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Dissociating mechanical from biochemical platelet activation: dimethyl sulfoxide modulates the platelet membrane to attenuate shear-mediated activation

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Abstract

Patients supported by implantable cardiovascular devices, including stents, prosthetic valves, and mechanical circulatory support systems, remain vulnerable to thromboembolic complications despite modern antithrombotic therapy. A central contributor is shear-mediated platelet activation, which differs in phenotype and pharmacologic sensitivity from activation induced by conventional biochemical agonists. Here we tested whether the platelet membrane can be perturbed to reduce shear-mediated activation, using dimethyl sulfoxide (DMSO) as a membrane-active probe. Gel-filtered platelets treated with DMSO were exposed to constant, dynamic ventricular-assist-device, and hyper-shear waveforms and assessed for thrombin generation, phosphatidylserine externalization, morphology, ultrastructure, and membrane probe anisotropy. DMSO at concentrations of 15% (v/v) or less reduced the measured shear-induced Platelet Activity State (PAS) across the tested shear regimes without overt membrane fragmentation under the tested conditions. Membrane anisotropy changed in a concentration-dependent, nonmonotonic pattern that coincided with the inhibitory concentration range. After shear exposure, DMSO-treated platelets retained a measurable arachidonic-acid-induced aggregatory response under the conditions tested. These findings provide functional evidence that shear-induced procoagulant activity can be attenuated while arachidonic-acid-induced activity remains detectable in the same platelet population, and they support membrane perturbation as a general strategy for studying, and potentially targeting, mechanically induced platelet responses, with membrane lipids such as cholesterol representing a plausible next class of fluidity-modulating agents.

Keywords: shear-mediated platelet activation; platelet membrane; mechanotransduction; membrane order; dimethyl sulfoxide; thrombosis

Introduction

Physical forces are fundamental to vascular physiology. In the circulation, blood flow imposes shear stress on the cells it carries, and altered shear is a well-recognized determinant of both thrombosis and hemostasis[1,2]. Physiologic shear helps maintain vascular homeostasis, but pathophysiologic shear, whether from vascular or valvular stenosis or from the supraphysiologic conditions generated by implanted mechanical circulatory support (MCS) devices, drives an increased risk of thrombosis[3,4].

Most prior work on shear-dependent platelet behavior has emphasized von Willebrand factor (vWF), including shear-dependent changes in vWF conformation and force-dependent binding to platelet receptors such as GPIb-alpha. However, platelet responses to free-flow mechanical loading also involve vWF-independent or adhesion-independent mechanisms, including mechanosensitive ion channels, extensional-strain sensing, cytoskeletal coupling, and changes in platelet membrane and receptor organization[4–8]. Accordingly, we use the term mechanical activation here to describe the functional platelet response to applied shear or related mechanical loading, without implying that a single membrane-intrinsic pathway accounts for all shear responses.

This distinction matters therapeutically. Conventional antiplatelet agents act primarily on biochemical pathways, including thromboxane, ADP-P2Y12, phosphodiesterase, and integrin signaling, and their effects under device-associated shear are strongly shear-regime dependent[9,10]. Aspirin reduces activation under some low-shear conditions but has limited efficacy under high or median device-derived shear[11]. A broader panel of clinically used agents showed similarly limited efficacy under the median DeBakey ventricular-assist-device waveform, although some agents retained activity under lower-shear conditions[10,12]. Thus, the most accurate statement is not that all

conventional agents fail under shear, but that their protection can be incomplete and dependent on the magnitude, loading history, and waveform of mechanical exposure.

Device thrombogenicity studies further show that reducing the platelet stress histories imposed by a mechanical circulatory support device can substantially decrease platelet activation, with reductions exceeding those observed with aspirin under comparable device-associated shear conditions[4,13–15]. Clinically, mechanical circulatory support is associated with a paradoxical coagulopathy involving both thrombosis and bleeding, and accumulated shear can reduce subsequent platelet aggregability through receptor downregulation and microparticle shedding[13,14]. These observations point to a mechanistic gap: device-generated forces can induce a procoagulant platelet phenotype while also impairing later platelet function[16,17].

This gap motivates a different target. The mechanical properties of the platelet, including membrane organization, cytoskeletal coupling, and membrane-associated mechanosensors, may influence sensitivity to applied force, and a membrane-directed perturbation could therefore serve as a probe of the mechanical arm of platelet activation. The membrane should not, however, be treated as an isolated sensor: force transmission plausibly depends on a coupled membrane, cytoskeletal, and protein system[8,18,19].

Dimethyl sulfoxide (DMSO) offers a well characterized chemical perturbation of membrane systems. Beyond its role as a solvent, DMSO is used as a cryoprotectant and permeabilizing agent, and molecular-simulation and model-membrane studies describe concentration-dependent changes in bilayer thickness, hydration, order, permeability, pore formation, and eventual disruption[20–25]. These studies are valuable mechanistic guides, but they do not establish that the same concentration regimes or molecular events occur in intact human platelets. DMSO also has

direct effects on platelet function, including aggregation and thrombin-related measurements, and these effects must be distinguished from membrane preservation[26–29].

Given evidence that platelets can sense supraphysiological mechanical loading through multiple membrane-associated pathways[6,8], we hypothesized that DMSO would alter platelet mechanical state and reduce shear-mediated activation while leaving at least one biochemical response measurable. We therefore examined the effects of DMSO on platelet morphology and ultrastructure, Platelet Activity State, phosphatidylserine externalization, shear-mediated activation under constant, dynamic, and hyper-shear conditions, membrane probe anisotropy, and arachidonic-acid-induced Platelet Activity State after shear exposure.

Results

DMSO preserves gross platelet morphology below overtly damaging concentrations

Platelet morphology assessed by scanning and transmission electron microscopy provided evidence of gross structural integrity versus overt activation or damage. By scanning electron microscopy, platelets exposed to DMSO at concentrations below 25% retained a rounded discoid appearance with visibly intact external membranes, without appreciable pseudopod formation, spreading, or microparticle shedding (Fig. 2A-G). Visible membrane damage became evident at 40% DMSO and above (Fig. 2H,I), where platelets underwent membrane fragmentation and disintegration. This morphology differed from the extended pseudopodia and flattened profile of sonication-activated platelets (Fig. 2J). Electron microscopy of this kind supports the absence of overt fragmentation; it does not by itself establish preserved membrane permeability, receptor function, cytoskeletal integrity, or procoagulant behavior. Intermediate DMSO concentrations produced limited subcellular ultrastructural changes, including an increased frequency of vacuolization, without overt fragmentation, so the inhibitory concentrations should not be regarded as structurally neutral.

Transmission electron microscopy allowed us to quantify internal ultrastructural change (Fig. 3). We assessed vacuolization by two complementary measures. The proportion of platelets containing vacuoles or dilated internal organelles was 27.4% in untreated controls and comparable at 1% DMSO (30.9%), then rose to a plateau at 5, 10, and 20% DMSO (58.7, 56.5, and 58.3%, respectively), and reached 100% at 40% DMSO, where extensive membrane disruption, blebbing, and fragmentation was apparent. The vacuolization area fraction, the percentage of platelet cross-sectional area occupied by electron-lucent vacuoles and dilated organelles, told a more restrained story: it remained near baseline from 0 through 20% DMSO (13.7, 11.7, 13.8, 15.9, and 10.6% at

0, 1, 5, 10, and 20% DMSO) and increased sharply only at 40% DMSO (47.8%). Thus, while intermediate DMSO concentrations increased the number of platelets showing some vacuolization, the extent of internal alteration per platelet stayed limited until concentrations reached the overtly damaging range. Detailed morphometric methods and additional images are provided in Supplementary Fig. S1 and Supplementary Table S1.

Low DMSO concentrations (below 5% v/v) do not increase resting platelet activation under static conditions

We next examined whether DMSO alters resting platelets in the absence of shear. Under static conditions, the measured Platelet Activity State did not increase at DMSO concentrations below 5% during the assay window (Fig. 4A). PAS increased between 10 and 40%, peaking near 20% DMSO, then declined at higher concentrations as platelet integrity was lost. Annexin V binding did not increase between 1 and 25% DMSO and rose at 40% (16.7%) and 60% (50.1%) (Fig. 4B). These findings define a concentration range in which the measured resting PAS and annexin V response remained near control values under the conditions tested; they do not exclude effects on aggregation or thrombin-related function reported at lower concentrations in other systems[27–29].

Constant and dynamic shear drive measured Platelet Activity State

To establish the activation baseline against which DMSO would be tested, gel-filtered platelets were exposed to constant (30 or 70 dynes/cm^2) or dynamic (50th-percentile DeBakey ventricular assist device) shear (Fig. 1) and sampled over time. Both regimens increased PAS progressively (Fig. 5). Relative to the 0-minute baseline, activation was significant by 2 minutes, reaching PAS

values of 0.10 ($p <= 0.01$), 0.42 ($p <= 0.0001$), and 0.22 ($p <= 0.01$) for constant 30 dynes/cm2, constant 70 dynes/cm^2, and dynamic shear, respectively. By 10 minutes, constant 70 dynes/cm^2 reached a PAS of 0.85 ($p <= 0.0001$) and dynamic shear reached 0.82 ($p <= 0.0001$), both higher than the 0.45 reached at constant 30 dynes/cm^2 ($p <= 0.001$). Constant high shear and dynamic device shear thus produced comparable end-point activation, while constant low shear produced substantially less, consistent with prior work showing that dynamic loading and shear-loading rate influence activation beyond stress magnitude and exposure time alone[31].

DMSO reduces measured shear-mediated Platelet Activity State across the tested shear regimes

DMSO pretreatment reduced the measured Platelet Activity State relative to untreated sheared controls under constant, dynamic device-derived, and hyper-shear conditions. Platelets pretreated with DMSO were exposed to constant and dynamic shear in the hemodynamic shearing device (HSD) and to hyper-shear (peak 100 to 1,000 dynes/cm^2) in the syringe-capillary platelet hammer, with hammer exposure matched for nominal stress accumulation (21,000 dynes*s/cm^2) to 5 minutes at 70 dynes/cm^2 in the HSD, while differing in peak stress, waveform, and exposure duration. Across each regime, DMSO at 15% or less reduced the response (Fig. 6). Under constant 70 dynes/cm^2, normalized PAS fell from 0.38 in the control to 0.08, 0.15, 0.13, 0.16, and 0.14 at 1, 3, 5, 10, and 15% DMSO, rising to 0.28 and 0.33 at 20 and 25% (Fig. 6A). Under constant 30 dynes/cm^2, PAS fell from 0.29 in the control to 0.17, 0.06, 0.02, and 0.10 at 1, 3, 5, and 10%, rising to 0.23 and 0.27 at 20 and 25% (Fig. 6A). Under dynamic device shear, PAS fell from 0.27 in the control to between 0.04 and 0.13 across 1 to 15%, rising to 0.20 and 0.22 at 20 and 25% (Fig. 6B). Under hyper-shear at 1,000 dynes/cm^2, PAS fell from 0.40 in the control to 0.30, 0.12, and 0.12 at 1, 3, and 5% DMSO (Fig. 6C). Above 15% DMSO, PAS values rose again but remained below

the untreated controls. The effect was therefore concentration-dependent and reproducible across the specific waveforms, exposure times, cumulative stresses, and PAS endpoint used here.

Phosphatidylserine (PS) externalization, detected by annexin V binding, is a marker of shear mediated platelet activation and a procoagulant platelet phenotype and typically increases under higher shear[33].

To test whether DMSO alters this response, annexin V binding was measured by flow cytometry after exposing untreated and DMSO-treated platelets to constant uniform shear (70 dynes/cm^2 for 10 minutes). DMSO did not produce a statistically significant change in the annexin V-positive fraction at any concentration tested: values were 83.70% at 5% DMSO and 81.54% at 10% DMSO versus 94.69% for the sheared control, returning toward control levels (90.73% and 94.66%) at 15% and 20% DMSO (Supplementary Fig. S4B). Under static conditions, annexin V binding was likewise unchanged across all DMSO concentrations (Supplementary Fig. S4A).

Platelet aggregation, a necessary step in clot formation, is another indicator of platelet reactivity and behavior. To understand platelet aggregation behavior upon treating them with DMSO, resting platelets in form of PRP were exposed to different DMSO concentrations (0-25% v/v), and their aggregatory behaviors were measured via Chrono-log aggregometer and in response to the following agonists: ADP (10 μM), collagen (2 μg/mL), arachidonic acid (AA) (0.5 mM), and ristocetin (1.25 mg/mL). DMSO-treated PRPs showed an overall decreased aggregation behavior in response to ADP and collagen, while the response to ristocetin was mostly preserved (Supplementary Figure S5). Full experimental details, representative traces, maximum aggregation amplitudes, lag times, number of donors, and statistical analyses are provided in Supplementary Figure S5 and its associated legend.

DMSO produces a nonmonotonic change in membrane probe anisotropy

To relate this functional effect to the membrane, we measured steady-state fluorescence anisotropy of the membrane probe TMA-DPH, a probe-specific readout of local membrane order and rotational mobility rather than a direct measure of whole-cell fluidity (Fig. 7).[32] At 1 to 5% DMSO, anisotropy remained near control values, with small numerical decreases at 3 to 5% (0.17, 0.16, and 0.16 at 1, 3, and 5% versus 0.17 for control). Anisotropy increased at 10 and 15% (0.21 at both; $p < 0.0001$) and returned toward control at 25% (0.18), producing a nonmonotonic, probe-specific concentration response. The concentration range over which anisotropy changed, roughly 3 to 15%, overlapped the range over which PAS was reduced (Fig. 6). This overlap is supportive but not mechanistic evidence: it is consistent with the hypothesis that a membrane mechanical perturbation can alter shear responsiveness, but it does not establish whether the anisotropy change is causal, whether decreases and increases act through the same mechanism, or whether DMSO acts on another membrane-associated or cytoskeletal target.

DMSO retains a measurable arachidonic-acid-induced Platelet Activity State response after shear exposure

We next asked whether DMSO-treated platelets retained a biochemical response after shear. Pretreatment with 5% DMSO reduced PAS under both constant shear (70 dynes/cm^2, HSD) and hyper-shear (800 dynes/cm^2, platelet hammer) at matched stress accumulation (Fig. 8A). When arachidonic acid (0.5 mM) was then added to these same hyper-sheared platelets, PAS increased substantially in both groups, from 0.53 to 0.89 in controls and from 0.06 to 0.85 in DMSO-treated platelets, with no significant difference between groups after agonist addition ($p = 0.875$) (Fig. 8B). Thus, under the conditions tested (0.5 mM arachidonic acid, the stated shear conditions, and

the assay window), DMSO-treated platelets retained a measurable arachidonic-acid-induced PAS response after shear exposure. The post-agonist responses were not statistically different, although formal equivalence testing was not performed. This is a functional dissociation within the measured assay system: shear-induced procoagulant activity was attenuated while arachidonic-acid-induced procoagulant activity remained detectable in the same platelet preparation. It does not establish that all mechanical and biochemical activation pathways are independent, nor that hemostatic function would be preserved in whole blood or *in vivo*. In parallel aggregometry, arachidonic-acid- and ristocetin-induced responses were not detectably reduced, whereas ADP- and collagen-induced responses were partially attenuated (Supplementary Fig. S5). Because formal equivalence testing was not performed, these responses are described as remaining measurable rather than fully preserved. Ristocetin is considered separately, as it tests the von Willebrand factor-GPIb axis and is not directly interchangeable with soluble agonists such as ADP, collagen, or arachidonic acid. Biochemical responsiveness is therefore agonist-dependent and should not be equated with preserved hemostatic function.

Discussion

Platelet activation and platelet damage are both important consequences of the supraphysiological forces generated by implantable cardiovascular devices. Prior work has demonstrated that shear-induced platelet responses differ from biochemical agonist responses in biomarker pattern and pharmacologic sensitivity[6,15,33]. Here, DMSO reduced the measured Platelet Activity State after constant, dynamic, and hyper-shear exposure while gross platelet morphology remained preserved and an arachidonic-acid-induced response remained detectable after shear. These results identify DMSO as a useful membrane-active probe for testing how mechanical perturbation changes platelet procoagulant behavior, rather than as a validated therapeutic agent.

The principal finding is a functional dissociation within the measured assay system: DMSO reduced shear-induced PAS but did not prevent a subsequent arachidonic-acid-induced PAS response. This supports the view that shear-induced and arachidonic-acid-induced procoagulant activity are not obligatorily coupled. It does not prove that all mechanical and biochemical activation pathways are independent, nor does it establish that normal hemostasis would be preserved in whole blood or *in vivo*. The result nonetheless motivates the possibility that a membrane-directed perturbation could complement, rather than simply replace, biochemically targeted antiplatelet therapy.

The agonist pattern observed here differs from the profile expected for pathway-selective antiplatelet agents. Aspirin suppresses cyclooxygenase-1-dependent thromboxane generation, whereas P2Y12 antagonists primarily inhibit ADP-dependent amplification, and effects on other agonists vary with concentration and assay conditions[34–37]. DMSO-treated platelets retained measurable responses to several agonists, although ADP- and collagen-induced aggregation was partially attenuated. This suggests that DMSO does not globally suppress platelet reactivity under

the tested conditions, but it does not establish therapeutic superiority, preserved hemostasis, or a membrane-specific mechanism; DMSO can affect receptors, ion channels, cytoskeletal coupling, osmotic state, and signaling, so the data support a membrane-associated perturbation rather than specifically a membrane-level mechanism.

Current antiplatelet drugs act on several biochemical pathways, including thromboxane, ADP-P2Y12, phosphodiesterase, and integrin signaling, and their effects under shear depend on the mechanical regime rather than being uniformly absent[12]. Aspirin retains measurable activity at some lower-shear conditions but is less effective at higher or median device-derived shear[10,11], and a broader panel of agents showed a similar pattern, with incomplete protection under median cfVAD shear[11,12]. The present results therefore support the possibility that a membrane-directed perturbation could complement biochemical antiplatelet therapy by acting on a step closer to where mechanical loading is sensed.

Nonmonotonic DMSO effects and membrane order

DMSO produced a nonmonotonic change in TMA-DPH anisotropy that coincided with the concentration range in which shear-induced PAS was reduced. Model-membrane simulations describe low-concentration bilayer thinning and changes in acyl-chain order, followed at higher concentrations by water defects, pore formation, lipid desorption, and bilayer disruption[21–25,38]. These models provide a plausible physical framework but should not be treated as direct evidence of pore formation or lipid desorption in the intact platelets studied here.

The concentration units require care in this comparison. Molecular-simulation papers generally report mole fraction or mol percent, whereas the present experiments report volume percent; these quantities are not interchangeable. In addition, the response of a complex platelet membrane depends on cholesterol, glycosphingolipids, membrane proteins, the cortical cytoskeleton, and the

open canalicular system, and DMSO effects on model membranes and erythrocytes can themselves be nonmonotonic and composition dependent[38,39]. The observation that 10 to 15% DMSO increased anisotropy while 3 to 5% slightly decreased it indicates that DMSO does not act as a simple one-directional fluidizer in this system. Anisotropy alone, however, cannot identify whether the relevant change is membrane fluidity, lipid packing, probe partitioning, membrane viscosity, membrane tension, or coupling to the underlying cytoskeleton. Future experiments should combine TMA-DPH with an independent probe or a direct mechanical assay and test whether washout reverses both anisotropy and shear sensitivity.

That TMA-DPH reported only a modest change is consistent with its localization near the headgroup region, which may make it sensitive to interfacial membrane packing; a core-localized probe might report a different magnitude[32,40,41]. The concentration-dependent, bidirectional behavior observed here is consistent with the idea that platelet shear responsiveness depends on a membrane mechanical state rather than on a single directional change in fluidity, while leaving the precise target unresolved.

The platelet membrane as part of a distributed mechanosensing system

Platelet stiffness and a rigid-body view of shear coupling

A complementary way to understand these findings is in terms of platelet mechanical compliance. The membrane and the platelet as a whole possess a measurable stiffness that governs how efficiently an external shear force couples into the cell. Using dielectrophoresis-mediated electrodeformation, we previously measured the stiffness of individual, minimally perturbed platelets and found a Young's modulus of approximately 3.5 kPa for resting platelets, rising to approximately 8.5 kPa when the platelets were stiffened by fixation; the accompanying modeling showed that treating the platelet as a rigid body substantially overestimates the mechanical stress

delivered to its membrane relative to a deformable model[70]. This motivates a simple rigid-body view of shear coupling: a stiff platelet subjected to shear couples efficiently to that force and experiences a larger transduced impact, whereas a more compliant, rubberized platelet deflects and dampens the same force, so that it is impacted but bounces off with reduced damaging effect. In this framework, any intervention that lowers platelet or membrane stiffness would be expected to raise the threshold for shear-mediated activation.

Membrane fluidization provides one route to this effect. Increasing bilayer fluidity is expected to lower the effective rigidity with which transmembrane proteins are held and to increase their lateral mobility, so that proteins acting as shear catch-and-transmit elements yield and reorient under load rather than rigidly transmitting or concentrating stress. The behavior is analogous to a flexible palm that bends in a strong lateral wind and springs back, versus a rigid trunk that resists, fractures, and is uprooted. Consistent with a direct mechanical effect, DMSO has been shown to alter the bending elasticity and shear modulus of both model membranes and human erythrocyte membranes in a concentration-dependent, and even biphasic, manner across the 1 to 10% range[71], paralleling the nonmonotonic anisotropy response observed here. DMSO may therefore act at two linked levels, changing the physical state of the bilayer and, through it, the stiffness of the platelet presented to shear. We did not measure platelet stiffness directly in this study, so this mechanistic chain remains a hypothesis to be tested; it is also consistent with the reduced efficacy of receptor-directed strategies under high shear, where progressive shedding of transmembrane receptors such as GPIb and integrin alphaIIb-beta3 limits approaches aimed at single receptors.

This stiffness-based interpretation unifies the present results with the broader behavior of membrane-directed interventions. Exogenous cholesterol, which changes membrane order and mechanics, was protective against shear-mediated activation, whereas fatty acids that sensitize the

membrane were not[69], indicating that the direction of the mechanical change, rather than lipid addition per se, determines the effect on shear responsiveness. Platelets also possess dedicated strain-sensing machinery that couples membrane deformation to activation[6], so the compliance of the membrane sets the input to these sensors. Taken together, the mechanical compliance of the platelet, set substantially by its membrane, is a modifiable determinant of shear-mediated activation.

A hallmark of platelet activation is the rapid stiffening and contraction of the actomyosin cytoskeleton that accompanies shape change and pseudopodia formation[72]. Because this transition occurs abruptly once an activation threshold is crossed, as it is under shear-mediated activation, the platelet behaves as though it sits near a mechanical tipping point between a resting and an activated state. Within the rigid-body view developed here, lowering platelet stiffness would move the platelet back from this tipping point into a safer operating regime in which platelet function is preserved but the tendency to activate in response to shear is reduced. On this reading, a membrane-directed or mechano-ceutical strategy does not globally suppress the platelet but shifts its operating point away from the shear-activation threshold.

The data are consistent with a model in which the platelet membrane participates in force sensing and signal transmission, but current evidence supports a coupled membrane, cytoskeletal, and protein system rather than a single distributed bilayer sensor. Platelets express mechanosensitive channels and show shear-dependent calcium entry that can be reduced by GsMTx-4[5], and extensional-strain studies further support adhesion-independent mechanical activation involving Piezo1-associated signaling, Pannexin1/P2X1 amplification, and cytoskeletal coupling[6,7,42]. Reviews of platelet mechanotransduction emphasize cross-talk among mechanosensitive receptors, calcium channels, the cortical cytoskeleton, and downstream signaling[8,18,19].

Membrane lipid composition and microdomain organization may influence these processes. Cholesterol-rich and sphingomyelin-rich platelet microdomains redistribute during activation and organize receptors and signaling proteins[8,43], and cholesterol depletion can reduce release of phosphatidylserine-exposing platelet extracellular vesicles without necessarily preventing phosphatidylserine exposure on the parent platelet[43–45]. These findings support a role for membrane organization in procoagulant output, but they do not establish that DMSO acts through cholesterol-dependent microdomains in the present experiments.

Beyond bulk membrane order, mechanotransduction may depend on the lateral organization and force-dependent behavior of membrane proteins[18,43]. GPIb-IX-V, GPVI, integrin alphaIIb-beta3, Piezo1, and cytoskeletal components may contribute to platelet responses under flow[8,46–48]. A membrane perturbation could therefore influence receptor mobility, microdomain organization, channel gating, cytoskeletal coupling, or membrane-protein force transmission[49]. The present data cannot distinguish among these mechanisms; the most defensible interpretation is that DMSO changes a membrane-associated physical state that correlates with reduced shear-induced PAS while leaving the tested arachidonic-acid response measurable.

DMSO and translational interpretation

DMSO is not a clinically selective antiplatelet agent on the basis of the present data. Prior work reports direct effects of DMSO on platelet aggregation, thrombin generation, membrane fluidity, and cryopreserved-platelet procoagulant activity[27–29,50–52]. The present experiments use acute exposure of gel-filtered platelets, whereas cryopreservation studies involve freezing, thawing, concentration, plasma reconstitution, vesiculation, and substantial membrane remodeling, and these systems should not be treated as equivalent. A recent whole-blood study reported that low-concentration DMSO can acutely promote red-blood-cell-dependent thrombosis under high-shear

flow, an effect not reproduced in isolated platelets or platelet-rich plasma[53]. Retention of biochemical responses in gel-filtered platelets therefore cannot be interpreted as clinical safety, because DMSO may produce distinct red-blood-cell-dependent effects in whole blood. The opposing responses observed in isolated platelets at higher DMSO concentrations and in whole blood at low DMSO concentrations emphasize that concentration, exposure time, red blood cells, and flow geometry strongly determine the phenotype, and these findings do not define a therapeutic DMSO window.

Membrane modulation as a class strategy: from DMSO to lipid-based agents

Membrane cholesterol is a plausible specific candidate for this membrane-directed approach. Cholesterol sets bilayer order, packing, and fluidity, and experimental and clinical studies have linked platelet cholesterol content or cholesterol-modifying treatment to changes in platelet reactivity, although the direction and magnitude depend on species, agonist, and method of lipid manipulation[54–57]. Statin studies provide clinical precedent that lipid-modifying drugs can alter platelet function, with lovastatin reported to normalize both elevated platelet membrane fluidity and hyperaggregation in hypercholesterolemic patients[56,59]; because statins have direct platelet actions beyond changing cholesterol, however, such studies do not establish platelet cholesterol as the selective mediator of shear responsiveness. In keeping with this framework, preliminary observations in our laboratory suggest that low-dose cholesterol modulation limits shear-mediated platelet activation[60,69], paralleling the effect of low-concentration DMSO reported here, a hypothesis that can be tested with controlled cholesterol depletion and repletion.

Taken together, DMSO should be viewed not as a therapeutic endpoint but as a proof of concept for a membrane-directed, or mechano-ceutical, strategy in which lipids such as cholesterol are titrated to raise the threshold for shear activation while retaining selected biochemical responses.

Defining the therapeutic window, agent selectivity, and behavior in plasma-containing and whole-blood systems for cholesterol and related lipid modulators is an important direction for future work.

Limitations

Several limitations should be emphasized. First, DMSO is a broad membrane-active perturbant rather than a selective mechanotransduction inhibitor, so its effects cannot be attributed to a single membrane target. Second, the study uses a limited biochemical challenge and a single PAS endpoint; preservation of an arachidonic-acid-induced PAS response should not be equated with preserved hemostasis, and additional assays testing ADP, thrombin, collagen-related agonists, TRAP, ristocetin and vWF-dependent responses, integrin activation, P-selectin exposure, aggregation, calcium signaling, and clot formation will be required to characterize the response more completely. Third, gel-filtered platelets omit plasma proteins, red blood cells, coagulation factors, and cellular interactions; whole-blood studies are especially important because DMSO interacts with red-cell membranes and because red cells alter platelet transport and shear exposure[53,61,62]. Fourth, the concentration-response experiments use different incubation durations across assays, which should be stated explicitly because DMSO effects may be time dependent. Fifth, the membrane mechanism remains inferential: TMA-DPH anisotropy provides a probe-specific readout of local membrane order, not a complete measure of membrane stiffness or fluidity, so the overlap between anisotropy changes and PAS inhibition is supportive rather than causal, and independent perturbations, washout studies, direct deformability or tension measurements, and rescue experiments are needed. Finally, several experiments have modest sample sizes; the principal effects appear large and internally consistent, but confirmation in larger donor cohorts with prespecified statistical models will strengthen the conclusions. At the highest

concentrations, DMSO also alters medium osmolality, and osmotic effects were not separately controlled; osmolality-matched controls will help distinguish membrane-specific from osmotic contributions.

This study shows that DMSO attenuates measured shear-induced Platelet Activity State in gel-filtered human platelets under defined constant, dynamic, and hyper-shear exposures. Under the tested conditions, selected biochemical agonist responses remained measurable, while TMA-DPH anisotropy changed nonmonotonically. These findings identify membrane perturbation as a useful experimental probe of shear-associated platelet procoagulant behavior, but they do not establish selective mechanotransduction inhibition, preserved hemostasis, or a therapeutic window. Larger donor studies, controlled cholesterol perturbation with rescue experiments, and validation in plasma-containing and whole-blood systems are required before membrane-directed agents can be considered therapeutic candidates.

Methods

Blood collection and platelet preparation

Blood was collected by venipuncture from healthy adult volunteers into anticoagulant citrate dextrose formula A (ACD-A, 10% v/v) under a University of Arizona Institutional Review Board-approved protocol (IRB #1810013264). All experiments were performed in accordance with relevant guidelines and regulations, and informed consent was obtained from all participants. Samples were centrifuged at 300 x g for 15 minutes to obtain platelet-rich plasma (PRP). PRP was utilized for aggregation studies outlined and for generation of gel-filtered platelets (GFP). For GFP, PRP was gel-filtered over a column of Sepharose 2B beads (Sigma-Aldrich) equilibrated with HEPES-modified Tyrode's buffer, pH 7.4 (Ca^{2+}-free, 0.1% fatty-acid-free bovine serum albumin)[63]. Gel-filtered platelets (GFP) were counted on a Z2 Particle Counter (Beckman Coulter) and diluted to the specified concentration. GFP were held at room temperature with occasional gentle mixing, and all experiments used GFP unless otherwise noted. Donors were healthy adults of both sexes who had not taken aspirin or other antiplatelet medications for at least 10 days before donation, and blood was processed within two hours of collection. Each experiment was performed on platelets from an individual donor (not pooled); the number of independent donors for each assay is given in the corresponding figure legend (typically n = 4 to 6). Exact male and female donor counts were not recorded at the time of the original experiments.

DMSO treatment

GFP (20,000 platelets/μL) were incubated for 10 minutes at 37 ℃ with DMSO (Sigma-Aldrich) at final concentrations of 1 to 60% (v/v) in HEPES-modified Tyrode's buffer, pH 7.4, and used immediately for shear exposure or activation assays. DMSO concentrations are reported on a volume/volume basis relative to the complete assay volume after all additions.

Exposure to constant and dynamic shear

GFP (20,000 platelets/μL) were exposed to uniform shear in the hemodynamic shearing device (HSD), a computer-controlled cone-and-plate/Couette viscometer described previously[31,64]. Constant profiles delivered a fixed amplitude (30 or 70 dynes/cm^2) with a reduced amplitude of 1 dyne/cm2 during sampling at 2, 5, and 10 minutes. The dynamic waveform was derived from the DeBakey ventricular assist device using the Device Thrombogenicity Emulator (DTE) methodology, in which computational fluid dynamics simulations capture platelet loading histories and trajectories through the device, and cumulative stresses are collapsed into probability density functions representing the device thrombogenic footprint[65]. A time-dependent waveform reconstructed from the 50th-percentile DeBakey device stress-loading history was extracted, scaled for the operating limits of the HSD, and looped at 110 cycles per minute with the same sampling pattern as the constant profiles (Fig. 1).

Exposure to hyper-shear (platelet hammer)

Hyper-shear was applied with the syringe-capillary shearing device (platelet hammer), a closed-loop recirculating syringe-capillary viscometer described previously (Sheriff et al., 2016)[66]. The device consisted of a Hamilton PSD/8 pump driving a 5 ml glass syringe connected to high-shear exposure tubing of internal diameter 0.366-0.8 mm. Platelets were ejected at controlled flow rates (up to 241 ml/min) to generate peak wall shear stresses of 100, 300, 600, 800, and 1,000 dynes/cm². Exposure time for each passage was defined as the dead volume of the tubing divided by the flow rate. Multiple passages were performed to achieve a constant cumulative exposure time of 3.33 s. Stress accumulation was calculated as the product of instantaneous shear stress and exposure time, yielding a cumulative stress of 21,000 dynes*s/cm² that was matched to 5 min at 70 dynes/cm² in the HSD. Between high-shear ejections, platelets were re-drawn into the syringe at low shear.

Platelet activation – Platelet Activity State Assay (PAS assay)

Platelet activation was measured with the Platelet Activity State (PAS) assay, a chromogenic prothrombinase-based assay in which acetylated prothrombin is converted to acetylated thrombin on the platelet membrane and detected with a chromogenic substrate; acetylation limits thrombin-mediated feedback activation[67]. Because prothrombinase assembles on negatively charged phospholipid surfaces, PAS is interpreted here as a measure of platelet-associated procoagulant activity rather than of every platelet activation pathway. Values for all sheared samples were normalized to a sonication positive control (10 s at approximately 10 W; Branson SLPt, Branson Ultrasonics). To confirm that DMSO did not interfere with the assay, control reactions containing DMSO at the same final concentrations but without platelets were run; no direct inhibition of the chromogenic substrate, acetylated thrombin, or the prothrombinase reaction was observed (Supplementary Fig. S3).

Platelet activation - Phosphatidylserine externalization by flow cytometry

Phosphatidylserine externalization was detected with annexin V-FITC, which binds phosphatidylserine with high affinity in the presence of Ca^{2+} [33,68]. Annexin V-FITC was added to samples at a 5:100 (v/v) ratio and incubated 30 minutes at room temperature, then fixed (4% paraformaldehyde with 0.1% BSA in PBS, 1:1 v/v) for 1 hour. Samples were analyzed on a FACSCanto II system (BD Biosciences), with activated platelets quantified as the annexin V-FITC-high population.

Scanning electron microscopy

Resting GFP (100 µL of 20,000 platelets/µL) were fixed in an equal volume of 2% (v/v) glutaraldehyde in HEPES-modified Tyrode's buffer, pH 7.4, on glass coverslips for 15 minutes, then washed through graded glutaraldehyde:water and dehydrated through graded ethanol:water and ethanol:hexamethyldisilazane series (5 minutes per stage, room temperature). Coverslips were air-dried overnight, sputter-coated with 4 nm gold in argon, and imaged on an Inspect F50 microscope (FEI).

Transmission electron microscopy and morphometry

GFP were incubated with DMSO for 30 minutes at 37 ℃, fixed (3% formaldehyde, 2.5% glutaraldehyde in phosphate buffer, pH 7.4) for 1 hour, and centrifuged (3,000 x g, 15 minutes). Pellets were washed twice in 0.2 M sodium cacodylate and stored at 4 ℃. Ultrathin sections (60 nm) were examined on an FEI Tecnai G2 Spirit BioTWIN at 80 kV with an AMT 2k CCD camera. Quantitative morphometry (proportion of platelets with vacuoles and vacuolization area fraction) was performed in ImageJ/Fiji 1.54p; details are given in Supplementary Fig. S1 and Table S1.

Membrane fluidity by fluorescence anisotropy

Membrane probe anisotropy was assessed by steady-state fluorescence anisotropy using 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), which localizes near the lipid-water interface and provides a probe-specific readout related to local membrane order and rotational mobility[32,40]. After DMSO treatment, platelets (100,000/uL) were incubated with 1 uM TMA-DPH at 37 ℃ for 5 minutes. Steady-state anisotropy was measured on a FilterMax F5 reader (Molecular Devices; excitation 360 nm, emission 430 nm) 10 minutes after probe addition and calculated as r = (I_para - I_perp) / (I_para + 2 x I_perp), where I_para and I_perp are the parallel and perpendicular emission intensities. DMSO was present during the optical measurement, and

anisotropy is reported as a probe-specific readout of local membrane order rather than an absolute membrane viscosity.

Platelet aggregation

Resting PRP and GFP were exposed to DMSO (0 to 25% v/v) and aggregation was measured with a Chrono-log aggregometer in response to ADP (10 μM), collagen (2 μg/mL), arachidonic acid (0.5 mM), and ristocetin (1.25 mg/mL). Representative traces, maximum aggregation amplitudes, lag times, donor numbers, and statistics are provided in Supplementary Fig. S5.

Statistical analysis

Analyses used Prism 8 (GraphPad). Normality was assessed by the Shapiro-Wilk test, which has limited power at small sample sizes; where n was small, effect sizes are emphasized alongside significance testing. Group comparisons used one-way or two-way ANOVA with Dunnett's multiple-comparisons correction against the specified control, or two-tailed t-tests where indicated; the specific test, number of independent donors, and comparison are stated in each figure legend. Data are presented as mean +/- SEM unless otherwise noted, and differences were considered significant at $p <= 0.05$, with exact p-values reported where available. The donor was the experimental unit for all functional assays; each independent donor contributed one value per condition. For the electron-microscopy morphometry, platelets within each micrograph were treated as technical replicates and the analysis is reported as qualitative and descriptive rather than as an independent biological comparison.

Figures

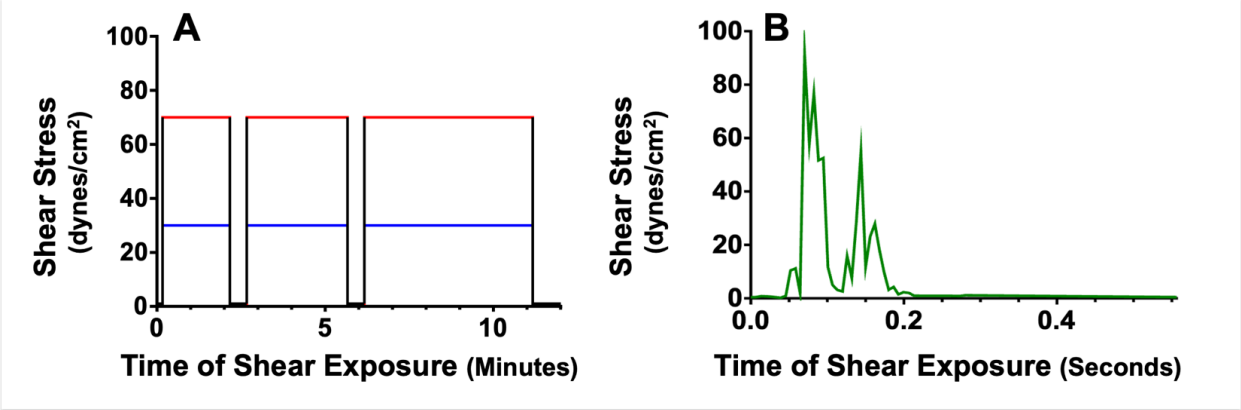


Figure 1. Constant and dynamic shear waveforms. Gel-filtered platelets were exposed for 0, 2, 5, or 10 minutes to (A) uniform constant shear at 30 dynes/cm^2 (blue) or 70 dynes/cm^2 (red), or (B) a repeating dynamic waveform (green) reconstructed from the 50th-percentile DeBakey device stress-loading history, looped at 110 cycles/min in the hemodynamic shearing device. Both waveforms included 1 dyne/cm^2 sampling intervals at each collection time.

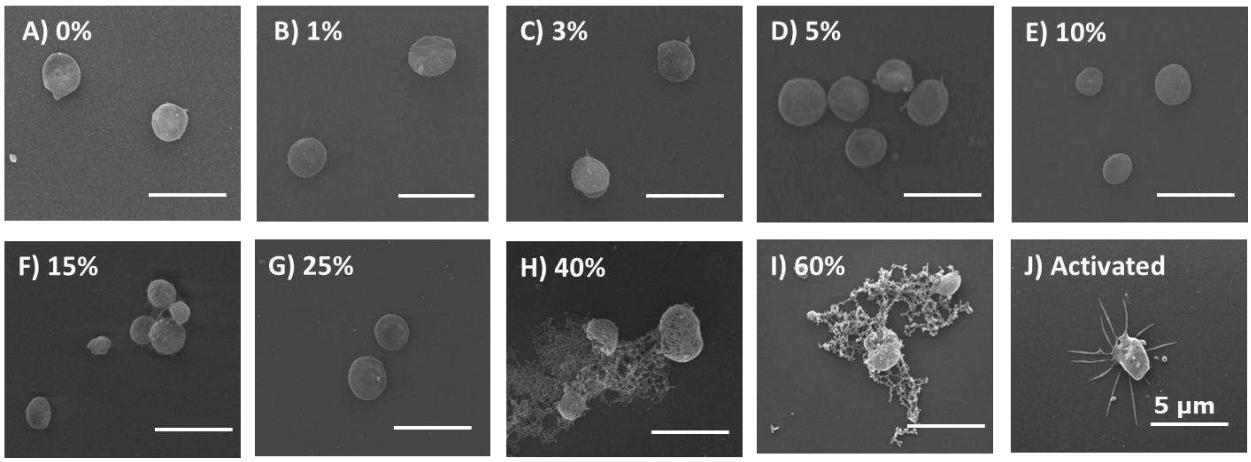


Figure 2. Scanning electron microscopy of DMSO-treated platelets. (A-I) Gel-filtered platelets exposed to the indicated DMSO concentrations (% v/v; 10 minutes, 37 ℃) and imaged by scanning electron microscopy; concentration is indicated at the top right of each panel. (J) Platelets after mechanical activation by sonication, shown for reference. Scale bars, 5 um.

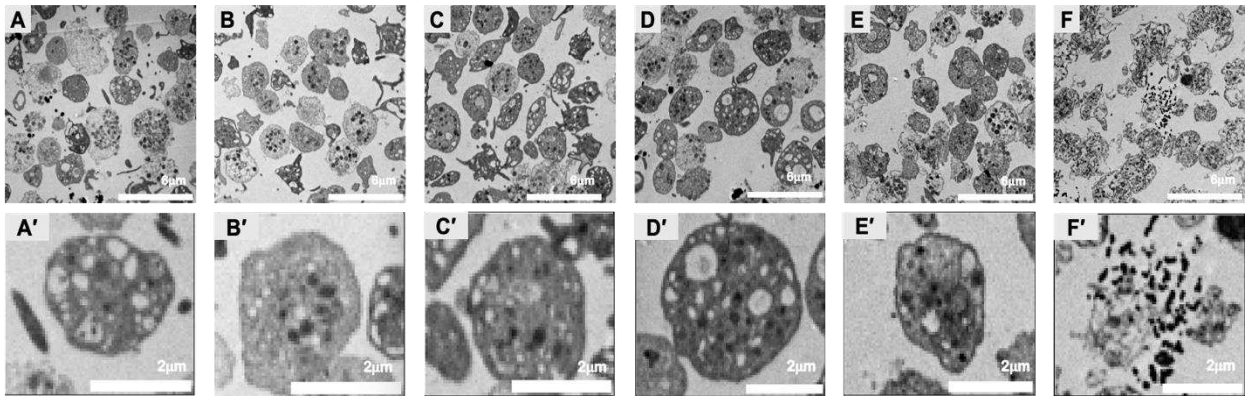


Figure 3. Transmission electron microscopy of platelet ultrastructure. Representative micrographs of gel-filtered platelets incubated with (A) 0%, (B) 1%, (C) 5%, (D) 10%, (E) 20%, and (F) 40% DMSO for 30 minutes at 37 ℃. Insets (A'-F') show higher-magnification views highlighting electron-lucent vacuoles and membrane features. Here, vacuolization increased with concentration whereas overt fragmentation was absent through 20% DMSO; at 40%, extensive vacuolization, membrane disruption, and fragmentation are evident. Scale bars, 6 um. Quantitative morphometry is provided in Supplementary Fig. S1 and Table S1.

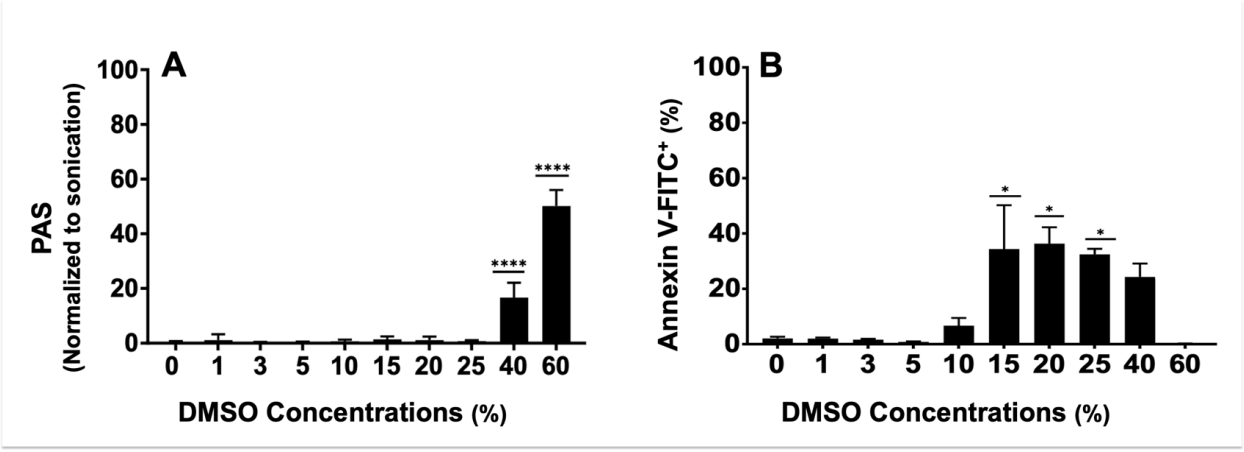


Figure 4. DMSO does not increase measured PAS or annexin V binding below 5% under the tested static conditions. (A) PAS-based platelet procoagulant activity in recalcified gel-filtered platelets after incubation with the indicated DMSO concentrations (% v/v; 10 minutes, 37 ℃). (B) Phosphatidylserine externalization measured as the annexin V-FITC-high population by flow cytometry. Values are mean +/- SEM (A, n = 6 donors; B, n = 6 donors; comparisons paired within donor). One-way ANOVA with Dunnett's test versus 0% control. **p <= 0.01; ****p < 0.0001.

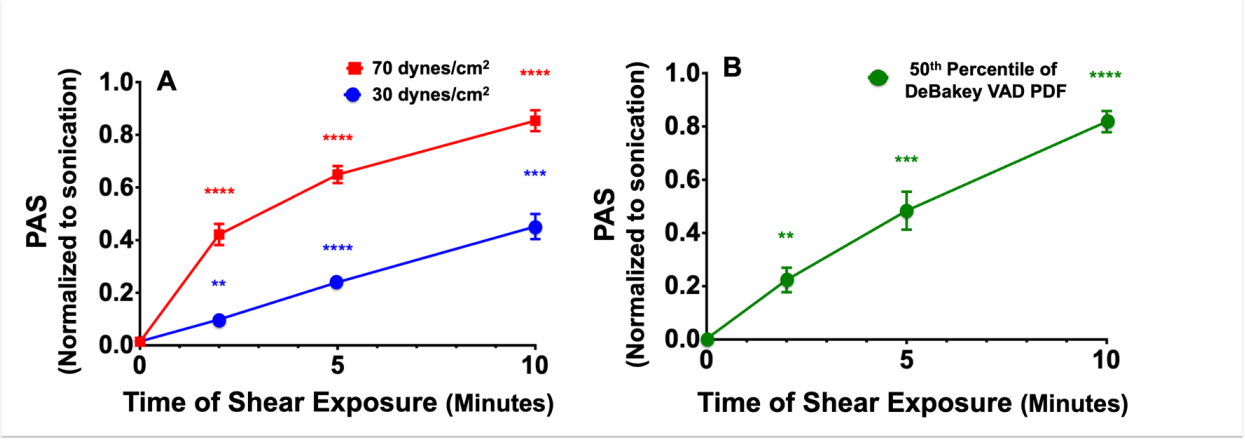


Figure 5. Shear-mediated platelet activation over time. PAS after (A) constant shear (30 or 70 dynes/cm^2) and (B) dynamic device shear, normalized to sonication. Values are mean +/- SEM (n = 6 donors for constant shear; n = 7 donors for dynamic shear). Two-way repeated-measures ANOVA with Dunnett's test versus 0-minute baseline for each condition. **p <= 0.01; ***p <= 0.001; ****p <= 0.0001.

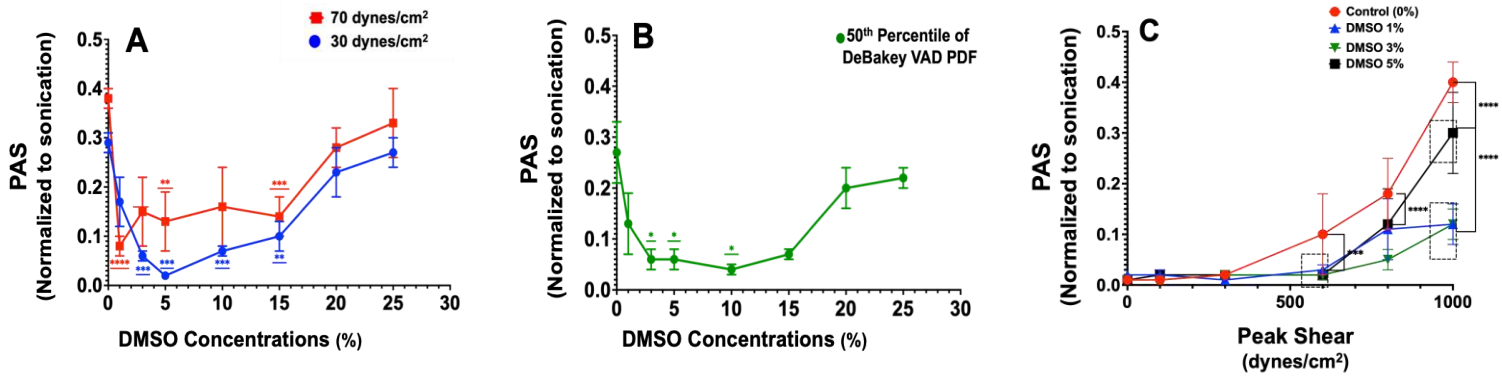


Figure 6. DMSO reduces measured shear-mediated Platelet Activity State across the tested shear regimes. Gel-filtered platelets were incubated with the indicated DMSO concentrations (% v/v; 5 minutes, 37 ℃) before shear. (A) Constant shear at 30 dynes/cm^2 (blue) or 70 dynes/cm^2 (red) and (B) dynamic device shear (green, 50th-percentile DeBakey profile, 110 cycles/min) in the hemodynamic shearing device for 5 minutes. (C) Hyper-shear at peak stresses of 100 to 1,000 dynes/cm^2 for 3.33 s total in the syringe-capillary platelet hammer (bottom panel). PAS normalized to sonication; values are mean ± SEM (n = 6 donors). One-way repeated-measures ANOVA with Dunnett's test versus control (comparisons paired within donor). *p < 0.05; **p <= 0.01; ***p <= 0.001; ****p <= 0.0001.

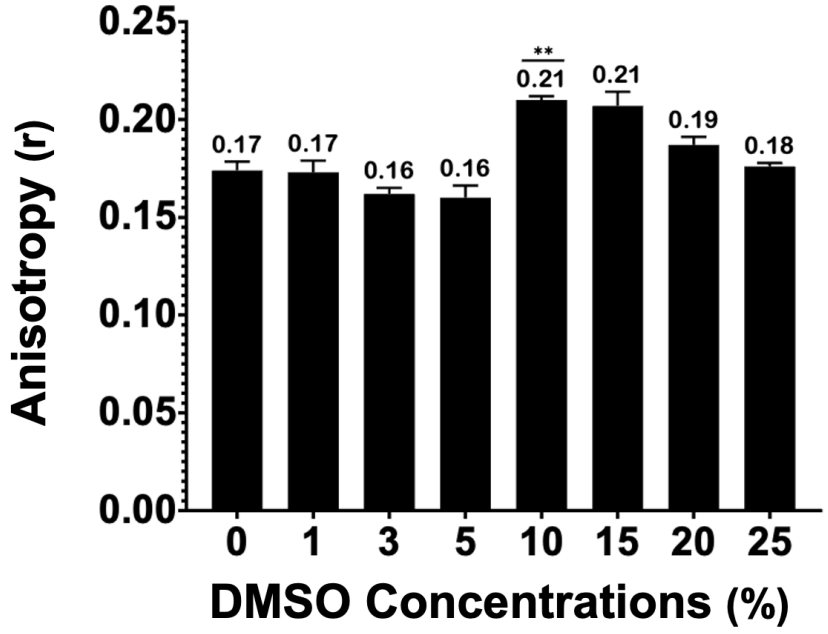


Figure 7. DMSO produces a nonmonotonic change in membrane probe anisotropy. Steady-state TMA-DPH fluorescence anisotropy (r), a probe-specific measure related to local membrane order and rotational mobility, in gel-filtered platelets after incubation with the indicated DMSO concentrations (% v/v; 10 minutes, 37 ℃). Values are mean ± SEM (n = 6 donors). One-way repeated-measures ANOVA with Dunnett's test versus 0% control (comparisons paired within donor). **p < 0.01.

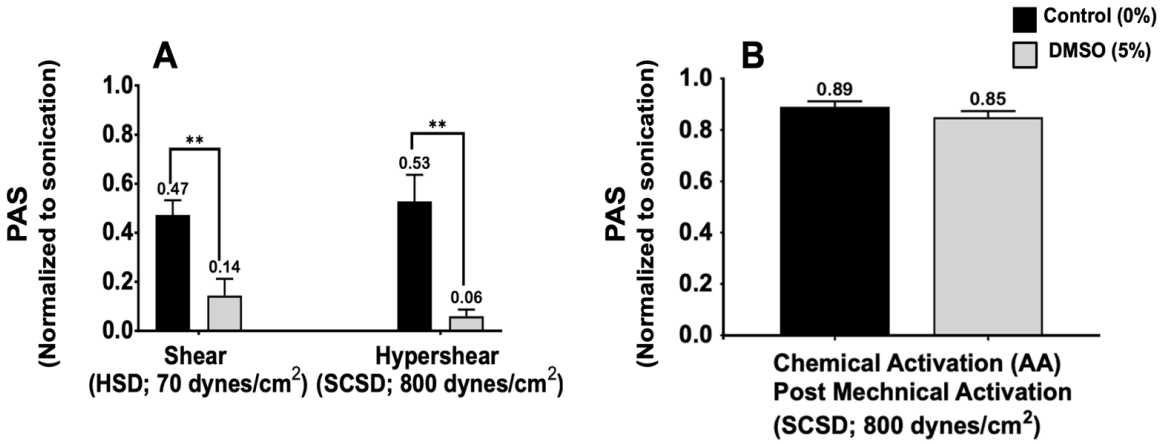


Figure 8. DMSO dissociates mechanical from biochemical platelet activation. Gel-filtered platelets were incubated with 5% DMSO (% v/v; 10 minutes, 37 ℃). (A) PAS after matched stress accumulation (21,000 dynes*s/cm^2) delivered by constant shear (HSD, 70 dynes/cm^2) or hyper-

shear (platelet hammer, 800 dynes/cm^2). (B) The same hyper-sheared platelets after biochemical activation with arachidonic acid (0.5 mM, 1 minute). Black bars, 0% DMSO control; gray bars, 5% DMSO. PAS normalized to sonication; values are mean +/- SEM (n = 4 donors). Paired two-tailed t-test. **$p < 0.01$ in (A); $p = 0.875$ in (B), not detectably different from control under the tested conditions; a $p = 0.875$ result does not establish equivalence.

Data availability

We confirm that we can honor the Data Availability Statement as written. All underlying datasets will be made available from the corresponding author upon reasonable request.

Author contributions

M.J.S., D.B., A.R. and P.L.T. conceived and designed the research. J.S. and P.L.T. fabricated the devices. S.S., P.L.T., L.V., J.T.Y., and C.E.O. conducted investigation and collected data. S.S., A.S., and P.L.T. analyzed data and prepared figures. S.S., A.S., P.L.T., K.R.M. and M.J.S. wrote and revised the manuscript. M.T.M. and C.A.A. contributed to membrane analysis and interpretation. J.E.I. conducted transmission electron microscopy and contributed to platelet biology interpretation. M.J.S. secured funding and provided overall project administration. All authors edited and approved the manuscript.

Declaration of Competing interests

None of the authors have any potential financial conflicts of interest related to this manuscript.

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Figure legends

Figure 1. Constant and dynamic shear waveforms. Gel-filtered platelets were exposed for 0, 2, 5, or 10 minutes to (A) uniform constant shear at 30 dynes/cm2 (blue) or 70 dynes/cm2 (red), or (B) a repeating dynamic waveform (green) reconstructed from the 50th-percentile DeBakey device stress-loading history, looped at 110 cycles/min in the hemodynamic shearing device. Both waveforms included 1 dyne/cm2 sampling intervals at each collection time.

Figure 2. Scanning electron microscopy of DMSO-treated platelets. (A-I) Gel-filtered platelets exposed to the indicated DMSO concentrations (% v/v; 10 minutes, 37 °C) and imaged by scanning electron microscopy; concentration is indicated at the top right of each panel. (J) Platelets after mechanical activation by sonication, shown for reference. Scale bars, 5 um.

Figure 3. Transmission electron microscopy of platelet ultrastructure. Representative micrographs of gel-filtered platelets incubated with (A) 0%, (B) 1%, (C) 5%, (D) 10%, (E) 20%, and (F) 40% DMSO for 30 minutes at 37 °C. Insets (A'-F') show higher-magnification views highlighting electron-lucent vacuoles and membrane features. Here, vacuolization increased with concentration whereas overt fragmentation was absent through 20% DMSO; at 40%, extensive vacuolization, membrane disruption, and fragmentation are evident. Scale bars, 6 um. Quantitative morphometry is provided in Supplementary Fig. S1 and Table S1.

Figure 4. DMSO does not increase measured PAS or annexin V binding below 5% under the tested static conditions. (A) PAS-based platelet procoagulant activity in recalcified gel-filtered platelets after incubation with the indicated DMSO concentrations (% v/v; 10 minutes, 37 °C). (B) Phosphatidylserine externalization measured as the annexin V-FITC-high population by flow cytometry. Values are mean +/- SEM (A, n = 6 donors; B, n = 6 donors; comparisons paired within donor). One-way ANOVA with Dunnett's test versus 0% control. **p <= 0.01; ****p < 0.0001.

Figure 5. Shear-mediated platelet activation over time. PAS after (A) constant shear (30 or 70 dynes/cm2) and (B) dynamic device shear, normalized to sonication. Values are mean +/- SEM (n = 6 donors for constant shear; n = 7 donors for dynamic shear). Two-way repeated-

measures ANOVA with Dunnett's test versus 0-minute baseline for each condition. **p <= 0.01; ***p <= 0.001; ****p <= 0.0001.

Figure 6. DMSO reduces measured shear-mediated Platelet Activity State across the tested shear regimes. Gel-filtered platelets were incubated with the indicated DMSO concentrations (% v/v; 5 minutes, 37 °C) before shear. (A) Constant shear at 30 dynes/cm2 (blue) or 70 dynes/cm2 (red) and (B) dynamic device shear (green, 50th-percentile DeBakey profile, 110 cycles/min) in the hemodynamic shearing device for 5 minutes. (C) Hyper-shear at peak stresses of 100 to 1,000 dynes/cm2 for 3.33 s total in the syringe-capillary platelet hammer (bottom panel). PAS normalized to sonication; values are mean ± SEM (n = 6 donors). One-way repeated-measures ANOVA with Dunnett's test versus control (comparisons paired within donor). *p < 0.05; **p <= 0.01; ***p <= 0.001; ****p <= 0.0001.

Figure 7. DMSO produces a nonmonotonic change in membrane probe anisotropy. Steady-state TMA-DPH fluorescence anisotropy (r), a probe-specific measure related to local membrane order and rotational mobility, in gel-filtered platelets after incubation with the indicated DMSO concentrations (% v/v; 10 minutes, 37 °C). Values are mean ± SEM (n = 6 donors). One-way repeated-measures ANOVA with Dunnett's test versus 0% control (comparisons paired within donor). **p < 0.01.

Figure 8. DMSO dissociates mechanical from biochemical platelet activation. Gel-filtered platelets were incubated with 5% DMSO (% v/v; 10 minutes, 37 °C). (A) PAS after matched stress accumulation (21,000 dynes*s/cm2) delivered by constant shear (HSD, 70 dynes/cm2) or hyper-shear (platelet hammer, 800 dynes/cm2). (B) The same hyper-sheared platelets after biochemical activation with arachidonic acid (0.5 mM, 1 minute). Black bars, 0% DMSO control; gray bars, 5% DMSO. PAS normalized to sonication; values are mean +/- SEM (n = 4 donors). Paired two-tailed t-test. **p < 0.01 in (A); p = 0.875 in (B), not detectably different from control under the tested conditions; a p = 0.875 result does not establish equivalence.

Supplementary Files

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