Stem Cell Composition of Umbilical Cord Blood Following Milking Compared with Delayed Clamping of the Cord Appears Better Suited for Promoting Hematopoiesis

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In comparing placental transfusion strategies, blood obtained from an umbilical cord that has been "milked" vs one in which clamping was simply delayed contains mesenchymal stromal cells in addition to solely hematopoietic stem cells, a composition more favorable for hematopoiesis, as suggested by its superior rescue of lethally irradiated bone marrow–depleted mice. *(J Pediatr 2019;■:1-5).*

The most often-cited benefit of delaying the clamping of the umbilical cord after birth is providing the newborn infant with a "placental transfusion" that increases the blood volume.[1] It is recognized that, in addition to infusing additional red blood cells, greater quantities of umbilical cord blood may contain cells "left over" from embryogenesis and organogenesis (eg, residual pluripotent stem cells) that entered the fetal circulation.[2-4] Placental transfusion has been shown to reduce the incidence of late-onset sepsis in preterm neonates, supporting the idea that umbilical cord blood has constituents that may help in ways beyond solely increasing intravascular volume—for example, improving immune function.[2]

There are 2 techniques presently in practice for placental transfusion: delayed cord clamping (DCC) and umbilical cord milking.[1-3] In DCC, the most commonly used method, clamping of the umbilical cord is delayed for 30-60 seconds while umbilical cord blood is "pumped" by the placenta into the infant, held at the level of the mother's heart. In umbilical cord milking, the intact umbilical cord is progressively squeezed toward the infant, actively pushing umbilical cord blood into the neonatal circulation before it is clamped.[4] This "low-tech" intervention allows for a faster (<20 seconds[4,5]) transfer of umbilical cord blood from the placenta to the neonate, an important consideration when timely resuscitation of a newborn infant is critical, for example, a depressed or premature infant. Studies comparing immediate cord clamping with umbilical cord milking have demonstrated that milking the cord results in higher systemic blood flow, higher blood pressure, higher urine output, fewer days on oxygen, decreased incidence of intraventricular hemorrhage, and a higher initial hematocrit.[4-8] In a large study comparing umbilical cord milking and DCC, hemodynamics and placental transfusion appeared to be better after the former.[9] Numerous studies have attested to the safety of umbilical cord milking.[5-7,10,11]

We became intrigued by reports from nearly 70 years ago suggesting that although both DCC and umbilical cord milking lead to increased initial hematocrit, umbilical cord milking is associated with a more sustained rise in red blood cell count compared with DCC.[12,13] Infants who received umbilical cord blood by milking the cord had higher hematocrit levels at 42 days after birth and lower requirements for blood transfusion. However, the physiological mechanism underlying this interesting finding has never been pursued. We speculated that umbilical cord milking may provide the recipient infant with blood more enriched in cells that could effect erythropoiesis. Therefore, we studied the content of umbilical cord blood from a series of healthy full-term infants assigned at random to receive umbilical cord milking or DCC.

Methods

Umbilical Cord Blood Collection

This randomized controlled prospective study was approved by the Institutional Review Board of the University of California San Diego (UCSD). Only newborn infants from full-term uncomplicated pregnancies (gestational age >37 and ≤42 weeks) were included. Exclusion criteria were parent or obstetrician refusal to participate, HIV or hepatitis

BPD	Bronchopulmonary dysplasia
BMT	Bone marrow transplantation
DCC	Delayed cord clamping
HSC	Hematopoietic stem cell
MSC	Mesenchymal stromal cell
UCSD	University of California San Diego

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infection or any other blood culture–positive bacterial infection, parental desire for umbilical cord blood banking, major congenital anomalies, severe maternal illness, placental abruption or previa, a ruptured uterus at delivery, or hemoperitoneum.

The umbilical cord was immediately clamped and cut close to the infant. The obstetrician then cut the distal 30 cm of the umbilical cord still attached to the placenta and deposited it into a sterile container before delivery of the placenta. That length of isolated umbilical cord was then subjected to a simulation of either DCC or umbilical cord milking. To emulate DCC, the contents of the isolated unclamped cord segment were allowed to passively drain by gravity into a sterile conical tube for 30 seconds. To mimic umbilical cord milking, approximately 20 cm of the isolated umbilical cord was squeezed ("milked") 3 times at 1-second intervals sliding the fingers toward the sterile container.

The time taken to collect the blood by simulated DCC or umbilical cord milking was recorded. The umbilical cord blood collected and analyzed was from the portion of the cord still connected to the placenta and represents the umbilical cord blood that the infant would have received had a placental transfusion procedure been performed. Immediate cord clamping was the standard of care at our institution at the time this study was performed.

The method of umbilical cord blood collection—DCC or umbilical cord milking—was assigned at random immediately before delivery as in our previous studies.[4,14] A block randomization scheme was used to ensure that an equal number of umbilical cord blood samples were obtained from cesarean and vaginal deliveries. No outcome data on the mothers or infants were collected. A sample size of 40 subjects (20 in each of the DCC and umbilical cord milking arms) was selected to account for potential dropout (eg, due to insufficient volumes for analysis), without loss of statistical power.

Umbilical cord blood was deposited into a 100-mL conical tube containing 11 mL of citrate-phosphate-dextrose solution (Sigma-Aldrich, St Louis, Missouri).

Determining Umbilical Cord Blood Stem Cell Composition by Flow Cytometry

Umbilical cord blood samples from each group (umbilical cord milking and DCC) were heparinized and placed on ice until the peripheral blood mononuclear cells were purified (within 12 hours of blood collection) using standard Ficoll-Paque gradient centrifugation. To determine the hematopoietic stem cell (HSC; $CD34^+$) population, 1×10^6 cells were immunostained with CD34-VioBlue antibody (catalog no. 130-095-393; Miltenyi Biotec, Bergisch Gladbach, Germany),[15] followed by identification and quantification on a BD FACSCanto flow cytometer (BD Biosciences, San Jose, California). To determine the mesenchymal stromal cell (MSC) population, 1×10^6 cells were stained with an MSC phenotyping antibody "cocktail" (Miltenyi Biotec; catalog no. 130-095-198) containing CD73-APC, CD90-FITC, CD105-PE, CD14-PerCP, CD20-PerCP, CD34-PerCP, and CD45-PerCP. Under the same conditions, the MSC cells were also identified as $CD73^+$, $CD90^+$, $CD105^+$, $CD40^-$, $CD20^-$, $CD34^-$, and $CD45^-$.[13] As a control, flow analysis was also performed using corresponding mouse IgG1 or IgG2a conjugated with APC, FITC, PE, PerCP, or VioBlue.

Statistical analyses were performed using SPSS v. 19 (IBM, Armonk, New York). The paired t test was used to compare normally distributed continuous data (stem cells and volume of cord blood), and the χ^2 test was used for categorical data (presence and absence of MSCs).

Transplantation of Umbilical Cord Blood into Lethally Irradiated Bone Marrow–Depleted NGS Mice to Determine Ability to Extend Lifespan

All animal experiments were performed according to protocols approved by the UCSD Institutional Animal Care and Use Committee. Immunoincompetent NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) were bred and maintained in a controlled barrier room in the animal care facility at UCSD. At approximately age 5 months, umbilical cord blood recipients were lethally irradiated (9.5 Gy) and subsequently retro-orbitally transplanted with 2000-4000 cells isolated from umbilical cord blood samples obtained following either DCC (n = 6 mice) or umbilical cord milking (n = 6). Recipients were maintained on antibiotic water (sulfamethoxazole and trimethoprim) and evaluated daily for signs of morbidity, weight loss, failure to groom, and splenomegaly. The time of death was noted and analyzed in a standard Kaplan–Meier survival curve. The experiment was terminated at 6 months posttransplantation. At the time of death, peripheral blood and bone marrow of the transplanted mice were evaluated for the presence of human-derived and mouse-derived HSCs based on cytometry for markers suggestive of the various HSC types listed below using antibodies specific for each species: CD34 cells, immature leukocytes (CD45), immature lymphocytes (CD3), B cells (CD19), T cells (CD4, CD8, and CD33); granulocytes (CD66b), natural killer cells (CD56), macrophages (CD11b and MAC1); erythrocytes (CD235a), and platelets (CD41b, CD42a, and CD61).

Results

Of the 40 umbilical cord blood samples obtained (20 umbilical cord milking, 20 DCC), 25 had sufficient volume (minimum 20 mL) for analysis (12 DCC samples and 13 umbilical cord milking samples). The time to collect umbilical cord blood following simulated umbilical cord milking was 5 seconds, and the average time to collect umbilical cord blood following simulated DCC was 28 ± 4 seconds. As illustrated in the Table, umbilical cord blood samples collected following DCC contained a greater percentage (as well as a larger volume) of HSCs than samples collected following umbilical cord milking. Most of the umbilical cord blood samples collected following umbilical cord milking also contained MSCs, in contrast to the composition of

Table. Composition of umbilical cord blood following DCC or umbilical cord milking

Variables	DCC (n = 12)	Umbilical cord milking (n = 13)	*P* value
Volume, mL, mean ± SD	61 ± 25	33 ± 18	.01
HSCs, % of cells per UCB sample, mean ± SD	47 ± 18%	21 ± 19%	.04
Samples with detectable MSCs, n	2/12	9/13	.02
MSC:HSC ratio	0.001 ± 0.003	0.118 ± 0.011	.02

UCB, umbilical cord blood.
The percentage of HSCs and MSCs shown reflects the percentage found in the total isolated fraction and the sorted peripheral blood mononuclear cell fraction.

umbilical cord blood following DCC, which rarely did. (The percentages of HSCs and MSCs in the Table represent those found in the total isolated peripheral blood mononuclear cell fraction.) Furthermore, the MSC:HSC ratio was higher following umbilical cord milking than following DCC, at a ratio known to be necessary for effective hematopoiesis.[16-26]

All neonates in both placental transfusion groups, including those with or without sufficient umbilical cord blood volumes for analysis, were simliar in terms of antenatal characteristics, gestational age, birth weight, perinatal complications, or maternal age. There were no differences in umbilical cord blood composition based on mode of delivery (5 samples in each group were obtained following cesarean delivery).

We tested our prediction that hematopoiesis would be functionally more effective using umbilical cord blood obtained by umbilical cord milking than by DCC. This was based on our finding that umbilical cord milking blood comprises MSCs in addition to HSCs, despite the fact that DCC-obtained umbilical cord blood contained a greater proportion and greater absolute number of HSCs. Bone marrow transplantation (BMT) was performed using umbilical cord blood from each category injected retro-orbitally into lethally irradiated NGS mice; 6 mice received DCC-obtained umbilical cord blood, and 6 received umbilical cord milking–obtained umbilical cord blood. Each mouse received a sample from a different delivery to rule out bias from a particular mother or neonate. The total number of cells injected into each mouse was rendered identical, so that only their composition varied: pure HSCs for the DCC-obtained blood and MSCs plus HSCs for the umbilical cord milking–obtained blood at a ratio of MSC:HSC as shown in the Table. (The samples of DCC blood that contained MSCs and the samples of umbilical cord milking blood in which MSCs could not be detected were not used for BMT, to avoid confounding the results.) The data are presented as Kaplan–Meier curves in the Figure. No mouse receiving DCC blood lived past 26 days, whereas 67% of the mice receiving umbilical cord milking blood lived 6 months, the point at which the experiment was terminated. The bone marrow of the surviving mice contained human HSCs. Of note, none of the nonirradiated NGS mice died after receiving umbilical cord blood from either source, supporting the absence of nonspecific toxicity of the specimens.

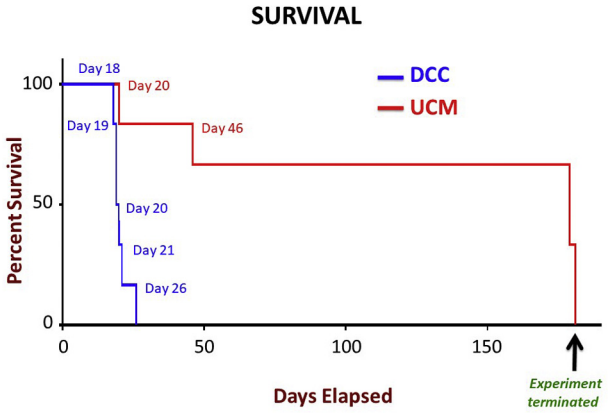

Figure. Survival of lethally irradiated NGS mice after BMT with umbilical cord blood obtained by DCC (*blue curve*; n = 6) or umbilical cord milking (*red curve*; n = 6). The experiment was terminated at 6 months post-BMT. The numbers indicate the day following transplantation on which an animal died. Mice that survived until the end of the study contained HSCs of human origin in their bone marrow at the time of sacrifice. As a control for nonspecific toxicity, no nonirradiated NGS mice died following transplantation of umbilical cord blood from either category.

Discussion

This report characterizes the differences in umbilical cord blood composition based on whether the placental transfusion was effected by umbilical cord milking or DCC. The umbilical cord blood produced by DCC contained predominantly HSCs, which should have ensured enhanced hematopoiesis but was not the case. Umbilical cord milking blood appeared to be superior in rescuing the survival of lethally irradiated bone marrow–depleted NGS mice. Our study suggests that effective hematopoiesis requires more than HSCs alone; MSCs are also required.[16-26] MSCs, a critical component of the normal hematopoietic niche, provide a number of essential factors, including Stem Cell Factor.[19-23] MSCs derived from human umbilical cord blood are particularly effective in abetting the proliferation, differentiation, and lineage commitment of HSCs. Our data showing the superior survival of lethally irradiated mice through reconstitution of their hematopoietic system by umbilical cord milking–derived umbilical cord blood in contrast to DCC-derived umbilical cord blood would support that assessment. In other words, we observed that umbilical cord blood–derived HSCs did not rescue a mouse with a lethally irradiated bone marrow unless a proper complement of MSCs were cotransplanted with them (ideally from the same sample)—a phenomenon predicted by recent studies of hematopoiesis.[18] Because umbilical cord milking–derived blood could rescue lethally irradiated bone marrow–depleted NGS mice more effectively than DCC-derived blood (despite

having more HSCs, supporting our prediction), we concluded that we were observing synergy between umbilical cord blood–derived HSCs and their accompanying "sister" stromal cells, which enhanced bone reconstitution and thus survival of the mice. Although it is true that other unknown (and hence uncontrolled) differences between DCC-derived blood and umbilical cord milking–blood could have been responsible for these outcomes, we were rigorous in our preparation to make sure that the only obvious difference—demographically and procedurally—was the composition of the BMT graft.

One explanation as to why umbilical cord milking yields a placental transfusion that contains more MSCs is simply the attendant mechanical disruption of the umbilical cord that dislodges MSCs from the stroma into the umbilical cord blood. Human umbilical cord blood–derived MSCs are found in high concentrations around the umbilical vessel endothelium and intima.[27] MSCs take up residence perivascularly, suggesting that they not only serve as pericytes, but also are critical for vascular endothelial cell function.[16-18] In fact, the interaction between vascular endothelial cells and MSCs may be critical to the each others' function and, in turn, for the normal functioning of perfused organs, including the bone marrow. In other words, it appears that MSCs are required for the normal function of any organ with a blood supply.[16-18] This insight also might explain why umbilical cord milking blood has been reported to be useful (sometimes even superior) in other perinatal conditions as well.[28]

Our study has several limitations related to the exigencies of dealing with patients in a clinical setting in real time. However, we do not believe that any these accommodations adversely impacted our observations and conclusions. Only well full-term infants were enrolled in the study before randomization, to exclude potential confounders; thus, the patient groups were demographically similar, and although some umbilical cord blood samples could not be analyzed because of insufficient blood volume collection, the number of such samples were equal in the 2 groups. Furthermore, the mode of delivery, sex, racial composition, and maternal age were balanced between the groups. We did not analyze the stem cell content of peripheral blood obtained directly from the infants after the placental transfusion for 2 reasons. First, we could not medically justify drawing large amounts of blood (at least 20 mL) from healthy infants to perform the necessary analyses. Second, the question we were asking involved the composition of the transfusate that an infant would receive, not confounded by the characteristics of a particular neonate's physiology. Thus, although the umbilical cord blood to be analyzed was taken directly from the placenta and the umbilical cord, it did represent the umbilical cord blood that infant would have received following an augmented placental transfusion procedure had the cord not been immediately clamped. (It is known that infused umbilical cord blood rapidly enters the peripheral circulation.)

It is true that during a physiological placental transfusion, the placenta is pumping blood into the higher resistance circuit of an infant, not represented by our experimental procedure in which umbilical cord blood in the DCC group is passively drained into a tube with no resistance; however, this difference would have impacted the volume and not the composition of the DCC samples. Indeed, the volume of umbilical cord blood following DCC was greater than that following umbilical cord milking (umbilical cord milking blood samples are likely less affected by an absence of resistance), perhaps accounting for the greater proportion of HSCs in the former compared with the latter. Nevertheless, this greater number of HSCs, without the presence of MSCs, was not sufficient to rescue the lethally irradiated mice, making our conclusions starker. The brief clamping of the relevant of umbilical cord segment to permit our analysis may have released MSCs into the umbilical cord blood, increasing the absolute number of MSCs in each group; however, this procedure was performed for all samples, thereby nullifying any differences.

Further studies, beyond the scope of this initial report, will be important; for example, examining the colony-forming units in the bone marrow of transplanted reconstituted irradiated mice (and identifying the cell types within those colonies based on various MSC:HSC ratios), determining whether DCC-derived umbilical cord blood can be made "therapeutic" in this BMT paradigm simply by adding MSCs and determining the minimum MSC content to stimulate hematopoiesis, characterizing the umbilical cord after extraction of blood by either placental transfusion technique, and correlating these preclinical findings in vitro and in mice by examining the peripheral circulation of human newborn infants for markers of all hematopoietic lineages.

It is worth noting that MSCs are thought to have additional benefits. For example, MSCs make a wide variety of trophic, anti-inflammatory, immunomodulatory, and proangiogenic cytokines and produce exosomes/microvesicles with potentially therapeutic cargo.[29] Studying those actions here is beyond the scope of this report. However, because umbilical cord milking provides MSCs in a manner not achieved by DCC, this technique might be expected to provide benefits for conditions that do not rely solely on hematopoiesis. For example, human umbilical cord blood–derived MSCs—more proliferative and less immunogenic than MSCs from other sources—have been suggested as a strategy for decreasing the severity of bronchopulmonary dysplasia (BPD) in extremely preterm infants based on their anti-inflammatory actions.[30] Human umbilical cord blood–derived MSCs attenuate inflammatory cytokines interleukin-6, interleukin-8, metalloproteinase-9, tumor necrosis factor α, and transforming growth factor β, which are increased in BPD, necrotizing enterocolitis, and sepsis.[31,32] In addition, these MSCs secrete anti-inflammatory factors, such as tumor necrosis factor–stimulated gene-6, and antioxidants, such as stanniocalcin-1. A phase I clinical trial in premature infants with BPD who received a tracheal infusion of MSCs evinced a rapid drop in their respiratory severity scores compared with controls. A recent meta-analysis of umbilical cord milking compared with immediate cord clamping

showed a reduction in BPD.[28] One could speculate that the improvement was due in part to an MSC-mediated attenuation of oxygen toxicity–induced injury and inflammation.[30]

Compared with DCC, umbilical cord milking would appear to offer all of the benefits of augmented placental transfusion, such as improved hemodynamics and organ perfusion, but with the additional benefit of providing a stem cell formulation that may improve hematopoiesis (based on the MSC:HSC ratio), as well as providing MSCs that can perform additional potentially therapeutic actions.[4,5,33] A large ongoing clinical trial comparing umbilical cord milking with DCC across many measures of neonatal health in preterm infants (PREMOD2; ClinicalTrials.gov NCT03019367) will address this speculation. ■

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