This report reviews the qualities and characteristics of the Liveyon PURE® products (PURE® and PURE PRO®). The data shows the viability of the cells before and after freezing, characterizes cell surface markers and growth factor expression, and the mixture of cells that make up the product’s composition.

Key terms: Regenerative medicine, umbilical cord blood, cell therapy, stem cells

There is great interest in cord blood (CB) for regenerative cell therapies. CB is an enriched source of stem cells, and also contains more mature cells that secrete a variety of growth factors, cytokines, and exosomes(1). CB is obtained from the umbilical cord and placenta, after a healthy, full-term baby is delivered. There is no risk to baby or mother. Since no embryos are destroyed in the collection of cord blood it avoids the controversies and ethical issues associated with embryonic stem cells. CB and other birth tissues are usually discarded after childbirth. However, now that their regenerative potential is recognized — hundreds of thousands of CB units are cryopreserved and ready for use.

There is a thirty-year history of CB therapies. Thus, more than 30,000 CB transplants have been performed to treat hematopoietic malignancies, marrow failure, immunodeficiency disorders, and inborn metabolic diseases (2). Most recipients (about 70%) did not have an HLA-matched sibling donor. Cord blood cells can have “reduced alloreactivity”; this permits transplant without the need to perfectly match tissues (3). In addition to hematopoietic stem cells (HSC), CB contains a variety of other beneficial cell types. These include endothelial progenitor cells (EPC) and mesenchymal stem cell (MSC). EPCs can help form new vasculature. MSCs can help form bone, muscle, cartilage and release a host of reparative growth factors and proteins. There are other types of beneficial cells in CB that are under current study (4, 5).

Historically, CB was used as a source HSC, used to replace blood and immune cells lost to disease or chemotherapy. Now it is understood that the variety of cells from CB can help repair damaged tissues in a variety of ways — through the modulation of immune responses and the release of reparative growth factors (e.g. VEGF, FGF, PDGF, and SCF). These paracrine and autocrine effectors may activate the host’s own stem cells for repair, replacement and regeneration.

Regenerative medicine is advancing daily. Here we characterize the Liveyon PURE® products. This data was obtained as part of the manufacturing validation process; to ensure that a safe and consistent product is made according to cGMP standards. Analyses include: cell viability, cell surface markers, growth factor and anti-inflammatory content, and cellular composition.

**Methods**

**Umbilical cord blood collection**

Cord blood was obtained through an unpaid, voluntary donation, after written maternal consent was obtained. Detailed information on donor medical history, health status, social and travel history was collected and reviewed. Maternal blood was collected +/- 24 hours of the baby’s birth and evaluated for infectious diseases, listed in Table 1. All results and assessments are reviewed by quality assurance personnel, in compliance with FDA Good Tissue Practice regulations and the standards of AATB (American Association of Tissue Banks).

**Processing of PURE PRO® and PURE®**

Quality control and quality assurance standards are rigorously maintained throughout all processes. Upon receipt, samples of the cord blood are removed for testing (sterility, cellular composition, viability, etc.). Cord blood is processed using our proprietary method; isolation

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**Table 1.** Infectious disease testing of maternal blood. *Ab* = antibody-based test, *NAT* = nucleic acid-based test
takes place in a certified cleanroom. The purified cells are counted and resuspended in cryopreservative media. Samples of the finished product are taken for sterility testing and endotoxin measurements; cell viability and hematology analysis are also performed (Figures 2, 3, and 4). The RBC counts and hemoglobin concentration are confirmed with a visual assessment (see Figure 1). The PURE\textsuperscript{®} and PURE PRO\textsuperscript{®} products are maintained at temperatures below 70\textdegree C for storage and shipping.

Liveyon PURE\textsuperscript{®} Cell Characterization
The PURE\textsuperscript{®} product was thawed by holding the vial in a gloved hand. Thaw time was about five minutes. Measurements of cell count, viability and size were determined using a Nexcelom Cellometer Auto 2000. The total number of nucleated cells was determined by staining with acridine orange and propidium iodide (AO/PI).

The thawed cells were also evaluated for growth factor content and cell surface markers (CD markers). Growth factors and anti-inflammatory proteins were determined by using ELISA kits purchased from R&D Systems (FGF2, EGF, VEGF, PGDF and IL-1RA). Flow cytometry was performed using a BD Accuri Flow Cytometer. Briefly, the PURE\textsuperscript{®} cell product was prepared for analysis by incubation with a FITC-, APC-, or PE-conjugated antibody, washed, and suspended in an appropriate buffer. Appropriate isotype-matched controls were also performed. Flow cytometry analysis was performed for CD19, CD31, CD34, CD45, CD73 and CD90.

To validate the consistency of the PURE\textsuperscript{®} product isolation process, 10 cord blood units were prepared for PURE PRO\textsuperscript{®}; 8 cord blood units were prepared for PURE\textsuperscript{®}. Statistical analysis (for average, standard deviation and range) was performed using standard methods.

Results

Viability
The Nexcellom automated cell counter uses dual fluorescence microscopy to count live and dead cells. AO enters all cells and binds to DNA; the count of green fluorescent AO-positive cells determines the total number of nucleated cells. PI permeates the membrane of dead cells, binds to DNA and fluoresces red; this enumerates dead cells. (Figure 2. represents cells from a single donor, before and after freezing. For this specific unit, the viability of the finished product was 94% prior to freezing and 87% post-thaw. To show the consistency of the PURE\textsuperscript{®} product, the viability pre- and post-thaw was

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<th>Table 2. Average viability of the PURE series product, n = 18</th>
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determined with 18 different batches of product (Table 2). The average viability after isolation of cells was 88.7% ±7.2%. After freezing and thawing, the average viability was 80.8% ±10.1%. These values exceed the FDA's Guidance for Industry, with a recommended cryopreservation result of at least 70% viability (in the Guidance for Allogeneic Placental/Umbilical Cord Blood for Hematopoietic and Immunological Reconstitution).

To further investigate the viability of the PURE® series, a post-thaw time course was performed. The product should be used immediately after thawing; however, it is of interest to determine the viability if application is delayed. Vials of PURE® series product were thawed, and remained at room temperature without any special incubation, pH adjustment, humidity control or washing procedure. The viability remains high, even 60 minutes post-thaw. Figure 3 also shows that the viability of the PURE® product (PURE) is essentially the same as the viability of the CB, upon receipt: 88.7% and 89.0%, respectively. This suggests that few live cells were lost during processing.

**Hematology analysis:** Hematology analysis was performed on CB upon arrival, during the PURE® isolation process, and on the finished products. A Sysmex NX-1000 automated hematology analyzer was used for these measurements. The results show a dramatic reduction of unwanted red blood cells and red blood cell progenitors (including nucleated RBC and reticulocytes). Hemoglobin is undetectable by Sysmex analysis. While RBC and RBC progenitors were below detectable limits, number of white blood cells was conserved. Other cell characterizations included: hematocrit, mean RBC size, mean corpuscular volume, mean corpuscular hemoglobin, platelet concentration, platelet volume, counts of neutrophils, lymphocytes, monocytes, eosinophils, basophils, and immature granulocytes.

**Growth factor analysis:** Growth factors include both protein hormones and cytokines. These stimulate proliferation as well as the differentiation and maturation of target cells. Cytokines are secreted proteins that play a role in immune and inflammatory responses. Interleukin-1 receptor antagonist (IL-IRA) is an example of an anti-inflammatory cytokine that plays a critical role in controlling detrimental effects of inflammation. Basic fibroblast growth factor (FGF2), is a strong mitogen that has broad effects in angiogenesis, wound healing and tissue repair. Epidermal growth factor (EGF) stimulates epidermal and dermal regeneration, partially through the regulation of fibroblast mitosis. Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis and vasculogenesis. It regulates the proliferation, migration and survival of endothelial cells. Platelet derived growth factor (PDGF) acts mainly on connective tissues as a potent mitogen, chemoattractant and vasoconstrictor.

**Figure 3.** The percentage of live cells was determined for cord blood (CB) and the fresh PURE product (PURE). This evaluation was also performed on the cryopreserved product immediately after thawing (0’) and 15’, 30’ and 60’ minutes after thawing. Columns correspond to the average viability; the bars indicate the standard deviation from of PURE product.

**Figure 4.** Hematological analysis of CB and PURE product. Samples of PURE product are evaluated during the isolation (“Early”, and “Late” in the isolation process, and as the finished product “PURE”). The left panel shows the WBC count, and the hemoglobin content. The right panel shows the cellular composition, during the processing and after freezing and thawing. The average value for 18 replicates is shown.
The Liveyon PURE® product was evaluated for these different growth factors and cytokines (Figure 5). Samples of 3x10^6 cells were taken from 18 different PURE® preparations and enzyme-linked immunosorbent assay (ELISA) were performed to assess quantities of each target of interest. The data illustrates that Liveyon PURE® product contains each of the growth factors and cytokines surveyed (Figure 5).

**Flow analysis:** Flow cytometry is a well-established method to identify and characterize cells. Cells in the PURE® product were evaluated for the surface markers CD19, CD31, CD34, CD45, CD90 and CD73. The data describes percentages of positive cells for each cell surface marker suggesting that the product contains B cells, endothelial cells, HSCs, MSCs, and a variety of immune cells.

**Discussion**

The purpose of this study was to characterize a heterogeneous population of cells in the PURE® and PURE PRO® cell suspension. Cell viability is high in all steps of the process: in the cord blood unit upon receipt, after product isolation, and after freezing and thawing (89.4%, 88.7% and 80.8%, respectively). All cell types exposed to processing, freezing and thawing have loss; this is true for delicate cord blood cells, also. Liveyon’s proprietary isolation and cryopreservation process maintains a high cell viability and reduces cell loss. Furthermore, the survival rate stays high, even when the thawed cells are maintained in non-ideal conditions (at room temperature, in cryopreservative).

Here we also characterize cell types present in the CB, and in the finished PURE® products. White blood cells from the CB are retained while the red blood cells are eliminated in Liveyon’s proprietary method. RBC precursors like nucleated RBC and reticulocytes are also removed; the hemoglobin levels drop to undetectable levels. Flow cytometry indicates the presence of stem and progenitor cells (CD34, 73 and 90); a variety of immune cells (CD45 and 19) and endothelial cells (CD31, 34). Analysis of replicate samples show that the product has great lot-to-lot consistency. The PURE® products are also consistent in their production of growth factors, and the potent anti-inflammatory IL-1RA.

CB are now being used in novel applications for nonhematopoietic diseases, cellular regenerative therapy, and immune modulation. A key element in advancing these applications is thorough characterizations: describing the cell preparation and composition in detail. Surprisingly, only a small minority of clinical studies characterize the stem cells used. For example, a meta-analysis by Murray et.al. (6) looked at studies where bone marrow aspires were used in the clinic. Less than 20% of the studies cited a cell count or characterization. In contrast, Liveyon provides a detailed description of cell viability, identity, growth factor expression, and safety testing. Moreover, Liveyon PURE® product offers convenience and ease of use. It is easily obtained via overnight shipping, can be stored at -80˚C for up to a year, and is ready for use in only a few minutes.

Presently, the use of umbilical cord cells is one of the most active areas in the regenerative medicine sector. There is already a strong record of safety (7, 8) and many clinical trials are underway to test applications and identify patients who could benefit. In summary, this evaluation of Liveyon PURE® suggests that it contains a high percentage of live cells to include stem cells and progenitor cells that play an important role in the regenerative medicine field. In addition, Liveyon PURE® contains a variety of growth factors and the potent anti-inflammatory IL-1RA which may help in healing and repair. The future of use of CB will extend far beyond its original use in hematopoietic reconstitution and become important in cellular repair, replacement, and regeneration.
References


Disclosure and disclaimer:

Disclosure: The flow cytometry, sterility testing and infectious disease testing were done independently by a third-party reference lab; Liveyon was the sponsor.

Disclaimer: Talk with a trusted healthcare professional before volunteering for a study or undergoing an experimental treatment.

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