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Metabolic tracing analysis reveals substrate specific metabolic deficits in platelet storage lesion

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Abstract

Background—Storage of platelets results in a progressive defect termed Platelet Storage Lesion (PSL). The PSL is characterized by poor platelet quality on a variety of assays. Metabolic defects are thought to underlie the PSL, thus this study was designed to quantitatively probe specific metabolic pathways over platelet storage.

Study Design and Methods—Relative incorporation of stable isotope labeled substrates was quantified by isotopologue analysis of key acyl-coenzyme A thioester products for fresh, viable (post-collection, day 2–5), and expired platelets (after day 5). We examined the incorporation of acetate, glucose and palmitate into acetyl- and succinyl-CoA via liquid chromatography-tandem mass spectrometry.

Results—Storage related defects in the incorporation of acetyl-CoA derived from acetate and palmitate were observed. Carbon derived from palmitate and acetate in succinyl-CoA was reduced over storage time. Glucose incorporation into succinyl-CoA increased in viable platelets, then decreased in expired platelets. Carbon derived from octanoate and pyruvate remained partially able to incorporate into acetyl- and succinyl-CoA in expired platelets, with high variability in pyruvate incorporation.

Conclusion—Isotopologue analysis is useful in probing substrate specific defects in the PSL.

Keywords

Platelet concentrate; platelet storage lesion; mass spectrometry; stable isotope labeling

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Introduction

Traumatic injury is the leading cause of death in the United States for patients between the age of 1 to 40 and a major cause of mortality in civilian¹ and military patients². Notably, 16% of the preventable deaths from injury are due to hemorrhage, highlighting the importance of achieving hemostasis and stabilization. In the Emergency department, patients are frequently coagulopathic³⁻⁴, and death by acute blood loss typically occurs within a 6-hour window from injury. Transfusion of platelets, plasma, and red blood cells (RBCs), with some controversy surrounding the ideal ratio, has demonstrated efficacy in restoring hemostasis⁵, but issues with supply, efficacy, and storage of platelets are a major challenge. The national blood supply has been under increasing stress due to increased medical demand, decreased donation, and increasingly stringent regulation to protect patients receiving transfusions⁶. Platelets are especially critical to meeting the demands of trauma medicine and are also increasingly subject to supply constraints due to their unique storage needs.

Collection and storage of platelets for use in hospital settings requires 1–2 days for microbiological testing before use. During storage, platelets intended for transfusion are kept at room temperature in gas permeable bags with constant agitation⁷. After 5–7 days platelets are no longer considered viable for human transfusion due to the risk of bacterial contamination⁸ leaving only a 3-day window for their use in some regulatory environments. This is considerably shorter than the 8–10 day normal *in vivo* turnover of platelets. In addition, even before the 5-day regulatory cut-off, platelet storage induces a time-dependent “platelet storage lesion” (PSL) which reduces the effectiveness of stored and transfused platelets⁹.

Importantly, the three functions -activation, aggregation, degranulation- whose deficiency defines the PSL, are all energetically demanding. The PSL has been shown to derive from dysfunctional metabolism, oxidative stress, and damage to the platelet during storage¹⁰. A challenge in addressing the PSL is that the metabolism of platelets is only partially understood¹¹. Importantly, platelets require tight homeostatic control of calcium, adenosine (ADP/ATP) and lipid metabolism and signaling to allow appropriate clotting responses upon activation. Platelets are anuclear but contain mitochondria and are capable of mitochondrial protein synthesis. A set of studies on platelet metabolism, utilizing radioactive tracers, have shown that platelets are capable of utilizing a number of substrates to maintain critical homeostatic processes including maintenance of lipid membrane components¹⁴⁻¹⁵. Modern liquid chromatography –mass spectrometry (LC-MS) now offers a highly sensitive and specific platform for performing metabolic studies with more throughput, sensitivity, and specificity than previous techniques¹⁶. Recently, Paglia, *et al.*, deployed sophisticated LC-MS based assays to interrogate the PSL, and observed a shift in platelet metabolism over days of storage, characterized by downregulation of the Krebs cycle, which was hypothesized to lead to eventual depletion of critical intermediate metabolites, membrane lipids, and a loss of function¹⁷. However, these previous studies examined the metabolites that build up during storage, and while absolute quantification of metabolites may provide insight into the metabolic state of a system, it is possible for compensatory metabolic actions to occur that maintain a metabolite pool by altering the utilization of other precursors¹⁸⁻¹⁹.

Therefore, in this study, we aimed to expand the previous work on platelet metabolism by using stable isotope labeling and mass isotopomer distribution analysis to probe the active metabolism of fresh, viable, and expired platelets. This provides orthogonal and additive information to existing studies using absolute quantification²⁰. Quantifying the relative enrichment of isotopologues enables evaluation of metabolic pathway activity, as isotopic labeling of a metabolite pool reveals the relative contribution of specific enzymes and pathways from a substrate to product pair²¹. Here, we have tested a panel of three isotopic tracers ($[^{13}\text{C}_2]$ -acetate, $[^{13}\text{C}_6]$ -glucose, and $[^{13}\text{C}_{16}]$ -palmitate) to examine metabolism of key carbon pathways by analysis of isotopic enrichment in various acyl-CoenzymeA (CoA) species.

Materials and Methods

Sample collection

Viable platelets were gathered from remnants of unused platelet concentrate (PC) (3 remnant bags across different days) used for transfusions at the University of Pennsylvania Trauma Center. Expired platelets (9 bags across different days) were obtained from the Blood Bank of the Hospital of the University of Pennsylvania. Fresh platelets (10 unique donors) were taken from the platelet rich plasma (PRP) of healthy donors of mixed sex, age, and blood type as previously described²².

Chemicals and reagents

Glucose, $[^{13}\text{C}_6]$ -glucose, sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate (NaHCO_3), calcium chloride (CaCl_2), magnesium chloride (MgCl_2), 5-sulfosalicylic acid (SSA), ammonium acetate, and trichloroacetic acid (TCA) were purchased from Sigma (MO, USA). $[^{13}\text{C}_2]$ -acetate and $[^{13}\text{C}_{16}]$ -palmitate were purchased from Cambridge Isotopes (MA, USA). 8.5 mL acid-citrate-dextrose Vacutainer® tubes (Sol A) were purchased from BD Biosciences (NJ, USA). All solvents and additives used including water, acetonitrile, methanol, and formic acid were Optima LC-MS grade purchased from Fisher Scientific (PA, USA).

Stable isotope tracing

Platelets were purified from blood or concentrate media by centrifugation with no brakes at $200 \times g$ for 10 min and the supernatant was discarded. The platelet pellet was resuspended in 1 mL pre-warmed Tyrode's solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO_3 , 3 mM CaCl_2 , and 1 mM MgCl_2) at 37°C containing 5 mM $[^{13}\text{C}_6]$ -glucose or 5 mM glucose in combination with either 1 mM $[^{13}\text{C}_2]$ -acetate or 100 μM $[^{13}\text{C}_{16}]$ -palmitate and transferred to a 1.5 mL microcentrifuge tube. Samples used for isotopic correction were treated with Tyrode's containing only 5 mM glucose. Suspended platelets were incubated with tracers for 1 hour at 37°C prior to quenching and acyl-CoA extraction.

Extraction and analysis of acyl-CoA thioesters

The extraction and LC-MS analysis have been described in detail previously¹⁸. Briefly, treated platelets were pelleted by centrifugation at $1,000 \times g$ for 2 min and resuspended in 1 mL of ice-cold 10% TCA followed by pulse-sonication for 30 s on ice using a sonic

dismembrator (Fisher), followed by a 10 min centrifugation at $15,000 \times g$. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by solid-phase extraction using Oasis HLB 1 cm³ (30 mg) SPE columns (Waters) as follows: tubes were conditioned with 1 mL of methanol followed by 1 mL of water. The collected supernatant was applied, washed with 1 mL of water, and eluted using 1 mL of methanol containing 25 mM ammonium acetate. Eluted compounds were evaporated to dryness under nitrogen and resuspended in 50 μ L of 5% SSA in water.

Injections of 20 μ L of the sample maintained at 4 °C were made from a Leap CTC autosampler (CTC Analytics, Switzerland) for LC-MS/MS analysis. Acyl-CoAs were separated using a reversed-phase HPLC Phenomenex Luna C18 column (2.0 mm \times 150 mm, pore size 5 μ m) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in 95:5 ACN/water (v/v) as solvent B, and 80:20:0.1 (v/v/v) ACN/water/formic acid as solvent C as previously described¹⁹. Gradient conditions were as follows: 2% B for 1.5 min, increased to 25% over 3.5 min, increased to 100% B in 0.5 min and held for 8.5 min, washed with 100% C for 5 min, before equilibration for 5 min. The flow rate was 200 μ L/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Sciex, Foster City, CA) in the positive electrospray (ESI) mode. The column effluent was diverted to the mass spectrometer from 8 to 18 min and to waste for the remainder of the run. The MS operating conditions were: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), gas 2 (15 units), and collision-induced dissociation (CID) gas (5 units). The ESI probe temperature was 450 °C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. A loss of 507 amu was monitored for each acyl-CoA, with a mass increase of 1 amu in both precursor and product ion for each increasing isotopologue as previously described²³.

Isotopologue and Statistical Analysis

Peak areas for acetyl-CoA (M+0–M+2) and succinyl-CoA (M+0–M+4) were integrated using Analyst 1.4.1 software to quantify ¹³C labelling of the acyl moiety by determining the mass isotopologue distribution. An isotopologue array generated for each acyl-CoA from each sample was multiplied by the inverse of the correction matrix determined experimentally from the platelets incubated with unlabelled glucose to determine the absolute enrichment of each isotopologue as described by Fernandez *et al.*²⁴ and implemented via a free on-line tool²⁵. This approach reduces the underestimation of isotopic labelling in highly labelled substrates. Therefore, results are presented as a percentage of the unlabelled isotopologue for each acyl-CoA, so that increasing percentages of enrichment in non-M+0 isotopologues indicates the presence of ¹³C derived from the labelled substrate investigated.

Results

Analysis of acetyl-CoA labeling patterns revealed that platelets incorporate into acetyl-CoA variable amounts of carbon derived from acetate, glucose and palmitate (Fig. 1). The utilization of acetate to produce acetyl-CoA was remarkably high (mean, SD; 64.9, 7.2% in

M+2% labeling) and had a strong reduction upon storage (Fig. 1A), with a decrease in the % labeling in acetyl-CoA from fresh to viable (51.7, 2.6%) to expired (31.4, 6.5%) platelets with a p-value < 0.01 by 2-way ANOVA with Dunnett's multiple comparisons test comparing fresh versus viable and expired platelets. A relatively smaller change in glucose utilization was observed over storage (M+2 % labeling for fresh 12.3,5.5%; viable 18.87,4.9%; expired 11.3,3.4%), and this change was only statistically significant in the labeling pattern observed for viable platelets compared to fresh (Fig. 1B). Acetyl-CoA produced from palmitate was moderate in fresh platelets (M+2 % labeling of 22.0, 5.8%) but markedly decreased in viable and expired plates (5.8, 4.3% and 10.9, 5.2%, respectively) (Fig. 1C).

Succinyl-CoA labeling provided complimentary information to the labeling observed in acetyl-CoA. The labeling patterns of succinyl-CoA were more starkly contrasting than for acetyl-CoA, possibly since succinyl-CoA is generally a smaller pool within cells. A similar decrease in succinyl-CoA labeling was observed from labeled acetate over storage. Interesting, the labeling for fresh platelets included high amounts of M+1, M+2, M+3 and M+4 labeling, indicating that carbon from acetate in succinyl-CoA was cycled around the Krebs cycle. Labeling of succinyl-CoA from glucose also agreed with the acetyl-CoA results, with a high level of M+2 labeling in viable platelets (24.0, 10%). Surprisingly, palmitate labeling into succinyl-CoA was higher than glucose in fresh (but not viable or expired platelets), suggesting that β -oxidation of fatty acids in fresh platelets contributed to carbon within the Krebs Cycle.

More recent work by Paglia, *et al.* suggested that the mode of platelet collection may affect their metabolism²⁶, so we explored the potential of using additional tracers to elucidate metabolic consequences of the PSL using the uniformly collected viable and expired platelets. Due to the results with palmitate, we further probed β -oxidation in viable and expired platelets via incubation with [¹³C₈]-octanoate (C8:0). This tracer allows circumventing the carnitine transport system for mitochondrial import. Fairly similar results were obtained using this tracer as compared to palmitate, with relatively minor labeling observed in succinyl-CoA. Expired platelets incorporated slightly less carbon from octanoate into acetyl-CoA than viable platelets, but not statistically so adjusting for the multiple comparisons used in this study. Using [¹³C₃]-pyruvate, we examined the ability of viable and expired platelets incorporate the tracer into acetyl-CoA without glycolysis as required for glucose. Quite interestingly, in expired platelets, pyruvate was able to partially but quite inconsistently label acetyl-CoA (viable vs expired 0.71, 2.12% vs 15.4, 17.0%). Labeling into succinyl-CoA with pyruvate confirms this observation, showed the same across-bag inconsistency, and demonstrates that the carbon derived from pyruvate transits the Krebs Cycle. Again, after adjusting for multiple comparisons, this relation was not statistically significant, but may warrant future study as this degree of variability was not observed with any other tracer. Regardless, pyruvate as a tracer was unique in its ability to incorporate into any metabolite in the expired platelets more so than viable.

Discussion

Metabolomics, and tracer studies in particular, have a particular promise in optimizing transfusion medicine. As highlighted in a recent review, the tractability of both RBCs and stored platelets to metabolomics studies indicates an open avenue of investigation into pressing questions regarding collection, inter-individual variability, storage conditions, and basic biology of RBC and platelet metabolism²⁷. A prime example of this convergence is the application of highly specific probes to reveal and quantify biochemical pathways in transfusion products, for example, the utilization of a stable isotope labeled citrate probe to elucidate an unappreciated citrate catabolic route in RBCs²⁸. Elucidation of these potentially extensive other pathways may provide storage additives, rescue substrates, or probes to quantify metabolic capacity.

Platelets are a particularly promising system for exploiting isotopic tracer analysis. Since they are anuclear, but contain mitochondria, it is possible to trace mitochondrial metabolism. Feasibility of *ex vivo* tracing is quite high, as platelets can be obtained from PRP in relatively high amounts even from pediatric blood draws²⁹. A major benefit of the metabolic tracer system is that it is self-normalizing, as each isotopologue is normalized to the entire analyte pool. Thus, tight normalization of the number of platelets across studies is not necessary and this avoids introducing less precise methods of normalization which may affect the results²³. One caveat of tracer studies is that in order to maximize reproducibility of the tracer incubation, a defined media is required. In our case, we used Tyrode's buffer, a buffered salt solution that is commonly used in platelet experiments, and allows complete control over carbon sources²³. The tight experimental control over the amount of labeled versus unlabeled carbon substrates allows a quantitative substrate to product relationship to be established, but is incompatible with the inherent variability in carbon sources in the blood metabolome. Notably, this protocol is also necessary to compare different times of platelet storage because over time in storage, substrates are metabolized.

A related limitation of our study is that we compared platelets across donors, thus introducing high inter-group variability into our comparison. This is not necessarily a flaw however, since this allows across donor and pool comparisons which were somewhat revealing in this study. Most notably, the isotopologue enrichment of pyruvate across expired pools was highly variable, with between 0–50% of both acetyl- and succinyl-CoA labeled depending on sample. This raises the question if substrate specific defects in the PSL may also be donor specific. Longitudinal studies within and across donors would be needed to address the provocative suggestion that certain platelet donors may have an ideal metabolomics wiring to accommodate storage, analogous to the possibility of variants in RBC donors with advantageous mutations in glycolytic pathways²⁷.

The high capacity for metabolism of acetate by fresh platelets was in line with previous evidence regarding the incorporation of acetate into platelets and other elements of blood¹⁴. Interestingly and not previously observed, the stark decay in the capacity for acetate metabolism to acetyl-CoA over storage indicates that this may be a pathway highly affected by the PSL. The remainder of our findings provides orthogonal confirmation of previous studies. The utilization of glucose was in good agreement with the previous study by Paglia

*et al.*¹⁷. Increased utilization of glucose into acetyl- and succinyl-CoA for the viable platelets corresponds to the timepoints in platelet storage where the TCA cycle becomes upregulated relative to other pathways.

We did not collect data on platelet function throughout this study. Thus, future studies combining substrate utilization and platelet function would be highly warranted if a substrate was found that even partially circumvented the PSL. Finally, differences in platelet metabolome by collection method has been observed²⁶, but our study was not originally designed to address this comparison.

An additional limitation of our study was the relatively small panel of tracers used and the few analytes considered. An expanding number of stable isotope tracers have been successfully employed in metabolic studies, and this system should be amenable to a wide variety of substrates. However, our study was limited by the choice of platform and analyte coverage. We conducted these experiments on a triple quadrupole mass spectrometer, which is only capable of monitoring relatively few analytes over a given run. This limitation is more acute when performing tracing studies, which require a number of transitions for each isotopologue for each analyte. The dissemination of high resolution mass spectrometers allows additional multiplexing to cover a wider range of analytes, as well as more complicated stable isotope labeling experimental designs incorporating differential neutron coded labels (e.g. ¹³C vs ¹⁵N vs ²H). A number of targeted and untargeted analytical methods may be useful in probing wider acyl-CoA metabolism³⁰, intra- vs extra-cellular metabolites³¹, or a much wider metabolite panel also examining glycolysis and other relevant pathways^{32–33}.

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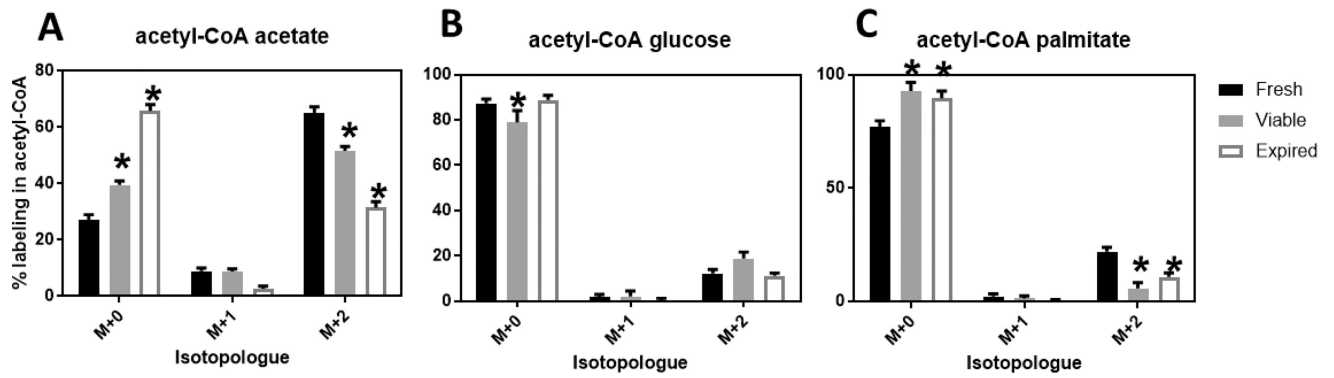


Figure 1. Relative enrichment as % labeling into acetyl-CoA from ^{13}C -labeled A) acetate, B) glucose, or C) palmitate. Fresh (n=10), viable (n=3), expired (n=9) platelets from different donors were compared using standardized treatments for 1 hour in Tyrode's buffer in the presence of the labeled substrate. * $p < 0.05$ for 2-way ANOVA with Dunnett's multiple comparison test against fresh platelets.

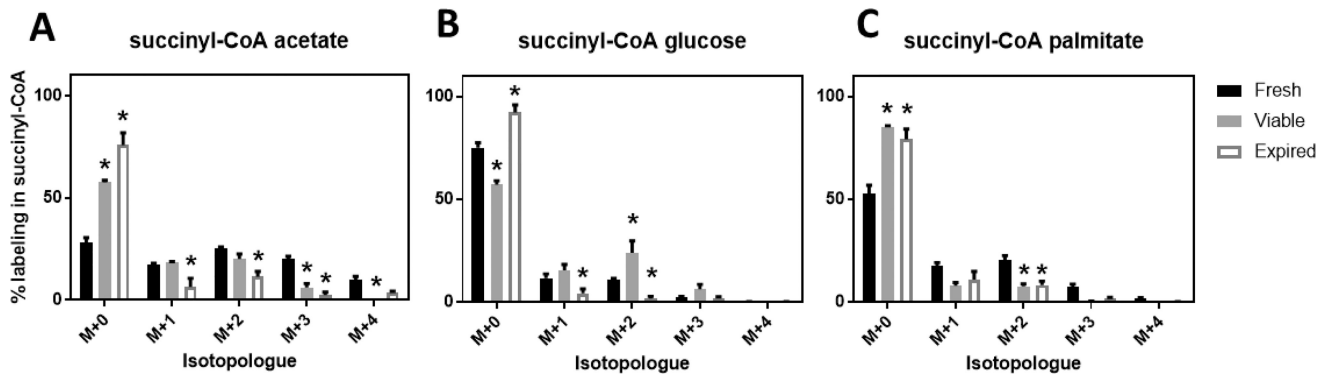


Figure 2.

Relative enrichment as % labeling into succinyl-CoA from ^{13}C -labeled A) acetate, B) glucose, or C) palmitate. Fresh (n=10), viable (n=3), expired (n=9) platelets from different donors were compared using standardized treatments for 1 hour in Tyrode's buffer in the presence of the labeled substrate. * $p < 0.05$ for 2 way ANOVA with Dunnett's multiple comparison test against Fresh platelets.

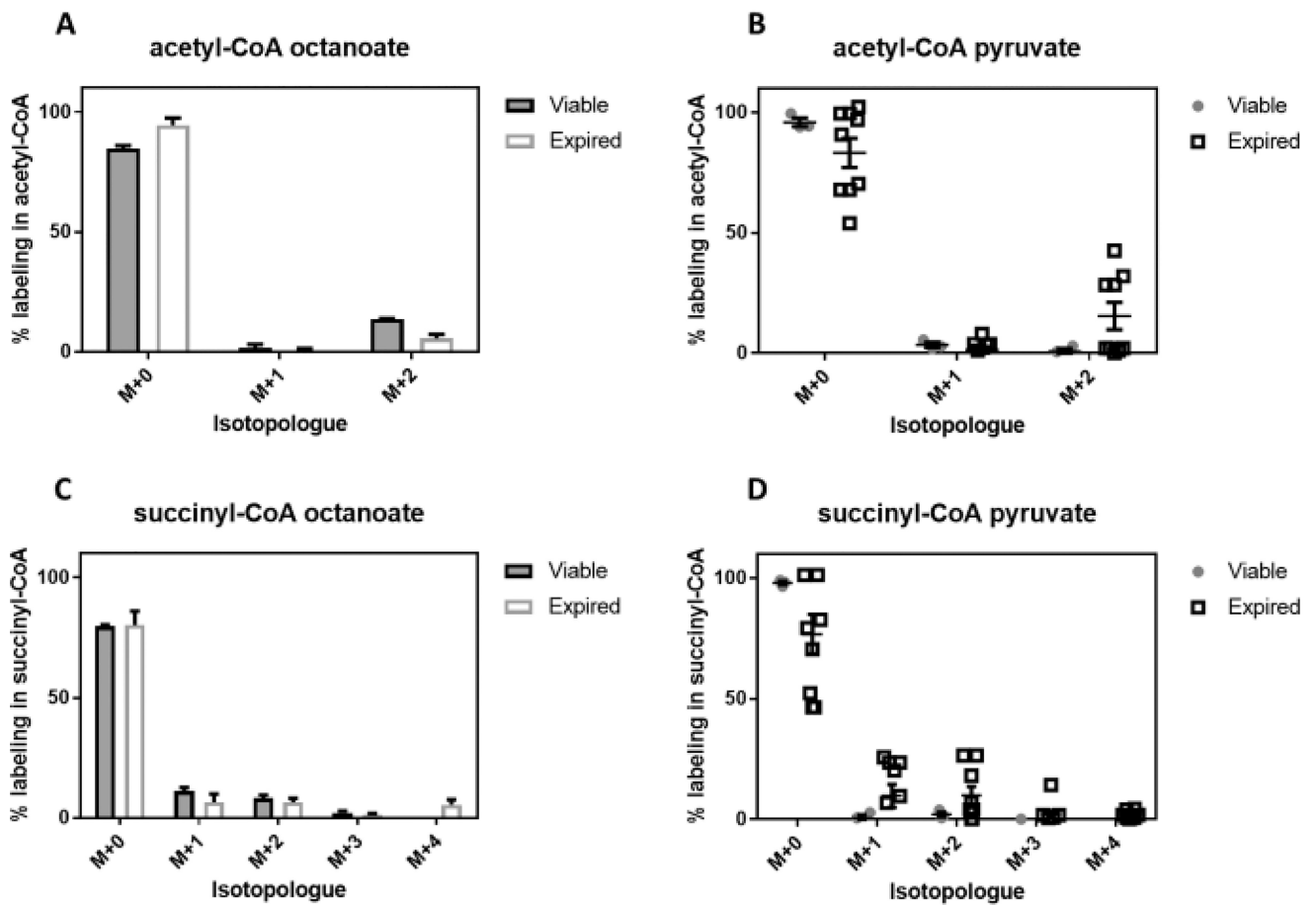


Figure 3.

Relative enrichment as % labeling into acetyl-CoA (A,B) and succinyl-CoA (C,D) from ^{13}C -labeled A, C) octanoate, B, D) pyruvate. Viable (n=3) and expired (n=9) platelets from different donors were compared using standardized treatments for 1 hour in Tyrode's buffer in the presence of the labeled substrate. Individual points in pyruvate labeling are broken out to demonstrate variability of labeling observed uniquely in pyruvate.