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Original Articles

A Novel Centrifugation Method Using a Cell Salvage Device Offers an Alternative to the Use of Leukocyte-Depleting Filters for Autologous Blood Transfusions

Michael Barchilon, BA;† Cristina Gaspar, MS, CCP;* Angela Mexas, PhD;†
Don Nieter, DVM, CCP-Emeritus*

**Cardiovascular Science Program, Midwestern University, Glendale, Arizona; and †College of Veterinary Medicine, Midwestern University, Glendale, Arizona, Midwestern University College of Health Sciences, Glendale, Arizona*

Abstract: Autotransfusion protocols often use the use of costly filters, such as leukocyte-depleting filters (LDFs), to minimize reinfusion of activated leukocytes and inflammatory mediators associated with reperfusion injury (RI). LDFs are used extensively in hospital settings; however, they represent an additional capital expenditure for hospitals, as well as a constraint on the reinfusion rate of blood products for health-care providers. We compared a commonly used LDF to a novel centrifugation method employing a widely used cell salvage device. Complete blood counts and enzyme-linked immunosorbent assays (ELISAs) measuring tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) were performed to compare the efficacy of these methodologies. The LDF removed, on average, 94% of all leukocytes, including 96% of neutrophils. The centrifugation method removed, on average, 89% of all leukocytes, including 91% of neutrophils and resulted in a highly concentrated red blood cell product. Our results suggest both methods offer equivalent leukocyte reduction. TNF-α was also comparably reduced following our novel centrifugation method and the LDF method and IL-2 levels were undetectable in all samples. These results indicate our novel centrifugation method may preclude the need for a LDF during select autotransfusion applications. **Keywords:** blood transfusion, cardiopulmonary bypass, inflammatory mediators (e.g, cytokines), leukocytes, leukocyte-depleting filter. *J Extra Corpor Technol. 2016;48:168–72*

A patient's blood is actively conserved during cardiopulmonary bypass (CPB) to reduce the potential for homologous blood transfusion(s). Administration of banked blood is associated with increased morbidity, mortality, and immunomodulation (1,2). However, even autologous blood reinfusion can elicit a systemic inflammatory reaction, increasing the patient's morbidity and mortality risk (3,4). One possible consequence of this inflammatory reaction includes reperfusion injury (RI), which may occur when blood flow returns to tissues following a period of ischemia. RI is attributed to a wide range of physiological changes including complement activation, cytokine release, and leukocyte activation (5). During open heart surgery, CPB isolates the heart and lungs from the systemic circulation, leaving these organs susceptible to this type of injury post-CPB. Autologous blood reinfusion commonly occurs during the post-CPB period. CPB and cell salvage devices expose the blood to nonbiological surfaces (including air) resulting in the activation of leukocytes and the subsequent release of cytokines, which are targeted for removal primarily through the development, validation, and use of leukocyte-depleting filters (LDFs) (6).

We developed a novel centrifugation method using a cell salvage device to determine if similar reductions in leukocyte and cytokine levels could be achieved without the use of a LDF. We evaluated erythrocyte, leukocyte, and cytokine levels in bovine blood after passage through a cell salvage device with either subsequent use of a

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Address correspondence to: Cristina Gaspar, MS, CCP, Cardiovascular Science Program, Midwestern University, Glendale, AZ 85308. E-mail: cgaspa@midwestern.edu
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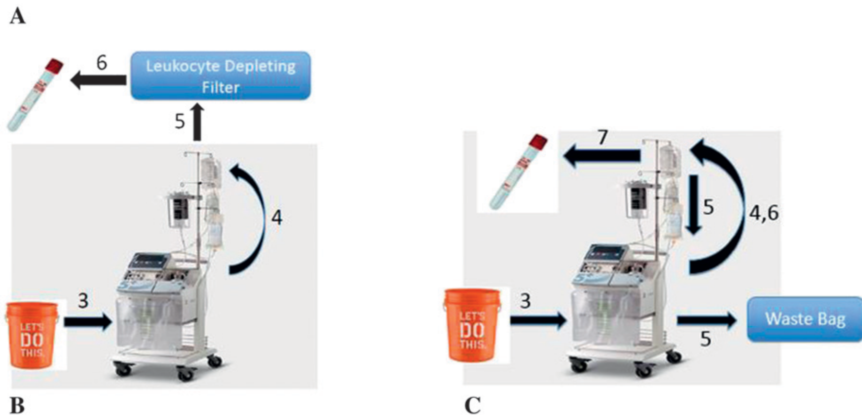

Figure 1. (A) Steps taken for both methodologies. (B) Depiction of steps taken for LDF method. (C) Depiction of steps taken for CENT method.

leukocyte depletion filter or centrifugation using our filter-sparing method.

MATERIALS AND METHODS

Figure 1 depicts the methodology. The study groups consisted of traditional cell salvage and washing followed by reinfusion through an LDF (LDF method) and traditional cell salvage followed by centrifugation and concentration (CENT method).

Approximately 18 L of bovine blood was collected from each of three cows being slaughtered to enter the local food supply and placed in three large plastic containers containing 60,000 units of porcine intestinal heparin (Sagent Pharmaceuticals, Schaumburg, IL) (Step 1) for anticoagulation. Baseline sample aliquots were collected for a complete blood count analysis and cytokine measurements from each container of bovine blood (Step 2). The heparinized blood was drawn into a Haemonetics Cell Saver 5+ (Haemonetics Corporation, Braintree, MA) until the 225 mL Latham bowl was adequately filled with erythrocytes as determined by the optical sensor of the machine operating in Automatic Mode (Step 3). The blood was washed with 1 L .9% saline and sent to the reinfusion bag in the Automatic Mode (Step 4) and samples were again collected for analysis. These steps were consistent between both methodologies.

During the LDF method, the processed blood was then passed through a Haemonetics LipiGard SB Filter (Haemonetics Corporation) from the reinfusion bag via gravity drainage (Step 5). Sample aliquots were collected for CBC and cytokine analysis (Step 6). The LDFs were weighed pre- and postuse to determine the amount of blood that would remain in the filter and therefore not reach the patient. The procedure was performed three times for each cow for a total of nine data points.

During the CENT method, the processed blood remained in the reinfusion bag while the Latham bowl was filled a second time with unprocessed blood and washed in Automatic Mode as described previously (Step 3). Before the "empty" phase, the machine was shifted into Manual Mode. The previously processed cells from the reinfusion bag were returned to the bowl and concentrated with the freshly washed cells of the second bowl. The return flow rate was gradually decreased to 25 mL/min to allow maximal concentration of erythrocytes and minimal cell spillage and loss. After maximal erythrocyte concentration was achieved in the Latham bowl, approximately 50 mL of the least dense cellular material overflowed into the waste bag (Step 5). The remaining concentrated contents of the bowl were sent to the reinfusion bag without additional washing (Step 6), and sample aliquots were collected for analysis (Step 7). The procedure was performed three times for each cow for a total of nine data points.

Table 1 lists the samples collected. Blood samples collected for CBCs (3 mL in ethylenediaminetetraacetic acid) were submitted to a commercial diagnostic laboratory for analysis (Antech Diagnostics, Phoenix AZ). For cytokine analysis, the samples (10 mL in 15-mL conical tubes) were

Table 1. Description of samples collected.

Sample	Label	Description
1	Baseline	Samples collected directly after heparinization
2	LDF	Samples collected after passage through the LDF
3	CENT	Samples collected after concentration and decanting of supernatant and buffy coat

Table 2. Raw laboratory values not accounting for RBC concentration.

Laboratory Value	Baseline	LDF	CENT	p Value (LDF vs. CENT)
Leukocyte count ($10^3/\mu L$)	4.9 ±.05	.4 ± .22	1.1 ± .18	.007
Erythrocyte count ($10^6/\mu L$)	7.4 ± .78	9.1 ± .55	15 ± .56	<.001
HCT (%)	38.3 ± 4.0	49.6 ± 2.7	82.1 ± 1.6	<.001
Neutrophil count per µL	1,825 ± 500	102 ± 75	351 ± 169	.025
Lymphocyte count per µL	2,501 ± 350	263 ± 129	658 ± 114	.011

Data presented as means ± SD.

centrifuged at 1,500 × g for 10 minutes at 4°C, and the serum was decanted and stored at −20°C for future use. Commercially available and validated enzyme-linked immunosorbent assay (ELISA) kits were used to measure tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) levels in serum samples at 1:2 dilutions, according to the manufacturer's instructions (Bovine IL-2 DuoSet Assay from R&D Systems, catalog no. DY2465; and Bovine TNF-α Assay from R&D Systems, Catalog no. DY2465). Each sample was measured in triplicate and the data were analyzed according to the manufacturer's instructions.

Two-tailed, Student's t tests assuming equal variance were performed to compare results obtained by each method. For each method, triplicate samples from each cow ($n = 3$) were averaged and these values were used for comparison between the cows.

RESULTS

Table 2 summarizes the number of leukocytes, erythrocytes, neutrophils, lymphocytes, and the hematocrit (HCT) measured in each sample at baseline and after the LDF and CENT methods. Erythrocyte and HCT values were significantly higher in the CENT method compared to the LDF method ($p < .001$). Leukocyte, neutrophil, and lymphocyte counts were significantly reduced from baseline in both groups but were higher in samples from the CENT method than those from the LDF method. Due to the hemo-concentrating effect of the CENT technique, we compared not only the absolute reduction in leukocyte numbers obtained, but also a relative measure of leukocyte depletion. The CENT method produced a very large increase in HCT (~114%) compared to a relatively minor increase (~30%) using the LDF method (Figure 2A). We calculated the expected leukocyte concentration associated with the CENT method (Figure 2B) (Expected WBCs/µL = $[HCT_{post\text{-}centrifuge} \times WBC_{baseline}]/HCT_{baseline}$) and compared this value with the actual measured count to report a relative leukocyte reduction for each method (Figure 2C). When considering the degree of hemo-concentration afforded by the CENT method, both methods offer comparable leukocyte reduction rates ($p = .14$), suggesting that leukocytes can be decanted from the cell salvage device without the use of an LDF. Similarly, the relative neutrophil reduction was not statistically different between each method (Figure 2D, $p = .09$). Comparable amounts of volume are lost from each method, as the LDF retains some volume during passage, and the CENT method spills some volume into the waste bag during processing (data not shown).

TNF-α levels measured by ELISA are represented in Figure 3. The second lot of bovine blood from Cow 2

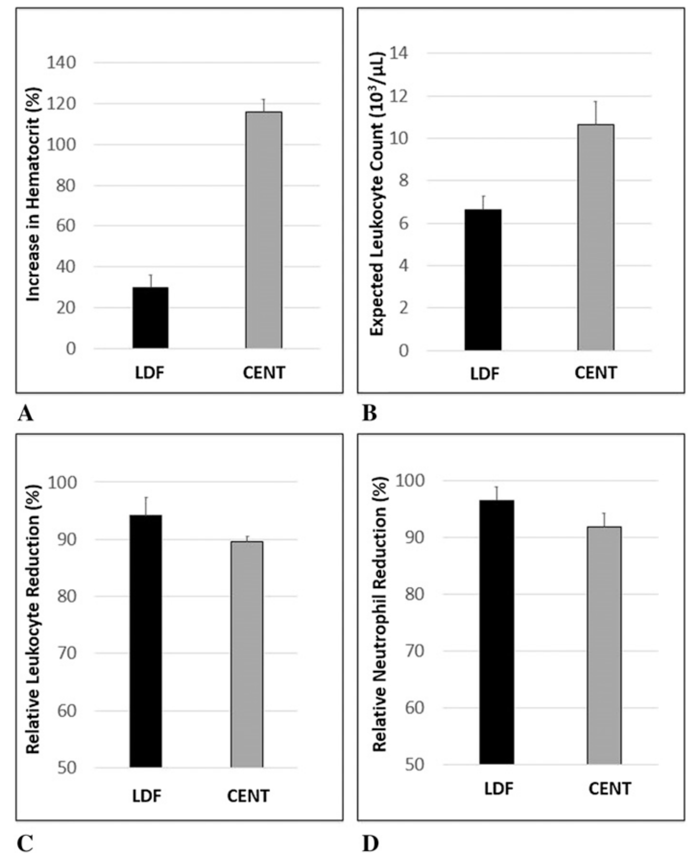

Figure 2. (A) Percent increase in HCT from baseline. (B) Expected leukocyte count when accounting for RBC concentration. (C) Relative leukocyte reduction when accounting for RBC concentration. (D) Relative neutrophil reduction when accounting for RBC concentration. Bars represent mean and standard deviation of three samples (baseline) or triplicate measures from three samples (LDF and CENT). There was no significant differences found in relative leukocyte reduction ($p = .14$) or neutrophil reduction ($p = .09$) between the two methods tested.

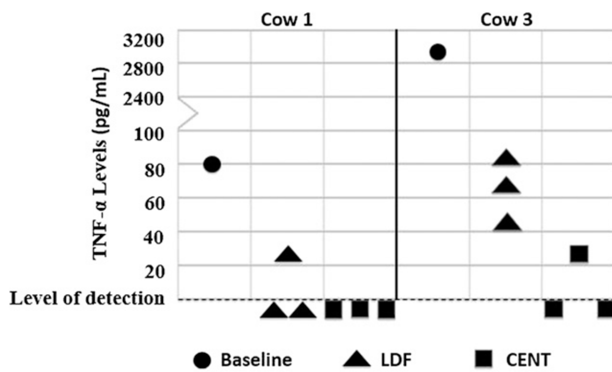

Figure 3. TNF-α levels. These depict the average of triplicate measures from two cow samples.

had undetectable amounts of TNF-α in every sample, including baseline, and is therefore not shown. Detectable TNF-α levels were present at baseline and reduced to detectable levels in four of the six LDF samples. TNF-α levels were only detectable in one of the six CENT samples, indicating the LDF and CENT methods were at a minimum comparable in the reduction of cytokine levels from baseline. Each symbol represents the average of triplicate measures for each sample. IL-2 levels were below the level of detection in all samples including baseline samples (data not shown).

DISCUSSION

Blood salvage and CPB procedures have been associated with systemic inflammatory reactions that can increase morbidity and mortality in already critical patients (7). Attempts to reduce activated white blood cells and inflammatory mediators prior to reinfusion through the use of LDFs have led to improved clinical outcomes in some (but not all) of the studies performed to date. Currently, justification for their use varies geographically and according to clinical circumstance (8–11). Furthermore, LDFs are not always readily available for use. The desire to contain health-care costs and improve blood management techniques prompted the development of our novel centrifugation method over 20 years ago and it was experimentally implemented during select adult open heart procedures. At the time, budget and research constraints restricted us from analyzing our method. Now, with the resources of our institution, we are able to study this method with bovine blood and our preliminary results are promising. Using our modified centrifugation method to decant the supernatant and buffy coat off of the transfusate, we successfully reduced white blood cell counts and TNF-α levels in bovine blood samples.

When comparing absolute leukocyte counts, it initially appears that the LDF method is more efficient in reducing leukocytes. However, during the CENT method one bowl is processed, sent to the reinfusion bag, and subsequently concentrated with a second bowl of collected and washed blood. In this regard, two times the amount of blood was processed to create a single reinfusion unit for the CENT method compared to the LDF method. Hence, an increase in leukocyte numbers would be expected in the CENT method. To compare the two methods and account for this volume difference, we used the final HCT from each method to normalize the data. This correlation has been similarly described in other studies (12). Considering the effect of erythrocyte concentration on leukocyte count when comparing the two methods, no statistically significant difference in leukocyte reduction was found. This supports our hypothesis that a novel centrifugation method can be used to reduce white blood cells without the use of an LDF.

TNF-α was measured because this cytokine has been implicated as a mediator of RI and has extremely widespread effects on inflammation, cellular proliferation, and differentiation (13). In the heart, reactive oxygen species are produced under ischemic conditions which subsequently increase TNF-α production, leading to contractile dysfunction, hypertrophy, fibrosis, and cell death (14). In the liver, RI seems to be similarly mediated, and both neutrophils and inflammatory mediators, including TNF-α, are known to play important roles (15,16). TNF-α levels were measured at a 1:2 dilution; a total of nine aliquots of blood were processed for each methodology. Because the second bovine's blood had undetectable levels, even at baseline, the data were not included. Therefore, only one out of six samples (16.7%) from the CENT method had measureable levels of TNF-α, while four out of six samples (66.7%) from the LDF method had measureable levels, suggesting that the CENT method adequately depletes harmful cytokines at least as well as the LDF method, although a larger sample size is needed for statistical confirmation. Although our results are limited to TNF-α values, these data suggest that applying the centrifugation method in place of a LDF may result in better clearance of other soluble cytokines that contribute to RI. Further studies expanding the scope of RI-marker analysis, such as measuring IL-1 and IL-6 levels, are warranted.

Exposure of blood to components of the CPB circuit, such as plastics and air, elicits an inflammatory response reflected in cellular and molecular alterations. We were interested in evaluating if the passage of blood through the cell saver device and/or LDF would result in increased levels of IL-2 in the reinfusion product. IL-2 was explored because of its ability to stimulate the activation of T cells ($CD4^+$, $CD8^+$) and the proliferation of natural killer cells and plasma cells (17). Zero samples had detectable IL-2 levels, including the baseline samples. The most likely

cause is that the healthy cows at slaughter did not have measurable circulating IL-2 levels to begin with. In humans, under normal, healthy conditions, IL-2 is only faintly detectable (18). Similarly, bovine IL-2 concentration is low in normal serum/plasma and may not be detected by conventional IL-2 assays developed for in vitro uses (19,20). We are therefore unable to conclude if either methodology would up-regulate or reduce IL-2 concentrations in vitro. The use of bovine blood for these experiments avoided the need for live animals and Institutional Animal Care and Use Committee approval, but limits the use of commercially available and validated assays for cytokine measures.

We recognize our limited sample size and outcome measures make broader extrapolations problematic. However, our data validate future exploration of this simple, cost-saving centrifugation method to offer an efficacious alternative to the use of expensive LDFs. Centrifugation may also provide additional benefits not found with the LDF method. The novel centrifugation method greatly reduces the volume of crystalloid wash solution returning to the patient and replaces it with hyper-oncotic packed red blood cells (RBCs). Less intravascular crystalloid is available to diffuse into tissues and increased intravascular colloidal osmotic pressure draws fluid into the intravascular space. Both of these factors work to minimize third-spacing of fluid and tissue edema. The centrifugation method we propose is not believed to add risk of injury or infection and simply omits the costly LDF.

Our findings offer a cost-saving alternative to the use of LDFs and support further work that would be aimed at objectively evaluating patient outcomes following the use of this centrifugation method in the clinical setting. Our novel centrifugation method produces a clean, concentrated, and relatively pure red blood cell product without the use of LDFs and may lead to comparable patient outcomes and cost savings in both veterinary and human medicine.

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