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hCG-Induced Ovulation in Thoroughbred Mares Does Not Affect *Corpus luteum* Development and Function During Early Pregnancy

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Contents

Our aim was to compare *Corpus luteum* (CL) development and blood plasma concentration of progesterone ([P_4]) in thoroughbred mares after spontaneous (Control: C) or human chorionic gonadotrophin (hCG)-induced ovulation. Lactating mares (C = 12; hCG = 21) were daily teased and mated during second oestrus *post-partum*. Treated mares received 2500 IU hCG i.v. at first day of behavioural oestrus when dominant follicular size was >35, ≤42 mm and mated 12–24 h after. Control mares in oestrus were mated with dominant follicular size ≥45 mm. Dominant follicle before ovulation, CL and gestational sac were measured by ultrasound and [P_4] by radioimmunoassay (RIA). Blood sampling and ultrasound CL exams were done at days 1, 2, 3, 4, 8, 12, 16, 20, 25, 30, 35, 40, 45, 60 and 90 after ovulation and gestational sac from day 12 after ovulation in pregnant (P) mares; non-pregnant (NP) were followed until oestrus returned. Data analyses considered four subgroups: hCG-P, hCG-NP, C-P and C-NP. Preovulatory follicular size was smaller in hCG mares than in C: 39.2 ± 2.7 mm vs 51.0 ± 1.8 mm ($p < 0.0001$). All hCG mares ovulated 24–48 h after treatment and presented similar oestrus duration as controls. *C. luteum* size in P mares showed the same pattern of development through days 4–35, presenting erratic differences during initial establishment. Thus, on days 1 and 3, CL was smaller in hCG-P ($p < 0.05$); while in hCG-NP, CL size was greater than in C-NP on day three ($p = 0.03$). *Corpus luteum* size remained stable until day 90 in hCG-P mares, while in C-P a transient and apparently not functional increase was detected on days 40 and 45 ($p < 0.05$) and the decrease from day 60 onwards, made this difference to disappear. No differences were observed in [P_4] pattern between P, or between NP subgroups, respectively. So, hCG-induced ovulation does not affect CL development, neither [P_4] during early pregnancy. One cycle pregnancy rate tended to be lower in hCG mares while season pregnancy rates were similar to controls.

Introduction

Ovarian follicular development and ovulation in mares have some unique characteristics compared with those of other large animals. Mares are seasonal polyoestrous animals; the duration of the oestrous cycle is about 20–21 days and is characterized by a relatively long period of oestrus (Hafez 1987). In most cases, a single cohort or wave of follicles develops during the oestrous cycle and the preovulatory follicle, recruited 13–14 days before ovulation, reaches a maximal size (≥45 mm) considerably larger than in other species. Ovulation in mares is not triggered by a typical luteinizing hormone (LH) surge. Instead, there is a progressive increase in LH during oestrus, which typically peaks 1 day after ovulation (Stabenfeldt et al. 1975). Because of the long oestrus duration it is difficult to determine when the mare should be bred during this period for optimum fertility. In attempts to decrease the number of inseminations or mating per cycle, and to synchronize ovulation time with breeding more accurately, human chorionic gonadotrophin (hCG) has been used widely on breeding farms. A single dose of 1500–3000 IU of hCG given to normal cycling mares on or after their second day of oestrus, will cause ≥80% to ovulate within 48 h, thus reducing considerably the oestrus duration time (1–3 days) and not affecting the pregnancy rate (Voss et al. 1975; Mc Cue et al. 2004). However, some negative effects have been reported, as for example loss of efficacy in inducing a timed ovulation with repeated use of hCG within the same season (Mc Cue et al. 2004), higher embryonic losses and lower birth rates (Zúñiga 2005). Because hCG-induced ovulation generally is applied before the dominant follicle reaches ovulation size, our hypothesis is that this follicle could generate a smaller and insufficient *Corpus luteum* (CL), affecting gestation maintenance.

The objective of this study was to evaluate the effect of hCG-induced ovulation over CL size and blood plasma concentrations of progesterone [P_4], during early pregnancy in thoroughbred mares.

Materials and Methods

Thirty-three thoroughbred lactating mares, between 5 and 15 years and weighing 450–550 kg were used. All mares were kept outdoors during the day and in stalls over night, fed on hay and concentrates, with water *ad libitum*. The breeding farm is located in El Monte, Región Metropolitana, Santiago, Chile.

Once parturition occurred, mares were teased daily with a stallion to determine the onset of oestrus. Foal heat was not used. Once the beginning of second *post-partum* oestrus was detected, daily transrectal ultrasonography was performed, measuring follicle size and evaluating uterine oedema development.

Ultrasound examinations were carried out with a real-time B-mode scanner, using a 5 MHz linear-array transrectal probe (Aloka, Tokyo, Japan). Follicle, CL and gestational sac sizes correspond to the mean values between longitudinal and transversal diameters.

From the total mares used, 21 received a single i.v. injection of 2500 IU of hCG (Chorulón®; Intervet, Santiago, Chile) when follicle size was >35 and ≤42 mm, with well developed uterine oedema and oestrus behaviour, covered by the stallion 12–24 h after treatment (hCG mares). The other 12, non-treated mares that presented natural spontaneous ovulation were considered

as control (C mares), and were covered by the stallion when oestrus behaviour, uterine oedema and dominant follicle size >45 mm were detected and mating was repeated every 48 h after ovulation occurred in this group. Not all mares did get pregnant during the studied oestrous cycle, situation that generated the distribution of pregnant (P) and non-pregnant (NP) females in four sub-groups: hCG-P, hCG-NP, C-P and C-NP.

After ovulation, ultrasound CL measurements were performed on days 1, 2, 3, 4, 8, 12, 16, 20, 25, 30, 35, 40, 45, 60, 75 and 90. Pregnancy diagnosis was made on day 12 after ovulation and confirmed on day 14. Measuring and evaluation of gestational sac were also performed from day 12 after ovulation onwards. In NP mares ultrasound evaluation and blood sampling finished once they returned into oestrus (day 16–20 after ovulation).

Progesterone assay

At the same time as ultrasound evaluation was performed, blood samples (5 ml) were collected with heparinized syringes, transferred into tubes and centrifuged at 1200 × g during 10 min. Plasma was stored at –20°C until assayed. Concentration of plasma progesterone was measured using a solid-phase ^{125}I-radioinmmunoassay technique specific for progesterone (Diagnostic Products Co.™, Coat-a-Count®, L.A., CA, USA). Both method and reagents were previously used and validated in mares (Reimers 1991). The intra- and inter-assay coefficients of variation were 4.9% and 8.5%, for high and medium [P_4] quality control samples (85.3 and 21.5 nmol/l), respectively.

Statistical analyses

The number of animals used (n) per group was defined according the variability presented in blood plasma progesterone concentration in cycling and pregnant mares ($\alpha = 0.05$ and $\beta = 0.8$).

Continuous variables

Preovulatory follicle size, CL size and oestrus duration, in hCG vs C mares were analyzed with one-tailed Wilcoxon matched pairs test. Follicular size at the beginning of the study in the four sub-groups (hCG-P; hCG-NP; C-P and C-NP) was tested by one-way analysis of variance (ANOVA). Progesterone data for the four subgroups were analyzed by one-way ANOVA for repeated measures.

Linear regression was used to determine relationship between preovulatory follicle size with CL size, and CL size with [P_4]. Correlation analysis was also applied to the latter two variables.

Nominal variables

Pregnancy ability, per cycle and for the season, in hCG vs C mares was analyzed with Fisher's one-way exact test.

p-values less than 0.05 were interpreted as significant. Data are presented as mean ± SD.

As it was possible to establish the number of services per cycle and per pregnancy, as well as the one cycle and season pregnancy rates in hCG vs C mares, these variables were analyzed with one-tailed Wilcoxon matched pairs test.

Results

Follicular size on first day of behavioural oestrus, was similar in all sub groups (hCG-P; hCG-NP; C-P; C-NP; $p > 0.05$; Fig. 1a).

All hCG mares (21) ovulated 24–48 h after treatment. Oestrus duration was not significantly affected by the hCG treatment (hCG mares: 4.7 ± 1.7 days; C mares: 5.6 ± 1.3 days; $p = 0.06$). Preovulatory follicle size was smaller in hCG mares than in C mares (39.2 ± 2.7 mm vs 51.0 ± 1.8 mm; $p < 0.0001$; Fig. 1b).

Corpus luteum size of non-pregnant mares was similar between subgroups, except on day three after ovulation, in which hCG-NP CL size was bigger than in C-NP ($p = 0.03$) (Fig. 2a). Blood plasma progesterone concentrations in NP-mares are shown in Fig. 2b.

Corpus luteum sizes of pregnant mares (hCG-P and C-P) are shown in Fig. 3a. CL size increased from day one until four after ovulation, though on days one and three after ovulation, CL size in hCG-P mares was smaller than in C-P mares. CL size then decreased until day 20 in both subgroups. In hCG-P mares increased from day 25 after ovulation and did not show important changes until last measurement on day 90. CL size in C-P mares also showed an increase on day 25 after

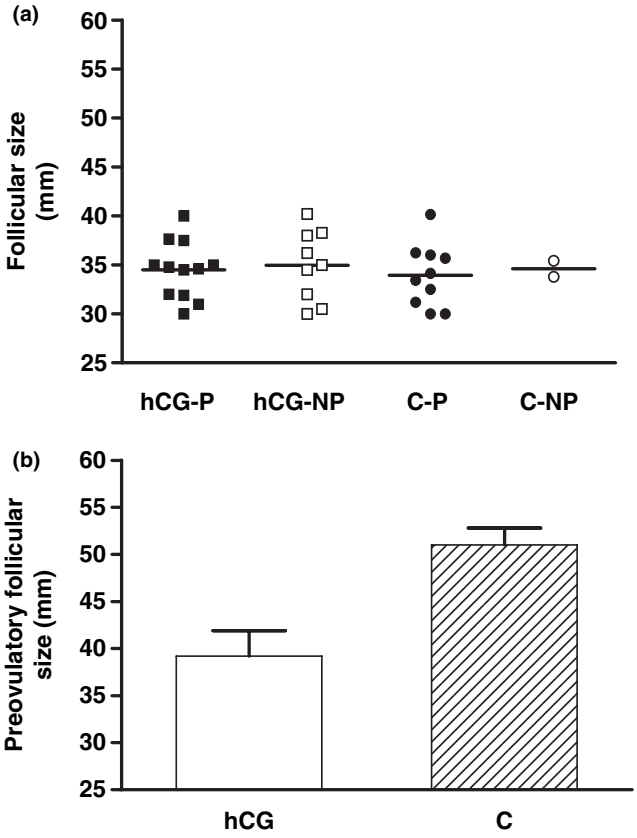

Fig. 1. Dominant follicular size on first day of oestrus (a) and preovulatory follicular size (b) in second *post-partum* oestruos cycle, in hCG treated and non-treated control mares

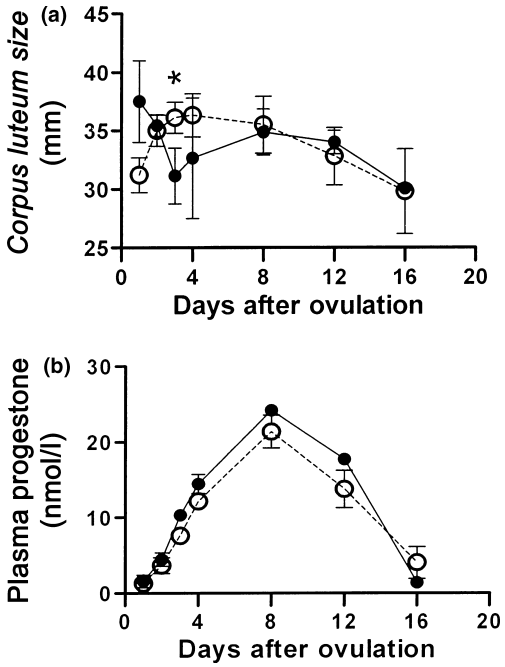

Fig. 2. *Corpus luteum* size (a) and plasma progesterone concentration (b) in non-pregnant mares treated and non-treated with hCG. Each point represents a mean ± SD. * $p \leq 0.05$. ○: hCG-NP CL; ●: C-NP CL

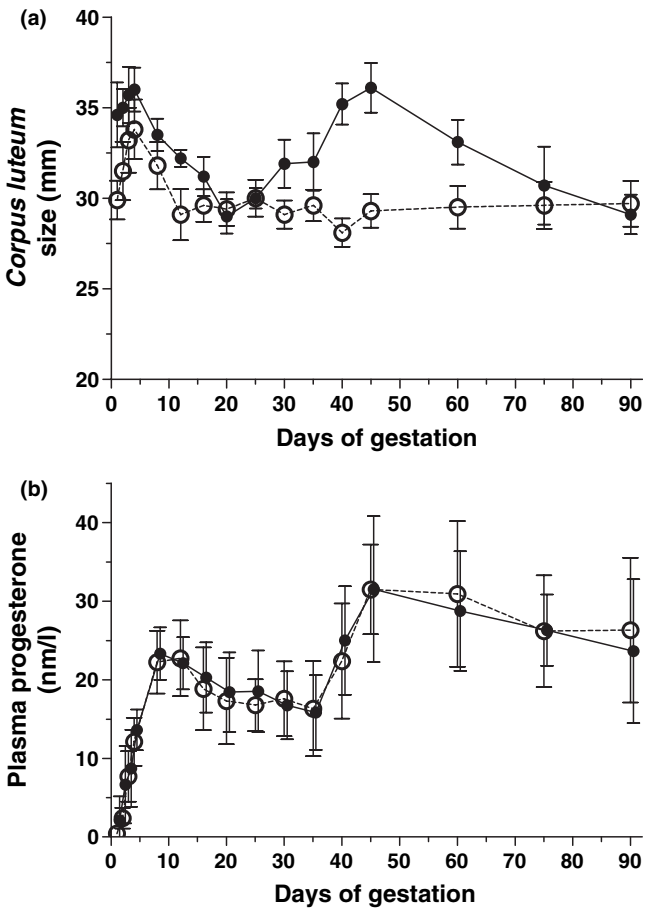

Fig. 3. *Corpus luteum* size (a) and blood plasma progesterone concentrations (b) in pregnant mares, treated and non-treated with hCG. Each point represents mean ± SD. ○: hCG-P; ●: C-P

ovulation, that continued until day 45, decreasing towards day 75 after ovulation. Consequently, on days 40 and 45 of gestation, CL size was larger in C-P mares than in hCG-P mares ($p < 0.05$), decreasing steadily from day 60 until 90, and becoming again similar in size to hCG-P from day 75.

No within-group differences were detected in blood plasma concentration of progesterone for hCG (hCG-P/hCG-NP) and Control (C-P/C-NP) mares until day eight after ovulation. The [P_4] increased in all subgroups between days one and eight after ovulation Thereafter, [P_4] in non-pregnant mares decreased reaching basal levels on days 16–20 after ovulation (Fig. 2b). In pregnant mares, [P_4] was maintained until day 12, where [P_4] showed a transient smooth decrease until day 35. From day 35 to 45, [P_4] increased and attained maximal values in hCG-P and C-P mares. It was maintained in both groups until day 60 and gradually declined thereafter until day 90 of gestation (Fig. 3b). No significant differences in [P_4] existed between pregnant subgroups.

No relationship was found between CL size and [P_4] values at different days after ovulation evaluated in pregnant, treated and non-treated (Fig. 3), nor in non-pregnant mares (Fig. 2).

Gestational sac diameter increased from day 12 until 60 of gestation. No differences were observed in gestational sac size and characteristics between hCG-P and C-P mares (Fig. 4). After day 60 of gestation, gestational sac measurement and evaluation of the entire structure could not be made because of the large dimensions of the foetus and gestational sac.

A positive, though weak relationship between preovulatory follicle size and CL size was only observed on day

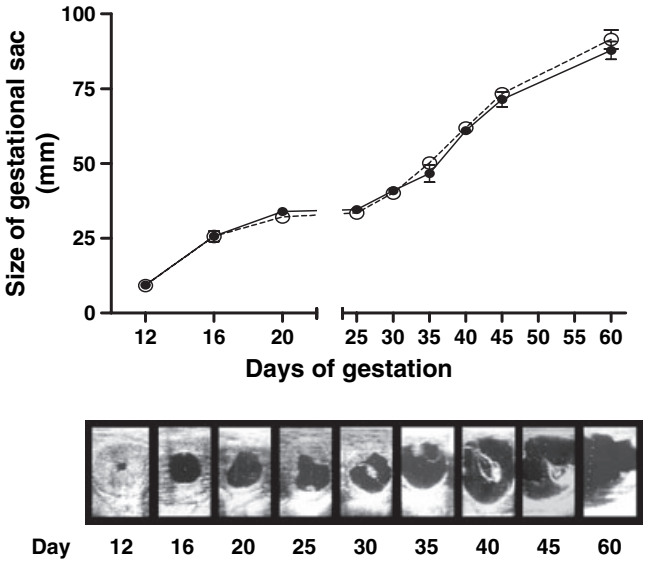

Fig. 4. Gestational sac size and ultrasound evaluation during early pregnancy in hCG treated and non-treated mares. Each point represents a mean ± SD. ○: hCG-P; ●: C-P. Photographic sequence of ecography images are representative of each date

one after ovulation ($r^2 = 0.278$; $p = 0.002$). No relationship between CL size and [P_4] was detected through lineal regression in hCG-P ($r^2 = 0.02$, $p = 0.07$), C-P ($r^2 = 0.006$, $p = 0.38$) and C-NP ($r^2 = 0.04$; $p = 0.55$) mares. In hCG-NP mares, a weak relation was observed ($r^2 = 0.09$, $p = 0.03$). Similar parameters were obtained through lineal regression on splitted periods and when correlation analysis was applied.

The number of services per cycle (hCG mares: 1.0; C mares: 2.0 ± 0.8; $p < 0.001$) and per pregnancy (hCG mares: 1.9 ± 0.6; C mares: 2.3 ± 0.8; $p = 0.01$) were significantly lower in hCG mares compared with controls. The one cycle pregnancy rate was 57.1% and 83.3% for hCG and C mares, respectively ($p > 0.05$). The pregnancy rate for the season was similar between both groups (hCG: 85.7%; C: 91.6%; $p > 0.05$).

Discussion

The present is, to our knowledge, the first study that evaluates the effect of hCG-induced ovulation over CL size and plasma progesterone concentrations during early pregnancy in mares. It supports previous observations on ovulation time after hCG- induction (Voss et al. 1974; Webel et al. 1977; Bollwein and Braun 1999; Mc Cue et al. 2004). Oestrus duration was slightly shorter in treated mares ($p = 0.06$), which differs from the reduction of 1–3 days in oestrus duration in hCG treated cycles previously reported (Voss et al. 1974; Webel et al. 1977; Watson and Hinrichs 1988).

In this study, preovulatory follicle sizes in treated mares were significantly smaller than in control mares and smaller than the preovulatory follicular sizes described for spontaneous ovulation (LeBlanc 1998). It is also important to mention that preovulatory follicle sizes at the time of induction were similar to those reported in other hCG-induced ovulation studies (Watson and Hinrichs 1988; Bollwein and Braun 1999; Kerban et al. 1999; Mc Cue et al. 2004).

Smaller preovulatory follicles originated smaller CL during first days after ovulation. These results are concurrent with a previous report (Bergfelt 2000) that indicated that maximum CL size represents 65–80% of preovulatory follicle size. This is in agreement with the smaller CL size in hCG mares when compared to control mares on days one and three. Afterwards, hCC and control mares showed the same CL sizes until day 35, suggesting a compensatory development in CL of induced mares. Greater CL sizes were measured in control mares on days 40 and 45 after ovulation, while in hGG-P mares CL sizes remain steadily. Similarly, primary *Corpus luteum* weight increases to a maximum value around day 40 after the end of oestrus (42 days after natural ovulation), and decreases smoothly until day 60, as reported by Squires et al. (1974). They also found that the weight of the primary CL in pregnant mares was not significantly different among days 18, 24, 30, 40, 50 and 60. These findings could reinforce our results in the sense that after primary CL is established in pregnant mares, there are no biologically important changes in its development during early pregnancy, no matter the variables measured are not the same.

The absence in CL size increase on days 40 and 45 after ovulation that was detected in hCG-P subgroup in the present study could be related to factors such as differences in the measurement of echogenicity and the way data is collected. On this respect, we calculated CL size as the mean value between the largest longitudinal diameter and the perpendicular transversal diameter, while Sevigna et al. (1999) used the largest cross-sectional area and its change was expressed in a volume ratio. On the other hand, according to Pierson and Adams (1995), tissue densities or tissue characteristics determined by ultrasonography can be observed, but are not readily quantitated by the human eye. No computer-assisted image analysis was made, as this study searched to be the most similar to veterinarian diagnosis usually do in their field work. It might be possible that our measurements could be more affected as the CL size enlarger. Another cause could be a different proportion between small dark lutein cells and large light-colour lutein cells (Van Niekerk et al. 1975), that could show different ultrasonographic images. If permitted, one could even speculate about a different response of hCG-P CL vs C–P CL to growing factors present by those dates of gestation (Murphy and Martinuk 1991). The [P_4] pattern during early pregnancy was similar to that described by Ganjam et al. (1975). However, results disagree with Burns and Fleeger (1975), who stated that [P_4] increases until day five, persists until day 30, increasing again until day 60 of gestation, and also with Holtan et al. (1975) report, where [P_4] showed an initial increase until day eight, followed by a transient decrease until day 28, increasing again afterwards reaching a maximum level of 15.2 ng/ml (48.3 nmol/l) on day 64 of gestation. The differences in [P_4] absolute values among our results and those previously published could be explained by the different analytic methods used.

Plasma progesterone concentration in non-pregnant mares showed the same pattern as pregnant mares until day eight after ovulation. Afterwards [P_4] in non-pregnant mares decreases reaching basal levels on days 16–20 after ovulation, as normally occurs when embryonic antiluteolytic stimuli are absent (Van Niekerk et al. 1975).

Results for CL size and [P_4] pattern in non-pregnant mares in the present study support Sevigna et al. (1999) previous publication, where negative pregnancy diagnosis on day 17 was related to changes in CL size and progesterone concentration at days 8–9 after ovulation. They also suggested that mares not pregnant on day 17 were probably not pregnant on days 8–9, eventually associated with the relation between the change in size of the CL and progesterone production on days 8–9, and the presence or absence of an embryo at that time.

Corpus luteum size and [P_4] pattern in the present study seem to have a similar pattern, though linear regression between both variables during the entire period did not show an important relation, except for the weak relation in hCG-P mares. This disagrees with Ginther (1995), who suggested that structural changes in CL are highly correlated with functional changes of it and that CL size and it echogenicity at ultrasound examination could be used as an indirect measure of the productivity of the luteal gland.

Results for CL size and [P_4] also allow to conclude that, higher embryonic losses and lower birth rates after hCG-induction previously reported by Zúñiga (2005), should not be associated with CL insufficiency, since [P_4] pattern in induced pregnant mares was completely normal.

Pregnancy rate was similar in both groups, however, when considered absolute values the one cycle pregnancy rate in hCG mares was 26.2% lower than in C mares, difference that could affect negatively the productive efficiency of the breeding farm. A possible explanation for the absence of statistical difference in pregnancy rate between the induced and spontaneous ovulation cycle could be the relative small number of animals used in this study.

The pregnancy rate for all the season was similar in both groups (hCG: 85.7%; C: 91.6%), these results are within the normal pregnancy rate range of 75–90% described by Pycock (2000).

The ability to maintain pregnancy was identical in hCG and in control mares as the same [P_4] pattern showed in both groups. Once mares were pregnant no differences were detected between pregnant subgroups. Nevertheless an important number of hCG mares (43%) were unable to get pregnant during the induced cycle.

Non-pregnant mares are often related to uterine pathology, or in rare cases, to primary luteal insufficiency (Ginther 1985). Both causes of pregnancy failure could be discarded, because mares were selected to exclude, as far as possible, uterine pathology as a cause of non-pregnancy. Added to this, primary luteal insufficiency, as a result of an under developed CL, is not involved in embryo loss before day 25 during the normal breeding season (Ginther 1992).

The important percentage of mares unable to get pregnant in the induced cycle suggests a possible negative effect of hCG over fertility in mares. The protocol used does not allow to solve this fact. However, an insufficient maturation of oocyte at ovulation time, when smaller preovulatory follicles were induced, could be considered as a possible cause. This assumption is based on a report from Goudet et al. (1998), which indicates that oocytes from bigger follicles at the end of oestrus are more mature than those from smaller ones. Consistent with Forde et al. (1987) suggestions that a combination of fertilization failure and early death of the fertilized ovum before it enters the uterus on day six is the major source of early reproductive wastage.

Results hereby presented support the conclusion that hCG-induced ovulation with dominant follicular size smaller than natural preovulatory size, does not affect CL development and function in early pregnant and in non-pregnant thoroughbred mares.

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