

# Renal Alterations in Prediabetic Rats With Periodontitis

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**Background:** Periodontitis was shown to have an impact on glucose levels in prediabetic and diabetic rats. The Zucker fatty rat (ZFR) is a well-characterized model of prediabetes presenting with impaired glucose tolerance, hyperinsulinemia, dyslipidemia, and moderate hypertension. The aim of the present study was to investigate whether periodontitis influences kidney changes in ZFRs.

**Methods:** Male adult ZFRs (N = 19) and their lean littermates (N = 18) were studied. Periodontitis was induced with ligatures in half of the ZFRs and lean rats, whereas the other half served as controls. After 4 weeks, the rats were sacrificed, and the kidneys, liver, and heart were removed and weighed. Kidneys were evaluated histologically for glomerular volume and renal mRNA levels of vascular endothelial growth factor (VEGF), VEGF receptor 2, transforming growth factor-beta, connective tissue growth factor, collagen IV $\alpha$ 1, fibronectin, and nephrin. Urinary albumin excretion and creatinine clearance were also evaluated.

**Results:** In prediabetic ZFRs, periodontitis was associated with kidney hypertrophy ( $P = 0.03$ ) and a tendency for increased glomerular volume ( $P = 0.06$ ). In lean littermates, elevated fibronectin mRNA levels ( $P = 0.03$ ) were noted in the presence of periodontitis.

**Conclusion:** Our findings suggest the participation of periodontitis in the development of early renal changes in ZFRs. *J Periodontol* 2008;79:684-690.

## KEY WORDS

Diabetes mellitus; diabetic kidney disease; periodontitis; prediabetes; rats.

Diabetic nephropathy is an important late complication in diabetes affecting approximately one-third of all diabetic patients. It is the main cause of end-stage renal failure and develops as a consequence of progressive damage to the renal glomeruli.<sup>1</sup> Growth factors, such as transforming growth factor-beta (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), and VEGF receptor 2 (VEGFR-2), have been implicated as potential pathogenic factors in the development of diabetic renal disease.<sup>2-4</sup> Although the mechanisms leading to diabetic renal injury are complex and not fully understood, hyperglycemia is considered a major risk factor.<sup>5</sup>

In a previous study,<sup>6</sup> ligature-induced periodontitis was associated with impairment in glucose tolerance in prediabetic rats. Although the mechanisms implicated in the effect of periodontitis on prediabetes are not well explored, glycemic alterations have the potential to affect prediabetes. Therefore, it is important to learn whether periodontitis-induced disturbances in glycemic control may be associated with the development of diabetic kidney disease. A correlation between severe periodontitis and kidney alterations has been suggested in patients with type 2 diabetes.<sup>7-9</sup> To our knowledge, no experimental study has addressed the role of periodontitis in initial renal changes.

The Zucker fatty rat (ZFR) was used in the present study because it is one of the most frequently used models of vascular complications in type 2 diabetes and is a well-established model of prediabetes,

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which is characterized by impaired glucose tolerance, hyperinsulinemia, hyperphagia, hyperlipidemia, and obesity that result from a mutation in the leptin receptor gene.<sup>10</sup>

The hypothesis is that periodontitis influences the occurrence of early organ alterations in prediabetes. The aim of the present study was to investigate if periodontitis plays a role in the development of renal changes in the pre-stage of type 2 diabetes. For this purpose, we evaluated renal morphologic (kidney weight and glomerular volume) and functional parameters (urinary albumin excretion [UAE] and creatinine clearance [CrCl]), in addition to renal gene expression of markers of renal permeability (VEGF, VEGFR-2, and nephrin) and matrix accumulation (TGF- $\beta$ , connective tissue growth factor [CTGF], fibronectin, and collagen IV $\alpha$ 1).

## MATERIALS AND METHODS

### Animals

Five-week-old male ZFRs (ZFR/Gmi-*fa/fa*; N = 24) and age-matched lean littermates (ZFR/Gmi-*+/fa*; N = 24) were investigated.<sup>5</sup> Animals were housed two per cage in a room with a 12-hour:12-hour artificial light cycle, a temperature of 21°C  $\pm$  1°C, and a humidity of 55%  $\pm$  5%. They had free access to tap water and to a powder diet<sup>||</sup> from the time of weaning, and cages with a wire mesh floor were used to ensure periodontitis-free rats as described previously.<sup>11</sup> The study complied with Danish regulations for the care and use of laboratory animals.

### Study Design

Prior to baseline, a periodontal evaluation was performed under general anesthesia (rodent mixture composed of one part of midazolam,<sup>¶</sup> one part of a solution containing fentanyl and fluanizone,<sup>#</sup> and two parts of sterile water, 1.8 ml/kg, subcutaneously). At baseline, rats were allocated randomly into the study groups (12 in each group): ZFRs with periodontitis (ZFR + P); ZFRs without periodontitis (ZFR); lean with periodontitis (lean + P); and lean without periodontitis (lean). Periodontitis was induced at baseline through placement of silk ligatures around both second maxillary molars as described previously.<sup>12</sup> The ligatures were checked every week to replace ones that were loose or lost. Body weight was monitored weekly. Four weeks after baseline, all animals were anesthetized and sacrificed by cervical dislocation. The right and left kidneys, liver, and heart were removed, dissected free of adipose tissues, and weighed. The left kidney and the poles of the right kidney were snap-frozen in liquid nitrogen. The middle of the right kidney was fixed in 4% paraformaldehyde for later histologic preparation. As described previously,<sup>6</sup> the heads were dissected free from connective tissues to allow morphologic mea-

surements of alveolar bone loss, and radiographs were taken to evaluate bone support.

### Determination of UAE and Serum and Urinary Creatinine Concentration

Urine samples were stored at -20°C until the assay was performed. Urinary albumin concentration was determined in 12-hour urine collections by a commercially available kit\*\* according to the manufacturer's instructions. The intra- and interassay coefficient of variation was <5% and <10%, respectively. Serum and urinary creatinine concentration was measured by an automated technique adapted from the method of Jaffé<sup>13</sup> and corrected for the prevailing glucose content because of interference in the Jaffé reaction. CrCl was calculated based upon 12-hour urine collections.<sup>13</sup>

### Glomerular Volume

The middle part of the right kidney containing the papilla was embedded in paraffin for light microscopy examination. Two-micron-thick sections were cut on a microtome<sup>††</sup> and stained with periodic acid-Schiff and hematoxylin. Mean glomerular tuft volume ( $V_G$ ) was determined from the mean glomerular cross-sectional area ( $A_G$ ) at a magnification of  $\times 400$  as described previously.<sup>14-16</sup> The areas were determined with a two-dimensional version of the nucleator<sup>‡‡</sup> as the average area of a total of 40 to 50 glomerular profiles.  $V_G$  was calculated as  $\beta/k \times (A_G)^{3/2}$ , where  $\beta = 1.38$ , which is the idealized shape of glomeruli, and  $k = 1.1$ , which is the size distribution coefficient.

### Quantitative Real-Time Polymerase Chain Reaction (PCR)

After homogenization of kidney tissue with the use of a mixer mill,<sup>§§</sup> total cellular RNA was extracted from renal cortical tissue.<sup>|||</sup> The quality of rRNA was estimated by agarose gel electrophoresis by the appearance of two distinct bands visible by fluorescence of ethide bromide representing intact rRNA. The amounts of RNA extracted were quantified by measuring the absorbance at 260 nm by spectrophotometry. Reverse transcription from RNA to DNA was performed with a kit<sup>¶¶</sup> under the following conditions: 25°C for 10 minutes, 48°C for 30 minutes, and 94°C for 29 seconds. PCR was performed in triplicate for each sample. Each well contained 25  $\mu$ l consisting of RNA, universal PCR mastermix, a primer of the target,

§ Harlan Scandinavia, Alleroed, Denmark.

|| Altromin 1314 fortified, Lage, Germany.

¶ Dormicum, Vetapharma, Leeds, U.K.

# Hypnorm, Roche, Basel, Switzerland.

\*\* Mouse Albumin ELISA Quantification Kit, Bethyl Laboratories, Montgomery, TX.

†† HM350, Microm, Walldorf, Germany.

‡‡ CAST, Olympus, Copenhagen, Denmark.

§§ MM301, Retsch, Haan, Germany.

||| 6100 Nucleic Acid PrepStation, Applied Biosystems, Foster City, CA.

¶¶ Multiscribe Reverse Transcriptase kit, Applied Biosystems.

i.e., VEGF,<sup>##</sup> VEGFR-2,<sup>\*\*\*</sup> TGF- $\beta$ ,<sup>†††</sup> CTGF,<sup>†††</sup> collagen IV $\alpha$ 1,<sup>§§§</sup> fibronectin,<sup>||||</sup> or nephrin,<sup>¶¶¶</sup> and a primer of the housekeeping gene.<sup>###</sup> Liver RNA was used as a negative control. Each real-time PCR ran at 50°C for 2 minutes, 95°C for 10 minutes, and for 40 cycles alternating between 95°C for 15 seconds and 60°C for 1.5 minutes.

### PCR Data Analysis

Data were analyzed with software.<sup>\*\*\*\*</sup> The output of amplification was measured as described previously<sup>13</sup> as the threshold cycle (Ct) value, defined as the cycle number at which amplification products are detected. The average of triplicates from each sample was used. The relative quantification of the target gene was calculated using the formula  $(1/2)^{Ct\text{-target gene} - Ct\text{-housekeeping gene}}$ .<sup>††††17</sup>

### Statistical Analysis

All statistical analyses were performed using a statistical computer program.<sup>††††</sup> Data are presented as mean  $\pm$  SEM. Normality was confirmed after checking all variables through probability plots, and all analyses were performed with the *t* test. Comparisons were made between lean + P and lean groups and between ZFR + P and ZFR groups. The level of significance was 0.05.

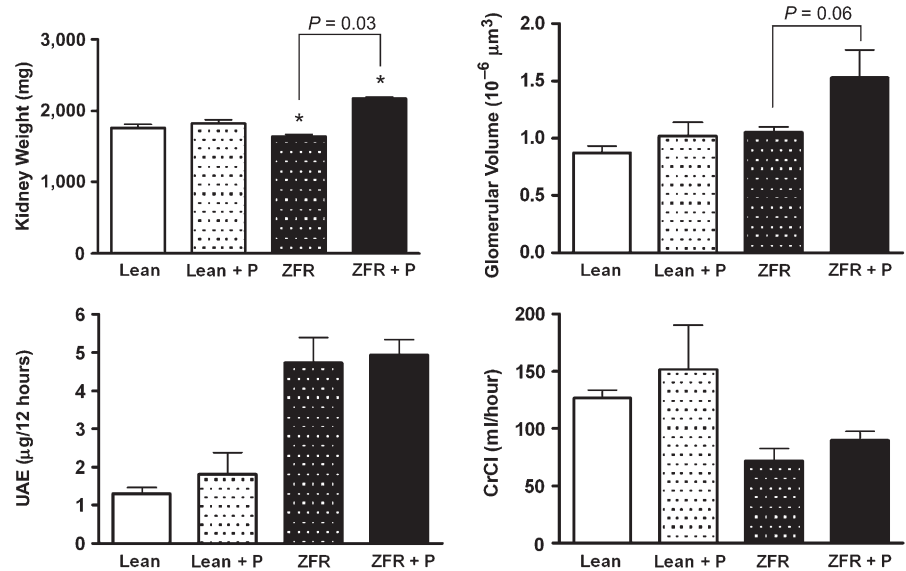
## RESULTS

Nineteen Zucker rats (nine in the ZFR + P group and 10 in the ZFR group) and 18 lean rats (eight in the lean + P group and 10 in the lean group) completed the protocol of the study. The other rats died as a consequence of weekly general anesthesia during the study.

### Kidney Weight, Glomerular Volume, UAE, and CrCl

Compared to ZFRs without periodontitis, ZFRs with periodontitis presented with an increased kidney weight ( $1,643.2 \pm 29.1$  mg versus  $2,173.5 \pm 218.4$  mg;  $P = 0.03$ ) and a borderline increase in glomerular volume ( $1.1 \pm 0.1 \times 10^{-6}$   $\mu\text{m}^3$  versus  $1.5 \pm 0.2 \times 10^{-6}$   $\mu\text{m}^3$ ;  $P = 0.06$ ). UAE and CrCl were not statistically different between the two Zucker groups (Fig. 1).

In lean rats, no statistically significant difference in kidney weight ( $1,765.0 \pm 4.4$  mg versus  $1,822.8 \pm 54.0$  mg) or glomerular volume ( $0.9 \pm 0.1 \times 10^{-6}$   $\mu\text{m}^3$  versus  $1.0 \pm 0.2 \times 10^{-6}$   $\mu\text{m}^3$ ) was found when comparing those with and without periodontitis. Again, no difference was found in UAE or CrCl between lean rats with and without periodontitis.



**Figure 1.**

Morphologic and functional renal parameters (mean  $\pm$  SEM). \* $P < 0.05$ .

### Renal Gene Transcripts

**Markers of renal permeability.** VEGFR-2 mRNA levels were comparable for ZFRs with or without periodontitis ( $5.0 \pm 0.4 \times 10^{-5}$  versus  $5.4 \pm 0.6 \times 10^{-5}$ ; Fig. 2). No significant differences in VEGF or nephrin mRNA levels were present between the two Zucker groups, although lower levels of both markers were seen for ZFRs with periodontitis ( $18.1 \pm 1.6 \times 10^{-5}$  versus  $14.9 \pm 1.1 \times 10^{-5}$  for VEGF and  $16.7 \pm 2.2 \times 10^{-6}$  versus  $13.3 \pm 2.2 \times 10^{-6}$  for nephrin). Comparable levels of the three evaluated markers of renal permeability were noted for lean rats with and without periodontitis ( $16.7 \pm 1.6 \times 10^{-5}$  versus  $16.3 \pm 1.0 \times 10^{-5}$  for VEGF,  $4.1 \pm 0.2 \times 10^{-5}$  versus  $4.5 \pm 0.3 \times 10^{-5}$  for VEGFR-2, and  $15.0 \pm 1.2 \times 10^{-6}$  versus  $15.1 \pm 1.3 \times 10^{-6}$  for nephrin; Fig. 2).

**Markers of renal matrix accumulation.** Non-significant elevations in mRNA levels of TGF- $\beta$  ( $8.4 \pm 0.4 \times 10^{-6}$  versus  $12.5 \pm 3.9 \times 10^{-6}$ ), fibronectin ( $15.9 \pm 8.2 \times 10^{-7}$  versus  $22.3 \pm 7.6 \times 10^{-7}$ ), and collagen IV $\alpha$ 1 ( $8.4 \pm 0.4 \times 10^{-6}$  versus  $12.5 \pm 3.7 \times 10^{-6}$ ) were noted in ZFRs with periodontitis compared to those without periodontitis (Fig. 3). CTGF mRNA levels were similar for the two Zucker groups ( $3.4 \pm 0.2$  versus  $3.6 \pm 0.5$ ).

## Mm 00437304, Applied Biosystems.

\*\*\* Mm 00440099, Applied Biosystems.

††† Mm 00441724, Applied Biosystems.

††† Mm 00515790, Applied Biosystems.

§§§ 185088267A, Applied Biosystems.

|||| 1546736, Applied Biosystems.

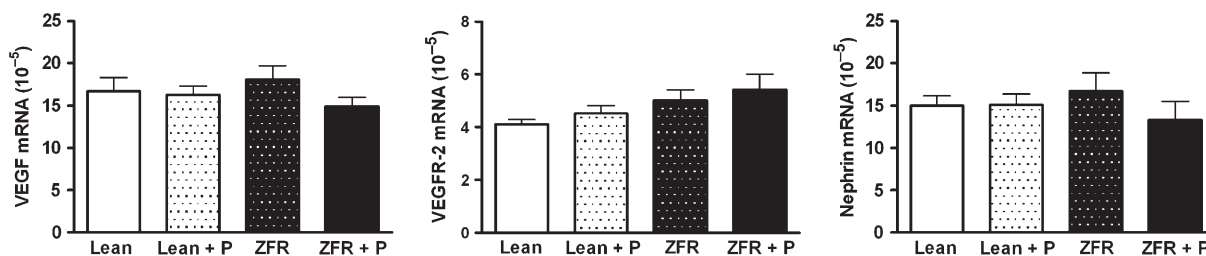
¶¶¶ Mm 497828, Applied Biosystems.

### 18S (4319413), Applied Biosystems.

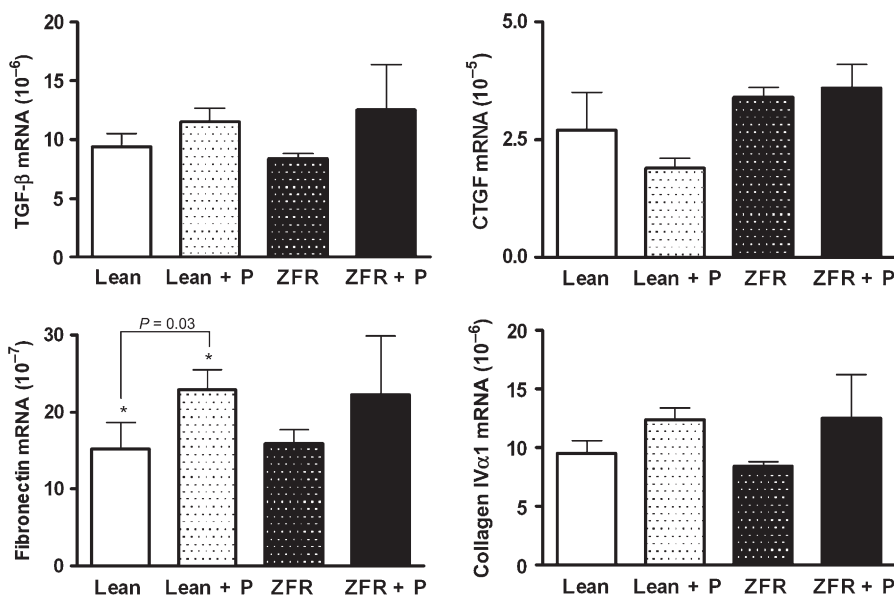
\*\*\*\* Sequence Detector, ABI Prism 7000, Applied Biosystems.

†††† Perkin-Elmer Cetus, Norwalk, CT.

†††† Statistical Analysis System (SAS) version 8.0, SAS Institute, Cary, NC.



**Figure 2.** mRNA expression of markers for renal permeability (mean ± SEM) \*P < 0.05.



**Figure 3.** mRNA expression of markers for renal matrix accumulation (mean ± SEM) \*P < 0.05.

Lean rats with periodontitis exhibited a statistically significant increase in fibronectin compared to lean rats without periodontitis ( $15.2 \pm 3.4 \times 10^{-7}$  versus  $22.9 \pm 2.6 \times 10^{-7}$ ;  $P = 0.03$ ). Although not statistically significant, lean rats with periodontitis presented elevated mRNA levels of TGF- $\beta$  ( $9.4 \pm 1.1 \times 10^{-6}$  versus  $11.5 \pm 1.2 \times 10^{-6}$ ) and collagen IV $\alpha$ 1 ( $9.5 \pm 1.1 \times 10^{-6}$  versus  $12.4 \pm 1.0 \times 10^{-6}$ ; Fig. 3).

**Liver and Heart Weights**

There was no statistically significant difference in liver weight for ZFRs with and without periodontitis ( $9,928.5 \pm 254.0$  mg versus  $11,072.0 \pm 917.1$  mg), whereas a borderline increase in heart weight was noted for ZFRs with periodontitis ( $705.2 \pm 22.7$  mg versus  $763.1 \pm 17.9$  mg;  $P = 0.06$ ). For lean rats, no statistically significant difference in liver weight ( $7,500.0 \pm 229.7$  mg versus  $8,153.2 \pm 322.7$  mg)

or heart weight ( $741.9 \pm 20.7$  mg versus  $744.9 \pm 27.4$  mg) was found between those with and without periodontitis.

**DISCUSSION**

This study is a continuation of a previously published study.<sup>6</sup> Thirty-seven rats were included, and data for body weight, food intake, glucose, insulin, glucose tolerance, insulin resistance, and alveolar bone loss and support were presented in the previous study. Briefly, there was not a statistically significant difference in final body weight for Zucker rats with and without periodontitis ( $328.6 \pm 10.1$  g and  $329.6 \pm 13.3$  g, respectively) or for lean rats with and without periodontitis ( $254.1 \pm 11.5$  g and  $252.6 \pm 5.2$  g, respectively). In ZFRs, periodontitis was associated with an

11% decrease in glucose tolerance, whereas it was associated with an 18% decrease in lean rats. Lean rats with periodontitis presented increased glucose concentration and insulin resistance and decreased insulin levels over time. Further, prediabetes increased the susceptibility to spontaneous and ligature-induced periodontitis.<sup>6</sup>

To our knowledge, this is the first study evaluating the development of early renal changes associated with periodontitis in ZFRs. In prediabetic ZFRs, kidney hypertrophy and a tendency to increased glomerular volume and heart weight were observed in association with periodontitis. For non-prediabetic Zucker rats, elevated levels of fibronectin mRNA levels were observed in the presence of periodontitis.

Diabetic kidney disease is characterized by the development of morphologic and functional alterations. Experimental animal models, including the ZFR, were

shown to mimic these changes.<sup>18-20</sup> The classic features of early diabetic kidney disease are renal and glomerular hypertrophy, which are believed to be caused, in part, by an increase in the number of capillaries to compensate for the microangiopathic changes.<sup>21</sup> Glomerular hypertrophy is usually associated with hyperfiltration and often results in microalbuminuria and increased CrCl. However, periodontitis was not associated with any of the evaluated functional renal changes in the present study. It can be speculated that the short-term nature of the study, together with the limited extension and severity of periodontitis, involving only two molars, could have contributed to the apparent lack of renal functional alterations. Further, the small sample sizes at the end of the study may have resulted in the lack of power to detect subtle renal changes. Although the chosen molecular markers did not reflect the reported morphologic changes, it is likely that periodontitis participated in the development of early diabetic kidney hypertrophy in the ZFR through other mechanisms.

The mechanisms responsible for the development of diabetic nephropathy are complex and not understood fully. Several growth factors have been suggested to take part in the pathways leading to renal changes.<sup>2,4</sup> Increased levels of VEGF and VEGFR-2 and decreased levels of nephrin, which is a molecule expressed in the glomerulus filtration barrier, diminished renal permeability in diabetes animal models.<sup>22-24</sup> Overproduction of TGF- $\beta$  and CTGF was linked to increased renal extracellular matrix formation, which is composed mainly of collagen and fibronectin.<sup>25,26</sup> In the present study, periodontitis was associated with increased fibronectin mRNA levels in Zucker lean rats, indicating increased renal matrix accumulation. No effect of periodontitis could be noted in any of the examined mRNA levels of markers for renal matrix accumulation or permeability in ZFRs. It was suggested that the peak of expression of growth factors in experimental models occurs before clinical alterations become evident.<sup>27</sup> Moreover, mRNA levels do not necessarily reflect protein levels, which were not investigated in the present study. Thus, the lack of statistical significance for the mRNA analyses in the present study does not exclude the possibility that the evaluated molecules could have participated in this process.

Few human studies have reported a correlation between periodontitis and diabetic kidney disease. Combined ischemic heart disease and nephropathy deaths were related significantly to the presence of severe periodontitis in patients with type 2 diabetes from the Gila River Indian Community.<sup>8</sup> In a more recent study<sup>9</sup> of the same population, the incidence of microalbuminuria and end-stage renal failure was related to the severity of periodontitis. Further, the concentration of immunoglobulin G antibody to *Porphyromonas gin-*

*ivalis* was correlated to albuminuria in lean patients with type 2 diabetes.<sup>7</sup> In a study<sup>28</sup> of a large sample of subjects without diabetes, periodontitis was associated with impairment of renal function. Although very early-stage kidney alterations were detected in the present study, our findings are in line with the above-mentioned results. Thus, based on the present experimental findings and on previous human investigations, it can be suggested that periodontitis has the potential to function as an aggravating factor for renal alterations.

The mechanisms underlying the influence of periodontitis in renal alterations are not clear. The chronic low-grade systemic inflammation characteristic of periodontitis may result in endothelial dysfunction, which has been associated with the development of nephropathy.<sup>29-31</sup> Moreover, the occurrence of repetitive bacteremia in the presence of periodontitis<sup>32</sup> may allow direct contact of periodontal pathogens with the glomerular tissues during renal blood filtration, possibly leading to cellular damage and consequent alteration in kidney function.<sup>28</sup> DNA from periodontal bacteria has been identified in atherosclerotic plaques and in abdominal aortic aneurysms, findings that may enhance the hypothesis of direct damage of periodontopathogens also in renal tissues.

Another interesting finding of the present study was the borderline significant cardiac enlargement noted in prediabetic rats with periodontitis. In humans, increased left ventricular mass was associated with periodontitis in hypertensive subjects<sup>33</sup> and in patients submitted to renal transplantation.<sup>34</sup> The ZFR is known to develop hypertension,<sup>35</sup> and although blood pressure was not measured in this study, the tendency for increased heart weight in ZFRs with periodontitis may indicate increased blood pressure in association with periodontitis in these rats.

## CONCLUSIONS

Ligature-induced periodontitis was associated with increased renal weight and a tendency toward increased glomerular volume and heart weight in ZFRs. In lean rats, an increased expression of fibronectin was detected in the presence of periodontitis. Our findings indicate the participation of periodontitis in the development and/or progression of early renal changes in ZFRs. The involved mechanisms are to be explored.

## ACKNOWLEDGMENTS

This study was supported by the Danish Dental Association, the National Research Council, and the Simon Spies Foundation, all in Copenhagen, Denmark; the Danish Diabetes Association, Odense, Denmark;

and Colgate-Palmolive, Lyngby, Denmark. The authors gratefully acknowledge the helpful assistance of Morten Grauballe, dentist, University of Copenhagen; Bo Bentzen, human biologist, University of Copenhagen; Karen Mathiassen, laboratory technician, University of Aarhus; and Kirsten Nyborg, laboratory technician, University of Aarhus. The authors report no conflicts of interest related to this study.

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Submitted August 3, 2007; accepted for publication October 26, 2007.