with the period of clinically relevant cytopenia, the predictive value of these assays for hematopoietic engraftment may be reduced because they do not provide functional information on the hematopoietic quality of the graft. The absence of functional hematopoietic information may result in the selection of low-potency stem cell products that fail to engraft in a patient despite a unit having a high cell count with an acceptable phenotype.

The colony-forming unit (CFU) assay is a hematopoietic functional assay that is often used to measure the function or potency of hematopoietic progenitors present in stem cell products. However, poor inter-laboratory reproducibility of the CFU assay even among experienced laboratories precludes universal implementation of this assay (1,2). As a consequence, the CFU assay fails to meet potency testing guidelines as set forth by the U.S. Food and Drug Administration (3). These guidelines require that a potency assay be capable of predicting therapeutic outcome, establishing industry release criteria and defining product expiration.

Reasonably good intra-laboratory reproducibility for the CFU assay has resulted in some investigators reporting that there is a good correlation between numbers of CFU-generating progenitors present in stem cell products and short-term hematopoietic reconstitution in autologous and allogeneic transplantation settings (4–9). Given that CFU assays performed at a single site can correlate with engraftment, it should be possible with stringent standardization of the method to improve inter-laboratory reproducibility so that results from different sites can be used to predict the *in vivo* efficacy of stem cell grafts for clinical applications.

The CFU assay takes advantage of the ability of a HPC to proliferate and differentiate to form a colony of cells committed to specific blood cell lineages. This *in vitro* assay is typically performed by removing an aliquot of cells from a stem cell product, preparing a working cell suspension, inoculating growth factor-containing semi-solid medium with a desired cell concentration and transferring cells and methylcellulose into culture dishes (Figure 1). The dishes are placed in a humidified incubator for a defined period. At the end of the culture period, the total number of colonies produced is counted microscopically and classified according to their morphologic features as burst-forming unit-erythroid (BFU-E), colony-forming unit erythroid (CFU-E), colonies containing granulocytes and macrophages (CFU-GM), and colonies containing granulocytes, erythrocytes, macrophages and megakaryocytes (CFU-GEMM). The type and number of the colonies obtained at the end of the culture period are driven by the amount and combination of growth factors present in the culture.

As a first step toward inter-laboratory standardization of the CFU assay, the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative designed a survey to evaluate current practices among different laboratories to identify sources of variability that may contribute to assay variability. The survey focused on practices associated with performing the CFU assay on fresh samples and was distributed internationally through membership rosters of the American Association of Blood Banks (AABB), International Society for Cell Therapy (ISCT) and European Group for Blood and Marrow Transplantation (EBMT) societies. Results from the survey expose highly variable laboratory practices, which support a need for the establishment of inter-laboratory standards for the CFU assay. Using survey results, we provide in this article suggestions for areas of practices to be considered for standardization

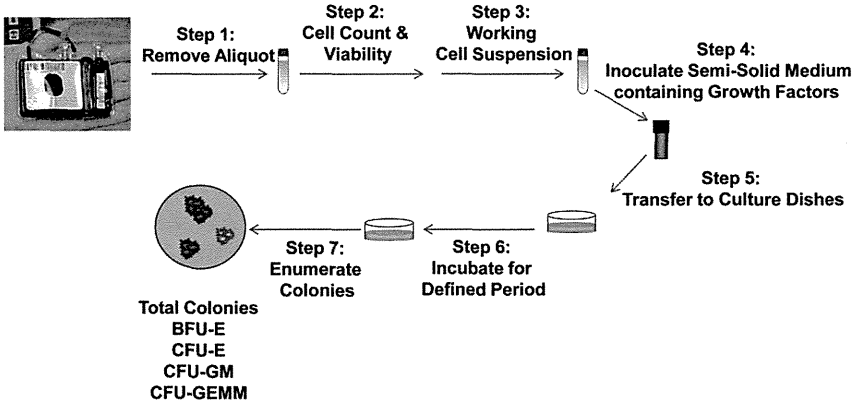

Figure 1. Basic steps for setting up the CFU assay. Step 1: An aliquot of cells is removed from a stem cell product. Step 2: A pre-dilution cell count and viability test are performed. Step 3: A working cell suspension is made from the aliquot of cells removed from step 1. Step 4: Semi-solid medium containing growth factors is inoculated with a defined volume of the working cell suspension. Step 5: The semi-solid medium containing growth factors and cells is transferred to a culture vessel. Step 6: The culture vessel is placed in an incubator for a defined period. Step 7: At the end of the culture period, the colonies are enumerated and differentiated.

of the CFU assay and how to address some of them to improve inter-laboratory assay precision.

Methods

The BEST Collaborative cellular therapy team used SurveyMonkey (http://www.surveymonkey.com/) to assess current laboratory practices for the CFU assay when performed on fresh samples. No attempt was made to assess laboratory practices for performing the CFU assay on previously frozen and thawed samples. The survey was designed to ask questions about how laboratories perform the CFU assay when evaluating HPCs in apheresis (HPC-A), marrow (HPC-M) and umbilical cord blood (HPC-C) products. The survey was designed with primarily closed-ended questions that provided one answer or multiple responses to a fixed set of possible choices. Skip Logic, or conditional branching, rules were also used to direct respondents through different questions based on their response to previous questions. Before wide distribution of the survey, the BEST membership pilot tested the survey to facilitate the removal of inconsistent questions.

The survey was widely distributed to members of the AABB, ISCT and EBMT. Individuals who responded to an invitation to complete the survey were directed to a specific page on the BEST Collaborative website. Each participant was provided with a brief explanation of the survey's purpose and a direct link to the survey. Participants were provided the option of providing contact information. Internet Protocol addresses were captured to allow tracking of results and to eliminate duplicate entries by participants at the same computer. After completion, respondents were not allowed to re-enter the survey. Surveys were collected over 5–6 months, and responses were collated into an Excel spreadsheet. Analysis of survey data was conducted after eliminating duplicate entries from the same institution or by different participants at the same computer.

Results

There were 105 individuals who initiated the survey. Of the 89 participants (n = 93 respondents) who provided their institutional affiliation, 56 resided in North America, 21 resided in Europe, seven resided in Asia, four resided in Australia and one resided in New Zealand. Because not all participants responded to each question, data are summarized by providing the number of respondents for each question in parentheses. Of the 105 respondents, 67.6% (n = 71) performed CFU assays, and 32.4% (n = 34) did not. The type of HPC product evaluated by their institution was specified by 67 respondents. Of these 67 respondents, 73%, 67% and 69% said they performed CFU assays on HPC-A, HPC-M and HPC-C.

Pre-dilution total nucleated cell counts

The first series of questions was designed to establish the time point at which laboratories removed an aliquot of cells from a product of HPC stem cells before cryopreservation and how pre-dilution cell counts and viabilities were performed. The more common practice (75% of 67 respondents) was to remove an aliquot of cells before rather than after the addition of dimethyl sulfoxide to hematopoietic stem cell products (Figure 1, step 1). Automated cell counts were performed by most laboratories (74% of 65 respondents); 26% performed manual cell counts on aliquots of cells removed from final products (i.e., pre-dilution cell count) (Figure 1, step 2). Replicate cell counts were performed by 48% (30 of 65) of laboratories to obtain average counts. When replicate counts were performed, 60% of respondents (n = 30) stated that the replicate count was done using an automated cell counter, whereas 40% of laboratories did a manual cell count. Less than half of laboratories surveyed performed replicate cell counts, and when replicate counts were performed, they were more likely to be done using an automated cell counter.

Viability testing

Of laboratories surveyed, 42 (65%; 65 total respondents) indicated that they used trypan blue, whereas 23 (26%) used 7-aminoactinomycin D (7-AAD) (Figure 1, step 2). Other viability stains used by participants included acridine orange/propidium iodide (AO/PI), AO/ethidium bromide (AO/EB) and erythrosin B. Most laboratories (72%) indicated that cells were incubated for a defined period when using either trypan blue or 7-AAD (Table I). Incubation times ranged from 1–10 min with trypan blue and 5–20 min with 7-AAD (Table I). No defined incubation times were given for AO/PI, AO/EB and erythrosine B. All viability assays performed with 7-AAD were analyzed using flow cytometry; 86% of viability assays performed with trypan blue were read microscopically. Three of 65 laboratories indicated that flow cytometry was used to evaluate cells stained with trypan blue.

Working cell suspension preparation

The aliquot that was used for performing a pre-dilution cell count and viability test was also the one used to prepare a working cell suspension to set up the CFU assay for 72% of respondents (n = 62). To prepare a working cell suspension for inoculating the semi-solid medium for CFU plating, 81% of

Table I. Practices for performing viability testing.

Stain	*Total	Defined Incubation Period for Cells and Viability Stain			*Incubation Period (min)									Stain	*Method used to Read Viability		
		Yes	No	N/A	1	2	5	10	15	20	2-3	2-5	N/A		Microscope	Fow Cytometry	N/A
Trypan Blue	42	29	12	1	15	2	8	2	0	0	1	1		Trypan Blue	36	3	3
7-AAD	20	16	3	1			2	6	1	6			1	7-AAD		17	3
AO/PI	1	0	1											AO/PI	1		
AO/EB	1	0	1											AO/EB	1		
Erythrosin	1	0	1											Erythrosin	1		

N/A, respondents did not answer question.
*Number of respondents.

laboratories (n = 62 respondents) said the aliquot came from the final stem cell product.

The preparation of the working cell suspension varied considerably from laboratory to laboratory (Figure 2). First, a large variety of pipetting devices was used. Air displacement pipettes were the most commonly used devices followed by serologic and positive displacement pipettes (Figure 2A). After making the working cell suspension, 39% of laboratories (n = 51 respondents) performed an additional cell count on the final dilution to verify the accuracy of the dilution. Second, the working cell concentrations prepared for inoculating semi-solid growth media were another source of variability. Working cell concentrations prepared by 45% of respondents were unique to their institution (Figure 2B). Finally, the calculations to determine the concentration of working cell suspensions were based on different cellular phenotypes that included TNCs, viable TNCs, total mononuclear cells (MNCs), total viable MNCs, $CD34^+$ and viable $CD34^+$ cells (Figure 2C).

Methylcellulose-based medium inoculation

Most survey participants (90%; n = 60 respondents) indicated that they purchased methylcellulose-based medium for the CFU assay; 5% said they used medium made in-house, and the remaining 5% indicated that they did not use methylcellulose-based medium. Batch preparations (i.e., methylcellulose +

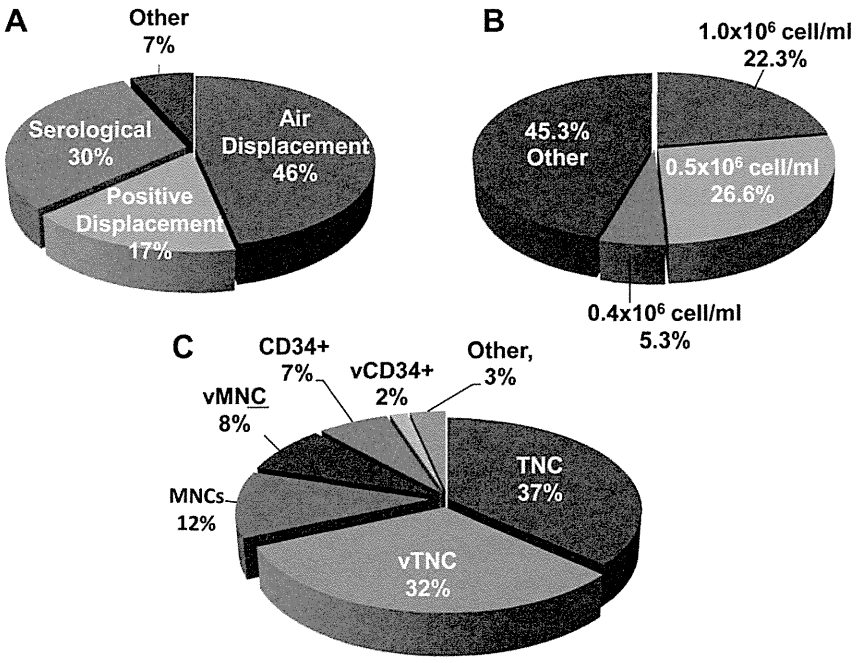

Figure 2. Techniques for preparing working cell suspensions for inoculating CFU semi-solid medium (n = 60 respondents). (A) Percent of survey respondents who use the indicated type of pipette devices to prepare a working cell suspension. (B) Percent of survey participants who use the indicated cell concentrations to inoculate the CFU assay. (C) Percent of laboratories that use the specified cell immunophenotypes to calculate the concentration of the working cell suspension.

cytokines + cells) sufficient to perform replicate platings were made by 76% of laboratories. The numbers of replicate plates per assay ranged from one to four. The most popular size of the culture plate was 35 mm followed by 24-well culture plates (Figure 3). Other types of culture plates reported included four-well and six-well plates, 10-mm dishes and flasks (Figure 3).

To transfer methylcellulose-containing cytokines and cells to culture plates or dishes, 61% of laboratories (n = 59 respondents) used a syringe, and 37% used a pipette. For laboratories using syringes, 81% (n = 36 respondents) used a blunt-ended needle to transfer medium to a culture plate. For the 21 laboratories that used a pipette, 38.1%, 9.5% and 47.6% employed an air-displacement, positive displacement or serologic pipette. One of 59 laboratories indicated that they used a 16-gauge blunt end cannula. One laboratory did not specify the type of pipette used.

The final total number of cells plated per dish or well varied from site to site. For laboratories using 35-mm dishes (45 of 57 respondents), the range of cells plated was 10,000–200,000 for HPC-A, 25,000–100,000 for HPC-M and 5000–100,000 for HPC-C. One laboratory indicated that the number of cells plated depended on the percentage of viable $CD34^+$ cells. For laboratories using 24-well plates, three of four laboratories indicated they plated 100,000 cells and 50,000 cells for HPC-A and HPC-M. Laboratories (59.6%; n = 57 respondents) tended to plate CFU based on viable cell counts. After plating cells, 26.3% of respondents indicated that they performed a cell count on the remaining working cell suspension to verify an accurate dilution.

Culture plates were placed in incubators at 37°C in 5% carbon dioxide (CO_2) at 95% humidity by most respondents. Culture plates were maintained at a temperature of 37°C by 98% of laboratories (n = 57 respondents) with one laboratory indicating culture plates were maintained at 22°C. Of laboratories, 93% said they used 5% CO_2/air mixture, and the remaining

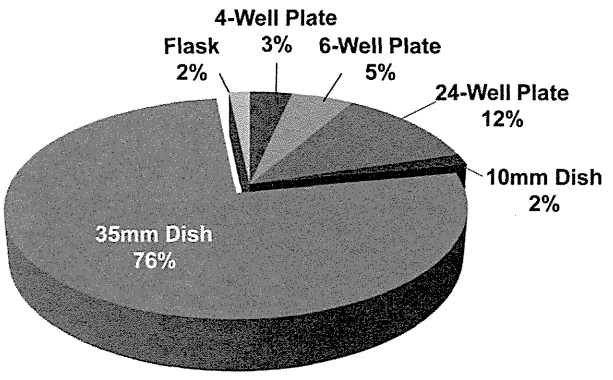

Figure 3. Types of culture vessels used for CFU plating (n = 59 respondents).

7% of respondents indicated that they used 7% CO_2/air 6% CO_2 + 10% O_2, 5.5% CO_2 or 5% CO_2 + 5% O_2. Most laboratories placed culture dishes within larger dishes along with a water dish to help maintain humidity. Of laboratories, 88% indicated that they used 95% humidity, one laboratory indicated a range of 50–100% humidity, another laboratory said that they were changing to 85% humidity and two laboratories did not know the humidity percentage of the incubator.

CFU readout

Culture periods for the CFU assay were predominately 14–16 days independent of whether the assay was used to measure colony formation in HPC-A, HPC-M or HPC-C Products (Table II). To assist in the enumeration and differentiation of colonies, 84% of respondents (n = 57) used a microscopic grid. The most popular type of microscope and magnification used to read colonies was an inverted light microscope and optical magnification of 400× (10× eyepiece and 40× objective lens), respectively (Figure 4). Colonies were most commonly scored and reported for CFU-GM (Table III). To calculate the total number of colonies present in a product, some laboratories (73%; n = 15 respondents) used the post-dilution cell count, and others (27%) used the pre-dilution cell count.

Validation and proficiency testing

Validation studies to test the linearity of tissue culture plates for determining at which cell dose a CFU culture plate or dish reached a saturation plateau were performed by 58% of respondents (n = 54). Only 37% (n = 57 respondents) had established criteria for determining whether a culture plate or dish was overgrown; the remaining 63% had no established criteria. The primary criterion for determining whether a CFU plate was overgrown was noted by 61% of 21 respondents to be overlapping colonies. Other criteria given to define if a culture plate was overgrown included the following: (i) colonies exceeded a maximum number per plate (e.g., >120, >150, 150–200, >300 colonies per plate), (ii) colonies were too numerous to count and (iii) phenol red in the methylcellulose turned yellow indicating that a change in the pH of the medium had occurred. If a CFU plate was determined to be overgrown, only 12.3% of laboratories (n = 57 respondents) repeated the assay by adding fewer cells per plate, 49.1% said that they did not repeat the test and the remaining 38.6% indicated that it was essentially not applicable to them.

Most laboratories (98%; n = 57 respondents) indicated that they participated in a proficiency

Table II. Incubation periods reported for CFU assay.

Cells per dish/well	<7 days	7–9 days	10–13 days	14–16 days	Other	N/A	Response count
HPC, apheresis	0.0% (0)	1.8% (1)	16.4% (9)	63.6% (35)	0.0% (0)	18.2% (10)	55
HPC, marrow	0.0% (0)	3.8% (2)	15.4% (8)	53.8% (28)	0.0% (0)	26.9% (14)	52
HPC, cord blood	0.0% (0)	3.8% (2)	11.3% (6)	58.5% (31)	0.0% (0)	26.4% (14)	53

testing program. The most commonly used proficiency testing program was from STEMCELL Technologies (59.6%) (Vancouver, BC, Canada) followed by the College of American Pathologists (36.8%). One laboratory indicated that their proficiency testing program involved the use of a third independent party to verify scoring; another laboratory indicated that they planned to participate in a program in the future. The average number of individuals trained to perform a CFU assay per laboratory was 4.1 (range, 1–10). Third-party formal training was attended by staff from 47.4% of reporting laboratories (n = 57 respondents).

Transplant outcome

Finally, laboratories were asked whether they observed a clinical correlation between engraftment and results obtained from CFU assays. Of 57 respondents who answered this question, 35% indicated that they saw a significant negative correlation with the length of peripheral blood cytopenias and their institutional CFU assay data. No significant correlation was seen by 19%, and the remaining 46% of laboratories indicated that they were unable to determine a correlation between engraftment and the CFU assay.

Discussion

Results from this study highlight that current inter-laboratory practices for setting up the CFU assay are highly variable at multiple steps of the procedure. Consequently, there are several technical aspects of the CFU assay that could be standardized. A list of recommendations for variables to be considered for standardization based on best laboratory practices or previously published data is presented in Table IV. For example, most laboratories (74%) performed automated cell counts, but a substantial number of laboratories (26%) indicated that they perform manual counts. Evidence is available that manual counts are less well controlled and are associated with more sources of error (i.e., preparing a dilution to charge a hemacytometer, manual counting, calculation errors) that lead to lower accuracy and precision than automated counts (10). Proficiency testing studies show automated methods have better inter-laboratory reproducibility than manual counts (1,11). Subsequently, one step to improve the standardization of the CFU assay would be for all laboratories to adopt well-controlled automated cell counts and eliminate the use of manual counts to perform cell counts on pre-dilution samples.

Viability testing is another step of the CFU assay that could be standardized or possibly eliminated. Answers regarding current laboratory practices for performing viability counts indicate that not only are different stains used among laboratories for conducting a viability assay, but also when the same viability stain is used by different laboratories the procedure is not the same. Among laboratories using the same dye, they reported the use of different incubation periods for staining cells and different techniques for assessing viable and non-viable cells (microscopically vs. flow cytometry). Despite reports that trypan blue is inferior to other vital stains (12–14), the results of this study show that trypan blue is the most commonly used stain (65% of respondents), whereas alternative stains such as AO/PI, AO/EB and erythrosin B are used by only a few

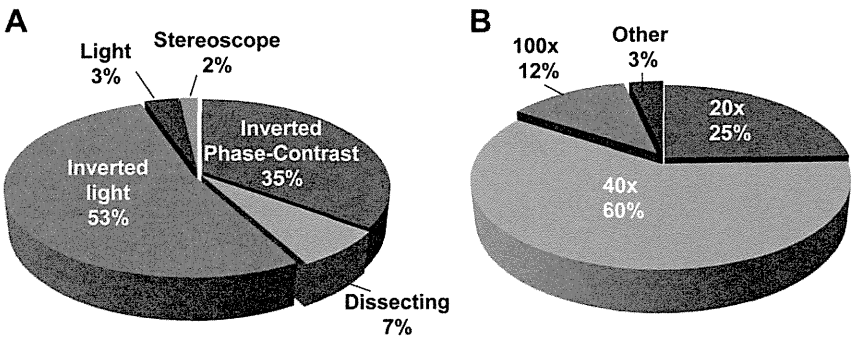

Figure 4. Microscopic readout for CFU. (A) Type of microscope used to read colonies. (B) Microscopic magnification used to read colonies.

Table III. Colony phenotypes reported by sites.

Colony type	Yes	No	Response count
BFU-E	78.3% (36)	21.7% (10)	46
CFU-GEMM	64.3% (27)	35.7% (15)	42
CFU-GM	94.2% (49)	5.8% (3)	52
Total Colony	61.9% (26)	38.1% (16)	42
Other			3[a]

[a]One site indicated they report CFU-E; a second, BFU-E and CFU-GM; a third site, that they do not report data to an outside institution.

sites. Given the variability associated with viability testing (i.e., trypan blue, AO/EB, AO/PI, 7-AAD) (15), we suspect that practices to integrate cell viability with cell counts contribute to CFU assay inter-laboratory testing variability. Based on the fact that the CFU assay inherently assesses cell viability (i.e., dead cells do not form colonies), one can argue that performing a viability assessment is redundant and unnecessary and introduces error into the final calculation for determining CFU counts.

Different strategies are used to calculate the concentration of the working cell suspension for inoculating the semi-solid medium for plating CFU. Some participants use TNC, total MNC and $CD34^+$ cell counts, whereas other participants couple these parameters with a cell viability determination to prepare a working cell suspension that is used to plate viable TNCs, total viable MNCs and viable $CD34^+$ cells. Current practices of using different cellular phenotypes among laboratories to plate cells make it difficult to compare and interpret inter-laboratory results. Likewise, the laboratory practice not to use the same aliquot to prepare the working cell suspension that is used to determine cell counts may contribute to inter-laboratory variability. The importance of accurate cell counts and a standardized strategy to prepare a working cell suspension to set up the CFU assay cannot be underestimated as the cell count ultimately is used to determine the total number of colony-forming progenitors in a product. Given the importance of performing accurate cell counts and the preparation of a working cell suspension, it would be prudent to verify the cell concentration of the working cell suspension with an automated cell counter.

Answers to questions pertaining to the inoculation of culture medium (i.e., transfer of cells into semi-solid medium), how semi-solid culture medium containing cells is transferred to culture plates and how culture plates are incubated and enumerated for colonies are also informative. Answers related to these steps of the CFU protocol provide insight into other areas of the assay that may represent additional sources of assay variability and potential target

Table IV. Prospects for standardization.

Pre-dilution cell count
- Enumeration of cells with automated cell counters
- Eliminate viability testing for determining the number of cells for CFU plating
- Same aliquot used to perform cell counts should also be used to set up the CFU assay

Preparation of working cell suspension
- Consensus on cell phenotype to be used to prepare working cell suspensions
- Define working cell concentrations specific for HPC(A), HPC(M), HPC(CB)
- Verification that an accurate dilution is made

Medium inoculation and plating
- Transfer semi-solid medium into culture plates with syringe or positive displacement pipette
- Define cell number to be plated per cm^2 for HPC(A), HPC(M), HPC(CB)
- Require same medium formulation with same cytokine cocktail
- Perform assay in triplicate
- Use same size tissue culture plates (e.g., 35-mm, 24-well plate)

CFC readout
- Require use of high-quality inverted microscope
- Define how to report colony results (i.e., total colonies)
- Use of an automated CFU counter

Quality control
- Linearity evaluation to determine when CFU plate is overgrown
- Define criteria for when CFU plate is overgrown
- Strategy for follow-up when a plate is overgrown

areas for standardization (Table IV). In the case of transferring semi-solid medium inoculated with cells, most (61%) laboratories (n = 59 respondents) use a syringe, whereas some use air-displacement pipettes (n = 8 respondents) and serologic pipettes (n = 10 respondents) to transfer methylcellulose medium. This is in light of available information demonstrating that the transfer of a viscous medium (i.e., methylcellulose) is best achieved using a positive displacement pipette (16) and a recommendation that transfer of methylcellulose into culture plates should occur with a syringe (17). Most laboratories indicated that they purchase culture media containing cytokines; other laboratories said that they use an in-house formulation. Acknowledging that different cytokine combinations and cytokine concentrations can affect colony growth and differentiation (18), it is important that all sites performing CFU assays to assess a stem cell product for clinical application use the same concentration and combination of cytokines. Enumeration of colonies using different types of microscopes and the fact that not all sites report colony numbers in an agreed-upon fashion also make it difficult to compare results. In particular, some sites report CFU-GM, whereas other sites report total CFU. Finally, the finding that 42% of sites (n = 42) have not established the linear range of their culture plates or dishes raises concerns about the accuracy of results being reported and

underscores the need for the establishment of assay standards.

In conclusion, the results of this survey represent a cross-sectional analysis of the current practices of clinical laboratories in the United States, Europe, Asia and Australia, and provide numerous areas of action for intervention. The identification of procedural differences among laboratories raises the question as to whether it is reasonable for an ad hoc international committee of experts in the field to develop a comprehensive set of international guidelines for performing a CFU assay, especially given that reasonably good intra-laboratory CFU reproducibility allows some investigators to report that there is a correlation between numbers of CFU generating progenitors present in stem cell products and short-term hematopoietic reconstitution (4). For the CFU assay to meet the requirements of a true potency assay for accuracy, precision, specificity, linearity and robustness as set forth by regulatory agencies (U.S. Food and Drug Administration and European Union), a standardization of procedural steps and integration of standards and controls that are currently commercially unavailable to run alongside test samples are necessary.

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LETTER TO THE EDITOR

Plasticizer concentration in cord blood cryopreserved with DMSO

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Plasticizers like di(2-ethylhexyl) phthalate (DEHP) have been used to increase the elasticity, transparency, durability and stability of the polyvinyl chloride used for blood bags and tubing for five decades.[1] DEHP can leach from the polyvinyl chloride, and its toxicity is an issue of concern.[2] There have been reports that DEHP, and its derivative mono(2-ethylhexyl) phthalate (MEHP), could disrupt the endocrine system.[3] Although agreement on the effect of DEHP on human health has not been reached, recommendations have been made to avoid exposing some sensitive populations to it, such as pregnant women who are carrying male fetuses, male neonates and peripubertal males.[4]

In our cord blood bank procedures, we routinely use DMSO, which is known as a solvent, for cryopreservation. Although the final concentration of DMSO is commonly between 5 and 10%, v/v, it is much higher when the cryoprotectant goes through tubing. The DMSO may cause the plasticizer to be released into the cell component.

Below we report the levels of DEHP measured in our frozen cord blood units. Our cord blood bank has been operated by the same research and technical team as a part of the Japanese Red Cross (JRC) blood center in Tokyo since 1995.

The cord blood was collected into a 200 mL collection bag containing 28 mL of citrate-phosphate-dextrose (Kawasumi, Tokyo, Japan), transferred to our facility and processed within 24 h. The processing of one unit involved the use of a 150 mL separation bag (Kawasumi), a bag connector made with acrylonitrile butadiene styrene (Terumo, Tokyo, Japan), syringes (Terumo), a winged needle set (Nipro, Osaka, Japan) and a freezing bag (F-025A, Nipro). The collection and separation bags were made of polyvinyl chloride with DEHP. The freezing bag was made of high-molecular-weight polyethylene and was DEHP free.

We used hydroxyethyl starch (HES40, Nipro) to sediment the RBCs, and the final DMSO concentration was 10%. Prior to 2008, we used Cryoserv (Nipro) and 10% Dextran40 (DextseranD40, Terumo) to prepare the cryoprotectant of 50% DMSO/5% Dextran40, and since 2008 we have used a 55%, w/v, DMSO/5% Dextran40 mixture (CryoSure-DEX40, WAK-Chemie, Steinbach, Germany).

We divided our processing procedures into three periods: (1) before 2008, when we used one syringe for each of the DMSO and the 10% Dextran40, and then mixed them together using a three-way stopcock of polycarbonate (Terumo) before adding the mixture to our cell fraction; (2) from 2008 to 2012, during which the original collection bag was used through to the end of the processing; and (3) since the start of 2012, when we began collecting the buffy coat fraction into a separation bag to process further. At this time the winged needle set, through which the 50% DMSO is added to the cell suspension, was changed to become a DEHP-free product.

The exact test with $\alpha = 0.05$ and power $= 0.8$, gave 27 as the total sample size necessary, using SAS (ver. 9.1.3, SAS Institute Inc., Cary, NC, USA). Therefore, for each of the three periods we chose nine cord blood units for the DEHP measurements.

A cord blood sample of 1 mL was mixed with 2 mL of acetonitrile in a glass tube. After centrifugation (5 min at 600 *g*), the supernatant was filtered. A HPLC was performed with an Alliance system 2695, 2998 PDA detector (Waters, Milford, MA, USA) and an Atlantis dC18 column (4.6 mm × 150 mm, 5 μm), at a flow rate of 1 mL/min. A sample of 10 μL was injected into the column at 40 °C. The eluent was monitored at 273 nm. Separations were performed with a mobile phase consisting of 1% acetic acid/acetonitrile mixture; 50% acetonitrile for the first 3 min and 100% acetonitrile after. MEHP and DEHP elucidate at 6.7 and 8.8 min, respectively. The DEHP (DOP standard, Wako Pure Chemical, Osaka, Japan) and MEHP (Wako Pure Chemical) were used as controls. The glassware was washed with acetone beforehand.

We determined the background level of DEHP in our procedures by keeping PBS instead of cord blood with 10% DMSO in the separation and freezing bags for 10 and for 20 min. The 10% DMSO/PBS in the separation bags contained an average of 1.7 mg/L of DEHP and in the freezing bags contained an average of 1.2 mg/L of DEHP, with no detection of MEHP.

Before processing and within 24 h from the time of collection, the cord blood contained 17.8 ± 2.7 mg/L of DEHP and 1.0 ± 0.2 mg/L of MEHP (average ± s.d., $n = 5$).

When standard DEHP and MEHP was added to the cord blood, the average recovery was 108.8% and 94.4%, respectively.

For the samples from the three periods, measurements were carried out twice, with the results shown in Figures 1 and 2. There were no differences in the DEHP and MEHP levels among the three periods by one-way analysis of variance analysis. As there were 17.8 mg/L of DEHP and 1.0 mg/L of MEHP in the cord blood at the start, with an average volume of 91.7 mL, and 16.5 mg/L of DEHP and 1.1 mg/L of MEHP in the final product of 25 mL, we can assume that the majority of the phthalates are carried over from when the cord blood was in the collection bag.

The irradiated RBC concentrates of the JRC, 20 days from collection, have been reported to contain up to 36 mg/L of DEHP and 3.3 mg/L of MEHP.[5]

The total amount of phthalates in one unit of cord blood (25 mL), 0.4 mg of DEHP and 0.03 mg of MEHP in average, is less than that of one bag of blood for transfusion. Assuming that one

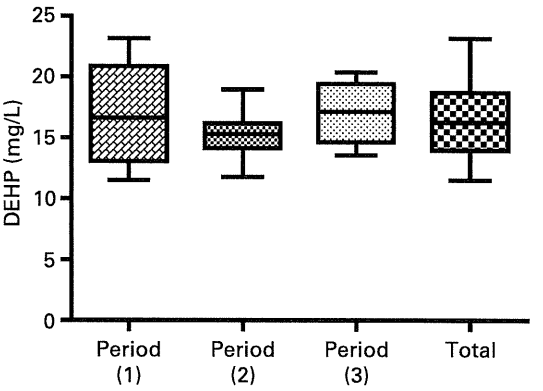

Figure 1. DEHP concentration in the cord blood units for transplantation. The average ± s.d. for periods (1), (2) and (3) ($n = 9$ for each) and for the total 27 samples were 17.2 ± 4.2, 15.3 ± 2.0, 17.2 ± 2.5 and 16.5 ± 3.1, respectively.

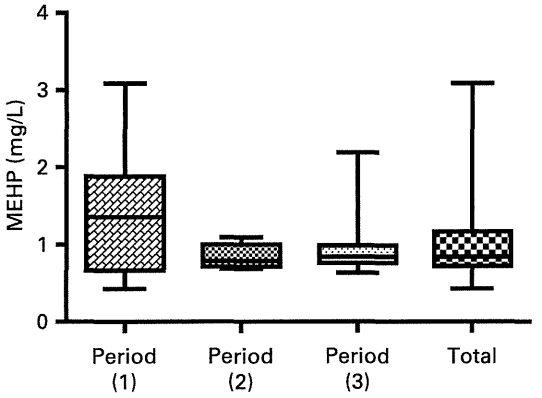

Figure 2. MEHP concentration in the cord blood units for transplantation. The average ± s.d. for periods (1), (2) and (3) ($n = 9$ for each) and for the total 27 samples were 1.4 ± 0.8, 0.9 ± 0.2, 1.0 ± 0.5 and 1.1 ± 0.6, respectively.

unit of cord blood, 25 mL, of the highest DEHP concentration is used for a baby of 3 kg, we can calculate that the recipient would receive 0.19 mg/kg of DEHP, which is lower than the parental tolerable intake value.[6] As the toxicity of MEHP has also been reported,[7] for sensitive populations the intake of phthalates should be a consideration.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTERNATIONAL FORUM

Should DEHP be eliminated in blood bags?

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Whole blood for transfusion was initially collected in glass bottles, but these are fragile, heavy to transport and prone to bacterial contamination. After the Second World War, Carl Walter experimented with plastics for collection and storage of blood, and found that di-ethylhexyl phthalate (DEHP) as plasticizer for polyvinyl chloride (PVC) had the most favourable properties [1, 2]. The breakage rate of frozen plasma in DEHP PVC is low, and the better storage properties of red cell concentrates in DEHP PVC are explained by leaching of plasticizer into the lipid bilayer of these cells, thereby improving membrane stability resulting in lower haemolysis rates [3]. However, ever since the 1970s, scientific and public concern has been raised on the toxicity of DEHP for transfused patients [4, 5]. DEHP and its metabolite mono ethylhexyl phthalate (MEHP) are associated with impairment of reproduction in animal models, resulting in testicular dysgenesis syndrome in (male) rodents, as reviewed in depth elsewhere [6], and particularly, neonates are susceptible to the risks of high exposure to DEHP. Due to the omnipresence of phthalates (not only DEHP), and some important differences in DEHP metabolism between animal models and humans [6], it is difficult to estimate the effect of DEHP toxicity in humans. A study of 234 young men showed no effect of MEHP in urine on their reproductive function [7], but others showed that DNA damage in sperm was associated with the MEHP concentration (after adjustment for other DEHP oxidative metabolites) [8]. *In vitro* human testis explants incubated with DEHP or MEHP showed that both significantly inhibited testosterone production [9], and a study of 881 men showed a 9% lower free androgen index between the lowest and the highest quartile of the proportion DEHP excreted as MEHP ($P = 0.02$) [10]. Also, an association was found between urinary MEHP concentrations and lower free T_4 and lower total T_3 thyroid hormones in adult men [11]. In addition to these effects on hormonal level in adult males, DEHP and its metabolites can have an effect on fetal development, with a shorter anogenital distance associated with higher metabolite concentrations in urine samples collected during pregnancy [12]. Others found no difference in T_4 and testosterone levels, as well as no effect on phallic length in 13 male adolescents 14–16 years of age that had underwent ECMO treatment in their neonatal period, as compared to age- and sex-matched controls [13]. Also, DEHP/MEHP exposure (as detected in cord blood) was significantly associated with a shorter pregnancy duration [14]. Despite these associations, they should be interpreted with care. DEHP is not the only phthalate that may cause these effects; studies that showed no association may not have been published; DEHP administered through the intestinal tract is converted to MEHP in a higher degree as by intravenously [15]; and because phthalates are everywhere in our environment, comparisons are less versus more, rather than absent versus present.

DEHP-plasticized PVC has long been used as standard material for a wide range of applications, like packing foil (including food), building material, toys and plastic devices. DEHP is considered to be a ubiquitous environmental contaminant and is present in air, dust, water, soil and food. The latter is considered to be the main source of DEHP intake for the general population. The current daily intake is estimated to be between 2 and 5 μg/kg for adults and between 4 to 8 μg/kg for children [16, 17]. Various measures to reduce the use of DEHP-plasticized PVC have indeed resulted in a 2-times lower exposure to DEHP in the general population over the last two decades [18].

DEHP is applied in many medical devices, and depending on the procedure, patients can be exposed to high (peak) concentrations of DEHP, in the order of 1 mg per kg of bodyweight. One of the sources of DEHP exposure is the leaching from the blood bags into the content of the container, particularly into lipophilic solutions such as plasma [19], but also red cells. Therefore some patient groups, including pregnant or nursing women and children, are considered to be most at risk of possible harmful effects attributable to DEHP exposure by medical treatments. DEHP is broken down into various metabolites. First, it is hydrolysed to MEHP and then further oxidized to mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP),

mono-(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono (2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) and mono[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) [20]. MEHP is believed to be the active metabolite related to male reproductive health, but also DEHP [9] as well as the various oxidized forms [12] has been found to have toxic properties. Obviously, the various compounds are closely related, although the half-time in circulation may differ due to different clearance rates [20]. It may therefore not be possible or necessary to make a distinction between DEHP and its metabolites; removal of DEHP from blood bags as a precautionary measure must thus be regarded as a way to reduce exposure to the active toxic metabolite.

A panel discussion at an expert meeting in October 2011 [the BEST Collaborative, www.bestcollaborative.com] revealed that the opinions whether or not the presence of DEHP in blood containers is an issue that needs to be debated, and whether time and money should be invested in developing and licensing alternatives, were diverse.

For this International Forum, experts were asked to provide current information on the use of plasticizers in blood bags, the status of regulations on the use of specific plasticizers and the perception of the risk of plasticizers. To place the exposure of DEHP in blood products versus medical devices and procedures in general, we further posed questions regarding the 'other' sources of DEHP, whether there are specific policies and how the use of DEHP in medical devices/procedures is regarded upon by advocate groups in the various countries.

Question 1

Do you currently use DEHP-plasticized PVC for storage of blood components? If so, which components? Have you introduced non-DEHP/non-PVC plastics for storage of any of your blood components? If yes, which blood components are stored in which plastic? Was concern relating to DEHP toxicity a contributing or decisive factor for selecting a different plastic, and why that particular one? Do you think it is possible to store red cells in non-DEHP containers with maintenance of sufficient quality?

DEHP-plasticized PVC is widely used for storage of red cell concentrates. Respondents are aware of the good storage characteristics (lower haemolysis rate) associated with the use of DEHP-containing bags, and many feel that there is no good replacement for DEHP in red cell storage containers. France reports that alternatives showed other problems, illustrating that one (known) risk should not be replaced by another (as yet unknown) risk. They also indicate that the presence of DEHP is a criterion when choosing new disposables. The American Red Cross collects almost half of their units in n-butyryl-tri-n-hexyl citrate (BTHC)-PVC systems (meaning that the container for red cells is made of this plastic), but they also remark that because DEHP is still present in most of the tubing and connectors, the red cells are still not truly 'DEHP free'.

In addition to BTHC, 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) as plasticizer for PVC is under development for use in red cell storage containers, but requires regular mixing to keep the haemolysis rate within the acceptance limits; also, the use of alternative additive solutions is suggested in combination with non-DEHP bags to maintain red cell quality similar to that in DEHP-containing bags. In any case, at this time, most respondents feel there is no easy or cost-effective way to replace DEHP from red cell containers.

For platelets, often other plastics are used (such as BTHC-PVC, trioctyl trimellitate (TOTM)-PVC, and polyolefin), but this is more related to the fact that these have better gas permeability to preserve platelet quality and/or come with the choice of apheresis technology, rather than the fact that the bags are DEHP free.

In none of the cases, was concern relating to putative toxicity of DEHP a leading factor in the selection of an alternative plastic.

Question 2

Do you have an active programme for replacing DEHP-containing plastics for blood containers with those that are DEHP free? If so, which criteria are used for selecting the new plastic; what are the requirements for in vitro and in vivo quality of the product and what are the requirements for DEHP content of the product, especially cellular components?

All except one of the countries indicate that they have no active programme to replace DEHP from blood bags. Sweden is probably most active; they work together with companies to develop alternatives for blood bags, preferably non-PVC without plasticizers. The requirements for blood components in such bags are the same as for the current components. Other countries also indicate that they would require that components stored in a new plastic should have the same, or even better, quality as those stored in DEHP-plasticized PVC. Concern is expressed that the defect rate, which is currently very low, might increase due to changes in the production methods and less experience with the new material. Both Australia and France include a statement on the application of DEHP in their tenders, and France formally asked suppliers to develop non-DEHP alternatives.

Although not explicitly stated by the respondents, it seems that a trade-off, that is, replacement of DEHP at the expense of a slight but still acceptable decrease in quality, is not considered acceptable.

Question 3

Is donor exposure to DEHP during apheresis a concern?

During the apheresis procedure, the blood comes in contact with long lines of PVC tubing, and depending on the components that are collected and the level of triglycerides, the donor can experience a spike in serum DEHP [21]. However, this normally resolved within 3 h with no lasting effects. In Belgium, the frequency of apheresis donation is limited to twice a month due to the DEHP concern. Other countries show awareness of the issue, and use single needle instead of dual-needle procedures, while others look into possibilities to replace PVC tubing in apheresis sets. In general, the DEHP burden of the donor is weighed against the total DEHP intake and is considered to be comparatively small and thus acceptable.

Question 4

In your country, are there specific patient groups for whom the use of non-DEHP and/or non-PVC for blood components is prescribed? If so, which ones? Do patients themselves ask questions or express concerns about the use of DEHP-plasticized PVC for storage of blood products?

For neonates who require large-volume transfusions, including exchange transfusions or ECMO, 'fresh' blood is prescribed. Other countries indicate that similar policies are also in place for adult patients requiring large-volume transfusions, for example dialysis, cardiac or trauma patients. However, in general, most countries indicate that DEHP concern is not the prime reason for using fresh blood, but do realize that the added benefit of fresh blood is the lower exposure to DEHP. It is probably only Canada where physicians are referred to a circular of information relating to DEHP, indicating that vulnerable patients should receive either fresh or washed products. In France and in the USA, there are guidelines that strongly recommend reducing the use of DEHP-containing devices when possible for at-risk patients; however, the reduction in DEHP specifically in the blood components is not mentioned. Some clinicians in the Netherlands and the USA have expressed concern relating to DEHP exposure to neonates, which has resulted in selection of non-DEHP equipment when treating these patients in the USA. In the UK, there was one patient question regarding allergy to DEHP. Except for this one question, all respondents indicated that patients have a low level of awareness regarding the presence of DEHP in blood bags.

Question 5

Do you currently use DEHP-free infusion systems for the administration of IV fluids? Does your hospital/country have specific policies with respect to the use of DEHP-containing plastics for medical devices?

Most countries have no specific regulations to phase out the use of DEHP for medical devices. A notable exception is France, where as of 2015, DEHP-containing devices are banned from neonatal, paediatric and maternity wards. Also, a ban on DEHP for all medical devices is under consideration. Denmark is looking to phase out the use of DEHP-free infusion systems, but only if suitable alternatives are present. Other regulating bodies advise to consider DEHP-free alternatives, for example the Ministry of Health Japan and the FDA in the USA, for certain patients at risk for DEHP-related toxicity.

In the absence of guidelines on the use of DEHP in medical devices, some respondents indicated that DEHP is still widely used in their ward, while others state that they are on all DEHP-free infusion systems. This is likely to be dependent on the type of hospital or ward; as one respondent noted, neonatal intensive care units generally choose non-PVC or non-DEHP products, while for general use, it is of less concern.

Question 6

To your knowledge, are advocate groups active in your country to promote the use of non-DEHP plastics? Do they focus on medical devices in general or do they pay special attention to blood bags in their arguments?

Advocacy groups are active in various countries. Groups in Australia, France and the USA have addressed transfusion products. In Brazil, it is the hospitals that take the lead in replacing certain ingredients from devices to treat patients,including DEHP, to have a sustainable and eco-friendly supportive care. In Denmark, some groups, including governmental fractions, are active in promoting the use of non-DEHP plastics, but have no specific focus on medical devices.

In summary, despite the recent publication of a number of papers debating the presence of DEHP in blood bags, there is little real concern on the presence of DEHP in transfusion equipment (devices, bags) for the general donor or patient population. Often, where possible, DEHP has been replaced from medical devices such as infusion sets, which already limits the exposure of patients to DEHP. Because of the beneficial effect of the leaching of DEHP on the storage properties of red cells, and the absence of readily available alternatives, there is little desire to move to non-DEHP-containing blood bags. We therefore conclude that, in the absence of real alternatives, DEHP should not be removed from blood bags.

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Question 1

All blood components supplied by the Australian Red Cross Blood Service (Blood Service), except apheresis platelets, are currently stored in DEHP-plasticized bags.

Apheresis platelets are stored in bags which are plasticized with butyryl trihexyl citrate (BTHC). The introduction of these bags was primarily based on the choice of apheresis technology. The supplier of this technology has advised that the choice of BTHC as their plasticizer is because of its superior platelet storage characteristics due to improved gas exchange.

The Blood Service has not evaluated non-DEHP bags with respect to their effect on the quality of stored red cells. It is our understanding that, to date, no currently

available alternatives to DEHP have been shown to provide the same protective effect on long-term stored red cells to enable the maintenance of equivalent quality and survival [1–3]. We are continuing to monitor the literature on this issue and actively follow-up our suppliers regarding developments in this area. All blood bag suppliers have active research in this area.

All blood bags used by the Blood Service are approved by the Australian regulator, the Therapeutic Goods Administration (TGA).

Question 2

The Blood Service does include consideration of the plastic/plasticizer as one of the criteria in the evaluation process of any potential new blood bags. We are currently defining specific criteria for the selection of a new plastic, and this will be incorporated into our next blood bag tender process which is currently underway. The absence of DEHP will be one of the selection criteria.

The Blood Service monitors the literature regarding the impact of DEHP and regularly engages with external clinicians and suppliers on this matter.

Question 3

As plastic disposable equipment, including tubing, used during apheresis procedures is made from plastics containing DEHP, donors can be exposed to DEHP due to leaching of the chemical from the PVC devices during the procedures.

Whilst DEHP exposure in plasmapheresis donors has been found to be similar to that of controls, higher levels of exposure have been reported in plateletpheresis donors [4]. Koch *et al.* measured urine levels of DEHP metabolites in donors undergoing plasmapheresis and either 'discontinuous' (single-needle) or 'continuous' (double-needle) plateletpheresis. The authors found that donors undergoing plateletpheresis had significantly higher exposure levels as compared with controls and with donors undergoing double-needle procedures having higher levels than those undergoing single-needle procedures. DEHP exposure in donors undergoing plasmapheresis procedures was similar to control levels. The authors concluded that the 'margins of safety might be insufficient to protect especially young men and women in their reproductive age from effects on reproductivity'. They recommended that plateletpheresis using discontinuous flow devices should be preferred.

In contrast, Buchta *et al.* measured serum DEHP levels in 36 donors undergoing either single-needle or double-needle plateletpheresis procedures using 3 different apheresis systems [5]. They showed a median increase in donor serum DEHP levels of 232 per cent compared with preprocedural levels, with a broad inter-individual variation. The total DEHP exposure was found to be independent of the apheresis technology used. DEHP levels returned to preprocedural levels within 3 hours after plateletpheresis. The authors concluded that the total dose of DEHP which was retained by the donor was within the normal range of DEHP exposure of the general population.

The risk of DEHP exposure is not limited to these apheresis procedures as people are constantly exposed to DEHP in their daily lives. As the very young are more vulnerable to the harmful effects of DEHP compared with adult blood donors, the risk to donors is considered to be comparatively small.

The Blood Services only uses single-needle apheresis procedures.

Question 4

There are no patient groups within Australia for whom the use of non-DEHP and/or non-PVC blood component storage bags is specifically prescribed. We are not aware of any specific patient groups or patients who have expressed concerns about the use of DEHP-plasticized PVC for the storage of blood components. The Blood Service does, however, provide blood with a shelf-life of less than 5 days for large-volume paediatric transfusions.

Question 5

The Australian Red Cross Blood Service is not involved in the selection of infusion systems for the country or for hospitals.

Question 6

At least two groups in Australia have considered the use of DEHP in medical devices. One group did report concerns about the margins for safety in certain transfusion recipients in 2006. The Therapeutic Goods Administration, in consultation with other international agencies, has progressed investigations regarding DEHP in medical devices.

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C. Buchta

Question 1
We use storage bags containing DEHP for leukapheresis products, as there are no alternatives; our patients are not within high risk groups, and they undergo three consecutive leukapheresis procedures and will get retransfusion of the product after spiking the cells with antigens (clinical trial); this low exposure is not relevant.

For patients within high risk groups, effective procedures to reduce DEHP loads in blood products have been described [1].

Question 2
We have no active programme for replacing DEHP-containing plastics due to the limited exposure of our patients (see question 1).

Question 3
Donor exposure during apheresis might be a concern in frequent aphereses in individuals with high lipids, for example lipid apheresis, but also in healthy donors who repeatedly donate. This we described in 2003 [2].

To my opinion, 'primum non-nocere' should apply to patients and healthy donors, although the total dose of DEHP retained by the donor is within the normal range of DEHP exposures of the general population.

Question 4
There is no evident awareness for DEHP exposure in Austria.

Question 5
There are some hospitals that developed policies, but to my knowledge, there are no official regulations.

Question 6
Not to my knowledge.

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Question 1
At BRC-F, DEHP-plasticized PVC is currently used for the storage of red blood cells and virus-inactivated plasma (methylene-blue plasma). The container for apheresis platelets contains butyryl-tri-n-hexyl citrate (BTHC) as a plasticizer. However, the tubing of the collection sets, the ACD container and the container with platelet additive solution (PAS) are not DEHP free. Buffy

coat-derived platelets are stored in a polyolefin container (PL2410, non-PVC), but the whole blood collection set, the PAS container and the pooling set contain DEHP. Granulocytes are stored in a container with BTHC as plasticizer.

DEHP exposure via blood transfusion represents a small part of the overall environmental exposure [1]. The use of fresh blood products in selected *at risk* patient populations (e.g. children undergoing exchange transfusion) can further decrease the impact of blood transfusion in the overall exposure to DEHP. A number of red blood cell containers with alternative plasticizers (e.g. BTHC) are available on the market. Future alternative containers will need to demonstrate good red blood cell stability during prolonged storage in addition to improved toxicity profiles.

Question 2

At present, there is no active programme for replacing DEHP-containing plastics for blood containers in our Blood Establishment.

Question 3

To limit donor exposure to DEHP, the frequency of apheresis procedures is limited to twice per month. At this frequency, DEHP exposure is considered to be far below the tolerable daily intake [1]. To further reduce donor exposure to DEHP, a single-needle (discontinuous) procedure is used to collect platelets by apheresis.

Question 4

In our region, there are no specific patient groups for whom non-DEHP or non-PVC blood components are prescribed. For neonates undergoing massive transfusion (e.g. exchange transfusion), fresh blood products are selected, thereby minimizing the exposure to DEHP.

Question 5

DEHP-free infusion systems for IV fluids are available on the market but not for all possible indications. Suppliers promote their use and hospital purchasing departments recognize their added value. Belgian legislation prescribes that medical devices are to be designed and produced in a way that minimizes the release of harmful substances. The use of DEHP-free systems is not obligatory but labelling of either the device or its packaging is imposed.

Question 6

To our knowledge, there are no advocate groups actively promoting the use of non-DEHP plastics. Unlike bisphenol-free baby products, DEHP-free medical devices are not receiving much attention in general public.

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S. Wendel, S. Biagini & P. Scuracchio

Question 1

According to Brazilian legislation, bags approved for use in haemotherapy are made of polyvinyl chloride (PVC) with addition of amounts of the following plasticizers: di (2-ethylhexyl) phthalate (DEHP), tri (2-ethylhexyl) trimellitate (TEHTM) and tri-octyl- trimellitate (TOTM) [1].

In our service, we currently use DEHP-plasticized PVC for red blood cells and plasma. For platelet concentrates originated either from apheresis or whole blood units, we use bags that do not contain DEHP. In this case, the plasticizer is the tri-octyl trimellitate (TOTM), so that the gas exchange is high enough for the standard platelet concentrates collected and also the DEHP content for platelets in plasma at the end of shelf-life is reduced by more than 90%.

On the other hand, red cells storage in TOTM-plasticized PVC showed a survival rate less than 75% when stored for 35 days, compared with DEHP-plasticized film and also a decrease in their viability [2, 3].

Question 2

There is no active programme for replacing DEHP-containing plastics for blood container with those that are DEHP free, yet.

Question 3

For collection of platelets and white blood cells, we use bags without DEHP, but plasticized with butyryl-trihexyl citrate (citroflex B-6; BTHC). However, the bags for red blood cells, plasma, therapeutic plasma exchange and associated disposable tubing contain DEHP-plasticized PVC. Although in these procedures, there is a concern about the donor exposure to DEHP during apheresis, it is important to highlight that these procedures are not performed so frequently. The highest exposure was found for donors undergoing plateletpheresis with dual-needle

technique, but even a very frequently donating donor (twice per month) receives a lower dose comparing with the tolerable daily intake [4].

Question 4

We believe that in Brazil, there are no policies for specific patient groups for whom the use of non-DEHP and/or non-PVC for blood components is prescribed. On the other hand, for vulnerable patients, some precautions are naturally taken reducing the exposure to DEHP. Patients considered as being at risk are neonates undergoing extracorporeal membrane oxygenation or exchange transfusion, patients on frequent haemodialysis, cardiac surgery and massive transfusion recipients (trauma). In the group of neonates and patients submitted to cardiac surgery, we always select fresh products and so, we can also reduce the exposure to DEHP plasticizers. No patients in our service have asked questions or concerns about the use of DEHP-plasticized PVC for storage of blood products and maybe most of these patients do not have any knowledge about them.

Question 5

Our hospital does not have any specific policies about the use of DEHP-containing plastics for medical devices, as well as there is no specific legislation which does not accept the use of these products for the administration of IV fluids.

Question 6

In Brazil, there are some hospitals that are trying to substitute specific materials and substances used in the treatment of the patients, which can offer a serious risk of contamination, for example mercury, PVC or DEHP plastics. The proposal is to transform the healthcare sector without compromising patient care and safety, so that it is ecologically sustainable and no longer a source of harm to people and environment.

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L. Thibault & M. Germain

Question 1

Héma-Québec is the sole provider of blood products in the Province of Quebec (Canada). Although Canada issued regulations in June 2011 regarding the use of phthalates in some manufactured products, biomedical instruments were exempted from those regulations [1]. All blood donations at Héma-Québec are collected and processed in DEHP-plasticized PVC. Red blood cell units and plasma prepared either by apheresis or processed from whole blood donations are currently stored in DEHP PVC storage bags. Buffy coat-derived platelet pools and thromb-apheresis products are stored in non-DEHP PVC containers plasticized with butyl-n-trihexyl citrate (BTHC).The choice of a non-DEHP PVC container for the storage of platelet products was mainly motivated by practical considerations, for example, the commercial availability of non-DEHP containers, the quality of stored products and the compatibility of collection sets with our processing equipment, rather than alleged safety or toxicity concerns that might be associated with DEHP-plasticized PVC.

Regarding the possibility of storing red cell concentrates in non-DEHP containers, Dumont *et al.* recently studied the *in vitro* parameters of red blood cells stored in PVC containers plasticized with DINCH, a non-DEHP plasticizer, and reported results comparable with those of red cells stored in DEHP-plasticized bags [2]. Interestingly, storage of red blood cells in DINCH-PVC containers required periodic mixing of the stored red blood cell

units to maintain adequate quality. It is well known that DEHP contributes to product quality by preventing excessive red blood cell haemolysis during storage. However, as exemplified by Dumont *et al.* and other published studies, substitutes to DEHP exist, so it might be possible to compensate for the protective effect conferred by DEHP.

Question 2

Currently, our organization does not have an active programme to replace blood collection sets and storage containers plasticized with DEHP with non-DEHP devices. Although alternatives to DEHP-plasticized bags exist for storing blood components, substitute products for collecting and processing blood products are either not readily available or simply non-existent.

Question 3

As manufacturer of blood components, we must ensure not only that the quality of blood products is optimal, but also that the safety of blood donors is not compromised. In this regard, repeated exposure to DEHP in frequent apheresis donors might be a concern. When this question was raised by one of our donors shortly after the implementation of the Canadian Phthalates Regulations in June 2011, we conducted a risk assessment in this matter and we concluded that DEHP exposure was not a cause for concern in apheresis donors, as the dose received through the course of one donation is much lower than the tolerable daily intake in adults. Furthermore, although the literature reports an increase in the DEHP blood levels following an apheresis procedure, the peak concentration is much lower than what happens in other medical procedures (e.g. haemodialysis) and the DEHP concentration in blood rapidly returns to its baseline level [3, 4]. Another study has reported that the levels of DEHP found in frequent plasma apheresis donors are not higher than levels measured in plasma donors prior to their first donation [5]. Finally, no ill effects attributable to DEHP have ever been reported in apheresis donors.

Question 4

In Canada, there are no designated patient groups which are prescribed blood products stored in non-DEHP containers. However, physicians can refer themselves to a section dealing with DEHP in our circular of information (blood product insert) that is distributed to hospital blood banks. For the most vulnerable patient groups such as foetuses, newborns or prepubescent male requiring massive transfusions, physicians are advised to use the freshest possible blood products or to remove a portion of the blood product supernatant before transfusion. To date, the presence of DEHP in blood products has not raised any requests for information or concerns from transfused patients.

Question 5

Infusion devices used by hospitals are mostly made up of PVC plasticized with DEHP. The Canadian Phthalates Regulations limit the allowable concentration of phthalates in the soft vinyl of children's toys and childcare articles only. Biomedical instruments were excluded from these regulations.

Question 6

To our knowledge, no group is currently lobbying to promote the use of DEHP-free products in our country.

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J. Georgsen

Question 1

Danish blood establishments use DEHP-plasticized PVC for storage of all blood components. Non-DEHP-plasticized blood bag systems were introduced in many Danish blood establishments a decade ago (Fenwal/Baxter, using BTHC or Butyryl Trihexyl Citrate) as a triple bag for SAGM RBC, FFP and BC. Although there were problems with unpleasant

smells, the usage was stopped as there were incidences of occupational dermatitis among staff members, and consequently, some blood establishments were sentenced to compensations.

There was and still is a public concern in Denmark regarding the use of DEHP and this was the main reason for using the triple bag in combination with another plasticizer. It was and has been the only blood collection set without DEHP that has been marketed in Denmark.

Currently, there seems to be no alternative to DEHP as plasticizer for blood bags.

Question 2

Danish blood establishments do not have an active programme for replacing DEHP-containing plastics for blood containers. The following criteria would have to be fulfilled before selecting DEHP-free plastics: 1. same or better quality of the blood components; 2. same or better performance regarding working environment.

Question 3

In donor apheresis procedures, a healthy individual is exposed to DEHP. Therefore, non-DEHP plasticizers for apheresis sets should be a priority.

Question 4

As the blood establishments in Denmark only use DEHP-plasticized blood bags, no patients are prescribed 'non-DEHP' blood components. We are not aware of patients who have expressed concerns about DEHP-containing blood. The public consider Danish blood components to be some of the safest in the world.

Question 5

DEHP-free infusion systems for administration of IV fluids are not used. The position of the Danish Health Authority and Medicines Agency is that DEHP should be phased out but only when alternatives that do not compromise health are available which currently does not seem to be the case.

Question 6

Consumer groups as well as parts of the parliamentarian establishment including the current Danish government are active in promoting non-DEHP plastics, for example in the EU, but without special focus on medical devices.

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S. Bégué & D. Dernis

Question 1

Up to now (November 2012), most of the disposables used by the French blood transfusion at the EFS are not DEHP free. The only exception is Terumo BCT's TACSI System used for preparation of whole blood buffy coat pooled platelet concentrates, as well as some platelet concentrate storage bags made out of polyolefin, PVC with BTHC and PVC plasticized with TOTM.

Blood products are variously concerned with DEHP, and we have identified three different situations:

(1) Products that are in continuous contact with DEHP-containing PVC, whether during process or during storage.
(2) Products that are being in contact with DEHP during the processing time, but not during long-term storage.
(3) Products that are never in contact with DEHP, whether during collection, process and storage.

Currently, EFS does not prepare any product entering in the third category.

All the RBCs are in the first category, and all the platelet concentrates and most of the therapeutic plasma are within the second category.

EFS uses DEHP-free TerumoBCT TACSI system for WB pooled platelet concentrates, but the advantage of this system needs to be put in perspective until the whole blood process, from collection up to storage is not entirely deprived from DEHP.

EFS has introduced non-DEHP/non-PVC plastic for storage, mainly for buffy coat pooled platelet concentrates and for single donor platelet concentrates collected with Fenwal (PL2410), Terumo BCT(PVC-BTHC) and Haemonetics apheresis systems. Here also, the collection systems are not totally DEHP free, and one can be concerned by the quantity of free DEHP brought by the system through tubing, connectors and other non-storage components. MacoPharma (PVC-TOTM) and Cerus (Polyolefin) have also DEHP-free platelet storage containers.

Despite controversy and a lack of definitive answers regarding prolonged effect of DEHP on humans [1], the EFS follows a precautionary approach and the presence of DEHP is a criteria when choice is made between disposables. Nevertheless, EFS is not willing to forgo from a blood component process on the sole basis of the lack of alternatives. Choice of the substitution material is a

supplier's responsibility over which EFS does not want to interfere. EFS has not defined any particular plastic for that purpose.

Recent scientific communications (2–4) show that long-term storage of whole blood filtration processed RBCs in DEHP-free (DINCH) disposable are within the French regulatory haemolysis cut-off (max 0,8% at 42 days) at the end of their storage time of 42 days. Other standard storage indicators from his same communication do not show any discrepancies between non-DEHP and classic containers.

Question 2

The EFS policy for reducing the use of DEHP-containing disposables can be declined through three axes:

(1) Express this requirement in the market tenders related to blood collection and process disposables
(2) To require from the suppliers the formal expression of their commitment in developing and promoting active research in DEHP-free substitutes.
(3) By regularly requesting information about these researches from the suppliers.

Regarding quality requirements, *in vitro* requirements for products prepared in DEHP-free disposables are not different from usual PVC DEHP-containing disposables. Non-DEHP containers must present the same qualities than the usual bags currently used, and stored blood products must present, up to the end of shelf-life the same characteristics and be in accordance with all regulatory requirements.

Question 3

EFS has an equivalent level of concern regarding blood donors and blood recipient's exposure to DEHP. Concerning the donors, we only have very scarcely expressed concerns about BPA and DEHP in apheresis systems. According to its will to tend to reduce the use of DEHP apheresis systems, EFS applies the same procurement policy for apheresis systems as for whole blood collection devices.

Question 4

Currently, in France, there are no regulatory requirements defining a specific patient group to whom non-DEHP blood components should be prescribed. In 2009, the AFSSAPS (at that time the name of French Health regulatory authority, now replaced by ANSM) identified hospitalized newborns in neonatology, children and prepubescent hospitalized in intensive care, haemodialysis or in long-term treatment as being a population where the risk to DEHP is maximal and to whom it should therefore be given preference to non-DEHP medical devices. A recent law voted at the end of 2012 defines a more restrictive framework to this recommendation.

Question 5

The law #2012-1442 voted on 24 December 2012 states that by 1 July 2015, DEHP-containing infusion disposables are to be banned in paediatric, neonatology and maternity wards. The article #4 of this law also states that the government has one year counting from now to address a report to the French parliament on endocrine disrupters and the advisability to ban DEHP from all medical devices.

Question 6

EFS is informed of the existence of one advocate group acting for the suppression of DEHP and phthalates in general from all blood components in France. We have no expressed concern of DEHP in plasma for fractionation from the LFB (Plasma Fractionation operator in France).

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E. Raspollini, S. Villa & P. Rebulla

Question 1

We currently use several DEHP-plasticized PVC containers for storage of blood components (Table 1).