Optimization of a Diagnostic Platform for Oxidation-Reduction Potential (ORP) Measurement in Human Plasma and Exploration of ORP after Trauma

David A. Polson

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Optimization of a Diagnostic Platform for Oxidation-Reduction Potential (ORP) Measurement in Human Plasma and Exploration of ORP after Trauma

David A. Polson

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1.0 Abstract:

Oxidation-reduction potential (ORP) measurement has demonstrated oxidative stress in patients with severe illness/injury. A new ORP diagnostic platform using disposable sensors (Supplemental Figure 1) has been validated by comparison to mass spectrometry, but the response of ORP to experimental positive and negative control conditions has not been determined. Furthermore, optimal methods of sample handling for ORP measurement have not been studied. We sought to optimize ORP measurement in human plasma, under controlled conditions. We hypothesized that freeze-thawing of the sample, storage of the sample for up to one month, and the method of sample anticoagulation would influence ORP levels. Furthermore, we hypothesized that ORP can detect incremental experimental changes that either increase or decrease redox state. Finally, we hypothesized that trauma injuries cause increased levels of reactive oxygen species (ROS) in the circulating bloodstream, and therefore would reflect higher ORP signals in plasma measurements in both human and mouse subjects.

We enrolled healthy human volunteers in a prospective observational study and measured ORP in plasma prepared with heparin or citrate anticoagulants directly after blood draw and up to 28 days later. Additionally, we evaluated the platform’s ability to detect an exogenous increase and decrease in ORP by performing both positive and negative control titrations using six different concentrations of hydrogen peroxide (H₂O₂) and two different concentrations of ascorbic acid respectively. Lastly, we compared human trauma plasma samples in an uncontrolled emergency room setting with healthy human plasma and then mimicked this experiment in a more controlled mouse model experiment. We found that fresh plasma better retains the ORP signal as compared to
freeze-thaw samples measured on subsequent days. We also found that the platform can detect exogenous, concentration-dependent oxidations with \( \text{H}_2\text{O}_2 \) and reductions with ascorbic acid. Furthermore, plasma prepared with heparin is more sensitive than using citrate anticoagulant when measuring ORP. Also, human trauma patients’ plasma (of varying degrees of trauma) samples were characterized by significantly higher ORP signals than healthy controls when measured in heparin anticoagulant, whereas mouse ORP signals did not change following a controlled, moderate traumatic brain injury. These data indicate that the diagnostic platform is capable of detecting exogenous increases and decreases in ORP signal from plasma samples with validity and is sensitive to different concentrations of positive and negative controls. We also show that plasma should be collected and centrifuged in heparin anticoagulant tubes and can be analyzed fresh or frozen for optimal results.

2.0 Introduction:

Reactive oxygen species (ROS) are byproducts of cellular metabolism, primarily via oxidative phosphorylation, as a result of mitochondrial respiration. ROS are essential signaling molecules under normal physiological conditions that contribute to a variety of biochemical pathways including but not limited to cell differentiation, gene transcription, and cell cycle progression\(^1,2\). However, ROS cause toxic effects as they accumulate. In biological systems, antioxidants produced internally or acquired by diet detoxify these reactive intermediates to limit damage to cells (Supplemental Figure 2). Either an increase in ROS generation or an impairment of antioxidant capabilities causes an imbalance known as oxidative stress.
Recent studies suggest that oxidative stress arises and/or is associated with different disease states including but not limited to trauma, diabetes, and severe infection (sepsis)\textsuperscript{3,4,5}. Oxidative stress is a common mechanism/characteristic of disease so measuring it could offer insight into the pathophysiological mechanism, severity of disease, and potential medical therapies. For example, making a measurement of ROS could aid to the triage process in emergency departments and lead to more informed triage decisions\textsuperscript{6}. Furthermore, with additional research, it is possible that oxidative stress can predict disease progression and be used for monitoring the pathophysiology of associated conditions\textsuperscript{6}. Measuring oxidative stress may also prove valuable in the development of antioxidant therapy applications. Generally, antioxidants are perceived as healthy compounds that exhibit preventative properties for a variety of neurodegenerative diseases, but little progress has been made with the medicalizing of them\textsuperscript{7,8,10}.

Oxidative stress can be quantified as the difference between oxidants and reductants (antioxidants) known as the oxidation-reduction potential (ORP)\textsuperscript{9}. ORP, measured as the electrical signal produced by oxidation of an electrode under standardized conditions, provides a rapid, composite measurement of oxidative stress without determining contributions of individual molecules. For clinical use, this aggregate measure of both oxidants and reductants, may provide more utility than other specific measures of oxidants alone. A novel platform has the ability to rapidly (in as little as 4 minutes) and easily (simple experimental procedure) determine ORP levels in biological samples such as plasma. Despite its potential application, we are not aware of any
studies that have optimized the best way for sample collection and storage conditions for reliable ORP measurements.

Previous validation experiments compared comprehensive plasma ORP measurements from this platform to mass spectrometry measurements to confirm that specific proteins were oxidized, but no prior experiments have titrated different levels of exogenous oxidizing agents or reducing agents to establish the performance characteristics of the diagnostic platform under controlled conditions. Understanding the limits of the diagnostic platform to detect incremental changes in plasma redox states is necessary for optimization of a protocol for sample handling and collection. These experiments also provided data that suggested trauma injuries in humans cause significant increase in ORP signals compared to healthy human counterparts, yet to our knowledge, there have been no experiments that measure ORP signal through time in a controlled setting. By tracking ROS through time after trauma in a controlled mouse model experiment, we could shed light on the pattern of ROS generation in trauma pathophysiology. We sought to optimize ORP measurements in human plasma, under controlled conditions. We hypothesize that freeze-thawing of the sample, storage of the sample for up to one month, and the type of anticoagulant used to collect whole blood would influence ORP levels. Furthermore, we hypothesized that ORP can detect incremental experimental changes that either increase or decrease redox state. Finally, we hypothesized that trauma injuries cause increased levels of ROS in the circulating bloodstream and therefore would reflect higher ORP signals in plasma measurements in both human and mouse subjects.
3.0 Methodology:

3.1 Subjects and Sample Collection:

3.11 Human Subjects and Sample Collection

This study received approval from the Institutional Review Board. Healthy human volunteers were recruited for this study for control plasma samples. Trauma patients were also recruited for this study out of the University of Vermont Medical Center Emergency Department for trauma plasma samples. Trauma injury was determined by emergency department staff and assigned an Injury Severity Score (ISS) (Supplemental Figure 3). Trauma injuries ranged from mild to severe. Whole blood samples were collected from healthy/trauma volunteers through a single intravenous blood draw. Whole blood was then transferred into both heparin and sodium citrate (3.2%) anticoagulant tubes and immediately centrifuged at 2000 rpm 4 °C for 10 min. After centrifugation, plasma samples were either aliquoted and stored at -80°C for future use or immediately prepared and analyzed through use of the diagnostic platform.

3.12 Mouse Subjects and Sample Collection

C57 Mice (control mice) were used for the duration of the mouse experiments. Blood collection in mice was performed using the cardiac puncture method in which whole blood collection occurred directly from the left ventricle of the heart through use of a syringe. All mice were euthanized prior to whole blood collection. Whole blood samples were placed in heparin anticoagulant tubes Heparin tubes were centrifuged (10 min, 2000 rpm, 4 °C). After centrifugation, plasma samples were analyzed through use of the diagnostic platform.
3.2 ORP Measurements:
Measurements were taken using the RedoxSYS™ platform from Aytu BioSciences®. Prior to ORP measurement, all platforms were measured for calibration (Supplemental Figure 4). Sensor chip side A was inserted into the device to ensure the device was within specification (100 mV ± 1) and then side B was inserted to further ensure the device was working within specification (300 mV ± 4.2). Following calibration check, plasma samples (30 µL) were pipetted onto the exposed filter paper reservoir of the disposable test sensors and then inserted in the platform for OPR measurements. Once applied to the reservoir, the sample flowed to the platinum electrode surface and current was measured in mV. The final reading was the aggregate measurement of the balance of oxidants and antioxidants in the plasma sample.

3.3 Experimental Design:
3.31 Control Time Course:
Following centrifugation, ORP signal was measured from human plasma collected in the sodium citrate (3.2%) tube and then in the heparin tube in duplicate to establish a time zero (fresh) measurement. The rest of the plasma was aliquoted into cryovials (100 µL aliquots) and stored at -80°C for later use. One aliquot of citrate derived plasma and one aliquot of heparin derived plasma were removed from the -80°C condition and placed at room temperature until they had completely thawed. The samples were analyzed in duplicate at different time points as follows: 2, 4, 6, 21, and 21 days after time zero measurement to determine stability of sample through time and through freeze-thaw event.
3.32 Elevated Time Course:
Following centrifugation, plasma samples were aliquoted into cryovials (100µL aliquots) as described above. Aliquots were then oxidized by adding 1% H₂O₂ (known as a potent oxidant). ORP signal was then measured from a citrate tube aliquot and a heparin tube aliquot in duplicate to establish a time zero (fresh) measurement. The rest of the aliquots were stored at -80°C for later use. As in the control time course, samples were again measured in duplicate at time points 2, 4, 6, 21, and 28 days after time zero measurement to determine stability and retention of an exogenously elevated ORP signal through time and through freeze-thaw event.

3.33 Positive Control Titration:
Following centrifugation, ORP signal was measured from both citrate and heparin plasma samples in duplicate. The remaining plasma was then titrated by adding increasing concentrations of H₂O₂, specifically 0.03, 0.1, 0.3, 1, 3, and 10% of H₂O₂ solutions. Each of these samples were then tested in duplicate to determine the platform’s ability to detect exogenous elevations in oxidative stress as compared to the control samples.

3.34 Negative Control Titration:
Following centrifugation, ORP signal was tested from both citrate and heparin plasma samples in duplicate. The remaining plasma was then titrated into 10 and 50 mM ascorbic acid solutions. Both of these samples were then tested in duplicate to determine whether control plasma could be reduced below the basal reading as determined by the platform’s ability to detect exogenous reductions in
oxidative stress of different concentrations as compared to the control sample. In addition to reducing basal plasma, another negative control titration was performed by first elevating (oxidizing) the plasma by adding 0.1% H$_2$O$_2$ prior to the addition of ascorbic acid (10 mM and 50 mM) in order to mimic an elevated signal of a clinical condition.

3.35 Human Healthy Control vs. Trauma Plasma
Following centrifugation, plasma ORP was measured from healthy, control volunteers in citrate and then in the heparin tube in duplicates. These data were then compared to ORP measurements obtained from consented trauma patients. Trauma patient plasma was also measured from citrate tube and then heparin tube in duplicates.

3.36 Mouse Healthy Control vs. Trauma Plasma Time Course
All healthy control mouse blood was collected via cardiac puncture and analyzed in duplicate using the diagnostic platform immediately following centrifugation. Trauma mice were induced a traumatic brain injury (TBI) via fluid percussion$^{25,26}$. Following fluid percussion, split into 2 groups; 4hr and 12hr groups. 4hr and 12hr mice were euthanized at their respective time points and blood was collected via cardiac puncture. Following centrifugation, plasma was immediate analyzed in duplicate using the diagnostic platform.

3.4 Statistical analysis:
Average ORP values are reported as mean ± standard error mean (s.e.m.) N represents number of replicates per experiment. All data was analyzed using two-way ANOVA statistical analyses with the exception of healthy human control vs. trauma patient data
which was analyzed via unpaired T-test. All analyses were conducted through use of GraphPad Prism.

4.0 Results:

4.1 Effects of Freeze-Thawing Human Plasma and Sample Storage:
Freeze-thawing control human plasma samples led to a decrease in ORP as determined by the novel diagnostic platform (Fig. 1A; Two-way ANOVA, $P < 0.05$, N=5). Freeze-thawing exogenously elevated plasma samples also led to a decrease in ORP (Fig. 1, B; Two-way ANOVA, $P < 0.05$, N=5).

4.2 Effects of Anticoagulant on ORP Values:
All control plasma samples measured in heparin anticoagulant showed a marked decrease in ORP as compared to citrate anticoagulant counterparts (Fig. 1A; Two-way ANOVA, $P < 0.05$, N=5). In exogenously elevated plasma however, the type of anticoagulant had no effect on the measurement (Fig. 1, B; Two-way ANOVA, ns, N=5).

4.3 Positive Control Titration on ORP Measurement in Human Plasma:
The addition of exogenous H$_2$O$_2$ (0.1%) to control human plasma to create different concentrations of H$_2$O$_2$-plasma solutions led to increased ORP signals as determined by the novel diagnostic platform (Fig 2; Two-way ANOVA, $P < 0.05$, N=5). However, the type of anticoagulant had no effect on different concentrations of exogenously elevated plasma and resulted in consistent ORP measurements (Fig 2; Two-way ANOVA, ns, N=5).
4.4 **Negative Control Titration on ORP Measurement in Human Plasma:**

The addition of exogenous ascorbic acid at two different concentrations (10 and 50 mM) to control human plasma significantly decreased ORP signals as determined by the novel diagnostic platform (Fig. 3A; Two-way ANOVA, \( P < 0.05 \), N=5). Control plasma measured in heparin anticoagulant also showed a decrease in ORP vs. citrate plasma counterparts when ascorbic acid was applied (Fig. 3, A; Two-way ANOVA, \( P < 0.05 \), N=5). The addition of exogenous ascorbic acid to plasma following the exogenous elevation of plasma to 0.1% H\(_2\)O\(_2\) also led to a decrease in ORP in comparison to the basal 0.1% H\(_2\)O\(_2\) measurement (Fig. 3B; Two-way ANOVA, \( P < 0.05 \), N=5). We found no difference in ORP values between elevated plasma prepared in heparin vs. citrate anticoagulant (Fig. 3B; Two-way ANOVA, \( ns \), N=5).

4.5 **Effects of Trauma on ORP Values**

Trauma injuries in humans of varying degrees led to a significant increase in plasma ORP signal compared to healthy human control plasma ORP readings when heparin anticoagulant was used (Fig. 4; Unpaired T-test, \( P < 0.05 \), N=5 healthy control, N=10 trauma). However, this increase as a result of trauma did not present itself with plasma measured in citrate anticoagulant (Fig. 4; Unpaired T-test, \( ns \), N=5 healthy control, N=7 trauma). With regard to mice, trauma injury had no effect on ORP signal (Fig. 5; Two-way ANOVA, \( ns \), N=5 healthy control, N=4 TBI 4hr, N=3 TBI 12hr).
5.0 Figures:

Figure 1. Effect of freeze-thawing human plasma in both citrate and heparin anticoagulant and the stability of human plasma in storage at -80°C on A, control human plasma (Two-way ANOVA; Anticoagulant $P < 0.05$, Time $P < 0.05$). and B, oxidized human plasma ($1\% \text{H}_2\text{O}_2$) up to 28 days (Two-way ANOVA; Anticoagulant $\text{ns}$, Time $P < 0.05$).
Figure 2. Effect of the addition of known oxidant $\text{H}_2\text{O}_2$ to human plasma at incremental concentrations in both citrate and heparin anticoagulants (Two-way ANOVA; Anticoagulant ns, $\text{H}_2\text{O}_2$ vs. ORP $P < 0.05$). The inset shows data from 0% – 0.3% $\text{H}_2\text{O}_2$ concentrations.
Figure 3. Effect of the addition of known reductant ascorbic acid to human plasma at two concentrations (10mM and 50mM) in both citrate and heparin anticoagulants on A, control human plasma (Two-way ANOVA; Anticoagulant $P < 0.05$), ascorbic acid vs. ORP $P < 0.05$) and B, oxidized human plasma (0.1% $H_2O_2$) (Two-way ANOVA; Anticoagulant ns, ascorbic acid vs. ORP $P < 0.05$).

Figure 4. Effect of traumatic injury to human plasma ORP signal in citrate and heparin anticoagulant (Unpaired T-test; Control vs. Trauma citrate ns, Control vs. Trauma heparin $P < 0.05$ (*)). Trauma ORP measurements were taken upon arrival to emergency department (time zero). Trauma injuries varied from mild to severe and trauma patients varied in age and sex. All readings were pooled.
6.0 Discussion:

Measurement of redox state has been around for quite some time. Originally, the standard potential of a redox system $E_0$ (from the Nernst-Peters equation) was measured by electrodes similar to those of the novel diagnostic platform. These electrodes however measured redox potential by implantation into subcutaneous tissue. While electrodes could also be placed in biological samples (similar to the diagnostic platform), issues arose in the reliability and reproducibility of the older methods that measured the entire redox state of the system not to mention that a much larger volume of a biological sample was needed to fully immerse electrodes. Other, more successful older methods were specific to the redox potential estimations by measuring individual molecules or groups of molecules from a sample as oppose to the entire sample as a whole. Among this was a fluorometric device that measured pyridine nucleotides and cytochromes. The issue with this method arose from the in vivo/in situ preparation of the sample. The method required that the sample be exposed so the fluorometric device’s primary use was to measure redox in animal models or preserved organ tissues.

The novel diagnostic platform measures redox state in biological samples ex vivo. It measures the entirety of the sample without focusing on individual types of molecules like the fluorometric method and several other older methods. Similar to the original electrode method, this platform has been validated with regard to accuracy through comparison to mass spectrometry in which specific protein show increased oxidation states in samples that elicit a greater ORP value as compared to control ORP samples. Furthermore, the platform’s use of disposable test sensors results in a reproducible and reliable method of ORP measurement as compared to previous
methods\textsuperscript{6}. The platform also provides a much quicker and cheaper alternative to current analyses of ORP including mass spectrometry\textsuperscript{15}.

This is the first study conducted to optimize a protocol for this novel point-of-care platform through from sample collection through measurement. Furthermore, the platform had not yet been validated through the addition of exogenous oxidants and reductants to control human plasma to measure changes in ORP level. We sought to optimize ORP measurement in human plasma, under controlled conditions. We hypothesized that freeze-thawing of the sample, storage of the sample for up to one month, and the method of sample anticoagulation would influence ORP levels. Furthermore, we hypothesized that the platform can detect incremental experimental changes that either increase or decrease redox state and therefore lead to misinterpretations of experimental findings. Our findings suggest that sample integrity exists through storage at -80°C conditions, however freeze-thaw cycles result in a significant decrease in the signal of both control and exogenously elevated plasma samples compared to fresh plasma counterparts. Also, the type of anticoagulant used for whole blood processing directly effects basal plasma ORP readings. With regard to our validation experiments, we found that the device accurately detects concentration dependent additions of both exogenous oxidants and reductants to human plasma samples.

6.1 Effect of Freeze-Thawing and Storage of Sample on ORP Values:

We conducted this set of experiments to test whether plasma samples’ redox state remained constant through freeze-thaw cycles and whether ORP signal remains stable
through time as a result of storage. We saw this as an essential experiment; the Freeman laboratory has collected and stored many frozen plasma samples from both control subjects and trauma patients but the validity of retrospective analyses would be compromised if freeze-thaw or storage affect ORP measurement. Figure 1A suggests that there is a significant deterioration (decrease) in the ORP signal (5-10 mV drop) as a result of freeze-thaw cycles in plasma samples collected in both citrate and heparin tubes as compared to fresh plasma ORP measurements. This drop in ORP signal remained constant through storage at -80°C conditions of up to one month in time (Figure 1A). This suggests that, though there is a drop in signal due to freeze-thawing, the samples remain stable when storage at -80°C. We also measured the stability of the ORP signal in samples elevated with exogenous H\textsubscript{2}O\textsubscript{2} (1%) to mimic the ORP signal obtained in a severe disease state such a sepsis or trauma (Figure 1B). Our results were identical to the control plasma time course where a significant drop in ORP occurred following freeze-thawing. This drop also remained constant through time. These data suggest that for optimal and most accurate results, fresh ORP measurements are preferred; we have concluded that frozen samples are incomparable to samples measured fresh.

A previous study measured the effect of storage time on sample integrity and concluded that samples stored at -80°C remain unchanged up to 6 months after initial blood draw\textsuperscript{6}. This result confirms our findings that plasma samples remain stable through proper storage. However, this study did not compare fresh plasma ORP readings with freeze-thawed plasma ORP readings. Since ORP signal significantly drops following a freeze-thaw cycle, we conclude that fresh ORP readings and freeze-thaw ORP readings are incomparable with regard to plasma.
6.2 Effect of the Anticoagulant on ORP Values:

In this study, we evaluated the influence of two different anticoagulants (heparin and sodium citrate) on ORP values measured in either citrated or heparinized human plasma. The use of an anticoagulant proves necessary due to the device limitation being unable to process whole blood samples; they coagulate in the disposable test sensor before reaching the platinum electrodes making it impossible to provide a reliable ORP measurement. Under normal circumstances, coagulation occurs when thrombin converts fibrinogen to fibrin. Once initiated, clot formation is driven by the activation of plasma glycoproteins that exhibit serine protease activity. These zymogens drive the reaction to completion and among them are thrombin and coagulation factors X and IX\textsuperscript{16}. Heparin effectively acts as an anticoagulant because of its ability to increase the activity of the inhibitory antithrombin enzyme. Upregulating of antithrombin activity inhibits factors X and IX activity to contribute to coagulation\textsuperscript{17,18}.

On the other hand, sodium citrate sequesters calcium ions in vitro\textsuperscript{19}. Calcium is a necessary second messenger in blood clot formation mediating the activation of zymogens into their active enzyme states including factor IX which in turn activates factor X\textsuperscript{16}. In the presence of sodium citrate, calcium required for blood clotting is neutralized. The activation of factors IX and X ceases as the calcium necessary for the reaction has been depleted leading to the overall inhibition of the coagulation pathway.

We found that plasma derived from heparin anticoagulant tubes had significantly lower baseline ORP measurements than plasma derived from citrate anticoagulant tubes. Figure 1A illustrates this difference and suggests that the difference in ORP signal between anticoagulant type remains regardless of freeze-thawing. The chelation of
calcium ions achieved by sodium citrate may lead to a significant increase in redox state while the potentiation of antithrombin via heparin does not have this effect. One study found that the administration of heparin during hemodialysis led to a significant decrease in the release of superoxide free radicals which suggests an alternate explanation for why baseline heparin ORP readings were lower than citrate\textsuperscript{20}.

Interestingly, following the addition of the oxidant H\textsubscript{2}O\textsubscript{2}, ORP measurements between heparin and citrate plasma reach a steady plateau (Figure 2). This suggests that plasma reaches a maximum oxidizable state that is consistent between heparin and citrate. This consistency between anticoagulants remains upon the reduction through ascorbic acid of highly elevated samples (Figure 3, B). But the difference in ORP between heparin vs. citrate exists after the addition of an exogenous reductant (in our case ascorbic acid) in basal samples without previous elevation. Figure 3A shows this pattern as heparin ORP signals remain significantly lower than citrate measurements even after the addition of ascorbic acid (10 mM and 50 mM). In other words, the difference in ORP is sustained by exogenous decreases in redox state yet abolished by exogenous increases in redox state. This suggests that as redox state increases, ORP measurements in heparinized plasma and ORP measurements in citrate plasma will ultimately converge whereas when redox state decreases, ORP values will remain definably different between the two types of anticoagulants.

6.3 Positive and Negative Control Validation of Platform:

No studies have tested the effects of exogenous oxidants and reductants on ORP signal when added to plasma samples. We saw this as an important set of data to deduce
the sensitivity of the platform and to ensure that the platform was actually sensing changes in redox state. First, we conducted a positive control titration using increasing concentrations of H₂O₂, a known oxidant. Our data suggest that the device can detect concentration dependent increases in ORP as a result of the addition of H₂O₂ (Figure 2). ORP measurements maxed out at around 230 mV and began to plateau by a concentration of 1% H₂O₂. Next, ascorbic acid was used on control plasma to determine the lower bounds of the device with regard to plasma. Figure 3A illustrates a significant reduction in ORP signal of control plasma after the addition of two different concentrations of ascorbic acid (10 mM and 50 mM), suggesting that plasma obtained from healthy (control) individuals also contain circulating oxidized proteins that can be further reduced. Furthermore, we confirmed that the platform could detect changes in ORP by first oxidizing control plasma with H₂O₂ (0.1%) and then adding ascorbic acid to decrease the signal as shown in Figure 3B. The data present a much more drastic decrease in signal in response to the ascorbic acid treatment used in comparison to the decrease seen in Figure 3A, suggesting that the changes in ORP are a direct result of changes in redox states. As more proteins become oxidized through H₂O₂ treatment leading to an overall greater potential for reduction after ascorbic acid treatment.

One similar study measured the effects of ascorbic acid on ORP signal of a phosphate-buffered saline (PBS) solution as a negative control experiment using the same platform. They produced similar results as they reported that the platform was able to detect incremental changes in ascorbic acid that resulted in the overall decrease in redox state¹. Our data further validate the ability of the device to detect decreases in ORP signal when plasma samples are exogenously treated with an antioxidant.
6.4 Effects of Trauma Injuries on ORP Values:

Several studies have tested the effect of trauma on plasma ORP in humans with this novel platform. In all cases, trauma has led to a significant increase in plasma ORP vs healthy control human subjects\textsuperscript{1,10,11}. In each of these studies, heparin was the anticoagulant used for sample preparation. We decided to verify these results and also to evaluate the effects of citrate on ORP in control and trauma plasma samples. Our data confirm the previous studies findings in heparin, however we didn’t see any change in ORP signal when using the citrate anticoagulant between trauma and healthy control volunteers. We believe that the lower basal readings in heparin allow for the detection of increases in ORP characterized by trauma injuries while citrate anticoagulant is not sensitive enough at these lower changes in ORP. If we recall the positive titration data (Figure 2), we did not see a difference between heparin and citrate anticoagulant. This is because at maximal H\textsubscript{2}O\textsubscript{2} concentrations, plasma ORP is maximized and a steady plateau is reached consistent between anticoagulants, denying statistical significance. Trauma injuries do not produce ORP readings nearly as high as the plateau. By our results, trauma ORP mimics a 0.03% H\textsubscript{2}O\textsubscript{2} plasma solution. At these low concentrations, a much greater change in ORP signal exists in heparin measurements than citrate which could account for why trauma goes undetected when using citrate anticoagulant.

Inclusion criteria for trauma patients consisted of any patient admitted to University of Vermont Medical Center Emergency Department requiring immediate medical attention as determined by the medical staff. Patients age and injury severity score (ISS) (when applicable) are listed in supplemental figure 5. ISS is a scoring system that adds the squares of the three highest abbreviated injury scale (AID) values (range 1-5 to score
different parts of the body) and is a statistic used almost exclusively for research methodology. It is a strong predictor of trauma severity and mortality\textsuperscript{21}. Included in this study were patients who suffered a mild to severe trauma of any kind. Previous studies stratified their results into different subsets of trauma. One such study separated multi-trauma patients by ISS where all patients who scored less than 16 were in a different from those who scored 16 or greater\textsuperscript{10}. As compared to control readings, both trauma groups showed significant increases in plasma ORP and the $\geq 16$ ISS trauma group was further elevated as compared to the $< 16$ ISS group. This suggests that, greater ORP values are associated with more severe trauma injuries and like the positive control titration, the platform can detect these incremental changes. While we did not stratify our data in this study, our data confirms previous results.

While trauma injuries take on many shapes, one particular study measured TBI specific trauma with regard to ORP in human plasma. The results were similar to other literature where trauma led to significant increase in plasma ORP signal\textsuperscript{1}. The platform’s ability to measure the difference between TBI and healthy status suggests application outside the hospital. TBI is a common injury acquired in contact sporting events including football, hockey, boxing, etc. Many patients come to the emergency department without actually suffering TBIs while many patients suffering TBI do not end up making it to the emergency department due to delayed or nonexistent symptoms. This platform offers a quick, convenient means to measure the severity of TBI before reaching the hospital which may help correct improper procedure following a head injury. Current biomarkers of TBI include a variety of cerebrospinal fluid and/or blood indicators\textsuperscript{22,23,24}. One more popular biomarker is the tau protein, a central nervous system protein that helps stabilize
microtubules. Tau increases in blood as a result of TBI$^{23,24}$. While this biomarker accurately indicates TBI, ORP measurement provides a much quicker, cheaper, and simpler means to the same end.

Stemming from the human TBI results reported by previous studies along with our own findings, we decided to undertake a controlled, mouse model time course experiment exploring the pattern of ORP at different time points after TBI. TBI was induced via fluid percussion which allows for the most consistent and controlled administration of TBI. A pendulum swings from 10 degrees (maximum injury with approximately 2:3 survival rate in mice) and strikes a piston of a fluid reservoir, sending a pressure pulse to the dura of the mouse (Supplemental Figure 6). This method does not induce skull fracture which attributes to its consistency$^{25,26}$. Following blood draw, the brains of the mice that received TBI treatment were examined to confirm TBI. Surprisingly, we found that ORP signal did not increase in TBI mice at 4 or 12 hours after trauma as compared to healthy control mice which contradicts previous studies and our results in human trauma patients$^{1,11}$. It is possible that fluid percussion induced mice experience TBI without severe neurological impairment or that mice have alternate redox systems than humans, both of which could explain this anomaly. Another explanation is the low N in our experiment. With increased measurements, we may potentially see a difference, though so far, that data suggests that the trauma pathophysiology of mice is different and therefore incomparable to the pathophysiology of humans in relation to ORP signals (Supplemental Figure 7).
6.5 Conclusion:

We were able to establish a protocol for the diagnostic platform to optimize ORP measurements. Freeze-thawing human plasma had a significant effect on ORP signal resulting in overall lower ORP as compared to fresh plasma readings; therefore, we suggest that plasma always be measured either fresh, frozen for the duration of an experiment for comparable results. Furthermore, we conclude that the proper storage of human plasma (-80°C) has no foreseeable effect on ORP signal, and one may retrospectively analyze plasma samples that have been stored for accurate results (assuming there is no cross comparison between fresh and freeze-thaw samples). Also, we suggest the usage of heparin anticoagulant over citrate anticoagulant when measuring the effects of different disease states on ORP due to heparin’s greater sensitivity to oxidizing effects at lower ORP levels. The use of citrate may result in nonsignificant results between disease state and healthy control.

We were able to validate the platform with regard to exogenous positive (oxidation) and negative (reduction) controls. We conclude that the device is performing within specification due to its ability to distinguish between exogenous oxidants and antioxidants. Furthermore, we found that it is sensitive to different concentrations of each.

Based on these data, we believe that this novel platform has a vast range of potential applications. With its ability to detect increases in ORP in patients suffering from trauma, it has clinical significance. Especially since it can measure ORP in as little as 4 minutes when alternate methods take much longer. Much literature associates increases in ROS as a characteristic of a variety of different disease/conditions. We have shown that trauma injuries reflect an increase in circulating ROS, and with the exploration of
other conditions, it is possible that the device will aid in other diagnoses. It may become an invaluable step during triage and disease progression monitoring. We suggest that future studies explore the effects of alternate disease states on ORP as determined by the diagnostic platform.

7.0 Supplemental Figures:
7.1 The Novel Diagnostic Platform

A. The diagnostic platform is a battery-powered device consisting of a heads up display and a reader slot for the disposable test strips. B. A disposable test strip is inserted into the reader slot of the device where a human biological substance is pipetted into the reservoir of the test strip. C. Once the sample crosses the bridge and reaches the electrodes, the device measures the current between the reference and working electrodes. Current is measured every 0.5 seconds resulting in a raw data trace. The final reading is the average of the last 10 seconds of readings⁹.
7.2 Endogenous Reactive Oxygen Species and Antioxidants

| ROS                      | Antioxidant              |
|--------------------------|--------------------------|
| Oxygen (Singlet)         | Glutathione              |
| Superoxide               | Catalase                 |
| Hydrogen Peroxide        | Superoxide Dismutase (SOD) |
| Hydroxyl Radical         |                          |

This table depicts the common endogenous ROS and antioxidants under normal physiological conditions. The flowchart depicts the ROS pathway from oxygen singlet to water (mediated by antioxidants)\(^9\).

7.3 Description of Injury Severity Score

| Region      | Injury description | Abbreviated Injury Scale (AIS) | Square of Three Major Injuries |
|-------------|--------------------|--------------------------------|--------------------------------|
| Head/Neck   | Traumatic Brain Injury | 3 | 9 |
| Face        | No Injury          | 0 |    |
| Thorax      | No Injury          | 0 |    |
| Abdomen     | No Injury          | 0 |    |
| Extremities | Fractured Humerus  | 2 | 4 |
| Skin        | No Injury          | 0 |    |

Total (ISS): 13

Sample injury severity score for a multi trauma patient with a head/neck injury and an extremity injury. Of the regions listed, an injury description is provided (or marked “no injury” if there is no injury for said region). Next, the injury is scored on the abbreviated injury scale (AIS). This is done by the attending physician and ranges from 1-5, 1 being mild and 5 being most severe. Finally, the highest 3 AIS scores are squared and reported in the final column where they are summed for the overall (ISS). An ISS greater than or equal to 16 is considered severe (life threatening)\(^21\).
7.4 Calibration Check Data

Machines retained calibration through time as determined by calibration chip side “A” and side “B.” Upper and lower Y-axis limits represent the acceptable boundaries of calibration values.
7.5 Trauma and Control Volunteer Demographics

|                | Citrate Trauma | Heparin Trauma | Control |
|----------------|----------------|----------------|---------|
| Age            | 34             | 53             | 27      |
| ISS            | 9              | N/A            | 13      |
| Age            | 56             | 18             | 50      |
| ISS            | 9              | 18             | 17      |
| Age Range:     | 27             | N/A            | 20-35   |

Citrate trauma subjects ranged from 27-80 years in age and had ISS scores ranging from 9-13. N/A denotes patients who were not assigned an ISS score due to early hospital discharge and were classified as mild trauma patients. Heparin trauma subjects ranged from 18-53 years of age and had ISS scores ranging from 1-18. Control subjects included 12 individuals ranging from 20-35 years of age. All three groups consisted of mixed male and female volunteers.

7.6 Fluid Percussion Diagram

Diagram of fluid percussion and its components^{26}. 
7.7 Mouse TBI Time Course Data

The data is inconclusive due to small N. However, as the data stands, there is no significance between Control, 4hr TBI, and 12hr TBI groups using a one-way ANOVA test (24 hr TBI group needs higher N for statistical analyses).
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