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Improved cord blood thawing procedure enhances the reproducibility and correlation between flow cytometry CD34+ cell viability and clonogenicity assays

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Umbilical cord blood (UCB) is cryopreserved and stored in cord blood banks (CBBs) for allogeneic transplantation in pediatric and adult patients [1]. It is known that cord blood unit (CBU) cell viability is deeply affected by both cryopreservation and thawing processes [2,3]. Cell viability is determined by flow cytometry using 7-Aminoactinomycin D (7-AAD) before and after cryopreservation. However, some authors report that the obtained data may be inaccurate when performing post-thawing assays due to membrane instability and cellular unspecific uptake of 7-AAD [4]. It is possible that this phenomenon varies during the staining protocols and, consequently, affects the flow cytometry results. On the other hand, clonogenic efficiency (CLONE; percentage of effective colonies from a known number of seeded CD34+ cells) is a complementary assessment to flow cytometry and provides information about cell viability, lineage commitment and proliferation ability. Therefore, it may be a more suitable quality control of the unit. The correlation between CD34+ cell viability, as assessed using flow cytometry and CLONE, is a subject under research and may depend on several pre-freezing variables [5,6]. We hypothesize that if the thawing and staining procedure-associated biases are removed, a real correlation factor may be determined.

Different solutions using Dextran 40, albumin and hydroxyethyl starch (HES) have been used in UCB thawing [7]. However, because there is no Dextran 40 available in our country, the initial CBU-attached segments-quality-control assays in our CBB were performed by diluting post-thawed blood in RPMI media with fetal bovine serum (FBS). Afterward, we validated a new thawing media composed of 0.6% HES and an elevated albumin concentration (4.2%) following previous reports [4,7]. We designed a systematic evaluation to determine the effect on cell viability measured, as by flow cytometry, using different membrane stabilizations and cell-staining incubation times compared with a sample that was non-stabilized and non-incubated after staining, referred to as standard tube (ST).

CBUs from our bank inventory were collected from healthy mothers who signed an informed consent form. As previously mentioned, the initial quality control assays were performed using 14 CBU attached segments thawed at 37°C in a serological bath. Blood was diluted in RPMI culture media with 10% FBS medium (1/10 dilution). An aliquot of this sample was transferred to a tube with counting beads, stained with CD45-fluorescein isothiocyanate/CD34-phycoerythrin and 7-AAD and incubated for 20 min at room temperature. Thereafter, an ammonium chloride solution was added, and the sample was incubated for 10 min for erythrocyte lysis. All the samples were analyzed with the International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol.

For the evaluation of the new thawing solution, nine segments from three CBUs were hand thawed. The blood was extracted and transferred to a tube where it was diluted 1:2 with thawing media (HES 0.6%, bovine serum albumin 4.2% in phosphate buffered saline). This sample was incubated for 5 min at room temperature and was diluted again 1:8 with the same media. Until this point, all the experimental units were identical. Because CBBs commonly stain for flow cytometry promptly after the thawing segments, we selected, as ST, the sample that was only incubated in thawing media the first 5 min and was then immediately stained to determine CD34+ cell viability.

Figure 1a shows the thawing protocol modifications that were performed based on the ST in two

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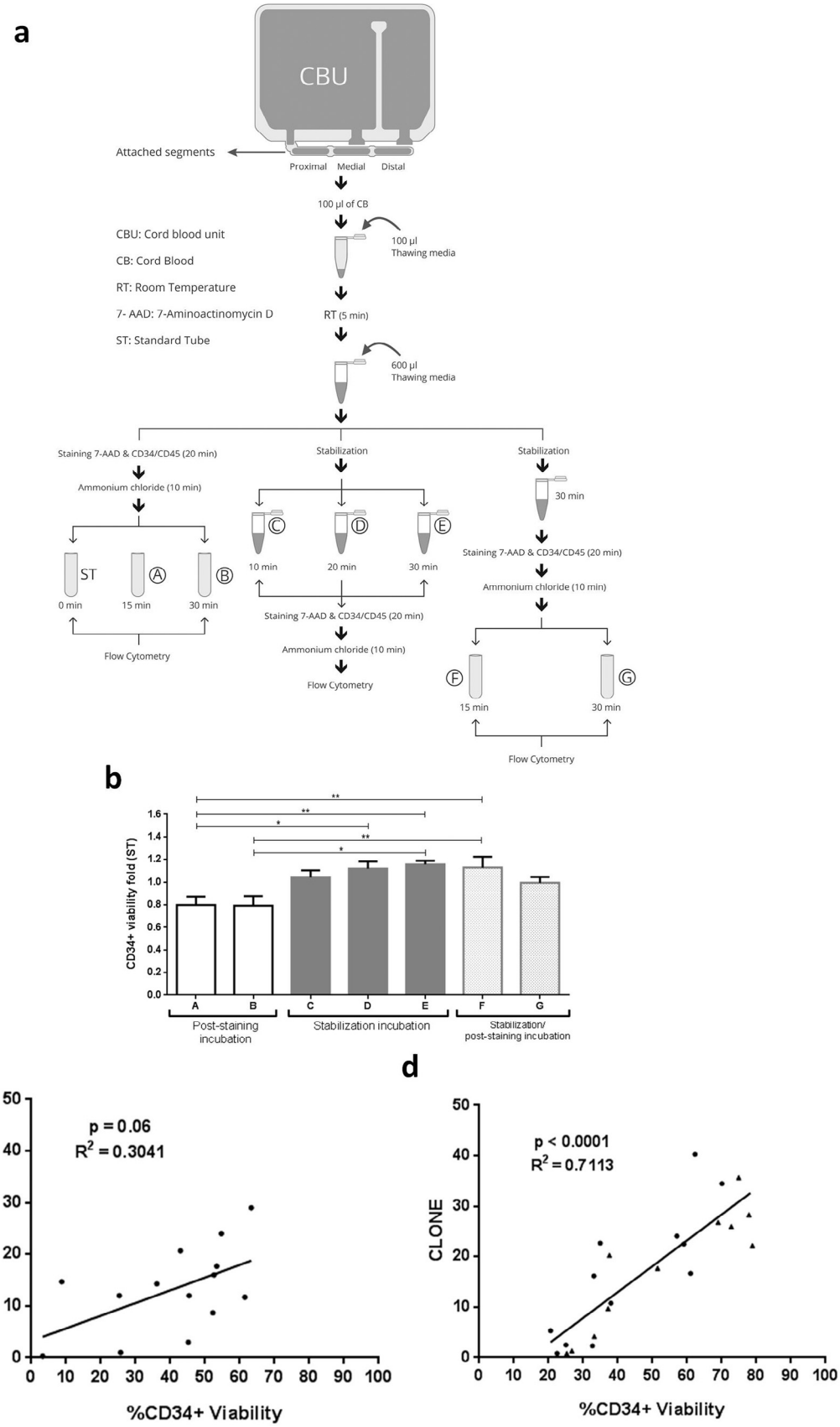
a
CBU
Attached segments
Proximal
Medial
Distal
100 µl of CB
CBU: Cord blood unit
CB: Cord Blood
RT: Room Temperature
7- AAD: 7-Aminoactinomycin D
ST: Standard Tube
100 µl
Thawing media
RT (5 min)
600 µl
Thawing media
Staining 7-AAD & CD34/CD45 (20 min)
Ammonium chloride (10 min)
Stabilization
Stabilization
30 min
ST
A
B
C
D
E
F
G
0 min
15 min
30 min
10 min
20 min
30 min
Flow Cytometry
b
CD34+ viability fold (ST)
Post-staining incubation
Stabilization incubation
Stabilization/ post-staining incubation
c
p = 0.06
R2 = 0.3041
CLONE
%CD34+ Viability
d
p < 0.0001
R2 = 0.7113
CLONE
%CD34+ Viability