Technical note: Determination of preanalysis storage temperature and time allowances for ex vivo innate immune responses

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ABSTRACT

Evaluation of ex vivo innate immune responses requires the transport of blood to a laboratory for analyses. Storage temperature and duration are highly variable in the published literature. The objective of the present study was to establish recommended storage temperature and duration for blood before analysis of several ex vivo innate immune responses in vitro. Peripheral blood was collected from 8 clinically healthy, 12-mo-old Holstein heifers and stored either on ice or at ambient temperature for 1.5, 3.5, 5.5, 7.5, 9.5, and 24 h before conducting analyses for lipopolysaccharide stimulated whole blood tumor necrosis factor- α (TNF- α) secretion, neutrophil L-selectin expression, and neutrophil phagocytic and oxidative burst capacities when exposed to an *Escherichia coli*. L-Selectin expression was greater in neutrophils stored on ice as early as 1.5 h after blood collection. In addition, when neutrophils were stored on ice, L-selectin expression at 24 h was not different from that at 1.5 h after blood collection. Measures of innate immune cell function, including whole blood TNF- α secretion and neutrophil phagocytic and oxidative burst abilities, were decreased in blood stored on ice. Both TNF- α secretion and neutrophil oxidative burst intensity did not differ at 24 h compared with 1.5 h after blood collection when blood was stored at ambient temperatures. These data indicate that both storage temperature and duration influence measures of innate immune responses. Therefore, storage durations should be minimized and storage protocols kept consistent to reduce the influences of storage conditions on experimental results.

Key words: assay, immune, temperature, time

Technical Note

Although it is always most optimal to assay biological materials as close to the time of collection as

possible, it is often convenient, even sometimes necessary, to store biological samples for some period until proper analyses can take place. This storage facilitates transport of samples to various locations and affords a limited amount of convenience regarding when samples must be assayed. As with all experiments involving the storage and later processing of biological materials, extra consideration is needed to ensure that measurements obtained are not greatly influenced by the storage process.

Ex vivo measurements of many innate immune responses are common research endpoints in a variety of fields of study including, but not limited to, immunology, nutrition, stress physiology, and welfare. Many of the innate immune responses are decreased when animals are stressed (Hulbert et al., 2011a,b), increased during infection and disease (Ballou et al., 2011), and are causative to the incidence and severity of peripartum clinical mastitis (Heyneman et al., 1990). Storage protocols for blood before ex vivo innate immune response analyses are quite variable (Chaveiro and Moreira da Silva, 2009; Keogh et al., 2011; Perdomo et al., 2011) or often not adequately described (Czeslick et al., 2006; Pang et al., 2009; Silvestre et al., 2011). Therefore, with minimal literature to support any specific storage protocol, the decision for various analyses lies with recommendations from assay manufacturers and the intuition of the investigators. The objective of the current study was to determine if temperature and duration of blood storage influence the values of various ex vivo innate immune responses.

All animal care and use was approved by the Texas Tech University (Lubbock) Animal Care Committee. Peripheral blood samples (two 10-mL heparinized and two 3-mL EDTA evacuated tubes) were collected from eight 12-mo-old Holstein dairy heifers. One evacuated tube with each additive was immediately placed on ice (**IC**; $0.2 \pm 0.39^\circ\text{C}$) and the other in an ice chest with no ice at ambient temperature (**AMB**; $21.9 \pm 0.51^\circ\text{C}$). Ex vivo innate immune responses were serially analyzed at 1.5, 3.5, 5.5, 7.5, 9.5, and 24 h after blood collection. Prior to aliquoting the blood at each time point, the blood tubes were mixed thoroughly on

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a laboratory rocker for at least 2 min and then immediately returned to their respective storage conditions. Ex vivo innate immune responses measured included diluted whole blood secretion of tumor necrosis factor- α (TNF- α) when stimulated with LPS, neutrophil surface L-selectin expression, and neutrophil phagocytosis and oxidative burst capacities when cocultured with an enteropathogenic *Escherichia coli*. The authors chose to sample up to 24 h after blood collection, as this time point represented a scenario where whole blood could be shipped overnight to accommodate various logistical challenges. Although heparin has been shown to affect immune cell morphology, and potentially function, in vitro and is not the recommended anticoagulant for measurement of functional ability of bovine leukocytes (Carlson and Kaneko, 1973), the authors chose to use heparin instead of EDTA or acid citrate dextrose, as the action of the latter anticoagulants relies on chelation of calcium. Cytokine release (Watanabe et al., 1996) and neutrophil phagocytosis and oxidative burst (Nunes and Demaurex, 2010) are calcium-dependent functions, and neutrophil phagocytosis is decreased when blood is diluted with EDTA or acid citrate dextrose when compared with heparin (Ducusin et al., 2001). Measurement of neutrophil L-selectin expression is not known to be adversely affected by calcium-chelating agents, and was performed on blood collected over EDTA.

All methodologies used in the current study have been described previously by Hulbert et al. (2011a,b) and will only be discussed briefly here. For measurement of whole blood secretion of TNF- α , 200 μ L of heparinized whole blood was cultured with 800 μ L of RPMI 1640 (no. 11875-085; Invitrogen Life Technologies Corp., Grand Island, NY) containing a final concentration of 1% antibiotic/antimycotic (no. 15240-062; Invitrogen Life Technologies Corp.) and 1 μ g/mL of LPS from *E. coli* O111:B4 (Sigma-Aldrich, St. Louis, MO), and incubated at 39°C in a water-jacketed incubator in an atmosphere of 5% CO₂ for 24 h. Following incubation, cultures were centrifuged at 1,000 $\times g$ for 15 min and the resulting supernatant was collected for TNF- α quantification using a commercially available ELISA kit (no. DY2279; R&D Systems Inc., Minneapolis, MN). The intra- and interassay coefficients of variation for the TNF- α ELISA were 4.2 and 5.4%, respectively. For quantification of L-selectin protein on the neutrophil surface, a mouse anti-bovine anti-CD62L IgG primary antibody (VMRD Inc., Pullman, WA) was diluted 1:200 in PBS and added to 100 μ L of whole blood collected over EDTA, and incubated in an ice bath for 1 h. Following incubation, red blood cells were hypotonically lysed and washed an additional 2 times before being resuspended in fluorescein-labeled secondary antibody [F(ab')₂ goat anti-mouse; AbD Ser-

otec Inc., Raleigh, NC] at a 1:400 dilution in 1 $\times$ PBS. Negative control samples for the neutrophil L-selectin expression assay included samples that received secondary antibody or 1 $\times$ PBS only. Samples were incubated with the secondary antibody in an ice bath for 1 h, and then washed 3 times using 1 $\times$ PBS. Samples were resuspended in 1 $\times$ PBS for flow cytometric analysis (Cell Lab Quanta SC; Beckman Coulter Inc., San Jose, CA). Neutrophils were gated based on side scatter and cellular volume. A small acquisition gate was placed in the middle of the first decade of the FL-1 histogram for neutrophils only. The photomultiplier tube (PMT) voltages were adjusted at the 1.5-h samples, so the peak FL-1 fluorescence intensities of the negative controls were in the small acquisition gate. The peak FL-1 fluorescence intensities of the negative controls of subsequent sampling times did not deviate from the original acquisition gate. The FL-1 geometric mean fluorescence intensity (GMFI) of cells determined to be neutrophils was reported. For measurement of simultaneous neutrophil phagocytic and oxidative burst capacities, 200 μ L of heparinized whole blood was chilled in an ice bath to quash cellular activity before addition of 40 μ L of 5 mM dihydrorhodamine 123 (DHR, no. D-23806; Invitrogen Life Technologies Corp.) in dimethyl sulfoxide and 40 μ L of heat-killed propidium iodide (PI)-labeled nonopsonized enteropathogenic *E. coli* harvested from the spleen of a septicemic calf. After the addition of DHR and PI-labeled *E. coli*, cultures were incubated in a 39°C recirculating water bath for 10 min to promote cellular activity, and immediately returned to the ice bath for 15 min to again quash cellular activity at a constant rate. Negative control samples for simultaneous flow cytometric measurement of neutrophil oxidative burst and phagocytosis included (1) blood cultured with DHR but no bacteria, which was incubated for 10 min at 39°C and (2) blood cultured with PI-labeled bacteria, which remained on ice. Red blood cells were hypotonically lysed, washed twice, and resuspended in 1 $\times$ PBS for flow cytometric analysis. Neutrophils were gated and negative controls used to set PMT voltage settings as described above. The peak FL-1 and FL-3 fluorescence intensities of the negative controls at all sampling times did not deviate from the acquisition gates set at 1.5 h. Neutrophils from *E. coli*-stimulated samples with FL-1 and FL-3 emissions greater than the oxidative burst and phagocytosis negative controls were considered positive for oxidative burst and phagocytosis, respectively. The GMFI for both oxidative burst-positive and phagocytosis-positive neutrophils were calculated.

Review of graphs of individual animal responses over time confirmed that all animals did not respond similarly to IC and AMB treatments, and that ranks

Figure 1. Effects of storage temperature and duration of storage on tumor necrosis factor- α secretion from whole blood stimulated with LPS when blood was stored on ice (IC) or at ambient temperature (AMB) before analysis. *Means across TEMP within a time point differ at $P < 0.05$. Temperature (TEMP) $\times$ time: $P = 0.83$; TEMP: $P < 0.001$; time: $P = 0.91$. Data are presented as means $\pm$ SEM.

of animals within treatments across time points were not highly correlated. Normality was confirmed before statistical analyses. All data were analyzed by ANOVA using the PROC MIXED in SAS (v9.2; SAS Institute Inc., Cary, NC) after fitting a linear model with the fixed effects of storage temperature, time, and the interaction of temperature $\times$ time. Compound symmetry and autoregressive (1) covariance structures were tested for modeling the correlation among repeated measures data, and covariance structure used was chosen based on the Bayesian information criteria. Animal variation within a treatment was also treated as a fixed effect to reduce between-treatment variation due to differences in animal responses, as differences between subjects were not of interest in the study. Pairwise comparisons between time points within a treatment and between treatments at each time point were performed using the Student's t -test. Differences between means were considered significant when $P \leq 0.05$. Data are reported as least squares means $\pm$ standard error of the mean.

Whole blood receiving the IC treatment and stimulated with LPS had decreased ($P < 0.0057$) TNF- α concentrations in culture compared with the AMB treatment at all time points; however, no differences ($P = 0.97$) were observed within either storage temperature over time (Figure 1). These data indicate that when whole blood is stored on ice before culture, the proportion of secreting leukocytes is diminished or the secretory ability of the leukocyte population is decreased, or both factors are occurring simultaneously.

Because no difference occurred within storage temperature over time, the results of the whole blood TNF- α assay remained consistent for at least 24 h following blood collection.

The GMFI of L-selectin protein on neutrophils was influenced by storage temperature; however, the effect of temperature varied over time (temperature $\times$ time, $P < 0.028$; Figure 2). L-Selectin GMFI was not largely influenced by duration of storage within either storage temperature, with the exception of a transient decrease ($P < 0.003$) at 7.5 h among neutrophils from IC, followed by an immediate return to baseline values. The L-selectin protein is known to be shed from the surface of neutrophils during various physiological conditions (McGill et al., 1996; Ferri et al., 2009). Due to the fact that a large magnitude of difference in L-selectin protein was observed as early as 1.5 h after blood collection, it can be concluded that neutrophils from IC blood do not produce additional L-selectin, but rather, AMB storage must induce shedding of the L-selectin protein on neutrophils. As the L-selectin protein was consistently greater in neutrophils from IC blood, it is recommended that blood be stored on ice before L-selectin quantification to capture an accurate estimate of physiological values.

The percentage of neutrophils from whole blood, positive for phagocytosis of an *E. coli*, did not differ ($P = 0.78$) between IC and AMB blood at 1.5 h (Figure 3a). The percentage of phagocytizing neutrophils from IC

Figure 2. Effects of storage temperature and duration of storage on geometric mean fluorescence intensity of neutrophils positive for expression of the adhesion molecule L-selectin when blood was stored on ice (IC) or at ambient temperature (AMB) before analysis. †Means within a temperature (TEMP) are different from 1.5-h measurement at $P < 0.05$. *Means across TEMP within a time point differ at $P < 0.05$. TEMP $\times$ time: $P < 0.028$. Data are presented as means $\pm$ SEM.

Figure 3. Effects of storage temperature and duration of storage on (a) percentage of neutrophils positive for phagocytosis of a pathogenic *Escherichia coli* and (b) geometric mean fluorescence intensity (GMFI) of phagocytosis-positive neutrophils when blood was stored on ice (IC) or at ambient temperature (AMB) before analysis. †Means within a temperature (TEMP) are different from 1.5-h measurement at $P < 0.05$. *Means across TEMP within a time point differ at $P < 0.05$. For percentage of neutrophils positive for phagocytosis, TEMP $\times$ time: $P < 0.001$. For GMFI of phagocytosis-positive neutrophils, TEMP $\times$ time: $P < 0.007$. Data are presented as means $\pm$ SEM.

blood was decreased ($P < 0.0027$) for the remainder of the study when compared with neutrophils from AMB blood. The percentage of phagocytizing neutrophils from AMB blood did not decrease throughout the observation period, whereas the percentage of phagocytizing neutrophils from IC blood decreased ($P < 0.0001$) as early as 5.5 h and continued to decrease through to 24 h after blood collection. A minimal increase ($P < 0.003$) in the percentage of phagocytizing neutrophils from AMB blood was observed at 9.5 and 24 h after

Figure 4. Effects of storage temperature and duration of storage on (a) percentage of neutrophils positive for oxidative burst response against an enteropathogenic *Escherichia coli* and (b) geometric mean fluorescence intensity (GMFI) of oxidative burst-positive neutrophils when blood was stored on ice (IC) or at ambient temperature (AMB) before analysis. †Means within a temperature (TEMP) are different from 1.5-h measurement at $P < 0.05$. *Means across TEMP within a time point differ at $P < 0.05$. For percentage of neutrophils positive for oxidative burst response, TEMP $\times$ time: $P < 0.001$. For GMFI of neutrophils positive for oxidative burst response, TEMP $\times$ time: $P < 0.001$. Data are presented as means $\pm$ SEM.

blood collection. Similar to the percentage of neutrophils phagocytizing at 1.5 h, no difference ($P = 0.60$) was observed in the GMFI of the phagocytosis-positive neutrophils between storage temperatures (Figure 3b). The GMFI of phagocytizing neutrophils from IC blood did not change ($P = 0.19$) over time. In contrast, GMFI of phagocytizing neutrophils increased ($P < 0.04$) at 9.5 and 24 h after blood collection in AMB blood.

Similar to the neutrophil phagocytosis data, both the percentage of neutrophils positive for oxidative burst

Table 1. Recommended storage duration and temperature for whole blood before analysis when stored either on ice (IC) or at ambient temperature (AMB) for ex vivo measures of the innate immune response

Variable	Recommended storage duration, ¹ h		Recommended temperature ²
	IC	AMB	
TNF- α ³	24 ⁴	24 ⁴	AMB
L-Selectin, GMFI ⁵	24 ^{4*}	7.5	IC
Phagocytosis, %	1.5	5.5	AMB
Phagocytosis, GMFI	24 ⁴	5.5	AMB
Oxidative burst, %	5.5	<1.5	AMB
Oxidative burst, GMFI	<1.5	24 ^{4*}	AMB

¹Recommended allowable storage duration of blood at each temperature based on the time point of the study at which each variable was different from 1.5 h (baseline) after blood collection, or the point at which storage duration at a temperature influenced the result.

²Recommended storage temperature based on which storage temperature provided the most stable values relative to 1.5 h after blood collection values.

³TNF- α = tumor necrosis factor- α .

⁴The study concluded at 24 h to simulate a possible scenario of shipping blood samples overnight. Therefore, 24 h may not represent the maximum allowable storage duration.

⁵GMFI = geometric mean fluorescence intensity.

*Differences ($P < 0.05$) were observed within a treatment during a single central time point, but subsequent measurements were not different ($P > 0.05$) from baseline values.

and the GMFI of the oxidative burst positive neutrophils were not different ($P = 0.35$) at 1.5 h after blood collection between storage temperatures (Figure 4a). The percentage of neutrophils positive for an oxidative burst was greater ($P < 0.011$) in AMB blood from 5.5 through 24 h after blood collection. Similar to the percentage of phagocytizing neutrophils, the percentage of oxidative burst-positive neutrophils from IC blood decreased ($P < 0.028$) at 9.5 and 24 h after blood collection. In addition, neutrophils from AMB blood had an increased ($P < 0.019$) oxidative burst percentage from 3.5 through 24 h postchallenge. With the exception of a transient decrease ($P < 0.0015$) at 5.5 h, the GMFI of oxidative burst-positive neutrophils from AMB blood did not change over time (Figure 4b). In contrast, the GMFI of oxidative burst-positive neutrophils from IC blood decreased ($P < 0.0004$) as early as 3.5 h and continued to decrease for the remainder of the observation period. The GMFI of oxidative burst-positive neutrophils from AMB blood was greater ($P < 0.0005$) from 3.5 h through 24 h after blood collection.

Considering the neutrophil phagocytosis and oxidative burst data together, it is clear that storage temperature and duration of whole blood significantly affects measured neutrophil function. Specifically, when blood is stored on ice, a rapid decrease occurs in the abilities of neutrophils to both phagocytize *E. coli* and produce an oxidative burst. It is well known that the phagocytic and oxidative burst capacities of neutrophils in an ice bath are almost nonexistent. Therefore, these data indicate that prolonged storage of neutrophils in whole blood on ice leads to a nonreversible decrease in the measured phagocytic and oxidative burst func-

tion. It can be assumed that the circulating neutrophil population comprises cells of all ages, with some cells being newly generated, and others becoming apoptotic. Conceivably, the newer neutrophils may have increased functionality over the older, near-death neutrophils, and these subpopulations may respond differently to storage at different temperatures. Regardless of the mechanism, it is recommended that whole blood be stored at ambient temperature for generally less than 7.5 h after blood collection before analyses for neutrophil phagocytic and oxidative burst capacities.

Although we cannot completely deduce the mechanism by which storage temperature and duration of whole blood influence ex vivo innate immunological responses, these data indicate that both storage temperature and duration of whole blood influence these measurements and caution should be exercised to minimize such effects. Recommended allowable storage duration of blood at each temperature (as outlined in Table 1) is based on the time point of the study at which each variable was different from 1.5 h (baseline) after blood collection, or the point at which storage duration at a specific temperature influenced results. As the study was only designed to simulate a potential overnight shipment of blood, it cannot be concluded that 24 h is the maximum allowable storage duration for these assays. It can, however, be concluded that, for those assays for which 24 h is listed as the allowable storage duration, storage up to 24 h appears to have little or no adverse effect on the variables measured in this study. The recommended storage temperature (as outlined in Table 1) is based on which storage temperature provided the most stable values relative to 1.5

h after blood collection values. Therefore, measures of induced cellular activity, such as whole blood secretion of TNF- α when stimulated with LPS and neutrophil phagocytic and oxidative burst capacities when cocultured with an *E. coli*, are decreased when whole blood is stored on ice. In contrast, when thermosensitive cell-surface proteins such as L-selectin are measured, storage on ice is necessary to maintain expression similar to in vivo levels. Generally, storage durations should be minimized and storage protocols kept consistent to reduce the influences of storage conditions on experimental results.

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