Increased Red Blood Cell Deformability Due to Isoxsuprine Administration Decreases Platelet Adherence in a Perfusion Chamber: A Double-Blind Cross-Over Study in Patients With Intermittent Claudication

By Piet A.M.M. Aarts, Jan Dirk Banga, Hans C. van Houwelingen, Robert M. Heethaar, and Jan J. Sixma

Platelet transport towards the vessel wall is influenced by the hematocrit, red blood cell (RBC) size, and shape. Recent in vitro studies have indicated that RBC deformability may also influence platelet transport. The observation that isoxsuprine, a known vasodilating drug, caused increased RBC deformability in vitro and decreased platelet transport in vitro prompted us to study the effects of this drug in vivo. The study was performed in a double-blind cross-over study of isoxsuprine vs placebo in ten patients with peripheral arterial insufficiency. RBC deformability was estimated from viscosity measurements using the blood viscosity equation of Dintenfass and expressed as $T$ value. Platelet transport was studied in an annular perfusion chamber according to Baumgartner. Human umbilical arteries were used as blood vessels. Perfusion studies were performed with whole blood or with RBCs of the patients mixed with normal platelets and plasma at a standardized hematocrit and platelet count. An increase in RBC deformability concomitant with a decrease in platelet adherence was observed in patients on isoxsuprine with a drop in $T$ value of $-0.06$ (from 0.91 toward 0.86), and a concomitant decrease in platelet adherence of 20% to 40%. These observations differed significantly from the results in the placebo group and showed a significant group-period interaction at the cross-over of medication (analysis of variance). The effects on platelet adherence were observed at high vessel wall shear rate ($1,800 \text{ s}^{-1}$) with perfusates consisting of patients’ RBCs and donor plasma and platelets at standardized hematocrit and platelet count. No differences were observed under these conditions at a shear rate of $300 \text{ s}^{-1}$. When whole blood of patients was used, nonsignificant effect was observed at shear rates of $300 \text{ s}^{-1}$ and $1,800 \text{ s}^{-1}$. This was probably caused by the added noise due to variations in hematocrit and platelet number. These data demonstrate that isoxsuprine increases RBC deformability, and they suggest the possibility of decreasing platelet-vessel wall interaction in vivo by manipulation of RBC deformability.

More recent studies have indicated that isoxsuprine may decrease blood viscosity. These effects were found in vitro and in vivo. Our objective was to extend our observations to the in vivo situation, and we achieved it by oral administration of isoxsuprine in a double-blind cross-over study of 12 patients suffering from intermittent claudication who were examined for five months. During the study, perfusion experiments were performed, and the patients were subjected to clinical examination and laboratory blood tests.

MATERIALS AND METHODS

Patients and clinical examinations. The study group consisted of 12 men, aged 50 to 72 years (mean $\pm$ SD 64 $\pm$ 6 years) with peripheral arterial insufficiency. Vascular surgery had been performed in two of them, vascular surgery combined with lumbar sympathectomy in one, and lumbar sympathectomy alone in two. All 12 men had stable intermittent claudication, and further vascular reconstructive surgery was not considered. All patients consented to cooperate in the study after being given detailed information. One of them died suddenly of a ruptured aneurysm three weeks after the start of the trial; another patient refused further participation in the study after three months because of upper abdominal discomfort. All patients were on oral anticoagulation with coumarin derivatives and continued to use this medication throughout the trial period.

The study period lasted 20 weeks and consisted of five episodes of four weeks. Clinical examinations including the completion of a standardized questionnaire and blood analysis were performed at the beginning and end of each episode. The first four weeks were considered a washout period in which all patients received placebo. The study period thereafter was divided in two periods of eight weeks. Each patient received either isoxsuprine or placebo during eight weeks and then switched to either placebo or isoxsuprine for the following eight weeks. Whether a patient first started with placebo and then isoxsuprine or the converse was selected at random. The medication consisted of identical capsules containing either 40 mg of isoxsuprine or placebo (courtesy of Duphar Nederland B.V., Amsterdam), taken orally two times daily throughout the study period. The testing procedure throughout the study period was as
follows: (a) measurement of brachial and ankle systolic blood pressure and determination of the ankle-arm pressure ratio (ankle-arm pressure index) at rest and after maximal walking distance; (b) the maximal walking distance was established on a treadmill using the modified Bruce protocol; (c) brachial and ankle systolic pressures and the pressure index were measured at one, three, five, and ten minutes after exercise.

The time schedule and the points at which clinical and laboratory examinations were performed are depicted in Fig 1 and were as follows: (a) treadmill tests and pressure measurements were performed the first and last day of the washout period and at the end of each eight-week period, during which the patient received either isoxsuprime or placebo according to a double-blinded randomized cross-over schedule (points 1, 2, 4, and 6); (b) laboratory tests were performed at the beginning and end of each four-week period (points 1 through 6).

**Viscosity measurements and determinations of RBC deformability from internal viscosity.** The viscosity of plasma, blood, and RBC suspensions was measured with a Couette type viscosimeter Contraves LS 30 (Contraves AG., Zurich) at a shear rate of 130 s⁻¹ as previously described. RBC deformability was determined according to Dintenfass. An empirical relation concerning the viscosity of blood and RBC suspension under Newtonian flow conditions was described by Dintenfass as:

\[
\mu_r = \mu_0 (1 - TH)^{-2.5}
\]

where \(\mu_r\) = viscosity of the suspension (mPas); \(\mu_0\) = viscosity of the suspending medium; \(H\) = hematocrit (vol/vol) and \(T\) is the so-called “Taylor factor,” a dimensionless parameter, related to RBC deformability. Increasing values of \(T\) indicate decreased RBC deformability (increased rigidity) and vice versa. The \(T\) value estimated by measurement of viscosity and \(H\) of a RBC suspension can thus be used as a parameter for RBC deformability. Prerequisites for the validity of this relation are normal human cells mean cell volume (mcv) between 85 and 100 fl and Newtonian behavior, or absence of RBC aggregation, which was achieved by measurement of RBC suspensions in buffer (absence of fibrinogen) at a shear rate of 130 s⁻¹. Differences in suspension viscosity caused by differences in RBC deformability were most pronounced at higher \(H\) values (\(H > 0.60\)); however, at \(H\) values >0.80, the couette flow is unstable. For this reason, we used suspensions of RBCs in isotonic saline (1 mL) with 0.60 < \(H\) < 0.80, at a uniform shear rate of 130 s⁻¹ (37 °C).

In a control experiment, using a Wells-Brookfield viscosity meter, a shear rate of 600 s⁻¹ was compared with the shear rate of 130 s⁻¹, used as standard for the clinical studies. The absolute value of \(T\) decreased from 0.91 ± 0.02 at 130 s⁻¹ to 0.80 ± 0.02 at 600 s⁻¹. The decrease by in vitro treatment with isoxsuprime was similar at both shear rates. Control experiments performed at a \(H\) of 0.40 demonstrated that the determination of \(T\) was independent of the RBC concentration.

**Platelet adherence experiments.** Platelet adherence studies were performed to find out whether changes in RBC deformability caused by drug treatment were large enough to affect platelet adherence and to have relevance for a thrombotic tendency.

Perfusion studies were carried out with the annular perfusion chamber of Baumgartner, using the steady flow system of Sakariasen. Arteries were isolated from the human umbilical cord (obtained immediately after birth), stored in buffer (0.2 mol/L of Tris, pH 7.4), and used within 24 hours, after a one-hour treatment with 0.1 mmol/L of aspirin to inhibit prostacyclin production by the vessel wall. Perusions were performed with whole blood of the patients and with recombined perfusates. These perfusates consisted of RBCs from the patient combined with plasma and platelets from a healthy donor with the same blood group. These two types of perfusion experiments were chosen so that we could discriminate between potential influences on platelet adherence: that of RBC deformability per se and that of action of isoxsuprime on platelets, plasma components, and subendothelium. Blood samples from patients and donors were drawn into one-ninth vol of 110 mmol/L of trisodium citrate. Platelet-rich plasma (PRP) was prepared from donor blood by centrifugation at 190 g for ten minutes (20 °C). The remaining blood was centrifuged at 3,000 g for 15 minutes (20 °C), to obtain platelet-poor plasma (PPP). The PRP and PPP were mixed to obtain standard PRP with 190,000 platelets per plasma. This standard PRP was mixed with washed, packed RBCs of the patients to obtain a standard \(H\) of 0.4. This standard \(H\) and platelet count was similar to those in previous studies. Packed and washed RBCs of the patients were obtained by centrifugation of patients’ whole blood for ten minutes at 3,000 g (20 °C). PPP was then removed, and two additional washes with isotonic saline containing 5 mmol/L of glucose were performed. The small buffy coat on top was removed carefully each time. Packing was done at 3,000 g for 15 minutes (20 °C).

Perusions were performed at vessel wall shear rates of 300 s⁻¹ and 1,800 s⁻¹. A vessel wall shear rate of 300 s⁻¹ was obtained with a perfusion chamber with a diameter of 6.1 mm (effective annular width 1.05 mm) at a flow rate of 35 mL/min. To obtain a vessel wall shear rate of 1,800 s⁻¹, a perfusion chamber with a diameter of 5.0 mm (effective annular width 0.60 mm) and a flow rate of 100 mL/min was used. Wall shear rates were calculated from flow equations of a Newtonian fluid through an annular slit similarly to calculations in our previous experiments. Recently, we used Laser-Doppler velocimetry to measure velocity profiles in the annular chamber from which vessel wall shear rates could be determined experimentally. This study showed that measured velocity profiles and derived values for wall shear rate agreed fully with theory. Perusions were performed at 37 °C for five minutes after prewarming at 37 °C for five minutes.

After perfusion, the vessel segments were fixed in 0.2 mol/L of phosphate buffer containing 2.5% glutaraldehyde and 2% osmium tetroxide and embedded in epon after dehydration as described by Baumgartner. Thin cross-sections, 1 μm thick, were cut on an ultratome (type 8800, LKB, Bromma, Sweden) and stained with methylene blue and basic Fuchsin (Merck, Darmstad, F.R.G.). Platelet adherence to the subendothelium was quantified by morphometry, based on counting 1,000 intersection points at a 10-μm distance, using a light microscope (Leitz-Wetzlar dialux 20 EB, E. Leitz GmbH, Wetzlar, F.R.G.) at 1,000× magnification according to Baumgartner. Platelet interaction was subdivided into contact platelets, spread platelets, or aggregates (>10 μm high). Total platelet adherence was expressed as percentage of coverage.

**Laboratory tests.** On each test day, a set of routine laboratory tests were performed. Five milliliters of whole blood, anticoagulated
with 2 mmol/L of potassium EDTA, was collected for determination of the hematocrit, mcv, hemoglobin concentration (Hb), leukocyte count (Coulter-counter, Model-S, Harpenden, England), and platelet count (Platelet Analyzer 810, Baker Diagnostics Ltd, Bethlehem).

Levels of β-thromboglobulin (β-TG) and platelet factor 4 in the plasma of the patients were determined with a β-TG radioimmunoassay (Amersham International plc, Amersham, England) and a PF-4 radioimmunoassay kit (Abbott Laboratories, North Chicago), respectively. Platelet aggregation by ADP was determined in a Payton Dual Channel Aggregation Module (Payton Associates, Inc, Scarborough, Canada) with 2.5 μmol/L and 5.0 μmol/L ADP (American Dade, Miami). Bleeding time was determined with Simplate-II device (General Diagnostics, Morris Plains, NJ).

Statistical analysis. All variables determined during the course of the study were tested. As a routine check, t tests between the groups (placebo-ixoxsuprine and isoxsuprine-placebo) were performed at successive test points. However, the individual t tests at respective test points were not related to each other and were not adequate to detect significant effects of the medication during the time course of the study. A more suitable method to test for correlation of the variables with the medication schedule is analysis of variance in repeated measures, which was computed with a BMD 4V-program. Only the data of points 3, 4, 5, and 6 were used, because points 1 and 2 gave no information about medication effect. Differences between isoxsuprine and placebo should result in a significant group-period interaction. Moreover, the signs of the interaction effect should be in accordance with the cross-over after point 4 (it should be either + + or − − + +).

Correlation between variables was computed in two different ways. First, all 60 data points (ten patients at six points) were used to construct scatter diagrams and to compute and test the correlation coefficient. The disadvantage of this method is that correlation may be due to either patient or treatment effect. To eliminate the patient effect, we computed the measure of decrease in time of a variable X at successive test points. However, the individual test at each point i, which yielded one figure per patient. A test for correlation between two parameters, X and Y, was performed by analysis of covariance, yielding information about whether correlation was due to the treatment or was also present within the two separate groups.

RESULTS

Viscosity measurements. Whole blood viscosity, plasma viscosity, and RBC deformability expressed as the mean value (±SD) for both groups during the course of the study are presented in Fig 2. RBC deformability showed significant changes congruent with the medication schedule. In addition, a significant group-period interaction was found by analysis of variance (P = .006). No significance was found for whole blood viscosity by BMD P4V; however, at test point 6, a significant difference (t test) occurred between isoxsuprine and placebo group (P = .005). However, when whole blood viscosity was normalized to a standard viscosity (a H of 0.4), a significant group-period interaction with a P value of .015 was found using equation 1. This is shown in Table 1. Plasma viscosity was constant and equal in both groups during the study.

Platelet adherence. The data on platelet adherence obtained in the various types of perfusion experiments during the course of treatment are listed in Table 2. A treatment effect with significant group-period interaction (P = .017) was seen in the perfusion experiments in which the RBCs of the patients were supplemented with plasma and platelets of normals at a wall shear rate of 1,800 s⁻¹ (Fig 3). The platelet adherence in these experiments was significantly lower with the RBCs of patients who used the active drug. In the other types of perfusion experiments, no significant effect of medication was found.

In the perfusion experiments with whole blood from the patients at a wall shear rate of 1,800 s⁻¹, a large proportion of the vessel surface was covered with platelet aggregates (see

<table>
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<tr>
<th>Table 1. Normalized Whole Blood Viscosity</th>
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<tr>
<td>Test Point</td>
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<td>1</td>
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<tr>
<td>2</td>
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<td>3</td>
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<td>6</td>
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Whole blood viscosity was normalized from the actual hematocrit to a standard value of 0.4 using eq. 1: μ = μ₀ (1−TH)⁻³ᵃ. Significance for group-period interaction: P = .016.
numbers in parentheses, Table 2) which, for unexplained reasons, decreased during the course of observation. An effect of isoxsuprine on aggregation formation cannot be ruled out completely; however, this was not significant because the placebo group also showed a decrease in aggregate formation. Isoxsuprine should then also work for at least eight weeks after cessation of administration, which seems ruled out completely; however, this was not significant for experiments with whole blood at wall shear rate of 1,800 s⁻¹, which yielded a set of 60 points (10 patients x 6 test points) for each type of perfusion experiment. Both variables were significantly correlated in three of four cases (recombined perfusates 1,800 s⁻¹ and 300 s⁻¹, and whole blood 1,800 s⁻¹) and not significant for experiments with whole blood at wall shear rate of 300 s⁻¹. The level of significance was highest for recombinant perfusates 1,800 s⁻¹ and 300 s⁻¹, and whole blood 1,800 s⁻¹ and not significant for experiments with whole blood at wall shear rate of 300 s⁻¹. The results obtained in all patients are shown in Fig. 4. The points in the graph are clearly separated into two groups, which indicates that the apparent relation between platelet adherence and RBC deformability is an effect of the medication (P < .011). Correlations within the groups were absent. These tests were not applied to the other type of perfusion experiments. We also tested for correlations by plotting all available values for platelet adherence v T, which yielded a set of 60 points (10 patients x 6 test points) for each type of perfusion experiment. Both variables were significantly correlated in three of four cases (recombined perfusates 1,800 s⁻¹ and 300 s⁻¹, and whole blood 1,800 s⁻¹) and not significant for experiments with whole blood at wall shear rate of 300 s⁻¹. The level of significance was highest for recombinant perfusates 1,800 s⁻¹ and 300 s⁻¹, and whole blood 1,800 s⁻¹. These data are presented in Fig. 5. Significant correlation was only found when all values (placebo and drug) were considered. Analysis of values obtained in the drug period and placebo period as separate sets yielded no correlation within these sets. To discover whether isoxsu-
prine had direct influence on platelet adherence, an experiment was performed with platelets and plasma of human volunteers to whom isoxsuprine was administered for four weeks. Platelet adherence studies were performed at the beginning and end of this period. In these studies, platelets and plasma were mixed with RBCs from healthy donors, not exposed to isoxsuprine at shear rates of 300 s\(^{-1}\) and 1,800 s\(^{-1}\); no effect of isoxsuprine could be detected (Table 3). Previous studies on in vitro treatment of RBCs with isoxsuprine have shown that the plasma concentrations achieved by in vivo administration are much too low to have an acute effect on deformability.

**Laboratory tests.** H, Hb content, mcv platelet count, and leukocyte count were not influenced by medication and had values within the normal range. The values of these variables showed a random variance throughout the study.

Aggregation studies with platelets of patients performed 30 minutes after blood collections showed normal aggregability with ADP (2.5 \(\mu\)mol/L and 5.0 \(\mu\)mol/L). Plasma analysis for the platelet-specific platelet factors β-TG and platelet factor 4 showed slightly enhanced values as compared with normal values, indicating some degree of in vivo platelet activation. Levels of β-TG ranged from 50 to 90 ng/mL of plasma (normal value < 50 ng/mL of plasma). Levels of platelet factor 4 ranged from 20 to 50 ng/mL of plasma (normal value < 20 ng/mL of plasma). No treatment effect was observed on these values. Platelet aggregation studies performed with platelets of the volunteers who had received isoxsuprine for four weeks were normal and identical before and after drug therapy.

**Clinical data.** Of the 12 patients who entered the trial, ten completed the full course. One patient died suddenly of a ruptured aneurysm in the fourth week of the washout period; a second patient completed the first eight-week episode, in which he took isoxsuprine but refused further cooperation because of upper abdominal discomfort. No adverse effects of either isoxsuprine or placebo was reported by the remaining patients. Nine patients reported that they had benefitted from the study, possibly because of the close medical attention and the concomitant training effect on their walking behavior. Three patients enlarged with claudication distance considerably during the study period. The ankle-arm pressure indices at rest and after maximal walking distance did not improve significantly in any of the patients and, in the patient who dropped out of the study after the isoxsuprine period, a marked deterioration of these values occurred. The maximal walking distance on the treadmill improved in three patients, in two of them during the placebo period and in the third patient during the isoxsuprine period of the study.

**DISCUSSION**

Behavior of RBCs in flowing blood, which is determined to an appreciable extent by \(H\) and RBC deformability, influences blood viscosity as well as platelet transport. In a previous report, we demonstrated that RBC deformability

<table>
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<tr>
<th>Shear Rate</th>
<th>Platelet Adherence Coverage (%)</th>
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<tbody>
<tr>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>300 s(^{-1})</td>
<td>44 ± 14</td>
</tr>
<tr>
<td>1,800 s(^{-1})</td>
<td>60 ± 12</td>
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ISOXSUPRINE REDUCES RED CELL DEFORMABILITY

..., perfusion experiments, in which only the platelets were in contact with the drug showed no effect (increase or decrease).

In the present study, correlation of RBC deformability and platelet adherence (Figs 4 and 5) could only be demonstrated when the RBC deformability was increased by isoxsuprine treatment, but not within the groups of pretreatment or posttreatment results. There are two possible explanations: first, the differences were caused exclusively by the influence of isoxsuprine on RBC deformability. Second, isoxsuprine affects both reduced deformability and platelet adherence by different independent mechanisms. The first explanation is more realistic, however, because theoretical considerations predict a close relation between RBC deformability and platelet adherence.

Marked improvement of the claudication distance was evident in three patients, and overall subjective benefit from the study period was reported by nine. It is well known, however, that close medical attention and walking training can be beneficial to patients with intermittent claudication and may lead to subjective improvement or even disappearance of intermittent claudication without objective alteration of the disease.\textsuperscript{19,20} The amelioration noted in our patients could not be explained by improvement of ankle-arch pressure indices at rest and after maximal walking distance and appeared to be due to subjective influences, as patients’ walking distances improved throughout the trial period, independent of the use of drug or placebo.

Although we did not expect a change in the disease state in this short study period, the results of the laboratory tests and perfusion experiments demonstrate a potentially important property of isoxsuprine, which is totally different from its putative vasodilating activity. Vasodilation, however, is not likely to be effective in this type of vascular diseases, as reported previously.\textsuperscript{21,22}

We conclude that eight-week oral use of isoxsuprine results in marked decrease in RBC deformability, which in turn causes a decrease in platelet adherence in an in vitro perfusion system. The potential beneficial effect of isoxsuprine in reducing platelet adherence in the course of vascular disease will have to be established in long-term studies.

APPENDIX

Correction of platelet adherence for \( H \) and platelet count. Recently, we described an empirical relation which gives the influence of wall shear rate, \( H \), and platelet concentration on platelet adherence in the annular perfusion chamber.\textsuperscript{23}

\[
\text{adh} = 0.83 \cdot 10^{-6} C_0 \cdot t \cdot \left( \gamma_w \cdot \gamma_H \right)^{0.53} \cdot 0.86H^{-0.60H^2}
\]

[2]

in which \( C_0 \) = platelet concentration (platelets per square centimeter), \( t \) = exposure time (s), \( \gamma_w \) = wall shear rate (s\(^{-1}\)) and \( H \) = hematocrit (volume fraction); \( \gamma_w \) is an arbitrary shear rate of \( 1 \text{ s}^{-1} \), which is introduced to make the exponent dimensionless. The constant of 0.83 \( \cdot 10^{-6} \) has the dimensions (cm \( \cdot \text{s}^{-1} \)).

This relation was used to calculate the platelet adherence at the standardized \( H \) and platelet count of 0.4 and 190,000 platelets per microliter, respectively, from the real measured

Table 4. Normalized Platelet Adherence in Whole Blood Perfusion Experiments

<table>
<thead>
<tr>
<th>Test Point</th>
<th>Time Course</th>
<th>Medication</th>
<th>Platelet Adherence (± SD)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>300 s(^{-1})</td>
</tr>
<tr>
<td>1</td>
<td>Start</td>
<td>Placebo</td>
<td>18 ± 4</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>24 ± 7</td>
<td>45 ± 18</td>
</tr>
<tr>
<td>2</td>
<td>4 wk</td>
<td>Placebo</td>
<td>21 ± 11</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>31 ± 10</td>
<td>50 ± 14</td>
</tr>
<tr>
<td>3</td>
<td>8 wk</td>
<td>Placebo</td>
<td>27 ± 6</td>
</tr>
<tr>
<td></td>
<td>Isoxsuprine</td>
<td>20 ± 16</td>
<td>36 ± 16</td>
</tr>
<tr>
<td>4</td>
<td>12 wk</td>
<td>Placebo</td>
<td>21 ± 6</td>
</tr>
<tr>
<td></td>
<td>Isoxsuprine</td>
<td>19 ± 6</td>
<td>36 ± 11</td>
</tr>
<tr>
<td>5</td>
<td>16 wk</td>
<td>Isoxsuprine</td>
<td>16 ± 3</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>23 ± 4</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>6</td>
<td>20 wk</td>
<td>Isoxsuprine</td>
<td>15 ± 4</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>23 ± 4</td>
<td>41 ± 5</td>
</tr>
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Platelet adherence was normalized to a standard hematocrit of 0.4 and a standard platelet count of 190,000/\(\mu\)L. An empirical relation, derived previously, which describes the dependence of platelet adherence on important parameters such as hematocrit, platelet count, and shear rate, was used for this purpose.\textsuperscript{18} The patients’ data and the empirical relationship are given in Appendix.
values of adherence, $H$, and platelet count. These latter data are listed in Table 5. In eq. 2, adherence is expressed as platelets per square centimeter, whereas in our experiments, percentage of coverage is used. For conversion, a relation between platelets per square centimeter and percentage of coverage as described by Bolhuis was used24 (1% coverage ~ 10$^5$ platelets per square centimeter).

ACKNOWLEDGMENT

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