Time-dependent hardening of blood clots quantitatively measured in vivo with shear-wave ultrasound imaging in a rabbit model of venous thrombosis

Etienne Mfoumou, Julien Tripette, Mark Blostein, Guy Cloutier

Division of Hematology, Department of Medicine, Sir Mortimer B. Davis Jewish General Hospital, Montreal, QC, Canada

Laboratory of Biorheology and Medical Ultrasonics, University of Montreal Hospital Research Center (CRCHUM), Montreal, QC, Canada

Department of Radiology, Radio-Oncology and Nuclear Medicine and Institute of Biomedical Engineering, University of Montreal, Montreal, QC, Canada

Abstract

Objective: Provide in vivo blood clot hardening evolution with ultrasound using supersonic imaging of shear waves.

Methods: We conducted a prospective study in flow stasis-induced venous thrombus within jugular veins of white female New Zealand rabbits. Blood clot elasticity was noninvasively measured in vivo using the Young's modulus (in kilopascals), on a 2-hour and a 2-week periods after thrombus induction. Monitoring was followed by a necropsy and ex vivo mechanical characterization to validate the existence and elasticity of explanted thrombi.

Results: Stagnant blood in the region of interest underwent clotting and progressive hardening with thrombus aging. The mean Young's moduli varied from 1.0 ± 0.6 kPa (at 10 min) to 5.3 ± 1.6 kPa (at 2 hours), then to 25.0 ± 6.8 kPa (at 14 days) post-surgery. Mean ex vivo moduli of 6.2 ± 0.7 kPa at 2 hours and 29.0 ± 2.4 kPa at 2 weeks agreed with in vivo measures.

Conclusions: Supersonic imaging of shear waves provides consistent quantitative non-invasive elasticity measurements not available with standard compression ultrasound imaging for diagnosing and following venous thromboembolism. This information translatable to humans could aid in determining whether continued anticoagulant treatment is necessary, especially in the setting of unprovoked venous thromboembolism.

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acute DVT and demonstrated the presence of a residual thrombus in about 50% of patients at 6 to 12 months. This residual thrombus persists indefinitely [14–16] and given that DVT may be contralateral in 50% of patients, residual vein thrombus may be considered a manifestation of an underlying prothrombotic tendency. Hence, an accurate quantitative mechanical characterization of this residual thrombus may offer an opportunity to predict recurrent VTE.

The present study uses, for the first time, the Supersonic Shear-wave Imaging (SSI) technology [17] for the measurement of time-dependent hardening of blood clots in vivo. This technique couples high-resolution anatomic images obtained with B-mode US, with mechanical information (Young’s modulus) provided by quantitative elastography. Our findings in an animal model support the hypothesis that mechanical characterization of a DVT is possible in vivo. Such a quantitative determination of blood clot hardening may define a risk factor, in the clinical setting, for deciding whether to continue oral anticoagulant therapy after an unprovoked venous thromboembolism, which is still controversial.

Materials and Methods

Animals

Experimental procedures, including ex vivo sample analyses, were conducted in accordance with guidelines of the Institutional Animal Care Committee of the University of Montreal Hospital Research Center. The investigation conformed with guidelines of the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996, Assurance Number A5377-01).

Nine healthy female New Zealand white rabbits, of 2.5 to 3.2 kg (mean ± SD, 3.0 ± 0.7 kg) in weight, were used in this study. Animals were purchased from Charles River Canada (St.-Constant, Quebec, Canada). The vendor provided health reports to certify that animals were free of pathogens. All rabbits were housed in separate cages (Lab Products Inc., Madison, WI) and water were provided ad libitum. Animals were purchased from Charles River Canada (St.-Constant, Quebec, Canada). The vendor provided health reports to certify that animals were free of pathogens. All rabbits were housed in separate cages (Lab Products Inc., Madison, WI) and water were provided ad libitum. Animals were acclimated to their environment 7 days before the beginning of experiments, for deciding whether to continue oral anticoagulant therapy after an unprovoked venous thromboembolism, which is still controversial.

Induction of Venous Thrombosis

The jugular vein was exposed using blunt dissection, including the distal bifurcation (Fig. 1). A slightly modified version of the model from Lorrain et al. [18] and the double-opposing inverted-sutures model [19] were used to induce venous thrombosis in the jugular vein. Two loose sutures were placed 2 cm apart, both proximally and distally on the segment of interest. This technique allows proceeding to the double ligatures without exposing the entire segment of interest. All other vessels connecting the selected jugular segment were ligated. On the segment of interest, the proximal suture on the jugular vein was placed first. A hypercoagulable state was promoted using a syringe, by aspirating the blood trapped in the segment of interest, keeping it 1-2 minutes in the syringe, and re-injecting it back while closing the distal suture, creating a stasis. As a direct result, a venous thrombosis was obtained from the combination of blood-flow stasis and increased coagulability. The incisions were then closed and the thrombus hardening was monitored by echography (SSI imaging), under general anaesthesia. On following days, US recordings were performed without anaesthesia expect for every third day, where animals received subcutaneous acepromazine (0.75 mg/kg) and were briefly anesthetized with isoflurane (2%) in oxygen (1 L/min) delivered with a face mask to permit blood sampling.
Shear Wave Elastography and Real-time Monitoring of Thrombus Hardening

In shear wave elastography, the scanner generates a remote radiation force through focused ultrasonic beams that induce the propagation of transient shear waves. An ultrafast imaging sequence is then performed to acquire successive radio-frequency (RF) data at a very high frame rate of a few kHz. The tissue displacement due to shear wave propagation is calculated using cross-correlation of successive RF echoes, and the shear wave velocity (and elasticity) is determined using a time-of-flight estimation [20]. In the current study, elastography with SSI was performed using the Aixplorer US scanner (Supersonic Imagine, Aix-en-Provence, France) equipped with a linear array transducer (SuperLinear 15-4). This transducer has a 4-15 MHz frequency band, and was attached to a stand-alone support equipped with a manually adjustable rotary system for appropriate positioning of the probe. This insured no motion of the probe during Doppler and elasticity recordings. Transversal and longitudinal views of the segment of interest were carefully interrogated in colour Doppler mode before and after surgery to insure accurate positioning of the probe on the animal. Images of the vein under analysis and of the adjacent artery were documented prior selecting the region of interest (ROI) for subsequent elasticity data collection.

After a few seconds of US recordings, when a stable colour-coded elasticity map was obtained, the mean value of the Young’s modulus within the ROI was recorded by processing the image with a dedicated Matlab software developed in-house (version 7.1, MathWorks Inc., Natick, MA, USA). The time-varying thrombus rigidity was then monitored by assessing its Young’s modulus at different stages (the higher is the Young’s modulus in kPa, the more rigid is the thrombus). Measurements were triplicated to insure consistency of results. The B-mode images of the Aixplorer scanner were complemented with B-mode images acquired with a high-frequency Vevo 770 US biomicroscope (Visualsonics, Toronto, ON, Canada) equipped with a 35-MHz single-crystal mechanical transducer (RMV-703). The focal length was 10 mm and a field of view of 12 mm × 12 mm was used. The close to four-time higher resolution of the Visualsonics scanner compared with the SSI scanner allowed confirming the identification of the thrombus location seen in elastography; also the higher blood clot echogenicity with frequency [21] helped guiding the thrombus selection. To guarantee comparability between measurements of different vessels, care was taken on maintaining the same US settings throughout experiments.

Typical representations of the ROI are shown for one rabbit in Fig. 2. Colour Doppler maps show the transversal view of the ROI, illustrating the flow inside the jugular vein and in an adjacent artery before (A) and after (B) the thrombus induction, using the Aixplorer SSI scanner. A longitudinal B-mode view of the jugular vein with a ligated end is also shown (C) using the high-frequency Visualsonics biomicroscope, after induction of venous thrombosis. This pre-investigation allowed identifying and documenting all details in a given ROI.

Haematological Analyses

Red blood cell (Rbc), white blood cell (Wbc) and platelet (Pl) counts, haemoglobin (Hb), hematocrit (Ht), prothrombin time (Pt) and fibrinogen concentration (Fib) were measured using standard haematology and clinical laboratory methods (at the University of Montreal Hospital, QC, Canada) on samples obtained by puncture of the central ear artery of the rabbit. Blood sample analyses were performed on the day of the experiment, and on the first and last days and every third day in the case of the 2-week monitoring period.

Euthanasia and Ex Vivo Validation of Explanted Thrombi

At the end of the 2-hour (short term) and 2-week (long term) monitoring periods, animals were euthanized by intravenous injection of 160 mg/kg sodium pentobarbital (Euthanyl, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada). Immediately after death,
thrombi were explanted and placed in Krebs solution. Necropsy observations allowed ocular verification of thrombus formation. In addition, to ensure the validity of the technique and because this is the first in vivo study on clotting blood shear wave elasticity imaging, the explanted thrombi were immersed in a homogeneous agar (number A-6924; Sigma Chemical, Saint-Louis, MO, USA) and gelatin (number G-2500 type A from porcine skin; Sigma Chemical, Saint-Louis, MO, USA) phantom and tested ex vivo with the same Aixplorer scanner, within 1 hour from the time of excision. The aim was to identify potential artefacts attributed to in vivo scans by using idealized ex vivo scanning conditions.

Results

In Vivo Elasticity Monitoring of Thrombi

Fig. 3 shows a typical evolution of the US elasticity modulus of an in vivo thrombus in rabbit #7 for the 2-hour follow-up period after thrombus induction. An increase in the spatially-averaged Young’s modulus is observed over time, as it can be observed on the SSI colour maps within the segmented ROI (dashed circles), corresponding to clotting blood. This is accompanied by the B-mode display of the ROI between 20 to 120 min post-surgery. As seen, the B-mode echogenicity did not vary significantly over time. The mean elasticity curves from measurements on all animals over ROIs selected on thrombi and within a reference region in the surrounding muscle are shown for the 2-hour and 2-week periods in Fig. 4A ($n=9$) and 4B ($n=6$), respectively. The Young’s modulus varied from $1.0 \pm 0.6$ kPa (at +10 min) to $5.3 \pm 1.6$ kPa (at +120 min), then from $7.9 \pm 2.8$ kPa (at +1 day) to $25.0 \pm 6.8$ kPa (at +14 days). We tested the rigidity data for changes over time with analyses of variance (ANOVA) computed using the generalized linear model in SPSS (v.17; SPSS Inc, Chicago, IL, USA), with repeated measures. On the first set of data immediately after surgery, measurements at +10 min have been taken as baseline measures, and the rigidity starts to be statistically different from this baseline after 50 min (see Fig. 4A). We did the same analysis for the following days (day #1 to day #14), with day #1 values as baseline, and this shows an increased significant rigidity after 5 days (see Fig. 4B). The ratio between the blood clots’ Young’s modulus to surrounding muscle’s Young’s modulus as a function of clot age is plotted in Fig. 5. Assuming that the muscle maintains its elastic properties over time (see Fig. 4), this ratio may be used in future studies to subdivide the thrombosis into acute, sub-acute and chronic stages for therapy planning (see Discussion).

Post-mortem Observations

Thrombi were confirmed at necropsy in all rabbits. A typical appearance of a thrombus is shown in situ in Fig. 6A, along with its excited appearance, B-mode and SSI images at the 2-hour (B- left) and 2-week (B- right) post-surgery. US images were acquired with thrombi
in the absence of risk factors for haemorrhage, be treated with indefinite anticoagulant therapy [26]. However, despite these recommendations, it is clear that the identification of patients particularly susceptible to recurrent VTE be identified. Indeed, the risk of recurrent thrombosis must be weighed against the risk of haemorrhage in deciding which patients should continue to receive anticoagulants. Two recent major clinical trials have attempted to identify such clinical risk factors [7, 27]. As a result, patients whose risk of rethrombosis would be greater than the risk of haemorrhage would benefit from continued anticoagulation. More importantly, other clinical data examining residual venous thrombosis by standard US, as a potentially important risk factor for recurrent VTE, has been published [28]. Therefore, this novel technology can be used as a “risk factor” for recurrent thrombosis and therefore aid in the decision as to which patients should remain on anticoagulants.

According to reported SSI ultrasound results of the current work, the Young’s modulus may become a new parameter to help clarify important properties of venous thrombi that could predict recurrent VTE. A monotonically increasing trend was observed in the first two weeks following thrombus induction. This can be explained by a combination of factors, among which the organization and transformation of the blood clot from a platelet-rich to dominant fibrin composition, with possible fibrin cross-linking [29]. In addition, because the clot rigidity increases monotonically with the fibrin content [30], it appears reasonable to use US elastography to characterize venous clots. Ultimately, a new and non-invasive parameter for assessing recurrent thrombosis risk can be developed. In addition, the SSI technology can likely enhance venous compression US [31] for non-invasive, quantitative and less operator dependent clinical assessment of biomechanical properties of thrombi.

The animal protocol investigated in this work revealed that the quantitative SSI method is capable of reflecting the existence and evolution of all analysed thrombi on a 2-hour and 2-week periods. It suggests that SSI can identify the blood clot regions. Further, close scrutiny of our data (Fig. 5) suggests that the variation of thrombus elasticity progresses in two different stages: one running from day #0 to day #7 (with few variances between Young’s modulus of different measures), and another phase from day #8 to day #14 (with larger variability likely associated with the heterogeneous nature of aged thrombi) [32]. Hence, day #7 appears to be a transition point between acute and chronic thrombi in rabbits. This is in agreement with existing transition points reported in the literature for mouse venous thrombosis (day #6) [33], rats (day #6) [34], and between acute and sub-acute thrombosis in humans (mean age of 5.7 days) [35]. Note that the proposed animal model was designed to create a blood clot incarcerated between two ligatures, thus inhibiting restoration of flow. We recognize this limitation for studying the effect of flow mediated agents on clot hardening and associated with the heterogeneous nature of aged thrombi [32].

**Haematological Parameters**

All haematological parameters, i.e. Rbc, Wbc, Pl, Hb, Ht, Pt and Fib were within the normal range of experimental rabbits reported by Mitruka and Rawnsley [22]. Their mean values are presented in Table 1.

**Discussion**

The risk of recurrent thrombosis in patients with unprovoked VTE has been estimated to be close to 30%, after eight years of discontinuation of anticoagulation treatment [9]. This risk can be abrogated through prolonged use of anticoagulants. Given that the risk of haemorrhage, for patients on anticoagulants, is approximately 1-2% per year [23–25], clearly the risk of thrombosis recurrence is of greater concern and has prompted recent guidelines, published by the American College of Chest Physicians (ACCP), to recommend that patients with un provoked VTE, in the absence of risk factors for haemorrhage, be treated with indefinite anticoagulant therapy [26]. However, despite these recommendations, it

![Fig. 5. Normalized elasticity modulus curves and their average plot for the 2-week monitoring period. Each normalized data represents the ratio of the clot’s Young’s modulus and the one of a surrounding muscle at the measurement time. The average curve represents the mean of n = 6 samples and error bars are the standard deviations on these samples between animals.](image1)

![Fig. 6. Evidence of thrombus formation after necropsy and ex vivo validation. (A) Typical thrombus appearance in situ. (B) Appearance of the explanted 2-hour old (left) and 2-week old (right) thrombi, together with conventional B-mode images (dynamic range 50 dB) and SSI elasticity mapping; the mean Young’s moduli of the explanted thrombi are 6.2 ± 0.4 kPa (at +2 hours) and 29.0 ± 2.4 kPa (at +2 weeks).](image2)
thrombolysis. The latter objectives could be addressed with the use of the animal model we proposed in [36].

There is a growing interest in the use of residual vein obstruction (RVO) to guide the duration of oral anticoagulant therapy (OAT) for uncomplicated DVT, toward recurrent VTE. Prophylaxis [9–11], While OAT has been shown to be effective for reducing the risk of recurrent VTE in the context of continued treatment, this advantage appears to be reduced in the case of treatment discontinuation [10]. However, the advantage of OAT is counteracted by major bleeding, which is the main adverse event for this therapy [10,11,37]. Therefore, given that RVO is time dependent and that the duration of anticoagulation might influence the risk of recurrent VTE, a real-time, non-invasive and quantitative assessment of the thrombus rigidity appears potentially useful in helping to tailor clinical decision making. For example, thrombus rigidity after three months of anticoagulation, a major time when decisions are made regarding continuing anticoagulation or not, may help in guide that decision, as recommended in the 2012 ACCP guidelines. It is unlikely that RVO will aid in the acute situation when medical thrombolysis or medical thrombectomy is used. Prospective studies are thus required to assess the importance of measuring the hardening of a thrombus with SSI in the context of a RVO, as an independent risk factor to evaluate the risk/benefit ratio of anticoagulation treatment in patients.

In conclusion, the present study is the first to monitor in vivo thrombus hardening using a non-invasive dynamic elastography technique, namely the SSI technology. We followed the thrombus formation during two weeks after its induction, and observed a constant increase of clot hardening. In vivo and ex vivo measurements were in acceptable agreement, demonstrating the validity of the SSI method in the context of VTE, especially with regard to its ability to potentially predict recurrent thrombosis.

Key points

- Ultrasonic elastography with supersonic shear wave imaging can monitor blood clot hardening in vivo
- Quantification of blood clot hardening can monitor the age of venous thrombosis
- Supersonic shear wave imaging, because it is a technique used in radiology imaging, can be translated to human diagnosis and treatment follow-up

Authors’ Contributions

E. Mfoumou, J. Tripette and G. Cloutier conceived and designed experiments. E. Mfoumou and J. Tripette performed experiments and analyzed data. G. Cloutier directed this research, contributed to agents/materials/analysis tools and equipments. E. Mfoumou, J. Tripette, M. Blosstein and G. Cloutier wrote the paper.

Conflict of Interest Statement

None to declare.

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