

Adiponectin and interleukin-6 levels in insulin-treated diabetic rats with experimental periodontitis

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Abstract: The aim of the study was to compare the serum levels of adiponectin and interleukin-6 (IL-6) in insulin-treated diabetic rats with or without periodontitis. Forty male Wistar rats were randomly divided into 2 groups (20 rats each): a) insulin-treated diabetic group (control, DI) and b) insulin-treated diabetic periodontitis group (test, DIP). Diabetes was induced, and insulin treatment was initiated on day 5. On day 16, periodontitis was induced in the DIP group. All rats were euthanized on day 77. Adiponectin and IL-6 were assessed on days 16 and 77. At the end of the experiment, 14 and 11 rats survived in the DI and DIP groups, respectively. Adiponectin levels were statistically significantly higher at the end of the experiment compared with levels on day 16 in the periodontitis group ($p < 0.05$), but not in the control group. At the end of the experiment, adiponectin levels were statistically significantly higher in the periodontitis group compared with the control group ($p < 0.05$). Within-group and between-group comparisons of IL-6 levels showed no statistically significant difference. In conclusion, serum adiponectin was increased in insulin-treated diabetic rats with periodontitis in comparison with insulin-treated diabetic rats, while IL-6 levels did not differ between groups.

Descriptors: Adiponectin; Rats; Diabetes Mellitus; Periodontitis; Insulin.

Introduction

Type 1 diabetes mellitus is characterized by a deficiency of insulin secretion, caused by a cellular-mediated autoimmune destruction of pancreatic β -cells.¹ Periodontitis is a chronic inflammatory disease involving tooth-supporting tissues.² There is an increased severity of periodontitis in individuals with poorly controlled diabetes.³ The periodontal conditions, when diabetes is controlled, are comparable with those of the general population.³

Adiponectin, a 30-kDa protein mainly secreted by adipocytes, has anti-inflammatory, antidiabetic, and anti-atherogenic properties.⁴ Research indicates that it is involved in the regulation of insulin sensitivity.⁵ Adiponectin levels are increased in type 1 diabetes⁶ and decreased in type 2 diabetes⁷ and in obesity.⁸ Adiponectin seems to be involved in bone metabolism. Adiponectin and adiponectin receptors, AdipoR1 and AdipoR2, are expressed in osteoblastic and osteoclastic cells.^{9,10} Positive

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Received for publication on Jul 30, 2011
 Accepted for publication on Nov 16, 2011

as well as negative effects on bone formation¹⁰ and osteoclast formation¹¹⁻¹³ have been reported for adiponectin. Adiponectin's role in periodontal disease has not been fully elucidated.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that has been reported to be elevated in type 2 diabetes¹⁴ and decreased in type 1.¹⁵ It is described as one of the osteoclast-inducing factors acting on osteoblasts.¹⁶ The hypothesis of this study was that adiponectin and IL-6 levels are different in insulin-treated diabetic rats with experimental periodontitis than in insulin-treated diabetic rats.

The purpose of the present study was to investigate the effect of experimental periodontitis on serum adiponectin and IL-6 levels in insulin-treated diabetic rats.

Methodology

Animals

Forty male Wistar rats (225-250 g) were randomly divided into 2 groups (20 rats each):

- diabetes induction and insulin administration (control, DI); and
- diabetes induction, insulin administration, and periodontitis induction (test, DIP).

Rats with probing depth > 0.5 mm were excluded.¹⁷ They were housed in stainless steel cages with no bedding material (2 per cage, 12-hour light/dark, room temperature 18-22 °C, relative humidity 55-65%). They were fed with a standard laboratory diet in powder form,¹⁸ given tap water *ad libitum* and acclimatized to the new environment for 7 days.¹⁹ The study protocol was in accordance with local laws and regulations, with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and with guidelines approved by the Council of the American Psychological Society (1980). The study was approved by the Ethics and Research Committee of the School of Dentistry, University of Athens, and by the Veterinary Directorate of the Prefecture of Athens (# K/3935/28-5-2008).

On day 1, the rats were anesthetized with an intramuscular injection of ketamine hydrochloride solution (100 mg/kg body weight, Imalgene® 1000, MERIAL, Lyon, France) and xylazine (10 mg/kg

body weight, Rompun®, Bayer HealthCare, Leverkusen, Germany). Type 1 diabetes was induced by the intravenous injection (into the tail vein) of streptozotocin (STZ) (Sigma, St. Louis, USA), 45 mg/kg body weight,²⁰ dissolved in citrate buffer (10 mM, pH 4.5). Rats with glucose levels > 300 mg/dL^{19,21} up to the 5th day were included in the study. Body weight and blood glucose were measured daily¹⁹ (glucometer Wellion® Linus, AgaMatrix Inc., Salem, USA) after a 12-hour nocturnal fast. The induction of diabetes was confirmed when glucose levels first exceeded 300 mg/dL during the first 5 days (G_d). On day 5, insulin (Protaphane®, Novo Nordisk A/S, Bagsværd, Denmark) treatment was initiated in both groups (subcutaneously, once/day). The dosage for each rat was adjusted daily.

On day 16, all rats were anesthetized, as mentioned above. Blood samples were collected from the tail vein and stored in Eppendorf tubes at -80 °C. In the DIP group, a sterile 4/0 silk ligature (Medipac®, Kilkis, Greece) was placed subgingivally around the maxillary right second molar.²² On day 77, blood samples were drawn from the tail vein and stored in Eppendorf tubes at -80 °C, and the rats were euthanized with ketamine hydrochloride solution (200 mg/kg body weight) (intramuscularly) and pentothal solution 10% (intraperitoneally).

Biomedical evaluation

Serum samples were analyzed for adiponectin and IL-6 levels by an Enzyme-Linked Immunosorbent Assay (ELISA) and read with Luminex 100 (Multiplexed Biomarker Immunoassays for Luminex® Instrumentation/xMAP® Technology - Luminex Corporation, Austin, USA). Adiponectin and IL-6 levels were determined with Milliplex™ Map Kits (Millipore Corporation, Billerica, USA) (Adiponectin, Rat CVD Panel 3, # RCVD3-89K; and IL-6, Rat Cytokine / Chemokine, # RCYTO-80K).

Histometric evaluation

The maxilla was placed in 10% neutral formalin for 48 hours and thereafter in neutral buffer EDTA, 0.5 M, for 1 month for progressive decalcification. Specimens were washed with running tap water,

separated along the midline, and embedded in paraffin. The sections from the right side (6 μm thick mesio-distally parallel to the tooth axis, semi-serial, longitudinal) were stained with hematoxylin-eosin. One section was selected from each specimen from the middle area of the tooth. It was photographed by light microscopy (Nikon Eclipse 80i, Nikon, Tokyo, Japan) and a digital camera (Nikon DS-2 MW, Nikon, Tokyo, Japan) and analyzed with Image Pro Plus 5.1 program (Media Cybernetics, Bethesda, USA). The distance from the cemento-enamel junction to the alveolar crest²² was measured. For each second right maxillary molar, the mesial and distal measurements were averaged (HR), and the median was calculated for each group. Measurements were repeated twice by the same examiner, blinded to the treatment groups, with 2 weeks between measurements. The average of these 2 scorings was documented. In almost 10% of the sites, the difference between the 2 scorings was 2-3%, while in most sites, the scorings were identical.

Statistical analysis

For statistical analysis, mean values and standard deviations or median (Q1-Q3) (when the normality assumption was not met) were calculated for weight, glucose levels, histological parameters, and serum adiponectin and IL-6 levels at specific time-points. 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 2 study groups at each time-point. Comparisons within groups between different time-points were performed by *t*-test for dependent variables or the Wilcoxon signed-ranks test. Results were significant if $p < 0.05$. Statistical analysis was performed with STATA 9.1 (Stata, Col-

lege Station, USA).

Results

At the end of the experiment, 14 rats (70%) in the DI and 11 rats (55%) in the DIP groups survived ($p = 0.33$).

There was no statistically significant difference in mean baseline (W_1) ($p = 0.64$) and final weight (W_{77}) ($p = 0.28$) between groups. For both groups, mean final weight (W_{77}) was statistically significantly increased compared with baseline weight (W_1) (DI: $p = 0.002$ and DIP: $p = 0.001$) (Table 1).

There was no statistically significant difference in median glucose levels between the groups on day 1 (G_1), day of diabetes confirmation (G_d), and day 16 (G_{16}) ($p > 0.05$). The same applied for median glucose levels on days 46 (G_{46}), 61 (G_{61}), 71 (G_{71}), and on the day of euthanasia (G_{77}) ($p > 0.05$). Median G_d glucose levels were statistically significantly higher compared with levels on days 16, 46, 61, 71, and 77 for both groups (for all occasions, $p < 0.05$) (Table 2).

Regarding the histometric evaluation, median values of HR were statistically significantly greater for the DIP [1045.7 (850.4-2217.7) μm] than for the DI [419.7 (360.6-459.1) μm] group ($p < 0.0001$).

Median adiponectin levels were not statistically significantly different between the DI and DIP groups on day 16 ($p = 0.14$), while they were statistically significantly higher for the DIP than the DI group on day 77 ($p = 0.046$). Concerning the DI group, median adiponectin concentrations did not differ statistically significantly between day 77 (final) and day 16 ($p = 0.4$). In contrast, median final adiponectin values were statistically significantly increased compared with those on day 16 for the DIP group ($p = 0.04$). Median IL-6 levels did not change

Table 1 - Mean weight \pm SD for groups DI and DIP on days 1 (W_1), 5 (W_5), 16 (W_{16}), and 77 (W_{77}) and average weight for the entire experimental period (W_m).

Groups ^a	W_1 (g)	W_5 (g)	W_{16} (g)	W_{77} (g)	W_m (g)
DI (n = 14)	253.43 \pm 5.60 ^c	233.86 \pm 22.67 ^b	231.43 \pm 23.51 ^b	273.57 \pm 25.28 ^{b,c}	249.10 \pm 23.64
DIP (n = 11)	257.82 \pm 25.56 ^c	237.64 \pm 6.87 ^b	237.64 \pm 7.54 ^b	286.36 \pm 32.86 ^{b,c}	257.82 \pm 29.6

^a No statistically significant difference between groups on days 1 (W_1), 5 (W_5), 16 (W_{16}), 77 (W_{77}), and the average weight (W_m) ($p > 0.05$). ^b Statistically significant difference when compared with W_1 within the group ($p < 0.05$). ^c Statistically significant difference when compared with W_{16} within the group ($p < 0.05$).

Table 2 - Median glucose levels (Q1-Q3) for each group (DI, DIP) at day 1 (G_1), day of diabetes confirmation (G_d), and days 16 (G_{16}), 46 (G_{46}), 61 (G_{61}), 71 (G_{71}), and 77 (G_{77}).

Groups ^a	G_1 (mg/dL)	G_d (mg/dL)	G_{16} (mg/dL)	G_{46} (mg/dL)	G_{61} (mg/dL)	G_{71} (mg/dL)	G_{77} (mg/dL)
DI (n = 14)	119.5 (94-128) ^b	456 (405-477)	212 (162-477) ^b	235.5 (151-456) ^b	201 (175-385) ^b	203 (170-377) ^b	173 (158-447) ^b
DIP (n = 11)	116 (109-128) ^b	391 (342-469)	194 (143-443) ^b	211 (149-489) ^b	193 (140-487) ^b	166 (153-447) ^b	172 (152-440) ^b

^aNo statistically significant difference between groups for any of the time-points examined ($p > 0.05$). ^bStatistically significant difference when compared with G_d ($p < 0.05$).

Table 3 - Median values (Q1-Q3) of serum adiponectin and IL-6 on the day of ligature placement (day 16) and the last day of the experiment (day 77) for groups DI and DIP.

Groups	Adiponectin ($\mu\text{g/mL}$)		IL-6 (pg/mL)	
	Day 16	Day 77	Day 16	Day 77
DI (n = 14)	14.7 (11-21.5)	10.4 (9.1-15)	253.2(24.4-1834.3)	48.9(24.4-643.6)
DIP (n = 11)	12.2(8.9-13.3)	16.6 (13.1-33.1) ^{a,b}	656.2(331.1-847.6)	902.3(90.8-2030)

^a Statistically significant difference between groups on the specific day ($p < 0.05$). ^b Statistically significant difference within the group, day 16 versus day 77 ($p < 0.05$).

statistically significantly between day 16 and day 77 in any group. Between-group comparisons also showed no statistically significant difference (Table 3).

Discussion

The present study compared serum adiponectin and IL-6 levels in insulin-treated diabetic rats with or without experimentally induced periodontitis. Diabetes induction was confirmed in rats 2¹⁹ and 3²¹ days after STZ injection. In the present study, day 5 was selected as the cut-off day. Since glucose levels were measured daily, the authors noticed that even though diabetes was confirmed in all rats by day 5, some rats did not exhibit glucose levels > 300 mg/dL at days 2 and 3 (data not shown), suggesting that 2 or 3 days might be a rather short time for STZ to act completely.

Both groups had similar glycemic states prior to insulin treatment. Insulin treatment significantly reduced glucose levels. However, glucose levels were not decreased to the levels documented in previous studies,^{19,21} despite daily adjustment of the insulin dosage.

The present study demonstrated that adiponectin levels were increased in insulin-treated diabetic rats in the presence of periodontitis, while IL-6 levels did not show significant changes. IL-6 promotes

osteoclast differentiation.¹⁶ Furugen *et al.*²³ found no significant difference in serum IL-6 levels in individuals with and without periodontitis, while higher levels have also been reported in periodontitis.²⁴ There is research showing that IL-6 inhibits adiponectin gene expression and secretion in 3T3-L1 adipocytes, indicating that it is implicated in adiponectin regulation.²⁵

The precise role of adiponectin in bone homeostasis has not been fully elucidated. Berner *et al.*⁹ showed that the addition of recombinant mouse adiponectin to the culture medium of murine osteoblasts enhanced their proliferation. Oshima *et al.*²⁶ noticed increased trabecular bone mass and decreased numbers of osteoclasts when mice were injected with adenovirus-expressing adiponectin. Their *in vitro* experiments showed that adiponectin suppressed the macrophage-colony-stimulating factor/receptor activator of nuclear factor-kB ligand (M-CSF/RANKL)-induced differentiation of mouse bone marrow stromal cells, as well as the differentiation of human CD14-positive peripheral blood mononuclear cells (PBMCs) into osteoclasts. Adiponectin treatment of CD14-positive cells also reduced the bone-resorption activity of osteoclasts. Their results suggest that adiponectin enhances bone mass by activating osteoblastogenesis and suppressing osteoclastogenesis. Other studies also confirm the

inhibitory effect of adiponectin on osteoclast formation^{11,12} Another study,¹⁰ however, suggested both positive as well as negative effects of adiponectin on bone formation. The positive effect on bone formation was by locally produced adiponectin through the autocrine/paracrine pathway and by circulating adiponectin via enhancement of insulin signaling through the indirect pathway. A negative action was reported through the direct pathway by circulating adiponectin. Adding to this complicated picture is the fact that Luo *et al.*¹³ showed that adiponectin increased osteoclast formation indirectly (via stimulation of RANKL and inhibition of osteoprotegerin production in osteoblasts).

The different actions of adiponectin may be partially explained by its various possible forms, without a full exploration of their biological activities.⁹ There are studies^{23,27,28} that have not shown statistically significant differences in serum adiponectin levels in periodontitis patients compared with healthy control individuals, while a recent article²⁹ showed a lower ratio of high-molecular-weight adiponectin to total adiponectin in patients with periodontal pockets; this ratio was independently asso-

ciated with periodontal condition.

In addition, the expression of AdipoR1 and AdipoR2 in gingival tissues has been shown to be reduced in periodontitis.³⁰ A decrease in the expression of the receptors leads to a decrease in adiponectin binding and thereby in its effects (adiponectin resistance).³⁰

Conclusion

In conclusion, within its limitations, this study showed that the serum levels of adiponectin were increased in insulin-treated diabetic rats in the presence of periodontitis, while serum IL-6 levels did not change. The role of adiponectin in periodontitis and its correlation with other factors needs to be further explored. Future studies are required to clarify the mechanisms by which adiponectin is implicated in periodontal diseases.

Acknowledgement

We express our gratitude to Mrs. K. Perrea for the assessment of the adiponectin and IL-6 levels.

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