Cryopreservation Prevents Iron-Initiated Highly Unsaturated Fatty Acid Loss during Storage of Human Blood on Chromatography Paper at \(-20^\circ C\)\textsuperscript{1,2}

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Abstract

Background: Fingertip prick whole blood collection on chromatography paper is amenable to high-throughput fatty acid (FA) profiling for large clinical and field studies. However, sample storage is problematic because highly unsaturated FAs (HUFA) in erythrocytes rapidly degrade in samples stored at \(-20^\circ C\).

Objective: The aim of the current study was to determine the mechanism of HUFA degradation and to develop prevention protocols.

Methods: Free fatty acid (FFA) standards and whole blood reference material from a single participant were used to examine sample storage at \(-20^\circ C\) for up to 90 d in triplicate. Iron chelation with deferoxamine (0–5000 \(\mu\)g), antioxidant protection with butylated hydroxytoluene (50 \(\mu\)g), cryopreservation with glycerol, and blood drying were examined using whole blood on chromatography strips. Biological replicate blood samples from additional participants \((n=6)\) with a range of \(\omega-3\) (n–3) HUFA concentrations were similarly assessed.

Results: FFAs were relatively stable when stored on chromatography strips at \(-20^\circ C\). Glycerol treatment prevented HUFA degradation in whole blood reference material for 30 d (45.6 \(\pm\) 0.4 to 46.8 \(\pm\) 0.1, means \(\pm\) SDs) compared to untreated saline controls (45.9 \(\pm\) 1.0 to 6.8 \(\pm\) 0.2). Pretreatment of paper for blood spots with deferoxamine and drying blood before storage slowed, but not entirely prevented, HUFA degradation over 30 d to 22% and 19% below baseline, respectively, compared to 86–92% in the controls. Protection against HUFA degradation with blood drying and glycerol treatment was confirmed in the biological replicate study and confirmed by prevention of cell lysis.

Conclusions: HUFA degradation during storage at \(-20^\circ C\) appears to be due to hemolysis and subsequent iron-initiated peroxidation. This degradation may be prevented by glycerol, iron chelation, and/or dried blood spotting. A more thorough understanding of methods to prevent degradation during storage is critical with increasing use of FA profiling in large clinical studies.

Keywords: storage, hemolysis, highly unsaturated fatty acid, peroxidation, deferoxamine, cryopreservation

Introduction

The FA composition of blood can provide information on dietary FA intakes (1) and disease risk (2). Developments in fast GC (3–5) and fingertip prick blood sampling for FA profiling have enabled higher analytic throughput for clinical studies (6–10). However, large-scale screening of FA profiles in clinical and field settings requires blood sample storage and can involve researchers unaware of the challenges with the storage of FAs in blood, particularly EPA (20:5n–3) and DHA (22:6n–3) because they are prone to oxidation. Ultra-cold storage can prevent degradation of highly unsaturated FAs (HUFA)\textsuperscript{3} (\(\geq 20\) carbons and \(\geq 3\) carbon-carbon double bonds) for many years, but access to and storage space in ultra-cold freezers can be limited. Commercial freezers (\(-20^\circ C\)) are more economical, and usually available, but the storage of isolated erythrocytes at \(-20^\circ C\) has been shown to result in the degradation of PUFA in as little as 2–4 wk of storage (11, 12). Despite these previously published observations, these insights continue to happen in recent clinical studies such as the Women’s Health Initiative (13).

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\textsuperscript{3} Abbreviations used: ARA, arachidonic acid; BHT, butylated hydroxytoluene; Fe\textsuperscript{3+}, ferric iron; HUFA, highly unsaturated FA.
Awareness and strategies to prevent sample degradation are becoming increasingly important because the use of blood FAs in large studies to assess diet intake is increasing and blood FAs are beginning to be used as a dietary/metabolic phenotype in genomic studies (14).

Interestingly, PUFA losses in plasma and serum stored at –20°C appear to be stable for 1–3 y (15, 16), but plasma FA composition can be influenced by recent dietary behavior, and erythrocyte FA composition is considered a better reflection of long-term dietary habits (2, 17, 18). Whole blood use for FA profiling is increasing because of fingertip prick sampling, but whole blood contains erythrocytes and therefore may also be at risk of HUFA degradation when stored at –20°C. The addition of butylated hydroxytoluene (BHT) to whole blood can partially prevent the loss of HUFAs in samples stored at –20°C (19), but identifying the specific mechanism of HUFA loss and developing additional strategies to protect samples are needed.

Presently, it is hypothesized that increased PUFA degradation during storage of erythrocytes and whole blood on chromatography paper at –20°C is potentiated by hemolysis. This hemolysis releases ferric iron (Fe³⁺) that can catalyze the peroxidation of HUFAs (20). The antioxidant BHT can prevent some PUFA loss, but PUFA loss may also be reduced by preventing hemolysis by drying blood samples or adding cryopreservants (glycerol) and by sequestering Fe³⁺ through iron chelation (deferoxamine). FFAs, a whole blood reference material from a single participant, and blood samples from a small group of young adults were used in as iron chelation, cryopreservation, and blood drying on HUFA stability. The determination of blood storage protocols that maintain erythrocyte and FA profile integrity independent of storage temperature conditions is necessary to enable large clinical and field studies.

**Methods**

**Study design.** The study design included an examination of the stability of individual FFA standards on chromatography paper, an assessment of strategies to prevent HUFA losses in blood using technical replicates of a human blood reference standard collected from a single male participant, and a final confirmation using biological replicate samples from 6 participants (details summarized in Figure 1). The overall study protocol received clearance from the University of Waterloo Human Ethics Committee, and the participants provided informed consent before participation.

**HUFA standard storage.** The oxidation of HUFAs on chromatography paper at –20°C during storage was first examined with use of arachidonic acid (ARA), EPA, and DHA FFA standards (U-72-A, U-99-A, and U-84-A; Nu-Chek Prep, Inc.). One microgram of each FA was applied to prerawed chromatography strips (Grade 3MM Chr; Whatman Ltd.) and stored at –20°C for up to 14 d to examine the susceptibility of these FAs to decreases. One set of chromatography strips was stored in open air at room temperature to serve as a positive control of HUFA susceptibility to degradation and another set was stored at –75°C to serve as a negative control. After 1, 3, 7, and 14 d, chromatography strips were prepared for FA analysis in triplicate by direct transesterification with 14% BF₃ in methanol as described previously (3).

**Mechanistic assessment of storage strategies.** A technical replicate design was used to screen the effect of numerous storage conditions. As such, –20–mL whole blood was collected from a single individual by venipuncture into EDTA-treated vacutainers by a trained technician to serve as a whole blood reference material. Aliquots of whole blood reference standard were used in triplicate to assess the effects of iron chelation, cryopreservation, and drying blood spots before freezing. Erythrocytes were also prepared to confirm a cryopreservation effect of glycerol. A set of whole blood vacutainers were centrifuged at 1400 × g, separated, and washed.

**Iron chelation.** The effect of iron chelation was examined with use of deferoxamine mesylate salt (Sigma Chemicals). Pretreated chromatography paper strips were prepared by applying 0-, 50-, 200-, 1000-, or 5000-µg deferoxamine diluted in 25-µL deionized water followed by drying. Whole blood samples were applied to the pretreated chromatography strips and stored together in a capped 20-mL test tube at –20°C between 3 and 90 d. On analysis days, samples were removed from storage and directly transisterified (3) for FA analysis.

**Cryopreservation of erythrocytes and whole blood.** Glycerol has been used to preserve frozen erythrocytes for transfusion purposes (21, 22), but the effect on FA composition has not been documented to our knowledge. Therefore, treatment of erythrocytes with glycerol was
performed according to previously published procedures (21, 22). Briefly, separated erythrocytes and 40% glycerol in saline (wt:vol) were mixed (1:1 vol:vol) and allowed to equilibrate for ~45min. Glycerol-treated erythrocyte samples were then aliquoted to cryovials in 200-μL portions and stored at −20°C between 3 and 90 d. A treatment with 100% saline was used as a control treatment. On analysis days, samples were removed from storage and 50-μL aliquots were directly transesterified (3).

The glycerol treatment of erythrocytes was then adapted to whole blood samples (25 μL) for the application of glycerol-treated whole blood to chromatography strips before storage at −20°C. Briefly, the hematocrit value of the blood sample was used to determine erythrocyte volume and 1:1 ratio (vol:vol) of 40% glycerol in saline (wt:vol) was added. Glycerol-treated whole blood (25 μL) was then applied to prewashed chromatography strips or chromatography strips washed and previously treated with either 50-μg BHT (Thermo Fisher Scientific, Inc.), 1000-μg deferoxamine, or the combination (BHT and deferoxamine). Whole blood treated with 100% saline solution applied to chromatography paper was also included as a control, but 100% saline–treated blood was also applied to chromatography paper pretreated with 25-μL 40% glycerol. Samples were then stored at −20°C between 3 and 90 d.

Drying of whole blood. The effect of drying blood on chromatography strips before storage was examined without or with pretreatment with either 50-μg BHT, 1000-μg deferoxamine, or the BHT and deferoxamine combination. Whole blood samples applied to chromatography strips were placed in a 20-μL test tube and dried under a stream of nitrogen for ~10 min until blood was sufficiently dried and absent of water. After drying, the test tube for each condition was capped and stored between 3 and 90 d at −20°C before FA determination.

Staining of erythrocytes in whole blood. To demonstrate the presence or absence of erythrocyte lysis during storage at −20°C, Giemsa solution (Sigma-Aldrich Canada Ltd.) was used as described previously (23, 24). Briefly, saline (control) and glycerol-treated whole blood samples were stored overnight at −20°C, thawed, and aliquots were spread across a microslide. For sample drying effects, whole blood was applied to microslides and frozen immediately or after drying and stored overnight at −20°C. After staining, erythrocytes were viewed under a Nikon Eclipse 50i microscope (Nikon Instruments) at 50× magnification with use of PixeLINK Capture OEM image capturing software (PixeLINK).

Confirmation of strategies to preserve whole blood FAs during −20°C storage. A trained technician collected fasting blood samples into an EDTA-lined vacutainer from 6 individuals (3 males, 3 females) recruited from the University of Waterloo campus (mean ± SD: 23.7 ± 1.4 y). Aliquots of whole blood (25 μL) were applied to washed chromatography strips pretreated with either BHT and deferoxamine or no antioxidant and dried before storage at −20°C for 90 d. After treatment of whole blood with either glycerol or saline control (as described above), separate aliquots of whole blood (250 μL) were stored in cryovials at −20°C for 90 d. Samples from conditions described above obtained after 0 and 90 d of storage were analyzed for FA compositions by GC after direct transesterification.

FA analyses. Whole blood stored in cryovials (50 μL) and on chromatography paper (25 μL) was directly transesterified by convection heating in 1-mL 14% boron trifluoride in methanol and 300-μL hexane (containing 3-μg 22:3n-3 ethyl ester as internal standard) at 95°C for 1 h. The resultant FA methyl esters were analyzed on a Varian 3900 gas chromatograph by fast GC as described previously (25).

Statistical analyses. Because of a lack of independent samples the effects of storage condition (i.e., antioxidant, glycerol, and drying treatments) within a single time point, and the effect of time within a single storage condition, were assessed individually by 1-factor ANOVA with a Levene statistic to test for unequal variance for the FAA standards and technical replicates of the whole blood reference material. In the presence of a significant Levene statistic (P < 0.05), a Welch correction was applied to the 1-factor ANOVA (P < 0.05). After a significant F value, post hoc comparisons were performed with use of Tukey’s honestly significant difference test for storage comparisons within a single time point and by Dunnett’s test for comparisons to the day 0 control within a storage condition. A paired (repeated measures) samples t test was used to test the effects of individual storage conditions over time in the biological replicates study, and an independent samples t test was used to compare storage conditions during a single time point. All statistical analyses were performed with use of SPSS version 15.0. Statistical significance was inferred as P < 0.05. Data are presented as means ± SDs.

Results

Storage of FFA standards on chromatography paper. The ARA, EPA, and DHA standards did not change from baseline during storage at either −20°C or −75°C throughout the entire 14-d storage period (Table 1). When stored in an open test tube at room temperature, ARA decreased by 17%, 61%, and 81%, EPA by 20%, 64%, and 87%, and DHA by 29%, 69%, and 91% after 3, 7, and 14 d, respectively.

Iron chelation of whole blood reference material on chromatography paper. Iron chelation with deferoxamine reduced degradation of HUFAs associated with storage at −20°C (Figure 2A). At baseline, HUFAs in all deferoxamine storage conditions did not differ (P = 0.11) and ranged from 54.3 ± 1.0 to 54.7 ± 0.4%.

Table 1 FFA standards of ARA, EPA, and DHA do not change during storage on chromatography paper at −20°C

<table>
<thead>
<tr>
<th>FAs</th>
<th>Storage</th>
<th>Baseline</th>
<th>1 d</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARA, μg</td>
<td>Open air</td>
<td>1.07 ± 0.04</td>
<td>n.d.</td>
<td>0.89 ± 0.05*</td>
<td>0.42 ± 0.04*</td>
<td>0.20 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>−20°C</td>
<td>1.02 ± 0.01</td>
<td>1.06 ± 0.02</td>
<td>1.09 ± 0.03</td>
<td>1.01 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−75°C</td>
<td>0.96 ± 0.10</td>
<td>1.07 ± 0.03</td>
<td>1.07 ± 0.03</td>
<td>1.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>EPA, μg</td>
<td>Open air</td>
<td>1.04 ± 0.04</td>
<td>n.d.</td>
<td>0.83 ± 0.04*</td>
<td>0.37 ± 0.03*</td>
<td>0.14 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>−20°C</td>
<td>1.02 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td>1.09 ± 0.01</td>
<td>1.01 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−75°C</td>
<td>0.97 ± 0.12</td>
<td>1.08 ± 0.03</td>
<td>1.08 ± 0.04</td>
<td>1.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>DHA, μg</td>
<td>Open air</td>
<td>1.08 ± 0.03</td>
<td>n.d.</td>
<td>0.77 ± 0.07*</td>
<td>0.33 ± 0.05*</td>
<td>0.10 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>−20°C</td>
<td>1.02 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td>1.10 ± 0.03</td>
<td>1.01 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>−75°C</td>
<td>0.97 ± 0.11</td>
<td>1.06 ± 0.03</td>
<td>1.07 ± 0.04</td>
<td>1.03 ± 0.00</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SDs, n = 3. *Different from baseline control, P < 0.05. ARA, arachidonic acid; n.d., not determined.
to 61.9 ± 2.0 μg/100 μL. HUFA concentrations in the control samples without deferoxamine treatment were significantly lower than in all deferoxamine-treated samples at all time points starting at 3 d. Although increased deferoxamine concentrations tended to provide increasing protection against HUFA degradation, HUFA concentrations with 50-μg deferoxamine and 200-μg deferoxamine did not differ at all time points (P = 0.15–0.87), nor did concentrations with 1000-μg deferoxamine and 5000-μg deferoxamine up to 14 d (P = 0.59–0.97) and near significance after 30 d (P = 0.06). HUFA concentrations with each deferoxamine storage condition became significantly lower than baseline values after 3 d with 50-μg deferoxamine (46.5 ± 3.9) and 200-μg deferoxamine (51.8 ± 0.6), after 7 d with 1000-μg deferoxamine (45.2 ± 5.4), and after 14 d with 5000-μg deferoxamine (40.5 ± 4.5).

**Cryopreservation with glycerol of erythrocytes and whole blood reference material.** Glycerol-treated erythrocytes stored at −20°C without an antioxidant prevented HUFA degradation for up to 90 d of storage (Table 2). Comparatively, in the saline-control condition, HUFAs decreased by 11% after 3 d and continued to decrease throughout the storage period to 95% below baseline concentrations after 90 d. The effect of glycerol treatment on HUFA concentrations and hemolysis alone and in combination with deferoxamine or BHT was examined in whole blood (Figure 2B). Some differences in baseline HUFAs existed between storage conditions, however, because comparisons are made back to baseline within a storage condition this was deemed acceptable. HUFA concentrations in the saline-control blood decreased from baseline (45.9 ± 1.0 μg/100 μL) beginning at 3 d (41.0 ± 3.1) and continuing to 90 d (4.5 ± 0.1) of storage. Glycerol treatment alone prevented significant HUFA decreases for 30 d (45.0 ± 1.0 μg/100 μL) of storage compared to baseline (46.8 ± 0.6) with significant declines shown after 90 d (41.9 ± 1.0). The glycerol-treated blood on BHT-treated paper resulted in HUFA concentrations that did not differ from those of glycerol-treated blood on paper without pretreatment (P = 0.45). Interestingly, the glycerol-treated whole blood on deferoxamine-treated chromatography paper appeared not to differ as well until day 90 when a significant decrease in HUFA concentrations was observed (30.9 ± 0.7 vs. 48.2 ± 1.0 μg/100 μL at baseline). Similarly, treatment of chromatography paper with glycerol before application of whole blood prevented degradation until 90 d of storage (33.2 ± 0.8) compared to baseline (43.4 ± 0.3).

**Drying of whole blood reference material on chromatography paper.** At baseline, N2 drying + BHT and N2 drying + BHT + deferoxamine samples were significantly higher than wet samples; however, similar to glycerol, this does not affect comparisons to baseline of HUFAs from a specific time point for the given storage condition. HUFA concentrations in wet samples decreased beginning at 7 d of storage (Figure 2C). Pre-drying the blood samples before storage slowed, but did not entirely prevent, the degradation of HUFAs because HUFA concentrations with drying alone were significantly lower than baseline from day 14 to 90. Paper treated with BHT and deferoxamine appears to provide additional protection to HUFAs because the HUFA concentrations remained similar to baseline until day 90. Drying and BHT-treated paper was particularly effective up to 30 d when HUFA concentrations were 50.8 ± 1.5 μg/100 μL compared to 45.7 ± 1.2 with drying and deferoxamine-treated paper and 40.7 ± 2.9 with drying alone.

**FIGURE 2** Treatment of human whole blood reference standard stored on chromatography paper with deferoxamine (A), glycerol (B), and drying (C) limits HUFA decreases for up to 90 d when stored at −20°C. Data points are HUFA means (μg/100 μL) of n = 3 technical replicates from a single participant, with error bars representing SDs. *Different from corresponding baseline, P < 0.05. Labeled means at a time without a common letter differ, P < 0.05. BHT, butylated hydroxytoluene; DFO, deferoxamine; HUFA, highly unsaturated FA; N2, nitrogen.

**Cryopreservation and drying prevent erythrocyte lysis.** Glycerol treatment was associated with the absence of erythrocyte lysis after storage at −20°C compared to lysis in the 100% saline-treated control as determined by Giemsa staining (Figure 3). Hemolysis was also observed with wet −20°C storage, but drying before −20°C storage prevented this hemolysis (Figure 3). However, drying resulted in “clumping” of the erythrocytes compared to the glycerol-treated whole blood.
Confirmation of HUFA changes in whole blood during storage with biological replicates. The protection against HUFA decreases during −20°C storage of dried blood spots by BHT and deferoxamine on chromatography paper, and of whole blood in cryovials by glycerol treatment, was confirmed in an additional 6 participants. The ω-3 status of the participants ranged from 19.5 to 35.0% n-3 HUFAs in total HUFAs (28.3 ± 6.0) or 2.3–5.7% of EPA and DHA in total FAs (3.8 ± 1.3) (data not shown). There was a consistent pattern of decline in the concentrations of HUFAs for both the dried blood on chromatography paper control (Figure 4A) and the saline-treated whole blood control (Figure 4B) after 90 d of storage at −20°C. HUFA concentrations were higher in blood stored for 90 d at −20°C on chromatography paper pretreated with 50-μg BHT and 1000-μg deferoxamine compared to control, but pretreatment did not fully protect against decreases in HUFA concentrations. Similarly, treatment of whole blood with glycerol before storage did not fully protect against decreases in HUFA concentrations. However, glycerol treatment provides clear protection against HUFA degradation with losses of 11% relative to baseline compared to 38% of HUFAs lost in the saline control.

Discussion

Presently, the stability of the FFA standards of EPA, DHA, and ARA (20:4n-6) on chromatography paper during storage at −20°C implicate the blood matrix in the degradation of HUFAs observed with erythrocytes and whole blood. Pretreatment of chromatography strips with 50–5000 μg of the iron chelator deferoxamine partially but not completely inhibited HUFA losses in a whole blood reference material during storage at −20°C in a concentration-dependent manner. The treatment of both erythrocytes and whole blood reference material with glycerol prevented hemolysis and HUFA losses during storage at −20°C for 90 d in cryovials and for 30 d on chromatography paper, respectively. This protection was duplicated by pretreating the paper with glycerol before blood application. Drying whole blood on chromatography paper before storage at −20°C also protected against HUFA losses, although not completely. Drying combined with pretreatment of chromatography strips with either deferoxamine or BHT can provide additional protection against HUFA losses, particularly with BHT. Glycerol treatment for protection against degradation in whole blood and deferoxamine treatment for protection against degradation in dried blood spots when samples are stored at −20°C was also confirmed in a sample of multiple participants.

In the present study, deferoxamine reduced HUFA losses to only 22% lower than baseline values compared to 92% lower in control samples after 30 d of storage at −20°C. This protection against HUFA degradation with deferoxamine provides clear evidence for an iron-mediated peroxidation mechanism. In healthy cells, ~3% of the hemoglobin-iron complex (HB-Fe3+) is converted to Hb-Fe3+ by O2, resulting in O2 radical formation (26). In addition, Fe3+ can accept a proton from H2O2 to form Fe3+ and the highly reactive -OH radical (20). The formation of Fe3+ on its own is able to act directly on PUFAs to cause peroxidation and FA loss (27). This reaction can be prevented through iron chelators that bind Fe3+ and essentially remove the free reactive iron from reacting with PUFAs. Deferoxamine was unable to fully prevent losses in HUFAs however, which suggests other initiators such as -OH and -O2 free radicals may be involved. Deferoxamine has been shown to prevent HUFA degradation for up to 1 y in erythrocytes stored at −50°C, but a control storage condition was not included (12). As such it is unclear whether prevention of FA degradation is due to the presence of deferoxamine or storage at −50°C compared to −20°C. Deferoxamine has also been shown to protect against lipid peroxidation in erythrocytes stored at 4°C (28).

Hemolysis occurs during storage at both −20°C and −75°C, however, storage of erythrocytes without antioxidant protection at no warmer than −80°C (29) maintains erythrocyte FA profiles for at least 4 y and possibly longer. This suggests that although lipid peroxidation reactions are highly active at temperatures as low as −20°C, storage at lower temperatures such as −75°C may be cold enough to slow peroxidation and maintain FA profiles for many years. Rapid degradations in HUFAs after 2 and 4 wk of storage at −20°C have been observed in erythrocyte total lipid (11, 13, 30) and phospholipid (12, 31) FAs. Conversely, no changes in plasma FAs occur when stored at −20°C (12, 15, 16), suggesting a mechanism of degradation inherent to the presence of erythrocytes in the blood samples.

No previous studies have attempted to determine the effects of erythrocyte or whole blood cryopreservation on HUFA

TABLE 2  Glycerol treatment of a human erythrocyte reference standard before storage in cryovials at −20°C prevents HUFA degradation for at least 90 d

<table>
<thead>
<tr>
<th>Cryopreservant</th>
<th>Baseline</th>
<th>3 d</th>
<th>7 d</th>
<th>14 d</th>
<th>30 d</th>
<th>90 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, μg/100 μL</td>
<td>53.0 ± 2.53</td>
<td>48.4 ± 1.19*</td>
<td>23.6 ± 0.07*</td>
<td>4.48 ± 0.76*</td>
<td>3.88 ± 0.06*</td>
<td>2.18 ± 0.16*</td>
</tr>
<tr>
<td>Glycerol, μg/100 μL</td>
<td>48.9 ± 9.68</td>
<td>49.0 ± 2.01</td>
<td>45.8 ± 1.16</td>
<td>41.9 ± 1.20</td>
<td>46.9 ± 1.10</td>
<td>44.2 ± 3.13</td>
</tr>
</tbody>
</table>

*Values are means ± SDs, n = 3. *Different from baseline control, P < 0.05. HUFA, highly unsaturated FA.
stability during storage at $-20^\circ$C. However, 2,2’-azobis (2-aminodipropyl) hydrochloride–induced hemolysis has previously been shown to increase FA peroxidation in erythrocyte membranes (32). Antioxidant/free radical scavengers such as curcumin (33) or thiol-containing drugs (34) can reduce or prevent peroxidation in hemolyzed erythrocytes. Our results suggest that freeze-thaw induced hemolysis is occurring before FA peroxidation because prevention of hemolysis can protect fully against HUFA degradation for at least 90 d. Erythrocytes stored at $4^\circ$C for extended periods of time may also undergo hemolysis resulting in lipid peroxidation (35), however, this process appears to take substantially longer than at $-20^\circ$C.

It is clearly shown here that iron chelation with deferoxamine and cryopreservation with glycerol protects against lipid peroxidation during long-term erythrocyte and whole blood storage, however, another potential prevention technique exists. The mechanism behind freeze/thaw-induced hemolysis may be directly due to the presence of water and its subsequent freezing in a whole blood or erythrocyte sample. The mechanisms for this have been reviewed (36) but are poorly understood. Briefly, ice crystal formation during freezing is thought to place mechanical stress on the erythrocytes, causing physical deformation to the lipid bilayer and eventual membrane rupture. The high volume of water in whole blood compared to erythrocytes suggests that whole blood may be more susceptible to hemolysis and possible HUFA degradation. Supporting evidence indicates that erythrocyte HUFA degrade after 2–4 wk of storage (11–13) compared to as little as 1 d in whole blood (37). Although small sample volumes may be responsible for increased HUFA losses during storage at $-20^\circ$C (13), EPA and DHA also degrade in larger volumes of whole blood in no less than 3 d (37). Presently, removal of water by drying blood spots before storage provided substantial protection against HUFA losses for up to 14 d during storage at $-20^\circ$C compared to only 3 d with samples stored wet.

Some minor losses were prevented by pretreatment of chromatography paper with deferoxamine and to a greater extent with BHT, suggesting that the drying process itself may also have caused hemolysis. Strong flows of nitrogen were applied to the blood samples. The air turbulence created may have damaged the erythrocyte bilayer and as a result released iron as excessive shaking or agitation of blood has been shown to result in significant concentrations of hemolysis (38). To combat this problem, air drying of whole blood on paper may reduce the turbulent flow experienced by erythrocytes and thereby reduce potential drying-induced hemolysis. This may increase oxygen-mediated lipid peroxidation, however, $>1$ d under these conditions may be required before substantial HUFA degradation occurs (19). Alternatively, interaction of erythrocytes with solid surfaces such as chromatography paper can cause damage resulting in cell lysis (39). BHT pretreatment of the chromatography strips provides further protection against HUFA degradation and, in support of our deferoxamine treatment experiment, suggests alternative peroxidation mechanisms in addition to iron-mediated pathways during storage at $-20^\circ$C.

The majority of the results of this study were made with use of technical replicates of a whole blood reference material collected from a single individual. This design was implicated to enable simpler comparisons between numerous different storage variables to observe the phenomenon and determine potential mechanisms of HUFA degradation. We confirmed the main findings in a small biological replicate study with participants with a range of $\omega$-3 HUFA concentrations. The HUFA degradation patterns observed in the present study resemble patterns observed in previous larger study samples (37, 40), but the assessment of storage methods in larger population studies is important because the iron and antioxidant status of individuals may differ and affect rates of HUFA degradation. Blood HUFA degradation observed during storage at $-20^\circ$C appears to be due to iron-mediated peroxidation pathways triggered by the release of iron during hemolysis. Storage of whole blood or erythrocytes in the presence of glycerol prevented hemolysis and HUFA degradation and improved the stability of samples stored at $-20^\circ$C.

Enabling blood storage at $-20^\circ$C may benefit field studies that require subzero preservation of non-FA analytes but do not have access to deep freezers. In addition, large sample numbers from clinical research can place pressure on storage capabilities, and blood storage at $-20^\circ$C can help to alleviate this problem. Dried blood spots on chromatography paper would require substantially less storage space than venipuncture collections, and pretreatment of chromatography paper with deferoxamine or BHT combined with dried blood spots can protect against HUFA degradation during storage periods of up to 30 d. Future research to assess the applicability of these methods for prevention of HUFA degradation should be conducted at room temperature and $4^\circ$C.

FIGURE 4 Preventing decreases of HUFAs in biological replicates from 6 human (3 males, 3 females) whole blood samples stored at $-20^\circ$C for 90 d. (A) HUFA decreases in dried blood spots on chromatography paper are less when paper is pretreated with BHT + deferoxamine; (B) glycerol pretreatment of whole blood stored in cryovials can prevent HUFA decreases. Black lines and data points are HUFA means ($\mu$g/100 mL) of 6 participants with error bars representing SDs. Gray lines represent technical replicates of a whole blood reference material collected from a single individual. This design was implicated to enable simpler comparisons between numerous different storage variables to observe the phenomenon and determine potential mechanisms of HUFA degradation. We confirmed the main findings in a small biological replicate study with participants with a range of $\omega$-3 HUFA concentrations. The HUFA degradation patterns observed in the present study resemble patterns observed in previous larger study samples (37, 40), but the assessment of storage methods in larger population studies is important because the iron and antioxidant status of individuals may differ and affect rates of HUFA degradation. Blood HUFA degradation observed during storage at $-20^\circ$C appears to be due to iron-mediated peroxidation pathways triggered by the release of iron during hemolysis. Storage of whole blood or erythrocytes in the presence of glycerol prevented hemolysis and HUFA degradation and improved the stability of samples stored at $-20^\circ$C.

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conclude, in samples to be stored at −20°C, glycerol cryopreservation prevents hemolysis can protect against HUFA degradation in whole blood and erythrocytes stored in cryovials, and drying of whole blood on chromatography paper pretreated with deferoxamine or BHT can reduce and possibly prevent the deleterious effects of hemolysis on blood HUFA composition.

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References
22. Valeri CR, Pivacek LE, Cassidy GP, Rago G. In vitro and in vivo measurements of human RBCs frozen with glycerol and subjected to various storage temperatures before deglycerolization and storage at 4 degrees C for 3 days. Transfusion 2001;41:401–5.