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DEPARTMENT OF SURGERY

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Hormonal strategies for follicular atresia induction and stimulation of emergence of a new follicular wave in mares

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Thesis presented to Post-Graduation Program of Anatomy of Domestic and Wildlife Animals, School of Veterinary Medicine and Zootechny – FMVZ, University of São Paulo – USP as a requirement to obtain the title of Doctor in Science.

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CERTIFICADO

Certificamos que a proposta intitulada "Novas estratégias hormonais para indução da atresia e estimulação da emergência de nova onda de crescimento folicular em éguas", protocolada sob o CEUA nº 3597020317 (ID 004938), sob a responsabilidade de **Luciano Andrade Silva** *e equipe; Ana Paula Reway; Ed Hoffman Madureira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 23/05/2018.

We certify that the proposal "New hormonal strategies to promote follicular atresia and to stimulate growth of a new follicular wave in mares", utilizing 20 Equines (20 females), protocol number CEUA 3597020317 (ID 004938), under the responsibility of **Luciano Andrade Silva** *and team; Ana Paula Reway; Ed Hoffman Madureira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/23/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2017 a 08/2019 Área: Anatomia dos Animais Domésticos E Silvestres

Origem:	Animais provenientes de outros projetos						
Espécie:	Equídeos	sexo:	Fêmeas	idade:	3 a 12 anos	N:	20
Linhagem:	Mestiços			Peso:	300 a 550 kg		

Local do experimento: Biotério de Teriogenologia - FZEA (Laboratório de Teriogenologia Dr. O. J. Ginther - ZMV - FZEA - USP).

São Paulo, 07 de agosto de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Prof. Dr._____

Institution:_____ Judgement:_____

Prof. Dr._____

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Prof. Dr._____

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LIST OF ABBREVIATIONS

AI – Artificial Insemination

E2 – Estradiol

ET – Embryo transfer

EV – Embryo vesicle

CL – Corpus luteum

DF – Dominant follicle

DHEA – Dehydroepiandrosterone

FSH – Follicle-stimulating hormone

FTAI – Fixed-Time Artificial Insemination

GnRH – Gonadotropin releasing hormone

hCG – Human chorionic gonadotropin

IM – Intramuscular

IGF – Insulin-like growth factor

IVF – In vitro fertilization

LH – Luteinizing hormone

OV – Ovulation

P4 – Progesterone

PGF2α – Prostaglandin F 2-alfa

StAR – Steroidogenic Acute Regulatory protein

TAI – Timed Artificial Insemination

VEGF – vascular endothelial growth fator

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REWAY, A.P. **Estratégias hormonais para a indução de atresia folicular e estimulação da emergência de nova onda de crescimento folicular em éguas.** 2021. 161p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

O desenvolvimento de um protocolo hormonal capaz de sincronizar o estro e a ovulação de éguas em tempo fixo tem sido desafiador. Os dois principais desafios a serem superados para garantir a eficiência desses protocolos são: (1) a promoção da atresia folicular e (2) a emergência do crescimento de uma nova onda folicular de forma sincrônica entre as éguas tratadas. Estudos prévios deste laboratório foram capazes somente de suprimir o crescimento folicular, por alguns dias, sem ocorrência de atresia e com posterior reestabelecimento do crescimento desses folículos estáticos. Esta condição não proporcionou um desenvolvimento folicular similar entre as éguas tratadas que permitisse sincronia de tamanho de folículos entre elas e, consequentemente, uma única indução da ovulação em tempo fixo. Este projeto teve como objetivo principal desenvolver um protocolo hormonal capaz de promover a atresia, a emergência sincrônica de uma nova onda folicular e a sincronização da ovulação em éguas. Cinco experimentos foram realizados, sendo: Capítulo 3: Experimento 1 - Estabelecimento da curva de concentração plasmática de progesterona (P4) diária em éguas castradas e inteiras utilizando dispositivo intravaginal como fonte exógena de progesterona. Experimento 2 - Avaliação de seis protocolos hormonais para indução de atresia folicular e sincronização de uma nova onda de crescimento folicular durante o período de estação reprodutiva. Experimento 3 - Reavaliação dos protocolos hormonais previamente testados no experimento 2 com reajustes na dose de P4 e na frequência de administração de estradiol (E2) para indução de atresia e sincronização de uma nova onda de crescimento folicular. Capítulo 4: Experimento 4 - Avaliação dos níveis plasmáticos de progesterona e do status funcional do corpo lúteo por meio de ultrassonografia Doppler em éguas cíclicas submetidas aos tratamentos utilizando diferentes fontes de progesterona exógena. Capítulo 5: Experimento 5 – Teste de eficiência da indução da ovulação em tempo fixo e da fertilidade em éguas cíclicas submetidas a protocolos hormonais para sincronização das ondas foliculares e da ovulação. Foi levantada a hipótese de que o grau de sincronia

do crescimento folicular dependeria da eficiência obtida na indução da atresia folicular devido ao tratamento hormonal realizado. Além disso, formulou-se a hipótese de que a função luteal dos CLs resultantes de ovulações após tratamentos hormonais apresentaria um elevado grau de vascularização e, consequentemente, as éguas apresentariam elevados níveis plasmáticos de P4. Outra hipótese consistia em ser possível realizar a inseminação artificial a tempo fixo em éguas e que os oócitos submetidos a tratamento hormonal para sincronização do estro e da ovulação seriam férteis. O efeito de novas combinações de fontes hormonais de progesterona e estradiol e a associação de baixas doses de hCG e deslorelina foram testados durante duas estações reprodutivas. Dois dos protocolos hormonais testados foram selecionados com base em sua eficiência e estudos de fertilidade desenvolvidos com eles (Capítulo 5; Experimento 5). O primeiro promoveu atresia folicular e os oócitos foram originados de folículos emergidos em uma nova onda de crescimento folicular. O segundo não foi capaz de promover a atresia e a emergência de uma nova onda folicular, porém provocou supressão do crescimento dos folículos e as ovulações ocorreram em folículos que retomaram o crescimento após a remoção da fonte de progesterona exógena. A função luteal pós-tratamentos hormonais permaneceu com boa capacidade de produção de P4. A fertilidade das éguas submetidas a esses protocolos hormonais, após inseminação artificial em tempo fixo 30 horas após a indução das ovulações, foi avaliada por meio de diagnóstico ultrassonográfico de gestação aos treze dias após a ovulação. As taxas de prenhez nos grupos tratados para sincronização da ovulação foram de 83% (15/18) e 72% (13/18), respectivamente, não sendo estatisticamente diferentes ($P > 0,05$). Entretanto, o diâmetro médio das vesículas embrionárias do grupo que não apresentou atresia folicular foi menor do que no outro grupo que foi capaz de promover atresia folicular ($P < 0,05$) e também menor do que a média citada pela literatura no décimo terceiro dia de gestação. O desenvolvimento embrionário pode ter sido comprometido no grupo cujos oócitos foram provenientes de folículos que tiveram seu crescimento retardado pela ação da progesterona exógena. Desta forma, as estratégias hormonais utilizadas foram capazes de desenvolver dois protocolos de tratamento hormonal capazes de sincronizar as ovulações para a realização da inseminação artificial em tempo fixo em éguas e obtiveram boas taxas de prenhez.

Palavras- chave: éguas, hormônios, sincronização, ovulação, inseminação artificial

Abstract

REWAY, A.P. **Hormonal strategies for follicular atresia induction and stimulation of emergence of a new follicular wave in mares.** 2021. 161 p. Thesis (Doctorate in Science) - School of Veterinary Medicine and Zootechny, São Paulo State University, São Paulo, 2021.

The development of a hormonal protocol capable of estrus synchronization and time-fixed ovulation induction in mares has been a challenge. The two main difficulties to achieve it are to promote follicular atresia and to induce the emergence of a new synchronic follicular wave among treated mares. Previous studies from our laboratory were capable, for a few days, to stop follicular growth with no occurrence of atresia and posterior reestablishment of the follicular growth. This condition did not permit a similar follicular development among the mares to permit only one treatment to induce ovulation in a fixed-time manner. The main objectives of this project were: (1) to develop a hormonal protocol capable to promote follicular atresia, and (2) the emergence of a synchronic follicular wave in mares. Five experiments were performed: Chapter 3: Experiment 1 – Daily plasma progesterone (P4) concentration curve in ovariectomized and cyclic mares treated with an intravaginal P4 releasing device. Experiment 2 – Evaluation of six hormonal protocols for atresia induction and synchronization of a new follicular wave. Experiment 3 – Test of two modified hormonal protocols for follicular atresia induction and synchronization of a new follicular wave. Chapter 4: Experiment 4 - Evaluation of plasma P4 levels and functional status of the corpus luteum (CL) using Doppler ultrasonography in cyclic mares submitted to treatments using different sources of exogenous P4. Chapter 5: Experiment 5 - Test of the efficiency of fixed-time ovulation induction and fertility in cyclic mares submitted to hormonal protocols for follicular wave and ovulation synchronization. It was hypothesized that the degree of synchrony of follicular growth would dependent on the efficiency obtained for induction of follicular atresia by the hormonal treatment performed. Besides, it was hypothesized that luteal function of CLs resulting from ovulations post-hormonal treatments would present high degree of vascularization and consequently the mares would present high plasmatic levels of P4. Another hypothesis was that it would be possible to perform fixed time artificial insemination in mares and its oocytes submitted to hormonal treatment for synchronization of estrus and ovulation would be fertile. The effect of new sources of

hormonal combinations of P4 and estradiol and the association of low doses of hCG and deslorelin were tested during two reproductive seasons. Two of the previously tested hormonal protocols were selected based on their efficiency for new studies of fertility (Chapter 5; Experiment 5). The first protocol promoted follicular atresia and the oocytes were originated from follicles emerged from a new follicular wave. The second protocol was not capable to promote neither atresia nor the emergence of a new follicular wave, however caused follicle growth suppression and ovulation of follicles that resumed its growth after exogenous P4 source removal. The luteal function post-hormonal treatments remained with a good capacity of P4 production. The fertility of mares submitted to hormonal treatment protocols for ovulation synchronization, after fixed time artificial insemination 30 hours after ovulation induction, was evaluated by ultrasound diagnosis of gestation thirteen days after ovulation. Pregnancy rates were 83% (15/18) and 72% (13/18), on both treatments and were not statistically different ($P > 0.05$). Nevertheless, the average diameter of the embryonic vesicles in the group not capable of causing follicular atresia was smaller than in the group that showed follicular atresia ($P < 0.05$), and even smaller than the average reported in the literature on the thirteenth day of gestation. Initial embryonic development may have been compromised when the oocytes resulted from follicles which presented suppressed growth by the action of exogenous P4. Therefore, the hormonal strategies used were able to develop two hormonal treatment protocols capable of synchronizing ovulations for fixed time artificial insemination in mares presenting satisfactory pregnancy rates.

Keywords: mares, hormones, synchronization, ovulation, artificial insemination

CHAPTER 1

1. INTRODUCTION

The use of horses still important for the development of livestock and agriculture activities in Brazil. Activities related to the Brazilian equine industry are responsible by the amount of $ 3 billion/year in the country economy and by around 3.6 million jobs. Brazil has the third largest equine herd in the world, after China and Mexico, with a total of 5.9 million of animals, but only 700.000 horses are registered in some of the official genealogical services (IBGE, 2017). The horse market showed 113% growth during the last decade. Data from MAPA (Ministry of Agriculture, Livestock and Supply; 2016) documented that there are 26 breeds of horses and six other pony breeds in the country. The leader herd in size is the breed Mangalarga Marchador with 644.000 horses, followed by the Nordestino breed (500.000). The Quarter Mile (346.936), Crioulo (322.000) and Mangalarga (100.000) complete the list of the five largest herds in Brazil. It is worth to mention that these numbers do not include the number of donkeys and hinnies, one of the most expressive growing herds in recent years (MAPA, 2016).

Producers and veterinarians are increasingly demanding foals born with superior genetic characteristics. One way to achieve this goal, the birth of genetically superior foals, is investing in reproductive biotechnologies because it provides faster and significant advances, not only for the required genetic and phenotypic characteristics but also to contribute to healthier animal selection. Thus, every new technique capable of reducing the interval of generations and increasing the number of stallions and mares has major importance for the Brazilian equine herd development (BORTOT, 2013). Due to popularization of current biotechnologies, as artificial insemination (AI) and embryo transfer (ET), these reproductive procedures are becoming more economically affordable (LOPEZ, 2019; MAPA, 2016).

The equine species presents some reproductive peculiarities compared to other livestock species of domestic mammals. The high variability of the estrus duration and the difficulty to predict the exact time of ovulation led to the need of development of methods to control the estrous cycle and ovulation (MELLO, et al, 2012). Besides, the implementation of commercial and accessible reproductive biotechnologies depends on the knowledge of reproductive anatomy and physiology, associated with good

management conditions and animal welfare (RODRIGUES et al., 2017; SILVEIRA, 2017; MARQUES et al, 2019).

Breeders have aimed to guarantee the genetic quality of their animals, which has resulted in increased use of AI technique in equines. Artificial insemination is a widely practiced reproduction technique that consists in deposition of equid semen, previously collected, evaluated and processed, in the reproductive tract of the mare. The benefits of AI include: accelerates the process of genetic improvement of the breeds, reduces the risk of accidents during natural cover, prevents infectious and contagious diseases, allows breeding of mares and stallions at different locations in the world, also facilitates the same stallion to generate more offspring and enables the use of frozen semen from stallions that have already died (BORDOT, 2013).

Artificial insemination is an important tool for genetic improvement, although it still has some limitations. Fixed time artificial insemination (FTAI) remains not practicable for horses yet (FANELLI et al., 2018). Synchronization of estrus and ovulation is an excellent tool to improve the use of timed artificial insemination (TAI). In bovine reproduction, estrus and ovulation synchronization is possible by using many different hormonal protocols allowing the application of TAI to manage big herds of cows without estrus detection (ALNIMER et al., 2009).

A new and efficient hormonal protocol for estrus synchronization and ovulation induction in mares will be extremely useful in the routine of equine breeding centers. An efficient protocol will be the one capable of stimulating ovulation of a considerable percentage of mares within a given period and presenting a small-time variation among these mares, allowing TAI to maximize pregnancy rates. There are some difficulties in the elaboration of these protocols for the equine species, for example, the adjustments of the hormonal combinations and its dosages: (1) to promote follicular atresia and, consequently, (2) to stimulate the emergence of a new synchronic follicular wave. Several characteristics of folliculogenesis in equine species difficult the elaboration of an efficient hormonal protocol and the main obstacles are: (1) the variable size of the ovarian follicles, (2) the different follicular population among mares and, mainly, (3) the different possible sizes of the dominant follicle at the time of ovulation (GINTHER, 1992).

Currently, there is no commercial progesterone (P4) releasing formulation available for use in mares, and capable of providing an adequate amount of this hormone within a predefined period of time. Estrus synchronization protocols require devices with constant P4 release concentration. For this reason, intravaginal bovine P4 release devices

have been alternatively used in mares. In several attempts to develop these hormonal protocols in mares, the results had been unsatisfactory, probably because the P4 commercial formulation used is not species specific, in other words, the release rate at the time of treatment probably was not effective for hypothalamus pituitary axis inhibition in this species (ROCHA FILHO et al., 2004; GRECO et al., 2008; SILVA et al., 2011; REWAY, 2017).

The optimization of reproductive results and the development of new drugs is the main goal of studies related to hormonal therapy in mares. However, estrous cycle manipulation in mares is not as efficient and routinely used as in cows. The costs of protocols already tested are also barriers to its use. Advances in this area would allow the establishment of follicular wave synchronization protocols that would assist the acquisition of better results using biotechnology tools in reproductive programs.

Based on the exposed, this study aimed to induce follicular atresia in mares, followed by the emergence of a new follicular wave, objecting to test the degree of synchronization of follicular growth using new hormonal combinations of P4 and estradiol (E2). This research was divided in three articles, which were represented by chapters three, four and five. Our initial experiments, described since chapter 3, were conducted to develop an estrus and ovulation synchronization protocol capable of promoting follicular atresia and synchronizing the stimulation of a new follicular wave. The first experiment evaluated plasmatic P4 release rate when using an adapted cow intravaginal P4 releasing device; supposedly follicular atresia would occur when plasma concentrations of P4 were maintained above 5 ng/ml at least during four consecutive days after the initiation of the hormonal treatment. The subsequent experiments tested six hormonal protocols for induction of follicular atresia and synchronization of a new follicular wave; then, two new protocols were tested in which adjustments were made. Presumably, the use of E2 in association with P4 at the time of the beginning of treatment would increase the efficiency of induction of follicular atresia. In chapter 4, an experiment evaluated the corpus luteum (CL) function in mares submitted to different hormonal treatments for estrus and ovulation synchronization. In chapter 5, an experiment evaluated the fertility rates of mares submitted to the hormonal protocols capable of promoting atresia and synchronization of the follicular wave. The results of this study will facilitate de execution of assisted reproduction procedures in equids and add, as an alternative of reproduction management, the technique of fixed-time artificial insemination (FTAI).

CHAPTER 2

2. LITERATURE REVIEW

The knowledge related to the factors that interfere in reproduction is crucial to successfully handle an equine herd. Therefore, the study of the reproductive physiology and peculiarities of the equine species is required to increase efficiency of assisted reproductive technique in equines. In order to efficiently synchronize ovulation in mares, for the purpose of providing satisfactory pregnancy rates, the fulfillment of four requirements is necessary. The first one involves providing a luteal period that mimics natural P4 levels of the estrous cycle. Also, it involves providing the ability of the exogenous source of P4 to change the frequency and amplitude of LH pulses in favor of the return to cyclicity. Second, all animals need to discontinue the luteal period synchronously, by interruption of exogenous P4 supply with or without administration of PGF2α, which causes a significant reduction in circulating P4 levels. The third requirement consists in synchronizing the emergence of the follicular wave. The synchronization of the follicular wave can be done in three distinct ways; by the aspiration of the ovarian follicles guided by ultrasound, by promoting ovulation of the dominant follicles, and by inducing follicular atresia, regardless the stage of development that the follicles may be found. Follicular atresia is expected after the luteal period induced by hormonal treatment. The fourth requirement is based on granting a synchronized pituitary surge of LH that can be achieved by administration of exogenous human chorionic gonadotropin (hCG), or a GnRH analogue or LH itself. Ovulations usually occur synchronously approximately 25 to 30 hours after the administration of ovulation inducers (MADUREIRA and MATURANA, 2012).

One of the most common methods of estrus synchronization in mares is the induction of luteolysis with the use of prostaglandins F2α (PGF) or its analogues. For the fastest return to estrus by luteolysis, the mare must have a PGF2α responsive CL. Better response of estrus synchronization using PGF2α treatment occurs when two applications are made with intervals of 14 days. This strategy promotes luteolytic effect in all animals at the second injection if the CL is not responsive at the first dose. After PGF2α application, onset of estrus can occur within 3-4 days and ovulation after 8-10 days (SAMPER, 2008).

Newcombe et al. (2008) tested the effect of different doses of cloprostenol, an analogue of PGF2α on ovulation induction in mares with follicles ≥ 28mm during diestrus. In this study, the authors found that the 625µg of cloprostenol anticipated ovulation, which occurred on average of 2.4 days after cloprostenol administration when follicles were ≥ 36mm in diameter and 8.7 days when follicles had a diameter ≥ 28mm. Another interesting aspect observed in this study was the total of 1234 studied estrus cycles, in which 25% of mares with 28mm follicles ovulated within three days and 50% of mares with follicles ≥ 36mm ovulated within two days after a 625µg of cloprostenol. In two other studies, Lindeberg et al. (2002) and Nielsen et al. (2008) found that PGF2α administration in diestrus mares may be associated with lower pregnancy rates. Lindeberg et al. (2002) reported that mares treated with PGF2α or PGF2α combined with hCG during the CL responsiveness period had lower pregnancy rates than the untreated control group. Similarly, Nielsen et al. (2008) found that estrus induction after PGF2α administration had a significant negative influence on pregnancy rate, with an average reduction from 61.4% to 39.1% after artificial insemination. These authors claimed PGF2α may exert a negative effect such as regression of follicles measuring 40 mm or even larger. Endocrinological disturbances may result from PGF2α treatment given in the presence of large follicles and this may lower pregnancy rate. The negative influence of PGF2α is not believed to be due to PGF2α by itself, whether the moment of the cycle when it is used (LINDEBERG et al., 2002 and NIELSEN et al., 2008).

A regular interovulatory interval extends from 16 to 25 days with a mean of 22 days, and it has been suggested that fertility of cycles with short interovulatory interval is lower. PGF2α are routinely used in equine ET programs, and the interval from one PGF2α treatment to the next ovulation can be as short as two days, but it may vary from 2 to 12 days (NEWCOMBE et al., 2008). Embryo recovery rate on donor mares with an interovulatory interval shorter than six days was lower in comparison with mares with longer interovulatory intervals. If a large follicle (≥ 30 mm) is undergoing atresia the interovulatory interval will be similar or even longer than non-treated mares (MELINA et al., 2019). One theory explains low fertility cycles with short interovulatory interval because of an increased postmating induced endometritis due to a short period of time between luteolysis and artificial insemination (PYCOCK et al., 2007). Another theory is based on a cattle study, which documented that oocytes from diestral follicles, secondary waves, or previous estrus, could not be viable due to their prolonged development and their extensive exposure to high P4 and low LH levels (INSKEEP, 2009).

The manipulation of the estrous cycle can also be done with estrogen and P4. This type of treatment aims to mimic a hormonal profile like the natural estrous cycle, culminating with obtention of a viable and good quality oocyte, causing endometrial edema, and inducing uterine characteristics needed for the development of pregnancy. The main obstacle to ovulation synchronization in mares is determining the dose of this combination of hormones (P4 and E2), the frequency and the appropriate time for its administration (FRITSCH, 2016).

Currently, on the market, the exogenous sources of progestogens available for horses are altrenogest (synthetic progestogen) and injectable P4 (natural P4 in oil solution). The use of altrenogest requires daily oral administration and the injectable P4 can be found in short action vehicle at a dose of 200 or 400 mg/per mare requiring administration every other day or in long action vehicle, administered every seven days at a dose of 1500 mg/per mare (ROCHA FILHO et al., 2004). These progestogens sources have satisfactory results in the maintenance of pregnancy and some studies have been developed to use them also for estrus synchronization in mares (ROCHA FILHO et al., 2004; GRECO et al., 2008; SILVA et al., 2011). The use of altrenogest daily is laborious but by the time the exogenous treatment of P4 ceases, the concentrations of the hormone in the blood will also decrease. On the other hand, the long-acting formulations of injectable P4, despite easiness to handle, maintains blood levels of P4 for periods as long as seven days. In addition, the injectable P4 vehicle can cause inflammatory reactions at the site of application and, consequently, some mares develop intolerant behavior to injections (BERGFELT, 2007). Squires (1993) suggests that estrus synchronization is effective with daily IM P4 application or its active synthetic derivative orally for periods ranging from 8 to 14 days.

Sharp (2000) defined that the P4 plasma level of 4 ng/ml is enough for pregnancy maintenance in mares supplemented with this hormone. Hughes et al. (1980) demonstrated that administration of 200 mg/day of P4 was capable to achieve the plasma level of 4 ng/ml, however, daily doses of 50 and 100 mg were not. Bergfelt & Ginther (1996) found that the physiological concentration of P4 in mares was close to zero on the day of ovulation and reached approximately 3 ng/mL on D2, 7 ng/mL on D4, 11 ng/ml on D7, with levels below 1 ng/mL characterizing the estrus period. Similar results were observed by Souza et al. (2008) in fillies of the Brazilian Equestrian breed in which the mean concentrations of plasma P4 found were 5.6 ng/mL on D4 post-ovulation and these

concentrations remained high until D14 returning to baseline values during the new estrus.

Another exogenous source of P4 that has been tested in mares are the slow-releasing intravaginal devices commonly used in bovine females. In mares, these devices have been used to regulate the first estrus of the reproductive season and as an attempt to synchronize the estrus. In these situations, after removal of the device, a new follicular wave is expected to develop and ovulation will later occur (NEWCOMBE et al., 2002). The selection of mares to be submitted to P4 treatment is especially important for the purpose of anticipating the reproductive season. Mares with an ovarian follicular population with an average size of less than 20 mm diameter may not respond to the treatment with P4 and continue in anestrus. In mares presenting follicles, which are on average 25 mm diameter, treatment with P4 may result in satisfactory results of estrus and ovulation synchronization (NEWCOMBE and WILSON, 1997). Some examples of commercial intravaginal P4 devices are: CIDR-B, PRID and CuMate. The devices contain between 1.55 and 1.90 mg of P4 and can elevate the serum concentrations of P4 in the mare to 3 to 5 ng/ml during approximately ten days. The response to the decrease in P4 levels after intravaginal device removal causes the development of new follicles and most mares present estrus and ovulation within eight days after intravaginal device removal (NEWCOMBE, 2002). One of the disadvantages of using these devices is the potential occurrence of vaginitis with mild to moderate secretion production. However, it has been demonstrated that this condition presents spontaneous resolution in most mares within 24 hours and its use is safe even in pregnant mares (NEWCOMBE, 2002).

Estradiol based compounds have also been tested on cyclic mares in estrus synchronization protocols. Administration of 17ß-estradiol (50 mg, IM) or estradiol cypionate (50 mg, IM) associated with PGF2α (without P4) on the day after ovulation caused follicular atresia. Treatments using the association of estradiol plus PGF2α can provide similar or even better results when combined with P4 (PINTO et al, 2004). The use of P4 combined with E2 was also tested for synchronization of estrus in mares. Larsen and Norman (2010) developed a protocol in which 89% of the mares were synchronized by administrating on day 0 (initiation of hormone treatment) 20 mL/per mare of estradiol benzoate associated with the insertion of intravaginal P4 device (1.72 g). However, the emergence of a new follicular wave and fixed time ovulation induction were not studied. The intravaginal device was maintained for 10 days and 7.5 mg PGF2α (dinoprost) was administered at the time of its removal. In this study, 100% of the mares presented

follicular atresia after the beginning of treatment, simulating a follicular aspiration, but without the need for the invasive procedure. For induction of ovulation, the recommendation is to use the inducing agents 48 hours after removal of the intravaginal P4 device. If the intention is to program ovulation in a group of equine females, the authors recommended transrectal palpation and stallion teasing 72 hours after removal of the P4 device. In this experiment, ovulation occurred in an interval between 5 and 8 days after finished the treatment with P4.

Newcombe et al. (2002) used a gradual release of P4 (2g) by intravaginal devices during 10 days in mares in anestrus and in transitional period, associated with 17ß-estradiol and PGF2α. In this study, the estrus began two days after removal of the device and follicles larger than 35 mm were detected 2.9 days after removal of the device. Most mares presented vaginal secretion and vaginitis with the use of this device.

Three other synchronization protocols were tested by Almeida et al. (2001). Different P4 sources were used during nine days. In the first group 0.5 mg/animal/day of melengestrol acetate was used; in the second group 0.045 mg/kg of body weight of oral altrenogest and in the third group a single dose of 3 mg of norgestomet IM plus subcutaneous implant of 3 mg of norgestomet. The second and third groups were effective in synchronizing the estrus and P4 at these doses did not interfere with pregnancy rates when compared to cycles which were not artificially manipulated.

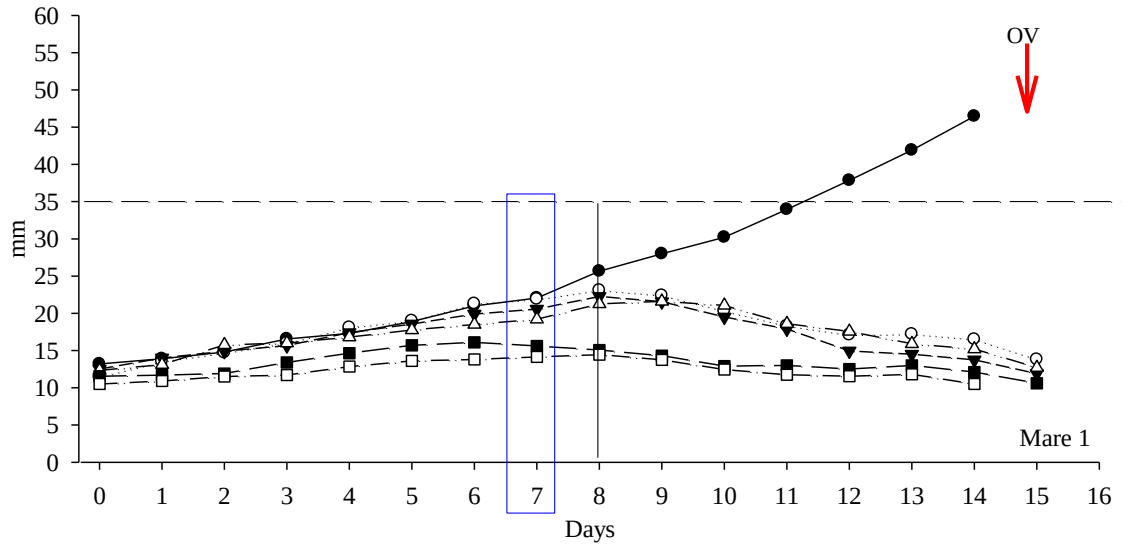
In a previous master's dissertation study performed at the Theriogenology Laboratory Dr. O.J. Ginther, Reway (2017) obtained 81.1% efficacy in synchronization of estrus and ovulation in mares as presented in table 2.1. In this study, the intravaginal devices used were the same as those used in cows, containing 0.96 g P4. Although follicular atresia did not occur, the growth of ovarian follicles was inhibited during the treatment period. Consequently, after the removal of the intravaginal devices there was no emergence of a new follicular wave, and the pre-existing follicles resumed their growth until the ovulation of the dominant follicle. The combination of P4 with E2 hormones at the beginning of the hormonal protocol was not tested. In addition, to achieve the efficiency of 81.1% ovulation synchronization, ovulation inductions were performed two times, adding more handling and ultrasound examinations of the mares.

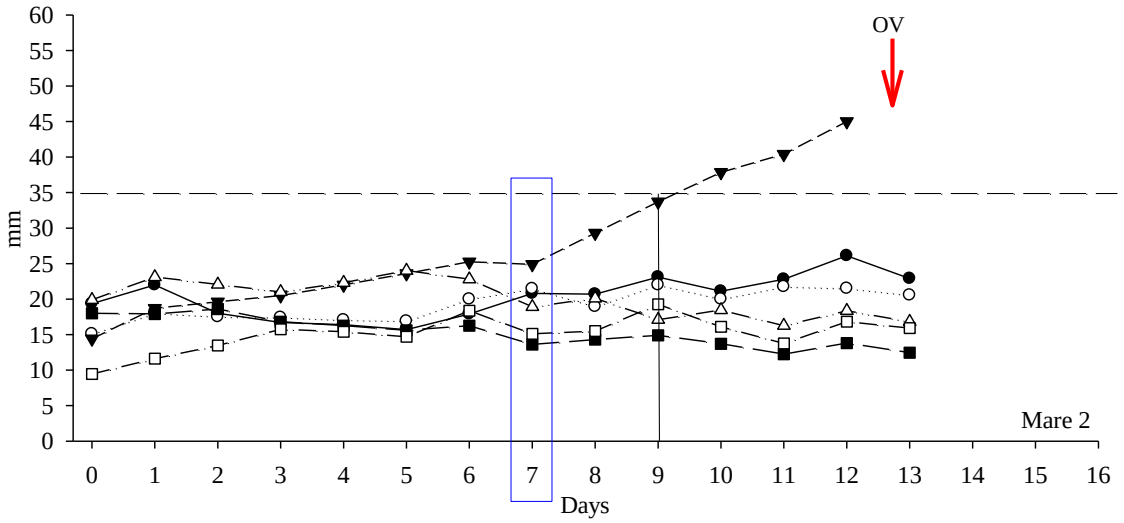
Table 2.1 Number and percentage of mares ovulating at the end of the hormonal protocol after ovulation induction at two different times (day 10 = D10 and day 12 = D12) in four experiments. On D10, ovulation was induced in all mares that presented pre-ovulatory follicle with diameter ≥35 mm and uterus with echotexture ≥3. On D12, a second ovulation induction was performed in mares which had not presented the criteria of ovarian and uterine appropriate characteristics for the induction performed on D10. The mares were randomly distributed and were at different days of the estrous cycle at the beginning of treatment (D0) in experiment I. In experiments II, III and IV the mares were selected after ovulation detection on days 5, 10 and 15 of the estrous cycle, when hormonal treatments were started in these three experiments, respectively (REWAY, 2017). OV = ovulation and No OV = no ovulation.

Experiments	D10		D12		Total	Total	Total
	OV	No OV	OV	No OV	OV	No OV	
I	10 (66.6%)	1 (6.6%)	2 (13.3%)	2 (13.3%)	12 (80%)	3 (20%)	15
II	5 (35.7%)	1 (7.1%)	5 (35.7%)	3 (21.4%)	10 (71.4%)	4 (28.6%)	14
III	7 (58.3%)	1 (8.3%)	3 (25%)	1 (8.3%)	10 (83.3%)	2 (16.7%)	12
IV	6 (50%)	0	5 (41.7%)	1 (8.3%)	11 (91.7%)	1 (8.3%)	12
Total	28(52.8%)	3 (5.7%)	15 (28.3%)	7 (13.2%)	43 (81.1%)	10 (18.9%)	53

Reway (2017) reported no regression of the dominant follicle and that there was a delay in follicular growth while the intravaginal P4 device (0.96 g) was used. It was observed that the day of the mare's estrous cycle when hormonal treatment started did not interfere with the results of the protocol and that in cyclic mares the treatment with intravaginal P4 device P4 (0.96 g) may be useful to suppress follicular growth, but it was not capable of causing follicular atresia nor the emergence of a new follicular wave. Three individual graphs illustrate follicular growth of three distinct mares submitted to the tested protocol in Figure 2.1. It was verified that the response of the mares to the intravaginal P4 device differs among them. In some mares, follicular growth almost ceased and in other mares there was only a slight deceleration of follicular growth. The daily average follicular growth of a mare is equivalent to 3 mm/day (GINTHER, 1992). The treatment with P4 caused a deceleration in follicular growth until D7. After D7, the follicles grew above the average reported by Ginther (1992). Moreover, the author of this study (REWAY, 2017) demonstrated that the association of two ovulation inducers (hCG and GnRH) was effective even when used at low doses, being able to promote ovulation at intervals between 24 to 48 hours after treatment. Based on the reported results, follicular atresia and the emergence of a new follicular wave are determinant events for new

follicles to grow with similar sizes to induce ovulation in a single day with greater efficiency.





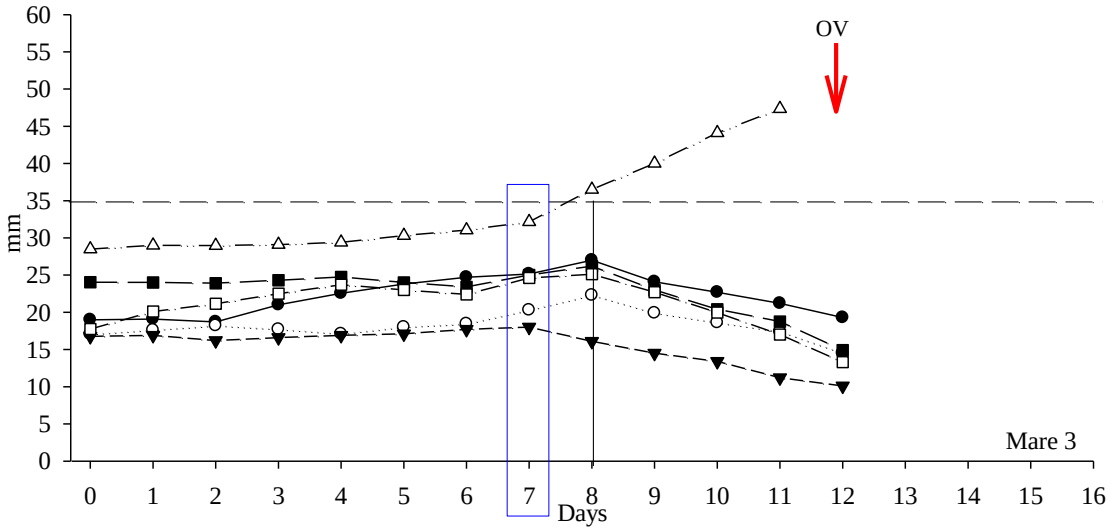


Figure 2.1 Graph representation of the ultrasonographic monitoring of the follicular dynamics of three mares used during Reway's experiment (2017). The day of the beginning of the protocol (D0) corresponded to days 5, 10 and 15 of the estrous cycle, respectively for mares 1, 2 and 3. The diameters of the three largest follicles of each ovary were measured daily. The graphs exemplify the effect of the intravaginal P4 device on follicular growth suppression. It is noted that this modulatory effect of P4 on follicular growth occurred in all three animals regardless of the day of the estrous cycle in which treatment was started. The blue rectangle indicates the day on which the P4 devices were removed. The day when the follicular divergence occurred is indicated by the vertical full line. The follicular diameter of 35mm is indicated by the horizontal dotted line. The red arrow indicates the day of ovulation.

The efficiency obtained in the hormonal treatment protocol tested by Reway (2017) would be reduced to 52.8% if ovulation induction has been performed only a single time, on D10. It was possible to increase the efficiency of the hormonal treatment protocol to 81.1% adding an alternative second ovulation induction. We performed transrectal ultrasound examination and then, first ovulation induction was executed when follicles were equal or greater than 35 mm diameter and endometrial edema on D10 was detected. A second ovulation induction for all the other mares not induced on D10 was performed with no other requirement on D12.

Based on the results obtained by Reway (2017), this research was designed to test if new hormonal combinations of P4 and E2 can induce follicular atresia and stimulate the emergence of a new follicular wave. The high degree of synchronization of follicular growth and the efficiency and temporal precision for ovulation induction at a fixed-time manner were the focus of the creation of the new hormonal protocols for mares.

REFERENCES

ALMEIDA, H.B., VIANA, W.G., ARRUDA, R.P., OLIVEIRA, C.A. Synchronization of estrus and follicular dynamics of Crioulas mares submitted to treatments with norgestomet, melengestrol acetate and altrenogest. **Brazilian Journal of Veterinary Research and Animal Science**, v. 38, n.6, 2001.

ALNIMER, M.A.; TABBAA, M.J.; ABABNEH, M.M.; LUBBADEH, W.F. Applying variations of the Ovsynch 320 protocol at the middle of the estrus cycle on reproductive performance of lactating dairy 321 cows during summer and winter. **Theriogenology**. v.72, p. 731-740, 2009.

ALVES, R. G. N.; QUEIROZ, A. R. R.; MIRANDA, C. D. M.; MURTA, D.C. R. X.; MURTA, D.V. F.; SANTOS, J.M.L.; CARNEIRO, J. A. M. Influence of the cyclicity on the pregnancy rate in animals submitted to the IATF program. **Brazilian Journal of Development**, v.5, n. 11, p. 24701-24706, 2019.

BERGFELT, D.R.; GINTHER, O.J. Ovarian, uterine and embryo dynamics in horses versus ponies. **Journal of Equine Veterinary Science**, v.16, n.2, p.66-72, 1996.

BERGFELT, D.R.; ADAMS, G.P. Ovulation and corpus luteum development. In: SAMPER, J.C.; PYCOCK, J.F.; McKINNON, A.O. **Current therapy in equine reproduction.** Hardcover: Sanders Elsevier, p. 1-13, 2007.

BORTOT, D. C.; ZAPPA, V. Aspects of equine reproduction: artificial insemination and embryo transfer: literature review. **Scientific Electronic Journal of Veterinary Medicine**, v. 11, n. 21, 2013.

FANELLI, D.; TESI, M.; ROTA, A.; BELTRAMO, M.; CAMILLO, F.; PANZANI, D. Studies on the use of PGF2α and GnRH analogues for Timed Artificial Insemination in jennies. **Journal of Equine Veterinary Science**, 2018.

FRITSCH, S.C. **Effect of administration of different doses of oestradiol followed by progesterone on the expression of endometrial estrogen and progesterone receptors in acyclic receptor mares. Master's Thesis.** FMVZ Unesp, Botucatu, 2016.

GINTHER, O.J. Sexual behavior Characteristics of the ovulatory season. In: **Reproductive Biology of the Mare: Basic and Applied Aspects**, 2nd ed. Wisconsin: Editor Equiservices, 1992.

GINTHER, O.J.; JACOB, J.C.; GASTAL, E.L.; GASTAL, M.O.; CARVALHO, G.R.; BEG, M.A. Temporal relationships and repeatability of follicle diameters and hormone concentrations within individuals in mares. **Reproduction of domestic animals**, v.44, p.92–99, 2009.

GRECO, G.M.; AVANZI, B.R.; FIORATTI, E.G.; QUEIROZ, F.J.R.; MEIRA, C.; ALVARENGA, M.A. Effect of interruption of progesterone treatment on acyclic receptor mares presenting the formation of accessory luteous bodies. **Acta Scientiae Veterinariae**, v.36 (Supl.2), 2008.

HUGHES, J.P., STABENFELT, G.H. E KENNEDY, P.C (1980) The estrous cycle and selected functional and pathologic ovarian abnormalities in the mare. **Veterinary Clinics of North America** (Large Anim. Pract.), v.2, n.2, p.225-239.

IBGE - **BRAZILIAN INSTITUTE OF GEOGRAPHY AND STATISTICS**. Effective of the equine herd. 2017. Available at:<https://sidra.ibge.gov.br/tabela/3939#resultado> Access in: June 20th 2019.

INSKEEP, E.K. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. **Journal of Animal Science** 82, p.24-39, 2009..

LARSEN, J.E.; NORMAN, S.T. The Synchronization of Oestrus and Ovulation in the Mare. **RIRDC - Rural Industries Research and Development Corporation**, Innovation for rural Australia, v. 10, n.202, p. 7-31. 2010.

LEY, M. B. Breeding in Mares for Equine Veterinarians, 1st Ed. Roca, São Paulo, p.184-191, 2006.

LINDEBERG, H., KOSKINEN, E., HUHTINEN, M., REILA, T., PERTTULA, H., KATILA, T. Influence of PG administration and follicle status on the number of conceptuses. **Theriogenology**; 58:571–574. 3. 2002.

LOPEZ, R.W. Continuously expanding, the equine market represents an annual income of R$ 16.5 billion in Brazil. **Attalea Agrobusiness Journal**, July, 2019.

MADUREIRA, E.H.; MATURANA, M.F. Technological advances in the use of drugs to control the reproduction of cows intended for TAI. Biotechnology of Bovine Reproduction, 5th **International Symposium on Applied Animal Reproduction** p.93-107, 2012.

MAPA. **MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY.** 2016. Review of the Horse Agrobusiness Complex Study. Available in: < http://www.agricultura.gov.br/assuntos/camar as-setoriais tematicas/documentos/camarassetoriais/equideocultura/anosanteriores/revisao-do-estudo-do-complexo-doagronegocio-do-cavalo/view>. Access in: January 27th 2020.

MELLO, C. M.; PAPA, F. O.; PUOLI FILHO, J. N. P.; ARAÚJO, G. H., DELL'AQUA JR., J. A. AND ALVARENGA, M. A. Efficiency of deslorelin acetate and equine pituitary extract in inducing ovulation in mares. **Veterinary and Zootechny**, 12: p. 392-398, 2012.

MARQUES, T.D.P; PESSOA, M.S, ABRÃO, F.O Nutritional aspects related to reproduction in horses. **Nutri Time Electronic Journal**. v. 16, n.03, 2019.

MELINA, P.; LUIS, L.; JUAN, C. A. Effect of the interval from Prostaglandin F2alpha treatment to ovulation on reproductive efficiency rates in a commercial Equine Embryo Transfer program. **Journal of Equine Veterinary Science**. 2019.

MEIRA, C. **Endocrinology of Reproduction, Follicular Dynamics, Superovulation and Embryo Transfer in Equine Species.** (Reproduction Area) - College of Veterinary Medicine and animal Sciences of Sao Paulo State University Julio de Mesquita Filho, Botucatu, SP, 2007.

NEWCOMBE, J.R.; WILSON, M.C. The use of progesterone releasing intravaginal devices to induce seasonally anoestrus Standardbred mares. **Equine Practitioners**, v.19, p. 13-21, 1997.

NEWCOMBE, J.R. Field observations on the use of progesterone releasing intravaginal device to induce oestrus and ovulation in seasonally anoestrus mares. **Journal of Equine Veterinary Science**, v. 22, p. 378-382, 2002.

NEWCOMBE, J.R.; JOCHLE, W.; CUERVO-ARANGO J. Effect of dose of cloprostenol on the interval to ovulation in the dioestrous mare: a retrospective study. **Journal of Equine Veterinary Science**, 28: 532-539, 2008.

NIELSEN, J.M., KOFOED BOCK, T.S.; ERSBOLL, A.K. Factors associated with fertility in horses in a Danish equine practice after artificial insemination with frozen-thawed semen. **Animal Reproduction Science**. 2008; 107: 336–337.

PINTO CR, BURNS PJ, WHISNANT S. Effects of single estradiol administration on follicular dynamics and luteal function of cyclic mares. In: International Symposium on Equine Embryo Transfer, 6, 2004, Rio de Janeiro. **Proceedings.** Rio de Janeiro: ISEET, 2004. p.92-94. Resumo.

PYCOCK, J.F. Therapy for mares with uterine fluid. In: SAMPER, J.C.; PYCOCK, J.F.; MCKINNON, A.O. **Current therapy in equine reproduction.** St. Louis, CA: Saunders, Elsevier; p. 96, 2007.

REWAY, A.P. **Development of a follicular wave synchronization protocol and determination of the ideal moment for ovulation induction in equine species**. Dissertation (Master's Degree in the Program of Domestic and Wild Animal Anatomy). University of Sao Paulo, FMVZ USP. São Paulo - SP, 2017.

ROCHA FILHO, A.N.; PESSÔA, M.A.; GIOSO, M.M.; ALVARENGA, M.A. Transfer of equine embryos into an ovulatory recipientes supplemented with short or long acting progesterone. **Animal Reproduction**, v. 1, n. 1, p. 91-95, 2004.

RODRIGUES, L. G. S.; RESENDE, V. C de S.; JÚNIOR, A. R. O.; SILVA, L.O.; MARIANO, L. B. Aspects of reproductive management of equine. **Nutritime Eletronic Journal**, on-line, Vicosa, v.14, n.2, p.5046-5053, abr. 2017.

SAMPER, J.C. Induction of estrus and ovulation: why some mares respond and others do not. **Theriogenology**, v.70, n.3, p.445–447. 2008.

SHARP, D.C. The early fetal life of the equine conceptus. **Animal Reproduction Science**, v.60-61, p.679-689, 2000.

SILVA, E.S.M.; FRADE, S.; FERREIRA, J.C.; PUOLI FILHO, J.N.P.; MEIRA, C. Effect of interrupting altrenogest treatment in non-cyclic recipient mares on pregnancy maintenance: partial results. **Resúmenes del II Congreso Argentino de Reproducción Equina**, 1º Ed. Editorial Universidad Nacional de Rio Cuarto, p.551-553, 2011.

SILVEIRA, L. L. Comparison between the first and second heat postpartum in Crioula and Thoroughbred mares. **Dissertation presented to obtain the master's degree in Animal Medicine: Equines. Federal University of Rio Grande do Sul.** Porto Alegre. 39p. 2017.

SOUZA, F.A., CANISSO, I.F. Biological aspects of aging in reducing reproductive efficiency in mares. **Tropical veterinary science**, v. 11, n. 1, p. 9 - 15. 2008.

SQUIRES, E.L. Progestin. In: MCKINNON, A.O. & VOSS, J.L**. Equine Reproduction.** Cap.33, p. 311-318. Philadelphia: Lea & Febiger. 1993.

CHAPTER 3

3. HORMONAL STRATEGIES TO INDUCE FOLLICULAR ATRESIA AND TO STIMULATE THE EMERGENCE OF A NEW FOLLICULAR WAVE IN MARES

SUMMARY

The development of a hormonal protocol, as the market protocols used in cows, capable of estrus synchronization and fixed-time ovulation induction in mares has been a challenge. Previous studies from our laboratory have developed a hormonal protocol capable of stopping follicular growth in mares for a few days, with no occurrence of follicular atresia and reestablishment of the follicular growth after hormonal treatment ended. Moreover, mares presented different follicular sizes in the beginning of the treatment and, after follicular growth reestablishment, the time to the follicle to reach the ovulatory size varied, making difficult to determine the exact time to ovulation induction in a fixed-time manner for all mares. The main objective of this study was to develop a hormonal protocol capable of promoting follicular atresia, and the emergence of a synchronic follicular wave in mares using different sources of progesterone (P4) combined or not with estradiol (E2). Three experiments were performed; the first was designed to establish the daily plasma P4 concentration curve in ovariectomized and cyclic mares using an intravaginal P4 releasing device as exogenous source of P4. The subsequent experiment evaluated six hormonal protocols for induction of follicular atresia and synchronization of a new follicular wave during the reproductive season. Finally, the third experiment evaluated two hormonal protocols, in which P4 and estradiol had some adjustments in dose and frequency of administration to improve the degree of follicular atresia and to synchronize a new follicular wave. Hormonal protocols were evaluated as efficient if presented these to two main criteria: at least 65% of mares should present follicles of ≥ 35mm diameter with uterus exhibiting echotexture ≥ 3 after 10 days from the beginning of treatment, and no more than 25% of mares could have ovulated spontaneously before the day determined for ovulation induction. The best hormonal protocols from experiments 2 and 3 were chosen based on the above criteria and were G2.2 and G3.1. The degree of synchrony of ovulation was not dependent on the efficiency

obtained for induction of follicular atresia. G2.2 protocol presented the highest degree of ovulation synchrony in treated mares, using an intravaginal device containing 0.96 g P4 without association with estradiol, but G2.2 protocol did not result in follicular atresia induction. In contrast, both follicular atresia induction and the emergence of a new follicular wave occurred when G3.1 protocol was used, but this hormonal treatment presented less efficiency regarding synchronization of ovulations. In conclusion, the combination of hormones using different P4 sources, prostaglandin F2α and estradiol was capable of promoting follicular atresia and the emergence of a new synchronic follicular wave in mares.

Keywords: mares, synchronization, ovulation, hormones

INTRODUCTION

The equine industry would benefit from hormonal treatments capable of synchronizing the follicular wave and, consequently, permitting the induction of ovulation in a fixed-time manner. These treatments can improve the efficiency of reproductive biotechniques in this species, such as embryo transfer (ET) and artificial insemination, respectively, by helping the recipient mares' management and determining the time for semen depositing with high accuracy. The long estrus period and the great variability of the time of ovulation in mares difficult the establishment of an efficient hormonal protocol for estrus and ovulation synchronization. For this reason, there are no efficient protocols available in the market for using in this species in comparison to the several protocols available for using in bovine females (BRADECAMP, 2011). Current methods for estrus synchronization in mares have reasonable accuracy to synchronize the estrus period. However, the exact time of ovulation is variable. Due to this fact, frequent reproductive exams are usually required, at least daily, by palpation and ultrasound to determine the moment of ovulation (LARSEN & NORMAN, 2010).

Synchronization of the follicular wave allows to perform fixed time mating or artificial insemination in mares. The protocols are based on removal of the suppressive effect of the dominant follicle, that can be achieved by physical ablation or by inducing ovulation (using LH or GnRH) or atresia (using P4 alone or combined with E2) of this follicle (GALLELI et al, 2019). Data collected by Reway (2017) reinforced the idea of the use of P4 in order to synchronize the follicular wave. Reway (2017) described the

action of an exogenous source of P4 (an intravaginal device), and the results indicated that the P4 intravaginal device was not capable of causing follicular atresia, but instead it delayed follicular growth. The P4 intravaginal device demonstrated to be effective for follicular growth suppression in mares.

Besides, in equine breeding programs the synchronization of the follicular wave will enable the use of advanced assisted reproduction techniques in a faster and more practical way. The advantage of efficiently manage large herds may reduce the number of unsuccessful artificial inseminations after multiple laborious examinations. In order to make it a reality, hormonal protocols allowing synchronization of ovulation in mares have been designed (FANELLI et al., 2018).

Several attempts have been made over the years to synchronize ovulation in a group of mares. Ultrasound-guided follicle ablation may be a feasible non-hormonal alternative for ovulation synchronization in mares (BERGFELT et al., 2007). The degree of ovulation synchronization was not significantly different between the follicle ablation group and the group that received an association of P4 and E2. Ovulations occurred within a two (56 and 70%) or a four days (83% and 90%) period.

In 2010, another attempt to synchronize mares' estrous cycle was made by Larsen and Norman, who developed a protocol in which 89% of the mares were synchronized by administering on day 0 (beginning of hormonal treatment) 20 ml of estradiol benzoate associated with the insertion of intravaginal P4 releasing device (1.72g). The intravaginal device was maintained for 10 days and at the time of removal was administered 7.5µg of cloprostenol IM. In this study, 100% of the mares presented follicular atresia after the beginning of treatment, simulating what occurs when follicular aspiration is performed, but without the need for the invasive procedure. For ovulation induction, Larson and Norman (2010) recommended to use ovulation inducing agents 48 hours after removal of the intravaginal P4 device. If one intenteded to program ovulation in a group of mares, the authors recommended transrectal palpation and ruffiation 72 hours after removal of the P4 device. In this experiment, ovulation of the group of mares occurred in an interval between 5 and 8 days after finishing treatment using P4 in association with E2 and PGF2α.

Another study performed by Kuhl et al. (2017) evaluated differences between two PGF2α analogues. Luprostiol (3.75 mg IM) and cloprostenol (30 µg IM) in combination with hCG were tested to induce estrus and ovulation in mares. It was observed a shorter time from injection of hCG until detection of ovulation using luprostiol (2.2 ± 0.2 days)

compared to d-cloprostenol (2.8±0.2 days). Induction of estrus with luprostiol led to a better synchronization of ovulation after treatment with hCG than cloprostenol without impairing fertility. In summary, for satisfactory levels of ovulation synchronization to be achieved for timing artificial insemination in mares, the window of a 2-day period for ovulation to occur still needs to be narrowed and greater accuracy achieved.

Based on the exposed, for better manipulation of the mares' estrous cycle, this research was conducted to promote follicular atresia, to stimulate the emergence of a new follicular wave and to test the degree of synchronization of follicular growth in mares using new hormonal combinations of P4 and E2. Moreover, this study aimed to describe the daily P4 plasmatic concentration curve produced by the P4 intravaginal releasing device in cyclic and ovariectomized mares. Also, to establish a hormonal protocol capable of synchronizing ovulation in mares for fixed-time insemination. It was hypothesized that follicular atresia would occur when plasma P4 levels were maintained above 5 ng/ml for at least four consecutive days since the beginning of the hormonal treatment. Also, that the use of E2 in association with P4 would increase the efficiency of follicular atresia induction and a new follicular wave would initiate immediately after removal or interruption of the exogenous source of P4. Lastly, it was hypothesized that the degree of follicular growth synchrony would depend on the efficiency previously achieved by the induction of follicular atresia.

MATERIAL AND METHODS

This study was conducted in accordance with the guidelines for the use of animals in experimentation of CONCEA and in accordance with the regulations of the Ethics Committee for the Use of Animals of the Veterinary Medicine and Animal Sciences School of the University of São Paulo (CEUA-FMVZ-USP Nº 3597020317).

The experiments were developed during the months of August 2017 to April 2019 at the Animal Sciences and Food Engineering School, FZEA-USP, Fernando Costa Campus, in Pirassununga - SP, Southeast Region of Brazil, latitude 21.98/21°59'46' S and longitude 47.425/47°25'33'O. Twenty cyclic and two ovariectomized mares aging from 6 to 14 years-old from the Laboratory of Theriogenology Dr. O. J. Ginther were used. The mares were kept exclusively on pasture of Mombaça grass (*Panicum sp* var. Mombaça), with water and mineral salt *ad libitum*. The mares received individual and daily

protein/energy concentrated maintenance supplementation. The body condition score was maintained between 6 and 7, on a scale from 1 to 9 (HENNEKE et al., 1983).

Ultrasonography

B-mode ultrasonographic data were collected using a Esaote equipment (MyLab30Vet, Italy) equipped with a multi-frequency linear transducer adjusted to 5 MHz. All the equipment settings were maintained the same for all exams.

In experiment 1, transrectal ultrasound exams were performed only to confirm the exact day of ovulation after transrectal palpation. In experiments 2 and 3, follicular growth dynamic was daily monitored by ultrasonography and follicles identity were maintained among exams. The diameters of the three largest follicles of the right and left ovaries, the diameter of the corpus luteum (CL) when present, and the uterine edema (echotexture; scores 1 to 4; GINTHER, 1992) were recorded and used for posterior analysis. The measurements were automatically calculated by positioning the ultrasound cursor perpendicular to the two largest diameters of the ovarian structure. The mean diameter was then calculated from these two values. Follicular data were plotted in individual graphs to observe the temporal and individual variations of follicular growth, therefore, for the study of follicular dynamics (GINTHER, 1992). Some variables related to follicular dynamics were collected in these individual plots, and they are listed below:

- Diameter of the largest follicle on day 0 (day 0 = beginning of the treatment).
- Growth rate of the largest follicle from day 0 until the day 8.
- Day of the follicular divergence. The day of follicular divergence corresponds to the day of the growth rate dissociation between the largest follicle and its subordinates, in other words, it is the moment when the subordinated follicles decrease their growth rate while the largest follicle continues to grow maintaining its growth rate (GINTHER, 1992).
- Diameter of the largest follicle on the day of follicular divergence.
- Day when the largest follicle reached 35mm in diameter (preovulatory follicle).
- Uterine echotexture when the largest follicle reached 35mm in diameter.
- Number of days to the largest follicle to reach 35mm in diameter after day 8.
- Number of days from the day when the largest follicle reached 35mm until ovulation.

- Number of days from day 0 to ovulation.
- Diameter of the preovulatory follicle one day before of ovulation day.

Experiment 1 - Daily plasma P4 concentration curve in ovariectomized and cyclic mares treated with an intravaginal P4 releasing device.

This experiment was performed to describe the daily plasma concentration of P4 after the administration of an exogenous source of P4 using an intravaginal releasing device containing 0.96 g of P4 (ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health). Two ovariectomized mares and two cyclic mares were used. The experiment started on the 10^{th} after ovulation in cyclic mares (day 0 = ovulation; 10^{th} after ovulation = D0). In the beginning of the treatment (D0), 0.25 mg of prostaglandin F2α IM was administered, and the P4 intravaginal releasing device inserted in both ovariectomized and cyclic mares. Daily blood samples were collected starting on the day of insertion of the P4 intravaginal device (D0) until two days after removal of the device (D10; Figure 3.1). Blood samples were collected after the P4 intravaginal device insertion on each mare by jugular venous puncture with a 30x18 needle in a vacutainer system (15 mL vacuolized tubes) and centrifuged for 10 minutes at 13500 G for subsequent isolation of plasma. The blood plasma samples were frozen at -20^{o}C until P4 quantification. Samples were sent to the Laboratory of Animal Endocrinology - UNESP - Araçatuba for P4 analysis by radioimmunoassay "Kit" (coat-a-count; DPC) technique, previously validated by Garbarino et al. (2004). P4 concentrations were expressed in ng/ml.

Experiment 2 - Evaluation of six hormonal protocols for follicular atresia induction and synchronization of a new follicular wave

Twenty cyclic mares were randomly distributed into six experimental groups (G2.1 to G2.6) in a 3 x 2 factorial experiment design during the 2017/18 breeding season. All the same 20 mares were randomly used in all experimental groups. Three different exogenous sources of progestogen (Figure 3.2) were tested combined or not with 20 mg/animal IM of 17β estradiol (17 Beta®; Botupharma, Brazil). The tested progestogen sources and doses were: 0.96 g of P4 intravaginal releasing device (ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health, Brazil), 1500 mg/animal IM of long

action P4 (P4-300®; Botupharma, Brazil), and oral altrenogest was daily at a dose of 0.045 mg/kg body weight (Botupharma, Brazil).

Experiment 3 - Test of two modified hormonal protocols for follicular atresia induction and synchronization of a new follicular wave

After the analysis of the results of experiment 2, two new hormonal treatment protocols were proposed to be tested aiming to improve the efficiency of the six previously tested protocols. Higher protocol efficiency was evaluated by means of higher level of follicular atresia associated with better ovulation synchronization. Eighteen cyclic mares were randomly distributed into two experimental groups (G3.1 and G3.2) during the 2018/19 breeding season. All the 18 mares were randomly used in both experimental groups (Figure 3.3). Exogenous sources of P4 (intravaginal device ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health) were tested, increasing the initial dose of P4 used in experiment 2 from 0.96 g to 1.92 g in both groups. In G3.1, the intravaginal P4 device was associated with two administrations of 20 mg/animal of 17β estradiol IM (17 Beta®; Botupharma, Brazil). The first dose was administered on D0 (beginning of treatment), and the second dose was administered two days after. The two administrations of 17β estradiol were performed to guarantee follicular atresia in this protocol. In G3.2, the 1.92 g of P4 intravaginal releasing device was used without the association with 17β estradiol.

STATISTICAL ANALYSIS

Data from all experiments were examined for normality with the Kolmogorov-Smirnov test. Data that were not normally distributed were transformed to natural logarithms. The end points were analyzed in a factorial 3 x 2 design by ANOVA using the SAS software (version 9.4; SAS Institute, Inc., Cary, NC) to determine the differences between treatments. The identity of the mares was added to statistical model. Paired and/or unpaired Student t-tests were used to locate differences between treatments when significant main effects or an interaction were obtained. Discrete data were analyzed by paired Student t-tests. The proportion data were compared with a chi-square test. A probability of $P \leq 0.05$ indicated a significant difference. All values are shown as the mean ± SEM.

RESULTS

Experiment 1

The daily plasma P4 concentration curve after insertion of an intravaginal device containing 0.96 g of P4 (ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health, Brazil) was determined (Figure 3.4). There was no confirmation of the hypothesis that follicular atresia would occur when plasma P4 levels were maintained above 5 ng/ml for at least four consecutive days since the beginning of the hormonal treatment.

In this experiment, we observed that daily plasma concentration of P4 from ovariectomized mares were like those obtained with cyclic mares. Besides, all mares had high plasma P4 concentrations on day 0. There was a decrease in plasma P4 concentration after the second day, however from the third to the eighth day, a plateau around 5ng/ml of P4 was maintained until the intravaginal device was removed. Cyclic mares presented higher plasma P4 concentration on day 0 compared to ovariectomized mares. After removal of the intravaginal device, plasma P4 concentrations decreased to less than 1 ng/ml in 24 hours in three of the four mares (Figure 3.4).

Experiment 2

The six hormonal protocols (groups G2.1 to G2.6) were tested and compared for the purpose of finding the best protocol attending the following criteria: promotion of a high degree of follicular atresia, a high degree of ovulation synchronization and also a precise time for induction of ovulation in a fixed time manner. The results are presented in Table 3.1.

According to our data, the diameter of the largest follicle on D0 showed no significant difference ($P = 0.167$) among the six groups (G2.1 to G2.6). In groups in which hormonal treatments used the association of a progestogen and E2 (G2.1, G2.3 and G2.5), at first detection the diameters of the ovulatory follicles were smaller (17.72 ± 0.92, 18.26 ± 0.94 and 15.71 ± 0.86, respectively) than in groups without association with E2 (G2.2, G2.4 and G2.6), possibly due to estradiol effect. Besides, in these groups 71.6% (43/60) of the follicles that ovulate emerged after the beginning of hormonal treatment (D0).

An independent effect of P4 treatment and an independent effect of E2 treatment occurred on the growth interval of the ovulatory follicle from its first detection to D8. This means that disregarding E2 treatment, the groups using three distinct sources of exogenous P4 presented different growth intervals due to the effect of the source of P4 used. Similarly, disregarding P4 treatment, groups using or not using E2 also presented different growth intervals due to the effect caused by E2.

In groups (G2.2, G2.4 and G2.6) in which P4 was used without the association with E2, 66.6% (40/60) of the ovulatory follicles were already present at the beginning of treatment (D0). The growth of the ovulatory follicle slowed down during treatment with exogenous P4 and these follicles resumed their growth after the removal of the exogenous source of P4. Due to this fact, a tendency was observed for the diameter of the ovulatory follicle on the day of deviation to be significantly larger in G2.2, without the use of E2 ($P = 0.065^{y}$).

On D8 the exogenous P4 source was removed, and at this time it was not observed a statistical difference among groups, regarding the ovulatory follicle diameter. However, effects of treatments with P4 and E2 ($P = 0.293^{yz}$) was noticed by the larger diameter of the ovulatory follicle on D8 presented by mares from the groups not treated with E2 (G2.2, G2.4 and G2.6).

The degree of follicular atresia was smaller ($P = 0.0001$) in the groups in which progestogen was not associated with E2: 15% (3/20), 10% (2/20) and 5% (1/20) in groups G2.2, G2.4 and G2.6, respectively. According to these data, it was found that the combination of the hormones P4 and E2 was responsible for atresia and stimulation of the growth of a new follicular wave. When P4 was not associated with estradiol, follicular atresia did not occur as expected.

In groups G2.1, G2.3 and G2.5, the follicles originated from a new follicular wave grew when the plasma concentrations of P4 were already decreasing and the oocytes from these ovulatory follicles possibly did not suffer the same influence of exogenous P4.

Growth rate and day of follicular deviation

Follicular growth rates from the beginning of hormonal treatment (D0) to the interruption of exogenous P4 (D8) did not differ ($P = 0.999$) between the six groups. Nevertheless, the effect of E2 treatment was detected and represented by higher follicular growth rate in groups G2.1, G2.3 and G2.5 from D8 to ovulation. The three highest

follicular growth rates were 2.63 ± 0.12, 2.6 ± 0.12 and 2.74 ± 0.11 mm/day respectively, when compared to the three lowest follicular growth rates from G2.2 (2.47 ±0.22), G2.4 (2.1 ±0.18) and G2.6 (2.55 ±0.12).

The average of daily growth rates from the six groups was smaller while the follicles were under the influence of exogenous progestogens until D8 compared to follicles after D8 until ovulation, but data showed no statistical difference (1.64 mm/day and 2.51 mm/day, respectively; $P > 0.05$). Therefore, it was observed that follicles grew at a rate approximately 65% higher, in other words, 0.87 mm/day more than when suppressed by the action of exogenous progestogen treatments.

Once the association of E2 included P4 in the treatments, the first detection of ovulatory follicles occurred approximately two to five days after the treatment started. As for the ovulatory follicles that did not suffer the action of E2, previous ultrasound detection was observed. Also, an effect of E2 treatment occurred in the day of follicular deviation. Approximately a two-day delay in follicular deviation occurred when compared to the groups in which E2 was not used.

The figure 3.6 shows the curve that represents the retrospective evaluation of the mean growth of the ovulatory follicles from the six groups. Between the groups treated with E2 and P4, the group using P4 releasing device had the devices removed on D8, this moment would correspond to day -4 in the graph (Figure 3.6). This may have implied in a smaller average size of the ovulatory follicles in this group, since promptly after the removal of the P4 device, on average, 3.6 ± 0.6 days these follicles ovulated. Conversely, for the groups in which the association of P4 with E2 was used, after the exogenous source of P4 ceased, the ovulatory follicles remained growing for more days until ovulation. On the follicle growth retrospective curve, for groups treated with P4 plus E2, the day exogenous P4 administration ceased corresponds approximately to day -8 in the graph. One can observe that after interruption of the P4 sources, then the ovulatory follicles have continued to grow for eight days until ovulation occurred (Figure 3.6).

Uterine echotexture

Uterine echotexture was evaluated on day when the ovulatory follicles reached diameter ≥ 35mm. In groups G2.1, G2.3 and G2.5, treated with E2, was observed that ovulatory follicles took more time to reach the diameter of ≥ 35mm, although there was no statistical difference between groups ($P > 0.05$).

However, it was observed an independent effect of P4 and independent effect of E2 treatment on the uterine echotexture. Without considering the E2 treatment, the three groups using three distinct sources of exogenous P4 were different. Thus, by excluding P4 treatments, the groups with or without E2 treatment also presented differences caused by the effect of E2. Groups in which P4 was administered without association with E2 (G2.2, G2.4 and G2.6) presented a lower degree of uterine echotexture (2.2 ± 0.28, 2.2 ± 0.27, 2.7 ± 0.27) when compared to groups in which E2 was used, although it was not statistically different ($P > 0.05$). The degree of uterine echotexture in groups G2.1, G2.3 and G2.5 were 2.8 ± 0.25, 2.3 ± 0.31 and 3.2 ± 0.21, respectively.

Time for ovulation induction

In groups in which P4 was associated with E2 (G2.1, G2.3 and G2.5), ovulations naturally occurred, on average, around D16 of treatment, approximately three days after than in the groups in which E2 was not administered. This effect of E2 at the moment of ovulation was represented by a delay on ovulation day. The ovulation detection of G2.1, G2.3 and G2.5 occurred on days 16.6 ± 1.06; 17.1 ± 1.41 and 15.9 ± 0.69, respectively.

The group presenting higher level of follicular atresia (G2.1) presented a large interval of occurrence of the ovulations, ranged from D8 to D30. The day of the first ovulatory event detected, on D8, was coincidental to the time of P4 intravaginal device removal. The subsequent ovulations continued to occur naturally for the next 22 days, until the last detection on D30.

In contrast, in G2.2, spontaneous ovulations occurred, on average, on day 11.6 ± 0.6. G2.2 was the group that most timely concentrated the spontaneous ovulations. A shorter range of time was observed in ovulations of G2.2, from day 9 to 19 (Figure 3.7). Day 8 would be the best indicated day to perform ovulation induction because it would facilitate mares' management by induction of ovulation on the same day of the intravaginal device removal. Besides, none of the 20 mares ovulated spontaneously until D8 and, therefore, none of the ovulations would be lost because its occurrence earlier than expected.

The number of mares and the respective days on which the follicles reached 35 mm diameter and the days of ovulations in groups G2.1 and G2.2 can be observed in Figure 3.7.

Experiment 3

The experiment 3 focused on the comparison between two new hormonal protocols for follicular wave synchronization. Comparative data obtained from these hormonal treatments after the adjustments to better synchronize the follicular wave and ovulation can be observed in Table 3.2. The data showed the effects of hormonal treatments in most of the variables analyzed between groups 3.1 and 3.2, and significant differences can be noticed.

The G2.1 group from experiment 2 underwent modifications and resulted in a new version of hormonal treatment now named G3.1. The P4 concentration was doubled and two doses of E2 were administered. This group received the association of 1.92 g P4 and one dose of 17β estradiol on D0 and another dose on D2. In G3.2 the hormonal protocol used a double dose of exogenous P4 (1.92 g) without the association with E2.

The highest degree of follicular atresia was found in G3.1, 94% (17/18), compared to the G3.2, 27.78% (5/18; $P < 0.0001$). At first detection, the mean diameter of the ovulatory follicles was 16.86 mm ± 0.84 and 19.36 mm ± 1.28 ($p = 0.068$) for G3.1 and G3.2, respectively. However, none of the ovulatory follicles in G3.1 could be detected at the onset of the hormonal protocol (D0), as the follicles emerged from the follicular wave subsequent to the atresia that resulted from the hormonal treatment. Differently, in G3.2, most of the ovulatory follicles (11/18) were already present on D0 and ovulations resulted from these follicles.

Suppression of follicular growth by the action of hormonal treatment resulted in an average daily growth rate of 1.39mm ± 0.29 and 1.83mm ± 0.15 for G3.1 and G3.2, respectively. After removal of the P4 intravaginal device (D8), both groups presented follicular growth approximately twice as fast, and the average follicular growth rate practically doubled for both, 3.08 mm ± 0.11 in G3.1 and 2.98 mm ± 0.2 in G3.2. On D8, the average diameter of ovulatory follicles in G3.2 was verified to be 30.66 mm ± 1.29, thus, in a few days these follicles would become pre-ovulatory ones, capable of responding to ovulation induction. Meanwhile, on D8 in G3.1, the mean diameter of ovulatory follicles was smaller 19.69 mm ± 1.37 ($P = 0.057$), and therefore, these follicles would take more days to ovulate, on average 9.83 ± 0.45 days after D8. For G3.2, this period from D8 to ovulation was significantly shorter, on average 5.22 ± 0.81 days ($P < 0.05$). The time when the follicles reached ≥ 35mm diameter in G3.1, on average, was on day 13.61 ± 0.53, four days later compared to G3.2, on day 9 ± 0.63. Also, in G3.1,

ovulations centered in a shorter range of time interval, from D13 to D21, while in G3.2 ovulations occurred from D7 to D23 (Figure 3.8). Since in G3.1 the follicles took more days to ovulate than in G3.2, the P4 plasma levels possibly no longer had any influence on the uterine echotexture aspects. In G3.1, on average, the degree of uterine edema was 3.66 ± 0.11, while in G3.2 it was 2.44 ± 0.33.

Data from each modified hormonal protocols (G3.1 and G3.2) were numerically compared with their original protocols from experiment 2 (G2.1 and G2.2). The G3.1 new hormonal protocol showed a higher degree of follicular atresia induction, reaching 94% (17/18; Table 3.2) compared to 75% (15/20; Table 3.1) in G2.1. For this reason, in G3.1 more mares (17/18) experienced a new synchronic follicular wave. As a consequence of follicular atresia, the moment when the follicles reached ≥ 35mm diameter was delayed, on average, one day (13.61 ± 0.53), while in G2.1 it occurred, on average, on day 12.9 ± 0.99. Regarding ovulations, all of them occurred spontaneously from D13 to D21 (Figure 3.9), and from D8 to D30, in G3.1 and G2.1, respectively. Although group G3.1 presented ovulations better concentrated compared to G2.1, the degree of synchronization of ovulations in G3.1 still needed to be improved (Figure 3.9).

In general, both groups G3.1 and G2.1, attended the criteria required for ovulation induction on D13: at least (12/18) 65% of follicles with diameter ≥ 35mm and maximum of 25% (4/18) of lost ovulations (Figure 3.9). The selection of G3.1 in detriment of G2.1 was motivated by the fact that G3.1 reached a higher degree of induction of follicular atresia. In order to future investigate fertility post hormonal treatment, G3.1 was expected to present better reproductive performance results as a consequence of better follicular wave and ovulation synchronization. Examples of the follicular dynamics of two mares submitted to both hormonal protocols, G3.1 and G2.1, are illustrated in Figures 3.11 A and B.

The G3.2 hormonal protocol presented a moderate increase in follicular atresia induction of 27.78% (5/18) of follicles (Table 3.2) compared to 15% (3/20) of follicles in G2.2 (Table 3.1). In contrast, the highest degree of follicular atresia resulted in less synchrony of ovulations in G3.2 compared to G2.2 (Figure 3.10). Regarding the moment of ovulation in G3.2, when follicular atresia was present, mares' ovulations occurred on day 16.6 ± 1.69, ranging from D14 to D23 (Figure 3.11 E) Mare 5 G3.2). Differently, when follicular atresia was absent in G3.2, mares' ovulations occurred on day 11.84 ± 0.68, ranging from D7 to D16 (Figure 3.11 D) Mare 4 G3.2). However, the results regarding time of ovulation from G3.2 were worse compared to G2.2, because in G2.2

ovulations occurred in a shorter period of time. In group G2.2, the mares ovulated among D9 and D19, whereas in G3.2 ovulations occurred from D7 to D23.

In addition, G2.2 was able to concentrate a higher number of follicles capable of ovulation induction than G3.2, due to lower degree of follicular atresia and the fact that only suppression of follicular growth was noted. For this reason, in G2.2, after interruption of exogenous P4 the follicles resumed its growth and were able to ovulate in better synchrony.

In G3.2, D11 was the day when at least 65% of follicles reached ≥ 35mm diameter, at this moment 72.22% of the mares (13/18) presented a pre-ovulatory follicle. Nevertheless, by D11 of the hormonal protocol, 27.77% (5/18) of ovulations had already occurred and would be lost (Figure 3.10). These data revealed the impossibility of strategically selecting an ideal day for induction of ovulations according to the proposed criteria. Therefore, as G2.2 fulfilled the proposed criteria, then G2.2 was selected in detriment of G3.2 to future research aiming to assess fertility post hormonal treatment.

For comparison purposes, examples of follicular dynamics of mares submitted to hormonal protocols of groups G2.2 and G3.2 are illustrated in Figure 3.5. The hormonal protocol of group G3.2 caused suppression of follicular growth in most mares, however, some mares presented follicular atresia.

Examples of the differences in mares' follicular dynamics submitted to G3.1 and G3.2 and G2.1 and G2.2 hormonal treatment protocols and its respective particular characteristics are illustrated by graphical representation in Figure 3.11. The first graph represents an example of the follicular dynamics of the G2.1; in A) Mare 1, the action of 0.96g of P4 associated with a single dose of E2 on D0 promoted follicular atresia in all monitored follicles. The first follicular wave of Mare 1 from G2.1 became a major and secondary follicular wave, in which the largest follicle reached characteristic size (≥ 23mm) for follicular deviation, but this follicle became atretic as well as its subordinates. Around D5 after the beginning of the hormonal treatment, in this same Mare 1 from G2.1, a new major and primary follicular wave was initiated when the P4 plasma levels were decreasing. In G3.1, the double dose of P4 associated with two administrations of E2 promoted follicular atresia of a 30 mm follicle. The first ovulatory follicle detection occurred on D6, and it was observed that the major primary follicular wave initiated one day later compared to G2.1. Among the similarities observed between groups G2.1 and G3.1 it was found that the follicular deviation day occurred three days after the removal of the P4 intravaginal device in both groups. In addition, both ovulations

occurred on D17. Differently, in Figure 3.11 the graphs representing C) Mare 3 from G2.2 and D) Mare 4 from G3.2, showed the absence of follicular atresia. In Mares 3 and 4, from groups G2.2 and G3.2, respectively, there was only the presence of a unique major secondary follicular wave. In Figure 3.11, graph E) Mare 5 from G3.2, the double dose of P4 maintained follicular growth static and after D8, the day of P4 intravaginal device removal, follicular atresia occurred. For this reason, in mare E it was observed the major secondary follicular wave probably started around D8. Ovulation in Mare 5 occurred on D16, due to static follicular growth caused by the action of P4, also due to a delay at the time the ovulatory follicular wave started. Besides, ovulation occurred six days after in Mare 5 than occurred in Mares 3 and 4, which exhibited just one follicular wave, thus the ovulatory follicle was already present since D0.

DISCUSSION

The higher values of plasmatic P4 observed in cyclic mares when the P4 intravaginal device was inserted compared to the values observed in ovariectomized mares can be explained by residual circulating P4 produced by the CL. Cyclic mares had started the treatment on day 10 of the estrous cycle; also, these mares presented on day 0 about two folds more plasmatic P4 when compared to ovariectomized mares. The values observed in plasma P4 concentrations found in cyclic mares can possibly be attributed to the endogenous production of P4 associated with treatment using an intravaginal device (0.96 g P4). Interestingly, after PGF2α administration, all four mares nearly equaled P4 plasma concentrations on D5 of the hormonal treatment. A plateau around 5 ng/ml of P4 was maintained in all treated mares, until the intravaginal device be removed. After D8, three of four treated mares (3/4) presented plasma P4 concentrations less than 1 ng/ml 24 hours after the removal of the intravaginal device.

Individual variation in plasma P4 concentrations may be found in a herd of cyclic mares, however, the values obtained from these two mares in experiment 1 are in accordance with the P4 values on diestrus phase described in the literature (ALLEN, 1987, GINTHER, 1986, PIMENTEL et al., 2014). There is a variation in the blood levels of P4 in mares during the different phases of the estrous cycle. The decrease in circulating LH concentrations observed one day after ovulation are associated with the post-ovulatory increase in blood P4 concentration (GINTHER, 2008). The P4 concentration

increases rapidly after ovulation, remaining high for 14-15 days in non-pregnant mares, reaching a maximum value, above 4ng/ml, four to seven days after ovulation. The levels of P4 decrease again from the fourth to the fifth days before the next ovulation, due to the CL lysis (PIMENTEL et al., 2014). Reference values of blood P4 concentration in cyclic, anestrus and pregnant mares are presented in Table 3.3. The results obtained in this experiment demonstrated that treatment using an intravaginal device 0.96 g of P4 in cyclic and ovariectomized mares is capable of maintaining the P4 plasmatic concentrations above 5 ng/ml. In experiment 2, regarding follicular growth rates and day of deviation, probably the antral follicles from non-E2 treated groups were sensitive to FSH and had already been recruited by the selection process. Before follicular selection, a group of follicles were growing in synchrony, until these follicles were succeeded by the growth of only one or eventually two follicles. In other words, the ovulatory follicles from non-E2 treated groups originated under the influence of exogenous P4 action. The follicular selection process occurs at the end of the common growth phase and the dominant follicle continues its growth at a continuous rate while the subordinate follicles begin to regress, this moment is known as the day of follicular deviation. The future dominant follicle often emerges approximately one day earlier than the future subordinate follicles, then the growth rates for the two follicles between emergence and deviation (six days later) did not differ, so the future dominant follicle presents a mean of 3 mm larger than the subordinate follicle at the beginning of deviation (23 vs. 20 mm of diameter). In this process, usually the largest follicle becomes dominant and continues to grow until it reaches a preovulatory diameter (≥ 30 mm) (GASTAL et al., 1999). Follicular deviation control depends on intrafollicular factors, such as growth factors, peptides, gonadotropin receptors, angiogenic factors and steroids, which are secreted differently in the future dominant and subordinated follicles. These intrafollicular factors can be determined by autocrine, paracrine, and endocrine regulatory effects (GURGEL et al., 2008). The complex interactions of systemic and local aspects of follicle deviation in mares happens when the wave stimulating FSH surge is declining and the future dominant follicle is near the beginning of diameter deviation. The insulin-like growth factor (IGF) system is a critical biochemical intrafollicular initiator of effects. IGFBPs (binding proteins) are degraded by proteases (BPPr) and free IGF-1 is released. The free IGF-1 stimulates the production of inhibin-A, activin-A, and vascular endothelial growth fator (VEGF) but does not have an immediate effect on E2 in mares. The combined changes in concentrations of these factors results in changes in follicles' responsiveness to the

concentrations of LH and FSH. This explains why the developing dominant follicle can continue growing in the presence of low concentrations of circulating FSH. Also, there are interactions among these factors, gonadotropins, and granulosa cells (GINTHER et al., 2004).

According to Antoniolli (2002), at a given moment, during the follicular growth wave, one or more follicles are selected to ovulate. This selection process involves the development of dominant follicles and atresia of the other follicles that are present in the same follicular wave. When the dominant follicle develops to a size in which it can ovulate, Antoniolli (2002) suggests that in the presence of high P4 levels, the dominant follicle cannot ovulate, but instead it becomes atretic and a new follicular wave begins. In experiment 2, the dominant follicle did not suffer atresia, even under the action of P4, and after removal of the exogenous source of P4, this follicle was able to grow and ovulate. Uribe-Velásquez et al. (2008), observed that the administration of CIDR in sheep on the day of ovulation inhibits the growth of the dominant follicle making it smaller than in the control group. The dominant follicle reached its maximum diameter, 5.5 mm, at day 5.4 in the control group, and 4.0 mm at day 5.7 in those submitted to exogenous P4 (CIDR). According to Ginther (1971), this inhibitory P4 mechanism on the dominant follicle can be observed when this hormone is administered both at the beginning and the end of the estrous cycle. Thus, the dominant follicle will remain its growth phase for a longer period, that is, this follicle will continue to grow at a slower rate. During the days when the follicle is under the action of P4, this follicle will grow until it reaches its maximum diameter and until the moment of ovulation. The action of P4 may be able to cause changes in the follicular fluid of the growing dominant follicle, and this P4 effects may compromise developing oocytes at the end of the estrous cycle (GINTHER et al., 1971).

The estrous cycle in equine species is characterized by ovarian follicular waves that follow the sequence: recruitment, selection, dominance, and ovulation (GINTHER et al., 2005). Estradiol is produced by the dominant follicle and GASTAL et al. (1999) reported that elimination of the dominant follicle results in decrease of estradiol and inhibin and increase of FSH. E2 levels decrease completely until the final development of the pre-ovulatory follicle; promoting expression of gonadotropin receptors and increasing follicle sensitivity to LH (GURGEL et al. 2008). Besides, the decrease in levels of circulating E2 also occurs when the development of new follicular waves is initiated. Decreased E2 concentration allows FSH to increase and new follicles to grow. Through

this hormonal control mechanism, the levels of FSH and E2 can be regulated (GINTHER et al. 2008). During follicular growth phase, the frequency of LH pulses is higher than during the luteal phase. This increased LH pulses' frequency contributes to ovarian follicular development (TSUKAMURA, 1998). Thereby, periods of high P4 concentrations can result in lower frequency of LH pulses, then a suppression of follicular growth eventually causes atresia of the dominant follicle (URIBE-VELÁSQUEZ et al., 2008). Nonetheless, in experiment 2, for those groups not treated with E2, there was no atresia of the dominant follicle, even though high levels of circulating P4 were observed during treatment. Also, for non-E2 treated groups, despite exogenous P4 action may had induced lower LH pulses' frequency, the dominant follicles became pre-ovulatory and were able to ovulate. So far, fertility of these ovulated oocytes after hormonal protocols still needs to be verified.

Administration of exogenous P4 has a dose-dependent effect on LH secretion in cows. Changes in the regulation of LH release due to P4 action may affect the development and the future capacity of the oocyte to be fertilized and result in pregnancy (KINDER et al., 1996). When the period of exposure to exogenous P4 concentrations is prolonged, persistent follicles can develop and may result in reduced fertility (KINDER et al., 1996). One study documented low fertility cycles due to ovulation from diestrus follicles of oocytes exposed to high P4 levels (INSKEEP, 2009). As seen in groups in which P4 was used without the association with E2, most of the ovulatory follicles were already present at the beginning of treatment (D0). Subsequently, these ovulatory follicles were able to resume their growth and ovulate after extensive exposure to the exogenous source of P4, although the fertility of these follicles remained unknown. In *Bos indicus* and *Bos taurus,* variations in P4 concentrations in estrus synchronization protocols indicate that high concentrations of P4 during the development of the ovulatory follicle may affect fertility. However, the influence of limited periods of low P4 concentrations on LH secretion, follicular growth, oocyte competence and fertility still need to be determined in the equine species. Bovine females *Bos indicus* are more sensitive to the effects of high P4 concentrations on pre-ovulatory period. Carvalho et al. (2008) found that high P4 plasma levels impair follicular development in *Bos indicus* females in a more accentuated way when compared to *Bos taurus*. The insertion of CIDR and the administration of E2 were performed and although the interval between the administration of E2 and the emergence of the follicular wave did not differ between the

groups (3.2 days), the pure *Bos indicus* females presented smaller dominant follicles at the end of the treatment with CIDR and lower pregnancy rates (CARVALHO et al. 2008).

In the hormonal protocols used in this study, two doses of PGF2α were administered. The first dose was administered at the beginning of the hormonal treatment to promote luteolysis of the CL from previous ovulations. In case these CLs were not mature enough to respond and undergo lysis due to the action of the first PGF2α dose, then the CLs would respond to a second PGF2α dose administered on day 8 at the time of P4 device removal. Some mares had large follicles at the time of P4 device insertion (D0) and ovulated even when submitted to exogenous P4 treatment. For this reason, a second dose of PGF2α on D8 was chosen to ensure the occurrence of luteolysis in a CL which did not respond to the first PGF2α dose on day 0. Phetudomsinsuk (2017) reported that interovulatory interval in mares treated with PGF2α (D6 after ovulation) was shorter than in the control group. This shorter interval might be because the growth rate of the dominant follicle in treated mares was higher than in the control. Phetudomsinsuk (2017) data contrasts with the study of Schauer et al. (2013), which found that PGF2α did not have any effect on follicle growth or ovulation. However, a dose of 5 mg PGF2α (dinoprost) on D6 after ovulation is recommended to fasten a return to estrous in mares without hastening ovulation. The distinct interovulatory intervals in mares after PGF2α administration possibly occur due to the existence of certain variables such as size of the remaining follicles in the ovaries (LINDEBERG et al., 2002; NEWCOMBE et al., 2008), degree of endometrial edema at the time of treatment (SAMPER, 2008) and dose of PGF2α used (NEWCOMBE et al., 2008). These results analyzed in combination with data from our study provide information revealing the potential for hormonal treatment to alter follicular growth rate. Daily growth rates of the ovulatory follicles under the effect of different sources of exogenous P4 did not differ among the six groups in experiment 2.

Uribe-Velásquez et al. (2008) observed different ($P < 0.0001$) follicular growth rates in sheep treated with CIDR when compared to the control group. The group of sheep treated with CIDR exhibited a lower follicular growth rate of 0.9mm/day. In contrast, the control group revealed a faster follicular growth rate of 1.7 mm/day. Therefore, Uribe-Velásquez et al. (2008) stated that in female sheep the growth of the dominant follicle, originated in the first follicular wave, was inhibited by the high plasma concentration of P4, obtained by the insertion of CIDR after ovulation. Data from this study demonstrated that in mares even P4 blood concentrations maintained above 5ng/mL for four

consecutive days were not capable of inhibited the dominant follicle, but these high P4 plasma levels also caused lower follicular growth rate. The growth rate of the ovulatory follicle, in non-E2 treated group from experiment 3, increased from 1.83 ± 0.15 to 2.98 ± 0.2 after the exogenous source of P4 was removed. Uribe-Velásquez et al. (2008) reported that the growth inhibition of the dominant follicle possibly occurred because P4 exerts negative feedback on the hypothalamus and pituitary gland, resulting in release regulation of gonadotropins. Martínez-García et al. (2007) also suggest that the increase in growth rate in some follicles occurs after the decrease in blood P4 concentrations.

During estrus, the dominant follicle develops and secretes E2. E2 concentrations are directly related to follicular activity and competence. In the absence of P4 (< 1 ng/ml), E2 secreted by the pre-ovulatory follicle induces relaxation of the cervix and vulva, stimulates secretions in the uterus, cervix, and vagina and facilitates sperm transport. In transrectal ultrasound examination, the dynamics of uterine echotexture is influenced by the different moments of the estrous cycle, due to predominant levels of ovarian steroids. The uterine edema is first visualized at the end of diestrus and it becomes more prominent in the progression of the estrous phase. Uterine edema decreases between 48 and 24 hours before ovulation and should not persist more than 36 hours after ovulation (GINTHER, 1986). Regarding uterine edema data, for the non-E2 treated groups, mares submitted to exogenous P4 hormonal treatments presented a lower degree of uterine edema when compared to the treatments in which E2 was included. This may have occurred because in groups in which the association of P4 with E2 was used, the ovulatory follicles only reached 35 mm around 12 days after the beginning of treatment, that is, four days after the cessation of the exogenous P4 action. Therefore, these mares exhibited uterine edema similar to the ones found in regular estrus phase, possibly because exogenous P4 action no longer interfered. Another possibility is that the administration of E2 has given the ovulatory follicle greater estrogenic competence compared to groups in which it was not administered.

By comparing our data with Larentis' et al (2020) findings, uterine edema probably occurred due to the absence of exogenous P4 effect, instead of exogenous E2 effect promoting better estrogenic follicular competence. In fact, Larentis et al (2020) demonstrated that exogenous administration of E2 and P4 can act directly in the mare's uterus even when ovarian hormonal activity is not involved. Mares exhibiting the condition referred as equine Turner's syndrome, that is, lack of one X chromosome (X0) were submitted to estrogen and P4 treatment. Subsequently, these mares were used as

recipients in equine ET programs. Larentis et al. (2020) provided evidence that the 17β E2 and long action P4 (P4-300®) can induce the formation of steroid receptors in the endometrium in X0 mares. This study demonstrated that the uterus of an X0 mare can respond to hormonal treatment even in the absence of hormonal ovarian activity. This may allow the successful use of acyclic or transitional mares or mules as ET recipients after hormonal treatment.

Another paper supports the idea that exogenous administration of E2 has not provided greater estrogenic competence to future ovulatory follicles (GASTAL, 2009). Gastal (2009) argued that the multiple ovulations in mares may be associated with higher E2 systemic concentrations and lower FSH levels. Thus, higher LH concentration preceding deviation seems to favor the development of multiple estrogen-competent ovulatory follicles. The synchronization of a uniform FSH surge during the common-growth phase in mares submitted to estrous cycle manipulation seems to induce waves in which the future dominant follicles have greater ovulatory capacity. Higher E2 produced by multiple ovulatory follicles from the preceding follicular wave were associated with lower FSH and it may be responsible for future smaller preovulatory follicles and subsequent double ovulation.

In both experiments 2 and 3, non-E2 treated mares from groups using P4 intravaginal device presented lower degree of uterine edema, probably because P4 systemic levels produced by this device remained high when the ovulatory follicles reached diameter ≥ 35mm, on average, 7.2 ± 0.85 days after the start of treatment (D0). One explanation for lower degree of uterine edema, in this group using P4 intravaginal device, is that P4 may have affected the ovulatory follicles and consequently the degree of uterine edema. The groups elected to conduct further experiments differed from each other on the degree of uterine edema. The first group elected due to the highest degree of follicular atresia was the group using the intravaginal P4 device plus E2, resulting in a higher average degree of uterine edema (2.8 ± 0.25). The second group, elected for presenting better ovulation synchronization, received no E2 treatment but also used the intravaginal P4 device; this group presented lower degree of uterine edema (2.2 ± 0.28). According to Cuervo-Arango & Newcombe (2010), the low degree of uterine edema in the pre-ovulatory phase, degree 1 or 2 considering score 1 to 5, may be responsible for the reduction in pregnancy rates.

Besides follicle and oocyte aspects, also it is essential to evaluate the uterine conditions of the mares, because after fertilization the uterus will be responsible for

providing the appropriate environment to maintain the pregnancy. The equine embryo secretes mainly E2 in early stages of pregnancy. On endometrium E2 stimulates the increase of uterine receptors for P4 (ZAVY et al., 1979). The manipulation of the estrous cycle in order to synchronize ovulation should mimic the endogenous hormonal concentrations, because E2 and P4 exert its action on the uterus by binding to receptors that regulate gene expression and protein synthesis (HARTT et al., 2005). Pre-exposure to estrogen increases expression of P4 receptors in the reproductive tract and, in addition, modulates gene expression which is important for establishment and maintenance of pregnancy (SPENCER; BAZER, 2002).

In physiological diestrus, uterine fluid accumulation can be suggestive of endometritis and the uterine environment becomes incompatible for embryo survival. Edema and uterine tonus should also be considered in the selection of embryo recipients (MOURA, 2012). Fleury et al. (2006) selected recipients mares on the day of inovulation (day 3 to day 8 post-ovulation) based on tonus and morphoechogenicity (echotexture) of the uterus. The tonus scores ranged from T1 (maximum tonus) to T4 (minimum tonus). Uterine echotexture was classified as E1 score (homogeneous image and higher echogenicity) to E4 (heterogeneous image and lower echogenicity). The pregnancy rates for mares with T1 (92%) and T2 (88%) were higher than for females with T3 (42%), as well as the pregnancy rates for mares with E1 (90%) and E2 (80%) were higher than for mares with E3 (52%) and E4 (10%). These findings of Fleury et al. (2006) led us to concern about the possibility of different pregnancy rates due to hormonal treatment described in experiments 2 and 3. This is particularly valid due to the results of different degrees of uterine edema detected in the groups elected for future studies regarding fertility.

In addition, on an ET program, uterine edema and length of the estrus period can also be correlated with the probability of pregnancy in recipient mares. Mares presenting uterine edema for three or more days during the estrus period preceding ET had a higher pregnancy rate (83.1%; 157/189) than recipients presenting less than three days of uterine edema (63.6%; 77/121). Also, pregnancy probability was lower in recipients showing no uterine edema during follicular phase (50% 11/22) (JACOB et al., 2019).

In experiment 2, the group using P4 intravaginal device in association with E2 presented a higher degree of follicular atresia, 75% (17/20) of mares presented follicular atresia. Therefore, as the minority (25%; 3/20) of the follicles that appeared in the first follicular wave became atretic in this group, subsequently a new wave of follicular growth

emerged while treatment with exogenous P4 was about to finish (Figure 3.11 A) Mare 1). Presumably, the oocytes from these ovulatory follicles should have good quality for fertilization. Nevertheless, this protocol showed the disadvantage of spontaneous ovulations occurring sparsely, in a dispersive pattern, from D8 to D30, which resulted in unsatisfactory degree of ovulation synchronization.

In the other groups from experiment 2, in which the association of P4 with E2 was used, the percentage of follicular atresia was lower, corresponding to 45% (9/20) and 70% (14/20) of mares submitted to injectable and oral exogenous source of P4, respectively (Table 3.1). Besides, ovulations detected in experiment 2 did not occur synchronously in these groups using P4 with E2. Consequently, these results did not enable to establish an ideal moment to induce ovulations according to the criteria of no more than 25% of ovulations occurring naturally before predicted. The hormonal protocols that associated E2 with P4 led mares to take more time to ovulate because of the follicular atresia and posterior emergence of a new follicular wave, the wave that originated the ovulatory follicle. Protocols in which P4 was used without E2 caused a suppression on follicular growth by the action of exogenous P4. In these protocols the follicles that existed at the beginning of treatment (D0) remained growing slowly (1.76 mm/day) compared to the average cited in the literature, 3 to 5 mm/day (DADARWAL et al., 2004).

Moreover, the protocol from experiment 2 using P4 intravaginal device without E2 had a lower atresia degree (15%; 3/20), that is, 85% (17/20) of mares had follicles containing oocytes that were already present at the beginning of treatment (D0). We have speculated that these oocytes showing decelerated growth since D0 may show aging aspects. 75% (15/20) of mares ovulated naturally within 4 days after D8. In this case, the ovulatory follicles originated from a follicular wave that had already appeared at the onset of treatment (D0) and ovulated immediately after the removal of the P4 intravaginal device (D8). Furthermore, oocytes from ovulations after non E2 treatment were exposed to exogenous P4 action for nine days, which delayed the natural moment of ovulation. However, this group showed greater efficiency and time accuracy for ovulation induction. Most of these ovulations, 75% (15/20), occurred spontaneously until D12, on average of 11.6 ± 0.68 days after D8 (Figure 3.10). In this group, 65% (13/20) of treated mares had a 35 mm pre-ovulatory follicle, that would be able to respond to ovulation inducing drugs, on the day of intravaginal P4 device removal (D8) (Figure 3.7; G2.2 A). The unsuccessful aspect of this protocol was the fact that 85% (17/20) of the follicles did not exhibit atresia as intended. The absence of both atresia and the synchronous emergence of a new

follicular wave could result in ovulation of follicles containing low fertility "aged" oocytes. However, there may be a probability that exogenous P4 action of the intravaginal device that maintained the growing follicles decelerated also decreased LH levels. In case this has happened, possibly the immaturity aspect of these oocytes was maintained. Consequently, fertilization of these oocytes would be possible. Inskeep's (2004) results disagree and documented that, in cattle, oocytes from diestrus follicles, secondary waves or previous estrus are nonviable due to their prolonged development and its extensive exposure to high P4 and low LH levels.

Results obtained from experiment 2 showed none of the six experimental groups presented both required criteria for a good hormonal protocol, that is, to induce follicular atresia and to synchronize the emergence of a new follicular wave and ovulations. According to Driancourt (2000), efficient protocols for ovulation synchronization should induce atresia of large follicles that are present in the ovaries, regardless its development stage. Also, these protocols should provide a new follicular wave recruitment, synchronized development of a new dominant follicle and ovulation at a predictable time. For this reason, emerged the need to develop a new strategy to improve the efficiency of the hormonal protocols previously performed. In this scenario the experiment 3 was designed. In experiment 3, the group using P4 intravaginal device with E2 was modified as an attempt to create a better new version. On this new protocol, the mares from this group were submitted to a higher P4 dose. The new P4 dose of the intravaginal device was increased from 0.96 to 1.92 g. In addition, two doses of 17β estradiol were administered, the first on D0 and the second on D2, aiming to induce follicular atresia in more than 75% of the follicles and consequently to improve ovulation synchrony. Similarly, the non-E2 treated group was modified and the new protocol used a 1.92 g P4 dose provided by the intravaginal device. This dose was used to verify the action of exogenous P4 alone on induction of follicular atresia and also to verify if a higher concentration of ovulations would occur in a shorter time interval compared to the previous treatment in experiment 2 (0.96 g P4).

The two new versions of hormonal protocols were tested during the 2018/19 reproductive season. Afterwards, the results were compared to evaluate the better hormonal protocol for ovulation synchronization. The most relevant aspects related to the variables analyzed were the degree of follicular atresia induction, higher number of pre-ovulatory follicles (≥ 35mm) detected in a short period of time and the degree of synchronization of ovulations.

Two aspects should be considered when comparing the results among all four groups; the fact that the hormonal treatment of groups from experiment 2 were performed in the 2017/18 reproductive season and the new version of the hormonal protocols were performed in the 2018/19 reproductive season. Also, that in groups from experiment 2 the number of mares per group was 20 and in experiment 3 the number of mares per group had to be reduced to 18. The same mares were used in all four treatments, except for two mares that needed to be excluded from the experiments.

In general, E2 causes atresia when used in the presence of FSH-dependent follicles because it promotes reduction of FSH concentrations due to negative feedback (BÓ et al., 1995). When E2 returns to basal levels, a new follicular wave occurs due to the new increase in FSH. In contrast, when E2 is administered in the presence of LH-dependent follicles, it may not induce atresia and may prevent the emergence of a new follicular wave until LH level has returned to basal levels. Hence, the use of combined E2 with progestogens can be more successful because it causes atresia of both FSH and LH-dependent follicles (DRIANCOURT, 2000). These observations explain the reason for choosing the basis of our protocols, whether treatment used exogenous P4 in association with E2 or without the use of E2.

Observing the follicular dynamics characteristics of all four selected groups studied in both experiments 2 and 3, it was noticed that the duration of gonadotropin suppression may be affected by the frequency of E2 administration, the dose of P4 and the individual characteristics among mares (Table 3.1 and 3.2). In accordance with MacMillan et al. (2003), the different mares' reproductive status on the first day of the ovulation synchronization protocols can also affect the duration of FSH and LH suppression.

In mares, the occurrence of a wave is defined as simultaneous follicular growth of follicles measuring approximately 6–13 mm in diameter. Major follicular waves develop a dominant follicle and smaller waves only generate small follicles (≤ 22.5 mm). Major follicular waves are subdivided into primary and secondary waves, according to the moment of its emergence. Major primary follicular waves occur near the middle of the diestrus, in which the dominant follicle ovulates at the end or near the end of estrus. When the emergence of the follicular wave occurs during estrus or beginning of diestrus a secondary follicular wave can originate a dominant diestrus follicle that may ovulate or regress. The follicular minor waves are characterized by the absence of a dominant follicle (SATUÉ and GARDON, 2020). In E2 treated group plus doubled P4 dose,

regardless the moment of the estrous cycle, hormonal treatment initiated a synchronous emergence of new major secondary follicular waves. The ovulatory follicles were detected on day 8, that is, when the P4 intravaginal devices were removed and the systemic concentrations of P4 would begin to decline, resembling the concentrations that naturally occur in the beginning of the diestrus. Simply synchronic emergence of follicular waves caused by this hormonal treatment was not a sufficient condition for the ovulatory follicles to ovulate within a relatively short time as expected.

In non-E2 treated group that used P4 intravaginal device (1.92 g), the follicular deviation occurred around day 7 (7.11 ± 0.75) after the beginning of the hormonal treatment, similar to the deviation day found in natural cycles, when it happens around six days after the emergence of major follicular wave. However, in this group, in the beginning of the hormonal treatment, a new follicular wave was not originated, in contrast, the exogenous P4 caused slower follicular growth. Follicular growth rates were reduced by the P4 action on both non E2 or E2 treated groups in association with P4 (1.92 g); growth rates were 1.39 ± 0.29 and 1.83 ± 0.15 mm/day from D0 to D8, respectively. Whereas, in natural cycles the dominant follicle continues to grow in response to the increased sensitivity to FSH. The dominant follicle grows at constant rate of 2.3 mm per day until it reaches a 40 mm diameter and in this stage of development the granulosa cells develop receptors for LH required for final oocyte maturation (GASTAL, 2009).

Follicular atresia, in non-E2 treated group that used P4 intravaginal device (1.92 g), occurred in five mares (27.78%; 5/18) and in these mares the dominant follicles reached the diameter ≥ 35mm on days 12, 13, 14, 15 and 20. Thus, follicular atresia postponed the moment when follicles reached pre-ovulatory size and ovulated (Figures 3.11 E) Mare 5). When a new follicular wave emerges after follicular atresia, the new group of growing follicles take more time to reach the ideal size and maturity to ovulate. The effect of P4 (1.92 g) caused suppression on follicular growth without causing atresia in 72.22% (13/18) of the follicles. These follicles reached diameter ≥ 35mm approximately on day 11, a few days earlier compared to when follicular atresia occurred. However, if one compares both P4 intravaginal device groups, from experiments 2 and 3, 72.22% (13/18) and 65% (13/20) of the dominant follicles reached the pre-ovulatory size at day 11 and 8, respectively. Therefore, the double the dose of P4 (1.92 g) promoted a greater inhibitory effect on the hypothalamic-hypophyseal axis and on the dominant follicle. A longer follicular growth phase and required three more days for the follicles to reach ≥ 35mm diameter. In comparison with ovine species, in sheep submitted to

exogenous P4 treatment using CIDR the length of dominant follicle growth phase is delayed for at least one day (URIBE-VELÁSQUEZ et al. 2008). Once the P4 supplementation ceases, there is a release of LH from the anterior pituitary gland, the so-called “rebound effect” that induces follicular maturation and ovulation (SAUTÉ and GARDON, 2020).

In experiment 3, the evaluation of the distribution of days in which follicles reached 35mm diameter and days in which ovulations occurred spontaneously demonstrated a higher concentration of ovulations in a shorter period of time in the new version of the protocol using the double P4 intravaginal device dose associated with two administrations of E2. Comparison of the distribution days when follicles reached 35 mm diameter in the new protocol using the double P4 intravaginal device dose without association with E2 demonstrated that follicles took more days to reach 35 mm. In addition, the distribution of days when ovulations occurred spontaneously showed asynchrony of the moment of ovulations in some mares when follicular atresia was presented. According to Antoniolli (2002), high P4 levels cause atresia of the dominant follicle and restart follicular growth. However, on both experiments 2 and 3, the groups using only P4 treatment without E2 showed low degree of follicular atresia. The group from experiment 3 using the intravaginal device with P4 double dose (1.92 g) without E2 presented only 27.78% (5/18) of mares showing atresia of all follicles. Once the dominant follicles did not suffer atresia by the action of P4, these follicles were able to ovulate after intravaginal device removal. The event of follicular atresia was responsible for the delay of ovulations. In contrast, when the P4 acted and promoted only suppression of follicular growth, ovulation happened approximately five days earlier. These data demonstrated that half of the P4 dose (0.92 g) enabled better conditions for ovulation synchronization. The previous hormonal protocol from experiment 2 showed that intravaginal P4 device containing 0.92 g concentrated ovulations in a shorter interval of time.

The event of ovulation was delayed in all four selected hormonal protocols from both experiments 2 and 3 when follicular atresia occurred. The treatment with intravaginal device as a source of exogenous P4 (0.96 g or 1.92 g) not associated with E2 promoted satisfactory synchronization of ovulation when there was no follicular atresia. Ovulations occurred, on average, approximately four to five days after P4 intravaginal device removal, regardless of the dose used.

Two different hormonal treatments demonstrated the possibility of ovulation synchronization: non-E2 treated group, from experiment 2, using P4 intravaginal device

(0.96 g) and the other group also using P4 intravaginal device, however, doubled P4 dose (1.92 g) in association two administrations of 17β E2 from experiment 3. These two hormonal protocols were able to synchronize ovulation in a satisfactory number of mares. These data provided information and new insights for future investigations regarding fixed time artificial insemination in equine reproduction. Equine timed artificial insemination (TAI) may allow to manage big herds without estrus detection. TAI in jennies has been recently described. Fanelli et al. (2019) submitted nine fertile jennies to three TAI protocols: PPG (PGF2α, PGF2α, GnRH), PGPG (PGF2α, GnRH, PGF2α, GnRH) and GPG (GnRH, PGF2α, GnRH). By comparing the three protocols of this study, synchronization and ovulation response were 55%, 67%, 89% and 50%, 63% 80%, respectively. Artificial Insemination (AI) was done with a fresh-diluted semen and the protocols showed a tendency for difference in pregnancy rates of the synchronized jennies (from 11% with PPG up to 56% with PGPG). Another important aspect observed was that follicle diameter or the presence/absence of a CL at the beginning of the treatment did not affect synchronization response or pregnancy rate. This study demonstrated a great progress with the possibility to synchronize and to confirm pregnancy in jennies using TAI.

In summary, the group using P4 (0.96 g) without E2 presented better ovulation synchronization, however, 85% (17/20) of the follicles did not exhibit atresia as intended. In comparison, 94.44% (17/18) of the follicles exhibited atresia on the group using E2 in association with intravaginal P4 device (1.92 g), and consequently, the oocytes were originated from a new follicular wave that emerged after the P4 levels were declining. These data may encourage the creation of new different strategies focusing on follicular wave and ovulation synchronization the in mares. The idea provided by the results found in our hormonal protocols may help to instigate the use of FTAI in large herd of mares.

CONCLUSIONS

The use of P4 intravaginal device containing 0.96 g without association with E2 decreased follicular growth rate, although it did not result in induction of follicular atresia in most treated mares.

Follicles that had its grown decelerated or temporarily blocked when submitted to the action of exogenous P4 were able to resume its growth, become pre-ovulatory and ovulate after ceasing P4 supplementation.

Follicular atresia occurred only in a small percentage of mares, ranging from 15 to 27%, when P4 plasma concentrations remained higher than 5 ng/ml for at least four consecutive days after the beginning of the hormonal treatment.

The association of P4 and 17β estradiol in the beginning of treatment was responsible for greater efficiency in induction of follicular atresia and the emergence of a new follicular wave.

The highest degree of ovulation synchronization occurred in mares submitted to the hormonal treatment using an intravaginal device containing 0.96 g P4 without association with E2.

Table 3.1 Effect of six hormonal treatments for induction of follicular atresia, stimulation of a new follicular wave and synchronization of ovulation. Twenty mares were treated randomly in all treatments.

Variable	G2.1	G2.2	G2.3	G2.4	G2.5	G2.6	*P-value*[1]
Diameters (mm)							
Largest follicle on D0	26.2 ± 1.5	24.6 ± 1.7	23.6 ± 1.2	25.6 ± 2.3	23.9 ± 1.6	26.9 ± 1.8	0.167
OvF detected on D0	17 ± 2.5ab	20.5 ± 1.7ab	18.6 ± 1.3ab	20.1 ± 1.46ab	14.1 ± 4.2b	20.4 ± 1.5a	0.030
OvF detected after D0	17.9 ± 0.9	21.3 ± 0.02	17.9 ± 1.3	14.6 ± 0.9	16.3 ± 0.8	17.9 ± 1.1	0.560
OvF at first detection	17.72 ± 0.92	20.67 ± 1.6	18.26 ± 0.94	18.53 ± 1.19	15.71 ± 0.86	18.92 ± 0.95	0.256[y]
OvF on D8 (P4 source removal)	25.4 ± 1.7	36.7 ± 1.6	29.2 ± 2.4	34.2 ± 2.5	23.1 ± 1.6	30.4 ± 2.3	0.293[yz]
OvF on deviation day	26.42 ± 1.17	33.16 ± 1.4	30.67 ± 0.97	32.64 ± 1.31	28.28 ± 1.14	31.19 ± 0.7	0.065[y]
Second largest follicle on deviation day	23.29 ± 0.9	23.7 ± 1.22	25.16 ± 0.96	25.71 ± 1.27	22.33 ± 1	25.9 ± 0.75	0.187
POF on day before ovulation	45.32 ± 1.25	43.93 ± 0.8	47.02 ± 0.9	46.24 ± 1.03	43.99 ± 1.12	42.82 ± 0.87	0.943[z]
Uterine echotexture (1-4)	2.8 ± 0.25	2.2 ± 0.28	2.3 ± 0.31	2.2 ± 0.27	3.2 ± 0.21	2.7 ± 0.27	0.573[yz]
Growth rates (mm/day)							
From D0 to D8	1.51 ± 0.19	1.78 ± 0.13	1.49 ± 0.19	1.74 ± 0.21	1.6 ± 0.14	1.77 ± 0.18	0.999
From D8 to ovulation	2.63 ± 0.12	2.47 ± 0.22	2.6 ± 0.12	2.1 ± 0.18	2.74 ± 0.11	2.55 ± 0.12	0.486[y]
Intervals (days)							
OvF growth from first detection to D8	5.31 ± 0.73	8.65 ± 0.24	6.64 ± 0.66	8.11 ± 0.52	4.52 ± 0.71	6.76 ± 0.6	0.236[yz]
OvF growth from D8 to ovulation	8.7 ± 1.09	3.6 ± 0.6	9.15 ± 1.44	6.2 ± 1.4	7.95 ± 0.69	6 ± 4.03	0.484[y]
Event detection day							
OvF first detection	5.2 ± 0.9	0.3 ± 0.2	4.3 ± 1.3	2.6 ± 1	5 ± 0.6	3.5 ± 0.8	0.081[y]
Deviation	9.6 ± 1.09	6.7 ± 0.5	10.35 ± 1.4	8.9 ± 1.3	10.6 ± 0.6	8.9 ± 1.01	0.596[y]
OvF ≥35mm	12.9 ± 0.99	7.25 ± 0.85	12.7 ± 1.42	10 ± 1.21	12.9 ± 0.64	10.8 ± 0.89	0.195[y]
Ovulation	16.6 ± 1.06	11.6 ± 0.68	17.1 ± 1.41	14.15 ± 1.38	15.9 ± 0.69	14 ± 0.9	0.348[y]
Ovulation with Fol atresia	16.53 ± 0.88	14.0 ± 2.5	17.77 ± 1.94	23 ± 1	16.42 ± 0.85	19 ± 0.00	SD
Ovulation without Fol atresia	17.0 ± 3.6	11.17 ± 0.53	16.54 ± 2.08	13.16 ± 1.34	14.83 ± 1.1	13.73 ± 1.1	SD
Number of mares with Fol atresia (n)	15/20 (75%)a	3/20 (15%)b	9/20 (45%)a	2/20 (10%)b	14/20 (70%)a	1/20 (5%)b	0.0001

[1]Interaction P-values. Upper lower-cases indicates groups differences. [Y]Effect of estrogen treatment was detected ($P<0.05$). [z]Effect of progestogen treatment was detected ($P<0.05$).
Fol: follicle; SD: Scarce data for statistical analysis; OvF: ovulatory follicle; POF: pre ovulatory follicle; P4: progesterone
SD: Scarce data for statistical analysis
NS: Not significant. * Tendency.
D0 = start of hormone treatment.
G2.1: the intravaginal devices of 0.96 g of P4 were inserted at D0 and 0.25 mg of prostaglandin F2α IM was administered; D8 – P4 device removal and new dose of 0.25 mg of PGFF2α IM.
G2.2: idem G2.1 with 17β estradiol IM administration at D0.
G2.3: D0 administration of 1500 mg/animal of P4 IM, 0.25 mg of PGFF2α ; D8 - new dose of 0.25 mg of PGFF2α IM.
G2.4: idem G2.3 with 17β estradiol IM administration atD0.
G2.5: D0 0.25 mg PGFF2α IM and oral daily administration of P4 (altrenogest 0.045 mg/kg live weight) over a period of nine days (D0 to D8); D8 - interruption of P4 oral administration and new dose of 0.25 mg PGFF2α IM.
G2.6: idem G2.5 with 17β estradiol IM administration D0.

Table 3.2 Effect of two hormonal treatment protocols, groups G3.1 and G3.2, for induction of follicular atresia, stimulation of a new follicular wave and ovulation synchronization. Eighteen mares were treated randomly in both treatments.

Variable	G3.1	G3.2	*P-value*
Diameters (mm)			
Largest follicle on D0	22.85 ± 1.58	24.25 ± 1.41	0.379
OvF detected on D0	.	19.77 ± 1.96	.
OvF detected after D0	16.86 ± 0.84	18.71 ± 1.38	0.281
OvF at first detection	16.86 ± 0.84	19.36 ± 1.28	0.068*
OvF on D8 (P4 source removal)	19.69 ± 1.37	30.66 ± 1.29	0.000
OvF on deviation day	24.88 ± 1.56	28.42 ± 1.03	0.057
Second largest follicle on deviation day	21.88 ± 1.05	25.43 ± 1.27	0.018
POF on day before ovulation	47.06 ± 0.96	44.39 ± 0.87	0.013
Uterine echotexture (1-4)	3.66 ± 0.11	2.44 ± 0.33	0.003
Growth rates (mm/day)			
From D0 to D8	1.39 ± 0.29	1.83 ± 0.15	0.228
From D8 to ovulation	3.08 ± 0.11	2.98 ± 0.2	0.689
Intervals (days)			
OvF growth from first detection to D8	1.28 ± 0.49	7.37 ± 0.62	0.000
OvF growth from D8 to ovulation	9.83 ± 0.45	5.22 ± 0.81	0.000
Event detection day			
OvF first detection	8 ± 0.53	2.61 ± 0.87	0.000
Deviation	11.11 ± 0.59	7.11 ± 0.75	0.000
OvF ≥35mm	13.61 ± 0.53	9 ± 0.63	0.000
Ovulation	17.83 ± 0.45	13.16 ± 0.83	0.000
Ovulation with Fol atresia	18 ± 0.44	16.6 ± 1.69	.
Ovulation without Fol atresia	15	11.84 ± 0.68	.
% of mares with Fol atresia (n)	94.44% (17/18)	27.78% (5/18)	0.000

Fol: follicle; SD: Scarce data for statistical analysis; OvF: ovulatory follicle; POF: pre ovulatory follicle; P4: progesterone
NS: Not significant. * Tendency.
D0 = start of hormone treatment.
G3.1: at D0 the intravaginal devices of 1.92 g of P4 were inserted and 0.25 mg of prostaglandin F2α IM and 20 mg 17β estradiol were administered; at D2 20 mg of 17β estradiol was administered; D8 – P4 device removal and a new dose of 0.25 mg of prostaglandin F2α IM.
G3.2: idem G3.1, but the same intravaginal P4 device was used without 17β estradiol association.

Table 3.3 Reference values of blood progesterone levels in cyclic, anestrus and pregnant mares (ALLEN, 1987, GINTHER, 1986, PIMENTEL et al., 2014).

Periods of P4 variation	P4 ng/mL
Diestrus phase	>4
Estrus phase	<1
6 to 14 days of gestation	8 – 15
30 to 35 days of gestation	4 – 6
40 to 150 days of gestation	8 – 25
160 to 280 days of gestation	>4
300 to 320 days of gestation	10
Hours before parturition	<10

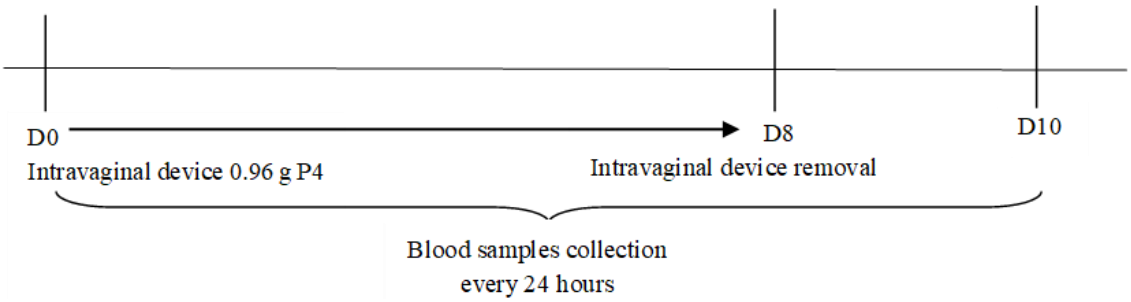


Figure 3.1 Experiment 1 design. On D0 (day of start of treatment) 0.25 mg of PGF2α (Sincrocio®, Ouro Fino Animal Health) was administered via IM and intravaginal device with 0.96g P4 was inserted and maintained until D8. Blood samples were taken every 24 hours from D0 to D10 for plasma analysis of P4 concentrations.

Figure 3.2 Schematic design of the hormonal treatment protocol for synchronization of the follicular wave used in the experiment 2. D0 = start of hormonal treatment. In order to avoid confusion among groups from different experiments the initial G followed by two numbers are going to be used. The letter G means groups, the number following letter G refers to all groups from experiment 2, and the second number represents one of the six different hormonal treatments performed. Example: G2.1 Group 1 experiment 2. G2.1: the intravaginal devices of 0.96 g of P4 were inserted at D0 and 0.25 mg of prostaglandin F2α IM was administered; D8 – P4 device removal and a new dose of 0.25 mg of prostaglandin F2α IM.

G2.2: idem G2.1 with 17β estradiol IM administration at D0.

G2.3: D0 administration of 1500 mg/animal of P4 IM, 0.25 mg of prostaglandin F2α; D8 - new dose of 0.25 mg of prostaglandin F2α IM.

G2.4: idem G2.3 with 17β estradiol IM administration at D0.

G2.5: D0 0.25 mg prostaglandin F2α IM and oral daily administration of P4 (altrenogest 0.045 mg/kg live weight) over a period of nine days (D0 to D8); D8 – interruption of oral administration of P4 and a new dose of 0.25 mg prostaglandin F2α IM.

G2.6: idem G2.5 with 17β estradiol IM administration at D0.

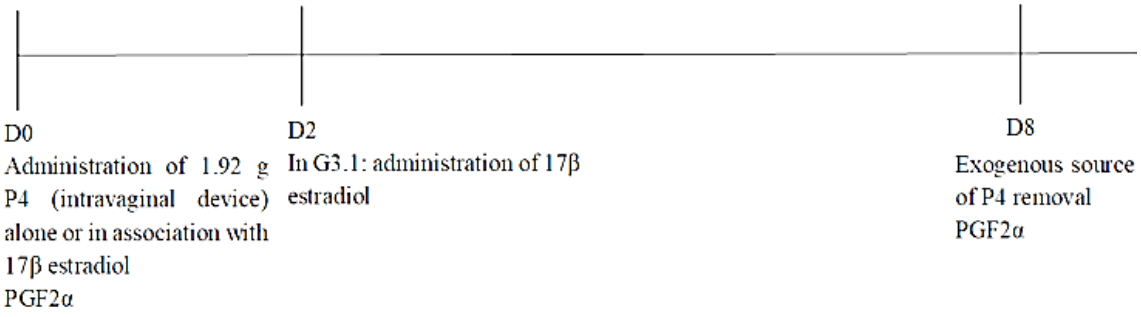


Figure 3.3 Schematic design of the hormonal treatment protocol for synchronization of the follicular wave used in the experiment 4. D0 = start of hormonal treatment.

G3.1: at D0 the intravaginal devices of 1.92 g of P4 were inserted and 0.25 mg of prostaglandin F2α IM and 20 mg 17β estradiol were administered; at D2 20 mg of 17β estradiol was administered; D8 – P4 device removal and a new dose of 0.25 mg of prostaglandin F2α IM.

G3.2: idem G3.1 without 17β estradiol association.

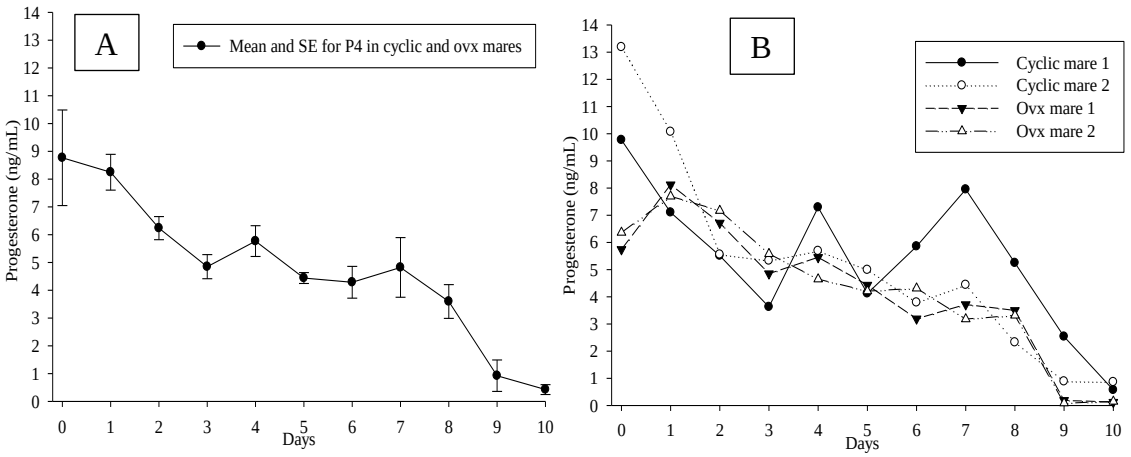


Figure 3.4 Daily plasma P4 concentrations. A) Means (±SE) for plasma progesterone concentration of four mares submitted to treatment with exogenous progesterone, the intravaginal devices containing 0.96 g of P4 were inserted on day 0 in two ovariectomized mares and two cyclic mares. The graph represents the release of progesterone into the plasma during the period of day 0 and day 10. The progesterone device was removed at D8. B) Individual graphic representation of plasma progesterone concentrations in two ovariectomized and two cyclic mares submitted to exogenous progesterone treatment with an intravaginal device containing 0.96 g of progesterone. The graph represents the release of progesterone into the plasma during the period of day 0 and day 10. The progesterone implant was removed at D8. Ovx = ovariectomized.

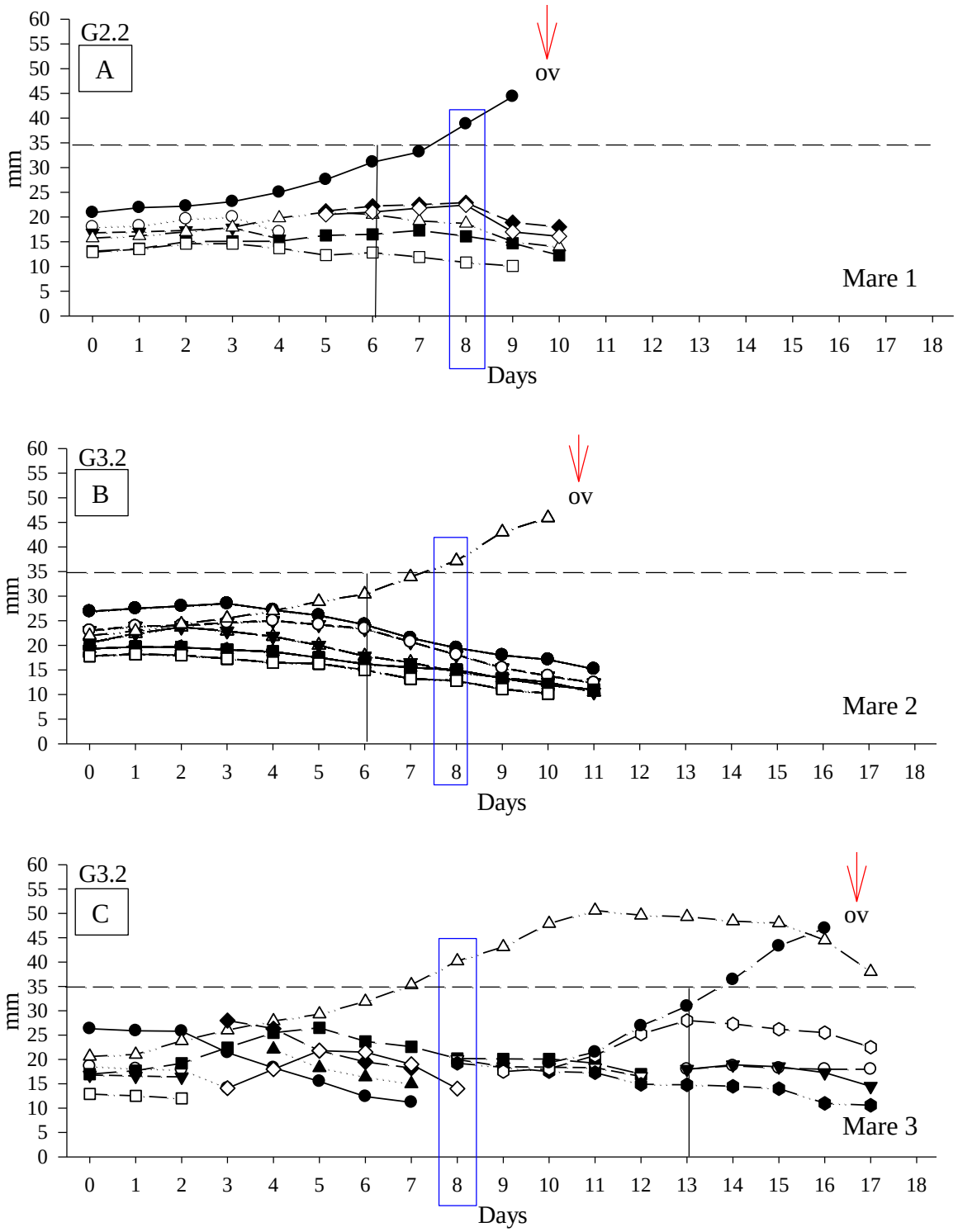


Figure 3.5 Graphic representation of the ultrasonographic follicular dynamics monitoring of mares submitted to G2.2 and G3.2 hormonal treatment protocols for comparative purposes among groups. The day the protocol started (D0) corresponded to the eleventh day (D10) of the estrous cycle. The diameters of the three largest follicles in each ovary were measured daily. The blue rectangle indicates the day that P4 intravaginal device was removed. The day when the follicular divergence occurred is indicated by the vertical full line. The moment follicles reached 35mm diameter is indicated by the horizontal dotted line. The red arrow indicates the day of ovulation.

A) Mare 1 - G2.2: the graph exemplifies the effect of the 0.96 g P4 intravaginal device inserted at D0, on follicular growth suppression. After the removal of the exogenous source of P4, ovulation of the pre-ovulatory follicle occurred at D10.

The graphs of mares 2 and 3 represent different examples of follicular dynamics of group G3.2, emphasizing the asynchrony at the time of occurrence of ovulations due to follicular atresia in mare 3 while in mare 2 only occurred suppression of follicular growth.

B) Mare 2 - G3.2: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, on follicular growth suppression. After the removal of the exogenous source of P4, a follicle that was already present at the beginning of the protocol (D0) ovulated at D11

C) Mare 3 - G3.2: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, on follicular growth suppression.and posterior occurrence of follicular atresia. After removal of the exogenous source of P4, a new follicular wave emerged and ovulation day was delayed compared to mares in which there was no follicular atresia submitted to the same protocol of group G3.2. Ovulation of the pre-ovulatory follicle occurred at D17.

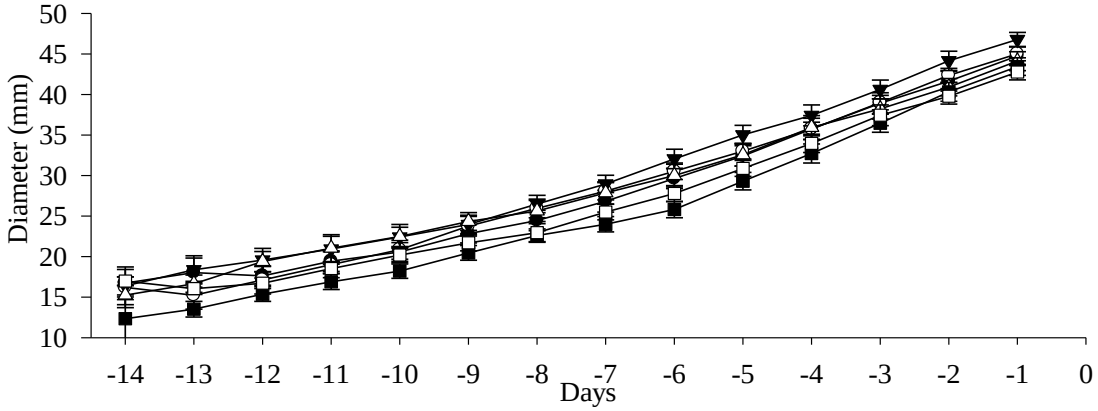


Figure 3.6 Graphic representation of the ultrasonographic monitoring of follicular dynamics with means and standard error of follicular growth of 20 mares from groups G2.1 to G2.6 used in experiment 2. The data were normalised for the day of ovulation (D0) and the two weeks before ovulation which corresponded to the fourteenth day (-D14). Average diameters and standard error of the ovulatory follicle measured daily were calculated. The graph shows that the daily growth rates of the ovulatory follicle under the effect of exogenous progesterone did not differ (P = 0.750) between the six groups (G2.1 = 1.51 ± 0.19; G2.2 = 1.78 ± 0.13; G2.3 = 1.49 ± 0.19; G2.4 = 1.74 ± 0.19; G2.5 = 1.6 ± 0.14); G2.6 = 1.77 ± 0.18) and after the interruption of exogenous P4 until the moment of ovulation there was a tendency for the difference (P = 0.08) between the average growth rates of the six groups (G2.1 = 2.63 ± 0.12; G2.2 = 2.47 ± 0.22; G2.3 = 2.6 ± 0.12; G2.4 = 2.1 ± 0.18; G2.5 = 2.74 ± 0.11; G2.6 = 2.55 ± 0.12). The mean daily growth rate of the groups (G2.1 to G2.6) under the influence of exogenous P4 (1.64 mm/day) was lower ($P < 0.05$) than the mean after the exogenous P4 interruption until ovulation (2.51 mm/day).

REFERENCES

ALMEIDA, H.B., VIANA, W.G., ARRUDA, R.P., OLIVEIRA, C.A. Synchronization of oestrus and follicular dynamics of Crioulas mares submitted to treatments with norgestomet, melengestrol acetate and altrenogest. **Brazilian Journal of Veterinary Research and Animal Science**, v. 38, n.6, 2001.

ANTONIOLLI, C.B. **Follicular development, follicular waves and estrous cycle manipulation.** 15f. Dissertation (Master in Animal Reproduction. Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, 2002.

AVANZI, B.R.; RAMOS, R.S.; ARAUJO, G.H.M.; FIORATTI, E.G.; TRINCA, L.A.; DELL'AQUA, J. A., … PAPA, F. O. Fixed-time insemination with frozen semen in mares: is it suitable for poorly fertile stallions? **Theriogenology**, v.83; n.9, p.1389–1393, 2015.

BERGFELT, D.R.; GINTHER, O.J. Ovarian, uterine and embryo dynamics in horses versus ponies. **Journal of Equine Veterinary Science**, v.16, n.2, p.66-72, 1996.

BERGFELT, D. R.; MEIRA, C.; FLEURY, J. J.; FLEURY, P. D. C.; DELL'AQUA, J. A.; ADAMS, G. P. Ovulation synchronization following commercial application of ultrasound-guided follicle ablation during the estrous cycle in mares. **Theriogenology.** v.68, p.1183-91, 2007.

BERGFELT, D.R.; ADAMS, G.P. Ovulation and corpus luteum development. In: SAMPER, J.C.; PYCOCK, J.F.; McKINNON, A.O. **Current therapy in equine reproduction.** Hardcover: Sanders Elsevier, p. 1-13, 2007.

BÓ, G.A.; ADAMS, G.P.; CACCIA, M. et al. Ovarian follicular wave emergence after treatment with progestogen and estradiol in cattle. **Animal Reproduction Science**, v.39, p.193–204, 1995.

BARBACINI, S.; ZAVAGLIA, G.; GULDEN, P.; MARCHI, V.; NECCHI, D. Retrospective study on the efficacy of hCG in an equine artificial insemination programme using frozen semen. **Equine Veterinary Education**. v.2, p.404–408, 2000.

BIANCHI, C.; BENAVENTE, M.A.; SIMONETTI, M.; ABA, M.A. Sychronization of time of ovarian folicular development in llamas (*Llama glama*) using a protocol based on GnRH and PGF2α. **Animal Reproduction Science**. n.192 p.200-205. 2018.

BRADECAMP, E. A. Pneumovagina. In: MCKINNON, A. O., et al. Equine Reproduction. 2nd ed. West Sussex, United Kingdom: Wiley-Blackwell; 2011. chap. 262. p. 2538–2544.

CARVALHO, J.B.P.; CARVALHO, N.A.T.; REIS, E.L.; NICHI, M.; SOUZA, A.H.; BARUSELLI, P.S. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus x Bos taurus, and Bos taurus heifers. **Theriogenology**, v.69, n.2, p.167–175, 2008.

CHANG, Z.; LIU, B.; YAO, L.; JIANG, G.M.; YU, J.; JING, H.T. Effects of FSH and the weather during induced ovulation and timed artificial insemination to increase jenny conception rates. **Science of Reproduction**. n. 9. 3220. 2019.

CUERVO-ARANGO, J., & NEWCOMBE, J. R. Risk Factors for the Development of Haemorrhagic Anovulatory Follicles in the Mare. ***Reproduction in Domestic Animals***, *45*(3), 473-480, 2010.

DADARWAL, D.; TANDON, S.N.; PUROHIT, G.N.; PAREEK, P.K. Ultrasonographic evaluation of uterine involution and postpartum follicular dynamics in French Jennies (Equus asinus). **Theriogenology**, in press, 2004.

DRIANCOURT, M.A. Regulation of ovarian folicular dynamics in farm animals. Implication for manipulation of reproduction. **Theriogenology.** v.54. n.6. p. 1211-1239. 2000.

FANELLI, D.; PANZANI, D.; ROTA, A.; SALARI, F.; MARTINI, M.; CAMILLO, F.A. New, Simplified 251 Protocol for Timed Artificial Insemination in Donkeys. **Journal of Equine Veterinary Science**, v. 66, p. 128-129, 2018.

FANELLI, D.; TESI, M.; ROTA, A.; BELTRAMO, M.; CAMILLO, F.; PANZANI, D. Studies on the use of prostaglandin F2a and gonadotropin-releasing hormone analogs for timed artificial insemination in jennies. **Journal of Equine Veterinary Science**, v. 74, p. 36-41, 2019.

FERREIRA JC, IGNACIO FS, MEIRA C. Doppler ultrasonography principles and methods of evaluation of the reproductive tract in mares reproductive tract in mares. **Acta Scientiae Veterinariae.** v.39, p.105 - 111. , 2011.

FLEURY, P.D.C.; ALONSO, M.A.; BALIEIRO, J.C.C. Evaluation of the recipient: effect of uterine characteristics and ovulation time. In: XVIII Annual Meeting of the Brazilian Embryo Technology Society, Araxá. **Acta Scientiae Veterinariae**, v.34 (supl. 1), p.502, 2006.

FRITSCH, S.C. **Effect of administration of different doses of oestradiol followed by progesterone on the expression of endometrial estrogen and progesterone receptors in acyclic receptor mares. Master's Thesis.** FMVZ Unesp, Botucatu, 2016.

GALLELLI, M.F.; BIANCHI, C.; TRASORRASA, V.; ZAMPINIA, E.; ABA, M.; MIRAGAYA, M. **Animal Reproduction Science**. n.208 p.105-106. 2019.

GARBARINO, E.J; HERNANDEZ, J.A; SHEARER, J.K; RISCO, C.A; THATCHER, W.W. Effect of lameness on ovarian activity in postpartum Holstein cows. **Journal of Dairy Science**; 87:4123–4131. 2004.

GASTAL, E. L., GASTAL, M. O., WILTBANK, M., GINTHER, O. J. Follicle deviation and intrafollicular and systemic estradiol concentration in mares. **Biology of Reproduction**, v. 61, p. 31-39, 1999.

GASTAL, E.L. Recent advances and new concepts on follicle and endocrine dynamics during the equine periovulatory period. **Animal Reproduction**, vol. 6, no. 1, p.144-158, 2009.

GINTHER, O.J. Influence of progesterone and number of corpora lutea on ovaries in sheep. Am. **Journal Veterinary Research.,** v.32, p.1987-1992, 1971.

GINTHER, O.J. Ultrasound imaging and reproductive events in the mare. Madison. **EquiServices**. p.377. 1986.

GINTHER O.J. Sexual behavior Characteristics of the ovulatory season. In: **Reproductive Biology of the Mare: Basic and Applied Aspects**, 2nd ed. Wisconsin: Publisher Equiservices, 1992.

GINTHER, O.J.; BEG, M.A.; GASTAL, E.L.; GASTAL, M.O. 45 Follicle dynamics and selection in mares. **Animal Reproduction**, v.,1 n.1, p.45-63, Oct./Dec. 2004.

GINTHER, O.J; GASTAL, E. L.; GASTAL, M.O.; BEG, M. A. Regulation of circulating gonadotropins by the negative effects of ovarian hormones in mares. **Biology of Reproduction**, v.73, p.315-323, 2005.

GINTHER, O. J. et al. Characterisation of pulses of 13,14- dihydro-15-keto-PGF2alpha (PGFM) and relationships between PGFM pulses and luteal blood flow before, during, and after luteolysis in mares. **Reproduction, Fertilility and Development**, v. 20, p. 684-693, 2008.

GRECO, G.M.; AVANZI, B.R.; FIORATTI, E.G.; QUEIROZ, F.J.R.; MEIRA, C.; ALVARENGA, M.A. Effect of interruption of progesterone treatment on acyclic receptor mares presenting the formation of accessory luteous bodies. **Acta Scientiae Veterinariae**, v.36 (Supl.2), 2008.

GURGEL, J. R. C.; VIANA, C. H. C; PEREZ, E. G.A; NICHI, M. Follicular dynamics in mares: intrafolicular aspects. **Brazilian Journal of Animal Reproduction**, v. 32, n. 2, p. 122-132, 2008.

HARTT, L.S.; CARLING, S.J.; JOYCE, M.M.; JOHNSON, G.A.; VANDERWALL, D.K.; OTT, T.L. Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. Reproduction, v.130, p.241-250, 2005.

HENNEKE, D.R.; POTTER, G.D.; KREIDER, J.L. AND YEATES, B.F. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. **Equine Veterinary Journal,** 15: 371- 372. 1983.

HUGHES, J.P., STABENFELT, G.H. E KENNEDY, P.C (1980) The estrous cycle and selected functional and pathologic ovarian abnormalities in the mare. **Veterinary Clinics of North America** (Large Anim. Pract.), v.2, n.2, p.225-239.

INSKEEP, E.K. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. **Journal of Animal Science**; v.82(ESuppl:E24e39), 2004.

KINDER, J.E., KOJIMA, F.N., BERGFELD, E.G.M., WEHRMAN, M.E., FIKE, K.E. Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. **Journal Animal Science**, 74 (1996), pp. 1140-1424.

KUHL, J.; AURICH, J.; & AURICH, C. Effects of the Prostaglandin F 2α Analogues Cloprostenol and Luprostiol in Combination With hCG on Synchronization of Estrus and Ovulation in Mares. **Journal of Equine Veterinary Science**. v.57, p.67–70, 2017.

JACOB, J.C.F.; GUERSON, Y.B.; FERRAZ, P.J. How to improve reproductive rates in an equine embryo transfer program. **Brazilian Journal of Animal Reproduction**, v.43, n.2, p.222-228, apr./jun. 2019.

LARSEN, J.E.; NORMAN, S.T. The Synchronization of Oestrus and Ovulation in the Mare. **RIRDC - Rural Industries Research and Development Corporation**, Innovation for rural Australia, v. 10, n.202, p. 7-31. 2010.

LARENTIS, G. R.; BOLL DE ARAUJO BASTOS, H.; MACHADO CENTENO, L. A.; LA CRUZ BUENO, V.; BRINGEL, B. A.; & MATTOS, R. C. Hormonal stimulation in a gonadal dysgenesis mare. **Journal of Equine Veterinary Science**, 2020.

LINDEBERG, H., KOSKINEN, E., HUHTINEN, M., REILA, T., PERTTULA, H., KATILA, T. Influence of PG administration and follicle status on the number of conceptuses. **Theriogenology**; 58:571–574. 3. 2002. A.R.G.

MACMILLAN K.L.; SEGWAGWE, B.V.E.;PINO, C.S Associatonns between the manipulation of follicular development and fertility in cattle. **Animal Reproduction Science**, v.78,p.327-344, 2003.

MARTÍNEZ-GARCÍA, J.A.; SÁNCHEZ-TORRES, M.T.; CORDERO, J.L. et al. Ovarian follicular dynamics after cauterization of the dominant follicle in anoestrous ewes. **Animal Reproduction Science**, v.98, p.225-232, 2007.

MOURA, J. C. A. DIAGNOSTIC IMAGING IN EQUINE REPRODUCTION "UTERINE CONTROL". Veterinary Medicine Academy of Bahia. **Animal**

Science, 22(1), VI North-East Animal Reproduction Congress, Fortaleza, CE.10 P. 2012.

NEWCOMBE, J.R.; WILSON, M.C. The use of progesterone releasing intravaginal devices to induce seasonally anoestrus Standardbred mares. **Equine Practitioners**, v.19, p. 13-21, 1997.

NEWCOMBE, J.R. Field observations on the use of progesterone releasing intravaginal device to induce oestrus and ovulation in seasonally anoestrus mares. **Journal of Equine Veterinary Science**, v. 22, p. 378-382, 2002.

NEWCOMBE, J.R.; HANDLER, J.; KLUG, E. Treatment of transition phase mares with progesterone intravaginally and with deslorelin or hCG to assist ovulations. **Journal of Equine Veterinary**

Science, v. 22, p. 57-64, 2002.

NEWCOMBE, J.R., JO¨CHLE, W., CUERVO-ARANGO, J. Effect of dose of cloprostenol on the interval to ovulation in the dioestrous mare: a retrospective study. **Journal of Equine Veterinary Science.** 28, 532–539. 2008.

NIELSEN, J.M., KOFOED BOCK, T.S., ERSBOLL, A.K. Factors associated with fertility in horses in a Danish equine practice after artificial insemination with frozen-thawed semen. **Animal Reproduction Science**. 2008;107:336–337.

PIMENTEL MML, SANTOS FA, DIAS RVC, MACEDO LB, FONSECA, ZAS, ANDRE WPP, RIBEIRO WLC. Monitoring of estral cycle of mares by vaginal cytology, ultrasonography and hormonal dosage. **Science Archives of Veterinary**. UNIPAR, Umuarama, v. 17, n. 1, p. 69-75. 2014.

PINTO CR, BURNS PJ, WHISNANT S. Effects of single estradiol administration on follicular dynamics and luteal function of cyclic mares. In: International Symposium on Equine Embryo Transfer, 6, 2004, Rio de Janeiro. **Proceedings.** Rio de Janeiro: ISEET, 2004. p.92-94. Abstract.

PHETUDOMSINSUK, K. Investigation into the effect of prostaglandin F2a, GnRH analogue and hCG on induction of ovulation in mares. **Thai Journal Veterinary Medicine**. v.47, n(4):p. 493-499. 2017.

RATTO, M.; CERVANTES, M.; NORAMBUENA, C.; SILVA, M.; MIRAGAYA, M.; HUANCA, W. Effect of location and stage of development of dominant follicle on ovulation and embryo survival rate in alpacas. **Animal Reproduction Science**. n.127 p.100-105. 2011.

REWAY, A.P. **Development of a follicular wave synchronization protocol and determination of the ideal moment for ovulation induction in equine species**. Dissertation (Master's Degree in the Program of Domestic and Wild Animal Anatomy). University of São Paulo, FMVZ USP. São Paulo - SP, 2017.

ROCHA FILHO, A.N.; PESSÔA, M.A.; GIOSO, M.M.; ALVARENGA, M.A. Transfer of equine embryos into na ovulatory recipientes supplemented with short or long acting progesterone. **Animal Reproduction**, v. 1, n. 1, p. 91-95, 2004.

SAMPER, J.C. Ultrasound appearance and uterine edema pattern for time ovulation in mares. **Proc. 43rd Annual AAEP** Convention 1997, 189-191.

SAMPER, J.C. Induction of estrus and ovulation: why some mares respond and others do not. **Theriogenology**, v.70, n.3, p.445–447. 2008.

SAUTÉ, K.; GARDON, J.C. Physiological and Clinical Aspects of the Endocrinology of the Estrous Cycle and Pregnancy in Mares. In: **Animal Reproduction in Veterinary Medicine.** IntechOpen. p. 1-33, 2020.

SCHAUER SN, GUILLAUME D, DECOURT C, WATSON ED, BRIANT C, AND DONADEU FX. Effect of luteinizing hormone overstimulation on equine follicleMaturation. **Theriogenology**. 79: 409-416. 2013.

SHARP, D.C. The early fetal life of the equine conceptus. **Animal Reproduction Science**, v.60-61, p.679-689, 2000.

SILVA, E.S.M.; FRADE, S.; FERREIRA, J.C.; PUOLI FILHO, J.N.P.; MEIRA, C. Effect of interrupting altrenogest treatment in non-cyclic recipient mares on pregnancy maintenance: partial results. **Resúmenes del II Congreso Argentino de Reproducción Equina**, 1º Ed. Editorial Universidad Nacional de Rio Cuarto, p.551-553, 2011.

SOUZA, F.A., CANISSO, I.F. Biological aspects of aging in reducing reproductive efficiency in mares. **Tropical veterinary science**, v. 11, n. 1, p. 9 - 15. 2008.

SPENCER, T.E.; BAZER, F.W. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. **Frontiers in Bioscience**, v.7, pg. 1879-1898, 2002.

SQUIRES, E.L. Progestin. In: MCKINNON, A.O. & VOSS, J.L. Equine **Reproduction.** Cap.33, p. 311-318. Philadelphia: Lea & Febiger. 1993.

TSUKAMURA, H. Neuroendocrine mechanism regulating luteinizing hormone secretion during periovulatory period: Negative and positive feedback actions of estrogen in the brain. **Journal of Reproduction and Development.**, v.44, p.j81-j90, 1998.

URIBE-VELÁSQUEZ, L.F.; OBA, E.; SOUZA, M.I.L. Follicular population and plasma progesterone (P4) concentrations in sheep subjected to different synchronization protocols. **Archives of Veterinary Medicine,** v.40, p.83-88, 2008.

VANDERWALL, D.K. Progesterone. In: MC KINNON, A.O.; SQUIRES, E.L.; VAALA, W.E.; VARNER, D.D. **Equine Reproduction**. 2 Ed. Ames:Blackwell Publishing, 2011, vol.2, cap.170, pg. 1637-1641.

VAUGHAN, J.L.; MACMILLAN, K.L.; ANDERSON, G.A.; D'OCCHIO, M.J. Effects of mating behavior and the ovarian follicular state off emale alpacas on conception. **Australian Veterinary Journal**. n.81 p 86-90. 2003.

ZAVY, M.T.; MAYER, R.; VERNON, M.W.; BAZER, F.W.; SHARP, D.C. An investigation of the uterine luminal environment of non-pregnant and pregnant pony mares. **Journal of Reproduction and Fertility Supplement**, v.27, p. 403-11, 1979.

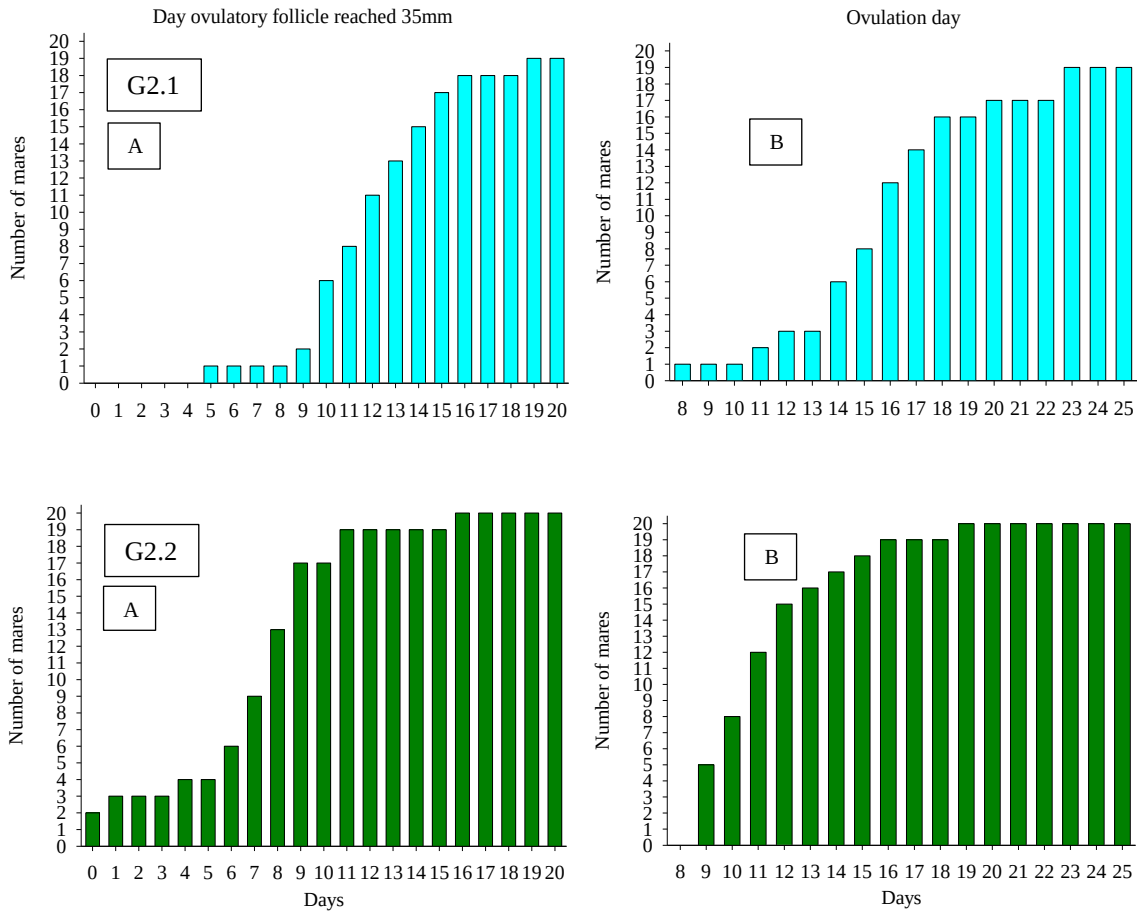


Figure 3.7 Cumulative distribution graph of the day when the ovulatory follicles in groups G2.1 and G2.2 reached 35mm (A; left), and the day when these follicles ovulated (B; right) after the hormonal treatment protocols.

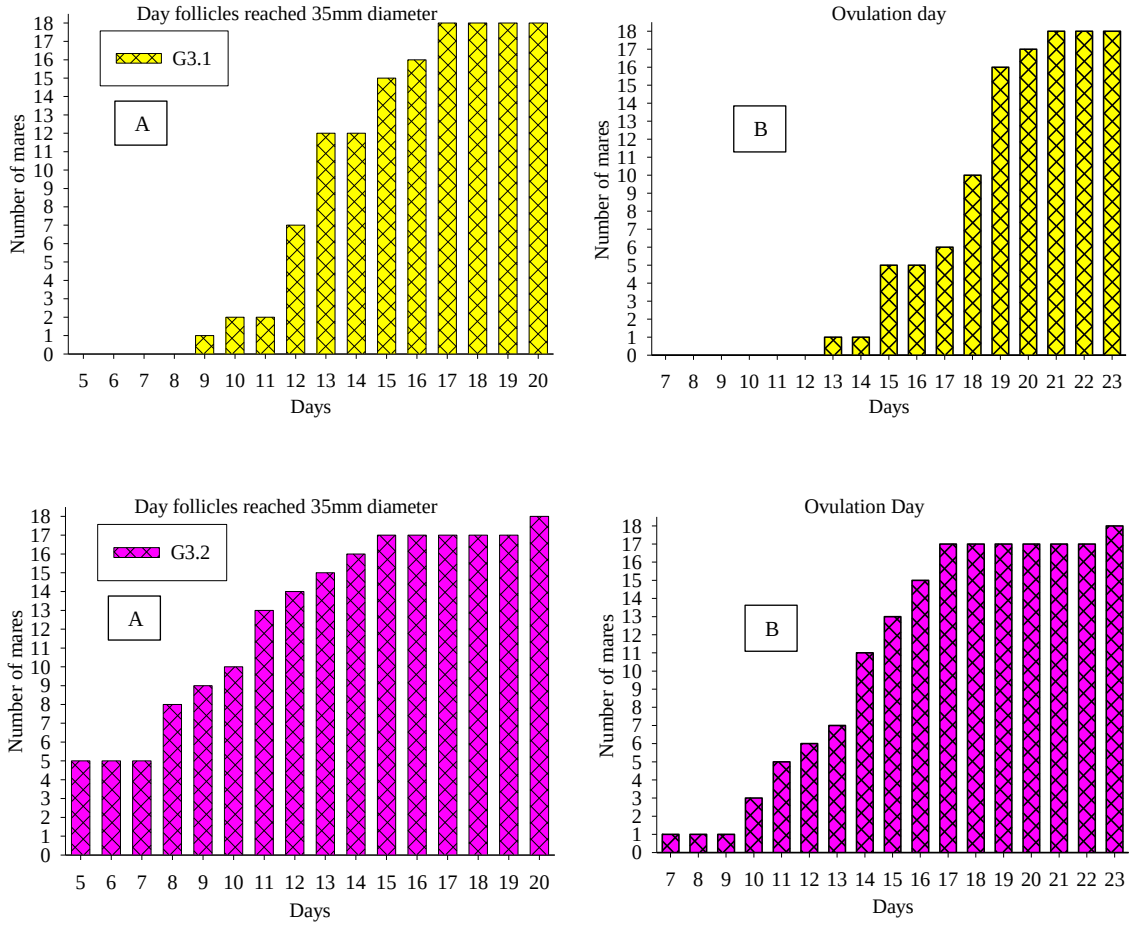


Figure 3.8 Cumulative distribution graph of the day when the ovulatory follicles of groups G3.1 and G3.2 reached 35mm diameter (A; left), and the day when follicles ovulated (B; right) after the hormonal treatment protocols.

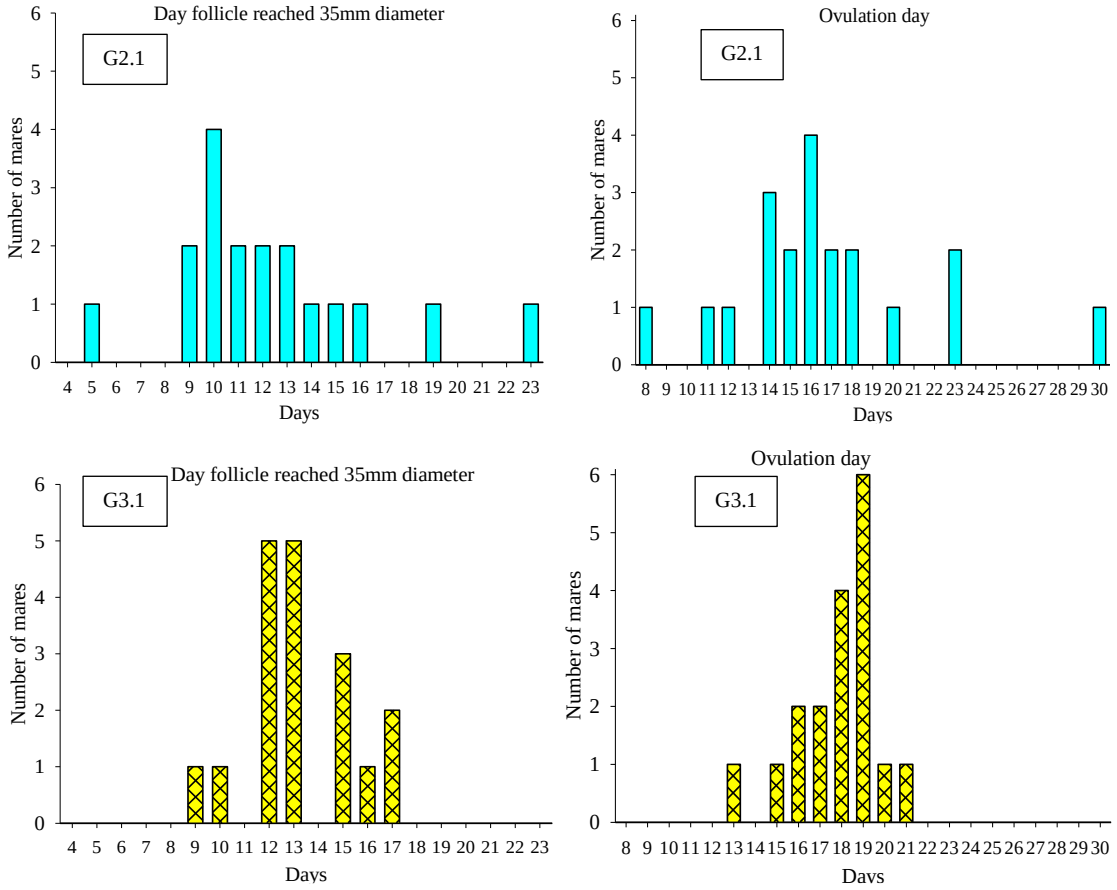


Figure 3.9 Graph of distribution of the days when ovulatory follicles of groups G2.1 and G3.1 reached 35mm diameter, and the day when follicles ovulated spontaneously after the hormonal treatment protocols.

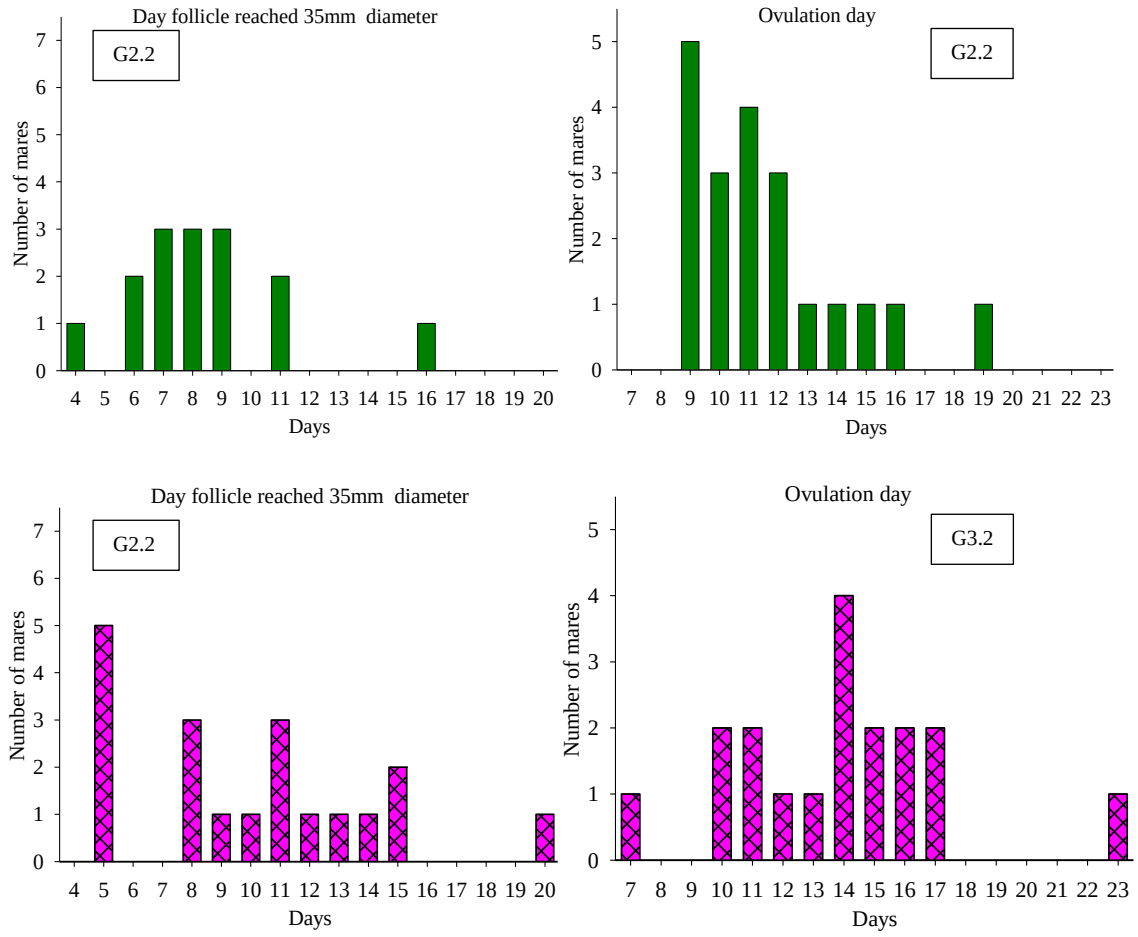
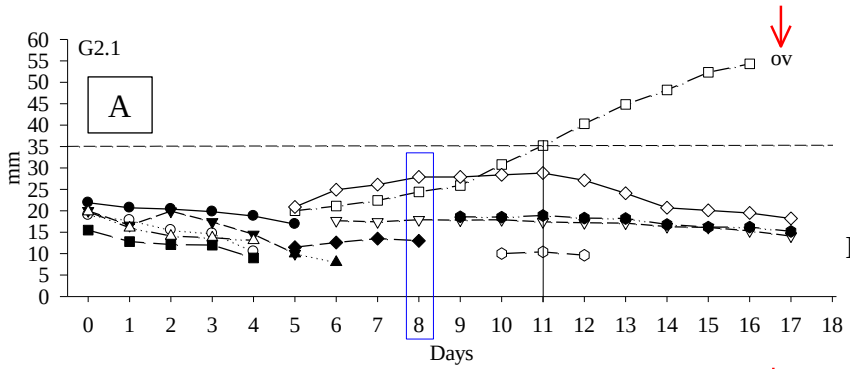
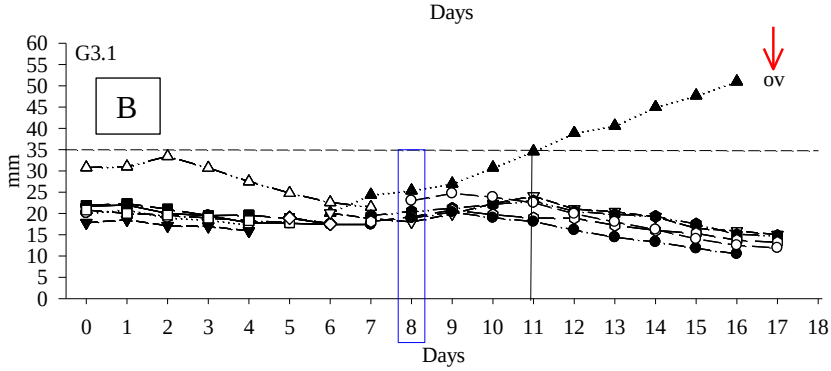


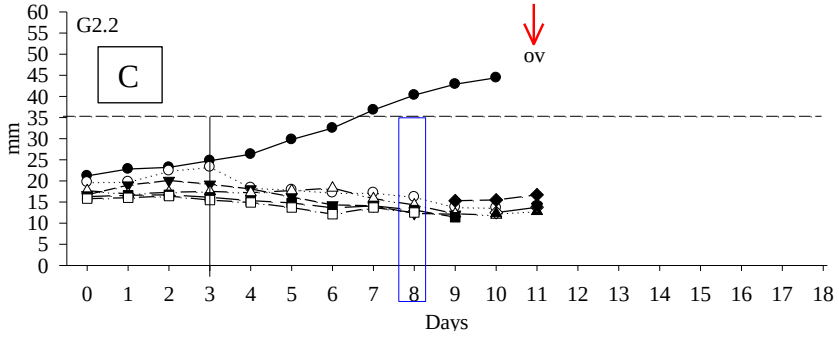
Figure 3.10 Graph of distribution of the day when the ovulatory follicles of groups G2.2 and G3.2 reached 35mm diameter, and the day when follicles ovulated spontaneously after the hormonal treatment protocols.



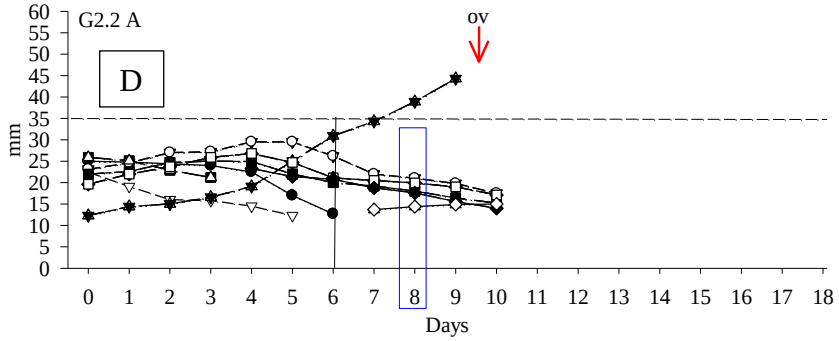
Mare 1



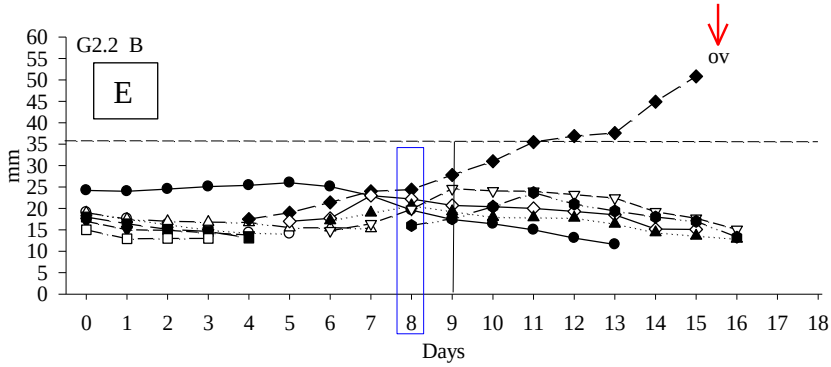
Mare 2



Mare 3



Mare 4



Mare 5

Figure 3.11 Graphic representation of the ultrasonographic follicular dynamics monitoring of mares submitted to G2.1 and G3.1, G2.2 and G3.2 hormonal treatment protocols for comparative purposes among groups. The day the protocol started (D0) corresponded to the eleventh day (D10) of the estrous cycle. The diameters of the three largest follicles in each ovary were measured daily. The blue rectangle indicates the day that P4 intravaginal device was removed. The day when the follicular divergence occurred is indicated by the vertical full line. The moment follicles reached 35mm diameter is indicated by the horizontal dotted line. The red arrow indicates the day of ovulation.

A) Mare 1 - G2.1: the graph exemplifies the effect of the 0.96 g P4 intravaginal device inserted at D0, in association with 17βestradiol, administered at D0, to induce follicular atresia. After the removal of the exogenous source of P4, the ovulatory follicle accelerated its growth rate and ovulated at D17.

B) Mare 2 - G3.1: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, in association with 17βestradiol, administered at D0 and again at D2, to induce follicular atresia. The largest follicle detected at D0 had approximately 30 mm in diameter and continued to grow until D2, but after the second dose of 17βestradiol at D2, the follicle suffered atresia. After the removal of the exogenous source of P4, the ovulatory follicle accelerated its growth rate and ovulated at D17.

C) Mare 3 - G2.2 : the graph exemplifies the effect of the 0.96 g P4 intravaginal device inserted at D0, on follicular growth suppression. After the removal of the exogenous source of P4, ovulation of the pre-ovulatory follicle occurred at D11.

The graphs of mares 4 and 5 represent different examples of follicular dynamics of group G3.2, emphasizing the asynchrony at the time of occurrence of ovulations due to follicular atresia in mare 5 while in mare 4 only occurred suppression of follicular growth.

D) Mare 4 - G3.2 A: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, on follicular growth suppression. After the removal of the exogenous source of P4, a follicle that was already present at the beginning of the protocol (D0) ovulated at D10

E) Mare 5 - G3.2 B: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, on follicular growth suppression.and posterior occurrence of follicular atresia. After removal of the exogenous source of P4, a new follicular wave emerged and ovulation day was delayed compared to mares in which there was no follicular atresia submitted to the same protocol of group G3.2.

CHAPTER 4

4. LUTEAL FUNCTION EVALUATION BY DOPPLER ULTRASONOGRAPHY AND PLASMA PROGESTERONE LEVELS IN MARES SUBMITTED TO HORMONAL TREATMENTS FOR SYNCHRONIZATION OF OVULATION

SUMMARY

The development of hormonal treatments and protocols capable of accurately manipulate the estrous cycle in mares still needs improvement. Moreover, is crucial to investigate the functionality of the corpus luteum (CL) formed after ovulation in mares submitted to those hormonal protocols. In bovine, there is a correlation between the quality of the CL and pregnancy rate. Also, plasma progesterone (P4) concentration still unknown in mares previously treated hormonally to synchronize ovulation. The best tool to evaluate luteal functionality is by determination of the CL's capacity to produce P4. In addition, the Doppler ultrasound examination allows the evaluation of luteal vascularization and can indirectly estimates the luteal function or its ability to produce P4. Based on the exposed, the present study aimed to evaluate the CLs resulting from the first ovulation that occurred after mares were submitted to six different hormonal protocols for estrus and ovulation synchronization. The influence of the different hormonal protocols on luteal function and its correlation with plasma P4 levels were analyzed. The collection of blood samples for plasma P4 analysis and the CLs ultrasonographic data were done eight days after ovulation. The CLs were evaluated by B-mode and Doppler ultrasonography. Then, the areas of luteal vascularization were estimated subjectively by visualization of color signals in the vascularized active portion of the CL. As for the objective analysis of the CLs' vascularization, the process consisted in extraction of images from recorded videos of the color Doppler ultrasound examinations, followed by counting of the colored pixels. Plasma P4 concentrations were analyzed by the radioimmunoassay kit (coat-a-count; DPC). Subsequently, the mean diameter, area, perimeter, objective and subjective analyzes of luteal vascularization and plasma P4 levels were compared among hormonal treatments. Although the morphological and vascularization characteristics of the CL formed after the six different

hormonal treatments did not differ, the average production of P4 was dependent on the hormonal treatment performed for synchronization of ovulation in mares.

Keywords: Doppler ultrasonography, hormonal treatment, ovulation synchronization, mares

INTRODUCTION

The new technologies in the field of equine reproduction contributes to better diagnosis, monitoring and prevention, enabling the improvement of the investigative work on physiological or pathological conditions. The number of studies using Doppler ultrasonography increased at the end of the 90s, but due to equipment costs and the need of a good prior training, the use of this technology remains limited in the equine breeding routine for mares (BOLLWEIN et al., 1998, MARTINOLI et al., 1998, NISWENDER et al., 1994, GINTHER, 2014).

The CL is a transient endocrine gland, which develops from the follicular cells. Follicle blood flow decreases immediately before ovulation, but then gradually increases as the area of CL grows and the concentrations of P4 rise. This gland not only provides steroids precursors, but also releases P4 into the systemic circulation (WEBB et al., 2002). Systemic levels of P4 reach its maximum peak 8 days after ovulation, then these levels of P4 start to decrease in parallel with the decrease of the CL area. However, in the luteolytic period the CL' blood flow decreases more slowly than the P4 systemic concentration (GINTHER, 2007b).

The function of the CL in mares can be estimated by Doppler ultrasonography. Doppler technology is based on frequencies of erythrocyte' echoes, and this frequency rises as the cells approach the transducer and declines when cells move on the opposite direction. The blood supply establishes the CL' functionality and the Doppler examination allows the visualization of its vascular perfusion. By evaluation of the vascular supply the current functional status of the CL can be determined, as well as its future viability (GINTHER, 2014). The color-Doppler mode represents, by different colors, the blood flow direction related to the face of the transducer, and it can be calculated by computer, based on the number of colored pixels and the estimation of the percentage of vascularization of a tissue (GINTHER, 2004).

The B-mode ultrasound enables the evaluation of the CL' size; however, the area and volume of the CL are not safe parameters to determine the production of P4 of this CL (VERONESI et al., 2002). By using color Doppler ultrasonography, the pattern of vascularization of the CL can approximately estimate the P4 production by this gland. Doppler ultrasonography is a non-invasive technique, becoming more accessible nowadays. This technique assesses the vascular function of the CL and correlates it with the P4 levels produced, since the circulating levels of P4 depend on the blood flow of the CL (GINTHER, 2006).

Luteal blood flow is positively correlated with circulating P4 in non-pregnant mares. So Doppler evaluation of CL' vascularization demonstrated to be a reliable tool for prediction of CL function, except during luteal regression (BOLLWEIN et al., 2002; GINTHER et al., 2007). Further, luteal blood flow profiles of pregnant and nonpregnant mares are similar approximately until day 12 of gestation, and after day 16 the differences start to be prominent (ŠICHTAŘ et al., 2013).

For correct evaluation of the ultrasound images of the CL, the operator needs practice to gain experience. For the purpose of performing a high-quality examination, it is necessary to combine knowledge about interpretation of the data as well as the correct configuration of the ultrasound equipment (GOMES, 2008).

Doppler ultrasound involves modalities as the spectral mode, the color-flow and the power-flow mode. For the spectral mode, the evaluation of the characteristics of the blood flow in an area, for example, of a blood vessel, consists in the right placement of the cursor upon the image that represents the lumen of this blood vessel. Blood flow volume (mL/min) can be automatic calculated by the US equipment if the insonation angle related to the vessel, diameter of the vessel, and mean velocity of the flow are known or able to be measured (SILVA, 2009). Color-flow mode focuses directly on the structure of interest and estimated perfusion can be provided by scoring or counting of colored spots of this area. An important advantage of this technique is that scores of perfusions can be done by different operators without knowledge of the source analyzed. Also, colored pixels from the images can be counted by a specific software (SILVA, 2009). Meanwhile, the advantage of the power-flow Doppler mode is the greater sensitivity to low flow, allowing the evaluation of blood vessels presenting slow flow or small diameter (GINTHER & UTT, 2004).

P4 production may be enhanced as a result of optimal CL blood flow in cyclic mares. Throughout the luteal phase, substantial micro vascularization occurs and then, in

the luteolytic period, such vascular perfusion begins to decline until it completely ceases (GINTHER, 2017). Furthermore, cyclic non-pregnant mares seem to experience significant changes of P4 production by the CL from one estrous cycle to another and/or at the end of the breeding season (PANZANI et al., 2017). Another interesting fact can be observed when there is a presence of an ipsilateral dominant follicle (DF) and a CL. Ipsilateral DFs and CLs present mutual positive effects represented by increased perfusion signals on both. A two-way positive effect between ipsilateral DF and CL may be caused by a common ovary arterial branch that supplies both structures. Besides, each structure might produce a substance (P4 or E2) that promotes a positive effect on the other one through a venoarterial pathway (GINTHER, 2019).

Equine reproductive biotechnologies, such as Doppler examination, still has many obstacles. However, the adequate use of these techniques may aggregate valuable information to the reproductive exams, as well as generate more positive results. The present study aimed to evaluate, by Doppler ultrasonography and determination of plasma P4 concentration, the effect of six different hormonal treatments on luteal characteristics on day 8 after ovulation. In order to determine a possible correlation between the luteal function vs. plasma P4 levels in mares submitted to hormonal protocols for synchronization of ovulation, description of the influence of different sources of exogenous P4 with/without 17β estradiol on the CL's function was provided. It was hypothesized that the functional status of the CL varies according to the effects of the six different hormonal treatments performed. Also, the other hypothesis considered that it would be possible, by means of Doppler ultrasonography exam, to estimate the levels of P4 produced by the CL post-hormonal treatments.

MATHERIAL AND METHODS

This study was conducted in accordance with the guidelines for the use of animals in experimentation of CONCEA and in accordance with the regulations of the Ethics Committee for the Use of Animals of the Veterinary Medicine and Animal Sciences School of the University of São Paulo (CEUA-FMVZ-USP No. 3597020317).

The experiment was performed during the months of August 2018 to April 2019 at the Animal Sciences and Food Engineering School, FZEA-USP, Fernando Costa Campus, in Pirassununga - SP, Southeast Region of Brazil, latitude 21.98/21°59'46' S and longitude 47.425/47°25'33'O. Twenty cyclic mares aging from 6 to 14 years-old from the

Laboratory of Theriogenology Dr. O. J. Ginther were used. The mares were kept exclusively on pasture of Mombaça grass (*Panicum sp* var. Mombaça), with water and mineral salt *ad libitum*. The mares received individual and daily protein and energy food supplementation. The body condition score was maintained between 6 and 7, on a scale from 1 to 9 (HENNEKE et al., 1983).

Ultrasonography

B-mode and color-Doppler ultrasonographic data were collected using an Esaote scanner (MyLab30Vet, Italy) equipped with a multi-frequency linear transducer adjusted to 5 MHz. All the equipment settings were maintained the same for all exams.

Initially, transrectal ultrasound exams of the mares were daily performed to confirm the day of ovulation. B-mode and color-Doppler ultrasonography were used to evaluate the CL functionality based on its morphology and vascularization characteristics on day 8 after ovulation. The diameters of the preovulatory follicles (POFs) one day before the day of ovulation and the diameters of the respective CLs on day 8 were recorded and used for posterior analysis. The measurements were automatically calculated by positioning the ultrasound cursor perpendicular to the two largest diameters of the ovarian structure. The mean diameter was then calculated from these two values. POFs and CLs data were analyzed in order to search for individual or treatment-derived variations.

The CL' Doppler scans were recorded on video. The scanning of the CL using color-Doppler was performed by the same observer for one minute across the entire CL area. The CL' vascularization was analyzed subjectively by visual evaluation of the amount of brightness of the colored Doppler signals in the entire CL structure using the recorded videos. In color-Doppler mode, the blood flow in the vessels is indicated by color signs and these signs were used to estimate the percentage of blood flow of the CL (FERREIRA et al., 2011). For subsequent objective analysis of the vascularized area (colored pixels) of the CL, we used color pixel analysis, in which a static image of the recorded videos was selected. This chosen image had to represent the largest cross section of the CL with distinct color areas. The number of colored pixels in the captured CL image was determined by an operator without knowledge of the obtained source. The percentage of colored pixels was calculated using the number of both colored and

uncolored pixels added together. All Doppler examinations were performed using constant ultrasound adjustments.

Experimental design

Twenty cyclic mares were randomly distributed into six experimental groups (G1 to G6) in a 3 x 2 factorial experiment design during the 2018/19 breeding season. All mares were used in all experimental groups and received 0.25 mg of prostaglandin F2α IM on day zero (D0) when treatments started. Three different exogenous sources of progestogen were tested combined or not with 20 mg/animal IM of 17β estradiol (17 Beta®; Botupharma, Brazil; Figure 4.1). The tested progestogen sources and doses were: 0.96 g of P4 intravaginal releasing device (ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health, Brazil), 1500 mg/animal IM of long action P4 (P4-300®; Botupharma, Brazil) and oral altrenogest was daily administered at a dose of 0.045 mg/kg body weight (Botupharma, Brazil).

The first ovulation detected after the hormonal treatments for ovulation synchronization was used as a marker of the Day 0 (D0) for this experiment. Doppler ultrasound examinations were performed to rapidly and non-invasively evaluate the vascularization of the CL and indirectly estimate its functionality. Data collection using Doppler ultrasound for CLs' analysis were performed on D8 and POFs' data were collected one day before ovulation. Ultrasonographic data were compared with the results obtained from the analysis of plasma P4 concentrations. For the analysis of plasma P4 concentrations, ten mares were randomly selected, the samples corresponded to the same ten mares of each group (G1 to G6), a total of sixty plasma samples were sent. All mares participated in all experimental groups (G1 to G6) and a total of 120 cycles were evaluated.

Blood samples were collected on the same day as the Doppler ultrasound examination (D8). All mares randomly participated in all experimental groups (G1 to G6). The samples were collected by jugular venous puncture with a 30x18 needle in a vacutainer system (15 mL vacuolized tubes) and centrifuged for 10 minutes in 13500 G for plasma isolation by pipetting. The blood plasma samples were frozen at -20^{o}C until P4 quantification. Samples from ten mares were randomly chosen and these samples

corresponded to the same mares from each group (G1 to G6), the selection totalized sixty plasma samples to be analyzed. Samples were sent to the Laboratory of Animal Endocrinology - UNESP - Araçatuba for P4 analysis by radioimmunoassay "Kit" (coat-a-count; DPC) technique, previously validated by Garbarino et al. (2004). P4 concentrations were expressed in ng/ml.

STATISTICAL ANALYSIS

Data were examined for normality with the Kolmogorov-Smirnov test. Data that were not normally distributed were transformed to natural logarithms. The end points were analyzed in a factorial 3 x 2 design by ANOVA using the SAS software (version 9.4; SAS Institute, Inc., Cary, NC) to determine the differences between treatments. The identity of the mares was added to statistical model. Paired and/or unpaired Student t-tests were used to locate differences between treatments when significant main effects or an interaction were obtained. Discrete data were analyzed by paired Student t-tests. The proportion data were compared with a chi-square test. A probability of $P \leq 0.05$ indicated a significant difference. All values are shown as the mean ± SEM.

RESULTS

Data obtained by subjective and objective analyses demonstrated that the quality and morphological characteristics of the CL from mares previously submitted to six different hormonal protocols did not differ ($P > 0.05$; groups G1 to G6). No difference was found in both subjective and objective analyses (colored pixels) for the characteristics of CLs vascularization ($P > 0.05$). Diameter, area and perimeter of the CL also revealed no differences among groups ($P > 0.05$) (Table 4.1). All analyses mentioned above were conducted after exclusion of data provided by mares presenting double ovulations.

Plasma levels of P4 produced in mares exhibiting single or double ovulation is presented in Table 4.1. In this study, for unknown reasons, the results of P4 analysis were equally overestimated for all mares. The mean values of P4 from mares that exhibited double ovulations reached a level twice higher (31.6 ng/mL) compared to mares presenting a single ovulation (16.6 ng/mL). Once mares with double ovulations were

excluded, groups G4 and G5 showed the highest values of plasma P4 levels (19.8± 1.7[a], 19.3± 1.5[a], respectively) compared to G1 (13.4± 1.1[b]ng/mL) (P = 0.44; Table 4.2).

In contrast, when all 20 mares/per group were included in the average calculations, groups G3 and G4 presented the highest diameter for the pre-ovulatory follicle (Table 4.1). Therefore, the effect of progestogen treatment was only detected by the increased preovulatory follicle diameter in groups G3 and G4 (47.0 ± 0.9[a], 45.8 ± 1.1[a], respectively; P = 0.977[z], Table 4.2), which used the exogenous P4 (P4-300®; Botupharma, Brazil). In summary, only the G4 showed positive effect of exogenous hormonal treatment using P4, which resulted both in the highest mean preovulatory follicle diameter and the highest mean of plasma P4 concentration.

The average rate of double ovulations was 19,2% (16/120), considering all the followed mares' estrous cycles from the six hormonal protocols (G1 to G6). Meanwhile, the average rate of double ovulations was 10% (6/60) considering only the ten randomly selected mares chosen for the analysis of plasmatic P4.

The percentage of double ovulations per group ranged from zero (0/20) to 20% (4/20). Among ten mares from each group, in G3, not a single mare exhibited double ovulations, whereas in groups G1, G2 and G6 the rate of double ovulations was 10% (2/20%). While groups G4 and G5 presented the higher rate of double ovulations (20%; 4/20).

DISCUSSION

Mares submitted to six different hormonal protocols for synchronization of the emergence of the follicular wave and ovulation were evaluated for purposes of comparison regarding the aspects of functionally of the CL. B-mode and Doppler transrectal ultrasound examinations were performed on the same day (D8) that blood samples for measurements of plasma P4 levels. Data compared the functional status of the CL based on its P4 production and vascularization. The CLs were evaluated eight days after ovulation post-hormonal protocols using different exogenous sources of P4 combined or not with estradiol. In accordance with Alonso (2013), the maximum degree of vascularization of the CL in cyclic mares occurs on the eighth day after ovulation, whether or not the mare is treated with hCG for ovulation induction. Whereas, Ginther et al. (2007) observed that the maximum luteal blood flow occurs on day 10 after ovulation; and Bollwein et al. (2002), when evaluating the estrous cycle of mares older than seven

years, observed that luteal vascularization increases until the seventh day after ovulation, then, it begins to decline.

Plasma levels of P4 were higher in hormonal treatments using an injectable exogenous source of P4 (P4-300®; Botupharma, Brazil) either combined or not with estradiol. The CLs originated eight days after this hormonal treatment (P4-300 with/without 17β estradiol) produced more P4 than groups using other sources of exogenous P4, as an example, the intravaginal device and oral altrenogest. Steroid hormone treatment may have influenced the composition and structure of the CL's cellular architecture. According to each hormonal treatment, a variation may have occurred in the proportion between large and small luteal cells, causing repercussions on P4 production (WITBANK et al., 2012).

Other studies investigated possible effects of hormonal treatments in CL parameters and P4 production (GOMES, 2008; MAIA 2015). The effect of an ovulation inducer agent, the deslorelin acetate, demonstrated no significant correlation among this drug, the CL vascularization and circulating levels of P4 (MAIA 2015). Medeiros et al. (2017) verified by Doppler ultrasonography and analysis of blood circulating P4 that superovulation protocols in mares, in which equine pituitary extract (EPE) was used, there was no effect of EPE treatment on luteal function ($P > 0.05$). Medeiros et al. (2017) compared mares treated or not with EPE, and there was no significant difference in serum levels of P4 (ng/mL): 11.97 ± 2.51; 11.64 ± 3, CL total area (cm^2): 5.24 ± 0.86; 5.91± 2.0, and percentage of vascularization of the CL: 82.66 ± 8.1; 83.16 ± 4, respectively for the control group and EPE group. Nonetheless, another investigation regarding the effect of other ovulation inducer agent, the hCG, may cause an increase in P4 production because its administration for ovulation induction has already been associated with a higher incidence of multiple ovulations (PERKINS and GRIMMET, 2001; VERONESI et al., 2003).

Among the sixty plasma samples analyzed, six (10%, 6/60) were obtained from mares that had double ovulation. Meanwhile, the double ovulation rate increased to 19.2% (16/120) considering all 120 mares' cycles evaluated. These results resemble the double ovulation rates reported by Ginther (1992) and Alonso (2013) in their studies, which ranged from 7 to 25%. It is noteworthy, that several investigations had already described various effects of ovulation inducers as possible contributors for occurrence of multiple ovulations in mares (PERKINS and GRIMMET, 2001; VERONESI et al., 2003). Segabinazzi et al (2015) showed a higher incidence of multiple ovulations in mares when

deslorelin acetate 750 µg or 1650 IU of human chorionic gonadrotophin (hCG) were used as inducers of ovulation in the presence of two or more follicles ≥ 35 mm, or only one follicle ≥ 38 mm diameter were observed. However, in this study there was no possible interference of ovulation inducers in multiple ovulation rates detected, because in this experiment these drugs were not used.

Despite the fact that in this study the morphology and vascularization of the CLs were not correlated with P4 production, attention should be drawn to the existence of other cellular and molecular mechanisms that may influence P4 plasma levels. Data from Hughes et al (2019) suggests a role for lipid mediators during luteal development, as regulators of P4 production and modulation of immune cell function. Lipids may be supportive or deleterious to luteal function and P4 production. Luteal steroidogenic cells are potent activators of T cells and one mechanism of the ability of luteal steroidogenic cells to alter the function of T cells is through production of paracrine lipid mediators. Metabolomic analysis of luteal tissue lipid mediators were used to identify lipid mediators (5-KETE and 15-KETE) as potential key regulators of luteal function and lifespan. Further investigation may elucidate these lipids as paracrine regulators of luteal steroidogenic, endothelial, and immune cell functions.

The concentrations of circulating P4 can be quite different among the studies (HOLTAN et al., 1979; ARRUDA et al., 2001; SOUZA et al., 2006). Arruda et al. (2001) reported a mean value of 4 ng/mL P4 on days 5 to 9 after ovulation. Holtan et al (1979) found approximately 10 ng/mL of P4 remained a constant value from day 5 to 14 after ovulation. Souza et al. (2006) observed higher levels of P4 on day 4 after ovulation in mares receiving hCG to induce ovulation or on day 1 after ovulation. The treated group presented 16.01 ng/mL of P4 and the control group 8.42 ng/mL, but on day 8 post-ovulation the P4 values were similar, with 14.85 and 16.08 ng/mL for treated mares vs. control group, respectively. In this study, P4 plasma concentration presented higher levels than expected, highlighting the highest mean value found was 22.66 ± 2.37ng/mL (P4-300 without 17βestradiol). The highest individual P4 plasma levels were detected in mares from group using altronogest and P4-300, both without 17β estradiol; these mares presented levels of 29.3 and 36.08ng/mL in a single and in double ovulations, respectively. The majority of P4 plasma levels found in treated mares, regardless the hormonal protocol used, exceeded the values reported in the literature (HOLTAN et al., 1979; ARRUDA et al., 2001; SOUZA et al., 2006).

It is known that multiple ovulation rates in mares can vary due to factors including race, age, reproductive status, pharmacological manipulation of the estrous cycle and individual characteristics (GINTHER et al., 1986). The highest incidence of multiple ovulations occurs in certain breeds as Draught horses and Thoroughbreds, reaching rates ranging from 15 to 22%, followed by Trotters, 13 to 15%, Apaloosas and Ponies, 8 to 11%. Mares aging from 2 to 5 years have a double ovulation rate ranging from 13 to 15%, then from 6 to 10 years it reaches 18% and this value increases to 24 to 35.1% when mares are older, 18 to 22 years (DAVIES MOREL et al., 2001; MORRIS et al., 2002). In cyclic mares, the average plasma P4 concentration is 3.77 ng/mL when only one follicle ovulates; also, P4 plasma profile differs if there is a double ovulation, an average 5.35 ng/mL, whether these ovulations occurred in a synchronic or asynchronic way (NAGY et al., 2004). Multiple ovulations may enhance the chance of pregnancy in embryo recipient mares and the probability of twin pregnancies after AI (PIMENTEL et al., 1995). Mares with two or more CLs at the time of embryo transfer (ET) present higher pregnancy rate (88.1%; 89/101; $P \leq 0.05$) than recipients having only one CL (71.5%; 178/249). However, the number of CLs does not affect early embryo loss, with rates ranging from 15.7% vs. 11.2% for embryo recipients with 1 or 2 CLs, respectively (CUERVO-ARANGO et al., 2017).

In this study, all mares that started the hormonal treatments were already cyclic and were randomly submitted to six different hormonal treatment protocols for synchronization of the follicular wave. As a result, these mares were distributed throughout each month of the reproductive season among the years of 2017 and 2018. There are previous studies describing the influence of the breeding season phase on the levels of circulating P4 (OKOLSKI et al., 1980; KING et al., 1988; TOWNSON et al., 1989). These authors agreed there is a progressive seasonal decline in plasmatic P4 in the autumn transition. The decrease in plasma P4 concentrations was described as a consequence of the reduced LH peak amplitude. However, other study showed that LH peak amplitude has no correlation with luteal P4 production, so additional explanations would be necessary concerning the seasonal effect on P4 plasma concentration (BRIANT et al., 2003). In our study, there were more cases of double ovulations in the autumn transition (66%; 4/6). However, due to the irregular ovulations distribution of treated mares during the months of the reproductive season, it was not possible to establish whether there were statistical differences in the P4 levels produced by the CLs throughout different months and transitional periods of the reproductive season.

Cyclic mares presenting high levels of plasmatic P4, similar to plasmatic P4 levels found in this experiment, may exhibit post-insemination fertility much more influenced by the interval between AI and ovulation than by the month of the breeding season in which AI is performed (BRANDÃO, 2003; XAVIER, 2010). Nunes et al. (2004) obtained 91.42%, 90% and 97.14% pregnancies at the 1st, 2nd cycles and at the end of the reproductive season, performing a single cooled semen/AI. In other words, the month of the reproductive season did not affect pregnancy rates. Brandão et al. (2003) performed single/cycle AI with fresh diluted semen and described conception rates of 61.11% (11/18), 84.21% (16/19), 55.56% (5/9), 53.85% (7/13), respectively, with no significant difference between them ($P > 0.05$); in 59 mares inseminated in November, December, January and February/March. In contrast, Valle et al. (2000) observed an effect of the bimester on the rate of conception within the reproductive season, in the first inseminated cycle of mares with cooled diluted semen. The experimental groups of Valle et al. (2000) were composed according to each bimester of the season: October/November, December/January and February/March, following ovulation data, and their results showed, in the same order of the bimesters cited, better conception rate in the first cycle of December/January (42.9%; 70.0%, 28.6%). However, regarding ovulatory follicle size and time of follicle growth, there was no difference among the groups ($P > 0.05$). Xavier et al (2010) findings corroborate Woods et al. (1990) studies, which evaluated the effect of the intervals of insemination/ovulation on fertility of mares inseminated with fresh diluted semen. The intervals were 48 and 24 hours before ovulation, and 48 and 72 hours, between the first and the second inseminations, and both of them concluded that regardless of the insemination/ovulation intervals, the inseminations closer to the time of ovulation are more effective. Besides, when a good follicular dynamics control is performed, the number of AIs/cycles can be reduced, especially when working with induction of ovulation (WOODS et al., 1990; XAVIER et al., 2010).

Panzani et al. (2017) used color Doppler ultrasound to evaluate mares' CLs during breeding season and the CL of the last cycle of the breeding season, from 6 to 18 days after ovulation. Thereafter, CLs analysis were compared to the respective plasma P4 concentration. The CLs parameters analyzed differed significantly ($P < 0.05$) between last cycle of the breeding season and previous cycles during breeding season. However, no significant differences were detected in P4 plasma concentration. The vascularization area of the CL of mares presenting < 1ng/mL of circulating P4 was significantly lower than the CL of mares presenting > 1 ng/mL of P4. In addition, Panzani et al. (2017)

reported mares exhibiting a pixel count of < 3473 pixels were prone to express < 1 ng/ml P4, while mares presenting levels > 1ng/ml P4 exhibited values seven times higher of pixels count.

Ginther et al. (2007) described a positive correlation between blood perfusion and luteal secretory function. The increase in CL vascularization and plasmatic P4 concentration was observed between D1 and D6 after ovulation and luteolysis was characterized by a progressive decrease in CL blood perfusion. However, our data was not able to correlate results obtained from the subjective and the objective analysis of CLs' vascularization with P4 plasma levels. For Ginther et al. (2007), Doppler ultrasonography supports the evaluation of the CL functional status, the selection of embryo recipients and pregnancy management. In contrast, the present study was not able to correlate the high rates of plasma P4 with the morphological and vascularization characteristics of the analyzed CLs. However, in an unexpected way, the effect of progestogen used on hormonal treatments was confirmed on P4 plasma levels and on the average diameter of pre-ovulatory follicle. Although this progestogen effect was not observed in the CLs' analysis. Arruda et al. (2001) verified that P4 plasma concentration does not depend on the size of the luteal gland, though the increase in the morphoechogenicity of the CL is associated with an increase in P4 plasma level. Another relevant aspect to be considered in this investigation was the evidence that there was no correlation between pregnancy rates after ET and the variables evaluated: morphoechogenicity, CL size and circulating P4 levels (ARRUDA et al., 2001).

The results of the present investigation revealed overestimated plasma P4 concentrations in all treated mares, regardless of the hormonal protocol which these mares were previously submitted. These findings suggested that despite overestimated plasma P4 values, supposedly not a single mare would present post-protocol luteal insufficiency, regardless the hormonal treatment performed. Webb et al. (2002) pointed luteal insufficiency and low levels of P4 production (≤ 2ng/ml) as the main causes of infertility and early embryo loss in mammals. However, the mares' CL begins the luteolysis process at day 14 and slowly reduces plasma P4 concentration. In women, the main etiology of the luteal phase defect is the supraphysiologic levels of steroids administration during an artificial cycle, it leads to changes in morphology, molecular phenotypes and endocrine features of granulosa and theca cells of the pre-ovulatory follicle. Prior to the LH surge, Steroidogenic Acute Regulatory protein (StAR) is virtually absent from the human granulosa cells, which are unable to synthesize P4 from cholesterol precursors.

Conversely, StAR is found in high concentrations in the pre ovulatory human theca cells (FATEMI et al., 2007). Moreover, it has been shown that the granulosa cell concentration of mRNA for the StAR, protein involved in transporting cholesterol from the external membrane to the internal membrane of mitochondria, increases after the LH surge in mares. Impaired CL function can be the result of improper development of the dominant follicle destined to become the CL or aberrant stimulation of a normally developed follicle. Both mechanisms result in a CL with deficiencies in P4 production (GERARD and ROBIN, 2019).

Low follicular stimulating hormone (FSH) and/or abnormal LH pulsatility has also been implicated as a potential cause of decreased levels of P4 produced by CL. Decreased P4 secretion by CL was observed in women presenting fixed and increased LH pulse frequency throughout the early follicular phase compared with women with normal luteal function who have an accelerating LH pulse frequency approaching ovulation (MESEN et al., 2015).

The LH has been suggested to increase simultaneously the expression of the encoding genes for StAR protein, P450 side chain splitting enzymes and 3β-deshidrogenase hydroxysteroid (ZIMRI et al, 2018). The CL in the mare is formed from the granulosa cells of the ovulatory follicle, and it is constituted by large and small luteal cells. Large cells produce P4, which acts through their specific cellular receptors (GALVAO et al., 2010). The entrance of the cholesterol into the mitochondria involves the acute steroid regulatory protein (StAR). This process represents a crucial step for steroid synthesis. Pregnenolone leaves the mitochondria and enters the reticulum smooth endoplasmic, where the enzyme 3β-deshydrogenase hydroxysteroid transforms it into P4 (SLOUGH et al., 2011). Furthermore, steroid hormones in follicular fluid dynamically change according to the presence or not of CL in the ovary of mares. Satué et al (2020) found in the follicular fluid of large and medium follicle sizes, E2 concentrations were significantly higher than in non-CL groups. Also, testosterone and androstenedione were significantly increased in larger follicles in the non-CL groups; also, intrafollicular dehydroepiandrosterone (DHEA) was significantly decreased as the follicular diameter increased on both groups. The hormonal treatments performed in this study may have altered LH pulsatity, either during the follicular growth phase in which the ovulatory follicle developed or at the moment of ovulation in which the CL was formed. It is possible that changes may have occurred at cellular level resulting in variations in P4 plasma levels.

Although there was no evidence of correlation between the characteristics of vascularization and morphology of the CL with its P4 production, verified by means of the B-mode and Doppler ultrasonography, other important aspects must be taken into consideration. The first one is the possibility that unknown molecular factors may have caused changes at cellular level, which could have affected the plasma concentrations of P4. Besides, it was not possible to establish whether there were influence in the P4 levels produced by the CLs throughout different months and transitional periods of the breeding season. Finally, the results demonstrated that regardless of the hormonal treatment preconized for synchronization of the emergence of the follicular wave and ovulation, high values of plasmatic P4 were found in all treated mares.

CONCLUSIONS

By performing B-mode and color Doppler ultrasound exams was not possible to estimate the levels of P4 based on the vascularization of the CL, at D8 after ovulation, originated from different hormonal protocols for synchronization of the follicular wave and ovulation in mares.

The functional status of the CL originated eight days post-ovulation, after different hormonal treatments, did not presented different morphology and vascularization characteristics.

Effect of exogenous progestogen treatment was confirmed on P4 plasma concentration produced by CLs, at D8 after ovulation, and on the average diameter of the pre-ovulatory follicle.

High P4 plasma concentration was detected in all treated mares

Table 4.1 Effect of six hormonal treatments on P4 plasma concentration and functional status of CLs formed eight days after ovulation, corresponding to each treatment (G1 to G6). Twenty mares were treated randomly in all hormonal protocols. Ten mares were randomly selected, then blood samples, correspondent to the same ten mares from each group (G1 to G6), were analyzed for measurement of P4 plasma concentrations.

Variable	G1	G2	G3	G4	G5	G6	Value of *P*
P4 plasm. c.	14.99 ± 1.82^{c}	16.77 ± 1.68^{bc}	14.90 ± 1.16^{c}	22.66 ± 2.37^{a}	20.44 ± 1.76^{ab}	19.37 ± 2.62^{abc}	0.0376
Diam POF	45.4 ± 1.3^{ab}	43.9 ± 0.8^{ab}	47.0 ± 0.9^{a}	45.8 ± 1.1^{a}	43.8 ± 1.1^{b}	42.4 ± 0.8^{b}	0.977^{z}
Diam CL	27.7 ± 1.38	26.4 ± 1.28	26 ±1.26	26.4 ± 1.16	27.6 ± 0.86	26.4 ± 1.42	0.939NS
Area CL	6.19 ± 0.62	5.66 ± 0.53	5.47 ±0.46	5.63 ± 0.45	6 ± 0.38	5.9 ± 0.65	0.934NS
CL Perimeter	92.5 ± 4.5	90 ± 4.29	90.3 ±3.96	90.8 ± 3.69	93.1 ± 2.92	90.37 ± 5.08	0.992NS
% vasc CL	53.5 ± 4.1	56.5 ± 3.84	61.2 ± 3.67	56 ± 3.98	54 ± 3.69	55 ± 3.16	0.735NS
Total N. Pixels	11022.8 ± 1373.908	12058.65 ± 1165.483	11735.05 ± 1101.446	12148.4 ± 1509.921	10756.5 ± 942.4482	10414.1 ±898.3151	0.8265NS
Total N. Pixels *X* Intens.	1387785 ± 144434.9	1444676 ± 156370.4	1425389 ± 129865.6	1462031 ± 170132.6	1323221 ± 111448.6	1288940 ±102698.3	0.9193NS
Total N. Pixels *X* Intens. *X* Area	8154813 ± 1036588	8430820 ± 1138242	8128798 ± 953040.6	8496453 ± 1167975	8164226 ± 788871.4	82404412 ± 1310858	0.5896NS

NS: Not significant.

C: concentration, CL: corpus luterum, diam: diameter, Intens: intensity, POF: pre-ovulatory follicle; Vasc: vascularization

[1]Interaction P-values. Upper lower-cases indicates groups differences. [Y]Effect of estrogen treatment was detected ($P < 0.05$). [z]Effect of progestogen treatment was detected ($P < 0.05$)

Table 4.2 Average (A) and StE (standard error) of plasma P4 levels produced by CLs formed eight days after ovulation corresponding to each treatment (G1 to G6). The same twenty mares were treated randomly in all treatments. Ten mares were randomly selected to analyze the plasma P4 concentrations

Animal	G1 P4	G2 P4	G3 P4	G4 P4	G5 P4	G6 P4	Total	*P*-value
Mare 1	15.62	16.68	18.92	14.65	13.39	19.96	·	·
Mare 2	14.81	14.49	8.66	15.29	14.5	11.82	·	·
Mare 3	15.47	11.22	18.27	23.3	20.92	22.03	·	·
Mare 4	28.59*	19.04	16.74	17.08	19.38	14.58	·	·
Mare 5	16.48	21.85	19.78	31.84*	30*	12.4	·	·
Mare 6	13.02	14.44	15.56	36.08*	27.28	29.3	·	·
Mare 7	6.48	15.79	14.79	24.98	23.66	23.98	·	·
Mare 8	17.07	28.23*	13.73	27.68	22.69	35.26*	·	·
Mare 9	12.08	16.49	10.57	14.85	18.08	10.06	·	·
Mare 10	10.28	9.56	12.07	20.91	14.56	14.31	·	·
A StE No*	13.4± 1.1^{b}	15.5± 1.2^{ab}	14.9± 1.1^{ab}	19.8± 1.7^{a}	19.3± 1.5^{a}	17.6± 2.1^{ab}	·	0.044^{z}
A StE Total	14.9± 1.8^{c}	16.7± 1.6^{bc}	14.9± 1.1^{c}	22.6± 2.3^{a}	20.4± 1.7^{ab}	19.3± 2.6^{abc}	·	0.0376
A StE *	28.59	28.23	0	33.96± 2.1	30	35.26	·	·
* rate	10%(1/10)	10%(1/10)	0	20%(2/10)	20%(2/10)	10%(1/10)	10%(6/60)	·

* Double ovulation, M: mean, SE: standard error, P4: progesterone

[1]Interaction P-values. Upper lower-cases indicates groups differences. [Y]Effect of estrogen treatment was detected ($P < 0.05$). [z]Effect of progestogen treatment was detected ($P < 0.05$).

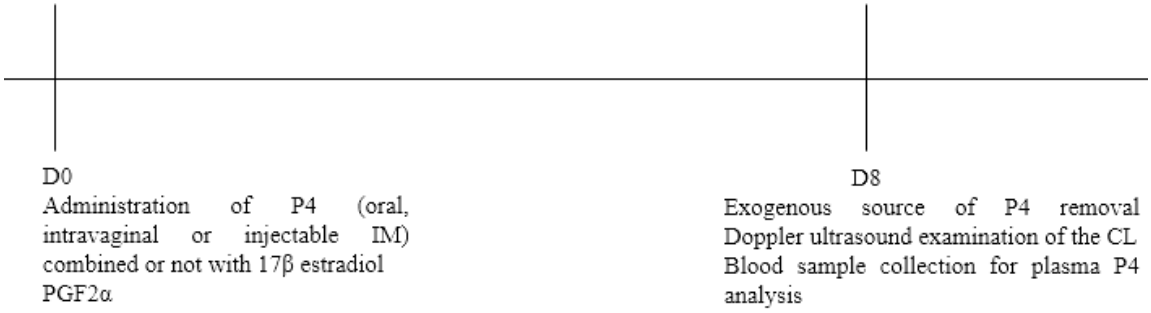


Figure 4.1 Schematic design of the hormonal treatment protocols for synchronization of the follicular wave used in this experiment for luteal function evaluation by Doppler ultrasonography. D0 = start of hormonal treatment. On day eight (D8) post-ovulation used as a reference, the functional status of the CLs were evaluated by transrectal examination, using B-mode and Color Doppler ultrasound. Also, blood samples for plasma P4 analysis were collected on the same moment (D8). The letter G means groups, followed by the number and respective description of one of the six different hormonal treatments performed. Example: G1 means Group 1: the intravaginal devices of 0.96 g of P4 were inserted at D0 and 0.25 mg of prostaglandin F2α IM was administered; D8 – P4 device removal and a new dose of 0.25 mg of prostaglandin F2α IM.

G2: idem G1 with 17β estradiol IM administration at D0.

G3: D0 administration of 1500 mg/animal of P4 IM, 0.25 mg of prostaglandin F2α; D8 - new dose of 0.25 mg of prostaglandin F2α IM.

G4: idem G3 with 17β estradiol IM administration at D0.

G5: D0 0.25 mg prostaglandin F2α IM and oral daily administration of P4 (altrenogest 0.045 mg/kg live weight) over a period of nine days (D0 to D8); D8 – interruption of oral administration of P4 and a new dose of 0.25 mg prostaglandin F2α IM.

G6: idem G5 with 17β estradiol IM administration at D0.

REFERENCES

ALONSO, M.A. **Effect of the application of hCG on different days of the estrous cycle on serum progesterone concentration and uterine and ovarian blood flow in mares.** 188f. Thesis (PhD in Animal Reproduction) - Faculty of Veterinary Medicine and Zootechny, University of São Paulo, São Paulo, SP. 2013.

ARRUDA, R. P.; VISINTIN, J. A.; FLEURY, J. J.; GARCIA, A. R.; CELEGHINI, E. C. C.; NEVES NETO, J. R. Are there correlations between size and morphoecogenicity of the corpus luteum detected by ultrasound and the plasma progesterone levels in equine embryo recipients? **Brazilian. Journal of veterinary Research and Animal Science.** São Paulo, v. 38, n. 5, p. 233-239. 2001.

BOLLWEIN, H.; MAYER, R.; WEBER, F.; STOLLA, R. Luteal blood flow during the estrous cycle in mares. **Theriogenology**, v.57, p.2043-2051, 2002.

BOLLWEIN, H., MAIERL, J., MAYER, R., STOLLA, R. Transrectal color doppler sonography of the A. Uterina in cyclic mares. **Theriogenology,** 49, 1483-1488. 1998.

BRANDÃO, F.Z.; SILVA FILHO, J.M.; PALHARES, M.S. et al. Effect of sperm concentration and number of artificial inseminations on the fertility of mares inseminated with fresh diluted semen. **Brazilian Veterinay Medicine and Zootechny Arquive**. v.55, p.61-67, 2003.

BRIANT, C.; OTTOGALI, M.; MOREL, M.; GUILLAUME, D. Use of GnRH antagonist, antarelix, associated or not with hCG, to control ovulation in cyclic pony mares. **Domestic Animal Endocrinology**. v.53. p.1–18. 2003.

CUERVO-ARANGO J, CLAES AN, RUIJTER-VILLANI M, STOUT TA. Likelihood of pregnancy after embryo transfer is reduced in recipient mares with a short preceding oestrus. **Equine Veterinay Journal**, v.50, n.3, p.386-390, 2017.

DAVIES-MOREL, M.C.G.; O'SULLIVAN, J.A.M. Ovulation rate and distribution in thoroughbred mare, as determined by ultrasonic scanning: the effect of age. **Animal Reproduction Science**, v.21, p.191–12, 2001.

FATEMI, H.M.; POPOVIC-TODOROVIC, B.; PAPANIKOLAOU, E.; DONOSO, P.; DEVROEY, P. An update of luteal phase support in stimulated IVF cycles. **Human Reproduction Update**, Volume 13, Issue 6, Pages 581–590, 2007.

GINTHER, O.J. Ultrasound imaging and reproductive events in the mare. Madison. **EquiServices**. p.377. 1986.

GÉRARD, N.; ROBIN, E. Cellular and Molecular Mechanisms of the Preovulatory Follicle Differenciation and Ovulation: What Do We Know in the Mare Relative to Other Species. **Theriogenology**, v.130, p.163-176, 2019.

GINTHER O.J. Sexual behavior Characteristics of the ovulatory season. In: **Reproductive Biologyof the Mare: Basic and Applied Aspects**, 2nd ed. Wisconsin: Editora Equiservices, 1992.

GINTHER, O. J., & UTT, M. D. Doppler ultrasound in equine reproduction: principles, techniques, and potential**. Journal of Equine Veterinary Science**, 24(12), 516–526. 2004.

GINTHER, O.J., GASTAL, E.L., GASTAL, M.O., UTT, M.D., BEG, M.A. Luteal blood flow and progesterone production in mares. **Animal Reproduction Science**, v.99, n.(1-2), p. 213-220. 2006.

GINTHER, O.J.; GASTAL, E.L.; GASTAL, M.O. et al. Luteal blood flow and progesterone production in mares. **Animal Reproduction Science**., v.99, p.213-220, 2007.

GINTHER, O.J., GASTAL, E.L. & GASTAL, M.O. Spatial relationships between serrated granulosa and vascularity of the preovulatory follicle and developing corpus luteum. **Journal of Equine Veterinary Science**. v.27, p.20-27 2007b.

GINTHER, O. J. How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. **Theriogenology**, 81(1), 112–125. 2014.

GINTHER, O. J. Systemic and intrafollicular components of follicle selection in mares. **Domestic Animal Endocrinology**, 59, 116–133. 2017.

GINTHER, O. J. Intraovarian spatial and vascular harmony between follicles and corpus luteum in monovulatory heifers, mares, and women. **Theriogenology**. v.128, p 31-39. 2019.

GOMES, T.J.S. **Equine corpus luteum vascular evaluation by power Doppler ultrasound.** 66f. Dissertation (Masters in Veterinary Medicine) - Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Lisboa. 2008.

HOLTAN DW, SQUIRES EL, LAPIN DR, GINTHER OJ. Effect of ovariectomy on pregnancy in mares. **Journal of Reproduction and Fertility Suppl,** v.27, p.457-63, 1979.

HUGHES C.H.K; BOSVIEL, R.; NEWMAN, J.W.; PATE, J.L. Luteal Lipids Regulate Progesterone Production and May Modulate Immune Cell Function During the Estrous Cycle and Pregnancy. **Frontiers of Endocrinoly** (Lausanne), v.10:662. 2019.

KING, S.S.; NEQUIN, L.G.; DRAKE, S.; HEBNER, T.S.; ROSER, J.F.; EVANS, J.W. Progesterone levels correlate with impending anoestrus in the mare. **Journal of Equine Veterinay Science**. v. 8. p.109–11. 1988.

MAIA, V.N. **Evaluation of vascularization and angiogenesis of mares' corpus luteum from natural or deslorelin acetate-induced ovulation.** 67f. Thesis (PhD in Tropical Animal Science) - Federal Rural University of Pernambuco UFRPE, Recife, PE. 2015.

MARTINOLI, C., PRETOLESI, F., CRESPI, G., BIANCHI, S., GANDOLFO, N., VALLE, M., DERCHI, L. (1998). Power Doppler sonography: clinical applications. **European Journal of Radiology**, 28, S133-S140, 1998.

MEDEIROS, M.C.R.; FARINASSO, A.; NERY, I.H.A.V.; SOUZA, H.M.; VASCONCELOS, J.L.A. et al. Study of follicular and corpus luteum vascularization of cyclic mares treated with equine pituitary extract using color-Doppler ultrasound. **Brazilian Archive of Veterinary Medicine and Zootechny,** 69 (5), 1089-1096 2017.

MIRO, J. Ovarian Ultrasonography in the Mare. **Reproduction in Domestic Animals**, v.47, p.30–33. 2012.

MORRIS, L.H.A., ALLEN, W.R. Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. **Equine Veterinay Journal**. v. 34, p. 51–60, 2002.

NAGY, P., HUSZENICZA, A.J., REICZIGEL, B.J., JUHÁSZ, M., KULCSÁR, A.K., ABAVÁRY, A.D., GUILLAUME, A.D. Factors affecting plasma progesterone concentration and the retrospective determination of ovulation time in cyclic mares. **Theriogenology**, v. 61, ed. 2–3 , p.203-214, 2004.

NISWENDER, G., JUENGEL, J., MCGUIRE, W., BELFIORE, C., WILTBANK, M. Luteal function: the estrous cycle and early pregnancy. **Biology of Reproduction**, 50, 239-247. 1994.

NUNES, D. B.; ZÚCCARI, C. E. S. N.; FERREIRA, C. S.; DE PAULA, F. A. L.; COSTA-E-SILVA, E. V. Fertility of chilled equine semen in commercial program using single artificial insemination per cycle. In: **Annual Meeting of the Brazilian Zootechnic Society**, 41, 2004, Campo Grande. Annals... Campo Grande. p.3. 2004.

OKOLSKI, A.; BIELAN˜SKI, A.B. Seasonal fluctuations of the plasma progesterone levels in mares with similar ovarian morphology. Int. Cong. **Animal Reproduction Artificial Insemination.** Madrid.;v. 4, p.13–5. 1980.

PANZANI, D., DI VITA, M., LAINÉ, A.-L., GUILLAUME, D., ROTA, A., TESI, M., … CAMILLO, F. 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**, v.56, p.35–39. 2017.

PERKINS NR, GRIMMETT JB. Pregnancy and twinning rates in Thoroughbred mares following administration of human chorionic gonadotrophin (hCG). **New Zealand Veterinary Journal**, v.49, p.94-100, 2001.

PIMENTEL, C. A.; TAROUCO, A. K.; HAMMES, A. M. Multiple ovulations in mares slaughtered in Pelotas-RS. **Rural Science**, Santa Maria, v. 25, n. 2, p. 271-275, May/ago. 1995.

SATUÉ, K.; FAZIO, E.; MEDICA, P. Can the presence of ovarian corpus luteum modify the hormonal composition of follicular fluid in mares? **Animals.** v.1*0*(4), p.646. 2020.

ŠICHTAŘ, J., HOŠKOVÁ, K., ŘEHÁK, D., VOSTRÝ, L., HÄRTLOVÁ, H. The luteal blood flow, area and pixel intensity of corpus luteum, levels of progesterone in pregnant and nonpregnant mares in period of 16 days after ovulation. **Czech Journal of Animal Science**. v.58 (11) :p. 512-519. 2013.

SILVA, L.A. **Uterine and ovarian vascular and architectural changes in equids and bovids; with emphasis on effect of the conceptus.** Dissertation presented to the graduate school of the University of Florida, 2009.

SOUZA, F.A.C.S. **Effects of human chorionic gonadotropin (hCG) on reproductive characteristics of female equines canditated to embryo recipients.** Dissertation (Masters in Animal Reproduction)- Veterinay Medicine and Zootechny School- São Paulo University, Pirassununga, 2006.

TOWNSON, D.H.; PIERSON, R.A.; GINTHER, O.J. Characterization of plasma progesterone concentrations for two distinct luteal morphologies in mares. **Theriogenology** 1989;32:197–204.

TOLGA, B.; STEVEN, L.YOUNG. Progesterone and the Luteal Phase: a requisite to reproduction. **Obstetrics Gynecolocy Clinic of North America.** v.42, n1, p.135–151.2015.

VALLE, G.R.; SILVA FILHO, J.M.; PALHARES, M.S. et al. Effect of the number of artificial inseminations on the fertility of inseminated mares with diluted semen, cooled to 14ºC and transported. **Brazilian Journal of Zootechny**, v.29, p.1721-1726, 2000.

VERONESI, M.C., GABAL, G., BATTOCHIO, M., MOLLO, A., SOLDANO, E., BONO, G., CAIROLI, F. Ultrasonographic appearance of tissue is a better indicator of CL function than CL diameter measurement in dairy cows. **Theriogenology**. v. 58, p.61-68. 2002.

VERONESI, M.C., BATTOCCHIO, M., FAUSTINI, M., GANDINI, M., CAIROLI, F. Relationship between pharmacological induction of estrous and/or ovulation and

twin pregnancy in the Thoroughbred mare. **Domestic Animal Endocrinology**, v.25, p.133-140, 2003.

WILTBANK, M.C.; SALIH, S.M.; ATILI, M.O.; LUO, W.; BORMANN, C.L.; OTTOBRE, J.S.; VEZINA, C.M.; MEHTA, V.; DIAZ, F.J.; TSAI, S.J.; SARTORI, R. Comparison of endocrine and cellular mechanisms regulating the corpus luteum of primates and ruminants. **Animal Reproduction.** v.9(3). p.242–259. 2012.

WOODS, G.L.; BERGFELT, D.R.; GINTHER, O.J. Effect of time of insemination relative to ovulation on pregnancy rate and embryonic loss rate in mares. **Equine Veterinary. Journal**. v.22, n.6, p.410-415, 1990.

XAVIER, I.L.G.S.; SILVA FILHO, J.M.; CARVALHO, G.R. et al. Effects of semen deposition site and insemination/ovulation interval on fertility of inseminated mares with fresh diluted semen. **Brazilian Journal of Zootechny**, v.39, p.512-519, 2010.

ZIMRI, C.V.; FLORES, A.C. Mare Reproductive Cycle: A Literature review. **Veterinary Abanico.** v.8(3): p.14-41. 2018.

CHAPTER 5

5. SYNCHRONIZATION OF ESTRUS AND OVULATION FOR FIXED TIME ARTIFICIAL INSEMINATION IN MARES

SUMMARY

Previous studies from our group have developed efficient hormonal treatments for synchronization of estrus and ovulation in mares. The first goal of this study was based on the investigation of the feasibility of inducing ovulation for artificial insemination (AI) at fixed time in mares. Subsequently, the second goal was focused on the evaluation of fertility rates post hormonal treatments in these mares. Eighteen cyclic mares were randomly assigned to two treatments and all of them were used in both experimental groups. In group 1 (G1), on day 0 (D0) mares received intravaginal devices containing 1.92 g (progestogen) P4, and injections of 0.25 mg of prostaglandinF2α (PGF2α) intramuscular (IM) and 20 mg of 17β estradiol (E2) IM on D0 and D2; on D8 the P4 intravaginal devices were removed and a new 0.25 mg dose of PGF2α IM was administered. Ovulations were induced with the combination of 1000UI of human chorionic gonadotropin (hCG) IM and 750µg of Gonadotropin-releasing hormone (GnRH) IM on D13 after the beginning of the hormonal treatment. In group 2 (G2), on day 0 (D0) mares received intravaginal devices containing 0.96 g P4 and an injection of 0.25 mg of PGF2α IM; on D8 the P4 intravaginal devices were removed, a new 0.25 mg dose of PGF2α IM was administered and ovulations were induced with the same drugs used in G1. Fixed time artificial insemination was performed on both groups 30 hours after induction of ovulation. The dose of fresh diluted semen used for AI contained at least 500 million sperm with progressive motility. Mares were evaluated by transrectal ultrasonographic examination thirteen days after ovulation for pregnancy diagnosis. Pregnancy rates were 83% (15/18) in G1 and 72% (13/18)) in G2, the results presented no significant statistical difference ($P > 0.05$). However, the mean diameter of embryonic vesicles (EVs) thirteen days after ovulation was larger in G1 (13.3 mm ± 0.74), ranging from 9.05 to 16.7mm, compared to G2 (10.5 mm ± 0.21), ranging from 6.8 to 14.8mm ($P < 0.0011$). Follicular atresia and emergence of a new follicular wave which originated the ovulatory follicles was observed in mares from G1. Follicular atresia did not occur in G2 and ovulations were derived from statics follicles that resumed its growth after removal

of the exogenous source of P4. This means that ovulations resulted from different situations post hormonal treatments; in G1 oocytes were originated from a new follicular wave, while in G2 oocytes were generated from follicles which had its growth suppressed by the action of exogenous P4 for nine days. Data have demonstrated the possibility to perform fixed time artificial insemination (FTAI) in mares using both hormonal protocols and achieving satisfactory fertility rates. Furthermore, this study also showed that fertility rate was not affected by neither of the hormonal treatments for synchronization of estrus and ovulation. However, embryonic development may have been compromised when oocytes came from static follicles that resumed its growth after P4 intravaginal device removal.

Keywords: synchronization, mares, hormones, fertility, fixed time artificial insemination

INTRODUCTION

Mares have a very variable period of estrus duration, on average from five to seven days, although it can vary from two to 14 days. Consequently, the prediction of the exact moment of ovulation is a time-consuming and laborious process. For this reason, the use of drugs for ovulation induction has become an extremely important tool in equine reproductive management (BEREZOWSKI et al., 2004). The manipulation of the estrous cycle in mares aims not only to synchronize the estrus, but also to be capable of programing synchronized ovulations. Dispersed occurrence of ovulations in a herd of mares is a challenge to be solved. Ovulations happening unexpectedly and out of schedule are major impediments in the equine reproductive season. Performing fixed time artificial insemination (FTAI) requires synchronization of the emergence of the follicular wave, followed by synchronic development of a pre-ovulatory follicle and ovulation. But this is not enough, the oocyte and the uterine environment of the mare must have the right conditions in order to produce and allow pregnancy maintenance. Attempts to create protocols for synchronization of ovulation in mares have already been described, showing variable results (FANELLI, 2018; GALLELI et al, 2019; CHANG et al., 2019, DOS REIS et al., 2020).

Currently, planning and scheduling of reproductive services consequently results in reduction of labor spent. The correct use of reproductive biotechnologies has facilitated

the routine of equine reproduction. Good examples of these procedures are ovulation induction and, in some cases, follicular ablation; used to suppress the effect of the dominant follicle and to produce a synchronous emergence of a follicular wave (SAMPER, 2008; GALLELI et al, 2019). Association of prostaglandinF2α (PGF2α) and Gonadotropin-releasing hormone (GnRH) analogues were used for synchronization of estrus and ovulation in jennies and showed satisfactory results (FANELLI, 2018). In mares, a hormonal protocol based on progestogen (P4)/ estradiol (E2) in association with gonadorelin acetate, demonstrated to be an effective tool for inducing cyclicity and ovulation, both at breeding and non-breeding seasons. The protocol included an intravaginal device containing 1 g of P4 for ten days. On the day of the P4 device removal also E2 benzoate and PGF2α were administered, followed by ovulation induction. Time of ovulations' occurrence were similar when the protocols were performed in mares at the breeding season (173.1 ± 68.8 h) and non-breeding season (192 ± 58.2 h) (DOS REIS et al., 2020).

The efficiency of ovulation induction in mares is maximized when the drug, the ovulation inducer, is administrated in the presence of a responsive pre-ovulatory follicle, uterine edema and cervical relaxation (McKINNON and McCUE, 2011). The most used hormonal agents available for ovulation induction in the equine industry are human chorionic gonadotropin (hCG) and GnRH analogues including deslorelin, buserelin, histrelin, tryptorelin acetate (FARIAS et al., 2016), gonadorelin, recombinant equine luteinizing hormone (LH) and prostaglandin analogues (TAZAWA et al., 2017). The election of the drug for ovulation induction is usually based on costs. That is the reason why without an understanding of physiology, follicular growth and the mechanisms that promote ovulation, failure in ovulation induction may occur. One of the most common ovulation inducers used in mares is hCG, a glycoprotein hormone produced by the women. The hCG has biological action similar to the endogenous LH, because it binds to LH receptors. hCG causes maturation of the oocyte and ovulation of the dominant follicle, usually on average of 36 to 48 hours after its administration. Also, hCG may have its effectiveness affected by the estrous cycle phase, follicular size and maturity (SAMPER, 2008). Other factors may influence the results of the efficacy of ovulation induction agents using hCG, for example: age of the mare, formation of anti-hCG antibodies, and period of the breeding season (GINTHER et al., 2009). Mares treated with hCG may produce a smaller corpus luteum (CL) due to the reduction in ovulation follicle diameter (URQUIETA et al., 2009). Bergfelt (2000) reported that the size of the CL corresponds

to 65 to 80% of the pre-ovulatory follicle size. According to Urquieta et al. (2009), hCG could have a negative effect on fertility, demonstrated by lower pregnancy rates of 57% when hCG was used as ovulation inducer; compared to 83% in the control group. This fact may be due to the smaller follicular diameter, it also may reflect in oocyte immaturity. Santos et al. (2018) suggested that association of hCG with desloreline may help minimize the problem of decreased fertility rate. The association of hCG with deslorelin as ovulation inducers may enhance oocyte maturation (SANTOS et al., 2018).

In assisted reproduction in women, the combination of hCG and deslorelin acetate induces and synchronizes ovulation effectively. It has been demonstrated that the association of GnRH analogue with hCG promotes positive effects in maturation of the in women (TEMPLETON et al., 1998). Without the use of GnRH analogues, there is an early elevation of LH in approximately 20% of women and, consequently, a lower efficacy of hormonal therapy due to lower oocyte quality and reduced number of embryos for selection. Therefore, in order to avoid the losses of the early LH elevation, hCG has been associated with GnRH to improve the production of oocytes, embryos and to increase the chances of pregnancy (TEMPLETON et al., 1998). Kasum et al. (2016) documented the combination of GnRH and hCG for in vitro fertilization (IVF) in women resulted in significantly higher number of excellent quality embryos and cryopreserved embryos, also better rates of live newborns. This study provided data that supports a prospect of greater chances of pregnancy in women presenting a history of low-income and/or immature oocyte recovery. In mares, satisfactory synchronization of estrus and ovulation will be represented by a large and selected number of mares ovulating within a short and determined period of time. A hormonal protocol that enables ovulation programming will improve the logistics in equine reproduction centers, therefore, FTAI could be implemented and become a reality also for equine species (MCKINNON & MCCUE, 2011). Thus, FTAI will help simplify the process for a proper time control for semen deposition in mares' uterus, especially artificial inseminations (Ais) using frozen semen or AIs performed using stallions presenting low fertility. Besides, susceptible mares with a history of post-breeding endometritis will certainly benefit with reduced number of breeding (TROEDSSON, 2006). The use of frozen semen still has some impediments regarding widespread commercial application. One of the obstacles of this procedure is the need for multiple examinations of the mares to detect the moment of ovulation; this process involves considerable effort and costs. Alternatively, mares' AIs using frozen semen can be performed as follows: two inseminations after ovulation

induction, timed at 24 and 40 hours. Nevertheless, the disadvantages of this technique can be major uterine inflammatory response post-insemination or even waste of semen (AVANZI et al., 2014). More practical FTAI protocols may contribute to spread the use of frozen semen in equine reproduction.

Currently, one interesting target of research is the increased demand for further investigation regarding the comparison of folliculogenesis and reproduction aspects in older women using the mare as an experimental model. These studies may help encourage the use of new reproductive strategies as FTAI for equine species. Also, this research area may help the recognition of the mare's value as a role model for women reproductive studies involving ovulatory follicle development and oocyte fertility in association with systemic and intrafollicular hormonal profiles (GINTHER et al., 2004, GINTHER et al., 2008, CARNEVALE, 2008).

Similarities in physiology and reproductive aspects between the mare and the woman, as follicular wave characteristics, age-associated alterations in endocrine and ovarian function, make the mare a great model to study the effects of maternal aging on reproduction. Assisted reproductive procedures in mares may provide an opportunity to better understand senescence alterations and to explore possible treatments (CARNEVALE et al., 2020).

Hormonal protocols used for synchronization of ovulation and FTAI in mares were able to suppress follicular growth by the action of exogenous P4. Follicles submitted to the action of exogenous P4 presented decelerated growth and were able to ovulate. However, the oocytes originated under these conditions may be susceptible to damages, which may endanger its future potential to develop an embryo (REWAY, 2017). Further investigation regarding these oocytes' quality may help clarify whether these gametes eventually appeared to have aged characteristics post treatments using P4. The unique similarities in reproduction physiology mare vs. women and the findings reported in hormonal protocols created for FTAI in mares could make the mare an excellent research model to investigate possible deleterious effects of maternal aging on the follicle, oocyte and early embryo.

Based on the data exposed, as an attempt to shorter the gap between insemination and ovulation, the main goal regarding manipulation of the mares' estrous cycle must be to establish a narrow window of time scheduled for ovulations to happen in synchrony. The differentiation of follicle cells, the expansion of cumulus cells, oocyte maturation, preparation for ovulation and luteinization, all these events must be coordinated.

Hormonal protocols must mimic the endogenous changes in order to result in fertile oocytes, formation of a functional CL and healthy uterus' conditions to support pregnancy. The objectives of this study were to determine (1) the efficiency of hormonal protocols for estrus and ovulation synchronization (2) the feasibility to perform FTAI in a herd of mares, and to test (3) the fertility of these mares post hormonal treatments. It was hypothesized that mares submitted to hormonal treatments for synchronization of estrus and ovulation would present synchronous ovulations allowing FTAI to be performed. In addition, the oocytes originated post hormonal treatments would be fertile.

MATERIAL AND METHODS

This study was conducted in accordance with the guidelines for the use of animals in experimentation of CONCEA and in accordance with the regulations of the Ethics Committee for the Use of Animals of the Veterinary Medicine and Animal Sciences School of the University of São Paulo (CEUA-FMVZ-USP No. 3597020317).

The experiments were conducted during the months of August 2018 to April 2019 at the Animal Sciences and Food Engineering School, FZEA-USP, Fernando Costa Campus, in Pirassununga - SP, Southeast Region of Brazil, latitude 21.98/21°59'46' S and longitude 47.425/47°25'33'O. Eighteen cyclic mares aging from 6 to 14 years-old from the Laboratory of Theriogenology Dr. O. J. Ginther were used. The mares were kept exclusively on pasture of Mombaça grass (*Panicum sp* var. Mombaça), with water and mineral salt *ad libitum*. The mares received individual and daily protein and energy concentrated maintenance supplementation. The body condition score was maintained between 6 and 7, on a scale from 1 to 9 (HENNEKE et al., 1983).

Ultrasonography

B-mode ultrasonographic data were collected using a Esaote equipment (MyLab30Vet, Italy) equipped with a multi-frequency linear transducer adjusted to 5 MHz. All the equipment settings were maintained the same for all exams.

Experimental design

Eighteen cyclic mares were randomly distributed into two experimental groups (G1 and G2) during the 2018/19 breeding season. All 18 mares were randomly used in both experimental groups. Different doses of exogenous source of P4 (intravaginal device ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health) were tested in association 17β estradiol IM or without the use of 17β estradiol. Mares were submitted to the following treatments: Group 1 (G1), on D0 (beginning of treatment) an intravaginal device containing 1.92 g P4 (ProgestarLeite® Pilot Lot 1; Inovare Biotechnology and Animal Health) was inserted, 0.25 mg of PGF2α and 20 mg 17β estradiol (17 Beta®Botupharma) were injected IM. Another dose of 20 mg 17β estradiol was IM injected on D2. On D8, a new dose of 0.25 mg of PGF2α IM was administered. Ovulation was induced on D13 using a combination of 1mL of hCG IM (1000UI Chorulon®, MSD Agroline) and 3mL of GnRH IM (750μG Sincrorrelin®, OuroFino) in all mares that presented pre-ovulatory follicle with diameter ≥35 mm and uterine echotexture score ≥3 (GINTHER, 1992). Group 2 (G2), on D0 an intravaginal device containing 0.96 g of P4 (ProgestarLeite® Pilot Lot 1; Innovare Biotechnology and Animal Health) was inserted and a 0.25 mg of PGF2α IM was injected. On D8, the P4 intravaginal device was removed, a new dose of 0.25 mg of PGF2α IM administered and ovulation was induced with the same combination and dose of the inducing agents and requirements used in group G1 (Figure 5.1).

Results from previous experiments (Chapters 3 e 4) were used to choose the two hormonal treatments to be tested in this study. This experiment described the efficiency of follicular wave and ovulation synchronization and pregnancy rates in mares in which FTAI was performed.

The hormonal treatments were initiated (D0) in different phases of the estrous cycle of the 18 mares. Mares were randomly distributed in both experimental groups; the beginning of treatment varied from day 0 (day of ovulation) until the 16th day of estrous cycle. The estrous cycle was divided into three periods as follow: D0 to D5, D6 to D10, and D11 to D16. This division into three periods aimed to determine whether the time of the estrous cycle, in which the protocol had initiated, presented any interference regarding the efficiency rates.

The day of ovulation induction needed to differ due to strategic reasons for both experimental groups. This approach was defined based on results from previous experiments (Chapters 3 and 4). Previous studies provided the best day for ovulation induction, revealing the highest number of mares suitable for ovulation induction in each

hormonal treatments. The fertility of oocytes submitted to distinct hormonal conditions was evaluated. The oocytes from G1 originated from a new follicular wave that emerged after the P4 levels declined. In G2, the oocytes originated from ovulations of follicles that already existed at the beginning of the hormonal treatment and remained slowly growing due to the effects of the exogenous P4. In G2, after P4 device removal, follicles were able to resume its growth and ovulate.

In addition, ultrasound monitoring for ovulation detection was performed every six hours to estimate the approximate interval, in hours, from the moment of ovulation induction until the time of ovulation. These monitoring were performed within 48 hours after ovulation induction or until its occurrence. FTAIs were performed in the time-interval as recommended by Loomis and Squires (2005), within 12 hours before ovulation or until six hours after (LOOMIS and SQUIRES, 2005). Therefore, FTAI was scheduled to be performed, using fresh semen, 30 hours post-ovulation induction.

The semen was collected from the same stallion presenting a recent history of good fertility. A simple routine examination post-collection and dilution with commercial semen extenders was performed before each AI. The sperm characteristics of total and progressive motility were classified in percentage (0 to 100%) and vigor (1 to 5) scores (CBRA, 1998). During evaluation of the motility and vigor, samples were taken for concentration measurement. For stallions, the average standard of sperm concentration ranges from 50 to 400 million per mL of ejaculate (LOVE, 2007). All mares were inseminated using fresh diluted semen provided by the same stallion, with a dose containing at least 500 million sperm presenting progressive motility, which is considered a particularly good value for the standards of the species. Each insemination dose was diluted in commercial milk-based medium (Botusemen®; Botupharma). The insemination volume was adjusted to approximately 20 mL. Brinsko (2006) states that most works use 250 to 500 million sperm with progressive motility/dose for AI, presenting no significant difference in pregnancy rates.

The efficiency of the protocols was evaluated according to the percentage of mares that responded to the hormonal treatment presenting the highest degree of ovulation synchronization and positive pregnancy rates.

STATISTICAL ANALYSIS

Embryonic vesicle (EV) diameter was examined for normality with the Kolmogorov-Smirnov test. Paired and/or unpaired Student t-tests were used to locate differences between treatments when significant main effects or an interaction were obtained. The proportion data were compared with a chi-square test. A probability of $P < 0.05$ indicated a significant difference.

RESULTS

Both hormonal protocols (G1 and G2) showed promising results and demonstrated to be efficient for ovulation synchronization, presenting a rate of 66.7% (12/18) and 72.2% (13/18), respectively. In G1 and G2, only five (5/18; 27.8%) and six (6/18; 33.3%) ovulations occurred earlier than expected and were lost, respectively. Examples of follicular dynamics of two mares submitted to hormonal protocols, groups G1 and G2, are illustrated in Figure 5.2, A and B.

The time-interval for ovulations to occur was the same for G1 and G2, from 30 to 48 hours. The approximate time when ovulations occurred after induction, in both groups G1 and G2 is demonstrated in Table 5.1 and Table 5.2, respectively. Estimated time-interval for ovulations that were actually used for FTAI was 18 hours, based on the difference between the first and last ovulations detected. Although, the real time-interval between ovulations occurrence must be considered as 23 hours, reminding the possible variation that ranged from 25 to 48 hours (Figure 5.3), due to ultrasound examinations performed every six hours. On average, both G1 and G2 presented good ovulation synchronization efficiency, but G1 presented numerically a higher efficiency (72.2%; 13/18; Table 5.1) compared to G2 (66.7%; 12/18) (Table 5.2). Efficiency of ovulation synchronization in groups G1 and G2, time of ovulations occurrence, number of lost ovulations obtained from all mares submitted to the FTAI are displayed on Table 5.5 and Table 5.6 (Appendices).

In group G1, when the protocol started in the period from D11 to D16, the efficiency of ovulation synchronization was lower (57.1%; 4/7) and the number of lost ovulations was higher (60%; 3/5) compared to other periods (Table 5.1). Group G2 showed the highest incidence of occurrence of ovulations before expected, between 12 and 18 hours after ovulation induction, when mares started treatment in the period from

D6 to D10. There were four lost ovulations in this period (D6 to D10; 66%; 4/6). Nevertheless, the rate of lost ovulations and the moment when the protocol G2 started (D0), in different periods of the estrous cycle, did not interfered with the results found for the efficiency of ovulation synchronization (G2; 66.7%; 8/12). In addition, most ovulations (55%; 10/18) were detected 42 and 48 hours after the ovulation induction (Table 5.4). The percentage of lost ovulations that occurred before expected in both groups, G1 and G2, are listed on Table 5.7.

The pregnancy rates in G1 were 83% (15/18) and 72% (13/18) in G2 ($P > 0.05$; Table 5.3). However, the mean diameter of the embryonic vesicles thirteen days after ovulation was bigger in G1 (13.3 mm ± 0.74) compared to G2 (10.5 mm ± 0.21; $P < 0.0011$). It is important to note the fact that G2 hormonal treatment was not able to cause follicular atresia and the emergence of a new follicular wave. In G2, the growth of the follicles was suppressed during treatment using P4. Then, the oocytes in G2 were originated from ovulations that resulted from follicles submitted to the effect of a nine-day period of exogenous P4 (0.96g).

In contrast, the hormonal treatment of group G1 induced follicular atresia and the emergence of a new follicular wave. In this group (G1), oocytes derived from ovulations of new follicles that emerged from a new follicular wave. For comparative purposes, Table 5.3 shows the pregnancy rates and diameters of the embryonic vesicles of both groups, G1 and G2. Also, pregnancy rates post FTAI in mares from groups G1 and G2 were compared to the period of time when the hormonal protocols began (D0), in different phases of the estrous cycle (D0-D5; D6-D10; D11-D16) and compared to the time of occurrence of ovulations after induction (Table 5.8).

There was no significant difference in the average diameter of embryonic vesicles (EVs) compared to the estimated time when ovulations occurred and neither compared to the time when the hormonal protocols started (D0) (Table 5.9). Nevertheless, EVs from G2 presented a proportional increase in the average diameter (9.95 ± 1; 10.35 ± 0.67; 11.16 ± 1.53) according to the period in which the hormonal treatment began (D0 to D5; D6 to D10 and D11 to D16, respectively).

The diameter of embryonic vesicles from each mare, detected on D13, on both hormonal protocols G1 and G2, are listed in Table 5.5 and Table 5.6, respectively. Mares' individual data regarding EVs diameters compared to the time when the protocols started, and the moment ovulations occurred also can be verified in Table 5.5 and Table 5.6. In order to better illustrate the initial development of the embryos (D13), Figures 5.4 (A to

H) show the images obtained by transrectal ultrasound B-mode examination of four EVS from G1 and G2.

An important issue to be considered for the successful implementation of FTAI in the equine breeding routine is the financial aspect involving this procedure. Economic viability and estimation of profits will be a critical point for FTAI to be accepted in the field of equine reproduction. The costs incurred to perform the hormonal treatments for FTAI per mare or per herd of mares are listed in Table 5.10 and Table 5.11. The costs were based on a survey, consulting the average market price, conducted from October 28th until November 14th of 2019, in the region of Pirassununga, state of São Paulo. Also, the additional expenses beyond the protocol costs are shown in Table 5.12 (Appendices; AGROLINE, 2019; BRAZIL FREE MARKET, 2019; HORSE CENTER, 2019).

DISCUSSION

This study was designed to test whether it is feasible, practical and economically viable to synchronize estrus and ovulation for fixed time insemination in mares. Another purpose of this experiment was to verify if FTAI was a good strategy to breed mares. The results of the fertility test post-insemination using fresh diluted semen demonstrated that FTAI may be a promising new option for equine reproduction.

In another study conducted by Reway (2017), the mares' response to the follicular wave synchronization protocol had already been proven not to be dependent on the phase of the estrous cycle in which the treatment was initiated. Previous experiments (REWAY, 2017) revealed the ovulation synchronization protocols may be performed in mares at any stage of the estrous cycle, as long as these mares were not in anestrus. The efficiency of the ovulation synchronization protocols starting at different phases of the estrous cycle were tested because on these designated days (5, 10 and 15) the ovarian microenvironment (follicular population and the presence and functionality of CL) may be quite distinct (GINTHER, 1992). Besides of that, on these days (5, 10 and 15), there was a possible distinct effect of the exogenous P4, consequently, it could have caused different degrees of follicular growth suppression. Possibly in the period from D11 to D16 there was a higher incidence of larger diameter follicles and the intravaginal device of 0.96 g P4 could not adequately suppress the growth of these follicles and their respective ovulations, so the ovulations were synchronized after its removal.

Regarding ovarian dynamics during the estrous cycle of the mare, ovulation corresponds to the beginning of the luteal phase (day 0) and the CL originated from this event produces P4 in increased amounts from day 2 to day 10 of the estrous cycle. This secretion remains constant until the twelfth day. Luteolysis occurs between the fourteenth and sixteenth day of the estrous cycle and causes a decline in plasma P4 concentrations (GINTHER, 1992). These events directly interfere with ovarian follicular growth patterns. In accordance with the results described by Reway (2017), this study also proved that ovulation synchronization protocols in mares can be initiated at different moments of the estrous cycle. Both results described demonstrated a good field applicability of these hormonal protocols.

Certain factors may influence pregnancy rate of mares submitted to artificial insemination, including the fertility of the mare and stallion, the type of semen used for insemination (fresh, cooled or frozen), the number of sperm/insemination dose, the time of semen storage before artificial insemination (SIEME et al., 2003). In this case, by using FTAI protocols in mares, important factors that may affect pregnancy rates are: synchronization and time of occurrence of ovulations, and also the fertility of oocytes. After FTAI, pregnancies diagnoses were performed by transrectal ultrasound thirteen days (D13) after ovulation detection in both groups (G1 and G2) to compare the fertility of the oocytes that had been submitted to the different hormonal treatment protocols.

The fertility test allowed the comparison of the efficiency of two elected hormonal treatments and verified the best results in artificial insemination at fixed time, by considering the following aspects: (1) Follicular atresia induction and synchronous emergence of a new follicular wave, (2) Greater time precision for ovulation induction in a short period of time, (3) Number of lost ovulations; occurred before predicted, and (4) Pregnancy rate at first artificial insemination/cycle.

The time when there was a higher incidence of ovulation is important to be evaluated because this time influences the election of the type of semen preservation used for FTAI in mares. The closer to ovulation the semen is deposited in the reproductive tract of the female, the greater are the chances of fertilization (HAFEZ, 2004). If AI is performed after ovulation, oocyte viability will be the major factor influencing the success or not of fertilization. Due to degenerative oocyte alterations from eight to ten hours after ovulation, it is recommendable to inseminate only until six hours after ovulation (WOODS et al., 1990). A process of nuclear degeneration followed by imperfect pairing of the chromosomes in the metaphyseal plate begins 12 hours after oocyte release, and

the fertilization of this oocyte might lead the embryo to death (HUNTER, 1990), or birth of animals with imperfect chromosomal complements. Consequently, artificial insemination needs to be performed at the appropriate time, ensuring both male and female gametes are viable by the time of fertilization (JACOB et al., 2000). FTAI was performed in all mares of both groups 30 hours after the induction of ovulations using hCG in association with deslorelin. Due to the viability period of the oocytes after ovulation, we considered that ovulations occurring 12 and 18 hours after induction were lost. All mares that ovulated between 12 and 18 hours, in G1 and G2, were not inseminated in that cycle. The lost ovulations were used as reference to start again the same protocol of hormonal treatment performed previously. All mares were submitted to single insemination at a fixed time per cycle and all mares participated of both hormonal protocols.

The spermatozoa are viable for fertilization in the reproductive tract of the mare for approximately 24 hours after insemination with cooled semen, or 48 to 72 hours after AI with fresh, fresh/diluted semen or natural cover (WATSON and NIKOLAKOPOULOS, 1996). Sieme et al. (2003) consider longevity of fresh semen in the genital system of equine females varies from 24 hours until seven days, with significant variations among stallions. The freezing process damages spermatic cell viability, reduces and decreases longevity and also impairs its fertilization capacity (BRINSKO and VARNER, 1993). According to Sieme et al. (2003) frozen semen viability is approximately 12 hours in the reproductive tract of the mare, and pregnancy rates increase from 30.8 to 61.9% by adding an AI six hours after ovulation instead of performing a single insemination within 12 to 24 hours pre-ovulation.

It can be speculated the possibility of using frozen semen combined with ovulation synchronization protocols of groups G1 or G2. In this example, artificial insemination would no longer be at a fixed time, instead, a group of mares would be selected for breeding purposes in a determined period. A group of cyclic mares would be submitted to the elected protocol (G1 or G2) and after induction of ovulations, AI with frozen semen in a larger number of mares would be possible within a time interval of 12 to 48 hours, reducing loss rates since ovulations identified at 12 and 18 hours would no longer be lost. This would require transrectal palpation and/or ultrasound examination for ovulations detection every six hours, and once ovulation was detected, then AI would be performed (MILLER, 2008). The serial examination program when using frozen semen is due to short-term sperm viability from six to 12 hours (VANDERWALL, 1997). Alternatively,

synchronized ovulations of groups G1 and G2 could be compatible with a two programmed inseminations protocol using frozen semen (Figure 5.5) (LOOMIS and SQUIRES, 2005). This protocol suggests the first AI must be performed 24 hours after ovulation induction, and a second insemination 12 hours after the first, this way no continuous monitoring is needed to detect ovulation and all mares are inseminated 12 hours before or until six hours after ovulation (LOOMIS and SQUIRES, 2005). Fertility rates in AIs/cycle using frozen semen on average vary from 30 to 40% (SAMPER et. al., 2001).

Due to ovulations distribution within 30 to 48 hours, fresh or cooled semen would be more appropriate for FTAI, based on the estimated viability time of equine male and female gametes. In this manner, FTAI performed 30 hours after ovulation induction would enable use of all ovulations occurring within 24 to 48 hours after induction (Figure 5.6). The programmed FTAI at hour 30 of the protocol would inseminate all mares until six hours post-ovulation or until 24 hours pre-ovulation. According to Ball (1998), AI must be performed within 24 hours prior to ovulation, because longer intervals leads to reduced fertility rates. In the event of working with a rare or high valuable semen, an alternative to use our protocols for FTAI is the addition of transrectal palpation or ultrasound examination 24 hours after ovulation induction, therefore, no mares ovulating before expected time would be inseminated.

Considering pregnancy diagnosis was performed thirteen days after ovulation detection, some embryonic vesicles in group G1 had a significantly smaller diameter than the reported average in the literature. The smallest EVs diameters observed were: 6.80, 7.30 and 8.95 mm. Hills (2011) investigated equine embryo initial growth and identified the following values: 3.64; 7.34; 10.43; 15.73mm from days 10 to 13, respectively. By comparing Hills' findings (2011) to our results in this experiment, one can notice some EVs in group G1 presented approximately a two-day delay in embryonic development and also, EVs diameters on D13 were similar to adequate size EVs on D11 of gestation. Moreover, regardless of the period the protocol was initiated in G1, the average EV diameters on D13 were smaller than the average of 15 mm reported in the literature (HILLS, 2011).

Results of this study indicated that fertility, evaluated thirteen days after ovulation, was not adversely affected by hormonal treatments. Groups G1 and G2 presented high fertilization rates after FTAI, despite the fact that G2 oocytes resulted from ovulatory follicles that were already present at the beginning of treatment (D0), and oocytes from

ovulatory follicles which originated when exogenous P4 concentrations were declining in G1. The initial embryonic development, therefore, could have been compromised when the ovulation came from static follicles that resumed their growth in G2, and the diameter of these EVs were similar to the EVs' diameter of mares aged ≥ 20 years (CUERVO-ARANGO et al., 2018). Several factors are discussed to clarify the variation in embryo diameter, for example, the exact interval between ovulation and flushing, the age of the mare, oocyte quality, oviductal and uterine environment and the use of frozen vs. fresh semen. All these factors play important roles in the variation in embryo diameter, development and size. According to Cuervo-Arango et al. (2018) young mares tended to produce larger embryos than older mares. In addition, Gutierrez-Adan et al. (2001) reported that bovine male embryos develop and grow faster than female embryos under in vitro production and culture conditions. In group G2, we have considered the possibility that follicles ovulated oocytes presenting aged characteristics. If there was no exogenous P4, ovulatory follicle growth would be faster and ovulation would have occurred a few days earlier. Studies evaluating senility effects on oocyte fertility are conducted in mares over 20 years of age used for reproductive purposes (DAVIES-MOREL, 2003; WILSON, 2007; CARNEVALE, 2008). There is a significant decrease in the number of viable foals born as the age of the reproducer increases, this occurs because in a 20-year-old mare, oocytes were formed during fetal life, therefore, these oocytes have a similar age (McDOWELL, 2008).

Currently, there are still no studies describing how exogenous P4 influences follicular growth delay, follicular fluid characteristics of the ovulatory follicle, and the possible P4 interference in cellular and molecular characteristics that might affect the oocytes quality and fertility. However, this study proved that the exogenous P4 effect from the intravaginal device containing 0.96 g did not adversely affected oocyte fertility.

Frequently, mares over 20 years of age are maintained for breeding purposes due to previous athletic performance and/or generation of superior quality foals, mainly used as donors in ET programs (LOSINNO and ALVARENGA, 2006; CARNEVALE, 2008). Reproductive senility is characterized by a decline in fertility. In mares, there is a low quality oocyte production caused by advanced age, which contributes to major decrease in fertility. In addition, a significant decrease in birth rates and viable foals occurs, particularly because of bivalent chromosome non-disjunction and premature separation of sister chromatids, associated with the occurrence of aneuploidy. Furthermore, senile oocytes may be compromised during meiosis, causing an imbalance in chromosomal

segregation. Chromosomal balancing error can lead to failure in fertilization, implantation, and embryonic loss (SOUZA and CANISSO, 2008). In old mares, oocytes remained for years in the prophase stage of the first meiotic division in the ovary, until activation of the second meiosis. In equines, the element that triggers the oocyte maturation resumption has not been clarified yet. Maturation and ovulation occur when the germinal vesicle is broken and a secondary oocyte is released in the metaphase stage of the second meiosis. After fecundation, the secondary oocyte concludes the second meiosis (HAFEZ, 2004; CURCIO et al., 2006). In old mares, prolonged exposure of oocytes to reduced ovarian pH may affect oocyte metabolism and interfere with fuse formation and follicular fluid characteristics (EICHENLAUB-RITTER, 1998). There are some morphological aspects of aged oocytes, as the presence of more vacuoles or vesicles in their cytoplasm due to the larger area occupied by endoplasmic reticulum, smaller area occupied by cortical granules and greater chromatin condensation, all signs of increased degenerative defects (ARMSTRONG, 2001). A new study, to evaluate follicular fluid of ovulatory follicles and morphology of the oocytes submitted to hormonal protocols for FTAI in mares, may contribute and provide additional information to help understanding the findings of this research.

The smaller EVs size in G2 suggested a developmental delay, and a similar problem may also be observed in embryos from old mares. In ET collections, between days 6 to 8 after ovulation, in embryos smaller than normal for their age, morphological abnormalities were detected, resulting in lower pregnancy rates after ET (SQUIRES et al., 1993). Carnevale (2006) noted three days after ovulation, embryos collected from oviducts of old mares had fewer cells and more morphological abnormalities than embryos of young mares, indicating 36 hours after ovulation, a developmental delay can already be detected in embryos from old mares. In our study, regardless of treatment performed for ovulation synchronization (G1 or G2), and different EVs sizes detected post-FTAI, fertility was not compromised until D13.

In another comparative study between oocytes from old mares and fillies, it was demonstrated that oocyte morphological differences may be associated with anaeuploidy, and oocytes from old mares may exhibit delayed metaphase II maturation (AGUIAR et al, 2002). According to Brinsko et al. (1995), lower pregnancy rates in old mares can result from the fertilization of oocytes without adequate meiotic maturation, because these oocytes require a longer period of time to mature until the second metaphase, when compared to oocytes from young mares. Additionally, aged oocytes may present a

mitochondrial DNA dysfunction. Mitochondrial damage can generate a decreased ATP production and an excessive presence of free radicals, responsible for damages in the mitochondrial oocyte membrane. (SOUZA and CANISSO, 2008). The formation of the meiotic and mitotic fuse and chromosomal segregation are active processes dependent on the energy generated by mitochondria, and the poor mitochondrial function increases the incidence of chromosomal abnormalities in embryos of old mares. In our study, there was no investigation about the presence of any type of genetic or mitochondrial abnormality in G1 or G2 embryos, the only noticeable alteration was related to EV morphology visualized by B-mode ultrasound: a reduced diameter of EVs in group G2 on the 13th day of gestation.

During oocyte growth and maturation, and at the time of ovulation, mitochondrial replication occurs, generating a finite mitochondrial reserve to be used in the first cell divisions of the embryo. Therefore, the initial development of the embryo is dependent on the satisfactory functionality of the mitochondria generated by the oocyte, because a new mitochondrial replication will not occur until later in the gastrulation phase (RAMBAGS et al., 2006). In this case, it is important to note that in G2, oocytes were under the influence of exogenous P4 during its growth and at the time of ovulation induction (D8). So, the delayed follicular growth could have generated oocyte alterations without causing damage to fecundation, but somehow negatively affected initial growth rate and development of the embryo. In group G1, due to follicular atresia and the emergence of a new follicular wave, ovulatory follicles grew when P4 plasma levels were decreasing. For this reason, in group G1, both ovulation induction at D13 and oocyte maturation occurred not influenced by exogenous P4, which means, under different conditions from G2, and possibly allowed a better initial development of the embryo and larger diameter of the EVs.

The prolonged estrus period in mares can reaches seven days, therefore, a delayed fertilization after ovulation can cause a premature meiosis resumption (HUNTER, 1990). Premature activation of the maturation factor (MPF) and the protein kinase A mitogen (MAPK) promotes a precocious oocyte maturation, in other words, this activation generates a mature oocyte before fertilization and compromises its quality (ANDERSON et al., 2009). In women submitted to hormonal therapy to conceive, the association of GnRH analogues with hCG promotes beneficial effects by preventing premature oocyte maturation (TEMPLETON et al., 1998). Without the use of GnRH analogues, there is an increase in LH and activation of oocyte maturation promoters prematurely, compromising

oocyte quality. In human IVF and in equine FTAI protocols (G1 and G2), a combination of deslorelin and hCG was used as an attempt to mimic natural oocyte maturation, to produce better quality embryos and higher pregnancy rates. (KASUM et al., 2016, REWAY, 2017).

Data acquired from this study, after FTAI in mares, revealed that the hormonal treatment used in group G1 may serve as a base for studies using old mares as senescence models for women in assisted reproduction. There are several similarities in age-related reproductive modifications, that would make the mare a valid model for human reproduction studies. Besides, the necessary time for ovulation to occur, after induction of oocyte maturation in vivo, is similar in mares and women; this may suggest a sequence of events that could be compared (CARNEVALE, 2008).

As age progresses, the mare presents cyclical and hormonal changes similar to those experienced by women. The first signs of reproductive senescence in both species are the shortening of the estrous cycle with high FSH concentrations. After, the cycles become longer with intermittent ovulations and high FSH and LH concentrations. Finally, in age-associated infertility there is follicular growth failure and poor oocyte quality in both species (CARNEVALE, 2008). Therefore, a more detailed analysis of oocyte quality and initial embryonic development, using G2 protocol as a basis, possibly will provide beneficial results for researches on age-related changes in both mares and women reproduction biotechnologies.

CONCLUSIONS

Hormonal protocols were able to synchronize ovulation in order to proceed artificial inseminations at fixed-time in mares.

The oocytes of mares submitted to hormonal treatments for synchronization of ovulation were fertile and the mares presented good pregnancy rates at thirteen days of gestation.

The embryonic vesicles derived from the fertilization of oocytes originated from follicles that suffered suppression of its growth due to the action of exogenous P4, resulted in smaller EVs diameters compared to EVs derived from the fertilization of oocytes that did not suffer the effects of P4.

Table 5.1 – Efficiency of ovulation synchronization and time of occurrence of ovulations in group G1 compared to the time the hormonal protocol started (D0) in different periods of the estrous cycle. In G1, number of mares = 18 mares; number of cycles = 23, due to six lost ovulations that occurred before expected (12 and 18 hours).

Prot. start (D0)	12h	18h	24h	30h	36h	42h	48h	Number of protocols/period	Lost ov.	Total cycles	Efficiency of ov. sync.
D0-D5	0	0	0	0	0	0	2/23	2	0 (0/2)	2	100%(2/2)
D6-D10	1/23	1/23	0	2/23	1/23	4/23	2/23	9	22.2%(2/9)	11	77.8%(7/9) 81.8%(9/11)
D11-D16	1/23	2/23	0	3/23	0	3/23	1/23	7	42.8%(3/7)	10	57.1%(4/7) 70%(7/10)
Total/cycle	8.7%(2/23)	13.1%(3/23)	0	21.7%(5/23)	4.4%(1/23)	30.4%(7/23)	21.7%(5/23)	23	21.7%(5/23)	23	78.3%(18/23)
Total/mare	11.1%(2/18)	16.7%(3/18)	0	11.1%(2/18)	0	33.3%(6/18)	27.8%(5/18)	18	27.8%(5/18)	18	72.2%(13/18)

Prot: protocol ov: ovulation sync: synchronization

Table 5.2 – Efficiency of ovulation synchronization and time of occurrence of ovulations in group G2 compared to the time the hormonal protocol started (D0) in different periods of the estrous cycle. In G2, number of mares = 18 mares; number of cycles = 24, due to six lost ovulations that occurred before expected (12 and 18 hours).

	Time of occurence of ovulations										
Prot. start (D0)	12h	18h	24h	30h	36h	42h	48h	Number of protocols/period	Lost ov.	Total cycles	Efficiency of ov. sync.
D0-D5	0	0	0	2/24	0	0	0	2	0 (0/2)	2	100% (2/2)
D6-D10	3/24	1/24	0	2/24	1/24	6/24	3/24	12	16.7%(4/24)	16	75%(12/16) 66.7%(8/12)
D11-D16	0	2/24	0	0	0	0	4/24	4	8.2%(2/24)	6	66.7%(4/6) 50%(2/4)
Total/cycle	12.5%(3/24)	12.5%(3/24)	0	16.7%(4/24)	4.1%(1/24)	25%(6/24)	29.2%(7/24)	24	25%(6/24)	24	75%(18/24)
Total/mare	16.7%(3/18)	16.7%(3/18)	0	11.1%(2/18)	0	33.3%(6/18)	22.2%(4/18)	18	33.3%(6/18)	18	66.7%(12/18)

Prot: protocol ov: ovulation sync: synchronization

Table 5.3 Pregnancy rate, average and standard error of embryonic vesicles diameter detected thirteen days after induction of ovulation in groups G1 and G2.

Groups	Pregnancy rate	Embryo Vesicle diameter D13
G1	83% (15/18)	13.3 mm ± 0.74 (9.05 -16.7mm)
G2	72% (13/18)	10.5 mm ± 0.21 (6.8 – 14.8mm)
P-value	> 0.05	< 0.0011

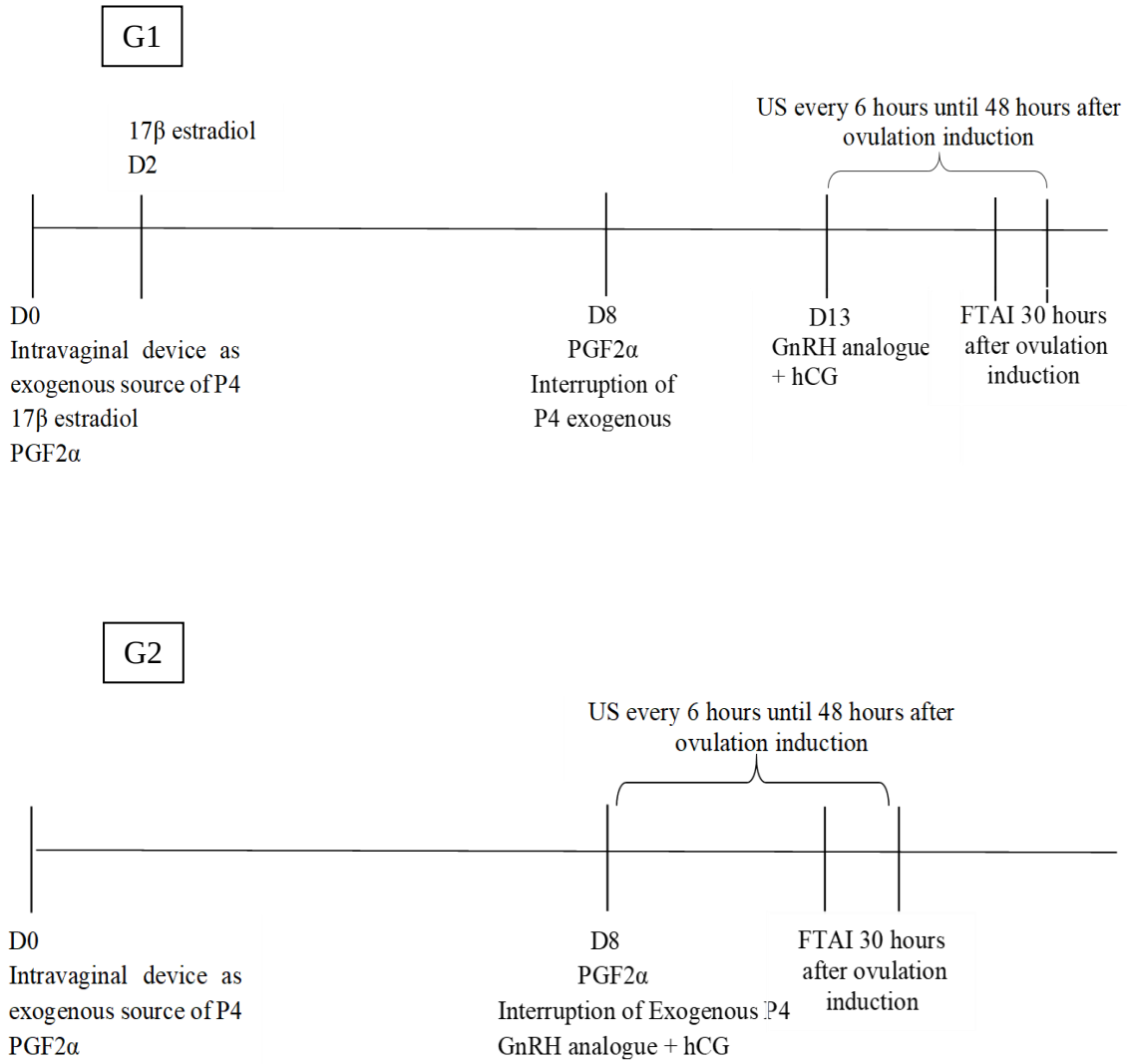


Figure 5.1: Schematic design of the hormonal protocols for follicular wave and ovulation synchronization in experiment 5. Eighteen mares were randomly distributed after ovulation detection (ovulation = day 0 of the estrous cycle). The hormonal treatment started from days 0 to 16 (D0 to D16) of the estrous cycle (D0 = day the treatment started). Ultrasound monitoring for ovulation detection was performed every 6 hours from the moment of ovulation induction, and until a 48 hour-period or until ovulation detection. FTAI were performed 30 hours after ovulation induction. US = ultrasound, FTAI = fixed time artificial insemination.

REFERENCES

AGROLINE. **Agricultural products.** Available at: https://www.agroline.com.br/produto/progestar-monodose-pct-10-unidades-boehringer-ingelheim-106830. Accessed on: October 28th. 2019.

AGUIAR, J. J.; LOSINNO, L.; KONCURAT, M. Nuclear, cytoplasmic and mitochondrial patterns of ovulated oocytes in young and mares. **Theriogenology**. v. 58, n. 2-4, p. 689-692, 2002.

ANDERSON, R.A., SCIORIO, R., KINNELL, H., BAYNE, R.A., THONG, K.J., DE SOUSA, P.A., and Pickering, S. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. **Reproduction.**; 138: 629–637. 2009.

ARMSTRONG, D. T. Effects of maternal age on oocyte developmental competence. **Theriogenology**, Amsterdam, v. 55, n. 6, p.1303-1322, 2001.

BALL, B.A. Evaluation and use of transported equine semen. In: Equine Assisted Reproductive Technology Workshop, 1998, Davis. **Proceedings…** Davis. p.18-24. 1998.

BEREZOWSKI, C.J., STITCH, K.L., WENDT, K.M., VEST D.J. Clinical comparison of 3 products available to hasten ovulation in cyclic mares. **Journal of Equine Veterinary Science**, v. 24, n.6, p. 231-233, 2004.

BERGFELT, D.R. Synchronization of estrus in the mare. In: **Equine Management and Artificial Insemination** . Philadelphia: Saunders, p.195-228.2000.

BERGFELT, D.R.; ADAMS, G.P. Ovulation and corpus luteum development. In: SAMPER, J.C.; PYCOCK, J.F.; McKINNON, A.O. **Current therapy in equine reproduction.** Hardcover: Sanders Elsevier, p. 1-13, 2007.

BRAZIL FREE MARKET. **Latin American online buying and selling community.** Available at: https://produto.mercadolivre.com.br/MLB-1172949492-ouro-fino-sincrorrelin-gnrh-_JM?quantity=1. Accessed on: October 28th. 2019.

BRINSKO, S.P.; VARNER, D.D. Artificial insemination. In: McKINNON, A.O.; VOSS, J.L. (Eds.) **Equine reproduction**. Philadelphia: Williams & Wilkins. p. 790-797. 1993.

BRINSKO, S.P.; BALL, B.A.; ELLINGTON, J.E.In vitro maturation of equine oocytes obtained from different age groups of sexually mature mares. **Theriogenology**. v. 44, n. 4, p. 461-469, 1995.

BRINSKO, S. P. Insemination doses: how low can we go? **Theriogenology,** v. 66, n. 3, p. 543-550, 2006.

CAMPBELL M. It‟s all in the timing: ovulation induction in the mare. **Veterinary Records.**, v.170, p.538-539, 2012.

CARNEVALE, E.M., COUTINHO DA SILVA, M.A., PANZANI, D., STOKES, J.E., SQUIRES E.L. Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. **Theriogenology**.n.64, p.519–27. 2005.

CARNEVALE, E.M. Reproductive Disorders. In: **Equine Geriatric Medicine and Surgery**. p. 193-200. 2006.

CARNEVALE, E.M. The mare model for follicular maturation and reproductive aging in the woman. **Theriogenology**, 69:23-30. 2008.

CARNEVALE, E. M.; CATANDI, G.D.; FRESA, K. Equine aging and the oocyte: a potential model for reproductive aging in women. **Journal of Equine Veterinary Science**, v.89, 103-122. 2020.

CBRA COLÉGIO BRASILEIRO DE REPRODUÇÃO ANIMAL. **Manual para exame andrológico e avaliação do sêmen animal**. 2. ed. Belo Horizonte: CBRA. p. 49. 1998.

CHANG, Z.; LIU, B.; YAO, L.; JIANG, G.M.; YU, J.; JING, H.T. Effects of FSH and the weather during induced ovulation and timed artificial insemination to increase jenny conception rates. **Science of Reproduction**. n. 9. 3220. 2019.

CUERVO-ARANGO, J.,;CLAES, A.; STOUT, T. A. E. Horse embryo diameter is influenced by the embryonic age but not by the type of semen used to inseminate donor mares. **Theriogenology**, 115, 90–93. 2018.

CURCIO, B.R.; LEON, P.M.M.; FREY JUNIOR, F.; NOGUEIRA, E.W.; DESCHAMPS, J.C. Equines: oogenesis, folliculogenesis and maturation. **Brazilian Journal of Animal Reproduction.** v.30, n.1/2, p.28-35. 2006.

DAVIES-MOREL, M.C.G. **Equine reproductive physiology breeding and stud management**. Wallingford: CABI Publishing, 374 p. 2003.

DOS REIS, W. V. A.; DE BRAGA, D.; JÚNIOR, M. V.; GHELLER, J. M.; DE DECO-SOUZA, T.; DA COSTA E SILVA, E. V.; MACEDO, G. G. P4/E2-based protocol for synchronisation of ovulation of mares during the breeding and non-breeding season. **Tropical Animal Health and Production.** v.52, p.2647–2651, 2020.

EICHENLAUB-RITTER, U. Genetics of oocyte ageing. **Maturitas**, Amsterdam, v. 30, n. 2, pp. 143-169, 1998.

FANELLI, D.; PANZANI, D.; ROTA, A.; SALARI, F.; MARTINI, M.; CAMILLO, F.A. New, Simplified 251 Protocol for Timed Artificial Insemination in Donkeys. **Journal of Equine Veterinary Science**, v. 66, p. 128-129, 2018.

FARIAS, L. D. et al. Induction of ovulation in mares: a review. **Brazilian Animal Reproduction Journal**, v.40, n.1, p.17-21. 2016.

FISSORE, R. A. et al. Mechanisms underlying oocyte activation and postovulatory ageing. **Reproduction**, London, v. 124, n. 6, p. 745-754, 2002.

GALLELLI, M.F.; BIANCHI, C.; TRASORRASA, V.; ZAMPINIA, E.; ABA, M.; MIRAGAYA, M. **Animal Reproduction Science**. n.208 p.105-106. 2019.

GINTHER O.J. Sexual behavior Characteristics of the ovulatory season. In: **Reproductive Biologyof the Mare: Basic and Applied Aspects**, 2nd ed. Wisconsin: Editora Equiservices, 1992.

GINTHER OJ, GASTAL EL, GASTAL MO, BERGFELT DR, BAERWALD AR, PIERSON RA. Comparative study of the dynamics of follicular waves in mares and women. **Biology of Reproduction,** 71:1195-1201. 2004.

GINTHER OJ, GASTAL MO, GASTAL EL, JACOB JC, BEG MA. Age-related dynamics of follicles and hormones during an induced ovulatory follicular wave in mares. **Theriogenology.** 2008.

GINTHER, O.J.; BEG, M.A.; GASTAL, E.L.; GASTAL, M.O.; COOPER, D.A. Treatment with human chorionic gonadotropin (hCG) for ovulation induction is associated with an immediate 17_-estradiol decrease and a more rapid LH increase in mares. **Animal Reproduction Science**, v. 114, p. 311–317, 2009.

GUTIERREZ-ADAN, A.; GRANADOS, J.; PINTADO, B.; DE LA FUENTE, J. Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. **Reproduction Fertility and Development**. v.13, p.361-365. 2001.

HAFEZ, E.S.E. Gametes transport and survival. In: Hafez, ESE. **Animal reproduction.** 7 ed. Barueri: Manole, 2004. chap.6, p.83-96.

HENNEKE, D.R.; POTTER, G.D.; KREIDER, J.L. AND YEATES, B.F. 1983. Relationship between condition score, physical measurements and body fat percentage in mares. **Equine Veterinary Journal,** 15: 371- 372. 1983.

HILLS, B. Equine ultrasound pregnancy images., In: **Equine reproduction**, 2nd 575 edn., 576 Eds: A.O. McKinnon, E.L. Squires, W.E. Vaala, and D.D. Varner, Ames, Iowa, USA. 577 Blackwell Publishing. 2011.

HORSE CENTER. **Veterinary Pharmacy.** Available at: https://www.farmaciahorsecenter.com.br/8-reproducao/17-946-estradiol-injetavel. Accessed on: October 28th. 2019.

HUNTER, R.H.F. Gamete lifespan in the mare's genital tract. Editorials. **Equine Veterinay Journal**, v.22, n.6, p.378- 379, 1990.

JACOB, J.C.F.; ALBUQUERQUE, F.T.; DOMINGUES, M.B. et al. Gestation rate in mares Mangalarga Marchador inseminated post-ovulation. **Brazilian Journal of Animal Reproduction**, v.24, n.1, p,51-55, 2000.

KASUM, M., KURDIJA, K., ORESKOVIC, S., CEHIC, E., PAVICIC-BALDANI, D., SKRGATIC, L. Combined ovulation triggering with GnRH agonist and hCG in IVF patients. **Journal Gynecological Endocrinology,** v. 32, n. 11. 2016.

LOOMIS, P.R.; SQUIRES, E.L. Frozen semen management in equine breeding programs. **Theriogenology**, v. 64, p. /480-491, 2005.

LOSINNO, L.; ALVARENGA, M.A. Critical factors in equine embryo transfer programs in Brazil and Argentina. **Acta Scientiae Veterinariae,** Porto Alegre, v.34 (Suppl1), p. 39- 49, 2006.

LOVE, C. C. Reproductive examination of the stallion: evaluation of potential breeding soundness. In: YOUNGQUIST, R. S.; THARELFALL, W. R. Current therapy in large animal. **Theriogenology.** 2nd. ed. Saint Louis: Elsevier-Saunders, 2007. p. 10-14.

McCUE, P.M., MAGEE, C., GEE, E.K. Comparison of compounded deslorelin and hCG for i nduction of ovulation in mares. **Journal of Equine Veterinary Science**, v.27, p.58-61, 2007.

McDOWELL, K. Factors affecting reproductive success in broodmares. Alberta Canada government department: **Agriculture and Rural**. p.52-6. 2008.

McKINNON, A.O., McCUE, P.M. Induction of ovulation, In "**Equine reproduction**", 2nd 575 edn., 576 Eds: A.O. McKinnon, E.L. Squires, W.E. Vaala, and D.D. Varner, Ames, Iowa, USA. 577 Blackwell Publishing; p.1858–1866. 2011.

MEYERS-BROWN, G.A.; McCUE, P.M.; TROEDSSON, M.H.T.; KLEIN, C.; ZENT, W.; FERRIS, R.A.. Induction of ovulation in seasonally anestrous mares under ambient lights using recombinant equine FSH (reFSH). **Theriogenology** v.80, p.456-462, 2013.

MEYERS-BROWN, G.A.; LOUD, M.C.; HYLAND, J.C.; ROSER, J.F. Deep anestrous mares under natural photoperiod treated with recombinant equine FSH (reFSH) and LH (reLH) have fertile ovulations and become pregnant. **Theriogenology.** v.98, p.108-115, 2017.

MILLER, C. D. Optimizing the use of frozen–thawed equine semen. **Theriogenology**, v.70. n.3, p.463–468.2008.

NEWCOMBE, J.R., CUERVO-ARANGO, J. Comparison of the efficacy of different single doses of buserelin with hCG for timed ovulation induction in the mare. **Animal Reproduction Science**, v 41, p.57, 2016.

RAMBAGS, B. P. B.; VAN BOXTEL, D. C. J.; THARASANIT, T. et al. Maturation in vitro leads to mitochondrial degeneration in oocytes recovered from aged but not young mares. **Animal Reproduction Science**. v. 94, n. 1-4, p. 359-361, 2006.

REWAY, A.P. **Development of a follicular wave synchronization protocol and determination of the ideal moment for ovulation induction in equine species**. Dissertation (Master's Degree in the Program of Domestic and Wild Animal Anatomy). University of São Paulo, FMVZ USP. São Paulo - SP, 2017.

SAMPER J.C. Management and fertility of mares bred with frozen semen. **Animal Reproduction Science.** v.68, p.219-228, 2001.

SAMPER, J.C., JENSEN, S., SERGENAT, J. Timing of induction of ovulation in mares treated with ovuplant or chorulon. **Journal of Equine Veterinary Science**, v.22, p.320-323, 2002.

SAMPER, J.C. Induction of estrus and ovulation: why some mares respond and others do not.**Theriogenology**, v.70, n.3, p.445–447. 2008.

SANTOS, R.S., MARCHIORI, M.O., BRUM, C., AMARAL, L.A., TORRES, A.J., BOFF, A.L.N., WALNUT, C.E.W. Effectiveness of Fertirel Acetate as an Ovulation Inducer in Mares. In: **35th Brazilian Congress of Veterinary Medicine**, 2008, Gramado. 35th Combravet, 2008.

SANTOS, G.O.; BASTOS, H.B.A.; SÁ, M.A.F.; JACOB, J.C.F.; WOLF, C.A.; MATTOS, R.C.; NEVES, A.P. Combination of hCG and Deslorelin Acetate on the Induction of Ovulation in Mares: Changes in Follicular Fluid Protein Profile. **Journal of Equine Veterinay Science**, 66, p.124-125, 2018.

SERITCH, P. Pregnancy Evaluation in the mare. In: SAMPER, J.C.; PYCOCK, J.F.; McKINNON, A.O. **Current therapy in equine reproduction.** Hardcover: Sanders Elsevier, p. 99-106, 2007.

SIEME, H.; SCHAFER, T.; STOUT, T.A. et al. The effects of different insemination regimens on fertility in mares. **Theriogenology**, v.60, n.6, p.1153-1164, 2003.

SINCLAIR, C.D., KOUBA, J.M., MURRAY, L.W., DOUTHIT, T.L., GRIEGER, D.M., WEBEL, S.K. Use of intravaginal triptorelin gel for ovulation induction in mares**. Journal of Equine Veterinay Science**, v.35, p.437-438, 2015.

SQUIRES, E.L. Progestin. In: MCKINNON, A.O. & VOSS, J.L. Equine **Reproduction.** Cap.33, p. 311-318. Philadelphia: Lea & Febiger. 1993.

SOUZA, F.A., CANISSO, I.F. Biological aspects of aging in reducing reproductive efficiency in mares. **Tropical veterinary science**, v. 11, n. 1, p. 9 - 15. 2008

TAZAWA SP, GASTAL MO, SILVA LA, EVANS MJ, GASTAL EL, Preovulatory Follicle Dynamics, Ovulatory and Endometrial Responses to Different Doses of hCG, and Prediction of Ovulation in Mares, **Journal of Equine Veterinary Science**. 2017.

TEMPLETON A, MORRIS JK. Reducing the risk of multiple births by transfer of two embryos after in vitro fertilization. **New England Journal Medicine**, v. 339, p.573–577, 1998.

TROEDSSON MHT, DESVOUSGES A, HENSEN PJ, BUHI WC. Equine seminal plasma proteins protect live spermatozoa from PMN-binding and phagocytosis, while providing a mechanism for selective sperm elimination of apoptotic and dead spermatozoa. **Animal Reproduction Science**, v.94, p.60-61, 2006.

URQUIETA, B., DURA,M.C. , COLOMA, I., PARRAGUEZ,V.H. hCG-Induced Ovulation in Thoroughbred Mares Does Not Affect Corpus luteum Development and Function During Early Pregnancy. **Reproduction in Domestic Animals**, n.44, p.859–864, 2009.

VANDERWALL, D.K. The challenges using frozen sêmen in horse breeding. In: Annual Conference of the society for theriogenology. Ottawa: Society for theriogenology. **Proceedings...** p. 178-79. 1997.

WATSON, E.D., NIKOLAKOPOULOS, E. Sperm longevity in the mare's uterus. **Journal of Equine Veterinary Science**, v.16, p.390-392, 1996.

WILSON, J. **Care of elderly horses**. University of Minnesota: Extension Services, Minneapolis, MN (www.extension.umn.edu/horse), 2007.

WOODS, G.L.; BERGFELT, D.R.; GINTHER, O.J. Effect of time of insemination relative to ovulation on pregnancy rate and embryonic loss rate in mares. **Equine Veterinary. Journal**. v.22, n.6, p.410-415, 1990.

YUAN, Y. Q. et al. Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. **Theriogenology**. v. 63, n. 8, p. 2147-2163, 2005.

Table 5.4 – Time of occurrence of ovulations after ovulation induction (time 0), using hCG + GnRH analogue, compared to the time when ovulations were detected for the first time by transrectal ultrasound scan.

	Lost Ovulations: 12 and 18h						
Groups	12h	18h	24h	30h	36h	42h	48h
G1 Total/number of mares	11.1% (2/18)	16.7% (3/18)	0	11.1% (2/18)	0	33.3% (6/18)	27.8% (5/18)
Total/cycles	8.7% (2/23)	13.1% (3/23)	0	21.7% (5/23)	4.4% (1/23)	30.4% (7/23)	21.7% (5/23)
G2 Total/number of mares	16.7% (3/18)	16.7% (3/18)	0	11.1% (2/18)	0	33.3% (6/18)	22.2% (4/18)
Total/cycles	12.5% (3/24)	12.5% (3/24)	0	16.7% (4/24)	4.1% (1/24)	25% (6/24)	29.2% (7/24)
Total/ number of mares	13.9% (5/36)	16.7% (6/36)	0	11.1% (4/36)	0	33.3% (12/36)	25% (9/36)
Total/cycles	10.6% (5/47)	12.8% (6/47)	0	19.1% (9/47)	4.2% (2/47)	27.7% (13/47)	25.6% (12/47)

H: hour

Table 5.5 - Efficiency of ovulation synchronization in group G1 compared to the beginning of the hormonal protocol (D0) at different phases of estrous cycle. Time of ovulations occurrence in 18 mares and 23 cycles. In group G1, due to five lost ovulations occurring before the expected time (12 and 18h), total number of cycles was 23, in order to submit all mares to FTAI.

	Protocol start		Lost ov.									
Animal	1 D0	2 D0	Yes	Time	No	24h	30h	36h	42h	48h	N. protoc.	Ov. synch. efficiency
Mare 1	D6	D6	X	18h	.	.	.	X	.	.	2	50%
Mare 2	D9	D8	X	12h	.	.	X	.	.	.	2	50%
Mare 3	D12	D13	X	18h	.	.	X	.	.	.	2	50%
Mare 4	D13	D12	X	18h	.	.	.	.	X	.	2	50%
Mare 5	D15	D12	X	12h	.	.	X	.	.	.	2	50%
Mare 6	D4	.	.	.	X	.	.	.	.	X	1	100%
Mare 7	D5	.	.	.	X	.	.	.	.	X	1	100%
Mare 8	D6	.	.	.	X	.	.	.	.	X	1	100%
Mare 9	D7	.	.	.	X	.	.	.	X		1	100%
Mare 10	D8	.	.	.	X	.	X	.	.	.	1	100%
Mare 11	D8	.	.	.	X	.	.	.	X	.	1	100%
Mare 12	D8	.	.	.	X	.	.	.	.	X	1	100%
Mare 13	D10	.	.	.	X	.	.	.	X	.	1	100%
Mare 14	D10	.	.	.	X	.	.	.	X	.	1	100%
Mare 15	D11	.	.	.	X	.	.	.	X	.	1	100%
Mare 16	D12	.	.	.	X	.	X	.	.	.	1	100%
Mare 17	D12	.	.	.	X	.	.	.	.	X	1	100%
Mare 18	D15	.	.	.	X	.	.	.	X	.	1	100%
D0-D5	2	0	2/2	0	2/2	0	0	0	0	2/23	2	100% (2/2)
D6-D10	9	2	2/9	1/12h;1/18h	7/9	0	2/23	1/23	4/23	2/23	11	81.8% (9/11)
D11-D16	7	3	3/7	1/12h;2/18h	4/7	0	3/23	0	3/23	1/23	10	70% (7/10)
Total/cycle	23	5	5/23	2/12h;3/18h	18/23	0	5/23	1/23	7/23	5/23	23	78.3% (18/23)
Total/mare	18	0	5/18	2/12h;3/18h	13/18	0	5/18	1/18	6/18	5/18	18	72.2%(13/18)

D: day, N: number, Ov: ovulation, Protoc: protocols, Synch: synchronization

Table 5.6 - Efficiency of ovulation synchronization in group G2 compared to the beginning of the hormonal protocol (D0) in different phases of estrous cycle. Time of ovulations occurrence in 18 mares and 24 cycles. In group G2, due to six lost ovulations occurring before expected time (12 and 18h), total number of cycles was 24, in order to submit all mares to FTAI.

	Protocol start		Lost ov.								Ov. synchr efficiency
Animal	1 D0	2 D0	Yes	Time	No	24h	30h	36h	42h	48h	N.protoc.	
Mare 1	D8	D10	X	12h	.	.	.	.	.	X	2	50%
Mare 2	D9	D8	X	18h	.	.	X	.	.	.	2	50%
Mare 3	D10	D9	X	12h	.	.	X	.	.	.	2	50%
Mare 4	D10	D11	X	12h	.	.	.	.	.	X	2	50%
Mare 5	D12	D12	X	18h	.	.	.	.	.	X	2	50%
Mare 6	D13	D12	X	18h	.	.	.	.	.	X	2	50%
Mare 7	D3	.	.	.	X	.	X	.	.	.	1	100%
Mare 8	D5	.	.	.	X	.	X	.	.	.	1	100%
Mare 9	D6	.	.	.	X	.	.	.	X	.	1	100%
Mare 10	D9	.	.	.	X	.	.	.	X	.	1	100%
Mare 11	D9	.	.	.	X	.	.	.	X	.	1	100%
Mare 12	D10	.	.	.	X	.	.	X	.	.	1	100%
Mare 13	D10	.	.	.	X	.	.	.	X	.	1	100%
Mare 14	D10	.	.	.	X	.	.	.	X	.	1	100%
Mare 15	D10	.	.	.	X	.	.	.	X	.	1	100%
Mare 16	D10	.	.	.	X	.	.	.	.	X	1	100%
Mare 17	D10	.	.	.	X	.	.	.	.	X	1	100%
Mare 18	D15	.	.	.	X	.	.	.	.	X	1	100%
D0-D5	2	0	0	.	2/2	0	2/24	0	0	0	2	100% (2/2)
D6-D10	12	4	4/12	3/12h;1/18h	9/12	0	2/24	1/24	6/24	3/24	16	75% (12/16)
D11-D16	4	2	2/4	2/18h	¼	0	0	0	0	4/24	6	66.7% (4/6)
Total/cycle	24	6	6/24	3/12h;3/18h	18/24	0	16.7%(4/24)	4.1%(1/24)	25% (6/24)	29.2%(7/24)	24	75% (18/24)
Total/mare	18	0	6/18	3/12h;3/18h	12/18	0	11.1%(2/18)	0	33.3%(6/18)	22.2%(4/18)	18	66.7%(12/18)

D: day, N: number, Ov: ovulation, Protoc: protocols, Synch: synchronization

Table 5.7 - Efficiency of ovulation synchronization in groups G1 and G2. Percentage of lost ovulations and loss rate on both hormonal protocols due to occurrence of ovulations before expected (12 and 18h). Ovulation induction (time 0) using hCG + GnRH analogue.

	Lost Ovulations: 12 and 18h		Loss rate	
Groups	12h	18h	Number of lost protocols	Efficiency of ovulation.synchronization
G1	11.1% (2/18)	16.7% (3/18)	27.8% (5/18)	72.2% (13/18)
G2	16.7% (3/18)	16.7% (3/18)	33.3% (6/18)	66.7% (12/18)
Total	13.9% (5/36)	16.7% (6/36)	30.6%(11/36)	69.4% (25/36)

H: hour

Table 5.8 Pregnancy rates post FTAI in mares submitted to protocols for ovulation synchronization, G1 and G2, compared to the time when the hormonal protocols started (D0), in different phases of the estrous cycle (D0-D5; D6-D10; D11-D16), and compared to the time of occurrence of ovulations after induction.

Groups	D0 - D5	D6 - D10	D11 - D15	Hour of ovulation/ Pregnancy rate	Total
G1	50% (1/2)	83.3% (10/12)	100% (4/4)	30h: 75% (3/4)	83% (15/18)
				36h: 100% (1/1)	
				42h: 83.3% (5/6)	
				48h: 85.7% (6/7)	
G2	100% (2/2)	77.7% (7/9)	57.1% (4/7)	30h: 40% (2/5)	72% (13/18)
				36h: 100% (1/1)	
				42h: 85.7% (6/7)	
				48h: 80% (4/5)	

D: day, FTAI: fixed time artificial insemination

Table 5.9 Average and standard error of embryonic vesicles diameter at D13 after FTAI in mares submitted to ovulation synchronization protocols, G1 and G2, compared to the time when hormonal protocols started (D0) in different phases of the estrous cycle and the time ovulations occurred after induction.

G1 Diameter EV D13	30h	36h	42h	48h	*P* Value	Average, StE and *P* value; EV/Start of the protocol
D0- D5	13.65	.	.	.		13.65
D6- D10	14.55	10.57	15.3	14		13.22 ± 0.73
	9.05	.	13.35	12.7		
	.	.	14.4	.		
	.	.	16.75	.		
	.	.	11.55	.		
D11- D16	.	.	.	13.4		13.26 ± 1.21
	.	.	.	10.2		
	.	.	.	16.15		
	.	.	.	13.3		
G1 Average and StE	12.41± 1.7	10.57	14.35± 056	13.36± 0.37	*P*= 0.41 NS	13.31 ± 0.74
Correlation EV/Ovulation hour					r= 0.37	*P* = 0.98 NS
G2 Diameter EV D13	30h	36h	42h	48h		Average, StE and *P* value; EV/Start of the protocol
D0- D5	.	.	.	8.95		9.95± 1
	.	.	.	10.95		
D6- D10	.	11.2	10.1	6.8		10.35± 0.67
	.	.	11.05	12.6		
	.	.	10.1	.		
	.	.	10.6	.		
D11- D16	11.1	.	11.45	.		11.16± 1.53
	14.8	.	7.3	.		
G2 Average and StE	12.95 ± 1.85	11.2	10.1 ± 0.6	9.82 ± 1.25	0.34 NS	10.52 ± 0.21
Correlation EV/ovulation hour					r= -0.92	*P* = 0.78 NS
G1 and G2 Average and StE	30h		36h	42h	48h	*P* Value
EV/ovulation.hour	12.63± 1.1		10.88±0.31	11.99± 0.81	11.9± 0.85	*P* = 0.88 NS
Correlation						r= 0.38
G1 and G2 Average and StE	D0- D5		D6- D10		D11- D15	*P* Value
EV/Start of the protocol	11.18 ± 1.36		12.03 ± 0.6		12.21 ± 0.98	*P* = 0.83 NS

NS: no significant statistical difference, D: day, EV: embryo vesicle, H: hour, Ov: ovulation, StE: standard error

Table 5.10 - Costs of products (R$) used for ovulation synchronization in mares submitted to G2 and G1 hormonal protocols. Price research performed in October 28th 2019.

Products	Price	Unit	Price/unit	Qt/protocol G1	Price/protocol G1	Qt/protocol G2	Price/protocol G2
Progesterone	114.00	1pack/10un	11.40/unit	2unit	22.80	1unit	11.40
Estradiol	71.50	50ml	1.43$/ml	4ml (40mg)	5.72	0	0
ProstaglandinF2α	76.95	50ml	1.54$/ml	2ml (0.25mg)	3.08	2ml (0.25mg)	3.08
Deslorelin	150.00	30ml	5.00$/ml	3ml (750µg)	15.00	3ml (750µg)	15.00
hCG	400.00	1box/25ml	16.00$/ml	1ml (1000UI)	16.00	1ml (1000UI)	16.00
	80.00	1unit/5ml					
Total	.	.	.	.	62.60	.	45.48

Qt: quantity

Table 5.11 - Costs of products (R$) used for synchronization of ovulation in mares submitted to G1 and G2 hormonal protocols. Price research performed in October 28th 2019.

Groups	Cost/mare	Cost/18 mares	Loss rate	Cost + loss rate	Pregnancy rate	Cost/pregnancy	Cost/18 pregnancies
G1	62.60	1126.80	375.22 (33.3%)	1502.02	83%	100.13	1802.34
G2	45.48	818.64	227.58 (27.8%)	1046.22	72%	80.48	1448.64

Table 5.12. Average market prices in the region of Pirassununga - SP, costs of FTAI protocols (G1 and G2) and extra procedures per mare, considering single/cycle insemination.

Procedures	Costs R$	FTAI protocols	
		G1	G2
Veterinary visit	200.00		
Veterinary travel	1/km		
Follicular control	200.00	62.60	45.48
Artificial insemination	200.00		
Semen collection	250.00		
Positive pregnancy diagnosos at 60 days	1500.00		
Total (without veterinary travel)	2350.00	2412.60	2395.48

FTAI: fixed time artificial insemination

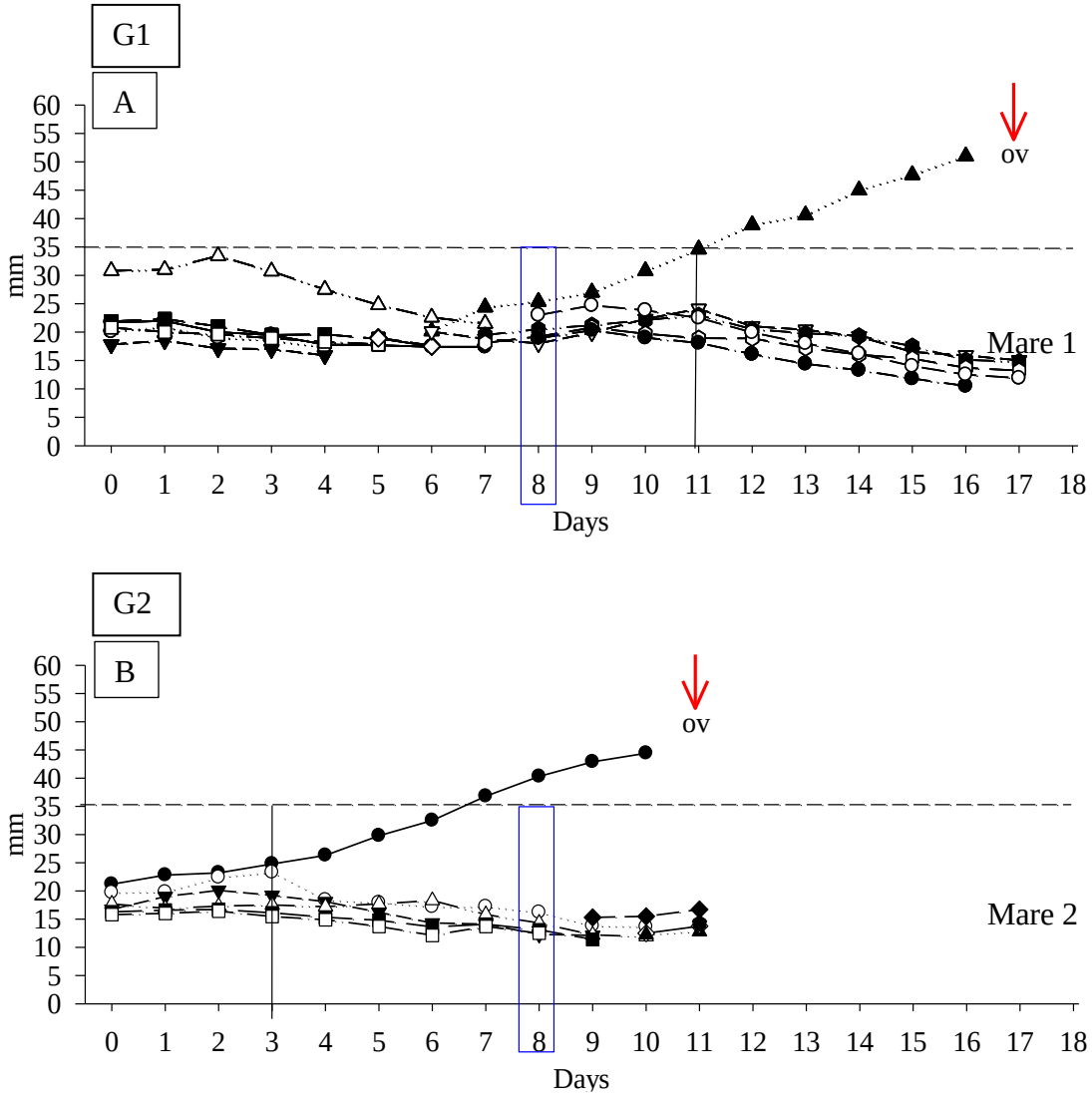


Figure 5.2. Graphic representation of the ultrasonographic follicular dynamics monitoring of mares submitted to G1 and G2 hormonal treatment protocols for comparative purposes among groups. The day the protocol started (D0) corresponded to the eleventh day (D10) of the estrous cycle. The diameters of the three largest follicles in each ovary were measured daily. The blue rectangle indicates the day that P4 intravaginal device was removed. The day when the follicular divergence occurred is indicated by the vertical full line. The moment follicles reached 35mm diameter is indicated by the horizontal dotted line. The red arrow indicates the day of ovulation.

A) Mare 1 - G1: the graph exemplifies the effect of the 1.92 g P4 intravaginal device inserted at D0, in association with 17βestradiol, administered at D0 and again at D2, to induce follicular atresia. The largest follicle detected at D0 had approximately 30 mm in diameter and continued to grow until D2, but after the second dose of 17βestradiol at D2, the follicle suffered atresia. After the removal of the exogenous source of P4, the ovulatory follicle accelerated its growth rate and ovulated at D17.

B) Mare 2 - G2: the graph exemplifies the effect of the 0.96 g P4 intravaginal device inserted at D0, on follicular growth suppression. After the removal of the exogenous source of P4, ovulation of the pre-ovulatory follicle occurred at D11.

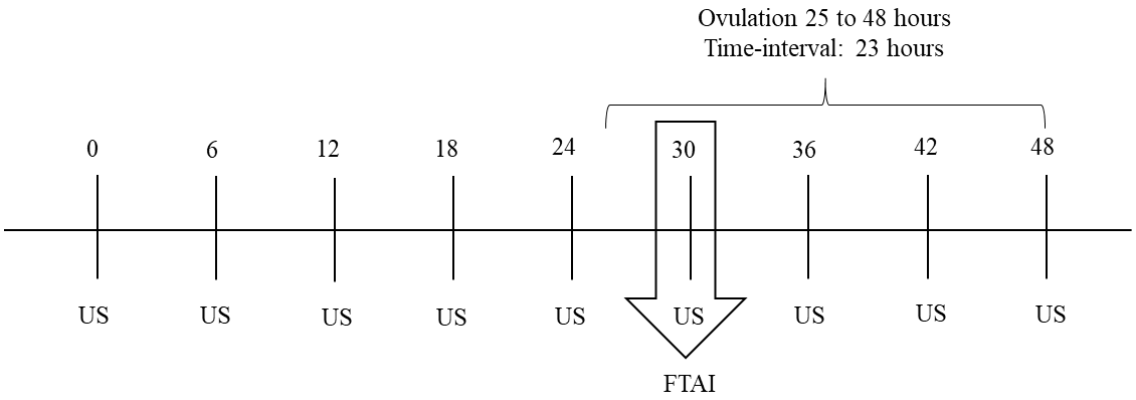


Figure 5.3 Schematic design of ultrasound examinations, every six hours, for ovulation detection post-induction. Estimated time-interval of 23 hours for useful ovulations for FTAI in mares submitted to hormonal protocols (G1 and G2) for ovulation synchronization. US = ultrasound, FTAI = fixed time artificial insemination.

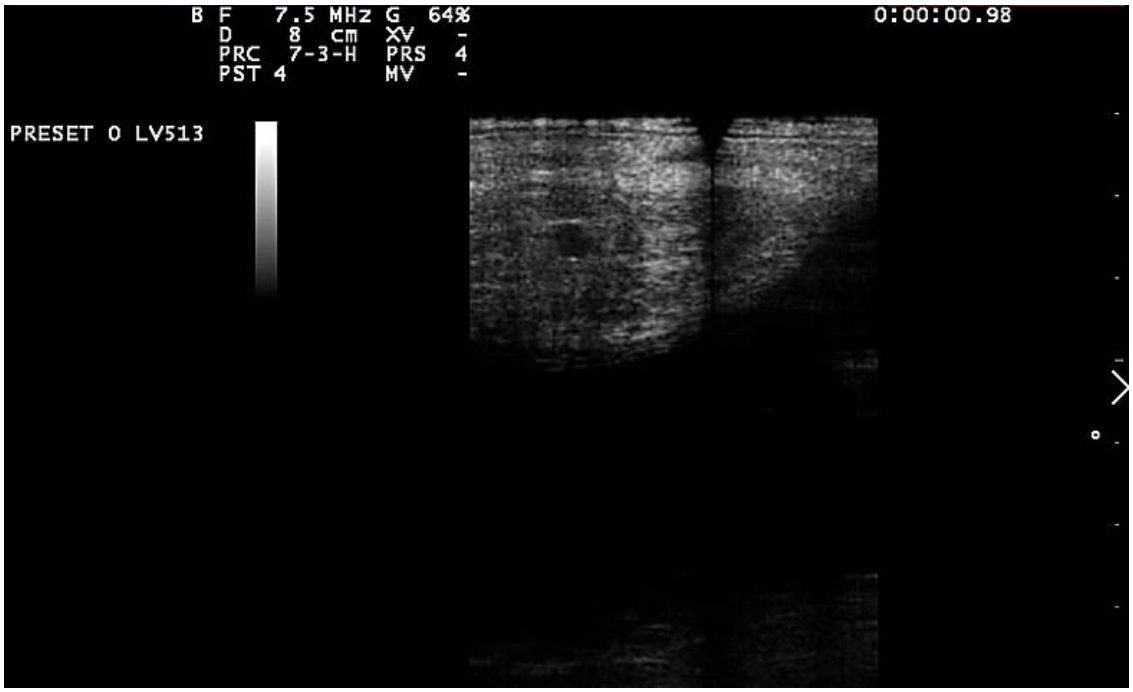


Figure 5.4 A) Mare 1: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 6.8mm diameter at D13 of gestation, submitted do hormonal protocol G2.

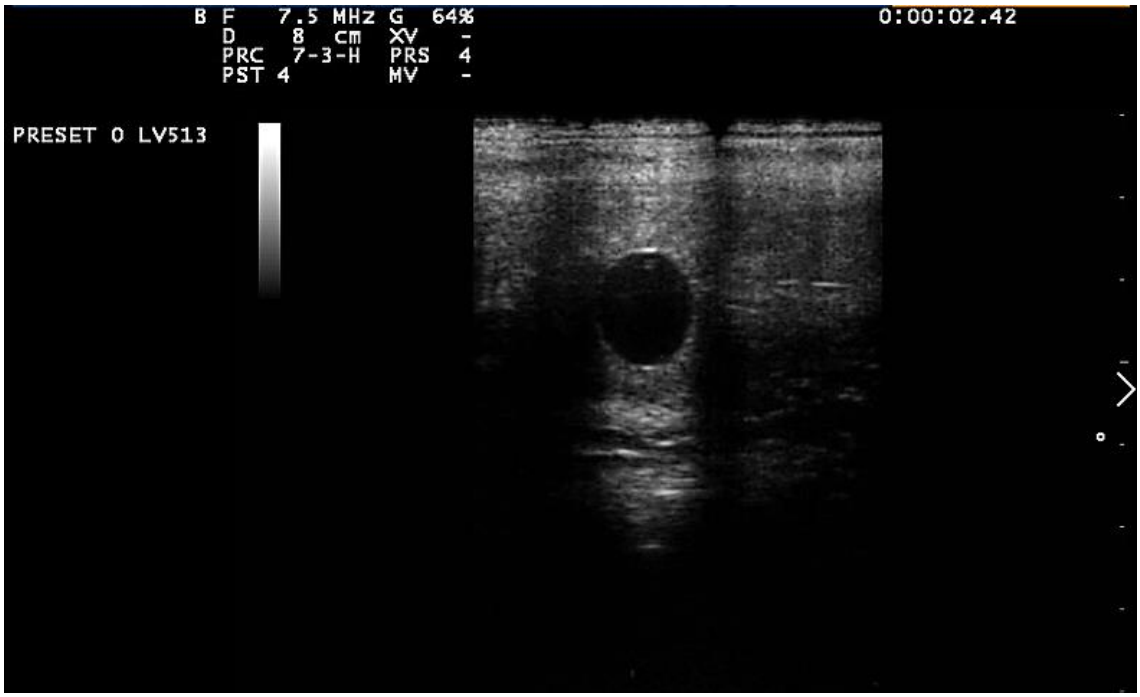


Figure 5.4 B) Mare 1: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 12.7mm diameter at D13 of gestation, submitted do hormonal protocol G1.

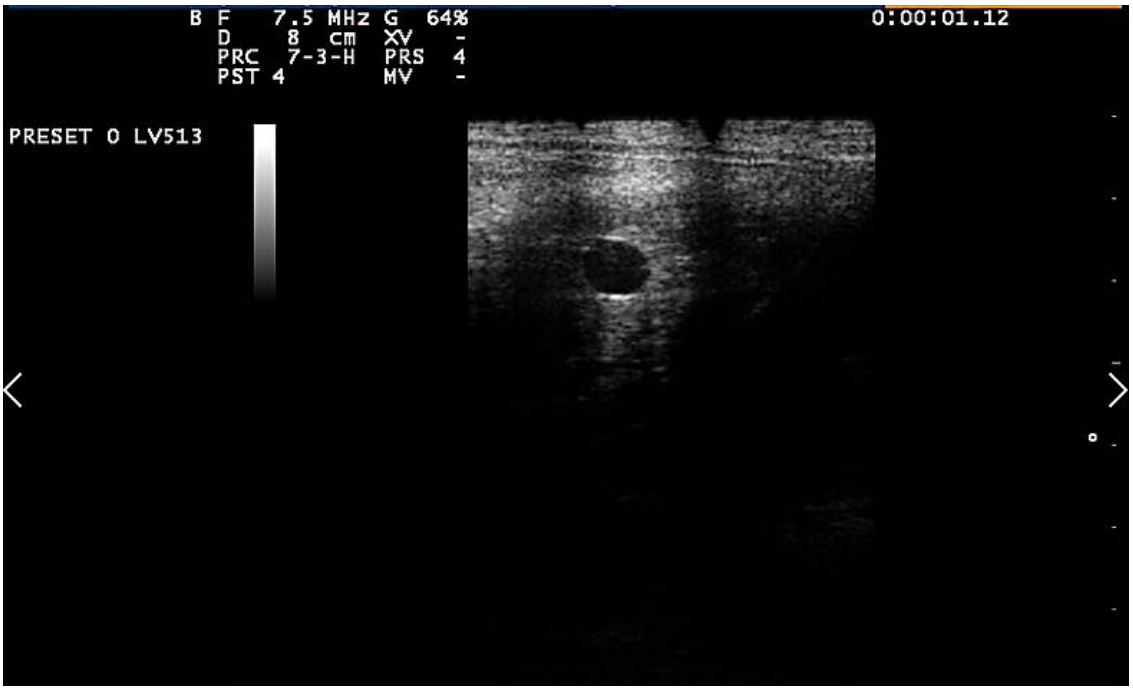


Figure 5.4 C) Mare 2: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 8.95mm diameter at D13 of gestation, submitted do hormonal protocol G2.

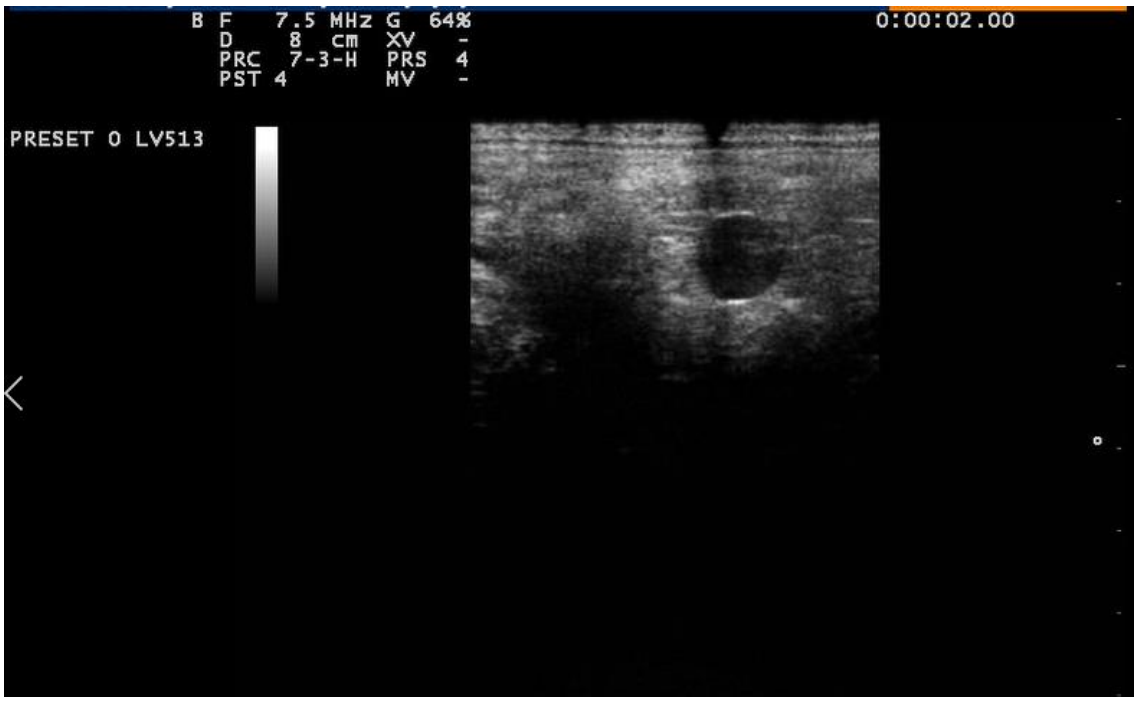


Figure 5.4 D) Mare 2: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 10.2mm diameter at D13 of gestation, submitted do hormonal protocol G1.

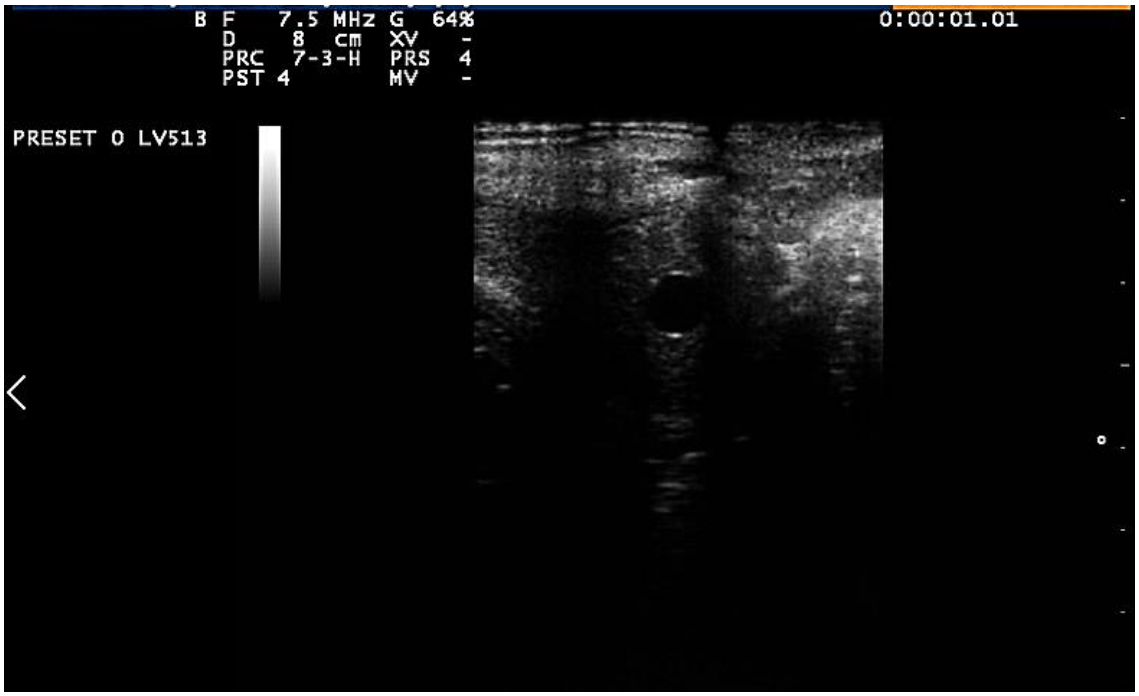


Figure 5.4 E) Mare 3: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 10.95mm diameter at D13 of gestation, submitted do hormonal protocol G2.

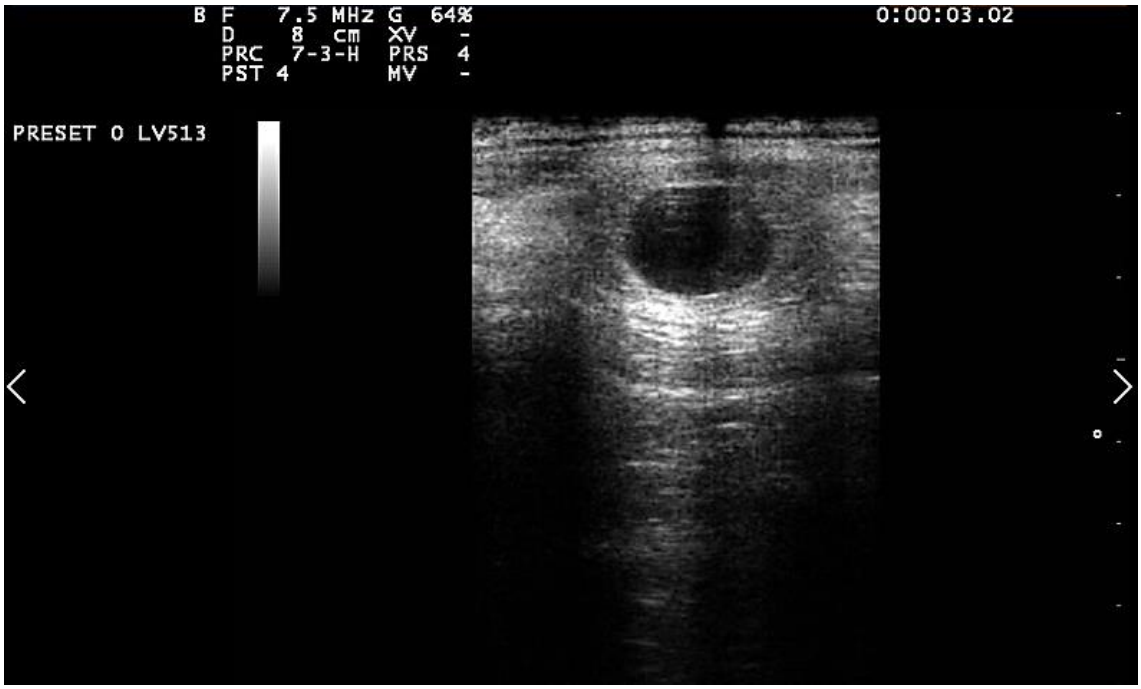


Figure 5.4 F) Mare 3: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 16.15mm diameter at D13 of gestation, submitted do hormonal protocol G1.

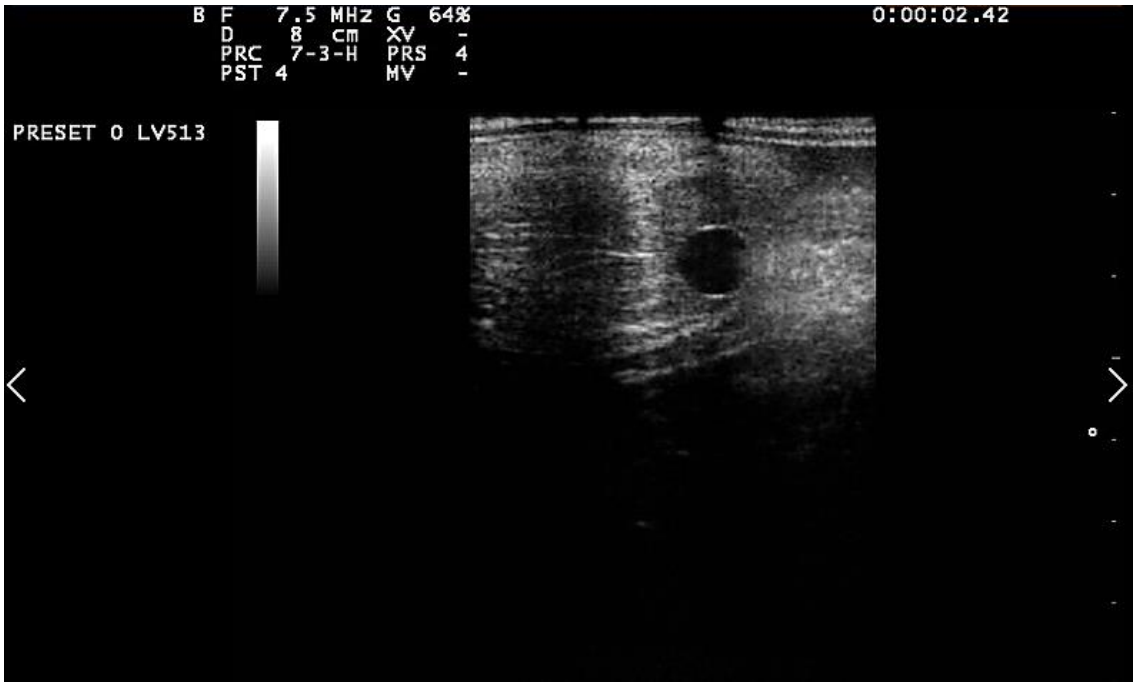


Figure 5.4 G) Mare 4: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 10.1mm diameter at D13 of gestation, submitted do hormonal protocol G2.

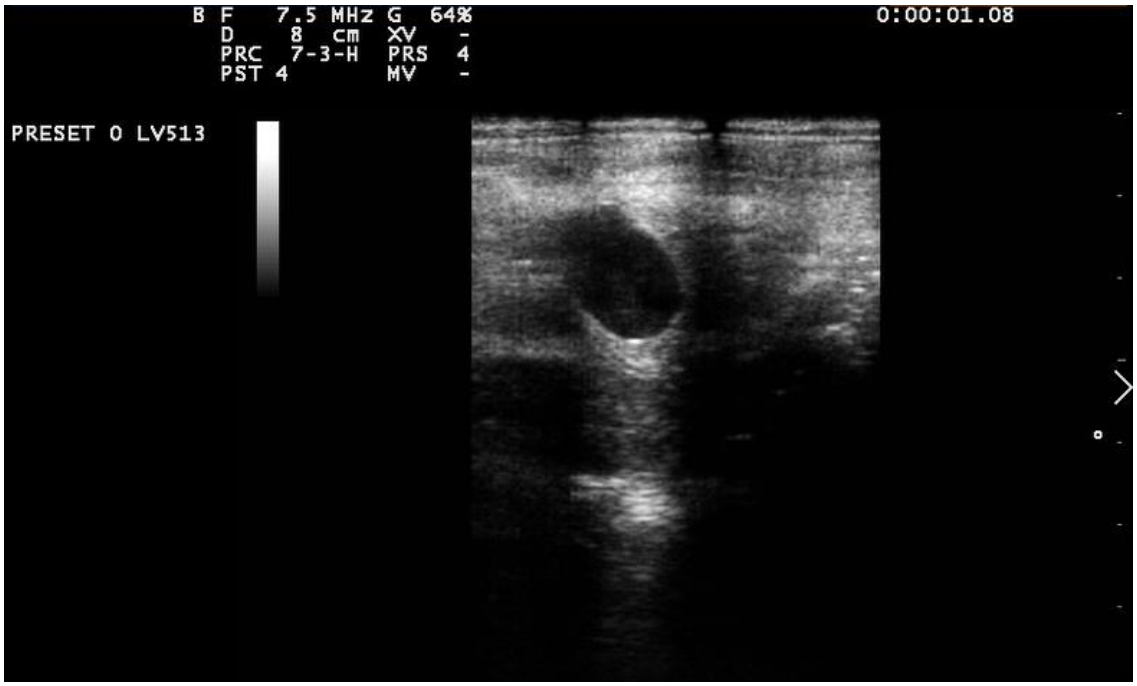


Figure 5.4 H) Mare 4: Transrectal ultrasound examination and visualization of an embryo vesicle (EV) with 14.55mm diameter at D13 of gestation, submitted do hormonal protocol G1.

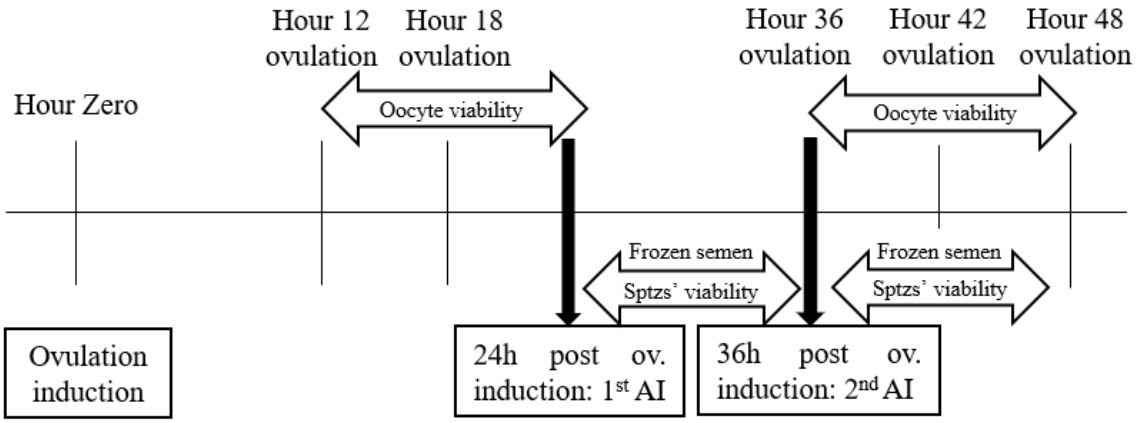


Figure 5.5 Schematic design of time of occurrence of ovulations for FTAIs using frozen semen. When hormonal protocols G1 and G2 are performed, the first AI could be performed 24 hours after ovulation induction. The second AI could be scheduled 36 hours after ovulation induction. Considering 12 hours of oocyte viability and six to 12 hours of viability of spermatozoa from frozen semen, this program for FTAIs using frozen semen in mares may help increase fertility rates and reduce labor.

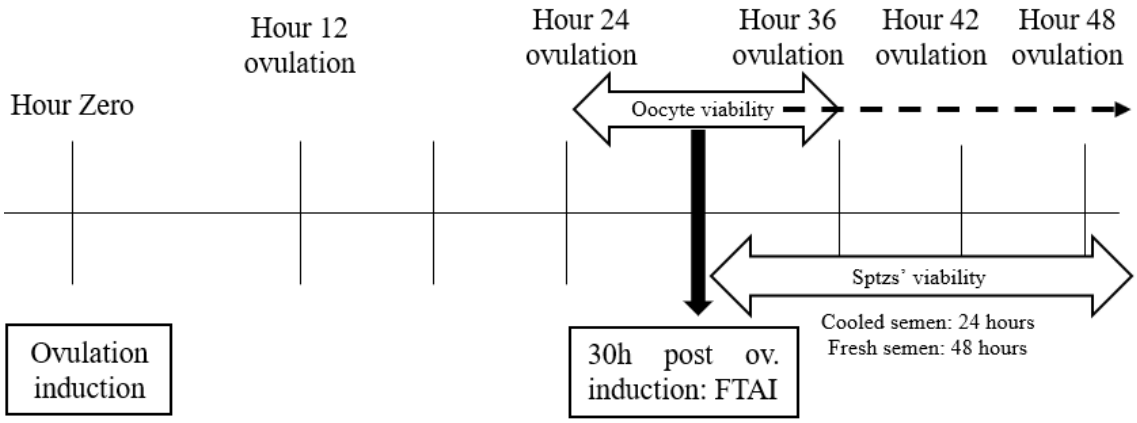


Figure 5.6 Schematic design of time of occurrence of ovulations for FTAIs using fresh or cooled semen. When hormonal protocols G1 and G2 are performed, the FTAI could be scheduled 30 hours after ovulation induction. Considering 12 hours of oocyte' viability and 24 or 48 hours of viability of spermatozoa from fresh and cooled semen, respectively, this program for FTAIs in mares may help increase fertility rates and reduce labor.

CHAPTER 6

GENERAL CONCLUSIONS

In summary, data from this study provided evidence that is possible to perform synchronization of estrus and ovulation in mares. Besides, mares submitted to hormonal treatments for ovulation synchronization presented satisfactory pregnancy rates at thirteen days of gestation. Therefore, these results set the stage for future experiments to further investigate refined hormonal protocols in order to implement a program for fixed-time artificial insemination in the field breeding routine for equine species.

Detailed exploration is needed to better understand the events that happened at cellular and molecular levels, which may influence the characteristics of oocytes originated post-hormonal treatments. Also, follicular fluid of mares submitted to ovulation synchronization protocols may help to clarify potential deleterious effects of delayed follicular growth and its repercussions on early concept development. Another important issue to be evaluated is the monitoring of gestational health and neonatal parameters of foals born from FTAI. The confirmation of births of healthy foals after synchronized inseminations in a large herd of mares would be crucial evidence that FTAI can effectively be recommended as a new strategy for equine reproduction.