Effect of Periodontitis on Insulin Resistance and the Onset of Type 2 Diabetes Mellitus in Zucker Diabetic Fatty Rats

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Background: Studies indicate that an association exists between periodontitis and type 2 diabetes mellitus (T2DM) and/or obesity, with chronic inflammation hypothesized as the common denominator. The purpose of this study was to determine the causal effect of periodontitis and the concomitant impact of diet on the onset of insulin resistance (IR) and T2DM using a rat model system that simulates human obesity and T2DM.

Methods: Twenty-eight, 5-week-old female Zucker diabetic fatty (ZDF, fa/fa) rats were divided into four groups of seven animals: high-fat fed–periodontitis (HF/P), high-fat fed–no periodontitis (HF/C), low-fat fed–periodontitis (LF/P), and low-fat fed–no periodontitis (LF/C). Periodontitis was induced by ligature placement. Fasting plasma insulin and glucose levels were measured, and glucose tolerance tests were performed to assess glucose homeostasis, IR, and the onset of T2DM. The level of tumor necrosis factor-alpha (TNF-α), leptin, triglycerides, and free fatty acids were determined in week 13 at sacrifice.

Results: HF/P rats developed more severe IR compared to HF/C rats (P<0.01) and LF/P or LF/C rats (P<0.001) as measured by fasting insulin levels and homeostasis model assessment analysis. The onset of severe IR occurred ~3 weeks earlier in HF/P rats compared to HF/C rats. HF/P rats developed impaired (110 to 125 mg/dl) and frank fasting hyperglycemia (>125 mg/dl) 2 weeks earlier than HF/C rats. There was no difference in the severity and onset of IR and T2DM between LF/P and LF/C rats. The level of TNF-α was significantly higher in HF/P rats compared to HF/C rats (P<0.01).


KEY WORDS
Animal studies; glucose tolerance; insulin resistance; periodontitis; type 2 diabetes mellitus.

Type 2 diabetes mellitus (T2DM) has become one of the main threats to human health in the 21st century, and it is estimated that by 2025 there will be 300 million people worldwide with T2DM; this is approximately double the number of people reported with the disease in the year 2000.1 The pathogenesis of T2DM involves complex interrelationships between genetic and environmental/acquired factors, and the activation of inflammatory pathways is widely believed to promote the development of T2DM.2,3 A major acquired factor in the pathogenesis of T2DM is obesity, which alters metabolic homeostasis and is closely linked with the increase in T2DM.4 Since 1980, the prevalence of obesity in United States adults has approximately doubled, and the prevalence of overweight children and adolescents has approximately tripled.5,6 This remarkable increase in obesity is directly linked to the consumption of energy-rich, high-fat (HF) foods (junk food) and increased sedentary lifestyles.7

It is believed that a close relationship exists among periodontitis, obesity, and T2DM, with chronic inflammation being the common denominator.8,9 The basic premise underlying this belief is that proinflammatory cytokines (and/or bacteria and their products) released locally in gingiva may enter the systemic...
circulation and influence tissues/organs in distant sites. At the same time, proinflammatory cytokines involved in T2DM may reach the gingival environment and aggravate the periodontal condition, resulting in a “bidirectional relationship” between periodontitis and T2DM.\textsuperscript{10-14} In addition, a number of cells, including adipose cells, especially visceral fat in obese individuals, produce the proinflammatory cytokine tumor necrosis factor-alpha (TNF-\textalpha{}), which induces insulin resistance (IR), thus contributing to the development of T2DM in obese subjects. Thus, there is a plausible rationale underlying the association among periodontitis, obesity, and T2DM.

A significant problem with investigating the relationship among these conditions is the difficulty in defining whether these associations are causal in nature; most of the data that suggest this interrelationship among periodontitis, T2DM, and obesity are based on epidemiologic or cross-sectional studies.\textsuperscript{9,15,16} Several prospective studies\textsuperscript{17-22} using human subjects demonstrated that the treatment of periodontitis led to improvement of glycemic control in subjects with T2DM. However, confounding factors, such as medications used for T2DM, body mass index, stage of T2DM, duration of T2DM, and onset/severity of periodontitis, are difficult to account for or are broadly controlled in such studies. Thus, causality among periodontitis, diabetes, and obesity remains unclear. A major drawback in determining the direct impact of periodontitis on the development or status of T2DM is the difficulty in testing human subjects because experimental induction of periodontitis is not possible. Thus, the use of an animal model system may be helpful in contributing to the understanding of periodontitis and its systemic effects. In this study, we used Zucker diabetic fatty rats (ZDF, \textit{fa/fa}), which model obese humans who develop IR and T2DM, in an attempt to directly assess the effect of periodontitis on the onset of IR and T2DM.

ZDF rats are a widely used, well-characterized model of obesity and T2DM in diabetes research.\textsuperscript{23-26} ZDF rats are a substrain of Zucker fatty rats (ZFR, \textit{fa/\textalpha{}}\textit{fa}) and have additional abnormalities that predispose them to T2DM.\textsuperscript{27,28} Unlike ZFR rats, which exhibit hyperinsulinemia, IR, and glucose intolerance but do not develop T2DM, ZDF rats develop T2DM. ZFR and ZDF rats have a point mutation in the leptin receptor (\textit{fa/\textalpha{}}\textit{fa}) that leads to impaired function of leptin signaling, whereas the heterozygote for the leptin receptor mutation (\textit{fa/\textalpha{}}\textit{fa}) is a lean control (Zucker lean control). Leptin has been implicated in the signaling network that modifies hunger and satiety;\textsuperscript{29} thus, ZDF rats exhibit hyperphagia.\textsuperscript{30} The male ZDF rat develops an age-dependent diabetes phenotype, with the onset of obesity at 5 weeks of age accompanied by a metabolic state of prediabetes with hyperinsulinemia and IR.\textsuperscript{25,30} Because of this rapid onset, male ZDF rats are not ideal to study the temporal development of IR. In contrast, female ZDF rats develop T2DM only after consuming a HF diet. Thus, female ZDF rats may be considered more useful in studying the development of IR and T2DM, primarily because of the slower onset, but also because they become insulin resistant upon consuming a HF diet, which models the disease progression in most humans in Western countries.\textsuperscript{31} Thus, female ZDF rats are an excellent model for prediabetes and T2DM in obese humans and are ideal to investigate the effect of periodontitis on the onset of IR and T2DM.

The purpose of this study was to determine the direct effect of periodontitis and the concomitant influence of a high-fat (HF) and low-fat (LF) diet on the onset of IR and T2DM using a rat model system that simulates human obesity and T2DM.

**MATERIALS AND METHODS**

**Animals**

Twenty-eight, 4-week-old female ZDF rats were purchased.\textsuperscript{1} These animals had four consecutive birthdays (seven per birthday), so that animals were of the exact same age at the time of the procedures. After arrival, each rat was placed in a separate cage with a wire mesh bottom with no bedding to avoid any bedding impaction to gingiva. Rats were fed a finely milled LF diet (12.3 kcal % fat)\textsuperscript{2} and autoclaved tap water \textit{ad libitum} during a 5-day acclimation. Animals were housed at a constant temperature (22°C) with humidity of 45% to 55% in a 12-hour light/dark cycle. Cages were changed daily because no bedding was used. The diet was changed to a finely milled HF diet (48 kcal % fat)\textsuperscript{5} for 14 rats 1 week after the ligature placement (week 2). The remaining 14 rats were maintained on a finely milled LF diet. Animals were sacrificed 13 weeks after ligature placement. One rat in the HF diet without periodontitis (HF/C) group did not recover well from anesthesia and died in week 3. Thus, the results presented here are from 27 rats, with the exception of the intraperitoneal glucose tolerance test (ipGTT) data, which are from 24 rats. The study was conducted in accordance with the University of Illinois Animal Care guidelines.

**Study Design**

The 14 rats in each of the two diet groups were placed into one of two groups in week 1 (5 weeks of age); seven animals received ligature placement (4-0 silk sutures were placed around maxillary second molars) and seven served as control. Thus, there were four groups of rats: HF diet with periodontitis (HF/P), HF/C, LF diet with periodontitis (LF/P), and LF diet without periodontitis (LF/C). General anesthesia was given by intraperitoneal injection of ketamine

\textsuperscript{1} Charles River Laboratories, Wilmington, MA.
\textsuperscript{2} Research Diets, New Brunswick, N.J.
\textsuperscript{5} Research Diets.
(7.5 mg/100 g body weight) and xylazine (1 mg/100 g body weight) for ligature placement and for the weekly ligature check. *Escherichia coli* lipopoly saccharide** (LPS; 10 ng in PBS) was soaked on the mesial and distal interproximal portion of ligatures once a week for the first 5 weeks at the time that ligature placement was confirmed. Ligature placement was checked, and loose/lost ligatures were replaced throughout the study except for the weeks when glucose tolerance tests were performed. Rats in the control groups were also anesthetized to control for any effect of the anesthetics.

**Determination of Plasma Fasting Glucose, Insulin Levels, and IR**

Fasting blood glucose levels following a 14-hour fast were determined in blood collected from nicked tails every week using a glucometer†† before general anesthesia was given. Following fasting glucose determination, ~200 to 250 μl tail blood was collected into heparinized tubes, and plasma was collected. The plasma samples were used to determine fasting insulin levels using an enzyme-linked immunosorbent assay (ELISA) kit.‡‡ IR was calculated from plasma samples collected every week using the homeostasis model assessment (HOMA-IR), where IR = (fasting glucose [mmol/l] × fasting insulin [mU/l])/22.5,32

**Serum TNF-α, Free Fatty Acid (FFA), Leptin, and Triglyceride Levels**

Concentrations of serum TNF-α,§§ FFA, leptin,¶¶ and triglycerides## collected at the time of sacrifice (week 13) were determined using ELISA kits (TNF-α and leptin) and colorimetric assays (FFA and triglycerides).

**Glucose Tolerance Test**

To determine the timing of onset of glucose intolerance and T2DM, iGTT was performed on samples collected from six rats per group at weeks 1, 4, 8, 10, and 12. Briefly, following a 14-hour fast, dextrose (2 g/kg body weight) was administered intraperitoneally, and the plasma glucose levels in tail blood were determined after 0 (baseline), 15, 30, 60, 90, and 120 minutes using a glucometer.***

**Alveolar Bone Loss**

Following sacrifice (week 13), maxillae were defleshed, and bone loss per tooth was assessed by stereomicroscopy††† and image analysis‡‡‡ as described by Tatakis and Guglielmoni33 with modification.

**Statistical Analysis**

The differences between groups were determined by analysis of variance with a post hoc Tukey test, with a significance level at P<0.05. Pearson’s correlation coefficient was used to determine the correlation between bone loss and the level of TNF-α, with a significance level at P<0.05.

**RESULTS**

**Alveolar Bone Loss Due to Periodontitis**

Alveolar bone loss was calculated as the area (mm²) bordered by the cemento-enamel junction, the crest of alveolar bone, and the mesial and distal line angles on the buccal and lingual sides of maxillary second molars (Fig. 1A). This area of bone loss obviously includes areas of connective tissue and epithelial cell attachment. Thus, the bone height in control groups (Fig. 1B) is considered close to normal. Ligated rats exhibited significantly more bone loss than non-ligated control animals at 13 weeks (P<0.001; Fig. 1). There was no significant effect of diet (HF versus LF) on the amount of bone loss at this time point (Fig. 1B). These data confirmed that ligature placement plus LPS induced periodontitis in these animals relative to controls.

**Effect of Periodontitis on Glucose and Insulin Levels**

Fasting glucose levels, following a 14-hour fast, for the first 10 weeks of the study are shown in Figure 2.
In samples collected at weeks 4 and 5, fasting glucose levels were statistically higher in HF/P rats than in HF/C rats \((P<0.001)\). As a group, HF/P rats developed impaired fasting glucose levels (110 to 125 mg/dl) by week 4 and developed frank fasting hyperglycemia \((\geq 126 \text{ mg/dl})\) by week 9. Although the difference in fasting plasma glucose between HF/P and HF/C rats seemed to be considerable at weeks 9 and 10, the difference was not statistically significant. The HF/C rats exhibited normal fasting glucose in weeks 4 and 5; they developed impaired fasting glucose by week 6 and remained in this range through week 10, reflecting the effect of a HF diet on glucose levels in female ZDF rats. In contrast, there was no difference between LF/P and LF/C rats and no difference in either group relative to baseline.

Figure 3 shows the results of fasting insulin levels (Fig. 3A) and fasting insulin and glucose levels (Fig. 3B) during all 12 weeks of the study. As shown in Figure 3A, plasma insulin levels were increased from baseline in HF/P and HF/C rats by week 4 of the study \((P<0.01)\) but not in LF/P or LF/C rats, reflecting the effect of HF feeding (Fig. 3A). Plasma insulin levels continued to increase in HF/P and HF/C animals during weeks 4 to 9. However, this increase was more rapid in HF/P rats compared to HF/C rats, and there was a significant difference in insulin levels between HF/P and HF/C rats in weeks 6 and 7. Later, insulin levels plateaued and began to decline after weeks 9 to 10 in HF/P and HF/C rats, reflecting a decline in beta cell function; this decline was accompanied by the onset of frank fasting hyperglycemia in HF/P and HF/C rats by 12 weeks (Fig. 3B). Insulin levels in LF/P and LF/C animals were significantly increased by week 12 relative to baseline \((P<0.05)\), but there was no difference between the groups (Fig. 3A).

### Effects of Periodontitis on IR

Because insulin levels may reflect differences in glucose levels and insulin sensitivity, we also assessed IR each week using HOMA-IR.\(^{32}\) As shown in Figure 4,
HOMA-IR analysis indicated that HF feeding was associated with an increase in IR in HF/P and HF/C rats compared to LF-fed animals. Further, HOMA-IR was significantly greater in HF/P rats at weeks 6 and 7 compared to HF/C rats \((P < 0.01)\). An average IR level \(\sim 100\) occurred by week 6 in HF/P rats, whereas the same level was not achieved until after week 9 in HF/C rats (Fig. 4). Thus, the development of IR was accelerated in HF/P rats compared to HF/C rats.

In contrast, there was a small, but gradual increase in IR in LF-fed animals with time (Fig. 4). HOMA values at 12 weeks were significantly greater in LF/P and LF/C rats compared to baseline (week 1; \(P < 0.05\)), but the difference between the groups was not statistically significant.

**Effects of Periodontitis on Glucose Intolerance and the Onset of Diabetes**

To further characterize the effect of periodontitis on the development of impaired glucose homeostasis and diabetes in ZDF rats, we performed ipGTT on six rats from each treatment group at weeks 1, 4, 8, 10, and 12. Glucose levels were measured at 0, 15, 30, 60, 90, and 120 minutes after intraperitoneal injection of dextrose (2 g/kg body weight) (Fig. 5).

Both HF groups and the LF/P rats exhibited impaired glucose tolerance at 4 and 8 weeks as evidenced by glucose levels between 200 and 300 mg/dl at 120 minutes. HF/P exhibited even greater glucose intolerance, with glucose levels >300 mg/dl at this time point at week 10 (Fig. 5). Glucose tolerance continued to deteriorate in HF/P and HF/C rats, which was consistent with the decline in insulin levels. Although not statistically significant, mean glucose levels trended to increased impairment in HF/P rats compared to HF/C rats at week 12.

**Effects of Periodontitis on Serum TNF-α Levels**

We hypothesized that increased IR is associated with an increase in the serum level of TNF-α, due in part to periodontitis in HF/P rats compared to HF/C rats. As shown in Figure 6, serum levels of TNF-α in samples collected at the time of sacrifice were greater in HF/P rats compared to HF/C rats \((P < 0.01)\). The average TNF-α level also seemed to be higher in LF/P rats compared to LF/C rats, but the difference between these two groups was not statistically different. The correlation between bone loss and the levels of serum TNF-α at sacrifice was statistically significant \((r = 0.67; P < 0.01)\). These results indicated that periodontitis contributed significantly to increased serum TNF-α levels in rats fed a HF diet.

Serum triglycerides, FFA, and leptin levels were also determined in samples collected at the time of sacrifice. There were no differences between rats with

*Figure 5.*  
Glucose concentration levels (y axis; mg/dl) from ipGTT performed at weeks 1, 4, 8, 10, and 12. Values for each time point (x axis; minutes) are expressed as mean ± SEM. Week 1 is a representative of 5-week-old ZDF rats prior to changing the diet.
or without periodontitis, and no difference was seen between HF and LF groups (data not shown).

DISCUSSION

The prevalence of T2DM has increased dramatically as the result of an increase in obesity caused by overly rich nutrition (HF diet/junk food) and a sedentary lifestyle. Although the association between obesity and T2DM is well recognized, underlying mechanisms remain poorly understood. Some studies indicated that adipose tissue, particularly visceral fat, produces cytokines and adipokines, such as TNF-α and resistin, which contribute to IR and the development of T2DM. Recent studies also suggested that there is a strong association between obesity and periodontitis, and it was shown that obese subjects have more severe periodontitis. Periodontitis is a chronic inflammatory disease triggered by bacterial products, such as LPS, and the subsequent exuberant inflammatory response to bacterial products leads to loss of alveolar bone. It was shown that subjects with periodontitis also have a higher concentration of proinflammatory cytokines, such as TNF-α, in sera and/or gingival crevicular fluid. Thus, a common denominator for periodontitis, T2DM, and obesity is chronic inflammation characterized by an increased expression of common proinflammatory cytokines. Therefore, it is reasonable to hypothesize that periodontitis and the attendant production of cytokines might contribute to the accelerated development of IR and T2DM in obese subjects. Thus, obese subjects with periodontitis may be at a greater risk for developing IR and, subsequently, T2DM than lean or even obese subjects with healthy gingiva. Based on the results of the present study, we suggest that obese individuals with periodontitis who consume a HF diet may be at risk for the accelerated development of severe IR and T2DM.

Most studies that have investigated the relationship between T2DM and periodontitis in humans were cross-sectional or retrospective in nature. In addition, it is difficult to measure the true effect of inflammation on comorbid conditions in human subjects with T2DM because they tend to be (or have been) on many medications, including some that reduce inflammation. There are many inherent variables and confounding factors involved in studies of diabetes in humans that make it difficult to determine the consequence of periodontitis on the development of IR and T2DM. In addition, it takes considerable time for humans to develop T2DM. Therefore, the development of an animal model system is essential in examining the effect of periodontitis on IR and the subsequent development of T2DM.

Liu et al. used male ZDF rats with fully developed diabetes to study the effect of induced inflammation on alveolar bone. In that study, ligatures soaked with periodontal pathogen were placed for 7 days, and changes were assessed in alveolar bone 4 days following the removal of ligatures. These investigators reported that T2DM caused impaired bone repair following the removal of ligatures. In a second animal study using Goto-Kakizaki rats, which are a non-obese model with characteristics of T2DM, bone loss was more severe in rats with T2DM than in control rats when periodontitis was induced by ligature placement. Both studies used rats that had already developed T2DM; thus, the onset of IR or T2DM was not determined. Recently, ZFR rats were used to investigate the relationship between prediabetes and periodontitis over a 4-week period following the placement of ligatures. At 4 weeks after the placement of ligatures, glucose intolerance was statistically higher in rats with periodontitis compared to rats without periodontitis. The investigators concluded that prediabetes worsened periodontitis; in turn, periodontitis was associated with deterioration of glucose metabolism as indicated by an increase in glucose intolerance. Collectively, the published data, in addition to that presented here, support the hypothesis that periodontitis aggravates glucose metabolism.

In the current study, we used an animal model of human obesity and determined the effect of periodontitis on the onset of IR and T2DM when rats were fed a HF or LF diet. Periodontitis was induced in animals as evidenced by an increase in alveolar bone loss in animals receiving a HF or LF diet. As previously reported, a HF diet promoted the development of T2DM in female ZDF rats, as reflected by the development of hyperglycemia and decreased insulin levels. In the present study, periodontitis in animals
receiving a HF diet accelerated the development of IR, as shown by changes in fasting insulin levels and HOMA scores and the development of fasting hyperglycemia. These results indicated that periodontitis can contribute to the development of IR and hyperglycemia in obese animals that are prone to the development of diabetes.

Based on results from a previous study\textsuperscript{31} and those presented here, it appears that a HF diet can promote IR. Studies indicated that there is a link between FFA and insulin signaling in human skeletal muscle\textsuperscript{48} as well as IR associated with high-lipid diets.\textsuperscript{49,50} An elevated level of plasma FFA almost completely abolishes the increase in insulin receptor substrate (IRS)-1–associated phosphatidylinositol-3 kinase and impairs IRS-1 tyrosine phosphorylation. This suggests that high lipid levels can cause IR. In addition, the infusion of lipid caused a 43% reduction in whole-body glucose disposal,\textsuperscript{48} again suggesting that increased lipid availability can lead to IR. Collectively, these results support our finding that IR was greater in ZDF rats fed a HF diet compared to an LF diet and that periodontitis in HF-fed ZDF rats accelerated the development of severe IR.

The effect of periodontitis on IR and glucose levels was most pronounced in animals receiving the HF diet, although bone loss was similar in animals with ligatures placed on HF and LF diets. This suggests that the effect of periodontitis on IR and glucose metabolism may be most apparent under conditions in which insulin action and/or secretion are partially impaired, including animals that are exposed to a HF diet. In animals with intact insulin secretory reserves and insulin-signaling activity, the effect of the level of periodontitis induced in the current study may not be sufficient to disrupt glucose homeostasis in a short amount of time.

Serum TNF-\(\alpha\) levels were significantly higher in HF/P rats compared to HF/C rats at sacrifice. Because HF/P and HF/C rats differ with respect to the presence or absence of periodontitis, it may be responsible for the difference in TNF-\(\alpha\) levels between these groups. The level of TNF-\(\alpha\) also tended to be higher in LF/P rats compared to LF/C rats, but this difference was not statistically significant. TNF-\(\alpha\) interferes with signal transduction downstream from the insulin receptor\textsuperscript{51,52} by promoting serine phosphorylation of IRS-1. This phosphorylation, in turn, impairs the auto-phosphorylation of the insulin receptor and subsequent signal transduction downstream of the insulin receptor.\textsuperscript{53} In the liver, increased TNF-\(\alpha\) levels may limit the ability of insulin to activate the signaling pathways required to mediate the effects of insulin on hepatic glucose production.\textsuperscript{54} In the context of the present study, it is reasonable to speculate that increased levels of TNF-\(\alpha\) may have contributed to the accelerated development of IR and the increased fasting glucose levels in HF/P animals compared to HF/C animals.

We did not observe statistically significant differences in glucose tolerance testing in HF/P rats compared to HF/C rats at 4, 8, 10, or 12 weeks, although there was a trend toward the development of severe glucose intolerance at weeks 10 and 12 in HF/P rats. Unfortunately, we did not perform ipGTT in weeks 6 and 7 when the differences in IR between HF/P and HF/C rats was the most pronounced; additional studies are needed to assess whether glucose tolerance is impaired at this time point. Also, although fasting blood glucose levels reflect the rate of hepatic glucose production, the response to glucose challenge also reflects the ability of insulin to regulate glucose uptake into other tissues, especially skeletal muscle. Thus, additional studies will also be needed to determine whether the effects of periodontitis (and TNF-\(\alpha\)) on insulin sensitivity are more marked in the liver or in skeletal muscle.

CONCLUSIONS

To the best of our knowledge, this was the first study to monitor the impact of periodontitis on the development of IR and T2DM over a prolonged period (13 weeks) in an experimental animal model. Our results indicated that periodontitis accelerated the onset of severe IR in ZDF rats fed a HF diet. Thus, a combination of periodontitis and diet played a role in accelerating the development of severe IR in this model.

Periodontitis accelerated the onset of severe IR in obese rats that have a predisposition to develop T2DM when fed a HF diet. Periodontitis may also affect the onset of T2DM in these animals. When fed a HF diet, the concentration of serum TNF-\(\alpha\) was significantly higher in rats with periodontitis and T2DM compared to rats with T2DM only. Although results from animal and human studies cannot be compared directly, the results from the current study suggest that periodontitis may precipitate the early onset of severe IR and, thus, T2DM in obese humans consuming a HF diet.

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