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Title:

Aggregatibacter Actinomycetemcomitans Infection Enhances Apoptosis *In Vivo* Through a Caspase-3 Dependent Mechanism in Experimental Periodontitis

Running title:

A.a. induced periodontal inflammation and apoptosis

Authors:

Jun Kang [1,2]

Beatriz de Brito Bezerra[3]

Sandra Pacios[2,5]

Oelisoa Andriankaja[2]

Yu Li[4]

Vincent Tsiagbe[4]

Helen Schreiner[4]

Daniel H. Fine[4]

Dana T. Graves[2#]

Affiliations:

[1]Department of Periodontology, School and Hospital of Stomatology, Peking University, Beijing, China

[2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA

[3] Prosthodontics and Periodontics Department, Piracicaba Dental School, University of Campinas, Piracicaba, Brazil

[4] Department of Oral Biology, New Jersey Dental School, University of Medicine and Dentistry of New Jersey, Newark, NJ

[5] Department of Periodontology, School of Dental Medicine, Universitat Internacional de Catalunya, Sant Cugat del Vallès, Spain

[#]**Corresponding author:**

Dana T. Graves

University of Pennsylvania School of Dental Medicine

240 South 40th Street

Philadelphia, PA 19104-6030 USA

Phone: (215) 573-8818

Email: *dtgraves@upenn.edu*

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ABSTRACT

The purpose of this study was to test the hypothesis that diabetes aggravates periodontal destruction induced by *Aggregatibacter actinomycetemcomitans* (*Aa*) infection. Thirty-eight diabetic and 33 normal rats were inoculated with *Aa* and euthanized at baseline, 4, 5 and 6 weeks after inoculation. Bone loss and the infiltration of polymorphonuclear leukocytes (PMNs) in gingival epithelium were measured in hematoxylin and eosin-stained sections. The induction of TNF-α was evaluated by immunohistochemistry and apoptotic cells by the transferase-mediated dUTP nick-end labeling (TUNEL) assay. After *Aa* infection, the bone loss in diabetic rats was 1.7-fold and PMN infiltration 1.6 fold higher than in normoglycemic rats ($P<0.05$). The induction of TNF-α was 1.5-fold higher and apoptotic cells up to 3 fold higher in diabetic vs. normoglycemic rats ($P<0.05$), respectively. Treatment with a caspase-3 inhibitor significantly blocked non-inflammatory cell apoptosis induced by *Aa* infection in gingival epithelium and connective tissue ($P<0.05$).). The results provide new insight into how diabetes aggravates Aa induced periodontal destruction in rats by significantly increasing the inflammatory response leading to increased bone loss and enhancing apoptosis of gingival epithelial and connective tissue cells through a caspase-3-dependent mechanism. Antibiotics had a more pronounced effect on many of these parameters in diabetic than normoglycemic rats suggesting a deficiency in the capacity of diabetic animals to resist infection.

Keywords: *Aggregatibacter actinomycetemcomitans*; diabetes; periodontal disease; apoptosis; host-pathogen interactions; animal models; caspase, inflammation, bone

INTRODUCTION

Periodontitis is one of the most prevalent infectious diseases worldwide. It is characterized by loss of supporting connective tissue and alveolar bone around the teeth *(36)*. Although triggered by a bacterial infection the destruction of periodontal tissue is caused by the inflammatory response to pathogenic bacteria. Immune mediators such as IL-1, TNF-α, IL-6 and RANKL have been found to be abundantly expressed in humans with periodontal disease and increased levels have been shown in the crevicular fluid from patients with periodontitis *(4, 13, 32)*. Animal studies have established cause and effect relationships between these cytokines and periodontal breakdown *(13, 24)*.

There are several types of periodontal diseases ranging from chronic periodontitis that affects adults to a form of aggressive periodontitis that primarily affects adolescents, localized aggressive periodontitis. Localized aggressive periodontitis (LAgP) is characterized by severe and rapid destruction of the supporting apparatus of the teeth, which may lead to tooth loss early in life *(3)*. *A. actinomycetemcomitans* (*Aa*) is commonly linked to LAgP *(3, 16)* and studies have shown that periodontal treatment leads to a reduction in its levels *(5, 31)*. Presence of *Aa* in periodontal pockets has also been considered an indicative of future disease progression *(9, 16)*. *Aa* has virulence factors such as leukotoxin and cytolethal distending toxin (CDT) that may contribute to its capacity to induce rapid tissue destruction by promoting apoptosis of a number of host cell types *(20)*.

A rat model has been developed to study pathogenic mechanisms of *Aa* induced periodontal tissue destruction *(8, 28, 38, 39)*. This model is characterized by infecting the animals with a rough strain of *Aa* that adheres to the oral epithelium and teeth *(7)*. Although it may not mimic the specific form of periodontal disease found in localized aggressive periodontitis in humans,

this model has provided insight into the colonization of the oral cavity by this bacterium and inflammation induced periodontitis *(28)*. However, relatively little is known about the local changes that are induced by this bacterium *in vivo*.

Periodontal disease is triggered by bacterial infection but the local inflammatory response has been shown to mediate the actual destruction of periodontal tissue. This response can be modulated by systemic conditions such as diabetes. Diabetes has been identified as one of the important risk factors for periodontitis increasing both its prevalence and severity *(26, 27, 40)*. One mechanism through which diabetes increases periodontal tissue loss and other diabetic complications is by exacerbating the inflammatory response to periodontal pathogens through increased oxidative stress, advanced glycation end products and expression of cytokines such as TNF-α *(12, 25, 33, 37)*.

Apoptosis is thought to contribute to periodontal disease progression. It has been suggested that apoptosis of epithelial cells may contribute to the loss of epithelial barrier function *(6)*. Moreover, loss of gingival fibroblasts has been shown to be one of the largest cellular changes that occurs with periodontal disease progression and may be associated with loss of connective tissue attachment *(29, 43)*. Infection by *Aa* has been shown to induce apoptosis in vitro *(21-23)*. However, relatively little is known about how it induces apoptosis *in vivo* and how a systemic condition such as diabetes affects *Aa* induced apoptosis and periodontal tissue destruction. Studies presented here address these issues using diabetic and matched normoglycemic rats, which are natural hosts of *Aa*. The results indicate that the effect of *Aa* infection on bone loss, TNF-α expression and apoptosis of epithelial cells and non-leukocytic gingival connective tissue cells is aggravated by diabetes. Moreover we demonstrate that apoptosis is induced by a caspase-3 dependent mechanism.

Materials and Methods

Animals

Goto-Kakizaki (GK) and normoglycemic control matched Wistar rats (5–10 weeks of age) weighing 150–250g were purchased from Charles River Laboratories (Wilmington, MA). The GK rat is a non-obese Wistar substrain that develops type 2 diabetes mellitus at age approximately 8 weeks. Rats were considered to be diabetic when glycated hemoglobin (HbAlc) levels exceeded 7.0%. During the experiments the HbAlc level in GK rats was typically 7.0-10.5%. All normoglycemic rats had HbAlc that ranged from 4.3 to 4.8%. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Aggregatibacter actinomycetemcomitans (Aa) Inoculation

Both diabetic (GK) and normal (Wistar) rats were inoculated with *Aa* as previously described *(39)*. To depress the 'natural' resident flora, rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days. During the last 2 days of antibiotics treatment, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex, Procter and Gamble, Cincinnati, OH). After a subsequent period of 3 days without antibiotics treatment, the rats were divided into 6 groups of approximately 7 rats each. The adherent *Aa* strain, Columbia University *Aa* clinical isolate #1,000 (CU1000NRif) was incubated in *Aa*-growth media with 35 mg/ml rifampicin (Sigma-Aldrich, St. Louis, MO) for 2 days. Adherent cells in the culture dishes were scraped into a solution of PBS plus 3% sucrose and minor adjustment was made by the addition of buffer to obtain 10^8 cells/ml (optical density$_{560}$ = 0.80). After fasting for 3 hours, rats received 10^8 *Aa* cells in 1g of powdered food supplemented with 3% sucrose. This protocol was followed for 4 days and repeated the next week for a total of 8 *Aa* inoculations in food *(39)*. During the first 4 days of the feeding rats also received 10^8 *Aa* in

PBS by oral gavage. After 1 hour, the inoculated food was removed and replaced with regular powdered food. Rats were euthanized four, five and six weeks after the inoculation period was completed. Baseline animals did not receive *Aa* in their food and were not inoculated with *Aa* but did receive powered food supplemented with 3% sucrose under the same conditions as experimental rats.

Treatment with Antibiotics and caspase-3 inhibitor

Four weeks after *Aa* inoculation two groups of rats received in their water a daily dose of kanamycin (20 mg) and ampicillin (20 mg) for 4 days with the intention to reduce the infection. Concomitantly, the oral cavities of the rats were swabbed with a 0.12% chlorhexidine gluconate rinse (Peridex, Procter and Gamble, Cincinnati, OH).

Caspase-3 inhibitor (Z-DEVD-FMK, SM Biochemicals, Anaheim, CA) was administered by intraperitoneal injection (1.5mg/kg). Control animals were injected with the same volume of vehicle (2% DMSO, MP Biomedicals, Solon, OH). Caspase-3 inhibitor begun one week prior to euthanasia and was injected daily until euthanized.

Sampling of total anaerobic bacteria (CFU) and Detection of *Aa* by PCR

Two microbial samples were collected, one after the inoculation of *Aa* and the other at the time of euthanasia. The rats were anaesthetized and their oral microflora was sampled with a cotton tip swab for soft tissue sampling, and a toothpick (Johnson & Johnson, Piscataway, NJ) for hard tissue sampling. Both samples were combined in tubes containing 1 ml PBS. Serial ten-fold dilutions were made and plated on trypticase soy agar (TSA) with 5% sheep blood (BD biosciences, San Jose, CA) for total anaerobe counts. Trypticase soy agar plates were incubated in an anaerobic atmosphere at 37^{o}C for 7 days to obtain total bacterial counts. To detect whether *Aa* was present in the samples DNA was prepared directly from the collected oral samples with a

DNA extraction kit (Qiagen, Valencia, CA) and subjected to polymerase chain reaction (PCR) analysis using forward and reverse primers (5'-GGAATTCCTAGGTATTGCGAAACAATTTGATC-3' and 5'-GGAATTCCTGAAATTAAGCTGGTAATC-3', respectively), which amplified a 262-base-pair PCR product from the *Aa* leukotoxin gene as previously described (10).

Level of Antibody to *Aa*

IgG antibody reactive with *Aa* was assessed by enzyme-linked immunosorbent assay (ELISA). Blood was collected by cardiac puncture and serum was obtained and stored at -20^{o}C. An *Aa* lysate was prepared and used to coat the wells of microtiter dishes (NUNC-ImmunoPlate with Maxi Sorp surface, Thermo Fisher Scientific, Rochester, NY). A standard curve was generated using purified rat IgG (Sigma-Aldrich, St Louis, MO) in carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich, St Louis, MO). Rat serum diluted 1/5 and 1/10 in blocking buffer was added to the wells coated with the *Aa* pellet lysate. The serum dilutions were added in duplicate wells, washed, incubated with rabbit anti-rat IgG-Fc conjugated to alkaline phosphatase (Bethyl Laboratories, Montgomery, TX) and quantified with p-nitrophenyl phosphate substrate (Sigma-Aldrich, Saint Louis, MO). Absorbance was read on a microplate reader at 405 nm.

Histomorphometric Analysis of Hematoxylin-/Eosin-stained Sections

Right maxillas were fixed in 4% paraformaldehyde at 4 ^{o}C for 48 hours and decalcified in 10% EDTA (pH 7.0) for 12 weeks. Paraffin-embedded sagittal sections were prepared at a thickness of 5 microns. The mid-interproximal region between 1st and 2nd molars was examined in each specimen and was established by being sectioned to a level where the root canal systems in adjacent teeth were visible. Two randomly chosen sections of each interproximal area were examined at 200×magnification. All data were analyzed by a blinded examiner who did not

know the group to which an animal belonged. Bone loss was measured as the distance between the cemento-enamel junction (CEJ) and the highest peak of the interproximal bone. The number of polymorphonuclear (PMNs) leukocytes was counted in the gingival epithelium at 600× magnification. The identification of these cells was confirmed by an experienced examiner.

Histomorphometric Analysis of TNF-α Immunohistochemistry-stained Sections

To evaluate the number of cells expressing TNF-α, sections were stained by immunohistochemistry with an antibody against TNF-α (IHCWORLD, Woodstock, MD). The number of positive cells was evaluated 1mm down from the level of bone crest apically in an area of periodontal ligament at between 1st molar and 2nd molar. Cell counts were obtained by one examiner and confirmed by a second independent examiner with similar results. Numbers of positive cells in epithelium and gingival connective tissue were evaluated based on a scale: 0, no positive cells; 1, 3 to 4 positive cells per field with weak immunostaining; 2, 4 to 10 positive cells per field with strong immunostaining; and 3, more than 10 positive cells per field with strong immunostaining. Sections were examined at 600×magnification.

Detection of apoptotic cells

Apoptotic cells were detected by an in situ transferase-mediated dUTP nick-end labeling (TUNEL) assay (DeadEnd™ Fluorometric TUNEL System kit, Promega, Madison, WI) following the manufacturer's instructions. This kit detects double-strand breaks in genomic DNA and identifies most stages of apoptosis. The fluorescein-12-dUTP-labeled DNA then was visualized directly by fluorescence microscopy. Additional counts were made to specifically avoid counting apoptotic leukocytes. This was accomplished by the TUNEL assay followed by immunofluorescence with an anti-CD18 antibody (Novus Biological, Littleton, CO). The number

of non-l eukocytic apoptotic cells (TUNEL+/CD18-) was counted at 200 X magnification with an immunofluorescent microscope using NIS Elements software (Nikon, Melville, N.Y) in epithelium or connective tissue above the alveolar bone crest. Cells counts were obtained by one examiner and confirmed by a second independent examiner with similar results.

Systemic Leukocyte Analysis

Rat lymph leukocytes were isolated and analyzed as previously described *(28)*. Single-cell suspensions were obtained from the submandibular and cervical lymph nodes. Lymphocyte populations were isolated by Ficoll-Hypaque density gradient centrifugation. Flow cytometry was conducted using anti-CD32 (clone D34-485) for blocking FcγII receptors, PE-conjugated anti-CD4 (clone OX-38), FITC-conjugated anti-CD3 (clone G4.18, BD Biosciences, San Jose, CA); FITC-labeled anti-FoxP3 (clone FJK-16s), and PE-conjugated anti-CD25 (clone OX39) from eBioscience (San Diego, CA); and anti-IA (clone 14-4-4S) from American Type Culture Collection (Manassas, VA). Blood was analyzed by HemaTrue Hematology Analyzer (HESKA, Loveland, CO). Total number of white blood cells, numbers of lymphocytes, monocytes and granulocytes, the percentages of lymphocytes, monocytes and granulocytes were analyzed.

Statistical analysis

Differences between two groups such as diabetic and normal were determined by Student's t test and between time points within a group by one way ANOVA except for evaluation of TNF-α. Differences in TNF-α values were determined by non-parametric analysis with Mann-Whitney U test. Significance levels were set at 5%.

RESULTS

Induction of Periodontal Disease

At baseline the antibody titer level in diabetic rats was low and increased after *Aa* infection so that at 6 weeks it was 32-fold higher than baseline ($P<0.01$) (Fig 1A). And for all *Aa* infected diabetic rats, the antibody titers rats were 18-fold higher than non-infected animals ($P<0.01$) (Fig 1B). Moreover, after infection diabetic animals had antibody titers level that were 2.3-fold higher than normoglycemic infected animals ($P<0.05$) (Fig 1B). Diabetic rats also showed a significant decrease in antibody titer level after antibiotic treatment ($P<0.05$) (Fig 1C).

The impact of *Aa* infection on total anaerobic bacteria levels in non-infected normoglycemic and diabetic rats as well as infected diabetic rats was also examined. Despite a trend towards increased levels of anaerobic bacteria in infected diabetic rats compared to normoglycemic the results were not significant differences (Supplemental Table 1). Similarly the percent rats exposed to *Aa* that had detectable infection was not higher between the normoglycemic and diabetic groups (Supplemental Table 2)

A number of parameters were evaluated to examine the impact of *Aa* infection on systemic leukocyte populations in diabetic animals. Following Aa infection there was no change in *Aa* infected compared to uninfected rats of MHC II positive cells, T cells, B cells or Treg cells for either normoglycemic or diabetic rats (Tables 1 and 2). However there was a slight reduction in the percent lymphocytes in the peripheral circulation of infected diabetic rats compared to infected normoglycemic rats and a 1.5 fold increase in the percent granulocytes in infected diabetic compared to infected normoglycemic rats ($P<0.05$) (Table 2).

Bone loss was induced in the diabetic rats as evidenced by an increase in the distance from CEJ to alveolar bone crest after 5 weeks ($P<0.05$) (Fig 2A and Supplemental Fig 1). In *Aa* infected diabetic animals there was a 1.8-fold increase in bone loss compared to non-infected diabetic rats at baseline. The bone loss of 1.7 fold higher in the infected diabetic compared to

infected normoglycemic rats (P<0.05) (Fig 2B). Antibiotic treatment significantly decreased bone loss in the diabetic rats (P<0.05) (Fig 2C).

PMN infiltration

The formation of a PMN infiltrate in gingival epithelium was assessed (Supplemental Fig 2). PMNs increased 7-fold (P<0.05) four weeks after *Aa* inoculation in diabetic rats (Fig 3A). Both normal and diabetic non-infected rats had similar levels of PMNs. After *Aa* infection PMN numbers increased 3.4-fold in normal rats, while it increased 5.3 fold in diabetic rats (P<0.05) (Fig 3B). The greater increase in PMNs in infected diabetes rats, compared to infected normal rats, is consistent with the significant increase (P<0.05) in blood granulocytes in infected diabetic rats. Antibiotic treatment significantly reduced the PMN infiltration in the diabetic rats (P<0.05) (Fig 3C).

TNF-α

TNF-α was measured in the gingival epithelium and connective tissue. In epithelium, TNF-α values of diabetic rats were significantly higher in those exposed to *Aa* inoculation compared to non-infected diabetic rats (P<0.05) and significantly higher than infected normoglycemic rats (P<0.05) (Fig 4B and Supplemental Fig 3). Antibiotic treatment resulted in a significant decrease in TNF-α expression in the epithelium of diabetic rats (P<0.05) but had no effect in the normoglycemic group (Fig 4C).

TNF-α was also measured in the gingival connective tissue. It significantly increased in diabetic rats 5 weeks after *Aa* infection (P<0.05) (Fig 5A) and was substantially higher than values found in infected normoglycemic rats (P<0.05) (Fig 5B). When treated with antibiotic there were no differences in TNF-a values in the connective tissue of diabetic and normal rats (P>0.05) (Fig 5C).

Induction of Apoptosis

Because apoptosis is thought to play an important role in periodontal disease progression we determined whether diabetic animals had significantly higher levels of apoptosis in the gingival epithelium (Supplemental Fig 4) and whether the increase was mediated by caspase-3 in both. Prior to *Aa* infection the level of apoptosis was low in both diabetic and normoglycemic groups. The onset of *Aa* infection significantly increased the level of apoptosis 2 to 3 fold in the normoglycemic rats and 12-fold in the diabetic with the difference between them being significant ($P<0.05$) (Fig 6A). The results were similar when presented as the percent gingival epithelial cells that were apoptotic or the number of apoptotic epithelial cells per um^2 (Fig 6B). The principal leukocytic cell type infiltrating *Aa*-infected gingiva was granulocyte. Apoptosis was evaluated as the percent of non-leukocytic TUNEL+/CD18- cells in the gingival epithelium. The number of TUNEL+/CD18- epithelial cells was significantly increased in both the normal and diabetic groups after *Aa* infection ($P<0.05$) (Fig 6C). The percent of apoptotic cells in diabetic animals was 2-fold greater than normoglycemic animals ($P<0.05$) (Fig. 6C). To assess the impact of inhibiting caspase-3/7, the specific caspase inhibitor DEVD was administered daily starting on week 4 and the number of TUNEL+/CD18- cells was counted one week later. *Aa* infection at this time point increased apoptosis of epithelial cells by 2.6-fold compared to baseline but antibiotic treatment had no significant effect in reducing these levels ($P>0.05$). Treatment with caspase inhibitor plus antibiotics reduced the number of apoptotic epithelial cells by reversing the impact of *Aa* infection to baseline levels ($P<0.05$) (Fig 6D).

Apoptosis was also examined in gingival connective tissue. Both normal and diabetic rats showed an almost 3 fold increase in apoptotic cells after infection when examined as the percentage of positive cells or as the number of apoptotic cells per area ($P<0.05$) (Fig.7A, B).

The total number of apoptotic cells in the gingival connective tissue of the diabetic group was more than 2-fold higher than the normoglycemic rats ($P<0.05$). The percent of TUNEL+/CD18- cells in the gingival connective tissue was measured. Following *Aa* infection values increased 3.9-fold ($P<0.05$) in diabetic but not in normal animals (Fig 7C). At 5 weeks *Aa* infection the percent of TUNEL+/CD18- cells significantly increased in diabetic group ($P<0.05$) (Fig 7D). Antibiotic treatment alone had no effect, but antibiotic treatment combined with caspase-3/7 inhibitor significantly blocked the increase in non-granulocytic cell apoptosis in the connective tissue ($P<0.05$) (Fig 7D).

DISCUSSION

The results here demonstrate that *Aa* infection significantly enhances PMN infiltration and TNF-α expression in both normal and diabetic rats. Moreover, each of these parameters was significantly greater in the diabetic animals, which agrees with the increased bone loss observed in the diabetic group here as well as in other studies *(17, 29, 30)*. Thus, diabetic rats exhibited greater inflammatory response compared to the normoglycemic group in response to a similar *Aa* inoculum.

Diabetes generally enhances inflammation by altering myeloid and lymphoid functions (12, 34). We found here that the local periodontal inflammatory response in diabetic animals was greater as evidenced by an enhanced expression of TNF-α and a larger PMN infiltrate and is consistent with findings in other models (15, 33). These local findings were in agreement with the significant increase in percent of whole blood granulocytes in diabetic rats post-infection. Elevated levels of antibody against *Aa* were also found in diabetic rats post-infection when compared to normal rats. The number of lymphocytes collected from whole blood, however, did

not exhibit the same trend, showing a significant decrease compared to normoglycemic rats after *Aa* infection. It is conceivable that this decrease in lymphocyte population could be a result of CDT-induced apoptosis. Alternatively, the decrease in lymphocyte population after *Aa* infection may be due to a proportional increase in granulocytes.

Aa infection has been shown to increase apoptosis *in vitro* but has not yet been tested *in vivo* in a periodontal model *(20)*. We demonstrate here that inoculating animals with *Aa* significantly stimulated apoptosis in both gingival epithelium and connective tissue of rats, especially in the diabetic animals. Other studies have also shown that apoptosis is significantly increased in diabetes when periodontal disease is induced in an animal model *(14, 29)*. There are several mechanisms through which *Aa* infection could enhance apoptosis in the rat. Our study indicates that the high rate of apoptosis in diabetic rats due to *Aa* infection is largely blocked by a caspase-3/7 inhibitor. It is possible that *Aa* through its cytolethal distending toxin (CDT) could stimulate apoptosis. CDT has been shown to induce apoptosis in epithelial cells, fibroblasts and endothelial cells *(19, 35)*. It has recently been shown that CDT induces apoptosis through a caspase-3 dependent pathway in immortalized gingival epithelial cells *(1)*. However, the other apoptosis inducing factor produced by *Aa*, leukotoxin A, appears not to stimulate apoptosis in rat cells *(20)*. Alternatively, *Aa* could induce apoptosis through indirect mechanisms. Interestingly, diabetic rats had significantly higher TNF-α levels and more apoptotic cells compared to normal rats after *Aa* infection. TNF-α has been shown to mediate both *P. gingivalis* and LPS induced apoptosis *in vivo (2, 11)*. Thus excessive production of TNF-α is another potential pathway through which diabetes could enhance apoptosis of epithelial and connective tissue cells thereby affecting the response to bacterial infection and may occur simultaneously with CDT induced apoptosis.

The impact of antibiotic treatment post-infection was also evaluated. Antibiotics have long been used as an adjunct therapy in the treatment of localized aggressive periodontitis *(18, 41, 42)*. We also examined the impact of antibiotic treatment on *Aa* antibody titer, alveolar bone resorption, PMN infiltration, TNF-α levels and apoptosis in *Aa* infected periodontium. For *Aa* antibody titer, PMN infiltration, TNF-α levels and apoptosis the diabetic rats showed a significant reduction with antibiotic treatment while these parameters were not reduced by antibiotic treatment in normoglycemic rats. These results suggest that there are anti-bacterial deficits in diabetic mice that contribute to greater induction of pro-inflammatory events stimulated by periodontal pathogens at the local level that can be reversed by antibiotic treatment.

In summary, the impact of diabetes on the periodontium was investigated in a relatively new model of periodontitis, oral inoculation of *Aa* in the rat, which has the advantage that the rat is a natural host of *Aa*. In this model, diabetes affected *Aa*-induced periodontal destruction by significantly increasing the inflammatory response leading to increased bone loss and apoptosis of gingival epithelial and connective tissue cells. The excessive production of TNF-α and the impact of CDT could be potential mechanisms through which apoptosis was induced at higher levels in diabetic animals. Antibiotics were able to reverse many parameters of the local host response in diabetic compared to normoglycemic animals suggesting that a component to the enhanced inflammatory response is due to a deficit in the capacity of diabetic animals to resist infection. This information provides valuable insight as to how diabetes may alter host-bacteria interactions in a way that promotes periodontal breakdown.

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372 **Table 1. Lymphocyte populations of *Aa* non-infected and infected rats**

	Normal		Diabetic	
	Non-infected	Infected	Non-infected	Infected
MHC II	55.9±11.7	44.1±14.8	38.3±6.8*	37.0±12.0
CD4+	31.5.1±11.4	32.4±13.0	42.1±9.3	42.7±13.9
CD8+	24.6±6.9	35.2±10.7	29.6±9.4	31.4±8.9
CD25+	5.2±0.9	6.7±2.8	5.4±0.8	7.9±2.4
FoxP3+	4.3±0.8	5.3±3.1	7.0±3.4	7.2±2.4

373 Lymphocyte populations from draining cervical and submandibular lymph nodes were analyzed
374 as described in MATERIALS and METHODS. Rats described in Fig.1 were examined for
375 lymphocyte populations according to status of infection. Each value is the mean of 5 to 7 rats ±
376 SE. * P<0.05, compared to normal rats.
377
378 **Table 2. Leukocytic cells of *Aa* non-infected and infected rats**

	Normal		Diabetic	
	Non-infected	Infected	Non-infected	Infected
WBC (10^3UI/mL)	7.9±2.9	7.8±4.7	6.9±3.2	8.0±4.0
Lymphocytes (10^3UI/mL)	6.2±2.5	5.8±3.3	4.4±1.0	5.2±2.8
Monocytes (10^3UI/mL)	0.4±0.1	0.3±0.2	0.4±0.3	0.4±0.2
Granulocytes (10^3UI/mL)	1.3±0.5	1.7±1.5	2.2±2.0	2.3±1.4
% Lymphocytes	77.0±5.5	76.0±10.5	69.0±13.8	65.6±13.5*
% Monocytes	4.8±1.0	3.5±1.2	4.8±1.4	4.5±1.8
% Granulocytes	18.1±4.6	20.6±9.9	26.2±12.5	30.0±13.4*

379 Leukocytic cells from peripheral blood were analyzed as described in MATERIALS and
380 METHODS. Rats described in Fig.1 were examined for leukocytic cells according to status of
381 infection. Each value is the mean of 5 to 7 rats ± SE. * P<0.05, compared to normal rats. WBC,
382 white blood cells.

383

384 **Figure Legends**

385 Figure 1. Diabetes increases the antibody titer to *Aa* in infected rats. The diabetic and normal rats
386 were infected orally with *Aa* and antibody (IgG) reactive with *Aa* was assessed by ELISA. After
387 4 weeks infection one group of rats was treated with antibiotics or equivalent vehicle alone. Rats
388 were euthanized at baseline and 4, 5 and 6 weeks after Aa inoculation was completed. (A)
389 Antibody titer levels in diabetic rats over time. (B) Antibody titer in non-infected (baseline) and
390 infected (4-6 weeks) normoglycemic and diabetic rats. (C) Effect of antibiotic treatment on
391 antibody titer in normoglycemic and diabetic rats. Each value in A, B and C is the mean of 5 to 7
392 rats ± SEM. *Significant difference between diabetics and normal rats (P<0.05). $^{+}$Significant
393 difference between diabetics rats in different groups (P<0.05).

394 Figure 2. Diabetes increases bone loss in *Aa* infected rats. The distance between CEJ to alveolar
395 bone crest was measured (A) CEJ to bone distance in diabetic rats. (B) CEJ to bone distance in
396 non-infected (baseline) and infected (4-6 weeks) normoglycemic and diabetic rats. (C) Effect
397 of antibiotic treatment on CEF to bone distance in normoglycemic and diabetic rats. Each value
398 is the mean of 5 to 7 rats ± SE. *Significant difference between diabetics and normal rats
399 (P<0.05). $^{+}$Significant difference between diabetics rats in different groups (P<0.05).

400 Figure 3. Diabetes increases the number of PMNs of *Aa* infected rats. The number of PMNs
401 infiltrating the gingival epithelium was measured. (A) PMN infiltration in diabetic rats over time.
402 (B) PMNs in non-infected (baseline) and infected (4-6 weeks) normoglycemic and diabetic rats.
403 (C). Effect of antibiotic treatment on PMN infiltration in normoglycemic and diabetic rats. Each
404 value in A, B and C is the mean of 5 to 7 rats ± SE. *Significant difference between diabetics
405 and normal rats (P<0.05). $^{+}$Significant difference between diabetics rats in different groups

406 (P<0.05).

407 Figure 4. Diabetes increases the TNF-α expression in the gingival epithelium of *Aa* infected rats.
408 TNF-α positive cells were detected by immunohistochemistry in histologic specimens using a
409 specific antibody. Rats described in Fig. 1 were examined for TNF-α expression using the
410 following scale that took both number of immunopositive cells and intensity of immunostaining
411 into account: 0: no positive cells; 1: 3 to 4 positive cells per field with weak immunostaining; 2:
412 4 to 10 positive cells per field with strong immunostaining; and 3: more than 10 positive cells per
413 field with strong immunostaining. (A) TNF-α in gingival epithelium of diabetic rats. (B) TNF-α
414 expression in non-infected (baseline) and infected (4-6 weeks) gingival epithelium in
415 normoglycemic and diabetic rats. (C). Effect of antibiotic treatment in normoglycemic and
416 diabetic rats. Each value represents the mean of 5 to 7 rats ± SEM. *Significant difference
417 between diabetics and normal rats (P<0.05). $^{+}$Significant difference between diabetics or normal
418 rats in different groups (P<0.05).

419 Figure 5. TNF-α expression is increased in gingival connective tissue of diabetic rats following
420 *Aa* infection. (A) TNF-α expression in gingival connective tissue of diabetic rats. (B)
421 TNF-α expression in non-infected (baseline) and infected (4-6 weeks) gingival connective tissue
422 in normoglycemic and diabetic rats. (C). Effect of antibiotic treatment in gingival connective
423 tissue of normoglycemic and diabetic rats. Each value represents the mean of 5 to 7 rats ± SEM.
424 *Significant difference between diabetics and normal rats (P<0.05). $^{+}$Significant difference
425 between diabetics or normal rats in different groups (P<0.05).

426 Figure 6. Diabetes increases the apoptosis of epithelial cells of *Aa* infected rats in a caspase-3
427 dependent manner. Apoptotic cells were detected in gingival epithelium by TUNEL staining in

epithelium in rats described in Fig. 1. In some groups rats were treated with a antibiotic or antibiotic plus capase-3 inhibitor starting at week 4. (A) Percent of apoptotic gingival epithelial cells per total number of gingival epithelial cells; (B) Total number of apoptotic gingival epithelial cells per area. C and D: Non-leukocytic cells were identified as CD18 negative and apoptotic cells identified as TUNEL positive. (C) TUNEL+/CD18- cells per total number of CD18- cells. (D) Rats at week 4 were treated with antibiotic or antibiotic plus caspase-3 inhibitor and euthanized a week after .TUNEL+/CD18- cells per total number of CD18- cells were counted. Each value is the mean of 5 to 7 rats ± SEM. *Significant difference between diabetics and normal rats ($P<0.05$). $^{+}$Significant difference between diabetics or normal rats in different groups ($P<0.05$). ^Significantly different between antibiotic and antibiotic plus caspase-3 inhibitor.

Figure 7. Diabetes increases apoptosis of cells in gingival connective tissue of *Aa* infected rats in a caspase-3 dependent manner. (A) Total apoptotic gingival connective tissue cells per total number of gingival connective tissue cells; (B) Total apoptotic gingival connective tissue cells per epithelial area. C and D: Non-leukocytic cells were identified as CD18 negative and apoptotic cells identified as TUNEL positive. (C) TUNEL+/CD18- cells per total number of CD18- cells in connective tissue. (D) Rats at week 4 were treated with antibiotic or antibiotic and caspase-3 inhibitor and euthanized a week after. The TUNEL+/CD18- cells per total number of CD18- cells were counted. Each value is the mean of 5 to 7 rats ± SEM. *Significant difference between diabetics and normal rats ($P<0.05$). $^{+}$Significant difference between diabetics or normal rats in different groups ($P<0.05$). ^Significantly different between antibiotic and antibiotic plus caspase-3 inhibitor.

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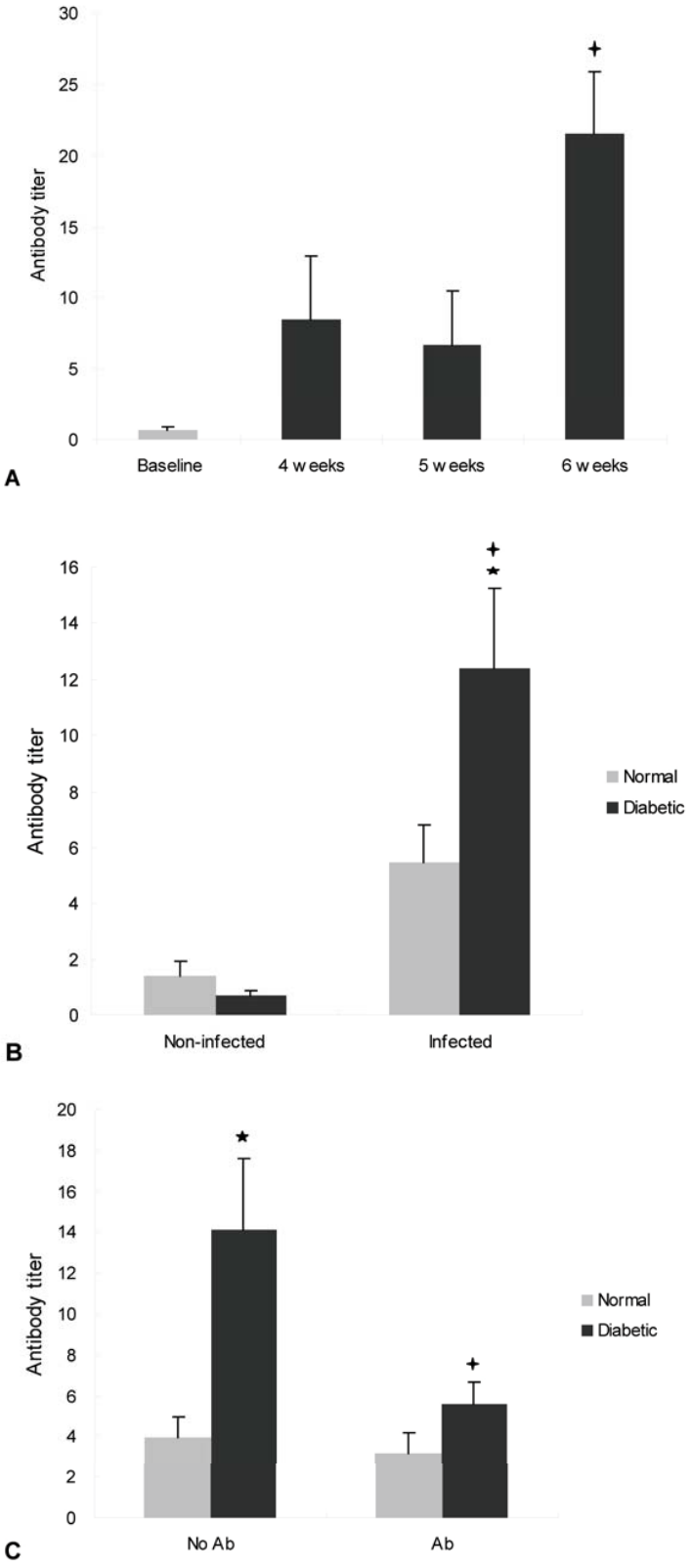
Fig 1.



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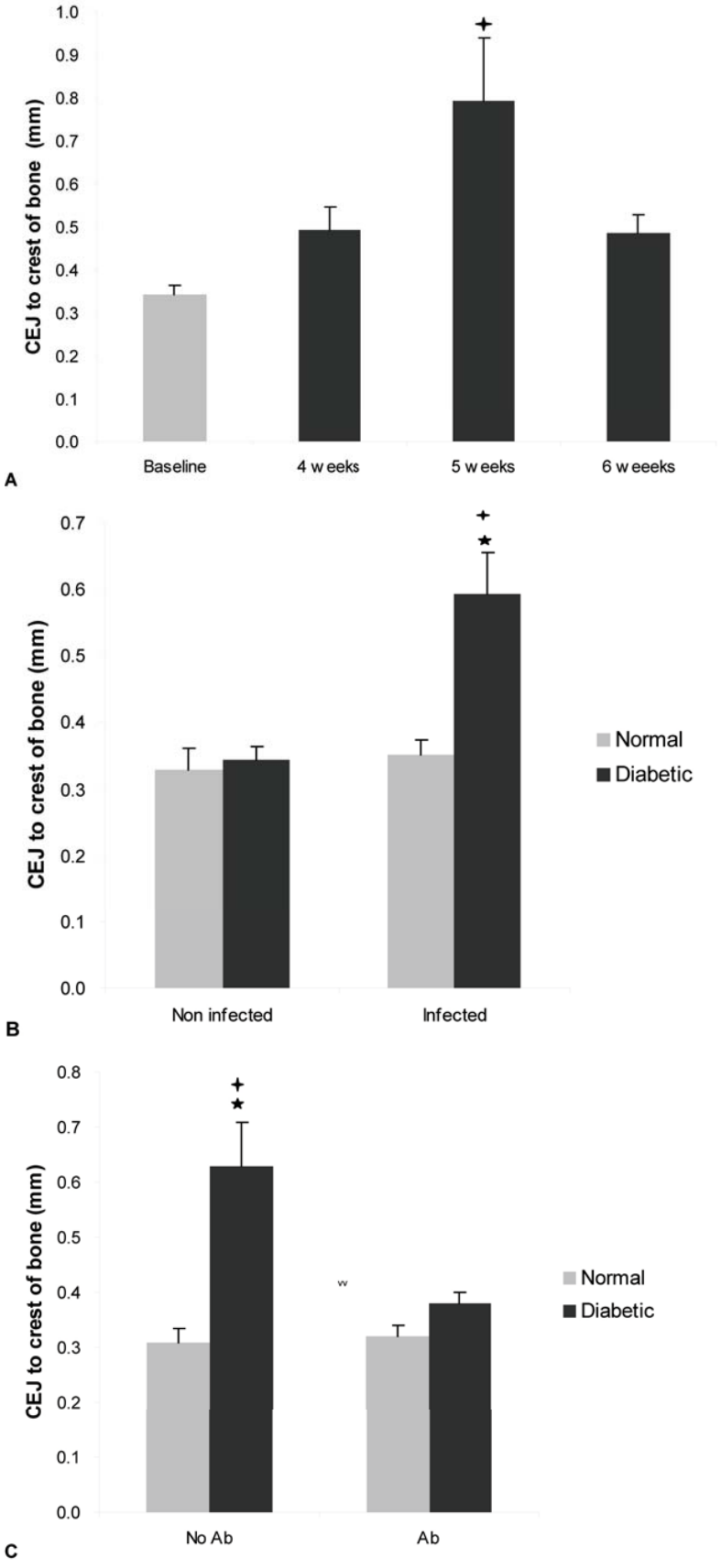

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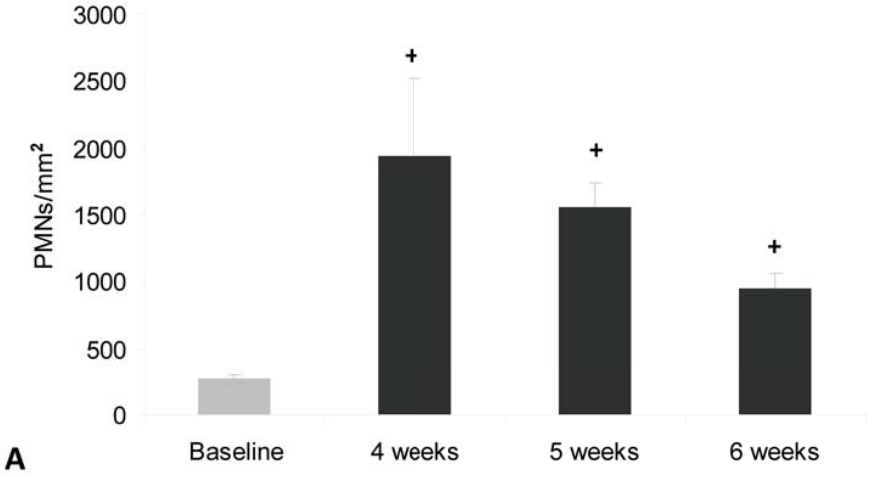

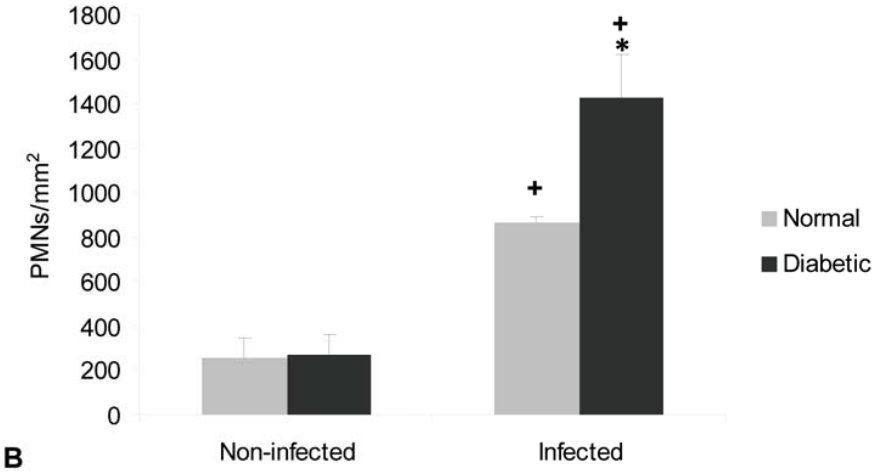

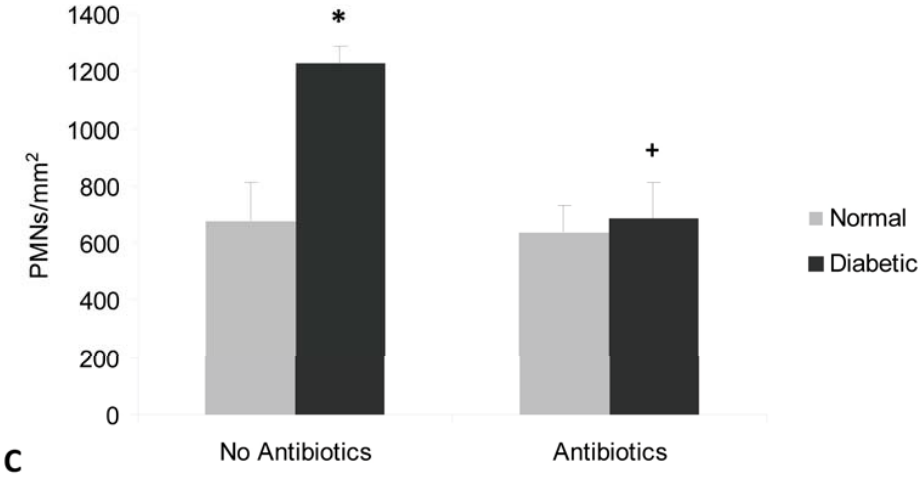

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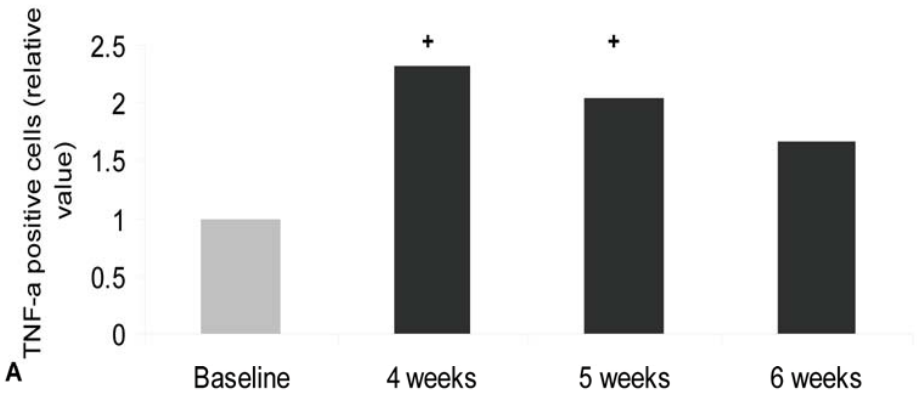

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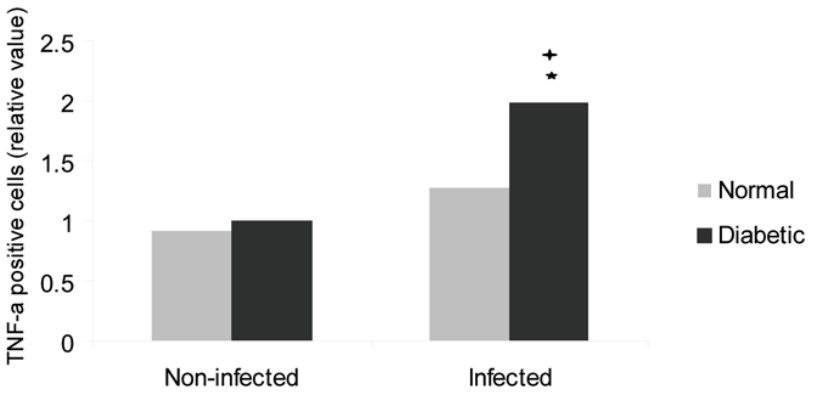

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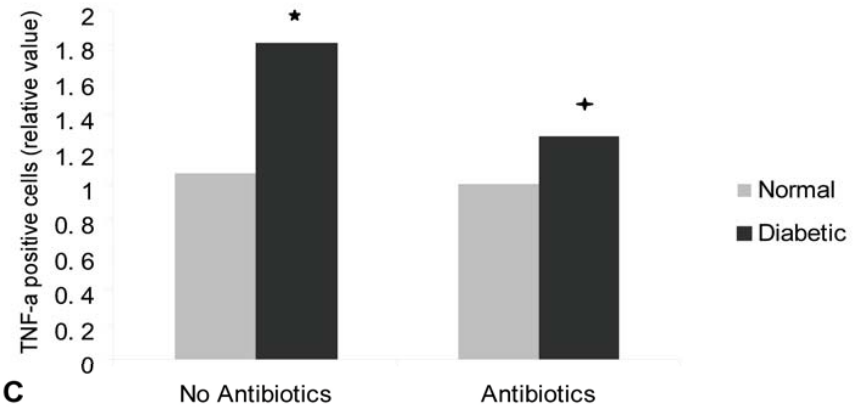

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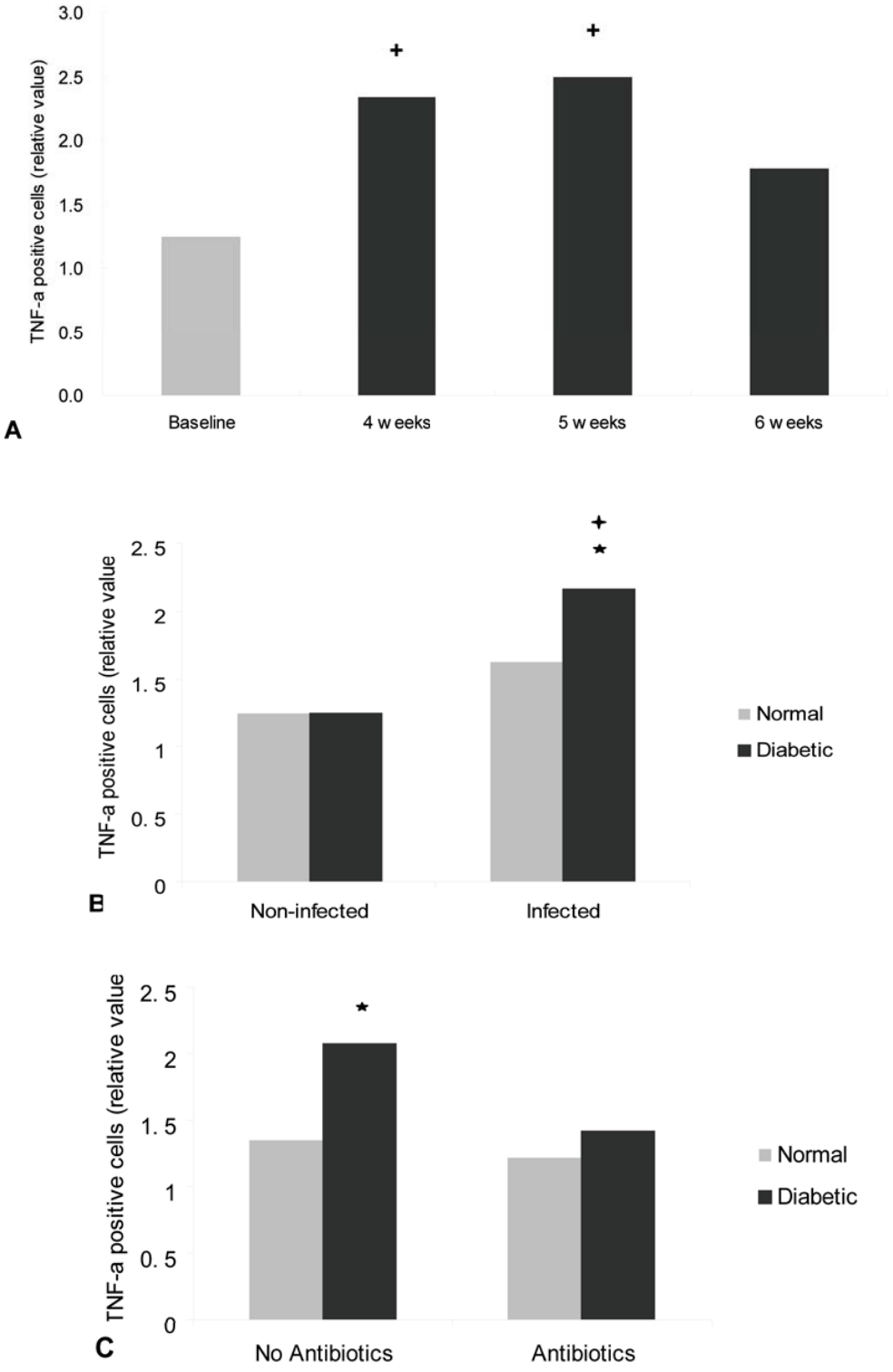

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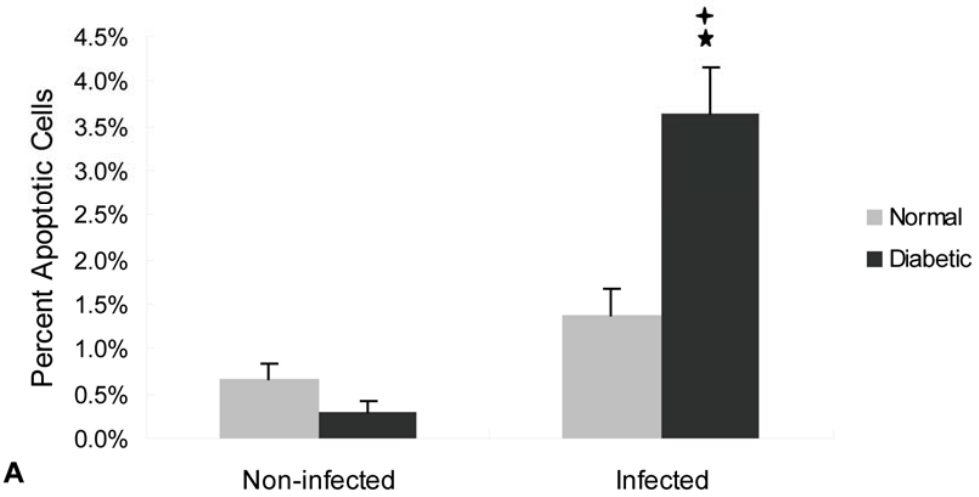

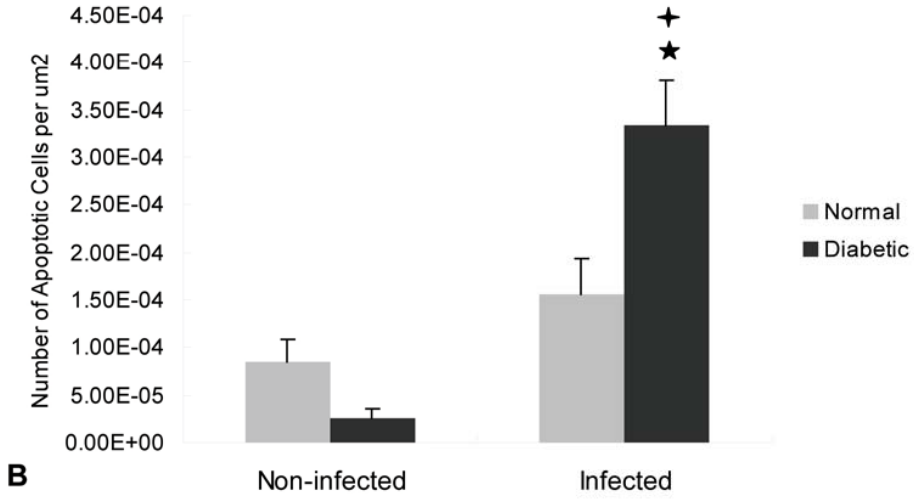

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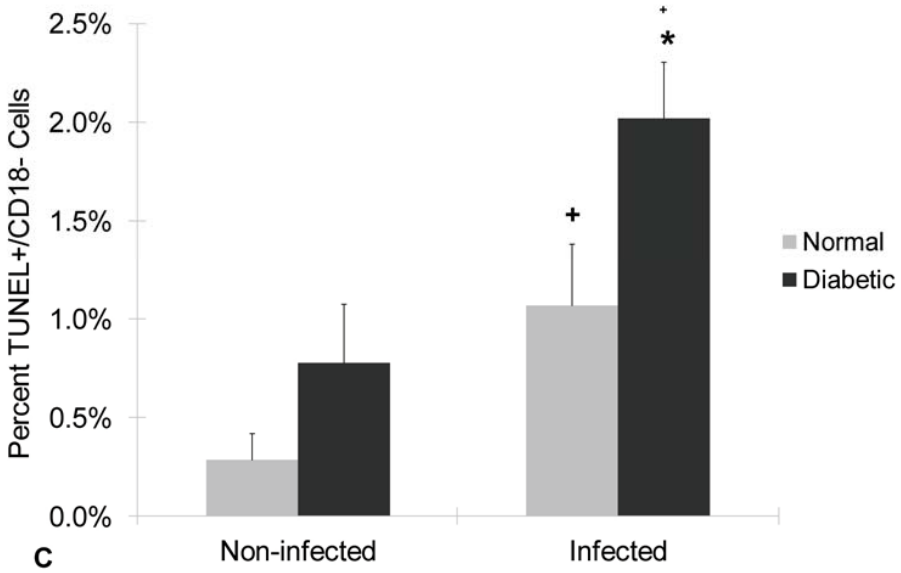

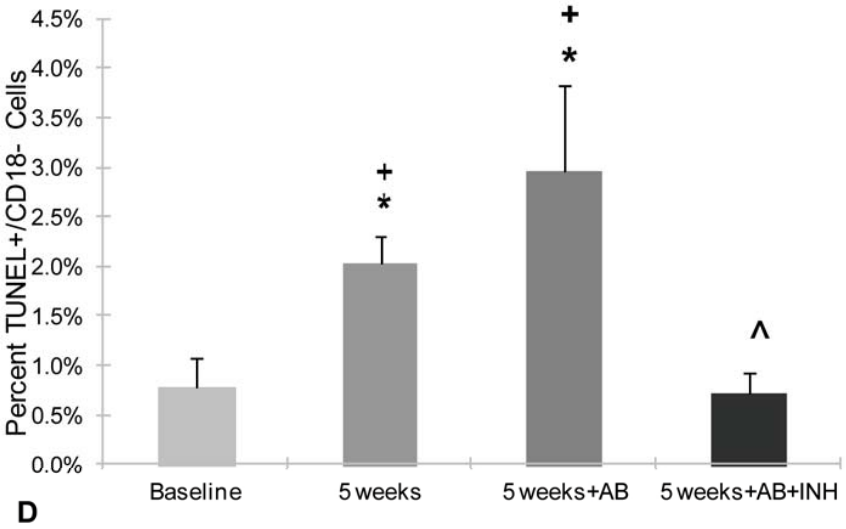

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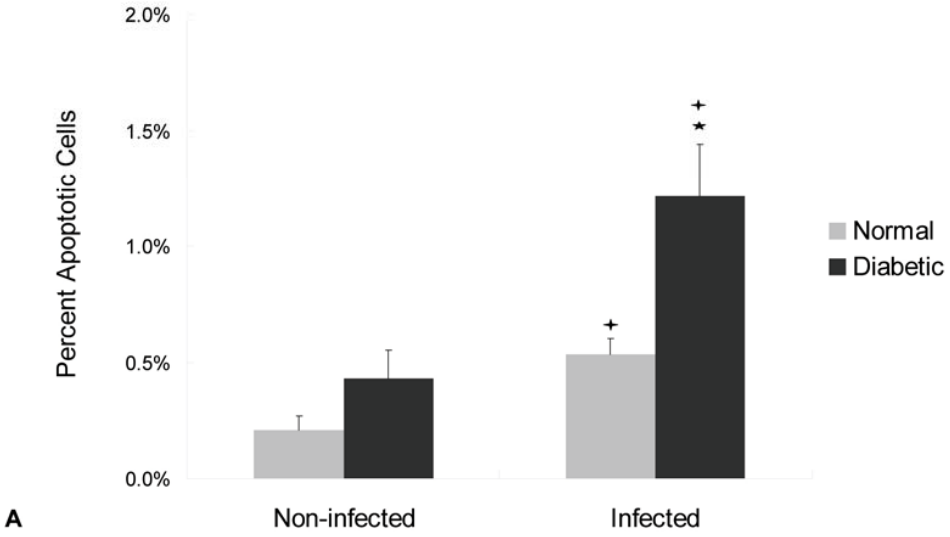

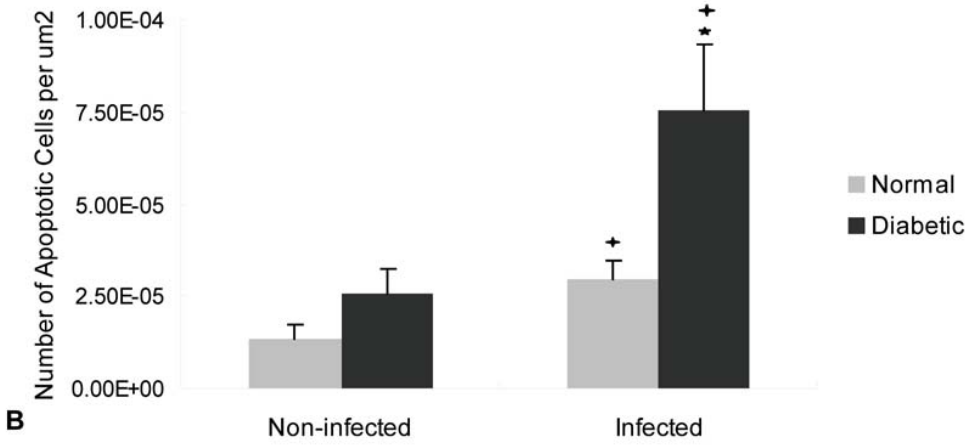

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