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Influence of saliva on the results of global laboratory coagulation tests

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ABSTRACT

Background: Oral wounds inevitably come into contact with saliva which can affect the time needed for bleeding to stop. The influence of saliva can be non-specific, related to dilution of blood, and/or mediated by salivary factors that affect haemostasis directly. The aim of this study was to assess if mixing blood with an individual's saliva would affect the rate of its coagulation measured by global coagulation tests, prothrombin time (PT) and activated partial thromboplastin time (APTT).
Methods: The study included 30 healthy non-smoking volunteers. Paired blood and unstimulated saliva samples were obtained from each participant and PT and APTT were determined in blood, blood + saliva and blood + water mixtures. Coagulation tests were performed using the mechanical clot detection method.
Results: PT was significantly longer in both blood + saliva and blood + water mixtures compared to blood alone. APTT was significantly longer only in blood + water mixture compared to blood.
Conclusions: Similarly prolonged PT in both mixtures suggests that both saliva and water prolong coagulation evenly due to their non-specific effect of blood dilution. The finding that APTT was significantly prolonged only when blood was mixed with water could indicate presence of tissue factor in saliva, however, in a concentration too low to influence the results of PT.

Keywords: Activated partial thromboplastin time, blood coagulation, blood coagulation tests, prothrombin time, unstimulated saliva.

Abbreviations: APTT = activated partial thromboplastin time; PT = prothrombin time; TF = tissue factor; tPA = tissue plasminogen activator.

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INTRODUCTION

Oral wounds, including wounds that are caused by surgical interventions in the oral cavity inevitably come into contact with saliva. Saliva may affect (prolong) the time needed for the bleeding to stop by diluting blood and thereby diluting procoagulant factors in blood. On the other hand, salivary influence on haemostasis could be more specific as it has been demonstrated that saliva contains cell-derived vesicles exposing procoagulant tissue factor (TF).[1–3] Berckmans *et al.*[2] proposed that salivary TF, as an additional source of extravascular TF, may serve to facilitate haemostasis as the first step in the process of wound healing. Besides reducing blood loss, this would also contribute to the non-specific host defence by reducing the risk of pathogens entering the blood. Wollein Waldetoft *et al.*[4] reported that saliva may induce clotting of plasma through both extrinsic and intrinsic pathways of coagulation. They associated this effect with the host defence mechanisms as clotting of plasma exudate during streptococcal pharyngitis or tonsillitis could contribute to the clearance of bacteria from the epithelial surface by entrapping bacteria in fibrin clots. Coagulation has also been implied as the simplest way in which the host is trying to limit the spread of tumour cells as it has been shown that tumour-associated macrophages express TF on their surface and that they may assemble the entire coagulation cascade and form cross-linked fibrin on their surface in apposition to the growing tumour.[5]

Besides playing a role in the initiation of coagulation, salivary TF could have the potential to enhance

The study was conducted at the Department of Laboratory Diagnostics, University Hospital Center Zagreb.
The study was reviewed and approved by the Ethics committee of the School of Dental Medicine, University of Zagreb.

clot contraction. It has been shown that activated monocytes promote clot contraction and that this ability is related to their TF expression.[6] Hypothetically, oral injuries might lead to increased level of TF in saliva by activating salivary monocytes and causing increased TF expression on their surface.

Berckmans *et al*.[2] also postulated that the reflex to put a bleeding finger into the mouth may be related to the ability of saliva to promote coagulation and stop the bleeding. This belief may also be encountered among laymen, e.g., diabetics who frequently prick their fingers for the purpose of monitoring blood glucose levels.

The aim of this study was to quantify the effect of saliva on the formation of blood clot by assessing if mixing blood with individual's saliva would affect the rate of its coagulation measured by global coagulation tests, prothrombin time (PT) and activated partial thromboplastin time (APTT).

MATERIALS AND METHODS

Subjects

The study was conducted at the Department of Laboratory Diagnostics, University Hospital Center Zagreb. Recruitment and sample collection was performed throughout a 3-week period, in July 2019. The study included 30 healthy non-smoking volunteers (63% women) who maintain good oral hygiene and health, and do not take any medications. Participants were aged 18–43 years (median 22, interquartile range 21–26).

The study was reviewed and approved by the Ethics committee of the School of Dental Medicine, University of Zagreb. All participants were provided with detailed explanation of the study and signed the informed consent. The study was performed according to the ethical standards of the Declaration of Helsinki.

Sample collection

Paired blood and saliva samples were obtained from each study participant. Blood was collected in one 4.5 mL 3.2% (0.109 M) sodium citrate vacutainer (Beckton Dickinson, United Kingdom). Whole unstimulated saliva was collected shortly after the blood draw by a 'spitting method' during 5 min. Saliva flow rate was calculated by dividing the amount of saliva with the collection period. Participants were instructed not to eat or drink anything except water for at least 30 min prior to collection.

Sample analysis

PT and APTT were measured in platelet poor plasma obtained after sample centrifugation at 2000 *g* for 15 min.

Blood was further divided into two aliquots. To assess the effect of saliva on global coagulation tests, an aliquot of 500 μL of blood was mixed with 100 μL of paired saliva, whereas 100 μL of distilled water was added to the other blood aliquot of 500 μL to control for the dilution of blood effect. PT and APTT were measured in the supernatants of the respective mixtures after centrifugation.

PT and APTT were determined using the mechanical clot detection method on the semiautomated dual-channel BEHRING Fibrintimer (Sysmed Lab, Europe Inc., Poland) analyser. PT was measured using the recombinant thromboplastin PT Innovin (Siemens Healthineers, Erlangen, Germany), whereas for APTT Dade Actin FS and Calcium Chloride (Siemens Healthineers, Erlangen, Germany) were utilized. All reagents were prepared according to the manufacturer's instruction and the protocol for PT and APTT recommended by the manufacturer was followed for analysis.

Specifically, for PT 100 μL of sample was pipetted into a disposable cuvette containing a metal mixing bar and incubated in the appropriate position of the analyser at 37 °C for one minute. For initiating the clotting reaction, 200 μL of PT Innovin was added and PT was measured in seconds. The formation of the fibrin clot causes stop of the stopwatch and indicates the PT. PT(%) (percentage activity of the normal value) was read from the calibration curve.

For APTT, 100 μL of sample and 100 μL of Dade Actin FS were pipetted into the measuring cuvette and incubated at 37 °C for three minutes. The addition of Calcium Chloride initiates the clotting reaction and equally, the clot formation stops the stopwatch. APTT results were expressed in seconds.

Additionally, pH of saliva was measured shortly after collection, using a benchtop pH-meter (Mettler Toledo, Switzerland).

Statistical analysis

Assessment of the normality of data was performed using the Shapiro–Wilk test. As the assumption of a normal distribution was rejected, Wilcoxon signed-ranks test was used to assess differences between the results of the coagulation tests obtained using blood samples and the results obtained using paired mixtures of blood with (a) saliva and (b) water. Seven results expressed as PT(%) in the blood group of measurements, one in blood + saliva and one in blood + water were >157.6. To be included in the statistical analysis these measurements were assigned a value of 157.6. Effect size was calculated using the formula $r = Z/\sqrt{N}$. Following criteria were applied to interpret the results: r = 0.1–0.3 = small, 0.3–0.5 = medium and >0.5 = large effect size.

After analysing the presumed deflection of the APTT and PT caused by mixing blood with saliva and water, we also analysed if there was a significant difference in the degree of this deflection between the two mixtures. In this way we wanted to control for the non-specific dilution effect of both saliva and water on the rate of coagulation process (water was used as control solution as it can influence coagulation by dilution, but has no components that could be directly involved in this physiological process). This comparison was performed using the Wilcoxon signed-ranks test.

Spearman's correlation was used to assess the association between the results of the coagulation tests with salivary pH and salivary flow.

Commercial statistical software IBM SPSS 22 was used for data analysis (IBM SPSS, Vermont, USA) with significance preset at $\alpha < 0.05$ for a two-sided test.

RESULTS

The results of Wilcoxon signed-ranks test showed a statistically significant difference in the results of PT measured in both blood + saliva and blood + water mixtures compared to matched blood samples (Table 1). PT in both mixtures was significantly prolonged compared to blood with large effect size. The difference in the APTT was statistically significant only for blood + water compared to blood with moderate effect size.

Descriptive statistics for the differences in APTT and PT after mixing blood with saliva or water with the values measured using blood as a referent solution are shown in Table 2. A positive median difference for PT(s) suggests that, on average, PT was prolonged in mixtures of blood both with saliva and with water. A negative median difference for PT(%) also indicates prolonged coagulation in both mixtures. However, the median difference for APTT(s) was positive for blood + water, whereas it carries a negative sign for blood + saliva. The results of Wilcoxon signed-ranks test used to evaluate the difference between median differences for a respective test were statistically insignificant.

Spearman's correlation revealed no significant association between the results of coagulation tests and salivary pH and flow.

Table 1. Descriptive statistics for the sample, N = 30

	Median	IQR	Min	Max	*P**	*r*
Blood						
APTT (s)	27.6	26.0–29.5	20.8	32.4		
PT (s)	9.6	9.0–9.9	8.2	11.2		
PT (%)	135.1	123.0–157.6	89.3	157.6		
Blood + saliva						
APTT (s)	27.4	26.2–29.6	23.0	38.9	1.000	<0.001
PT (s)	10.6	9.7–11.2	8.7	12.8	<0.001	−0.861
PT (%)	104.3	88.8–129.7	65.4	157.6	<0.001	−0.807
Blood + water						
APTT (s)	28.4	27.2–31.2	24.2	34.0	0.010	−0.473
PT (s)	10.6	9.9–11.3	8.7	14.7	<0.001	−0.874
PT (%)	104.3	87.8–122.4	51.5	157.6	<0.001	−0.844

IQR = interquartile range; Min = minimum; Max = maximum; APTT = activated partial thromboplastin time; PT = prothrombin time; s = seconds; *r* = effect size.
*Wilcoxon signed-ranks test for comparison to blood without saliva and water.

Table 2. Descriptive statistics for the differences in APTT and PT results for blood mixed with saliva or water from the APTT and PT results measured on paired blood samples, N = 30

	Median	IQR	Min	Max	*P**	*r*
Difference between blood + saliva and blood						
APTT (s)	−0.7	−1.8 to 2.8	−5.2	15.2		
PT (s)	1.0	0.6 to 1.3	−0.2	2.9		
PT (%)	−27.9	−33.9 to (−7.2)	−61.8	7.5		
Difference between blood + water and blood						
APTT (s)	1.1	−0.2 to 2.7	−4.5	7.2	0.177	−0.247
PT (s)	0.8	0.5 to 1.6	0.2	5.9	0.333	−0.177
PT (%)	−22.7	−41.8 to (−11.7)	−106.1	0.0	0.388	−0.158

IQR = interquartile range; Min = minimum; Max = maximum; APTT = activated partial thromboplastin time; PT = prothrombin time; s = seconds.
*Wilcoxon signed-ranks test.

DISCUSSION

The aim of this study was to examine if mixing blood with individual's saliva would affect the rate of its coagulation measured by global haemostatic tests, PT and APTT.

PT was significantly and similarly prolonged in both blood + saliva and blood + water mixtures in comparison to the PT of blood alone. Similarly prolonged time needed for the blood to clot in both mixtures could be related to the diluting effect of water and saliva. The expected presence of TF in saliva and its influence on coagulation was likely overridden by the activity of the recombinant human TF added to the mixture to conduct the test. Our finding that the APTT was significantly longer from the APTT of blood only for the blood + water mixture could be interpreted as confirmatory for the presence of TF in saliva, however, in a concentration too small to be reflected in the result of the PT test. Because reagents for the APTT are used to evaluate coagulation abnormalities in the intrinsic pathway and therefore do not contain TF, the TF supposedly present in saliva might have acted as an additional initiator of coagulation when APTT was performed in blood + saliva mixtures and in this way, acting procoagulantly, counteracted the diluting (anticoagulant) effect of saliva.

Comparison of the degree of deflections in the results of APTT and PT caused by mixing blood with water and saliva did not reveal statistically significant differences. With regards to APTT, this analysis showed that the results of APTT measured in blood + saliva mixtures diverged from the APTT of blood in a wider range than the results of the APTT measured in blood + water mixtures. In other words, even though APTT values measured on blood + saliva samples were more frequently lower than those measured on blood alone (i.e., APTT was shorter), positive deflections in several individual blood + saliva samples (reflecting prolonged APTT) were greater than positive deflections in the APTT of paired blood + water samples. Because water was used to control for the diluting effect, this finding might imply the presence of certain additional anticoagulant properties of saliva. Anti-clotting activity is a well-known property of saliva of blood-feeding insects such as ticks, mosquitos and flies. The importance of such substances for these organisms is self-evident. For humans, slowing down the haemostatic process in the mouth by salivary anticoagulants (above the haemostasis deceleration caused by the unavoidable dilution of blood) carries no benefit so these substances could possibly have other physiological roles, if present (possibly immunomodulatory). While straightforward plasmin-like proteolytic activity in human saliva has not been confirmed, plasminogen activation system has been shown to be active, particularly tissue plasminogen activator (tPA) originating from oral epithelial cells and from salivary glands[7]. Plasmin is best known as a fibrinolytic enzyme but has been shown to cleave and inactivate coagulation factors FV, FVIII, FIX and FX in vitro so it possesses the potential for anticoagulant activity in vivo, however, of unknown physiological significance[8]. Based on the report by Virtanen *et al.*[7], it is possible that individuals' saliva may show higher tPA activity than expected, even exceptionally high. Hypothetically, if some of our participants had higher activity of tPA, this could contribute to longer APTT in blood + saliva in comparison to blood + water. Because of the slower coagulation process, the possible anticoagulant effect of tPA could be reflected in the APTT results more than in the results of PT testing as performing PT leads to faster clotting. In addition, with regards to the extrinsic pathway of coagulation, plasmin may have a procoagulant activity as well as it has been shown to inactivate the anticoagulant tissue factor protein inhibitor[8]. The described interpretation of our results is based solely on the available scientific literature because we were unable to measure concentrations (activity) of salivary factors that may affect blood coagulation. This presents a limitation of this study.

In summary, the results of this study suggest (confirm) that saliva may influence coagulation process in a way which is beyond a mere effect of blood dilution. The results of APTT testing in combination with PT results are suggestive of the presence of TF in saliva and its procoagulant activity. However, it seems that inherent effect of saliva on blood coagulation in some individuals might be predominantly anticoagulant.

Statistically significant effect of saliva on blood coagulability measured using coagulation tests was small when expressed in seconds. In the oral cavity, the shortening or prolongation of bleeding in the mouth due to the contact of the wound/blood with saliva would be dependent on the volume of saliva mixed with blood, and on the resultant of the interplay between procoagulant and anticoagulant factors originating from both saliva and from blood/wounded tissue. Except for the floor of the mouth where blood may be able to mix with a somewhat greater volume of saliva, the blood/the wounds located at most other sites of the oral cavity come into contact with a thin layer of saliva covering oral surfaces[9]. Differences in the ratio of procoagulant/anticoagulant factors in saliva covering different oral surfaces might contribute to the probability of a site-dependent salivary influence on haemostasis in the mouth.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Data Availability Statement

All data from this research are available from the corresponding author upon reasonable request.

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