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Effects of Plasma Sample Handling On Equine Insulin Assays

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EFFECTS OF PLASMA SAMPLE HANDLING ON EQUINE INSULIN ASSAY

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Abstract

Often, the assumption is made that the results of the various laboratory tests being run are accurate, and that abnormal results reflect a physiologic change occurring in the patient. Unfortunately, this assumption is not always true. Inaccurate results, for various reasons, are an inherent part of diagnostic testing. Having inaccurate results is often worse than having no results at all, because they can lead to an incorrect diagnosis or result in unnecessary testing, wasting the client's financial resources, or potentially expose the patient to unnecessary risks in pursuit of the cause of an abnormality that does not exist. With appropriate attention, these can be minimized. The purpose of this study was to evaluate how the time from blood collection to centrifugation effect equine plasma insulin values. For this study, jugular blood samples were collected from 10 horses. Twelve 5 mL Idexx EDTA tubes were collected one time from each horse. Blood tubes were stored in a 4°C refrigerator until being centrifuged for plasma collection. Samples were centrifuged at various intervals from the time of collection: 15 min post collection, and then at 4, 8, 12, 24, and 36 hr 2500 rpm for 10 minutes and plasma collected. Plasma insulin was evaluated by two separate laboratories, Lab A and Lab B. ANOVA single factor was utilized to determine whether time from sample collection to centrifugation was significantly different. Data was analyzed at P=0.05 for all statistical tests. Labs A and B plasma insulin concentrations were compared at each sample time using a two-sample T-test assuming unequal variances for the means. There was no significant difference between centrifuge times within each lab (P= 0.982). However, when

comparing each sample time between laboratories, there was a significant difference at 4 hrs and 36 hrs (P= 0.024 and P=0.038). Further research is needed to evaluate when time from blood collection to centrifugation affects equine plasma insulin values in order to assure when veterinarians would know if a sample were to be viable.

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Chapter I: Introduction

Diagnostics are an aid in medicine to provide a diagnosis and proper treatment. To properly utilize diagnostics the understanding of how to perform them must be known. A sample starts with collection. Each type of sample is typically collected and analyzed or evaluated in a specific way. When samples are not collected, handled, or analyzed properly, then misdiagnosis can occur, and patients are prescribed the wrong medication or treatment. After collection the next step is sample handling. Handling errors can occur in the clinic, during storage or shipping, or at the lab. Sample collection and handling requires precision, patience, and attention to detail. The purpose of this study was to evaluate how time from blood collection to centrifugation affect equine plasma insulin values. For this study, jugular blood samples were collected from 10 horses. Twelve 5mL Idexx EDTA tubes were collected one time from each horse. Blood tubes were stored in a 4°C refrigerator until being centrifuged for plasma collection. Samples were centrifuged at various intervals from the time of collection: 15 min post collection, and then at 4, 8, 12, 24, and 36 hr 2500 rpm for 10 minutes and plasma collected. Plasma insulin was evaluated by two separate laboratories. ANOVA single factor was utilized to determine if centrifuge time was significant. Data was analyzed at P=0.05 for all statistical tests. Labs A and B plasma insulin levels were compared at each sample time using a two-sample T-test assuming unequal variances for the means.

Based on previously published literature, it was expected that after 18 hrs delay from collection to centrifugation, insulin concentrations would decrease. Results from this study could impact sample handling in the veterinary field. If a critical time period

could be identified, then veterinarians and technicians would know whether or not they had to rush to get samples centrifuged between farm or house calls.

Chapter II: Literature Review

Introduction

Diagnostics are an aid in medicine to provide a diagnosis and proper treatment. One blood sample will let a veterinarian evaluate red blood cells (RBC), white blood cells (WBC), electrolytes, glucose, kidney, liver, and pancreatic enzymes, among other things. Diagnostics are used daily to evaluate the health of people or animals. If diagnostics were not available, issues like blood clots could not be located, heart function could not be evaluated, liver failure could not be detected until it is too late. Diagnostics are available to aid doctors and health staff in treating patients to the best of their ability.

Sample Collection

There are a variety of specimen samples that can be used to diagnose animal health in veterinary medicine, including skin, hair, saliva, fecal, urine, and blood. Each type of sample is typically collected and analyzed or evaluated in a specific way. When samples are not collected, handled, or analyzed properly, then misdiagnosis can occur, and patients are prescribed the wrong medication or treatment. Sample collection and handling requires precision, patience, and attention to detail.

When collecting a skin sample, questions that need to be answered include: what type of tissue is being collected? How does the sample need to be preserved? How many samples need to be collected? These tissue samples need to be biopsied or cut with a scalpel rather than scissors. Forcep use needs to be avoided or done very carefully as pulling on the sample can damage the cells. If tissue samples are not handled or fixed

properly, they will be deemed unsuitable for evaluation (Woldermeskel, 2019). Fixing a sample is done to preserve the cells and components in a life-like state. If this is not done properly, the cells could be altered or decay, resulting in misdiagnosis or inconclusive results.

Hair is a strong, stable tissue that is simple to collect, transport, and store (Pfeifer, 2022). Hair samples are collected to evaluate mites, perform dermatophyte cultures, and for toxicological studies. This allows for diagnosing different mite species, or conditions such as ringworm and rain rot. Even though hair is a strong tissue, damage should be minimized so that microscopic evaluation can take place. To that end, rubber coated forceps are recommended for collection (Wesche & Casey, 2005). Hair needs to be dry, but does not need to be cleaned before collection. Collection areas should not be shaved to prevent removal of mites, or bacteria with clippers. The follicle needs to be obtained along with the full hair strand. Hair that will be cultured for dermatophytes needs to go into the correct transport media as quickly as possible to prevent contamination to the hair and to the media. If it is being examined microscopically, it needs to be fixed to a microscope slide.

Fecal exams can reveal a lot of information regarding patient health. There are many diagnostic tests that can be conducted using fecal material, and a variety of collection methods. Some tests require swabs that are to be placed in a specific media or fresh fecal samples in leakproof containers. Specific medias might be required to aid or prevent the growth of bacteria. Others support the testing of microbes within the gut. A full sample in a leak proof container needs to be contaminate free to prevent new bacteria being introduced. These are needed to evaluate consistency, color, and aid in microscopic

evaluations of microbes and live bacteria. Fecal diagnostic tests require the correct volume of material, depending on the test being conducted. Also, for some tests, samples must be evaluated within 24 hrs of collection, while others could be refrigerated until submitted. All samples should be handled with care and should be contaminant-free, meaning without grass, litter, shavings, or foreign bacteria being introduced. The best samples are collected directly from the rectum.

Animal urine collection is a vital part of veterinary practice for ascertaining animal health and in scientific investigations for assessing the results of experimental manipulations (Kurien, Everds, & Scofield, 2004). Untainted animal urine collection is very challenging, especially with small rodents, and is an almost impossible task under conditions of microgravity. The fundamental aspects of urine collection include ease of collection, quality of sample, prevention of contamination, severity of procedures used, levels of pain caused to the animal and refinement of methods to reduce stress, pain, or distress (Kurien, Everds, & Scofield, 2004). A small quantity of urine will suffice for qualitative urinalysis, which includes the measurement of urinary pH, protein, glucose, bilirubin, hemoglobin, ketone, urobilinogen, and creatinine levels. The best way to obtain sterile urine is by cystocentesis. While this technique is the most sterile way to collect urine, it can result in a false positive for blood cells or bacteria if the patient is not prepped properly. The second-best collection method is a voided, or ‘free-catch’ sample, where the urine is collected in a sterile or clean container as it is leaving the body. The third, but least desirable, method is collecting the urine out of a container or off the floor. Obtaining urine by free catch or one of the later methods tends to be less stressful and painful to the animal than cystocentesis (Kurien, Everds, & Scofield, 2004), but are more

prone to sample contamination. Contamination can easily occur from introduction of hair, bacteria from unclean skin, or material from personal protective equipment, PPE, falling into the collection container.

Blood collection is a common procedure in laboratory animal and wildlife research as well as in captive animal care. Refinement, as part of the 3Rs (replacement, reduction, and refinement of animal use), should be considered when selecting a blood collection site and how the blood will be collected. This suggests, when collecting blood from animals, it's important to consider collection sites that are the least painful/distressful for them and ensure that the appropriate blood volume is collected. Positive reinforcement training (rewarding the animal for completing the behavior or task asked of them) may also be used to reduce their stress and allow for them to remain awake during collection (U.S Department of Agriculture, 2021). When stress is elevated during blood collections it leads to elevated glucose, cortisol, WBC, and liver enzymes. Sedation or general anesthesia of the patient can provide effective restraint. However, effects of homological variables have to be considered. When a patient is under anesthesia the RBC count, hemoglobin concentration, and HCT will decrease. Physical restraint, such as placing the patient in a sack with only the site for venipuncture (limb or tail) protruding or placing a hood on the patient, may be adequate for small animals and stocks and chutes for large animals in some cases, but subtle ‘reactive’ movements, especially in response to the needle entering the skin, may frustrate effective venipuncture and the excitement response mediated by increased catecholamine secretion in the restrained, conscious animal may change the hematological values. The skin should then be cleaned using an alcohol solution (e.g., 70% ethanol or 100% methanol), or a

detergent followed by an alcohol solution to decrease risk of bacteria entering the bloodstream. The alcohol should be allowed to ‘air dry’ prior to the venipuncture because contamination of the sample with alcohol may cause some hemolysis (Elizabeth, 1994). Povidone iodine should not be used as a cleaning agent, as the residue may interfere with some biochemical assays (Elizabeth, 1994). Once the skin has been cleaned, the collector should avoid touching the immediate area to prevent contamination of the site. In most cases, a needle and syringe are used to collect the sample of blood. The size and gauge of the needle and the size and volume of the syringe should be appropriate for the size of the vessel and the volume of blood to be collected to prevent hemolysis or lysis of the cells. In some circumstances a butterfly catheter may be more suitable for venipuncture than a needle, particularly when animals are not anesthetized and may move during the procedure. The butterfly catheter provides increased stability and is less likely to come out of or lacerate the vein if the patient moves, allowing less chance for hemolysis. Both the needle and syringe should be sterile. Needles should not be reused between animals because of contamination of samples and possible transmission of disease. Align the needle, with the bevel facing upwards, and the syringe with the direction of the vein and at an angle of approximately 15° to horizontal. This technique helps prevent outside cells being introduced to the sample. When inserting bevel down the needle will slice rather than puncture, this causes more pain for the animal and drags along the skin. Avoid lateral movement of the tip of the needle as laceration of the vein may result with subsequent hemorrhage and hematoma formation (Clark, Holz, & Duignan, 2017). Commercially available evacuated blood tubes, commonly known as Vacutainers®, are an alternative to using a needle and syringe to collect the blood sample. When the cap of the tube is

punctured by the specially designed double-ended needle, which is already placed in a vein, the negative pressure withdraws blood from the vein into the tube. The Vacutainer® is set up to draw the appropriate amount of blood with this feature lysis is less likely to occur due to underfilling. Evacuated blood tubes may be of use in larger animals but are inappropriate for use in small animals as the pressure of the vacuum collapses the vein and precludes withdrawal of blood. Once the sample of blood has been collected, it must be mixed with an anticoagulant to prevent clotting. Blood collection tubes are distinguished by top color, which identifies tube additives. Tube additives prevent or activate clotting and determine the final blood product (e.g., whole blood, serum, plasma) submitted for testing. Serum samples are required for blood chemistry panels, serology, immunology, and most endocrinology tests. Serum is the fluid portion of the blood that has had fibrinogen removed during clotting. Blood for serum testing is collected in red or red/gray (tiger or marble) top tubes (Poulin, 2017). Plastic red top and marble (tiger) top tubes contain a gel to separate serum from the clot and a clot activator but no anticoagulants or preservatives. Plasma is required for blood chemistry, coagulation, platelet function, and some toxicology tests. Plasma is the liquid portion of the blood from which the red and white blood cells and platelets have been removed. Plasma consists primarily of water with dissolved proteins, hormones, lipids, enzymes, salts, carbohydrates, vitamins, and waste materials (Poulin, 2017). Anticoagulant tubes include lavender (purple), green, and light blue. Tubes with red and red/gray (marble or tiger) tops do not contain anticoagulant additives (Poulin, 2017). Several anticoagulants are commercially available, including ethylenediaminetetraacetic acid (EDTA), lithium heparin and sodium citrate. EDTA provides the best preservation of cell morphology and

should be the anticoagulant routinely employed for hematology. In heparinized blood samples, leukocytes may aggregate (clump) and cells stain poorly with Romanowsky stains (Lewis, 1975). Consequently, heparin is not recommended as an anticoagulant for routine hematological assays in mammals due to cell clumping. Several sizes of anticoagulant tubes, including 10 mL, 5 mL, 2 mL and 0.5 mL volumes are commercially available and the appropriately sized tube should be selected for the volume of blood collected. Laboratory needs should also be considered. Significant underfilling of tubes may result in artifactual changes in the shape of erythrocytes (e.g., echinocytosis). To minimize hemolysis of the sample, remove the needle from the syringe and gently expel the blood into the tube containing the anticoagulant. Gently roll and/or rock (end to end) the tube so that the blood is thoroughly mixed with the anticoagulant. Vigorous shaking may cause hemolysis and should be avoided (Clark, Holz, & Duignan, 2017). If the blood is squirted through the needle into a tube, shaken too vigorously, subject to delayed processing or exposed to temperature extremes the erythrocytes will lyse (i.e., hemolysis), which may result in spurious laboratory data such as decreased hematocrit and increased mean corpuscular hemoglobin concentration. Hemolysis may also interfere with some biochemical assays. Experimental investigation of the effect of hemolysis on biochemical analysis of canine serum samples found that hemolysis consistently interfered with the analysis of creatinine kinase, lactate dehydrogenase, aspartate aminotransferase, lipase, and albumin, all of which increased with increasing hemolysis (Clark, Holz, & Duignan, 2017). To prevent hemolysis, let the sample sit in a vertical position for 15 to 20 min at room temperature, then centrifuge at 2500 revolutions per minute for 10 to 15 min.

Sample Handling

Blood samples are the most common specimen sent to diagnostic laboratories to be analyzed (Lima-Oliveira, Lippi, Salvagno, Picheth, & Guidi, 2015). Types of sample handling issues can include miss labeling, handling, storage, and shipping. When poor sample handling occurs it affects diagnosis, management, and treatment of a patient.

Major types of specimen labeling errors are associated with a small number of common causes. Specimen labeling errors have significant consequences for patient care, for healthcare management and for increasing costs that are often unaccounted for. Specimen labeling errors may be prevented by adhering to appropriate policies as well as unique educational programs, marketing strategies and other techniques. However accurate and timely labeling of specimens is an integral part of patient identification and cannot be taken for granted as a "given" in the total examination process. Ensuring accurate specimen labeling is critical because errors resulting from a failure in this step can, at best, provide results of no clinical value and, at worst, lead to the most adverse of patient outcomes (Kahn, 2005).

Inadequate mixing of whole blood with an anticoagulant is a handling issue that can result in clots or artifacts in the sample (Clarizio & Pohlman, 2021). Shaking of blood tubes, forcing blood through needles, vigorous expulsion into tubes, can cause shearing of red blood cells (hemolysis) and platelet clumping (Hematology, 2020). Cell preservation is optimal in anticoagulants preventing clotting. High concentrations of EDTA are hypertonic in comparison to red blood cells, so if only a small amount of blood is collected (e.g., 0.5 mL) and placed into a standard 5 mL EDTA tube, the red blood cells

will shrink. Rarely, exposure of blood to EDTA can result in the binding of antibodies in the animal's plasma to platelets or red blood cells. The consequence of antibody binding to platelets is that platelets aggregate, resulting in a falsely decreased platelet count. With antibody binding to red blood cells, red blood cells aggregate and mimic agglutination, which is a key feature that is usually diagnostic for immune-mediated hemolytic anemia. In this scenario, it is an artifact but can lead to an erroneous diagnosis of immune-mediated disease (Schaefer et al., 2009). Binding of EDTA-dependent antibodies to platelets and red blood cells can be overcome by collecting blood into citrate anticoagulants, however, routine collection of blood into citrate is not recommended due to the dilution of blood by liquid citrate (Hematology, 2020). Collection directly into a vacutainer tube (allowing the vacuum to draw the appropriate amount of blood) is optimal, but difficult to achieve in small or pediatric patients with small veins that collapse easily. Difficult venipuncture, particularly through a small gauge needle, can result in shearing of red blood cells (artifactual hemolysis), which affects cell counts and mimics true intravascular hemolysis. Collection of a small blood volume (e.g., 0.5-1 mL) with placement into a standard 5 mL EDTA tube will cause shrinkage of red blood cells, because EDTA is hypertonic. This will cause a false decrease in the mean cell volume (MCV) and false increase in mean cell hemoglobin concentration (MCHC) of red blood cells. Crenation (shrinking) of red blood cells will also be evident on the blood smear. This is a common artifact that is seen in hematologic samples.

Sample Storage

For specimen integrity and accurate test results, blood samples must be stored in specific ways. Storage of blood samples may lead to changes in biochemical as well as physical properties because of storage conditions. Depending on the sample use, one of three temperatures will typically be specified for blood sample storage: room temperature, refrigerated, or frozen. Room temperature is specified as between 15 and 30°C; refrigeration temperature is between 2 and 10°C; frozen temperature is at or below -20°C (Neoteryx, 2017). Rehak and Chiang (1988) found that significant differences in creatinine, glucose, phosphorus, potassium, aspartate aminotransferase, and alanine aminotransferase concentrations occurred over a range of temperatures. Glucose values suffered a drastic decrease while phosphorus values increased rapidly at temperatures greater than 22ºC. Potassium concentrations spiked at 3ºC and 38ºC. Both aminotransferases increased after being stored at temperatures greater than 22ºC, but they experienced a constant decrease at 38ºC. Creatinine values increased rapidly at storage temperatures greater than 25ºC (Rehak & Chiang, 1988). Glucose levels were significantly different after being stored for 4 hrs. Potassium, creatinine, and total protein results were significantly different when tested at 32 hours; calcium at 40 hrs; albumin and alanine aminotransferase at 48 hrs; and CO2 at 56 hrs (Boyanton & Blick, 2002). Tayal, Gupta, and Goswami (2009) found that even if samples are centrifuged as soon as samples are received in the clinical laboratory and stored at –20°C, there was progressive deterioration of glucose, AST, ALT, creatinine, and potassium after 72 hrs.

Sample Transportation

Transportation of blood samples is a major part of the preanalytical pathway and can be major in delaying laboratory results to the clinicians. Due to increasing volume of samples submitted by clinicians, it is important to have the sample delivered to the laboratory as quickly as possible in order to measure analytes within the established stability time, maintaining fast turnaround times and ensuring sample integrity. Furthermore, the transportation process itself must be firmly controlled to assure that the analyses requested are not affected by temperature, agitation, or other physical or biological influences. Finally, transportation logistics must be well arranged to satisfy sample flow from hospital wards and/or clinicians, while at the same time matching sample reception with the workflow at the laboratory in terms of numbers of samples, peak arrival during daytime, etc. Any delay in the time frame from blood collection to centrifugation to analysis, or any deviation from standard transportation conditions, could potentially alter laboratory results and subsequently have a negative impact on patient safety. For samples being analyzed in-house, manual transportation by trolley or by hand (e.g., by the phlebotomist or the clinician drawing a STAT sample) are both very reliable and common, as no technical instruments are involved. They are slow and if emergency samples are carried by hand, the time spent with sample transportation is time lost to perform other tasks. Another hurdle is the tendency to gather samples at the wards to send them in batches to the laboratory. This perhaps saves transportation labor, but the first sample collected may have been waiting for hours at the ward before it is transported to the lab. Also, if samples are received batch-wise, it is not possible to maintain a “first-in-first-out” process, as the laboratory does not know in which order the samples were

collected (Nybo, Cadamuro, Cornes, Rioja, & Grankvist, 2019). External samples are transported in a variety of ways, by car, in boxes, using drones, or even by planes and trains. If the samples are properly protected from temperature deviation and agitation, none of these transportation forms should affect the samples significantly. It is, however, crucial to monitor these conditions. Optimally, transportation should be performed under the same temperature conditions as storage before and after analysis until the analysis quality control has been performed and approved. Ideally, the time and temperature of transport boxes should be logged (Nybo, Cadamuro, Cornes, Rioja, & Grankvist, 2019).

Lab Procedures for Blood Samples

The total testing process (TTP) of a laboratory is a complex procedure that includes three phases: the pre-analytical, analytical, and the post-analytical. In all phases of TTP, quality indicators are used by health laboratories based on the requirements of the International Organization for Standardization. Laboratory errors can occur at any stage of the pre-analytical phase to the post-analytical phase of the TTP. Pre-analytical errors include all errors that occur prior to analysis. Some of the pre-analytical errors include hemolyzed sample, insufficient sample, incorrect label, incorrect requisition, clotted samples and tubes broken in centrifuges. The impact of pre-analytical error occurs in the analytical and the post-analytical stage. At the analytical phase, where analysis takes place, non-conformity with quality control, calibration failure, random and systematic errors can occur (Teshome, Worede, & Asmelash, 2021).

Centrifuging a sample takes place in the pre-analytical phase. The purpose of centrifugation is to remove the liquid portion from the solids. When a sample is

centrifuged too fast, this will cause the red blood cells to rupture and create hemolysis. When centrifuged too slowly, the cells and liquid do not separate. If a sample is stored too long and centrifuge time is delayed, it has been shown to decrease plasma assay concentrations. When samples are held in a refrigerator as whole blood, proteins and chemical components such as ATP, glucose, creatinine, or albumin start to deteriorate. Waiting too long to centrifuge samples can cause changes in lab results.

Insulin Assays

Insulin blood test measures the amount of insulin in blood. Insulin is a hormone that helps move blood sugar, known as glucose, from the bloodstream into cells. Glucose comes from the foods that are eaten and drank and is the body's main source of energy. The stability of insulin in blood samples is a commonly discussed issue in both human and veterinary medicine. Equine insulin blood levels are used to assess dysregulation of insulin. This can lead to the diagnosis of equine metabolic syndrome, assess the laminitis risk in equine patients, or determine insulin resistance in patients. Optimal pre-analytical handling of samples is essential for valid analytical results, but this is often not feasible in the daily routine of an ambulatory practice. Factors that can impact insulin are diet, pregnancy, stress, and illness. Animals or humans that are pregnant, on high energy forage, stressed, or ill will have a higher plasma insulin reading. Analytical factors that can hinder results are quality control standards not being met, the wrong buffer is used, or dilution of the sample is calculated wrong. Radioimmunoassay (RIA) is a common procedure used to determine insulin values. This assay is very sensitive and specific. To use RIA, an antigen has to have radioisotope or radioactive

element. An antibody is then introduced, and they bind. The test then measures the binding of the radioactive antigen. To prepare for RIA a tracer agent or radioactive element is incubated. If incubated at the wrong temperature and not used in the right time frame, it can result in a decreased concentration (EMD Millipore Corporation , 2017). Once incubation is completed the agents are washed to remove any unbound antigens. Once washed the tracer is ready to use with the antibody. If the tracer is not set up for the specific species, then results may be falsely elevated or decreased. Other things that can hinder assay results are storage of samples, like discussed above. A new pipet should be used when preparing each individual sample. If a pipet is not exchanged for a new one each time the samples are contaminated.

The objective of this study was to determine how delay from blood collection to centrifugation affects equine insulin concentrations. Based on the literature review, it is expected that after 18 hrs from draw time, insulin concentrations will start to decrease. Results from this study could impact sample handling in the veterinary field. If a critical time period could be identified, then veterinarians and technicians would know whether or not they had to rush to get samples centrifuged between farm or house calls.

Chapter III: Methods

This study was approved by the Murray State University Institutional Animal Care and Use Committee (protocol 2023-018; Appendix A).

Animals

Ten horses, five mares and five geldings, were selected from the Murray State University Rudolph Equine Educational Center's herd (Table 1). Routine management was not changed during the study, and included use in university classes as well as equestrian team practices and competitions. Horses were fasted for twelve hours prior to collection. Horses maintained in stalls received regular turnout in paddocks or round pens as appropriate. Feeding twelve hours prior consisted of mixed grass hay or pasture forage, depending on housing, and concentrates as needed to maintain body weight. Concentrates consisted of either Kalm'N EZ® (Tribute Equine Nutrition®, Upper Sandusky, OH, USA), or Bulk Custom Horse Pellets® (Stockdales®, Paris, TN, USA) (Table 2).

Table 1: Equine Demographics

Horse	Age	Breed	Sex
Ellie	15	Quarter Horse	Mare
Maggie	16	Quarter Horse	Mare
Dottie	15	Quarter Horse	Mare
Phoebe	16	Quarter Horse	Mare
Pixie	14	Quarter Horse	Mare
Boone	15	Quarter Horse	Gelding
Plaino	18	Quarter Horse	Gelding
Shooter	17	Thoroughbred	Gelding
Ben	20	Thoroughbred	Gelding
Lark	19	Quarter Horse	Gelding

Table 2: Feeding Rations for Horses Used in Study

Horse	Feed Type	kg of grain/day
Ellie	Tribute	3.2 kg
Maggie	Custom Pellets	2.7 kg
Dottie	Custom Pellets	2.7 kg
Phoebe	Custom Pellets	3.2 kg
Pixie	Custom Pellets	3.2 kg
Boone	Tribute	1.6 kg
Plaino	Tribute	3.2 kg
Shooter	Tribute	3.2kg
Ben	Tribute	3.2 kg
Lark	Custom Pellets	2.7 kg

Data Collection

Before the study all horses had a physical exam including temperature, respiration, and heart rates checked (TPRs). Sample collection occurred in December, at the end of a 16-week semester. Horses were caught and blood samples were collected in their respective pastures or stalls. Jugular blood samples were collected using a twenty-gauge needle and a vacutainer (BD Vacutainer®, BD Biosciences, CA, USA) to reduce stress associated with blood collection. Twelve 5mL Idexx EDTA tubes were collected one time from each horse. Blood tubes were stored in a refrigerator (4°C) until being centrifuged for plasma collection. Samples were centrifuged at 2500 rpm for 10 minutes (LW Scientific, Inc.; LWS M24 Combo®) and plasma collected at various intervals from the time of collection: 15 min post collection, and then at 4, 8, 12, 24, and 36 hr. post collection. Plasma was stored in (GE Appliances chest freezer/-29°C).

Plasma analysis was performed at two different laboratories. At Lab A, insulin concentrations were determined by radioimmunoassay using the EMD Millipore Human Insulin Specific Radioimmunoassay Kit (Human Insulin-Specific RIA | HI-14K (emdmillipore.com)). Serially diluted standards were run at the beginning of the assay. Control samples were run at the beginning, middle, and end of the assay. Each test tube contained assay buffer (100 uL), standard, control, or unknown sample (100 uL), insulin antibody (100 uL), and hydrated insulin 125I tracer (100 uL). Tubes were vortexed for ~10 sec before incubating overnight (12-20 hrs.) at 4°C. After incubation, a precipitating reagent (1 mL) was added, tubes were vortexed for ~10 seconds, and incubated for 20 min in a refrigerator. Tubes were centrifuged (2000 – 3000 x g) at ~6°C for 22 min. The solution in the tubes was decanted, leaving the pellet. Tubes were placed in a gamma counter and counted for one minute. Results were calculated using conversion software.

At Lab B, insulin concentrations were determined by radioimmunoassay using the EMD Millipore Human Insulin Specific Radioimmunoassay Kit (Human Insulin-Specific RIA | HI-14K (emdmillipore.com)). Serial dilution standards were run at the beginning and end of the assay. Serial dilution standards were run at the beginning of the assay. Each test tube contained the equine plasma sample (100 uL), insulin antibody (100 uL), and hydrated insulin 125I tracer (100 uL). Tubes were vortexed for 2 – 3 seconds. Tubes were then incubated overnight (20 – 24 hours) at room temperature. After incubation, a precipitating reagent (1 mL) was added, tubes were vortexed for 2 – 3 seconds, and then incubated for 20 minutes in a refrigerator. Tubes were centrifuged (2000 – 3000 x g) at 10°C for 20 minutes. The solution in the tubes was decanted, leaving the pellet. Tubes were placed in a gamma counter and counted for one minute. Results were calculated

using conversion software.

Statistical Analysis

Statistical analysis was performed on the effects of centrifuge times as well as comparison between lab procedures on equine insulin plasma samples using Microsoft Excel (version 16.0.1). Descriptive statistics were utilized to summarize data points and to determine if outliers existed within the data set. Outliers were detected by using the IQR (Interquartile Range) and confirmed by using z-scores compared to the means of the sample population. One outlier was detected and was removed from the final analysis. To determine if time to centrifugation was significant, a one-way ANOVA was utilized. Data was analyzed at P=0.05 for all statistical tests. Labs A and B plasma insulin levels were compared at each sample time using a two-sample T-test assuming unequal variances for the means.

Chapter IV: Results and Discussion

When comparing each centrifuge time within each lab there was no significant difference from time 15 min to time 36 hrs. For both Lab A and Lab B, there were no significant differences detected between each centrifuge time (P=0.9858 and P=0.9967, respectively) (Table 3 and Table 4). The lack of a difference between times shows that centrifuging a sample up to 36 hrs after collection had no effect on equine plasma insulin concentrations. These results suggest that insulin is stable in whole blood when stored in a 4°C container for up to 36 hrs. This could impact how veterinarians in the field handle samples. If they had access to a cooling container, a veterinarian would not need to hurry back to a clinic to collect plasma from the samples. The veterinarian and owner could be confident that diagnostic results for plasma insulin would not be negatively affected so long as the samples were kept at 4°C. However, further studies need to be performed to determine when stability from collection time is affected. Not all veterinarians have access to a cooling container while in the field.

Table 3: One Way ANOVA for Lab A Centrifugation Times from Blood Collection

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	87.754	5	17.550	0.126	0.986	2.409
Within Groups	6683.848	48	139.247			
Total	6771.6	53				

Table 4: One Way ANOVA for Lab B Centrifugation Times from Blood Collection

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.628	5	2.526	0.067	0.997	2.477
Within Groups	1356.329	36	37.676			
Total	1368.956	41				

Results from Lab A and B appeared to be different for insulin concentrations. A two-sample T-test confirmed significant differences existed at times 4 hrs and 36 hrs between the laboratories (P=0.0236 and P=0.0384, respectively; Table 5). While there was no difference in plasma insulin concentration from time to centrifugation, there was significant variability between laboratories. This could be explained with the difference in protocols between laboratories. Lab A incubated the samples over night at -20°C they then vortexed the samples and incubated them at 4°C for 20 min before analysis, while Lab B incubated the samples at room temperature for 20 to 24 hrs they then vortexed the samples and incubated them at 4°C for 20 min before analysis. Previous research has shown that insulin is not stable at room temperature (Ellis, Evans, Livesey, & Yandle 2001). Based on this, results from Lab A may be more dependable than those from Lab B. While this may explain the differences at 4 hrs and 36 hrs, it would be expected that the other times would have been affected as well. It is possible that samples to one of the laboratories may have thawed during transportation, which may have affected insulin concentrations. For each time, plasma from one tube was collected and sent to Lab A, while plasma for Lab B was collected from a different tube. It is possible that plasma samples sent to Lab B were slightly hemolyzed as compared to other samples, which could also have affected the results. It is also possible that sample mishandling occurred at the laboratory. Seeing the differences in labs it would be recommended that

veterinarians stay with one lab once they send out a sample for a patient. Further research would need to be performed to determine if hemolysis or mishandling occurred during collection or shipping, rather than the difference actually being related to laboratory procedures.

Table 5: Centrifuge time comparison between Lab A and Lab B

	Lab A		Lab B		
	Mean	Variance	Mean	Variance	*P*
15 min	32.9556	200.5265	22.7444	66.5703	0.08352
4 hrs	35.1067	129.5641	22.6333	94.3575	0.023643*
8 hrs	31.1867	128.7285	22.2778	81.6969	0.08526
12 hrs	32.5056	123.3172	22.9667	68.9225	0.05677
24 hrs	33.0022	122.6843	23.4111	92.1061	0.06724
34 hrs	34.4167	130.6604	23.2444	87.3903	0.0384*

Chapter V: Conclusion

Results from this study suggest that equine plasma insulin levels are stable in whole blood while being stored at 4°C for up to 36 hrs. This data should help veterinarians improve sample handling to achieve accurate diagnostic results. They now know that it is possible to delay centrifuging blood samples that are going to be evaluated for plasma insulin for up to 3 days, so long as the samples are kept cool. Further research should be conducted to determine when stability is affected, evaluate the effect of storing whole blood samples at different temperatures before centrifuging, and should evaluate other blood components as well.

Future research also needs to be carried out to determine the reason for the differences between the two laboratories. Since only two of the six times were affected, it is difficult to determine if lab protocols are the cause, or if another factor is at play. Results from such research could also help laboratories to ensure they are utilizing the most effective and accurate methodology.

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APPENDIX A: IACUC APPROVAL

MURRAY STATE UNIVERSITY
Institutional Animal Care and Use Committee

October 10, 2022

Dr. Terry Canerdy
Veterinary Technology Program
Murray State University
100 Animal Health Technology Center, Carman Pavilion
Murray, KY 42071

Dear Dr. Canerdy:

The Murray State University Institutional Animal Care and Use Committee (IACUC) has approved your research protocol for the course titled, "Effects of Plasma Handling on Equine Insulin assays."

The protocol timeline is approved through August 2023. Please use the Animal Use Report (attached) to keep up-to-date information about the animals. At the termination of the protocol, you will need to complete the Conclusion Report (attached) and list final information concerning the animals.

If you have any questions, please contact me at 270-809-3534

Sincerely,

Kristi Stockdale

Kristi Stockdale
IACUC Coordinator

cc:
IACUC File