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Diabetes increases interleukin-17 levels in periapical, hepatic, and renal tissues in rats

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ABSTRACT

Objectives: This study aimed to evaluate the association between endodontic infection and diabetes on interleukin-17 levels in periapical, hepatic, and renal tissues of rats.
Design: Forty male rats were divided into groups: normoglycemic rats (N), normoglycemic rats with apical periodontitis (N-AP), rats with experimental diabetes (ED), and rats with experimental diabetes and apical periodontitis (ED-AP). Diabetes was induced by intravenous streptozotocin injection, and blood sugar levels were monitored to confirm disease development. Apical periodontitis (AP) was induced by pulp exposure to the oral environment during 30 days. After 30 days, hepatic and renal tissues were obtained, and IL-17 levels were quantified by ELISA. The right hemi-jaw was used to quantify IL-17 levels by immunohistochemistry. The values obtained in parametric tests were tabulated and analyzed statistically by analysis of variance (ANOVA) and Tukey tests, and the values obtained for scores were statistically analyzed by using the Kruskal-Wallis and Dun tests. The level of significance was set at 5%.
Results: ED and ED-AP groups expressed significantly higher IL-17 levels in both hepatic and renal tissues ($p < 0.05$), compared to N and N-AP groups. Apical periodontitis (AP) in ED-AP group was significantly more severe than that in N-AP group ($p < 0.05$). Furthermore, there was a significantly larger increase in the IL-17 levels in ED-AP group compared to N group ($p < 0.05$).
Conclusion: Our results indicate that diabetes increases IL-17 levels in hepatic and renal tissues and also enhances IL-17 production in apical periodontitis area of rats.

1. Introduction

Apical periodontitis (AP) is an infectious disease characterized by the destruction of periradicular tissues and is mediated by cytokines secreted from immunocompetent cells that infiltrate the periapical tissues in response to intracanal bacterial infection (Kawashima et al., 1996). Previous studies which investigated the association between oral infections and diabetes have shown that AP and periodontal diseases increased triglyceride levels (Cintra et al., 2013), blood glucose concentrations (Cintra, Samuel, Facundo et al., 2014), serum inflammatory cells (Cintra, da Silva Facundo et al., 2014), as well as altered organs weight of diabetic rats (Cintra et al., 2017). It's well established that diabetes alone exerts deleterious effects in some organs, including kidney and liver, as a consequence of elevated blood glucose levels (Lee et al., 2008). However, to the best of our knowledge, none of them has ever investigated the impact of AP in the inflammatory mediators in those organs of diabetic rats.

Interleukin-17A (IL-17) is the founding member of a novel family of pro-inflammatory cytokines consisting of at least six members, IL-17A-17F (Aggarwal & Gurney, 2002). IL-17 has potent effects on numerous cells of the innate immune system, particularly on the granulocyte lineage, and it is important in bridging the adaptive and innate immune systems (Yu & Gaffen, 2008).

Many reports describe the presence of IL-17 in AP (Marçal et al.,

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2010; Xiong, Wei, & Peng, 2009) and it is known to contribute to the exacerbation of chronic AP (Colić et al., 2014), as it might be linked to the acute process of AP (Marçal et al., 2010). Furthermore, increased IL-17 serum levels have also been previously reported in a study investigating the association between AP and periodontal disease in rats (Cintra, Samuel, Azuma et al., 2014). IL-17 plays an active role in diabetes pathogenesis (Kumar, Natarajan, & Shanmugam, 2013; Wang, Chueh et al., 2011; Wang, Yang et al., 2011), especially in relation to its deleterious role in maintaining the integrity of pancreatic islets and in glycemic control (Kumar et al., 2013; Sumarac-Dumanovic et al., 2013; Wang, Chueh et al., 2011; Wang, Yang et al., 2011). Diabetes is further associated with increasing the severity and progression of AP (Armada-Dias et al., 2016; Fouad & Burleson, 2003; Iwama et al., 2003), resulting in recovery failures following non-surgical endodontic treatments (Brito, Katz, Guelmann, & Heft, 2003; Wang, Chueh et al., 2011; Wang, Yang et al., 2011). Considering that IL-17 has an important role in both diseases, diabetes and AP, it would be interesting to evaluate if diabetes can potentiate the production of IL-17 locally in AP site. It is therefore important to understand the relationship between diabetes and AP with respect to the systemic effects in rats. The aim of this study was to evaluate the influence of diabetes on AP progression and on IL-17 levels in periapical tissues, and also evaluate the association of diabetes and/or AP on IL-17 levels in hepatic and renal tissues.

2. Materials & methods

2.1. Induction of diabetes, apical periodontitis, and sample preparation

Male Wistar rats (*Rattus norvegicus albinus*, n = 40) that weighed 250–300 g and were 6 weeks of age were used in the study. The rats were housed in mini-isolators for rats (Alesco, São Paulo, Brazil), kept in temperature-controlled rooms (25 ± 1 °C) and given *ad libitum* access to water and food. The experimental protocol was approved by and conducted in accordance with guidelines of the institutional ethical committee, and in accordance with the U.K. Animals (Scientific Procedure) Act, 1986.

The rats were fasted overnight (14–16 h), and the tail-tip blood used to measure blood glucose levels using a blood glucose monitoring system (Accu-Check® Performa, Roche Diagnostics Corporation, IN, USA). The rats were subsequently intramuscularly anesthetized with 87 mg/kg ketamine (Francotar, Virbac do Brasil Ind. Com. Ltda., São Paulo, Brazil), and 13 mg/kg xylazine (Rompum, Bayer S.A., São Paulo, Brazil). The rats were then randomly assigned into four groups (n = 10 rats/group): normoglycemic rats (N); normoglycemic rats with apical periodontitis (N-AP); rats with experimental diabetes (ED), and rats with experimental diabetes and apical periodontitis (ED-AP). The, either citrate buffer solution or streptozotocin (Sigma- Aldrich Corp., MO, USA) was injected into the penile vein. The citrate buffer solution (0.01 M, pH 4.5) was injected in groups N and N-AP, and streptozotocin, which was dissolved in citrate buffer solution at 35 mg/kg body weight and used for the experimental induction of diabetes, was injected in groups ED and ED-AP (Cintra et al., 2013; Cintra, Samuel, Facundo et al., 2014).

Six days after diabetes was induced, blood samples were collected from each rat and their blood glucose levels determined. Rats with blood glucose levels > 200 mg/dL were used in this study (Cintra et al., 2013; Cintra, da Silva Facundo et al., 2014; Garber, Shabahang, Escher, & Torabinejad, 2009).

Once hyperglycemia was confirmed, the animals were anesthetized as previously described, and endodontic infection was induced in groups N-AP and ED-AP as follows: pulps of the first upper right molars were exposed to the oral eviroment, using surgical round burs (Broca Ln Long Neck-Maillefer, Dentsply Ind. Com. Ltda, Rio de Janeiro, Brazil), for 30 days (Cintra et al., 2013; Cintra, da Silva Facundo et al., 2014; Garber et al., 2009). The rats were killed with an overdose of the anesthetic solution after 30 days. The left kidney and a liver fragment from each rat were immediately collected and preserved in liquid nitrogen to avoid cytokine degradation prior to the determination of IL-17 levels in the hepatic and renal tissues.

2.2. ELISA

Kidney and liver tissue fragments were used to quantify IL-17 levels by the enzyme-linked immune-sorbent assay (ELISA) capture technique. Briefly, 0.2 g of tissues and 800 µl of sterile phosphate-buffered saline (PBS), pH 7.0, were kept on ice and ground in a tissue homogenizer (Ultraturrax T8, IKA, Germany) for approximately 5 min. The resulting homogenate was thereafter centrifuged (10,000*g*, 15 min, 4 °C) and the supernatant immediately stored at −80 °C (Revco, Twinsburg, Ohio, USA). To determine the IL-17 levels, 100 µl of the supernatant was assessed using ELISA commercial kits (Rat IL-17A ELISA MAX™ Deluxe, cat #437904; Biolegend, San Diego, CA, USA) following the manufacturer's instructions.

2.3. Histological analysis

Maxillae from sacrificed rats were removed, post-fixed in neutral buffered formalin for 48 h, decalcified during 4 weeks in buffered (pH 8) 10% EDTA (Sigma Chemical Co, St Louis, MO, USA), rinsed in sterile water, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Serial slices (5 µm) were prepared in a mesiodistal plane and stained with hematoxylin and eosin. The slices were examined sequentially under an optical microscope (DM 4000 B, Leica, Wetzlar, Germany) and the intensity and extension of the inflammatory infiltrate evaluated. The average number of cells per field and the extension beyond the apical foramen (AP groups) were considered. The number of cells in each experimental group (n = 10/group) was calculated (100× magnification). The intensity of inflammation was graded as follows: absent (0 to few inflammatory cells, score = 1), mild (< 25 cells, score = 2), moderate (25–125 cells, score = 3) and severe (> 125 cells, score = 4). The extension of inflammation was graded as follows: absent (score = 1), mild (inflammatory cells extending up to 300 µm beyond the tooth apical foramen, score = 2), moderate (inflammatory cells extending up to 600 µm beyond the tooth apical foramen, score = 3), and severe (inflammatory cells extending more than 600 µm beyond the tooth apical foramen, score = 4).

For AP and AP-O groups, the area of periapical lesion associated with the distal root of the maxillary first molar was histometrically meausured. The area was calculated by rounding up the lesion boundary, considering the outer external surface of alveolar bone, and it was expressed in square micrometers. For each rat, 5 serial histological sections were histometrically measured by an image processing system that consisted of a light microscope (DM 4000 B, Leica, Wetzlar, Germany), color camera (DFC 500, Leica), color image processor (Leica Qwin V3 software Leica Microsystems, Wetzlar, Germany) and a personal computer (Intel Pentium 4, 2.80 GHZ, Windows XP SP3). The AP areas were determined for each slice, and the average value (mean ± standard deviation) was calculated for each experimental group.

2.4. Immunohistochemistry

For immunohistochemical reactions, antigen retrieval was achieved by immersing the histological slices in buffer solution (Diva Decloaker®; Bio- care Medical, CA, USA) in a pressurized chamber (Decloaking Chamber®, Biocare Medical, CA, USA) at 95 °C for 10 min. The slices were rinsed with 0.1 M PBS (pH 7.4) at the end of each stage of the immunohistochemical reaction. The histological sections were immersed in 3% hydrogen peroxide for 1 h and in 1% bovine serum albumin for 12 h to block endogenous peroxidase activity and nonspecific sites, respectively. The histological slices containing samples from all the experimental groups were divided into three batches, and each batch was incubated with one of the following primary antibodies:

rabbit anti-rat IL-17 (SC-7927, 1:100, Santa Cruz Biotechnology, CA, USA). The primary antibodies were diluted in PBS with 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) and were placed at the slices in a moist chamber for 24 h. The histological sections were incubated with a biotinylated secondary antibody (SC-2041, 1:100; Santa Cruz Biotechnology, CA, USA) for 2 h and subsequently treated with streptavidin–horseradish peroxidase conjugate for 1 h (Universal Dako Labeled Streptavidin-Biotin kit®, Dako Laboratories, CA, USA). The reaction was developed using the 3,3′-diaminobenzidine tetrahydrochloride chromogen (DAB chromogen kit®, Dako Laboratories, CA, USA) and counterstained with Harris's hematoxylin.

2.5. Immunohistochemical analysis

Histological sections were analyzed under bright field microscopy by a certified histologist (E.E.). The values for each section were measured three times by the same examiner on different days to reduce data variations. Positive immunostaining was defined as a brownish color in the cell cytoplasm. All of the histological sections were analyzed in the periapical region of the distobuccal root with a 250× magnification. Each analysis field displayed a 600–800 μm size and was positioned across the mesiobuccal root apex so that the entire root periapical region was contained in the analysis.

The scores were assigned as follows (Garcia et al., 2013): 1, complete absence of immunoreactive cells; 2 (low IR), a few immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immunoreactive cells); 3 (moderate IR), a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 4 (high IR), a large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immunoreactive cells).

2.6. Statistical analysis

The total assessed values obtained in parametric tests were tabulated and analyzed statistically by means of analysis of variance (ANOVA) and Tukey tests. The values obtained for scores were statistically analyzed by Kruskal-Wallis and Dun test. The level of significance was set at 5% in all the test

3. Results

The results showed that diabetic rats (ED and ED-AP) had significantly higher IL-17 levels in the hepatic and renal tissues as compared to the normoglycemic rats (N and N-AP) ($p < 0.05$) (Table 1). The presence of AP did not, however, significantly change the levels of IL-17 in the hepatic or renal tissues of diabetic and normoglycemic rats ($p > 0.05$) (Table 1).

The values obtained in the histological scores are shown in Table 2. No inflammation was observed in the periapical tissues of the normoglycemic rats or in rats with experimental diabetes without AP (N and ED) (Fig. 1). The groups with endodontic infection (N-AP and ED-AP) showed that AP was restricted to the periapical tissues. Furthermore, these lesions were mainly composed of neutrophils and mononuclear cells. Histologically, the inflammatory infiltrate of ED-AP group was significantly more severe than in the N-AP group ($p < 0.05$). Histometrically, the ED-AP group showed a larger AP covered area as compared to the N-AP group ($p < 0.05$) (Fig. 2 and Table 2).

Table 1
Summary of IL-17 expression levels in the experimental groups.

Groups	IL-17 LEVELS (pg/ml)	
	Renal tissue	Hepatic tissue
N	266.6 ± 63.3[a]	400.8 ± 94.5[a]
N-AP	271.8 ± 59.8[a]	416.2 ± 157.4[a]
ED	384.3 ± 96[b]	604.7 ± 116.4[b]
ED-AP	364.6 ± 63.1[b]	520.5 ± 129[b]

*Different superscript letters (a, b) in columns represent significant difference ($p < 0.05$).

Table 2
Representation of scores assigned to the criteria of intensity of the inflammatory infiltrate and inflammation extent and means of bone loss (μm).

Histological parameters	Scores	GROUPS			
		N	N-AP	ED	ED-AP
Inflammatory infiltrate	1 (absent)	10/10	0/10	10/10	0/10
	2 (mild)	0/0	1/10	0/10	0/10
	3 (moderate)	0/10	7/10	0/10	4/10
	4 (severe)	0/10	2/10	0/10	6/10
	Median	1[a]	3[b]	1[a]	4[c]
Inflammation extension	1 (absent)	10/10	0/10	10/10	0/10
	2 (mild)	0/10	3/10	0/10	0/10
	3 (moderate)	0/10	5/10	0/10	2/10
	4 (severe)	0/10	2/10	0/10	8/10
	Median	1[a]	3[b]	1[a]	4[c]
Bone loss	Mean (μm)	0[a]	0797712.1[b]	0[a]	0985946.7[c]

*Different superscript letters (a, b) in horizontal lines represent significant difference ($p < 0.05$).

The immunostained slices showed that a brownish color was observed in the extracellular matrix of some cells. The immunostaining was predominantly observed at the periodontal ligament, alveolar bone margins and the AP sites. The presence of IL-17 was observed in the following immunostaining patterns: low in N, low to moderate in ED, moderate in N-AP, moderate to high in ED-AP. The distribution of scores and the immunoreactivity patterns representative of each experimental group are shown in Table 3 and in Figs. 1 and 2. The AP groups (N-AP and ED-AP) had higher IL-17 levels compared to the groups without endodontic infection (N and ED) ($p < 0.05$). The experimental diabetes groups (ED and ED-AP) had higher IL-17 levels compared to the normoglycemic groups (N and N-AP) ($p < 0.05$). ED-AP group showed higher IL-17 levels compared to N-AP group ($p < 0.05$) (Fig. 2 and Table 3).

4. Discussion

Rats used in this study had uniform body weights and were initially normoglycemic. Diabetes mellitus was induced by injecting a subset of the rats with streptozotocin, and glucose levels were subsequently observed to be approximately 6-fold higher than in normoglycemic rats. Diabetic rats exhibited intense thirst, polyuria, and apathy. The overall metabolism of streptozotocin-induced diabetic rats is very similar to that of diabetic human patients (Kohsaka, Kumazawa, Yamasaki, & Suda, 1996). Male rats were used because male rodents tend to be more susceptible to streptozotocin-induced diabetes (Deeds et al., 2014). This decreased sensitivity experienced by females may be attributed to estradiol's ability to protect pancreatic β cells from apoptosis induced by oxidative stress (Le May et al., 2006). Blood glucose levels were consistently higher in the diabetic model group rats as compared to the normoglycemic control group, indicating that hyperglycemia persisted in the diabetic rats.

A model of oral infection was used as previously described (Cintra et al., 2013; Cintra, da Silva Facundo et al., 2014; Cintra, Samuel, Azuma et al., 2014; Cintra, Samuel, Facundo et al., 2014; Garber et al., 2009). A previous study reported maximal active lesion expansion and bone destruction between days 7 and 15 after pulp exposure in a rat model system in which periapical lesions had been induced (Kohsaka et al., 1996).

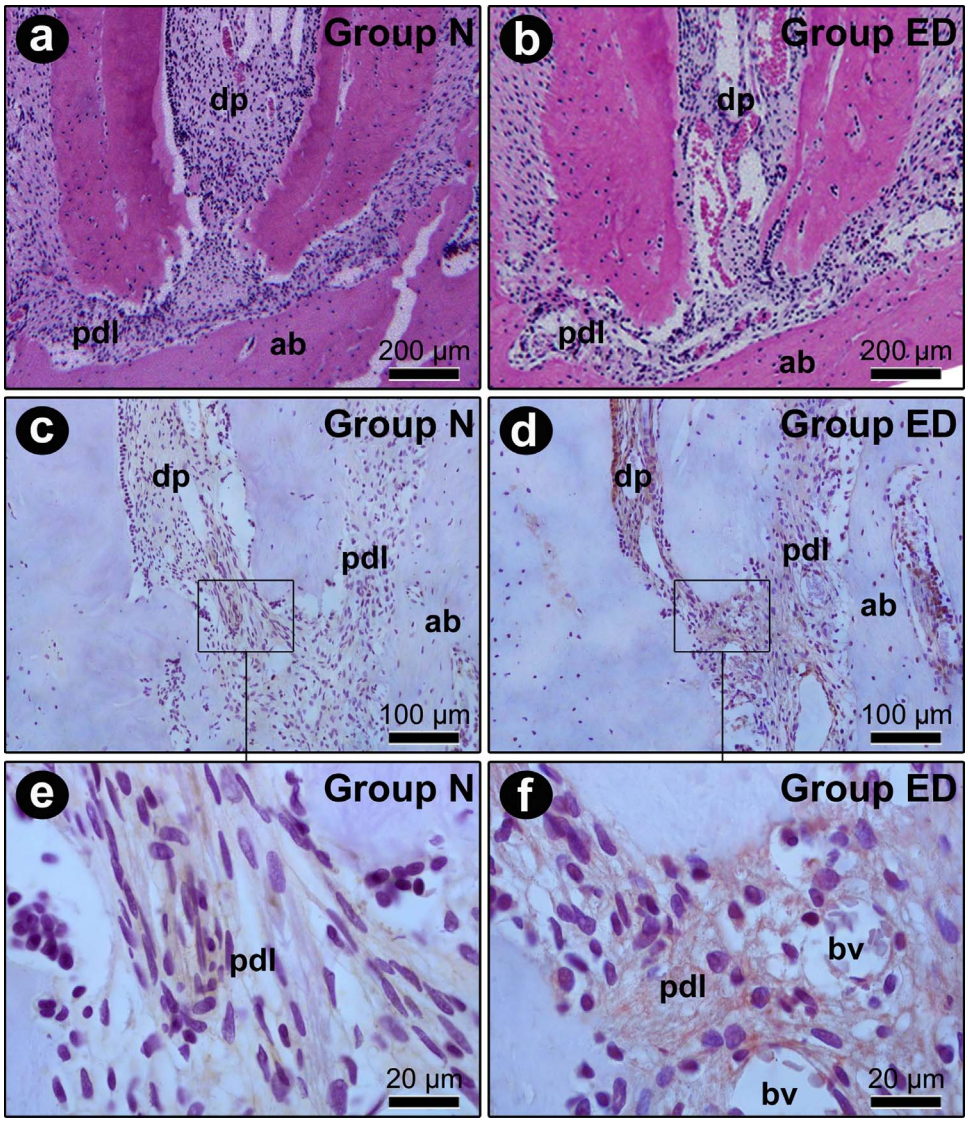

Fig. 1. Representative photographs of histological (a and b) and immunohistochemical analysis (c–f). (a) Periapical region of rats in the control group (hematoxylin-eosin staining, ×100). (b) Periapical region of diabetic rats without pulp exposure (hematoxylin-eosin staining, ×100). (c and e) IL-17 expression in the periapical tissues of rats in the control group (×100 and ×400, respectively). (d and f) IL-17 expression in the periapical tissues of rats in the diabetic rats (×100 and ×400, respectively). pdl, periodontal ligament; dp, dental pulp; ab, alveolar bone; bv, blood vessel.

To evaluate if AP may potentiate the deleterious effects caused by diabetes on systemic health, we evaluated the hepatic and renal tissues. Those two different organs were chosen because they may reflect the systemic effects caused by diabetes. The liver is an insulin-dependent organ that plays a key role on the homeostasis of glucose, and which can be severity affect by diabetes (Seifter & England, 1982). In addition, one of the most frequent deleterious effects of diabetes is the development of diabetic nephropathy, which is a consequence of renal injury caused by hyperglycemia (Greene et al., 2005). To date, there are no studies that quantified IL-17 levels in hepatic or liver tissues of diabetics.

The results of this study are consistent with studies, where increased IL-17 levels have been reported in lymphoid and pancreatic tissues (Simoni et al., 2011), as well as in the bloodstream (Boehm, Rosinger, & Sauer, 2009; Jain et al., 2008; Sumarac-Dumanovic et al., 2013). Although many studies have shown positive results in the improvement of diabetes by inhibiting IL-17 (Amirshahrokhi & Ghazi-Khansari, 2012; Emamaullee et al., 2009; Wang, Chueh et al., 2011; Wang, Yang et al., 2011; Zhang, Huang, Sun, Tian, & Wei, 2012) and increased levels of IL-17 associated with loss of glycemic control (Kumar et al., 2013; Simoni et al., 2011), the immunological mechanism of this cytokine in the pathogenesis of diabetes mellitus is still unclear.

Our results showed that AP did not impact the levels of IL-17 in hepatic and renal tissues, irrespective of the presence or absence of diabetes. Some reports have however shown that periodontal disease was capable of altering IL-17 serum levels in both normal (Schenkein et al., 2010) and diabetic patients (Santos et al., 2010). This is an important comparison because it is known that periodontal disease pathogenesis is similar to that of AP, and is represented by the response of the body against an aggressor agent, resulting in the release of inflammatory mediators and subsequent bone resorption (Silva, Garlet, Fukada, Silva, & Cunha, 2007). These results suggest that further studies are required to understand the influence of AP in general health during the presence or absence of systemic diseases.

The histological finding showed that diabetes accelerated the development and progression of AP in rats with the presence of an inflammatory infiltrate containing neutrophils and mononuclear cells, which is consistent with other studies (Armada-dias et al., 2006; Cintra, da Silva Facundo et al., 2014; Iwama et al., 2003). These results suggest that there is an intense inflammatory response in conditions of hyperglycemia, which is also in agreement with other findings in the literature (Cintra, da Silva Facundo et al., 2014; Iwama et al., 2013).

An increase in IL-17 levels in the periapical region of normoglycemic rats with AP [N-AP] compared to control group (N) ($p < 0.05$) and an increase in IL-17 levels in diabetic groups with apical periodontitis (DE-AP) compared to diabetic rats (DE) were observed in this study. The increase of IL-17 levels in the presence of AP found in our study is

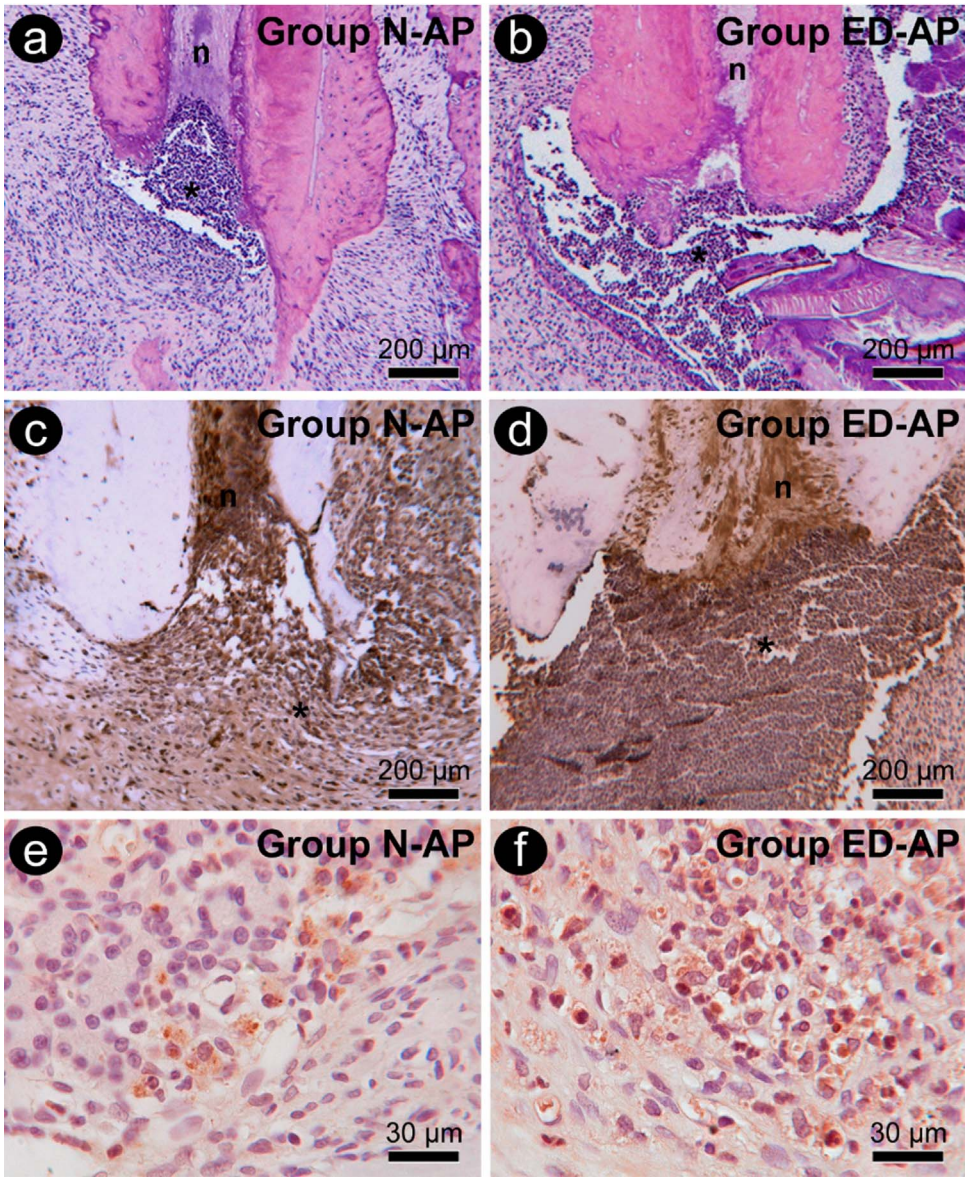

Fig. 2. Representative histological photographs (a and b) and immunohistochemical analysis (c–f). (a) Periapical region of normal rats with pulp exposure (hematoxylin-eosin staining, ×100). (b) Periapical region of diabetic rats with pulp exposure (hematoxylin-eosin staining, ×100). (c and e) IL-17 expression in the periapical tissues of normal rats with pulp exposure (×100 and ×400, respectively). (d and f) IL-17 expression in the periapical tissues of diabetic rats with pulp exposure (×100 and ×400, respectively). n, necrosis; * Site of infiltration of inflammatory cells.

Table 3
Representation of IL-17 immunoreactivity scores in the N, N-AP, ED, ED-AP groups.

Scores of IL-17 immunoreactivity	GROUPS			
	N	N-AP	ED	ED-AP
1 (Absent)	0/10	0/10	6/10	0/10
2 (low)	0/10	2/10	4/10	0/10
3 (moderate)	0/10	8/10	0/10	4/10
4 (high)	0/10	0/10	0/10	6/10
Median	1^{a}	3^{b}	1^{a}	4^{c}

*Different superscript letters (a, b) in horizontal lines represent significant difference ($p < 0.05$).

consistent with other studies in the literature (Marçal et al., 2010; Xiong et al., 2009). This increase may be explained because AP is characterized by a persistent migration infiltration of inflammatory cells such as neutrophils, lymphocytes, plasma cells, macrophages and mast cells (Takahashi, 1998). Previous investigations suggest that CD4+ T lymphocytes are the predominant infiltrating inflammatory cells present in AP and develop a role in this disease (Colić et al., 2007; Kawashima et al., 1996). IL-17 is a proinflammatory cytokine that is primarily secreted by T lymphocytes, which mediate the adaptive immune system (Iwakura, Ishigame, Saijo, & Nakae, 2011). In addition, IL-17 promotes the expansion and recruitment of cells of the innate immune defense system to amplify inflammation and neutrophil mobilization (Cua & Tato, 2010). It has been therefore suggested that IL-17 develops a protective role against infection by stimulating bone resorption in the periapical tissues (Xiong et al., 2009). Until now, there have been no studies comparing the amount of IL-17 present in the periapical region of diabetic rats with normoglycemic rats. The results of this study showed that there is an increase of IL-17 levels in the periapical region of diabetic rats without AP (ED) as compared to control rats (N) ($p < 0.05$). In addition, an increase of IL-17 levels in AP rats with experimental diabetes (ED-AP) was observed as compared to the normoglycemic rats with AP (N-AP) ($p < 0.05$). Studies show that diabetes acts in development, progression and severity of apical periodontitis (Armada-Dias et al., 2006; Iwama et al., 2003), increased risk of tooth loss (Brito et al., 2003; Wang, Chueh et al., 2011; Wang, Yang et al., 2011), as well as increased release of IL-17 (Marçal et al., 2010; Xiong et al., 2009). These findings are extremely important, since they suggest that IL-17 is a cytokine which is expressed in the development of AP during diabetes. Thus, other studies should be performed to better understand the mechanisms responsible for exacerbating bone resorption in diabetics.

5. Conclusion

The present study suggests that diabetes increases IL-17 levels in hepatic and renal tissues, as well as enhances the IL-17 production in AP.

Conflict of interest

None.

Ethical approval

Approved by Animal and Ethical Committee of the College of Dentistry Araçatuba, São Paulo State University [00540-2012].

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