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IL-17 Levels in Diabetic and Non-diabetic Experimental Periodontitis: a Pilot Study

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Abstract: Although the clinical relationship between periodontitis and diabetes mellitus (DM) is well established, few investigations have focused on the immunoinflammatory responses in sites with periodontitis in subjects with DM. Because of the potential association between periodontitis and diabetes, the aim of this study was to examine the serum levels of IL- 17 in diabetic and non-diabetic rats with experimental periodontitis before, and after non-surgical periodontal treatment, in an attempt to establish a correlation between diabetes and periodontitis utilizing IL-17 as a marker. Materials and Methods: This study was conducted on 60 male rats, which were divided randomly into 2 equal groups, each with 30 rats. In the first group, only experimental periodontitis was induced, while in the second group both experimental periodontitis and diabetes were induced. Estimation of the serum levels of IL-17 was done using the ELIZA technique. Results showed that non-surgical periodontal therapy tended to reduce serum IL-17 levels in diabetic and non-diabetic periodontitis groups. Conclusion: In view of the very high prevalence of both periodontitis and diabetes and their potentially severe repercussions, inflammatory cytokines such as IL-17 may substantiate increased risk of the systemic inflammatory burden.

Key words: IL-17; experimental; periodontitis; diabetes.

INTRODUCTION

Periodontitis is a local inflammatory process mediating destruction of periodontal tissues triggered by bacterial stimuli. However this disease is also characterized by presence of systemic inflammatory host response (Noack *et al* 2001). Numerous reports demonstrated a correlation between periodontal disease (PD) and many other systemic diseases including diabetes mellitus (DM). Although the mechanism is still poorly defined, one mechanism may be a "spill over" effect, in which inflammatory cytokines produced during chronic inflammatory responses influence susceptibility to systemic disease (Genco 2005).

Exciting new evidence has emerged related to the production of inflammatory cytokines by a subset of $CD4^+$ T-helper (Th) cells. This population plays an essential role in regulating autoimmune disease and inflammation by secreting a novel proinflammatory cytokine, IL-17; hence these cells are termed "Th-17" (Dong 2006). Although Th17 cells have been shown to play important roles in host defense against infection by recruiting acute inflammatory cells, experimental evidence supports a pathological role of Th17 cells during numerous systemic and organ-specific autoimmune diseases (Curtis and Way 2009).

For many years, periodontitis was described as an imbalance between Th1 and Th2 cytokine profiles (Gemmell and Seymour 2004). Recently, this concept has been defined by the discovery of Th17 and T regulatory cells and their related cytokines in periodontal lesions (Takahashi *et al* 2005; Gaffen and Hajishengallis 2008; Cardoso *et al* 2009). IL-17 was found to be locally produced by T cells in periodontal lesions. It was also reported that this cytokine may exacerbate the inflammatory reactions both directly and indirectly via inflammatory mediators from gingival fibroblasts in periodontal tissues (Takahashi *et al* 2005). However studies still debate whether the dominant role played by IL-17 in the pathogenesis of PD is host-protective or destructive (Kramer and Gaffen 2007).

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Recent studies suggested that Th-17 cells are involved in the pathogenesis of diabetes, and that development of Th-17-targeted therapeutic agents may be of benefit in this disease (Emamaullee *et al* 2009). The relationship between periodontitis and diabetes has been extensively investigated over the past twenty years. It has been suggested that there is a two-way relationship between diabetes mellitus (DM) and periodontal disease. Accordingly, DM is a known risk factor of PD (Mealey 2006; Bascones-Martinez *et al* 2011) but, also vice versa, PD may complicate the severity of diabetes by worsening the degree of glycemic control (Grossi and Genco 1998; Donahue and Wu 2001).

Literature reviews have indicated that the cytokine induced inflammatory state in periodontitis can contribute to the overall low-grade inflammation that occurs in diabetes and increased serum concentrations of acute-phase response markers and cytokines have been observed in diabetic patients (Santos-Tunes *et al* 2010). On the other hand sustained hyperglycemia in DM can impair host defense and wound healing, prolong the inflammatory response and induce microvascular alterations (Pontes-Andersen *et al.* 2007).

In experimental periodontitis and diabetes research, rodents have been the most used animals due to their short generation time, small size, ability to work with large numbers, easy access, handling, and housing, and lower cost compared to other species (Madden and Caton 1994; Chen and Wang 2005). In addition, they are often superior to in vitro or clinical trials in addressing mechanistic properties and serve as an essential link between hypothesis and human patients (Graves *et al*. 2008).

Since IL-17 was implicated in the pathogenesis of both periodontitis and diabetes, and since mechanisms underlying the association between PD and diabetes remain somewhat controversial, we sought to investigate a possible role of IL-17 as a link between PD and diabetes in rat models.

The aim of this study was therefore to examine IL-17 levels in diabetic and non-diabetic rats with ligature-induced periodontitis before and after non-surgical periodontal therapy in an attempt to establish a correlation between diabetes and periodontitis utilizing IL-17 as a marker.

MATERIALS AND METHODS

Animals:

This study was conducted on 60 male rats (100-120g). The animals were kept in plastic cages with access to food and water ad libitium in a temperature-controlled room with a standard 12/12h light- dark illumination cycle. The cages were kept under hygienic conditions and away from any source of chemical contamination according to the animal house protocol approved by the Ethical Committee of the National Research Center, Cairo, Egypt. Before the surgical procedures, all the animals were allowed to acclimatize to the laboratory environment for a period of 5 days.

Experimental Design:

The animals were divided randomly into 2 groups of 30 rats each:

- Group (1) (n=30) was considered the periodontitis group in which experimental periodontitis was induced.
- Group (2) (n=30) was considered the periodontitis and diabetes group in which both experimental periodontal disease and diabetes were induced.

Protocol of Experimental Diabetes Induction:

Diabetes was induced by intraperitoneal injection of Streptozotocin (Sigma, St Louis, MO, USA) to overnight fasted rats. Streptozotocin was used in multiple low doses (40 mg/kg for 3 days) which resulted in a slow immune-mediated form of type 1 diabetes (Brondum *et al*. 2005). Streptozotocin was freshly prepared immediately before injection (José *et al.* 2005; Omer *et al.* 2005).

Blood samples were collected from the retro- orbital plexus of veins under ether anesthesia once after 72 hours and again at the end of the experiment before animal sacrifice to determine the blood glucose levels. Glucose levels measurement was done with a calibrated blood glucose meter to confirm the establishment of diabetes.

The normal blood glucose level in rats is between 50-135 mg/dL. Blood glucose levels above 250 mg/dL or higher were considered hyperglycemic (Leob and Quimby 1989). Seventy two hours after streptozotocin injection, glucose solution was administered to the diabetic animals to prevent secondary hypoglycemia which is fatal. Experimental diabetes was induced before induction of periodontal disease because diabetes takes about 48-72 hours to occur (José *et al* 2005).

Protocol of Experimental Periodontal Disease Induction:

General anesthesia was administered by a combination of ketamine© (0.4 ml/kg) with xylazine© (0.2 mL/kg) via an intra-muscular injection. For all animals of both groups 4/0 sterile silk© ligatures were tied on the necks of mandibular first molars on both sides in the submarginal area and kept in position to promote microbial dental plaque accumulation and inflammation resulting in periodontal breakdown and induction of experimental periodontitis. The ligature model is based on environmental changes with growth of indigenous bacteria as a consequence of tying ligatures around molars. The ligatures were inspected repeatedly during the course of the study; because the molars move in an occlusal direction as a result of continuous tooth eruption and the ligatures tend to become loose or lost after 1 to 2 weeks (Page and Schroeder 1982; Nociti *et al* 2000). After appearance of the signs of inflammation such as redness, edema and bleeding at the 7^{th} day of periodontitis induction, a blood sample was taken from both groups to measure the level of IL-17 and these measurements were used later as the baseline (control) measurements. After two weeks in addition to the previously mentioned signs of inflammation (redness, edema and bleeding) loss of gingival tissue attachment occurred denoting establishment of periodontal disease. The ligatures were then removed in all animals and another blood sample was taken to measure the level of IL-17 in both groups. After ligature removal, scaling and root planning (SRP) was initiated for the mandibular molars using manual #13–14 mini five curettes (Hu-Friedy Co. Inc., Chicago, IL, USA) by performing 10 distal–mesial traction movements in the buccal and lingual aspects. The interproximal areas were scaled with the same curettes using cervicoocclusal traction movements. After SRP, irrigation of the submarginal areas with 1mL of saline solution was done. SRP was performed by the same experienced operator (Fernandes *et al* 2009).

Animals of both groups (1 and 2) were randomly allocated, using a computer-generated table, to the SRP treatment and the measurement of IL-17. For better standardization, animal 1 was the first choice, followed by 2 and 3, respectively. Blood samples for IL-17 measurement were taken at 4 time intervals from both groups: 1 week following ligature- induction of periodontitis (baseline samples), 2 weeks after periodontitis induction (before SRP) then one month and two months after SRP.

Laboratory Investigations:

Blood samples were allowed to clot and then the serum was separated by centrifugation. Serum samples were then stored at -20^0 C and thawed immediately before assay. Measurement of IL-17 was done according to the manufacturer's recommendations using the ELISA (Enzyme linked immunosorbent assay) technique using the Murine IL-17 ELISA kit supplied by ID Labs Inc. Biotechnology Kit in Canada (P.O.Box 1145, stn CSC. London on N6A 5K2). Method of detection of rat IL-17 was a solid phase sandwich ELISA technique. The results were reported as the concentration of IL-17 per milliliter of serum (Jeffrey *et al* 2007). Samples with cytokine levels below the detection limit of the assay (16pg/mL) were scored as 0 pg/mL). At the end of the study, animal scarification was done using ether anesthesia followed by cervical dislocation.

Statistical Analysis:

Data management and analysis were performed using SAS (vs. 8.2) and SPSS (vs. 17). Differences between groups were performed using the Mann-Witney test. Changes overtime were tested using the Friedman test. All p-values are two-sided. P-values < 0.05 were considered significant (Dawson and Trapp 2001).

Results:

Clinically the ligature-induced periodontal disease resulted in signs of inflammation such as edema, redness, and bleeding after 1 week in addition to attachment loss of tooth gingival tissues at the end of the 2nd week. There were no dropouts during the course of the study period. 60 samples were analyzed at baseline (1 week after periodontitis induction), 2 weeks after periodontitis induction (before SRP), then one and two months after periodontal therapy.

Data are presented in tables 1 and 2 in the form of mean and standard deviation (SD) values. Figures (1) and (2) display the IL-17 levels (pg/mL) in both groups at all time periods.

Table (1) displays the means and standard deviation values of serum IL-17 levels in group (1), the periodontitis group.

The table demonstrates that in group 1 the mean level of IL-17 was 23.7 ± 6.4 at baseline, which changed to a recorded mean of 323.9 ± 130.3 after 2 weeks showing a statistically significant increase of IL-17 levels between 1week (baseline) and 2 weeks measurements (P value < 0.05). This resulted in a percentage of change of 12.67% between one and two weeks as shown in table (3).

On the other hand the mean level of IL-17 one month after therapy changed to a recorded value of 182 ± 52.4 showing a significant decrease with P-value < 0.05. This resulted in a percentage of change of 43.8% between 2 weeks and 1 month measurements (Table 3).

Results of IL-17 measurements 2 months after therapy showed the mean level of IL-17 to be 57.7 ± 16.7 demonstrating a statistically significant decrease of IL-17 levels 2 months after therapy (P < 0.05), recording a 68.3% of change between 1 and 2 months and a 82.25% of change between 2 weeks and 2 months (Table 3).

Table (2) displays the mean and standard deviation values of serum IL-17 levels measured in group 2 taken at baseline (1 week from periodontitis induction), 2 weeks (before SRP) and one and two months after periodontal therapy. The table shows that in group (2), the mean level of IL-17 was 378.5 +155.7 which changed after 2 weeks to a mean of 630.2 ± 203.9 showing a statistically significant increase of IL-17 levels between baseline and 2 weeks measurements with a P value < 0.05. This resulted in a percentage of change of 19.36% between one and two weeks measurements (Table 3).

On the other hand a recorded mean of 457.5 +232.5 was obtained when IL-17 levels were measured one month after therapy showing no significant difference between two weeks before and one month after therapy (P value = 0.959), resulting in a percentage of change of 27.41% between both time periods (Table 3).

However, 2 months after therapy the mean of IL-17 levels was changed to 226.3 ± 85.4 showing a statistically significant lower value than the mean level of IL-17 levels measured at 2 weeks before therapy and 1 month after therapy (P< 0.05).

The percentage of change calculated was 50.53% between 1 and 2 months after therapy and 64.09 % between 2 weeks (before SRP) and 2 months after therapy (Table 3).

When the Friedman test was used to record the difference between the groups at each time period, statistical significant results were obtained between both groups at all time periods (Table 4).

Table 1: Shows the results obtained in group (1) the Periodontitis group.

	Mean	Standard deviation	P-value
1 week after periodontitis induction (baseline)	23.7	6.4	-------
2 weeks after periodontitis induction	323.9	130.3	< 0.05
One month after therapy (SRP)	182	52.4	< 0.05
Two months after therapy (SRP)	57.7	16.7	< 0.05

P values < 0.05 are considered significant

Table 2: Shows the results obtained in group (2), the Periodontitis and Diabetes group.

	Mean	Standard deviation	P-value
1 week after periodontitis induction (baseline)	378.5	155.7	------
2 weeks after periodontitis induction	630.2	203.9	< 0.05
One month after therapy (SRP)	457.5	232.5	0.959
Two months after therapy(SRP)	226.3	85.4	< 0.05

P-values < 0.05 are considered significant

Table 3: Shows the percentage of change in the two groups at the different time periods.

	Group (1)	Group (2)
From 1 week to 2 weeks	12.67 %	19.36%
From 2 weeks to 1 month	43.80 %	27.41 %
From 1 month to 2 months	68.30 %	50.53 %
From 2 weeks to 2 months	82.25 %	64.09 %

Table 4: Shows the difference between the groups at each time period.

	P-value
1week (baseline)	---------
2 weeks (before SRP)	0.02
One month after therapy	0.01
Two months after therapy	0.01

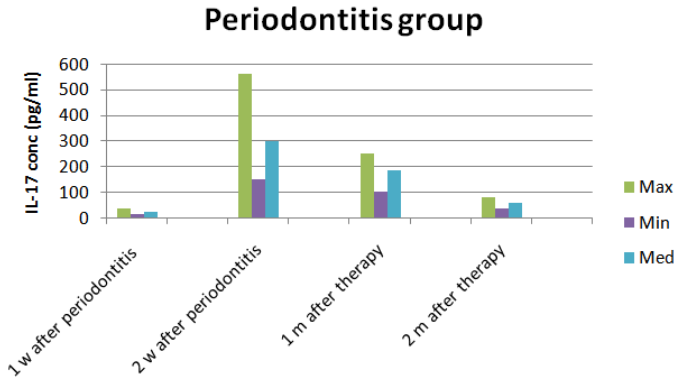

Fig. 1: Displays the maximum, minimum and median values of IL-17 concentration in group 1(the periodontitis group) at all time periods.

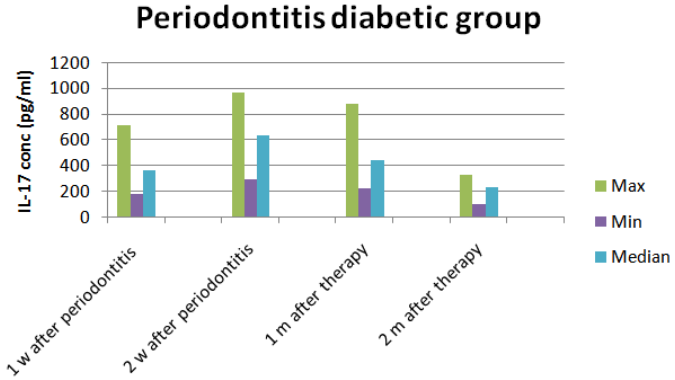

Fig. 2: Displays the maximum, minimum and median values of IL-17 concentration in group 2 (the periodontitis diabetic group) at all time periods.

Discussion:

The present study demonstrated that non-surgical periodontal therapy tended to reduce serum IL-17 levels in diabetic and non-diabetic groups.

Although the association between periodontitis and diabetes has been discussed in several reviews in recent years, none have focused on the use and contribution of rodent models (Pontes-Andersen *et al* 2007). Moreover few studies have investigated the effect of Th17 cells and Th-17 related cytokines in the host-immune defense mechanism of periodontitis and diabetes (Zhao *et al* 2011).

To our knowledge this is the first study to measure IL-17 levels in rats before and after periodontal therapy. The decision of choosing rats to test our hypothesis is that data from animal model research is a valid and powerful tool for testing hypothesis that cannot be addressed in humans. Moreover the breakthrough leading to the discovery of the Th17 lineage came from murine models (Graves *et al* 2008).

Few rodent investigations have explored the consequences of periodontitis for diabetes and their results indicated clearly that periodontitis can become a health hazard for diabetes, however the exact mechanisms are still to be unrevealed (Pontes-Andersen *et al* 2007). The use of ligatures in rats as experimental models was realized because many of the same series of events occur in these animals as in the non-human primate (Graves *et al* 2008).

Results of the present study demonstrated higher levels of IL-17 in the periodontitis group 2 weeks after induction of periodontitis than those measured at baseline or after periodontal therapy.

Although the role of IL-17 in the pathogenesis of PD is poorly understood, numerous studies indicated a potential role of IL-17 mediated inflammation in the initiation and progression of PD, suggesting that Th17 cells may contribute to the pathogenic tissue destruction that occurs in PD. IL-17 was detected in periodontal tissue biopsies and levels of IL-17 was significantly higher in gingival crevicular fluid (GCF) and culture supernatants of gingival cells in periodontitis patients compared to control samples (Takahashi *et al* 2005; Vernal *et al* 2005).

Moreover peripheral blood mononuclear cells from gingivitis and periodontitis patients stimulated with a Porphyromonas gingivalis (Pg) antigen secreted IL-17 (Oda *et al* 2003; Kramer and Gaffen 2007).

The results of our study are in accordance with Trombone *et al* 2009, who demonstrated high levels of the cytokines IL-1β, TNF-α, IL-17, in mice with induced periodontitis.

With regards to human studies, Lester *et al* 2007 found higher concentrations of IL-23, IL-17, IL-1beta, IL-6 and Interferon-gamma in moderate clinical attachment loss (CAL) sites than normal CAL sites suggesting the possibility that IL-23/IL-17 immune response was present in chronically inflamed gingiva. Moreover Johnson *et al* 2004 found that the IL-17 concentrations changed as a consequence of the progression of gingivitis to periodontitis suggesting a role of this cytokine in this progression. Although the studies by Johnson *et al* 2004 and Lester *et al* 2007 were done on human subjects, in relation to this conducted animal study, their observations support the findings of the present results.

The present findings demonstrated serum levels in diabetic rats with periodontitis to be higher than those of the non-diabetic group. Moreover the P values for the periodontitis diabetic group were higher than the periodontitis group at all time periods.

Several studies reported that the precise biological mechanisms that could explain the elevated levels of IL-17 in diabetes remain to be truly assessed. Evidence on the association between diabetes and periodontitis supports the concepts of increased severity of periodontitis in diabetic subjects (Salvi *et al* 2008). Inoue *et al* 1997 found pocket probing depths of diabetic rats to be significantly deeper than those of controls at one and 3 months after streptozotocin injection. Diabetes is believed to increase the systemic inflammatory burden in periodontitis (Iacopino 2000; Mealey and Oates 2006,) through a number of cellular and molecular alterations that take place in the periodontium as a consequence of sustained hyperglycemia (Pontes-Andersen *et al* 2007).

One hypothesis is the indirect damage produced by hyperglycemia through the non-enzymatic glycation of proteins and the subsequent accumulation of advanced glycation end products (AGEs) in periodontal tissues (Grossi and Genco 1998), and the other is a direct cellular damage through stimulation of intracellular pathways (Bascones-Martinez *et al* 2011). In addition to the stimulation of proinflammatory mediators by pathogens in periodontal sites, AGEs, when attached to its receptors (RAGE), also stimulate the overproduction of proinflammatory cytokines, including IL- 17 (King 2008). The binding of these molecules to neutrophils produces a hyperinflammatory state that amplifies the response to cytokines (Bascones-Martinez et al 2011).Supporting these findings, experimental studies in rodents also reported elevated expression of AGEs and RAGE in diabetic gingival tissues (Lalla and Schmidt 1998). Results from rodent studies also demonstrate higher amount of plaque and increased number of gram-ve anaerobes in diabetic rats compared to non-diabetic rats (Takai *et al* 1986). The increased number of Gram-ve anaerobes in diabetic rats and the fact that mononuclear cells stimulated with a Pg antigen secreted IL-17 (Oda *et al* 2003) could provide an explanation for the higher IL-17 levels in diabetic rats in the present study.

The results of the present study reinforce basic findings from human and rodent studies that report impaired host defense upon pathogens, prolonged inflammatory response and cytokine imbalance as ultimate consequences of hyperglycemia in the periodontium (Graves *et al* 2004; Pontes-Andersen *et al* 2007).

The present study demonstrated significant reduction of IL-17 levels one and two months after non-surgical periodontal therapy, in non- diabetic periodontally affected animals. However when IL-17 levels were measured in the diabetic periodontitis group one month following therapy, non-significant results were obtained. Significant results in the latter group were obtained when IL-17 levels were measured 2 months after periodontal therapy, denoting significant reduction in these levels when compared with those before therapy.

Few studies have evaluated the effect of periodontal therapy on the local levels of inflammatory markers in diabetic and healthy subjects and conflicting results have been shown (Talbert *et al* 2006; Navarro-Sanchez *et al* 2007; and Correa *et al* 2008).

While some authors suggested that the short-term effect of non-surgical periodontal therapy can be distinguished as early as one month, others suggested that healing after non-surgical therapy is gradually taking place over several months (Tervonen *et al* 1991). This might explain the non-significant results seen in the study only one month after non-surgical periodontal therapy. The high IL-17 levels in the periodontitis diabetic group suggests a potential hyperactivity of TH17 cells in this group that may favor periodontal tissue destruction and increase the systemic inflammatory burden (Duarte *et al* 2010), which in all probability retained the high levels of IL-17 yielding non-significant results one month after therapy. Since IL-17 secretion triggers neutrophils and macrophages recruitment for subsequent pathogen clearance (Bascones-Martinez 2011), the significant reduction of IL-17 levels 2 months after therapy may indicate a near complete resolution of inflammation.

In the present study IL-17 levels were still higher 2 months after therapy than the levels measured at baseline denoting the presence of a mild inflammatory host response. This might have been caused as a result of impaction of food, hair or bedding material where studies have shown that even a small amount of bacteria can elicit a host response in rodents (Pontes Andersen *et al* 2007).

With regards to human studies, Duarte *et al* 2010 measured serum concentrations of both TNF- and IL-17 after non-surgical periodontal therapy in human subjects and demonstrated significant reduction in both cytokines six months following therapy, which is in line with the results of the present study.

Nevertheless, current evidence on the impact of periodontal therapy on systemic markers of inflammation should be classified as preliminary and warrant further investigations.

Conclusion:

Within the limitations of this study it appeared that non-surgical periodontal therapy tended to reduce IL-17 serum levels. Because evidence suggests that chronic inflammation of the periodontium has far-reaching consequences for overall health, and in view of the very high prevalence of both periodontitis and diabetes and their potentially severe repercussions, inflammatory cytokines such as IL-17 may be important links between oral and systemic disease. The findings of this study may therefore suggest that high serum levels of IL-17 may substantiate increased risk of systemic inflammatory burden, and may be useful when developing therapeutic agents that target the Th17 effector molecule, IL-17.

Recommendation:

Further studies and larger sample sizes are required to assess the role of this cytokine in the periodontal lesions of diabetic subjects and determine the impact of periodontal treatment on the levels of this cytokine as well as on the glycemic control in diabetic subjects. Moreover although animal models have been successful in advancing our understanding of basic immunologic mechanisms, we need to conduct more human studies to fully realize the potential benefits of immunology for human health.

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