



Effect of the subdose of human chorionic gonadotropin applied in the *Hou Hai* acupoint on ovulation induction in mares

[Efeito da subdose de gonadotrofina coriônica humana aplicada no acuponto *Hou Hai* sobre a indução da ovulação em éguas]

M.O. Ribeiro¹, M.A.A. Silva², L.D.P. Maggitti Junior², P.A. Bezerra³,
R.F. Bittencourt⁴, M.A.R. Feliciano¹, A.L.A. Santana¹,
P.D.A. Silva⁵, L.P. Barbosa^{1*}

¹Centro de Ciências Agrárias, Ambientais e Biológicas - Universidade Federal do Recôncavo da Bahia - Cruz das Almas, BA

²Aluno de pós-graduação - Escola de Medicina Veterinária e Zootecnia - Universidade Federal da Bahia - Salvador, BA

³Aluna de graduação - Centro de Ciências Agrárias, Ambientais e Biológicas - Universidade Federal do Recôncavo da Bahia - Cruz das Almas, BA

⁴Escola de Medicina Veterinária e Zootecnia - Universidade Federal da Bahia - Salvador, BA

⁵Aluna de pós-graduação - Faculdade de Ciências Agrárias e Veterinárias - Universidade Estadual Paulista Júlio de Mesquita Filho - Jaboticabal, SP

ABSTRACT

The objective of this study was to evaluate the effects of an hCG sub dose applied at the *Hou Hai* acupoint on corpus luteum (CL) quality and ovulation induction in mares. Fifteen crossbred mares were distributed in randomized blocks and used in three periods with each period employed as the blocking factor in three treatments: T1 = 1500 IU of hCG via intravenous (IV); T2 = 450 IU of hCG applied at the false acupoint (IV); and T3 = 450 IU of hCG applied at the *Hou Hai* acupoint. Mean diameter of the CL, serum concentration of progesterone (P₄), vascularization of the pre-ovulatory follicle and CL were evaluated. Females administered 450 IU of hCG at the *Hou Hai* acupoint exhibited greater ovulation rates (33.33%) 48h after induction; The minimum number of colored pixel (NCP) of the pre-ovulatory follicle of control females was superior (40.33) to that of mares administered 450 IU of hCG IV at the false acupoint (36.84) and similar to that of those administered hCG at the *Hou Hai* acupoint (39.31). Further, moderately positive correlations were found between the CL diameter and the P₄ concentration on D8 (P<0.05). IV administration of 450 IU of hCG or at the *Hou Hai* acupoint was efficient at inducing ovulation and ensuring the quality of CL in mares.

Keywords: Doppler, equine, pharmacopuncture, hormone therapy, progesterone

RESUMO

O objetivo foi avaliar os efeitos de uma subdose de hCG aplicada no acuponto *Hou Hai* na qualidade do corpo lúteo (CL) e na indução da ovulação em éguas. Quinze éguas mestiças foram distribuídas em blocos ao acaso, sendo o período utilizado como fator de blocagem, em: T1 = 1500 UI de hCG por via intravenosa (IV); T2 = 450 UI de hCG aplicado no falso acuponto (IV) e T3 = 450 UI de hCG aplicada no acuponto *Hou Hai*. Avaliou-se diâmetro médio do CL, concentração sérica de progesterona (P₄), vascularização do folículo pré-ovulatório e do CL. As fêmeas que receberam 450 UI de hCG no acuponto *Hou Hai* apresentaram maiores taxas de ovulação (33,33%) 48h após a indução. O número de pixels coloridos (NPC) mínimo do folículo pré-ovulatório das fêmeas do grupo controle foi superior (40,33) ao das éguas que receberam 450 UI de hCG IV no falso acuponto (36,84) e semelhante ao das éguas que receberam hCG no acuponto *Hou Hai* (39,31); correlações moderadamente positivas foram encontradas entre o diâmetro do CL e a concentração de P₄, ambos no D8 (P < 0,05). A administração IV de 450 UI de hCG ou no acuponto *Hou Hai* foi eficiente na indução da ovulação e na garantia da qualidade do CL nas éguas.

Palavras-chave: Doppler, equino, farmacopuntura, hormônio terapia, progesterona

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*Autor para correspondência (corresponding author)

E-mail: larissa@ufrb.edu.br

INTRODUCTION

Reproductive biotechniques in equine culture, such as artificial insemination with fresh, cooled, or frozen semen, and oocyte or embryo transfer, require the use of ovulation inductors (Berezowski *et al.*, 2004) to trigger ovulation (Farias *et al.*, 2016). This is because the estrus period in this species is prolonged (Ferris *et al.*, 2012) and highly variable (Berezowski *et al.*, 2004).

Human chorionic gonadotropin (hCG), which is similar to the luteinizing hormone (LH), is the most commonly used ovulation inducer in mares. In fact, it promotes 100% ovulation within 48h after being applied (Awan *et al.*, 2016). hCG also increases progesterone production (P₄) shortly after ovulation, consequently leading to a better pregnancy rate and conception (Köhne *et al.*, 2014). However, there is no consensus on the effective hCG dose for promoting ovulation in less than 48h (Silva *et al.*, 2016). Although high doses are commonly used, the application of sub doses of hCG may reduce costs and potentially minimize concerns regarding anti-hCG antibody formation (Gastal *et al.*, 2006; Morel and Newcombe, 2008), thereby enabling successive applications during the reproductive season.

In addition to the traditional intravenous (IV), intramuscular (IM), and subcutaneous (SC) routes of drug administration, studies examining drug applications to a specific acupoint, known as pharmacopuncture, have been performed in goats (Cardoso *et al.*, 2018) and sheep (Souza *et al.*, 2019), enabling the use of lower doses with similar results. The *Hou Hai* acupoint is linked to reproductive organs. According to Lin *et al.* (2006), this acupoint is easily accessible as it is located between the ventral base of the tail and the dorsal limit of the anus, making the technique viable for routine use in the field.

In this context, the use of low-dose hCG at the *Hou Hai* acupoint may improve or promote an effect similar to that of the traditionally used dose (1500 IU). Accordingly, the objective of the present study was to evaluate the effects of sub dose hCG on corpus luteum (CL) quality and ovulation induction after application at the *Hou Hai* acupoint in mares.

MATERIALS AND METHODS

The study was conducted at Fazenda Experimental da Escola de Medicina Veterinária e Zootecnia da Universidade Federal da Bahia-UFBA, in the city of Entre Rios-BA located at latitude 11°56'31" South and longitude 38°05'04" West and 152 m above sea level. The climate is tropical and the region has an annual mean rainfall of 1550mm and a mean temperature of 24°C, which reaches a minimum of 16.8°C and a maximum of 32.5°C (Somar..., 2012). The project was approved by the Comissão de Ética no Uso de Animais (CEUA) of UFBA, under registration number 67/2016.

Fifteen crossbred mares in reproductive activity, considered healthy after physical and obstetrical examination, were used in this study. The females presented body condition scores between 4 and 6 according to Henneke *et al.* (1983) and were submitted to a semi-intensive maneuver system in a pangola (*Digitaria decumbens*) and brachiaria (*Brachiaria decumbens*) grass pasture. Ground-corn based supplementation (57.7%), soybean meal (40.8%), and mineral salt (1.5%) were also offered twice per day (500g/animal). Water was supplied *ad libitum*.

The mares were used in three periods, distributed in randomized blocks with crossover designs; the period was used as a blocking factor in three treatments: T1 (control) (n = 15) = 1500 IU of hCG (Chorulon™, MSD, Brazil) applied via IV at the jugular vein; T2 (n = 15) (false acupoint) = 450 IU of hCG applied via IV at the jugular vein; and T3 (n = 15) = 450 IU of hCG applied in the *Hou Hai* acupoint.

The females were monitored every 48h by performing a B-mode ultrasonography examination with the first ovulation of the reproductive season discarded to avoid the transition phase and confirm the reestablishment of the reproductive activity in the mount season. Eight days after the first ovulation, all mares received 5mg of dinoprost tromethamine (Lutalyse™, Pfizer, USA) via the IM route, as a luteolytic agent. After PGF_{2α} application, females were monitored daily by B-mode ultrasonography. The mares that presented dominant follicle, with a diameter greater than 35mm, and the presence of uterine edema grade 3, were administered hCG to induce ovulation,

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according to the dose and treatment route described above.

Applications of hCG by IV at the jugular vein and the *Hou Hai* acupoint were preceded by site sterilization with 70% alcohol. Application at the *Hou Hai* acupoint, located between the ventral base of the tail and the dorsal limit of the anus, was accomplished using a 1-mL syringe coupled to a 16 G catheter at 45° with a 45-mm depth. The mean diameter (mean of the larger horizontal measure and the larger vertical measure) and vascularization (number of colored of minimum, maximum, and average pixels and heterogeneity) of the pre-ovulatory follicle and CL on days 0 (ovulation day D0), 2 (D2), 4 (D4), and 8 (D8); ovulation rate; interval between induction and ovulation; ovulation rate at 48h after induction; and serum concentration of P₄ (D8 only) were evaluated.

After application of the ovulation inducer, the B-mode ultrasonography evaluations were performed at 12h intervals twice per day until the approximate moment of ovulation, associated with disappearance of the follicle image (anechoic) and replacement with the CL image (hypoechoic). To follow the CL development, average diameters were measured in the B-mode and vascularization in the Doppler ultrasonography on days 0 (ovulation day D0); 2 (D2); 4 (D4); and 8 (D8) after ovulation. An ultrasonography device equipped with color Doppler (Mindray Z5™, model DP 2200 VET, China) coupled with a linear transrectal transducer (5.0hz) was used to evaluate blood perfusion (follicular and luteal vascularization) by visualizing color changes corresponding to blood flow throughout the follicular wall and luteal parenchyma.

The pre-ovulatory follicle and CL images obtained by color Doppler imaging were analyzed using Image ProPlus™ software (Media Cybernetics Inc., San Diego, CA, USA) at Laboratório de Ultrassonografia do Setor de Reprodução Animal da Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho”. A tool within the software was used to count the vascularized areas of the pre-ovulatory follicle and the CL to obtain the average number of colored pixel [NCPs; exotexture data, heterogeneity of pixel

(standard deviation NCPs)], and minimum and maximum NCPs.

The serum concentration of P₄ was measured eight days after ovulation via the collection of blood samples from the jugular punctures using anticoagulant vacuum tubes. After collection, blood was centrifuged (300 × g for 15min) to obtain the serum, which was transferred to 2mL polypropylene microtubes. The samples were labeled, frozen at -20°C, and delivered to the Laboratório de Reprodução Animal do Departamento de Zootecnia da Universidade Federal de Viçosa for analysis. A chemiluminescence-based commercial kit (Access Progesterone™, Beckman Coulter, CA, USA) was used according to the manufacturer's recommendations to determine the P₄ concentration.

The normality of the data was assessed by the Shapiro–Wilk test, analysis of variance, and the Tukey test at 5% probability for the parametric data (average diameter and vascularization of follicle at the time of induction, interval between inducer application and ovulation, average diameter and vascularization of CL at D0, D2, D4, and D8). For the non-parametric data (ovulation rate and ovulation rate after 48h of inducer application), the Kruskal–Wallis test was used at 5% probability. Pearson correlation was used to assess the correlations among the average diameter of the CL, CL blood flow, and the P₄ serum concentration on D8.

RESULTS

The average diameter of the pre-ovulatory follicle, the ovulation rate, and the interval between inducer application and ovulation did not change (P>0.05) upon reducing the dose or changing the route of administration of hCG. However, the application of 450 IU of hCG at the *Hou Hai* acupoint increased (P<0.05) the percentage of females that ovulated 48h after induction (Table 1). The administration of a sub dose of hCG at the false acupoint and the *Hou Hai* acupoint did not influence (P>0.05) the mean diameter of CL at any of the evaluation time points (D0, D2, D4, and D8). Furthermore, it did not increase or decrease (P>0.05) the serum P₄ concentration (Table 2).

Table 1. Pre-ovulatory follicle, ovulation rate, and moment of ovulation of the mare with ovulation induced by the sub dose of human chorionic gonadotropin (hCG) at the *Hou Hai* acupoint

Variables	1500IU of hCG intravenous	450IU of hCG intravenous	450IU of hCG of <i>Hou Hai</i> acupoint	P-value
Diameter pre foll (mm) ¹	37.0±3.40	38.0±3.80	39.6±4.50	0.774
Ovulation rate (%)	100.00	86.67	86.67	0.342
IBIO (h) ^{2 a}	41.60±6.19	42.55±18.87	46.29±24.84	0.744
Ov>48h (%) ^{3 b*}	0.00	13.33	33.33	0.043

¹ pre foll = pre-ovulatory follicle; ² IBIO = interval between the inductor and ovulation; ³ Ov>48h = Ovulation above 48hours after induction; ^a Parametric data, refer to the mean ± standard deviation and were not influenced by the treatments, analysis of variance (P>0.05); ^b Non-parametric data; * A difference was observed using the Kruskal Wallis test (P<0.05).

Table 2. Development of the corpus luteum and serum progesterone concentration of mares with ovulation induced by sub doses of human chorionic gonadotropin (hCG) at the *Hou Hai* acupoint

Variables	1500 IU of hCG intravenous	450 IU of hCG intravenous	450 IU of hCG at <i>Hou Hai</i> acupoint	P-value
CL D0 (mm) ¹	22.9±3.4	25.5±5.0	27.4±6.2	0.062
CL D2 (mm) ²	30.4±6.8	31.7±5.0	31.2±7.1	0.884
CL D4 (mm) ³	28.9±6.3	29.5±5.1	28.7±5.2	0.939
CL D8 (mm) ⁴	26.9±4.6	24.4±3.7	25.6±5.1	0.397
P ₄ D8 (ng/mL) ⁵	5.24±2.23	4.43±3.81	5.40±2.81	0.687

¹ CL D0 = corpus luteum on ovulation day; ² CL D2 = corpus luteum 2 days after ovulation; ³ CL D4 = corpus luteum 4 days after ovulation; ⁴ CL D8 = corpus luteum 8 days after ovulation; ⁵ P₄ D8 = progesterone concentration 8 days after ovulation; Data are expressed as mean ± standard deviation and did not differ between treatments, analysis of variance (P>0.05).

A significant (P<0.05) correlation was found between CL diameter and the serum concentration of P₄ (R² = 0.30) in the mares administered 1500 IU of hCG IV on D8 (Figure 1a). A significant (P<0.05) correlation was also observed between the average NCP and the P₄ concentration on D8 (R² = 0.48) in the mares administered 450 IU of

hCG IV at the false acupoint (Figure 1b).however, there was no correlation between these variables in mares administered 450 IU of hCG at the *Hou Hai* acupoint. Such finding suggests that the behavior of one variable did not influence that of another (P>0.05).

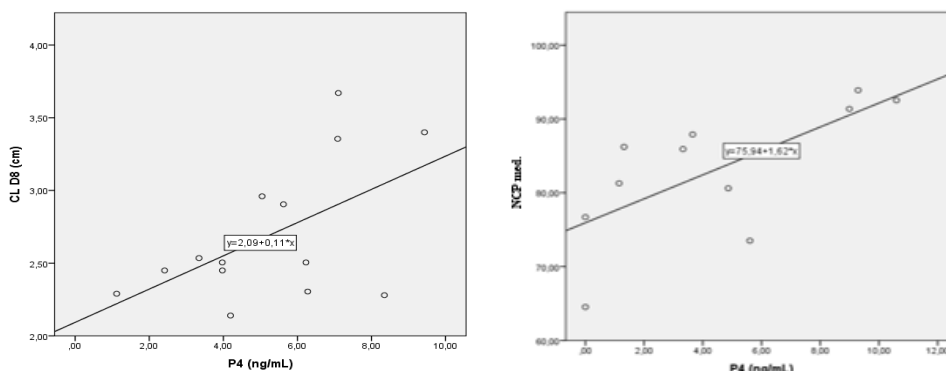


Figure 1. a) Moderate positive correlation between the corpus luteum (CL) and serum progesterone concentration (P₄) eight days after ovulation for crossbred mares with ovulation induced using 1500IU of human chorionic gonadotropin via intravenous administration. b) Moderate positive correlation between mean number of colored pixels (NCP med.) and serum progesterone concentration (P₄) eight days after ovulation for crossbred mares with ovulation induced using 450IU of human chorionic gonadotropin via intravenous administration at the false acupoint.

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The administration of 450 IU of hCG IV at the false acupoint reduced blood perfusion in the pre-ovulatory follicle as demonstrated by the minimum NCP ($P < 0.05$) (Figure 2a). However, the

average and maximum NCPs and heterogeneity were similar between the treatments ($P > 0.05$), indicating that the hCG dose and application route had no influence (Figure 2b, c, and d).

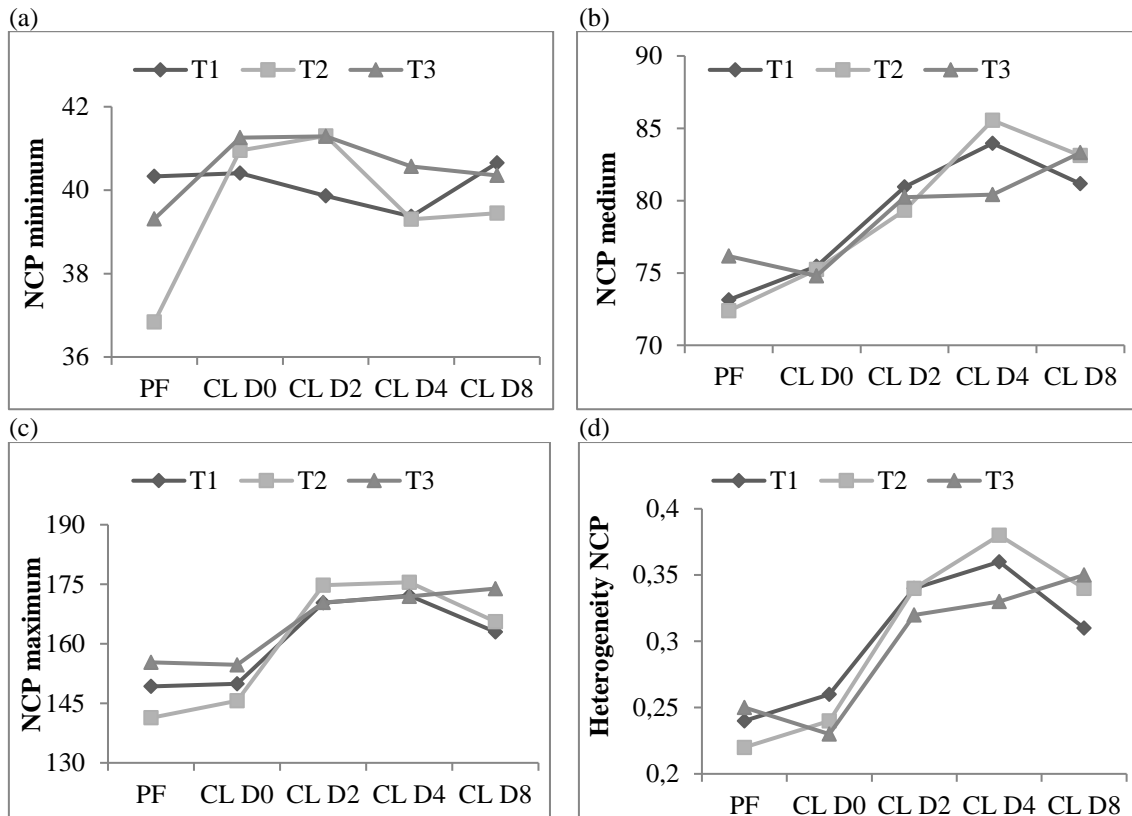


Figure 2. Minimum (a), medium (b), maximum (c) and the heterogeneity of the number of colored pixels (NCP) of the pre-ovulatory follicle (PF) and corpus luteum (CL) on days 0 (D0), 2 (D2), 4 (D4), and 8 (D8) after ovulation, which were evaluated in crossbred mares. T1 = 1500 IU of hCG IV; T2 = 450 IU of hCG IV; T3 = 450 IU of hCG at the *Hou Hai* acupoint. A statistical difference was observed for PF by the Tukey test ($P < 0.05$) (a). No difference was found between treatments for CL D0; CL D2; CL D4; and CL D8, analysis of variance ($P > 0.05$) (b, c, d).

DISCUSSION

The results obtained for the pre-ovulatory follicle diameter, ovulation rate, and the interval between induction and ovulation indicate that 450 IU of hCG may be used to induce ovulation in mares administered IV at either the false acupoint or the *Hou Hai* acupoint (Table 1). These results corroborate those reported by Cardoso *et al.* (2018), who obtained similar results for the different application routes in goats, and those reported by Luna *et al.* (2008), who demonstrated that the use of hormones at doses lower than conventional doses produced similar results.

In the present study, the pre-ovulatory follicles had a mean diameter similar to those (38.3mm at 2500 IU and 39.1mm at 2500 IU) reported by Gastal *et al.* (2006). However, these values are below those reported for mares with spontaneous ovulation that normally exhibit smaller diameters. This is because the application of hCG in mares promotes ovulation in pre-ovulatory follicles and accelerates follicular maturation and ovulation (Cuervo-Arango and Newcombe, 2008).

In this study, the mean ovulation rate of 91.1% of that predicted was observed (Table 1). According to Awan *et al.* (2016), an ovulation rate between 75 and 100% can be obtained after hCG

administration. Morel and Newcombe (2008) reported ovulation rates of 92.5% and 92.4% in mares treated with 750 IU and 1500 IU of hCG, respectively. In both studies, the hCG doses used to induce ovulation were higher than those used in the present study at both the false acupoint (IV) and the *Hou Hai* acupoint, demonstrating that it is not necessary to use elevated doses to promote ovulation in mares.

Both the 1500 IU high dose and the 450 IU low dose of hCG induced ovulation 48h after treatment (Table 1) as effectively as the high doses of hCG that were previously reported (Gastal *et al.*, 2006; Samper, 2008; Romano *et al.*, 2015). According to Samper (2008), the average response time to hCG treatment is approximately 36h, ranging from 12 to 48h when doses between 1500 IU to 3300 IU are administered via IM or IV. Other studies have reported response times longer than 36h. Romano *et al.* (2015) induced ovulation in mares with 750 IU, 1500 IU, and 2500 IU of hCG administered via IV and observed response times of 41h, 38h, and 41h, respectively.

The low dose of hCG applied at the *Hou Hai* acupoint significantly increased the percentage of females that ovulated 48h after hCG application (Table 1). Although ovulation tends to occur 48h after induction (Samper, 2008; Morel *et al.*, 2010; Romano *et al.*, 2015), it is not uncommon for some mares to ovulate after this interval. Gastal *et al.* (2009) observed a 52% ovulation rate 48h after inducing ovulation in mares with 500 IU (low dose) of hCG via IV. According to Samper (2008), it is important to specify the ovulation time, especially when cooled semen is used to inseminate a female, a process that must be performed between 12 and 24h before ovulation.

The use of a low dose of hCG as an ovulation inducer at the false acupoint (IV) or *Hou Hai* acupoint was as efficient at promoting the development of CL as the 1500 IU dose applied via IV. Such finding is elucidated by the diameter of the CL evaluated from the day of ovulation (D0) until eight days after ovulation (D8) and the concentration of serum P₄ (Table 2). The largest CL diameter was observed on D2 after ovulation in all treatments, accompanied by discrete and gradual decreases until D8. Previously, Urquieta *et al.* (2009) used 2500 IU of hCG to induce ovulation in mares. As a result, CL diameter was found to decrease from D4 onward; however, the

CL diameters were greater (36mm) than those observed in our study. A similar CL diameter on D2 was also reported in a study by Cuervo-Arango and Newcombe (2013).

The average serum concentration of P₄ on D8 was 5.02ng/mL, indicating that the mares showed functional CLs. Further, a P₄ concentration above 2ng/mL in the blood of the equine species indicates functional CL. According to Grizendi and Fernandes (2015) and Sieme *et al.* (2016), P₄ levels below 2ng/mL are characteristic of luteal insufficiency. According to Sieme *et al.* (2016), the P₄ level required to maintain gestation in the initial third is above 4ng/mL, a concentration exceeded by those of all treatments in this study. However, three mares (one in each treatment group) presented serum concentrations of P₄ below 2ng/mL, suggesting that 6.6% of the mares did not have a functional CL.

Because hCG is a glycoprotein with luteotropic action and biological activity like that of the luteinizing hormone (LH) (Newcombe, 2011), which is essential to the luteogenic process and adequate functioning of the CL, its use might improve CL development and consequently guarantee maximum functionality regarding P₄ production. In mares, previous studies have suggested a positive correlation between hCG administration, the plasma concentration of P₄, and fertility (Fleury *et al.*, 2007). Further, due to the elevated progesterone production immediately after ovulation, which consequently improves the gestation rate and concept development, Köhne *et al.* (2014) suggests the use of hCG as an ovulation inducer.

A moderate positive correlation was found between the CL diameter and the serum concentration of P₄. However, this was only observed in females whose ovulation was induced with 1500 IU of hCG IV. The correlation between CL diameter and serum P₄ concentration is unclear. Some studies also reported a positive correlation (Arruda *et al.*, 2001), similar to the observed in the present study. However, some studies have reported no correlation (Nagy *et al.*, 2004; Rodrigues *et al.*, 2014), as observed in this study in females administered 450 IU of hCG at the false acupoint or the *Hou Hai* acupoint. Nagy *et al.*, 2004) affirmed that the variation in P₄ concentration may be influenced by individual

factors, such as weight, age, metabolism, and number of hormone receptors.

CL diameter is less frequently used as a single indicator of CL functionality. However, its functionality can be considered when evaluations of CL size are added to echogenicity or vascular perfusion. In this study, a moderate positive correlation between the mean NCP of CL and serum P₄ concentration was observed on D8, but only in females administered 450 IU of hCG via the IV route. The existence of this correlation indicates that the use of Doppler ultrasonography is a viable option for detecting a functional CL with the colored pixel count, associated with the morphoecogenic CL evaluation, an analysis that can reduce costs, as it is impossible to immediately quantify the serum concentration of P₄.

The neovascularization formed after ovulation is essential for transporting supplies to newly developed CL cells, facilitating their synthesis and secretion of P₄ (Ferreira-Dias *et al.*, 2006). Positive correlations between blood flow and P₄ concentration in mares administered hCG as an ovulation inducer were observed in the present study and those by Romano *et al.* (2015) and Ginther *et al.* (2007).

A significant change in the minimum NCP was observed in the treatments for the pre-ovulatory follicle tissue. In fact, the group of females administered 450 IU of hCG IV presented a smaller value. However, the average and maximum NCP of the follicle, and the average, minimum, and maximum NCP of the luteal tissue were not influenced by the treatments on days 0, 2, 4, and 8 (post ovulation). Similarly, significant modifications to the pixel heterogeneity between the evaluated treatments have not been verified (Figure 2a, b, c, and d). The average, minimum, and maximum NCPs increased gradually from D0 (ovulation day) to D8 post ovulation, demonstrating better luteal tissue vascularization independent of the hCG dose and the application route.

Follicular growth, ovulation, and the formation of the corpus luteum can be studied echographically and be correlated with endometrial modifications (Valentin, 2006). According to Valentin (2006), CL studies seek to evaluate vascularization with colorful doplervelocimetry, among other goals, to

define normal CL, by associating its morphology and function. In this study, by associating CL size with P₄ production and the alteration in the average number of colored pixels from CL, we can infer that the formed CLs were functional and could maintain a gestation independent of hCG dose and application route. This fact is favorable as the use of an hCG dose lower than what is commonly used to induce ovulation enables reduced protocol cost and decreased risk of anti-hCG antibody formation, which were proposed by Gastal *et al.* (2006) and Morel e Newcombe (2008).

Similar to the present study, Romano *et al.* (2015) did not observe any differences in luteal blood perfusion between mares administered multiple doses of hCG and those with spontaneous ovulation. Nevertheless, the vascular changes that occur naturally in the ovary during the cycle are evident, and the evaluation with colorful doplervelocimetry of the ovarian tissue enables the observation and quantifiable estimation of the vascular changes of structures during analysis in real time (Carvalho *et al.*, 2008).

CONCLUSION

The IV application of 450 IU of hCG at the false acupoint or the *Hou Hai* acupoint was found to be efficient in inducing ovulation in mares and assuring the quality of CL, ultimately proving that the hCG dose commonly used in this species is in excess. The use of ultrasonography with color Doppler is efficient at predicting CL functionality, thereby enabling real-time evaluation of the vascularity and diameter of CL. B-mode ultrasonography allows for field research when the P₄ serum concentration cannot be determined.

CONFLICT OF INTEREST STATEMENT

We confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support that could have influenced the outcome of this work.

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