, perhaps due to the higher number of fibroblasts, macrophages, neutrophils and vascular endothelial cells.

The systemic distribution of IL-6 was mostly diffused without well-defined orientation, excepting seven cases where it was found a cluster distribution (fig. 1).

The specific features of chronic pulpitis samples were as follows: high level of collagen fibers in central pulp area, fibroblastic growth, disordered collagen fibers, chronic inflammatory infiltrate, diffuse calcification around blood vessels (fig. 2 and 3).

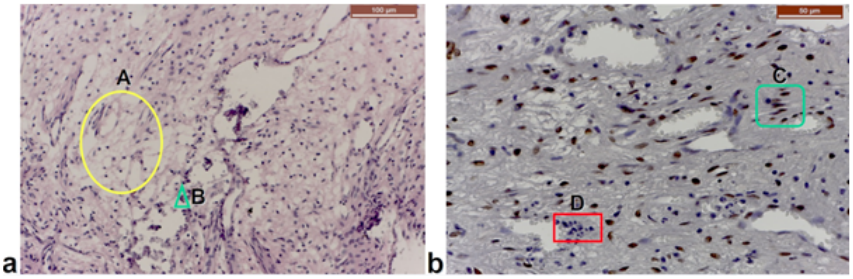

Fig. 4. Acute pulpitis (a), pulp edema (A) with dissociation of pulp tissue, leukocytes exudates (B) and pulp congestion (Col. HE, x200), respectively acute pulpitis (b) with IL-6 mild expression (C)(*brown surface staining*), IL-6 found in cytoplasm of macrophages, lymphocytes, fibroblasts, neutrophils (D) (IHC, anti IL-6, x400)

In chronic pulpitis samples have been identified the peripheral odontoblastic layer. The specific features of acute pulpitis samples were as follows: vascular congestion, pulp edema, leukocytes exudate, cells detritus (fig. 1 and 4).

The recorded data were statistically analysed. The mean rank of acute pulpitis was higher comparing with chronic pulpitis. Test Mann-Whitney (comparing the mean scores values between study groups) did not found the existence of significant statistical differences (U 153.5, $p = 0.101 > 0.05$) (table 2).

Table 2

TESTS STATISTICS[a]

	scor
Mann-Whitney U	153.500
Wilcoxon W	453.500
Z	-1.642
Asymp.Sig. (2-tailed)	**.101**

a. Grouping Variable: Pulpitis

For category score zero, mean values rank of chronic pulpitis is equal to mean values rank of acute pulpitis. Related to samples classified in score zero, there is no significant statistical differences between chronic pulpitis and acute pulpitis (U 12.0, $p = 1 > 0.05$). For category score one, mean values rank of chronic pulpitis is higher comparing to mean values rank of acute pulpitis. Related to samples classified in score one, were found significant statistical differences between chronic pulpitis and acute pulpitis (U 4.0, $p = 0.046 < 0.05$). For category score two, the mean values rank of chronic pulpitis is also higher than acute pulpitis. Significant statistical differences were found between chronic pulpitis and acute pulpitis classified as score two (U 4.0, $p = 0.017 < 0.05$). In score three category, the mean values rank of chronic pulpitis is lower comparing with acute pulpitis, without significant statistical differences (U 5.0, $p = 0.258 > 0.05$).

Despite high number of studies describing pulp pathology in relation to clinical and therapeutical issues, a few number of studies are focused on the relation between morphopathological diagnostic and presence of IL-6 associated with inflammatory processes in pulp tissue [3,7,15,20-24]. The results of our study, related to localization, distribution, number, presented similar values with those described by literature data. In this context, we must highlight a few relevant aspects regarding the correspondences between histopathological and immunological issues. The release of IL-6 is stimulated by peptidoglycans derived from gram positive bacteria in carious dentine; the concentration of IL-6 released by pulp cells is dependent by action time and peptidoglycans dose [15]. Tokuda M. et al. (2001) demonstrated the stimulation of IL-6 secretion in pulp human cells under the action of lipopolysaccharides Prevotella intermedia. The fibroblasts are implied in the pathogenesis of pulp inflammation through IL-6 production, accordingly to Lin S.K. et al. (2002). The release of excessive IL-6 levels conducts to transformation of acute reversible phase of pulp inflammation in chronic ireversible phase [25]. Elsalhy M. et al. (2013) found significant higher levels of IL-6 in pulp samples of teeth exposed to deep dental caries. The ratio IL-6/IL-10 is significantly higher in pulp tissue with ireversible pulpitis, comparing with pulp tissue derived from teeth with deep dental caries. The author considers that ratio IL-6/ IL-10 can be considered a marker of pulp inflammation in samples of pulp tissue derived from teeth with deep dental caries [22]. The increase of IL-6 levels is also demonstrated by Barkhordar RA et al. (1999) in pulp tissue collected from teeth with deep dental caries. Also in this study, mean value of IL-6 levels was 36 +/- 3.9 pg/mg protein in samples with chronic pulpitis and only 0.01 +/- 0.02 pg/mg protein in healthy pulp tissue samples. Park H.S. (2002) determined, using ELISA test, levels of IL-6 in dental pulp with chronic pulpitis. The mean level of IL-6 in sample with tissue affected by pulp inflamation was 43.62 pg/mg protein while control group (healthy pulp tissue) had a mean value of 24.41 pg/mg protein. In same study, author found the existence of PMN inflammatory infiltrate in tissue affected by pulp inflammation and the absence of PMN infiltrate in control group. Also Nakanishi T. et al. (1995) showed the existence of higher levels of IL-6 in samples with pulp inflammation comparing with healthy pulp tissue.

Despite the detection of IL-6 both in incipient and advanced stages of pulp inflammations, the exact role of these cytokines in the pathogenesis and progression of pulp inflammation is not well defined.

Conclusions

The mean values rank of scores for acute pulpitis is higher comparing with chronic pulpitis. For score one with U 4.0, $p = 0.046 < 0.05$ and score 2 with U 4.0, $p = 0.017 < 0.05$, values rank in chronic pulpitis is higher comparing with values rank in acute pulpitis. The mean rank for score 3 was lower in chronic pulpitis, comparing with acute pulpitis, without significant statistical differences - U 5.0, $p = 0.258 > 0.05$. The number of IL-6 positive cells in study group was higher comparing with control group ($p < 0.05$). Higher frequency of IL-6 was recorded in pulp cell-rich zone of crown dental pulp. The systemic distribution of IL-6 was mostly diffuse without well-defined orientation.

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