Sex-Related Differences in Platelet Aggregation: Influence of the Hematocrit

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A number of investigators have recently reported that human female platelets have increased reactivity in vitro compared to platelets from age-matched males. A possible explanation for these observations is that the lower hematocrit in females results in a larger volume of distribution of the anticoagulant, with resultant higher calcium and citrate concentrations. To examine this possibility, we performed adenosine diphosphate (ADP) and collagen aggregations on platelets obtained from 11 male and 11 female healthy subjects. When aggregations were performed on blood samples using the standard amount of 3.8% sodium citrate as an anticoagulant (9:1, v:v blood:anticoagulant), the female platelets were significantly more reactive than the male platelets over a wide range of ADP and collagen concentrations (p < 0.01). The same subjects were then retested, but on this occasion the citrate concentration was adjusted for the hematocrit according to a mathematically derived standard curve. The previously noted increased reactivity of female platelets disappeared, and there was no significant difference in reactivity to either ADP or collagen between the male and female platelets. The reported in vitro sex differences in platelet reactivity is contributed to by an artifact caused by differences in the size of the citrate distribution compartment. It is possible that a similar phenomenon is responsible for the increased platelet reactivity reported in certain disease states, which frequently have a concomitantly associated anemia.

THERE HAVE BEEN a number of recent reports of sex-related differences in sensitivity to aggregating agents of male and female platelets. Ramwell and associates found that platelets from male animals of various species are more sensitive to low levels of aggregating agents than platelets from female animals.1 These investigators also reported that human female platelets are more sensitive to threshold levels of aggregating agents than platelets from age-matched males, an observation that has now been confirmed by a number of other groups.2, 4

The relevance of in vitro tests of platelet function to thrombogenesis is uncertain. Furthermore, the results obtained may be influenced by a variety of in vitro conditions unrelated to platelet reactivity in vivo. One important variable that could influence platelet reactivity in vitro is the plasma calcium concentration. It is standard practice in most laboratories to collect whole blood into a fixed ratio of citrate anticoagulant without modifying the amount according to the patient's hematocrit. Indirect evidence indicates that citrate is excluded from blood cells so that its plasma concentration is directly proportional to the hematocrit.5 Since the ionized calcium concentration has been reported to affect platelet aggregation,6 it is possible that the reported differences in platelet aggregation between male and female platelets is an in vitro artifact caused by differences in the plasma citrate concentration. To investigate this possibility, we have compared platelet aggregation in vitro in males and females before and after correction for the hematocrit.

MATERIALS AND METHODS

Subjects were 11 males (age 31-35, mean 31) and 11 females (age 21-55, mean 33). No woman was taking oral contraceptives or was pregnant, and no subject had received medications of any kind for 2 wk prior to blood sampling. On the morning of the testing, the subjects were fasted, and 40 ml of blood was collected into sodium citrate (3.8%; 9:1, v:v), and 5 ml were taken into EDTA for hemoglobin and hematocrit determination and platelet count. The same subjects were then retested, but the final citrate concentration was adjusted according to the result of the hematocrit determination (see below).

Correction of Citrate Concentration for Hematocrit

Citrate is excluded from blood cells and its volume of distribution (D) in plasma can be calculated from the following formula: D = 1 - hematocrit (Appendix 1). This was used to construct a correction curve, which was applied to standardize the plasma citrate concentration over a range of hematocrit values. The adjustment of citrate concentration was accomplished as follows: blood was collected into 3.8% sodium citrate (9 vol of blood to 0.9 vol of sodium citrate) for platelet aggregation studies and a sample was collected into EDTA. A hematocrit determination was performed on the EDTA specimen and the plasma citrate concentration adjusted from the standardization curve by adding more citrate to the sample of blood for platelet aggregation studies (Appendix 1).

Platelet Aggregation Studies

Platelet aggregation studies were carried out in a Payton aggregometer (Payton Associates, Scarborough, Ontario). The final platelet concentration in the platelet-rich plasma (PRP) was corrected to 200,000/μl with the patient's own platelet-poor plasma (PPP). The aggregating agents were adenosine diphosphate (ADP) (Sigma Associates, Washington, D.C.) and acid-soluble collagen. The ADP was diluted in sodium chloride and stored in aliquots at −70°C. Bovine acid-soluble collagen was prepared as described7 and stored at 4°C. A single batch of ADP and collagen was used for all experiments.

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SEX DIFFERENCES IN PLATELET AGGREGATION

All aggregations were performed in the same aggregometer at 37°C using 1 ml siliconized cuvettes, siliconized rotating bars (10,000 rpm).

The aggregometer recorder was set at 0% and 100% using PRP and PPP, respectively, before each aggregation experiment. Varying concentrations of aggregating agent were added to the cuvette at time 0 and the percent maximum collagen aggregation defined as the maximum aggregation that occurred within 5 min of adding the aggregating agent. The percent maximum ADP aggregation was defined as the maximum primary wave that occurred within 5 min of addition of the aggregating agent. Concentrations of ADP were selected that only produced a primary wave. For the purposes of these experiments, secondary aggregation was defined as an initial wave of aggregation followed by an increase in aggregation of greater than 5%.

The initial dilution of aggregating agent was chosen at random for each aggregation study. Depending on the degree of aggregation observed, the technologist then selected a higher or lower dilution of aggregating agent. Because of limited quantities of platelet-rich plasma obtained from each person, not every dilution of aggregating agent was tested for each platelet preparation.

Statistical Analysis

Comparisons between the aggregation responses of the 22 individuals with and without correction for hematocrit were made using a single dose-response model that best fitted the observed data. The examination of the curves for each individual demonstrated that the percent aggregation, "100P" (where 0 < p < 1), in relationship to an increasing concentration of aggregating agent, "D," invariably formed a sigmoidal-shaped curve. The data were therefore transformed so the response was linearly related to the logarithm of the dilution. The optimal transformation was determined to be logit.5 The linear model was then used.

Logit P = A + B log, D where logit P = log(P/Q) with Q = 1 - P.

Estimates of the parameters A and B were obtained using weighted least squares.

Comparisons between sexes prior to and following correction for hematocrit were made using the estimates of the slope "B" and the estimates of the dilution, D50, that would result in a 50% aggregation. The value C = log,D50 was chosen rather than the intercept "A" to draw inferences about shifts in the fitted lines. Two sample unpaired t tests were performed under the assumption of equal variances. We tested (A) whether the change in the transformed response per unit change in dose was the same for both males and females, and (B) whether the dose required to produce 50% aggregation was the same for both sexes.

RESULTS

Figure 1 illustrates the correction curve used to standardize the final concentration of citrate according to the size of its compartment of distribution.

Figure 2 shows the relationship between percent aggregation and ADP concentrations for platelets prepared from blood with citrate concentration unadjusted for hematocrit in male and female subjects.

Platelets from the female subjects aggregated to a greater extent than did platelets from male subjects over a wide range of ADP concentrations. When aggregation with ADP was performed on platelets prepared from blood samples in which the citrate concentration was corrected for hematocrit, the difference in aggregability was no longer evident (Fig. 2B).

The percent maximum aggregation (mean ± SE) of platelets prepared from the unadjusted citrate samples

*An angular transformation was also considered. However, based on the appropriate tests, the logit transformation resulted in the better fit.
The slopes of the aggregation–dilution relationship for both the male and female precorrection and postcorrection were not significantly different from each other (Table 1). In contrast, the dose of aggregating agent required to produce 50% total aggregation was significantly different ($p < 0.01$ for collagen, and $p < 0.001$ for ADP) for platelets from female subjects compared to platelets from male subjects when collected in the standard fashion. However, following correction for the hematocrit, there was no significant difference in the concentration of aggregating agent required to produce 50% aggregation.

**DISCUSSION**

Recently, platelets from human females have been reported to have increased aggregability in vitro compared to platelets obtained from male subjects.²-⁴ The possibility that a sex-related difference in aggregation of human platelets might be caused by differences in the citrate concentration was initially suggested by Hardisty and associates a number of years ago.⁹ They observed a relationship between the amount of ADP necessary to initiate secondary aggregation and the hematocrit, but did not attempt to reverse this effect by adjusting the citrate concentration according to the hematocrit.⁹ The results of our study indicate that the hematocrit has an important effect on the measured platelet reactivity in vitro, for both collagen and ADP induced aggregation. Platelet aggregation caused by ADP and collagen was greater over a wide range of aggregating agent concentrations in a group of premenopausal women compared to males of similar age when blood was collected in the standard manner. When the studies were repeated after correcting the citrate concentration according to the hematocrit, the difference in platelet reactivity was no longer evident.

Because of the considerable interindividual differences in platelet reactivity, the potential existed for differences in reactivity to exist, yet not be identified.

**Fig. 3.** The relationship between the percent aggregation (ordinate) and dilution of collagen (abscissa) in males (●―●) and females (○―○) when whole blood is collected in the standard fashion (A) and after correction for the hematocrit (B). Each point = mean ± SE; $n = 6$.

from male and female subjects plotted as a function of collagen dilution is shown in Fig. 3A. Once again, the platelets from the female subjects aggregated to a greater extent than did platelets from the male subjects, but this difference was no longer evident when the studies were repeated following adjustment for the hematocrit (Fig. 3B).

| Table 1. Results of Statistical Analysis Comparing Male and Female Platelet Aggregation Before (pre) and Following (post) Correction of Citrate Concentration for Hematocrit |
|-----------------|------|--------|--------|--------|--------|--------|
| Response        | Sex  | Colagen |        | ADP    |        |        |
|                 |      | Pre     | Post   | Pre    | Post   |        |
| Slope           | Male | -3.20 (1.19) | -2.63 (1.53) | 1.17 (0.22) | 1.19 (0.20) |
|                 | Female | -3.84 (2.43) | -3.12 (1.25) | 1.19 (0.25) | 1.23 (0.23) |
|                 | $t$ Value | 0.79 | 0.82 | -0.27 | -0.41 |
| C-50            | Male | 5.43 (0.52) | 5.50 (0.53) | 5.86 (0.31) | 5.95 (0.45) |
|                 | Female | 6.10 (0.43) | 5.84 (0.38) | 5.16 (0.33) | 5.61 (0.51) |
| $t$ Value       |      | 3.31* | 1.60 | 5.07† | 1.69 |

C-50 represents the log dilution of aggregating agent required to produce 50% aggregation.

* $p < 0.01$.
† $p < 0.001$. 

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(beta error). To limit this variation, the data were analyzed following logistic transformation. While a significant difference \((p < 0.01)\) in platelet reactivity existed between males and females when blood was collected in the standard fashion, no significant difference existed following correction for hematocrit. With the sample size tested, a difference of 12\% in platelet reactivity could be present and not be detected. To reduce this difference to 6\%, a sample size of 108 individuals would be required.

These observations indicate that the reported sex-related differences in platelet aggregation is contributed to by differences in the volume of distribution of the anticoagulant. The method used to correct for citrate concentration is simple and can be readily employed in a routine laboratory by collecting whole blood into 3.8\% sodium citrate \((9:0.9, v:v)\), performing a microhematocrit, and then adjusting the citrate concentration by the addition of more citrate according to the correction curve.

It is possible that the increased aggregation reported in a number of disease states could be related to the associated anemia rather than to a true increase in platelet reactivity in vitro. Our findings indicate that the influence of the patient's hematocrit should be taken into account when performing platelet aggregation studies.

APPENDIX 1

The final citrate concentration was corrected to that which would be present in a blood sample with a hematocrit of 50\% collected in the standard fashion \((9:1, v:v, blood:sodium citrate)\). Therefore, a 10-ml sample of whole blood and anticoagulant would contain 4.5 ml of plasma and 1 ml of sodium citrate (final citrate concentration is 6.72 mg). The total amount of citrate required for any blood sample is given by the formula:

\[
\text{Citrate} = \text{volume of blood} \times \frac{(1 - \text{Hct})}{4.5}
\]

The further amount of citrate to be added to each 9-ml whole blood sample collected into 0.9 ml 3.8\% sodium citrate is:

\[
2 \times \frac{(1 - \text{Hct})}{4.5} - 0.9 \text{ ml}
\]

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