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Effect of Supplemental Garlic on the Incidence of Anemia in Horses

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EFFECT OF SUPPLEMENTAL GARLIC ON THE INCIDENCE OF ANEMIA IN HORSES

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EFFECT OF SUPPLEMENTAL GARLIC ON THE INCIDENCE OF ANEMIA IN HORSES

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For my Mom, Dad, and brother Jared,
for supporting and believing in me no matter what

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Victoria Willis May 2021 44 Pages

Directed by: Phillip Gunter, Todd Willian, Martha Day, and Noah Ashley

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Garlic (*Allium sativum* L.) is a spice that has been used for centuries for medicinal purposes or flavoring in food. Garlic has also been commonly used as a fly and pest control for horses and is still commonly used for that purpose today. Recent research has shown garlic may cause Heinz body anemia in horses. The purpose of this study was to evaluate the incidence of Heinz body anemia in horses supplemented with varying rates of garlic. This study included 12 horses divided into 4 groups (control and 3 supplement rates: low (0.0625 g/kg), medium (0.125 g/kg), high (0.1875 g/kg)) that were provided garlic for 74 days. Blood samples and weights were taken on day 0, 25, 50, and 74. Data were analyzed using Proc Mixed of SAS as a completely randomized design, $\alpha = 0.05$. Garlic affected red blood cells ($P = 0.0278$) and platelets ($P = 0.0058$). Hemoglobin ($P = 0.0740$) and Heinz bodies ($P = 0.4055$) were not significantly affected by garlic. Overall, these results show that red blood cell counts and platelets were affected by garlic supplementation, but hemoglobin and Heinz body counts were not. Our findings indicate that garlic supplementation may be safe for horses, but further research is needed.

INTRODUCTION

This project is focused on finding the effect of garlic on the incidence of anemia, specifically Heinz body anemia in horses. Garlic has been used by horse owners for years as a fly control agent, the theory most horse owners has is that the garlic may not only expel a scent from the horses' body that repels flies but also will be in their manure and could lower the fly count in their barn. The problem with garlic is that it comes from the same family as onions, and onions are known to cause a noninfectious hemolytic anemia in horses (Lassen and Swardson, 1995). Anemia can result in problems including weakness, lethargy, reduced exercise tolerance, tachycardia, and tachypnea (Lassen and Swardson, 1995). Since horses are more than just pets but are also athletes and work animals, anemia can be a big issue and easily preventable if it is caused by something that can be easily removed from their feed.

Garlic comes from the same family as onions (Amaryllidaceae) and has a component in it that causes anemia in the horse. Anemia can be defined as a decrease in red blood cell mass leading to a reduction in measured erythrocyte concentration, hemoglobin concentration, and hematocrit below the reference interval (Schwarzwald, 2013). Heinz body anemia is a type of anemia where a Heinz body, which is an irreversible clump of denatured hemoglobin that has attached itself to the cell membrane of an erythrocyte, attaches itself to the cell wall of a red blood cell and causes the body to dispose of the red blood cell since it is damaged. This leads to a decreased number of red blood cells over time as the body will not be able to produce enough new cells to replace the damaged red blood cells.

LITERATURE REVIEW

Garlic

Garlic (*Allium sativum* L.; Family: Amaryllidaceae) is a spice that has been used for centuries to treat humans and animals for various ailments or to flavor food. The *Allium* species can be used to lessen the risk of diabetes and cardiovascular diseases, protect against infections by activating the immune system and have antimicrobial, antifungal, anti-aging as well as anti-cancer properties (Abd-Elhakim et al., 2020). Garlic has also been commonly used as a fly and pest control for horses and is still commonly used for that purpose today. Recent research has shown that garlic may cause anemia and more specifically Heinz body anemia in horses.

Chemical Composition

Yusuf et al. (2018) reported that the chemical composition of powdered garlic on a dry matter basis contained 4.55 mg/100g moisture, 73.22 mg/100g carbohydrates and 15.33 mg/100g crude protein. The crude fat was 0.72 mg/100g while crude fiber was 2.10 mg/100g and ash was 4.08 mg/100g. They found that garlic contained 10.19 mg/100g potassium, 26.30 mg/100g calcium, 10.19 mg/100g phosphorous, 5.29 mg/100g iron, 0.001mg/100g magnesium, 0.34 mg/100g zinc and 0.001 mg/100g manganese. Lead and cobalt concentrations were below detectable levels. Garlic was found to be acidic (pH = 3.91) and contained 4.21mg/100g alkaloids, 3.54 mg/100g tannins, 0.64 mg/100g carotenoids (Yusuf et al., 2018). Stahl and Sies (2003) reported that carotenoids are pigments that play a major role by protecting plants against the photooxidative processes.

Yusuf et al. (2018) further reported that garlic contained 0.80 mg/100g saponin, 5.56 mg/100g flavonoids, 0.04 mg/100g steroids and 0.02 mg/100g cardenolides. Moghimipour and Handali (2014) stated that saponins are bitter but are known for their biological uses including anti-inflammatory and antimicrobial properties as well as others. Flavonoids, according to Panche, Diwan and Chandra (2016), are a group of naturally found substances that have varying structures and can be found in fruit, vegetable, grain, bark, roots, stems, flowers, tea, and wine. The flavonoids are the main component of the garlic that contain the health benefits.

Potential uses

Historically garlic has been known for its antimicrobial and antiparasitic properties (Williams, 2013) which are two areas that equine owners and managers focus on regularly. It would streamline management if they could provide the horse with a daily supplement to address these issues and provide support with a rotational and targeted deworming program as well as a topical antifungal treatment rather than only use dewormer and topical treatments. In the horse industry, garlic has been historically fed for fly and insect control/repellent (Williams, 2013). Garlic, with varying scientific proof, also stimulates immune function, mitigates the effect of oxidants, provides antimicrobial and antiviral protection, enhances foreign compound detoxification, improves radioprotection, restores physical strength, improves resistance to various stresses and has anti-aging effects (National Research Council, 2009), and has the potential to reduce the risk of cardiovascular disease and cancer.

Efficacy

Velerio and Maroli (2005) studied the effectiveness of garlic oil when applied to the skin of humans to deter sand flies and found that 1% and 0.005% garlic oil dilution provided 97 and 40% repellent effectiveness, respectively. Even though garlic in many forms has been added to horse feed for respiratory health, antiparasitic, or fly control, there is minimal research supporting whether garlic helps horses in any of these ways. In a study by Hein et al. (2005) the effectiveness of garlic to repel mosquitos was conducted in humans. They found that when evaluating the number of bites a patient had while taking the garlic supplement versus while taking the placebo, there was no significant difference between the two treatments. The researchers also analyzed the number of mosquitoes that consumed blood during the visit with the human subjects; there was no significant difference in the number of unfed mosquitoes between the two experimental groups. Prior to the human subjects entering the space with the mosquitoes, the mosquitoes were weighed to see if there was a difference in how much blood was consumed during the visit between the experimental groups. Again, they found that the weights of the mosquitoes were not different between the two groups. At the end of the study, the study did not provide enough strong evidence that garlic acted as an effective deterrent against mosquitos.

Safety

There have been some drug interactions with garlic that have been reported in other species. Results for garlic and drug interactions have varied due to differences in garlic consumption and processing; multiple studies have been performed with an interaction between the organosulfur constituents of garlic and various drugs (Zhou et al., 2007). The concern for drug occurs in -SH group containing compounds such as enzymes that are most commonly found in the liver, this organ is also the major location of drug metabolism (National Research Council, 2009). Along with these potential drug interactions, toxicity can occur, and clinical signs can include gastric irritation, decreased sperm production, Heinz body anemia, and occupational asthma (Williams, 2013). A Canadian study (Person et al., 2005) found that when horses were fed freeze dried garlic at more than 0.4 g/kg per day, horses developed Heinz body anemia. In this study, horses fed garlic showed an increase in mean corpuscular volume, Heinz body score, platelet count, serum–free and total bilirubin concentration, and decreases in red blood cell count, blood and mean corpuscular hemoglobin concentration, and serum haptoglobin concentration. In a report by Aslani et al. (2005), the authors identify onion (*Allium cepa*) toxicosis to be characterized by hemolytic anemia that is accompanied by the formation of Heinz bodies in the animals' erythrocytes. The authors go on to explain that the toxic components of all varieties of onions are called aliphatic sulfides, which consist of allyl and propyl di-, tri, and tetrasulfides; of these compounds the allyl are the most potent. This toxin causes a reduction in the activity of glucose-6-phosphate dehydrogenase in the red blood cells; this process interferes with the regeneration of glutathione which is required to prevent oxidative denaturation of hemoglobin. When the hemoglobin is denatured, it will precipitate on the surface of the red blood cells (the Heinz bodies) which then activates intra- and extravascular hemolysis (Aslani, 2005).

Recommended Dosage

NRC (2009) states that though more data are needed, intake levels of 15 mg/kg BW/day of dried garlic powder on a long–term basis is unlikely to result in a risk of an adverse event in horses under normal circumstances and that amounts reaching 90 mg/kg BW/day may not be associated with any adverse events in healthy, non-exercising adult horses (Williams, 2013).

Blood Work

Complete Blood Count (CBC) tests provide owners/researchers/veterinarians with an overarching understanding of the horses' blood content in reference to the red blood cells, white blood cells, platelets, biochemical analysis and electrolytes, providing objective information about the horses' overall health.

According to Messer (1995) the CBC (Hemogram) will show the following components of the red blood cells: Packed Cell Volume (PCV) – percentage of the blood that is made up of red blood cells (south mountain equine); can vary from 30% to 45% (Messer, 1995). Red Blood Cell (RBC) Count – number of red blood cells in a given volume, typically 1 litre, of blood (south mountain equine). The average RBC ranges between 6×10^6 - 12×10^6 (Messer 1995). According to South Mountain Equine, a low PCV and RBC count normally indicate anemia. An increase in PCV and RBC count may be due to several factors, including dehydration or anxiety prior to taking the blood sample (south mountain equine). Mean Corpuscular Volume (MCV) – average volume of each red blood cell sampled (south mountain equine). The average MCV ranges between

36fL and 52fL (Lassen and Swardson 1995). Differences in the average red blood cell volume explain why the PCV and RBC count do not always match and can also be used to help identify causes of anemia (South Mountain Equine). Hemoglobin (Hb) – the average reference range for hemoglobin is 12g/dL – 18g/dL (Messer, 1995). For a horse to perform properly normal concentrations of Hb are necessary. Lassen and Swardson (1995) state that the amount of hemoglobin found in the blood is provided by 2 measurements: MCHC and the concentration of hemoglobin within the red blood cells. Heinz bodies are also formed by the precipitation of denatured hemoglobin (Lassen and Swardson, 1995). Mean Corpuscular Hemoglobin (MCH) – average amount of hemoglobin in each red blood cell (South Mountain Equine). The average MCH ranges between 12.3pg-19.7pg (Lassen and Swardson, 1995). Mean Corpuscular Hemoglobin Concentration (MCHC) – amount of hemoglobin in the circulating blood (South Mountain Equine). Hemoglobin is in red blood cells and allows them to carry oxygen through the body. The average MCHC ranges between 34g/dL-39g/dL (Lassen and Swardson, 1995). These measurements can be useful in identifying causes of anemia or in identifying mineral deficiencies, such as iron (Messer, 1995). Hematocrit (HCT) – one of the main variables that is used to determine if a horse is anemic, and if it is seen to be below the reference level (Schwarzwald, 2013), which ranges from 32% to 52% (Lassen and Swardson, 1995). Factors including hydration, exercise, excitement, or stress can also affect HCT values, so it is not always the best indicator of anemia (Schwarzwald, 2013).

A CBC also provides information about the white blood cells in the body, of which there are five types, the information from the white blood cells will provide the veterinarian with more information about the horse's baseline health, and the results for this will consist of: White Blood Cell Count (WBC) – total number of white blood cells in the blood (South Mountain Equine). Average WBC levels range between 6 x 10^3/µL – 12 x 10^3/µL (Messer, 1995). Increases in the WBC (leukocytosis) are most commonly a result of bacterial or viral infection, stress, drug administration or immune mediated disease (South Mountain Equine). A decrease in the total numbers of white blood cells (leukopenia) may be due to overwhelming bacterial or viral infection, bone marrow disease or endotoxemia (South Mountain Equine). Neutrophils – the most common white blood cells in the horse (South Mountain Equine). The average number of neutrophils range from 2.5 x 10^3/µL to 6.2 x 10^3/µL (Messer, 1995). According to South Mountain Equine a low number of neutrophils (neutropenia) is most often a result of an increase in demand for them. Neutrophilia is an increase in the numbers of circulating neutrophils. This is most often due to bacterial or viral infection, injury, stress or drug administration. Some bone marrow conditions may result in overproduction of neutrophils and result in neutrophilia. Monocytes – important in the breakdown of damaged tissues and the destruction of microbes (South Mountain Equine). The average absolute number of monocytes in a horse's blood sample ranges from 0 x 10^3/µL to 1 x 10^3/µL (Messer, 1995). South Mountain Equine states that an increase in monocyte numbers (monocytosis) may indicate bacterial infection, chronic inflammation or stress. Monocytosis may also be seen during the recovery phase following viral infection. Monocytopenia, low numbers of circulating monocytes, is not clinically significant as no monocytes may be found in the examination of blood from clinically normal horses. Eosinophils – these cells are most commonly associated with parasitic disease and with allergic conditions (South Mountain Equine). The number of eosinophils in a sample can range from 0 x $10^3/\mu L$ to 1 x $10^3/\mu L$ (Messer, 1995). Increased numbers (eosinophilia) may be due to parasitism or hypersensitivity (allergic) reactions. Basophils – rarely found in blood samples. When found in blood samples the appropriate range is 0 x $10^3/\mu L$ to 0.2 x $10^3/\mu L$ (Messer, 1995). When they are found in increased numbers (basophilia), this may indicate long standing allergic disease or ongoing recovery from colic (South Mountain Equine). Lymphocytes – cells with the greatest responsibility for managing the immune system (South Mountain Equine). The average lymphocytes range between 1.2 x $10^3/\mu L$ and 5.0 x $10^3/\mu L$ (Messer, 1995). Lymphocytosis (increased numbers of lymphocytes) can be caused by excitement and exercise or some cancers. Lymphocytosis is a common incidental finding in young horses (South Mountain Equine).

The third component found in a CBC is the platelet counts. Platelets have a number of important functions including blood clotting and the release of various beneficial chemicals at the site of injuries (South Mountain Equine). Platelets in each sample range between 100 x $10^3/\mu L$ and 350 x $10^3/\mu L$ (Messer, 1995). In some cases, toxins or some cancers result in decreased platelet production from bone marrow. An increase in numbers of circulating platelets is rarely seen. This is most commonly due to bacterial infection (south mountain equine).

Anemia

Anemia is a problem in horses or any living organism, for the body to function properly it needs to have oxygen transported through the body regularly, and consistent transport is necessary for adequate athletic performance. Schwarzwald (2013) defines anemia as a decrease in red blood cell mass leading to a reduction in measured erythrocyte concentration, hemoglobin concentration, and hematocrit below the reference interval. Anemia is functionally characterized by a reduced oxygen-carrying capacity of the blood. There are mechanisms the body has in place to protect itself and try to survive when under anemic stress. The first response to anemia according to Schwarzwald (2013) is to increase the 2,3-biphosphoglycerate (2,3-BPG; formerly called 2,3-diphosphoglycerate, 2,3-DPG) content in the red blood cells. What is thought to cause the increase in 2,3-BPG production is the change in intercellular pH that causes the oxygen affinity of hemoglobin to decrease, which leads to more oxygen being readily released in the tissue. The second way a horse's body will work to combat anemia to keep their body functioning normally is to dilate their blood vessels. The process will begin with blood viscosity, a measurement of the thickness and stickiness of blood, providing a direct measure of the ability of blood to flow through the blood vessels. Viscosity will decrease at low erythrocyte concentrations. Erythrocytes are a red blood cell that is typically a biconcave disc without a nucleus. Erythrocytes contain the pigment hemoglobin, which imparts the red color to blood, and transports oxygen and carbon dioxide to and from the tissues. Schwarzwald (2013) notes that tissue hypoxia activates mechanisms of local blood flow regulation and causes the vessels to dilate. The result of this is a decrease in peripheral vascular resistance, a baroreceptor-mediated sympathetic activation, and increase cardiac output, and improvement in oxygen delivery to the tissue according to Schwarzwald (2013). The changes that the body undergoes in the vascular tissue causes an alteration in distribution of blood throughout the body. Instead of the blood being distributed throughout the body to the vital (heart, brain, muscles) and non-vital (skin and intestines) tissues, it is redirected to the vital tissues that are oxygen dependent. Though the body can’t support itself long term with these mechanisms, this is how it adapts to survive until blood chemistry returns to normal. Due to these function’s anemia, even severe, can be tolerated as long as it meets the following criteria: does not occur acutely and is not associated with acute hemolysis or blood loss, there is no loss of blood volume, no other disease process or conditions (such as infection, fever, severe injury, exercise, agitation, or advanced pregnancy) are present which increase oxygen demand Schwarzwald (2013).

Diagnosis of Anemia

Clinical Signs:

Many of the clinical signs of anemia are caused by diminished oxygen-carrying capacity as well as the associated compensating mechanisms of the body. The detection of anemia is based on the severity as well as rate of development; for example, horses with chronic anemia tolerate lower red blood cell mass than horses with rapidly evolving, acute anemia. Mild anemia cases will be harder to detect as the clinical signs will be subtle (Schwarzwald 2013). Schwarzwald (2013) explains that the total red cell volume and hemoglobin concentrations are correlated with the exercise capacity of the horse. While a horse is working, the oxygen demand in the tissue increases, small reductions in the red cell mass decrease the level of exercise intensity at the point tissue hypoxia will occur, which results in lower exercise capacity. Schwarzwald (2013) also describes severe anemia as depression, weakness, tachypnea (abnormally rapid breathing), tachycardia (rapid heartbeat that may be regular or irregular but is out of proportion to age and level of exertion or activity), hyperkinetic pulses (increase in the velocity of the upstroke and amplitude, and pale or icteric mucous membranes).

Hematology:

For diagnosis of anemia, Schwarzwald (2013) describes the hematocrit, hemoglobin concentration, and erythrocyte concentration levels found when a CBC are below the normal reference range. Defining clinical anemia is challenging as factors such as: hydration and splenic contraction in response to exercise, excitement, and/or stress can affect the hematologic values. Due to these varying factors that can affect these values, changes in the hematocrit and hemoglobin may not show the actual change in oxygen-carrying capacity in the blood. Traditionally, to establish etiology and development of anemia is to determine if it is regenerative (a corrective reticulocyte count above 1%) or non-regenerative (a corrective reticulocyte count below 1%: Hadley, 2002). Identifying regenerative anemia is challenging in horses because release of reticulocytes in the blood is extremely minimal compared to other species. Even though the number of reticulocytes released is low when a horse is under severe stress in terms of low red blood cell production, the reticulocytes that are released into the blood stream will have unique characteristics and can be detected.

Blood smears:

A blood smear is a simple way to evaluate the blood when trying to diagnose anemia. A wedge smear uses EDTA-anticoagulated whole blood and is made using two clean glass slides. Once the smears are made, the slides are air dried then stained with

Romanowsky-type stains, but new methylene blue stain can be used to detect Heinz bodies (Bettger et al., 2005).

Bettger et al. (2005) noted that Heinz body prevalence was subjectively scored according to their prevalence (a score of 0 = no visible projections on smear, 1 = 1 or 2 projections/smear, 2 = 1 or 2 projections/ocular view, 3 = up to 10 projections/ocular view, 4 = approximately 75% of all RBCs with visible projections, and 5 = virtually all RBCs with visible projections).

Table 1. Common findings on peripheral blood smears in anemic horses - (Schwarzwald, 2013)

Finding	Description / Appearance	Cause
Rouleaux formation	Erythrocytes lining up in rows, very prominent in equine blood	Normal
Howell-Jolly bodies	Round, basophilic inclusion bodies, found near the periphery of the erythrocytes	DNA remnants, found in 0.1% of normal erythrocytes; more often with severe (regenerative) anemia
Heinz bodies	Pale-pink (Romanowsky stain) or dark-blue (new methylene blue stain) round protrusions of the erythrocyte membrane or bodies within the cell	Denatured aggregates of hemoglobin due to oxidative damage (maple leaf, onion, or phenothiazine toxicity)
Eccentrocytes ('hemighosts')	Erythrocytes with small peripheral cytoplasmatic clearing	Alterations in the erythrocyte membrane and condensation of hemoglobin in the remainder of the cell due to severe oxidative damage (maple leaf toxicity)
Schistocytes (erythrocyte fragments)	Small, irregularly shaped erythrocyte fragments with two to four angular or pointed projections	Endothelial damage and alterations in the microcirculation, associated with DIC, neoplasia, or inflammation of very vascular organs (liver spleen, lung, kidney, bone marrow, placenta)
Autoagglutination	Irregularly shaped erythrocyte clusters of different sizes	Immune-mediated hemolytic anemia, adverse effect of unfractioned heparin, occasionally incidental finding as a result of anti-erythrocyte IgM antibodies present in normal animals
Echinocytes	Uniformly spaced, pointed membrane projections	Crenation artifact, electrolyte depletion
Macrocytosis	Large erythrocytes	Regenerative anemia, prolonged storage of blood
Microcytosis	Small erythrocytes	Iron deficiency, chronic blood loss
Spherocytes	Small round erythrocytes with lace of central pallor (difficult to detect in horses)	Result from the partial removal of red cell membrane by macrophages secondary to antibody or complement attachment (immune-mediated) or oxidative damage (maple leaf toxicity)
Parasites	Teardrop-shaped inclusions with two or four ('maltese cross') organisms per cell	*Babesia caballi* or *Theileria equi*

Causes of anemia:

Schwarzwald (2013) stated that anemia can be caused by a variety of factors: hemorrhage (blood loss anemia), red blood cell destruction (hemolytic anemia), or lack of erythrocyte production (hypoproliferative anemia). These three types of anemias fall into two categories; regenerative: blood loss anemia and hemolytic anemia and non-regenerative: hypoproliferative anemia.

Anemia can have a variety of causes according to Lassen and Swardson (1995), however, there are three main causes: blood loss, hemolytic, and hypoproliferative (Table 2). Blood loss can then be broken down into two subcategories of chronic and acute (Table 2). Chronic blood loss anemia consists of parasitism, gastrointestinal neoplasia, and gastric ulcers; whereas acute blood loss anemia is disseminated intravascular coagulation, trauma or surgery, immune-mediated thrombocytopenia, rodenticide toxicity from warfarin, guttural pouch mycosis, congenital factor deficiency (Factor VIII:C and multiple factors) and equine purpura hemorrhagica.

Hemolytic anemia (Table 2) is broken down into two subcategories: noninfectious and infectious. Noninfectious hemolytic anemia consists of neonatal isoerythrolysis, ingestion of red maple leaf and onions, incompatible blood transfusion, phenothiazine toxicosis, penicillin administration, monensin toxicosis, immune-mediated hemolytic anemia, hemolytic anemia secondary to lymphoma, secondary to systemic organophosphate treatment for cutaneous habronemiasis and glucose-6-phosphate dehydrogenase deficiency. Infectious hemolytic anemia is cause by equine infectious anemia virus, babesia equi or caballi and clostridial infections. The last main cause of anemia is hypoproliferative and can be caused by anemia of chronic inflammation, chronic renal failure, idiopathic aplastic anemia, familial hypoplasia of standardbreds and bone marrow neoplasia (Lassen and Swardson, 1995).

Table 2. Causes of anemia in athletic horses – (Schwarzwald, 2013)

<table>
<tr><th colspan="2">Regenerative Anemia</th><th>Non-regenerative Anemia</th></tr>
<tr><th>Blood Loss Anemia</th><th>Hemolytic Anemia</th><th>Hypoproliferative Anemia</th></tr>
<tr>
<td>Acute:
• Trauma
• Surgery
• Guttural pouch mycosis
• Severe exercise-induced pulmonary hemorrhage
• Internal abdominal or thoracic bleeding
• Coagulation disorders
Chronic
• Gastrointestinal (ulceration, neoplasia, parasites)
• Respiratory (ethmoidal hematoma, guttural pouch mycosis, pulmonary hemorrhage)
• Urogenital
• Coagulation disorders</td>
<td>Infectious:
• Equine Infectious Anemia (EIA)
• Piroplasmosis (Babesia caballi, Theileria equi)
• Anaplasmosis (Anaplasma phagocytophilum)
Immune-mediated hemolytic anemia (IMHA)
• Secondary IMHA (clostridial or streptococcal infection, penicillin, lymphoma, purpura hemorrhagica)
• Autoimmune hemolytic anemia
• Transfusion incompatibility
Oxidative injury (Heinz body anemia and methemoglobinemia)
• Maple leaf toxicity
• Onion/garlic poisoning
• Phenothiazine poisoning
Others
• Exercise-induced intravascular hemolysis
• Terminal liver failure
• Snake bite
• Intravenous administration of concentrated dimethyl sulfoxide (DMSO) or hypotonic solutions</td>
<td>Anemia of inflammatory (chronic) disease:
• Chronic infection
• Chronic inflammation
• Severe trauma
• Neoplasia
Nutritional deficiencies
• Iron deficiency (chronic hemorrhage, rarely nutritional)
• Folate deficiency
Immune-mediated hypoproliferative anemia
• Pure red cell aplasia by treatment with erythropoiesis-stimulating agents
• Immune-mediated hemolytic anemia affecting erythroid precursors
Infectious
• Equine infectious anemia
Others
• Chronic organ dysfunction
• Aplastic anemia
• Myelophthisic disorders
• Myelodysplastic syndromes</td>
</tr>
</table>

Heinz body anemia:

Herman and Javaid (2020) explain Heinz bodies to be present when there is oxidative injury to an erythrocyte. A Heinz body itself is an irreversible clump of denatured hemoglobin that has attached itself to the cell membrane of an erythrocyte. Though how Heinz bodies are formed is not completely understood, the assumption is that amino acid substitutions in the beta-chains of the hemoglobin polypeptides allow them to precipitate within the red blood cell and form the Heinz bodies.

Herman and Javaid (2020) note that Heinz bodies and their formation on the cell membrane can cause a multitude of problems but one of the first problems is that the Heinz body itself, on the membrane wall, increases the rigidity of the cell and can make the cell more likely to fragment in the spleen. The macrophages in the spleen work to remove the damaged cells which in turn forms bite cells. Bite cells are the cells that had the Heinz bodies attached to them but when the Heinz body was removed, it left a 'bite' out of the cell. Due to the increased destruction of cells by removing the Heinz body, this may lead to oxidant-induced hemolytic anemia. Along with that, Heinz bodies can cause intravascular lysis of red blood cells which in turn can lead to hemoglobinemia and hemoglobinuria due to the production of reactive oxygen species. Lastly, Heinz bodies can cause the production of methemoglobinemia or brown plasma which can occur in animals from oxidant-induced injury of iron in hemoglobin, Fe^{2+} becomes oxidized to Fe^{3+} which produces methemoglobin (Herman and Javaid, 2020).

Schwarzwald (2013) describes Heinz body anemia as the result of ingesting toxic levels of maple leaves, wild onions, phenothiazines, or methylene blue (Table 1). When a horse has Heinz body anemia there is oxidative damage to erythrocytes that leads to hemolytic anemia characterized by the presence of Heinz bodies and eccentrocytes on the blood smear. When a horse has Heinz body anemia the clinical signs can vary depending on type and amount of toxin, time since exposure, and occurrence of complicating factors like pigment nephropathy and acute renal failure, but horses usually present with weakness, depression, anorexia, exercise intolerance, signs of hematuria, colic, ataxia, or acute death. For identifying Heinz body anemia in the laboratory setting in moderate to severe cases hyperbilirubinemia, hemoglobinemia, and hemoglobinuria are found as well as Heinz bodies and eccentrocytes can be seen on the blood smears. To treat Heinz body anemia the first step is to remove the source of the toxin, after removing the toxin other treatments consist of administering activated charcoal through a nasogastric tube to decrease the absorption, IV fluid administration to support renal function, and administering high doses of vitamin C.

Past Studies:

A study was conducted by Bettger et al. (2005) in Canada to determine if horses would consume enough garlic voluntarily to produce Heinz body anemia. The study was conducted over a 71-day period where 2 horses consumed amounts of garlic reaching 0.25 g/kg of garlic twice daily. The researchers concluded that horses will voluntarily consume enough garlic to cause Heinz body anemia through analysis of the horses' blood work. The two horses that consumed the garlic developed hematologic and biochemical indications of Heinz body anemia. These indications were shown by increases in their Heinz body scores (HBS), mean corpuscular volume (MCV), mean corpuscular hemoglobin, platelet count, serum unconjugated, total bilirubin concentrations. The conclusion was also represented by decreases in red blood cell count (RBC), blood hemoglobin concentration, mean corpuscular hemoglobin concentration and serum haptoglobin concentration (Bettger et al., 2005).

Overall garlic has been and continues to be used for a variety of functions in the human and animal industries, from flavoring to medical supplements to support western medicine. But, as helpful as garlic is to the body, it can be just as harmful by causing anemia, specifically Heinz body anemia. Anemia is detrimental for the horse that is idle in its work and may just be a pasture pet but can lead to other adverse side effects in the working and sport horses. Even though garlic can be positive in helping horses some medically, it seems to be better to find another way to support horses, so they don't get the side effects of anemia in the process.

MATERIALS AND METHODS

Horses:

Twelve horses were used in this study (Table 3). Horse ages ranged from 5-19 years old. Horses' weights ranged from 370 kg to 598 kg and there were a variety of breeds consisting of quarter horses (5), thoroughbred (2), Warmblood (2), Appaloosa (2), and Pony of the Americas (1).

Table 3: Horse Information

Name	Sex	Age	Breed	Treatment Group
Ollie	Gelding	Unknown	POA	High Garlic
Jasper	Gelding	Unknown	Warmblood	Moderate Garlic
Willie	Gelding	13	Quarter Horse	Low Garlic
Nica	Mare	Unknown	Thoroughbred	Control
Poke	Gelding	5	Appaloosa	High Garlic
Teddy	Gelding	19	Quarter Horse	Moderate Garlic
Autumn	Mare	Unknown	Thoroughbred	Low Garlic
Wesley	Gelding	13	Quarter Horse	Control
Rosie	Mare	16	Quarter Horse	High Garlic
Lady	Mare	11	Quarter Horse	Moderate Garlic
Brandy	Mare	5	Appaloosa	Low Garlic
Captain	Gelding	Unknown	Warmblood	Control

Groups:

Horses were randomly divided into 4 different groups (n=3). Control (treatment 1) and three experimental groups: low garlic – (0.0625 g/kg BW), moderate garlic – (0.125 g/kg BW), high garlic – (0.1875 g/kg BW).

Diet:

Each horse received a balanced diet based on their weight and base nutritional needs. All 12 horses received 1 tablespoon of molasses at each feeding, the purpose of the molasses was to make the diet with the garlic more palatable so the horses would be more willing to eat the mixture. Garlic was top dressed then grain/garlic/molasses was mixed together prior to feeding. Horses were fed twice a day, being fed out of feed bags so the horses consumed only what they were supposed to consume and to prevent spillage and loss of feed. Garlic treated horses received their weighed-out garlic for their treatment group twice daily. Horses were provided pasture and hay *ad libitum* throughout the study. The horses had automatic waterers in their fields that were checked daily to make sure they were working properly so that horses always had access to water.

Housing:

Horses lived in 2 pastures, one mare field (5) and one gelding field (7) for the duration of the study.

Procedure:

The study was conducted over 74 days, and blood and weights were collected on day 0, day 25, day 50, and day 74.

Complications:

At the beginning of this study 3 (2 high and 1 moderate garlic) horses rejected the food/garlic combination and had to be slowly introduced that took about 10 days for them to become adjusted and consume the amount randomly selected for them.

One control horse had colic symptoms on day 10 and was brought into a stall for observations, received half of its diet. It was able to be turned back out on day 11 after the afternoon feeding period and was increased back to full ration the morning of day 12.

On day 15 another control horse was brought into the barn due to a laceration on the left forearm for treatment and to prevent dirt from entering the wound. The horse stayed in the barn until day 23.

One control and one moderate garlic were brought in due to excessive cold/wet conditions. These two horses were brought in on day 23 and turned back out on day 24.

On day 50 a high garlic treated horse was brought in due to sarcoid tumors growing excessively and needing daily treatment and stayed in for 24 days.

While horses were in, they were provided with the same free choice hay that the rest of the horses in the study were provided.

Collection:

Horses were fed their morning diet. Blood was collected from each horse's jugular into a vacutainer tube in the field. The blood was stored in a cooler with ice until the end of the blood draw and was then stored in a refrigerator until being taken to the lab (Murray State University Breathitt Veterinary Center) for analysis. Blood was transported the day after collection.

Data Analysis:

The data were analyzed with SAS 9.4 using the Proc Mixed procedure (specifically designed to fit mixed effect models. It can model random and mixed effect data, repeated measures, special data, data with heterogeneous variances and autocorrelated observations) as a completely randomized design. The main effects include treatment and the date of blood draw and their interaction. PDIFF (requests that p-values for differences) option of LSMEANS (Least square means) was used to separate means when protected by F-test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

WBC:

White blood cell count was not affected by date (*P* = 0.3302) or treatment (*P* = 0.0899). White blood cell count averaged 73.7475 µL (Table 3). However, a trend was detected for WBC between low and medium rates of garlic supplementation with WBC count being greater at the low garlic rate compared with the medium garlic rate. Messer (1995) reported that WBC count ranged between 6000 and 12000 µL. These results are in agreement with Bettger et al. (2005), who reported no difference in WBC count between horses that were provided garlic and those that were not. They also reported no impact from garlic provision over the course of the study. These results may be happening because even though the body isn't undergoing any disease or viral infection it is dealing with an increased stress level since the blood and bodily functions are trying to compensate for the garlic affecting the blood parameter levels.

RBC:

Treatment (*P* = 0.0277) impacted overall RBC count which averaged 7496458.5/L RBC's (Table 4). Red blood cell count for moderate and high garlic supplementation rates did not differ and were less than control, high rate of garlic was less than low rate of garlic, but medium and low rates were not different (Figure 1). Date (*P* = 0.3082) did not alter RBC. Bettger et al. (2005) had similar results finding that red blood cell count in their study decreased significantly in horses that were supplemented with garlic and remained low for the remainder of the study. The RBC count was below the reference limit from days 49 to 71. No changes were observed in the RBC count of control horses. For both studies, RBC count decreased which continues to confirm that garlic supplementation does decrease RBC count.

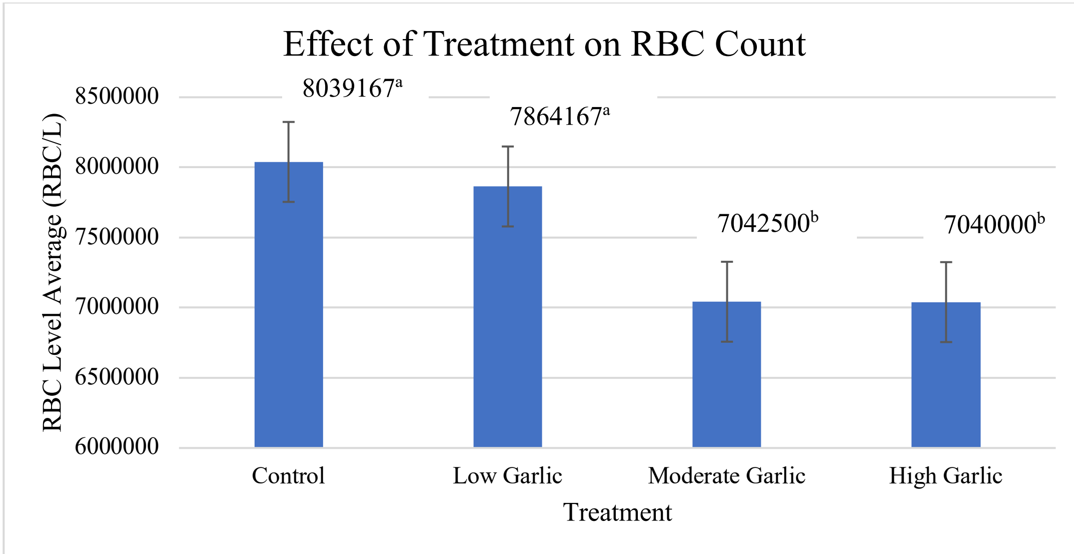

Figure 1: Effect of treatment on RBC

Control – 0g/kg garlic, Low garlic – 0.0625g/kg garlic, Moderate garlic – 0.125g/kg garlic, high garlic – 0.1875g/kg garlic

[a,b] means within a row without a common superscript differ ($P < 0.05$)

Hemoglobin:

Hemoglobin was not affected by date (*P* = .5139) or treatment (*P* = 0.0740). The average hemoglobin level was 12.936g/dL (Table 4). However, a trend was noted for treatment such that hemoglobin was lower for medium and high rates of garlic when compared with control (Figure 2). Bettger et al. (2005) found that blood hemoglobin concentration decreased in garlic treated horses and was below the lower reference limit from days 49 to 68. In control horses, hemoglobin remained unchanged and within the reference range. In this study we found that the decrease in hemoglobin was not significant but there was a trend that follows the results from the Bettger study.

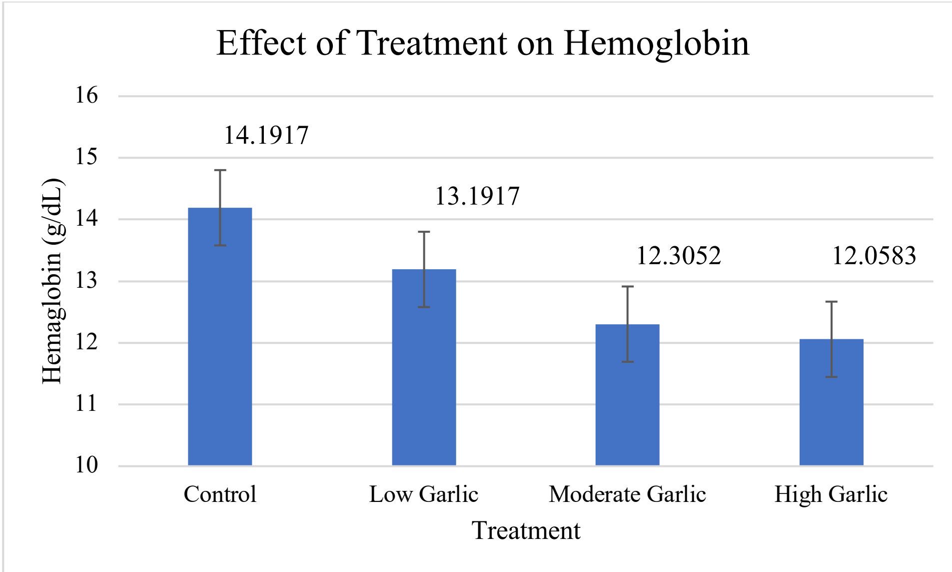

Figure 2: Effect of treatment on hemoglobin concentrations.

Control – 0g/kg garlic, Low garlic – 0.0625g/kg garlic, Moderate garlic – 0.125g/kg garlic, high garlic – 0.1875g/kg garlic

HCT:

Treatment (*P* = 0.1569) did not affect HCT level. A trend (*P* = 0.0729) was noted for date where HCT and an increasing trend over the study period that HCT level for day 0 was less than day 25 and 50. The average HCT was 37.02% (Table 5). Bettger et al. (2005) found that the HCT decreased significantly in garlic treated horses and approximated the lower reference limit through day 68. The HCT of control horses did not change over time and was maintained within the reference range. In this study the results we have are opposite results. Bettger et al (2005) follows what would be expected of horses moving towards anemia since the HCT levels decreased overtime. In this study the horses have results the opposite from what would be expected. We expected to see HCT levels to decrease as garlic supplemented levels increased as well as HCT levels decrease throughout the study. Since we did not see any horses become anemic then that explains why HCT levels were not below the reference limit, even though we had a decrease in RBC.

MCV:

Blood draw date (*P* = 0.1084) did not affect MCV level in the blood. Blood MCV control treatment levels averaged 49.7354 fL (Table 4), which falls within the range reported by (Lassen and Swardson, 1995). A trend was noted for treatment (*P* = 0.0974) effect on MCV level where medium garlic was greater than control and low rate of garlic supplementation. Bettger et al. (2005) found that the MCV increased significantly in garlic treated horses and reached peak values on days 68 and 71; the upper reference was exceeded from days 42 to 71. In control horses, MCV remained unchanged and within the reference range. They may have seen this effect due to the higher amounts of garlic fed. They also compared the garlic treated horses to control horses whereas in the current study we compared different levels of garlic to each other and a control as well as horses in our high garlic treatment group were fed less garlic than the amount of garlic fed in the Bettger study.

MCH:

Blood draw date (*P* =0.7867) did not affect MCH levels. Treatment (*P* = 0.0194) altered blood MCH values, with an average at 17.772pg (Table 4). Mean corpuscular hemoglobin was greatest for medium garlic which did not differ from high garlic, both were greater than control, medium garlic was also greater than low garlic, but low and high garlic did not differ (Figure 3). Bettger et al. (2005) found that the MCH increased significantly in garlic treated horses; the MCH in garlic treated horses was greater than the upper reference limit from days 59 to 71. Though the results between the Bettger study and this study differ slightly, in this study the MCH value did not continuously increase but rather decreased some from moderate garlic to high garlic, and it still was all greater than the control. The MCH may be increasing but that doesn't mean that their oxygen carrying capacity is as well as we see that RBC count is decreasing and MCH is increasing so the hemoglobin is inundating the cells so they can try and make up for the lack of oxygen being transported through the blood from the decrease in RBC.

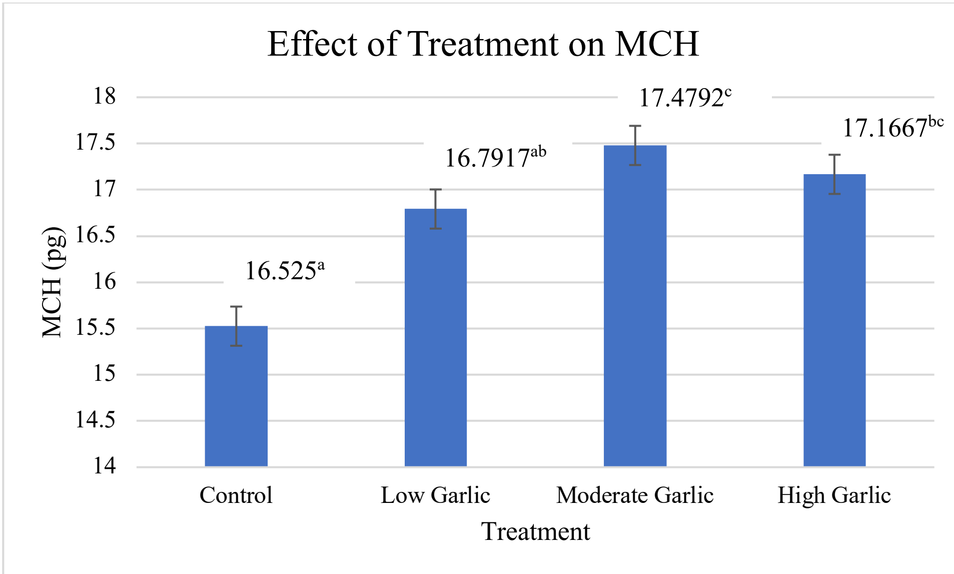

Figure 3: Effect of treatment on MCH count

Control – 0g/kg garlic, Low garlic – 0.0625g/kg garlic, Moderate garlic – 0.125g/kg garlic, high garlic – 0.1875g/kg garlic

[a,b,c] means within a row without a common superscript differ ($P < 0.05$)

MCHC:

Treatment (*P* = 0.2842) did not affect MCHC blood levels. Date of the blood draw (*P* < 0.0001) affected MCHC levels in the blood with an average of 34.233/dL (Table 5). Blood MHCH levels on day 25 and 74 were not different and were greater than MCHC levels on day 0 and 50, which did not differ. While MCHC levels differed by date they fell within the range reported by Lassen and Swardson (1995). Bettger et al. (2005) found that the MCHC of their garlic treated horses decreased on day 28 and reached a minimum on day 49. What we found in this study and what Better et al. found in theirs is different. In their study the MCHC levels continuously decreased, and, in this study, it decreased, increased, then decreased again. It makes sense for the MCHC levels to decrease consistently through the study if the hemoglobin decreases consistently because it's the mean concentration of the hemoglobin so the Bettger et al. study follows that logic unlike this study. The MCHC may have fluctuated due to the decrease in RBC's could cause a decrease in hemoglobin levels thus a decrease in MCHC, then the body would try to make more hemoglobin but because there was not as many RBC's the hemoglobin would become highly concentrated in the few RBC's causing the MCHC to then increase again.

Platelets:

Platelets were unable to be analyzed due to clumped platelets from some of the horses. Factors that could cause platelets to clump without the rest of the blood sample clotting (being unreadable) includes repeated blood draws, changes in blood flow, a delay in the blood analysis or temperature of storage conditions (Gardón et al., 2017). Bettger et al. (2005) found that platelet count increased significantly in garlic treated horses peaking on day 55; platelet counts remained within the reference range through day 71. No change in platelet count was found in control horses.

Neutrophils:

Neutrophils were not affected by date (*P* = 0.2881), but a trend was noted for treatment (*P* = 0.0597) and had an average of 3929.48 µL (Table 4), with control and high garlic being greater than medium and low garlic. Bettger et al. (2005) found that in both groups of horses, no differences were found between treatments or changes over time in total cell counts of neutrophils. Because neutrophils are a type of WBC, the results from both studies are not a surprise. In these studies horses were not infected with a virus or a bacterium where neutrophils would increase, therefore neutrophil levels did not change substantially from garlic treatment.

Lymphocytes:

Lymphocyte levels were not affected by blood draw date (*P* =0.6446). Treatment (*P* = 0.0461) impacted lymphocyte levels with an average of 2652.61 µL (Table 4) where low garlic had greater lymphocyte levels than the control, with medium and high garlic rates being intermediate and not different than control or low garlic. Bettger et al. (2005) found that lymphocyte count decreased significantly in garlic treated horses; day 0 lymphocyte counts for garlic treated horses were greater than the upper reference limit. No changes in lymphocyte counts were found in control horses. Because lymphocytes are most responsible for immune function the results from either study doesn't show the response to garlic necessarily but rather the average horses in each treatment normal lymphocyte level in response to an infection or just where its level normally is. The horses in this study may have been different but all stayed within the normal range.

Monocytes:

Treatment (*P* = 0.0045) impacted overall Monocyte levels in the blood with an average level of 295.00 µL (Table 4). Monocyte level for the control and low garlic as well as for the control and moderate garlic did not differ. Control and high levels of supplemented garlic did differ with monocyte levels in high garlic being higher. Monocyte levels in low garlic and moderate garlic treatments also differed, with moderate garlic treated horses having lower monocyte levels in the blood. The levels of monocyte content in the blood did not differ between low garlic and high garlic. Monocyte levels between moderate garlic and high garlic did differ with high garlic having a higher monocyte count. A tendency was detected for changes in monocyte levels due to date of the blood draws (*P* = 0.0933), such that day 0 was greater day 25, 50 and 74. Bettger et al. (2005) found that in both groups of horses, no differences were found between treatments or changes over time in total cell counts of monocytes. Monocytes become more prevalent when tissue break down is increased and decreases when tissue breakdown slows down. For what is known about the effects of garlic on horse's monocyte results should be reflected like the Bettger et al. study. In this study, there was a horse in the high garlic treatment group that had sarcoid tumors, so that could have increased tissue breakdown in new tumors were developing as well as other horses in the study could have underlying problems we do not know about. With that said, all the levels were within the recommended levels.

Eosinophils:

Eosinophil concentrations were not affected by date of blood draw (*P* = 0.2358) or treatment (*P* = 0.5814) that averaged 451.35 µL. Bettger et al. (2005) found that in both groups of horses, no differences were found between treatments or changes over time in total cell counts of eosinophils. The results from both studies follow logic because eosinophils are affected mainly by parasitic infection.

Basophils:

Basophil levels were not impacted by blood draw date (*P* =0.8147) or treatment (*P* = 0.2048). Bettger et al. (2005) found that in both groups of horses, no differences were found between treatments or changes over time in total cell counts of basophils. This study agrees with the Bettger study and these results make sense because Basophils are rarely found in a basic blood sample.

Heinz Bodies:

Heinz body levels were not affected by treatment (*P* = 0.4055) or date of blood draw (*P* = 0.4055) where the average level of Heinz bodies was 0.0208 Heinz Bodies present. Bettger el al. (2005) found a significant increase in mean Heinz body score in garlic treated horses. Mean Heinz body score increased on day 28, peaked on day 59, and remained high through day 71, compared with the baseline value. In control horses, the Heinz body score varied from with no significant change over time. In this study only one horse had a Heinz Body score of 1 on day 50 in the high garlic group but it disappeared by day 74. So, based on this study it looks as though there is progress towards finding an amount of garlic to feed that may not cause Heinz body development.

Table 4: Effect of Treatment on Blood Chemistry

Variable	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM
WBC	7330.83/µL	7745.83/µL	6768.33/µL	7650.00/µL	280.255
RBC	8039167/L^{a}	7864167/L^{ab}	7042500/L^{bc}	7040000/L^{c}	284939.5
Hemoglobin	14.1917g/dL	13.1917g/dL	12.3042g/dL	12.0583g/dL	0.6108
HCT	39.19%	38.18%	36.39%	34.90%	0.01413
MCV	48.7083fL	48.5750fL	51.7333fL	49.9250fL	0.9458
MCH	16.5250pg^{a}	16.7917pg^{ab}	17.4792pg^{c}	17.1667pg^{bc}	0.2118
MCHC	33.9750g/dL	34.6667g/dL	33.8583g/dL	34.4333g/dL	0.3297
Platelets		231042/µL	218375/µL	243417/µL	12896.75
Neutrophils	4240.83/µL	3971.67/µL	3435.42/µL	4070.00/µL	204.6575
Lymphocytes	2299.17/µL^{a}	2976.67/µL^{b}	2686.25/µL^{ab}	2648.33/µL^{ab}	162.985
Monocytes	284.17/µL^{ab}	314.17/µL^{ac}	241.67/µL^{b}	340.00/µL^{c}	18.0292
Eosinophils	478.33/µL	436.67/µL	367.08µL	523.33/µL	80.236
Basophils	35.8333/µL	36.1667/µL	37.9167/µL	48.8333/µL	10.6207
Heinz Bodies	0	0	0	1	0.04167

^{a,b,c} means within a row without a common superscript differ ($P < 0.05$)

Table 5: Effect of Blood Draw Date on Blood Chemistry

Variable	Date 0	Date 25	Date 50	Date 74	SEM
WBC	7560.0µL	706.67µL	7186.67µL	7700.83µL	280.255
RBC	7329167/L	7227500/L	7481667/L	7947500/L	289939.5
Hemoglobin	13.3167g/dL	12.2125g/dL	12.8083g/dL	13.4083g/dL	0.610825
HCT	35.05%	36.84%	40.30%	35.87%	0.01413
MCV	48.2083fL	51.0333fL	48.8833fL	50.8167fL	0.945825
MCH	16.9917pg	16.9125pg	17.1667pg	16.8917pg	0.211775
MCHC	35.2917g/dL[a]	33.2333g/dL[b]	35.1250g/dL[a]	33.2833g/dL[b]	0.329725
Platelets	190917/µL		195083/µL	338583/µL	13055
Neutrophils	3907.50/µL	3865.42/µL	3696.67/µL	4248.33/µL	204.6575
Lymphocytes	2825.83/µL	2537.92/µL	2619.17/µL	2627.50/µL	162.985
Monocytes	335.00/µL	288.33/µL	275.83/µL	280.83/µL	18.029152
Eosinophils	445.83/µL	312.92/µL	540.83/µL	505.83/µL	80.236
Basophils	37.667/µL	39.0833/µL	43.6667/µL	38.3333/µL	10.62065
Heinz Bodies	0	0	1	0	

[a,b] means within a row without a common superscript differ ($P < 0.05$)

CONCLUSION

The rates of garlic fed to horses did affect some individual blood component levels, but incidence of Heinz body anemia was not detected. Heinz bodies were detected in the blood of one horse; however, formation was minimal and not different from the other horses. However, as the dose of garlic fed increased, the concentration of RBC decreased, and hemoglobin tended to decrease. Mean Corpuscular Hemoglobin increased with an increase in the amount of garlic fed. Garlic does alter some blood component levels but most of the changes stayed within the reference levels even though they were affected. We found that when first introduced the garlic reduced the MCHC levels but then it returned to original levels in the next two blood draws, showing that the body was able to recover and potentially compensate for how the garlic affects the blood.

Future studies need to be done with lower amounts of garlic, to represent what an owner would actually feed, to confirm that Heinz bodies will not form at these lower garlic levels. These future studies also need to continue to evaluate the health of the red blood cells and hemoglobin to make sure that it is safe for the horses at the lower levels.

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Appendix I

INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE

DATE: October 12, 2020

TO: Dr. Phillip Gunter
FROM: Western Kentucky University (WKU) IACUC
PROJECT TITLE: Effect of supplemental garlic on the incidence of anemia in horses
SUBMISSION ID: 20-08

ACTION: APPROVED
APPROVAL DATE: October 12, 2020
EXPIRATION DATE: October 12, 2023

Thank you for your submission of New Project materials for this project. The Western Kentucky University (WKU) IACUC has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a project design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission based on the applicable federal regulation.

Please note that any revision or new funding awards to previously approved materials must be approved and/or recognized by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All UNANTICIPATED PROBLEMS, SERIOUS, and UNEXPECTED adverse events must be reported promptly to this office. Please use the appropriate reporting forms for this procedure.

All NON-COMPLIANCE issues or COMPLAINTS regarding this project must be reported promptly to this office.

This project requires continuing review by this committee. Please use the appropriate forms for this procedure when requesting revisions. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of 10/12/2023.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Robin Pyles at (270) 745-3360 or ori@wku.edu. Please include your project title and reference number in all correspondence with this committee.

- 1 -

Appendix II

WKU

A LEADING AMERICAN UNIVERSITY WITH INTERNATIONAL REACH
COLLEGE OR EXTENDED CAMPUS

To: Victoria Willis
801-05-2942
Agriculture

From: Dr. Ranjit Koodali, Dean
The Graduate School

Cc: Dr. Jennifer Gill
Dr. Fred Degraves
Deanna Durrant

Date: July 23, 2020

Congratulations! The Graduate Student Research Committee of the Graduate Council has recommended that you be awarded $2,535 for your research project. Enclosed are two copies of your contract with your account number. ***In order to have access to your account, you must sign and return the Initial Contract to the Graduate School.*** Purchase equipment, supplies, etc. with the assistance of the departmental office associate who is knowledgeable of placing purchases using online requisition procedures.

DO NOT LOSE YOUR ACCOUNT INFORMATION. You will need your account number for all requisition orders placed for your research project. It is the student's responsibility to keep up with information provided regarding the research project; this includes account number, contracts, etc.

If travel expenses are part of your authorized grant expenditures, you will need to send a copy of your travel expense voucher through the Graduate School Office for approval (See "Procedures for Travel Expense Vouchers"). For services rendered by outside parties, you will need to file a Payment Authorization Form through the Graduate School Office (See "Procedures for Payment Authorization Forms").

This grant has an expiration date of 07/23/2021. You are requested to abide by the conditions of the research contract. Once the grant has expired, provided you have not filed and been approved for an extension, any monies not used will be returned to the Graduate Student Research budget. The Graduate School Office is not responsible for reminding you to file an extension. All expired accounts will be closed promptly.

Complete and return the Final Contract with your final report and itemized spreadsheet at the conclusion of your research. If your research results in publication, you may wish to give Western Kentucky University's Graduate Student Research Committee recognition (See "Guidelines for Grant Funding" for recommended wording).

If you have any questions concerning your grant, please contact Jennifer Paul in the Graduate School Office at 270-745-3482.

The Spirit Makes the Master
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phone: 270.745.2446 | **fax:** 270.745.6950 | **e-mail:** graduate.studies@wku.edu | **web:** www.wku.edu/graduate