3-1-2016

The Mare as a Model for Luteinized Unruptured Follicle Syndrome: Intrafollicular Endocrine Milieu.

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Recommended Citation

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15 Running title: Intrafollicular endocrine milieu of LUFs

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17 Keywords: equine, growth factors, HAF, hormones, LUF, reLH

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23

24 **Abstract**

25 Luteinized unruptured follicle (LUF) syndrome is a recurrent anovulatory dysfunction that

26 affects up to 23% of women with normal menstrual cycles and up to 73% with endometriosis.

27 Mechanisms underlying the development of LUF syndrome in mares were studied to provide a

28 potential model for human anovulation. The effect of extended increase in circulating LH

29 achieved by administration of recombinant equine LH (reLH) or a short surge of LH and

30 decrease in progesterone induced by prostaglandin $F_2\alpha$ ($PGF_2\alpha$) on LUF formation (Experiment

31 1), identification of an optimal dose of COX-2 inhibitor (flunixin-meglumine, FM; to block the

32 effect of prostaglandins) for inducing LUFs (Experiment 2), and evaluation of intrafollicular

33 endocrine milieu in LUFs (Experiment 3) were investigated. In Experiment 1, mares were treated

34 with reLH from Days 7−15 (Day 0 = ovulation), $PGF_2\alpha$ on Day 7, or in combination. In

35 Experiment 2, FM at doses of 2.0 or 3.0 mg/kg every 12 h and hCG (1500 IU) were administered

36 after a follicle ≥32 mm was detected. In Experiment 3, FM at a dose of 2.0 mg/kg every 12 h

37 plus hCG was used to induce LUFs and investigate the intrafollicular endocrine milieu. No LUFs

38 were induced by reLH or $PGF_2\alpha$ treatment; however, LUFs were induced in 100% of mares

39 using FM. Intrafollicular $PGF_2\alpha$ metabolite (PGFM), $PGF_2\alpha$, and PGE_2 were lower and the ratio

40 of PGE_2:$PGF_2\alpha$ was higher in the Induced LUF group. Higher levels of intrafollicular E2 and

41 total primary sex steroids were observed in the Induced LUF group along with a tendency for

42 higher levels of GH, cortisol, and T; however, LH, PRL, VEGF-A, and NO did not differ

43 between groups. In conclusion, this study reveals part of the intrafollicular endocrine milieu and

44 the association of prostaglandins in LUF formation, and indicates that the mare might be an

45 appropriate model for studying the poorly understood LUF syndrome.

46

Introduction

Anovulation is one of the main causes of infertility in women and females of many domestic species. One of the types of anovulatory dysfunction is luteinized unruptured follicle (LUF) syndrome, which has been reported in women (Marik & Hulka 1978, Koninckx *et al.* 1981, Hamilton *et al.* 1985, Hulka 1985, Katz 1988, Check 2007), mares (Kaiser *et al.* 1999, Gastal *et al.* 2006, Ginther *et al.* 2007*a*, Cuervo-Arango & Newcombe 2009), cattle (Peter 2004), llamas (Adams *et al.* 1991), rhinoceroses (Stoops *et al.* 2004), and elephants (Lueders *et al.* 2011). LUFs, also known as hemorrhagic anovulatory follicles (HAFs) in veterinary medicine, occur when the preovulatory follicle fails to rupture or ovulate and the antrum gets increasingly filled with blood. LUF/HAF is the most common form of anovulation in mares. LUF syndrome during the breeding season is considered a serious economic concern for the equine industry. Similarly, anovulation can have significant financial implications for women undergoing assisted reproductive techniques (Eijkemans *et al.* 2005). Therefore, it is important to investigate the mechanisms of development of LUF syndrome. Greater knowledge about the pathophysiology of LUFs can be applied to prevent their occurrence and thus to develop safe and effective treatments to optimize reproductive health in both animals and humans.

The ultrasonographic morphological characteristics of naturally occurring or induced LUFs are similar in women (Priddy *et al.* 1990, Zaidi *et al.* 1995) and mares (Coetsier & Dhont 1996, Cuervo-Arango & Newcombe 2012). LUF formation involves the development of well-vascularized luteal tissue, as indicated by echotexture and color Doppler signals in both women and mares (Zaidi *et al.* 1995, Ginther *et al.* 2007*a*). In the absence of ovulation, the antrum of the follicle becomes permeated with blood, which appears as echogenic foci and fibrin-like strands on a B-mode ultrasonogram. Due to similarities in antral follicular dynamics (Ginther *et al.*

70   2004, Baerwald 2009, Gastal 2011) and LUF morphology between species, we have proposed

71   the mare as an appropriate model for understanding LUF syndrome in women.

72   The reported incidence of LUFs in women is highly variable. LUFs have been documented to

73   occur in 11-23% of women with normal menstrual cycles (Vanrell *et al.* 1982, Kerin *et al.* 1983,

74   Dal *et al.* 2005), 13-73% of women with endometriosis (Kaya & Oral 1999), and 4-58% of

75   women with unexplained infertility (Koninckx & Brosens 1983, Kugu *et al.* 1991). In addition to

76   this, LUFs have been reported in women with pelvic inflammatory disease (Hamilton *et al.*

77   1986) and inflammatory arthritis (Smith *et al.* 1996). LUFs are highly repeatable across cycles

78   (79-90%), resulting in recurrent anovulation (Hamilton *et al.* 1986, Qublan *et al.* 2006) and

79   infertility.

80   In cycling mares, the incidence of LUFs is also highly variable (5-25%; Lefranc & Allen

81   2003, Ginther *et al.* 2008*a*, Cuervo-Arango & Newcombe 2009, 2010). A 5% incidence of LUFs

82   has been reported during the early ovulatory season, followed by 20% during the late

83   reproductive season (Gastal *et al.* 1998). Similarly, LUF syndrome has been shown to occur

84   more often in older mares and be recurrent in some individuals (>50% of the estrous cycles),

85   encompassing much or all of the breeding season (Ginther *et al.* 2007*a*, Cuervo-Arango &

86   Newcombe 2009, 2010). Therefore, recurrent LUFs result in prolonged periods of anovulation

87   and long interovulatory intervals (Ginther *et al.* 2007*a*).

88   The systemic and intrafollicular endocrine milieu associated with LUF syndrome is poorly

89   understood. Knowledge about the endocrine changes associated with LUF formation is lacking

90   (Hamilton *et al.* 1985, Ginther *et al.* 2007). The use of luteinizing hormone (LH) during early

91   proestrus in rodents has successfully induced LUFs (Plas-Roser *et al.* 1985, Mattheij & Swarts

92   1995). Similarly, the use of luteinizing hormone releasing hormone, human chorionic

93   gonadotropin (hCG), and human menopausal gonadotropin has been associated with LUFs in

94   women (Bergquist & Lindgren 1983, Ghanem *et al.* 2009), and hCG in guinea pigs (Westfahl

95   1988). Continued investigations are required to determine the effects of chronic administration of

96   LH on the incidence of LUFs in mares and women. It has been well documented that a $PGF_2\alpha$

97   injection causes immediate release of LH, resulting in induction of ovulation in several species

98   such as cows (Hafs *et al.* 1975), sows (Srikandakumar & Downey 1989), and mares (Gastal *et al.*

99   2005). In a recent study, administration of $PGF_2\alpha$ with or without complete ablation of antral

100   follicles increased LH concentrations early in the ovulatory wave and also during the

101   preovulatory period and were associated with a high incidence of LUFs in mares (Ginther *et al.*

102   2008*b*). Although it is not known exactly what physiological mechanism (interference with

103   luteinization or maturation of granulosa cells) might be disturbed when LH levels are affected at

104   the beginning of the follicular wave or during the ovulatory period, there is enough evidence to

105   allow the test of a hypothesis of LH participation in LUF formation.

106   The advances in knowledge regarding LUF syndrome in women and animals have been slow,

107   in part because of the difficulty of predicting the occurrence of such ovarian structures.

108   Therefore, the use of pharmacologically induced LUFs is a promising way to study this

109   syndrome. Use of pharmacological agents in fertility or superovulatory treatments has increased

110   the occurrence of LUFs and/or anovulatory follicles in women (Martinez *et al.* 1991, Ghanem *et*

111   *al.* 2009) and mares (Lefranc & Allen 2003, Ginther *et al.* 2008*a*, Cuervo-Arango & Newcombe

112   2010, Meyers-Brown *et al.* 2011). Similarly, intrafollicular or systemic administration of

113   prostaglandin inhibitors has been shown to cause luteinization of follicles in rats (Armstrong &

114   Grinwich 1972), rabbits (Salhab *et al.* 2003), mares (Watson & Sertich 1991, Ginther *et al.*

115   2008*a*, Cuervo-Arango & Domingo-Ortiz 2011), and women (Killick & Elstein 1987, Priddy *et*

116   *al.* 1990, Jesam *et al.* 2010, 2014). In mares, flunixin meglumine (FM) at a dose rate of 1.7 or 2.0

117   mg/kg body weight has been shown to induce LUFs in 73-83% of mares, respectively (Cuervo-

118   Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz 2011). Furthermore, intrafollicular

119   administration of prostaglandins has successfully prevented FM-induced LUF formation,

120   allowing subsequent ovulation and conception in mares (Martínez-Boví & Cuervo-Arango

121   2015). In women, NSAIDs have been used experimentally to inhibit ovulation, and a dose-

122   dependent effect has been observed (Athanasiou *et al.* 1996, Bata *et al.* 2006, Jesam *et al.* 2010,

123   2014). Therefore, pharmacological approaches to inhibit ovulation and induce LUF formation,

124   such as the use of COX-2 inhibitors, may serve as an effective model to elucidate the

125   pathophysiology of LUF syndrome.

126   The objectives of the experiments conducted in this study were to investigate: the role of

127   reLH and $PGF_2\alpha$ in the formation of LUFs (Experiment 1), the optimum dose of FM required to

128   experimentally induce LUFs (Experiment 2), and the intrafollicular endocrine milieu

129   (Experiment 3) in induced LUFs in the mare. The hypotheses tested were that: (1) reLH, when

130   administered during diestrus alone or along with $PGF_2\alpha$, would increase the incidence of LUFs;

131   (2) higher doses of FM would increase the incidence of LUFs; (3) inhibition of intrafollicular

132   prostaglandin synthesis would be associated with LUF formation; (4) intrafollicular

133   prostaglandins ($PGF_2\alpha$ and PGE_2) concentrations would be decreased during systemic FM

134   treatment; (5) imbalance in the intrafollicular endocrine milieu is associated with LUF formation.

135

136   **Materials and Methods**

137

138   *Animals*

139   Mares ($n = 36$) were evaluated during two ovulatory seasons (April to October) in the northern

140   hemisphere and handled in accordance with the United States Department of Agriculture Guide

141   for Care and Use of Agricultural Animals in Research. This study was approved by the Southern

142   Illinois University Institutional Animal Care and Use Committee (IACUC, 10-041). The mares

143   were Quarter-Horse type breed, 5−15 years of age, weighed 400−550 kg, had docile

144   temperament, and did not present any apparent abnormality of the reproductive tract as

145   determined by ultrasonographic examination. Mares were reused in subsequent experiments after

146   ultrasonographically confirming at least two normal ovulatory cycles in between experiments.

147   All mares had good body condition score (average score ~6-7; score 1 = emaciated and score 9 =

148   obese; Henneke *et al.* 1983) throughout the study. Mares were kept under natural light in pasture

149   with free access to water and trace-mineralized salt.

150

151   *Experiment 1. Effect of reLH and PG on LUF formation*

152

153   *Animals and treatments*

154   On Day 7 (Day 0 = ovulation), mares ($n = 30$) were randomly divided into six different treatment

155   groups to receive either saline, $PGF_2\alpha$ (5 mg i.m.; Lutalyse, Pfizer Animal Health, Kalamazoo,

156   MI, USA), reLH (0.5 mg or 1.0 mg i.v.; AspenBio Pharma Inc., Castle Rock, CO), or a

157   combination of $PGF_2\alpha$ and reLH. The groups were: Control (10 ml saline, i.v.), LH 0.5 (0.5 mg

158   of reLH), LH 0.5 + PG (0.5 mg reLH and 5 mg $PGF_2\alpha$), PG (5 mg $PGF_2\alpha$), LH 1.0 (1 mg reLH),

159   and LH 1.0 + PG (1 mg reLH and 5 mg $PGF_2\alpha$). Mares were treated with $PGF_2\alpha$ (5 mg/mare)

160   only once on Day 7 and with reLH once every day from Days 7−15.

161

162   *Ultrasonographic examinations and end points*

163   Transrectal ultrasonographic examinations were performed daily from Day 0 until 4 days after

164   the next ovulation or the beginning of LUF formation. A LUF was detected ultrasonographically

165   by the presence of a thick and echoic follicle wall, suggestive of luteinization, decreased

166   turgidity, echoic foci and/or fibrin septae in the antrum, a gel-like substance in antrum, and/or

167   pronounced serration of the granulosa layer around the whole follicle, as previously described

168   (Ginther *et al.* 2006, Cuervo-Arango & Newcombe 2012). A series of comparative ultrasound

169   images of LUFs in women and mares is given for illustration purposes regarding the similarities

170   shown by both species (Fig. 1). For transrectal scanning, a duplex B-mode (gray-scale) and

171   pulsed-wave color-Doppler ultrasound instrument (Aloka SSD-3500; Aloka America,

172   Wallingford, CT, USA) equipped with a finger-mounted 3.5−10 MHz convex-array transducer

173   was employed. The color-flow mode was used to display follicle, corpus luteum (CL), and LUF

174   blood flow as previously described (Ginther *et al.* 2007*a*, 2007*b*). Constant color-gain, velocity,

175   and filter settings were used for all Doppler examinations. The entire follicle, CL, and LUF were

176   scanned in a gradual, steady motion several times.

177   Follicle diameter was calculated from the average of height and width of the antrum at the

178   apparent maximal area from two frozen images. The largest follicle was measured on each day of

179   examination. CL diameter was measured throughout the study. In addition, endometrial

180   echotexture was scored from 1 to 4 (minimal to maximal) during each examination, based on the

181   extent of anechoic areas of the endometrial folds (Ginther & Pierson 1984). Follicle wall blood

182   flow was quantified in follicles ≥28 mm until ovulation or LUF formation, using color-flow

183   Doppler as previously described in mares (Acosta *et al.* 2004) and women (Campbell *et al.*

184   1993).

185

186 ***Experiment 2. Optimum dose of flunixin-meglumine (FM) to induce LUF***

187

188 *Animals and treatments*

189 At the beginning of estrus, cycling mares ($n = 18$) with a growing follicle ≥ 32 mm (Hour 0), in

190 the presence of endometrial edema (echotexture score ≥ 3) were administered 1500 IU of hCG

191 (Chorulon®, i.v.; Intervet Inc, Millsboro, DE). Immediately, mares were randomly divided ($n =$

192 6 mares/group) into three treatment groups (FM-2, FM-3, and Control), and treatments were

193 started. All groups were treated every 12 h with FM or saline until Hour 36. FM-2 and FM-3

194 groups received 2.0 or 3.0 mg/kg of body weight of FM (Flunixiject™, i.v.; Henry Schein®

195 Animal Health, Dublin, OH), respectively, and the Control group received 10 ml of saline (i.v.)

196 solution. Mares were monitored for any adverse effects from the above mentioned doses of FM,

197 as the normal dose was exceeded (1.1 mg/kg of body weight per day).

198

199 *Ultrasound evaluations*

200 Mares were scanned every other day from Day 12 after ovulation until a follicle reached $25-27$

201 mm in diameter; subsequently, scans were conducted daily. Mares with a ≥ 32-mm follicle were

202 randomly allocated to a treatment group and scans were performed: every 12 h from Hours $0-36$,

203 every 2 h between Hours $36-48$, and every 12 h from Hours 60-96. Mares with more than one

204 ≥ 32-mm follicle at the beginning of treatment were not included in the study.

205   Follicle diameter, follicle blood flow, LUFs, and endometrial echotexture were evaluated

206 using the same ultrasound methodology as in Experiment 1. The thickness of the follicle wall

207 (granulosa layer) was determined by averaging measurements made on three different locations

208 (Gastal *et al.* 1998, 2006). In addition, the following qualitative B-mode characteristics of the

209 preovulatory follicle were recorded from Hour 0 until ovulation or LUF formation: 1) presence

210 of echoic foci floating in the antrum, 2) detection of follicle wall serration (i.e., irregular or

211 notched surfaces of the granulosa layer; Gastal *et al.* 2007), and 3) loss of spherical shape.

212

213 ***Experiment 3. Systemic and intrafollicular hormones and growth factors***

214

215 *Animals and treatments*

216 Cycling mares ($n = 23$) with a growing follicle ≥28-mm were scanned daily after Day 15 using

217 B-mode and color-Doppler ultrasonography (Gastal *et al.* 1998, 2006) until a follicle ≥32 mm

218 was detected. When the largest follicle reached ≥32 mm (Hour 0) and the score for endometrial

219 echotexture was between 3 and 4 (estrus-like), mares were randomly assigned into two treatment

220 groups: Control ($n = 11$) and Induced LUF ($n = 12$). Mares received an injection of 1500 IU of

221 hCG and were treated twice daily with saline (10 ml, i.v.; Control group) or FM (2.0 mg FM/kg

222 of body weight; Induced LUF group) until 36 h after hCG injection.

223

224 *Collection of follicular fluid and end points*

225 Follicular fluid was collected from the preovulatory follicle in the Induced LUF and the Control

226 groups by ultrasound-guided transvaginal follicle aspiration at Hour 36 after hCG injection as

227 described (Gastal *et al.* 1995, 1999*b*). The aspirated follicular-fluid was immediately processed

228 in a refrigerated centrifuge (1500 xg for 10 min), and 10 ml of the supernatant was stored at

229 −20ºC until hormone assays were performed. Follicle diameter, follicle blood flow, and

230 endometrial echotexture were measured, as described in Experiment 1.

231

232 ***Blood samples and hormone assays***

233 Jugular blood samples were collected in heparinized tubes, immediately placed in ice water bath,

234 processed in a refrigerated centrifuge (1500 xg for 10 min), decanted, and stored (-20°C) until

235 analyzed. For Experiment 1, samples were collected daily between Days $7-15$. For Experiment

236 2, samples were collected every 12 h between Hours $0-36$ and then every 2 h until ovulation or

237 LUF formation. For Experiment 3, samples were collected every 12 h between Hours $0-36$. For

238 Experiment 1, systemic LH was assayed from Days $7-9$ to investigate the effect of treatment on

239 the increase in circulating LH, and progesterone (P4) was assayed from Days $7-15$. For

240 Experiment 2, systemic PGFM (prostaglandin $F_2\alpha$ metabolite) and P4 were assayed from Hours

241 $0-38$. For Experiment 3, PGFM was assayed in both plasma and follicular fluid; additionally,

242 follicular fluid was assayed for $PGF_2\alpha$, prostaglandin E_2 (PGE_2), estradiol 17-β (E2), P4,

243 testosterone (T), LH, nitric oxide (NO), vascular endothelial growth factor-A (VEGF-A),

244 cortisol, prolactin (PRL), and growth hormone (GH). Furthermore, the ratios of PGE_2:$PGF_2\alpha$,

245 E2:P4, E2:T, and P4:T were calculated. Total primary sex steroids were calculated by combining

246 the E2, P4, and T.

247   Plasma P4 concentrations were determined using a solid-phase radioimmunoassay kit

248 containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A Count Progesterone, Diagnostic

249 Products Corporation, Los Angeles, CA, USA) as described and validated for mare plasma

250 (Ginther *et al.* 2005). Plasma LH was assayed using an equine ELISA kit (Endocrine

251 Technologies, INC., Freemont, CA, USA). Plasma and follicular fluid PGFM, $PGF_2\alpha$, PGE_2, and

252 E2 concentration was determined using ELISA kits (Neogen Co., Lexington, KY, USA) after

253 extraction with diethyl-ether, as previously described (Ginther *et al.* 2010). Intrafollicular LH,

254   GH, cortisol, PRL, and testosterone concentrations were determined by ELISA kits (Endocrine

255   Technologies, INC., Freemont, CA, USA). Intrafollicular NO was estimated using a colorimetric

256   kit (Cayman Chemical Company, Ann Arbor, MI, USA). Intrafollicular VEGF-A was assayed by

257   ELISA kit (Kingfisher Biotech, Inc., Saint Paul, MN, USA). All the assays were performed

258   following the manufacturers' protocol and were validated for equine follicular fluid by using

259   multiple dilutions and pilot assays to determine the optimal dilutions required for the hormone

260   concentration to be within the detection range of the assay. The intra-assay CVs and sensitivities

261   for the different hormone assays were as follows: P4, 8.7% (experiment 1), 6.3% (experiment 2),

262   6.6% (experiment 3), and 0.02 ng/ml; PGFM, 6.3% (experiment 2) and 3.2% (experiment 3), and

263   20 pg/ml; $PGF_2\alpha$, 6.1% and 2 pg/ml; PGE_2, 2.6% and 0.1 ng/ml; E2, 4.9% and 0.1 ng/ml; NO,

264   5.1% and 20 μM/ml; LH, 4.5% (plasma) and 8.9% (follicular fluid), and 0.25 ng/ml; GH, 5.2%

265   and 0.25 ng/ml; cortisol, 9.5% and 1 ng/ml; testosterone, 6.1% and 0.1 ng/ml; and VEGF-A,

266   3.6% and 28.5 pg/ml.

267

268   *Statistical analyses*

269   The Shapiro-Wilk test was used for testing normal distribution of the data. Data not normally

270   distributed were transformed to log or rank before any statistical analyses. Dixon's test was used

271   to identify outlier observations, which were excluded from any statistical analyses. Sequential

272   data were analyzed by one-way ANOVA for main effects of group, time, and group by time

273   interaction. The analyses were done using SAS PROC MIXED (Version 9.2; SAS Institute, Inc.)

274   with a REPEATED statement to minimize autocorrelation between sequential measurements.

275   When a group effect or interaction was obtained, differences among groups within time points

276   were further analyzed. Tukey's test was used between time points within a group to identify

277   significant differences. A probability of $P < 0.05$ indicated that a difference was significant and

278    $P > 0.05$ or < 0.1 indicated that results tended to be different. Data are presented as mean $\pm$

279   S.E.M.

280

281   **Results**

282

283   *Experiment 1. Effect of reLH and PG on LUF formation*

284   No LUFs were observed in this experiment, regardless of treatment. More specifically, reLH

285   (0.5−1.0mg) given for 9 days during the diestrous phase and/or PG administered on Day 7 did

286   not induce LUF formation. Furthermore, reLH had no effect on any other endpoint. The diameter

287   of the largest follicle increased ($P < 0.0001$) for all groups, but no overall group effect was

288   observed during the treatment period (data not shown). A group-by-day interaction ($P < 0.02$)

289   was potentially caused by the greater diameters in the PG groups when compared with the

290   Control and LH groups. After combining the reLH (LH 0.5 + LH 1.0) groups and PG (LH

291   0.5+PG, LH 1.0+PG, and PG) groups, a larger ($P < 0.05$) follicle diameter was seen on Days

292   13−16 for the PG group when compared with the Control and LH groups (Fig. 2A). The growth

293   rate of the largest follicle from Days 7−16 was greater ($P < 0.001$) in the PG group than in the

294   Control and reLH groups (Table 1). In addition, maximum follicle diameter during the treatment

295   period and on Day 16 was greater ($P < 0.01$) in the PG group compared to the Control and reLH

296   groups. The percentage of blood flow of the dominant follicle 3 days before ovulation did not

297   differ ($P > 0.05$) among groups.

298        $PGF_2\alpha$ had a marked effect on CL diameter and half-life, which affected the plasma P4

299   concentrations and shortened the interovulatory interval (IOI; Table 1). As expected, CL

300   diameter and P4 concentration decreased ($P < 0.007$) faster in PG-treated groups, compared to

301   the other groups (data not shown). The Control and reLH groups did not differ throughout

302   treatment, whereas PG-treated groups had a smaller CL diameter on Day 12 ($P < 0.0001$).

303   Therefore, the reLH (0.5 and 1.0 mg) groups and the PG groups were combined for further

304   analyses (Fig. 2B). The Control and reLH-treated groups had a larger ($P < 0.0001$) CL diameter

305   and greater P4 concentration from Days 9−15 than the PG group (Fig. 2B, C). The reLH

306   treatment had no effect on CL diameter or P4 concentration when compared to the Control

307   group. P4 concentration was greater ($P < 0.0002$) on Day 12 and lower ($P < 0.004$) on the day of

308   the beginning of luteolysis in the Control and reLH groups compared to the PG-treated group

309   (Table 1). Also, the mean day of the beginning of luteolysis (day before P4 was <1.0 ng/ml) was

310   earlier ($P < 0.0001$) in the PG-treated group compared to the Control and reLH-treated groups

311   (Table 1). Endometrial echotexture was greater ($P < 0.002$) in the PG (3.3 ± 0.1) versus Control

312   (2.9 ± 0.1) and reLH (2.8 ± 0.1) groups. Plasma LH concentrations from Days 7−9 increased (P

313   < 0.03) for reLH (3.7 ± 0.7 ng/ml) and PG (3.0 ± 0.6 ng/ml) groups; however, an increase was

314   not observed in the Control group (1.8 ± 0.2 ng/ml).

315

316   ***Experiment 2. Optimum dose of flunixin-meglumine to induce LUF***

317   In the Control group, ovulation was detected at 38.7 ± 0.7 h (range, 36 to 40 h) in all mares. In

318   FM groups, both doses (2 and 3 mg/kg of body weight) resulted in induction of LUFs in 100% of

319   the animals; no complications were observed in any animal after FM treatment. LUFs were first

320   observed in treated mares at 49.2 ± 1.9 h (range, 44 to 60 h). No difference was observed in the

321   time of first detection of LUFs and plasma PGFM concentration between FM treated groups.

322   Therefore, FM groups were combined for further analyses (i.e., Induced LUF group). The plasma

323   PGFM concentration was lower ($P < 0.05$) in the Induced LUF group when compared to the

324   Control group at Hour 24 (Fig. 3). Plasma P4 concentration was not different ($P > 0.05$) between

325   groups from Hour 0 to 36 (data not shown).

326   Follicle diameters of Induced LUF and Control groups were not different ($P > 0.05$) between

327   Hours 0−38. However, Induced LUFs grew to a larger ($P < 0.009$) diameter from Hours 0−60

328   (Fig. 4A). Follicle wall serration, follicle wall thickness, and follicular shape (round/irregular)

329   were not different between Induced LUF and Control groups up to Hour 38. The first increase (P

330   < 0.05) in follicle wall serration occurred between Hours 0−24 in both groups (Fig. 4B). Follicle

331   wall thickness and follicle wall serration continued to increase between Hours 38−60 for the

332   Induced LUF group (Fig. 4B, C).

333   Follicle blood flow (Fig. 5D) increased differentially between groups after the beginning of

334   treatment (time effect, $P < 0.001$; interaction, $P < 0.02$). The follicle blood flow of the largest

335   follicle was greater ($P < 0.02$) in the Induced LUF group at Hour 38 than in the Control group.

336   Follicle blood flow increased earlier in the Control group (Hour 12) compared to the Induced

337   LUF group (Hour 24).The number of echoic foci in the follicular fluid before ovulation or LUF

338   formation was lower ($P < 0.0001$) in the Control group (0.8 ± 0.3) than in the Induced LUF

339   group (3.5 ± 0.2). Endometrial echotexture (overall score, 3.8 ± 0.1) was not different between

340   groups.

341

342   ***Experiment 3. Systemic and intrafollicular hormones and growth factors***

343   Plasma PGFM concentration was lower ($P < 0.0002$) at Hour 24 in the Induced LUF group

344   versus the Control group (Fig. 5A). Overall, the follicle diameter tended ($P < 0.07$) to be greater

345   in the Induced LUF group when compared with the Control group at Hours 0−36 (Fig. 5B). The

346   follicle diameter was greater in the Induced LUF group at Hour 24 ($P < 0.02$) and Hour 36 ($P <$

347   0.05). Follicle diameters at Hours 12, 24, and 36 increased in both groups, when compared to

348   Hour 0. Follicle blood flow did not differ between groups (Fig. 5C), but increased earlier in the

349   Control group (Hour 12) compared to the Induced LUF group (Hour 24). In addition,

350   endometrial echotexture did not differ between groups; however, a faster decrease ($P < 0.05$) in

351   edema score was detected between Hours 24−36 in the Control group (Fig. 5D).

352   Differences in follicular fluid hormone concentrations were observed between the Control

353   and Induced LUF groups (Fig. 6). PGFM concentration was lower ($P < 0.004$) in the Induced

354   LUF group (49.6 ± 2.0 pg/ml) versus the Control group (102.3 ± 20.5 pg/ml; Fig. 6A). $PGF_2\alpha$

355   was lower ($P < 0.0006$) in the Induced LUF group (0.013 ± 0.005 ng/ml) compared to the

356   Control group (22.4 ± 6.1 ng/ml; Fig. 6B). Also, PGE_2 concentration was lower ($P < 0.004$) in

357   the Induced LUF group (0.35 ± 0.05 ng/ml) compared to the Control group (24.7 ± 8.5 ng/ml;

358   Fig. 6C). The PGE_2:$PGF_2\alpha$ ratio was higher ($P < 0.03$) in Induced LUF (93.0 ± 43.0) compared

359   to Control (3.6 ± 1.5) mares (Fig. 6D).

360   Differences in follicular fluid primary sex steroids and their ratios were detected between

361   treatment groups (Fig. 7). Intrafollicular E2 concentration was greater ($P < 0.02$) in the Induced

362   LUF group (1993.0 ± 325.6 ng/ml) compared to the Control group (1114.3 ± 173.0 ng/ml). P4

363   was not different between groups (Fig. 7B); however, T concentration tended ($P < 0.1$) to be

364   greater in the Induced LUF group (Fig. 7C). Furthermore, the total primary sex steroid

365   concentration was greater ($P < 0.009$) in the Induced LUF group (3340.6 ± 350.4 ng/ml) versus

366   the Control group (2047.4 ± 320.4 ng/ml; Fig. 7D). The ratios of E2:P4 and P4:T tended ($P <$

367   0.1) to be lower in the Induced LUF group (Fig. 7E, F); however, the E2:T ratio was not different

368   between groups (Fig. 7G).

369   The follicular fluid LH, PRL, VEGF-A, and NO concentrations did not differ between the

370   Control and Induced LUF groups (Fig. 8A, B, E, F). However, the concentration of cortisol

371   tended ($P < 0.09$) to be higher in the Induced LUF group (6.8 ± 1.9 ng/ml) compared to the

372   Control group (3.2 ± 0.5 ng/ml; Fig. 8D). Also, the follicular GH levels tended ($P < 0.07$) to be

373   higher in the Induced LUF (0.59 ± 0.05 ng/ml) versus the Control group (0.48 ± 0.05 ng/ml; Fig.

374   8C).

375

376   **Discussion**

377   The pathophysiologic mechanisms underlying LUF syndrome in mares, women, or other species

378   are not known. This series of experiments was conducted to gain insight about the effects of

379   exogenous and endogenous LH and $PGF_2\alpha$ on LUF formation, as well as the effects on

380   intrafollicular prostaglandins and other hormones in induced LUFs when a COX-2 inhibitor

381   (FM) was used. This study is apparently the first to report and compare a wide range of

382   intrafollicular biomarkers between ovulatory follicles and LUFs. Thus, the results herein

383   presented are clinically relevant to veterinary and human medicine.

384   Low levels of follicular fluid prostaglandins and PGFM in mares with induced LUFs were

385   the most consistent and novel finding in this study (Experiment 3). Our hypotheses that

386   intrafollicular prostaglandins would be inhibited by systemic FM administration and that

387   inhibition of intrafollicular prostaglandin synthesis would be associated with LUF formation

388   were therefore substantiated. Intrafollicular $PGF_2\alpha$, PGFM, and PGE_2 were consistently

389   decreased in mares of the Induced LUF group. Similar results have been shown in the follicular

390   fluid of women treated with various NSAIDs (Priddy *et al.* 1990). Prostaglandins are vital for the

391   ovulatory process in vertebrates (reviewed in Murdoch *et al.* 1993). It has been reported that

392   intrafollicular prostaglandins increased 36 h after hCG treatment in mares (Watson & Sertich

393   1991, Sirois & Dore 1997). A recent study (Martínez-Boví & Cuervo-Arango 2015)

394   demonstrated the importance of prostaglandins in the ovulation process in mares by using

395   intrafollicular injection of a supra-physiological cocktail of PGE_2 and $PGF_2\alpha$ (500 μg and 125

396   μg, respectively) to reverse the anovulatory effects of FM. Another novel finding from our study

397   was the increased intrafollicular PGE_2:$PGF_2\alpha$ ratio in mares with induced LUFs. High

398   intrafollicular PGE_2:$PGF_2\alpha$ ratio has been associated with lower pregnancy rates in humans

399   (Smith *et al.* 1991). These findings corroborate the role of lowered intrafollicular prostaglandins

400   in LUF formation and support the concept that an altered PGE_2:$PGF_2\alpha$ ratio may be caused by a

401   compensatory mechanism when prostaglandin synthetase is blocked. These results highlight the

402   importance of the mare as a possible animal model to study LUF syndrome in women.

403   Increased follicular fluid E2 in mares with induced LUFs was another novel finding in this

404   study. We have previously reported that mares had higher E2 concentrations 3 days before the

405   beginning of LUF formation (Ginther *et al.* 2007a). Likewise, higher plasma E2 concentrations

406   have been described in women with spontaneous LUFs (Hamilton *et al.* 1985). However, no

407   differences in intrafollicular E2 concentrations were previously documented in women following

408   NSAID treatment (Priddy *et al.* 1990). Estradiol-mediated stimulation of PGE_2 synthesis in

409   preovulatory follicles in mice (Toda *et al.* 2012), may explain the increased PGE_2:$PGF_2\alpha$ ratio in

410   mares of the Induced LUF group. Furthermore, intrafollicular total primary sex steroids were

411   higher in the Induced LUF group, although no differences were found in P4 concentrations.

412   Greater overall steroid concentrations can be due to low levels of intrafollicular prostaglandins

413   since prostaglandins decrease intracellular transport of cholesterol, reduce cellular cholesterol

414   uptake, and lower the activity of steroidogenic enzymes (reviewed in Niswender *et al.* 2000).

415 Higher intrafollicular E2 levels in Induced LUF mares were consistent with a more differentiated

416 endometrium with higher uterine edema.

417   LH, VEGF-A, NO, and PRL do not seem to be involved in LUF formation as the levels were

418 not different between Induced LUF and Control groups. Cortisol and GH levels only tended to be

419 higher in the Induced LUF group. Cortisol is thought to be involved in controlling the

420 inflammatory process of ovulation (Espey & Lipner 1994, Andersen 2002). The role of cortisol

421 in LUF formation is unclear at this point. It is possible that over-inhibition of the inflammatory

422 process results in anovulation. Growth hormone has been shown to increase steroidogenesis in

423 granulosa cells, and GH receptors (GHR) have been detected in granulosa cells in mice (Silva *et*

424 *al.* 2009).

425   In this study, higher doses of FM were used in an attempt to increase the incidence of LUFs.

426 Our hypothesis was not substantiated because both 2 mg and 3mg doses of FM induced LUFs in

427 all mares (100% success rate). Nevertheless, the use of FM and hCG provides a reliable model

428 for the study of LUF syndrome (Experiment 2). FM treatments were administered every 12 h and

429 no adverse effects were noted in any animal. The percentage of LUFs in our study was higher

430 than the 83% incidence of LUFs reported following FM treatment in mares (Cuervo-Arango &

431 Domingo-Ortiz 2011) and the 36% incidence following treatment with higher doses of COX-2

432 inhibitor (meloxicam) in women (Jesam *et al.* 2014). The increased incidence of LUFs in the

433 present study was, in part, attributed to a higher dose of FM in our study compared to previous

434 study (Cuervo-Arango *et al.* 2011), and/or to a lower dose (1500 IU) of hCG in our study versus

435 2500 IU in a previous study (Cuervo-Arango *et al.* 2011, Cuervo-Arango & Domingo-Ortiz

436 2011). Therefore, it seems that for proper experimental induction of LUFs, an optimum balance

437　between ovulatory stimulus (hCG) and an adequate decrease of intrafollicular prostaglandin (by

438　the use of COX-2 inhibitor) must be achieved.

439　　　The systemic concentration of PGFM was reduced in FM treated mares at Hour 24 in

440　Experiment 2 and at Hours 12 and 24 in Experiment 3. These results were consistent with

441　previous reports in mares (Ginther *et al.* 2011, Cuervo-Arango *et al.* 2011). Plasma PGFM

442　concentrations are indicative of systemic $PGF_2\alpha$ concentrations, since $PGF_2\alpha$ is rapidly

443　metabolized in the body (Shrestha *et al.* 2012). Lower systemic PGFM levels were also an

444　indicative of the effectiveness of FM treatment in blocking prostaglandin synthesis in our study.

445　　　In Experiment 1, we aimed to induce LUFs by injecting reLH between Days 7−15 with or

446　without treating with $PGF_2\alpha$ on Day 7. However, our hypothesis was not supported because

447　reLH did not induce any LUFs. Furthermore, no differential effect on dominant follicle growth,

448　CL development, or plasma P4 was seen by the use of reLH. $PGF_2\alpha$ decreased the CL lifespan

449　and plasma P4, and shortened the IOIs. LH has been shown to be important for the establishment

450　of follicle dominance in mares (Gastal *et al.* 1999*a*, 2000). Daily injections of eLH at the

451　preovulatory phase, after a 32-mm follicle was detected, failed to induce anovulatory follicles in

452　mares (Schauer *et al.* 2013). Similarly, in our study, the reLH treatment did not affect follicular

453　growth before and after deviation phases, nor did it induce anovulation (LUF). Although the

454　results of our experiment are not similar to what has been described in women (Bergquist &

455　Lindgren 1983) and rats (Mattheij & Swarts 1995), it seems to be premature to assume that LH

456　does not affect LUF formation in mares. The dose and frequency of LH administrations were not

457　evaluated in our study. Continued titration studies are required to fully understand the role of LH

458　in LUF formation.

459   In summary, COX-2 inhibitors used in conjunction with hCG can be used to

460   pharmacologically induce LUFs with 100% success in mares. We postulated that LUFs result

461   from decreased intrafollicular prostaglandin concentrations and/or altered prostaglandin

462   synthesis, as indicated by disparity in $PGE_2:PGF_2\alpha$ ratio. Increased intrafollicular E2 was

463   associated with LUF formation; however, further studies are necessary to ascertain the cause-

464   effect relationship and also to understand the role of testosterone, cortisol, and GH. The effect of

465   LH on LUF formation remains unclear. This study further encourages the use of intrafollicular

466   versus systemic biomarkers for evaluating ovulatory disorders. Finally, results from this study

467   suggest the use of the mare as a potential model for investigating anovulatory infertility in

468   women.

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Figure Legends

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751 **Figure 1.** A series of comparative ultrasound images of preovulatory follicles and LUFs in four

752 women (A–D) and one mare (E–H). Images were obtained before ovulation in women (A) and a

753 mare (E), and various stages of LUF formation. Large diameter, thicker and echogenic follicle

754 wall (luteinized; long arrows), and echoic foci and fibrin-like strands (short arrows) in the

755 follicle antrum can be observed in different degrees in LUFs in women (B–D) and mare (F–H).

756

757 **Figure 2.** Mean ($\pm$ S.E.M.) diameters of the largest follicle and CL, and corresponding

758 concentrations of P4 for Days 7−15 after ovulation (Day 0) for the Control, LH combined, and

759 LH + PG combined groups. Arrow indicates beginning of treatments on Day 7. The probabilities

760 for a group effect (G), day effect (D), and group-by-day interaction (GD) are shown. An asterisk

761 (*) indicates days of a significant difference ($P < 0.05$) between groups and a pound mark (#)

762 indicates a difference that approached significance ($P < 0.1$).

763

764 **Figure 3.** Mean ($\pm$ S.E.M.) $PGF_2\alpha$ metabolite (PGFM) concentration for the Induced LUF group

765 (flunixin meglumine treatments combined) versus the Control group (saline). The probabilities

766 for a group effect (G), hour effect (H), and group-by-hour interaction (GH) are shown. An

767 asterisk (*) indicates hours of a significant difference ($P < 0.05$) between groups.

768

769 **Figure 4.** Mean ($\pm$ S.E.M.) follicle diameter, follicle wall serration, follicle wall thickness, and

770 follicle blood flow for the Induced LUF group (flunixin meglumine treatments combined) versus

771 the Control group (saline). The probabilities for a group effect (G), hour effect (H), and group-

772   by-hour interaction (GH) are shown for Hours 0−38. An asterisk (*) indicates the first increase

773   ($P < 0.05$) within a group.

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775   **Figure 5.** Mean (± S.E.M.) plasma $PGF_2\alpha$ metabolite (PGFM) concentration, follicle diameter,

776   follicle blood flow, and endometrial echotexture score for the Induced LUF group (flunixin

777   meglumine treatment) versus the Control group (saline). The probabilities for a group effect (G),

778   hour effect (H), and group-by-hour interaction (GH) are shown. An asterisk (*) indicates hours

779   of a significant difference ($P < 0.05$) between groups, and a pound mark (#) indicates a

780   difference that approached significance ($P < 0.1$) between groups.

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782   **Figure 6.** Mean (± S.E.M.) follicular fluid concentrations of $PGF_2\alpha$ metabolite (PGFM), $PGF_2\alpha$,

783   PGE_2, and PGE_2:$PGF_2\alpha$ ratio for the Induced LUF group (flunixin meglumine treatment) versus

784   the Control group (saline). Bars with different superscripts within an endpoint are different ($P <$

785   0.05).

786

787   **Figure 7.** Mean (± S.E.M.) follicular fluid concentrations of E2, P4, T, and total primary sex

788   steroids for the Induced LUF group (flunixin meglumine treatment) versus the Control group

789   (saline). Bars with different superscripts within an endpoint are different ($P < 0.05$), and a pound

790   mark (#) indicates a difference that approached significance ($P < 0.1$) between groups. NS, non-

791   significant.

792

793   **Figure 8.** Mean (± S.E.M.) follicular fluid concentrations of LH, cortisol, GH, VEGF-A, and NO

794   for the Induced LUF group (flunixin meglumine treatment) versus the Control group (saline).

795   Bars with a pound mark (#) indicate a difference that approached significance ($P < 0.1$) between

796   groups.

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