

Using Redox Potential as a Feasible Marker for Banked Blood Quality and the State of Oxidative Stress in Stored Red Blood Cells

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Abstract

Background Stored Red Blood Cells (RBCs) may undergo oxidative stress over time, with functional changes affecting critical tasks such as oxygen delivery. Central to these changes are oxidation-reduction (redox) reactions and the redox potential (RP) that must be maintained for proper cell function. RP imbalance can lead to oxidative stress that may contribute to storage lesions and transfusion-related morbidities. Direct measures of RP may allow for evaluation of erythrocyte quality and enable corrections of RP prior to transfusion.

Methods Multiple random RBC segments were tested, ranging in age from 5 to 40 days at 5 day intervals. RP was recorded by measuring open circuit potential of RBCs using novel nanoporous gold electrodes with Ag/AgCl reference. RP measures were also performed on peripheral venous blood samples from 10 healthy volunteers. RP measures were compared between groups of aged RBCs, and with volunteer blood.

Results Stored RBCs show time-dependent increases in RP. There were significant differences in Day 5 RP compared to all other groups ($p \leq 0.005$), Day 10-15 vs ages \geq Day 20 ($p \leq 0.025$), Day 20-25 vs Day 40 ($p = 0.039$), and all groups compared to healthy volunteers. RP became more positive over time suggesting ongoing oxidation as RBCs age. However, storage time alone does not predict the ultimate RP value measured from a given unit.

Conclusions There are significant differences in RP between freshly stored RBCs and all others, with RP becoming more positive over time. However, storage time alone does not predict RP, indicating RP screening may be important independent of storage time and may serve as a marker of RBC quality and state of oxidative stress. RP measurements may also provide a target by which to restore RP balance in aged pRBCs, improving their clinical effectiveness while reducing associated morbidities.

Background

Oxidation-Reduction (Redox) reactions, including those involving reactive oxygen species (ROS), lie at the heart of nearly every biochemical process occurring within the body and within biologically active environments, such as that present within units of stored blood(1, 2). These redox reactions reflect the overall oxidative stress of the environment and involve the transfer of electrons between oxidants and reductants. The continuous measurement of these combined ubiquitous reactions in blood, Red Blood Cells (RBCs), and other biologic fluids can be termed ambient redox potential (RP). As redox potential is the balance of all oxidants and reductants present, it is a measure of electron pressure in the system much in the same way as pH is the measure of proton pressure (balance of acid and base) in a system. This balance may be especially important with regard to the health and viability of RBCs, with multiple manuscripts describing oxidative injury as a contributing factor in red cell storage lesions (2–4), and evidence that the addition of antioxidant species to stored RBCs may decrease oxidative stress (5),

reduce cell damage by free radicals (6), and preserve red cell energy, redox metabolism, and overall RBC quality (7).

Despite the seeming importance and implications of utilizing RP measures, there are currently few means by which to make the measurement directly, and most investigators have relied historically on isolated redox species and secondary markers of oxidative stress such as glutathione couples (8), malondialdehyde (MDA) (2), and estimates of oxidative injury in processed samples (2, 8, 9), among others, instead of evaluating the state of overall oxidation-reduction balance and oxidative stress directly via measurements of RP. In addition, while measurements of oxidative stress and redox potential are optimally made at the bedside and/or immediately upon sample collection, few, if any, measures of redox state and oxidative stress can be made at the point-of-care (POC) without sample processing. However, with the use of novel nanoporous gold electrodes our group has described previously (10, 11), we are able to make direct measurements of oxidative stress via RP, at the point of care that could provide new insight into the overall redox state, and degree of oxidative stress, present in RBC units.

To this end, we report on the evaluation of RP measurements in multiple random units of RBCs by measuring RP in segments taken from blood banked RBC units, ranging in age from 5 to 40 days at 5 day intervals (day 5, 10, etc.), and additionally in peripheral venous blood samples taken from healthy volunteers. We sought to not only record RP measures in RBC of varying age, but to investigate whether there was a statistically significant change in oxidative stress as measured by RP (whether more oxidative or reductive) over time among stored RBC units, compare these results to the RP of peripheral venous blood of healthy volunteers, and evaluate the degree of RP variation among RBCs of similar age. We hypothesize that RP measurements will increase among RBC units overall the longer they are stored, indicating ongoing and increasing oxidation, and that the RP values of stored RBCs will be higher than that of peripheral blood of healthy volunteers.

Methods

Blood

Red Blood Cells Additive Solution-3 (AS3) Leukocytes Reduced were obtained from the American Red Cross and stored at 1–6 C in accordance with AABB Standards (12). Samples of approximately 1 mL were taken from segments of multiple, independent RBC units, ranging in age from 5 to 40 days at 5 day intervals, and used for testing. In addition, 10 whole blood samples of approximately 2–3 mL were taken from 10 separate healthy adult volunteers via peripheral venipuncture and placed into sodium heparin tubes. After sample collection, approximately 1 mL was immediately taken for redox potential measurement as described below. This study was approved by the University of Michigan Institutional Review Board.

Nanoporous Gold Electrode Fabrication:

The nanoporous (np) gold electrode fabrication has been previously described (10), and all electrodes utilized for this study were fabricated from the same stock and batched materials. Briefly, fabrication consists of overlaying a nanoporous gold structure onto gold coated slides, precut to an approximate dimension of 1 inch x 6 mm. The nanoporous gold structure was obtained by dealloying gold leaf (Manetti 12 karat white gold) in nitric acid and rinsing with deionized water, which produces a complex matrix of nanopores with diameters of approximately 20–50 nanometers each. The combined gold slide with overlaid np structure was then treated under ultra-violet light for 4 hours and the resultant np gold coated electrode was then covered with Teflon tape containing a 1/8 inch diameter hole punch in the center to provide a defined area and region for redox testing. We have previously demonstrated this electrode to have excellent intra-rater reliability, while also resistant to biofouling (10).

Redox Potential (RP) Measurement:

RP measurements were obtained and processed immediately at time of sample collection. Direct measurement of RP was performed by measuring open circuit potential (OCP) of the RBC sample via the np gold electrode, with Ag/AgCL reference, using a ParstatMC™ multi-potentiostat (Princeton Applied Research, Oak Ridge, TN). RP measurements and age of the stored blood sample were recorded, and samples were placed in one of 5 groups based on age. These are: 1) 5 days, 2) 10 to 15 days, 3) 20 to 25 days, 4) 30 to 35 days, and 5) 40 days. The RP values of blood from healthy volunteers were determined as a reference for circulating RP values in healthy subjects. Redox potential measurements were not adjusted for pH.

RP Statistical Analysis:

Linear regression was performed on all samples collected and an R^2 value calculated, producing a fitted regression line utilizing Excel and analyzed with SAS9.4 statistical programming software (SAS Institute Inc.). In addition, Mann-Whitney U-test with Holm Sequential Bonferroni Adjustment for multiple comparisons was used to evaluate statistical differences between the groups defined by age. The level of significance was set at $\alpha = 0.05$, and this data was also analyzed using SAS statistical software.

Results

A total of 32 samples from random RBC units were tested for RP, with sample distribution as noted in **Table 1**. An additional 10 peripheral venous samples from healthy volunteers were also tested as a reference for circulating RP values in healthy individuals (Median: -93 mV, Range: -111 to -68 mV). Median and interquartile ranges (IQR) for each group of aged RBCs tested is also noted in **Table 1**. Linear regression analysis was performed on the samples collected to investigate the overall relationship between RP values (i.e. change in oxidation over time) and age of banked RBCs, noting an R^2 value of 0.62.

Overall, stored RBCs show time-dependent increases in RP with a reasonable goodness of fit based on linear regression. There were significant differences in Day 5 RP compared to all other groups ($p \leq 0.005$).

In addition, Day 10–15 RP values noted significant difference when compared to all ages ≥ 20 days ($p \leq 0.025$), and Day 20–25 RBCs when compared to Day 40 ($p = 0.039$). All groups were noted to have a significantly more positive RP measurement when compared to peripheral venous blood from healthy volunteers. RP values became more positive over time suggesting an increase in oxidation with greater RBCs storage time. However, storage time alone did not predict RP, as there were RBC units of greater storage age that maintained RP values less than that of median RP values in RBC units with less storage time. This is demonstrated in **Fig. 1**, where stored RBCs show time-dependent increases in RP, becoming more positive over time, and suggesting an increase in oxidation as RBCs age. However, while the median change is significant, RP values are demonstrated that are found to be less than that of the median RP of a group composed of less aged RBCs, indicating that these individual units are less oxidized than both the RBC units of similar age and some of the RBC units that have aged less than that particular unit.

Discussion

Given the delicate redox balance that must be maintained in biologic systems, alterations in blood redox state may also directly affect the overall health and viability of banked blood, contributing to storage lesions due to increases in overall oxidative stress (13), potentiation of red cell lysis in oxidized states (14), and impact on clot formation and contraction (15). This also includes decreases in red cell deformability that can be present in states of increased oxidative stress (16). As a result, these alterations can contribute direct effects on systemic coagulopathy, as well as impairments in the ability of red cells to traverse the microcirculation and provide effective systemic oxygen delivery. Despite the seeming importance of making direct assessments of redox state to assess the viability of banked blood, there are currently few, if any, means by which to make these measurements in the clinical setting, and none by which to make them at the point of care (POC), immediately upon sample collection where these measurements could make the most impact. However, with the use of novel nanoporous (np) gold electrodes our group has described previously (10, 11), we can now make direct measurements of the redox state, via RP, at the point of care that could provide new insight into overall oxidative stress, and the degree of oxidative injury, present in RBC units and in circulating blood. The value measured reflects the overall (ambient) redox balance (RP) arising from the sum of metabolically active oxidant and reductant species contributing to the signal. The more positive the RP, the more oxidized the sample, and the more negative the RP, the more reduced (anti-oxidized) the sample. Having a direct measure of the oxidative state of RBCs available during storage and prior to transfusion could enable more advanced monitoring and utilization of banked RBCs.

Although RBC transfusions are vital in cases of trauma and hemorrhagic shock, multiple studies indicate that the administration of older blood units, as much as 14 days and older, can increase the risk of transfusion related lung injury (17) and increase the risk of adverse clinical outcomes and mortality in critically ill patients, especially if multiple transfusions of RBCs are required (18, 19). These effects are attributed to storage lesions that accumulate over time as RBCs are stored, and include altered redox states (increased oxidation/oxidative injury), with multiple reports describing oxidative injury and

changes in redox state as a major contributing factor in red cell storage lesions (2–4). Also, while a previous study of oxidation-reduction potential in stored RBCs did show an increase in oxidation during storage, a small number of RBC units were tested and only units from day 1 and day 42 were included, with no evaluation of units between these extremes of age (20). In addition, redox potential was only measured in the supernatant, which may not provide the best measurement for assessing the RBC unit as a whole, as we have noted dampened/altered RP signals measured from plasma vs whole blood samples taken simultaneously from the same blood draw in previous studies (11).

Indeed, the data presented here support the concept as the overall RP of banked blood increases across the units tested reflecting an overall increase in oxidation present, and thus oxidative stress, as they age. When compared to the circulating redox state of healthy volunteers (representing fresh blood from healthy donors), all RBC units tested were found to have a median RP that was more positive (more oxidated), even when compared to RBCs on day 5 of storage. While time of storage appears to contribute, the discrepancy between healthy volunteers and day 5 of storage may also be related to the way in which the RBCs are processed and stored, producing a more oxidized environment from the onset of collection and processing. Variables contributing to this include temperature and pH variations, as well as the anticoagulant utilized in collection, and could result in an increase in the initial level of oxidation present at the onset of RBC storage (21).

However, age alone does not accurately predict the RP of any individual unit of RBCs. While data presented here demonstrates oxidation increases over time as RBCs are stored, there can also be significant variation in the RP status of any given donor due to multiple variables including age, state of health, comorbid conditions, and medications the subject may be taking at the time of donation. One study of 15 donors noted a smaller increase in malondialdehyde (MDA) and less decline in antioxidant capacity in blood taken from the same donors after receiving a 10 day regimen of antioxidant supplementation vs blood collected from these individuals prior to initiating the antioxidant regimen (22). Baseline variation in the RP values of peripheral blood taken from the healthy volunteers in our study also provides evidence to indicate that individual variations in baseline RP status, or oxidative state, exist throughout the population. This may have implications for donor screening and allow for better characterization of blood throughout initial processing and storage.

It should be noted that length of storage of RBCs is not itself necessarily a predictor of adverse clinical outcomes of transfusion. Randomized clinical trials in the settings of critically ill adults (23), cardiac surgery (24), and adult hospitalized patients (25), in which subjects were assigned to transfusion of either short duration storage RBCs or prolonged duration stored RBCs, have not demonstrated differences in mortality or secondary outcomes such as length of stay, or transfusion reactions. The generalizability of these trials to the treatment of specific patients has been questioned on the basis of a number of clinical and methodologic issues, including inconsistency in the definition of short, standard, and prolonged storage; heterogeneity of case mix; multiple transfusions; and dichotomization of the continuous variable of storage duration (26). Notably, none of these trials utilized any direct measure of RBC unit quality. However, a recent trauma study analyzing data from the Pragmatic Randomized Optimal Platelet and

Plasma Ratios (PROPPR) trial reported an increased likelihood of 24-hour mortality in patients receiving massive transfusions (> 10 units) of RBCs if older than 22 days (27). In addition, a second study analyzing PROPPR data used a scalar metric (Scalar Age of Blood Index, SBI) that accounted for the distribution of the blood ages of all transfusions received by each patient and found that a higher, more positive SBI (indicating older RBC units were used) was associated with both 24-hour and 30 day mortality, despite adjustments for total units received and clinical covariates (28). Therefore, an open question remains whether there are critical aspects of the RBC storage lesion that can adversely impact clinical outcomes for specific patients.

While the data presented here provides further evidence that progressive oxidation occurs over time when RBCs are stored, it also suggests that RP screening of banked blood could be important independent of age and may serve as a precision medicine marker of RBC quality and blood oxidation state allowing for more selective and efficient use of RBC utilization. The ability to determine the RP status of RBC units in the blood bank, or at the point of care, could provide a useful blood “vital sign” to evaluate the health and oxidative state of RBCs. With a direct measure of the redox status, and oxidative stress of any given RBC unit planned for transfusion, therapeutic interventions could be delivered to improve the redox state, such as antioxidant therapies (e.g. Vitamin C, N-acetylcysteine, and others) that have been shown to reduce oxidative stress in RBCs (5, 6), reduce cell damage by free radicals (22), and preserve red cell energy and overall RBC quality (7). Although evaluating a limited number of single pRBC units over time, our colleagues at Virginia Commonwealth University have also reported evidence that the addition of Vitamin C may help to stabilize the redox state over the duration of pRBC storage and that progressive oxidation occurs in these units (29). Furthermore, RP values may also serve as a gauge for providing *systemic* antioxidant therapy in patients receiving large transfusions if their systemic RP values increase significantly after resuscitation with RBCs. In fact, traumatic injury itself can produce negative effects on the health and viability of circulating blood by causing changes in circulating redox state, as well as the induction of platelet activation that can stimulate the production of reactive oxygen species (ROS) that alter the systemic redox state and promote the adhesion and activation of additional neutrophils, platelets, and endothelial cells, stimulating the extrinsic coagulation pathway and ultimately leading to dysregulation of the coagulation cascade (30). Given that 33% of patients suffering from trauma and hemorrhage present with coagulopathy on hospital admission (31), if the redox state of banked blood units given to these patients is also altered, the negative effects of storage lesions could be amplified, worsening systemic oxidation and oxidative stress. Therefore, having bedside measurements of RP at the point of care could add a new dimension to patient monitoring that could improve both banked blood viability, its effectiveness when given to those in need of transfusion, and the overall health and function of circulating blood in these patients.

There are a number of important limitations to this study. Overall, the total number of units sampled was relatively small. Sampling was done from the RBC segments and not directly from the blood bag itself where RP may have been different. Current studies are underway that include direct sampling from the blood bag itself. We performed only single RP measures on each sample, however our previous work has demonstrated excellent reproducibility of measurement (10, 11). Given this and the small sample size of

the segments we did not feel it necessary to perform duplicate measures. We did not measure RP of RBCs at day zero, although they were not available due to the time required for processing and subsequent delivery to the blood bank after initial collection from blood donors. While we did measure RP in fresh whole blood of non-acutely ill/injured volunteers, this blood was not processed in the same way that occurs with blood donation. As mentioned earlier, such processing could change RP, and we decided to make direct measures of RP from these samples as the result would more likely reflect circulating RP values of patients receiving transfusions. Lastly, we made no additional measures of oxidative stress or RBC damage such as fragility-deformability or oxygen carrying capacity (p50). Thus, it is not possible to know with certainty the extent of storage lesions present at RP values measured in this study.

Conclusions

There are significant differences in RP between freshly stored RBCs and all others, with RP becoming more positive over time. However, storage time alone does not predict RP, indicating RP screening may be important independent of age and may serve as a marker of RBC health. Targeting RP may enable the use of antioxidant therapies to restore RP balance in stored RBCs, as well as systemically in those receiving multiple transfusions, improving the clinical effectiveness of RBCs and potentially reducing associated morbidities.

Abbreviations

ROS - Reactive Oxygen Species

RBCs - Red Blood Cells

RP – Redox Potential

POC – Point of Care

np – nanoporous

SBI – Scalar Age of Blood Index

Declarations

Ethics approval and consent to participate

Although this study involves the use of samples from human blood products, ethics approval and consent was waived given all samples were taken from banked blood units and thus did not require an IRB protocol.

Consent for publication

Not Applicable

Availability of data and materials

The majority of data generated or analyzed during this study are included in this published article. Any data not included is available from the corresponding author on reasonable request.

Competing interests

Authors Daniels, Collinson, and Ward hold a patent for the nanoporous gold electrodes used in this study; however, they receive no financial benefits or incentives from this work. The authors have no other conflicts or competing interests to disclose.

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1. NIH K12 Award through the Pediatric Critical Care and Trauma Scientist Development Program (PCCTSDP):

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2. University of Michigan - Fast Forward Medical Innovation Kickstart Award:

Financial support for redox electrode and platform development

3. Michigan Center for Integrative Research in Critical Care (MCIRCC):

Provided lab space for performing measurements

4. Department of Pediatrics/Division of Pediatric Critical Care Medicine:

Provided start-up funds that supported material and lab costs as well as lab assistant support, in addition to protected time for pursuing research.

Authors' contributions

RCD – Principal Investigator overseeing all aspects of redox electrode production, testing, and data collection. RCD also manufactured some electrodes used in the study and aided in testing the banked RBC units. He performed a majority of data interpretation, statistical analysis, and final graph/figure production. As the primary author, RCD drafted and edited the vast majority of submitted manuscript.

HJ – Laboratory associate in RCD's lab who manufactured the majority of electrodes used. HJ performed the the majority of banked RBC unit testing. HJ also collected the data and performed some early data interpretation and graphical data representation.

RDD – As director of our blood bank RDD helped to coordinate access to banked RBCs and aided in sample collection. RDD also reviewed the manuscript and provided revisions as appropriate.

MC – Co-inventor of the nanoporous gold redox electrode with whom RCD communicates as needed regarding ongoing redox electrode development and testing.

KW – Mentor for RCD who reviewed data acquisition and analysis and who also provided some written contributions and revisions to the manuscript after review.

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Tables

Table 1. RP measurements among samples and distribution of RBC samples tested, grouped by stored RBC unit age.

Age (Days)	Samples	Median (IQR)
5	6	-60 (-71 to -37)
10-15	8	7 (-14 to 12)
20-25	6	33 (19 to 55)
30-35	9	48 (33 to 68)
40	3	103 (81 to 109)

RP Sample Distribution and Values per RBC Group

Figures

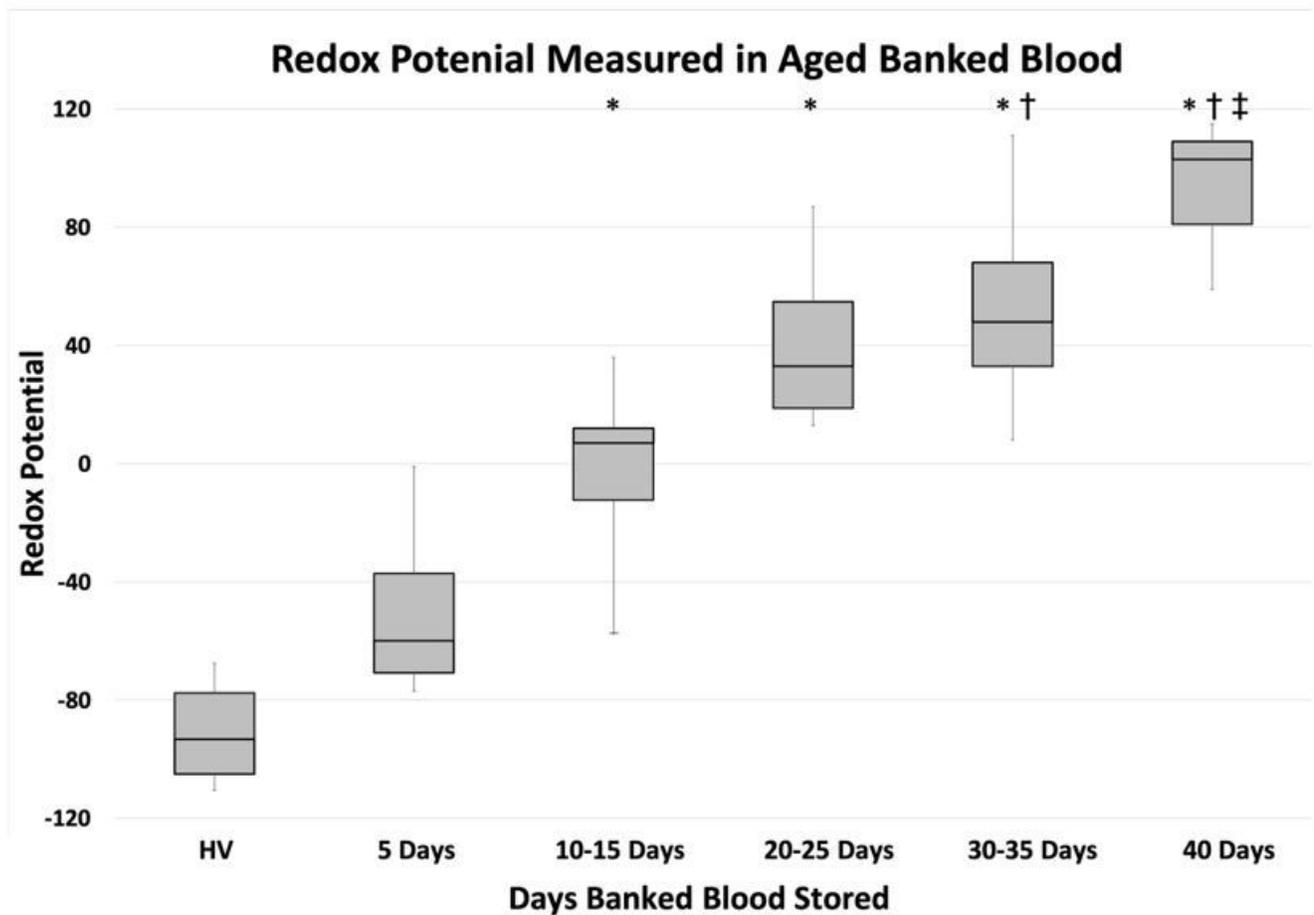


Figure 1

Redox Potential (RP) measured from multiple random units of Red Blood Cells (RBCs), ranging in age from 5 days to 40 days of age (n=32 total), along with RP measures of peripheral venous blood taken from 10 volunteers (HV). Aged RBC samples were placed into one of 5 groups depending on duration of storage: 5 Days, 10-15 days, 20-25 days, 30-35 days, and 40 days. Redox potential becomes progressively more positive (more oxidized state) over time, with 5 day-old RBCs having an RP closest to that of the peripheral blood of healthy human volunteers (HV). * Significant Difference from Day 5 RBCs † Significant Difference from Day 10-15 RBCs ‡ Significant Difference from Day 20-25 RBCs Mann-Whitney U-Test with Holm Sequential Bonferroni Adjustment

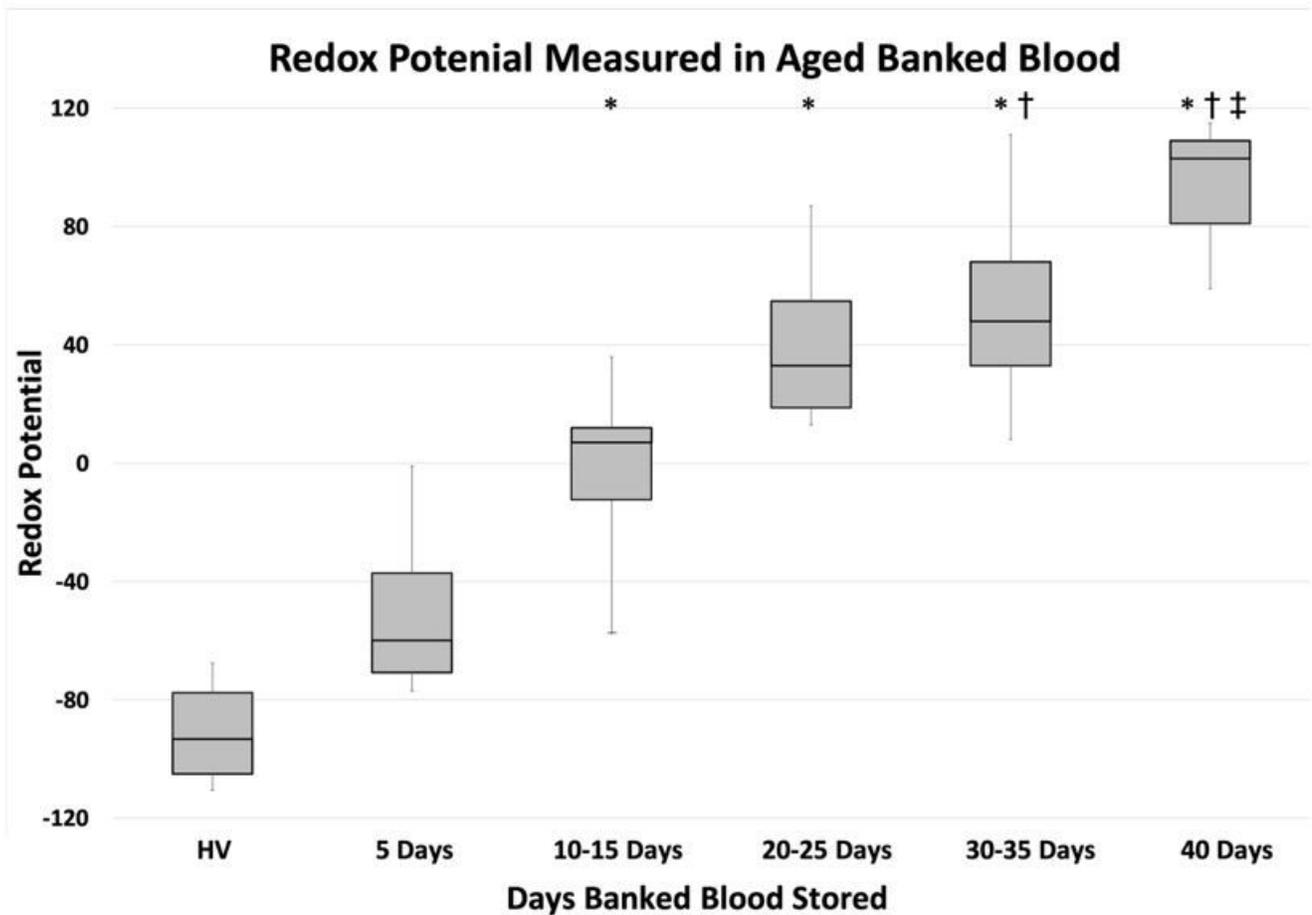


Figure 1

Redox Potential (RP) measured from multiple random units of Red Blood Cells (RBCs), ranging in age from 5 days to 40 days of age (n=32 total), along with RP measures of peripheral venous blood taken from 10 volunteers (HV). Aged RBC samples were placed into one of 5 groups depending on duration of storage: 5 Days, 10-15 days, 20-25 days, 30-35 days, and 40 days. Redox potential becomes progressively more positive (more oxidized state) over time, with 5 day-old RBCs having an RP closest to that of the peripheral blood of healthy human volunteers (HV). * Significant Difference from Day 5 RBCs † Significant Difference from Day 10-15 RBCs ‡ Significant Difference from Day 20-25 RBCs Mann-Whitney U-Test with Holm Sequential Bonferroni Adjustment