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Biological activity using dynamic speckle in blood serum of mares in different reproductive stages

Atividade biológica por speckle dinâmico em soro sanguíneo de éguas em diferentes estágios reprodutivos

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Highlight:
Biological activity is captured by the biospeckle, regardless of the reproductive stage.
An ascending bioactivity curve occurred between 5 and 10 min of analysis.
The activity peak occurred within 15 and 25 min of analysis, reaching zero after 45 min.
The presence of bioactive molecules influences the biospeckle pattern detected.

Abstract

This study aimed to evaluate the biological activity in blood serum plasma samples from mares at different reproductive stages using dynamic speckle analysis. For this purpose, samples were collected from 40 Mangalarga Machador mares from the Formoso 2S horse farm in Cajueiro, AL. The mares were classified into four groups of ten animals each according to the pregnancy stage: ten empty mares (not pregnant), ten pregnant mares in the early third of pregnancy between one month and four months, ten pregnant mares in the final third of the pregnancy between seven and eleven months, and ten lactating mares (within two months post-partum). The biological activity response using a dynamic speckle (biospeckle) was obtained by capturing images reflected by a coherent light (diode laser with a wavelength of 532 nm and intensity of 3 mW) on a sample using a CCD (*charge coupled device*) camera. This data was analyzed using image processing techniques through the computational application Speckle-THSP-MCO-Descritores (STMD), applying THSP (time history speckle pattern) methodology, which evaluates the temporal evolution of the interference image from spreading over the sample surface over time. A coherence matrix generated from the THSP was used to present the intensity module dispersion, which provided the bioactivity data. These data were then processed using the program OriginPro 8. Ink, generate graphs and compare the results from the different groups under study. A general biospeckle signature was observed regardless of the mares' reproductive stage considered in this study. A short phase of movement of the samples associated with an accommodation of the drop on the slide was observed. It was followed by an ascending curve starting between 5 and 10 min of observation, reaching

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a peak within 15 and 25 min, and finally decayed uniformly until almost zero after 45 min. The group of pregnant mares in the final third of pregnancy presented superior bioactivity compared to pregnant mares in the early third of pregnancy. The curve observed for the group of lactating mares is similar to the curve obtained for the group of pregnant mares in the early third of pregnancy. Bioactive molecules act as dispersion elements of coherent light incident on a sample. The variation inherent to the presence of bioactive molecules in the different stages evaluated influenced the biospeckle pattern detected in each sample. It was concluded that the biological activity peaks of the blood plasma samples from the mares evaluated in this study using the dynamic speckle analysis technique were different for both amplitude and time of occurrence, according to the different reproductive stages.
Key words: Biospeckle. Equine. Blood.

Resumo

Objetivou-se com esse estudo, avaliar os picos de atividade biológica de amostras de plasma sanguíneo de éguas em diferentes estágios reprodutivos, por análise de speckle dinâmico. Foram utilizadas amostras de 40 éguas da raça Mangalarga Marchador pertencentes ao Haras Formoso 2S localizado na cidade de Cajueiro – AL. As éguas foram divididas em 4 grupos de 10 animais cada, classificados de acordo com a fase de gestação, sendo 10 éguas vazias (não prenhes), 10 éguas prenhas no terço inicial de gestação entre 1 e 4 meses, 10 éguas em terço final de gestação entre 7 e 11 meses e 10 éguas em fase de lactação (até o segundo mês pós-parto). A obtenção da leitura de atividade biológica por speckle dinâmico (biospeckle) foi feita através da captura de imagens refletidas por uma luz coerente (laser de diodo com comprimento de onda de 532 nm e intensidade de 3mW) por meio de uma câmera CCD (*charge coupled device*) nas amostras de soro sanguíneo. Esses dados foram analisados através de técnicas de processamento de imagens pelo aplicativo computacional Speckle-THSP-MCO-Descritores (STMD), empregando-se o método de THSP (*time history speckle pattern*), que avalia a evolução temporal da figura de interferência a partir do espalhamento pela superfície da amostra ao longo do tempo. A partir do THSP foi gerada uma matriz de co-ocorrência, usada para apresentar o MDI (módulo de dispersão de intensidades), que forneceu os dados da bioatividade das amostras. Os dados obtidos foram então tratados pelo programa OriginPro 8.Ink, para geração de gráficos e comparação dos mesmos entre os grupos estudados. Observou-se que, independente do estágio reprodutivo das éguas considerado nesse estudo, existe uma assinatura geral de atividade captada pelo biospeckle. Ocorreu uma curta fase de movimentação das amostras, provavelmente associada à acomodação da gota na lâmina, seguida de uma curva ascendente iniciada entre o minuto 5 e 10 de avaliação, que alcança seu pico em uma faixa de tempo entre o minuto 15 e 25, decaindo de maneira uniforme até praticamente se anular quando passados 45 minutos de análise. O grupo de éguas em terço final de gestação apresentou bioatividade das amostras superior, quando comparadas às éguas que estão em terço inicial. A curva observada no grupo de éguas em lactação se assemelha um pouco à curva do grupo de terço inicial. A variação inerente à presença de moléculas bioativas nas diferentes fases avaliadas, que atuam como elementos dispersores de uma luz coerente incidida nas amostras, aparentemente influenciam no padrão de biospeckle detectado em cada uma delas. Conclui-se que os picos de atividade biológica de amostras de plasma sanguíneo de éguas avaliadas pela técnica de análise de speckle dinâmico, mostram-se distintos tanto em amplitude quanto em tempo de ocorrência, de acordo com os estágios reprodutivos das fêmeas.
Palavras-chave: Biospeckle. Equino. Sangue.

Introduction

The speckle pattern depictures the interference generated by the scattering of light after interacting with a physical environment. Since this measurement is greatly influenced by the parameters of the analyzed material, such as molecular characteristics and bioactivity, the speckle can be classified as static or dynamic. Over the past decades, the use of dynamic speckle or biospeckle, has gained prominence as an alternative to analyze activity

in biological samples. The increasing interest in this method lies in the fact that it provides fast and reliable tests, allowing a range of possibilities regarding the study of biological systems, extending from the characterization of seed bioactivities to animal body fluids (Braga, 2017).

The biospeckle principle is based on the physical phenomenon of light interference, which occurs when two or more waves overlap, producing a pattern that can be classified as destructive or constructive (Chang, 2005). Regarding the different body fluids analysis, the superposition pattern observed is the result of the interaction between the electromagnet wave (laser light) and the sample. Hence, when laser light is applied to the examined sample, the scattered light projection surfaces create an interference pattern called a dynamic speckle or biospeckle (Briers, 1975).

Acknowledging that the sample is active over time, and its biological elements (atoms, molecules, cells, and tissues) are responsible for the laser light scattering, which creates interference that results in a granular speckle pattern (Braga, 2017). In the literature, bio-speckle was first referred to not long after the laser was created (Briers, 1975).

Concerning the application of the introduced technique, it becomes clear that each component in the sample can modify the resulting pattern, and be considered a dispersing element capable of influencing the resulting outcome. Therefore, it is possible to consider that metabolic and hormonal variations related to the reproductive stage of mares can be factors that cause a change in serum activity capable of being captured by dynamic speckle. The hormonal variance mentioned is already applied for this purpose, including progesterone, stroma sulfate, and other hormones in the circulating blood plasma, to identify changes in the reproductive and gestational cycle of these animals (Hafez & Hafez, 2004).

This study aimed to evaluate the biological activity peaks in blood plasma samples from mares at different reproductive stages, using dynamic speckle analysis.

Methodology

Blood serum samples were acquired from 40 Mangalarga Machador mares from the Formoso 2S horse farm in Cajueiro-AL. The mares were classified into four groups of ten animals, each according to the pregnancy stage: ten empty mares (not pregnant); ten pregnant mares in the early third of pregnancy between one month and four months; ten pregnant mares in the final third of the pregnancy between seven months and 11 months; and ten lactating mares (within two months post-partum). The samples were collected in order by the experimental group, starting with the empty females, followed by the ones in the 1/3 initial stage of pregnancy, then the 1/3 final gestation ones, and finally, those that were in lactation. Blood was obtained with a venous access catheter in the left jugular vein using a vacuum tube collection system without anticoagulant (Vacutainer). Subsequently, the samples were forwarded to the Laboratory of Animal Physiology and Parasitology at the *Universidade Federal de Alagoas* (UFAL)-Arapiraca Campus, in a hermetically sealed thermal box with a temperature of approximately 5 °C. All ten samples from each group were centrifuged at the same time for ten minutes, with 40 s of acceleration and 60 s of automatic braking, at 3600 revolutions per minute (RPM), generating a gravitational force of 2561 g. This procedure causes the blood contents (red blood cells, white blood cells, platelets, and plasma) to separate because the spinning movement forces the higher density particles away from the rotation axis. Thus, the blood cells are moved to the bottom of the tube away from the plasma, which retains the other molecules that can be analyzed (proteins, hormones, and other biochemical elements).

The samples were transferred to 1 mL Eppendorf microtubes and stored under the same storage

conditions as previously mentioned, in the thermal box, to the Biospeckle Laboratory at *Núcleo de Ciências Exatas* (NCEx), UFAL-Arapiraca Campus. Utilizing a 10 µl fixed automatic volume pipettor with disposable tips, the aspirated content of the sample was placed onto a slide, which was then carefully positioned in the alignment center of the coherent light and the camera that captures the images in the assembled analysis system (Figure 1) permitting then to start the analysis through the incidence of a diode laser with a wavelength of 532 nm and intensity of 3 mW. Preliminary tests verified that the samples did not present any significant activity after 45 min under laser incidence; therefore, it was defined as the data gathering set time for all 40 samples.

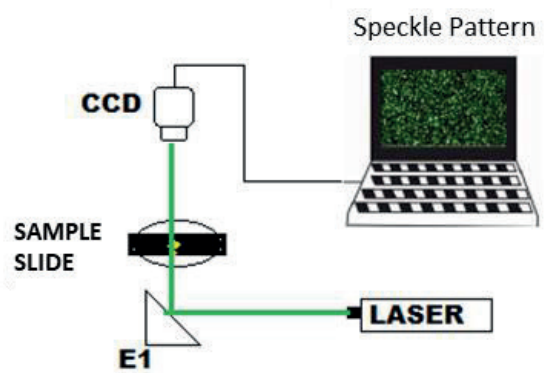

Figure 1. Assembled apparatus to obtain the speckles from the different experimental samples.

The image acquisition system (speckles) consisted of a charge-coupled device(CCD) camera connected to a computer, a laser light, and a mirror (E1) for reflection (Figure 1). Different configurations were previously tested, regarding the quantity and distancing of reflective mirrors as well as the distancing of the sample support apparatus (slide) to the image acquiring element (camera), to obtain a better performance and design a methodology that could be followed throughout the experiment because there is no literature to be referenced on this specific purpose.

The dynamic speckle sample peak acquisition was achieved by capturing the images reflected by the laser using a camera CDD. To enhance the bioactivity monitoring efficiency 1-minute videos were recorded, resulting in 45 videos per sample, 450 videos per experimental group, and the total of 1800 videos to be later examined and fragmented in frames, using the command prompt of the FFMPEG tool (ffmpeg -i “video1” -r 7 “pasta1” \% d.png), generating around 400−420 images, for each video.

The examination of the captured image patterns was performed using a computational application called Speckle-THSP-MCO-Descriptors (STMD), developed by the Biospeckle Laboratory at NCEx, UFAL-Campus Arapiraca (Lucena, Ferreira, Oliveira, & Lima, 2012), which employs the time-history speckle pattern (THSP) method, which assesses the dynamic speckle temporal evolution of the sample surface. Based on this, the co-occurrence matrix (MCO) was established, making it possible to define the intensity dispersion module (MDI) and entropy.

Finally, the data were processed using OriginPro 8. Ink software, to create plots and compare results between the investigated groups.

Results and Discussion

Figures 2 and 3 show the averages of the normalized data of the intensity dispersion module (MDI) of blood plasma samples from Mangalarga Marchador mares in different reproductive stages. They are presented separately and combined, respectively.

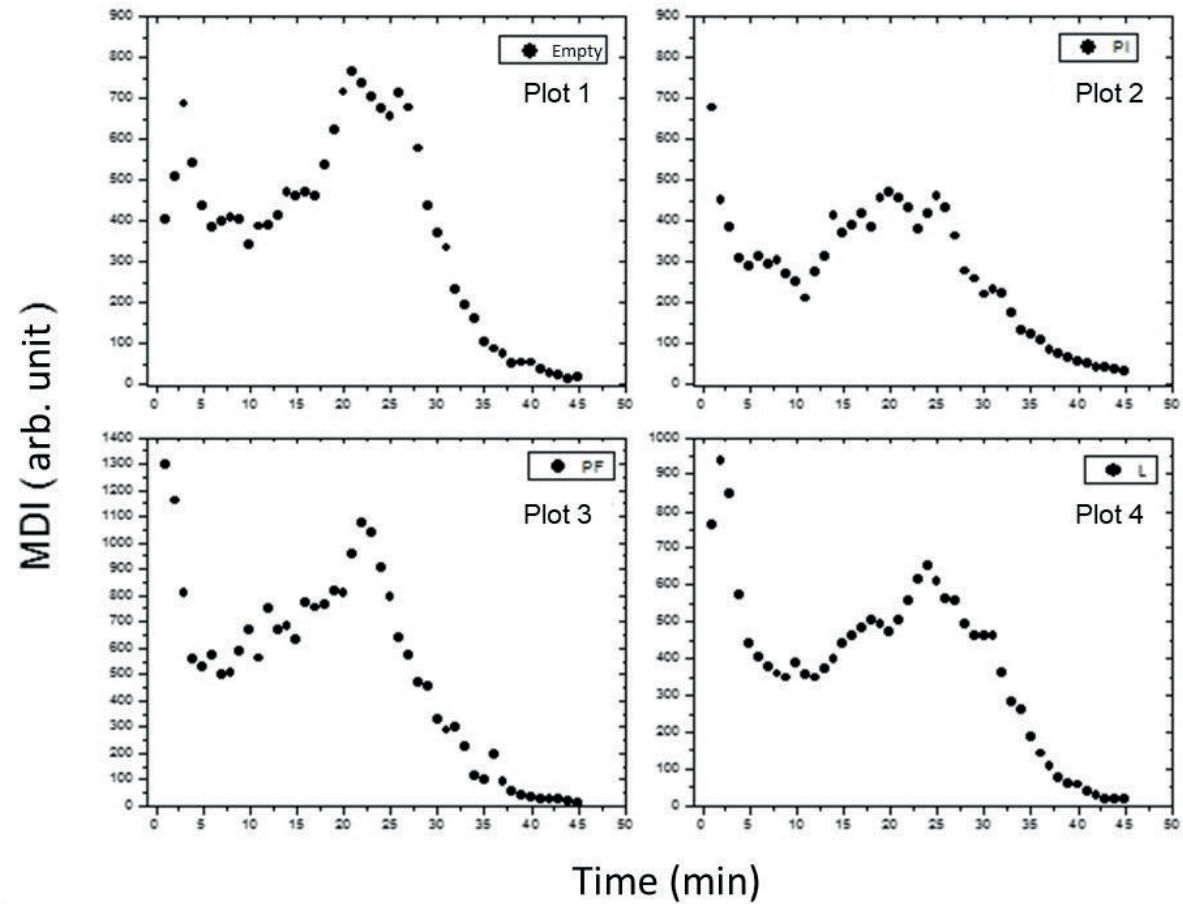

Figure 2. Isolated MDI plots as a function of time (45 min) with dynamic speckle averaged values from the Mangalarga Marchador mares blood plasma samples in different reproductive stages, whether being not pregnant (Empty-Plot 1), early third of pregnancy (PI-Plot 2), final third of the pregnancy (PF-Plot 3), or lactating (L-Plot 4).

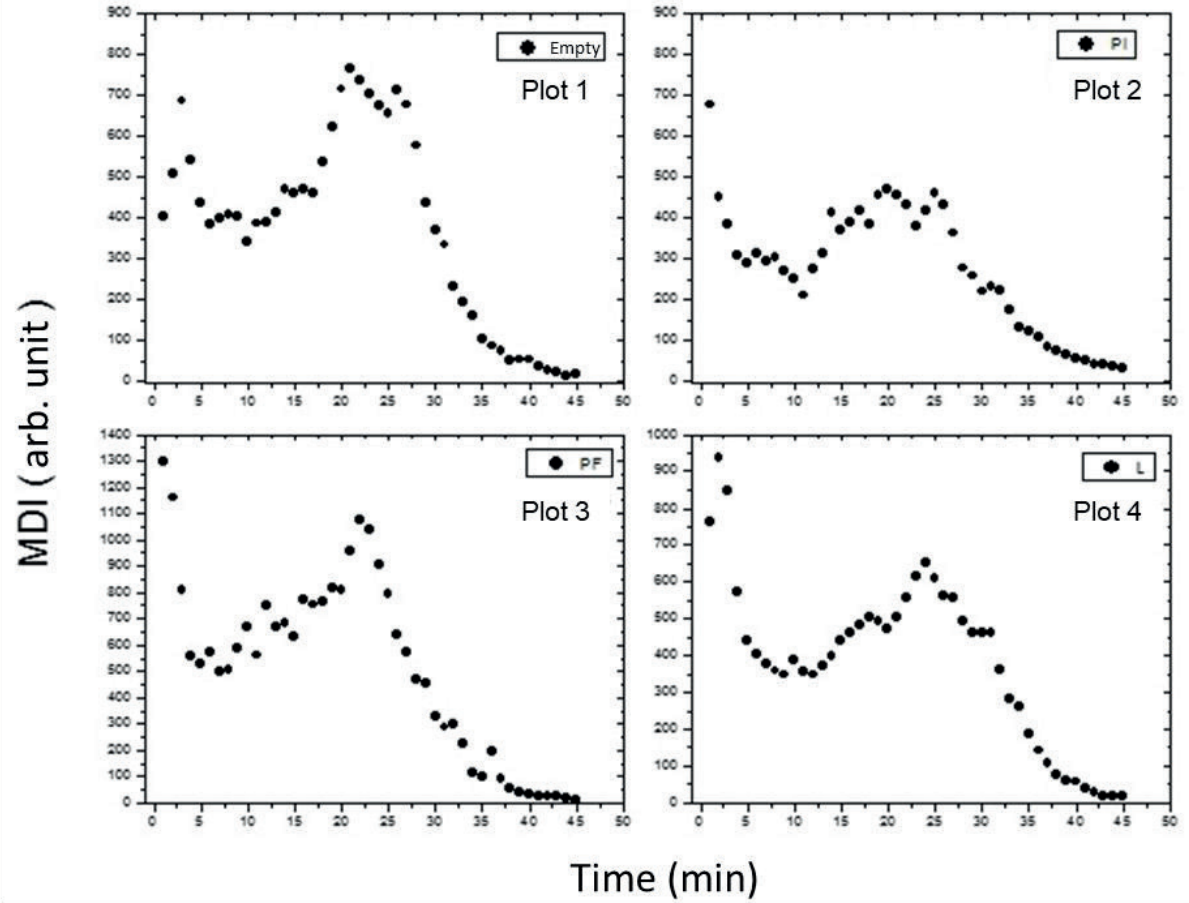

Figure 3. MDI combined plot as a function of time (45 min) with dynamic speckle-averaged values from the Mangalarga Marchador mares blood plasma samples in different reproductive stages, whether being pregnant (Empty), early third of pregnancy (PI), final third of pregnancy (PF), or lactation (L).

It is noticeable from Figures 2 and 3 that regardless of the mares' reproductive stage, there is a general activity signature captured by the dynamic speckle evaluation system. It is possible to observe that at the beginning of the assessment, a short motion peak rises, which is associated with the settlement of the drop in the slide, followed by an ascending curve that starts between the 5th and 10th minute, reaching its maximum value between minutes 15 and 25, and then decayed uniformly until almost reaching zero after 45 min.

The blood plasma is mostly composed of water (90%), but it also contains other extremely relevant bioactive elements that represent approximately 7−8% of plasma constituents (Diaz Gonzalez & Scheffer, 2003). Although the laser incidence did not increase the temperature of the samples, it is natural for a drop to dehydrate over time, which would momentarily increase the concentration of bioactive substances, increasing biological activity due to the gathering of these light scattering elements, in every tested group. Following the increase in molecular movement, a decrease in activity develops due to the degradation of these constituents, which are associated with the metabolic processes that occur. It explains the response pattern found in this study.

Among the bioactive elements are generating coherent light dispersion, plasma proteins, multiple ions, glucose, lipids, amino acids, vitamins, gases, and hormones. Biochemical variations in plasma

reflect the metabolic condition of the animal's tissues, which could reveal and evaluate not only physiological stages but also tissue damage as well as the animals' adaptations to metabolic imbalances and nutrition (Diaz Gonzalez & Scheffer, 2003).

Albumin stands out among these constituents because it is responsible for 80% of the blood plasma osmolarity. It acts as the main carrier protein of fatty acids, amino acids, calcium, and hormones Diaz Gonzalez & Scheffer, 2003). Therefore, this could influence the noticeable variations within the dynamic speckle activity in mares at different reproductive stages. However, a study by Andreazzi et al. (2015) observed that there was no significant difference in the serum levels of this protein when comparing empty and pregnant mares.

Likewise, Penteado, Vaz, Lacerda, Santana and Summa (1999), and Harvey, Pate, Kivipelto and Asquith (2005) reported in their investigations, constant serum levels during pregnancy. Campelo (2008) studied BH and Bretão mares and observed lower albumin levels through the final period of pregnancy in both breeds. However, these results were attributed to the differences in nutritional conditions throughout the experimental period.

Moreover, the biochemical constituents, glucose, are distinctly present in the results. However, studies have revealed that both pregnant and empty mares present no changes in these element levels (Andreazzi et al., 2015).

With all considered, the determination of hormonal dosage is the most reliable way to classify mares in different reproductive stages, especially the analysis of estrogen, progesterone, and prostaglandin, among others (Meirelles, Alonso, & Affonso, 2017; González, 2006). Alternative testing techniques combined with biospeckle is necessary because the oscillation of these hormones over the gestational and lactational period is significant, as observed in the related literature. For instance, in mares, stromal sulfate can be identified at 40 days of gestation, when the foal starts to produce and release it into the mother's bloodstream, increasing further within 75 and 100 days of pregnancy. It is possible to identify this hormone in urine, milk, or the mother's blood plasma, both in horses and other domestic animals (Hafez & Hafez, 2004). Thus, stromal sulfate present in blood plasma could be one of the bioactive elements that influence variations in the dynamic speckle pattern of samples from pregnant mares, especially in the initial third of gestation.

The results of the group in the initial third of pregnancy exhibited an earlier maximum peak than the group that was not pregnant. Since there is a higher gestational hormone concentration, stroma sulfate, for example (Hafez & Hafez, 2004), these substances may increase the speckle scattering pattern due to its higher activity. After the eighth day of gestation, it is possible to identify estrogen in the embryo, and the serum concentration increase depends on the size of the fetus (Meirelles et al., 2017). Nonetheless, the first pregnancy weeks present low estrogen levels in the maternal serum, evidencing that the uterus absorbs the estrogen produced by the fetus.

During the initial pregnancy phase, the estrogen levels are low, around (25 + \ - 16 pg/mL), and the increase in ovarian activity affects these levels, increasing them within the first 35 and 60 days. Around the 80th day, there is an estrogen level development, and after 210 days of gestation, it peaks (130/400 pg/mL) due to fetal gonad hypertrophy (Allen, 2001).

The primary role of the high amount of estrogen produced by pregnant mares is to promote fetal development, acting actively on the blood flow of the uterus and the placenta (Allen, 2001). Bollwein, Mayer, Weber, and Stollar (2002) described similar findings, stating that this hormone has a uterotonic function and an impairing effect on progesterone. The authors also associate the increase in blood flow with the increase in estrogen concentrations during the estrous cycle, especially during pregnancy.

Radioimmunoassay, ELISA, or chemiluminescence tests are the most widely used to measure progesterone, on samples collected within the 16th to the 22nd day after ovulation, when the rate of this hormone is higher than 2 ng/mL in pregnant mares. Some authors do not consider this method reliable enough because empty mares exhibit a more extensive luteal phase, which may produce a false-positive result (Hafez & Hafez, 2004). According to Matta (2013), this hormone level increases throughout the interval between days 40 and 60 of gestation as a result of the first active corpus luteum. It remains elevated until day 150 and then rises further at the end of pregnancy. Progesterone is possibly related to the blood serum biological activity in the final third of the gestation group, measured by the dynamic speckle in this study.

Comparing the results from the group of mares in the initial third of gestation with those in the final third of gestation group, it is noticeable that, on average, the activity in the latest group is higher than in the earliest. An increase in progesterone concentration can be observed in the second and third trimester of the mares' gestational phase when steroid precursors transported to the uterus are metabolized within the fetoplacental tissue (Ousey et al., 2003; Allen, 2001). When the mare approaches the last pregnancy weeks, progesterone levels rise rapidly, peaking within two and three days before labor, and decaying in the few last hours before birth (Mc Kinnon & Voss 2011).

As previously mentioned, mares in the final pregnancy phase showed higher bioactivity than those in the initial phase, which could be related to fewer observable bioactive hormones in the final period of pregnancy, causing the plasma to display more dispersible elements in the speckle imaging because there is more available space in the sample for the molecular motion. The reversed effect can be seen in the samples from the initial pregnancy period.

At the same time, the results from the mare in the lactation phase group peaked later than in any other group. Oxytocin is a gestational hormone, but it is also present in the lactation phase, as it promotes breast milk release, and it is produced primarily by the neurohypophysis (Mitchell, Fang, & Wong, 1998). It is also one of the main substances responsible for uterine contractions during birth, producing peaks of extreme contractions and expulsion at the delivery time (Vivrette, Kindahl, Munro, Roser, & Stabenfeldt, 2000). During lactation, growth hormone (GH) and prolactin act directly on the secretory tissue of the mammary gland.

The curve for the lactating mares group is somewhat similar to the curve of the group in the initial phase. However, at this lactating stage, the main active hormone is prolactin, which directly affects milk production and secretion. Other hormones, such as progesterone and estrogen, are present during this phase and the initial pregnancy phase (Mc Kinnon & Voss 2011; Hafez & Hafez, 2004). The empty mares group results show a descending curve, very similar to the one produced by the group in the final period of gestation. This is probably due to the influence of various reproductive hormones, as they experience estrous cyclic activity, with an emphasis on estrogen in the follicular phase and progesterone in the luteal phase (Hafez & Hafez, 2004). As mentioned before, both hormones are also in the final pregnancy stage.

It is noteworthy that other hormones are also associated with equine reproduction, such as eCG and prostaglandins. However, the amount circulating varies significantly, making it difficult to detect in the plasma. The eCG hormone, equine chorionic gonadotropin, is secreted by developing endometrial chalices. These chalices originate in the binucleated trophoblast cells that occupy the base of the pregnant horn. Additionally, this hormone is exclusive to equine pregnancy (Meirelles et al., 2017). Between the 37th and the 41st day after ovulation, it is possible to identify it in the

bloodstream, and it quickly approaches its peak of 40−180 IU/mL at around 55−75 days. However, chalice degeneration and absence of eCG occurs between 100 and 140 days of gestation, where their levels are no longer detectable in the circulation (Allen, Wilsher, Stewart, Ousey, & Fowden, 2002).

Prostaglandins (PGs) are not stored in cells, and their production is carried out by enzymes according to the body's needs. For this reason, they are difficult to measure in peripheral plasma (Ousey, 2004). The most influential PGs during pregnancy and birth are prostaglandin F2α (PGF2α) and E2 (PGE2). The uteroplacental tissue is responsible for the production and release of these PGs in the amniotic, allantoid fluid and umbilical and uterine circulation during the middle and the final third of pregnancy, but still, the concentrations remain low in the plasma (1−2 ng/mL); hence this hormone may not affect the biological activity of the sample too much (Ousey, 2004).

Finally, it is worth mentioning that, regardless of the reproductive stage, the biological activity in the blood serum of mares is captured by the biospeckle, and the predominant hormones in the different reproductive phases, act as bioactive molecules and influence the response pattern detected in the evaluation.

Conclusion

In conclusion, the biological activity peaks of the mares' blood plasma samples, assessed using the dynamic speckle analysis technique, are distinct both in amplitude and time of occurrence, according to the reproductive stages of the females.

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