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BRIEF REPORT

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Salivary tissue factor induces thrombin generation in a diurnal rhythm

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Abstract

Background: Upon tooth extraction, extravascular tissue factor (TF) initiates coagulation to arrest bleeding. Additionally, saliva is in constant contact with the wound and contains extracellular vesicle-derived procoagulant TF. Since the duration of postextraction bleeding is highly variable between patients, we hypothesized this may be caused by variation in saliva-derived TF-induced clotting activity.

Objectives: We aimed to assess the variability of saliva-induced thrombin generation (TG) in healthy individuals.

Methods: TG was performed according to the calibrated automated thrombinography (CAT) method. Diluted saliva was added (instead of recombinant TF and phospholipids [PL]) to normal pooled plasma (NPP) in the absence/presence of anti-TF antibodies. Saliva was collected from healthy individuals in the morning, afternoon and evening.

Results: Addition of saliva to NPP induced TG curves similar to those induced by r-TF and PL. Moreover, addition of anti-TF antibodies abolished saliva-induced TG, indicating TF-dependence. A large inter-individual variability (peak CV 31%, range 73-220 nmol/L thrombin) in saliva-induced TG was observed. Interestingly, within subjects, saliva-induced TG was significantly ($P = 0.009$) increased in the morning (167 ± 40 nmol/L thrombin) compared to the afternoon (124 ± 39 nmol/L thrombin) and evening (123 ± 38 nmol/L thrombin). This diurnal variation was not attributable to gingival stimulation or damage induced by tooth brushing.

Conclusions: We identified a diurnal rhythm in salivary TF activity that may have implications for tooth extraction and dental surgery, as performing invasive procedures in the morning may be beneficial for rapid coagulation. Future studies should correlate salivary TF to clinical outcome (ie, postextraction bleeding) and assess a possible relation with bacterial status in the oral cavity.

KEYWORDS

dentistry, diurnal rhythm, saliva, thrombin generation, tissue factor

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Essentials

- Large inter-individual variation in duration of bleeding after tooth extraction.
- Saliva is in contact with oral wound area and contains procoagulant tissue factor (TF).
- Salivary TF induces thrombin generation (TG).
- Saliva-induced TG has a diurnal rhythm with increased procoagulant activity in the morning.

1 | INTRODUCTION

Postextraction bleeding is a frequently encountered (incidence up to 26%) complication in dental practice, defined as continuous bleeding for more than 8-12 hours after dental extraction.[1,2] Upon tooth extraction, extravascular tissue factor (TF) is released from damaged endothelium of the wound. TF is known as the key initiator of the coagulation cascade to arrest bleeding. Besides blood, another autologous body fluid, namely saliva, is in constant contact with the wound. Saliva was previously demonstrated to contain extracellular vesicle-derived TF[3,4] capable of triggering coagulation, as apparent from a shortened clotting time of autologous plasma and whole blood.[5] TF in saliva is bound to the membrane of exosomes released from epithelial cells.[5] Since the duration of postextraction bleeding is highly variable between patients, we hypothesized that, besides the influence of blood-borne coagulation factor levels, comorbidities, and medication,[6] there may be variation in salivary TF-induced clotting activity. To obtain more mechanistic insight into the procoagulant effect of saliva, we measured thrombin generation (TG). TG is more informative than conventional clotting assays, which only reflect a small part of the coagulation process; more than 95% of all thrombin is formed after the initiation phase, when plasma has already clotted. In contrast, in TG measured by calibrated automated thrombinography (CAT) the entire process of the formation of the bulk of thrombin via feedback on factors V, VIII, and XI, as well as the effects of anticoagulant pathways and thrombin inhibition by protease inhibitors is visualized in the thrombogram.[7,8] Therefore, in the current study we used a modified TG assay to assess the variability of saliva-induced TG in a healthy population.

2 | MATERIALS AND METHODS

2.1 | Saliva sample collection

Saliva was collected from 13 healthy individuals, who did not either eat, drink, or smoke for 30 minutes and thoroughly rinsed their mouths with water prior to collecting ~1 mL of saliva into a plastic sterile tube. Saliva samples were immediately placed on ice and stored at −20°C until use. For the intra-individual variation in saliva-induced TG, samples were collected at three time points, in the morning (7-9 AM), afternoon (11 AM-1 PM), and evening (5-7 PM).

2.2 | Saliva-induced TG

TG was measured using the CAT method (using recombinant [r-]TF) as previously described by Hemker et al.[9] Directly prior to addition to the measurement wells, saliva was homogenised by vortexing at 850 rpm for 5 minutes and serially diluted 10-, 5-, 3.75-, or 2.5-fold (for dose response curve) or diluted 5-fold (for inter- and intra-individual variation experiments) with bovine serum albumin (BSA) buffer (5% BSA, 20 mmol/L Hepes, 140 mmol/L NaCl, 0,02% NaN_3, pH 7.35). To study saliva-induced TG, 20 µL diluted saliva (known to contain phospholipids (PL)[10]). was added to 80 µL normal pooled plasma (NPP), in the absence or presence of 100 ug/mL anti-TF antibodies (rabbit polyclonal antibodies against TF were a generous gift of Dr. W. Kisiel, University of New Mexico, Albuquerque, NM, USA). Regular TG curves were obtained by addition of 20 µL CAT trigger solution containing 1 pmol/L r-TF (Innovin, Dade-Behring, Germany), of which concentration and activity were previously determined by comparison to recombinant TF with known activity (kindly provided by Dr. Y Nemerson [Mount Sinai Medical School, New York, NY, USA] obtained as described in[11]) and 4 µmol/L PL.

3 | RESULTS AND DISCUSSION

Addition of saliva to NPP dose-dependently induced TG curves similar to those induced by the combination of r-TF and phospholipids (Figure 1A). Based on comparison of TG curves induced by saliva or r-TF, we estimated the concentration of TF in saliva to be in the order of 1-2 ng/mL, consistent with literature.[5,12]

To confirm that TF was indeed the procoagulant factor in saliva that triggered TG, we compared TG from the same saliva sample (from five donors) in the absence and presence of anti-TF antibodies (Figure 1B). Although thrombin was still generated in the presence of the antibodies, the lag time of this TG curve was substantially prolonged. The residual TG was comparable to that in the absence of tissue factor, and can hence be attributed to contact activation. Altogether we concluded that the saliva-induced TG is indeed TF-dependent. Moreover, the large variation between TG curves observed for the five donors in this experiment led us to further explore the inter- and intra-individual variation of saliva-induced TG curves.

In 13 healthy individuals, a large interindividual variability in saliva-induced TG was observed, with coefficients of variation (CVs) of 31% for peak (range 68-242 nmol/L thrombin), 6% for ETP (range 1271-1678 nmol/L thrombin·min) and 24% for lag time (range

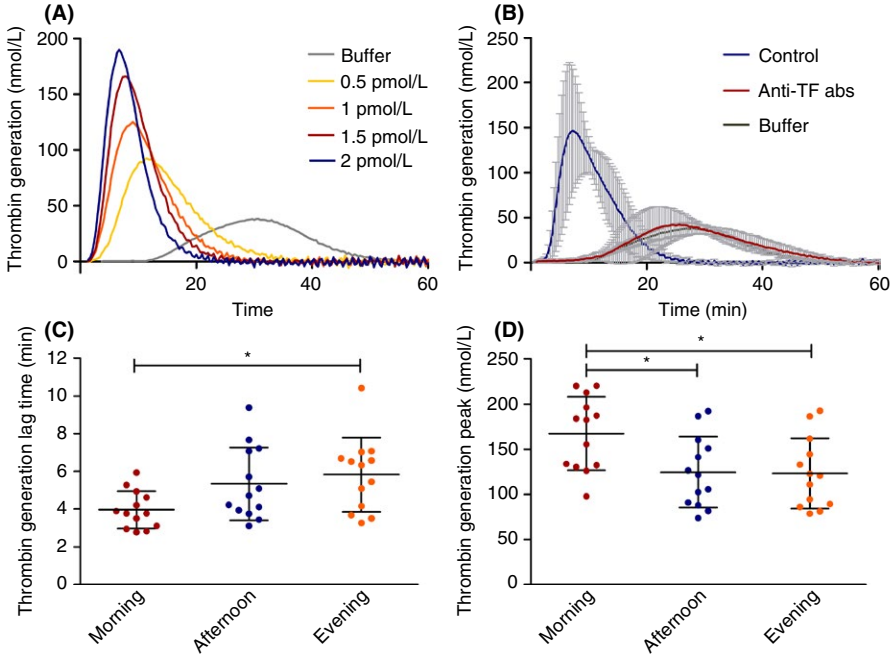

FIGURE 1 Saliva-induced TG in healthy individuals. (A) Saliva dose-dependently induces TG curves. Estimated concentrations of TF were based on comparison with curves generated from r-TF in conventional TG. Saliva dilutions were: 1/120, 1/60, 1/45, and 1/30 for the estimated 0.5, 1, 1.5, and 2 pmol/L TF indicated in the figure, respectively. Buffer condition does not contain saliva, TG for this control is induced by contact activation. (B) Addition of anti-TF antibodies (abs, 100 μg/mL) abolishes (TF-dependent) TG, demonstrating TF-dependency of saliva-induced TG. Buffer condition does not contain saliva, TG for this control is induced by contact activation. (C, D) TG induced with saliva samples collected from 13 healthy donors in the morning (7-9 AM), afternoon (11 AM-1 PM) and evening (5-7 PM) shows large interindividual variation and a diurnal rhythm. The TG parameters lag time (C) and peak (D) are presented as mean ± SD and were compared by one-way ANOVA with post hoc Bonferroni test if normally distributed, or by non-parametric Kruskal-Wallis with concurrent post hoc Dunn's test

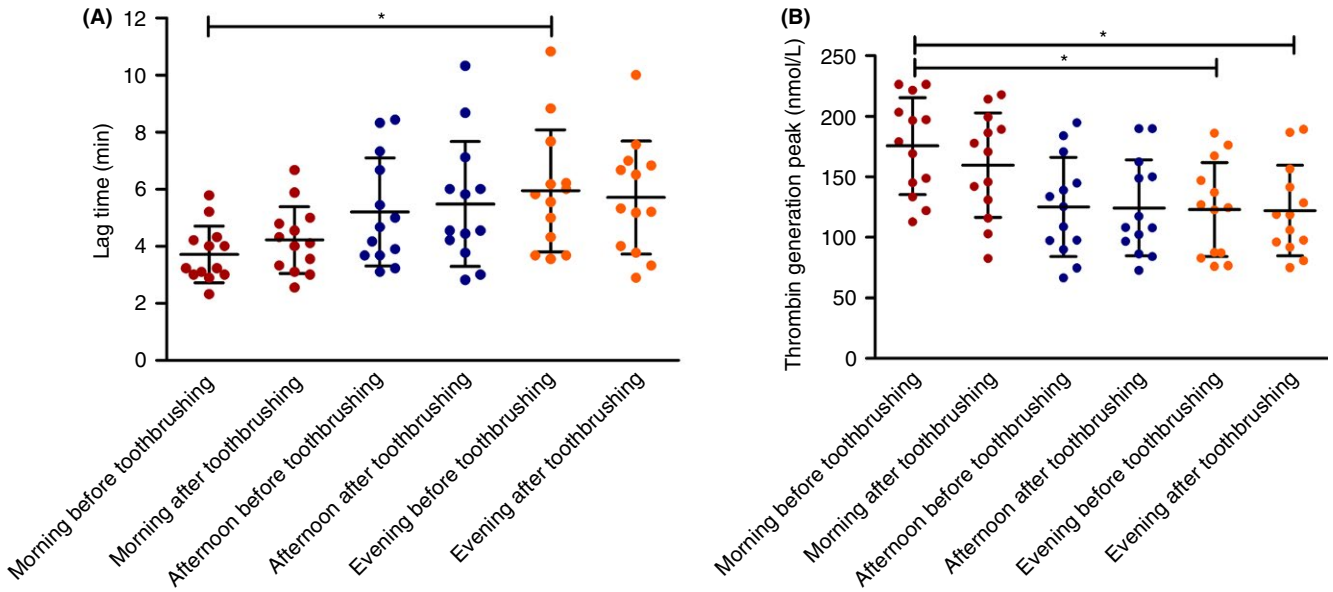

FIGURE 2 Toothbrushing does not influence TF-induced TG. TG induced with saliva samples collected from 13 healthy donors before and after toothbrushing at three time points (morning 7-9 AM, afternoon 11 AM-1 PM, and evening 5-7 PM) shows that (A) TG lagtime is shorter and (B) TG peak is higher in the morning compared to the evening but does not change as a result of gingival stimulation/possible damage induced by toothbrushing. Lag time (A) and peak (B) are presented as mean ± SD and were compared by one-way ANOVA with post hoc Bonferroni test if normally distributed, or by non-parametric Kruskal-Wallis with concurrent post hoc Dunn's test

2.67-7.33 minutes). Saliva was collected from these individuals at three time points, in the morning (7-9 AM), afternoon (11 AM-1 PM), and evening (5-7 PM). Interestingly, we found that, within individuals, the saliva-induced TG occurred significantly faster ($P = 0.03$ for lagtime) and was significantly increased ($P = 0.009$ for peak) in the morning (lag time 3.9 ± 1.0 minutes, peak 186 ± 40 nmol/L thrombin) compared to the afternoon (lagtime 5.3 ± 1.9 minutes [not significantly increased], peak 124 ± 39 nmol/L thrombin) and evening (lag time 5.6 ± 1.2 minutes, peak 120 ± 43 nmol/L thrombin) (Figure 1C, D).

Our findings raised the question what could cause a higher salivary TF concentration in the morning compared to the rest of the day. One factor that we hypothesized could cause the diurnal rhythm in saliva-induced TG was toothbrushing, as this could stimulate and/or damage the gingiva to release TF. To investigate this, 13 healthy individuals donated saliva before and after tooth brushing (without toothpaste and with concurrent rinsing with water), at the three time points mentioned earlier. Again, we observed a significant ($P = 0.002$) difference in TG between morning (lagtime 4.0 ± 1.0 minutes, peak 168 ± 41 nmol/L thrombin) and evening (lagtime 5.8 ± 1.9, peak 123 ± 39 nmol/L thrombin), but TG parameters did not differ before and after toothbrushing (Figure 2).

When humans or animals have a wound, they instinctively expose their blood to saliva (eg, by licking). Saliva contains growth factors and histatin, which promote wound healing[13,14] and provide antimicrobial activity.[15] In addition, saliva can serve as an additional source of extravascular TF to promote hemostasis. Besides reducing blood loss, rapid clot formation contributes to innate immunity and host defence, by decreasing the risk of pathogens entering the blood.[5]

A previous study by Hell et al.[16] reported on the procoagulant effect of extracellular vesicles in amniotic fluid, as measured by a modified TG assay. The current report is the first we are aware of that evaluated thrombin generation induced by saliva. A potential cause for the observed increased salivary TF activity early on the day may be that morning saliva is concentrated, as no food or beverages are consumed during the night, comparable to albumin levels in urine.[17] However, in the current study, subjects were asked to rinse their mouth before collecting saliva, also in the morning. Therefore, the contribution of this overnight concentration effect on morning salivary TF concentration is questionable.

A diurnal rhythm as observed for TF in saliva, but with a morning nadir and an increase towards the evening, is well known from several hormones (eg, cortisol[18]), peripheral blood cells (eg, lymphocytes and monocytes[19]), and inflammatory cytokines (eg, IL-6[20]). Moreover, various hemostatic factors are known to be subject to circadian variations. For instance, FVII and TF pathway inhibitor (TFPI) peak in the morning.[21] Hence, evidence suggests the existence of a relatively procoagulant status in the morning, supported by a preponderance of thromboembolic events early on the day.[22] Further studies are required to study possible associations of salivary TF levels and activity with factors potentially causative of this within-day variation, such as melatonin, cortisol, and adrenaline. Another interesting question for further studies is whether the coagulation potential of saliva depends on the type of stimulus inducing its secretion, for instance chemosensory (ie, smell of food) and masticatory stimuli or anxiety (which induces hyposalivation).[23]

A final factor to consider as a cause for increased TF activity in the morning is the oral microbiome. Oral streptococci were previously demonstrated to induce (endothelial) tissue factor activity.[24] During the day, toothbrushing and food intake are known to alter the composition of the oral biofilm compared to the night, during which the low saliva flow creates a hospitable environment for, amongst others, streptococci.[25,26] In support of this hypothesis, a previous study reported that the use of prophylactic antibiotics prior to tooth extraction was independently associated with a higher risk of postoperative bleeding.[6] Therefore, it would be interesting to assess a possible relation between salivary TF concentration and bacterial status in the oral cavity.

From a clinical perspective, the observed diurnal rhythm in salivary TF activity may have implications for tooth extraction and other dental surgery, as performing invasive procedures in the morning may be beneficial for rapid coagulation. This may be particularly relevant for patients with a known bleeding tendency, for instance those on oral anticoagulants, to reduce the risk of prolonged postprocedure bleeding. Future studies should assess whether bleeding after dental procedures varies depending on the time of day, and if so, correlate salivary TF activity to postprocedure bleeding.

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RELATIONSHIP DISCLOSURE

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

L.N. van der Vorm, J.E.I.G. Brouwers, C. Mondria, and J.A. Remijn were involved in concept and design of the research. L.N. van der Vorm and C. Mondria performed the experiments and analyzed the results. L.N. van der Vorm, J.E.I.G. Brouwers, C. Mondria, P.G. de Groot and J.A. Remijn, interpreted the data. L.N. van der Vorm drafted the manuscript. B. de Laat, P.G. de Groot and J.A. Remijn critically revised the manuscript. All authors approved the final manuscript.

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