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Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of *Porphyromonas gingivalis*

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***Background*: *Porphyromonas gingivalis* is a major aetiological agent of periodontitis, a destructive disease affecting the tooth-supporting tissues. Recent reports have indicated that high-molecular-weight molecules from cranberry juice concentrate can prevent the attachment of human pathogens to host tissues.**

***Objectives*: The aim of the present study was to investigate the effect of non-dialysable material (NDM) prepared from cranberry juice concentrate on growth, biofilm formation and adherence properties of *P. gingivalis*.**

***Methods*: The effect of cranberry NDM on biofilm formation was studied using a polystyrene microplate assay and by scanning electron microscopy. The effect of cranberry NDM on the attachment properties of *P. gingivalis* was evaluated by a microplate assay in which mammalian proteins were immobilized into wells.**

***Results*: Our results indicated that cranberry NDM is a potent inhibitor of biofilm formation by *P. gingivalis*. However, it has no effect on growth and viability of bacteria. Cranberry NDM also prevented significantly the attachment of *P. gingivalis* to surfaces coated with type I collagen, fibrinogen or human serum.**

***Conclusions*: Our data suggest that cranberry constituents may have a beneficial effect for the prevention and treatment of periodontitis by reducing the capacity of *P. gingivalis* to colonize periodontal sites.**

Keywords: periodontitis, periodontopathogens, bacterial colonization, biofilms

Introduction

Periodontitis is an inflammatory disorder leading to the destruction of tooth-supporting tissues, including the periodontal ligament and the alveolar bone, and is caused by a specific group of Gram-negative anaerobic bacteria.[1] The continuous challenge to the host immune system by periodontopathogens and their products induces a number of host-mediated destructive processes.[2] Much evidence points to *Porphyromonas gingivalis* as the key pathogen in chronic periodontitis.[1] The colonization of subgingival sites by this bacterium is a critical step in the initiation of periodontal diseases. *P. gingivalis* is well known to express a number of adhesins, associated with either the outer membrane or fimbriae, that promote its adhesion to tooth surfaces, gingival epithelial cells, basement membrane components, erythrocytes and oral bacteria.[3]

The cranberry is a native North American fruit for which a number of studies have reported benefit properties for human health. Cranberries are particularly rich in various polyphenolic compounds, including flavonoids, phenolic acids and complex phenolic polymers.[4] It has been reported that high-molecular-mass proanthocyanidins (condensed tannins) from cranberry juice inhibit the adherence of uro-pathogenic fimbriated *Escherichia coli* and thus protect against urinary tract infections.[5] Furthermore, a high-molecular-weight cranberry fraction was also reported to inhibit the sialic acid-specific adhesion of *Helicobacter pylori* to human gastric mucosa, a critical step for gastric ulcer development.[6] In the area of dental research, it has been reported that a non-dialysable material (NDM) prepared from cranberry juice concentrate inhibits the coaggregation of many oral bacteria[7] and prevents mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) biofilm formation.[8–10] To our knowledge, no study has investigated the effect of cranberry constituents on adherence and growth of periodontopathogenic bacteria. In the present study, we hypothesized that high-molecular-weight cranberry constituents may have a

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beneficial effect in the prevention of periodontitis by reducing growth and adherence of *P. gingivalis*.

Materials and methods

Cranberry fraction

Juice concentrate from the American cranberry *Vaccinium macrocarpon* was kindly provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, USA). The juice was exhaustively dialysed (5 days) in 14 000 MW cut-off dialysis bags at 4°C against distilled water and then lyophilized. The NDM was dissolved in distilled water prior to use. Chemical analyses of cranberry NDM were realized by Robin Roderick (Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoid of sugars and acids and contains 0.35% anthocyanins (0.055% cyanidin-3-galactoside, 0.003% cyanidin-3-glucoside, 0.069% cyanidin-3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside and 0.086% peonidin-3-arabinoside) and 65.1% proanthocyanidins. This latter class of complex phenolic polymers was enriched 125-fold in comparison with the original juice concentrate (data not shown).

Bacteria and culture conditions

P. gingivalis ATCC 33277 was used throughout the study. Bacteria were routinely grown in Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with haemin (10 μg/mL) and vitamin K (1 μg/mL) (THB-HK) and incubated for 24 h in an anaerobic chamber ($N_2/H_2/CO_2$ 75:10:15) at 37°C.

Growth

The effect of cranberry NDM on growth of *P. gingivalis* was determined using a plate diffusion assay. The cranberry NDM powder was sterilized by a 48 h treatment in 70% ethanol. The cranberry NDM was then diluted in sterile 50 mM phosphate-buffered saline (PBS, pH 7.2) to obtain final concentrations ranging from 0 to 500 μg/mL. Samples (50 μL) of the dilutions were placed in glass penicylinders (8 by 8 mm; Bellco Glass Inc., Vineland, NJ, USA) on 3% blood (sheep)-supplemented THB-HK agar plates that had been inoculated by spreading 100 μL of an overnight culture of *P. gingivalis*. After 7 days of incubation at 37°C under anaerobiosis, growth inhibition on the agar surface was evaluated visually by comparing with the control (PBS alone).

Biofilm formation, desorption and viability

A 24 h culture of *P. gingivalis* in THB-HK was diluted in fresh broth medium to obtain an optical density at 655 nm (OD_{655}) of 0.07. Samples (100 μL) were added to the wells of a 96-well tissue culture plate (Sarstedt, Newton, NC, USA) containing 100 μL of serial dilutions (0–500 μg/mL) of sterile cranberry NDM in THB-HK. Control wells with no NDM were also inoculated. After incubation for 48 h at 37°C under anaerobic conditions, spent media and free-floating bacteria were removed by aspiration using a 26G needle and the wells were washed three times with distilled water. The *P. gingivalis* biofilms were stained with 0.4% Crystal Violet (100 μL) for 15 min. The wells were washed four times with distilled water to remove the unbound Crystal Violet dye and dried for 2 h at 37°C. After adding 100 μL of 95% (v/v) ethanol to each well, the plate was shaken for 10 min to release the stain from the biofilms and the absorbance at 550 nm (A_{550}) was recorded. A preliminary assay revealed that NDM did not affect the reading of the absorbance values at 550 nm. Assays were run in triplicate and the means ± SD of two independent experiments were calculated.

The effect of cranberry NDM on biofilm formation by *P. gingivalis* was also investigated by scanning electron microscopy. *P. gingivalis* was inoculated as above (2 mL/well) in 35 mm dishes in the absence and presence of cranberry NDM at 125 μg/mL. After 48 h of incubation, spent media and free-floating bacteria were removed. The biofilms on each plate were fixed overnight in fixation buffer [4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3]. Samples were dehydrated through a graded series of ethanol (50%, 70%, 95% and 100%), critical point dried, gold sputtered and examined with a JEOL JSM-35CF scanning electron microscope operated at 15 kV.

The capacity of cranberry NDM to promote desorption of a *P. gingivalis* biofilm was investigated. Briefly, a 48 h biofilm of *P. gingivalis* was prepared as above and treated for 2 h with the cranberry NDM at final concentrations ranging from 0 to 250 μg/mL. Following these treatments, the biofilms were stained with Crystal Violet.

The effect of cranberry NDM on *P. gingivalis* viability was investigated using the tetrazolium sodium 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT; Sigma-Aldrich Canada Ltd, Oakville, Ontario) reduction assay. Briefly, XTT was dissolved in PBS at 1 mg/mL and menadione was prepared in acetone at 1 mM. The XTT/menadione reagent was prepared fresh and contained 12.5 parts XTT/1 part menadione. A 48 h biofilm of *P. gingivalis* was prepared as above and treated with cranberry NDM (0–250 μg/mL) for 4 h (anaerobiosis, 37°C) prior to adding 25 μL of XTT/menadione. After 1 h at 37°C, the absorbance at 490 nm (A_{490}) was read using a microplate reader.

Adherence to protein-coated surfaces

Wells of a flat-bottomed microtitre plate (MaxiSorp; Nalge Nunc International, Rochester, NY, USA) were filled with 100 μL of rat tail type I collagen [BD Sciences, Bedford, MA, USA; 1 mg/mL in 0.01% (v/v) acetic acid], human fibrinogen (ICN Biomedicals, Aurora, OH, USA; 1 mg/mL in 50 mM carbonate buffer pH 9) or human serum [20% (v/v) in 50 mM carbonate buffer pH 9], and the plate was incubated overnight at room temperature. The protein solution was then removed by aspiration using a 26G needle and 0.05% glutaraldehyde (100 μL) was subsequently added. After 45 min at room temperature, the glutaraldehyde was removed and the wells were washed twice with distilled water. Bacteria from a 24 h culture were harvested by centrifugation (10 000 **g** for 15 min) and suspended in PBS to a concentration of 10^{10} bacteria/mL, as determined with a Petroff–Hausser counting chamber. Equal volumes of bacteria and cranberry NDM at 250, 125, 50, 12.5, 2.5 or 0.5 μg/mL were mixed. After 10 min, 100 μL of the mixture was added to the protein-coated wells. The plate was incubated at 37°C for 1.5 h with gentle shaking every 20 min. After the attachment period, unbound bacteria were removed by aspiration using a 26G needle and the wells were washed three times with PBS containing 0.01% Tween 20 to minimize non-specific hydrophobic interactions. Adherent bacteria in the wells were fixed with methanol for 15 min, extensively washed with distilled water and then stained with 0.4% Crystal Violet (100 μL) for 15 min. Wells were rinsed with distilled water and dried at 37°C for 2 h. After adding 100 μL of 95% (v/v) ethanol to each well, the plate was shaken to release the stain. The A_{550} was recorded using a microplate reader. Assays were run in triplicate and the means ± SD of two independent experiments were calculated.

Cell-surface hydrophobicity

The relative cell-surface hydrophobicity of *P. gingivalis* cells treated with the cranberry NDM fraction (0–125 μg/mL) was determined by

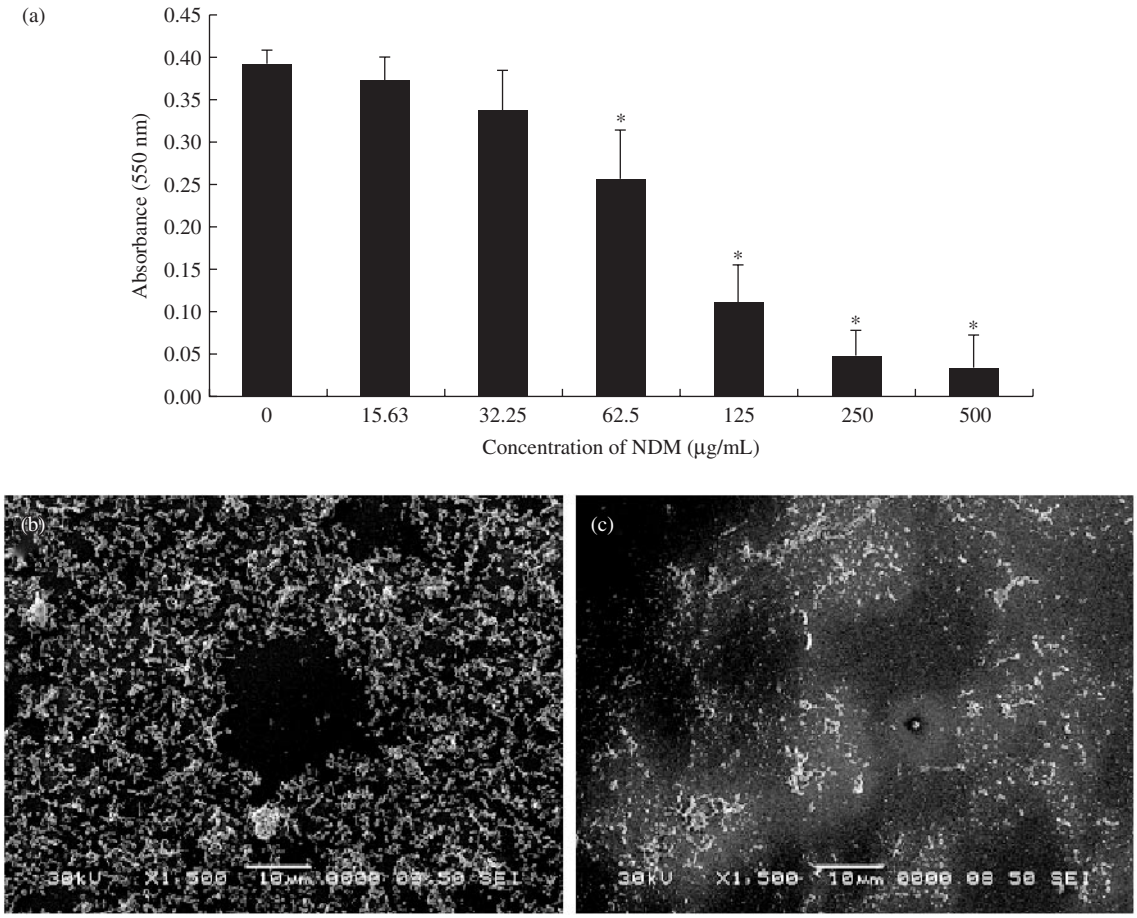

Figure 1. Effect of cranberry NDM on biofilm formation by *P. gingivalis*. Polystyrene microplate assay; *significantly different from control at $P < 0.05$ (a). Scanning electron photomicrographs of *P. gingivalis* biofilm formed in the absence (b) and the presence (c) of cranberry NDM at 125 μg/mL; magnification of ×1500.

measuring their absorbing property to *n*-hexadecane using the method of Rosenberg *et al.*[11]

Statistical analyses

Differences between means were analysed for statistical significance using the Student's *t*-test. Differences were considered significant at the 0.05 level (*P* value).

Results

The cranberry NDM was added at various concentrations in THB-HK to determine its inhibitory effect on biofilm formation by *P. gingivalis.* The formation of biofilm was dose-dependently inhibited by the cranberry fraction (Figure 1a). A significant inhibition ($P < 0.05$) was observed when cranberry NDM was used at a concentration of 62.5 μg/mL and higher. Scanning electron microscopy confirmed the inhibitory effect of the cranberry fraction on *P. gingivalis* biofilm formation. In the control well, *P. gingivalis* formed a uniform three-dimensional structure (Figure 1b). However, when cranberry NDM was added at 125 μg/mL, very few *P. gingivalis* cells were observed attached on the plate (Figure 1c).

The plate diffusion assay revealed that the cranberry NDM had no effect on the growth of *P. gingivalis* even at a concentration of 250 μg/mL. In addition, the XTT reduction assay indicated that at the same high concentration, a 4 h treatment with the cranberry fraction did not affect the viability of a 48 h biofilm of *P. gingivalis*. Lastly, such a treatment did not cause any desorption of the *P. gingivalis* biofilm.

Using a microplate assay in which proteins were immobilized onto the bottom of wells, *P. gingivalis* was found to attach to a similar extent to type I collagen, fibrinogen and human serum (Figure 2). The cranberry NDM caused a significant inhibition of *P. gingivalis* attachment to all three proteins when added at 50 μg/mL. We investigated whether the cranberry NDM inhibits biofilm and adherence of *P. gingivalis* through a modification of the cell-surface hydrophobicity. Untreated cells showed a percentage hydrophobicity of 21. Incubating cells for 1 h in the presence of cranberry NDM, up to a concentration of 125 μg/mL, did not modify the cell-surface hydrophobicity of *P. gingivalis*.

Discussion

Colonization and subsequent biofilm formation by *P. gingivalis* in subgingival sites is the initial step in the pathogenesis of periodontitis. Thereafter, *P. gingivalis* together with other Gram-negative bacterial species stimulate the host defence system leading to an overproduction of a large variety of inflammatory mediators, including interleukin-1β, tumour necrosis

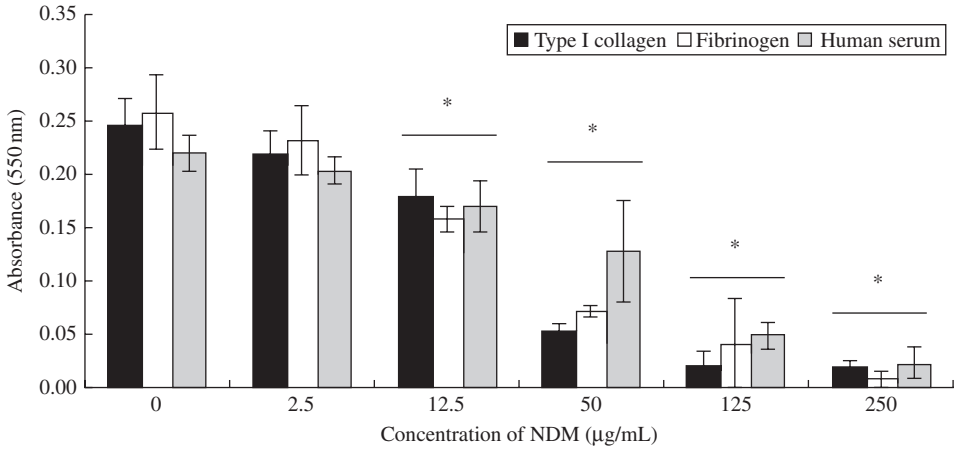

Figure 2. Effect of cranberry NDM on attachment of *P. gingivalis* to polystyrene surfaces coated with type I collagen, fibrinogen and human serum. *Significantly different from control at $P < 0.05$.

factor-α and prostaglandin E_2.[2] We have previously showed that a high-molecular-weight fraction prepared from cranberry juice concentrate could inhibit lipopolysaccharide-induced pro-inflammatory cytokine and chemokine production by human macrophages.[12] In the present study, we investigated the inhibitory effects of this cranberry fraction on growth, biofilm formation and adherence properties of *P. gingivalis*.

The growth and viability of *P. gingivalis* were found to be unaffected by cranberry NDM for which the chemical analysis indicated that it is enriched in complex phenolic polymers. This supports the study of Ahuja *et al.*,[13] who reported that cranberry juice concentrate had no antibacterial activity on *E. coli*.

Biofilms, which are defined as structured microbial communities attached to surfaces, play an important role in most bacterial infections of the human body. In the oral cavity, biofilms allow bacteria to evade immune defences and to better resist mechanical removal and chemotherapeutic agents. Yamanaka *et al.*[10] and Steinberg *et al.*[8] previously reported the capacity of cranberry NDM to prevent biofilm formation by oral streptococci, including *S. mutans* and *S. sobrinus*. In the present study, we showed that cranberry NDM could also prevent the formation of *P. gingivalis* biofilm at a concentration of 62.5 μg/mL and higher. However, the cranberry fraction did not show any capacity to desorb a preformed biofilm of *P. gingivalis*. Although they used a much higher concentration of cranberry NDM (2 mg/mL), Steinberg *et al.*[9] reported a desorption effect on *S. sobrinus* biofilm. *P. gingivalis* possesses multiple structures and components for binding to host cells and proteins. Using a microplate assay in which type I collagen, fibrinogen or serum proteins were immobilized onto the bottom of wells, we demonstrated that cranberry NDM could inhibit the attachment of *P. gingivalis*. Such an inhibition was also observed with another Gram-negative periodontopathogen, *Fusobacterium nucleatum* subsp. *nucleatum* (data not shown). The mechanism involved in the inhibition of biofilm formation and adhesion of *P. gingivalis* appears not to involve a modification of the cell-surface hydrophobicity, as previously reported for the inhibition of oral streptococci biofilm.[10] Additional studies will investigate whether cranberry constituents interfere with a specific receptor-ligand interaction involved in *P. gingivalis* attachment. Indeed, proanthocyanidins with unique A-type linkages have been isolated from cranberry fruit and were found to attach to specific fimbrial adhesins on *E. coli*.[5]

To prevent periodontal disease progression, mechanical procedures are used to remove the dental biofilm. Although these procedures are effective in managing the majority of periodontitis patients, there are situations in which conventional therapy does not always achieve the desired clinical outcome. Control of disease in individuals with significantly increased risk for periodontitis (smokers, diabetics or individuals possessing genetic predisposition) or who do not respond to conventional therapy may require adjunctive treatments, such as use of antimicrobials or host modulators. The inhibition of adhesion is an attractive target for the development of new therapies in the prevention of bacterial infections, particularly infections of mucosal surfaces. Our results brought clear evidence that cranberry compounds can inhibit biofilm formation and adherence of *P. gingivalis*. The fact that cranberry NDM acts by preventing bacterial adhesion rather than by inhibiting growth may represent an advantage since bacteria cannot develop resistance. Considering that cranberry NDM was previously reported to inhibit proteinases of *P. gingivalis*,[14] this cranberry fraction may offer new perspectives for the prevention/treatment of *P. gingivalis*-associated periodontitis.

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Transparency declarations

None to declare.

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