Cross Talk between Apical Periodontitis and Metabolic Disorders: Experimental Evidence on the Role of Intestinal Adipokines and Akkermansia muciniphila

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Abstract

Introduction: Infection and dysbiosis present a close relationship with metabolic diseases although the influence of apical periodontitis (AP) in this context needs further investigation. This study evaluated the influence of AP in a rat model of metabolic syndrome induced by 10% fructose supplementation. Methods: Male Wistar rats were used. Animals that received a high-fructose diet (HFD, n = 30) or filtered water (control, n = 30) were subdivided into the following groups: (1) without induction of AP (no AP, n = 10 each), (2) with AP induction 2 weeks before euthanasia (AP 14 days, n = 10 each), and (3) with AP induction 4 weeks before euthanasia (AP 28 days, n = 10 each). Results: HFD triggered metabolic syndrome, as indicated by the induction of overweight and hyperglycemia, besides polydipsia, regardless of the AP induction. Serum or intestinal tumor necrosis factor, interleukin 1 beta, and interleukin 6 levels were undetectable, regardless of the experimental group. Serum leptin and adiponectin levels were significantly increased in the HFD group without AP induction. The intestinal levels of leptin were significantly increased in the groups with 28 days of AP induction despite HFD. A significant elevation of liver glutathione levels was observed in animals submitted to HFD and AP for 14 days. AP induction (14 or 28 days) led to pulp and periapical tissue inflammation without any influence of HFD. Either HFD or AP induction led to dysbiosis, as indicated by a significant reduction of fecal A. muciniphila expression. Conclusions: We provide novel evidence that AP can have systemic impacts on metabolic disorders, likely by modulating intestinal metabolism and microbiota. (J Endod 2019;45:174–180)

Key Words

Adipokines, Akkermansia muciniphila, apical periodontitis, fructose, gut, metabolic syndrome

Significance

We provide novel evidence that AP can have systemic impacts on metabolic disorders, likely by modulating intestinal adipokines and microbiota.

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The close relationship among obesity, low-grade inflammation, and microbiota sparked our interest to evaluate the role of apical periodontitis (AP) in the outcome of systemic metabolic diseases. AP is associated with a complex microbiota; because this lesion has no epithelial barrier, oxidative stress products and inflammatory cytokines can easily reach the bloodstream (11). There is a lack of scientific studies on AP and its systemic effects. Thus, the present study investigated the cross talk between systemic inflammatory changes and intestinal dysbiosis, and their correlation with AP outcomes in a rat model of metabolic syndrome induced by a high-fructose diet.

Material and Methods

Animals

Male Wistar rats (8 weeks old, total N = 60 animals) weighing 240–280 g at the onset of the experiments were obtained from the Central Animal House of the Pontificia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. The animals were housed under standard conditions of temperature (22 ± 2 °C), light (12-hour light-dark cycle), and humidity (50%–70%) in ventilated cages with autoclaved wood chip bedding. They received a standard rat chow diet (Nuvilab; Nuvital Nutrients, Colombo, PR, Brazil) with free access to filtered water or 10% fructose solution depending on the experimental group. The experimental protocols followed the current Brazilian guidelines for the care and use of animals for scientific and didactic procedures from the National Council for the Control of Animal Experimentation. The local animal ethics committee evaluated and approved all of the protocols (CEUA 14/00428). We followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to report in vivo experiments (12). The number of animals and the intensity of noxious stimuli were the minimum necessary to show consistent effects.

Induction of Metabolic Syndrome and Experimental Groups

Metabolic syndrome was induced as described previously (13). The rats received 10% fructose in the drinking water for 8 weeks. The control animals received filtered water during the same period. Rats and chow were weighted (g) 3 times a week. The water was refilled, and the consumed volume (mL) was measured every day; the results were pooled weekly. Glucose levels were measured at the end of the experiments with a digital glucometer (Accu-Check III; Boehringer Mannheim, Mannheim, Germany). Animals submitted to a high-fructose diet were pooled weekly. Glucose levels were measured at the end of the experiments (12). The number of animals (N) observed), and mouse/rat leptin catalog number RRP300 (no significant cross-reactivity was observed), and mouse/rat leptin catalog number M080 (no significant cross-reactivity was observed). The results are expressed in pg/mL or pg/mg tissue for serum and intestine, respectively.

Determination of Oxidative Stress

After euthanasia, the livers and hearts were immediately collected for the determination of catalase and reduced glutathione activities, 2 classic indicators of tissue oxidative stress, as described previously (14).

Histopathologic Analysis

To confirm the induction of tooth pulp inflammation, the mandibles were collected and fixed in 10% neutral-buffered formalin solution. The samples were decalcified with 17% EDTA (pH = 7.0). The paraffin blocks containing the maxillae were serially cut (6-μm thickness) in the longitudinal plane. The sections were stained with hematoxylin-eosin and examined under light microscopy. A microscope (Axio Imager A1; Carl Zeiss, Oberkochen, Germany) coupled to an image capture system (Axio Vision Rel. 4.4 Software Multimiedia, Carl Zeiss) was used (200× magnification). Periapical events were qualitatively analyzed as previously described (15). Histologic slides (3 fields in each) were evaluated for every tooth, which included the root dentin, the apical foramen, and the periapical tissues. The intensity of the inflammatory infiltrate was classified according to the following scores: 0, absent; 1, discrete; 2, moderate; or 3, severe. An experienced pathologist (F.D.M.M.) in addition to an experienced endodontist with advanced histology skills (J.A.P.F.) evaluated the slides in a blinded manner.

Evaluation of Fecal A. muciniphila

The day before the euthanasia, = 4 g feces was collected from each animal. Cellular DNA was extracted using the Wizard Genomic Purification Kit (Promega, Madison, WI) according to the protocol suggested by the manufacturers. One milliliter of the extracted DNA was used in a total volume of 12.5 mL GoTaq qPCR Master Mix (Promega). The primer used to amplify A. muciniphila was F: 5′AGGCGAGGAAATCTAAAA−3′ and R: 5′GGTTGGCTGCTTACATT−3′, which correspond to nucleotides 1231−1250 (sense) and 1395−1414 (antisense) (accession number NR_074436.1) in a concentration of 0.64 μmol per reaction. Quantitative polymerase chain reaction amplification was confirmed with the amplicon by melting curve analysis (melting temperature = 88.7). The specific parameters of the primers for bacterial detection were evaluated using the BLAST search program (Basic Local Alignment Search Tool; NCBI, NIH, Bethesda, MD). Negative (0.9% sodium chloride) and positive controls (the oligo sequence design according to the sequence of the primers) were used in each quantitative polymerase chain reaction. To evaluate the concentration of bacteria and the limit of detection, a serial dilution was made with the positive control, with

were collected to evaluate the levels of tumor necrosis factor (TNF), interleukin (IL) 1 beta, IL-6, leptin, and adiponectin. The samples were stored at −80°C until use. The following cytokines and adipokines were analyzed by sandwich enzyme-linked immunosorbent assay using Duoset kits according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN): rat TNF-α catalog number DY510 (no cross-reactivity with human or porcine TNF-α with 2.4% of cross-reactivity with mouse TNF-α), rat IL-6 catalog number DY506 (no cross-reactivity with human or porcine IL-6 with 0.7% of cross-reactivity with mouse IL-6), rat IL-1β/IL-2F2 catalog number DY501 (no cross-reactivity with human or porcine IL-1β/IL-2F2, with 0.79% of cross-reactivity with mouse IL-1β/IL-2F2), rat total adiponectin/Acrp50 catalog number RRP300 (no significant cross-reactivity was observed), and mouse/rat leptin catalog number M080 (no significant cross-reactivity was observed). The results are expressed in pg/mL or pg/mg tissue for serum and intestine, respectively.
concentrations varying from $10^4$–$10^7$. All samples were analyzed in duplicate. To confirm that the amplified product was from *A. muciniphila*, we performed specific control sequencing for this bacterium.

**Statistical Analysis**

The results are expressed as the mean ± the standard error of the mean. Our data were submitted to the Bartlett test for equal variances that indicated a Gaussian distribution for the results. Data were subjected to 2-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test (GraphPad Software Inc, San Diego, CA). *P* values <.05 were considered statistically significant. The variations in the experimental sample size are described in each figure legend.

**Results**

We used a model of metabolic syndrome induced by the supplementation with 10% fructose (HFD) to evaluate the cross talk between metabolic changes and AP in rats. Regarding the general metabolic alterations, the groups submitted to HFD for 8 weeks displayed an increase of body weight gain, irrespective of the AP induction. The increased body weight gain in HFD groups was significant from 40 days after the onset of experiments when compared with control animals with or without AP (Fig. 1A). HFD elicited hyperglycemia in all animals submitted to the HFD, confirming the induction of type 2 diabetes (Fig. 1B). This evidence was supported by data showing an increase of water intake in HFD groups, regardless of the AP intervention (Supplemental Fig. S1A–D is available online at www.jendodon.com).

Next, we examined the serum and intestinal levels of cytokines and adipokines. The proinflammatory cytokines, namely TNF, IL-1β, and IL-6, were undetectable in either the serum or intestine of any experimental groups (data not shown). However, there was a significant increase in the serum levels of the adipokine leptin in the HFD group, without AP induction (Fig. 2A). Moreover, the intestinal levels of leptin were significantly increased in the groups with 28 days of AP induction, regardless of the supplementation with 10% fructose (Fig. 2B). The levels of adiponectin were significantly increased in the serum of the HFD group without AP induction (Fig. 2C) although no variation in the intestinal levels of this adipokine was noticed in any other groups (Fig. 2D). Considering the relationship between metabolic syndrome and oxidative stress, we also assessed the activities of catalase and glutathione in the liver and heart. No significant alteration was noted when comparing the different experimental groups, except a significant elevation of glutathione levels in the liver of animals submitted to HFD and AP for 14 days (Supplemental Fig. S2A–D is available online at www.jendodon.com).

Considering the induction of AP, the histologic analysis indicated that control groups (without coronal opening) showed no alterations in the pulp or periapical tissues (Fig. 3A and B). It is noteworthy that 100% of these teeth presented healthy pulp and periapical tissues without any significant effect of HFD (Fig. 3G). Those teeth submitted to coronal opening (14- or 28-day opening) showed necrosis in the coronal region in 100% of the cases (Fig. 3C and E). Additionally, the groups with 28 days of AP induction showed root pulp necrosis in 100% of the cases (Fig. 3E and F), apart from the supplementation with 10% fructose. Data analysis revealed a time-related increase of periapical inflammation, with mild periapical inflammation at 14 days and intense periapical inflammation at 28 days (Fig. 3G).

The fecal expression of *A. muciniphila* was reduced in all of the experimental groups with HFD, with or without AP induction, in relation to the control animals. This decline was significant in animals that received 10% fructose supplementation and had been submitted to pulp exposure for 14 and 28 days. The groups with pulp exposure for 14 or 28 days without HFD also displayed a significant reduction of *A. muciniphila* expression in feces (Fig. 4).

**Discussion**

This study investigated the influence of AP in a model of metabolic disease induced by long-term ingestion of fructose in rats. For this purpose, we investigated the influence of AP on inflammation, oxidative

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) The time-related body weight changes in the control or HFD groups. The *dotted lines* represent interventions to induce AP at 28 or 14 days. (B) Glycemic levels at the end of experiments. **A significant difference in relation to the control group (*P* < .01). The columns represent the mean of 10 animals, and the vertical lines indicate the standard error of the mean.
stress and dysbiosis related to metabolic syndrome, as well as the influence of fructose-induced metabolic syndrome on AP development. The present findings carry significance on the potential systemic repercussions of AP.

Our data show that 10% fructose supplementation for 8 weeks was able to induce metabolic syndrome in rats. This model is well accepted, and it has been widely used for investigating the complications related to metabolic syndrome in rodents (16, 17). Data show that fructose supplementation induced marked body weight gain, which was more evident within ≈ 6 weeks of experiments, even with a decrease in the average food intake in the HFD groups, since the 3rd week. Moreover, the glycemic levels were increased, and the water consumption was higher in all HFD groups when compared with controls from the beginning of the experiments, confirming diabetes-related polydipsia (13). From the present results, it is possible to conclude that AP induction did not alter fructose-induced type 2 diabetes features, such as overweight, hyperglycemia, or polydipsia. Conversely, it was shown that periodontitis induction impaired glucose metabolism and insulin resistance in Zucker prediabetes fatty rats (18, 19). Nevertheless, AP and periodontitis display different pathogenesis and progression, which might partly justify the present discrepancy. Furthermore, genetic models of diabetes greatly differ from diet models, such as the one used by us.

As a next step, we evaluated the effects of HFD and AP induction on serum and intestinal levels of cytokines and adipokines. TNF, IL-1β, and IL-6 are well-known proinflammatory cytokines that exert a relevant role in local AP development, besides metabolic syndrome (20, 21). In this study, the levels of these cytokines were undetectable in either the serum or the intestine in any experimental group, confirming previous studies (21–23).

The adipose tissue itself can synthesize proinflammatory cytokines, named adipokines, such as leptin and adiponectin (21). Leptin is increased in obese individuals and is present under proinflammatory states (24). Adiponectin has an opposite role; it has anti-inflammatory effects and regulates insulin sensitivity (25). We found an increase in serum leptin and adiponectin levels in the HFD group compared with control animals. Additionally, there was an increase of intestinal leptin levels in groups with AP induction for 28 days, an effect that tended to be higher in animals submitted to HFD. An elevation of serum leptin levels in animals submitted to HFD was quite expected, whereas the adiponectin raise might represent a compensatory response to overcome leptinemia (26). However, to our knowledge, this is the first experimental evidence showing that AP induction alters the intestinal leptin levels. Partly upholding our data, periodontitis induction in a rat model of spontaneous type 2 diabetes led to an elevation of serum leptin levels when compared with diabetic rats without periodontal disease (27).
A previous study from our group showed a significant alteration of glutathione activity in the livers of animals submitted to chronic intake of 20% glucose solution and AP induction for 21 days (14). Here, the induction of liver oxidative stress was observed only in animals submitted to HFD and AP for 14 days, as indicated by an increase in glutathione levels. The other experimental groups with HFD only or HFD plus AP for 28 days did not display any significant change of this parameter. Accordingly, a previous study also showed unaltered glutathione or catalase activity in the liver of fructose-supplemented rats (28). This might suggest that liver

Figure 3. Representative images of histologic analysis (hematoxylin-eosin staining). (A) The control sample; the arrowhead shows the normal pulp tissue (100 ×). (B) The HFD sample; the arrowhead shows normal peripical tissue with a preserved periodontal ligament. No bone resorption is observed (200 ×). (C) The control AP 14-day sample showing coronal opening access ( ● ), abscess inside the root (arrowhead), and mild inflammation at the periapical area (*) (40 ×). (D) HFD AP 14-day sample showing mild inflammation (*) at the periapical area without bone resorption (200 ×). (E) Control AP 28-day sample showing coronal opening access ( ● ), totally necrotic pulp tissue, abscess at the periapical area (arrowhead), and extended inflammatory process with bone resorption (*) (40 ×). (F) HFD 28-day sample showing severe and extended inflammation (*) with abscess formation (arrows) (200 ×). (G) Data showing the time-related inflammatory scores at the periapical area in groups AP 14 days and AP 28 days. Significantly different in relation to control (**P < .01). The columns represent the mean of 7–10 experiments, and the vertical lines indicate the standard error of mean.
oxidative stress is an early event when metabolic disease and AP are present, preceding the intestinal adipocyte changes.

The present study also evaluated the possible influence of fructose-elicited metabolic syndrome on AP development. For this purpose, we used the classic AP model induced by the first molar tooth pulp exposure to the oral environment for 14 and 28 days (29). We opted to induce periapical lesion in a single tooth in order to evaluate the interaction among 2 variables (AP and HFD). The induction of periapical lesion in multiple elements is able to raise cytokine levels per se (30), which would be an interference to the analysis. We observed healthy pulp and periapical tissues in those groups without coronal opening despite the fructose supplementation. On the other hand, after 14 days of AP induction, it was possible to observe necrotic coronal pulp tissue and mild inflammation at the periapical area. After 28 days of AP induction, the pulp tissues were necrotic, with severe periapical inflammation and the presence of an abscess in most samples. The present set of results indicates a time-dependent progress of AP-related inflammation. HFD did not influence the extent of pulp or periapical inflammation at 14 or 28 days. It is tempting to conclude that AP leads to systemic alterations, but the induction of metabolic syndrome by fructose ingestion did not impair the progression of AP.

The predominant bacterial phyla in the bowel are Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (31). Another phyla present in minor extent is Verrucomicrobia, which encompasses the gram-negative bacteria A. muciniphila (32). A. muciniphila resides at the gut mucus layer, and its levels are inversely correlated with the presence of metabolic diseases and chronic inflammation (33). Moreover, these bacteria are present in healthy subjects controlling fat storage, adipose tissue inflammation, and glucose metabolism, with overall favorable metabolic effects (9). Our results showed a reduction of fecal A. muciniphila levels in all of the experimental groups with HFD and/or AP induction. A decrease of A. muciniphila expression might be predictable in groups submitted to HFD because these bacteria are down-regulated under metabolic diseases and inflammatory alterations (9). However, as far as we know, this is the first experimental evidence showing the influence of AP on fecal A. muciniphila expression.

Figure 4. Fecal Akkermansia muciniphila expression is significantly reduced in all HFD and AP groups in relation to the control group (**P < .01). The columns represent the mean of 5–10 experiments, and the vertical lines indicate the standard error of mean.

Conclusion

We provide evidence that AP is able to alter systemic parameters related to metabolic syndrome by showing that AP per se leads to intestinal leptinemia and dysbiosis. In contrast, HFD-induced metabolic changes did not appear to impair AP progression, at least in our experimental paradigm. Future animal studies are needed to investigate whether extended protocols of fructose supplementation might affect AP outcomes besides the influence of fructose-induced metabolic changes on periapical tissue healing.

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://doi.org/10.1016/j.joen.2018.10.013).

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