Am J Transl Res 2022;14(3):1628-1639
www.ajtr.org /ISSN:1943-8141/AJTR0140212

Original Article

Time dependent changes in the bioenergetics of peripheral blood mononuclear cells: processing time, collection tubes and cryopreservation effects

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Received October 30, 2021; Accepted February 6, 2022; Epub March 15, 2022; Published March 30, 2022

Abstract: Objectives: Bioenergetic measurements in peripheral blood mononuclear cells (PBMCs) using high-throughput respirometry is a promising minimally invasive approach to studying mitochondrial function in humans. However, optimal methods for collecting PBMCs are not well studied. Methods: Bioenergetics and viability were measured across processing delays, tube type and cryopreservation. Results: Storage of collection tubes on dry ice resulted in unrecoverable samples and using the Cell Preparation Tube (CPT™) significantly reduced viability. Thus, storage in Sodium Citrate (NaC) and ethylenediaminetetraacetic acid (EDTA) tubes were studied in detail. Cell viability decreased by 0.5% for each hour the samples remained on wet ice prior to processing while cryopreservation decreased viability by 9.6% with viability remaining stable for about one month in liquid nitrogen. Adenosine triphosphate linked respiration (ALR) and proton-leak respiration (PLR) changed minimally while maximal respiratory capacity (MRC) and reserve capacity (RC) decreased markedly with collection tubes stored on wet ice over 24 hrs. Changes in respiratory parameters were more modest over the first 8 hours. Manipulations to replace media did not attenuate changes in respiratory parameters. Cryopreservation decreased ALR, MRC and RC by 17.20, 95.30 and 54.92 pmol/min, respectively and increased PLR by 2.65 pmol/min. PLR, MRC and RC changed moderately during the first month in liquid nitrogen for freshly frozen PBMCs. Conclusions: Our results suggest that bioenergetics in PBMCs vary based on the processing time from specimen collection and preservation method. Changes in bioenergetics can be minimized by processing samples with a minimal time delay. Changes in viability are minimal and may not correspond to changes in bioenergetics.

Keywords: Cryopreservation, high-throughput respirometry, mitochondria, peripheral blood mononuclear cells, viability

Introduction

The mitochondria, the powerhouse of the cell, can be disrupted in many common diseases, including psychiatric [1-4] and neurodegenerative disorders [5], persistent systemic inflammation [6], cardiac disease [7], cancer [8] and diabetes [9]. One of the challenges of mitochondrial research is measuring mitochondrial function since such measurement typically involves invasive procedures such as skin or muscle biopsy. However, measuring mitochondrial function in peripheral blood mononuclear cells (PBMCs) has been proposed as a biomarker of bioenergetic health for a wide range of conditions [10], including adiposity [11], porphyria [12], postoperative cardiac surgery [13] and diabetic neuropathy [14].

Measurement of mitochondrial function in fresh human tissue has been facilitated by the development of the Seahorse 96 XF Analyzer (Seahorse Bioscience, Inc., North Billerica, MA), a high-throughput respirometer. The Seahorse analyzer measures oxygen consumption rate (OCR) in real-time using a 96-well plate in a wide range of intact living cell types [15, 16]. We have used Seahorse analysis to assess mitochondrial abnormalities related to autism spectrum disorder (ASD) using lymphoblastoid

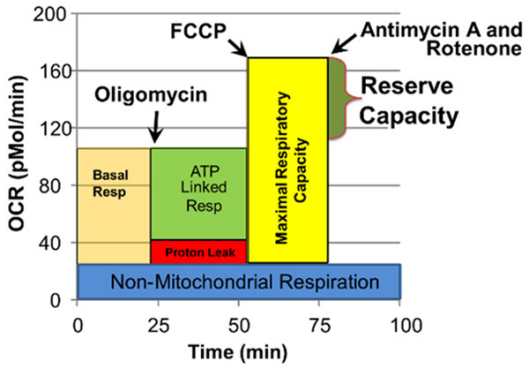

Figure 1. Seahorse assay and various mitochondrial respiratory parameters.

cell lines (LCLs) [17-24] as well as PBMCs [25] and have linked variation in mitochondrial function in PBMCs to immune abnormalities [26] and prenatal environmental exposures [27, 28].

Oxygen consumption rate (OCR) is measured to determine mitochondrial activity. Three OCR values are measured over 18 minutes to determine mitochondrial activity for each segment of the assay. Regents are added to determine parameters of mitochondrial activity. Basal Respiration is the difference between baseline OCR and non-mitochondrial OCR. Oligomycin, which is a complex V inhibitor, is added to determine the portion of Basal Respiration that is ATP-Linked Respiration and Proton-Leak Respiration. Carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore, is added to collapse the inner membrane gradient, driving the mitochondria to respire at its maximal rate. This determines Maximal Respiratory Capacity. Antimycin A and Rotenone, are complex III and I inhibitors, which stop mitochondrial respiration to determine the non-mitochondrial respiration. Reserve Capacity is the difference between Basal Respiration and Maximal Respiratory Capacity.

The Seahorse assay is a 4-step process which monitors OCR three times during four distinct periods of time in response to various reagents that activate or inhibit the electron transport chain (ETC) (**Figure 1** and **Table 1**); from which several key parameters are derived (**Table 2**). We have recently examined the reliability of the Seahorse assay, finding excellent intraclass correlation coefficients across parameters for measures in PMBCs [29].

One of the important unanswered questions when obtaining PBMCs for assessment of mitochondrial function is how sensitive the PBMCs are to transport and storage. From a practical standpoint it is not always possible to assay PBMCs immediately since blood collection can be distant from the analysis laboratory, especially in the medical setting. Thus, we examined several different methods of collection and the sensitivity of mitochondrial parameters to the timing of performing the Seahorse assay after collections. We also examined the effect of cryopreservation on mitochondrial parameters to understand whether samples can be reliably stored and processed at a later time. This is especially important when collecting samples from remote sites across the country or across the world.

Material and methods

This study was approved by the Institutional Review Board at Phoenix Children's Hospital (Phoenix, AZ) under protocol #20-163. Participants provided written informed consent. After collection, blood samples were stored and processed in several manners to test PBMC processing methods.

Experimental design

The requisite amount of blood was collected in 4 mL ethylenediaminetetraacetic acid (EDTA) or 4 mL Sodium Citrate (NaC) Vacutainer tubes and/or 4 mL Mononuclear Cell Preparation Tubes (CPT). Tubes were immediately chilled on ice and then processed using the various methods outlined in **Tables 3**-**6**. The number of tubes drawn is provided in the parentheses in the tables.

In general, experiments were divided by (1) the time tubes spent on ice, between collection and processing (**Tables 3**-**5**) and (2) the effect of cryopreservation of PBMCs (**Table 6**). To look at the effect of ice on the preservation of the collected blood, we examined the preservation after 24 hrs on ice using various blood collection tubes (**Table 3**) as well as manipulating the plasma (**Table 4**) to determine if it would improve cell preservation. In three experiments (Exp 1-3), only viability was examined.

We then studied changes in viability and mitochondrial respiration using various types of

Table 1. Oxygen Consumption (OCR) measurements during the standard Seahorse Assay

Assay Measurement	
Baseline OCR	Baseline OCR is measured before introducing any reagents.
OCR after oligomycin	Oligomycin, a complex V inhibitor, shuts down the production of ATP so the OCR related to ATP production can be determined.
Maximal OCR	Carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) is used to collapse the mitochondrial inner membrane gradient, inducing the mitochondria to function at its maximum extent possible.
Non-Mitochondrial OCR	Antimycin A and Rotenone are added to shut down ETC complex activity to measure OCR from non-ETC processes.

Table 2. Mitochondrial respiratory measurements derived from the Seahorse Assay

Derived Measurement	Measurement Meaning and Calculation Equation
ATP-Linked Respiration	The fraction of basal OCR that is attributed to ATP production. *ATP-Linked respiration = Baseline OCR - OCR after oligomycin*
Proton-Leak Respiration	The amount of basal OCR that is associated with protons leaking through the inner mitochondrial membrane in order to control oxidative stress. *Proton-Leak Respiration = OCR after oligomycin - Non-Mitochondrial OCR*
Maximal Respiratory Capacity	The maximum respiratory rate of the ETC. This parameter is thought to be sensitive to deficits in mitochondrial biogenesis, mtDNA damage and/or inhibition of ETC function. *Maximal Respiratory Capacity = Maximal OCR - Non-Mitochondrial OCR*
Reserve Capacity	The amount of extra ATP that can be produced by oxidative phosphorylation when there is a sudden increase in energy demand. This parameter is an index of mitochondrial health; when it becomes negative, the mitochondrion is unhealthy leading the cell towards apoptosis. *Reserve Capacity = Maximal OCR - Baseline OCR*

Table 3. 24-hour experiments with various tube types

Time	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
Fresh	CPT (6)	NaC (6)	NaC (2); ED (6)	NaC (6)	NaC (4); ED (4)	NaC (8)
24-hours	CPT (4)	NaC (8)	NaC (2); ED (6)	NaC (6)	NaC (4); ED (4)	NaC (3)

For yellow highlighted cells, there is no mitochondrial respiration data. For Red highlighted cells there is no data. Number of replicate tubes in parentheses. NaC, Sodium Citrate Vacutainer collection tubes; ED, ethylenediaminetetraacetic acid Vacutainer collection tubes; CPT, Mononuclear Cell Preparation Tube.

Table 4. Plasma Replacement on a Subset of 24-hour Experiments

Time	Exp 1	Exp 2	Exp 4
Fresh	CPT (6)	NaC (6)	NaC (6)
24-hours	Plasma Remove (2) Not Removed (1)	Plasma Not Removed (2) Plasma Removed Replaced w/DMEM No Mix (2) Replaced w/DMEM Mixed (2) Replaced w/PBMC Wash Buffer (2)	Plasma Not Removed (2) Replaced with Seahorse Media/Mix (2) Seahorse Media/No Mix (2)

For yellow highlighted cells, there is no mitochondrial respiration data. Number of replicate tubes in parentheses. NaC, Sodium Citrate Vacutainer collection tubes; ED, ethylenediaminetetraacetic acid Vacutainer collection tubes; CPT, Mononuclear Cell Preparation Tube.

tube, which remained on wet ice for up to 12 hrs. Although CPT tubes were used to collect blood in Exp 13, because of the initial viability in findings with the CPT tube, its mitochondrial respiration and viability was not studied in Exp 13. There was one participant who provided samples for all the non-cryopreservation experiments.

Finally, we investigated the effect of cryopreservation of PBMCs under various conditions including tube types, storage time, delay in processing, removal of plasma and spin vs no spin (**Table 6**). The process of cryopreservation requires cooling down of the PBMCs overnight to -80°C before storing in liquid nitrogen. To determine if any changes were due to the cryo-

Table 5. 12-hour experiments with various tube types

Time	Exp 7	Exp 8	Exp 9	Exp 10	Exp 11	Exp 12	Exp 13
1-hour			NaC (2); ED (2)	NaC (2); ED (2)			
2-hour			NaC (2); ED (2)	NaC (2); ED (2)			
3-hour			NaC (2); ED (2)	NaC (2); ED (2)			
4-hour	NaC (1)	NaC (1); ED (1)	NaC (2); ED (2)	NaC (2); ED (2)			
5-hour					NaC (1); ED (1)	NaC (1); ED (1)	NaC (1); ED (1); CPT (1)
6-hour	NaC (1)	NaC (1); ED (1)			NaC (1); ED (1)	NaC (1); ED (1)	NaC (1); ED (1); CPT (1)
7-hour					NaC (1); ED (1)	NaC (1); ED (1)	NaC (1); ED (1); CPT (1)
8-hour	NaC (1)	NaC (1); ED (1)			NaC (1); ED (1)	NaC (1); ED (1)	NaC (1); ED (1); CPT (1)
12-hour	NaC (1)	NaC (1); ED (1)					

Number of replicate tubes in parentheses. NaC, Sodium Citrate Vacutainer collection tubes; ED, ethylenediaminetetraacetic acid Vacutainer collection tubes; CPT, Mononuclear Cell Preparation Tube.

Table 6. Cryopreserved cells from various tube types and delays from processing

Isolated	Exp 14				Exp 15		Exp 16		Exp 17		Exp 18	
	Tube	Time	Plasma Removed	Spin	Tube	Time	Tube	Time	Tube	Time	Tube	Time
Fresh	NaC (10)	0 d			NaC (2)	0 d	ED (2)	0 d	ED (2)	0 d	ED (2)	0 d
	ED (4)	0 d			NaC (2)	61 d	ED (2)	9 d	ED (2)	1 d	ED (2)	3 d
	NaC (2)	69 d			NaC (4)	55 d	ED (2)	23 d	ED (2)	4 d		
	NaC (2)	88 d										
24-hours	NaC (4)	0 d										
	ED (4)	0 d										
	NaC (1)	63 d	No	No								
	NaC (1)	88 d	No	No								
	NaC (1)	88 d	No	Yes								
	NaC (1)	88 d	Yes	Yes								
	ED (1)	63 d	No	No								
Isopropanol							ED (2)	9 d				

PBMCs were isolated in three ways: fresh, after 24 hrs on wet ice or use of isopropanol during cryopreservation. For the PBMCs on ice for 24 hrs several methods were used to determine if PBMCs could be preserved better, including removing plasma and spinning down the sample after collection. In experiment 17, PBMCs were examined after cryopreservation but before storage in liquid nitrogen. Number of replicate tubes is provided in parentheses. NaC, Sodium Citrate Vacutainer collection tubes; ED, ethylenediaminetetraacetic acid Vacutainer collection tubes. In experiment 16, some of the PBMCs were frozen using an isopropanol bath.

preservation process versus the storage process, in Exp 17 we examined the PBMCs before being stored in liquid nitrogen. Four different individuals provided samples for these cryopreservation experiments.

Blood collection and processing

Samples were processed by centrifuging at 1500 g for 10 mins at 4°C to separate plasma either after the stated delay or for the samples in which plasma was removed, within 30 minutes of collection. Just prior to Seahorse assay, plasma was removed and replaced with room temperature wash buffer containing Ca^{2+}/Mg^{2+}-free PBS with 0.1% BSA and 2 mM EDTA. Diluted blood was then layered on top of Histopaque-1077 (Sigma Aldrich, St. Louis, MO, USA) and centrifuged at 400 g for 30 mins at room temperature. PBMCs were washed twice with wash buffer and counted using a hemocytometer. Isolation procedure duration was 90-120 mins.

Cryopreservation

PBMCs were stored by suspending in culture medium (90% FBS/10% DMSO) and initially frozen down to -80°C using Mr Frosty cryofreeze container (Thermo Fisher Scientific, Waltham, MA). In experiment 16, some of the PBMCs were frozen using an isopropanol bath. After overnight storage at -80°C PBMCs were transferred to liquid nitrogen for long-term storage.

Mitochondrial respiration assay

PBMCs were placed in assay media (unbuffered RPMI supplemented with 1 mM pyruvate, 2 mM glutamate and 25 mM glucose) that was warmed to 37°C and pH adjusted to 7.4 prior to cell suspension. XFe96 plates (Seahorse Bioscience, Billerica, MA) were prepared by adding 25 µL of 50 µg/mL Poly-D-lysine (EMD Millipore, Billerica, MA) for two hours, washing with 250 µL sterile water and drying in a laminar flow hood overnight prior to seeding with 4×10^5 viable PBMCs per well. After seeding, the plates were spun with slow acceleration (4 on a scale of 9) to a maximum of 100 g for 2 min and then allowed to stop with zero braking (Eppendorf Model 5810R Centrifuge). The plate orientation was reversed, and the plate was spun again in the same fashion. Prior to Seahorse assay, XFe96 wells were visualized using an inverted microscope to ensure PBMCs were evenly distributed in a single layer and viability of the cells was confirmed by trypan blue exclusion. For each experimental condition, at least four replicate wells were measured simultaneously to improve assay reliability. Runs with clear measurement probe failure, reagent injection failures, or non-physiology measurements (ALR or PLR <-1 pmol/min) were eliminated.

Statistical analysis

Analyses were performed using PASW Statistics 18 (IBM SPSS Statistics, Armonk, NY). Graphs were produced using Excel version 14.0 (Microsoft Corp, Redmond, WA). As mentioned above, mitochondrial measures were obtained in at least quadruplicate, so a mixed-model linear regression was used to account for both within-subject variation from repeated mitochondrial measurements on the same individual and the between-subject variation from experimental variables. Interactions were considered where appropriate and the final models were simplified to eliminate any non-significant higher order interactions. In general, an alpha limit of 0.05 was used for significance.

Results

24 hour delay: crushed ice vs dry ice

Dry ice was used to chill tubes in Exp 3. The NaC tube cracked and the cells in the EDTA tube hemolyzed, resulting in unusable samples.

24 hour delay: tube resilience

All (100%) of the 23 NaC and 10 EDTA tubes which were on crushed ice for 24 hours remained intact but only 75% (3 of 4) of the CPT tubes remained intact after 24 hours on crushed ice.

24 hour delay: viability

The initial analysis with NaC, EDTA and CPT tubes demonstrates a significant tube type interaction (EDTA, NaC, CPT) by 24 hrs preservation [$F(2,21) = 13.75$, $P<0.01$] which was clearly due to a significant reduction in viability with the CPT tubes with 24 hrs delay in processing (**Figure 2A**). A revised analysis which removed the CPT tubes from the analysis demonstrated no significant difference between tubes [Mean (SE): EDTA 93.0% (3.3%) vs NaC 90.1% (1.4%)] or 24 hrs delay in processing [Mean (SE): Fresh 93.6% (2.6%) vs 24 hrs 89.5% (2.6%)].

24 hour delay: buffer mixture

To help sustain cell viability over 24 hrs, we added various cellular supportive media to determine if they would improve viability as well as removed plasma to prevent any plasma factors from damaging immune cells. As seen in **Figure 2B** there was no significant effect of adding media, mixing, or removing plasma from the samples on the cell viability.

The 24-hour time span: viability

Viability was not different between the EDTA and NaC tubes but did linearly decrease over 24 hrs. Viability of fresh PBMC was estimated to be 94.1% (SE 2.0%) [$F(1,44) = 3131.26$, $P<0.001$] with a 0.5% (SE 0.2%) decrease for each hr delay in processing [$F(1,44) = 5.52$, $P<0.05$; **Figure 2C**].

The 24-hour time span: bioenergetics

ALR was not significantly different between the EDTA and NaC tubes. As seen in **Figure 3A**, ALR changed over 24 hrs in a curvilinear manner, first increasing with a peak at about 12 hrs and then decreasing slowly [Linear $F(1,323) = 7.62$, $P<0.01$; Curvilinear $F(1,323) = 9.13$, $P<0.01$].

Using the NaC tube as compared to the EDTA tube increased PLR by 7.31 (SE 2.26) pmol/min

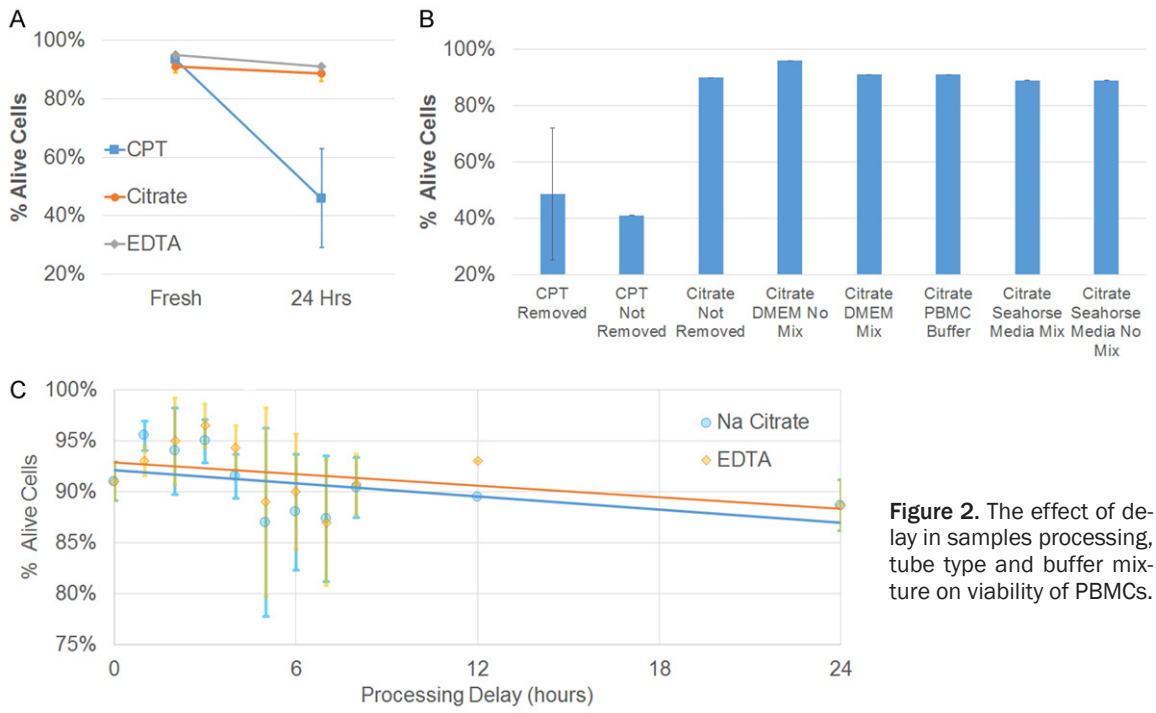

Figure 2. The effect of delay in samples processing, tube type and buffer mixture on viability of PBMCs.

[F(1,323) =10.48, P=0.001]. As seen in **Figure 3B**, there was a curvilinear change in PLR over 24 hrs [Linear F(1,323) =10.81, P=0.001; Curvilinear F(1,323) =13.44, P<0.001] but this curvilinear change was only significant for the NaC tube [Interaction Linear F(1,323) =12.93, P<0.001 Curvilinear F(1,323) =11.09, P=0.001].

Using the NaC tube as compared to using the EDTA tube increased PLR by 70.63 (SE 30.51) pmol/min [F(1,323) =5.36, P<0.05]. As seen in **Figure 3C**, there was a curvilinear change in MRC over 24 hrs with an initial increase, peaking at about 8 hrs and then decreasing for the EDTA tube [Linear F(1,323) =4.94, P<0.05; Curvilinear F(1,323) =13.27, P<0.001]. Rather than increasing initially, MRC in the NaC tube curvilinearly decreased over 24 hrs without peaking first [Interaction Linear F(1,323) =4.45, P<0.05].

Using the NaC tubes as compared to the EDTA tubes RC increased by 59.65 (SE 23.96) pmol/min [F(1,323) =6.20, P=0.01]. As seen in **Figure 3D**, there was a curvilinear change in RC over 24 hrs for the EDTA tube with an initial increase which peaked at about 8 hrs and then decreased [Linear F(1,323) =3.86, P=0.05; Curvilinear F(1,323) =13.66, P<0.001]. Rather than increasing initially, RC in the NaC tube decreased over 24 hrs without peaking first [Interaction Linear F(1,323) =3.73, P<0.05].

The 8-hour time span: bioenergetics

ALR was not significantly different between the EDTA and NaC tubes. ALR increased by 2.3 (SE 0.87) pmol/min for every hr delay in processing [F(1,299) =6.74, P=0.01; **Figure 4A**].

PLR was not significantly different between the EDTA and NaC tubes. PLR decreased by 0.67 (SE 0.18) pmol/min for every hr delay in processing [F(1,248) =14.12, P<0.001; **Figure 4B**].

MRC was significantly different across the two tubes. Using the NaC tubes increased MRC by 58.70 (SE 21.61) pmol/min [F(1,300) =7.38, P<0.01]. As seen in **Figure 4C**, there was a curvilinear change in MRC over the first 8 hrs, which for the NaC tube initially increased, peaking at about 4 hrs and then decreased [Linear F(1,300) =9.01, P<0.01; Curvilinear F(1,300) =7.66, P<0.01]. The peak was slightly later for the ETDA tubes at about 7 hrs [Curvilinear by Tube Interaction: F(1,300) =6.86, P<0.001].

RC was significantly different across the two tubes. Using the NaC tube increased RC by

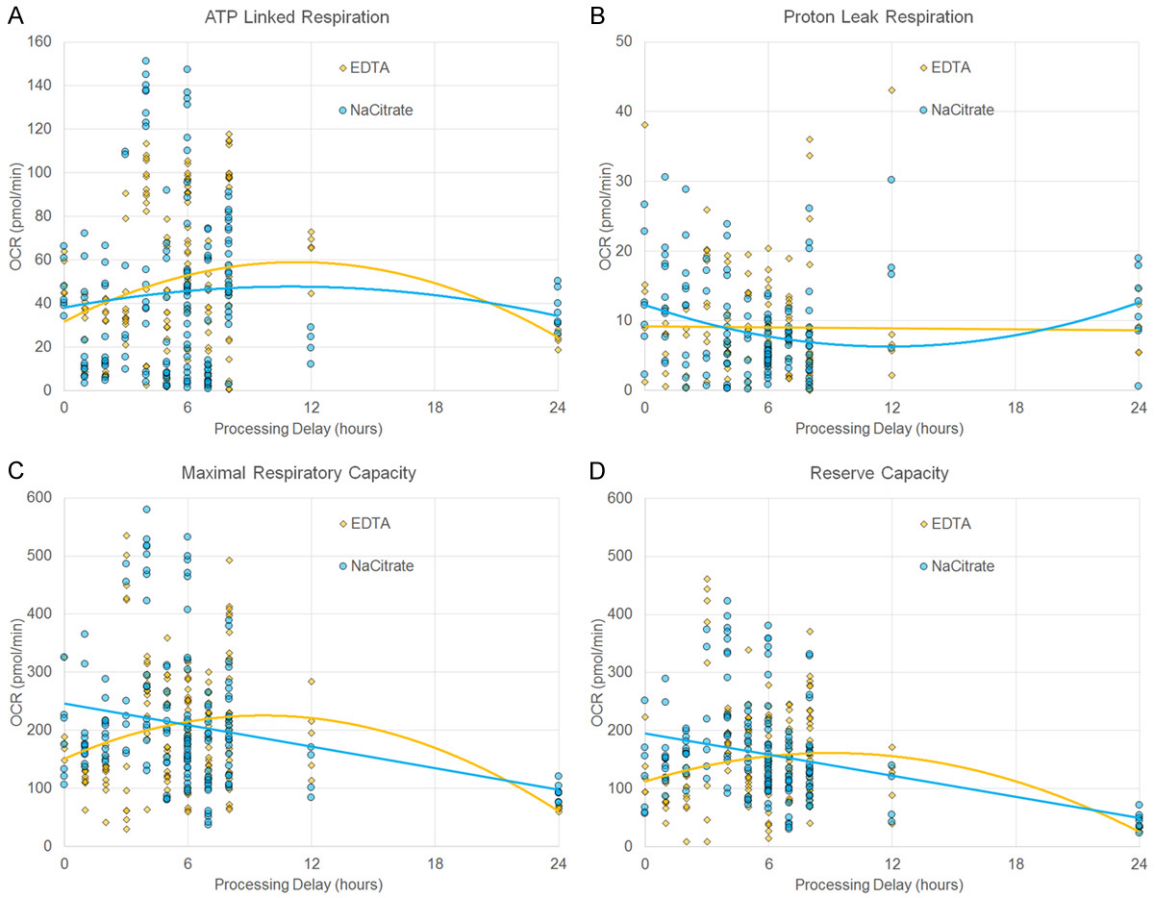

Figure 3. Change in mitochondrial respiratory parameters with various delays in processing over 24 hours.

56.47 (SE 16.82) pmol/min [F(1,300) =11.28, P=0.001]. As seen in **Figure 4D**, there was a curvilinear change in RC over the first 8 hrs, which for the NaC tubes initially increased, peaking at about 4 hrs and then decreased [Linear F(1,300) =12.67, P<0.001; Curvilinear F(1,300) =11.73, P=0.001]. This peak was slightly later for the EDTA tubes at about 7 hrs [Curvilinear by Tube Interaction F(1,300) =8.41, P<0.01].

Cryopreservation: viability

The standard "Mr Frosty" freezing process decreased viability by 9.6% (SE 2.7%) [F(1,28) =6.74, P<0.05] while isopropanol decreased viability by 14.3% (SE 6.4%) [F(1,28) =4.94, P<0.05]. Removing plasma in the samples in which processing was delayed by 24 hrs increased viability by 14.1% (SE 6.6%) [F(1,28) =4.54, P<0.05]. Storing samples in liquid nitrogen was associated with a non-linear change in viability [Linear: F(1,28) =6.50, P<0.05; Curvilinear F(1,28) =13.76, P=0.001]. As seen in **Figure 5A**, viability was relatively stable for a little over a month, after which it started to decrease significantly.

Cryopreservation: bioenergetics

Cryopreservation decreased ALR by 17.20 (SE 1.83) pmol/min [F(1,164) =88.60, P<0.001] and delaying processing time by 24 hrs decreased ALR by 17.11 (SE 3.30) pmol/min [F(1,158) =26.74, P<0.001; **Figure 5B**]. Removing and spinning the samples increased ALR by 45.41 (SE 11.58) pmol/min [F(1,164) =15.37, P<0.001] and 52.21 (SE 11.58) pmol/min [F(1,164) =20.32, P<0.001], respectively.

Cryopreservation increased PLR by 2.65 (SE 1.16) pmol/min [F(1,160) =5.26, P<0.05]. Delaying processing time by 24 hrs altered the samples by changing PLR with prolonged freezing [Delay by Linear Interaction: F(1,159) =19.13, P<0.0001; Delay by Curvilinear Inter-

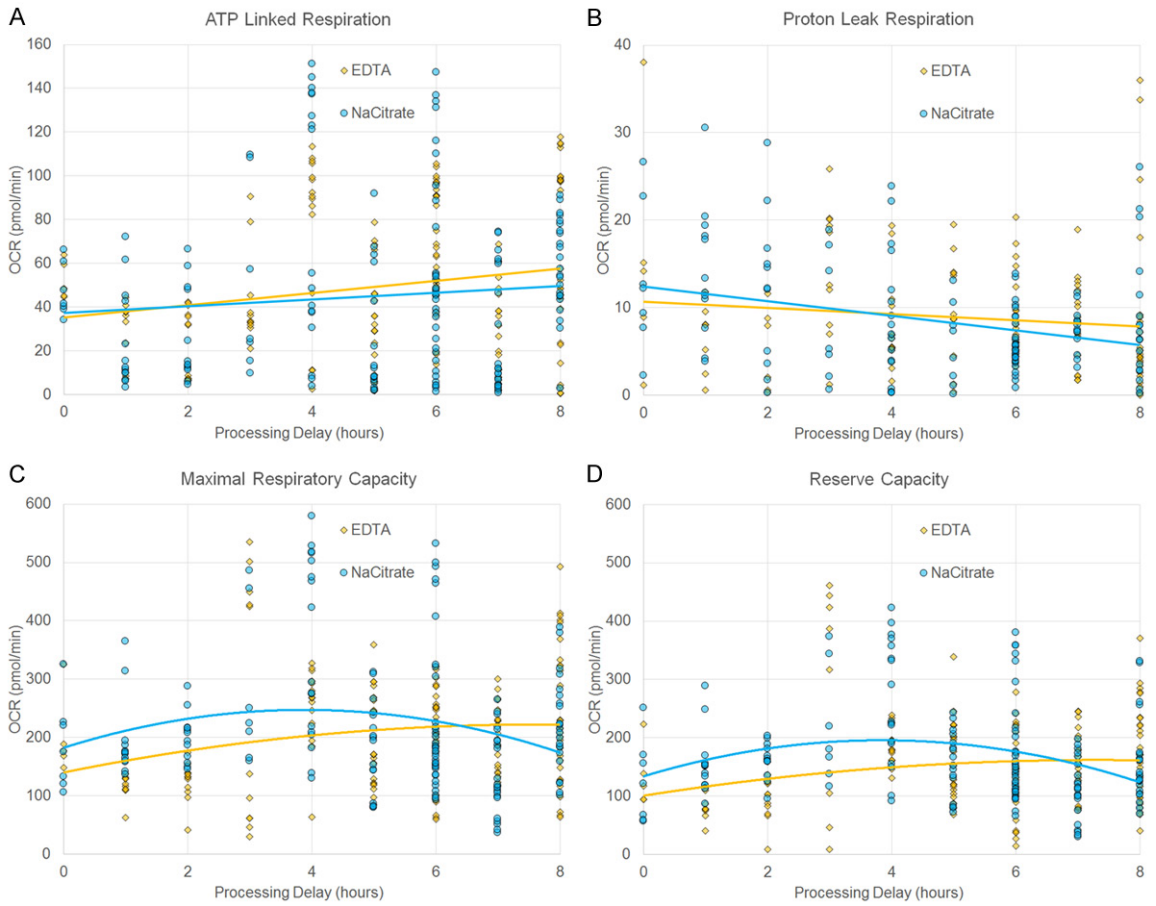

Figure 4. Change in mitochondrial respiratory parameters with various delays in processing over 8 hours.

action: F(1,160) =11.26, P=0.001]. As seen **Figure 5C**, for freshly frozen samples, PLR increased with the number of days frozen, while for samples which were not frozen for 24 hr, PLR decreased at first, until about 50 days and then started to increase. Removing and spinning the samples increased PLR by 13.07 (SE 5.31) pmol/min [F(1,154) =6.05, P=0.01] and 22.80 (SE 5.31) pmol/min [F(1,154) =18.42, P<0.0001], respectively. PBMC processing in the NaC tubes increased PLR by 4.85 (SE 1.64) pmol/min [F(1,70) =8.76, P<0.01].

Cryopreservation decreased MRC by 95.30 (SE 10.60) pmol/min [F(1,165) =80.86, P<0.0001] and delaying processing by 24 hrs decreased MRC by 59.82 (SE 11.76) [F(1,165) =25.86, P<0.0001]. The MRC changed in a curvilinear fashion with days in liquid nitrogen [Curvilinear F(1,166) =9.64, P<0.01] with an initial decrease in MRC, especially in the PBMCs which were delayed in processing for 24 hrs, followed by an increase in MRC after about 40 days in liquid nitrogen (See **Figure 5D**). Removing or spinning the sample did not significantly affect MRC.

Cryopreservation decreased RC by 54.92 (SE 16.49) pmol/min [F(1,166) =1109, P=0.001]. RC changed over number of days frozen in a curvilinear fashion [Linear F(1,164) =30.13, P<0.0001, Curvilinear F(1,163) =26.58, P< 0.001] with this change being different in those PBMCs frozen fresh and those frozen after a 24 hr delay [Linear F(1,164) =8.03, P<0.01, Curvilinear F(1,163) =8.04, P<0.01]. As seen in **Figure 5E**, PBMCs in which processing was delayed for 24 hrs demonstrated a gradual decrease in RC with number of days in liquid nitrogen, while PBMCs cryopreserved fresh demonstrated an initial decrease in RC until about 40 days at which time RC increased.

Discussion

This study aimed to determine if variations in collecting, handling, and processing PBMCs

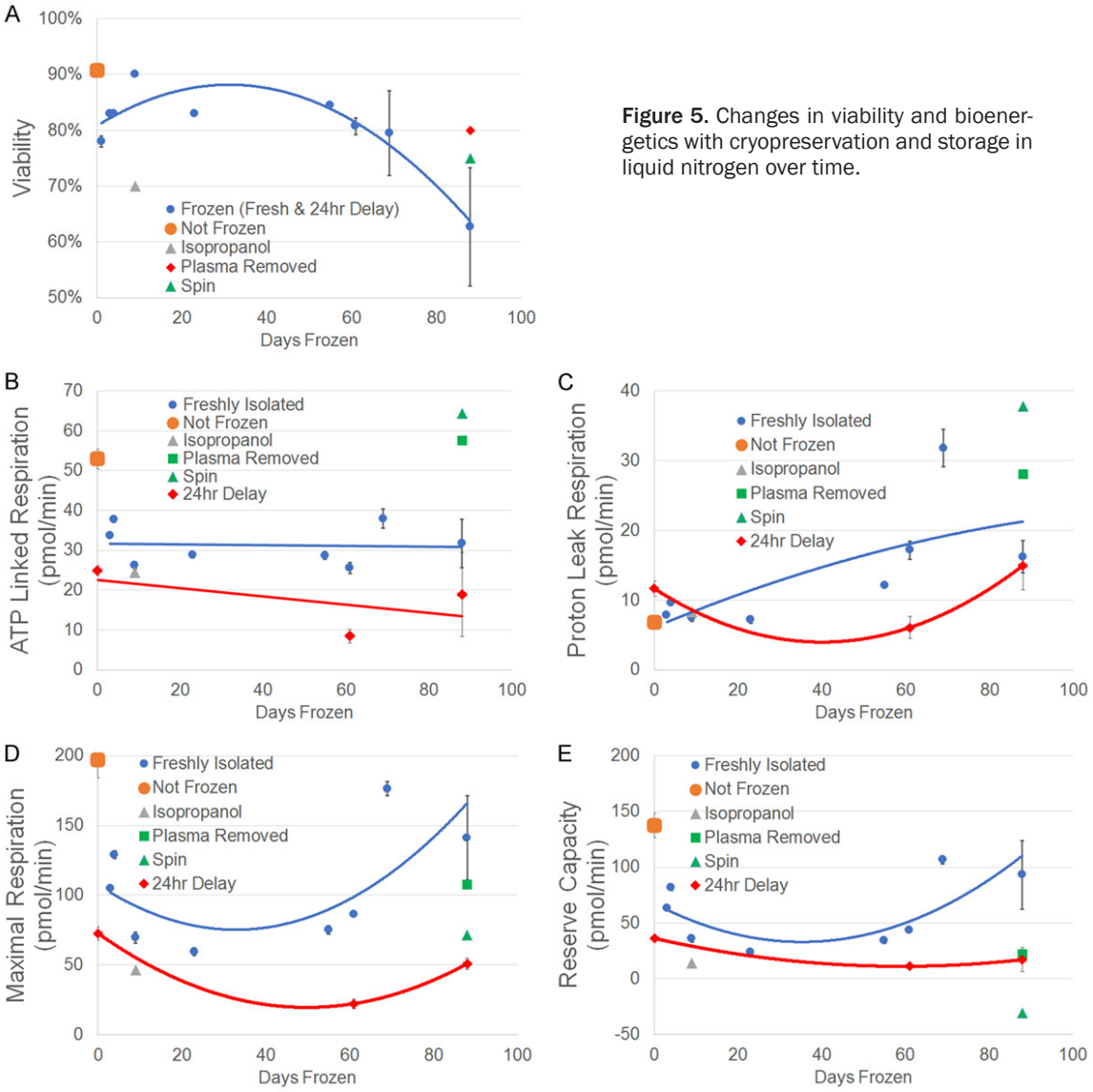

Figure 5. Changes in viability and bioenergetics with cryopreservation and storage in liquid nitrogen over time.

affected their viability and bioenergetics. This study examined two different blood collection tube types commonly used to collect blood samples (i.e., NaC and EDTA) as well as a specialized blood tube for collecting immune cells (i.e., CPT). This study examined various ways of storing collected blood, prior to processing, which included storing the collection tubes on dry ice and wet ice as well as the effect of cryopreservation and short- versus long-term storage on the viability and bioenergetics of PBMCs. In addition, this study looked at a few variations in processing the samples before storage, in order to improve viability and bioenergetics. To this end, we performed 18 separate experiments on samples from 5 different individuals.

Initial experiments demonstrated two important findings: (1) using dry ice to store blood collections tubes results in significant failure and inability to recover viable samples, and (2) using the CPT tubes resulted in more failures than expected as well as a significant decrease in viability of the PBMCs after 24 hrs on wet ice. Thus, the majority of the remaining experiments were performed on the NaC and EDTA collection tubes either on wet ice or cryopreserved.

Viability of PBMCs was not affected by whether the EDTA or NaC tubes were used for collection and storing the tubes on wet ice for 24 hrs had a marginal but significant effect on viability with a 0.5% (SE 0.2%) loss in viability

for each hr on wet ice. The cryopreservation process decreased viability by 9.6% (SE 2.7%) with this viability relatively stable for about one-month in liquid nitrogen. While manipulating the samples by replacing serum with various media did not change viability for samples stored on wet ice for 24 hrs, when they were analyzed after the 24 hrs of storage, removing plasma or spinning the samples did improve viability if the samples were cryopreserved for a prolonged period of time. These manipulations were not performed on samples that were cryopreserved fresh, so it is not known if this technique could be used on freshly cryopreserved samples to extend their viability with prolonged liquid nitrogen storage. Using isopropanol in the cryopreservation process significantly decreased viability.

Bioenergetic parameters were affected by several factors. First, consistent differences were seen when using different collection tubes. Using the NaC tubes increased PLR in two of the three bioenergetic analyses while MRC and RC were increased in all three bioenergetic analyses when the NaC tubes were used. In addition, changes in bioenergetics over time on wet ice were different for NaC and EDTA tubes. Thus, it is essential for any study to use consistent blood collection tubes to confidently compare bioenergetics across different blood draws.

Bioenergetics of PBMCs stored on wet ice changed over 24 hrs. This was examined on two time scales 0 hrs to 24 hrs and 0 hr to 8 hrs in order to better understand the dynamic changes in bioenergetics. Over 24 hrs on wet ice, both ALR and PLR varied throughout the 24 hr period with values after 24 hrs on wet ice presenting similar to baseline. However, both MRC and RC significantly decreased after 24 hrs on wet ice, making these measurements questionable after such a delay. Similarly, ALR and PLR changed a small amount after 8 hrs on wet ice but MRC and RC demonstrated more substantial changes. These changes were not linear, with an initial increase in these parameters followed by a peak and then a reduction. Samples collected in NaC tubes demonstrated a peak at about 4 hrs while those collected in EDTA tubes demonstrated a peak at about 7 hrs. The non-linear effects in this data are interesting with several possible mechanism in play, but the fact remains that these changes are seen even within this limited storage time, suggesting that collected samples should be analyzed sooner than later to obtain accurate results. Given that changes in samples collected in the EDTA tubes were slower, we believe the EDTA is the preferred collection tube type.

Finally, the cryopreservation process was found to have a significant effect on bioenergetics with a significant decrease in ALR, MRC and RC and increase in PLR. Cryopreserving samples that had been on wet ice for 24 hrs magnified these effects on bioenergetics. Bioenergetics did change with time stored in liquid nitrogen with these effects being more minimal within the first month. Manipulating the sample by removing serum or spinning the samples prior to storing on wet ice for 24 hrs did affect bioenergetics. Given that PLR was significantly increased, it is unlikely that these effects were beneficial as an increase in PLR suggests increased mitochondrial oxidative stress.

There are several limitations to this study, including only having a few participants as well as only performing limited manipulations on limited samples. In the future, research should continue to study these differences using more participants to establish a reliable curve.

Conclusions

With variation in bioenergetics dependent on time and tube type used for collection, it is important to determine how much time, from collection to processing, specimen will need to be kept on ice. Keeping track of the time between collection and processing is crucial as we continue to investigate these relationships and establish reliability with data from more participants. Until further information is available, it appears that EDTA tube collections within a few hours may be optimal if samples cannot be immediately processed. Additionally, it is important to realize that viability and bioenergetics do not always correspond as bioenergetics appears to demonstrate complex non-linear changes over time which are not always reflected in viability.

Acknowledgements

We would like to thank Amrit Ammanamanchi for his technical help with this project.

Disclosure of conflict of interest

None.

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