UNIVERSITÉ LAVAL | Faculté de médecine dentaire

Development of an *in vitro* hyperglycemic system to explore periodontal inflammation

Final report

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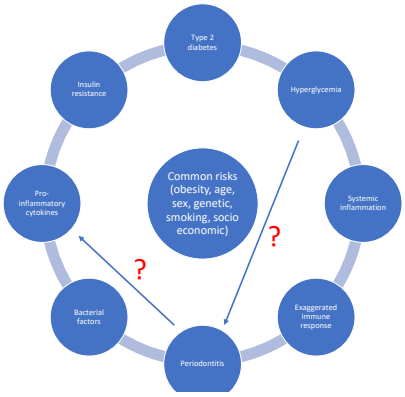
Background and relevancy: Periodontitis is an irreversible chronic inflammatory disease induced by the infiltration of immune cells into the gingival sulcus. While periodontitis etiology is multifactorial, polymicrobial and host-specific, its pathogenesis is inflammatory. Periodontitis is caused by the presence of dysbiotic bacterial microbiota (e.g. plaque biofilm) in the gingival sulcus. Subgingival bacterial biofilm formation is facilitated by bacterial coaggregation partners such as *Fusobacterium nucleatum*. In addition, dysbiotic plaque biofilm shows increased abundance of periodontal pathogenic bacteria, among others *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*. The duration and severity of the disease can be modified by several systemic factors that modulate host-bacterial interactions, for example, type 2 diabetes, a chronic metabolic disease characterized by hyperglycemia and insulin resistance. Hence, hyperglycemic patients have more risk to develop periodontitis than normoglycemic individuals. Conversely, periodontitis may affect diabetes outcomes indicating that hyperglycemia and periodontitis are interconnected in a two-way relationship. However, mechanisms to explain how hyperglycemia affects the regulation of the inflammatory response during periodontitis are still scarce (as shown in the conceptual model on the right). Noteworthy, type 2 diabetes does not always induce the development of periodontitis, suggesting that other factors, including host-periodontopathogen interactions, may be involved in the development and progression of periodontitis in relation to a hyperglycemic environment.



Conceptual model connecting type 2 diabetes and periodontitis. Modified from Kocher 2018.

Microbial ecology studies of subgingival biofilms collected from patients with periodontitis revealed that *Fusobacterium nucleatum* and *P. gingivalis* are implicated in the pathogenicity of periodontitis. In addition, *P. gingivalis* is also known to orchestrates physiological changes within the biofilm making it more virulent and is thus considered a key pathogen for the development and progression of periodontitis. Furthermore, subgingival plaque biofilm microenvironment is important for the interaction between microbial communities as well as between microbial communities and the host. When the homeostatic state within the microbiome is broken, dysbiosis sets in and disrupts the microbial ecosystem contributing to the development of periodontitis. Inhibitory metabolites, among others hydrogen peroxide (H_2O_2), produced by commensal bacteria are consequently important for the ecological balance of a microbial ecosystem by selecting for the growth of specific species.

We hypothesize that a hyperglycemic microenvironment can modulate the regulation of the inflammatory response during periodontitis. The objectives of this research project are: **1)** Investigate the influence of hyperglycemia on the secretion of cytokines by a cell coculture model following the stimulation of periodontal inflammation with a dysbiotic biofilm (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*); and **2)** Study the impacts of a commensal bacterium (*Streptococcus oralis*) on dysbiotic biofilm-induced periodontal inflammation in a hyperglycemic microenvironment.

Methodology:

Cell Culture

Human immortalized gingival keratinocytes (hTERT TIGKs, ATCC #CRL-3397) were cultivated in keratinocytes-serum free media (Gibco) supplemented with 100 µg/ml penicillin/streptomycin + 0.25 µg/ml amphotericin B at 37°C in 5% CO_2. To mimic *in vitro* normoglycemia and hyperglycemia, the cells were continuously maintained in media in which the glucose concentration was set at 5.79 mM (normoglycemia) and 25 mM (hyperglycemia).

Bacterial culture

Streptococcus oralis (ATCC #35037) was cultivated in Brain Heart Infusion (BHI) at 37°C in aerobic condition. *Fusobacterium nucleatum* (ATCC #25586) et *Porphyromonas gingivalis* (ATCC #33277) were cultivated in Todd Hewitt Broth (THB) supplemented with 1% hemin and 0,5% vitamin K_1 at 37°C in anaerobic condition (80% N_2, 10% CO_2 and 10% H_2).

Coculture biofilms-keratinocytes

An insert cell culture system (BrandTech Scientific) was used to coculture the bacterial biofilms with the gingival keratinocytes.

Mono-specie (*S. oralis*, *F. nucleatum* or *P. gingivalis*), 2-species (*F. nucleatum* + *P. gingivalis*, *P. gingivalis* + *S. oralis* and *F. nucleatum* + *S. oralis*) and 3-species (*S. oralis* + *F. nucleatum* + *P. gingivalis*) biofilms were allowed to grow on one part of the insert system. The gingival keratinocytes were cultivated on the other part of the insert system. Twenty-four hours before the coculture, the keratinocytes were washed and their media replaced with culture media containing 5.79 mM (normoglycemia) and 25 mM (hyperglycemia) glucose without antibiotic-antimycotic. Both insert parts were then assembled for the coculture of the biofilms with the keratinocytes (as shown on the right).

https://shop.brandtech.com/media/catalog/product/b/r/brand_insert_2in1_plate_image.jpg?width=700&height=700&store=brandtech_en&image-type=image

Cytokines quantification

Interleukin-6 (IL-6) and interleukin-8 (IL-8) pro-inflammatory cytokines in the cell culture supernatants were quantified by ELISA accordingly to the manufacturer's protocol (R&D Systems).

Results:

Biofilms formation

First, we have determined if the coating of the inserts was required for bacterial adherence. We found that coating of the insert parts for 2 hours with artificial saliva enhanced bacterial adherence compared to uncoated insert parts.

Given that the obtention of colony forming unit (CFU) of *F. nucleatum* and *P. gingivalis* on blood agar is challenging, we normalized the number of bacteria by using the optical density of each strain in solution at 660 nm. For biofilms formation, the optimal ODs determined were: 0.5 for *S. oralis*, 0.25 for *F. nucleatum* and 0.5 for *P. gingivalis*.

We then optimized the seeding sequence of each bacterial strains for the formation of 2- and 3-species biofilms. Several combinations and sequences were tested. For the 2-species biofilm, *F. nucleatum* and *P. gingivalis* had to be seeded together and then incubated for 48 hours in anaerobic condition. For the 2-species biofilm containing *S. oralis*, *S. oralis* was seeded on the insert and incubated for 24 hours in aerobic condition following be the seeding of *P. gingivalis* or *F. nucleatum* and incubation for 48 hours in anaerobic condition.

Regarding the 3-species biofilms, we found that the seeding of *S. oralis* on the insert and its incubation for 24 hours in aerobic condition following by the simultaneous seeding of *F. nucleatum* and *P. gingivalis* and then incubation for 48 hours in anaerobic condition was optimal.

Cells and bacteria position in the insert coculture system

The insert coculture system is composed of two parts (plate and insert). We then optimized the position of the cells and bacteria within the coculture system. The endpoint was the measurement of IL-6. We

found that the cultivation of the keratinocytes in the insert and the growth of the biofilms in the plate increased IL-6 secretion compared to the opposite setup (e.g. biofilm in the insert and keratinocytes in the plate).

Contact time between the coculture system parts

We next determined the optimal contact time between the insert and the plate for IL-6 measurement. We had performed kinetics (0, 4h, 6h and 24h) with the keratinocytes cultivated in the insert and the biofilms grown in the plate. 24 hours contact between the insert and the plated resulted in more than 80% of keratinocytes death for all biofilms tested. We found that 6 hours was optimal for the secretion of IL-6 by the keratinocytes following contact with the biofilms compared to 4 hours.

Keratinocytes pro-inflammatory cytokines secretion following stimulation with the biofilms in normoglycemic and hyperglycemic environments

First, we have quantified IL-6 and IL-8 secretion by unstimulated keratinocytes in 5.79 mM and 25 mM glucose. Level of IL-6 and IL-8 were the same in both normoglycemic and hyperglycemic conditions indicating that 25 mM glucose did not increase pro-inflammatory cytokine production in our in vitro coculture system.

We then quantified the keratinocytes IL-6 and IL-8 secretion following stimulation with the mono-, 2- and 3-species biofilms for 6 hours. For the mono-specie biofilms, *P. gingivalis* and *F. nucleatum* are greater stimulators of IL-6 and IL-8 secretion then *S. oralis*. Level of IL-6 and IL-8 secreted were the same between 5.79 mM and 25 mM glucose. Stimulation of the keratinocytes with the 2-species and 3-species biofilms resulted in the same level of IL-6 and IL-8 secreted, either in 5.79 mM and 25 mM glucose. However, we observed a great data variability in the level of IL-6 and IL-8 secreted that may suggest that the biofilms formed were not similar between experiments. These data suggest that hyperglycemia did not impact pro-inflammatory cytokines secretion in our in vitro coculture system. These results also suggest that *S. oralis* was not able to counterpart the pro-inflammatory potential of *P. gingivalis* and *F. nucleatum* in 2-species or 3-species biofilms.

Perspectives:

Generation of reproductible multi-species biofilms is challenging. In future work, we are planning to improve the multi-species biofilm formation by working on the OD of the bacterial solutions as well as on the seeding sequence. We are also planning to test *Streptococcus salivarius* and *Streptococcus gordonii* as an alternative for multi-species biofilms containing *S. oralis*. Finaly, the scientific manuscript containing the results described above is under writing and should be submitted for publication within the year.