"Revised"

Repeatability of preovulatory follicular diameter and uterine edema pattern in two consecutive cycles in the mare and how they are influenced by ovulation inductors

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Abstract

Follicular diameter is used as a guiding tool to predict ovulation in the mare. However, the great range in preovulatory follicular diameter makes prediction of optimal breeding time based on follicular diameter unreliable. Uterine edema pattern is also useful to determine the best time to breed, since intensity of edema tends to dissipate as ovulation approaches, however not every mare follows this pattern. The aims of this study were to assess the repeatability of preovulatory follicular diameter and uterine edema pattern in two consecutive spontaneous cycles and to determine how induction treatments (hCG, $PGF_2\alpha$ and GnRH analogues) influence them. 53 mares were followed during two consecutive cycles and scanned 3 times a day from 2 to 3 days before ovulation. During the first cycle, mares had a spontaneous ovulation and in the consecutive cycle mares

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received either: a) no hormonal treatment; b) 1500 IU hCG; c) 125 to 250 µg Cloprostenol or d) 2.1 mg Deslorelin implant. Mares ovulated consistently from similar follicular diameters in two consecutive spontaneous cycles ($r = 0.89$; $P < 0.000$). All three induction treatments had a significant effect on reducing the preovulatory follicular diameter ($P < 0.005$). Mares showed fair correlation in uterine edema patterns in both consecutive non-induced cycles ($r = 0.71$; $P < 0.005$). In conclusion mares in consecutive cycles ovulated from consistent follicular diameters. Follicular diameters recorded from previous ovulations can be relied on to predict the optimal breeding time in successive cycles especially in mares that ovulate from unusually small follicles.

Key words: Mare; Follicular diameter; Repeatability; Uterine edema; Cycle.

1. Introduction

Follicular diameter is largely used in practice as a guiding tool to predict ovulation in the mare. It is the simplest and probably most extensively used clinical criterion. Some work has claimed follicular diameter to be a good predictor of time of ovulation [1] when using a certain type of breed. However the great range of follicular preovulatory diameter in the mare, 24 h prior to ovulation from 34 to 70 mm [2], 22 to 65 mm (Newcombe unpublished) renders this criterion unreliable to estimate the optimal breeding time. This large range is mainly due to factors such as time of year [3] with larger follicles early in the season (April to June), number of preovulatory follicles (double preovulatory follicles are smaller than single [3]), breed [2] (large draught breeds such as Clydesdale, Shire, Irish Draught, Welsh Cob with larger follicles than ponies, Thoroughbred (TB) or Standardbred) and individual variation within a breed (Newcombe unpublished). More recently, it has

been shown that induction of ovulation with hCG is hastened from smaller preovulatory follicles than in non-induced cycles [4].

The use of follicular size could only be useful as guide when not to mate after ovulation. A field study showed that none of 181 mares ovulated before reaching a follicle diameter of 35 mm, [5]. However, if 35 to 40 mm were the cut point for mating, mares with preovulatory follicles reaching > 50 mm would be several days away from ovulation. There exists the common belief that the diameter of the preovulatory follicle tends to be similar to that of the previous cycle. Therefore, if records were available of previous cycles for any given mare it could be good practice to rely on the diameter of the last follicle recorded before ovulation was detected at previous cycles. However, there is to date no scientific evidence to support such breeding management.

Uterine edema pattern is also used by some clinicians to aid the estimation of optimal breeding time. The ultrasonic anatomy of the uterus is thought to be dependent on the prevailing circulating levels of ovarian steroids [5,6]. The uterus during the follicular phase develops engorged endometrial folds which result in a typical ultrasonic image, so called “cart wheel” effect that represents the alternate and intertwining of echogenic and hypoechogenic areas. Uterine edema usually becomes less prominent as ovulation approaches [6-7-8]. Clinical observations however show that this pattern is not followed by every mare, and some mares may ovulate when still showing prominent uterine edema, conversely others may not show any uterine edema in the preovulatory period [8]. There have been few studies on uterine edema pattern, and there seems to be a lack of understanding on factors affecting it, which may account for its individual variation in the mare. Similarly, there is no evidence whether mares follow consistent patterns in subsequent cycles.

The aims of this study were two fold: a) to assess the degree of repeatability in diameter of the preovulatory follicle and uterine edema pattern in two non-induced consecutive cycles in a mixed population of mares; and b) to investigate whether this repeatability is affected by hormonal induction treatments (human chorionic gonadotropin, Deslorelin a GnRH analogue and Cloprostenol, a $PGF_2\alpha$ analogue). A secondary objective was to determine whether mares have preference to ovulate at any particular time of the day and whether this preference will be consistent in consecutive cycles.

2. Materials and Methods

2.1. Animals

A total of 53 mares from various breeds (TB, Standardbred, Irish Draught, and Warmblood), age-range from 2 to 26 years during the breeding season 2007 (March to August) were used in the study. The animals belonged either to the Warren House equine fertility clinic (resident mares used as recipients for commercial embryo transfer) or to private clients (mares as donors for embryo transfer or for AI) which were temporarily resident at the clinic. Mares were pastured while diestrus and in early estrus and were stabled as ovulation approached so they could be examined 3 times a day. Mares were fed grass while at pasture and on hay, cereal concentrate and beet pulp while stabled.

2.2. Experimental design

All mares used in the study were followed for at least 2 consecutive cycles which included transrectal ultrasound examinations at 8 h intervals for at least 24 h prior to ovulation. All 53 mares had one spontaneous cycle (in which no hormonal treatment

was used to induce ovulation). In the following cycle, mares were allocated to one of the following treatment groups:

- Control group (n = 20): no hormonal treatment was used to induce ovulation as in first cycle (spontaneous cycle).
- hCG-induced cycle (n = 14): mares were administered 1500 IU of hCG (Chorulon®) subcutaneously when they were in estrus with a follicle > 35 mm (all follicular diameters at the time of hCG induction ranged from 35 to 40 mm).
- $PGF_2\alpha$-induced cycle (n = 13): mares were given 125 to 250 µg of Cloprostenol (0.5 to 1 ml of Estrumate®) while in diestrus and with presence of a diestrous follicle ≥ 25 mm. (follicular diameters at the time of $PGF_2\alpha$ treatment ranged from 25 to 37 mm). Different dosage of Cloprostenol varied upon clinical grounds (1 ml dose was used in 4 mares to lyse corpora lutea aged 6 o 7 days, whereas 0.5 ml dose was used to induce ovulation in mares with corpora lutea older than 8 days).
- Deslorelin-induced cycle (n = 6): mares were subcutaneously implanted with 2.1 mg Deslorelin-implant (Ovuplant®) while in estrus with presence of a follicle > 30 mm (follicular diameters at the time of Deslorelin implant ranged from 30 to 35 mm).

Only mares that had single ovulations in both consecutive cycles were included in the study.

2.3. Ultrasound examination

Data used in the study (follicular diameter, uterine edema score and time of ovulation) were obtained by transrectal ultrasound performed with an ultrasound machine (Midray ®) equipped with a 7.5 MHz linear probe. Examinations were

performed every other day until the mare was known to be in estrus with a follicle larger than 30 mm. A mare was considered to be in estrus when she showed visible uterine edema, positive teasing behaviour and relaxed cervix by manual vaginal examination. From then on mares were examined daily until approaching ovulation (usually until 2 to 3 days prior to ovulation) and from then every 8 h until ovulation was detected. Decision when to examine 3 times a day was made upon by assessment of follicular consistency at rectal palpation and after mares had been given hCG or Ovuplant.

2.4. Follicular diameter and ovulation

Follicular diameter was obtained from average of 2 linear measurements of the antrum taken at right angles when the image of the follicle was maximum as described by [3] using the electronic callipers. For data analysis and to minimize error in measurement, preovulatory diameter was obtained from the average of follicular diameters at each of the last three examinations before ovulation (it is known that follicular diameter remains constant in the last 36 to 24 h prior to ovulation [1]. Often at the last examination before detection of ovulation, a follicle with irregular shape and/or collapsing was difficult to measure accurately. On these occasions, data for that reading were excluded from the average and only the previous two examinations were used.

Ovulation was detected as per rectal palpation and ultrasonography: absence of the previously recorded follicle and presence of a hypoechoic area within the same ovary. The time of ovulation was recorded as having occurred either by 8 a.m., 4 p.m. or 12 a.m. For data analysis of ovulation time, cycles induced with hCG and

148 Ovuplant were not included in the analysis as the time of treatment may determine
149 time of ovulation.
150
151 *2.5. Uterine edema*
152 Uterine edema was subjectively assessed visually as per transrectal ultrasonography
153 of the horns and uterine body. Uterine edema scores were given on a scale from 0 to
154 3 in increments of 0.5 depending on the size and prominence of endometrial folds.
155 The score of 0 corresponded to that of a diestrous-like echotexture with no
156 ultrasonographic presence of endometrial folds and a score of 3 was given to mares
157 with maximal endometrial folding. Four readings of uterine edema score were taken
158 for each mare around the preovulatory period (one every 8 h for the last 24 h prior
159 to, and at the time of ovulation). Preovulatory uterine edema pattern was compared
160 between consecutive cycles within each mare taking into account any effect of
161 treatment.
162
163 *2.6. Statistical analysis*
164 Paired t-test was used to analyse effect of induction treatments on preovulatory
165 follicular diameter. Strength of agreement (repeatability) between preovulatory
166 follicular diameters of two spontaneous (non-induced) consecutive cycles was
167 assessed by Pearson's correlation and probability plot.
168 Chi-square analysis was used to assess the preferred time of ovulation and its
169 repeatability between consecutive cycles.
170 Strength of agreement of uterine edema pattern between non-induced consecutive
171 cycles was estimated by Spearman's ranked correlation analysis.
172

3. Results

Mares ovulated consistently from similar preovulatory follicular diameters in two consecutive spontaneous cycles ($r = 0.89$; $P < 0.000$, **Fig. 1**). The mean and median difference in follicular diameter between the two consecutive cycles was - 0.3 mm and 0.0 mm respectively. The probability plot (**Fig. 2**) showed that 60 % of the mare population will ovulate in a consecutive cycle from a single follicle of a diameter as little as - 0.8 to 1.7 mm (95 % CI) difference when compared with the previous cycle (rest of percentiles are shown in **Table 1**).

Induction with hCG, $PGF_2\alpha$ and Ovuplant had a significant effect on reducing the preovulatory follicular diameter ($P < 0.001$, $P < 0.000$ and $P < 0.005$ respectively) when compared with the non-induced consecutive cycle (**Fig. 3**). Mean preovulatory follicular diameters for each group are shown in **Table 2**.

Mares induced with $PGF_2\alpha$ took an average of 6.7 ± 0.7 days from treatment to ovulation. Cloprostenol dose did not affect either length of interval to ovulation or follicular diameter difference between the induced and non-induced cycles ($P > 0.05$). Mares induced with hCG and Ovuplant took 60 ± 8.9 h and 39.7 ± 2.3 h from treatment to ovulation respectively.

Mares did not consistently ovulate at the same time of the day in consecutive cycles ($P > 0.05$). Of 33 mares analysed, 14 (42 %) ovulated in the same time period in both consecutive cycles, whereas 19 (58 %) did not.

There was no significant ($P > 0.05$) tendency of mares to ovulate at any particular time of the day. Of all cycles analysed ($n = 86$) 34.9, 39.5 and 25.6 % ovulated by 8 a.m., 4 p.m. and 12 a.m. respectively.

Mares showed a fair correlation in uterine edema patterns in both consecutive non-induced cycles ($r = 0.71$; $P < 0.005$). Mares induced with any of the treatments

showed higher edema scores than when non-induced (**Fig. 4a)**, this tendency was only significant for the $PGF_2\alpha$-group ($P < 0.03$) (**Fig. 4b**).

4. Discussion

The main objective of this study was to show whether mares ovulated consistently from similar follicular diameters in consecutive cycles. Although it is a common practice to look back to records of preovulatory diameter from previous cycles to aid the estimation of optimal breeding time, the literature had to date no evidence to prove this. Therefore this study is the first to show a good degree of repeatability in follicular size between consecutive cycles. Although some mares had a discrepancy in diameter of more than 5 mm (n = 3), most of mares ovulated follicles within 3 mm difference in consecutive cycles (n = 17) (**Fig. 2**). Provided the huge range in preovulatory follicular diameters of the mare population (34 to 70 mm) [2], and in our study (ranging from 36 to 59 mm), an existing record of previous cycles is useful for the practitioner, especially in those mares that ovulate from unusually small follicles.

Recent work has also shown a profound effect of some hormonal treatments (hCG) which provoked cessation of follicular growth after induction, therefore hastening ovulation from follicles of smaller diameter than those in spontaneous cycles [4]. Our data is in agreement with the latter study and mares induced with 1500 IU of hCG ovulated from significantly ($P < 0.001$) smaller follicles. These mares received the induction treatment when they had a follicle of 35 to 40 mm. Follicles induced with hCG ovulated from an average diameter of 38.8 ± 0.83 mm compared with 44.2 ± 1.22 mm in spontaneous cycles (**Table 2**). Hence hCG had a great effect on stopping follicular growth since most mares (n =11) did not grow follicles larger

than 42 mm between induction and ovulation. In 3 mares however, hCG failed to stop follicular growth and these mares took an average of 120 ± 12 h from induction to ovulation when compared with 44 ± 1.6 h in the other hCG-induced cycles. It is worth noting that each of these 3 mares reached preovulatory diameters of > 50 mm after hCG treatment. The difference in follicular diameter of these 3 mares between hCG-induced and spontaneous cycles was ≤ 4 mm.

In the same way, Deslorelin implants (Ovuplant) induced ovulation from significantly smaller follicles in treated cycles when compared with spontaneous cycles (**Table 2**). It was not surprising that Ovuplant provoked a similar effect in stopping follicular growth like hCG as the implant acts as GnRH agonist. The subsequent release of LH would have the same effect on the follicle as hCG. Mean follicular diameter in the Ovuplant group was the smallest (34.2 mm, **Table 2**) in part due to the smaller range in follicular diameter at the time of treatment (30 to 35 mm). Although only 6 mares were given Ovuplant, all of them responded even from small follicles, and ovulated in 39.7 ± 2.3 h from treatment. These results support the clinical evidence of the reliability of Ovuplant to induce ovulation from smaller follicles (< 35 mm).

Most striking was however, the effect of Cloprostenol on inducing ovulation from follicles of smaller diameters (**Fig. 3**). There is no reference in the literature of such effect. There is though, evidence of the ovulatory effect of $PGF_2\alpha$ and its analogues in several species [9-10-11-12] including equine [13-14-15]. It was shown that Luprostiol (a $PGF_2\alpha$ analogue) had the ability to induce a release of LH, FSH and GnRH into the pituitary and peripheral venous blood in mares [16]. The mechanism by which Cloprostenol was able to reduce preovulatory follicular diameter in induced-cycles is not known, but ability to induce a release of GnRH might result in

248 a similar effect to that of hCG and Ovuplant. The $PGF_2\alpha$-induced group had a
249 greater variation in preovulatory follicular diameters from induction to ovulation
250 than the hCG and Ovuplant groups (S.E.M. 1.77, 0.83 and 0.67 for the $PGF_2\alpha$, hCG
251 and Ovuplant groups respectively, **Table 2**). This larger variation in the $PGF_2\alpha$
252 group might account for the greater follicular diameter range of the largest follicle at
253 the time of treatment (25 to 37 mm) and variation in length of interval from $PGF_2\alpha$
254 treatment to ovulation (interval ranged from 2 to 10 days). Although we have
255 previously shown (unpublished data) that Cloprostenol dose has an effect on interval
256 to ovulation, the difference between doses used in this study probably was not
257 sufficient to significantly affect the interval length in such a small number of mares.
258 Some workers have reported that ovulation tends to occur at night [17], and there is
259 the common belief that mares are animals of nocturnal ovulation. The latter study
260 only included data from 7 mares during 3 or more estrous cycles (n = 25 cycles).
261 Although 23 out of 25 mares ovulated between 11 p.m. and 7 a.m. it is a bold
262 statement to say that mares have preference for nocturnal ovulation. Indeed, other
263 studies [18,19] could not find such association nor could this one ($P > 0.05$).
264 Although there was a slight tendency of mares ovulating during the period 8 a.m. to
265 4 p.m. (39.5 % of all cycles analysed), this association was not significant ($P >$
266 0.05). From the results of this study it can be concluded that mares do not have
267 preference to ovulate at any particular time of the day nor follow the same patter in
268 consecutive cycles.
269 The uterine edema pattern of two spontaneous consecutive cycles was shown to be
270 fairly correlated ($r = 0.71$, $P < 0.005$). Uterine edema intensity tended to dissipate as
271 ovulation approached (**Fig. 4a**) for all treatment and spontaneous groups. This is in
272 agreement with other studies [6-7-8]. The reason why edema disappears before

ovulation seems to be that the increasing amount of preovulatory estrogens up-regulates the endometrial progesterone receptors allowing the progesterone to work through these receptors to dissipate edema prior to ovulation [6]. The preovulatory source of progesterone is intrafollicular which increases in concentration in preovulatory follicular fluid as ovulation approaches [20]. As endometrial progesterone receptors are up-regulated by estrogens, they are able to respond to low amounts of progesterone and subsequently dissipate uterine edema [6]. The reasons for variation in uterine edema pattern within mares and why they tend to be consistent in consecutive cycles needs to be elucidated, but perhaps individual variation in edema scores accounts for different steroids concentrations in individual mares which then remain consistent in consecutive cycles.

An interesting observation was that treatment of mares in consecutive cycles with induction agents increased uterine edema scores in the peri-ovulatory period when compared with non-induced cycles (**Fig. 4a**). This increase was only significant in the $PGF_2\alpha$-induced group ($P < 0.03$) (**Fig. 4b**). The mechanism by which these treatments affect uterine edema pattern remains unknown. A possible explanation could be at the endometrial progesterone receptor level which might be affected by difference in either steroids concentrations or length of period under its influence due to effect of ovulatory treatments.

In conclusion mares are likely to ovulate from similar follicular diameters and have consistent uterine edema patterns around the peri-ovulatory period in two spontaneous consecutive cycles. Ovulation induction treatments have an important effect on reducing the preovulatory follicular diameter.

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Tables

Table 1. Percentile values of mares (n = 20) ovulating in two consecutive non-induced cycles.

percentil	lower bound	follicular diameter difference (mm)	upper bound
10	- 5.9	- 4.1	- 2.3
20	- 4.3	- 2.8	- 1.3
30	- 3.2	- 1.9	- 0.5
40	- 2.4	- 1.1	0.3
50	- 1.6	- 0.3	1.0
60	- 0.9	0.5	1.8
70	- 0.1	1.3	2.6
80	0.7	2.2	3.7
90	1.7	3.5	5.3

Each percentile value represents the percent of mares that will ovulate from preovulatory follicles in two consecutive cycles with a difference in diameter (mm) equal or less to the value (follicular diameter difference) shown in the table. Upper and lower bounds show the range in difference of follicular diameter with a confidence interval of 95 %.

Table 2. Mean ± S.E.M. preovulatory follicular diameter for spontaneous (sp) and induced cycles.

non-induced		hCG		$PGF_2\alpha$		Ovuplant	
sp	sp	sp	induced	sp	induced	sp	induced
46.03^a	46.33^a	44.18^a	38.82^c	47.77^a	39.42^d	40.92^a	34.25^b
± 1.42	± 1.34	± 1.22	± 0.83	± 2.0	± 1.77	± 1.38	± 0.65

Different superscripts show significant difference within groups: $P < 0.005$ (a,b), $P < 0.001$ (a,c) and $P < 0.000$ (a,d). Non-induced (n = 20), hCG-induced (n = 14), $PGF_2\alpha$-induced (n = 13) and Ovuplant-induced (n = 6).

Illustrations

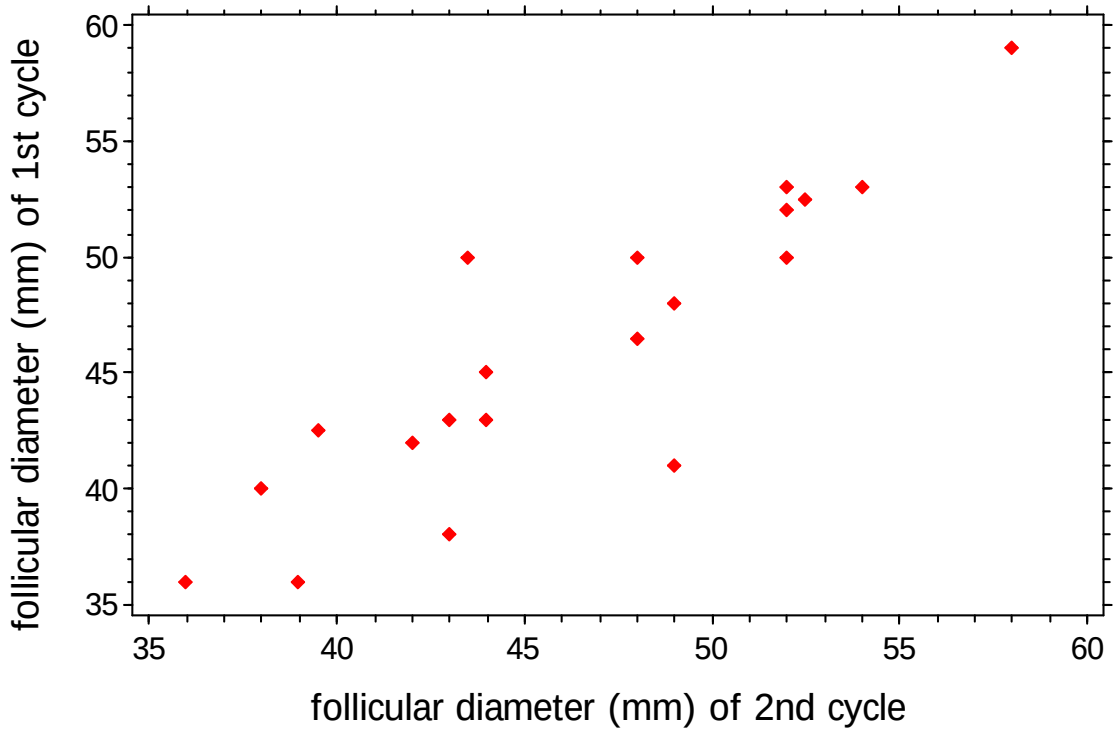

Fig. 1. Scatter plot of preovulatory follicular diameters in two consecutive spontaneous cycles. Each symbol represents the preovulatory follicular diameter (mm) for two non-induced (spontaneous) consecutive cycles (n = 20). Pearson's correlation coefficient showed a good level of agreement between preovulatory follicular diameters of two consecutive cycles ($r = 0.89$; $P < 0.000$).

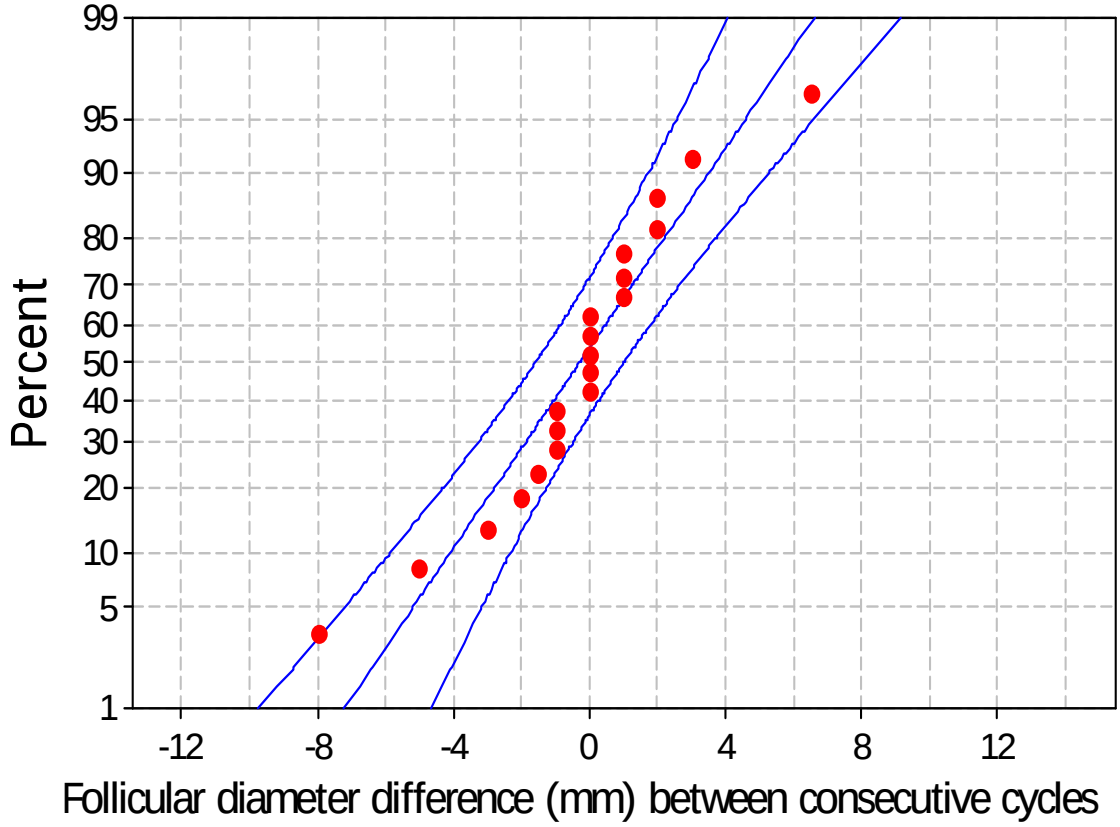

Fig. 2. Probably plot of follicular diameter differences between two non-induced consecutive cycles (n = 20 mares). Each point represents the difference (mm) between follicular diameters of two consecutive spontaneous cycles. Outer lines show the upper and lower bounds for expected follicular diameter difference of two consecutive cycles in the mare population (CI 95 %).

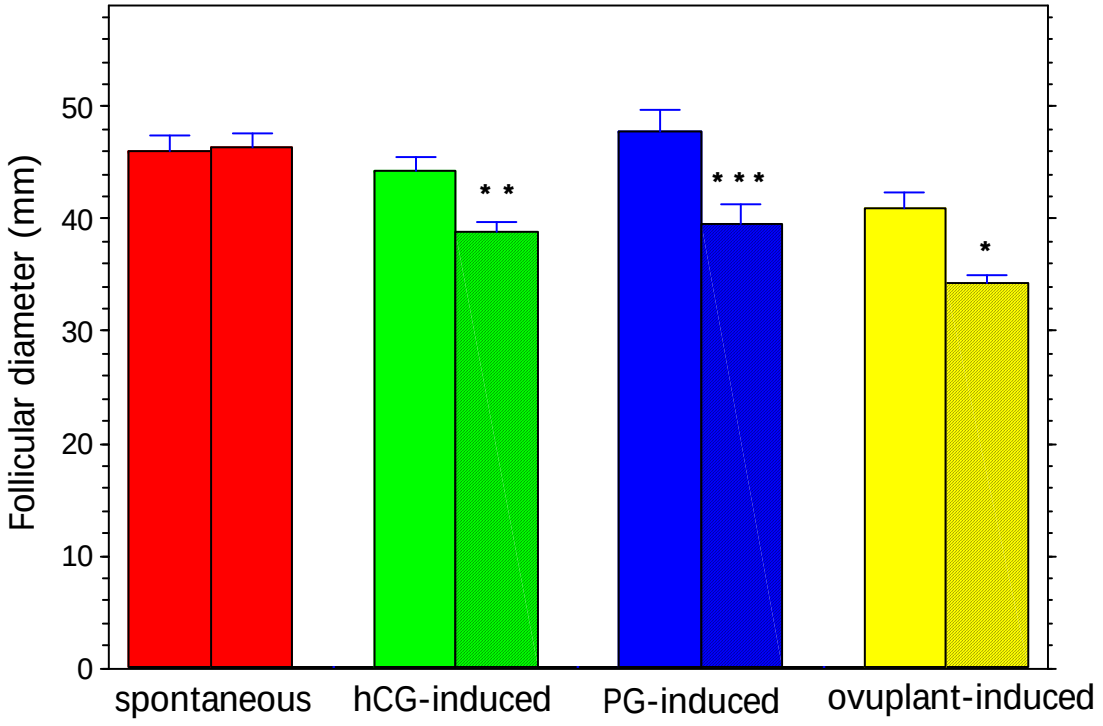

Fig. 3. Effect of hCG, $PGF_2\alpha$ (PG) and Ovuplant on preovulatory follicular diameter. Bars represent mean follicular diameter ± S.E.M. (whiskers) for each treatment group. Each treatment group includes one spontaneous cycle (non-striped bars) and one induced cycles (striped bars) with hCG, $PGF_2\alpha$ and Ovuplant. For each treatment group both cycles were consecutive. The spontaneous group is formed by two non-induced cycles. Significant difference is shown as $P < 0.005$ (*), $P < 0.001$ (**) and $P < 0.000$ (***).

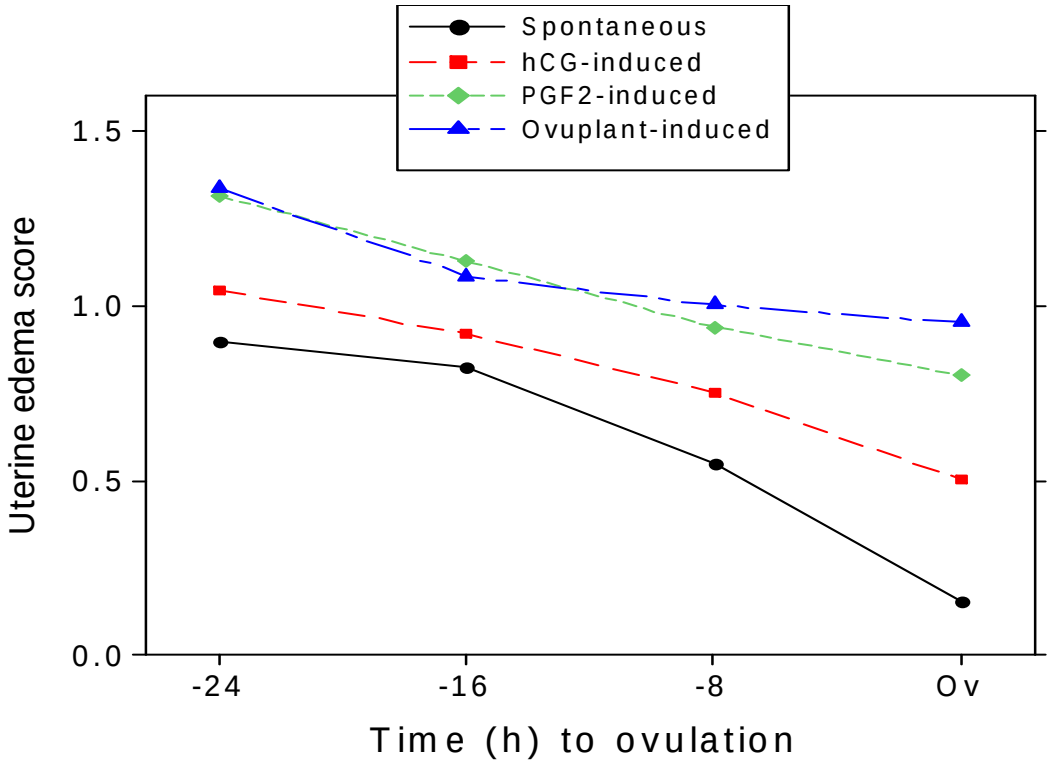

Fig. 4a. Uterine edema score in the preovulatory period. Lines connect mean uterine edema scores for each examination point (24, 16, 8 h prior to ovulation and when ovulation (Ov) was first detected). The "spontaneous" line connects mean uterine edema scores of all non-induced cycles for all groups (n = 73).

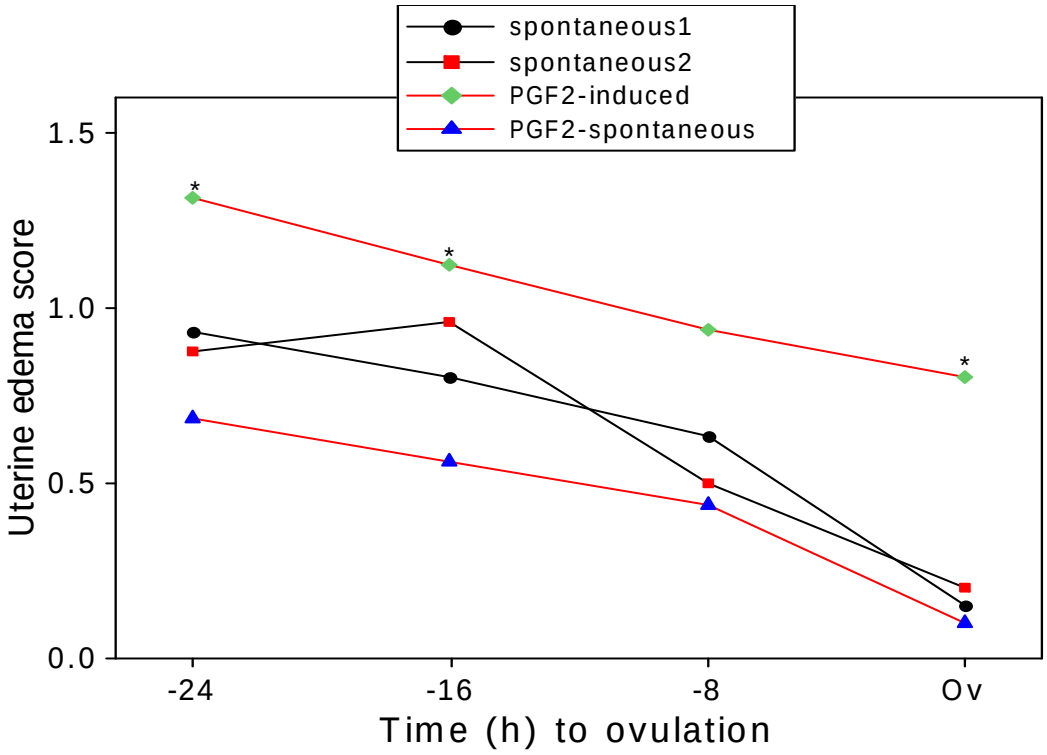

Fig. 4b. Uterine edema scores in the preovulatory period of mares with spontaneous cycles and induced with Cloprostenol ($PGF_2\alpha$). Each line connects mean uterine edema scores for each examination point of two consecutive cycles for the spontaneous (inner lines) and $PGF_2\alpha$-induced groups (outer lines). Significant difference between examination points of consecutive cycles within the same group (PGF2α-induced versus non-induced) is shown as (*) $P < 0.05$.