

Decontamination of Two Umbilical Cord Blood Grafts Prior to Autologous Administration

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ABSTRACT

Umbilical cord blood (UCB) is proving to be a valuable resource of stem cells and is currently being used for a variety of oncological and hematological pathologies and metabolic disorders; in addition, new clinical trials are showing promising results in certain neurological, autoimmune and developmental disorders. More recently, the US Food and Drug Administration (FDA) has granted approval for the clinical use of cellular therapies with UCB-based products and new therapeutic utilizations are being studied for regenerative medicine; all these developments will increase the utilization of “off-the-shelf” UCB units.

As a drawback, contamination of UCB grafts is a significant occurrence (upwards of 5% in most analysis) and, even though it consists mainly of non-pathogenic bacteria, it can raise serious questions regarding intravenous UCB administration, especially in patients who are not receiving coincidental antibiotic coverage.

Here we report the successful decontamination of two UCB grafts prior to administration without compromising the viability of the stem cells administered, and propose to apply the same principle and procedure to any contaminated graft.

Keywords: cord blood stem cells, stem cell graft, umbilical cord blood, UCB, graft contamination, UCB stem cells, ASD, decontamination of cord blood.

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Article received on the 21st of November 2022 and accepted for publication on the 21st of December 2022

INTRODUCTION

Since the first successful administration of stem cells from UCB in 1988 (1, 2), banking of cord blood in public and family stem cell banks, together with bone marrow donor registries, has greatly attenuated the difficulty of finding stem cell donors for transplantation, so that more than 40.000 UCB grafts were administered since then (3). Being harvested much easier than bone marrow and having better graft-versus-leukemia and graft-versus-host characteristics, UCB has also showed some important limitations in transplant procedures: a smaller number of total nucleated cells (TNCs), stem cells and delayed engraftment, which often prompts the use of two or more UCB grafts; and also microbiological contamination of the graft.

The UCB advantage is likely due to the presence of young pluripotent stem cells, which have not been exposed to post-natal environmental and infectious factors suspected to induce modification of the immune system, and the relative T cell immaturity from UCB can translate in better immunologic tolerance and modulation of existing T cells in recipient (4). This is in line with the less stringent matching requirements for UCB donors and the observed better graft-versus-host (GVHD) properties for UCB and the graft-versus-leukemia effect observed in children (5-7). UCB has T cells with lower cytotoxic activity and higher activation-induced apoptosis compared to T cells from peripheral blood (8) and the different cytokine profiles (e.g. IL-7, IL-15, IL-12, etc) are the probable factors influencing the activity and fate of T cells from administered UCB grafts.

Umbilical cord blood is also successfully used for treating metabolic disorders (Hurler and Sanfilippo syndromes, Krabbe disease) in early childhood, with 75% being disease-free at five years after transplant (9); and outside the field of transplantation was successfully administered for neurological disorders (cerebral palsy, neuron motor disease) and developmental disorders such as autistic spectrum disorders (ASD), developments which will most likely be followed by increased UCB utilization.

The usefulness of a UCB graft for transplantation is typically judged by its ability to engraft before day 45 post-transplantation and is evaluated

before cryopreservation by the generation of colony-forming units (CFUs) at two weeks (3); after decryogenation, evaluation with an aldehyde dehydrogenase test was proposed (10). In order to predict the UCB quality prior to harvesting –afterwards verified by the number of CD34+ and CFUs– it was observed that a younger gestational age (34-37 weeks) was a common positive predictor in some studies while the importance of other factors differed (11, 12). Shorter interval (<10 hours) to processing, higher birthweight (>3500 g), larger collection volumes (>80 mL) and Caucasian race correlated with better UCB grafts (11); while cesarean delivery and male babies had better prediction value than the UCB volume in another study (12). Interesting to mention in this context, male babies are also more prone to ASD.

A larger UCB collection volume was also associated with a lower rate of contamination in more than 10.000 UCB samples harvested between 2010 and 2020 in a German bank, with 12% of UCBs of less than 60 mL and 6% of UCBs with volumes of more than 60 mL being contaminated, yielding an overall contamination rate of 8% and a correlation coefficient $r = -0.9$ between UCB volume and the presence of contamination (13). The same study showed that cesarean deliveries had a much lower contamination rate (1.4%) than vaginal deliveries (9.7%). Also, a difference between contaminating bacteria was shown, so that *Bacteroides* species was practically absent in C-section deliveries, with *Propionibacterium acnes* (40%) being much more frequent in C-section than vaginal, and the remaining bacteria, including *Staphylococcus* (35%), *Bacteroides* (23%), *Enterococcus* (22%) and *Escherichia* (14%), having higher percentages in vaginal delivery vs cesarean section – where only *Staphylococcus* (34%) was comparable (13).

The contamination rates of UCB vary substantially among stem cell banks (between 1-15%), however a common characteristic is that its rate decreased from the 1990s to 2010, most likely due to better practices and standards; this fact alongside above data (e.g., difference between cesarean vs vaginal deliveries) allow us to infer that most instances of contamination of the UCB grafts occur during cord blood sampling at birth and less during cryopreservation procedures (14-18). Another aspect is the detection of contamination, which is influenced by the tes-

ting methods used (e.g., 10 mL anaerobic culture bottles have more positive results than 1 mL bottles, plasma vs culture medium, needle gauge, etc (16, 19-23). Most contaminations involve bacteria normally populating the skin or mucosa *Stafilococcus*, *Peptostreptococcus*, *Streptococcus anginosus*, *Propionibacterium*, etc), which are not normally pathogenic (16, 17, 19, 24), but also *Bacteroides*, *Pseudomonas*, *Actinobacter*, *Clostridium*, *Bacillus* which can be more problematic (18, 24). □

MATERIALS AND METHOD

Informed consent was obtained from the parents prior to preparation and administration of UCB grafts, which was done as part of the CORDUS clinical trial, registered on clinicaltrials.gov with NCT04007224 and approved by The National Committee on Bioethics IS/4/12.02.2020.

An important aspect of decontamination is the killing of bacteria without affecting the viability of stem cells, and we have obtained that by using antibiogram-based antibiotic treatment of the UCB graft and verified it by testing for sterility of decontaminated graft via Bactalert cultures and the viability of stem cells from UCB post-decontamination with Trypan Blue staining and flowcytometry testing with 7-amino-actinomycin D (7-AAD).

We based our approach on well-established facts: i) antibiotics act selectively on bacterial protein synthesis (16, 30 and/or 50 S ribosomal sub-units) inhibiting bacterial wall formation and do not alter mammalian or human cells, which lack such organelles (25-28) ii) it is current practice in laboratories to protect human cell cultures against bacterial contamination by adding a mixture of penicillin and streptomycin (P&S), which was shown to be much better tolerated than amphotericin B or gentamycin for exposures of 24 hours and more (29-31).

We have added a very small amount of antibiotic to the UCB graft after decryogenation and the addition of dextrane and albumin was done according to the Rubinstein protocol (32). The antibiotic dose was calculated such that the concentration achieved in the bag was up to 10 times the minimal inhibitory concentration (MIC) which kills 100% of the respective bacterial isolates (MIC100) as per antibiogram and the literature and confirmed by clinical practice.

Adding the antibiotic early after decryogenation allowed it to act on the contaminating bacteria before the graft was administered, so that in most likelihood they were killed before infusion (confirmed by bacterial cultures); at the same time, the relatively short exposure time – between 1-2 hours – at relatively high antibiotic concentration is having minimal impact on the stem cells (confirmed by viability tests).

Viability and cell count was performed on the resulting graft before (Trypan Blue) or after (flow cytometry) UCB graft administration, and bacterial cultures were performed from the UCB graft wash (centrifugation supernatant), with results being read in the Bactalert system one week after inoculation. Patients also received the respective antibiotic either orally three days before and three days after UCB administration, or in one dose of intravenous antibiotic prior to UCB administration, which was preceded by administration of the Duke University premedication according to the respective protocol (33). □

RESULTS

Both contaminated UCB grafts (presented below as A and B) were used for autologous administration in two children with ASD and without prior immune suppression; one of the kids was previously treated twice with autologous bone marrow stem cells.

Each case represents a different scenario – one in which the antibiogram was performed on the identified bacteria before cryopreservation, and the other in which the antibiogram was performed after cryogenation, on a portion of the decryogenated graft.

In both cases, the cultures performed on the graft after decontamination for the bacteria which were initially reported were negative, and the viability of stem cells in both grafts was very good.

A. First contaminated UCB graft with an initial harvested volume of 113.9 mL – which was reduced after treatment with hydroxyethylstarch, so that the final graft volume was approximately 25 mL – was placed in a dual cryo bag of 20 + 5 mL, with 10% DMSO, 1% dextran. TNC 790×10^6 WBC (TNCd corrected for nRBC) 470×10^6 ; CD45 viability 99% and a CD34+ number of 1.1×10^6

The cord blood tested negative for Anti-Hbc, Anti-HCV, Anti-HIV 1/2, but prior to cryogenation the bacterial test was positive for *Propionibacterium acnes*, and the antibiogram performed with three antibiotics showed that it was sensitive to Penicillin G and clindamycin but resistant to metronidazole.

The patient had a body weight of 32 kg, blood group A II and Rh+, and blood testing prior to administration showed normal values for complete blood count, erythrocyte sedimentation rate, liver and kidney function tests, lactate dehydrogenase, sodium, potassium, serum protein electrophoresis, IL-6; abnormal values for ferritin 5.2 ng/mL (lower limit normal 13.7 ng/mL), TNF-alpha 10,5 pg/mL (ULN 8.1), serum albumin 53.8% (LLN 55.8%).

The patient tested negative for IgEs to penicillin and was started on clindamycin 300 mg PO q12 h three days prior to the procedure, and also three days after. Additional HLA-A, HLA-B, and HLA-DRB1 typing of both patient's peripheral blood and a contiguous segment attached to the UCB graft confirmed the identity of the autologous graft.

Clindamycin 0.1 mg was added to the UCB (25 mL) following decryogenation and after adding dextrane and albumin according to the Rubinstein protocol and before first centrifugation. Ensuing cell viability was good, exceeding 90% for TNCs.

Bactalert cultures – both aerobic and anaerobic – were performed from the decryogenated UCB and were negative at one week and 10 days.

Overall, there were no adverse reactions observed acutely; patient's TNF-alpha (7.6 pg/mL) IL-6 (1.5 pg/mL) and ferritin (20.1 ng/mL) were normal at one month after administration, normal serum albumin 56.6%; and six months post administration a quantifiable degree of progress was reported on the underlying ASD pathology.

B. The initial volume of UCB harvested was 116 mL, with WBCs 73.9×10^6 /mL, viability 94%. Before cryogenation, testing of the harvested UCB showed a positive aerobic culture and *Staphylococcus milleri*/*Streptococcus anginosus* (Sa) was identified, but no antibiogram was performed at that time; anaerobic culture was negative.

The literature shows that Sa is susceptible to all third-generation parenteral cephalosporins, except ceftazidime with 100% sensibility (34).

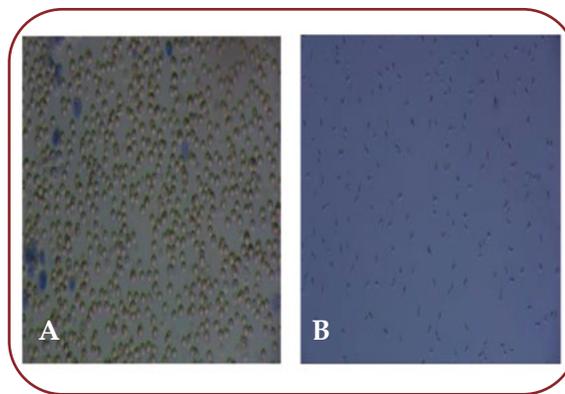


FIGURE 1. A. Trypan Blue, live RBCs, dead cells (blue); B. Trypan Blue, 200 x mesenchymal stem cells

Minimum inhibitory concentrations (MIC50 and MIC90) for ceftriaxone are established at 0.25 and 0.5 mg/L, and a concentration of ceftriaxone above 1 mg/L ensures that 100% of isolates are killed, which corresponds to 0.001 mg/mL, 0.01 mg/10 mL and 0.02 mg/20 mL.

At presentation, the patient had blood group B III Rh+ and a body weight of 23 kg; the identity of the autologous graft was confirmed via HLA-A, HLA-B, and HLA-DRB1 typing of both patient's peripheral blood and a cryotube from the UCB graft.

Prior to UCB administration, the patient's blood testing showed normal liver function tests, sodium, potassium, calcium, ESR, ferritin and high hematocrit 41.7% (N < 40%), platelets 535×10^3 /mm³ (N < 450.000), CRP 0.104 mg/dL (N < 0.1) and alpha-2 globulins 13.3% (N < 11.8%); low creatinine 0.29 mg/dL (N > 0.31) and gamma-globulins 9.8% (N > 11.1%).

In this case, we used a different strategy, which was possible given the very high number of TNCs (over 1.000×10^6); we "sacrificed" the smaller portion (5 mL) of the dual bag in order to perform two tests: most of the volume (4,5 mL) was combined with approximately 5 mL Dextrane 20% + albumin and 0.1 mg Ceftriaxone and the approximately 10 mL of decryogenated treated cord blood was inoculated in a Bactalert aerobic bottle for 10 days; the remaining small amount (approximately 0,5 mL) was used to perform antibiogram testing.

The antibiogram showed that bacteria was sensitive to 13 Antibiotics, including Ampicillin, Oxacillin, Penicillin, Clindamycin, Daptomycin, Gentamycin, Amoxicillin+clavulanic acid, Ciprofloxacin, Moxifloxacin, Rifampin, Tetracy-

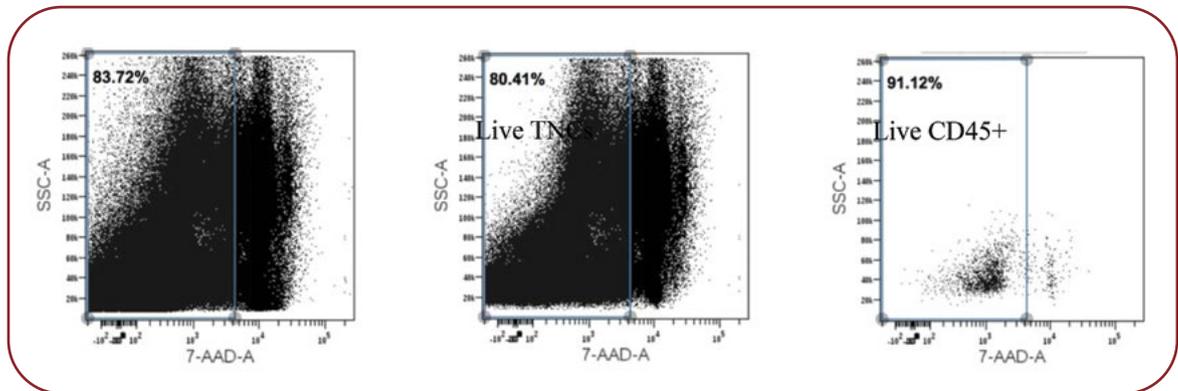


FIGURE 2. Flow cytometry dot plots depicting cell viability of UCB cells and fraction of viable CD45 + cells

cline, Mupirocin, Imipenem, and resistant to Azythromycin and Erythromycin.

The Bactalert aerobic bottle inoculated with cord blood + Ceftriaxone 0.1 mg showed no growth after one week, so we proceeded with the UCB graft treatment.

The day of administration the remaining UCB (20 mL bag) was decryogenated according to the Rubinstein protocol, and after dextrane and albumin we added Ceftriaxon 0.2 mg, which was more than 10 times the concentration of antibiotic able to kills all Sa strains (MIC100) (34).

Subsequent TNC for the administered graft was 850×10^6 , with a viability of 85% on Trypan Blue and about 83% on flowcytometry.

We used 10 mL of supernatant from the decryoogenated graft to innoculate another Bact-alert aerobic bottle for culture, and the result came negative after one week of incubation.

Although the patient had a history of allergies, including atopic dermatitis, there was no known antibiotic allergy and, after premedication with antihistamine and hydrocortisone, Ceftriaxone 1000 mg was given iv in 100 mL normal saline in 30 minutes prior to administration of the UCB graft. During the administration, the patient had mild facial flushing, which resolved spontaneously in a few minutes after occurrence without administering additional medication. For this patient, behavioral progress was reported within days of administering the UCB, very early compared to other treated kids, and we surmised that it was probably due to the high number of TNCs contained by the respective UCB graft.

Blood tests repeted at one year post UCB administration showed normal TNF-alpha (6.4 pg/mL), normal ESR (3 mm/h), normal neo-

pterin (4.9 nmol/L) and improved value for alpha-2 globulin fraction (12.4% vs 13.3% initially) and the child continued to show cognitive and behavioral progress. □

DISCUSSION

Utilization of contaminated grafts in immune-suppressed patients was previously reported (considered safe because transplanted patients have wide spectrum antibiotic coverage while immunosuppressed), and while the vast majority of patients survived without serious side effects (35), there is at least one reported fatality following *Pseudomonas cepacia* inoculation (24); so, it is probably safer to eliminate the potentially infectious agent prior to intravenous administration while making sure that the viability of stem cells is not compromised. This step becomes more stringent in non-conditioned (non-immune-suppressed) patients treated with UCB for metabolic and neurologic conditions (cerebral palsy, autistic spectrum disease, etc.) in whom antibiotic coverage is not administered (patients receiving stem cell treatments, not transplants).

Compared to treating cellular cultures with Penicillin + Streptomycin (P/S) to prevent bacterial contamination (in the absence of contamination), our procedure concerns cell cultures which are already contaminated. Treating the decryogenated UCB with antibiotics which were proven to be non-toxic (e.g., excluding amphotericin B or gentamycin), for less than two hours, allows for good viability of stem cells while eliminating the contaminating bacteria.

Regarding the possible scenarios, for UCB grafts with low volume (<60 mL) we should em-

ploy rapid tests for detecting contamination, such as the ratio of nicotinic acid/nicotinamide (36) or bacterial identification by 16 S sequencing (37), and very importantly before cryogenation an antibiogram should be performed for each contaminated graft, because it gives prompt and important information and spares graft material.

Another possible path for contaminated grafts for which no antibiograms were done prior to cryogenation, in order to preserve the cryogenated graft material as much as possible, we can only perform an antibiogram from the segment attached to the UCB graft or from a cryotube (without performing blood cultures for verification). Even in the absence of negative bacterial culture before administration, we are reasonably assured that no live bacteria will be infused considering that we can achieve high antibiotic concentrations (more than MIC100) in the administered graft.

These possible paths are summarized in Figure 3.

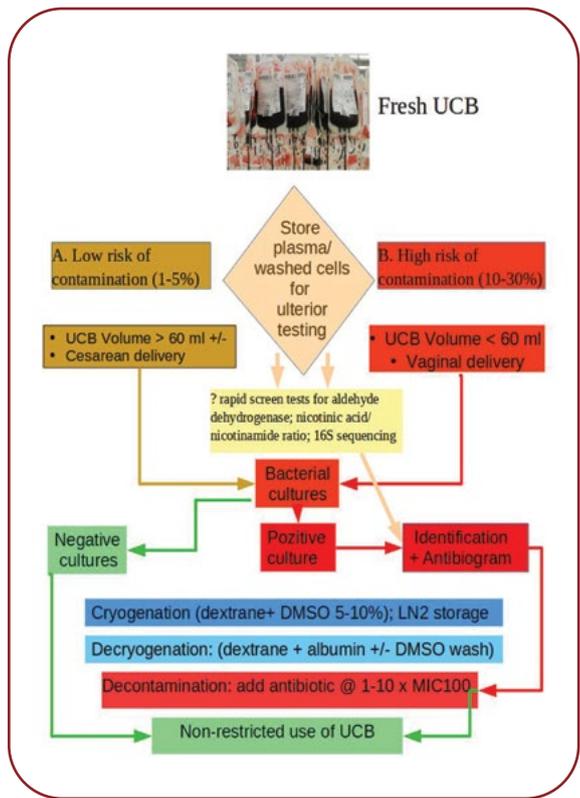


FIGURE 3. Flowchart for testing and decontamination of UCB grafts

Another aspect is that, in some cases, bacteria which are present before cryopreservation will not be viable or grow/multiply after decryogenation (19, 38, 39), which raises the possibility that some UCB grafts contain inhibitory plasmatic factors (21), very likely T cells active against the respective bacteria which can eventually be expanded and used.

Expansion of viable and functional UCB stem cells ex-vivo using small molecules (nicotinamide, StemRegenin-1, UM171) was successfully demonstrated (6). In this instance, the primary goal for administration of expanded UCB units was to address the shortcomings of UCB transplantation (delayed engraftment, higher rates of acute GVHD and infections with higher associated treatment-related mortality), while preserving the advantages of lower rates of relapse and chronic graft-versus-host disease, objective which was safely accomplished in several clinical trials (40-43).

Expanded UCB was also used to lower the severity of viral infections after stem cell transplantation and improve the outcome of transplant-related viral infections (44) by expanding UCB stem cells in the presence of viruses such as Epstein-Barr and adenovirus, viruses for which cytotoxic T lymphocytes specific were demonstrated in the expanded grafts (45).

Future research in this direction may show whether this approach can be used outside transplantation procedures (with or without prior immune suppression) for other severe viral infections (Ebola, SARS-CoV-2, HIV, etc) and perhaps in infections with bacteria resistant to multiple antibiotics. □

CONCLUSIONS

Decontamination of UCB grafts which tested positive for bacteria can be successfully done prior to their administration by adding the antibiotic shown in the antibiogram without compromising the viability of the stem cells from the graft; it should be added in the first steps after decryogenation in order to allow time for the antibiotic to act before the administration of the graft.

If there is no antibiogram done prior to cryopreservation, it can be performed from a decryogenated portion of the graft prior to administration. High concentrations of the antibiotic, well

above the established bactericidal concentrations for the respective species and which cannot be attained *in vivo*, can be obtained for a limited time – up to two hours – in the small volume of the decryogenated graft without producing important damage to the administered stem cells.

Future research may determine if expanding a UCB graft in certain conditions (Antigen + cytokines, etc) is a useful strategy for treating infections with difficult-to-treat viruses and bacteria.

Graft decontamination is especially needed in non-transplant administration of stem cells, when patients are not usually given antibiotics and intravenous administration of a live bacteria is not a reasonable risk, and an antibiotic concentration of 1-10 x MIC100 acting on the decryogenated graft for 1-2 hours is proposed as

being effective for bacterial decontamination and safe for the stem cells. □

Informed consent: obtained prior to the described procedures from the parents of both children

Conflicts of interest: none declared.

Financial support: none declared.

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Acknowledgements: Flow cytometry measurements were performed by Dr. Livia Sima in the dedicated core facility of the Institute of Biochemistry of the Romanian Academy, using the 3-laser FACSVerse instrument and the FACSuite software.

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