Contraction of Blood Clots Is Impaired in Acute Ischemic Stroke

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Objective—Obstructive thrombi or thrombotic emboli are the pathogenic basis of ischemic stroke. In vitro blood clots and in vivo thrombi can undergo platelet-driven contraction (retraction), resulting in volume shrinkage. Clot contraction can potentially reduce vessel occlusion and improve blood flow past emboli or thrombi. The aim of this work was to examine a potential pathogenic role of clot contraction in ischemic stroke.

Approach and Results—We used a novel automated method that enabled us to quantify time of initiation and extent and rate of clot contraction in vitro. The main finding is that clot contraction from the blood of stroke patients was reduced compared with healthy subjects. Reduced clot contraction correlated with a lower platelet count and their dysfunction, higher levels of fibrinogen and hematocrit, leukocytosis, and other changes in blood composition that may affect platelet function and properties of blood clots. Platelets from stroke patents were spontaneously activated and displayed reduced responsiveness to additional stimulation. Clinical correlations with respect to severity and stroke pathogenesis suggest that the impaired clot contraction has the potential to be a pathogenic factor in ischemic stroke.

Conclusions—The changeable ability of clots and thrombi to shrink in volume may be a novel unappreciated mechanism that aggravates or alleviates the course and outcomes of ischemic stroke. The clinical importance of clot or thrombus transformations in vivo and the diagnostic and prognostic value of this blood test for clot contraction need further exploration. (***Arterioscler Thromb Vasc Biol*. 2017;37:271-279. DOI: 10.1161/ATVBAHA.116.308622.)**

Key Words: blood coagulation ■ clot retraction ■ stroke ■ thrombosis

Stroke is a leading cause of death and disability worldwide[1,2] and accounts for 1 in every 20 deaths in the United States each year.[3,4] Despite this clinical importance and numerous studies on circulatory disorders in the brain vessels, the pathogenesis of stroke remains largely unclear, and the results of stroke prophylaxis and treatment are unsatisfactory.[5] Brain damage caused by abnormalities of cerebral blood flow is often ischemic by nature[2] and is associated with thrombosis or thromboembolism, indicating involvement of hemostatic reactions.[6,7] Hypercoagulability and changes in blood rheology in combination with local dysfunction and destruction of endothelium comprise a pathogenic basis for ischemic stroke.[6,8–10] In addition to disorders of plasma and vascular hemostasis, platelet activation plays a role in ischemic stroke.[6,10,11] Platelet stimulation and aggregation are followed by release of potent triggers of the coagulation cascade and secondary platelet activation.

See cover image

A main physiological activator of platelets is thrombin, an enzyme that forms in the blood irrespective of the initial cause of intravascular blood clotting. Concomitant with platelet activation, thrombin converts fibrinogen, a soluble plasma protein, into an insoluble fibrin gel made of a filamentous network.[12–14] Fibrin sticks to activated platelets via the integrin receptor αIIbβ3 to form a platelet–fibrin meshwork comprising the structural basis of a hemostatic clot or an obstructive thrombus.[12,15,16] When the blood clot is formed in a tube, it undergoes volume shrinkage, and liquid serum is expelled. This process is called clot contraction or retraction.[17] The cellular and molecular composition of the blood clot has been found to influence the rate and extent of clot contraction.[18] Importantly, contraction occurs not only in vitro but also inside a vessel if a clot or thrombus is formed.[19] The importance of clot contraction in vivo is based on at least 2 mechanisms: (1) at the site of tissue injury, the active decrease in clot size reduces blood loss by pulling together wound edges and (3) blood flow is recovered past a contracted formerly obstructive thrombus, the latter being hypothetically an important compensatory or adaptive mechanism in thrombotic states.[17,19,20]

Despite potential medical significance, clot contraction has not been studied systematically in clinical settings mainly because of no standardized and automated methods for registration and quantification of this process. Previously, a limited number of studies assessed extent of clot contraction in vitro subjectively as a visible ratio of the volume of serum

Received on: June 10, 2016; final version accepted on: November 17, 2016.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.308622/-/DC1.

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Nonstandard Abbreviations and Acronyms	
NIHSS	National Institutes of Health Stroke Scale
RBC	red blood cell
TF	tissue factor
TIA	transient ischemic stroke
TOAST	Trial of Org 10172 in Acute Stroke Treatment
TRAP	thrombin receptor–activating peptide

and the reduced volume of the clot. It is not surprising that the parameters of clot contraction under various circumstances are hardly comparable and highly scattered with the normal range of the extent of contraction varying from 20% to 90%. In this work, we use a recently described instrumental technique[18] that enabled us to quantify the kinetics of blood clot contraction in normal and disease states.

The goal of this work was to evaluate the potential pathogenic importance of clot contraction in acute ischemic stroke by correlating the parameters of clot contraction with clinical and laboratory characteristics.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Comparison of Hemostatic and Hematologic Profiles in Stroke Patients and Healthy Donors

Hemostatic and hematologic parameters for stroke patients and healthy donors are shown in Table 1. Comparison of these parameters revealed an elevation in fibrinogen and D-dimer levels and a decrease in the rate of ADP-induced platelet aggregation in stroke patients (Table 1). In addition, stroke patients had a reduced platelet count and increased fibrinogen level, hematocrit, erythrocyte sedimentation rate, and leukocyte count. In vitro model experiments with variation of the platelet count and fibrinogen levels suggest that clot contraction can be directly modulated by these blood constituents. In particular, clot contraction was enhanced by the increasing number of platelets (Figure IIA in the online-only Data Supplement), whereas fibrinogen had a dose-dependent inhibitory effect (Figure IIB in the online-only Data Supplement), consistent with the changes in blood composition observed in the stroke patients. Because the molecular and cellular composition of the blood impacts the process of clot contraction,[18] these differences collectively suggest that clot contraction has the potential to differ in healthy donors and stroke patients.

Characterization of Clot Contraction in Stroke Patients

Automated optical tracking of clot size (Figure 1A and 1B) revealed a 60% reduction in the average extent of clot contraction at the end point in stroke patients relative to healthy donors (Figure 1C), which resulted in stroke patients having a significantly larger clot size. Stroke patients also had a reduced average velocity and area over the curve while having a prolonged lag time (Figure 1D and 1E). Cross-correlation of clot contraction parameters revealed that extent of clot contraction, average velocity, and area over the curve were all strongly correlated with each other, suggesting a mechanistic relationship between the parameters. Interestingly, although lag time and extent of clot contraction were not significantly correlated in healthy donors, there was a significant negative correlation between these 2 parameters in stroke patients, suggesting that reduced platelet activity increases the lag time and decreases the extent of clot contraction (Tables I and II in the online-only Data Supplement).

To see whether patients experiencing a transient ischemic stroke (TIA) have more normalized clot retraction, blood

Table 1. Hemostatic and Hematologic Parameters in Stroke Patients and Healthy Subjects

Parameters (Normal Ranges Are Shown in Parentheses)	Healthy Subjects (n=79)	Patients With IS (n=85)
Hemostatic parameters		
aPTT (26–36), s	32.1±0.5	35.4±2.4
Prothrombin ratio (70–130), s	103.6±1.5	105.8±2.8
INR (0.85–1.15)	0.99±0.01	1.00±0.02
Fibrinogen (1.8–4.0), g/L	2.7±0.1	3.3±0.2*
Thrombin time (14–21), s	17.7±0.2	17.4±0.4
D-Dimer (0–0.5), µg/mL	0.23±0.03	1.7±0.4‡
Antithrombin III (80–120), %	90.5±1.0	94.1±4.9
Plasminogen (80–135), %	111.8±2.9	109.8±4.3
Protein C (70–130), %	104.3±2.1	103.7±2.9
Maximal ADP-induced platelet aggregation (60–90), %	64.3±1.6	62.0±4.4
Rate of ADP-induced platelet aggregation (30–45), %/min	36.6±1.0	24.3±2.4§
Hematologic parameters		
Platelet count (180–320), $\times 10^9$/L	286±10	252±8*
Mean platelet volume (8.6–12.6), fL	9.5±1.4	8.4±0.1†
Red blood cells (3–5), $\times 10^{12}$/L	4.5±0.1	4.5±0.1
Hematocrit (36–48), %	38.5±0.5	41.6±0.6‡
Hemoglobin (120–160), g/L	134.1±1.8	139.3±2.2
Color index (0.85–1.05)	0.93±0.01	0.92±0.01
Mean cell volume (80–100), fL	85.6±0.8	92.2±0.9§
Mean cell hemoglobin (30–35), pg	32.1±0.4	30.9±0.3†
Leukocytes (4–9), $\times 10^9$/L	5.8±0.2	7.8±0.2§
Eosinophils (0.5–5), %	3.0±0.3	2.0±0.1*
Monocytes (3–11), %	6.2±0.2	8.1±0.3§
Lymphocytes (19–37), %	34±1	23±1§
Basophiles (0–1), %	0.4±0.1	0.49±0.02
Neutrophils (47–78), %	58.8±1.1	66.3±1.0§
ESR (0–15), mm/h	8.5±1.0	19.4±2.1‡

aPTT indicates activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; INR, international normalized ratio; and IS, ischemic stroke. *P<0.01, †P<0.05, ‡P<0.001, §P<0.0001.

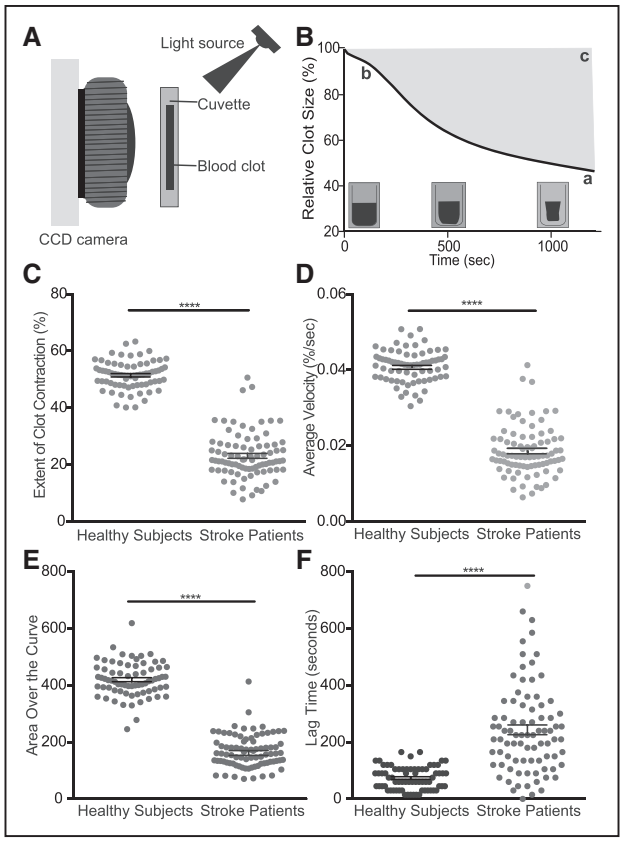

Figure 1. Optical tracking system used for measurements of clot contraction. **A**, The optical analyzer (side view) used to measure light scattering during the process of clot contraction. **B**, The changes in relative clot size are converted into a kinetic curve that can be analyzed for (a) the extent of clot contraction at 20 min, (b) the lag time or the time to 5% contraction, and (c) the area over the curve (AOC) that characterizes the entire process of clot contraction and integrates the extent and rate of contraction and the lag time. Optical tracking was used to assess differences in between healthy subjects and stroke patients in (**C**) extent of clot contraction, (**D**) average velocity of contraction, (**E**) AOC, and (**F**) lag time. Parameters for healthy subjects and stroke patients were compared using an unpaired, 2-tailed Student *t* test. ****$P<0.0001$.

samples from patients with TIA were analyzed. It was found that TIA patients also had a significantly reduced extent of contraction, average velocity, lag time, and area over the curve ($P<0.01$ for average velocity, $P<0.0001$ for all other parameters), whereas clot contraction in TIA patients did not significantly differ from that of ischemic stroke patients (Table III in the online-only Data Supplement).

Kinetic Phase Analysis of Clot Contraction in Stroke Patients

It has been shown previously that clot contraction in the blood of healthy subjects occurs in 3 phases: initiation of contraction (phase 1), linear contraction (phase 2), and mechanical stabilization (phase 3).[18] Regression analysis conducted on average kinetics curves (Figure 2) revealed that stoke patients and healthy donors did not have a difference in the duration of the different phases or in the rate constant associated with phase 1 (Table 2). However, the rate constants of the linear phase 2 and the exponential phase 3 were significantly reduced by 2 times and 10 times, respectively, in stroke patients compared with healthy subjects, indicating impairment of the mechanisms of compaction (phase 2) and stabilization of the clots (phase 3).

Correlation Between Contraction Parameters and Laboratory Tests

Because the observed differences between clot contraction in healthy subjects and stroke patients were likely related to disorders of hemostasis and changed blood composition, we investigated correlations between the extent of clot contraction and hemostatic, hematologic, and biochemical parameters (Table IV in the online-only Data Supplement). The main finding was that the extent of clot contraction moderately but significantly correlated with the platelet count in stroke patients. We also revealed slight but significant correlations between the extent of clot contraction and prothrombin time (prothrombin index and international normalized ratio) and hematocrit in healthy subjects, whereas these correlations were not present in stroke patients. In stroke patients, only monocyte count weakly but significantly correlated with the extent of clot contraction ($r^2=0.2839$; $P<0.001$).

Analysis of Platelet Function and Morphology in Stroke Patients

Because platelets are critical for the contractile force generated during clot retraction, we studied the functionality of platelets in ischemic stroke using flow cytometry–based evaluation of the baseline activity of quiescent platelets and their responsiveness to chemical activation. We used thrombin receptor–activating peptide (TRAP) to mimic the effect of thrombin on protease-activated receptors without a risk of fibrin formation that would complicate the assay. Platelet reactivity was assessed by surface expression of P-selectin and by the ability to bind fibrinogen as a measure of the integrin αIIbβ3 activation. In addition, isolated unstimulated platelets from ischemic stroke patients were studied with scanning electron microscopy to assess their functional status reflected by their shape. In stroke, platelets were initially activated as revealed by a higher

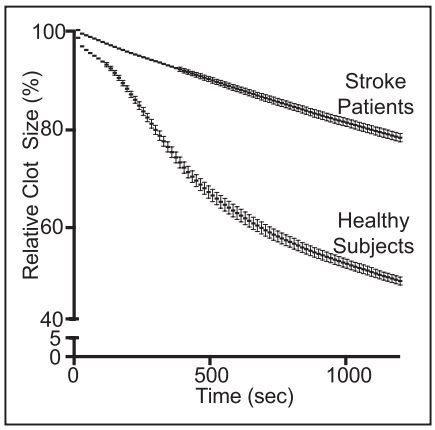

Figure 2. Averaged kinetic clot contraction curves obtained in the blood of stroke patients (n=85) and healthy subjects (n=79). Optical tracking was used to measure the relative changes in clot size during 20 min with 15-s intervals. Data are represented as mean±SEM.

Table 2. Phase Kinetic Parameters of Clot Contraction in Stroke Patients and Healthy Subjects

Rate Constants	Healthy Subjects	Stroke Patients
Phase 1, 1/s	0.023±0.006	0.014±0.005
Phase 2, %/s	−0.073±0.002	−0.0210±0.0003*
Phase 3, 1/s	0.0021±0.0004	0.00029±0.00006*

*P<0.0001.

level of P-selectin expression in unstimulated cells (Table 3; Figure III in the online-only Data Supplement). This observation was confirmed by the frequently observed shape change and formation of filopodia in platelets from stroke patients (34%) compared with morphologically altered platelets from healthy subjects (9%, P<0.0001; Figure IV in the online-only Data Supplement). In response to TRAP-induced stimulation, platelets from the blood of ischemic stroke patients had a significantly lower expression of P-selectin compared with TRAP-activated normal platelets. TRAP-induced stimulation also resulted in reduced fibrinogen-binding capacity compared with the TRAP-activated normal platelets. Collectively, these results indicate that in ischemic stroke, platelets are initially partially activated and have a reduced responsiveness to a thrombin-like stimulus. It is noteworthy that the ratio of activated versus quiescent cells for both fluorescent markers was ≈2- to 4-fold higher in healthy donors compared with stroke patients, suggesting, in combination with the reduced exposure of P-selectin and fibrinogen-binding activity, that the stroke platelets have a substantially decreased overall activation potential. As a matter of fact, contraction in the group of stroke patients studied for platelet functionality was significantly impaired with an average extent of 28±7% versus 51±1% for control (P<0.0001).

Analysis of Clot Contraction Based on the Severity of Stroke

The National Institutes of Health Stroke Scale (NIHSS) is a validated clinical methodology to assess the severity of stroke in scale from 0 to 42, where <15 corresponds to a minor-to-moderate stroke and >15 corresponds to a moderate-to-severe or severe stroke. Modified Rankin scale measures rank the level of disability after stroke with 0 representing no symptoms and 5 representing severe disability.[21] The Alberta Stroke Program Early CT score value is inversely related to the area of damage as determined through a topographical evaluation of damage.[22] Within the cohort of patients in this study, the NIHSS value correlated strongly with modified Rankin scale score and inversely with Alberta Stroke Program Early CT score (Table V in the online-only Data Supplement).

The stroke patient population was divided based on NIHSS value to compare those with a value <15 to those with a value >15. We found that patients with more severe strokes (NIHSS >15) had a moderately but significantly increased extent of clot contraction, average velocity, area over the curve, and a reduced lag time when compared with patients with minor and moderate stroke (NIHSS <15; Figure 3). It is noteworthy that regardless of stroke severity the patients had a significantly reduced extent of clot contraction compared with healthy subjects, and neither subgroup of stroke patients differed significantly from the total stroke patient population (Tables VI and VII in the online-only Data Supplement). Hemostatic, hematologic, and biochemical parameters were compared for the populations of stroke patients divided based on NIHSS score (Table VII in the online-only Data Supplement). Patients with an NIHSS score >15 had a faster activated partial thromboplastin time and increased fibrinogen level, D-dimer level, platelet count, leukocyte count, and protein S-100 level.

Analysis of Clot Contraction Based on Pathogenesis of Stroke

To reveal whether the notable alterations in clot contraction in stroke patients had a pathogenesis-related significance, we investigated differences between subpopulations of stroke patients as defined by the TOAST classification (Trial of Org 10172 in Acute Stroke Treatment; Table 4), which allows for a distinction to be made between the following pathogeneses of ischemic stroke: (1) large-vessel atherosclerosis, (2) cardioembolism, 3) small-vessel occlusion, (4) stroke of other determined pathogenesis, and (5) stroke of undetermined pathogenesis.[23] Because of the small number of patients with TOAST subtypes 3 to 5, we preceded with analysis of clot contraction in patients with cardioembolic and atherothrombotic strokes. Assessment of extent of clot contraction based on stroke subtype revealed that there was a lower extent of clot contraction in patients with cardioembolic strokes when compared with patients with atherothrombotic strokes (Figure 4).

Table 3. Functional Characterization of Platelets Isolated From the Blood of Ischemic Stroke Patients and Healthy Donors Before and After Stimulation of the Cells With TRAP*

	P-Selectin Expression			Fibrinogen-Binding Capacity		
	Quiescent Platelets	TRAP-Activated	Activated/Quiescent Ratio	Quiescent Platelets	TRAP-Activated	Activated/Quiescent Ratio
Healthy donors (n=11)	3.3±0.8	83.6±3.7	≈25	1.0±0.3	58.3±5.6	≈58
Ischemic stroke patients (n=11)	9.9±5.1†	71.1±7.3‡	≈7	1.1±0.4	42.9±7.4§	≈39

TRAP indicates thrombin receptor–activating peptide.
*Numbers (mean±SEM) represent relative flow cytometry counts (%) for the fractions of platelets bearing fluorescently labeled fibrinogen or antibodies to P-selectin.
†P<0.05 between the donors and patients.
‡P<0.01 between the donors and patients.
§P<0.001 between the donors and patients.

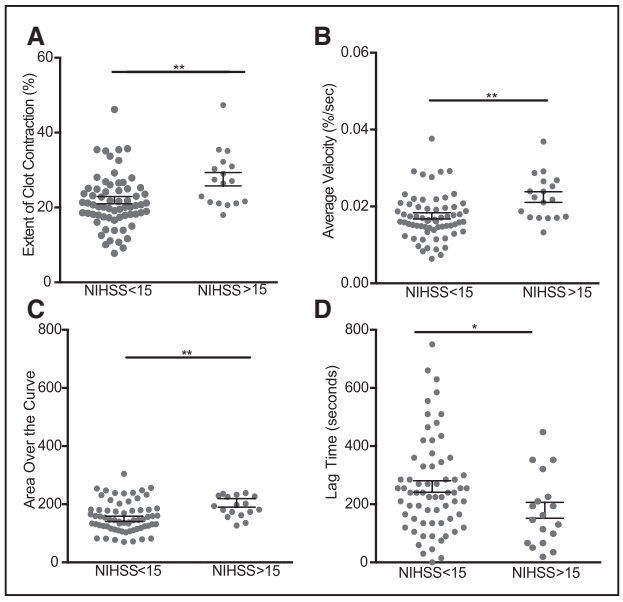

Figure 3. Clot contraction parameters in the blood of 2 groups of ischemic stroke patients segregated based on the National Institutes of Health Stroke Scale (NIHSS) values: <15 (minor-to-moderate stroke) and >15 (severe stroke). Optical tracking was used to assess differences in (**A**) extent of clot contraction, (**B**) average velocity of contraction, (**C**) area over the curve, and (**D**) lag time. Parameters for stroke patients were compared using an unpaired, 2-tailed Student *t* test. **P*<0.05, ***P*<0.01.

Discussion

Stroke is a leading cause of death and disability worldwide.[4] Although the pathogenesis of ischemic stroke is complex and heterogeneous, a common link between the different pathogeneses is increased activity of the coagulation and fibrinolytic systems[10] in conjunction with macro- and microvessel disruption[9] manifesting in thrombotic events. A thrombus or thrombotic embolus can result in blockage of blood flow to a portion of the brain; size and location of the thrombotic event influences stroke severity and functional outcome.[24] Brain tissue is a prominent source of tissue factor (TF),[25] and locations of brain damage are associated with high levels of TF and other procoagulant compounds,[26] which can intensify hypercoagulability and potentially lead to exhaustion of anticoagulant mechanisms and functional impairment of platelets.[27]

Information gleaned from in vitro and ex vivo studies of clot structure has been widely used to inform clinical findings.[28] Thrombi collected from ischemic stroke patients have a platelet–fibrin network interspersed with cells, and those rich in red blood cells (RBCs) are generally more susceptible to thrombolysis.[29] In ischemic stroke, the structure of thrombi is nonhomogenous, but, on average, RBCs comprise 34% of the volume,[30] which can influence the process of clot contraction.[18] Interestingly, fibrin clots formed in the blood from ischemic stroke patients had abnormal fibrin ultrastructure that could influence contraction of the fibrin–platelet network.[28] Collectively, this provides the need for an examination of clot contraction in samples collected from patients with ischemic stroke.

Blood clot contraction remains the least studied stage of blood clotting. This work is the first systematic assessment of clot contraction in ischemic stroke. The main finding of this work is that clots formed from the blood of patients with ischemic stroke have a reduced extent of clot contraction when compared with clots formed from the blood of healthy subjects (Figure 1). This result is somewhat unexpected as ischemic stroke is associated with a procoagulant activity,[9] but the aggregate of laboratory values collected from this cohort of ischemic stroke patients sheds some light on potential mechanisms for impaired clot contraction.

Platelets contain actomyosin machinery that generates contractile forces[31–33] that are propagated through the clot via

Table 4. Clinical Classification of Stroke Patients Enrolled in This Study

TOAST Classification				
	Cardioembolic	Large Artery Atherosclerotic	Small-Vessel Occlusion	Total Patients
Number of patients, n (%)	33 (39)	45 (54)	6 (7)	84 (100)
Male sex, n (%)	13 (39)	25 (56)	3 (50)	41 (48)
Age, y	69±2	68±1	60±4	68±1
Ischemic stroke classifications				
NIHSS score at the time of admission (median; 1st, 3rd quartile)	9; 4.5, 17	7; 3, 13	4; 3.5, 6	7; 4; 13.5
mRs score (median; 1st, 3rd quartile)	3; 2, 4.5	3; 2, 4	1; 0.75, 1.75	3; 2, 4
ASPECT score (median; 1st, 3rd quartile)	7; 3, 8	8; 3, 8	9; 9, 9	7; 3.5, 8.5
OCSP classification, n (%)				
TACI	9 (27)	10 (22)	0	19 (23)
PACI	20 (60)	21 (47)	1 (17)	42 (50)
LACI	4 (12)	13 (29)	2 (33)	19 (23)
POCI	0 (0)	1 (2)	3 (50)	4 (5)

ASPECT indicates Alberta Stroke Program Early CT; LACI, lacunar infarct; mRs, modified Rankin scale; NIHSS, National Institutes of Health Stroke Scale; OCSP, Oxfordshire Community Stroke Project; PACI, partial anterior circulation infarct; POCI, posterior circulation infarct; TACI, total anterior circulation infarct; and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

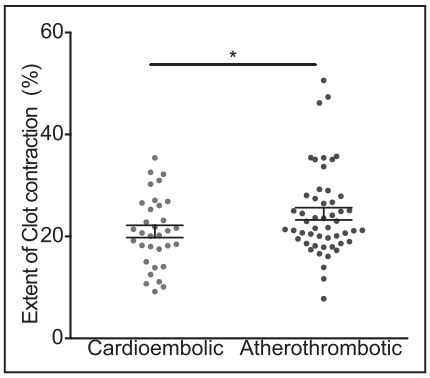

Figure 4. Clot contraction parameters in the blood of patients of clinical subpopulations of ischemic stroke as defined by TOAST classification (Trial of Org 10172 in Acute Stroke Treatment). The extent of clot contraction is reduced in patients with cardioembolic type strokes (n=33) compared with atherothrombotic type strokes (n=45). *P<0.05.

platelet–fibrin interactions.[34,35] Therefore, reduced contraction in stroke patients is likely because of reduced number and functionality of platelets (Table 1). We confirmed the mechanistic importance of the quantitative and qualitative changes in platelets for clot contraction through in vitro experiments with varying platelet counts (Figure IIA in the online-only Data Supplement) and through flow cytometry (Table 3; Figure III in the online-only Data Supplement) and scanning electron microscopy (Figure IV in the online-only Data Supplement) of platelets from stroke patients. On the basis of the surface expression of P-selectin, fibrinogen-binding capacity, and morphology, it has been found that platelets in the blood of stroke patients are initially activated and, more importantly, have a dramatically reduced capacity to respond to a thrombin-like activating stimulus. This is consistent with an earlier report that states that platelets from ischemic stroke patients are insensitive to thrombin in vitro,[27] which may result from cleavage of protease-activated receptors, metabolic exhaustion, or other mechanisms. Although a reduced platelet count in stroke is moderate (Table 1) and can have a relatively minor contribution to impaired clot contraction, it is platelet dysfunction that is likely to be the major pathogenic mechanism for reduced clot contraction observed in the blood of stroke patients. Disruption in both platelet number and function results in partial contractile inefficiency.

The prolonged lag phase observed in ischemic stroke (Figure 1) could be, in part, because of increase of antithrombin III activity after activation of the clotting cascade (Table 1), which inhibits thrombin and other clotting factors,[36] resulting in the slightly prolonged activated partial thromboplastin time observed in stroke patients (Table 1). Reduced thrombin activity strongly correlates with impaired clot contraction and could be a factor here despite addition of exogenous thrombin to initiate clotting.[18]

Increased fibrinogen level that can accompany ischemic stroke[37] (Table 1) can suppress clot contraction, which was confirmed in the in vitro experiments (Figure IIB in the online-only Data Supplement). Higher fibrinogen concentration results in increased fibrin mass and more fibers not associated with activated platelets/platelet aggregates, which can impede clot contraction.[12,18] In stroke patients, fibrin and RBCs can undergo oxidative and proteolytic changes that result in the formation of densely matted deposits of fibrin and RBCs, which have been implicated to play a role in persistent thrombi.[38,39] The abnormal fibrin structure associated with ischemic stroke[40–42] results in lower clot permeability and a slower rate of lysis.[42]

Contractile forces that are generated by platelets are able to compress RBCs tightly into the core of clots.[19] Compacted RBCs form a tessellated network and acquire a polyhedral shape, resulting in the terminology polyhedrocytes.[19] Polyhedrocytes have been found inside coronary thrombi extracted from patients with myocardial infarction,[43] confirming that intravascular clots undergo contraction. Increased hematocrit results in a reduced extent and rate of clot contraction,[18] and the higher hematocrit in stroke patients (Tables 1 and 2; Figure 2) is consistent with this inhibitory effect and could be additional mechanism of impaired contraction in stroke patients.

Based on this data analysis, we propose that collectively the reduced platelet count, increased hematocrit, and increased fibrinogen contribute to the impaired mechanism of clot contraction, which results in vessel occlusion and brain infarct. The compromised contraction of an intravascular clot or thrombus may play an important role in the pathogenesis of ischemic stroke. Although severity of stroke and infarct size are largely determined by the diameter and location of the occluded vessel, the ability of the thrombi to contract either more or less can aggravate or alleviate the course of ischemic brain damage. For example, if clot or thrombus contraction results in a 50% reduction in the vessel cross-sectional area, this would translate to a reduction of blood flow to 24% of that with no clot, whereas the impaired contraction seen in the stroke patients, resulting in an 80% reduction in the vessel cross-sectional area, would reduce the flow rate to 4% of the original.

Interestingly, clot contraction is also reduced in TIA similar to the reduction seen in ischemic stroke patients (Table III in the online-only Data Supplement), indicating that clot contraction is impaired in these patients. TIA is characterized as a focal neurological deficit that lasts <24 hours and, although ischemic in nature, does not result in infarction.[44] Patients with a recent TIA are at a high risk for the occurrence of an ischemic stroke,[45,46] and 15% of ischemic stroke patients presented with a previous TIA.[47] Our findings suggest that a premorbid status and predisposition to thrombosis, such as are seen in TIA and ischemic stroke, are critical determinants for the ability of clots to contract rather than a transitory episode of cerebral vascular occlusion and ischemia that may or may not result in persistent thrombosis or thromboembolism. Although rapid treatment of TIA has the potential to help reduce the incidence of ischemic stroke,[48,49] the TIA samples in our study were examined before the administration of any anticoagulant, thrombolytic, or antiplatelet drugs.

Our results show that despite an overall impairment of contraction in stroke patients, severe strokes had a relative increase in the extent of clot contraction compared with minor/moderate stroke (Figure 4). NIHSS score >15 also had a higher Protein S-100 level, a molecular marker of brain damage,

which is correlated with infarct volume, functional outcome, and ongoing ischemia.[50] We propose that in some cases of stroke with focal brain damage, the contractile activity of clots and thrombi can be improved as a potential compensatory mechanism to decrease the consequences of thrombosis and restore blood flow. In patients with severe ischemic brain damage, the blood–brain barrier is disrupted,[51] and massive amounts of TF are released into systemic circulation, resulting in activation of the extrinsic pathway of blood coagulation.[52–54] It has been shown that the level of brain damage is associated with the amount of TF that is released.[55,56] Also, ischemic stroke patients have increased TF-bearing microparticles[57] and TF expression on monocytes.[58]

We hypothesize that increased TF could potentially cause a secondary wave of thrombin generation, resulting in a faster activated partial thromboplastin time (Table VI in the online-only Data Supplement) and higher fibrinogen turnover in severe stroke patients.[59] Thrombin can induce highly active platelets in severe ischemic stroke patients, which can be determined through expression of P-selectin.[60,61] Platelet hyperactivity in combination with increased platelet count (Table VI in the online-only Data Supplement) could explain the increased extent of contraction in patients with more severe stroke compared with less severe stroke (Figure 3).

We propose a dual role of clot contraction in the ischemic stroke (Graphical Abstract in the online-only Data Supplement). Impaired clot contraction results because of changes in blood composition. This impaired clot contraction has the potential to be a pathogenic factor that aggravates the course of disease by increased blockage of blood flow. Enhanced clot contraction occurs in atherothrombotic patients compared with cardioembolic patients (Figure 4). This supports the idea that TF can potentially enhance contraction in subpopulations of stroke patients because atherothrombotic stroke patients have increased TF[62] and D-dimer, creating a thrombogenic state,[63] and increased P-selectin expression on platelets, indicative of hyperactivity.[64,65] Cardioembolic strokes are not a result of platelet hyperfunction but rather arise because of blood stasis.[66] This in conjunction with marginally enhanced clot contraction in severe strokes supports the notion that an increased thrombogenic state may result in the enhanced contraction observed here. This enhanced clot contraction has the potential to act as a compensatory or adaptive factor in ischemic stroke. However, this potential requires further examination.

In summary, the pathophysiological significance of clot contraction in thrombotic states has been explored. Clot contraction is associated with the restoration of blood flow past otherwise obstructive thrombi, potentially influencing the outcome of thrombosis.[17,19,20] However, compressed, dense thrombi are more impermeable and may be resistant to fibrinolysis. Consequently, clinical consequences of clot contraction in vivo may be ambiguous, resulting in either aggravation or relief of the thrombotic state. Our results reveal that further systematic studies of the clinical importance of clot contraction are worthwhile, as they could inform a novel pathogenic mechanism and result in potential laboratory tests that improve diagnosis of and prognosis in thrombotic conditions such as ischemic stroke.

Acknowledgments

We thank Dr Fazly Ataullakhanov (HemaCore, Ltd) for providing the Thrombodynamics Analyser System used in this study and Drs Timur Demin and Marat Saikhunov for providing clinical data. Scanning electron microscopy was performed at the Interdisciplinary Center for Analytical Microscopy of Kazan Federal University.

Sources of Funding

The work was supported by National Institutes of Health grants HL-090774, UO1HL116330, and T32 H10791, National Science Foundation grant DMR1505662, the Program for Competitive Growth at Kazan Federal University, and American Heart Association grant 16PRE30260002 (V. Tutwiler).

Disclosures

None.

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Highlights

- Blood clot contraction is an understudied aspect of blood clotting and thrombosis, and this work is the systematic study of clot contraction in a thrombotic state.
- Extent of clot contraction is reduced in the blood of ischemic stroke patients compared with healthy subjects.
- The clot contraction in ischemic stroke is likely weakened because of platelet dysfunction combined with altered blood composition.
- Impaired clot contraction may be an important pathogenic factor in thrombosis.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Contraction of Blood Clots Is Impaired in Acute Ischemic Stroke

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Arterioscler Thromb Vasc Biol. 2017;37:271-279; originally published online December 1, 2016;
doi: 10.1161/ATVBAHA.116.308622
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Methods and Materials

Contraction of Blood Clots is Impaired in Acute Ischemic Stroke

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Patients and inclusion criteria

Informed consent was obtained from patients suffering a recent stroke and healthy donors under the approval of the Ethical Committee of the Interregional Clinical Diagnostic Center (*ICDC, Kazan, Russia*) and in accordance with the Declaration of Helsinki. Informed consent was obtained from a legal representative if the stroke patients were physically and/or mentally incapable of giving consent. 96 patients with acute ischemic stroke (85 studied for clot contraction and 11 studied for platelet functionality) and 11 patients with transient ischemic attack (TIA) were enrolled within 12 hours after the onset of clinical symptoms prior to receiving any medication. Diagnosis of ischemic stroke or TIA was confirmed for all of the subjects based on clinical assessment and magnetic resonance imaging (MRI). Stroke and TIA patients were excluded from this study if by the time of examination they received anticoagulant, thrombolytic or antiplatelet drugs.

The average age in the stroke patients' main group (68±9 years, n=85) did not quite match the average age of the control group comprising healthy donors (58±13 years, n=79, M±SD). However, in a separate study it was shown that the extent and rate of clot contraction in a group of 16 elderly patients with an average age of 65±11 years did not differ from the younger healthy control subject used as a control in this study, but was significantly different from the ischemic stroke patients of the same age (Figure S1).

Acute ischemic stroke causal subtypes were defined using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) for all the patients. The NIH Stroke Scale (NIHSS) was used to measure stroke severity. Patients were also classified according to the Oxfordshire Community Stroke Project (OCSP). We used modified the Rankin Scale (mRS) to measure the degree of disability in patients before hospital discharge. Volume of damage was assessed using the Alberta Stroke Program Early CT score (ASPECTS). Clinical characteristics of the stroke patients can be found in Table 1.

Blood collection and sample processing

Venous blood samples were collected within 6 hours after the onset of stroke symptoms. Blood was drawn under aseptic conditions without venous stasis using vacutainers, stored at room temperature and analyzed within 4 hours for both stroke patients and healthy donors. Blood samples were collected into 3.2% trisodium citrate 9:1 by volume (S-Monovette tubes, Sarstedt, Germany). One blood sample was used directly to examine the blood clot contraction. Another citrated blood sample was centrifuged (1500g, 10 min) to obtain platelet-poor plasma (PPP) and used for blood coagulation tests. A third blood sample was stabilized with K_3-EDTA (1.6 mg/mL final concentration) and used for hematological tests. A non-stabilized whole blood sample was mixed with a clotting activator silicate (Sarstedt, Germany) and allowed to clot for 20-30 minutes at 37°C followed by centrifugation (2000g, 10 min) to obtain serum which was used for biochemical blood tests.

Coagulation, hematological, and biochemical tests

An automated coagulometer Sysmex CA-1500 (Sysmex, Canada) was used with fresh citrated plasma samples for the following tests: activated partial thromboplastin time (aPTT), prothrombin time, INR, fibrinogen concentration, concentrations of antithrombin III, plasminogen, protein C, and D-dimer concentration. Cell count was performed in EDTA-treated whole blood samples with an ABX Pentra 60 cell counter (hematology analyzer) (Horiba, Japan).

The following parameters were analyzed: erythrocyte count, mean corpuscular volume, hematocrit, hemoglobin, mean corpuscular hemoglobin, leukocyte count, monocyte count, neutrophil count, lymphocyte count, eosinophil count, basophil count, platelet count, and mean platelet volume .

Blood chemistry analyses were performed with RX Imola (Randox, UK) and Advia 1200 (Siemens, Germany) analyzers (Siemens, Germany). Serum concentrations of albumin, total serum protein, total bilirubin, glucose, alanine aminotransferase, aspartate aminotransferase, creatinine, urea, magnesium ion, sodium ion, and potassium ion were measured with RX Imola. Cholesterol, triglycerides, low density lipoprotein cholesterol, and high density lipoprotein cholesterol analyses were performed on Advia 1200. Serum S-100B protein concentration was assessed by immunoassay with Cobas e411 (Roche, Switzerland).

Continuous optical tracking of contracting blood clots in vitro

Optical tracking of contracting blood clots was performed using a novel automated assay[1] that was applied in clinical settings for the first time. Samples from patients and healthy donors were activated under standard conditions with 1 U/ml thrombin (Sigma-Aldrich, cat #T8885) and 2 mM $CaCl_2$ (final concentrations). Activated samples were quickly transferred to 12x7x1 mm transparent plastic cuvettes which were pre-coated with a residual layer of 4% Triton X-100 (Sigma-Aldrich, cat #90002-93-1) in phosphate buffered saline to prevent sticking of the clot to the chamber. Cuvettes were then added to the Thrombodynamics Analyzer System (Figure 1A) where a thermostatic chamber kept the samples at 37°C. Images of the clots were taken every 15 seconds for 20 minutes to track the changes in clot size based on the light scattering properties of the clot through the use of a light emitting diode and a CCD camera. Following data collection and computational processing, a kinetic curve (relative clot size vs. time) was plotted from the data and assessed for the following parameters: extent of clot contraction at 20 minutes, the lag time (time to reach 5% contraction), the average velocity of contraction, and the area over the curve (AOC) (Figure 1B). The AOC is an informative parameter that characterizes the intensity of entire process of clot contraction by integrating the extent and rate of contraction and the lag time.

Non-linear regression analysis of the kinetics of clot contraction

The kinetic curves of clot contraction were analyzed using a piecewise function in Prism GraphPad 6.0, as previously described.[1]

$$Relative\ Clot\ Size = \begin{cases} t_0 < t < t_1 & y_0 - (y_0 - y_1)(1 - e^{-k_1(t)}) \\ t_1 < t < t_2 & y_1 - k_2 t \\ t_2 < t < t_{end} & y_2 - (y_2 - y_3)(1 - e^{-k_3(t)}) \end{cases}$$

where t_1 and t_2 correspond to time points at which the first derivative of the curve reaches a local minimum or maximum; y_1 and y_2 correspond to the extent of contraction at these time points, whereas y_0 and y_3 correspond to the extent of contraction at the

beginning and end time points. Three phases of clot contraction have been recently revealed [1] and the rates and rate constants of contraction for each phase were determined through curve fitting with the piecewise function. This analysis allows for the abnormalities in clot contraction to be localized to a specific phase of contraction.

Effects of platelet count and fibrinogen concentration on clot contraction in vitro

To obtain samples with varying platelet counts, platelet-rich plasma was combined with platelet-poor plasma in increasing volume fractions to obtain the desired platelet concentration. To obtain samples with varying fibrinogen concentrations, isolated platelets were collected through centrifugation of platelet-rich plasma at 640g for 10 minutes in the presence of PGE_1 (1 µg/ml final concentration), they were washed and re-suspended in a modified Tyrode's buffer with increasing concentrations of purified human fibrinogen (Hyphen Biomed, France). Clot formation and contraction was initiated with 1 U/ml thrombin and 2 mM $CaCl_2$ (final concentrations) and the extent of contraction was measured after 20 minutes of incubation at 37°C. Three independent clot contraction experiments were performed for each experimental condition.

Platelet isolation by gel-filtration

To perform studies on isolated platelets from healthy subjects or ischemic stroke patients, citrated blood was centrifuged at room temperature at 200g for 10 min to obtain platelet-rich plasma (PRP). Platelets from PRP were isolated by gel filtration on Sepharose 2B (GE Healthcare, Sweden) equilibrated with Tyrode's buffer (4 mM HEPES, 135 mM NaCl, 2.7 mM KCl, 2.4 mM $MgCl_2$, 5.6 mM D-glucose, 3.3 mM NaH_2PO_4, 0.35 mg/ml bovine serum albumin, pH 7.4). Platelet count was performed in a hemocytometer with a 400× magnification. Isolated platelets were used within 3 hours after blood collection.

Flow cytometry of quiescent and activated platelets

Isolated platelets were studied before and after activation with thrombin-receptor activation peptide (TRAP) (Bachem Americas Inc., USA) added at 50 µM for 3 min at room temperature. To quantify expression of P-selectin and fibrinogen-binding capacity, platelets (200,000 in 50 µl) were incubated at room temperature for 10 min either with anti-human CD62P phycoerythrin-labeled murine antibodies (BD Biosciences, USA) (0.045 µg/ml final concentration) or with Alexa fluor 488-labeled human fibrinogen (5 µg/ml final concentration) (ThermoFisher Scientific, USA). After incubation with the labeled ligands, the cells were analyzed using FacsCalibur flow cytometer equipped with BD CellQuest™ software (BD Biosciences, USA). Platelets were gated based on the size and granularity using Forward Scatter (LFS) and Side Scatter (LSS) channels and 5,000 cells were counted in each sample. For detection of platelets bearing Alexa fluor 488-labeled fibrinogen and anti-CD62P PE-labeled antibodies we used two channels with green and yellow filters, respectively. To quantify expression of P-selectin and fibrinogen-binding capacity, platelets (200,000 in 50 µl) were incubated at room temperature for 10 min either with anti-human CD62P R-phycoerythrin-labeled murine antibodies (BD Biosciences, USA) (1.5 µl per sample) or with Alexa fluor 488-labeled human fibrinogen (5 µg/ml final concentration) (ThermoFisher Scientific, USA).

FlowJo X software was used for data analysis (Fig. S3). Paired samples of isolated platelets from 11 stroke patients and 11 healthy donors were analyzed in parallel.

Scanning electron microscopy of platelets

Isolated platelets (1,000,000 in 100 µl of phosphate buffered saline, pH 7.4) were fixed in a 2% glutaraldehyde solution (final concentration) for 90 minutes at room temperature. Fixed platelets were layered on a carbon filter (0.4 µm pore size) and centrifuged at 150g for 7 min. The samples were rinsed three times for 5 min with the phosphate buffered saline, dehydrated serially in 30, 50, 70, 50, 90, 95 vol% and three times with 100 vol% ethanol, then dried overnight with hexamethyldisilazane (HMDS). A thin film of gold-palladium was layered on the samples using a sputter coater (Quorum Q 150T ES, Quorum Technologies, UK). Micrographs were taken using a scanning electron microscope Merlin (Zeiss, Germany)(Fig. S4). Isolated platelets from 3 healthy donors and 6 stroke patients were prepared for scanning electron microscopy. Not less than 10 randomly selected images were analyzed for each platelets preparation and the total number of cells counted was 892 and 311 for stroke patients and healthy subjects, respectively.

Statistical analysis

All statistics were completed using Prism GraphPad 6.0. Samples were analyzed for statistical significance using two-tailed unpaired t-tests or a chi-square test with alpha=0.05 between control donors and stroke patients or between subtypes of stroke. A Pearson correlation test was used to determine the coefficients of correlation and significance of correlations for parametric data and a Spearman correlation was used for non-parametric data. All data is presented as mean±SEM unless indicated otherwise. Significance is represented as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

References

1. Tutwiler V, Litvinov RI, Lozhkin AP, Peshkova AD, Lebedeva T, Attaullakhanov FI, Spiller KL, Cines DB, Wiesel JW. Kinetics and mechanics of clot contraction are governed by the molecular and cellular composition of the blood. *Blood*. 2016;127(1):149-159.

Supplementary Material

Contraction of Blood Clots is Impaired in Acute Ischemic Stroke

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Supplemental Tables

Table SI. Correlation coefficients between clot contraction parameters in healthy donors

	Extent of contraction	*Lag time*	*Average velocity*	*Area over the curve*
Extent of contraction	-	0.011	0.968****	0.733****
Lag time	0.011	-	0.184	-0.298*
Average velocity	0.968****	0.184	-	0.641****
Area over the curve	0.733****	-0.298*	0.641****	-

Table SII. Correlation coefficients between clot contraction parameters in stroke patients

	Extent of contraction	*Lag time*	*Average Velocity*	*Area over the curve*
Extent of contraction	-	-0.516****	0.990****	0.687****
Lag time	-0.516****	-	-0.456****	-0.430***
Average velocity	0.990****	-0.456****	-	0.737***
Area over the curve	0.687****	-0.430****	0.737****	-

Table SIII. Clot contraction parameters in healthy subjects, patients with TIA, and stroke patients, total and based on NIH Stroke Scale

	Healthy Subjects (n=79)	*TIA (n=11)*	*Total Stroke (n=85)*	*NIHSS <15 (n=63)*	*NIHSS>15 (n=17)*
Extent of contraction, %	51±1	25±3	23±1	22±1	28±2
Lag time, sec.	74±5	226±43	243±17	260±20	179±27
Average velocity, %/sec.	0.041±0.001	0.020±0.003	0.019±0.001	0.018±0.001	0.022±0.001
AOC, a.u.	420±7	207±31	162±8	150±9	205±15

Table SIV. Correlation coefficients between hemostatic parameters and extent of clot contraction

Parameters	*Healthy subjects*	*Stroke patients*
aPTT	0.089	0.175
Prothrombin time	0.306**	0.049
Thrombin time	0.134	0.181
Fibrinogen	-0.073	-0.205
Platelet count	0.089	0.244*
Hematocrit	-0.253*	0.045

Table SV. Correlation coefficients between clinical end point parameters

	NIHSS	*mRs*	*ASPECTS*	*TOAST*	*OCSP*
NIHSS	-	0.68****	-0.69****	-0.20	-0.64****
mRs	0.68****	-	-0.46**	-0.19	-0.52****
ASPECTS	-0.69****	-0.46**	-	0.26	0.74****
TOAST	-0.2	-0.19	0.26	-	0.32**
OCSP	-0.64****	-0.52****	0.74****	0.32**	-

**P<0.01; ****P<0.0001

Table SVI. Significance for comparison of clot contraction parameters in healthy subjects and stroke patients based on NIH Stroke Scale

	NIHSS <15 vs. NIHSS >15	*NIHSS <15 vs. Healthy Subjects*	*NIHSS <15 vs. Total stroke*	*NIHSS >15 vs. Healthy Subjects*	*NIHSS >15 vs. Total stroke*	*Healthy vs. Total stroke*
Extent of contraction, %	*	****	ns	****	ns	****
Lag time, sec.	ns	****	ns	****	ns	****
Average velocity, %/sec.	*	****	ns	****	ns	****
AOC, a.u.	*	****	ns	****	ns	****

Table SVII. Comparison of laboratory parameters based on NIH Stroke Scale

Parameters (normal ranges are shown in parentheses)	*NIHSS <15 (n=63)*	*NIHSS >15 (n=17)*
Hemostatic Parameters		
aPTT (26-36), sec.	32.2±0.5	29.0±0.8**
Prothrombin ratio (70-130), sec	108.8±3.0	99.5±0.2
INR (0.85-1.15)	0.97±0.01	0.99±0.01
Fibrinogen (1.8-4.0), g/L	2.9±0.1	3.4±0.3*
Thrombin time (14-21), sec.	17.4±0.4	17.4±1.4
D-dimer (0-0.5), μg/ml	0.6±0.09	1.7±0.3***
Antithrombin III (80-120), %	91.4±6.0	100±8.4
Plasminogen (80-135), %	106.6±5.6	116.2±6.5
Maximal ADP-induced platelet aggregation (60-90), %	64.8±5.3	50.3±5.7
Rate of ADP-induced platelet aggregation (30-45), %/min.	23.8±2.5	19.8±3.3
Hematological Parameters		
Platelet count (180-320), $\times 10^9$/L	250±9	263±16**
Red blood cells (3-5), $\times 10^{12}$/L	4.6±0.08	4.4±0.16
Hematocrit (36-48), %	41.7±0.7	40.9±1.5
Hemoglobin (120-160), g/L	138.8±2.6	141.5±4.1
Color index (0.85-1.05)	0.92± 0.01	0.94±0.01
Mean cell volume (MCV) (80-100), fL	91.1±0.9	94.7±1.4
Mean cell hemoglobin (MCH) (30-35), pg	30.1±0.3	31.2±0.5
Leukocytes (4-9), $\times 10^9$/L	7.6±0.2	8.7±0.5*
Eosinophils (0.5-5), %	2.1± 0.1	1.7±0.2
Monocytes (3-11), %	8.2±0.3	7.8±0.7
Lymphocytes (19-37), %	23.2±1	23±2.2
Basophiles (0-1), %	0.49±0.02	0.45±0.05
Neutrophils (47-78), %	66±1.1	67±2.4
PMNLs (1.5-8),$\times 10^9$/L	4.9±0.2	6.1±0.5*
Erythrocyte sedimentation rate (ESR) (0-15), mm/hr	15.9±1.7	21.8±3.9
Biochemical Tests		
Triglycerides (<150), mg/dL	150±9	110±8*
Protein S-100 (<0.12), μg/L	0.09±0.1	0.18±0.05**
AST (10-36), U/L	22.7±1.1	28.1±3.2*
Glucose (70-100), mg/dL	122±3.6	140±10.8*

Supplemental Figures

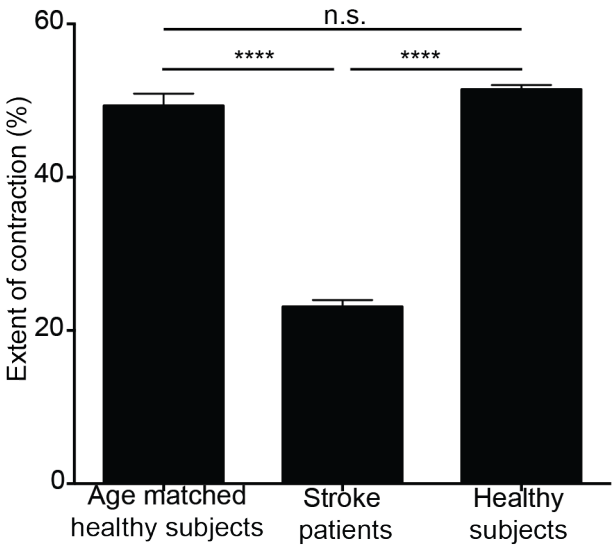


Figure SI. Clot contraction in age matched samples. Extent of clot contraction was compared for the healthy subjects included in this study (58±13 years, n=79), stroke patients (68±9 years, n=85), and a healthy subjects population age matched to the stroke patients (65±11 years, n=16). Parameters were compared using an ANOVA. ****P<0.0001

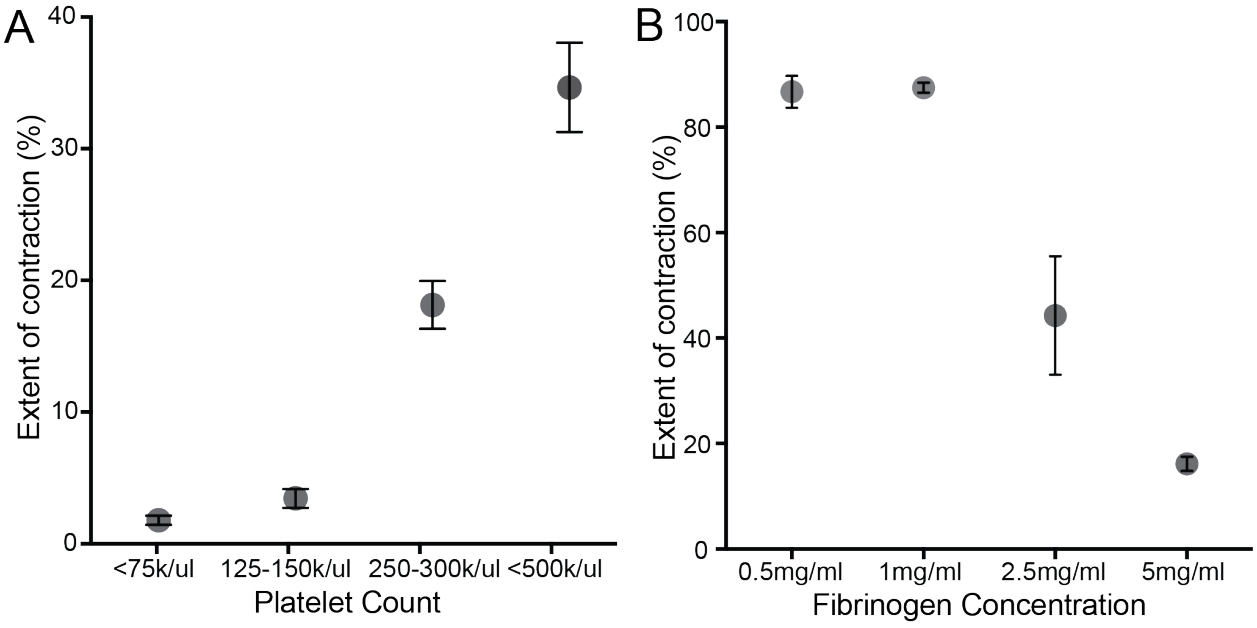


Figure SII. Effects of platelets (A) and fibrinogen (B) on clot contraction in vitro. Optical tracking was used to access the effects of platelets and fibrinogen across low, normal, and high values on the final extent of clot contraction in vitro. The results are averaged from 3 independent experiments for each experimental condition.

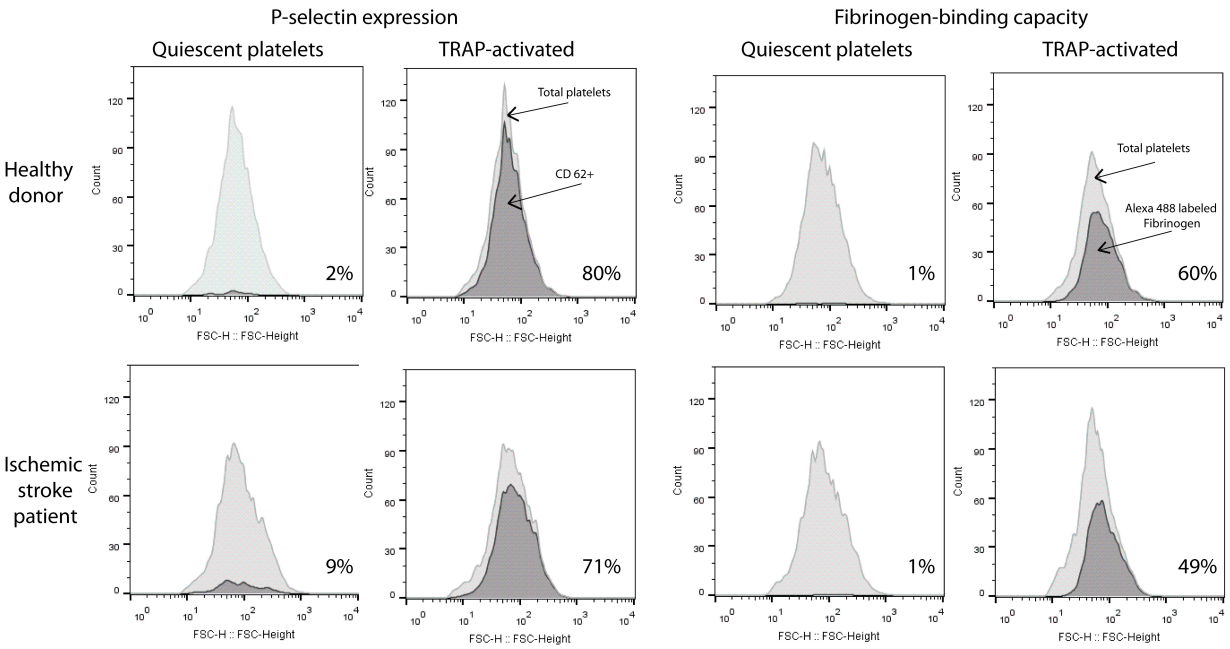


Figure SIII. Representative raw data from flow cytometry of platelets isolated from the blood of a healthy donor and an ischemic stroke patient under various experimental conditions. The platelets were incubated with either anti-human CD62 phycoerythrin-labeled antibodies or Alexa fluor 488-labeled human fibrinogen before and after activation with thrombin receptor-activating peptide (TRAP). Each plot represents the peak of counts for a fraction of fluorescing platelets superimposed on the peak of total platelet counts (around 5,000 total counts each). Numbers (%) represent a portion of the fluorescing platelets.

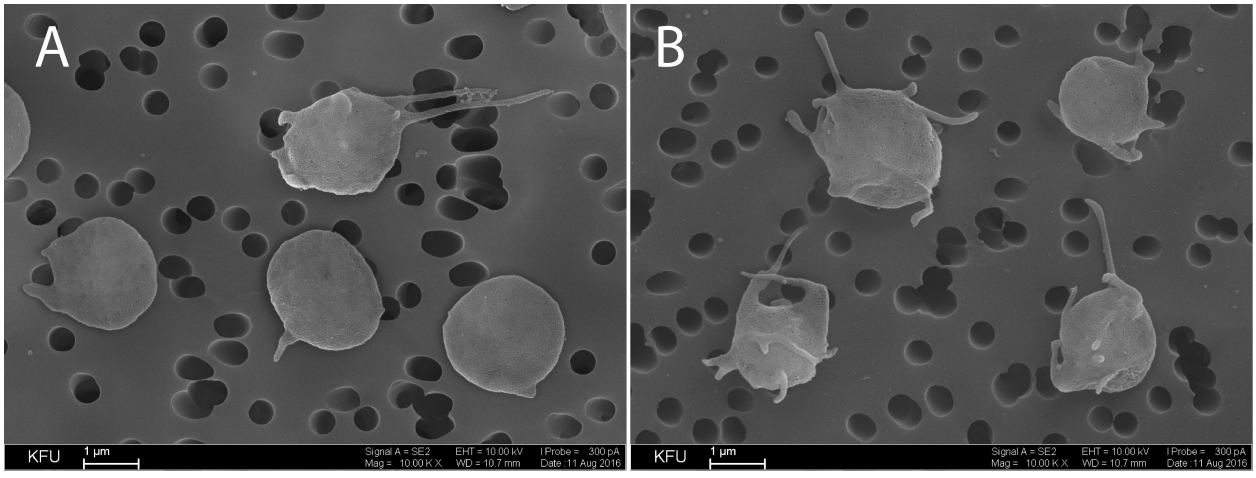
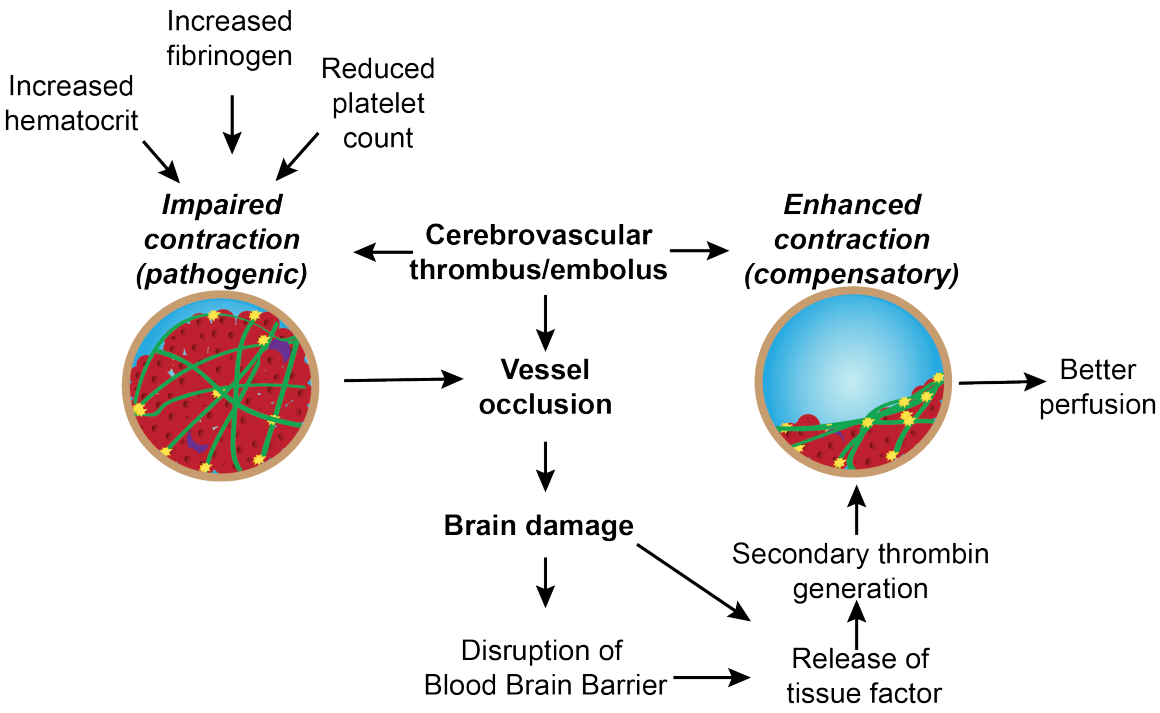


Figure SIV.Representative scanning electron micrographs of unstimulated platelets isolated from the blood of a healthy donor (**A**) and an ischemic stroke patient (**B**), showing a higher degree of spontaneous activation of the unstimulated (quiescent) platelets in ischemic stroke reflected by the shape change and formation of filopodia.



A hypothetical pathogenic role of clot contraction in ischemic stroke. The pathogenic basis of ischemic stroke is formation of a thrombus or thrombotic embolus blocking a brain vessel and causing focal brain damage**.** Contraction of the intravascular clot or thrombus is impaired in stroke patients due to platelet dysfunction and reduced platelet count, increased hematocrit, and increased fibrinogen. This has the potential to aggravate occlusion of a blood vessel and brain damage. In some cases, brain infarct results in the release of coagulation factors, such as tissue factor, which may trigger a secondary wave of thrombin formation, leading to an enhanced extent of clot contraction that may provide a compensatory mechanism to improve the cerebral blood flow.