

The Effect of Experimental Periodontitis on the Serum Levels of Leptin and IL-18 in Insulin-Treated Diabetic Rats

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Abstract: The aim of this study in rats was to assess the effect of ligature-induced periodontitis on insulin-treated type 1 diabetes in terms of serum levels of leptin and interleukin-18 (IL-18). Twenty five male Wistar rats were studied, out of 40 initially used: (1) 14 rats with insulin-treated diabetes (control, DI) and (2) 11 rats with combination of insulin-treated diabetes and periodontitis (test, DIP). For all rats, type 1 diabetes was streptozotocin-induced and insulin treatment started on day 5. For DIP group, periodontitis was induced by ligation on day 16. Serum levels of leptin and IL-18 were assessed on days 16 (baseline) and 77 (final) by using multiplex immunoassay. All rats were sacrificed on day 77 (end of the study). Insulin treatment in the newly-induced diabetes significantly increased serum leptin levels and significantly reduced serum IL-18 levels. In the newly-induced diabetes, the combination of the continuation of insulin treatment and periodontitis non-significantly increased serum leptin and IL-18 levels. Serum IL-18 levels were significantly higher for the combination of periodontitis and insulin-treated diabetes as compared to insulin-treated diabetes alone. Within its limits, this study in rats showed that experimental periodontitis induced changes in serum biomarker levels suggestive of alterations in the systemic inflammation generated by insulin-treated type 1 diabetes.

Keywords: Diabetes Mellitus, Type 1, Insulin, Periodontitis, Interleukin-18, Leptin, Biologic Markers

1. Introduction

Diabetes is a heterogeneous group of metabolic diseases, where hyperglycemia arises from deficient insulin secretion, insulin activity or both. Chronic hyperglycemia damages tissues and organs in the long-term. The etiopathogenesis of diabetes involves processes that range from autoimmune destruction of the pancreatic b-cells resulting in insulin deficiency to disorders resulting in insulin resistance. These disorders are based on deficient insulin activity. Inadequate insulin secretion and/or reduced response to insulin lead to deficient insulin activity. Impaired insulin secretion and

deficient insulin activity might coexist. Type 1 (T1) and 2 (T2) diabetes differ in pathogenesis, clinical manifestation and disease progression. The immune-mediated T1 diabetes results from a cellular mediated autoimmune destruction of the b-cells of the pancreas. [1]

Periodontitis is defined as “a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by progressive destruction of the tooth-supporting apparatus”. [2] The relationship between diabetes and periodontitis has been studied mostly for T2 diabetes, whereas there is limited information on T1 diabetes and periodontitis. [3] In T2 diabetes, hyperglycemia is

associated with adverse periodontal outcomes and severe periodontitis has negative impact on glycemic control. [4]

Biologic markers (or biomarkers) are implicated in the host immune response and diabetes. [5-10] In T1 diabetes, changes in serum levels of biomarkers might occur. [11-13] In periodontitis, the local inflammation might induce systemic inflammation at certain grade [14] and serum biomarker levels might be altered. [10] Advanced glycation end products, as a result of chronic hyperglycemia, are related to the increase of inflammation biomarkers, which then affect other biomarkers. [15] Leptin and interleukin-18 (IL-18) are related to T1 diabetes [11, 13, 16, 17] and inflammation. [6, 18] The impact of insulin treatment on serum biomarkers levels has been earlier addressed. [11, 16, 19] When the insulin-treated diabetes is combined with periodontitis, data on serum biomarkers level changes with insulin treatment are very limited.

The limited data on the relationship between insulin-treated T1 diabetes and periodontitis led the authors to examine whether the systemic burden of insulin-treated T1 diabetes is altered by periodontitis. Periodontal tissue loss, time of T1 diabetes onset and diagnosis, glycemic control, medication and diabetes complications might be difficult to control in studies in humans. In animal studies, the induction of experimental T1 diabetes, the initiation of insulin treatment and the induction of experimental periodontitis at specific time points prevent such limitations. The ligature-induced periodontitis model [20, 22] and the streptozotocin (STZ)-induced diabetes model [20, 22, 23] have been studied in rats. The STZ-induced diabetes is T1 diabetes. Rats were studied in the present experiment.

The following hypotheses were examined in this study: (1) insulin treatment affects the circulating biomarkers in experimentally-induced T1 diabetes, (2) the combination of experimental periodontitis and insulin treatment affects the circulating biomarkers in T1 diabetes and (3) the addition of experimental periodontitis to experimentally-induced and insulin-treated T1 diabetes affects the circulating biomarkers.

The aim of the present study was to assess the effect of experimental periodontitis on the serum levels of leptin and IL-18 in rats with newly-induced and insulin-treated T1 diabetes.

2. Materials and Methods

2.1. Animals

Forty adult male Wistar rats (225-250 g) were used for the present randomized, parallel-arm 77 day experiment. The animals were randomly classified into two groups of 20 each: experimental diabetes and insulin administration (DI, control) and experimental diabetes, insulin administration and experimental periodontitis (DIP, control). The study followed the guidelines approved by the Council of the American Psychological Society (1980) and the European Communities Council Directive of 24 November 1996 (86/609/EEC). It was approved by the Ethics and Research

Committee of the National and Kapodistrian University of Athens and the Veterinary Directorate of the Prefecture of Athens.

Information on initial evaluation, housing and breeding, diabetes induction, body weight and glucose evaluation, insulin administration, tooth ligation, histologic evaluation of the alveolar bone loss and sacrifice were analyzed in an earlier study involving the same animal sample. [23] In summary, a 45 mg/kg STZ intravenous injection was used to induce diabetes. All rats received STZ injection on day 1. Successful diabetes induction was verified if serum glucose levels were >300 mg/dl up to day 5, measured with a glucometer. On day 5, insulin (Protaphane®, Novo Nordisk A/S, Bagsværd, Denmark) treatment was initiated in all rats (subcutaneously, once/day). The dosage for each rat was adjusted daily. Periodontitis was induced at the maxillary right second molar in DIP rat group. Intraculcular placement and ligation (ligation) of 4/0 silk suture was used for periodontitis induction. The rats belonging to DIP group were kept ligated from day 16 (ligation day) and thereafter for 61 days. DI group was not ligated. All animals were sacrificed on day 77 (end of the study). The maxillary right second molar was studied histometrically for all animals. The presence of periodontitis was assessed histometrically based on the distance between the cemento-enamel junction and the alveolar crest mesially and distally of this tooth. [23] Serum glucose levels were assessed daily (77 glucose assessments per animal).

For each animal, two venous blood samples (3.5 ml each, after overnight fasting) were collected on days 16 and 77, respectively. The blood samples were used to assess the serum levels of leptin and IL-18. Serum separator tubes were used and samples were left to clot for two hours at room temperature. Then centrifugation of the samples followed at 3000 rpm for 15 minutes. Serum was removed, aliquoted and stored in Eppendorf tubes at -80°C. For each animal, serum levels of leptin and IL-18 were assessed on days 16 (baseline values) and 77 (final values).

2.2. Biomedical Evaluation

The analysis of the serum samples was performed by using multiplex immunoassay and read with Luminex 100 (Multiplexed Biomarker Immunoassays for Luminex® Instrumentation/xMAP® Technology-Luminex Corporation, Austin, USA). The levels of leptin and IL-18 were determined with Millipex™ Map Kit (Millipore Corporation, Billerica, MA, USA, Rat Cytokine/Chemokine, RCYTO-80K, RCYTO-80K-PMX). The animal was the unit of measurement for the biomarker values.

2.3. Statistical Analysis

For statistical analysis, mean values and standard deviations or median values and 1 and 3 quartiles (Q1-Q3) (when the normality assumption was not met) were calculated for serum leptin and IL-18 levels at two timepoints (day 16-baseline, day 77-final). The t-test or Mann-Whitney

test for normally or non-normally distributed continuous variables was used for comparison of the above-mentioned measurements between the two study groups at each time-point. Comparisons within groups between different timepoints were performed by t-test for dependent variables or the Wilcoxon matched pairs signed-ranks test. Statistical analysis was performed with STATA 9.1 (Stata, College Station, TX, USA). The level of statistical significance was set at 5% ($p=0.05$).

3. Results

At the end of the study, 14 rats (70%) in DI and 11 rats (55%) in DIP group survived. Survival rate ($p=0.33$), baseline and final weight and glycemic state prior to insulin onset were similar between groups. There was statistically significant weight gain at final evaluation for both groups. Diabetes was induced in all animals. Insulin treatment statistically significantly reduced glucose levels in both groups. Periodontitis was induced in all ligated rats. There was no statistically significant difference in median glucose levels between the groups on day 1, on the day of diabetes confirmation and on days 16, 46, 61, 71 and 77. [23]

Baseline biomarker levels were similar between groups (table 1). At final evaluation, there were statistically significant differences between groups for IL-18 (Mann-Whitney $z=2.08$, $p=0.04$) (table 2). Comparison between final and baseline values for each biomarker and group showed that in DI final values as compared to baseline were statistically significantly higher for leptin and statistically significantly lower for IL-18 (table 3).

Comparison of the change in values (between final and baseline evaluation) for all biomarkers between groups revealed the following. Statistically significant difference in level change between the groups was found for IL-18. The median IL-18 change was negative for DI and positive for DIP. The absolute value of the median IL-18 change was statistically significantly higher for DI than DIP (table 4).

Table 1. Comparison of the baseline (at day 16) values of leptin and IL-18 between animal groups by using Mann-Whitney test.

Agent	Animal group		p-value
	DI (n=14)	DIP (n=11)	
Leptin	1,976.9 (954.2-5,265.7)	1,461.4 (621.7-2,773.3)	0.55
IL18	181.8 (133.9-240.4)	122.1 (81.6-202.6)	0.06

DI: diabetes induction and insulin administration (control),

DIP: diabetes induction, insulin administration and periodontitis induction (test).

Table 2. Comparison of the final (at day 77) values of leptin and IL-18 between animal groups by using Mann-Whitney test.

Agent	Animal group		p-value
	DI (n=14)	DIP (n=11)	
Leptin	3,491.8 (2,338-5,946.6)	3,176.6 (755.6-4,366.9)	0.38
IL18	61.2 (41.1-132.8)	169.9 (68.9-194.5)	0.04

DI: diabetes induction and insulin administration (control),

DIP: diabetes induction, insulin administration and periodontitis induction (test).

Table 3. Comparison between the final and baseline values of leptin and IL-18 in each animal group by Wilcoxon matched pairs signed ranks tests.

Agent	Animal group	
	DI	DIP
(pg/ml)	n=14	n=11
Leptin _b	1,976.9 (954.2-5,265.7)	1,461.4 (621.7-2,773.3)
Leptin _f	3,491.8 (2,338-5,946.6)	3,176.6 (755.6-4,366.9)
p-value	0.01	0.09
IL-18 _b	181.8 (133.9-240.4)	122.1 (81.6-202.6)
IL-18 _f	61.2 (41.1-132.8)	169.9 (68.9-194.5)
p-value	0.01	0.59

Leptin_b, IL-18_b: Baseline (at day 16) values of leptin and IL-18, accordingly.

Leptin_f, IL-18_f: Final values (at day 77) of leptin and IL-18, accordingly.

DI: diabetes induction and insulin administration (control),

DIP: diabetes induction, insulin administration and periodontitis induction (test).

Table 4. Comparison of the changes (between final and baseline evaluation) in leptin and IL-18 between animal groups by using Mann-Whitney tests.

Agent	Animal group		p-value
	DI (n=14)	DIP (n=11)	
(pg/ml)			
ΔLeptin	916.8 (26.6, 2,718.1)	2,179.7 (110.5, 2,485.2)	1.00
ΔIL18	-95.5 (-143.3, -79.5)	20.1 (-53.2, 92.7) ^a	0.009

^a statistically significant difference between groups DI and DIP (Mann-Whitney $z=2.63$, $p=0.009$).

Δ: change between final (day 77) and baseline (day 16) evaluation.

DI: diabetes induction and insulin administration (control),

DIP: diabetes induction, insulin administration and periodontitis induction (test).

4. Discussion

The present study compared serum leptin and IL-18 in the newly STZ-induced and insulin-treated T1 diabetes combined or not with ligature-induced periodontitis around one tooth. In the newly-induced and insulin-treated T1 diabetes, the continuation of insulin treatment for two more months significantly increased serum leptin levels and significantly reduced serum IL-18 levels. In the newly-induced and insulin-treated T1 diabetes, the combination of the continuation of insulin treatment for two more months and the induction of periodontitis non-significantly increased serum leptin and IL-18 levels. In the newly-induced and insulin-treated T1 diabetes, the continuation of insulin treatment for two more months either combined or not to periodontitis increased serum leptin levels. The leptin increase was significant only in the absence of periodontitis. Serum IL-18 levels were significantly higher for the combination of periodontitis and insulin-treated T1 diabetes as compared to insulin-treated T1 diabetes alone. The change in IL-18 levels was significantly lower and the change in leptin levels was non-significantly higher when the continuation of insulin treatment for two more months was combined with the induction of periodontitis as compared to the continuation of insulin treatment alone.

In newly STZ-induced T1 diabetes in rats, the present serum leptin levels were higher relatively long after insulin treatment initiation than soon after it. This finding is in agreement with results by Soliman [16] in newly STZ-

induced T1 diabetes in rats, where serum leptin was higher at 21 than at 2 days of insulin treatment. Thus, in newly STZ-induced diabetes in rats, the present study showed that the continuation of insulin treatment for two more months led to higher serum leptin at 72 (day 77) than at 11 days of insulin treatment (day 16), whereas Soliman's [16] study showed that the continuation of insulin treatment for 19 more days led to higher serum leptin at 21 than at 2 days of insulin treatment. Comparing study design between the present study and the study by Soliman, [16] it is similar for both studies concerning animal model, gender, diabetes induction method and insulin treatment and it differs in study duration. Specifically, the experiment ended at 77 days after STZ injection in this study and at 21 days after diabetes induction in Soliman's [16] study. The agreement in leptin findings between the two studies becomes even more important since they have similar study design.

In newly diagnosed children with T1 diabetes, serum leptin levels were significantly lower prior to insulin treatment initiation than 5 days after. In children with T1 diabetes receiving insulin treatment for a time period more than 2 years, the levels of serum leptin were significantly higher compared with those of age-matched healthy children. In undercontrolled diabetic children who had higher circulating hemoglobin A1c (HbA1c) concentrations and were then oversubstituted by insulin, leptin levels were elevated. [11]

The present increase in serum leptin levels with insulin treatment is explained by the fact that insulin stimulates leptin synthesis and secretion. [24] In this study, the increased insulin, as a result of insulin treatment, enhanced leptin secretion and led to elevated serum leptin. On the other hand, leptin plays important role in the regulation of glucose metabolism through food-intake independent mechanisms. [25] Leptin administration reduced hyperglycemia in mice with STZ-induced diabetes, independent of leptin signaling in the liver. [26]

The addition of periodontitis to insulin-treated diabetes led to non-significantly greater leptin increase (by 2.37 times). This might be explained through the link of leptin to inflammation. Leptin, as a proinflammatory cytokine, affects the secretion of acute-phase reactants, such as interleukin-1 (IL-1) and tumor-necrosis factor- α (TNF- α), and promotes T helper 1 (Th1)-cell differentiation. Proinflammatory mediators that upregulate leptin expression, such as TNF- α and IL-1, help in turn in the creation of a loop of acute phase reactants that influence each other in promoting the development of chronic inflammation. [6] Serum leptin levels increased after exposure to inflammatory stimuli [4] and in experimental acute inflammation. Serum leptin was associated with inflammatory biomarkers [6] and was positively related to the active phase of rheumatoid arthritis. [7] In the periodontal tissues, leptin upregulates the synthesis of proinflammatory and proteolytic molecules [8]. In humans, serum leptin was significantly elevated in periodontitis [27] and positively related to the severity of periodontal loss. [28] Serum leptin in non-obese individuals was significantly

elevated in periodontitis, as assessed in a systematic review and meta-analysis. [29]

Serum IL-18 levels were significantly higher in mice with experimentally-induced T1 diabetes [17] and in T1 diabetes patients as compared to controls. [29-31] Despite the IL-18 increase in T1 diabetes, the serum levels of IL-18 binding protein (IL-18BP) and IL-37, which are negative regulators of IL-18 function, were similar for T1 diabetic individuals and controls. [30] On the other hand, there was positive correlation between serum IL-18 and IL-18BP levels and significant increase of the free, unbound IL-18 in T1 diabetes. [31] Serum IL-18 was higher in T1 diabetes patients with poor than good glycemic control. [29-31] Specifically, serum IL-18 levels in T1 diabetes patients were positively correlated with glucose levels [28] and with HbA1c. [30, 31] Serum IL-18BP levels were positively correlated to HbA1c levels in T1 diabetes patients. [31] Moreover, the serum IL-18 levels were significantly elevated in early T1 diabetes stages in humans, [32] where proper glycemic control might have not been achieved. These findings reveal a link between IL-18 and glycemic control in T1 diabetes. In the present study, in the newly-induced T1 diabetes the continuation of insulin treatment for two more months led to reduction of the glucose levels. The present significant reduction in serum IL-18 levels with the continuation of insulin treatment is explained by the reduced glucose levels achieved with insulin treatment.

The addition of periodontitis to insulin-treated diabetes changed the type of effect exerted on serum IL-18 levels by increasing them. In the combined insulin-treated diabetes and periodontitis group, the non-significant increase in IL-18 with time and the significantly higher IL-18 as compared to insulin-treated diabetes alone might be attributed to the ligation-induced inflammation. Periodontitis patients had increased IL-18 [18] and periodontitis was positively associated with IL-18 levels in patients planned for diagnostic coronary angiography. [33] IL-18 is implicated in the pathogenesis of inflammatory diseases, [31] enhances immune reactions related to both Th1 and T helper 2 (Th2)-cells and enhances the superiority of Th1 over Th2 cells. [34] The balance between the immune reactions related to Th1 cells and those related to Th2 cells is important in the pathogenesis of periodontitis. The present findings might be the result of synergistic and antagonistic effects among the various mediators of inflammation in periodontitis. The present IL-18 change observed was significantly greater (by 4.75 times) for the insulin-treated diabetes alone than for the combination of insulin-treated diabetes and periodontitis. The type of IL-18 change differed between the two groups, which partly explains this finding. It seems that the effect of insulin-treated diabetes on IL-18 was stronger than the effect of the combination of insulin-treated diabetes and periodontitis. The addition of periodontitis alleviated the effect of insulin-treated diabetes on IL-18.

In summary, the present study in rats found the following. In the newly-induced T1 diabetes the continuation of insulin treatment for two more months induced changes in the levels

of circulating biomarkers implying regulation of the glycose levels. The addition of periodontitis to newly-induced and insulin-treated T1 diabetes induced changes in the levels of circulating biomarkers suggestive of alterations in the systemic inflammation generated by insulin treated T1 diabetes.

The present findings should be interpreted with caution since periodontitis was induced around one tooth, which might have affected the level of systemic inflammation, the number of animals studied was relatively small and the findings might differ in naturally occurring diabetes and periodontitis in humans. In future studies another time point of the assessment of the biomarkers might be added, specifically the day of diabetes induction. The three time points of biomarkers assessment would allow intragroup comparisons before insulin treatment initiation, soon after it and relatively long after it.

5. Conclusion

Within its limits, this study in rats showed that experimental periodontitis induced changes in serum biomarker levels suggestive of alterations in the systemic inflammation generated by insulin-treated T1 diabetes.

Statement of Any Potential Source of Funding and Conflict of Interest

The authors declare that they have no conflicts of interest and that no funding was used for this study.

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