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Influence of two ovulation-inducing agents on the pituitary response and follicle blood flow in mares

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ABSTRACT

The objective of the current study was to evaluate the effects of deslorelin and hCG, two ovulation-inducing therapies, on LH surge and follicle vascularity in mares. Thirty mares were either treated with 1.5 mg IM of deslorelin, 2,500 IU IV of hCG or 2 mL IM of NaCl 0.9% (GnRH, hCG and Saline groups, respectively). Power-flow Doppler examination and blood collection were performed every hour during the first 12 hours after treatment (H0) and every six hours between hours 12 (H12) and 30 (H30) after treatment. Moreover, endpoints were evaluated every hour through the last six hours before ovulation (OV-6 to OV-1). In GnRH group, plasma LH concentration progressively increased ($P < 0.001$) during the first 6 hours after treatment and remained high ($P > 0.1$) until OV-1. A significant increase in LH concentrations was first detected ($P < 0.05$) at 24 hours after treatment in hCG group, while no changes ($P > 0.1$) on LH levels were found during H0-H30 and between OV-6 and OV-1 in the Saline group. Independent of the treatment, significant variations on the percentage of the follicle wall with Doppler signals were not observed ($P > 0.1$) throughout the entire experiment. A weak correlation between the preovulatory follicle vascularity and the plasma LH concentration was found in GnRH, hCG and Saline groups ($r = +0.29$, $+0.29$ and -0.23, respectively; $P < 0.0001$). These results described for the first time the immediate and continuous pituitary response to ovulation-inducing therapy with injectable deslorelin. Moreover, spontaneous and induced ovulations were not preceded by an increased follicle vascularity, which differs from previous reports in large animals.

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1. Introduction

Determining the most appropriate moment to perform natural or artificial mating is essential to maximize conception rates through the breeding season in mares [1]. However, the long physiologic duration of the equine estrus, when compared to other domesticated livestock [2-4], makes it difficult to accurately predict the time of ovulation [5]. Currently, the use of ovulation-inducing therapies [6] and the visualization of impending ovulation indicators by B-mode ultrasound examination [7] has been applied to ensure breeding at the appropriate time.

In mares, ovulation-inducing therapies with deslorelin or hCG stimulate the final maturation and the rupture of the follicular wall within 48 h [1,8,9]. Deslorelin, a synthetic GnRH agonist [1,10], acts on the adenohypophysis inducing the pulsatile release of endogenous LH and the subsequent ova maturation [11]. Differently, hCG binds to follicular LH receptors due to its structural similarity to the equine gonadotropin molecule [12,13], prompting an analogous LH-activity [14].

Transrectal Doppler ultrasonography has been recently used to study the blood flow of the follicle wall in mares [15,16], cattle [17,18], ewes [19], canines [20], and women [21]. Changes in the ovarian vascular network during estrus allow the delivery of essential components for follicular maturation and steroidogenesis [22]. Therefore, Doppler ultrasonography has the potential to be an efficient real-time method for *in vivo* evaluation of the functional status of preovulatory follicles. However, the current literature remains unclear about the follicle hemodynamics during the

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impending ovulation phase in mares. Initial studies described an increased follicle vascularity through the preovulatory period [16,23] followed by an abrupt decrease of the Doppler signals in the follicle wall during the last four hours before ovulation [7]. In contrast, the occurrence of ovulation apparently was not affected by the absence of changes on the follicular vascularity in mares treated with hCG [24] or recombinant equine LH [15]. Moreover, the effect of synthetic GnRH agonist agents on the blood flow of dominant follicles has not been described in mares.

Studies with cattle and mares have suggested a correlation between the LH surge and the blood-flow of follicles after ovulation-inducing therapy [25-28]. Considering the distinct biologic activity of hCG and GnRH analogs on final follicular maturation, we have hypothesized that the hemodynamics of dominant follicles are dependent on the ovulation-inducing agent. Therefore, the primary purpose of the present study was to characterize the influence of treatment with hCG and deslorelin on the vascularity of preovulatory follicles in mares. The specific goals were: a) To describe the relationship between changes in the percentage of follicle wall with power-flow signals and the concentrations of LH during the impending ovulation phase, and b) to evaluate the effectiveness of Doppler technology for predicting spontaneous and induced ovulations in mares.

2. Material and methods

2.1. Animals and experimental groups

Thirty cycling mixed breed mares with 5–15 years of age, weighting 250–380 kg, were used for the present study. Mares were handled in accordance with the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (protocol number 139/2012). The study was conducted during a single mid-breeding season (from January to March) in the Reproduction Center of the Department of Animal Reproduction and Veterinary Radiology at the São Paulo State University, Brazil (Latitude 22° 53′ 09″ and Longitude 48° 26′ 4″). All mares were maintained on grass hay, pelleted feed and trace mineralized salt with free access to water. The age of mares was estimated from dental characteristics [29]. Score for body condition was evaluated according to Henneke et al. (1983) and for all mares, it remained high (score $\geq$ 7) during the experiment.

B-mode ultrasonography examination was done once a day to monitor follicular development. Mares showing a pre-ovulatory follicle $\geq$35 mm of diameter associated with endometrial edema were assigned into three experimental groups. The first group (n = 10) received GnRH (1.5 mg of Deslorelin, IM); the second group (n = 10) received hCG (2500 IU of hCG, IV); and third group (n = 10) received a Saline (1.5 mL of NaCl 0.9%, IM) and served as a control group in our experiment. Only mares that ovulated between 30 and 48 h after treatment were used in the GnRH and hCG groups. All mares from the Saline group had spontaneous ovulation. Mares with double ovulations or anovulatory hemorrhagic follicles were not used in the study. The moment immediately before treatment was considered H0.

2.2. Data collection

Blood collection and Doppler ultrasonography examination were performed to characterize the changes in plasma LH concentration and vascularity of the preovulatory follicle. Data collection was performed every one hour during the first 12 h of the study and every six hours from hours 12 (H12) to 30 (H30) after treatment. Additionally, endpoints were evaluated hourly from H30 until the detection of a corpus luteum (CL) in mares treated with GnRH or hCG. In the Saline group, mares were scanned with B-mode ultrasonography every 4 h from H30 to the visualization of impending ovulation signals within 12 h. After that, endpoints were evaluated hourly, and mares that ovulated in less than 10 h were included in the study. Impeding ovulation was predictable by the combination of a thick and echogenic granulosa, decreased turgidity, loss of spherical shape, detached granulosa segments and echoic spots in the antrum [30-33].

2.3. Doppler ultrasonography examination

Vascularity of the preovulatory follicles was estimated using a pulsed-wave color Doppler ultrasound instrument (SONOACE PICO, Medison Brazil Ltda) equipped with a linear endocavitary transductor of 5–9 MHz (LV5-9CDn, 60 mm). Doppler settings were maintained constant during the entire study. Power-flow mode function was used to display blood-flow signals from the vessels of the follicle wall. The entire follicle was scanned in a slow and continuous motion. Scans of 1 min were recorded in a portable computer equipped with a video capture device (Pinnacle Studio 9, Ottawa, ONT, Canada). The follicular vascularity of each film was posteriorly estimated by two independent operators blind to treatment or ovulation. A similar subjective methodology has been used for studying blood-flow of follicles in mares [16,34] and heifers [18]. The vascularity of preovulatory follicles was scored considering the percentage (0%–100%) of follicle wall circumference with Doppler signals during the film clip analysis, as described previously for large animals [7,18,24,34].

Supplementary video related to the Doppler ultrasonography examination of the follicle can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.05.032

2.4. Plasma LH concentration

Blood samples were collected immediately before each Doppler ultrasonography examination by jugular venipuncture into heparinized tubes for the measurement of plasma LH concentration. Blood was centrifuged (1500×*g*/10 min) and plasma was stored at −20 °C until assayed.

Hormonal assays were performed in the School of Animal Sciences at Louisiana State University Agricultural Center, Baton Rouge, USA. Plasma samples were assayed by radioimmunoassay, as previously validated for mares [35]. The double-antibody assay was based on anti-equine chorionic gonadotropin primary antiserum generated in a rabbit and radio-iodinated ovine LH tracer. The intra- and inter-coefficient of variation were 3.2% and 7.0%, respectively. The sensitivity of the assay was 0.4 ng/mL.

2.5. Statistical analysis

The first part of the data was normalized to the moment immediately before treatment (Hour 0) and ended at Hour 30. The last six hours before the CL visualization (OV-6 to OV-1) were used for retrograde statistical analysis of the ovulation-impending phase. A Shapiro-Wilk test was performed to visualize the normal distribution of the data. Mixed-model analysis for repeated measures (SAS PROC MIXED-Version 9.2 SAS Institute, Inc, Cary, NC) was used to compare the means of each response variable between groups and time and their interaction. Post hoc analyses were conducted using a Tukey test. No statistical differences between operators, among hours or an operator-by-hour interaction were

detected (n = 2; r = 0.72; P > 0.1) for preovulatory follicles score. Therefore, the average was used for statistical analysis of blood-flow data. The level of significance was defined at 0.05. Data is presented as a mean ± standard error of the mean.

3. Results

The interval from H0 until CL detection was longer (P < 0.001) in Saline group (80.00 ± 3.38 h) than in GnRH and hCG groups (40.06 ± 1.05 and 37.70 ± 0.47 h, respectively).

Effect of treatment (P = 0.002), hour (P < 0.001) and their interaction (P < 0.001) was found for plasma LH concentration between H0 and H30 (Fig. 1). In GnRH group, a progressive increase on LH concentration was observed during the first 6 h after treatment. A significant decrease (P < 0.01) was detected from H12 to H18 (13.4 ± 0.2 and 11.1 ± 0.5 ng mL^{-1}, respectively). However, the LH concentration remained higher (P < 0.04) than the observed at H0 (5.6 ± 0.4 ng mL^{-1}) in mares treated with deslorelin. In hCG group, concentrations of LH remained low during the first 12 h post-treatment (4.0 ± 0.2 ng mL^{-1}, P > 0.1) and a significant increase (P < 0.001) was first detected at H24 (6.7 ± 0.4 ng mL^{-1}). The plasma LH concentration did not change during the first 30 h post-treatment (4.9 ± 0.3 ng mL^{-1}, P > 0.1) in mares from Saline group. Also, LH levels remained constant (P > 0.1) between OV-6 to OV-1 in mares treated with deslorelin, hCG or NaCl 0.9% from (8.5 ± 0.9, 10.4 ± 0.7 and 12.7 ± 0.9 ng mL^{-1}, respectively; Fig. 2).

The mean concentration of LH in GnRH group was greater (P < 0.05) than in hCG and Saline groups during the first 12 h after treatment (11.2 ± 0.7, 4.01 ± 0.2 and 4.6 ± 0.3 ng mL^{-1}, respectively). In addition, the LH concentration was greater in GnRH group than in hCG and Saline groups within the H5-H12 and H7-H9 intervals, respectively (P < 0.01, Fig. 1). However, no differences (P > 0.1) between GnRH and hCG groups were detected for LH concentrations between H12 and H30 (8.3 ± 0.8 ng mL^{-1}). Effect of treatment was not detected (P > 0.1) for plasma LH concentration during the last six hours before ovulation (Fig. 2).

No effect of treatment, hour and their interaction (P > 0.05) was observed for the follicle vascularity during the first 30 h after treatment (Fig. 3) and during the last six hours before ovulation (Fig. 4). Based on examinations performed hourly or at each six hours no difference between groups (P > 0.1) was detected for follicle vascularity on H0-H12 (34.05± 0.8%, ranged from 10.0 to 87.5%) and H12-H30 (37.1± 1.5%, ranged from 15.0 to 90.0%). In addition, independent of the treatment, the vascularity of the preovulatory follicle remained constant and low (37.4± 1.5%; P > 0.1) during the last six hours before ovulation. A weak correlation between the follicle vascularity and the plasma LH concentration was found from H0 to H30 in GnRH, hCG and Saline groups (r = +0.29, +0.29 and −0.23, respectively; $P < 0.0001$).

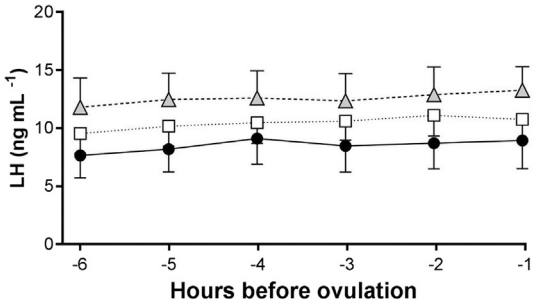

Fig. 2. Mean (±s.e.m.) for plasma LH concentration (ng mL^{-1}) in mares treated with 1.5 mg of deslorelin (●), 2.500 IU of hCG (□) or 2 mL NaCl 0.9% (▲).

4. Discussion

Doppler ultrasonography has become one of the best and most reliable techniques for *in vivo* evaluation and study of the ovarian hemodynamics in livestock [15,18]. The present study described for the first time the lack of association between the follicle vascularity and the LH surge before spontaneous or induced ovulation in cycling mares. Our findings allow the reevaluation of previous concepts considered definitive regarding the physiology of the final stages of follicular maturation in the species.

This is the first report of vascularity analysis of the equine preovulatory follicle using Power-flow imaging. The conventional Color-flow function has been originally used for general examinations of the ovarian hemodynamics in large animals [16]. However, due to its greater sensitivity to weak Doppler signals from vessels with small caliber and low blood flow [36], Power-flow function has recently been used in mares for direct analysis of the luteal tissue [37] and the endometrium [38,39]. Moreover, a similar approach was successfully applied to characterize the blood-flow of dominant follicles during estrus in cattle [18,27].

A daily increase in vascularity of the dominant follicle as it matures before the first ovulation [16] and during the last portion [7] of the ovulatory season was previously reported in mares. However, despite the greater sensitivity of the current approach and the shorter interval between examinations, changes on follicle vascularity were not detected before ovulation in the present study. Under the current experimental conditions, our findings demonstrated that low and unchanged vascularity did not prevent the ovulatory capability of dominant follicles, contrasting the results

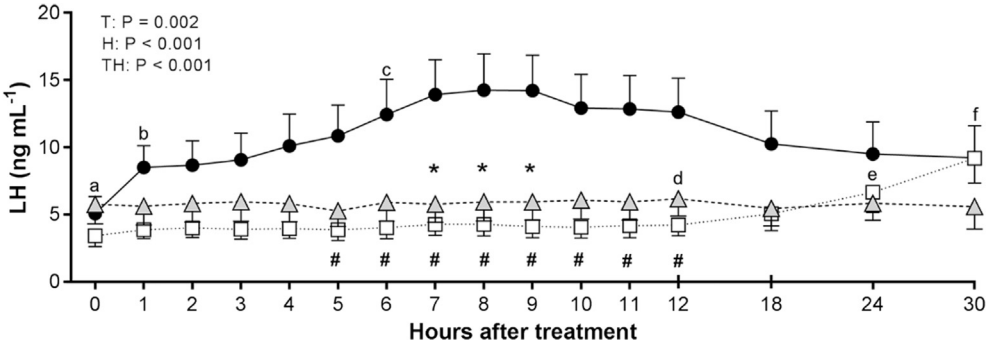

Fig. 1. Mean (±s.e.m.) for plasma LH concentration (ng mL^{-1}) of mares treated with 1.5 mg of deslorelin (●), 2500 IU of hCG (□) or 2 mL NaCl 0.9% (▲). The main effect of treatment (T), hour (H) and interaction (T: H) are shown. H0 = moment immediately before treatment. ^{a,b,c} are different ($P < 0.05$) within the GnRH group. ^{d,e,f} are different ($P < 0.05$) within the hCG group. # Indicates difference between the GnRH and hCG groups within the same hour. * Indicates difference between the GnRH and Saline groups within the same hour.

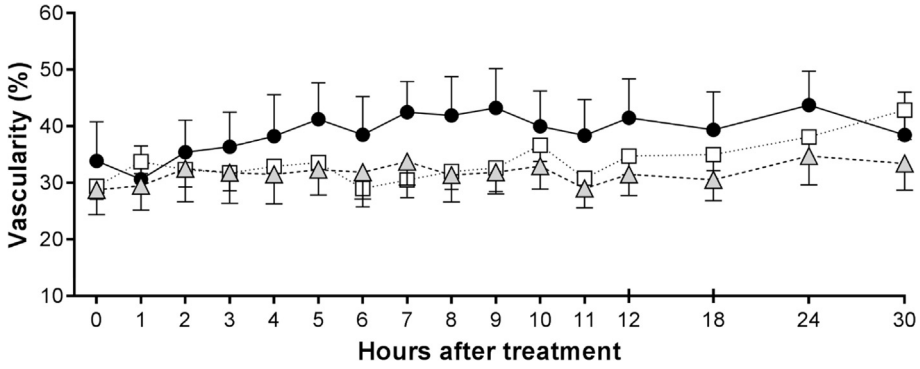

Fig. 3. Mean (±s.e.m.) for vascularity of preovulatory follicles (%) in mares treated with 1.5 mg of deslorelin (●), 2500 IU of hCG (□) or 2 mL NaCl 0.9% (▲).

and interpretation suggested by Ref. [23]. In this regard, the establishment of pregnancy, but not the occurrence of ovulation, has been associated with a high percentage of follicle wall circumference with Doppler signals [24]. Moreover, the final maturation of the follicle has not been correlated with changes on its vascularity in mares later submitted to oocyte recovery procedures [16]. Additionally, ovulation failure has not been reported in synchronized beef cows with impaired follicle vascularity [40].

The final growth and maturation of the dominant follicle has been associated with an increased release of LH [41,42], reaching maximum concentration 24 h after spontaneous ovulation in mares [11,43]. The effect of ovulation-inducing agents on the plasma LH concentrations was expected due to the distinct biological activity between hCG [12-14] and deslorelin [11]. In the present study, the ovulation-inducing therapy with a GnRH analog triggered an immediate pituitary response as previously reported in cattle. However, a single peak of LH has been detected immediately after the administration of gonadorelin in heifers [27], while LH increased progressively over time and remained high until ovulation in mares treated with deslorelin. In contrast, a greater rate of increase in endogenous LH concentration was observed only 24 h after hCG treatment. The increase in LH response at a later time (H30) was expected in mares from the hCG group. In addition to LH-activity on gonadal receptors [44-46], hCG induces a subsequent release of gonadotrophins when the dominant follicle reaches maturity [45,47,48].

5. Conclusion

Under the current experimental conditions, a single ovulation-inducing therapy with deslorelin or hCG induced a progressive LH rise without affecting the blood flow of dominant follicles. Our study demonstrated for the first time that a continuous and low follicle vascularity did not prevent the occurrence of ovulation in mares. Therefore, the real-time analysis of the follicle wall with color Doppler signals did not assist in predicting the time of induced or spontaneous ovulation. The outcomes of a reduced vascularity of preovulatory follicles on the final stages of oocyte maturation and on the functionality of the subsequent CL must be investigated.

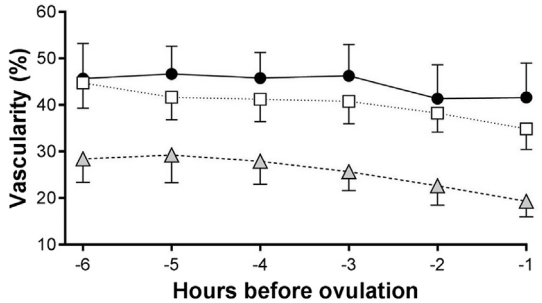

Fig. 4. Mean (±s.e.m.) for vascularity of preovulatory follicles (%) in mares treated with 1.5 mg of deslorelin (●), 2.500 IU of hCG (□) or 2 mL NaCl 0.9% (▲).

Acknowledgments

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