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# The Effect of Red Maple Leaf Toxicosis on Reduced Glutathione Levels in Equine Erythrocytes in Vitro

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THE EFFECT OF RED MAPLE LEAF TOXICOSIS ON REDUCED GLUTATHIONE LEVELS IN EQUINE  
ERYTHROCYTES *IN VITRO*

by

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Submitted in partial fulfillment of the requirements  
For graduation with Honors

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**Abstract:**

Red maple leaf toxicosis is an equine blood disorder resulting from the consumption of wilted red maple (*Acer rubrum* L.) leaves by horses. Compounds within the leaves of red maple have oxidative effects on equine erythrocytes and can cause hemolysis of erythrocytes, the conversion of hemoglobin to methemoglobin, and the production of Heinz bodies. Reduced glutathione is important in the protection of equine erythrocytes from these oxidative events; however, in the presence of red maple toxin, glutathione is rapidly oxidized and is unavailable. The objective of this study is to determine whether the presence of vitamin C alters levels of reduced glutathione for equine erythrocytes *in vitro*. Equine erythrocytes were sampled and treated with red maple leaf extract alone and in combination with vitamin C. Erythrocyte suspensions were then measured for concentration of reduced glutathione with the use of spectrophotometry. The results show a difference between reduced glutathione levels in control blood samples and blood samples treated with extract from wilted red maple leaves. Limited data shows an effect of vitamin C on samples with and without red maple leaf extract. These results are supported by other studies where oxidative damage in erythrocytes of other species causes decreased reduced glutathione concentration.

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## Introduction

Oxidative stress is damaging to erythrocytes (red blood cells). It causes the formation of complexes of iron and products of hemoglobin denaturation that cause abnormalities of the erythrocyte membrane and contribute to premature erythrocyte destruction (Claro *et al.* 2006). Essentially, oxidation causes the iron molecules within the cells to change form and lose their function (Bennati *et al.* 1983). The once-functional hemoglobin is converted to methemoglobin which lacks the ability to effectively carry oxygen. In addition, oxidation can result in the lysis of blood cells (hemolysis) due to the damage and stress placed upon them by the oxidizing agent (Boyer *et al.* 2002). When the functionality of red blood cells is reduced due to damage from an oxidant, symptoms of anemia are expressed, which include weakness, lethargy, depression, and in extreme cases, death (Boyer *et al.* 2002). Oxidative damage to erythrocytes can result in a variety of physical and chemical changes including methemoglobinemia, hemolysis, Heinz body formation and changes in glutathione levels.

Methemoglobin has been studied in relationship to oxidation of blood cells in past experiments. Inal *et al.* (2004) studied the oxidative effects of isosorbide dinitrate (ISDN) which is a drug used in the treatment cardiovascular diseases. The study used methemoglobin concentration as well as reduced glutathione concentration as their measurements of oxidative effects. While the cardiovascular treatment is effective for cardiovascular disorders in humans, it causes many side effects due to the oxidative stress it places on cells. Inal *et al.* (2004) wanted to determine the extent of these possible side effects so as to associate them with possible risks along with prescribing the ISDN medication. An additional objective was to determine whether an antioxidant (vitamin E) would alter levels of oxidation. The ISDN group

showed much higher oxidized glutathione (GSSG) levels than the control group and slightly higher GSSG levels in comparison to the ISDN + vitamin E group (Inal *et al.* 2004). These results support the hypothesis that oxidized glutathione levels increase with oxidative stress and vitamin E helps reduce the effects of oxidative stress.

Oxidative stress can also be demonstrated by quantifying levels of methemoglobin production. Mansouri (1989) explored the cellular biochemical processes of methemoglobin and how it is reduced by NADH-methemoglobin reductase after being incubated with sodium nitrite solution, which causes oxidation of erythrocytes. As the oxidative chemicals increase in concentration, the oxidative effects of methemoglobin production are also increased (Mansouri, 1989).

Robin *et al.* (1987) examined differences between methemoglobinemia in horses as compared to humans. Previous studies noted differences between horses and humans in the presence of methemoglobin build up in their blood supply (Robin *et al.* 1987). They found that, in horses, as antioxidant concentration increases, the methemoglobin concentration decreases. Conversely, as the oxidant concentration increases, the concentration of methemoglobin increases (Robin *et al.*, 1987). This behavior of the erythrocytes is the same as human erythrocytes, but there were some differences between human and horse erythrocytes not relevant to this study.

Red maple leaves, when wilted, can cause serious damage to horses' erythrocytes if ingested (Boyer *et al.* 2001). This syndrome is referred to as red maple leaf toxicosis and is due to a currently unidentified toxin in red maple leaves that has strong oxidative effects on equine

erythrocytes. As in humans, horses can suffer from methemoglobinemia when their erythrocytes have been damaged by the red maple oxidizing agent.

Accompanying the methemoglobinemia in red maple toxicosis are morphological changes in erythrocytes. These changes include the formation of cellular membrane inclusions called Heinz bodies (Claro *et al.*, 2006; Webster, 1949). The identification and quantification of Heinz bodies can be representative of the oxidative damage done to erythrocytes and, therefore, can be useful measurements for oxidative damage. One study was done with the objective of exploring Heinz body anemia and methemoglobinemia as it relates to red maple leaf toxicosis. In the study, two ponies were used as subjects and both died after being given dried red maple leaves orally. Before death, they showed clinical signs including polypnea, tachycardia, weakness, icterus, cyanosis, and brownish discoloration of the blood and urine (George *et al.*, 1982). In humans, Heinz body anemia is generally associated with drug-induced oxidative damage (Hopkins and Tudhope, 1974).

Beyond methemoglobinemia and Heinz body formation, another measurement of oxidative stress can be found through the measurement of the amount of reduced glutathione (also referred to as GSH) present in blood samples. Glutathione is a tripeptide molecule made up of three amino acids: glutamate, cysteine, and glycine. Under physiological conditions, glutathione is anionic. Cell regulation of glutathione occurs via the thiol-disulfide exchange equilibria. Reduced glutathione and is considered a major cellular conjugator (Rahman *et al.*, 2007). Evidence suggests gene expression may play a crucial factor in determining the sensitivity of cells to a broad range of toxic chemicals (Rahman *et al.*, 2007). Intra-cellular synthesis of GSH depends on ATP for two steps. GSH can also be generated by reducing

oxidized glutathione (GSSG) in the presence of NADPH. In the body, NADH can be hydrogenated to NADPH and then contribute to this process (Morris *et al.*, 2014). This information would later be utilized in carrying out the plate reader assay for measurement of GSH.

Glutathione exists in the cells of all mammals at millimolar (mM) concentrations. The functions of glutathione include amino acid transport, maintenance of protein sulfhydryls reduction status, and defense against oxidizing molecules. While low levels of glutathione in cells is not lethal, irreversible cell damage can result (Baker *et al.*, 1990). Previous studies have concluded that reduced glutathione, alone and without additional factors, protects hemoglobin from oxidation (Hill *et al.*, 1964).

Previous studies have been done to show glutathione concentration change as a result of oxidative damage. Phenylhydrazine-mediated induction of haem oxygenase activity in rat liver and kidney development of hyperbilirubinemia was studied using glutathione concentration as the marker for oxidative damage (Maines and Veltman, 1983). The results showed that GSH concentration decreased from 5.20 mM to 3.12 mM in the control and phenylhydrazine-treated samples, respectively (Maines and Veltman, 1983).

A possible treatment for red maple toxicosis is the use of intravenous vitamin C (ascorbic acid) and E (alpha-tocopherol) after ingestion of the wilted leaves (Claro *et al.* 2006). However, this treatment cannot completely reverse the effects of oxidation from the toxin. Some effects of vitamin C that are important for erythrocyte metabolism include regulation of the balance between hemoglobin and methemoglobin and transmembrane efflux of reducing molecules into the extracellular space (Benatti *et al.*, 1983). Research was conducted at Otterbein University to test and study whether or not vitamin C and E, if given preventatively through

dietary supplements, are capable of reducing hemolysis in equine erythrocytes exposed to the toxin. This research has provided evidence for reduction of methemoglobin production in toxin-affected erythrocytes after dietary supplementation of the antioxidants (O'Callaghan, 2014).

### **Objective**

Red maple leaf toxicosis in horses is a debilitating condition. It has been reported that horses that ingest dried maple leaves experience severe oxidation of erythrocytes leading to hemolytic anemia and methemoglobinemia (Agrawal et. al, 2013; Boyer et al, 2002; Witonsky et al, 2001). Horses that ingest at least 1,500mg leaf tissue per kg of body weight wilted red maple leaves likely die due to oxidant-induced damage to erythrocytes caused by free radicals (Agrawal et al., 2013; George et al., 1982). The goal of this research is to determine if there are common baseline levels of GSH in equine erythrocytes, and then to determine if red maple leaf extract treatment results in a change to these baseline levels, and whether the antioxidant vitamin C can prevent the oxidation of reduced glutathione (GSH) to oxidized glutathione in cells experiencing oxidative stress. Levels of GSH (glutathione) will be measured in equine erythrocytes *in vitro* via spectrophotometric assays to analyze oxidative effects. The addition of vitamin C is expected to significantly reduce oxidation as indicted by higher levels of reduced glutathione. This study may prove to be beneficial in the control and/or prevention of red maple leaf toxicosis and forms of oxidative stress in other species (Maars et al., 1986).

## **Methods**

**Leaf Tissue:** Red maple leaves were collected from plants grown in Otterbein University's research greenhouse and at the Otterbein Community Garden in Westerville, OH. Plant tissue was dried in a desiccator, ground with mortar and pestle, and stored in a refrigerator at 4° C until use. Within two hours before use, the dried leaf tissue was removed from the refrigerator and 0.05 g was added to 500 µl of distilled water. The tubes were each vortexed thoroughly and then allowed to sit for a minimum of 5 minutes to allow the leaf tissue to leach its extract into the water. The sample were then centrifuges for 5 minutes at 13,200 rpm. After removing the tubes from the centrifuge, the supernatant was pipetted into a separate 1.5 mL microfuge tube. The tubes of leaf tissue were then centrifuged again at 13.2 rpm for 5 minutes, and supernatant was collected. This process was repeated until all supernatant was clear and removed from the top. The specified amount of leaf extract was then added to erythrocyte samples as indicated below.

**Blood:** Blood samples of up to 60 mL were collected from selected healthy horses, as approved by Otterbein's Animal Care and Use board (see appendix A). All horses used in the study were housed at the Austin E. Knowlton Equine Center in Westerville, OH. Horses ranged in age from 10-23 years of age and include both mares and geldings. An 18-guage needle with needle guide into a heparinized 10mL vacutainer was used to draw blood from the jugular vein of the horse. One needle was place in the jugular vein and up to 8 vacutainers were filled with blood at the time of sample collection. Blood samples were immediately placed in an insulated pack with two frozen ice packs while they were transported roughly three miles to the Science

Center at Otterbein University. There, samples were centrifuged for 10 minutes at 3400 rpm. The plasma supernatant was removed from the blood samples. Some of the samples were separated in 1mL aliquots and placed at -80° C until later use. If the erythrocyte sample was to be used in the next few hours, the erythrocytes were washed in phosphate buffered saline solution two times (Horn *et al.*, 1991). The final blood volume of erythrocytes was suspended in 67 mM phosphate buffer with dextrose (pH 7.4). At that point, the blood could be used immediately for treatments, incubation or evaluation. Blood samples that were not being actively worked with were stored at 4 ° C in the laboratory (Mansouri, 1989).

**Treatment of erythrocytes with vitamin C:** In cases where preincubation with vitamin C was indicated, whole blood samples were incubated in six separate groups for 0, 30 and 60 minutes with 10% vitamin C solution (90 mmol) at room temperature and at 37° C prior to addition of the toxin (Claro, *et al.* 2006). The vitamin C solution was made with 0.1585 gm of L-ascorbic acid ordered (Sigma Aldrich) in 10 mL of KPE (define KPE) buffer (0.1 M potassium phosphate buffer with 5mM EDTA disodium salt).

**Extract Treatment:** With preliminary testing, we found that 20 minutes of incubation with red maple leaf extract produced significant amounts of oxidation; hence, samples were incubated at room temperature with red maple extract for 20 minutes in control and vitamin C treated groups (See Figure 3). A solution was made from the red maple extract with 200 µl of leaf tissue in 800 µl of KPE buffer (See Appendix 1). One hundred 100 microliters of this leaf tissue solution were pipetted into 0.5 mL of pre-incubated blood samples. After incubation, the samples were centrifuged at 3500 rpm for 10 minutes at 4° C (Rahman *et al* 2006).

**Reduced Glutathione Measurement:** Upon removal from the centrifuge, the samples were prepared for spectrophotometric analysis with a Biotrak II plate reader (Biochrom Ltd., Cambridge, England). The plasma supernatant was discarded and the erythrocyte pellet was resuspended in 4 volumes of 5% metaphosphoric acid working solution (prepared fresh) and kept at 0 to 4 ° C. The suspension was mixed and centrifuged at 3000 g at 4 ° C for 10 minutes. The upper, clear, aqueous portion was collected to be used in the plate reader assay. GSH levels were measured with the spectrophotometer with the addition of 5,5'-dithiobis (2-nitro benzoic acid; DNTB) at 412 nm (Claro, *et al.* 2006; Griffith, 1980). Procedures for the assay were done as indicated by Rahman, *et al.* (2006), which is a modification of Vandeputte's methods (1964). Twenty microliters of KPE buffer was added to well A (blank) and 20 microliters of each standard (See GSH standards) was added to well B-H in column 1, then 20 microliters of the sample to wells A-H in columns 2 and 3. The assay is kinetic so this must be done quickly. 120 microliters of 50/50 solution DTNB and glutathione reductase were then added to each of the wells in the three columns. Precisely 30 seconds later, 60 microliters of NADPH solution was added to each of the wells in all three columns and spectrophotometer readings began. Readings were taken every 17 to 22 seconds for 2-3 minutes until a significant and relatively uniform yellow pigmentation in all wells was visible. The data was copied into excel where it would be recorded and later analyzed. The GSH standard concentrations were serially diluted to give concentrations of 13.2, 6.6, 3.3, 1.65, .825, and 0.4125 nM/mL. Readings were taken from these standards for each sample put in the plate reader. The sample readings' slope (rate of yellow pigmentation development over time) was compared with the slopes of the known GSH values, and in that manner GSH value for each sample was calculated.



## Results

The objective of this research was to quantify levels of reduced glutathione in normal blood and then determine how levels are altered following the addition of oxidizing agents and antioxidants *in vitro*. Results showed that blood drawn from healthy horses have GSH concentrations that vary greatly and range from 4.3 to 37.3 mM/ul (Table 1). Therefore, blood samples' GSH concentrations cannot simply be compared to one another directly. After the assay for GSH had been performed several times with reproducible result, control readings were taken from blood samples without treatment vitamin C or an oxidizing agent. Figure 1 shows an example of the data from the GSH assay. A blood sample without vitamin C or extract treatment showed a rate of change of pigmentation (optical density) per second of 1086.1 OD/s. Refer to Figure 2 for an understanding of how the slope from Figure 1 is used to calculate GSH concentration of the sample. The slope from sample in Figure 1 is substituted for the y-variable in the equation for the GSH standard line in Figure 2. The equation for that line was then solved for x and results showed a GSH concentration of 33 mM/uL.

Blood samples were then incubated with oxidizing agents (red maple leaf extract) for different amounts of time ranging from 0 to 60 minutes. The results consistently showed that the greatest effect on both control and extract-treated samples was seen after 20 minutes of incubation (Figures 3,4,5). Based on this data, in later experiments, the chosen incubation time with the red maple leaf extract was 20 minutes.

In more advanced trials, four groups of samples were tested. These included blood without either vitamin C or red maple leaf toxin (RMLT), blood with no vitamin C but with RMLT, blood with vitamin C and no RMLT, and blood with vitamin C and RMLT. Results across

three trials indicated that GSH levels in blood with RMLT (no vitamin C) decreased when compared to blood without RMLT or vitamin C (Table 2, Figures 6,7).

Results were less consistent across three trials with the other treatments of blood with vitamin C added. In some cases, blood with vitamin C had higher GSH levels, even when RMLT was also added (Figure 6). However, in other cases, vitamin C-treated blood had lower GSH concentrations (Figure 7).

Overall, information gained from these experiments show that GSH concentration in horse blood is not constant from horse to horse or even within the same horse from day to day. Further, we learned that normal, healthy horses can have blood with GSH concentrations across a large range. It was established that red maple leaf extract does have oxidative effects on equine erythrocytes and that oxidative damage includes reduction of reduced glutathione concentration. Further steps were taken to observe the effect of vitamin C on oxidative damage to erythrocytes but inconsistent results were yielded.

## **Discussion**

The initial measurements of reduced glutathione concentration in equine erythrocytes showed highly varied levels of GSH in the cells. These readings very varied even if the same horse was used at the same time on a different day. However, there did seem to be a normal range in which different samples from different horses at different times of day would remain. Future research with regard to reduced glutathione concentration in equine erythrocytes could determine if these concentrations are dependent on the time of day, temporal proximity to meal time, or other environmental factors.

It is important to understand that the concentrations of reduced and oxidized glutathione (GSH and GSSG, respectively) are inversely related. Their sum remains constant within the cells, but depending on oxidative damage, less of the reduced glutathione will exist in the cell. Perhaps there are other aspects of the horses' natural environment on that have oxidative effects on their blood cells. Perhaps these environmental effects played a role in varying the concentration of GSH in untreated erythrocyte samples. Is there a draw back to having too much GSH? Would the cell be operating on negative feedback to lower the GSH concentration if it gets too high? Is the real problem that GSSG is too high? Things to consider.

The effect of the oxidizing agents on reduced glutathione levels was as expected in that, when erythrocytes were exposed to an oxidizing agent, the concentration of GSH decreased. However, when vitamin C was added to the samples, results were inconsistent. In the first trial, concentrations of GSH were higher in samples that had been incubated with vitamin C prior to oxidant treatment. This would indicate that, as predicted, perhaps vitamin C had protective

effects on equine erythrocytes and can prevent the oxidative damage (lower GSH concentration) from oxidizing agents (red maple leaf extract). However, in the second trial, the opposite effect was seen. From this data one must consider the reasons behind the inconsistency. Other studies evaluating the micromethod assay for determination of glutathione have acknowledged that even with commonly-used assays, results can vary and show inconsistency (Eyer and Podhradsky, 1985).

This research relates to other projects that have been done because there have been several other studies on humans which investigate the oxidative damage done by some monitoring damage to erythrocytes. It is widely known that red maple leaf toxicosis is a problem in horses and veterinarians currently use vitamin C intravenously for treatment of symptoms (Brown, 2007). This is the first research to consider the oxidative effect on glutathione in particular in the equine species.

It should be reasonable to expect similar results from equine erythrocytes as have been seen in human erythrocytes. One study found that the mechanism of the equine erythrocyte KCL co-transporter is the same as in humans: stimulated by high pressure and modified by inhibitors of cellular protein kinases/phosphatases (Gibson and Hall, 1994).

It is also important to consider the clinical relevance of research like this. According to a previous study, the two most important compounds from red maple leaves associated with red maple leaf toxicosis are gallic acid and pyrogallol. These two compounds reach their peak levels in the horses' blood 24 – 48 hours after digestion (Agrawal *et al.*, 2012). If this is the case, then perhaps it would be reasonable to expect an intravenous injection of vitamin C immediately

following ingestion of wilted red maple leaves to prevent oxidative effects at their peak level of absorption.

In another previous study, vitamin C was used as a physiological reducing agent. Vitamin C can have both prooxidant and antioxidant effects. The prooxidant capacity of vitamin C is due to the reduction of transition metal ions, but the mechanisms for antioxidant effects were still unknown (Baysal *et al.*, 1989). Other studies acknowledge that the biological reactions involved with vitamin C are complex and the molecular mechanisms are not fully understood (Benatti *et al.*, 1983). Accurate measurement of GSSG levels has been a difficult process because of the low amounts normally available in cells and the lack of sensitivity of chemical reactions within available assays (Tietze, 1968).

Perhaps one could also consider other reasons for clinical signs due to red maple leaf toxicosis. For example, if there is Heinz body formation or any physical change in the cell shape of erythrocytes, that would change the viscosity of the blood and its ability to flow through the body (Braasch, 1969).

Future endeavors could take this research in one of several possible directions, some of which have already been stated. In general, though, a greater understanding of glutathione behavior in equine erythrocytes may be a good place to start.

A more accurate interpretation of the results may be available if GSSG content was measured in addition to GSH concentration in order to provide a total amount of glutathione in both reduced and oxidized forms. Another study measured glutathione content in human erythrocytes and used GSSG measurements in their methods (Guntherberg and Rost, 1965).

While erythrocytes are ideal model organisms for this type of research, it may be advantageous to examine other types of tissue like the livers of horses that have died from red maple leaf toxicosis to track the oxidative damage and effects throughout different tissues, as glutathione is present in high concentrations in many tissues including the liver, according to one study (Shaik and Mehvar, 2006).

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Table 1. Among six trials testing the GSH concentration in control blood samples, GSH levels ranged from 4.3 – 37.3 mM/microliter. Range = 33 mM/microliter; n=6. Average = 21.48

Trial	GSH Concentration (mM/microliter)
1	33.45
2	25.79
3	7.15
4	20.9
5	37.3
6	4.3

Table 2. The change in optical density/s (slope) is a reflection of how much GSH is present in each of the samples. Higher slope is associated with higher concentration of GSH. Where Vitamin C treatment is indicated, the samples were preincubated with vitamin C solution for 60 minutes at room temperature prior to adding the red maple leaf extract (RMLT).

Treatment	Slope (Change in Optical Density/ s)
Control, Control	517.92
No Vitamin C WITH RMLT	413.36
Vitamin C treated, NO RMLT	529.18
Vitamin C treated WITH RMLT	645.59

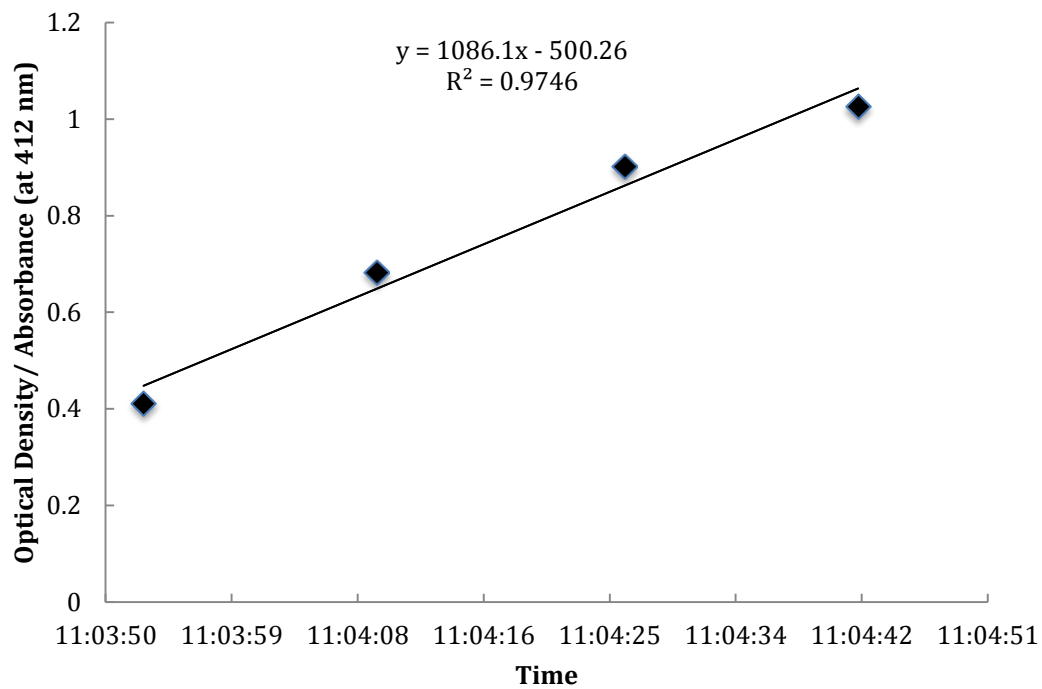


Figure 1. Control readings were taken from blood samples not treated with vitamin C or with an oxidizing agent. The slope of this curve indicates the rate of development of yellow pigment while photospectrometer measurements were taken with the plate reader over the course of about 2 minutes. This was done to establish the normal blood parameters for GSH levels. See Figure 2 for a better understanding of how GSH levels are then calculated.

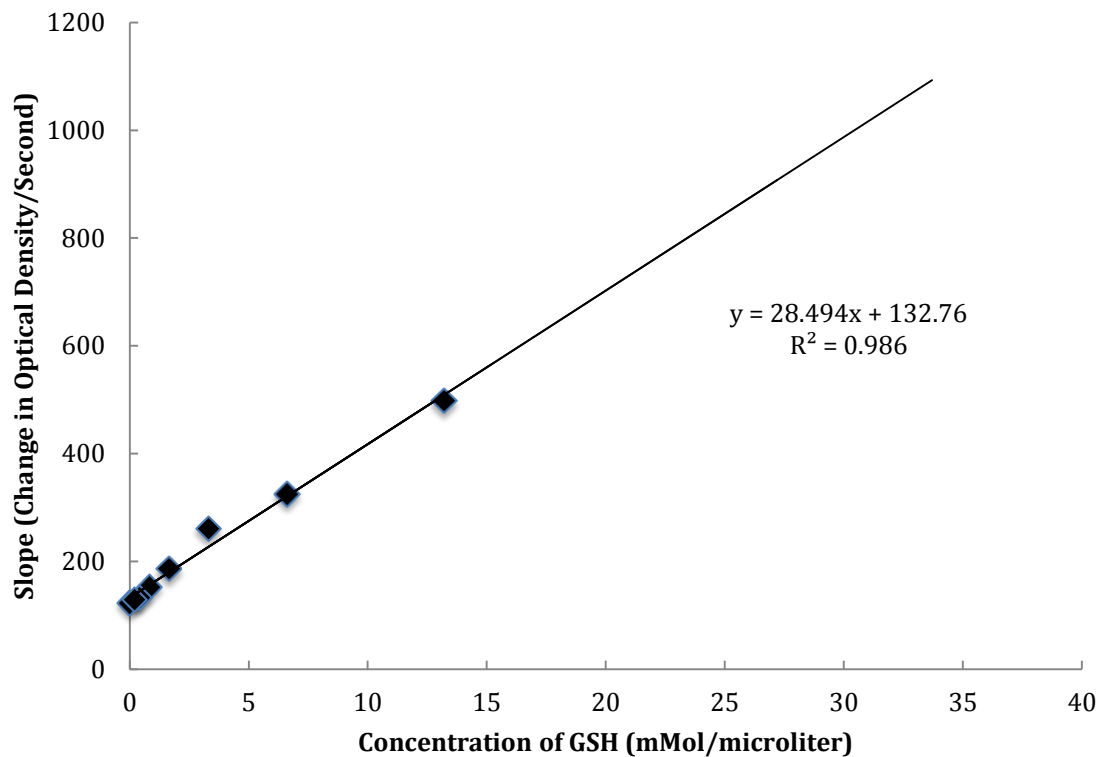


Figure 2. Known concentrations of reduced glutathione were used to create solutions with known GSH values. Their measurements were used to create a graph used for projection of GSH concentration related to rate of development of yellow pigmentation in the plate reader by photospectrometry. In this case, the slope from Figure 1 was inserted for the Y-variable in the equation for this trend line, and solved for X. The solution for X represents the GSH concentration of blood. The concentration of GSH for this sample was calculated to be 33.46 mM/microliter.

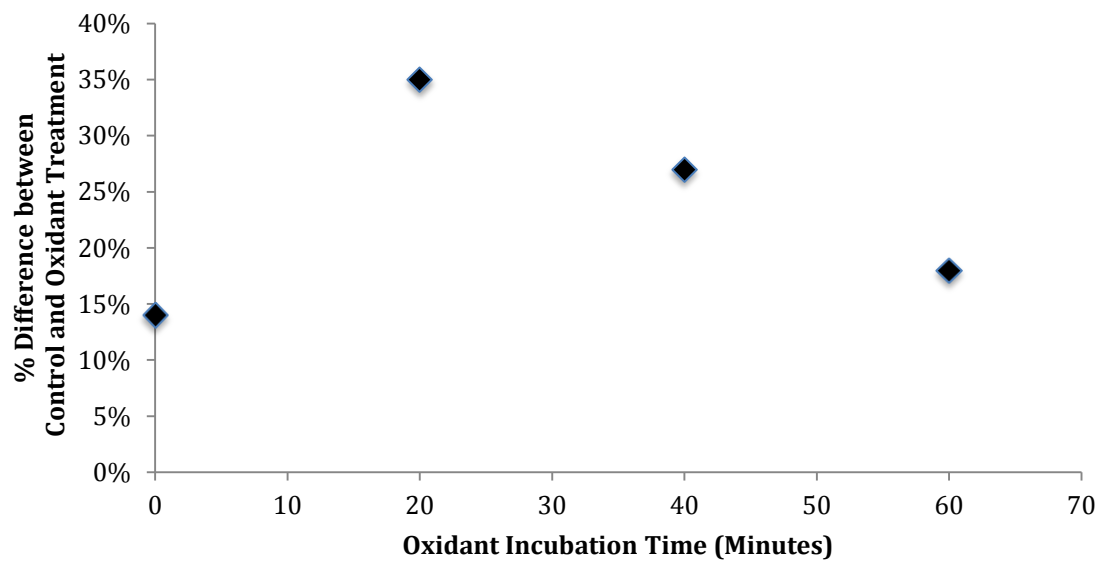


Figure 3. The greatest effect of the oxidizing agent (in this case, Red Maple “A”) was seen after 20 minutes of incubation when compared with samples incubated for 0, 40, and 60 minutes.

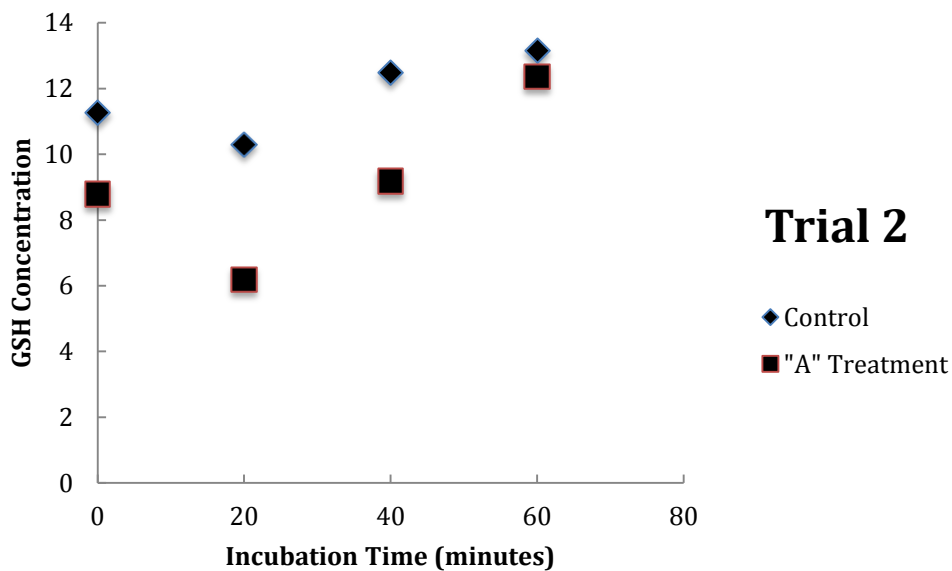
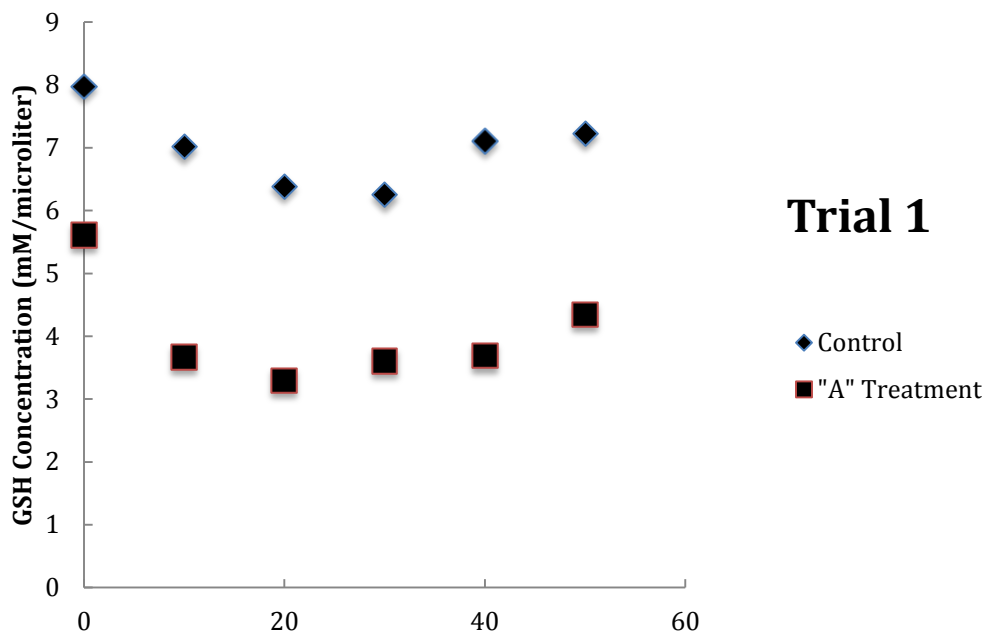


Figure 4. After 20 minutes of incubation, the greatest effect on GSH levels in both control and extract-treated samples was seen. As seen in Trial 1, Trial 2 shows the greatest effect on GSH levels in control and extract-treated samples was observed after 20 minutes of incubation. This information was used to determine incubation time with the oxidizing agent for remainder of experiments involved with this study.



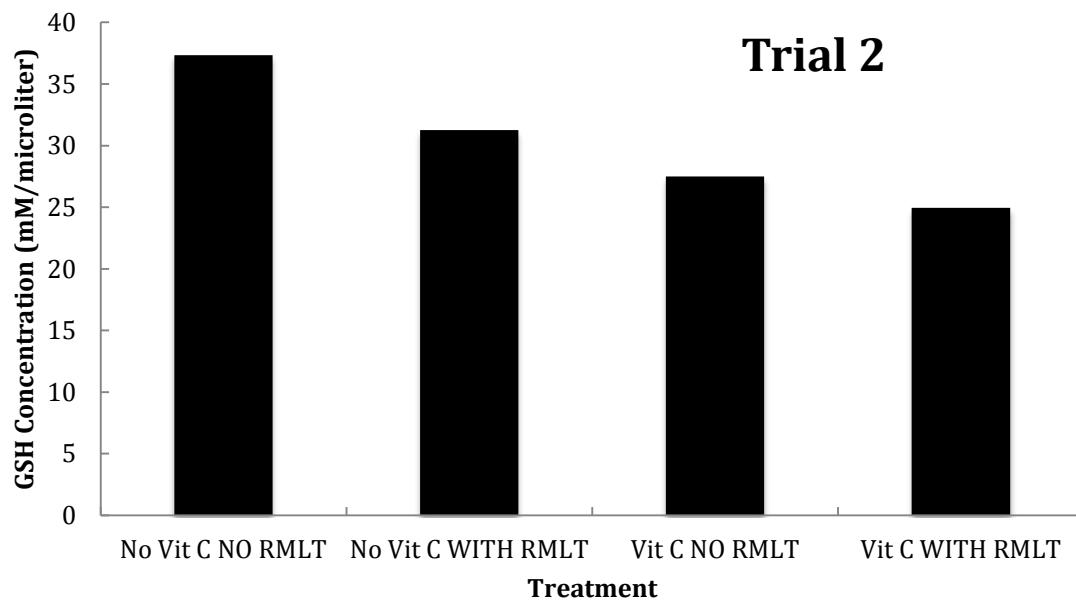
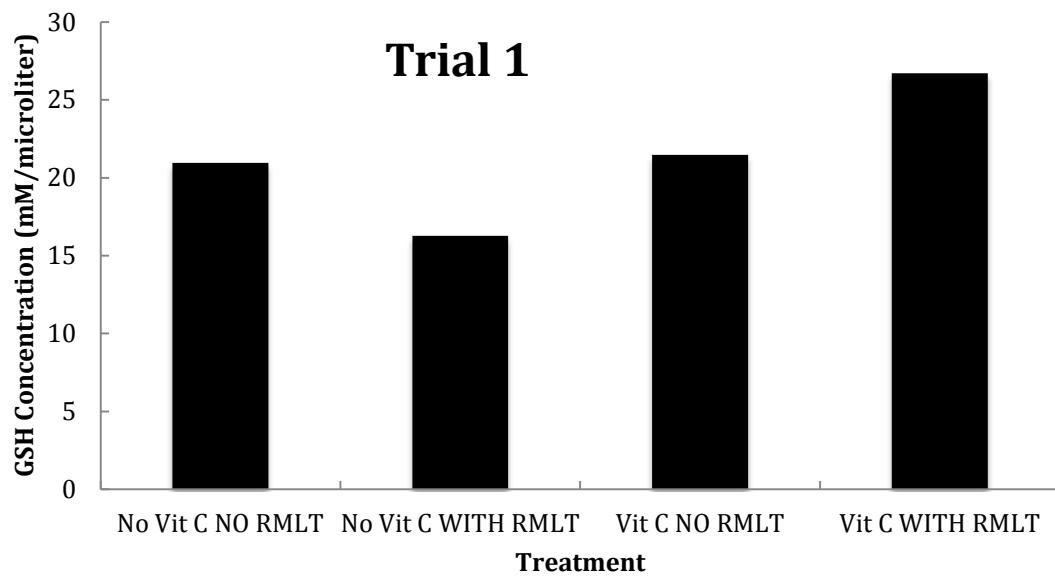


Figure 5. The concentration of GSH in samples treated differently varies. Here, the highest concentration of reduced glutathione is seen in the blood treated with both vitamin C and the red maple leaf extract (RMLT). Trial 2 with the same treatments as Trial 1 yielded different results.

**Appendix A:**  
**ACUC Approval Form**

**Official Memo:** Protocol Approval Confirmation

**Date:** 1/8/14

**Protocol Approval Number:** 2014-01-02-01

**Participant:** Madeline Rohl

**Faculty:** Dr. Jeff Lehman

**Project Title:** Effect of vitamins C and E on oxidative processes in equine erythrocytes

**Approved By:** Animal Care and Use Committee 2013 - 2014

**Committee Chair:** Dr. Sheri Birmingham

## Appendix 1

Below are the procedure to make the buffers required in the study:

**KPE buffer:** Prepare KPE buffer by doing the following: KPE = 0.1 M potassium phosphate buffer with 5mM EDTA disodium salt, pH 7.5. KPE buffer should be made of fresh each time from the stored solutions A and B. Make solution A by adding 6.8g  $\text{KH}_2\text{PO}_4$  to 500mL  $\text{dH}_2\text{O}$ . Store at 4 degrees C. Make solution B by adding 8.5g  $\text{K}_2\text{HPO}_4$  or 11.4g  $\text{K}_2\text{HPO}_3 \cdot 3\text{H}_2\text{O}$  to 500mL  $\text{dH}_2\text{O}$ . Store at 4 degrees C. Make 0.1M phosphate buffer by adding 16 mL of solution A to 84 mL of solution B. Adjust the pH to 7.5 and then add 0.327g EDTA sodium salt.

**GSH standard solutions:** Use the following instructions for preparing the GSH standard solutions of known concentrations: Prepare GSH stock solution by dissolving 1mg GSH/mL KPE, aliquot and store at -20 degrees C for up to several weeks. Dilute stock 1:100 with KPE to make a working solution of 10 micrograms/mL. Dilute 800 microliters of the working solution with 200 microliters of KPE to make the top standard concentration (26.4 nM/mL, 13.2 nM/mL, 6.6 nM/mL, 3.3 nM/mL and so on to 0.103 nM/mL).