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Effect of hCG in the Presence of hCG Antibodies on the Follicle, Hormone Concentrations, and Oocyte in Mares

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Contents

Follicle blood flow, follicular-fluid and plasma hormone concentrations, and oocyte quality were studied 30 h after an ovulation-inducing hCG treatment when the pre-ovulatory follicle was 32 mm. Mares were grouped as positive (n = 16) and negative (n = 44) for hCG antibodies before the experimental hCG treatment. Percentage of the follicle wall with blood flow signals was less ($p < 0.05$) in the antibody positive group than in the negative group. The concentrations of follicular-fluid oestradiol and free IGF1, and plasma oestradiol were greater ($p < 0.001$), and follicular-fluid progesterone ($p < 0.001$) and plasma LH ($p < 0.02$) were less in the antibody-positive group than in the negative group. For recovered oocytes at 30 h (n = 37), the antibody-positive group had fewer ($p < 0.001$) mature (MII) oocytes than the antibody-negative group. Results were attributable to highly effective neutralization of the hCG in the antibody-positive group.

Introduction

Treatment with hCG is used frequently in mares when the pre-ovulatory follicle is 32–35 mm to induce ovulation in approximately 48 h or to induce maturity of the oocyte for collection by aspiration of follicle contents 28–36 h after treatment (reviewed in Ginther 1992). Owing to high molecular weight (36.5 kDa) and glycoprotein properties of hCG, antibodies may develop following hCG treatment during repeated oestrous cycles (Roser et al. 1979; Wilson et al. 1990). Some reports have indicated a reduction in the efficacy of hCG in inducing ovulations when given during several oestrous cycles (Sullivan et al. 1973; Wilson et al. 1990; McCue et al. 2004), whereas others have indicated that refractoriness does not develop from repeated hCG treatments (Loy and Hughes 1966; Michel et al. 1986; Gastal et al. 2006b). No information is available on the maturity and quality of collected oocytes after an hCG treatment in the presence of circulating hCG antibodies.

Increased follicle blood flow along with a rapid increase in LH at the terminal stage of follicle maturation is pertinent for meiosis resumption and completion of oocyte maturation. In women, perifollicular blood flow was estimated by Doppler ultrasonography prior to oocyte recovery and indicated that oocytes recovered from follicles with greater blood flow resulted in better embryos and more pregnancies after embryo transfer (Chui et al. 1997; Bhal et al. 1999, 2001; Coulam et al. 1999; Du et al. 2006). In mares, increased blood flow to the pre-ovulatory follicles 30 h after hCG injection was associated with greater pregnancy rate (Silva et al. 2006). The extent of follicle blood flow in hCG antibody-positive vs antibody-negative mares after hCG treatment is unknown.

Our previous study (Ginther et al. 2007) considered the relationships between ultrasound morphology of maturing follicles, expansion and apoptosis of cumulus and granulosa, blood flow of the follicle, and concentrations of follicular-fluid factors in relation to oocyte maturity 30 h after hCG treatment. The maturity of the recovered oocytes was determined by stereomicroscopic examination for the presence (mature) or absence (immature) of a polar body in the perivitelline space. Further examinations of the recovered oocytes were carried out to determine the meiotic stage by using immunofluorescent probes and confocal microscopy (Siddiqui et al. 2008). As a result, four oocytes that were initially categorized as immature by stereomicroscopic examination were found subsequently to be in the germinal vesicle stage or with obscured morphology and were classified as atypical. Greater follicular-fluid progesterone and lower oestradiol and free IGF1 concentrations were associated temporally with maturation of the oocytes. At the time of these studies, the presence or absence of hCG antibodies was not considered, owing to technical limitations.

The purpose of the present study in mares was to investigate the effect of an ovulation-inducing dose of hCG in the presence vs absence of hCG antibodies on blood flow of the pre-ovulatory follicle, follicular-fluid hormone concentrations, systemic oestradiol and LH concentrations, and maturity and quality of recovered oocytes at 30 h post-treatment.

Materials and Methods

Animals

Mares were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Information regarding experimental conditions, including feeding and management of the mares (large ponies), has been reported (Ginther et al. 2007). Briefly, mares (n = 60) in good body condition with a growing 28 mm follicle 15 days after ovulation were scanned daily by B-mode ultrasonography until a ≥ 32 mm follicle was detected. Each mare with a ≥ 32 mm follicle was treated with 2500 IU of hCG (i.v.). Doppler ultrasonographic evaluation of follicle blood flow was done just before collection of follicular-fluid and recovery of oocytes by transvaginal follicle aspiration 30 h after the experimental hCG injection. A total of 38 of the 60 mares were exposed to hCG one or more times between 18 and 125 days before the experimental hCG treatment.

Detection of hCG antibodies

Antibodies to hCG were assayed in the blood samples that were collected in the previous study (Ginther et al. 2007) immediately before the experimental hCG treatment (hour 0) and 30 h post-treatment (hour 30). The assay used a direct binding procedure as reported (Roser et al. 1979) with the following modifications. The hCG (5 μg; potency 11900 IU/mg; CR-125, National Hormone and Peptide Program, Torrance, CA, USA) was iodinated (^{125}I) by the iodogen method (Matteri et al. 1987). The reaction mixture was passed through Sephadex G-25 M (PD-10 column; Amersham Biosciences AB, Uppsala, Sweden) to recover the iodinated hCG. The plasma samples (50 μl) were pipetted into assay tubes followed by addition of 100 μl of phosphate buffered saline EDTA buffer (0.05 M, pH 7.4) and 75 μl of ^{125}I-labeled hCG solution (15 000 cpm). The tubes were vortexed and incubated at 4°C for 72 h followed by addition of 800 μl of 66% aqueous solution of dioxane (catalog # D111, Fisher Scientific, Fair Lawn, NJ, USA). A white flocculent precipitate formed immediately. The tubes were centrifuged at 1750 g for 30 min and decanted, and the precipitate in the tubes was counted in a gamma counter. Results were calculated as percentage ^{125}I hCG bound to plasma, thereby reflecting the amount of hCG antibodies in the plasma by the percentage of hCG-binding capacity.

Samples from two stallions and a filly that had not been exposed to hCG were used as negative controls; the percentage of non-specific hCG binding activity was ≤ 8% for each of the three animals. In addition, the samples from 22 of the 60 mares that were not exposed to hCG during the previous and present ovulatory season, according to retrospective examination of mare records, had a non-specific hCG binding capacity of 2–8%. Therefore, plasma samples of the mares with ≤ 8% binding capacity were considered negative for hCG antibodies. The remaining samples were from 38 mares that were exposed to hCG one or two times during the 125 days before the experimental hCG treatment. Sixteen of the 38 (42%) hCG-exposed mares had ≥ 57% hCG binding capacity at hour 30 and 22 had ≤ 8% binding capacity. Therefore, mares with ≥ 57% binding capacity were considered positive for hCG antibodies. In this way, mares were divided retrospectively into antibody-positive (n = 16) and antibody-negative (n = 44) groups.

Doppler ultrasonography of the follicles

Doppler ultrasonographic examination of the follicles was performed by using a duplex B-mode and pulsed-wave colour-Doppler ultrasound instrument (Aloka SSD-3500; Aloka, Wallingford, CT, USA) equipped with a finger-mounted 7.5-MHz convex-array transducer (UST-995-7.5). The colour and spectral Doppler ultrasonographic procedures have been described (Ginther and Utt 2004; Ginther 2007). Briefly, prior to oocyte recovery, the percentage of the follicle wall with blood flow signals was estimated while the entire follicle was being scanned in a slow continuous motion several times, as previously reported (Gastal et al. 2006a). The velocity end points from spectral Doppler ultrasonography of a vessel in the wall of the pre-ovulatory follicle were peak systolic velocity (PSV), end diastolic velocity (EDV), and time-averaged maximum velocity (TAMV) (Fig. 1). The vessels did not accommodate an angle cursor, and therefore the velocities were considered relative between groups rather than as actual velocities. The follicle blood perfusion end points were pulsatility index (PI) and resistance index (RI) and were computed from the velocities, as described (Ginther 2007). A Doppler spectrum of three cardiac cycles was generated (sample gate = 1.5 mm) from the most prominent colour spot of the follicle wall and one of the cycles was used for spectral measurements. The mean values of three such measurements were used in the statistical analyses.

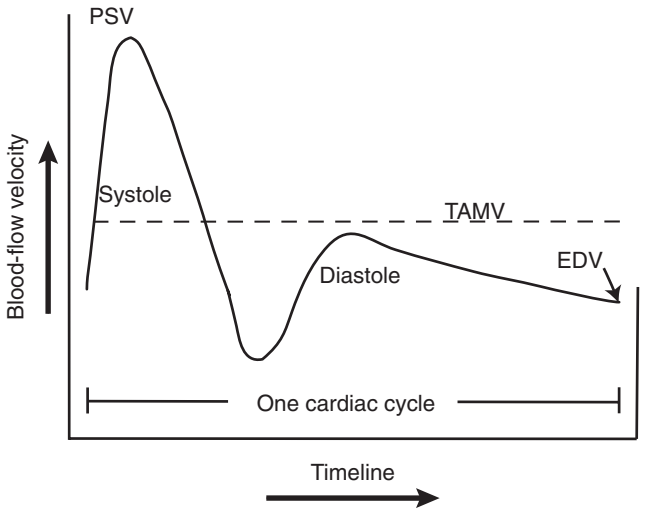

Fig. 1. Velocity spectrum (schematic) or an arterial pulse for a cardiac cycle showing locations of peak systolic velocity (PSV), time-averaged maximum velocity (TAMV), and end diastolic velocity (EDV). Adapted from Ginther (2007)

Collection of follicular-fluid and oocytes

Follicular-fluid and oocytes were collected by transvaginal follicle aspiration 30 h after the experimental hCG treatment as described (Ginther et al. 2007). Briefly, follicular-fluid was aspirated and the follicle wall of the aspirated follicle was lavaged with 180 ml of PBS per follicle. The aspirated follicular-fluid and lavaging fluid were held separately in a 37°C water bath. The follicular-fluid was searched for an oocyte or cumulus oocyte complex (COC). Then the fluid was centrifuged (500 g, 10 min), and 10 ml was stored at –20°C until assay. The lavaging fluid was filtered (70 μm, BD Falcon Cell Strainer; BD Biosciences Discovery Labware, Bedford, MA, USA) and searched for a COC or oocyte with a stereomicroscope (Bausch & Lomb, Inc., New York, NY, USA). The cumulus of the oocyte was removed by using 0.05% hyaluronidase and repeated pipetting. The denuded oocyte was fixed in microtubule stabilizing buffer by incubating for 1 h at 38°C and stored at 4°C until immunofluoroscent staining as described (Siddiqui et al. 2008).

Assays of follicular-fluid and systemic hormones

Oestradiol, progesterone, testosterone, and free IGF1 in follicular-fluid samples were assayed by radio- or enzyme-immunoassays, as validated in our laboratory for equine follicular fluid (Donadeu and Ginther 2002). The intra-assay CV and sensitivity for follicular-fluid oestradiol and testosterone, respectively, were 7.7% and 0.5 pg/ml and 1.9% and 0.01 ng/ml. The intra- and interassay CV and sensitivity for follicular-fluid progesterone and free IGF1 were 4.4%, 15.4%, and 0.06 ng/ml and < 1.0%, 9.4%, and 0.02 ng/ml, respectively. Plasma samples were collected in heparinized tubes just before treatment at hour 0 and just before follicle aspiration at hour 30. Plasma concentration of oestradiol was measured by a double-antibody radioimmunoassay kit, and LH concentration by radioimmunoassay, as described and validated in our laboratory for mare plasma (Donadeu and Ginther 2002; Ginther et al. 2005). The intra-assay CV and sensitivity for oestradiol and LH were 7.1% and 0.2 pg/ml and 7.3% and 0.2 ng/ml, respectively.

Plasma hCG concentrations were determined, using a two-site immunoradiometric assay kit containing capture antibody-coated tubes and ^{125}I-labeled detection antibody (DSL 8300; Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The kit-supplied standards (3–1000 mIU/ml) and experimental plasma samples were pipetted (50 µl) into antibody-coated tubes, mixed with 200 µl of assay buffer, and incubated for 30 min at room temperature. After decanting and washing the tubes with wash buffer, ^{125}I-labeled detection antibody (200 µl) was added to each tube, and the tube was vortexed and incubated for 30 min at room temperature. The tubes were decanted, washed with wash buffer, drained, and counted for 1 min in a gamma counter. Serial concentrations of hCG (15–1000 mIU/ml) in PBS containing 0.1% gelatin were processed as for the experimental samples and resulted in a displacement curve that was similar to the standard curve. None of the serial concentrations of equine LH (0.78–100 ng/ml) in PBS were detectable in the assay, indicating that the hCG assay did not cross-react with equine LH. The intra-assay CV and sensitivity were 15.7% and 0.2 mIU/ml, respectively.

Oocyte maturity and quality

An oocyte was recovered from 37 of 60 mares. The immunofluoroscent staining, confocal microscopy, and the criteria for oocyte development and quality were described previously (Siddiqui et al. 2008). Briefly, oocytes were immunolabeled with primary antibody (monoclonal mouse anti-α and -β-tubulins) followed by the secondary antibody (goat anti-mouse antibody conjugated with fluoroscein isothiocyanate), phalloidin conjugated with tetramethylerhodamine isothiocyanate, and Hoechst 33342, respectively, to visualize microtubules, microfilaments and chromatin. The oocytes were classified as metaphase I (MI, immature; n = 4), metaphase II (MII, mature; n = 29) and atypical (n = 4). Two of the atypical oocytes had an amorphous mass of microtubules near the condensed chromatin and may have been in the germinal-vesicle stage. The other two had a disorganized meiotic spindle, and it was not determined whether the oocytes were viable with capability for subsequent development. Comparisons of the frequencies of the three oocyte classifications were made between mares in the antibody-positive and antibody-negative groups.

Statistical analyses

End points for follicle blood flow and follicular-fluid and systemic hormone concentrations were compared between antibody-positive and antibody-negative groups by ANOVA (SAS, version 9.1.3; SAS Institute Inc., Cary, NC, USA). Frequency data for oocyte quality and maturity were analyzed by Fisher's exact test. A probability of $p \leq 0.05$ indicated that a difference was significant. Data are presented as the mean ± SEM, unless otherwise indicated.

Results

Each of the 16 mares that were positive for hCG antibodies did not have detectable hCG 30 h after the hCG treatment, whereas each of the 44 mares that were negative for hCG antibodies had detectable hCG at hour 30 (Fig. 2). The percentage of hCG binding capacity in the antibody-positive group at hour 30 (62.0 ± 2.8%) was less ($p < 0.02$) than at hour 0 (71.0 ± 3.0%).

Percentage of follicle wall with blood flow was significantly less for the antibody-positive group than for the negative group; data and probabilities are shown (Table 1). The spectral Doppler end points (PSV, EDV, TAMV, PI, RI) did not differ between the positive group and the negative group.

The mean values of the follicular-fluid hormone concentrations and the probabilities of a difference between the antibody-positive and negative groups are shown (Table 1). The follicular-fluid hormone concentrations were greater for oestradiol and free IGF1 and lower for progesterone in the antibody-positive group than in the antibody-negative group. Follicular-fluid testosterone concentration did not differ between groups. Plasma concentration of oestradiol at hour 30 was significantly greater and LH was significantly lower in the antibody-positive group (Fig. 2).

The oocyte-recovery rate between hCG antibody-positive (44%) and negative mares (68%) was not different ($p = 0.09$). The hCG antibody-positive group had significantly fewer MII oocytes and more oocytes were atypical than in the antibody-negative group; data and probabilities are shown (Table 1). The frequency of MI oocytes was not different between groups.

Discussion

The effect of exposure to a previous hCG treatment or the presence of hCG antibodies on the reliability of ovulation induction by hCG administration has not been resolved adequately (see Introduction). Most of the reports on the efficiency of hCG for induction of ovulation following previous hCG exposure did not have information on the presence of hCG antibodies. In

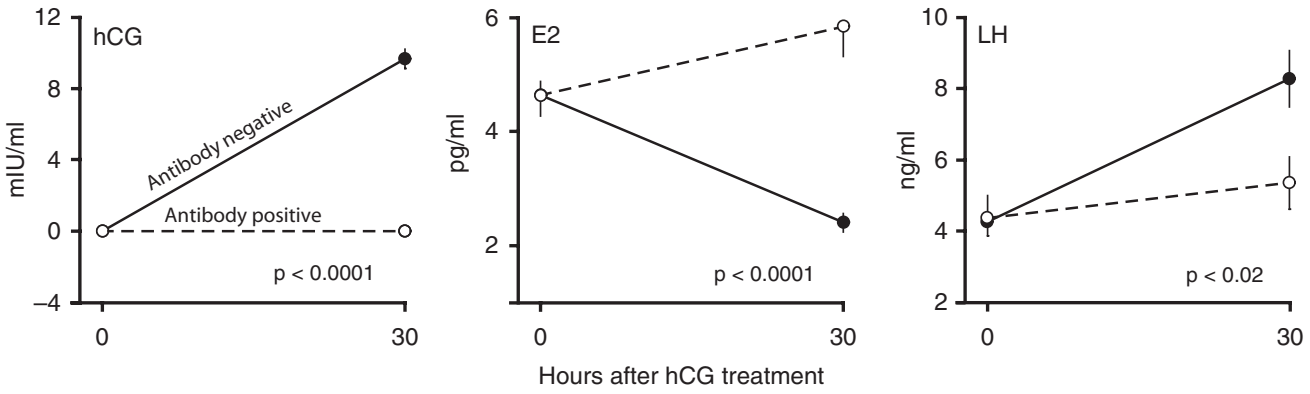

Fig. 2. Means (± SEM) for plasma concentrations of hCG, oestradiol (E2), and LH after treatment with an ovulatory dose of hCG when the pre-ovulatory follicle was ≥ 32 mm (hour 0) in mares that were positive for hCG antibodies (n = 16) vs negative for hCG antibodies (n = 44). The interaction of group and hour was significant for each end point, and the probability for difference between groups at hour 30 is shown

Table 1. Follicle haemodynamics, follicular-fluid and plasma hormone concentrations, and oocyte maturity and quality 30 h after hCG treatment in hCG antibody-positive mares vs antibody-negative mares

	Presence of antibodies to hCG		
Characteristics	Positive (n = 16)	Negative (n = 44)	p-values
Follicle blood flow (% of wall)	68.6 ± 4.3	76.0 ± 2.2	< 0.05
Spectral-Doppler end points			
PSV (cm/s)	9.7 ± 0.8	10.0 ± 0.5	NS
EDV (cm/s)	5.0 ± 0.4	5.3 ± 0.3	NS
TAMV (cm/s)	6.3 ± 0.5	6.8 ± 0.3	NS
PI (index)	0.70 ± 0.04	0.69 ± 0.03	NS
RI (index)	0.45 ± 0.02	0.46 ± 0.02	NS
Follicular-fluid hormones			
Oestradiol (ng/ml)	1153 ± 80	785 ± 30	< 0.001
IGF1 (ng/ml)	29.6 ± 3.6	11.4 ± 1.1	< 0.001
Progesterone (ng/ml)	440 ± 60	1245 ± 100	< 0.001
Testosterone (ng/ml)	15.5 ± 0.9	15.5 ± 0.5	NS
Maturity and quality of oocytes			
Mature (MII stage)	14% (1/7)	93% (28/30)	< 0.001
Immature (MI stage)	28% (2/7)	7% (2/30)	NS
Atypical	57% (4/7)	0% (0/30)	< 0.001

NS, not significant. PSV, peak systolic velocity; EDV, end diastolic velocity; TAMV, time-averaged maximum velocity; PI, pulsatility index; RI, resistance index.

one study (Wilson et al. 1990), the incidence of ovulation within 48 h after hCG treatment, despite the presence of hCG antibodies, was 34%. In the present study, 42% of 38 mares that were previously exposed to hCG before the experimental treatment were positive for hCG antibodies. Apparently, the variable effectiveness of repeated hCG treatment is attributable to production of antibodies only in some mares (e.g. 42%; present study), and successful ovulation induction in some mares despite high levels of antibodies (e.g. 34%; Wilson et al. 1990). However, further study is needed with various hCG doses and intervals from previous exposures, with measurement of the hCG antibody production for each mare.

The hCG antibodies were completely effective in neutralizing the hCG by 30 h, as indicated by non-detectable hCG in the 16 mares that were positive for antibodies. The 9% reduction in hCG binding capacity from 71% to 62% between hours 0 and 30 in the 16 mares provides an apparent indication of the amount of hCG that was bound by the antibodies. In this regard, it has been noted that the amount of hCG that could be bound by the amount of antibodies that are stimulated with repeated conventional hCG treatment is potentially much greater than the amount of hCG injected (Roser et al. 1979). In the present study, antibody tests were not made during the interval between hours 0 and 30, and the number of hours required for neutralization of hCG is not known. Information regarding the extent of antibody neutralization by various doses of hCG and time factors involved in antibody formation and hCG neutralization is an area of research with potential applied application.

The decrease in the percentage of the follicle wall with Doppler signals of blood flow in the antibody-positive group indicates that the antibodies neutralized the hCG so that hCG was not available for hastened development of the follicle and its oocyte. Increased blood flow in the pre-ovulatory follicle during follicle (Gastal et al. 2006a) and oocyte (Ginther et al. 2007) maturation following hCG treatment has been previously reported. The results are also consistent with the reports that the percentage of blood flow in the follicle wall was associated with better outcomes in assisted reproduction in women (Bhal et al. 1999, 2001; Coulam et al. 1999) or with improved pregnancy rate after natural breeding in mares (Silva et al. 2006).

The greater concentrations of oestradiol and free IGF1 and lower concentration of progesterone in the follicular fluid of the positive-antibody group vs the negative group are consistent with the stage of maturity of these same follicles and oocytes in the previous report (Ginther et al. 2007). The role of increased progesterone and decreased oestradiol and free IGF1 in the final oocyte maturation process was reviewed in the previous report (Ginther et al. 2007).

The reported (Ginther et al. 2007) reduced concentration of plasma and follicular-fluid oestradiol in mares with mature oocytes 30 h after hCG treatment is consistent with the lower oestradiol in mares that were negative for hCG antibodies in the present study. Treatment with hCG is known to reduce the

pre-ovulatory concentrations of oestradiol (Gastal et al. 2006a). The hCG-induced oestradiol decrease is related to the up-regulation and down-regulation of 17-β hydroxysteroid dehydrogenase enzymes in follicle cells of hCG-treated mares (Brown et al. 2004, 2007) and a decrease in mRNA expression for aromatase enzyme in the granulosa cells in cattle (Ndiaye et al. 2005). The previous report (Ginther et al. 2007) did not provide information on plasma concentrations of LH 30 h after hCG treatment. In the present study, the LH concentration was greater in the group that was negative for hCG antibodies. The increase in LH after hCG treatment in the antibody-negative group compared to the positive group is consistent with a report (Evans et al. 2006) that conventional hCG treatment resulted in an increase in LH within 24 h. The reduction in oestradiol after hCG treatment presumably favours an increase in the rate of output of LH, similar to the reported temporal relationships between oestradiol and LH before spontaneous ovulations (Ginther et al. 2006).

The maturity and quality of oocytes were adversely affected by the presence of circulatory hCG antibodies at the time of hCG treatment. In the seven mares with high hCG binding capacity (positive-antibody group), only one oocyte (14%) compared to 28 of 30 (93%) in the negative group was mature by 30 h after hCG treatment. The mature oocyte in the positive-antibody group may have been from a mare that would have ovulated soon after hour 30 without the aid of hCG treatment; if so, binding of the hCG would have been inconsequential. The cytoskeletal system of the atypical oocytes has been described (Siddiqui et al. 2008). Two of the atypical oocytes were in an apparent germinal vesicle stage. The viability of the other two atypical oocytes is not known, but they had not reached maturity, as indicated by non-detection of a polar body.

In conclusion, treatment with hCG in the presence of hCG antibodies prevented the ability of the hCG to increase blood flow of the follicle wall, lower the concentration of oestradiol and free IGF1 and raise the concentration of progesterone in the follicular fluid, lower the concentration of oestradiol and raise the concentration of LH in the plasma, and increase the maturity and quality of the oocyte. These results are interpretable on the basis of neutralization of the molecules in the administered hCG and indicate that a history of previous hCG treatment should be a consideration in using hCG for oocyte collection programs. For both hCG-induced oocyte maturation and follicle ovulation, the results indicated that development of hCG protocols that would reduce the incidence of antibody formation and a rapid and simple method for quantitating hCG antibodies would have practical applications.

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Author contributions

All the authors participated in the design of the study. Siddiqui MAR is the senior author of the manuscript, specifically participated in study design, oocyte collection procedure, processing and confocal microscopy, data analysis and drafting the manuscript. Gastal EL was involved in project planning Doppler ultrasonography, transvaginal follicle aspiration for obtaining follicular-fluid samples and oocyte, and revised the manuscript draft. Gastal MO was involved in B-mode and Doppler ultrasonography of the mares, plasma and follicular-fluid sample collection procedure and processing, preparation of figures and revision of the draft. Beg MA was responsible for hormone analysis including detection of hCG antibodies, revision of the draft. Ginther OJ was the group leader, supervised all aspects of the research work and revised the final version of the manuscript.

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