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Evaluation of the Stability of Fatty Acids in Erythrocytes from Human Umbilical Cord

Angela M. Jaramillo[1] · María L. Garmendia[2] · Patricio Muñoz[3] · Alicia Corbari[3] · Rodrigo Valenzuela[3] · Paola Casanello[4,5]

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Abstract The interest in the amount of polyunsaturated fatty acids (PUFA) in the umbilical cord blood (UCB) is increasing, but the stability of erythrocyte PUFA in these samples during storage and washing of the erythrocytes has not been directly evaluated. The purpose of this study was to analyze the effect of the lapse of time on the fatty acid (FA) content from UCB sample collection and maintained at 4 °C (0–12 h) until erythrocyte separation and washing. Palmitic acid (16:0), stearic acid (18:0), 18:1n-7/n-9, linoleic acid (18:2n-6), arachidonic acid (20:4n-6), 22:4n-6, eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and docosahexaenoic acid (22:6n-3) together accounted for 87% of the FA profile in the umbilical vein erythrocytes. No difference was observed in the concentration of any of the FA studied, nor in the sum of saturated fatty acids (SFA), PUFA, or LC-PUFA in umbilical erythrocytes obtained at delivery and stored up to 12 h before the separation of erythrocytes. However, if a washing step was included in the processing of the erythrocytes, a decrease in the concentration of 16:0, 18:0, 18:3n-3, 20:4n-6, 22:4n-6, total SFA, PUFA, LC-PUFA, and n-6 LC-PUFA was evidenced, compared to unwashed erythrocytes. The FA concentration in umbilical cord erythrocytes did not change between samples stored from 0 to 12 h until erythrocyte separation. Erythrocyte washing before storage decreased the concentration of significant individual and total SFA, PUFA, and LC-PUFA. These results should be considered when planning the collection of UCB samples for the study of fatty acid concentration due to the nonscheduled timing of deliveries.

Keywords Erythrocytes · Fatty acids · PUFA · Stability · Umbilical cord blood

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✉ Paola Casanello
pcasane@uc.cl

[1] PhD Program in Nutrition and Foods, Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

[2] Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

[3] Department of Nutrition, Faculty of Medicine, University of Chile, Santiago, Chile

[4] Department of Obstetrics, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

[5] Department of Neonatology, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

Abbreviations

ALA	alpha-linolenic acid (18:3n-3)
ARA	arachidonic acid (20:4n-6)
DGLA	dihomo-gamma-linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (22:6n-3)
DPAn-3	docosapentaenoic acid (22:5n-3)
EPA	eicosapentaenoic acid (20:5n-3)
FAME	fatty scid methyl esters
FID	flame ionization detector
LC-PUFA	long-chain polyunsaturated fatty acid(s)
LNA	linoleic acid (18:2n-6)
MUFA	monounsaturated fatty acid(s)
n-3	n3 fatty acids
n-6	n6 fatty acids
NEFA	non-esterified fatty acid(s)
PAM	palmitic acid (16:0)
PBS	Phosphate buffered saline
SFA	saturated fatty acid(s)
STA	stearic acid (18:0)
UCB	umbilical cord blood

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Introduction

Both n-6 and n-3 long-chain polyunsaturated fatty acids (LC-PUFA) have important functions on metabolism, inflammation, and cell differentiation (Kim et al., 2006; Marion-Letellier et al., 2015; Mozaffarian and Wu, 2012). Likewise, n-6 and n-3 LC-PUFA such as arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA) have structural and functional activities in the brain (Innis, 2007) and therefore are especially important during fetal development and the first years of life (Koletzko et al., 2001). ARA can be obtained from linoleic acid (18:2n-6, LNA), and EPA and DHA derivate from alpha-linolenic acid (18:3n-3, ALA) after complex processes of derivation and elongation, principally in the liver (Zhang et al., 2016). In diets with high input of n-6 FA, the synthesis of ARA from LNA is higher than that of DHA from its precursor ALA (Metherel et al., 2018). On the other hand, DHA intake, mainly from fish consumption, is very low in Western diet, compared to the intake of LNA from vegetable oils and ARA from eggs and meat (Simopoulos, 2016).

During pregnancy and lactation, women can store LC-PUFA to ensure an adequate flow of ARA and DHA to the fetus and the newborn (Lauritzen and Carlson, 2011). During the period of embryonic (from implantation to the eighth week of development) and later fetal (from the ninth week of gestation until delivery) development, the placenta increases the transfer of ARA and DHA to the embryo and fetus through specific transporter proteins (Lewis and Desoye, 2017). In normal pregnancies, the free FA content in maternal plasma is higher; also, placental and endothelial enzyme activity increase to enable taking up fatty acids from maternal lipoproteins such as triglycerides and phospholipids. These FA can be bound to intracellular fatty acid binding proteins (FABP) and used for placental metabolism or to reach the fetal circulation helped by placental membrane transport proteins such as the plasma membrane FA binding protein (FABPpm), FA translocase (FAT/CD36), and fatty acid transporter proteins 1–4 (FATP) (Duttaroy, 2009; Larque et al., 2011; Lewis et al., 2018).

There is increasing number of studies on the amount of PUFA in the umbilical cord blood because of the fact that the FA content in the umbilical blood is an indicator of the FA that the fetus received in the prenatal period and a proxy to their concentration in fetal tissues (Buyukuslu et al., 2017).

On the other hand, maternity ward services work 24/7, and thus the study of cord blood PUFA in the offspring at birth requires safe storage of the samples to accurately measure the FA profile, independent of the time of delivery. This is especially relevant with samples that are collected at night, where there is normally no research staff available to process them until the next day. A few studies have analyzed the effect of different temperatures (room temperature, 4 °C, −20 °C, −80 °C) and time of storage of adult whole blood (Rogiers, 1978) and red blood cells (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006; Metherel et al., 2013) on the FA content of these samples (Metherel and Strak, 2016). However, the stability of erythrocyte PUFA in cord blood erythrocytes has not been directly evaluated.

The aim of this study was to analyze the effect of the lapse of time from cord blood sample collection and subsequent maintenance at 4 °C (0–12 h) until erythrocyte separation and determination of the FA concentration of these samples. We, therefore, designed a study with a group of healthy pregnant women who had term vaginal deliveries and evaluated both the effect of storage time of cord blood samples kept at 4 °C before separating the erythrocytes as well as the effect of washing the erythrocytes on the stability of the different FA present in umbilical cord erythrocytes.

Materials and Methods

Study Participants

Pregnant women ($n = 6$) at the time of delivery in the maternity ward of the Hospital Dr. Sótero del Río, Santiago, Chile, were invited to participate in this study. Inclusion criterion was that the delivery occurred between 8:00 AM and 10:00 AM. Exclusion criteria included preterm deliveries, and mothers or newborns with medical, obstetrical, or neonatal complications.

Ethical Aspects

At the time of the recruitment, before delivery, the invited patients were given general information about the study, and all participants signed the written, informed consent approved for this study by the ethics committee of the Southeast Metropolitan Health Service. This study was conducted under the criteria in the Declaration of Helsinki.

Sample Collection and Preparation of Umbilical Cord Erythrocytes

Once the baby was delivered and the cord clamped, 25 mL of blood was extracted by puncturing the umbilical vein, before the placenta was delivered. Five BD Vacutainer spray-coated K_2-EDTA tubes were filled with 5 mL of umbilical vein blood. Each tube was processed at different times: The first tube was processed immediately after the blood collection (time 0 h, control). The second, third, fourth, and fifth tubes were kept at 4 °C and processed 3, 6, 9, and 12 h after collection.

The sample processing consisted of centrifuging the cord blood for 10 min (2500 rpm, 4 °C). After centrifugation, the plasma and buffy coat were removed with a micropipette, and an aliquot of 500 μL of the red cell fraction was obtained from the center of the cell column with a clean tip and stored in a previously labeled cryotube (unwashed sample). The rest of the red cell fraction was washed with cold phosphate-buffered saline (PBS, 4 °C) until reaching a red cell fraction to PBS ratio of 1:3. The tube's content was mixed with gentle inversion movements, centrifuged for 5 min (1400 rpm, 4 °C), and the supernatant was removed. The washing process was repeated once more and, afterward, the washed red cell fraction was stored in a previously labeled cryotube (washed sample). The unwashed and washed cryotubes—a total of 10 from each patient (one washed and one unwashed sample for each time point)—were stored at −20 °C for 3 months before quantification.

Lipid Extraction and Fatty Acid Methyl Ester (FAME) Preparation and Analysis

The frozen samples were thawed at room temperature. Four-hundred and fifty milliliters from each cryotube was used for total fat extraction. Quantitative extraction and separation of total lipids from erythrocytes were carried out according to Bligh and Dyer (1959) with ice-cold chloroform/methanol (2:1 by vol). FAME from erythrocytes were prepared according to Morrison and Smith (1964). Boron trifluoride (12% methanolic solution) as catalyst and methanolic sodium hydroxide (0.5 N) were used. FAME samples were extracted with dichloromethane and later separated and quantified by gas–liquid chromatography (Agilent model 7890 A, CA, USA) using an Agilent J&W GC capillary column (Agilent DB-FFAP, 30 m × 0.25 mm; I.D. 0.25 μm) and a flame ionization detector (FID). The injector temperature was set at 250 °C and the FID temperature at 300 °C. The oven temperature at injection was initially set at 95 °C and was programmed to increase to 240 °C at a rate of 5 °C/min. Hydrogen was used as the carrier gas (35 cm/s flow rate) in the column and the inlet split ratio was set at 5:1. The identification and quantification of FAME were achieved by comparing the retention times and the peak area values (%) of the samples with those of a commercial lipid standard (Nu-Chek Prep Inc., Elysian, MN, USA). As an internal standard, 23:0 was used (Nu-Chek Prep Inc.). An Agilent Chemstation Software (Palo Alto, CA, USA) data system was used for peak analysis. The concentration (nmol/mL) of each FA was calculated by multiplying the μg/mL value by a factor related to its molecular mass.

Statistical Analyses

Descriptive statistics are presented as mean ± SD of the concentration (nmol/mL) of each FA. The effect of time and wash and their interaction on the FA content in umbilical vein erythrocytes was investigated/explored using two-way ANOVA. Student's *t*-test was used to analyze the differences in the FA content between unwashed and washed umbilical red blood cells at each time point (0, 3, 6, 9, and 12 h) when the effect of the washing was significant in the two-way ANOVA analyses. Stata version 15.1 (Texas, USA) was used to conduct statistical analyses.

Results

The mean fat percentage extracted from umbilical erythrocytes was 0.39 ± 0.17 of the sample's weight, which corresponds to 1.72 ± 0.76 g of umbilical erythrocytes. The analytical coefficient of variation of the principal FA was between 1% and 5% for palmitic acid (16:0, PAM), 2% and 11% for stearic acid (18:0, STA), 3% and 9% for 18:1n-7/n-9, 2% and 6% for linoleic acid (18:2n-6, LNA), 4% and 10% for dihomo-gamma-linolenic acid (20:3n-6, DGLA), 6% and 9% for arachidonic acid (20:4n-6, ARA), 1% and 5% for EPA, 1% and 11% for 22:4n-6, 1% and 11% for docosapentaenoic acid (22:5n-3, DPAn-3), and 5%and 11% for docosahexaenoic acid (22:6n-3, DHA). All coefficients of variation were under 12%, which shows/proves the acceptable accuracy of the measurements.

The characteristics of the six newborns and their mothers are reported in Table 1. The gestational age at birth was between 35 and 40 weeks. Birth weight range was 2525–4010 g, and Apgar scores at 1 and 5 min were between 7 and 9.

In Table 2, the FA concentration (nmol/mL) in unwashed umbilical erythrocytes at each time point is reported. The FA profile from umbilical red blood cells included 21 fatty

Table 1 Clinical characteristics of the newborns and their mothers

	Mean ± SD or % (n)
Mother	
Parity, number of children	1.6 ± 1.2
Caesarean section, % (*n*)	33% (2)
Newborn	
Female sex, % (*n*)	33% (2)
Gestational age at birth, weeks	37.6 ± 1.9
Birth weight, kg	3.41 ± 0.54
Birth length, cm	49.0 ± 2.3
Birth weight, *z*-score	0.84 ± 1.2
Apgar at 1 min, score	8.5 ± 0.8
Apgar at 5 min, score	8.8 ± 0.4

Data are mean ± SD or percentage (number) for 6 dyads. The birth weight *z*-score adjusted for sex and gestational age was calculated using references from Intergrowth-21st.

Table 2 Fatty acid concentration in unwashed umbilical erythrocytes at each time point

Fatty acid	Time (h)				
	0	3	6	9	12
Saturated fatty acids (SFA)					
12:0	4.14 ± 2.52	5.21 ± 1.20	3.14 ± 1.71	3.02 ± 2.36	4.63 ± 1.01
14:0	28.9 ± 6.30	31.0 ± 6.7	32.6 ± 12.2	32.7 ± 7.8	43.1 ± 33.9
16:0 (PAM)	1003.7 ± 143.2	1076.0 ± 150.4	1100.9 ± 213.9	1142.1 ± 184.3	1100.8 ± 190.4
18:0 (STA)	265.9 ± 40.2	279.1 ± 47.8	293.4 ± 66.2	312.9 ± 77.0	290.4 ± 62.1
20:0	7.96 ± 1.13	8.46 ± 0.80	8.34 ± 1.39	8.98 ± 1.36	8.90 ± 2.11
22:0	17.2 ± 1.9	18.3 ± 1.9	18.3 ± 2.9	18.6 ± 4.5	18.8 ± 2.7
24:0	60.4 ± 11.0	61.0 ± 9.9	661.9 ± 9.5	63.8 ± 9.3	64.0 ± 12.6
Total SFA	1388.5 ± 183.3	1479.3 ± 202.8	1519.3 ± 297.6	1582.3 ± 266.4	1530.8 ± 291.3
Monounsaturated fatty acids (MUFA)					
16:1	38.8 ± 10.5	53.9 ± 15.8	55.2 ± 17.2	62.4 ± 20.6	66.6 ± 45.9
18:1n-7/n-9	466.7 ± 86.3	532.8 ± 89.1	547.1 ± 118.8	578.8 ± 117.0	580.6 ± 176.7
20:1n-9	4.85± 1.89	5.31 ± 1.77	4.98 ± 2.01	5.50 ± 1.86	8.22 ± 8.23
22:1n-9	8.44 ± 2.80	9.50 ± 4.88	9.44 ± 4.36	7.92 ± 2.98	9.08 ± 1.46
24:1	41.50 ± 9.4	43.6 ± 5.3	43.1 ± 6.8	44.9 ± 5.2	45.4 ± 8.0
Total MUFA	560.8 ± 106.0	645.3 ± 110.5	659.8 ± 142.8	699.7 ± 145.8	709.9 ± 234.9
Polyunsaturated fatty acids (PUFA)					
18:2n-6 (LNA)	155.3 ± 32.1	184.1 ± 65.9	187.8 ± 42.1	206.8 ± 80.0	183.8 ± 54.9
18:3n-6	2.52 ± 1.41	4.47 ± 2.74	5.57 ± 4.39	7.76 ± 6.71	7.11 ± 7.49
18:3n-3 (ALA)	17.5 ± 14.9	15.0 ± 10.9	20.2 ± 14.1	21.6 ± 17.0	20.9 ± 16.6
20:3n-6 (DGLA)	73.5 ± 22.5	80.4 ± 37.9	83.2 ± 29.2	88.3 ± 41.1	75.1 ± 31.9
20:4n-6 (ARA)	348.3 ± 139.0	352.6 ± 184.2	373.7 ± 171.1	388.7 ± 202.0	336.3 ± 189.9
20:5n-3 (EPA)	2.75 ± 1.68	2.73 ± 2.39	4.20 ± 3.24	2.43 ± 2.70	5.03 ± 4.79
22:4n-6	74.2 ± 28.6	68.8 ± 34.5	73.9 ± 33.9	75.1 ± 36.6	65.4 ± 37.7
22:5n-3 (DPAn-3)	6.62 ± 2.96	6.59 ± 4.03	7.19 ± 4.15	9.22 ± 3.41	7.57 ± 5.31
22:6n-3 (DHA)	95.4 ± 44.7	94.2 ± 60.3	96.1 ± 60.2	101.7 ± 66.1	88.3 ± 66.1
Total PUFA	774.7 ± 259.3	807.7 ± 371.5	849.5 ± 328.5	899.7 ± 406.3	787.7 ± 364.7
Total LC-PUFA	600.9 ± 229.8	605.5 ± 311.7	637.8 ± 289.2	665.6 ± 337.5	577.8 ± 323.2
Total n-6 LC-PUFA	496.1 ± 185.0	501.9 ± 251.3	530.3 ± 228.7	552.2 ± 274.2	476.9 ± 255.4
Total n-3 LC-PUFA	104.8 ± 49.1	103.5 ± 66.5	107.5 ± 65.0	113.3 ± 69.8	100.9 ± 70.6
n-6/n-3 LC-PUFA ratio	4.98 ± 0.82	5.31 ± 1.09	5.52 ± 1.33	5.29 ± 1.85	5.65 ± 1.75

Total MUFA = 16:1 + 18:1n-7/n-9 + 20:1n-9 + 22:1n-9 + 24:1. Total SFA = 12:0 + 14:0 + 16:0 PAM +18:0 STA + 20:0 + 22:0 + 24:0. Total PUFA = 18:2n-6 LNA + 18:3n-6 + 18:3n-3 ALA +20:3n-6 DGLA +20:4n-6 ARA + 20:5n-3 EPA + 22:4n-6 + 22:5n-3 DPAn-3 + 22:6n-3 DHA. Total LC-PUFA = total n-3 LC-PUFA + total n-6 LC-PUFA. Total n-3 LC-PUFA = 20:5n-3 EPA + 22:5n-3 DPAn-3 + 22:6n-3 DHA. Total n-6 PUFA = 20:3n-6 DGLA +20:4n-6 ARA + 22:4n-6. The n-6/n-3 LC-PUFA ratio are total n-6 LC-PUFA/total n-3 LC-PUFA. Data are mean ± SD (newborns, n = 6) of concentration (nmol/mL).

ALA, alpha-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA, long chain polyunsaturated fatty acid; LNA, linoleic acid; PAM, palmitic acid; STA, stearic acid.

acids. At the time of collection (0 h), the most representative SFA in the profile were PAM and STA, which together accounted for 47% of the whole FA profile. The most abundant monounsaturated fatty acid (MUFA) was18:1n-7/n-9, representing 17% of the FA profile.

The most abundant n-6 FA were LNA, ARA, and 22:4n-6, which together accounted for 21% of the FA profile. The most represented n-3 FA were ALA, EPA, DPA, and DHA, together accounting for 4% of the profile.

The concentrations of the FA (nmol/mL) in neonatal erythrocytes exposed to a washing step before their quantification are presented in Table 3.

The results of the two-way ANOVA (evaluated the effects of time, washing of erythrocytes, and their interaction on the FA content in neonatal erythrocytes) are shown in Table 4. The time (time effect in two-way ANOVA) and the interaction between time and wash (interaction effect in two-way ANOVA) did not have any effect on any individual FA or on

Table 3 Fatty acid concentration in washed umbilical erythrocytes at each time point

Fatty acid	Time (h)				
	0	3	6	9	12
Saturated fatty acids (SFA)					
12:0	11.3 ± 16.2	5.99 ± 3.80	8.07 ± 7.10	9.61 ± 8.38	8.29 ± 8.22
14:0	55.6 ± 58.3	32.7 ± 10.2	52.8 ± 51.0	31.7 ± 5.2	51.4 ± 45.0
16:0 (PAM)	1017.2 ± 307.1	864.3 ± 183.5	904.2 ± 197.7	833.3 ± 138.7	1033.8 ± 170.3
18:0 (STA)	247.9 ± 53.6	224.7 ± 31.8	222.1 ± 39.2	200.7 ± 48.0	279.7 ± 89.8
20:0	7.18 ± 2.62	6.43 ± 1.55	7.48 ± 3.05	5.85 ± 0.82	8.39 ± 3.51
22:0	13.2 ± 2.9	13.2 ± 3.6	13.6 ± 1.94	12.4 ± 3.21	14.8 ± 3.24
24:0	43.0 ± 10.7	44.7 ± 13.6	43.2 ± 7.1	40.7 ± 10.2	47.9 ± 14.1
Total SFA	1397.6 ± 421.9	1192.3 ± 228.8	1251.7 ± 258.6	1138.9 ± 185.3	1446.9 ± 311.9
Monounsaturated fatty acids (MUFA)					
16:1	65.7 ± 61.0	37.4 ± 14.5	63.0 ± 62.4	41.1 ± 30.6	65.0 ± 72.8
18:1n-7/n-9	643.1 ± 340.6	472.1 ± 128.3	620.1 ± 360.9	487.0 ± 119.6	660.3 ± 427.0
20:1n-9	13.0 ± 14.8	7.57 ± 2.96	13.3 ± 16.6	7.02 ± 3.52	12.7 ± 15.9
22:1n-9	14.3 ± 6.5	11.9 ± 6.0	12.2 ± 5.8	10.5 ± 3.8	10.0 ± 3.6
24:1	30.8 ± 5.8	31.6 ± 9.1	31.4 ± 4.3	29.6 ± 11.1	34.6 ± 5.6
Total MUFA	767.1 ± 411.9	560.7 ± 149.0	740.3 ± 433.8	575.6 ± 150.0	782.9 ± 516.2
Polyunsaturated fatty acids (PUFA)					
18:2n-6 (LNA)	183.1 ± 99.6	133.8 ± 50.9	207.9 ± 143.3	161.6± 78.4	165.3 ± 63.3
18:3n-6	6.26 ± 6.31	3.76 ± 3.21	22.8 ± 46.0	4.39 ± 6.18	3.88 ± 5.96
18:3n-3 (ALA)	13.6 ± 7.88	6.76 ± 1.73	6.53 ± 6.67	11.1 ± 10.2	12.3 ± 7.03
20:3n-6 (DGLA)	48.0 ± 33.5	50.9 ± 35.0	48.0 ± 32.5	53.7 ± 34.3	54.7 ± 28.4
20:4n-6 (ARA)	206.6 ± 157.7	240.1 ± 185.6	227.9 ± 172.2	245.2 ± 173.6	252.5 ± 165.4
20:5n-3 (EPA)	25.2 ± 59.2	3.13 ± 2.88	27.2 ± 56.9	3.62 ± 3.57	2.66 ± 2.19
22:4n-6	45.3 ± 34.9	52.5 ± 41.0	48.6 ± 42.3	47.2 ± 45.1	56.8 ± 37.2
22:5n-3 (DPAn-3)	7.50 ± 9.34	4.79 ± 4.12	8.10 ± 6.87	5.24 ± 3.21	4.60 ± 4.13
22:6n-3 (DHA)	62.4 ± 46.6	65.7 ± 61.1	79.1 ± 50.4	70.8 ± 55.8	70.2 ± 56.1
Total PUFA	596.9 ± 277.6	561.1 ± 363.9	676.7 ± 332.6	602.1 ± 282.0	622.0 ± 258.6
Total LC-PUFA	395.1 ± 258.1	417.4 ± 321.1	439.1 ± 262.6	426.0 ± 300.0	441.6 ± 282.5
Total n-6 LC-PUFA	299.9 ± 224.4	343.7 ± 259.4	324.6 ± 244.8	346.2 ± 246.3	364.1 ± 227.8
Total n-3 LC-PUFA	95.2 ± 89.5	73.6 ± 65.8	114.4 ± 86.6	79.8 ± 59.0	77.5 ± 61.5
n-6/n-3 LC-PUFA ratio	5.23 ± 2.72	6.00 ± 2.12	3.97 ± 2.97	4.73 ± 2.25	6.13 ± 3.98

Data are mean ± SD (newborns, n = 6) of concentration (nmol/mL). The identification of the fatty acids is as shown in Table 2.

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; LNA, linoleic acid; PAM, palmitic acid; STA, stearic acid; total LC-PUFA, total long chain polyunsaturated fatty acid(s); total MUFA, total monounsaturated fatty acid(s); total PUFA, total polyunsaturated fatty acid(s); total SFA, total saturated fatty acid(s).

the total SFA, total MUFA, and total PUFA in umbilical erythrocytes (Table 4, Fig. 1a,b). However, there was a significant effect of washing the erythrocytes on the content of 16:0 PAM ($p = 0.003$), 18:0 STA ($p = 0.001$), total SFA ($p = 0.003$), 18:3n-3 ALA ($p = 0.004$), 20:4n-6 ARA ($p = 0.007$), 22:4n-6 ($p = 0.032$), total PUFA ($p = 0.015$), total LC-PUFA ($p = 0.013$), and total n-6 LC-PUFA ($p = 0.006$) (Table 4, Fig. 2a, b. *Post hoc* analyses showed that washing decreased the concentration at 3 h of 16:0 (211.7 nmol/mL, p-value = 0.026), 18:0 (54.3 nmol/mL, p-value = 0.021), total SFA (286.9 nmol/mL, p-value = 0.022), and 18:3n-3 ALA (8.29 nmol/mL, p-value = 0.048), at 6 h for 18:0 (71.3 nmol/mL, p-value = 0.023) and 18:3n-3 ALA (13.3 nmol/mL, p-value = 0.028), and at 9 h for 16:0 (308.7 nmol/mL, p-value = 0.004), 18:0 (112.2 nmol/mL, p-value = 0.006), and total SFA (443.3 nmol/mL, p-value = 0.003).

Discussion

To our knowledge, this is the first study to analyze the stability of FA in umbilical cord erythrocytes after storage at

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Table 4 Estimated effect of time and washing on the most relevant fatty acid concentration in umbilical erythrocytes

Fatty acid	Estimated effect	*F*	*p*-Value
16:0 (PAM)	Time effect	0.43	0.785
	Wash effect	**9.51**	**0.003**
	Interaction effect	1.30	0.284
18:0 (STA)	Time effect	0.62	0.648
	Wash effect	**12.56**	**0.001**
	Interaction effect	1.52	0.211
18:1n-7/n-9	Time effect	0.46	0.761
	Wash effect	0.35	0.556
	Interaction effect	0.68	0.606
18:2n-6 (LNA)	Time effect	0.44	0.778
	Wash effect	0.44	0.510
	Interaction effect	0.65	0.627
18:3n-3 (ALA)	Time effect	0.51	0.727
	Wash effect	**8.84**	**0.004**
	Interaction effect	0.28	0.890
20:4n-6 (ARA)	Time effect	0.08	0.988
	Wash effect	**7.72**	**0.007**
	Interaction effect	0.07	0.990
20:5n-3 (EPA)	Time effect	0.72	0.582
	Wash effect	1.77	0.189
	Interaction effect	0.71	0.589
22:4n-6	Time effect	0.00	1.000
	Wash effect	**4.83**	**0.032**
	Interaction effect	0.16	0.957
22:5n-3 (DPAn-3)	Time effect	0.32	0.866
	Wash effect	1.08	0.303
	Interaction effect	0.55	0.698
22:6n-3 (DHA)	Time effect	0.06	0.992
	Wash effect	2.97	0.090
	Interaction effect	0.05	0.995
Total SFA	Time effect	0.54	0.704
	Wash effect	**9.23**	**0.003**
	Interaction effect	1.28	0.291
Total MUFA	Time effect	0.47	0.759
	Wash effect	0.17	0.678
	Interaction effect	0.69	0.604
Total PUFA	Time effect	0.15	0.961
	Wash effect	**6.27**	**0.015**
	Interaction effect	0.09	0.984
Total LC-PUFA	Time effect	0.06	0.993
	Wash effect	**6.54**	**0.013**
	Interaction effect	0.05	0.995
Total n-6 LC-PUFA	Time effect	0.07	0.991
	Wash effect	**7.99**	**0.006**
	Interaction effect	0.08	0.987
Total n-3 LC-PUFA	Time effect	0.21	0.932
	Wash effect	1.00	0.322
	Interaction effect	0.17	0.951

(Continues)

Table 4 Continued

Fatty acid	Estimated effect	*F*	*p*-Value
n-6/n-3 LC-PUFA ratio	Time effect	0.52	0.719
	Wash effect	0.05	0.816
	Interaction effect	0.49	0.745

Total MUFA = 16:1 + 18:1n-7/n-9 + 20:1n-9 + 22:1n-9 + 24:1. Total SFA = 12:0 + 14:0 + 16:0 PAM +18:0 STA + 20:0 + 22:0 + 24:0. Total PUFA = 18:2n-6 LNA + 18:3n-6 + 18:3n-3 ALA +20:3n-6 DGLA +20:4n-6 ARA + 20:5n-3 EPA + 22:4n-6 + 22:5n-3 DPAn-3 + 22:6n-3 DHA. Total LC-PUFA = total n-3 LC-PUFA + total n-6 LC-PUFA. Total n-3 LC-PUFA = 20:5n-3 EPA + 22:5n-3 DPAn-3 + 22:6n-3 DHA. Total n-6 PUFA = 20:3n-6 DGLA +20:4n-6 ARA + 22:4n-6. The n-6/n-3 LC-PUFA ratio are total n-6 LC-PUFA/total n-3 LC-PUFA. Analyses were done with two-way ANOVA and using FA concentration as nmol/mL. p-value < 0.05 was considered significant.

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DPAn-3, docosapentaenoic acid; EPA, eicosapentaenoic acid; LNA, linoleic acid; PAM, palmitic acid; STA, stearic acid; total LC-PUFA, total long-chain polyunsaturated fatty acids.

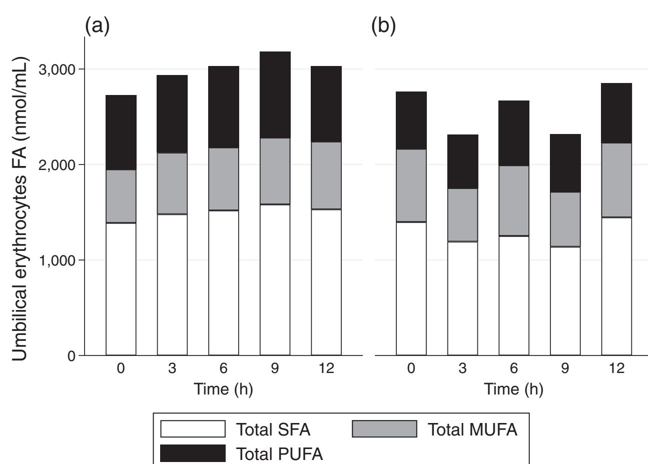

Fig. 1 Total saturated fatty acid (SFA), total monounsaturated fatty acids (MUFA), and total polyunsaturated fatty acids (PUFA) at each time point in (a) unwashed and (b) washed umbilical cord erythrocytes. Total SFA = 12:0 + 14:0 + 16:0 PAM +18:0 STA + 20:0 + 22:0 + 24:0. Total MUFA = 16:1 + 18:1n-7/n-9 + 20:1n-9 + 22:1n-9 + 24:1. Total PUFA = 18:2n-6 LNA + 18:3n-6 + 18:3n-3 ALA + 20:3n-6 DGLA + 20:4n-6 ARA + 20:5n-3 EPA + 22:4n-6 + 22:5n-3 DPAn-3 + 22:6n-3 DHA. Values are the mean of concentrations (nmol/mL) of six newborns

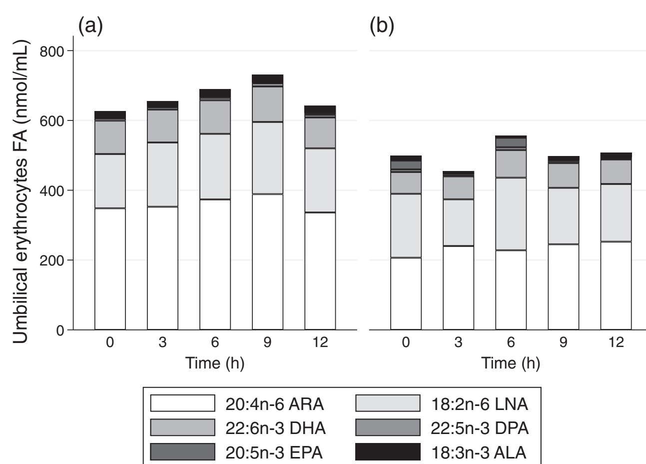

Fig. 2 Most relevant n-6 and n-3 polyunsaturated fatty acids in (a) unwashed and (b) washed umbilical cord erythrocytes. ALA, 18:3n-3 alpha-linolenic acid; ARA, 20:4n-6 arachidonic acid; DHA, 22:6n-3 docosahexaenoic acid; DPA, 22:5n-3 docosapentaenoic acid; EPA, 20:5n-3 eicosapentaenoic acid; LNA, 18:2n-6 linoleic acid. Values are the mean of concentrations (nmol/mL) of six newborns

4 °C until processing. Our results show that there is no effect of storage time (up to 12 h at 4 °C) or the interaction between time and wash on the erythrocyte FA concentration in the cord blood samples. We observed an effect of the erythrocyte washing in 16:0 (PAM), 18:0 (STA), total SFA, 18:3n-3 (ALA), 20:4n-6 (ARA), 22:4n-6, total PUFA, total LC-PUFA, and total n-6 LC-PUFA concentration. The concentration of these FA decreased after washing at 3, 6, and 9 h.

A previous study had analyzed whole blood samples collected in heparin tubes and preserved for 48 h at 4 °C before quantifying their FA content. That study did not show any significant change in the amount of non-esterified FA (NEFA) and PAM, STA, 18:1n-9, LNA, and ALA fatty acid contents in whole blood from healthy adults. However, LC-PUFA (Rogiers, 1978) were not analyzed. Metherel et al. (2013) found lower EPA and DHA concentrations when samples of whole blood were kept at room temperature, 4 °C, or −20 °C for 7, 60, and 180 days, the higher effect being at −20 °C. Di Marino et al. (2000) reported a significant increase in 16:0 and 18:1n-9 and a decrease in ARA, 22:4n-6, DPAn-3, and DHA in red blood cells stored at −20 °C without any antioxidant for 6 months. Magnusardottir and Skuladottir (2006) observed a lower amount of DHA and higher PAM, STA, and 18:1n-9 concentration in red blood cells stored for 17 weeks at −20 °C without any antioxidant. Umbilical erythrocytes in our study were stored at −20 °C for 10 weeks, and all samples were processed at the same time; nevertheless, the storing temperature affected the amount of PUFA in all samples, as previously described.

Lipid peroxidation can affect PUFA with three or more carbon–carbon double bonds, being also a target for free-radical attacks. Lipid peroxidation includes three steps: initiation, propagation, and termination (Halliwel, 1989). Hemoglobin breakdown or hemoglobin degradation can promote the initiation of lipid peroxidation (Gutteridge, 1986; Trotta et al., 1983). Likewise, the hydroperoxides formed during initiation can be destabilized, facilitating the propagation of this peroxidation (Halliwel, 1989). Initiation and propagation could be promoted by the higher concentration of O_2^- (Halliwel, 1989) in the solution (PBS) used to wash the erythrocytes. The termination of lipid peroxidation should take place by antioxidant enzymes in the erythrocytes (superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx]) (Yoshioka et al., 1979) or antioxidants such as vitamin E present in the lipid fractions of umbilical erythrocytes, (Yoshioka et al., 1979), and vitamin C and E in the plasma (Jacob, 1995). The termination of lipid peroxidation by antioxidant vitamins could be affected by the complete removal of the plasma during the washing of erythrocytes. Conversely, the lower values determined as a result of washing may not be related to increased lipid peroxidation at all; instead, it could be the result of incomplete removal of PBS during the wash. If some PBS were to get "stuck" to the erythrocytes after centrifugation, then this would result in a diluted erythrocyte fraction compared to unwashed erythrocytes. This interpretation cannot be completely discarded with these results. Another important observation is that the PUFA content is positively related to lipid peroxidation (Clemens et al., 1987), especially important in umbilical blood due to its higher LC-PUFA content. In this study, the lipid peroxidation or conditions such as storage temperature and the absence of antioxidants could have affected the PUFA or LC-PUFA concentration in a significant manner, as reflected in the decrease of 20:4n-6 (ARA), 22:4n-6, total PUFA, total LC-PUFA, and total n-6 LC-PUFA.

In this study, the samples were stored without any antioxidant. Magnusardottir and Skuladottir (2006) found higher conservation of the FA concentration when butylated hydroxytoluene (BHT) was added to the red blood cells stored for 17 weeks at −20 °C. Metherel et al. (2013) described a protective effect on EPA and DHA when using BTH in whole blood samples kept at −20 °C for 7–60 days. However, Fernandez et al. (1993) reported lower ARA concentration after 48 h of storage at 4 °C in the presence of the antioxidant acid-citrate-dextrose on fresh samples of sheep erythrocytes. The absence of antioxidant in our samples could have affected the amount of PUFA, particularly total n-6 LC-PUFA, due to the fact that both washed and unwashed samples did not have antioxidants and were kept at the same temperature and processed at the same time.

The significant differences in washed versus unwashed samples found only at some time points could be due to the statistical test used. The two-way ANOVA for repeated measures does not take into account that the samples for each subject between time points are related. Therefore a sensibility analysis using generalized estimating equations (GEE) was performed, which allowed considering repeated measurements. In this analysis, we found differences only for the washed samples, in the same fatty acids plus 22:6n-3 (DHA), which was close to the statistical significance when using two-way ANOVA. We preferred using two-way ANOVA for these analyses because of its extended use in publications of experimental studies and owing to the sample size of this study.

In this group of healthy newborns, we found that PAM, STA, 18:1n-9, LNA, ARA, 22:4n-6, and DHA in washed umbilical erythrocytes accounted for 87% of total FA in the profile. The FA in erythrocytes are derived from phospholipids (85%), triacylglycerol (13%), and cholesteryl esters (2%). The phospholipids constitute the lipid class with the lowest amount of the essential fatty acids LNA and ALA and the highest amounts of their derivatives ARA, EPA, and DHA (Rise et al., 2007). In this study, phospholipids were not separated from the other lipid classes, but total FA were extracted and trans-esterified from red blood cells. Red blood cells have higher levels of ARA, DPA, and DHA compared to plasma, LDL, or HDL compartments. A recent publication described the same FA in erythrocytes from adult subjects although in different percentages (Brenna et al., 2018). In relation to umbilical erythrocytes, a study in Brazilian healthy newborns reported that these FA represent 88% of the FA (Assumpção et al., 2017). Another study performed in Polish newborns found lower EPA (0.3%), 22:4n-6 (1.7%), and DPA n-3 (0.4%) and higher LNA (6.5%), ALA (0.3%), 20:3n-6 (1.9%), ARA (10.4%), and DHA (3.6%) compared to the current results (Pankiewicz et al., 2007). Although there are many other studies reporting FA concentrations in different components of the umbilical blood, these two studies measured FA in washed umbilical erythrocytes, similar to our study. Additionally, all the subjects were healthy newborns from mothers without complications and without FA supplementation during gestation. Nevertheless, the extraction and methylation protocols were different between these studies. We found a 0.8% and 0.2% higher LNA and EPA and 7.0%, 2.6% and 0.01% lower ARA, DHA and ALA compared to the Brazilian and Polish newborn umbilical erythrocytes. In our results, ARA and DHA were 7.0% and 2.6% lower and EPA was 0.2% higher than the data from these populations. In Chilean mothers at 6th month of pregnancy, a study showed higher ARA and LNA and lower ALA, EPA, and DHA intake based on daily recommendations (Barrera et al., 2018). It is possible that these differences could be due to dietary (Simopoulos, 2011) and genetic factors (Ameur et al., 2012).

All the analytical intra-assay coefficient variability was below 12%. Harris and Thomas (2010) studied the analytical variability for EPA and DHA measured twice in the same sample. Their study showed that the analytical coefficient variability mean for EPA and DHA was 3.9%. In our study, we found the analytical coefficient variability mean was 3.0% for EPA and 5.0% for DHA. Although these values are slightly higher than those reported by Harris and Thomas, in this study total fat from erythrocytes was extracted, whereas the cited authors separated fatty acids from erythrocyte phospholipids. In another study, the intra-assay coefficient variability for ARA, DHA, PAM, STA, and 18:1n-9 FA was 2.09%, 2.38%, 1.29%, 1.78%, and 0.91%, respectively (Magnusardottir and Skuladottir, 2006). In the latter study, the samples were erythrocytes from adult women.

In conclusion, if umbilical erythrocytes are separated from umbilical whole blood until 12 h after collection and washed before storing at −20 °C without any added antioxidant, significant changes are observed in 16:0 (PAM), 18:0 (STA), 18:3n-3 (ALA), 20:4n-6 (ARA), 22:4n-6, total SFA, total LC-PUFA, and total n-6 LC-PUFA concentrations. To our knowledge, this study is the first to describe the effect of time and sample wash on umbilical vein erythrocytes. Owing to the characteristics of human deliveries, which have normally no time schedule, these results could be very useful for research groups interested in cord blood sample collection and later FA quantification.

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Conflict of Interest The authors declare that they have no conflict of interest.

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