Risk factors for the development of haemorrhagic anovulatory follicles (HAFs) in the mare

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Abstract

Haemorrhage into the dominant follicle during the reproductive season is a subtle but definitive cause of infertility in the mare population. This condition however can be of high relevance for an individual in which its incidence is abnormally high. Little is known about the nature and factors affecting the incidence of haemorrhagic anovulatory follicles in the mare. The objectives of the study were to define and characterize the ultrasonographic development and incidence of haemorrhagic anovulatory follicles and to investigate possible risk factors influencing its occurrence. Detailed reproductive and ultrasound records of seven mares studied during their entire reproductive lives (> 10 years and 612 oestrous cycles) were analysed retrospectively and computed into a statistical mixed model. Of all animal studied, two mares were found to have an unusually high incidence of haemorrhagic anovulatory follicles of about 25 %. Time of

season and use of induction treatments (Cloprostenol) were found to influence its incidence. It appears that early-enhanced stimulatory effect of LH on an ovary with presence of small and immature follicles might increase the risk of ovulatory failure of those follicles later in the cycle. Mares during the months of highest follicular activity (May to August) and after treatment with hormones to induce oestrus and ovulation are at greater risk to develop haemorrhagic anovulatory follicles. The potential relevance of this study is two folds: clinical relevance for the practitioner to better understand this condition and so improve reproductive management of mares with abnormally high incidence; and to provide useful insights for researchers willing to further investigate the nature of this phenomenon.

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Introduction

Failure to ovulate the dominant follicle and subsequent persistence of the anovulatory unruptured structure has been reported to occur in several domestic animal species and in women. The species in which this phenomenon has been demonstrated as naturally occurring are cows (Garverick 1997; Peter 2004), mares (Newcombe 1987, Ginther 1992; Ginther *et al*. 2007) and women (Marik and Hulka 1978; Katz 1988; Zhu 1989; Toda 1990). Research studies on methods of contraception and ovulatory process have also shown experimentally-induced unruptured follicles in rabbits (Salhab *et al.* 2003; Grinwich *et al.* 1972), rats (Armstrong and Grinwich 1972) and women (Killick and Elsein 1987). It seems that this phenomenon might occur naturally in all domestic species, however only in species like the cow and the mare in which follicular dynamics

are easily and routinely followed ultrasonographically, allow sufficient number of observations to characterize this syndrome.

A distinct cause of ovulatory failure observed in the mare is haemorrhage of the dominant follicle(s) with subsequent organization of follicular contents and, in most occasions, luteinization of follicular wall without previous follicular collapse. This condition has been referred to in different ways: first reference to what seemed to be the same sort of follicle was back in the 40's (Burkhardt 1948) where it was described as occurrence of large persistent follicles during the months of October to November. Later in the century they were given the name of "autumn follicles" (Knudsen and Weiert 1961) as they were reported to occur more frequently at the end of the ovulatory season. Ginther (1979) was the first to describe the macroscopic features of this anovulatory follicle which was defined as blood-filled structures with presence of luteal tissue in the surrounding wall and so termed "haemorrhagic follicles". With the advance in imaging techniques in the 80's, several studies reported occurrence of this anovulatory condition in different population of mares (Ginther and Pierson 1984 and 1989; Townson and Ginther 1988; Carnevale *et al*. 1989; Ginther 1992).

The fate of the haemorrhagic follicles will further classify them into luteinized or non-luteinized unruptured follicles depending on the degree of luteinization of granulosa cells and ability of secreting progesterone as evidenced by macroscopic and hormonal studies (McCue and Squires 2002). The latter study named them as "persistent anovulatory follicles" and found that approximately 90 % of them developed luteal tissue. Assessment of echodensity and thickness of granulosa layer of anovulatory follicles viewed on ultrasound can be used to differentiate presence of luteal tissue. In fact, human studies based on ultrasound and histology evidence have reported the development and ultrasonographic appearance of luteinized follicles in a similar way to

that described in mares (Coualaum *et al*. 1982; Toda 1990). This condition in women was termed luteinized unruptured follicle (LUF) syndrome and resembles that seen in mares.

Most recently controlled studies have shown in detail hormonal profiles and Doppler ultrasonographic characteristics of the development of haemorrhagic anovulatory follicles in the mares (Ginther *et al*. 2006; Ginther *et al*. 2007). The later studies found only subtle differences in follicular wall vascularity between ovulatory and haemorrhagic follicles during the 3 days prior to ovulation / beginning of haemorrhage. Hormonal profiles on LH, FSH and progesterone did not however reveal any significant difference.

The main relevance of this condition lies in the failure of collapse of the dominant follicle with consequently no release of the oocyte and therefore impossibility of fertilization and pregnancy unless it is accompanied by a normal ovulation of another follicle. However, due to the low incidence of haemorrhagic anovulatory follicles (HAFs) reported as low as 5-8 % (Ginther and Pierson 1989; McCue and Squires 2002) the overall impact on fertility is low. Nevertheless it can be frustrating for the practitioner when dealing with pre-ovulation breeding (natural mating and AI with chilled semen) when the mare is bred on the basis of a normal dominant follicle which then fills with blood and luteinize without rupturing, leaving that cycle with no chance of conception. The relevance of this condition on fertility can increase dramatically in individuals that tend to have reoccurrence of HAFs in subsequent cycles (Ginther *et al*. 2006).

The mechanisms of development of HAFs in the mare remain unclear. It has been proposed that the incidence is higher during the autumn months and in mares aged > 20 years (Ginther *et al*. 2007).

To better understand this phenomenon in the equine species, this study focuses on risk factors affecting the development of haemorrhagic anovulatory follicles. In order to do so, detailed reproductive ultrasonographic records of 7 individual mares' breeding lives (> 10 years) were analysed retrospectively.

Materials and methods

Animals

Clinical records from 7 mares (aged from 12 to 26 years) were analysed for > 10 years (range 10 to 18 years) from 1990 to 2007 (a total of 612 oestrous cycles; Table 1). These mares were at least followed at 24 h intervals during the peri-ovulatory period but often 3 times a day. The mares were either resident at a veterinary clinic (used as donor and recipient mares for embryo transfer programme or other reproductive procedures). The mares were bred in the clinic and were mostly Irish Draft (mare details are shown in Table 1).

Records

Records were obtained from transrectal ultrasonographic examinations performed at least once daily during oestrus and up to three times daily as ovulation approached. The ultrasound equipment changed over the years, but was equipped always with a linear probe of 7.5 MHz. All observations were taken by the same operator. The use of hormonal treatments to induce oestrus and ovulation (Cloprostenol and/or hCG) was recorded in every case. The end points recorded were:

Ovulation: detected as per rectal palpation and ultrasonography by absence of the previously recorded follicle and presence of a hypoechoic area within the same ovary as described by Newcombe (1996). Confirmation of ovulation was by the later presence of an echoic CL. The date of ovulation was recorded as the day in which it was first detected. An ovulation could be classified in three categories:

- Spontaneous: when no hormonal treatment had been given since the previous ovulation.
- PG-induced: when oestrous signs and ovulation followed the administration of ~~Cloprostenol (~~a PGF analogue~~)~~ Cloprostenol ~~during dioestrus~~ (Estrumate®, Schering-Plough Animal Health Ltd, Welwyn Garden City, AL7 1TW, UK~~;)~~ ~~(dose ranged from~~ 25 µg to 1 mg~~, of Cloprostenol given~~ subcutaneously). This hormonal treatment was used to lyse CL and/or luteinized follicles in order to induce oestrus. The variation in dose was due to parallel clinical trials involving different Cloprostenol doses. An ovulation was classified as PG-induced if the administration of Cloprostenol was followed by clinical signs of luteolysis (endometrial oedema, oestrus behaviour and/or cervix relaxation). The interval from Cloprostenol administration to ovulation could be short but was always < 11 days.

Haemorrhagic anovulatory follicle (HAF): detected by transrectal ultrasonography as described in Ginther (1992). In brief, the previously fluid-filled follicle of anechoic echotexture fills with echogenic specks which float freely in the follicular fluid ~~(Figure 1a)~~ and swirl if balloted, and without follicular collapse the granulosa layer becomes increasingly echodense and deeper. ~~(Fig 1d).~~ The number and echodensity of the intrafollicular specks increase but still have a mobile/swirling appearance. The follicle diameter increases and eventually the contents acquire a static organised appearance (Fig 1a-f~~f~~). Luteinization of the follicle was assumed when follicular wall became

highly echoic and 'thickened', the uterus acquired a dioestrus-like echotexture, on manual examination *per vaginam*, the cervix contracted and increased in tone as following normal ovulation. Additionally the mares then experienced what appeared to be a normal dioestrus period and in most instances did not return to oestrus for at least two weeks unless this dioestrus was forshortened by prostaglandin ~~administration .~~

When a luteinized follicle was concurrent with a normal ovulation and development of a CL, then a clinical diagnosis of luteinization was dependent on the progressive ultrasonic changes in the follicle as described previously. Follicles that haemorrhaged but showed no clinical or ultrasonic signs of luteinization were excluded from the study. These types of follicles followed the same course and appearance of follicles that subsequently luteinized but failed to develop a hyperechoic "thickened" neither follicular wall nor an organised cavity. In the absence of a concurrent normal ovulation, the mare remained in a clinical non-luteal phase.

As with ovulating follicles, HAFs were classified as occurring either spontaneously, or induced after administration of Cloprostenol (as above). Intervals from induction treatment to HAF were recorded (the interval from Cloprostenol to HAF development was less than 12 days in all cases). Some HAF occurred during the same cycle of a normal ovulation. For data analysis, the date of HAF was estimated on the day the follicle filled with echodense specks (day of expected ovulation: day 0 as in Fig~~ure~~ 1a).

Endometrial oedema: degree of endometrial folding was subjectively assessed by transrectal ultrasonography. Increasing scores of 0.5 were given to the uterus from 0 (no endometrial folding coincident with dioestrus-like echotexture) to 3 (maximum endometrial folding).

Follicular diameter: Largest follicular diameter at the time of Cloprostenol administration and immediate preovulatory follicular diameters were measured and recorded as shown previously (Cuervo-Arango and Newcombe 2008).

Study design

For data analysis the experimental unit used was the "cycle" which could be either "ovulatory" (when no HAF developed during one inter-ovulatory period) or "haemorrhagic" (when single or multiple HAFs whether accompanied by ovulation(s) or on its own developed and luteinized during an inter-ovulatory period).

S~~Data were analysed for s~~tatistical analysis of the data was performed by using a mixed model, with three fixed factors:

- Induction treatment (spontaneous or induced with Cloprostenol)
- Time of season (winter: December to March; early: April to July; or late in the ovulatory season: August to November).
- Age (young: 2-8; middle age: 9-14 and old: > 15 years old)

And corresponding two-way/three-way interactions using the procedure MIXED of SAS® (SAS System, Release 8.2, SAS Institute Inc., Cary, NC, 1999). In addition, because of the great range in Cloprostenol doses used for induction treatment, the mean Cloprostenol dose and largest follicular diameter at the time of treatment for both HAF and non-HAF induced cycles were tested for difference by Mann-Whitney non-parametric test and 2-sample t-test respectively.

Results

Only one out of the 7 mares studied over 10 years had no recorded HAF. Four mares had between 2.8 and 7.5 % of HAF incidence and the remaining 2 had an incidence as high as 24.7 and 25.3 % (Table 1). The recurrence rate after the first HAF cycle in the same year was 0 % (mean age of first HAF cycle 6.44 ± 1.3 years). However the recurrence rate of HAF at some oestrous cycle during the lifetime was 100 % in all mares. Due to the low number of HAF cycles in mares 3 to 6, the analysis of risk factors for HAF development was performed only in data from mares 1 and 2.

The two mares with highest incidence (mares 1 and 2) were more likely to have HAF cycles after induction with Cloprostenol ($P < 0.000$) than in spontaneous cycles (Table 1). There was a significant effect of Cloprostenol dose on HAF incidence ($P = 0.004$): the Cloprostenol dose used in non-HAF cycles averaged 254.5 ± 21.5 µg compared with 375 ± 35 µg in HAF cycles (Fig 2). The mean largest follicular diameter at the time of Cloprostenol (PG) administration in HAF cycles (15.6 ± 1.4 mm) was non-significantly smaller than in non-HAF cycles (18.8 ± 1.9 mm) ($P > 0.05$). Interval from PG administration to ovulation / beginning of HAF was not different (7.1 ± 0.24 and 7.7 ± 0.26 days respectively). Effect of induction with hCG on HAF was not estimated due to the low number of cycles induced with this hormone (4 % of all oestrous cycles).

There was an effect of time within year ($P = 0.004$): higher during early (April to July) and late in the ovulatory season (August to November) than in the winter months, with HAF incidences of 31, 28.1 and 11.7 % respectively (Fig 3). Incidence of HAF amongst different age groups was not different ($P > 0.05$): 23.5, 25.7 and 26.1 % for the young, middle age and old periods respectively (Fig 4). However Mares 1 and 2 had only 1 HAF cycle (2.4 % incidence) during the first 3 years of their reproductive lives (2 to 4, 41 oestrous cycles).

223 The likelihood of having two consecutive HAF cycles was 31.5 %. If the cycle
224 consecutive to HAF was induced with PG, the probability increased to 40 %, whereas if
225 no induction treatment was used, a new HAF cycle developed only in 9 % of the times.
226
227 The uterine oedema patterns of HAF cycles without concurrent ovulations (n = 34 HAF
228 cycles) were compared with those of non-HAF cycles (n = 30 chosen randomly); no
229 significant difference ($P > 0.05$) at any observation time between the two types of cycles
230 was found.
231 Mare 1 and 2 had a total of 80 HAFs of which 40 % had solitary HAF(s) only without
232 concurrent ovulation whereas the remaining 60 % had normal ovulation(s) during the
233 same cycle. Details of different types of HAF cycles are shown in Table 2.
234
235 **Discussion**
236
237 This study intended to investigate the risk factors associated with the development of
238 haemorrhagic anovulatory follicles in the mare. This anovulatory condition is difficult
239 to research, in part due to the low overall incidence in a given population of mares and
240 also because of the little knowledge of its nature. The present study has allowed the
241 possibility to investigate in depth the occurrence of this phenomenon mainly by long-
242 term analysis of the ovarian activity of two mares with abnormally high HAF incidence
243 (> 24 %) over all their entire reproductive life.
244
245 <u>Possible mechanisms of ovulatory failure</u>
246

247 The most obvious factor affecting HAF incidence of this study was the use of
248 Cloprostenol to induce the following oestrus. Yet the mechanisms by which
249 administration of prostaglandin during the luteal phase increases the likelihood of
250 developing HAFs remains unclear.

251 It is known that the LH surge initiates a cascade of proteolytic activity (Robker *et al*.
252 2000) driven by enzymes such as matrix metalloproteinases (MMPs) and plasminogen
253 activators (PA) / plasmin which is required in the tissue remodelling accompanying the
254 ovulatory process (Smith *et al*. 2002). The same preovulatory LH surge induces
255 prostaglandin synthesis by equine granulosa cells (Sirois and Dore 1997) which has
256 been proven in numerous species to be essential for ovulation to occur since
257 administrations of prostaglandin inhibitors, given either intra-follicular or systemically,
258 caused luteinization of follicles without previous rupture and oocyte release (Salhab *et*
259 *al*. 2003; Grinwich *et al*. 1972; Armstrong and Grinwich 1972; Killick and Elsein
260 1987). It remains still undefined but it is hypothesized that prostanoids may regulate
261 various MMPs and PA (Li *et al*. 2006).

262 Incidence of a similar condition described in humans (luteinized unruptured follicle
263 syndrome, LUF) is increased in women who have undergone super-ovulation
264 programmes with Clomiphene (a steroid receptor inhibitor which increases circulating
265 concentrations of FSH and LH) (Zhu 1989). On the basis of that link and studies that
266 showed the effect of LH/hCG on metabolism of intrafollicular $PGF_2\alpha$ and PGE_2
267 (Channing 1973), human researchers have hypothesized that if LH/hCG stimulation of a
268 follicle occurs too early, premature luteinization can result with a consequent increased
269 PGE_2 synthesis, decrease in $PGF_2\alpha$ synthesis and inhibition of follicular rupture
270 (Coulam *et al*. 1982).

Allen (1979) showed that pregnant pony mares under the constant effect of eCG presented ovaries with haemorrhagic follicles which luteinized subsequently. This effect of eCG on the ovaries from day 50 to 100 approximately has also been shown in Thoroughbred and Irish Draught mares (Newcombe, ~~unpublished~~personal communication 2007). The continuous stimulatory effect of eCG LH-like activity on small follicles (Urwin and Allen 1982) could be inducing haemorrhage and luteinization of follicles in a similar way that HAF occurs in cyclic mares.

Due to the low number of HAF cycles in mares 3 to 6, the following analysis of risk factors for HAF development was performed only in data from mares 1 and 2.

Risk factors

Multiple ovulators

It is worth noting that the mares with highest HAF incidence (mares 1 and 2) were also the ones with highest multiple ovulation rate (Table 1). These mares often had triple ovulations and when a follicle(s) haemorrhaged, it was accompanied in the majority of cases by one or two ovulations (Table 2). This increase in number of follicles passed the point of follicular deviation in HAF cycles was significantly higher than in mares with lower HAF rate (Table 2). Perhaps these mares had an intrinsic higher gonadotrophin circulating concentration in early stages of follicular development. Hormonal studies during the early stages of follicular development remain to be done to elucidate this enigma.

Month

There is the belief that HAF cycles tend to occur during the autumn (Burkhardt 1948; Ginther *et al*., 2006) and have on that basis been termed "autumn follicles" (Knudsen and Weiert 1961). Although the highest incidence of HAF cycles occurred during the month of November, the low number of cycles in that month may have precluded it from being statistically significant. It also could be argued that the higher HAF incidence experienced at the end of the season might be due to the fact that reproductively "normal mares" become pregnant early in the season whereas the problematic mares are left to the end. Apart from that peak during November, it was observed that the majority of HAF cycles concentrated around the months of maximal follicular activity (May to August) coincident with the highest circulating LH monthly mean values (Turner *et al*. 1979).

Age

There was no significant effect of age on HAF incidence. It appeared to be though a protective effect against HAF development during the first few youngest years of age (only one HAF in mares 1 and 2 over the 41 oestrous cycles during the age period 2 to 4; Fig 4). Research on effect of aging on follicular dynamics and gonadotrophin concentrations tell us that middle age mares (15 to 19 years) have higher circulating FSH than young mares (5 to 7 years) while LH remains similar. In older mares (> 20 years old) both FSH and LH are raised (Carnevale *et al*. 1993). Interestingly, the only mare older than 20 years (mare 3) in the present study had her first HAF cycle recorded at 19 years old. In the following 7 years, aged 20 to 26, 26.1 % of her cycles (n = 21)

developed HAFs spontaneously (without use of induction treatments). Not surprisingly this mare had the highest spontaneous HAF rate of all the mares studied.

Induction treatments

The present results suggest strong evidence of a link between HAF and use of induction treatments (PGF analogue, especially when high doses are used > 300 μg) which have been proven to induce both a direct release of LH in the horse (Jöchle *et al*. 1987) and indirect increase in LH as the negative feed back of progesterone is released after PGF-induced luteolysis. On the basis of the latter study it could be hypothesized that the increased LH concentration following induction with PGF analogue could interfere with intra-follicular metabolism of prostanoids and proteolytic enzymes in an immature follicle. It would however have to be a slow process as the mean interval from Cloprostenol administration to HAF development was 7.7 ± 0.26 days. However it seems that this effect is rather synergistic in mares that are somehow predisposed to develop HAFs. Preliminary results of hormonal controlled studies have also shown a link between HAF and use of prostaglandin in the mare (Ginther *et al*. 2008) which supports this theory.

Clinical relevance and conclusions

There is little a veterinarian can do after detecting a follicle which develops haemorrhage and subsequent luteinization (especially in mares that have been already

345 mated) but make arrangements for the next cycle to breed again. It is important to bear
346 in mind that some follicles before undergoing follicular collapse during the ovulatory
347 process can look exactly like a follicle about to haemorrhage (Fig 1a-b), however unlike
348 HAF, the rupturing follicle will collapse completely giving the appearance of a
349 hypoechoic area for at least 12 to 15 h, after which they may fill quickly with blood to
350 resemble a HAF in early stages. It is therefore important for this purpose to examine
351 mares at least twice daily to avoid mistaking a normal ovulation/corpus
352 haemorrhagicum for a HAF.
353 Assuming the fact that the mare cannot conceive following haemorrhage into the
354 follicle, most practitioners would try to induce the following oestrus with prostaglandin.
355 On the basis of our results and especially if the mare is known to have high percentage
356 of HAFs cycles, the next cycle preferably should not be induced. In the case of
357 necessity of using induction treatment, it is recommended to administrate the lowest
358 luteolytic dose of PGF (e.g. 25 to 125 µg Cloprostenol / 0.1 to 0.5 ml Estrumate) to
359 minimize likelihood of recurrence. It is worth highlighting that mares 1 and 2 were
360 more than 4 times more likely to develop consecutively a new HAF after Cloprostenol
361 induction than in spontaneous cycles. These two mares had a fairly good fertility in non-
362 HAF cycles (and HAF cycles with concurrent normal ovulations), therefore it is still
363 possible to get this type of mares pregnant as long as they have a ovulation from a
364 ruptured follicle.
365 In conclusion this study has provided some useful data for the practitioner in
366 understanding this anovulatory condition in the mare and new insights for prospective
367 researchers involving hormonal studies to elucidate the proposed effect of $PGF_2\alpha$ on the
368 pathogenesis of HAF development.
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