Winter 12-15-2020

THE EFFECT OF HOLDING TIME OF WHOLE MARE BLOOD ON CONCENTRATION OF PROGESTERONE

Clarissa Menefee
menefeecc@jacks.sfasu.edu

THE EFFECT OF HOLDING TIME OF WHOLE MARE BLOOD ON CONCENTRATION OF PROGESTERONE

THE EFFECT OF HOLDING TIME OF WHOLE MARE BLOOD ON CONCENTRATION OF PROGESTERONE

By

Clarissa Catherine Menefee, Bachelor of Science

Presented to the Faculty of the Graduate School of

Stephen F. Austin State University
In Partial Fulfillment

Of the Requirements

For the Degree of

Masters of Science

Stephen F. Austin State University

December 2020

THE EFFECT OF HOLDING TIME OF WHOLE MARE BLOOD ON CONCENTRATION OF PROGESTERONE

By

Clarissa Catherine Menefee, Bachelors of Science

APPROVED:

_____
Stephanie Jones, Ph.D., Thesis Director

_____
John Mehaffey, Ph.D., Committee Member

_____
Candis Carraway, Ph.D., Committee Member

_____
Sharon Rice, Committee Member

_____

Pauline M. Sampson, Ph.D.

Dean of Research and Graduate Studies

Abstract

The objective of this study was to evaluate if holding time had an effect on mare whole blood progesterone levels. This research was completed using 12 mares, with blood being collected on multiple cycles to get a total of 28 separate blood collections. At each collection 5 vials of blood were taken for different time treatments of 0, 12, 24, and 48 hours. The 0 hour was centrifuged within 30 mins of collection and the other 4 were refrigerated at 4℃ until the specific time treatment was finished. After they were centrifuged all the samples were stored in the freezer at -22℃ until they were transported to the lab. A total of 140 data points was analyzed for this research. On further analysis of the results, the conclusion is that there is no effect of time on concentration of progesterone in the whole blood of mares.

Acknowledgements

The author would like to express her sincere thanks to God, her family and several influential individuals in her life. Special thanks to Dr. Stephanie Jones for all of her help and guidance as her major professor on this research project. Not only is she a great mentor, but also a great friend. She would also like to thank her committee members, Dr. John Michael Mehaffey, Dr. Candis Carraway, and Sharon Rice, for their knowledge and help during this research project.

Great appreciation goes to the Stephen F. Austin State University Agriculture department for the many years of knowledge and learning they have provided. She was privileged to make many friends and learn so much in these past 4 years.

Lastly, thank you so much to all her friends and family for the continual support and motivation to complete this research. Without them Clarissa would not be the person she is today.

Table of Contents

List of Tables

List of Figures

Chapter I

Background

Introduction

In previous research, there have been few articles published providing information on mare reproduction and analysis of progesterone concentration in circulating blood. The published research is outdated and most of the laboratory testing methods that were used are no longer the equivalent to current testing methods, which utilizes improvement in the technology available. There is little modern or current research available regarding progesterone concentration in circulating blood of mares utilizing more modern blood testing techniques (Oltner and Edquist, 1982, Wiseman, *et al*. 1983). Another aspect of the blood testing process is the amount of time that passes between collection and when the whole blood sample is centrifuged and stored until laboratory analysis is performed. Currently, there is no standard or generally accepted set time that is recommended for handling whole blood samples and analysis for progesterone concentration determination in the equine reproduction industry. Most researchers assume processing of whole blood into serum or plasma as soon as possible is the best option, but at what time period does the change in the amount of hormone concentration and degradation cause a significant difference in the whole blood sample. This analysis is necessary because in whole blood, progesterone levels can be degraded over time. Red blood cells and other cellular elements can cause the progesterone to

deteriorate over time. Time and temperature of whole blood can also determine how quickly the progesterone will be diminished in the whole blood sample. Due to the physiological significance of progesterone in the mare, this study is very relevant to horse breeders and producers. Horses required adequate progesterone concentrations to maintain the conceptus in the first trimester of pregnancy and sometimes may be deficient in this hormone during this critical period. Early pregnancy loss is common in mares and is still not completely understood (Irvine, *et al*. 1990). Most researchers suspect the early embryonic loss is due to low circulating progesterone concentrations (Asbury, C. A.). When this deficiency in available circulating progesterone occurs, there may be instances in which the mare's embryo is aborted. Due to the risk of spontaneous abortion in the mare, producers collect whole blood samples to ensure the mare's primary corpus luteum is producing adequate levels of circulating progesterone concentration to maintain a pregnancy. If a mare is determined to have reduced, levels of circulating progesterone concentration, then the mare owner may be advised to provide the mare with oral supplementation of progesterone (Mottershead, 2004). Supplementation of oral exogenous progesterone is an added cost to the producer and should not be used unless the mare is justly in need of it. There can also be negatives to orally supplementing exogenous progesterone to mares, and this is another reason as to the importance to have accurate and precise analysis and laboratory methods used to determine the results of progesterone concentrations in bred mares. New research needs to be performed in order to provide additional information to that which is currently lacking in the scientific

literature. Does whole blood samples collected from mares need to be centrifuged immediately after collection for storage and handling or is it acceptable to wait a day or more before sending samples for centrifugation and testing? Will various holding times of refrigerated whole mare blood effect the reported concentration of progesterone and potentially effect recommendations for oral progesterone supplementation in pregnant mares. These questions and others related to holding times of mare whole blood will be answered in this research.

Another factor regarding progesterone testing is a wide range of different laboratory equipment utilized to measure the amount of progesterone in equine blood samples. Outdated methods involved the use of chromatography paper and a spectrophotometer. As research was been performed in this area, innovative lab methods have been discovered. Radioimmunoassay (RIA) and enzyme linked immunoassay (ELISA) methods are the most commonly used lab blood testing systems today (Senger, 2005). Most current research uses a solid phase RIA with differing sensitivities. These modern analysis methods and the resultant research researchers are performing is an important addition into the scientific literature, because it can give researchers new insight into how quickly the circulating progesterone is degrading specifically in whole blood collected from mares, and may possibly open up opportunities for further research in other species.

Objectives

The objective was to analyze the effect of holding time on whole blood progesterone levels collected from cycling mares during the diestrus period. Blood was collected from the jugular artery or vein of mares in the diestrus period with a mature corpus luteum present on either ovary. Five 5 mL vials of blood were collected consecutively from each mare from a single venipuncture at each blood draw. The five vials of whole blood were allocated into a holding time treatment groups, which consisted of 0, 6, 12, 24, and 48 hours. After the blood collection, each vital of blood was stored in the refrigerator at 4℃ for the allotted holding time treatment, samples were centrifuged, serum was separated into a storage tube, and then the serum samples were stored at -20℃ until further analysis was performed. The serum samples were sent to the Animal and Food Sciences Lab at Louisiana State University and assayed using a solid phase RIA to determine progesterone concentrations across the allocated holding time treatment groups. This data should indicate a point in which the progesterone concentration started to degrade over a specific holding time period. This research study will support the creation of new standards on timing and centrifugation of equine whole blood for progesterone concentration analysis.

Significance

This research is significant as majority of research on progesterone testing in the blood of mares is over 30 years old. There has been some, more current research published, but it is minimal when compared to the collective literature on the topic. New research is important as there is no current standard in the time period between blood sample collection and centrifugation of the blood sample in the modern animal science community. Although hormone analysis in blood samples has become more and more precise and accurate, there needs to be a protocol in place before the blood comes to the laboratory to allow for direct comparison across research publications. Researchers should report in publications the holding time in which mare whole blood samples are stored under refrigeration and the time period between storage and centrifugation, due to the know effects of red blood cells and other cellular elements causing progesterone to deteriorate over time. These are important factors regarding the handling and processing of whole blood samples that could possibly affect the concentration of progesterone determined by further analysis. Knowledge of the potential effects of blood sample handling on progesterone levels and relative amounts of degradation in the circulating hormone before being sent to the laboratory for analysis may influence how producers and veterinarians handle and store their blood samples after collection. Knowledge of the importance of circulating progesterone levels for pregnancy maintenance in mares, the instances that the analysis methods used should be as accurate as possible, as it will help

provide the equine industry with relevant research for those that are concerned with breeding and foaling mares.

Chapter II

Literature Review

Introduction to Progesterone

Progesterone, also known in the family of progestins, is an important hormone for pregnancy. Progestins are a form of steroid hormone that is derived from acetate and cholesterol (Bhurke, *et al.*2016*)*. Progesterone is a critical hormone in mammals for initiation and maintenance of pregnancy (Owens *et al*., 1980). The most prominent ovarian progestin is progesterone (Ginther, 1992). There are two temporary endocrine structures that reside in the ovary. These two structures are the follicle and the corpus luteum. The primary hormones produced from these two structures are estrogen and progesterone (Senger, 2005). The corpus luteum is the major producer of progesterone in the ovary (Graham and Clarke, 1997). These structures must constantly function as the hormones are always being degraded in the body, and it is important to consider the half-life of progesterone. Steroid hormones are degraded in the liver and excreted by the kidneys, which directly effects the half-life of the hormone (Senger, 2005). Some hormones have longer half-lives than others, due to the specific structure of the hormone. According to research performed by Ginther (1992), progesterone was given to ovariectomized mares to determine how quickly the hormone degraded in the body. Progesterone concentration peaked at an average of 15 minutes post injection and has returned to base line levels by 60 minutes.

Structure of Progesterone

To have a better understanding of the progesterone hormone, it is necessary to look at the chemical makeup of this steroid hormone. Steroid hormones are very complex and share a common type of nucleus called the cyclopentanoperhydrophenanthrene nucleus. When looking at the structure of progesterone it is comprised of 21-carbons, which are derived from cholesterol (Senger, 2005). With cholesterol serving as the basic building block, biosynthesis of the progesterone molecule can begin. Cholesterol is transformed into pregnenolone, which can then form both corticosteroids and progesterone (Ginther, 1992).

Functions of Progesterone

Progesterone has many functions in most mammals. The primary functions include: helping with the release of oocytes, maturing embryos for implantation, and pregnancy maintenance. Another function of progesterone is to support lobular-alveoli development in the mammary gland in order to prepare for milk production once the fetus has been born. Lastly, progesterone is present in the brain to moderate the display of sexual behavior and seeking of a mate (Graham and Clarke, 1997).

In the role of 'the hormone of pregnancy', progesterone is also important in the determination of how the uterus interacts with the embryo. Following fertilization, the embryo moves into the uterus to mature and grow during gestation. The uterine lining must be prepared and receptive to receive and support this embryo. Progesterone plays a

very important role in ensuring the uterine lining or endometrium is equipped for the embryo. Progesterone and estradiol work together to ensure the endometrium is in the correct condition (Bhurke, *et al.* 2016). In early pregnancy, progesterone is solely being produced and secreted by the primary corpus luteum, and in past research it was thought that after the first month of gestation the primary corpus luteum would regress and the secondary corpus lutea would take over producing the majority of the progesterone. This was proven in later research to be false, and the primary corpus luteum is still active and non-regressed all the way through day 180 of gestation (Ginther, 1992). This is why the corpus luteum has an important role in maintaining pregnancy.

Cyclicity in the Mare

When discussing breeding and reproduction, it is important to know that mares are seasonal breeders. The mare will only cycle in the long days of the year because they are seasonally polyestrus (Plotka, *et al*. 1972). This means that during the specific long day breeding season the mare will cycle multiple times. The reason behind this is because of the photoperiod effect on the brain. As the day length increases, the mare's retina is stimulated by the increase in daylight which decreases melatonin production. This causes a chain reaction of hormones to be released from the hypothalamus, so the mare will start cycling. Long day breeders can begin to cycle as early as March and continue to cycle until July to August if no pregnancy has occurred. The estrous cycle for a mare is

approximately 21 days on average, but may last anywhere from 15 - 26 days (Senger, 2005).

Progesterone Production

In the mare, as in other species, progesterone is produced from the corpus luteum (CL). These structures on the ovary can be palpated or seen via ultrasound. The importance of the corpus luteum to mares and all species is their production of progesterone for pregnancy maintenance (Santos, *et al.* 2015). The corpus luteum is made from the shell that is left when the egg is ovulated from a dominant follicle. Granulosa cells in the dominant follicle are then converted into large luteal cells as the corpus hemorrhagicum converts into a corpus luteum. These large luteal cells produce progesterone for the embryo in the first crucial days of development. If the dominant follicle does not have enough granulosa cells, this causes there to be fewer large luteal cells, which in turn will not produce sufficient progesterone to support the pregnancy (Ginther, 1992). According to Ginther and Santos (2015), the output of progesterone will increase as well as the vascularity as the CL develops. The primary CL that develops from the remnants of the dominant follicle, which released the ovum that is now fertilized and forming an embryo, will not regress until around day 180. The primary CL secretes the majority of progesterone into the ovarian venous system until around day 80 when there are sufficient accessory CL's to support in the production of progesterone (Ginther and Santos, 2015).

Mares are known to have issues and situations in which progesterone concentrations are not adequate; therefore, blood testing for progesterone analysis is traditionally involved, which occurs a few days post breeding to check and ensure that the mare is producing sufficient progesterone to support the growing embryo and pregnancy. Luteal deficiency in the mare can pose a significant problem and lead to spontaneous abortion of the fetus. Mares have a 10-15% higher chance of abortion compared to other species. This loss usually occurs in the first 40 days of gestation and is thought to be cause by low progesterone levels (Allen, W. R. 2001). Because of this concern, there is a necessity to test mare circulating progesterone levels during the breeding season. The current recommendation or standard minimum requirement for circulating progesterone concentration in the pregnant mare's blood is at least 3 - 4 ng/mL is needed to support a pregnancy (McCue, 2018, Mottershead, 2004, Serum Progesterone, Watson, *et al*. 1995).

Progesterone Supplementation

Progesterone supplementation in the equine industry is a widely used alternative when a mare is low in progesterone or to suppress estrus behavior (McCue, P. M.). Oral supplementation of progesterone is usually given in the form of Regumate, which is the product name of the synthetic hormone altrenogest. Altrenogest is a synthetic version of the native progesterone hormone in the mare. This product is used to synchronize estrus, inhibit sexual behavior and maintain pregnancy (Mottershead, 2004).

Uses of these hormones can have adverse reactions to the humans handling them, it is common for the supplementation to be unnecessary and can cause adverse reactions to the fetus. Because supplementation of progesterone in mares for pregnancy maintenance has to be done for 120 days, the exposure of the person handling the drug is high (McCue, P. M.). Regumate can transfer through unbroken skin and can cause adverse effects such as abnormal or absent menstrual cycles in women and decreased libido in men who came in contact with this hormone. Because of the possible negative effects, it is important to wear gloves when working with this product and to be careful not to get it on exposed skin (FDA). In a lot of cases producers put mares on Regumate without even checking the blood progesterone levels to see if it is necessary for that specific mare. Testing the blood is important, to know exactly what the progesterone concentration is because supplementation is costly to the producer and can have negative effects on fetal fillies specifically. Fetal fillies exposed to Regumate during development are prone to clitoromegaly. This can persist in the filly through the first breeding season. Fillies treated with Regumate when in utero had no effect on functional reproduction on those females. Treatment of mares pregnant with Regumate that are pregnant with fillies are at a higher risk of androgenizing the foal. Caution should be used when putting these mares on Regumate, so that there are no adverse effects on the foal (Naden, *et al*. 1990).

Blood Testing Overview

The majority of published research on testing equine blood progesterone levels is outdated, and there can be a lot of variation in testing methods utilized in modern analysis and publications. When looking through the published literature regarding the analysis of equine progesterone levels, research has come a long way and significant advances have been made. In the past, progesterone analysis has been performed on the blood through chromatographic partition on filter paper with ultraviolet absorption spectroscopy. This method was used in the early 1950-60's before there were less time-consuming options available (Edgar and Ronaldson 1958, Short, 1958). In more recent developments, there was the creation of radioimmunoassay (RIA) for hormone detection using extraction and non-extraction methods. This was the older form of RIA used when it was first developed. This is still not a very widely used form of progesterone testing currently. In this type of testing, antiserum is made from other animals that are immunized against different forms of the progesterone hormone to make it possible to find the progesterone in the blood of the animal being tested (Breuel, *et al.* 1988, Holdsworth, 1980, Nagy, *et al,*1998, Relave, *et al.* 2007, Urwin and Allen, 1983, Wiseman, *et al.* 1983). Because of the older methods of RIA, there can be a lot of variation in hormone detection. Some studies for example used goats to create the antiserum for the direct RIA progesterone test, while others used mice or sheep (Breuel, *et al.* 1988, Holdsworth, 1980). Considering modern research methods used within the last 10-20 years or so, there has

been more published use of solid phase RIA and enzyme-linked immunoassay (ELISA). Solid phase RIA is used as it is a faster and more reliable method of progesterone testing. Most current research uses solid phase RIA, although ELISA is accurate, most researchers seems to prefer solid phase RIA specifically (Brito, *et al*. 2017, Brogan *et al*. 2016, Ginther, *et al*. 2007, Ishak *et al*. 2017, Santos, *et al*. 2015). Coat-A-Count is one of the most widely used solid phase RIA tests available to producers (Brogan, *et al.* 2016). These tests may not be difficult, but with the wrong technique or misuse, the tests can provide skewed results. Because of this, there is a need for care to be taken in the use of testing methods utilized for equine reproductive health. This can be made easier by publishing research and reporting the most reliable options in order to streamline the process. Determining the amount of progesterone in the blood of mares with rapid immunoassay tests could influence or resolve some of the problems with equine reproduction today (Relave, *et al.* 2007).

Progesterone Degrading in Different Species

Different species have differing amounts of hormone degradation over time. Bovine and ovine species have an increased tendency for progesterone to decline more rapidly than other species. For example, ovine blood stored for 24 hours at 22 ℃ will have a 50% reduction in progesterone than that when tested immediately (Wiseman, *et al.* 1983). On the other hand, there was no significant loss of progesterone in the stored whole blood of horses, dogs, llamas, or pigs for the same time and temperature (Castro, *et*

al. 2004). In a study reported by Holdsworth (1980), progesterone levels were measured in bovine blood plasma and preserved whole blood by a direct radioimmunoassay. The samples were first measured to see how room temperature storage of whole blood would degrade progesterone. There was a significant decline in progesterone when the samples were left at room temperature overnight, but this decline was reduced if the samples were stored at 4℃ instead. The use of direct assay, for progesterone detection decreased the complexity of testing for progesterone and increased the speed of analysis.

Sensitivity to Time and Temperature

In a study conducted by Castro *et al.* (2004), radioimmunoassay was performed on bovine blood to test progesterone levels according to different treatments on time and temperature. This study used a solid phase RIA with a sensitivity of 0.1 ng/mL. Six mature cows were used to collect blood in the luteal phase. The blood was collected into 12 separate containers where half of them were left at ambient temperature and the other half were placed in an icebox. Then the blood from each treatment was centrifuged at 1,4,8, and 24 hr. The results of this study showed that temperature and time affected the progesterone concentration significantly. At the 8-hour mark, on whole blood at room temperature, the progesterone content had already dropped 40%. Whereas in refrigerated samples, the decline was first observed at hour 8. This is just one example of how the testing of blood can be very sensitive to both temperature and time, and this is why it is

important to know the correct techniques to keep the reported progesterone concentration of whole blood samples accurate.

Oltner and Edqvist (1982) studied how plasma progesterone levels are affected during storage of heparinized blood in dogs, horses, cows, and pigs. The samples were taken from all animals with one being whole blood and the other being serum. One sample of blood from each animal was centrifuged immediately to create a 0-hour plasma sample to indicate the 100% progesterone level for comparison. This enables the conclusion of the testing results at the end of the research to indicate how much the same animals blood progesterone level degraded. Blood samples were then incubated for 2, 4, 6, 24, 48, and 120 hours at room temperature or refrigerated at 4°C. RIA was used to analyze the blood, where all samples from a specific animal were tested in the same assay to avoid any interassay variation. In the case of the mare in this study there was a significant decrease in progesterone levels when the blood was left at room temperature when compared to the refrigerated samples.

Another example of testing the effects of time and temperature on serum and plasma progesterone levels was done by Vahdat *et al.* (1979). This was similar to previous research where blood is collected and then put into different treatment groups. Blood was collected from cows that were 7 months pregnant. Each cow had four serum and four plasma samples taken. All blood taken was put into the different incubation time intervals. These intervals were 0, 6, and 24 hours. The samples that represented the 0

hour were held for no more than 30 minutes before being centrifuged and then stored at -20℃. The other samples were then incubated at 40℃ for 6 or 24 hours or at 4℃ for 24 hours. Following incubation, samples were centrifuged and stored at -20℃ until they were assayed. The results indicated that progesterone concentrations decreased significantly in the 6 hours the samples were incubated in serum and plasma compared to the blood that was tested within 30 minutes. There was also a greater amount of degradation in the plasma samples compared to that of the serum samples. The reasoning behind this observation is that there could be an interaction between the progesterone molecule and the blood cell surface that causes an increased amount of hormone degradation.

RIA VS. ELISA

Radioimmunoassay (RIA) and enzyme linked immunoassay (ELISA) are two modern hormone testing methods. In recent years, there has been studies performed using ELISA in equine research (Panzani, *et al*. 2017). Although when looking at research performed by Nagy et al. (1998), shows that solid phase RIA is superior to the ELISA when looking specifically at progesterone in mares. A disadvantage of the ELISA, is that although it may be accurate, it has low precision on detecting the progesterone hormone in mare blood. The main disadvantage of RIA is having to work with radioactive isotopes. That is why researchers have moved more toward the ELISA because it is less work for people in the lab and it is less hazardous. Both of this blood testing methods are

very important in the field of research, but when specifically talking about mares the RIA is the most valid test to choose. When doing research where absolute values are needed, for example, looking at the amount of circulating progesterone in the blood, is when the RIA is superior to the ELISA and will give the most accurate results for researchers.

Conclusion

Progesterone is an important hormone in all animals and specifically in mares. Production of this hormone can be the limiting factor when talking about maintaining pregnancy. Mares are known for their lack of progesterone production and are prone to abortion more than any other domesticated species. Because of this a lot of mares are put on supplementation of progesterone when it is not needed. Mares need to have blood tests performed to know if supplementation is needed. That is why reliable tests are especially important to equine producers. There is a lot of information currently available about progesterone testing and how hormones degrade in different species. The problem is that most of this research is very old, outdated and not specific to equine. In recent years, there has been a lack of new research performed evaluating the accuracy of modern solid phase RIA methods. This is the method most producers and researchers are currently using. Another problem that needs to be addressed is what is the standard procedure for handling and centrifugation of the whole blood prior to progesterone analysis. From all of the literature reviewed previously, it becomes apparent that there are no current standards for blood handling. When should you centrifuge the blood? What temperature does it

need to be kept or stored at to prevent hormones from degrading prior to progesterone analysis? These are all questions that need answers to help researchers and producers make decisions and know they have the most accurate results in their hands in order to do so. Research is not at a loss of having different types of blood handling and testing methods, but more at a loss on which ones are the most accurate and trustworthy to use.

Chapter III

Materials and Methods

This research project was conducted at the Stephen F. Austin State University (SFA) Equine Center in Nacogdoches, TX under IACUC approval # 2020-02. All lactating and nonlactating, cycling quarter horse and paint mares were used out of the SFA mare herd. Enrolled mares were between the ages 4 to 21 with an average age of 13.75 ± 5.23 years old. All mares were fed to National Research Council (NRC) guidelines healthy with a good body condition score or 5 or greater. This research was conducted during the spring breeding season, March through July of 2020. Mares were scanned with ultrasound periodically to detect and map any and all preovulatory dominant follicles on either ovary. Ovulation and the formation of a primary CL were observed at least once before the mare would be considered cycling and would be eligible to be enrolled into this research study. The mare was considered to be eligible for this research on her second cycle of the breeding season and blood collection was performed once the mare was determined to be five days post ovulation. Over the course of this research, there were 28 different collections from the 12 mares giving us a total of 140 data points. Blood was collected on 12 mares, in the mid-luteal phase, with 5 samples being taken per collection from each mare to assess the amount of progesterone being produced. All 5 samples were taken with one venipuncture using the vacutainer blood

collection system to collect whole blood from the jugular artery or vein into non-heparinized blood tubes on each individual mare 5 days post ovulation. Blood was collected from some enrolled mares in this study for multiple cycles. Due to the cyclicity of the mare, if the mare remained nonpregnant and cycling, the mare may be reenrolled in the study and used for subsequent blood collections over multiple months. Each new estrous cycle and ovulation resulted in the formation of different CL and production of progesterone even in the same mare. Comparisons were made between time of testing, concentration of progesterone and time effects on the circulating progesterone concentration of the different blood storage holding times. Each mare's 5 blood tubes per collection of blood was labeled and refrigerated immediately after collection. The holding time intervals were 0, 6, 12, 24 and 48 hours. The 0 hour was centrifuged within 30 mins of the blood being collected. All samples other than the 0 hour were refrigerated at 4℃ for the specific time treatment being applied. After each tube had completed the specific holding time, the blood sample was centrifuged and frozen at -22℃ until being sent to the lab for testing (Mukasa-Mugerwa, *et al*. 1989). All the frozen blood samples were transported in to Louisiana State University in Baton Rouge, LA for further analysis.

Progesterone was analyzed at the Louisiana State University Laboratory with solid phase radioimmunoassay using commercially available kit reagents validated for equine plasma (Progesterone Double Antibody, 125I RIA Kit, MP Biomedicals, Inc.,) (Oberhaus, E. 2017).

Statistical analysis of serum progesterone concentration data was performed using the GLM procedure of the Statistical Analysis System (SAS Inst. Inc.). The model includes horse, hour, month, age and horse by age interaction. Analysis of data using LSMEANS, MEANS, and using Bonferroni's adjustment. LSD was run to get a t test with an alpha of 0.05.

Chapter IV

Results and Discussion

The overall objective of this study was to determine the effects of storing whole mare blood under refrigeration for various time intervals would affect the concentration of progesterone in the blood using modern analysis methods. According to the data, progesterone concentration was similar across the holding times of whole blood and in the degradation of progesterone in whole mare blood. Previous research on this topic is limited when looking at the mare specifically. The results from this collaborate with results of Oltner and Edquist (1982), whom reported that there is a slight variation in progesterone levels over time, but there is no significant decrease in progesterone concentration. The authors suggested that this may not be the case, because that research study only had a sample size of 4 mares compared that in the current study which included 28 mares. The other research specifically focused on equine blood progesterone levels degrading over time was reported by Wiseman *et al.* (1983) that also collaborates the findings that equine blood progesterone levels do have slight variation over time, but there is no significant change in progesterone concentration reported. In contrast to research reporting the degradation of progesterone in the whole blood from beef cows was measured. There was a significant increase in the variation in progesterone concentration over time in bovine whole blood. This leads to the query of the differences among bovine and equine blood in order to stimulate different reported rates of

degradation for progesterone concentration to be degraded significantly more in the cow than the horse.

Figure 1. Progesterone Concentration Value Over Time

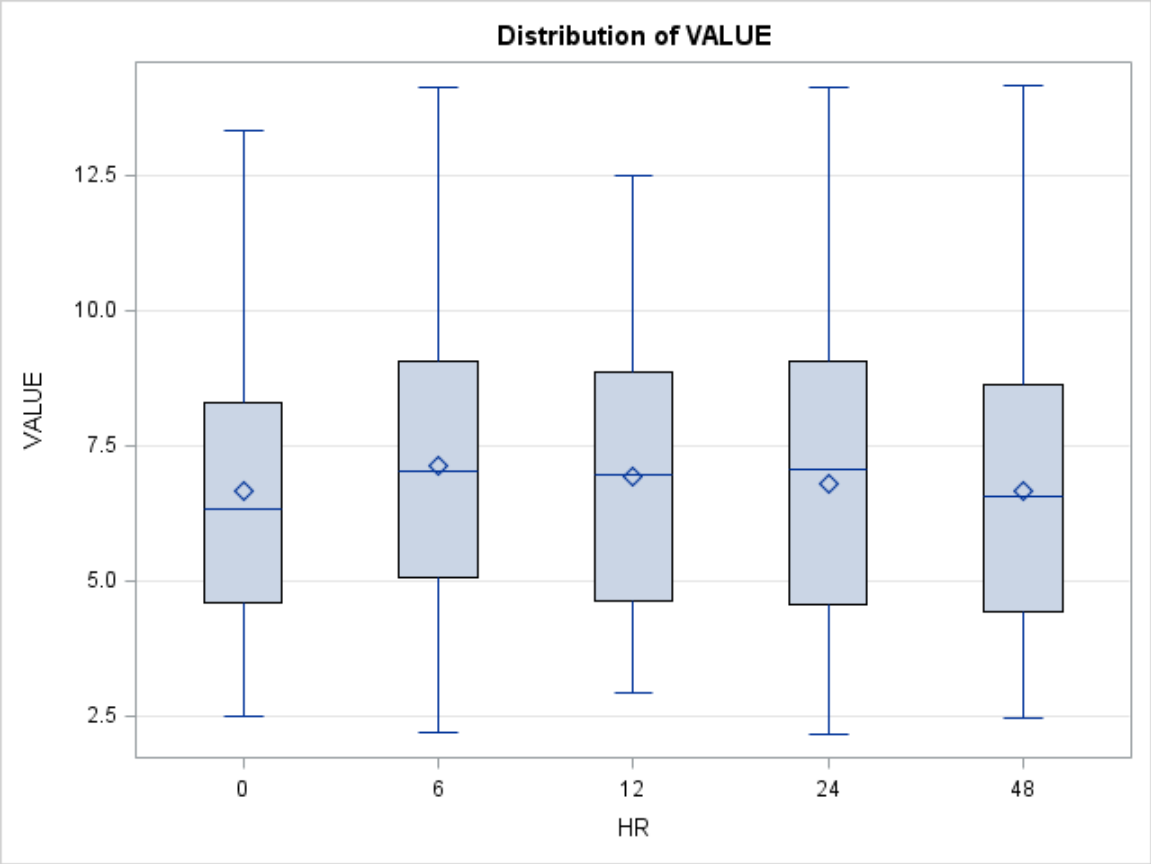

Table 1. Progesterone Concentration over Hour Treatments

	Treatment					
Item	0	6	12	24	48	*P*-Value
Progesterone Concentration	6.67 ± 2.63	7.14 ± 2.74	6.93 ± 2.54	6.81 ± 2.81	6.65 ± 2.77	1.00

Overall there are slight numerical variations in overall mean of the samples, but no significant difference ($p = 1.0$) was found across the holding times. This data indicates that various holding times between blood collection and centrifugation does not affect subsequent progesterone concentration. A numerical increase was found from the 0 hour to the 6-hour sample, then the progesterone concentration decreased at the 12-hour sample. It is very interesting that the mean progesterone levels increase before it decreased back to the previous concentration. This data rejects the hypothesis that the progesterone levels would significantly decline over the allotted amount of time.

Chapter V

Summary and Conclusion

Overall, there were no significant differences discovered by this research in holding times of whole mare blood on progesterone concentrations. From this study conclusions can be made that if stored in the refrigerator, mare blood progesterone levels do not significantly decrease over a 48-hour period. Although this research did not support the initial hypothesis that blood progesterone levels would significantly decline over time, it is still important information for the equine industry. With this new information equine producers can recognize that blood samples can be accurate when assayed even if the samples were to be delayed during shipped to the laboratory for analysis. This research is more trustworthy compared to previous literature due to the increased sample size. In the past research, there may have been four horses enrolled for this type of study, where as in this study, 12 horses were used multiple times to provide a total of 140 data points. This larger sample size causes the results reported in this study to be more accurate and worthier of consideration.

These results give rise to new questions such as: why mare blood does not significantly degrade over time unlike cattle or other species. This research is useful for equine producers who are actively breeding mares and would be concerned with progesterone levels for recently bred mares. With the new knowledge gained from this research, new standards of blood handling can be implemented and will provide

producers peace of mind knowing that the results of the blood samples sent for analysis are accurate. An increase in testing of progesterone levels in mares could reduce the number of mares that receive recommendation for oral progesterone supplementation when it is not justified, which also decreased cost for the producer and decreases the chance of exposure to synthetic hormones for the individual administering the medication to the mare. This may also eliminate the need for excess usage and expenditure for progesterone supplementation when the mare may not need it for pregnancy maintenance.

Literature Cited

Allen, W. R. (2001). Luteal deficiency and embryo mortality in the mare. Reproductive Domestic Animals, 36, 121-131.

Asbury, A. C. Pregnant mares. American Association of Equine Practitioners. https://aaep.org/horsehealth/pregnant-mares

Bhurke, A.S., I.C. Bagchi, M.K. Bagchi (2016). Progesterone-regulated endometrial factors controlling implantation. American Journal of Reproductive Immunology, 75, 237-245. doi:10.1111/aji.12473

Breuel, K. F., D. M. Henricks, S. L. Gray, J. C. Spitzer, and T. Gimenez (1988). Effect of holding time and temperature of bovine whole blood on concentration of progesterone, estradiol-17 beta and estrone in plasma and serum samples. Theriogenology, 30(3), 613–627.

Brito, L. F. C., C.A. Wolf, J.M. Baldrighi, and O.J. Ginther. (2017). Effect of GnRH and hCG on progesterone concentration and ovarian and luteal blood flow in diestrous mares. Animal Reproduction Science, 176, 64–69. https://doi.org/https://dx.doi.org/10.1016/j.anireprosci.2016.11.010

Brogan, P. T., H. Henning, M. de Ruijter-Villani, and T.A.E. Stout. (2016). Relationship between colour flow Doppler sonographic assessment of corpus luteum activity and progesterone concentrations in mares after embryo transfer. Animal Reproduction Science, 166, 22–27. https://doi.org/https://dx.doi.org/10.1016/j.anireprosci.2015.12.010

Castro, T. de, N. Benquet, E. Rubianes, L. Valdez, and M. Rodriguez (2004). Decline in assayable progesterone in bovine serum under different storage conditions. Tropical Animal Health and Production, 36(4), 381–384. https://doi.org/http://www.kluweronline.com/issn/0049-4747/contents

Edgar, D.G. and J.W. Ronaldson. (1958). Blood levels of progesterone in the ewe. J. Endocrinology, 16, 378-384.

Farm manager's guide to progesterone assays. Bet Labs. https://betlabs.com/farm-managers-guide-to-progesterone-assays/

FDA Animal Drug Safety Communication: FDA highlights potential health risks to people exposed to altrenogest products for horses and pigs. (2018). FDA Center of Veterinary Medicine. https://www.fda.gov/animal-veterinary/cvm-updates/fda-animal-drug-safety-communication-fda-highlights-potential-health-risks-people-exposed

Ginther, O.J. (1992). Reproductive biology of the mare. Basic and applied aspects, 2nd ed. Equiservices Publishing, Cross plains, WI, USA, 233-390.

Ginther, O. J., M. D. Utt, M. A. Beg, E. L. Gastal, and M. O. Gastal (2007). Luteal blood flow and progesterone production in mares. Animal Reproduction Science, 99(1–2), 213–220. https://doi.org/http://dx.doi.org/10.1016/j.anireprosci.2006.05.018

Ginther, O.J. and V.G. Santos (2015). Natural rescue and resurgence of the equine corpus luteum. Journal of Equine Veterinary Science, 35, 1-6.

Graham, J.D. and C.L. Clarke (1997). Physiological action of progesterone in target tissues. The Endocrine Society, 18 (4), 502-519.

Holdsworth, R. J. (1980). Measurement of progesterone in bovine plasma and preserved whole blood samples by a direct radioimmunoassay. British Veterinary Journal, 136(2), 135–140.

How can you be sure that your mare no longer needs progesterone supplementation? https://www.vetmed.ucdavis.edu/sites/g/files/dgvnsk491/files/local_resources/pdfs/no-longer-needs.pdf

Irvine, C. H. G., P. Sutton, J. E. Turner, P. E. Mennick (1990). Changes in plasma progesterone concentrations from days 17 to 42 of gestation in mares maintaining or losing pregnancy. Equine Veterinary Journal, 22 (2), 104-106.

Ishak, G. M., E.L. Gastal, M.O. Gastal and S.T. Bashir. (2017). Pre-ovulatory follicle affects corpus luteum diameter, blood flow, and progesterone production in mares. Animal Reproduction Science, 187, 1–12. https://doi.org/https://dx.doi.org/10.1016/j.anireprosci.2017.09.003

McCue, P. M. (2018). Progesterone therapy. Select Breeders Services.

McCue, P. M. Regumate. Colorado State University, 1-2.

Mottershead, J. (2004). Does my mare need regumate? Equine-Reproduction.com

Mukasa-Mugerwa, E., T. Bekele and O.B. Kasali (1989). Veterinary Research Communications 13, 75. N rlhttps://doi.org/10.1007/BF00366855lnn

Naden, J., E. L. Squires, T. M. Nett (1990). Effect of maternal treatment with altrenogest on age at puberty, hormone concentration, pituitary response to exogenous GnRH, oestrous cycle characteristics and fertility of fillies. Journal of Reproductive Fertility, 88, 185-195

Nagy, P., L. Solti, M. Kulcsár, J. Reiczigel, G. Huszenicza, K. Abaváry, and A.Wölfling (1998). Progesterone determination in equine plasma using different immunoassays. Acta Veterinaria Hungarica, 46(4), 501-513.

Oberhaus, E. L. (2017). Evaluation of a Role for Prolactin in the Recrudescence of Ovarian Activity in Seasonally Anovlatory Mares. LSU Doctoral Dissertation. 4328.

Oltner, R. and L. E. Edquist (1982). Changes in plasma progesterone levels during storage of heparinized whole blood from cow, horse, dog and pig. Acta Veterinaria Scandinavica, 23(1), 1–8.

Owens, R. E., J.L. Fleeger, P.G. Harms, D.T. Atkins, and C.H. Rahe (1980). Time-dependent loss of radioimmunoassayable levels of progesterone following ambient temperature incubation of heparinized bovine blood. Theriogenology, 13(4), 305–309.

Panzani, D., A. Rota, A.L. Lainé, D. Guillaume, F. Camillo, I. Vannozzi., M. Tesi. (2017). Corpus Luteum Vascularization and Progesterone Production in Autumn and Winter Cycles of the Mare: Relationship Between Ultrasonographic Characteristics of Corpora Lutea and Plasma Progesterone Concentration in the Last Cycles Before Anestrus. Journal of Equine Veterinary Science, 56, 35–39. https://doi.org/https://dx.doi.org/10.1016/j.jevs.2017.05.001

Plotka, E.D., D.M. Witherspoon, and C.W. Foley. (1972). Luteal function in the mare as reflected by progesterone concentrations in peripheral blood plasma. American Journal of Veterinary Research, 33 (5), 917-920.

Relave, F., R.C. Lefebvre, S. Beaudoin and C. Price (2007). Accuracy of a rapid enzyme-linked immunosorbent assay to measure progesterone in mares. The Canadian Veterinary Journal 48(8), 823–826.

Santos, V.G., E.M. Bettencourt and O.J. Ginther (2015). Hormonal, luteal, and follicular changes during initiation of persistent corpus luteum in mares. Theriogenology, 83, 757-765.

Senger, P.L. (2005). Pathways to Pregnancy and Parturition, 3rd ed. Current Conceptions Inc, Redmond, OR, USA.

Serum progesterone. Colorado State University. http://csu-cvmbs.colostate.edu/documents/ria-serum-progesterone.pdf

Short, R.V. (1958). Progesterone in blood I. the chemical determination of progesterone in peripheral blood. Journal of Endocrinology 16, 415-425.

Urwin, V. E. and W.R. Allen (1983). Follicle stimulating hormone, luteinising hormone and progesterone concentrations in the blood of Thoroughbred mares exhibiting single and twin ovulations. Equine Veterinary Journal, 15, 325-329. doi:10.1111/j.2042-3306.1983.tb01813.x

Vahdat, F., S.D. Johnston, C.L. Ketelsen, J.P. Hurtgen and H.L. Whitmore (1979). Effect of time and temperature on bovine serum and plasma progesterone concentration. Theriogenology, 12(6), 371–374. Vahdat, F., B.E. Seguin, S.D. Johnson, J.P. Hurtgen, and H.L. Whitmore (1981). Decline in assayable progesterone in bovine plasma: effect of time, temperature, anticoagulant, and presence of blood cells. American Journal of Veterinary Research, 42(3), 521–522.

Volkmann, D. H. (2006). The effects of storage time and temperature and anticoagulant on laboratory measurements of canine blood progesterone concentrations. Theriogenology, 66(6/7), 1583–1586. https://doi.org/10.1016/j.theriogenology.2006.01.024

Watson, E. D., C. Broadley and M. Colston (1995). LH and progesterone concentrations during diestrus in the mare and the effect of hCG. Theriogenology, 43(8), 1325–1337.

Wiseman, B. S., G.F. Sargent, D.J. Kesler, N.S. Scheffrahn, D.L. Vincent and P.J. Thomford (1983). Changes in porcine, ovine, bovine and equine blood progesterone concentrations between collection and centrifugation. Animal Reproduction Science, 5(3), 157–165.

Vita

After completing her work at Menefee Homeschool, Tenaha, Texas in 2016, Clarissa Catherine Menefee entered Stephen F. Austin State University at Nacogdoches, Texas. She received the degree of Bachelor of Science in Agriculture from Stephen F. Austin State University in December of 2018. In January of 2019, she entered the Graduate School of Stephen F. Austin State University and received the degree of Masters of Science in December of 2020.

Permanent Address: 1744 M.L. Anderson Dr.

Tenaha, TX 75974

JAS Style Manual

This thesis was typed by Clarissa Catherine Menefee