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EFFECTS OF TRANSPORT TEMPERATURE ON THE STABILITY OF INFLAMMATORY, HEMOSTASIS, ENDOTHELIAL FUNCTION, AND OXIDATIVE STRESS PLASMA BIOMARKER CONCENTRATIONS

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Abstract

BACKGROUND—A number of studies in critically ill patients are conducted outside the hospital. Specimens should ideally be transported from out-of-hospital setting to a laboratory using dry ice, but this approach is expensive and may not be feasible in some circumstances. We, therefore, examined the impact of temperature during transport of specimens on the precision of biomarker concentrations.

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OBJECTIVE—To determine the effects of transport temperature conditions on biomarker concentrations in specimens processed within 1 hour of collection.

METHODS/PATIENTS—We simulated transport by storing specimens at four temperature conditions: packaged at -80°C (control), on dry ice (-79°C), on cold gel packs (4°C), and at room temperature (RT, 21°C). We examined 8 biomarkers spanning 4 signaling domains - inflammation, hemostasis, endothelial dysfunction, and oxidative stress. We calculated mean, median and percent difference for each biomarker concentration compared to the control transport temperature at -80°C in 26 subjects (16 hospitalized with severe sepsis and 10 non-hospitalized volunteers).

RESULTS—Patients with severe sepsis had log-fold higher median concentrations of IL-6, hs-CRP, D-dimer, E-selectin, sICAM-1, and sVCAM-1 compared to non-hospitalized volunteers ($p<0.05$). When specimens were combined, we observed a 7% difference in the mean and median IL-6, hs-CRP, D-dimer, PAI-1, E-selectin, s-ICAM, s-VCAM, and nitrite concentrations for dry ice and cold gel packs transport compared to transport at -80°C ($p>0.05$). Larger differences (up to 12%) were observed when biomarker concentrations for PAI-1 and s-VCAM at room temperature were compared to transport at -80°C ($p>0.05$).

CONCLUSIONS—Select inflammatory, coagulation, endothelial dysfunction, and oxidative stress biomarkers can be transported at 4°C on gel packs for 24 hours with minimal effects on precision.

Keywords

sepsis; IL-6; hs-CRP; sICAM-1; sVCAM-1; E-selectin; nitrate; PAI-1

INTRODUCTION

Characterization of circulating biomarker concentrations in both healthy and disease states is important to understand signaling mechanisms and for the development of personalized therapies. However, appreciable changes in biomarker concentrations may occur if the specimen is not handled appropriately. Specifically, suboptimal transport conditions may directly affect the concentration of the biomarker. Other factors that may introduce imprecision include differences in specimen collection techniques, delayed specimen processing, and inadequate storage conditions (1–3).

There has been an increase in studies conducted on critically ill patients in out-of-hospital settings. These studies are important to understand the disease and biomarker trajectories before hospital admission and following hospital discharge. Due to feasibility issues, blood specimens collected from patients in out-of-hospital settings may be stored at suboptimal temperature conditions. For instance, studies conducted in patients transported to the emergency department may require the storage of their specimens for several hours either in the field or an ambulance. The fore-mentioned environments may have drastically different temperatures. Similarly, a multicenter study examining mechanisms underlying poor long-term outcomes of critically ill patients may require collecting a specimen at the participant's home or in a nursing facility and shipping the sample by overnight courier to the coordinating center. Although dry ice is commonly used to ensure optimal temperature and maintain the integrity of the sample, it is a hazardous material, and individuals must receive

special training in its use. An alternative has been to store specimens at room temperature or use gel or ice packs that maintain the temperature at approximately 4°C. However, the effect of temperature on biomarker concentrations is poorly understood.

Previous studies have mainly selected common hematological and clinical chemistry analytes to examine the effects of temperature on analyte stability. These studies examined effects of temperature in the setting of delaying specimen processing, such as centrifugation, or storing the specimens following specimen processing over short and long intervals (4, 5). For studies conducted in critically ill patients, initial specimen processing can usually be performed promptly, but transport may be delayed. Identifying the effects of temperature during transport on biomarker stability is important to ensure high-quality specimen collection for analysis and the integrity and validity of clinical and research results.

We sought to determine the effects of transport temperature conditions on biomarker measurement in specimens obtained from non-hospitalized volunteers and hospitalized patients with severe sepsis processed within 1 hour of collection. We selected biomarkers from 4 signaling domains - inflammation, hemostasis, endothelial dysfunction, and oxidative stress and 4 transport temperature conditions (dry ice, cold gel pack, and room temperature, and -80°C) that were relevant to samples collected in out-of-hospital studies.

MATERIALS AND METHODS

Study Participants

The study was approved by the Institutional Review Board of the University of Pittsburgh, and before enrollment into the study, all participants provided written informed consent. We enrolled 16 patients with severe sepsis in the intensive care unit (ICU). We defined severe sepsis as infection with acute organ dysfunction in accordance with the 2001 International Consensus Criteria (6, 7). We also enrolled 10 non-hospitalized volunteers who did not report any symptoms of an acute infection.

Sample Collection and Processing

We collected six vacutainer tubes of blood (~30 ml) from each patient and volunteer. The blood collection included: two 4.5mL lithium heparin (PST™ Tube, 76 units, reference #367962), two 4.5 ml sodium citrate (3.2%, reference #366415) and two 6.0 mL K₂EDTA (10.8 mg, reference #367863) tubes of blood (Becton Dickinson and Company, Franklin, NJ). We standardized blood collection for the severe sepsis patients and volunteers. For severe sepsis patients, we collected venous blood via indwelling catheters after discarding the initial 10 ml of blood. For volunteers, trained phlebotomists collected blood via a peripheral venipuncture. We immediately transported all specimens to the laboratory for processing. We centrifuged all specimens at 1300g for 10 minutes at 18–25°C within 1 hour of collection. We aliquoted the plasma from the lithium heparin, sodium citrate, and K₂EDTA tubes into 0.5mL cryovials.

Simulation of Transport Conditions

To simulate transport conditions, we selected -80°C (freezer) as the control and compared the following 3 conditions, dry ice, cold gel pack, room temperature against the control (1, 8). We placed the 0.5mL cryovials that contained plasma aliquots (lithium heparin plasma, sodium citrate or K_2EDTA) inside of 100-cell specimen boxes. We placed the control box directly into a -80 freezer and packaged the test boxes into Styrofoam shippers for 24 hours at the following temperatures: dry ice, cold gel pack (12 packs at $2-8^{\circ}\text{C}$), and room temperature. Following the 24-hour transport simulation, we measured the temperature via a digital thermometer, removed the 100-cell specimen boxes from the Styrofoam shippers and stored them at -80°C until batch analysis.

Human Plasma Biomarker Assays

We used the Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA) to measure interleukin (IL)-6, plasminogen activator inhibitor (PAI)-1, endothelial selectin (E-selectin), soluble intracellular adhesion molecule (sICAM)-1, and soluble vascular adhesion molecule (sVCAM)-1. Specifically, the plasma type and methodology used were: for IL-6 and PAI-1, K_2EDTA and multiplex immunoassays, Bio-Rad Laboratories, Hercules, CA; for E-selectin, sICAM-1, and sVCAM-1, K_2EDTA and multiplex assays, R&D Systems, Minneapolis, MN, respectively.

We used the nitrate/nitrite colorimetric kit (Cayman Chemical, Ann Arbor, MI) to measure nitrate in lithium heparin plasma specimens spectrophotometrically (Synergy™ Mx Multi-Mode Microplate Reader, BioTek Instruments, Inc., Vermont). We used the AU5800 automated analyzer (Beckman Coulter, Brea, CA) to measure high-sensitivity C-reactive protein (hs-CRP) in lithium heparin plasma specimens. We used the STA-R® Evolution analyzer (Diagnostic Stago, Inc., Parsippany, NJ) to measure D-dimer in sodium citrate plasma specimens. The assays were performed in our Molecular Core Laboratory or at the University of Pittsburgh Medical Center, Presbyterian Hospital Automated Testing Laboratory.

We selected the above biomarkers because they are common mediators measured in observational studies and clinical trials in critically ill patients. Thus, the findings of this study can provide guidance as to which temperature condition is most appropriate to ship specimens.

Statistical Analyses

We compared biomarker concentrations for patients hospitalized with severe sepsis and volunteers. We used the Wilcoxon rank sum test for analysis because the data was not normally distributed. We calculated the mean and median concentrations for each biomarker for all four conditions. We determined biomarker stability by calculating the percent change for each biomarker for each of the four conditions. Specifically, we categorized the biomarker concentration observed for the 3 transport temperature conditions compared to control temperature as small differences ($< \text{or } =5\%$), moderate differences ($6-15\%$) and large differences ($>15\%$). The analyses were performed using both GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com), and

SAS 9.1 (SAS Institute, Cary, NC). We had 80% power to detect a difference of 0.7 SD and defined statistical significance at $p < 0.05$.

RESULTS

Patient characteristics

The mean age ($\pm$ standard deviation [SD]) for the 16 severe sepsis patients was 57.4 ± 14.4 years. Eight (50%) were women, and all patients were white (self-reported race). Pneumonia, intra-abdominal infection, and skin and soft tissue infections were the most common infections, occurring in 5, 4, and 2 patients, respectively. As expected, all patients had, at least, 1 organ dysfunction. The majority (91%, 14 out of 16 patients) had acute kidney injury, 8 (50%) had an acute respiratory failure and received mechanical ventilation, and 7 (43.7%) had septic shock and received vasopressors. The 10 volunteers were <45 years of age, all were women, and none reported taking medications for chronic diseases. None of the volunteers reported recent symptoms consistent with an infection.

Differences in biomarkers concentrations between severe sepsis patients and volunteers

As expected, several circulating plasma biomarker concentrations were elevated in the patients with severe sepsis compared to the volunteers for samples transported at -80°C (Table 1). For example, compared to volunteers, patients with severe sepsis had higher median concentrations of IL-6 (115 vs. 2.5 pg/ml, $p < 0.0001$), hs-CRP (6.7 vs. 0.18 mg/dl, $p < 0.0001$), E-selectin (40 vs. 25 ng/ml, $p = 0.03$), sVCAM-1 (8142 vs. 780 ng/ml, $p < 0.0001$), sICAM-1 (937 vs. 295.3 ng/ml, $p = < 0.0002$) and D-dimer (3.5 vs. 0.49 $\mu\text{g/ml}$, $p = 0.001$). The median circulating nitrate concentrations were also higher, but the results did not reach statistical significance.

Effect of transport temperature on plasma biomarker concentrations

The temperature around the dry ice was -79°C , and most of the dry ice was still solid in the shipping containers following the 24-hour transport simulation period. The temperature around the cold gel packs ranged from $2-7^{\circ}\text{C}$ (mean 4°C ; SD 1.4) at the time of packaging and ranged from $8-19^{\circ}\text{C}$ (mean 13.4°C ; SD 3.03) at the end of the simulation. The room temperature in the air conditioned room, where the experiment was conducted, ranged from $20-25^{\circ}\text{C}$ (mean 22°C ; SD 1.5) at the start and from $20-22^{\circ}\text{C}$ (mean 21°C ; SD 0.82) at the end. The control temperature was maintained at -80°C throughout the experiment. The concentration of each biomarker and the four transport temperature conditions are shown in Supplemental Table 1.

For inflammatory markers (Table 2), such as IL-6 and hs-CRP, we observed a small, non-significant, ($< 5\%$) difference in mean and median concentrations when simulating transport with dry ice ($p = 0.41$ and 0.55 ; $p = 0.95$ and 0.78), cold gel packs ($p = 0.43$ and 0.77 ; $p = 0.94$ and 0.78), and room temperature (0.61 and 0.77 ; 0.99 and 0.78), compared to -80°C transport. For hemostasis markers, such as D-dimer, we observed a small, non-significant difference ($< 2\%$) in mean and median concentrations for all three conditions (dry ice: $p = 0.86$ and 0.78 ; cold pack gel: $p = 0.98$ and 0.78 ; room temperature: $p = 0.84$ and 0.89). Furthermore, there were small differences ($< 2\%$) in PAI-1 concentrations when transport

was simulated using dry ice ($p=0.62$ and 0.88) and cold gel packs ($p=0.80$ and 0.88). However, we observed moderate to large differences (19.8% and 11.1%) in mean and median concentrations when transport was simulated using room temperature compared to -80°C transport ($p=0.11$ and 0.88). For endothelial dysfunction markers, such as E-selectin, sVCAM-1, and sICAM-1, we observed small differences ($<4\%$) that were not statistically significant when transport was simulated using dry ice ($p=0.83$ and 0.77 ; $p=0.98$ and 0.77 ; and $p=0.94$ and 0.77) and cold gel packs ($p=0.85$ and 0.88 ; $p=0.73$ and 0.88 ; and $p=0.82$ and 0.88). However, moderate differences for E-selectin and sVCAM-1 (3.6–8.1%) were observed when transport was simulated using room temperature compared to -80°C transport ($p=0.74$ and 0.77 ; $p=1.00$ and 0.77).

DISCUSSION

Transport of blood specimens under sub-optimal temperature conditions may compromise biomarker stability. To our knowledge, this is the first study that collectively examines the effects of transport temperature conditions on biomarkers implicated in infection and severe sepsis. In the present study, we investigated an understudied area in biomarker research, the effect of temperature during ‘transport’, which is the critical interval between specimen processing and storage. We observed small, non-significant differences in IL-6, hs-CRP, D-dimer, nitrate, and sICAM-1 concentrations when transport was simulated using dry ice, cold gel packs, or room temperature compared to -80°C . In contrast, large appreciable differences in concentrations in PAI-1 and moderate differences in E-selectin, and sVCAM-1 were observed when transport was simulated at room temperature compared to -80°C .

Previous studies have focused on the effects of temperature on laboratory analytes, such as general clinical chemistry and hematological tests in two settings: i). Delayed specimen processing after phlebotomy (i.e. centrifugation); and ii). Short, moderate, or long- term storage periods over days, months, or years (see Supplemental Table 2) (5, 9–12). The results of these prior studies and our study suggest that transporting specimens in cold gel packs at 4°C may not affect the results of select biomarkers.

We noted small differences in IL-6 concentrations among the four transport conditions (dry ice, cold gel pack, room temperature and -80°C). Along similar lines, Friebe et al. examined temperature effects during a 24-hour storage period on IL-6 plasma concentrations and reported comparable concentrations amongst plasma specimens stored at room temperature, -20°C and -70°C (13). We are not aware of studies assessing the effect of transport temperature conditions on hs-CRP. We observed small differences in hs-CRP concentrations among the four transport conditions, similar to results reported by Aziz and colleagues (14).

We observed moderate differences in E-selectin, and sVCAM-1 concentrations when transported for a 24-hour period at room temperature compared to -80°C . Similarly, Hartweg and colleagues reported no differences in concentrations of these biomarkers among specimens stored at 4°C , 21°C , and 30°C compared to specimens stored at -80°C .

Our D-dimer findings confirm and extend the previous report of Vukovich et al. on the stability of sodium citrate plasma D-dimer concentrations as the researchers reported no

difference in patient specimens stored at room temperature for 24 h or in liquid nitrogen (15). Plasma nitrate was not influenced by transport temperature as evidenced by comparable concentrations among the different transport temperature conditions, in line with similar observations by Moshage et al., which reported comparable recovery of nitrite and nitrate from spiked plasma stored at both 4°C and room temperature (16).

Strengths of our study include that we selected biomarkers that comprise 4 signaling domains relevant to critical illness. We included both, patients in the ICU who were diagnosed with severe sepsis and volunteers with no signs of acute infection. Thus, we examined the effects of transport temperature conditions across a wide biomarker concentration range. The inclusion of critically ill patients who had elevated concentrations of biomarkers allowed us to determine if the increased production of the biomarkers would be missed if the specimen was transported under certain temperature conditions. Our study models biomarker testing that is performed using outpatients specimens in a setting outside of the hospital (e.g. personal home, long-term care facility, nursing home). Specifically, in these settings, study coordinators are trained to perform minimal specimen processing (short spins via centrifuge and aliquoting via plastic transfer pipettes), and the specimens are then transported overnight to the coordinating research site's laboratory.

Our study has limitations. First, our sample size allotted us 80% power to detect a difference of 0.7 SD. Thus, larger differences, such as differences observed in PAI-1 concentrations among specimens measured at -80°C and room temperature, may not be significant due to inadequate power. However, the differences in most biomarkers measured at -80°C and gel packs were small. Studies that use gel packs to transport specimens should be careful in interpreting their results. For example, if a study shows large differences (e.g., log-fold differences) in biomarker concentrations between patients, then these differences cannot be solely due to differences in temperature during shipping. However, if a study shows small differences (e.g., <5%) in biomarker concentrations between patients, these differences could be attributed to differences in temperature during shipping. Second, we did not match the control group to the sepsis group because the main objective of the study was to examine the effect of temperature conditions across a range of biomarker concentrations. Third, we simulated room temperature transport conditions in an air-conditioned room and may not be generalizable to temperature during transport as seasonal changes in temperature may occur.

Our results have important implications for out-of-hospital studies measuring these plasma biomarkers, eliminating the need to transport specimens on dry ice. Our results suggest that IL-6, hs-CRP, E-selectin, sVCAM-1, sICAM-1, PAI-1, nitrate, and D-dimer concentrations are affected minimally when the specimen is transported at 4°C. While many of these biomarkers may be stable even at room temperature, the environmental temperature may vary significantly during transportation, particularly during summer. Thus, gel packs are a viable alternative to dry ice to transport specimens for studies that require measurement of these biomarkers in critically ill patients in the pre-hospital or after hospital discharge setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1Circulating biomarker concentrations (mean, median, interquartile range) at -80°C transport^{*}

Biomarkers	Volunteers	Severe Sepsis Patients	p-value
	N mean, median (IQR)	N mean, median (IQR)	
IL-6, pg/mL	10 3.1, 2.5 (1.9, 3.1)	12 404, 115 (36, 529)	<0.0001
hs-CRP, mg/dL	10 0.26, 0.18 (0.08, 0.39)	16 8.2, 6.7 (4.7, 9.5)	<0.0001
E-Selectin, ng/mL	10 26, 25 (17, 38)	12 63, 40 (33, 104)	0.03
sVCAM, ng/mL	10 884, 780 (649, 1148)	12 9546, 8142 (5343, 12091)	<0.0001
sICAM, ng/mL	10 355, 295 (190, 390)	11 990, 937.7 (721, 1400)	0.0002
PAI-1, ng/mL	10 14, 14 (11, 17)	12 18, 18 (11, 22)	0.20
D-dimer, $\mu\text{g/mL}$ FEU	4 0.50, 0.49 (0.41, 0.61)	15 6.2, 3.8 (2.1, 11)	0.001
Nitrate, μM	7 3.8, 0.80 (0.41, 7.6)	12 7.2, 5.9 (1.7, 12)	0.13

^{*} For each specimen the total specimens may not equal n=10 volunteers and n=26 severe sepsis patients because a specimen for the specific biomarker was not available for the temperature condition and a result was not obtained.

Table 2

Percent Difference In Biomarker Concentrations: Dry Ice, Cold Pack and Room Temperature Conditions Compared to -80°C^a .

Biomarker	n ^	Median [IQR ^β] Mean [SD]		
		Dry Ice	Cold Gel Pack	Room Temperature
Inflammation				
IL-6, pg/mL ^a	22	0 (−7.4,17.3) 5.0 (19.3)	−0.8 (−8.0, −14.7) 2.5 (19.3)	−0.4 (−12.7,14.9) 2.2 (24.2)
hs-CRP, mg/dL ^b	26	−0.5 (−3.1, 2.0) 0.3 (7.6)	−0.4 (−2.3, 0.5) −0.9 (2.8)	−0.1 (−1.8, 1.7) 0.1 (3.1)
Endothelial Function, ng/mL				
E-selectin ^c	22	−2.7 (−9.0, 3.1) −1.3 (10.1)	−5.5 (−7.6, 1.2) −2.5 (10.8)	−6.0 (−9.3, 5.2) −3.6 (10.1)
sVCAM-1 ^d	22	−6.7 (−12.4, 3.7) −3.1 (14.0)	−3.4 (−8.2, 5.3) −3.1 (20.0)	−9.5 (−12.3, 0.3) −8.1 (13.1)
sICAM-1 ^e	22	−2.8 (−5.4, 1.4) −1.1 (7.3)	−2.6 (−7.1, −0.4) −3.3 (6.7)	−2.4 (−5.9, 4.8) −1.4 (8.0)
Hemostasis				
PAI-1, ng/mL ^f	21	−1.4 (−7.7, 11.7) 1.5 (12.9)	−0.1 (−10.6, 7.1) 2.0 (14.8)	11.1, (5.8, 31.2) 19.8 (24.9)
D-dimer, µg/mL FEU ^g	26	0.6 (−2.8, 5.0) 1.6 (14.8)	0 (−1.9, 6.0) 1.8 (19.1)	0 (−1.3, 8.9) 1.6 (14.9)
Oxidative Stress				
Nitrate, µM ^h	20	2 (1, 4) 1.5 (1.5)	−4 (−5.3) −2.3 (3.7)	−5 (−8, 5) −2.4 (1)

^aThe biomarker concentration is reported as percent change from the -80°C biomarker concentration. The percent change was calculated as follows: Dry ice (Cold gel pack or Room temperature) transport biomarker concentration subtracted from -80°C transport biomarker concentration divided by -80°C transport biomarker concentration multiplied by 100.

^β_{IQR} – Interquartile Range.

[^] For each specimen the total specimens may not equal n=26 because a specimen for the specific biomarker was not available for the temperature condition and a result was not obtained. IL-6, Interleukin- 6; hs-CRP, high sensitivity C-Reactive Protein; sVCAM, soluble vascular adhesion molecule; sICAM, soluble intercellular adhesion molecule 1; and PAI-1, Plasminogen activator inhibitor-1. The following are the mean and median p-values for each biomarker and dry ice, cold gel pack and room temperature vs -80°C transport:

^a dry ice p=0.41 and 0.55, cold gel pack p=0.43 and 0.77, room temperature p=0.61 and 0.77;

^b dry ice p=0.95 and 0.78, cold gel pack p=0.94 and 0.78, room temperature p=0.99 and 0.78;

^c dry ice p=0.83 and 0.77, cold gel pack p=0.85 and 0.88, room temperature p=0.74 and 0.77;

^d dry ice p=0.98 and 0.77, cold gel pack p=0.73 and 0.88, room temperature p=1.00 and 0.77;

^e dry ice p=0.94 and 0.77, cold gel pack p=0.82 and 0.88, room temperature p=0.87 and 0.77;

^f dry ice p=0.62 and 0.88, cold gel pack p=0.80 and 0.88, room temperature p=0.11 and 0.88;

^g dry ice p=0.86 and 0.78, cold gel pack p=0.98 and 0.78, room temperature p=0.84 and 0.89; and

h
dry ice p=0.72 and 0.77, cold gel pack p=0.57 and 0.77, room temperature p=0.24 and 0.77.

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