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BRIEF REPORT

Extracellular vesicles from amniotic fluid, milk, saliva, and urine expose complexes of tissue factor and activated factor VII

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Abstract

Background: Tissue factor (TF) is expressed in the adventitia of the vessel wall and on extracellular vesicles (EVs) in body fluids. TF and activated coagulation factor (F) VII(a) together form the so-called extrinsic tenase complex, which initiates coagulation.

Aim: We investigated whether EVs in amniotic fluid, milk, saliva, and urine expose functional extrinsic tenase complexes that can trigger coagulation.

Methods: Milk, saliva, and urine were collected from healthy breastfeeding women ($n = 6$), and amniotic fluid was collected from healthy women undergoing routine amniocentesis ($n = 7$). EVs were isolated from body fluids by size exclusion chromatography (SEC) and clotting experiments were performed in the presence and absence of antibodies against TF and FVIIa in normal plasma and in FVII-deficient plasma. The ability of body fluids to generate FXa also was determined.

Results: Amniotic fluid, milk, saliva, and urine triggered clotting of normal plasma and of FVII-deficient plasma, which was almost completely inhibited by an anti-FVII antibody and to a lesser extent by an anti-TF antibody. Fractionation of body fluids by SEC showed that only the fractions containing EVs triggered clotting in normal plasma and FVII-deficient plasma and generated FXa, which again was almost completely inhibited by an anti-FVII antibody and partially by an anti-TF antibody.

Conclusion: Here we show that EVs from amniotic fluid, milk, saliva, and urine expose complexes of TF and FVIIa (i.e., extrinsic tenase complexes) that directly activate FX. Based on our present findings we propose that these EVs from normal body fluids provide hemostatic protection.

KEYWORDS
body fluids, coagulation factor VII, extracellular vesicles, extrinsic tenase complexes, tissue factor

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1 | INTRODUCTION

The “extrinsic tenase” complex consists of activated coagulation factor VII (FVIIa) and tissue factor (TF), its transmembrane receptor. Together they form a two-subunit enzyme that initiates coagulation.[1,2] Under physiological conditions, these two subunits are spatially separated. Whereas FVII(a) is present in blood, TF is expressed in the subendothelial adventitia, epithelial tissues, and by highly vascularized organs such as brain, heart, and lungs.[3] Upon vascular damage, FVII(a) binds extravascular TF, thereby initiating coagulation. Specifically, this extravascular expression of TF is known as “hemostatic envelope.”[4]

Tissue factor is also present on extracellular vesicles (EVs) in human body fluids such as amniotic fluid, milk, saliva, and urine.[5–7] At present, there is only circumstantial evidence that such TF-exposing EVs (TF-EVs) contribute to hemostasis. For example, amniotic TF-EVs may reduce blood loss during delivery,[5] milk TF-EVs may seal bleeding wounds of sore nipples and reduce gastrointestinal bleedings in newborns,[6] saliva TF-EVs may promote hemostasis when licking a wound,[7] and urine TF-EVs may limit hematuria.[7]

In 1985, Pusey et al. showed that human amniotic fluid contains an enzymatic activity that directly activates factor X (FX).[8] Therefore, we hypothesized and investigated whether EVs in normal human body fluids expose functional extrinsic tenase complexes, that is, EVs exposing complexes of TF and FVIIa. Here we demonstrate that the investigated body fluids contain EVs exposing functional extrinsic tenase complexes and we propose that these EVs provide hemostatic protection.

Essentials

- The extrinsic tenase complex consists of tissue factor and activated coagulation factor VII.
- Extracellular vesicles from normal body fluids expose extrinsic tenase complexes.
- These extracellular vesicles directly activate factor X.
- These extracellular vesicles may provide hemostatic protection.

2 | METHODS

2.1 | Body fluids collection, handling, and storage

Amniotic fluid was collected from seven healthy pregnant adult women (median age: 32 years; range: 25–37 years) at routine amniocentesis at median 22 weeks of gestation (range: 15–28 weeks). Amniotic fluid was centrifuged at 400*g* for 10 min and then at 1550*g* for 20 min to remove cellular components. Milk, plasma, saliva, and urine were collected from six healthy breastfeeding adult women (median age: 33 years; range: 28–37 years) who had been breastfeeding for median 6 weeks (range: 4–12 weeks). Milk and plasma samples were collected, prepared, and stored as described previously.[9] Saliva and urine were centrifuged at 400*g* for 10 min and then at 1550*g* for 20 min to remove cells and cell debris. After processing, all aliquots were stored at −80°C until use. Samples were collected with approval from the Ethics Committee of the Medical University of Vienna (#1892/2020 and #1721/2015). Informed consent was signed by study participants before sample collection.

2.2 | Isolation of extracellular vesicles from body fluids

Frozen stored body fluids were thawed in a water bath at 37°C, and fractionated by Sepharose CL-2B size exclusion chromatography (SEC; GE Healthcare; Telos column: 15 ml, Kinesis) to isolate EVs. Briefly, 500 μl of body fluid was diluted with 500 μl 0.9% NaCl before loading on a SEC column. After the loaded sample entirely entered the column, the column was eluted with 0.9% NaCl until in total 26 fractions of each 500 μl were collected. Fractions 8–10 were pooled as EV-containing fractions, and fractions 18–20 pooled as control fractions, respectively. The pooled fractions were used for the subsequent experiments immediately.

2.3 | Blood plasma clotting assay

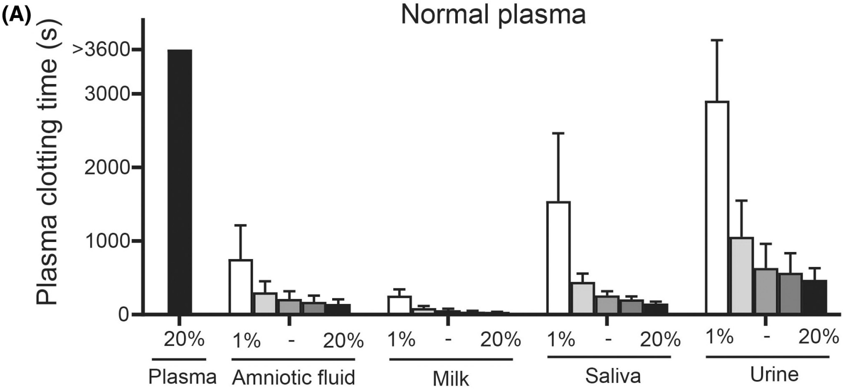
A blood plasma clotting assay, also referred to as fibrin generation test, was performed with body fluids or fractions thereof as described previously.[7]

2.4 | Chromogenic activated factor X generation assay

Seventy-five μl of diluted body fluid samples (10% in 0.9% NaCl, vol/vol) or 75 μl of pooled SEC-isolated EV fractions were incubated in the presence of absence of an anti-TF antibody (clone HTF-1, eBiosciences; final concentration, 30 μg/ml) or an anti-FVII antibody (clone CLB VII-1, Sanquin; final concentration, 30 μg/ml) at 37°C for 5 min. Fifty μl of human coagulation FX (16.8 nM final concentration) and $CaCl_2$ (3.3 mM final concentration) and 25 μl of a chromogenic substrate for activated FX (S-2765™, Chromogenix, Instrumentation Laboratory; 400 μM final concentration) were added to the samples. Activated FX generation was kinetically measured every 1 min at 37°C for 1 h on a SpectraMax i3 microplate detector Molecular Devices) at a wavelength of 405 nm. Optical density changes (ΔOD) measured at the beginning 15 min were used for calculation of ΔOD per minute. Measurements without purified FX were used as negative control.

2.5 | Fibrinolysis assay

The fibrinolytic potential of body fluids was assessed by immersing fibrinogen clots into the respective body fluid using methodology previously used to assess the lytic activity of pancreatic fluid.[10]



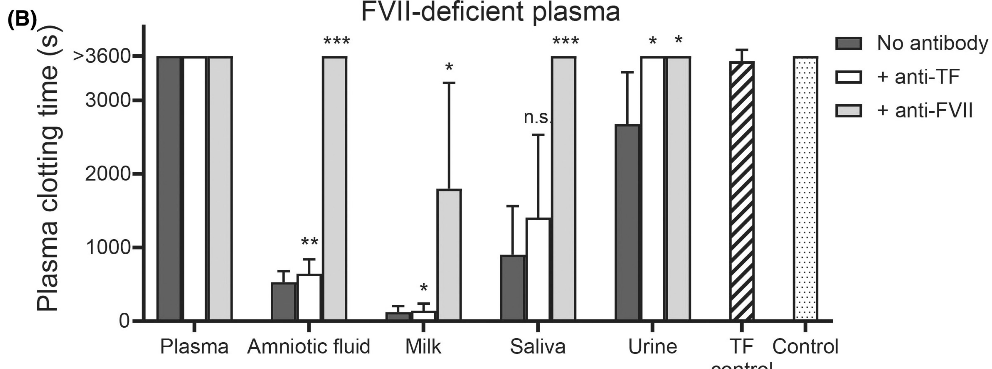


FIGURE 1 Body fluids initiate coagulation of normal and factor VII-deficient plasma. A, Amniotic fluid, milk, saliva, urine, and plasma (control; $n = 5$) were added to normal plasma at final concentrations of 1%, 5%, 10%, 15%, and 20% (vol/vol). B, We arbitrarily chose 5% (vol/vol) as the final concentration of body fluids for further studies. Body fluids (amniotic fluid $n = 7$; other body fluids $n = 6$) were added to coagulation factor VII (FVII)-deficient plasma in the absence or presence of anti-tissue factor (TF)- or anti-FVII antibodies. Innovin (final concentration 1 pM), an artificial mixture of TF and phospholipids, was used as TF control. Addition of Innovin to FVII-deficient plasma did not initiate coagulation, confirming the FVII deficiency; 0.9% NaCl was used as a negative control. *$p < .05$; **$p < .01$; ***$p < .001$.

2.6 | Statistics

Data were analyzed by paired sample *t* test (SPSS version 25.0 software, SPSS Inc.). A probability value of <.05 was considered statistically significant. Continuous variables are shown as mean ± standard error of the mean (SEM), unless stated otherwise.

3 | RESULTS AND DISCUSSION

To investigate whether human body fluids initiate coagulation, amniotic fluid, milk, saliva, and urine were added to normal plasma. Addition of all body fluids shortened the plasma clotting time in a dose-dependent manner (Figure 1A). Milk was the most coagulant body fluid, followed by amniotic fluid, saliva, and urine. As expected, plasma from the same healthy individuals, which is known not to contain TF-EVs, was not coagulant in our assay. Figure 1B shows that the body fluids also initiated coagulation of FVII-deficient plasma, which was almost completely inhibited by an anti-FVII antibody. An anti-TF antibody was less effective than an anti-FVIIa antibody, possibly due to competition with soluble FVII(a).

Next, body fluids were fractioned by SEC to investigate whether the functional extrinsic tenase complexes are present on EVs. Of the 26 SEC fractions of each body fluid, the fractions known to contain EVs (i.e., fractions 8–10) were pooled. As a control, fractions 18–20, which contain small particles with a diameter <70 nm and soluble proteins, were pooled.[11] Fractions 8–10, but not fractions 18–20, initiated coagulation (Figure 2A), showing that indeed EVs expose the tenase complexes. Based on an Innovin titration curve, we estimated the EV-associated TF activity of amniotic fluid, milk, saliva, and urine in normal plasma (deficient in TF) and the tenase activity in FVII-deficient plasma, which is deficient in both TF and FVII(a). The ability to trigger coagulation was highest for milk and amniotic fluid derived EV-containing fractions, followed by saliva and urine in normal- and FVII-deficient plasma (Figure 2B,C). These findings indicate that body fluids contain EVs exposing functional extrinsic tenase complexes.

The body fluids also efficiently activated FX to FXa in a chromogenic assay, with milk again being the most potent activator, followed by amniotic fluid, saliva, and urine (Figure 3A). Consistent with our previous results, the SEC-isolated EVs from milk and amniotic fluid had the highest tenase activity, followed by saliva and urine, which was inhibited by an anti-VII antibody and an anti-TF

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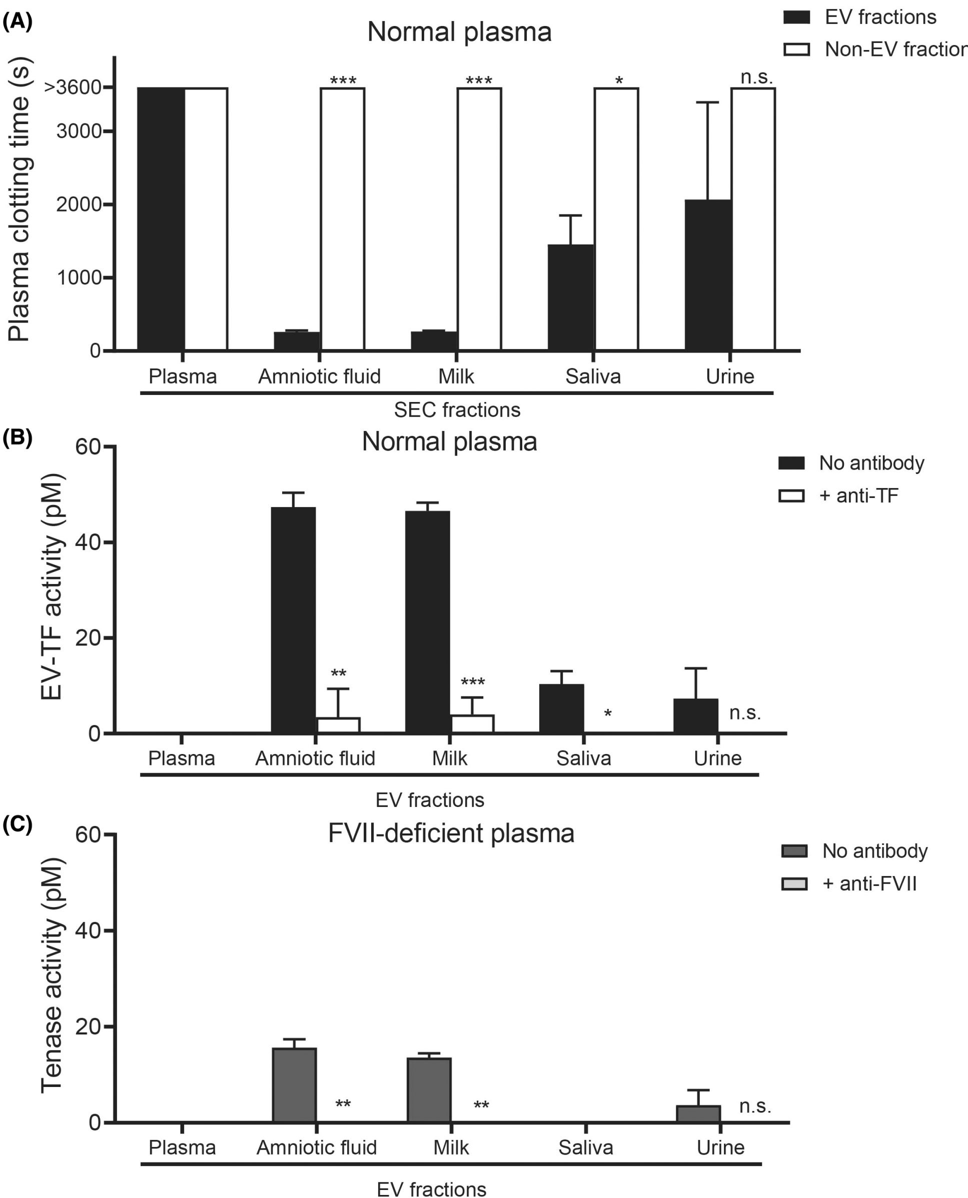

FIGURE 2 Isolated extracellular vesicles from body fluids initiate coagulation of normal and factor VII-deficient plasma. A, Body fluids were fractionated by size exclusion chromatography (SEC). Extracellular vesicle (EV)-containing fractions (fractions 8–10) and non-EV-containing control fractions (fractions 18–20) from amniotic fluid, milk, saliva, urine, and plasma (control; $n = 1$) were added to normal pool plasma at a final concentration of 20% (vol/vol). B, EV-containing fractions (fractions 8–10) were added to normal plasma (which is physiologically deficient in TF) and the EV-associated TF activity was measured and (C) the extrinsic tenase activity was measured in FVII-deficient plasma (which is deficient in both, TF and FVII). $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; n.s., non-significant.

antibody (Figure 3B–D). No FXa was generated in absence of purified FX (Figure 3E), confirming that the chromogenic substrate is indeed specific for FXa, and that relevant quantities of zymogen FX or FXa are absent from the body fluids.

Taken together, we demonstrate that normal human body fluids contain extrinsic tenase complexes that are present on EVs. These EVs efficiently trigger FXa generation and clotting of plasma.

Two recombinant forms of FVIIa (eptacog alfa and eptacog beta) are used to treat or prevent bleeding in patients with hemophilia that have developed inhibitory antibodies,[12,13] because high-dose recombinant FVIIa is able to induce hemostasis by directly activating FX, thus bypassing large parts of the coagulation cascade, and thereby limiting blood loss.[14] Here we hypothesize that body fluids may act as "natural bypassing agents." In fact, there is even direct clinical evidence to support this hypothesis. In 1934, Alphons Solé reported that application of human milk-soaked tamponades on sites of injury effectively stops ongoing external bleedings in hemophiliacs.[15]

The question then is why normal body fluids contain functional extrinsic tenase complexes. Body fluids may provide hemostatic protection, particularly in situations that challenge the coagulation system. An example is childbirth, because delivery is associated with

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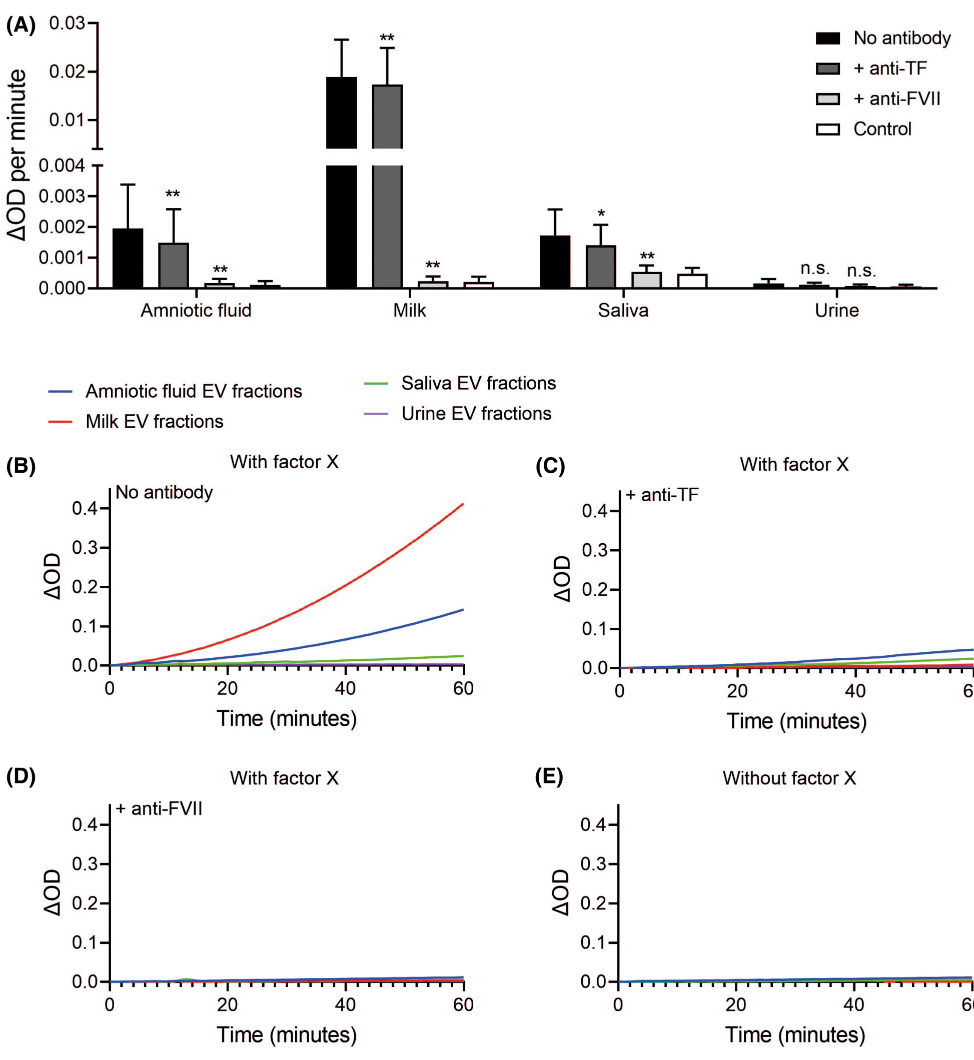

FIGURE 3 Isolated extracellular vesicles from body fluids directly activate factor X. A, The ability of body fluids to directly activate factor X (FX) was measured with a chromogenic assay as described in Methods. A, FXa generation was measured in amniotic fluid ($n = 7$), milk, saliva, and urine ($n = 6$; final concentration 5%, vol/vol) after addition of recombinant FX in the absence or presence of inhibitory antibodies against tissue factor (TF) or FVII. B–E, Extracellular vesicles (EVs) were isolated from body fluids by size exclusion chromatography and FXa generation was measured in (B) the absence and (C) the presence of an inhibitory antibody against TF and (D) an inhibitory antibody against FVII. E, In the absence of FX, no FXa was generated. *$p < .05$; **$p < .01$; n.s., non-significant.

vascular- and tissue damage leading to blood loss. To investigate such a possible hemostatic effect of amniotic fluid during delivery, we added amniotic fluid to human wound blood, which already contains endogenous coagulant TF-EVs from the wound, as described previously.[7,16] Addition of amniotic fluid shortened the clotting time of wound blood (data available upon request) indicating that amniotic fluid promotes hemostasis even when TF from the wound is present already, and thus the contact between amniotic fluid and blood may be beneficial to reduce blood loss of the mother during and after delivery.

Of note, excessive clotting can be devastating not only intravascularly but also in extravascular systems. Obstructions of the upper airways, urinary tracts, and milk ducts by blood clots are potentially life-threatening complications. For that reason, we hypothesized that the coagulant properties of body fluids may be counter-balanced by fibrinolysis. We therefore generated fibrin clots and incubated these clots in body fluids in the presence and absence of aprotinin, a plasmin inhibitor, and measured both clot weight and concentration of D-dimer, a fibrin split product, after 24 h. Milk almost completely lysed blood clots, which was inhibited by aprotinin, indicating the presence of plasmin. Also, saliva and urine were potent fibrinolytics. In contrast, amniotic fluid contained no detectable fibrinolytic activity (Figure 4A,B). These results suggest that the prothrombotic properties of specific body fluids may be counter-balanced by their fibrinolytic properties, which in turn may protect the patency of semi-closed structures in, for example, airways, urinary tracts, and milk ducts.

In conclusion, normal human body fluids contain extrinsic tenase complexes that are exposed on the surface of EVs. Based on our present findings we propose that these EVs provide hemostatic protection.

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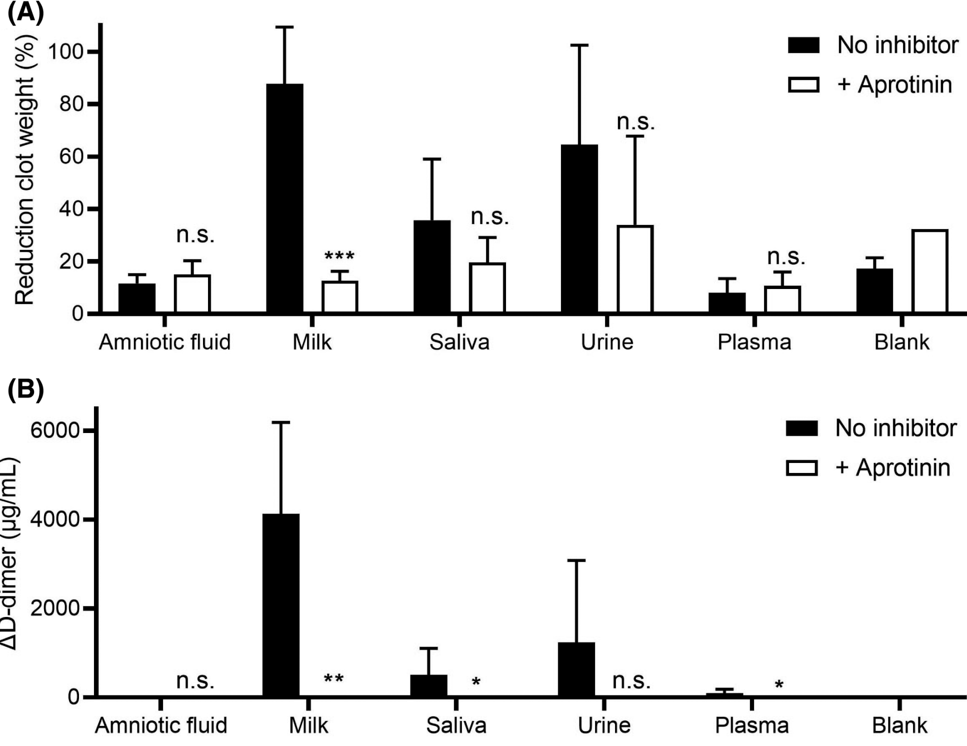

FIGURE 4 Fibrinolytic potential of body fluids. The fibrinolytic potential of body fluids was investigated as described previously[10]. Briefly, generated fibrin clots were incubated with amniotic fluid ($n = 7$), milk, saliva, urine, and plasma (control; $n = 6$) without or with aprotinin (a fibrinolysis inhibitor) for 24 h and the mass of the remaining clot was estimated by weighing and compared to the original weight (A). In addition, D-dimer concentration was measured before and after clot incubation with body fluids for 24 h without or with aprotinin. Buffer (0.9% NaCl) was used as blank. *$p < .05$; **$p < .01$; ***$p < .001$; n.s., non-significant.

AUTHOR CONTRIBUTIONS

Y.H. designed and performed experiments; R.N. and J.T. designed experiments; Y.H., G.Y.-S, I.P., C.A., T.L., A.R., J.T., R.N. analyzed data; J.T., A.R., G.Y.-S. and T.L. acquired samples; R.N. and J.T. supervised the study; Y.H., J.T., and R.N. drafted the manuscript; R.N., J.T., T.L., A.R., G.Y.-S., C.H., C.A. and I.P. provided administrative and technical support; all authors took part in reviewing and editing the entire manuscript and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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