

Stress-Induced Alterations in Coagulation: Assessment of a New Hemoconcentration Correction Technique

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Objective: For the examination of psychological stress effects on coagulation, the Dill and Costill correction (DCC) for hemoconcentration effects has been used to adjust for stress-induced plasma volume changes. Although the correction is appropriate for adjusting concentrations of various large blood constituents, it may be inappropriate for time-dependent or functional coagulation assays. Two new plasma reconstitution techniques for correcting hemoconcentration effects on stress-induced changes in coagulation were compared with the DCC. **Methods:** Blood was collected from 31 men during baseline, the Trier Social Stress Test (TSST), and after 20-minute recovery. For the reconstitution techniques, TSST plasma samples were reconstituted with either baseline plasma or physiological saline equal to the amount of plasma lost during stress. **Results:** Uncorrected activated partial thromboplastin time (APTT) decreased, whereas fibrinogen, factor VIII clotting activity (FVIII:C), D-dimer and prothrombin time (PT%) increased significantly during the TSST. The DCC produced a significantly greater decrease in APTT during stress compared to uncorrected APTT, a significant decrease in PT% compared to uncorrected PT%, and stress D-dimer and fibrinogen and FVIII:C being no different than baseline. APTT, fibrinogen, D-dimer and PT% after saline reconstitution were not different from baseline, whereas FVIII:C after saline reconstitution remained elevated. APTT, PT%, fibrinogen and D-dimer after plasma reconstitution were no different from uncorrected values, whereas FVIII:C remained significantly elevated. **Conclusions:** The observed changes in coagulation are likely in part a consequence of stress and hemoconcentration, but the DCC seems to be an inappropriate hemoconcentration correction technique of time-dependent assays. The saline reconstitution technique may be more biologically relevant when examining stress-hemoconcentration effects on coagulation. **Key words:** acute psychological stress, coagulation, hemoconcentration.

APTT = activated partial thromboplastin time; **BP** = blood pressure; **CHD** = coronary heart disease; **DBP** = diastolic blood pressure; **FVIII:C** = factor VIII clotting activity; **Hgb** = hemoglobin; **Hct** = hematocrit; **HR** = heart rate; **PT%** = prothrombin time percentage; **SBP** = systolic blood pressure; **TSST** = Trier Social Stress Test.

INTRODUCTION

Cardiovascular disease is the underlying cause of more than one third of the deaths in the United States, with coronary heart disease (CHD) and stroke being the first and the fourth leading causes of death, respectively (1,2). Traditional risk factors, such as hypertension, elevated serum cholesterol level, body mass index, diabetes, and smoking, do not fully explain CHD risk (3). One factor suggested to explain additional risk is psychosocial stress that may precipitate atherothrombotic events and CHD, especially myocardial infarction (4). A number of pathophysiological mechanisms have been suggested to explain this link, including elevated coagulation activity (5–8), hemoconcentration (i.e., when the ratio of blood cells to plasma increases), and increased blood viscosity (9,10), all contributing

to exacerbated shear stress imposed on atherosclerotic plaques and promoting plaque ruptures (5).

The mechanistic underpinnings of acute stress-induced coagulation activation are not fully understood but may involve hemoconcentration. During acute psychological stress, an increase in blood pressure (BP) pushes intravascular fluid into interstitial spaces, whereas molecules of high molecular weight (i.e., >69 kDa) cannot pass through capillary walls. Of particular importance, most hemostasis factors (e.g., clotting factors) have molecular weights greater than 69 kDa and thus are too large to pass through vascular pores when intravascular fluid is forced into extravascular spaces during stress (11). The effects of stress and hemoconcentration on hemostasis are often overlooked in laboratory studies and may be one of the most important shortcomings of studies examining the effects of acute mental stress on hemostasis. Only a few known studies have examined stress-hemoconcentration effects on hemostasis (12–14). Two of these studies suggest that stress-induced changes in coagulation markers do not depend on hemoconcentration. However, one study (12) covaried for changes in hematocrit (Hct), which only controls for variation in Hct change rather than actually correcting for plasma volume shifts (15). In the second study, Hct and plasma volume changes during stress were not significantly correlated with changes in any coagulation measure (13). However, correlations are less powerful than arithmetic adjustment for plasma volume shifts (16).

In addition to concentrations of clotting factors, hemoconcentration could influence clotting time by allowing clotting factors to be closer to each other, platelets and the endothelium, thereby hastening their opportunity to become activated and produce clotting. In a study by von Känel and colleagues (14), the effects of stress and hemoconcentration on several coagulation parameters were corrected arithmetically using the widely accepted formula by Dill and Costill (15). Adjustment for hemoconcentration did not significantly alter factor VIII clotting activity (FVIII:C), FXII:C, von Willebrand Factor, or D-dimer

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levels but resulted in a significant decrease in fibrinogen, FVII:C, activated partial thromboplastin time (APTT) (i.e., indicating a shortened clotting time of the intrinsic pathway), and prothrombin time activity percentage (PT%) (i.e., indicating prolonged clotting time of the extrinsic pathway). The adjusted result for APTT is puzzling because one would expect a decrease in fibrinogen to be accompanied by a prolongation in APTT. That is, a reduction in a clotting factor should be associated, with a slower clotting response. Moreover, the formula by Dill and Costill (15) is designed to mathematically adjust the *concentration* of a physiological parameter based on changes in plasma volume and has no time component. Therefore, the use of such mathematical correction for these measures is questionable.

A more biologically appropriate method of correcting for plasma volume shifts when examining clotting time and clotting activity may be to physically correct for plasma volume changes by reconstituting plasma samples that exhibit stress-induced decreases in plasma volume with a relevant fluid (e.g., physiological saline or the person's own plasma from baseline) to correct for any fluid movement out of the vascular compartment into the interstitial space. Physiological saline is representative of the filtrate that is lost during acute stress. The person's own plasma from baseline is relevant because it is endogenous to the individual and has, theoretically, a lesser amount of coagulation activity than plasma collected during a stressor. Similarly, given that most clotting assays require a dilution of plasma before analysis, previous research has examined the effects of plasma dilution on several coagulation parameters. For instance, in an exponential fashion, progressive dilutions with saline from 0% to 90% reduced fibrinogen concentration and FVII:C and increased PT in seconds and APTT (17). Other studies have only examined one dilution (e.g., 80%) and found that fibrinogen, FII:C, FVII:C, FIX:C, and FX:C all decrease by approximately the same ratio (18). However, no known studies have examined the influence of small dilutions, such as those corresponding to the plasma volume losses that are typically observed during acute stress, nor have any known studies examined dilution series in the context of acute stress reactivity.

To test the efficacy of these reconstitution techniques, we recently conducted a pilot study that compared them with arithmetic adjustment (19). PT in seconds and APTT as well as calculated plasma volume were determined at baseline and immediately after a mental arithmetic stressor. Plasma samples obtained immediately after the stressor were reconstituted with baseline plasma or physiological saline equal to the amount of plasma lost during the stressor. APTT during stress was not significantly different than APTT at baseline when adjusting for plasma volume changes arithmetically or with either reconstitution technique. On the other hand, PT during stress became significantly longer than PT during baseline when adjusting arithmetically but no longer differed from PT during baseline when adjusting with either reconstitution technique (19). These preliminary results support the notion that mathematical adjustment overcorrects for plasma volume shifts when examining PT. However, this study had important shortcomings, including

low power due to a very small sample size ($n = 10$) and only examining male university students.

To help explain why acute stress may trigger acute coronary syndromes, the goal of the present study was to more fully explain whether stress-induced hypercoagulation is due to hemoconcentration, actual coagulation activation, or a combination of both. We improved on the limitations of previous research by applying this reconstitution technique to plasma obtained in a larger sample of healthy men with a broad age range. Specifically, we examined the effects of stress and hemoconcentration on APTT, PT%, FVIII:C, fibrinogen concentration, and D-dimer concentration by comparing arithmetic adjustment to reconstitution with an individual's own plasma obtained at baseline and reconstitution with physiological saline. Given previous findings (14,19), we hypothesized that reconstituting plasma collected during a stressor with the person's own plasma from baseline or with physiological saline such that plasma volume is equal to plasma volume at baseline will result in coagulation parameters being closer to baseline than uncorrected values, with the exception of FVIII:C because elevated FVIII:C has survived arithmetic adjustment for plasma volume shifts in previous research (14). Consistent with our suggestion that physical reconstitution may be a more appropriate method of adjusting for plasma volume shifts when examining time-dependent assays, we also hypothesized that reconstitution of plasma collected during a stressor will result in APTT and PT% being closer to baseline values than APTT and PT% corrected arithmetically, whereas reconstitution will result in fibrinogen, D-dimer, FVII:C, and FVIII:C having values similar to values corrected arithmetically. Finally, we had the exploratory aim of examining the efficacy of the correction techniques after 20 minutes of recovery after termination of the stressor.

METHODS

Participants

Study participants were 31 nonsmoking and nonobese (body mass index $<30 \text{ kg/m}^2$) men between 20 and 50 years of age who were in excellent physical and mental health, as confirmed by an extensive health questionnaire and telephone interview. Specific exclusion criteria, as obtained by subjects' self-report, were regular strenuous exercise, smoking, use of over-the-counter or prescribed medication, alcohol and illicit drug abuse, heart disease, elevated blood sugar level and diabetes, elevated cholesterol level, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, and current infectious diseases. Participants were asked to refrain from exercising and from drinking alcohol for 24 hours before their testing session. The current study is part of a larger, ongoing study examining the effects of flavanols on psychobiological stress reactivity that will be described elsewhere. For the parent study, participants were randomly assigned to ingest 50 g of dark chocolate either with or without flavanols. For the purpose of the current study, participants were enrolled from February 2010 to August 2010. The purpose of the current study was to examine a new method of correcting for stress-hemoconcentration effects, and no effects of flavanols on stress and hemoconcentration were expected. Controlling for flavanol condition indeed did not significantly alter any effects of the plasma manipulations. Therefore, all results are presented without controlling for flavanol condition. The study protocol was approved by the Ethics Committee of the State of Zurich, Switzerland.

Procedure

On arriving to the laboratory at 10 AM, informed consent was obtained. Participants were seated in a comfortable chair in a quiet room and were

provided a standardized breakfast (jelly sandwich and water). After breakfast, an indwelling venous 20-gauge catheter was inserted in a suitable vein in the ante-cubital fossa. Participants then consumed dark chocolate, followed by a 2-hour rest period. Next, participants performed the Trier Social Stress Test (TSST), which includes a 3-minute preparation phase, a 5-minute free speech phase, and a 5-minute mental arithmetic task all performed in front of an audience (20). The TSST has been shown to evoke robust responses in many of the same hemostasis markers investigated in the present study (14,21–23). After the task, subjects remained seated for a 20-minute recovery period. Blood samples for hemostasis and hemoconcentration measures were collected immediately before the preparation phase of the TSST (“baseline”), 3 minutes after the TSST (“poststress”), and 20 minutes after the TSST (“recovery”). Finally, the catheter was removed, and participants received 175 Swiss Fr compensation.

Hemodynamic Measures

As a manipulation check for the psychological stressor, systolic BP (SBP) and diastolic BP (DBP) were measured by Omron sphygmomanometry device (Omron 773; Omron Healthcare Europe B.V., Hoofddorp, the Netherlands) 10 minutes before, during the speech and mental arithmetic portions of the TSST, and 15 minutes after the TSST. Heart rate (HR) was measured continuously via a portable HR monitor (LifeShirt; Vivometrics, Ventura, CA).

Hemostasis Measures

Venous blood was drawn into polypropylene tubes containing 3.8% sodium citrate (Sarstedt, Numbrecht, Germany). Samples were centrifuged for 20 minutes at 2000g. Plasma was then aliquoted into polypropylene Eppendorf tubes (Eppendorf North America, Hauppauge, NY) and stored at -80°C until assayed. All coagulation assays, except for D-dimer, were determined using the BCS coagulation analyzer (Dade Behring, Liederbach, Germany). FVIII:C, APTT, and PT% were determined by standard coagulometric methods using factor-deficient standard human plasma and reagents (Dade Behring). Plasma fibrinogen levels were determined using a modified Clauss method (Multifibrin U; Dade Behring). Plasma D-dimer concentrations were measured by means of an enzyme-linked immunosorbent assay (ZYMUTEST D-dimer; HYPHEN BioMed, Neuville-sur-Oise, France). Interassay and intra-assay coefficients of variation were less than 10% for all coagulation measures.

Hemoconcentration Measures

Blood was collected into 2.74-mL ethylenediaminetetraacetic acid-coated Monovettes (Sarstedt) for hemoconcentration measures. Hct and hemoglobin (Hgb) concentrations were determined with a Sysmex KX-21N Counter (Sysmex Digitana AG, Horgen, Switzerland). Hct levels were calculated from the red blood cell concentration, and Hgb concentrations were determined by the cyanmethemoglobin method. Plasma volume changes were calculated arithmetically from Hct and Hgb values at baseline, poststress, and recovery (16). Given that changes in the actual size of red blood cells can affect the packed cell volume of Hct, Hgb is used in the plasma volume equation to control for possible changes in mean corpuscular volume (24).

Hemoconcentration Correction Techniques

All plasma samples were thawed in a water bath. Then, samples from poststress and recovery were aliquoted into three 650- μL portions, which were subjected to the following conditions: a) Changes in hemostasis markers were corrected arithmetically by the formula by Dill and Costill (15). b) Plasma samples were reconstituted back to baseline plasma volume with the participant's actual plasma from baseline. c) Plasma samples were reconstituted back to baseline plasma volume with physiological saline.

Arithmetic Hemoconcentration Correction

Values of hemostasis parameters were arithmetically corrected to reflect plasma volume shifts. The calculation for estimating plasma volume changes incorporates both Hct (to determine the percentage of plasma volume) and Hgb

(to control for hemoconcentration-induced changes in red blood cell volume) before and after each manipulation (15). The equation is as follows:

$$\begin{aligned} BV_A &= BV_B \times (Hgb_A/Hgb_B) \\ CV_A &= BV_A \times (Hct_A/100) \\ PV_A &= BV_A - CV_A \\ \% \Delta PV &= 100 \times (PV_A - PV_B)/PV_B \end{aligned}$$

where BV is blood volume, CV is red blood cell volume, PV is plasma volume, Hgb is hemoglobin, Hct is hematocrit, subscript B refers to baseline sample, subscript A refers to the period (poststress or recovery) sample, BV_B is taken as 100, and PV_B is $100 - Hct_B$.

Corrected values for hemostasis measures were calculated from the measured levels during each period and the estimated percentage change in plasma volume. The equation is as follows:

$$C_C = C_U \times [1 - (\% \Delta PV / 100)],$$

where C_U is measured coagulation parameter during each period.

Plasma Samples Reconstituted With Baseline Plasma

The 650 μL of plasma from poststress and recovery was reconstituted with an amount of plasma from baseline so that plasma volume at poststress and recovery became equal to plasma volume at baseline. This amount was determined from the following formula:

$$\text{Reconstitution amount} = [(PV_B \times CV_A)/(PV_A \times CV_B) - 1]/650,$$

It was expected that plasma volume would increase to above baseline levels at recovery in some individuals. When this occurred, plasma was not reconstituted. Plasma volume was not expected to increase immediately at poststress. However, this occurred in one individual, and therefore, plasma was not reconstituted for this participant. Therefore, the sample size for analyses comparing plasma manipulations was 30.

Plasma Samples Reconstituted With Physiological Saline

Plasma samples from poststress and recovery were reconstituted with physiological saline (0.9% NaCl; Laboratorium Dr. G Bichsel AG, Interlaken, Switzerland) via the same method as in the plasma reconstitution manipulation.

Unmanipulated Plasma Samples

Plasma was not manipulated by reconstitution, and arithmetic correction for plasma volume shifts was not applied to hemostasis measures.

Statistical Analyses

Data were analyzed using SPSS (version 17.0) statistical software (SPSS, Inc, Chicago, IL). Data are presented as mean \pm standard error of the mean. All tests are two-tailed with level of significance set at $p < .05$. The Kolmogorov-Smirnov test was used to ensure a normal distribution of hemoconcentration, hemostasis, and hemodynamic measures. Fibrinogen values did not follow a normal distribution. Conventional transformations did not result in normal distributions. Therefore, z scores were computed. All other variables followed a normal distribution.

A series of repeated-measures analyses of variance (ANOVAs) were conducted for HR, DBP, SBP, Hct, and plasma volume with time (baseline, poststress, and recovery) as the independent variable. Planned within-subjects contrasts were used to compare each level of time, using the Bonferroni adjustment for multiple comparisons. Next, for hemostasis variables, baseline variables with values equal to actual baseline values were created for each plasma manipulation condition. These variables were needed to compare differences in plasma manipulations from baseline to poststress. Based on previous findings (23), it was expected that recovery plasma volume would not be significantly different from baseline plasma volume. Therefore, when recovery plasma could not be reconstituted because calculated plasma volume at recovery was greater than or equal to baseline plasma volume, values equal to uncorrected recovery values were included for each reconstitution condition at recovery. Then, a series of three (time: baseline versus poststress versus recovery) by four (plasma manipulation) repeated-measures ANOVAs were conducted. Planned within-subjects contrasts were used to compare levels of hemostasis parameters in each

plasma manipulation at poststress and recovery to levels at baseline and to each other, using the Bonferroni adjustment for multiple comparisons. The Huynh-Feldt correction was applied to account for violations of the sphericity assumption. Fibrinogen was not normally distributed, and transformations did not render it normal. Thus, a series of Friedman tests for repeated measures for each plasma manipulation were conducted for fibrinogen, followed by Wilcoxon signed rank posttests with Bonferroni adjustment for multiple comparisons (eight comparisons; to be significant, p had to be $< .00625$). Results are presented as mean (standard deviation).

RESULTS

Subject Characteristics

Table 1 contains subject characteristics of the entire sample and for each flavanol condition. No differences were observed between flavanol conditions for any subject characteristic.

Hemodynamic and Hemoconcentration Measures

Table 2 contains HR, BP, Hct, and plasma volume data for each time point. Because of equipment failure or experimenter error, data were unavailable for 3 participants for HR and 6 participants for BP, leaving 28 and 25 participants for analysis, respectively. HR and BP were averaged for each period. Repeated-measures ANOVA indicated a significant main effect of time for HR ($F(2,54) = 75.29, p < .001$), SBP ($F(2,48) = 95.2, p < .001$), DBP ($F(2,48) = 103.99, p < .001$), Hct ($F(2,60) = 80.77, p < .001$), and plasma volume ($F(2,60) = 81.74, p < .001$). HR and BP increased significantly from baseline to poststress and remained somewhat elevated at recovery (p values $< .001$). Hct increased significantly from baseline to poststress ($p < .001$) and was significantly less than baseline level at recovery ($p = .02$), whereas plasma volume decreased significantly from baseline to poststress ($p < .001$) and was not significantly different from baseline at recovery ($p = .14$).

Hemostasis Measures

The time-by-plasma manipulation interaction violated the sphericity assumption for each hemostasis measure. Therefore, Huynh-Feldt corrected degrees of freedom and F statistics are reported for each measure. Table 3 summarizes the relative change from baseline at poststress for each plasma manipulation.

Activated Partial Thromboplastin Time

Because of laboratory error, APTT was not assessed at poststress for one participant and at recovery for another. In addition, sufficient plasma for all manipulations was not obtained in 3 subjects, leaving a sample size of 26. Repeated-measures

TABLE 2. Hemodynamic and Hemoconcentration Measures

Measure, M (SD)	Baseline	TSST	Recovery
Heart rate, beats per min	71.9 (14.6)	94.9 (21.2) ^a	76.6 (15.9) ^a
SBP, mm Hg	124.4 (9.6)	159.6 (16.5) ^a	131.4 (11.8) ^a
DBP, mm Hg	70.2 (10.0)	97.8 (13.9) ^a	77.2 (11.1) ^a
Hematocrit, %	43.2 (2.2)	44.7 (2.1) ^a	42.9 (2.0) ^b
Plasma volume, %	56.8 (2.2)	53.3 (2.4) ^a	57.3 (2.2)

M = mean; SD = standard deviation; TSST = Trier Social Stress Test; SBP = systolic blood pressure; DBP = diastolic blood pressure.

^a Significantly different from baseline at $p < .001$.

^b Significantly different from baseline at $p = .02$.

ANOVA indicated a significant time-by-plasma manipulation interaction ($F(2.69,61.89) = 34.80, p < .001$). Figure 1 shows that APTT decreased significantly from baseline (34.4 ± 0.8) to poststress when uncorrected ($33.1 \pm 0.8, p < .001$), corrected arithmetically ($31.1 \pm 0.8, p < .001$), or corrected with baseline plasma reconstitution ($33.1 \pm 0.8, p < .01$) but that it was not significantly different from baseline when corrected with saline reconstitution ($33.9 \pm 0.9, p = .08$). At poststress, there was no difference between uncorrected APTT and APTT corrected with baseline plasma reconstitution ($p = .87$). However, arithmetic correction resulted in APTT becoming significantly shorter than uncorrected APTT and APTT corrected with baseline plasma reconstitution (p values $< .001$). APTT at recovery was not different from baseline for any plasma manipulation condition (p values $> .23$).

Prothrombin Time

Repeated-measures ANOVA indicated a significant time-by-plasma manipulation interaction ($F(3.29,75.63) = 18.33, p < .001$). Figure 2 shows that PT% increased (i.e., clotting time of the extrinsic pathway accelerated) from baseline (86.9 ± 2.4) to poststress when uncorrected ($88.8 \pm 2.4, p = .01$) and significantly increased when corrected with baseline plasma reconstitution ($89.7 \pm 2.2, p < .001$). PT% at poststress was not significantly different from baseline when corrected with saline reconstitution ($86.8 \pm 2.3, p = .65$) but was significantly less

TABLE 3. Change Relative to Baseline After Stress for Each Plasma Manipulation

Measure	Uncorrected	Dill and Costill (15)	Plasma Reconstitution	Saline Reconstitution
APTT	-	--	-	=
PT%	+	+	=	-
D-dimer	+	+	=	=
FVIII:C	+	+	+	=
Fibrinogen	=	=	=	=

APTT = activated partial thromboplastin time; FVIII:C = factor VIII clotting activity; PT% = prothrombin time percentage.

For APTT, - indicates a significant decrease relative to baseline, whereas -- indicates a significant decrease relative to uncorrected APTT after stress.

Numbers in parentheses are references, unless otherwise indicated.

TABLE 1. Baseline Demographic and Cardiovascular Characteristics

Variables, M (SD)	Total (N = 31)	Flavanol (n = 19)	No Flavanol (n = 12)
Age, y	32.5 (9.8)	30.4 (10.0)	33.8 (9.7)
BMI, kg/m ²	23.8 (3.1)	24.0 (3.1)	23.4 (3.2)
SBP (baseline), mm Hg	124.4 (9.6)	126.5 (12.1)	122.4 (10.2)
DBP (baseline), mm Hg	70.2 (10.0)	71.8 (12.1)	69.1 (9.0)

M = mean; SD = standard deviation; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure.

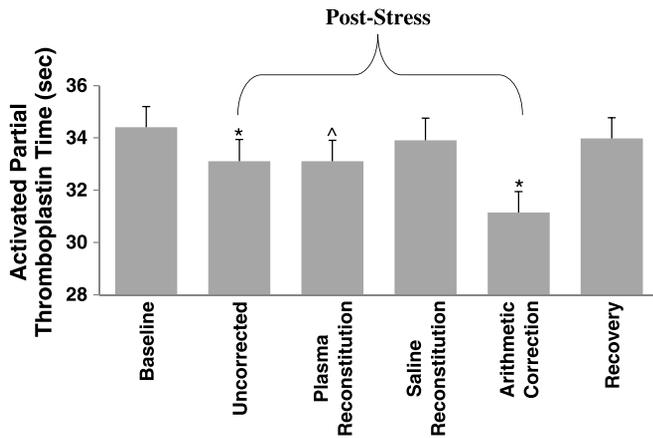


Figure 1. Differences in activated partial thromboplastin time change across conditions. *Significantly different from Baseline, Saline Reconstitution and Recovery at $p < .001$. ^Significantly different from Baseline, Arithmetic Correction and Recovery at $p < .01$.

than PT% at baseline when corrected arithmetically (83.5 ± 2.4 , $p < .001$). There was no significant difference between uncorrected PT% and PT% corrected with baseline plasma reconstitution ($p = .18$). PT% at recovery was not different from baseline for any plasma manipulation condition (p values $> .24$).

Factor VIII Clotting Activity

Because of laboratory error, FVIII:C values were outside the acceptable range for two participants at poststress. Sufficient plasma was not obtained for all manipulations at poststress in 3 participants and at recovery in 2 participants, leaving a sample size of 23. Repeated-measures ANOVA indicated a significant time-by-plasma manipulation interaction ($F(1.77,39.1) = 4.91$, $p = .015$). Figure 3 shows that FVIII:C increased significantly from baseline (106.0 ± 7.0) to poststress when uncorrected (115.1 ± 8.5 , $p = .008$), corrected with baseline plasma reconstitution (118.4 ± 7.6 , $p < .001$), or corrected with saline reconstitution (113.4 ± 7.6 , $p < .001$) but was not significantly different from baseline when corrected arithmetically (108.2

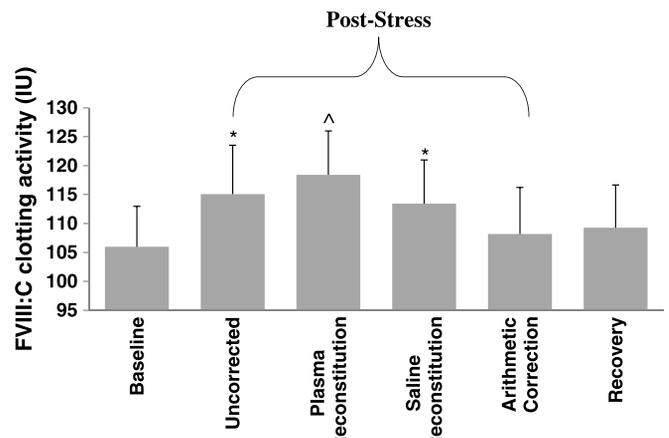


Figure 3. Differences in factor VIII clotting activity change across conditions. *Significantly different from Baseline, Arithmetic Correction and Recovery at $p < .001$. ^Significantly different from Baseline, Arithmetic Correction, Saline Reconstitution and Recovery at $p < .001$.

± 8.1 , $p = .85$). FVIII:C corrected arithmetically was significantly less than FVIII:C corrected with plasma reconstitution ($p = .001$) and uncorrected FVIII:C ($p < .001$). However, uncorrected FVIII:C was not significantly different from FVIII:C corrected with saline reconstitution ($p = .50$). FVIII:C at recovery was not significantly different from baseline for any plasma manipulation condition (p values $> .14$).

D-Dimer

Repeated-measures ANOVA indicated a significant time-by-plasma manipulation interaction ($F(1.57,40.84) = 6.90$, $p = .005$). Figure 4 shows that D-dimer increased from baseline (155.1 ± 12.2) to poststress when uncorrected (176.7 ± 12.3 , $p = .017$) or corrected with baseline plasma reconstitution (177.7 ± 13.4 , $p = .021$) but was not significantly different from baseline when corrected arithmetically (166.4 ± 11.8 , $p = .24$) or when corrected with saline reconstitution (164.3 ± 11.2 , $p = .11$). There was no significant difference between uncorrected D-dimer and D-dimer corrected with baseline plasma reconstitution ($p = .80$). Likewise, there was no significant difference between D-dimer corrected arithmetically and D-dimer corrected with saline reconstitution ($p = .63$). D-dimer at

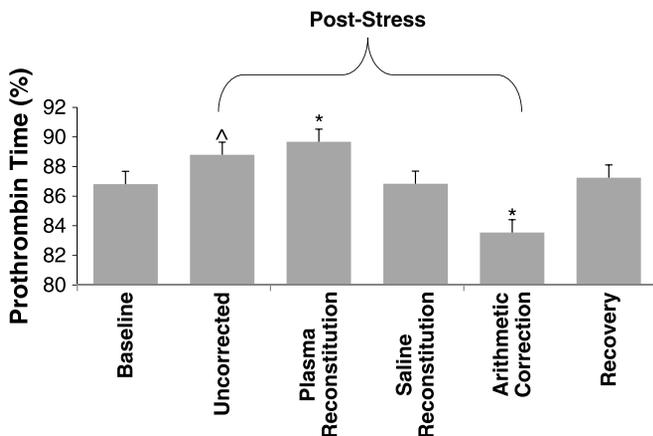


Figure 2. Differences in prothrombin time change across conditions. *Significantly different from Baseline, Uncorrected, Plasma Reconstitution and Recovery at $p < .001$. ^Significantly different from Baseline, Saline Reconstitution and Recovery at $p < .02$.

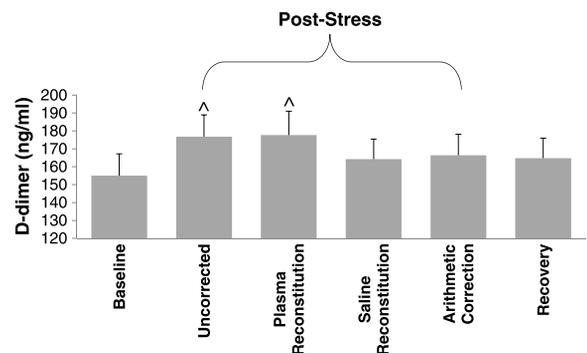


Figure 4. Differences in D-dimer change across conditions. ^Significantly different from Baseline, Saline Reconstitution and Arithmetic Correction at $p < .04$.

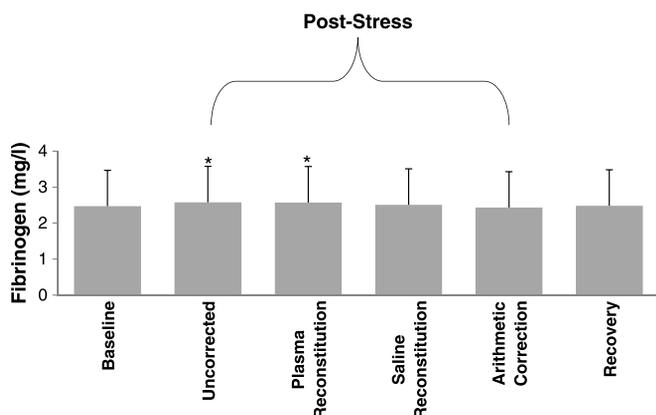


Figure 5. Differences in fibrinogen change across conditions. *Significantly different from Baseline at $p < .001$.

recovery was not different from baseline for any plasma manipulation condition (p values $> .21$).

Fibrinogen

Because of laboratory error, fibrinogen was not assessed at poststress for one participant. Sufficient plasma was not obtained for all manipulations at poststress in 3 participants and at recovery in 2 participants, leaving a sample size of 25. Friedman test was significant for uncorrected ($\chi^2 = 23.63$, $df = 2$, $p < .001$), plasma reconstitution ($\chi^2 = 23.24$, $df = 2$, $p < .001$), and arithmetic correction ($\chi^2 = 7.52$, $df = 2$, $p = .023$), whereas it was nonsignificant for saline reconstitution ($\chi^2 = 3.83$, $df = 2$, $p = .15$). As shown in Figure 5, after Bonferroni correction, Wilcoxon signed rank tests suggested that fibrinogen increased from baseline (2.39 ± 0.38) to poststress when uncorrected (2.57 ± 0.53) or corrected with plasma reconstitution (2.60 ± 0.55 , p values $< .001$) but was not significantly different from baseline when corrected arithmetically (2.42 ± 0.40 , $p = .02$) or corrected with saline reconstitution (2.43 ± 0.54 , $p = .07$). At recovery, fibrinogen was not significantly different from baseline for any plasma manipulation (p values $> .31$).

DISCUSSION

As expected, the TSST evoked significant changes in hemodynamic activity. Increases in BP during acute stress force plasma into interstitial spaces. Therefore, the experimental setting evoked the hemodynamic background for the hemoconcentration mechanisms under study. Moreover, Hct increased and plasma volume decreased during the TSST. Plasma volume, not Hct, returned to baseline levels 20 minutes after the stressor. Without correction for stress-induced hemoconcentration, APTT decreased and FVIII:C increased from baseline to poststress and returned to baseline levels at recovery, suggesting a transient elevation of activity of the intrinsic pathway of the coagulation cascade during stress that quickly returned to resting levels. Similarly, PT% increased from baseline to poststress, suggesting elevated activity of the extrinsic pathway of the coagulation cascade during stress. However, PT% shifted to an intermediate level at recovery that was not significantly different from baseline or poststress. D-dimer concentration increased significantly

from baseline to poststress and remained marginally elevated at recovery, whereas fibrinogen increased significantly from baseline to poststress and returned to baseline levels at recovery. However, the purpose of this study was to further understand the role that stress and hemoconcentration has on the changes in these coagulation parameters. The results of this study suggest that arithmetic correction is appropriate for concentrations and saline reconstitution is appropriate for time-dependent/functional assays, whereas plasma reconstitution is not appropriate for either.

When examining the different methods of correcting for stress and hemoconcentration, reconstitution with the participant's own plasma from baseline had no substantial effect on APTT, PT%, and D-dimer. Essentially, the values for APTT, PT%, and D-dimer during stress were not different than uncorrected values. Hence, hemostasis activity seemed to be unchanged by the baseline plasma reconstitution manipulation, suggesting that reconstitution with the person's own plasma from baseline did not significantly alter the plasma's environment. Although this technique brings additional amounts of clotting factors (e.g., D-dimer, fibrinogen) into the sample, the concentration actually decreases. To show this, we mathematically computed what the postreconstitution concentrations should be for D-dimer after baseline plasma reconstitution. However, these computed values were not significantly different from the values after baseline plasma reconstitution (analyses not shown). Unfortunately, this computational check cannot be performed with APTT, PT%, or FVIII:C because they are not concentrations. In addition, the findings for APTT and PT% are at odds with our previous study (21), which showed that APTT and PT in seconds were not different from baseline after baseline plasma reconstitution. However, examination of the means from our previous study suggests that the pattern of results was the same as in the current study. The most likely explanation for this discrepancy is that our previous study did not have enough statistical power ($n = 10$).

Conversely, arithmetic correction with the formula by Dill and Costill (15) and reconstitution with physiological saline both resulted in marked changes in APTT, PT%, and D-dimer. For APTT, arithmetic correction resulted in clotting time of the intrinsic pathway becoming even faster, suggesting that hemoconcentration masks part of the accelerated clotting time. For PT%, arithmetic correction resulted in a lower percentage, suggesting that clotting time of the extrinsic pathway is actually slower at poststress than at baseline when accounting for hemoconcentration. Saline reconstitution, on the other hand, resulted in APTT and PT% being no different from baseline, suggesting that hemoconcentration accounts for the observed decreases in clotting time of both the intrinsic and extrinsic pathways of the coagulation cascade, respectively. Finally, for D-dimer and fibrinogen concentration, both arithmetic correction and saline reconstitution removed the effects of stress, suggesting that the increase in D-dimer and fibrinogen concentration during acute stress is accounted for by hemoconcentration. With saline reconstitution, the absolute number of molecules did not change, but the concentration decreased significantly more than it did

with the plasma reconstitution. Therefore, the apparent increase in fibrinogen and subsequent fibrin formation (as indicated by D-dimer concentration) may be by a consequence of hemoconcentration and not necessarily because of actual activation of the coagulation system. It is not plasma, per se, that is lost during stress, but it is filtrate (or intravascular fluid) that moves across the capillary pores into the interstitial space. Saline more closely represents this filtrate, whereas the person's plasma from baseline still has clotting factors, proteins, and other large molecules in it. If there were genuine, or actual, coagulation activation, significant changes from baseline to poststress would still be expected even after saline reconstitution (such as seen with FVIII:C).

Compared with APTT, PT%, and D-dimer, FVIII:C at poststress was significantly greater after both reconstitution techniques than at baseline but was even greater after plasma reconstitution than after saline reconstitution. Given that the concentration of FVIII should decrease after either reconstitution method, its activity should also theoretically decrease. However, this was not the case. One possibility is that elevated catecholamine levels at poststress could stimulate FVIII:C regardless of concentration changes, indicating genuine activation of the intrinsic pathway of the coagulation system. Indeed, epinephrine infusion markedly increases FVIII:C (25). Conversely, FVIII:C at poststress corrected arithmetically was not significantly different than baseline, suggesting that hemoconcentration accounted for the change in FVIII:C. However, this interpretation is confounded by the limitations of the formula by Dill and Costill (15), which is only designed to correct for concentration changes that accompany plasma volume shifts.

Acute stress has been suggested to be a trigger of atherothrombosis and subsequent acute coronary syndromes. Defining mechanisms of this link is a critical issue in cardiovascular behavioral medicine. One plausible link is through altered hemostasis activity. Disruption of hemostatic balance (i.e., balance between coagulation and fibrinolysis) is a crucial factor in atherosclerotic development and CHD. If a plaque ruptures, vessel occlusion is more likely if the ratio of prothrombosis activity (i.e., promoting clot formation) to fibrinolysis activity (i.e., promoting clot dissolution) is high. During psychological stress both prothrombosis and fibrinolysis activity increases, but prothrombosis activity usually increases to a greater extent than fibrinolysis activity. The resulting net hypercoagulable state may put an individual at greater risk for thrombotic coronary occlusion after plaque disruption, especially in individuals with atherosclerosis or endothelial dysfunction (26,27).

The results of the present study suggest that hemoconcentration may contribute to stress-induced changes in hemostasis activity. Contracted plasma volume may temporarily provide a prothrombotic intravascular environment by allowing clotting factors to be physically closer together and through greater exposure of the endothelium to prothrombotic molecules. By increasing the relative concentration, not the absolute number of cells, the endothelium still "sees" more of that molecule. Moreover, when their concentrations increase, clotting factors have greater opportunity to act on each other and on platelets,

which in turn can hasten clotting (e.g., APTT and PT). Such an intravascular environment resulting from acute stress may promote atherogenesis or trigger an atherothrombotic event, especially in patient populations, pointing to the clinical significance of correcting for stress-hemoconcentration.

Several limitations of the current study must be noted. First, we used a small sample of convenience from a larger, ongoing study. However, the purpose of this portion of the larger study was to simply compare the plasma volume correction techniques. Second, collection and measurement errors occurred, leaving an even smaller sample size for HR, SBP, DBP, APTT, FVIII:C, and fibrinogen analyses. Third, our results cannot be generalized to women or individuals with atherothrombotic disease. This is especially important given that hypercoagulability can be especially harmful in individuals who have developed coronary artery disease. As a first step in bringing this issue further to light, we focused on a relatively homogenous sample of men. Future research ought to particularly examine the influence of hemoconcentration on stress-induced coagulation changes in such patient populations. Fourth, the reconstitution techniques used in this study cannot be applied to individuals who show an increase in plasma volume. For instance, one participant did not show the expected decrease in plasma volume after the TSST, although BP and HR increased in the expected direction. This participant's plasma volume increased 1.2% from baseline to poststress, whereas plasma volume decreased 6.1%, on average, for the entire sample. Therefore, no physical manipulations of his plasma were made, and hemostasis measures were not examined. Additional studies are needed to untangle why most, not all, individuals are susceptible to hemoconcentration effects during stress. Studies are necessary to determine how to correct for such plasma volume increases, or "hemodilution," that may occur, as well as whether such correction is even necessary. Finally, although the present study demonstrated that reconstituting affected plasma samples with saline was effective in correcting hemoconcentration effects on most of the coagulation parameters examined, future studies are needed to specifically examine whether varying the saline reconstitution amount (i.e., reconstitute with 50%, 100%, and 150% of plasma loss) changes clotting in a dose-response manner.

In conclusion, the different hemoconcentration correction techniques yielded different results. Mathematical adjustment is viable when examining stress-hemoconcentration effects on large molecular weight substances, such as D-dimer, but is problematic when examining stress-hemoconcentration effects on clotting time and clotting activity. The saline reconstitution technique may be a more biologically relevant correction method when examining stress-hemoconcentration effects on clotting time and clotting activity, whereas the plasma reconstitution technique had little effect on clotting time and activity. Altogether, the results of this study suggest that stress-induced changes in coagulation are a product of both hemoconcentration and actual coagulation activation. Future investigations should continue to explore interrelationships between hemoconcentration, clotting factor activity and clotting time.

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