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The effects of intrauterine infusion of peanut oil on endometrial health, salivary cortisol and interovulatory period in mares

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Abstract

Intrauterine infusion of peanut oil at Day 10 post-ovulation has been reported to prolong dioestrus in mares. However, the effects of peanut oil treatment on the endometrium and whether the technique is painful have not been assessed. The objectives of this study were, (i) to determine the effect of intrauterine infusion of peanut oil on endometrial health, (ii) to determine whether use of intrauterine peanut oil is painful and (iii) to confirm that peanut oil causes prolonged dioestrus. Six mares aged 3-12 years old were used in a cross-over design with each mare administered both 1 ml of intrauterine peanut oil and a sham treatment on different oestrous cycles. The effect of intrauterine infusion of 1 ml peanut oil or sham treatment were measured using interovulatory period, uterine fluid accumulation as determined by transrectal ultrasonography, serum progesterone levels, endometrial Kenney biopsy scores and histological features, endometrial eosinophil numbers and salivary cortisol measurements. The individual mare response to intrauterine infusion of peanut oil was variable. Peanut oil infusion did not statistically prolong the luteal phase, nor elevate salivary cortisol levels but did cause superficial erosion of the endometrial surface epithelium in all mares and significantly increased eosinophil numbers in the endometrium (P=0.0068). The Kenney grade for biopsies from 2/6 mares worsened transiently following infusion. In conclusion, intra-uterine peanut oil does not statistically increase the duration of the luteal phase but results in an inflammatory response and increase in endometrial eosinophil numbers suggesting treatment may be associated with a hypersensitivity-type reaction. Those contemplating using peanut oil to suppress oestrus should also be aware of the legislative and regulatory implications.

Introduction

Oestrus-related behavioural issues in mares can disrupt athletic performance [1-6]. Altrenogest (Regumate Equine[1]) is probably the drug most commonly used to suppress oestrus in mares. Internationally, its use in mares is not allowed by some governing bodies (e.g. the British Horseracing Authority [7]), but is allowed by others (e.g. the FEI, under certification, [8]; New South Wales Racing [9], and the Hurlingham Polo Association [10]). However, the use of Regumate Equine[1] is not unproblematic since it has the potential to cause positive drug test results for in-contact horses via feed contamination [11], and poses risks to pregnant women, women of childbearing age, and those with certain types of tumour and thrombo-embolic disease. Furthermore, it requires daily administration, which can be burdensome to some commercial operations.

Injectable Altrenogest may provide reliable, short-term suppression of the behavioural signs of oestrus, and avoid some of the problems associated with handling the oral product [6, 12]. Such a product (Readyserv[2]) is currently licensed in Australia. The use of medroxyprogesterone acetate (MPA) has been shown to be ineffective in suppressing oestrus in mares [3, 12, 13]. Repeated injections with low dose intravenous [14] or high dose intramuscular [15] oxytocin prolongs dioestrus (thereby suppressing oestrus) in up to 70% of mares. However, protocols require daily injections for 7-29 days [14, 15] which is challenging for some owners, with some additionally considering the protocol a welfare concern. Injection of human Chorionic Gonadotrophin during dioestrus also potentially prolongs dioestrus, but has only been assessed in a small number of mares [16]. Gonadotrophin releasing hormone (GnRH) vaccines (reviewed in [4]) can be effective in suppressing oestrus [5, 17]. However, there is individual variation in response to treatment with some (particularly older mares) requiring repeated vaccinations, and other mares

entering prolonged (> 12 months) suppression of reproductive cyclicity [4, 17]. This may be undesirable in a commercial context, particularly if the owner wishes to breed the mare immediately following retirement from competition.

Reports of non-medicinal methods of oestrus suppression include the insertion of a marble into the mare's uterus [18-20], manual disruption of an early embryo (to induce pseudo-pregnancy) [2]; and, anecdotally, covert ovariectomy. Intrauterine marbles suppress oestrus unreliably [19, 20] have been reported to fracture [21], to be associated with colic [22] and can damage the endometrium, impacting upon future fertility. There are also ethical issues associated with failure to declare the insertion of an intrauterine marble, during competition, or at sale. Establishing pregnancies in order to kill the embryos is unlikely to be viewed by the general public as ethically acceptable practice [2]. Ovariectomy not only renders the mare irreversibly infertile, but also surgical risks which may be difficult to justify in an ethical harm:benefit analysis, particularly since ovariectomy does not always abolish oestrus behaviour [23].

In 2011, intrauterine infusion of fractionated coconut oil or peanut oil at Day 10 post-ovulation was reported to cause prolonged dioestrus in mares [24]. Potentially, this method of oestrus suppression has the advantages of not requiring medical treatment at the time of competition; of being non-painful; of not carrying drug-associated risks to in-contact humans or horses, and not causing long-term disruption to the reproductive cycle.

Peanut oil is a more probable candidate for oestrus suppression via prostaglandin synthesis regulation than coconut oil, since peanut oil is comprised of mono- and poly-unsaturated fatty acids (PUFAs) [25], whereas coconut oil is comprised primarily of saturated fatty acids [26].

Notably, the second most abundant fatty acid in peanut oil is omega-6 PUFA, linoleic acid, which has been shown to modulate prostaglandin synthesis and influence the relative production of PGF and PGE in ruminant endometrial cells. If these observations in ruminants are applied to mare endometrial cells, it is possible that exposure of equine endometrial cells to linoleic acid could decrease the synthesis of PGF and subsequently inhibit luteolysis [27]. Anecdotally, peanut oil is being used in clinical practice as a method of oestrus suppression in mares, following the publication of the paper of Wilsher and Allen in 2011[24]. However, a 2016 paper [28] showed that intrauterine coconut oil causes an inflammatory reaction in the endometrium, which raises the possibility that treatment with intrauterine plant oil can have a detrimental effect on endometrial health and subsequently future fertility. Furthermore, no studies have been reported assessing whether the intrauterine infusion of either coconut or peanut oil is painful for mares. This paper therefore aimed to investigate the clinical suitability of intrauterine administration of peanut oil as a reversible, welfare-friendly and ethical method of oestrus suppression in mares. The objectives of the study were (i) to determine the effect of intrauterine infusion of peanut oil on endometrial health, (ii) to determine whether use of intrauterine peanut oil is painful and (iii) to confirm that peanut oil causes prolonged dioestrus.

2. Materials and Methods

2.1 Mares

All animal work was performed in accordance with the Animals (Scientific Procedures) Act 1986 guidelines set by the Home Office and Ethics Committee of the Royal Veterinary College (PPL 70/8577). Six mares were identified as being suitable for inclusion in the study following a clinical reproductive examination, and grading of a screening uterine biopsy

sample as Kenney Grade I or IIa. The mares were aged between 3 and 14 years old. Two were Dartmoor ponies (history of donation of multiple embryos); one Standardbred type (no history of foaling); two warmbloods (one maiden, one pluriparous) and one Morgan (who had donated multiple embryos and foaled herself once). The study took place in the physiological breeding seasons across two consecutive years. All mares were kept at grass. Before the start of the experiment, mares were accustomed for 4 days to entering the examination stocks for up to 15 minutes, to rectal examination, and to having saliva swabs taken (see below), in order to minimise/ eliminate the potentially confounding stress which those procedures might cause.

2.2 Study design

All six mares were used according to a cross-over design. For the cortisol and efficacy studies, randomisation of treatment order was included with 3 mares receiving a sham treatment at oestrous one and oil treatment in oestrous two and a further 3 mares receiving oil treatment in oestrous one and sham treatment in oestrous two. For the assessment of endometrial health, all six mares had control biopsies collected at the oestrus prior to both the oestrous periods referenced above. Randomisation for this part of the study was not possible as pre-oil samples were required as controls.

Following initial induction of oestrus by intramuscular injection of 125-250 mcg cloprostenol (Estrumate[3]), each mare had pre-treatment endometrial biopsy samples taken during oestrus (see below for biopsy methods). No further treatments were carried out in the oestrus period in which the pre-treatment endometrial biopsy samples were collected. Having acquired these baseline, pre-treatment endometrial biopsy samples, experiments were undertaken across two subsequent oestrus periods according to the cross over design above.

147

148 *2.3 Monitoring and manipulation of the reproductive tract*

149 Reproductive status including return to oestrus, ovulation and evaluation of the uterus was
150 monitored by a combination of rectal examination, transrectal ultrasonographic evaluation of
151 the reproductive tract, and biweekly serum progesterone sampling (see below). Biweekly
152 serum progesterone continued throughout the initial post-treatment return to oestrus,
153 subsequent dioestrus, and until subsequent return to oestrus had been demonstrated, up to a
154 maximum of 60 days. Ten days after ovulation, mares received either an intrauterine 'sham'
155 treatment or peanut oil treatment according to the cross over design (n=3 received sham
156 treatment at this first cycle later followed by oil treatment at a subsequent cycle and n=3
157 received an oil treatment at this first cycle, later to have a sham treatment at a subsequent
158 cycle). The mare was placed in stocks, her rectum manually evacuated, her tail wrapped in a
159 clean rectal glove and bandaged, and her vulva and perineum washed with dilute
160 chlorhexidine gluconate solution (Hibiscrub[4]) until scrupulously clean, rinsed with water, and
161 dried. An AI pipette was introduced through the mare's dioestrus cervix using a conventional
162 sterile embryo transfer technique [29] taking care not to digitally penetrate the cervical canal,
163 and to minimise trauma to it. The peanut oil was infused as follows: 3.0 ml Peanut oil
164 (Arachis Oil BP20089[5], Table I) taken from a 5 ml aliquot which had been sterilised using a
165 Millex-GP Syringe 0.22 µm Filter Unit10[6,] was loaded by aspiration into a sterile AI pipette
166 with a syringe attached. One ml of oil was deposited into the uterine body. The fact that the
167 oil had been loaded by aspiration into the distal end of the pipette ensured that the full 1ml
168 was deposited. The catheter was not flushed, due to the potentially confounding,
169 inflammatory effects on the uterus of air or flushing liquids. The catheter was withdrawn,
170 and the uterus massaged per rectum to ensure that the oil was distributed throughout the

uterus. As a control (sham), the same procedure was performed but no oil (or any other fluid) deposited into the uterine body.

When mares received a sham treatment, they were monitored until 240 minutes post-treatment for any behavioural signs of discomfort, or vulval discharge. During this time mares were kept in a familiar stall or paddock, with their normal companion. Saliva samples were collected to measure salivary cortisol (see below).

When mares received intrauterine peanut oil, they were monitored until 240 minutes post-treatment for any behavioural signs of discomfort, or vulval discharge. Saliva samples were collected to measure salivary cortisol (see below). Twenty-four hours post treatment, the mare's reproductive tract was examined by rectal palpation and ultrasonography. The ultrasonographic appearance of the corpus luteum and the uterus, and the depth and echogenicity [30] of any free intrauterine fluid were assessed and recorded, as was the presence of any vulval discharge. These examinations were performed for 3-5 days or until no abnormalities were recorded.

When progesterone values fell to 0-1 ng/ml following the oil treatment, suggesting a return to oestrus, this was confirmed by palpation and ultrasound imaging of the reproductive tract. Endometrial biopsies were taken at the first oestrus immediately following oil infusion according to the criteria and technique described above. This time point was chosen for post-treatment biopsies as it reflected when mares might be assessed for future breeding use following intrauterine treatment in a clinical setting. Additionally, to check for infection, in addition to endometrial biopsies endometrial swabs were taken (using a guarded technique) at this oestrus in three mares that had free fluid in the uterus 24 hours following administration

of the oil. (Swabbing was only performed in mares with significant ultrasonographically visible free fluid in the uterus, because swabbing in the absence of clinical suggestion of endometritis was not written into the experimental licensing protocol). If the Kenney grade attributed to the post-treatment biopsy sample was the same or better than the pre-treatment result from the same site in the same mare, no further biopsies were taken. Where the Kenney grade for the post-treatment sample was worse than that of the pre-treatment sample, further biopsies were taken at the next oestrus period (2/6 mares).

2.4 Endometrial biopsies

Pre-treatment (control) and post-oil treatment endometrial biopsies were taken from each mare as follows. Reproductive status was monitored using rectal palpation, ultrasound imaging of the reproductive tract, and progesterone assay, as described above. Pre-treatment and post-treatment endometrial biopsies were taken from each mare in oestrus, i.e. when she had at least one ovarian follicle of ≥35mm diameter; significant uterine oedema, and a relaxed cervix, and recorded serum progesterone levels were 0-1ng/ml. One biopsy was taken from the base of each uterine horn using Equivet endometrial biopsy forceps (Kruuse UK[6]), and conventional biopsying technique [31] under light sedation (40 µg/kg i.v. Romifidine, (Sedivet 1% Injection[7]). Endometrial biopsies were individually preserved in 10% buffered formalin. Each sample was attributed a random code for labelling. The date, identity of the mare, sample site (right or left horn), and code were recorded by the person taking the biopsies. All endometrial biopsies were submitted to the Royal Veterinary College Diagnostic Laboratory, processed to paraffin wax and sectioned at 6 µm using standard techniques, then examined and graded using the Kenney and Doig system (1986) by a specialist veterinary pathologist (KCS), who was blinded to the identity of the mare and the stage of treatment. Eosinophil counts were performed by counting the absolute number of

221 eosinophils in ten randomly selected sections of endometrium examined at x400
222 magnification.

223

224 *2.5 Saliva sampling and Cortisol Assay*

225 An initial, baseline saliva sample ('Paddock') was taken from the mare at rest (i.e. in a
226 familiar paddock or stable, in familiar company) by holding a Salivette[8] swab between locked
227 forceps and gently rolling the swab around the mare's tongue and between her tongue and
228 cheeks for approximately one minute. The Salivette was returned to ice for ≤ 4 hours before
229 transportation to the laboratory. Mares were brought into the breeding barn in the company of
230 a familiar companion, to minimise stress. The mare was then placed in stocks, her rectum
231 manually evacuated, her tail wrapped in a clean rectal glove and bandaged, and her vulva and
232 perineum washed with dilute chlorhexidine gluconate solution (Hibiscrub[4]) until scrupulously
233 clean, rinsed with water, and dried. With the mare in stocks, a second saliva swab was taken
234 ('pre-treatment'). The mare was then treated either with sham infusion, or with infusion of
235 peanut oil as above. Additional saliva swabs were taken at 10, 30, 45, 60, 90, 120, 180 and
236 240 minutes post sham/oil treatment, when the mares was in her familiar stable or paddock,
237 with her familiar companion.

238

239 Additionally, three mares underwent a further study of cortisol reactions to being placed in
240 stocks, as follows. Mares had a baseline saliva sample taken at rest. They were then led into
241 the stocks and had a second swab taken whilst in the stocks (in the absence of a rectal
242 examination), 15 minutes after the first sample was taken. The mares were removed from the
243 stocks and had further swabs taken at 45, 75, 195 and 255 minutes after the first sample.

244

Salivette swabs inside their collection tubes were stored on ice until transported to the laboratory. Tubes were then centrifuged for 10 minutes at 1000xg. Recovered saliva was transferred to a 1.5 mL centrifuge tube and stored at -20°C until analysis. All saliva samples were frozen within 4 hours of collection. Salivary cortisol analysis was carried out using an enzyme immunoassay based on a competitive format (Salimetrics, State College, PA) as described by the manufacturer. Briefly, saliva samples were thawed, vortexed, and centrifuged at 1500xg to precipitate mucins. Samples and cortisol-HRP conjugate were added to a microtiter plate pre-coated with monoclonal anti-cortisol antibodies. The plate was incubated for 1 hour at room temperature. Each well was washed 4 times with phosphate-based wash buffer. Tetramethylbenzidine substrate was added, and the plate was incubated in the dark for an additional 25 minutes at room temperature. Stop solution was added and the optical density was read at 450nm on a Infinite M200 Pro plate reader 11[10]. Samples were analysed in duplicate. The sensitivity was 0.007 µg/d, intra-assay coefficient of variation was 5.74% and inter-assay coefficient of variation was 5.16%.

2.6 Progesterone assays

Twice weekly serum samples were collected from mares starting at ovulation immediately prior to oil infusion. Progesterone was determined by competitive immunoassay (Immulite Progesterone) and measured on an Immulite 1000 analyser at Rossdales Laboratories, as previously described [32].

2.7 Statistical analysis

Statistical analysis was performed in GraphPad Prism 6[11]. Normality testing was performed on all data sets. Inter-ovulatory period was compared using a paired t-test. Differences in

salivary cortisol in experiment 1 were assessed using a repeat measures two-way ANOVA and post hoc Bonferroni's multiple comparisons test with the source of variation defined as time and treatment and comparing all time points to sample 1 ('Paddock'). Differences in salivary cortisol in experiment 2 were assessed using a Friedman test with a post hoc Dunn's multiple comparison test. A comparison of endometrial Kenny Grade and eosinophil counts before and after infusion of the oil was made using a Wilcoxon matched-pairs signed rank test.

3. Results

3.1 Efficacy of treatment

There was no significant difference in interovulatory period when mares received a sham (n=6) or intrauterine peanut oil infusion infusion (n=6) (Supplementary Figure 1, P=0.8433). The mean +/- SE inter-ovulatory period for mares receiving sham treatment was 23+/- 2.1 days and for oil treatment 28.2+/- 5.8 days. In four mares, intrauterine oil infusion did not extend the interovulatory period beyond what would be expected under physiologically normal conditions (interovulatory periods of 14, 20; 21 and 22 days) (Fig. 1, Mares I-IV). Two mares that received intrauterine peanut infusion experienced prolonged interovulatory periods of 45 and 47 days (Fig. 1, Mares V, VI).

3.2 Uterine response to treatment

No intrauterine fluid was detected using ultrasonography in any of the mares during oestrus prior to pre-treatment control biopsies being taken, prior to sham treatment, or prior to oil treatment. One of six mares exhibited opaque vulval discharge twenty four hours after oil treatment, which was not obvious 48 hours after treatment. Ultrasonographic examination of

the reproductive tract 24 hours after oil infusion demonstrated a 'delineating' pattern in the uterine horns of all six mares (Fig 2A). In 4/6 mares, in whom oil treatment was not associated with a prolonged interovulatory period, hyperechoic free fluid of 0.5-3cm depth was imaged within the uterine lumen 24 hours after treatment (Fig. 2B). No treatment was given to clear this fluid. Endometrial swabs taken at the beginning of the next oestrus from 3/4 of the mares who had free fluid in the uterus 24 hours after oil infusion, were found to be negative for pathogenic bacteria and fungal growth when cultured for 48 hours under aerobic and anaerobic conditions on 5% sheep blood agar, MacConkey agar, and Staphylococcus/Streptococcus selective agar, all at 37°C. The fourth mare (with a fluid depth of 0.5cm) was not swabbed, for the reasons related to licensing explained above. Culture of the peanut oil from the same batch used to infuse the mares was also negative for bacterial and fungal growth

Endometrial biopsies (n=6 mares, one each from left and right horn) collected prior to peanut oil infusion showed no evidence of significant inflammatory or glandular disease. Following intrauterine oil infusion, endometrial biopsies were collected at the next return to oestrus. This ranged from 10 to 40 days following oil infusion. There was no significant difference in the endometrial Kenney Grade before and after intrauterine infusion of peanut oil (n=6, p=0.999) (Table II). There was no change in the endometrial Kenney Grade for biopsies from both left and right uterine horns before or after intrauterine peanut oil infusion in 4/6 mares (Table II). In 2/6 mares, the Kenney grade for one of the two biopsies collected from each mare post-treatment transiently worsened from grade I to grade IIa, returning to pre-oil classification by the second oestrus period post oil infusion (Table II). None of the mares biopsied post oil infusion showed significant endometrial inflammatory or glandular disease, consistent with the failure to culture pathogens from endometrial swabs. The four mares that

returned to oestrus rapidly after oil treatment all showed multifocal erosion of the surface epithelium, and in some cases this was associated with scattered subjacent or transmigrating neutrophils, consistent with surface or intraluminal irritation (Figure 3A-D). Two mares only showed small or rare surface erosion of the epithelium. As a direct result of the prolonged dioestrus experienced by these two mares, these biopsies were collected significantly longer after oil infusion (37, 40 days). The presence of eosinophils in endometrial biopsies was noted in 4/6 mares post peanut oil infusion but only 1/6 mares prior to infusion of the peanut oil (Fig. 4A). Eosinophil counts were quantified in endometrial sections pre and post peanut oil infusion. The median number of eosinophils in the endometrium was significantly increased post peanut oil infusion ($p=0.0068$) when compared to numbers prior to the oil infusion (Fig. 4B).

3.3 Stress response to treatment

In order to determine whether the infusion of peanut oil was painful, salivary cortisol was monitored prior and immediately following the intrauterine infusion of peanut oil or sham treatment. There was no significant difference in the salivary cortisol levels in mares receiving intrauterine peanut oil or sham treatment at any time point (Fig. 5A). There was a significant increase in salivary cortisol following placement of the mares into the stocks and a rectal examination (pre-treatment) when compared to salivary cortisol levels measured in the paddock for both sham and oil groups (Fig. 5A). This rise in salivary cortisol was sustained at 10 minutes post oil/sham treatment but then dropped back to paddock levels for the remainder of the measurement period. To further explore whether the transient rise in salivary cortisol was due to restraint or rectal examination, a second cortisol experiment was performed whereby salivary cortisol was measured in the paddock, after placement in stocks (but with no rectal examination performed) then at 30, 60, 180 and 240 minutes after removal

from stocks, to mimic a selection of the sampling time in Fig 5A. There was no transient rise in salivary cortisol following placement in stocks alone (n=3 mares) (Fig 5B). There was a significant decrease in salivary cortisol at 180 minutes post removal from stocks.

One mare who did not experience an extended interovulatory period following treatment exhibited behavioural signs of mild discomfort (elevated tail, vulval 'winking') from 10-30 minutes following oil infusion. No behavioural signs of discomfort (elevated tail, vulval 'winking') were observed in the remaining 5 mares following either the sham or peanut oil infusion.

Discussion

The primary purpose of this paper was to investigate the effects of intrauterine peanut oil - a treatment which had previously been reported to be an efficacious method of suppressing oestrus in mares - on endometrial health, and salivary cortisol levels. Intrauterine infusion of peanut oil caused some superficial erosion of the surface epithelium of the endometrium in all mares. This was most pronounced in mares who did not undergo a prolonged luteal phase, and thus were biopsied much later in relation to the day of treatment. Though the superficial nature of the damage makes it likely to repair spontaneously, the licensing constraints of this project meant that we do not know for certain that a repair process occurred, or how long it takes. Whilst it persists, the superficial erosion of the epithelium may compromise mares' endometrial immune defence system, and make them more prone to endometrial infection caused, for example, by environmental contaminants which access the reproductive tract.

Four of six mares also appeared to exhibit an immunological reaction to the peanut oil, as evidenced by ultrasonography, histology and bacteriology. The ultrasonographic appearance of oil in the uterus immediately and in the days after infusion - a 'delineating' pattern

believed to be caused by the hyperechoic oil lining the endometrial folds (which were themselves not pronounced because the mares were in dioestrus) - was very similar in this study to that reported by Diel de Amorim et al [28]. The hyperechogenicity, distribution and volume of this pattern allowed it to be easily distinguished from non-oil, free fluid in the uterus. None of the mares who underwent a prolonged luteal phase following oil treatment in this study exhibited free intrauterine fluid on ultrasonography 24 hours after oil infusion. Conversely, all of the mares who did not undergo a prolonged luteal phase did exhibit intrauterine fluid following treatment. Ultrasonographically detected intrauterine fluid can be either infectious or sterile. Possible sources of infection include contamination with environmental pathogens during the catheterisation of the cervix, and bacterial contamination with the oil. Mares were prepared for catheterisation according to standard procedures which are practiced during successful embryo transfer by the authors. Guarded endometrial swabs taken from three of the mares who had free fluid in the uterus 24 hours after oil infusion were negative for pathogenic bacteria (the fourth mare was not swabbed). The fact that none of the mares underwent a short luteal phase on the sham cycle suggests that contamination due to poor technique was unlikely. Bacteriological culture of the oil was negative. This is consistent with the findings of Diel de Amorim et al [28], who cultured their coconut oil to rule out infection as a cause of treated mares undergoing shortened luteal phases, and also got negative culture results

Diel de Amorim et al [28] reported a lymphoplasmocytic inflammatory cell infiltration and neutrophilic inflammation of the stratum compactum of the endometrium following intrauterine coconut oil infusion, with occasional eosinophils seen. The inflammatory response to intrauterine peanut oil infusion seen in this study was predominantly eosinophilic, with an increase in the number of eosinophils observed in the endometrium following

intrauterine peanut oil infusion. This is consistent with previous reports that eosinophils are found only occasionally in endometrial biopsies from clinical normal mares [33], but are frequently associated with an acute immune reaction (e.g. to seminal plasma [34]), and in cases of pneumovagina / pneumouterus [31] . Indeed, an acute immunological response to oil in the uterus has been described previously [35].

Although negative culture results from the mares and the oil make it unlikely, we cannot definitively rule out infection, which subsequently resolved, as the cause of the fluid which was imaged in the uterus post treatment. Nonetheless, the combination of the ultrasonographical, biopsy and laboratory results in 4/6 mares who did not undergo a prolonged luteal phase in response to treatment are more suggestive of a transient, sterile, eosinophilic, hypersensitivity-like endometrial inflammation, reaction to the peanut oil, although to make this conclusion, this would need to be assessed immediately following treatment. Either way, this uterine inflammation presumably provokes a release of PGF2α from the endometrium, that out- competes any potential anti-luteolytic effects of the peanut oil fatty acids, leading to luteolysis.

The clinical significance of this eosinophilic infiltrate in some mares following intrauterine infusion of peanut oil needs to be further investigated. We do not know whether repeated intrauterine infusions of peanut oil in such mares are likely to result in a gradual desensitisation to treatment, or, conversely, in an increased sensitisation, with an associated possible risk of a more systemic reaction. Previous research on intrauterine inflammatory reactions in the mare suggests that treatment with steroidal or non-steroidal anti-inflammatory drugs [36, 37] at the time of intrauterine peanut oil infusion could dampen /

abolish the hypersensitivity-like, eosinophilic response, thereby increasing the likelihood of mares responding to treatment. However, this possibility is currently unproven and requires further research. Furthermore, any injection (however well tolerated) constitutes a welfare harm, and the ethical justification of inflicting that harm in order to improve the chances of an otherwise unsuccessful treatment working when a non-painful, efficacious, licensed alternative treatment is available is doubtful.

One of the aims of this study was to determine whether intrauterine infusion of peanut oil is painful for mares. This was assessed using a combination of observations of behavioural indicators of stress / pain and measurements of salivary cortisol as an indicator of stress [38, 39]. The fact that one mare who did not undergo a prolonged interovulatory interval following treatment exhibited behavioural signs of mild discomfort (elevated tail, vulval 'winking') from 10-30 minutes after oil infusion should not be ignored. However, in 5/6 mares the oil treatment appeared to be well-tolerated, with no behavioural indicators of stress / pain (for example kicking/stomping feet, listlessness, reluctance to eat, abnormal facial expressions) being observed. Furthermore, the cortisol results show that there was no stress response associated with intrauterine infusion of oil itself. However, there was a stress response associated with the rectal examination which was performed prior to oil infusion, as confirmed by the additional experiment undertaken to differentiate between the effects of restraint in stocks and rectal examination on stress. This is consistent with a finding recently reported by others [39] for lactating and non-lactating pregnant mares, and, to the authors' knowledge, is the first demonstration of a stress response to rectal examination in non-pregnant mares.

This study took as its starting point the fact that intrauterine infusion of peanut oil had been previously shown to be an effective method of oestrus suppression in mares. Our primary aims were to assess the effects of that treatment on endometrial health and salivary cortisol. It is noteworthy, however, that the efficacy of intrauterine peanut oil at prolonging dioestrus was significantly lower in this experiment than in the one previous report [24]. In that study, luteal persistence for 30 days was reported in 11/12 mares following treatment. In the present study, intrauterine oil infusion was associated with increased interovulatory periods (of 45 and 47 days) in 2/6 mares, however when taking into account the 4/6 mares that did not respond to treatment, this observation was not statistically significant. Such variability of responses between mares has also been reported for other methods of oestrus suppression (e.g. [4, 15, 18, 19]). It is also consistent with the recent work of Diel de Amorim et al [28], which failed to reproduce the results which Wilsher and Allen [24] obtained with coconut oil. Furthermore, since mares are known to undergo spontaneously prolonged luteal phases [40, 41], it is possible that the prolonged interovulatory period in 2/6 mares was not actually caused by the oil treatment.

The variability between our results and those of Wilsher and Allen [24] could potentially be explained by a difference in the exact composition of the peanut oil used in the two studies. All experiments described in this paper used a standardised batch of peanut oil (Arachis Oil BP2008[5)], which was batch tested for fatty acid composition (Table I), in order to enable regulatory bodies to assess its permissibility. It is impossible to accurately compare the composition of this batch with that of the peanut oil used by Wilsher and Allen [24] because, although those authors provided a table of the general composition of peanut oil, the paper did not describe the exact composition of the batch which they used. Nonetheless, when one compares the generic composition provided by Wilsher and Allen [24] and the composition

of the peanut oil which we used, there do not seem to be differences sufficient to account for a disparity in response. For example, it would be unlikely that the slightly higher levels of oleic acid in the batch used in this study (69.8% versus range 36.4-67.1%) would lead to more rapid luteolysis. Previous studies have shown that if one exposes pregnant ewe endometrial cells to increasing concentrations of oleic acid, the ratio of PGF2α:PGE2 moves in favour of PGE2 [42]. If one applies this concept to the non-pregnant mare endometrium, the oil used in this study, if anything, should have lengthened the period to luetolysis.

Another possible reason that mares might have failed to enter prolonged dioestrus following treatment would be if the process of infusion itself caused luteolysis, via a prostaglandin release provoked by cervical stimulation [29]. Wilsher and Allen [24] used Wilsher forceps [43] to facilitate oil infusion. In the present study, a conventional, commonly-used non-surgical embryo transfer technique [29] was used to pass the pipette through the cervix, as this technique is what would more likely be used by clinicians in general practice. The technique used in this study was also used in the study on intrauterine coconut oil by Diel de Amorim et al [28]. It is unlikely that the difference in technique for cervical catheterisation between this study and that of Wilsher and Allen [24] resulted in a prostaglandin release which would account for the failure of luteostasis in 4/6 mares. The operator has years of successful experience with non-surgical embryo transfer using the technique adopted in this study. More importantly, if luteolysis was being caused by insertion of the pipette through the cervix, one would have expected that to occur during the sham treatment as well oil treatment, whereas in fact no shortening of the inter-ovulatory period following sham treatment was recorded.

In addition to the clinical information provided by this study, those contemplating using intrauterine peanut oil to suppress oestrus in mares should be aware of the legislative and regulatory implications. Intrauterine peanut oil is likely to be classified as a medicine by medicines regulatory authorities. It is currently unlicensed for oestrus suppression, whereas licensed products (e.g. Altrenogest, Regumate Equine[1] and Readyserv[2]) are available. Furthermore, peanut oil might also be considered to be a medicine by sport regulatory authorities. In that case, its use might be prohibited during competition, though its use prior to the competition period (meaning that mares were still in dioestrus at the time of competition) might be permitted – this needs regulatory clarification.

Conclusions

The results of this study suggest that, like intrauterine infusion of coconut oil [27] intrauterine infusion of peanut oil is at least temporarily detrimental to endometrial health. Veterinarians recommending the use of intrauterine peanut oil infusion should be aware that neither this study nor the papers published by Wilsher and Allen [24] and Diel de Amorim et al [28] included any assessment of pregnancy rates in mares bred when they returned to oestrus after oil treatment. Until this data is made available by future research, the long-term implications of intrauterine peanut oil infusion for fertility are unproven. Furthermore, similar to recent work using intrauterine treatment with coconut oil [28], this study failed to demonstrate that intrauterine peanut oil is an efficacious method of oestrus suppression.

Acknowledgments

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Competing interests: None

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Manufacturers' details

1. MSD Animal Health Walton Manor, Walton, Milton Keynes MK7 7AJ UK
2. Ceva Animal Health Pty Ltd, 11 Moores Road, Glenorie NSW 2157 Australia
3. MSD Animal Health Walton Manor, Walton, Milton Keynes MK7 7AJ UK
4. Regent Medical Ltd, Medlock Street, Oldham, Lancs, OL1 3HS, UK
5. Augustus Oils Ltd, Augustus House, Mill Lane, Alton, Hants, UK
6. Merck, Suite 21, Building 6, Croxley Green Business Park Watford Hertfordshire WD18 8YH United Kingdom
7. Kruuse UK Ltd.12 Sherburn Network Centre, Lancaster Close, Sherburn in Elmet, North Yorkshire LS25 6NS UK

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641 **8.** Boehringer Ingelheim Limited, Ellesfield Avenue, Bracknell, Berkshire RG12 8YS,
642 UK

643 **9.** Sarstedt AG&Co, D-51588 Numbrecht Germany

644 **10.** Tecan, Seestrasse 103, 8708 Männedorf, Switzerland
645
646 **11.** GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA

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650 **SUMMARY OF FIGURES AND TABLES:** 2 tables and 5 figures

651

652 **FIGURE LEGENDS**

653

654 **Table I:** Composition of Peanut Oil (Arachis Oil BP2008, batch PE108505), measured by
655 gas liquid chromatography. SFA indicates saturated fatty acid, MUFA indicates
656 monounsaturated fatty acid, PUFA indicates poly-unsaturated fatty acid.

657

658 **Table II:** Endometrial histological features prior to and following infusion of peanut oil.
659 Results for the left horn and right horn are shown as l/r. * indicates a valid biopsy was not
660 read. [a]Biopsies taken at an oestrus prior to infusion of peanut oil. [b,d]Biopsies taken at first
661 oestrus following infusion of peanut oil. [c]Biopsies taken at second oestrus following infusion
662 of peanut oil.

663

664 **Figure 1:** Serum progesterone in mares I-VI (Table II) administered 1 ml intrauterine peanut
665 oil on day 10 post ovulation (indicated by *). Day 0 is the day of ovulation immediately prior
666 to administration of the oil.

667

668 **Figure 2:** Ultrasound images taken 24 hours after the intrauterine infusion of peanut oil. The
669 image on the left was taken from a mare, who underwent a prolonged interovulatory period
670 following intrauterine peanut oil infusion. This shows oil (hyperechoic) delineating the
671 dioestrus endometrial folds of the right uterine horn as it spreads and is trapped between

672 them. The image on the right is taken from a mare, who returned to oestrus within 4 days
673 following treatment. Note the measurable quantity (>2cm) of hyperechoic fluid within the
674 lumen of the uterine horn, which is believed to represent a sterile inflammatory reaction to
675 the oil infusion.

676

677 **Figure 3:** Endometrial biopsies pre- and post-oil administration. H and E stained
678 representative sections in mares that had short (top (Mare I) and middle (Mare II)) and long
679 (bottom panel (Mare VI)) inter-ovulatory periods following oil infusion. **Top panel** pre-oil:
680 intact endometrial surface epithelium with scattered stromal leucocytes; post-oil: endometrial
681 surface erosion with small to moderate numbers of stromal leucocytes. **Middle panel** pre-oil:
682 intact endometrial surface epithelium with small numbers of stromal leucocytes; post-oil:
683 endometrial surface erosion with small to moderate numbers of stromal leucocytes. **Bottom**
684 **panel** pre-oil: intact endometrial surface epithelium with scattered stromal leucocytes; post-
685 oil: intact endometrial surface epithelium with scattered stromal leucocytes.

686

687 **Figure 4 (A)**. Endometrial biopsy showing eosinophilic infiltration of superficial stroma with
688 associated oedema. Eosinophil arrowed. H&E x400. (**B).** Eosinophil numbers in the
689 endometrium prior to and following the administration of intrauterine peanut oil (n=11
690 sections, line indicates median value).

691 **Figure 5: Figure 5 (A)**. Salivary cortisol measured prior to and following the administration
692 of 1 ml intrauterine peanut oil (n=6) or sham (n=6) procedure. Saliva samples were taken in
693 the paddock prior to moving the mares into the stocks (paddock), after restraint in stocks,
694 rectal examination and preparation of the vulvar region and immediately prior to
695 administration of sham or peanut oil (pre-tx), and 10-240 minutes following the

696 administration of oil (black bars) or sham delivery (grey bars). **B**. Salivary cortisol was
697 measured in a paddock, after restraint in the stocks (pre-tx) and at 30-240 minutes following
698 removal from the stocks (n=3 mares). Cortisol was measured using an enzyme immunoassay
699 as described in materials and methods. * indicates $p<0.05$ and *** $p<0.001$ compared to the
700 paddock sample.

701

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Table II Endometrial histological features prior to and following infusion of peanut oil. Results for the left horn and right horn are shown as l/r. The * indicates a valid biopsy was not read. [a]Biopsies taken at an oestrus prior to infusion of peanut oil. [b]Biopsies taken at first oestrus following infusion of peanut oil. [c]Biopsies taken at second oestrus following infusion of peanut oil. [d]Biopsies taken at first oestrus post infusion of intrauterine peanut oil.

	Kenny Grade			Erosion of surface epithelium[d]	Eosinophil Count Pre oil Infusion LH/RH	Eosinophil Count Post oil Infusion LH/RH
	Pre-Oil[a]	Post-Oil 1[b]	Post-Oil 2[c]			
Normal interovulatory period						
Mare I	I/IIa	IIa/IIa	I/I	Multifocal	1/0	0/1
Mare II	IIa/IIa	IIa/IIa	n/a	Multifocal	2/3	20/30
Mare III	I/I	IIa/I	I/I	Multifocal	0/0	24/16
Mare IV	I/*	I/I	n/a	Multifocal	2/*	6/0
Prolonged interovulatory period						
Mare VI	I/IIa	I/I	n/a	Small	10/16	12/20
Mare VI	I/I	I/I	n/a	Rare	0/1	2/0

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Table I: Composition of Peanut Oil (Arachis Oil BP2008, batch PE108505) as measured by gas liquid chromatography. SFA indicates saturated fatty acid, MUFA indicates monounsaturated fatty acid, PUFA indicates poly-unsaturated fatty acid.

Fatty Acid	**Classification**	**Systematic Name**	**Total fatty acids (%)**
Palmitic Acid	SFA	Hexadecanoic acid	6.24
Stearic acid	SFA	Octadecanoic acid	1.77
Oleic acid	MUFA Omega-9	9-Octadecenoic acid	69.77
Linoleic acid	PUFA Omega-6	9,12-Octadecadienoic acid	12.26
Linolenic acid	PUFA Omega-3	9,12,15-Octadecatrienoic acid	0.22
Arachidic acid	SFA	Eicosanoic acid	0.92
Eicosenoic acid	MUFA	(z)-icosa-11-enoic acid	2.86
Behenic acid	SFA	Docosanoic acid	2.75
Erucic acid	MUFA Omega-9	*cis*-13-docosenoic acid	0.45
Lignoceric acid	SFA	Tetracosanoic acid	2.05

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Figure 5

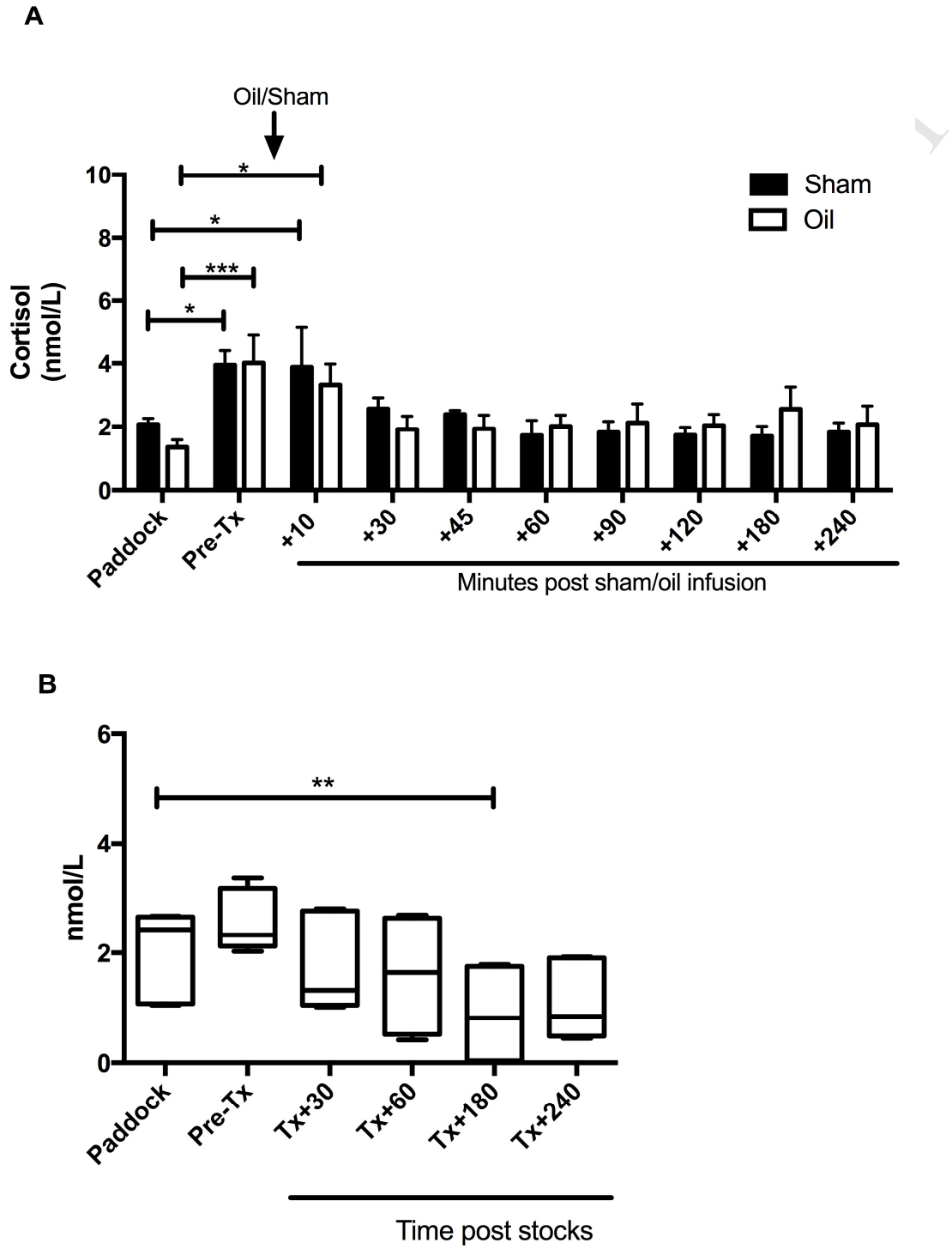
A
Oil/Sham
Sham
Oil
Cortisol (nmol/L)
10
8
6
4
2
0
*
*
***
*
Paddock
Pre-Tx
+10
+30
+45
+60
+90
+120
+180
+240
Minutes post sham/oil infusion
B
nmol/L
6
4
2
0
**
Paddock
Pre-Tx
Tx+30
Tx+60
Tx+180
Tx+240
Time post stocks

Figure 1

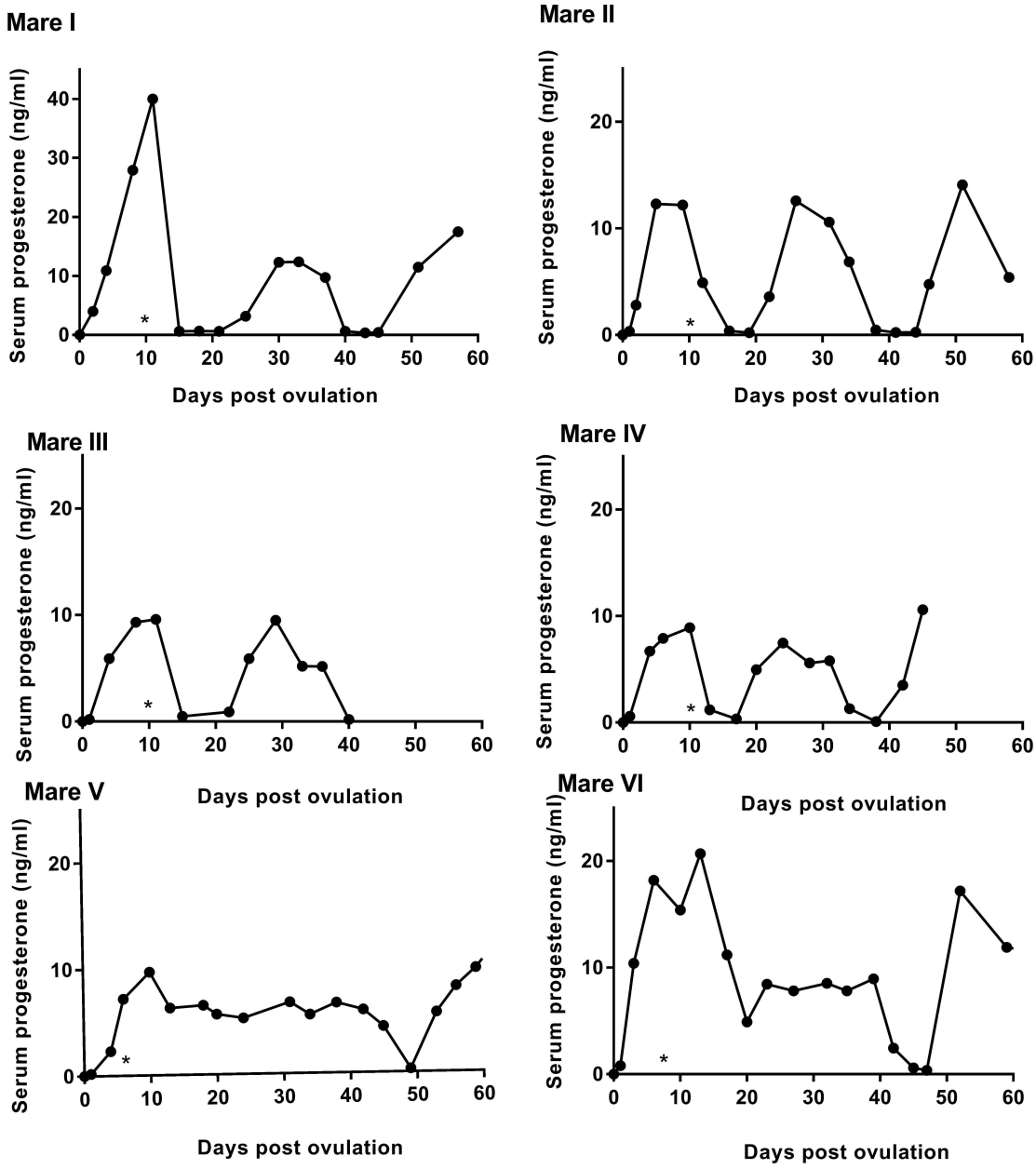
Mare I
Serum progesterone (ng/ml)
Days post ovulation
Mare II
Serum progesterone (ng/ml)
Days post ovulation
Mare III
Serum progesterone (ng/ml)
Days post ovulation
Mare IV
Serum progesterone (ng/ml)
Days post ovulation
Mare V
Serum progesterone (ng/ml)
Days post ovulation
Mare VI
Serum progesterone (ng/ml)
Days post ovulation

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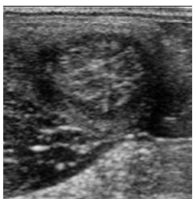
Figure 2

A



B

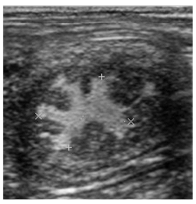


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Figure 3

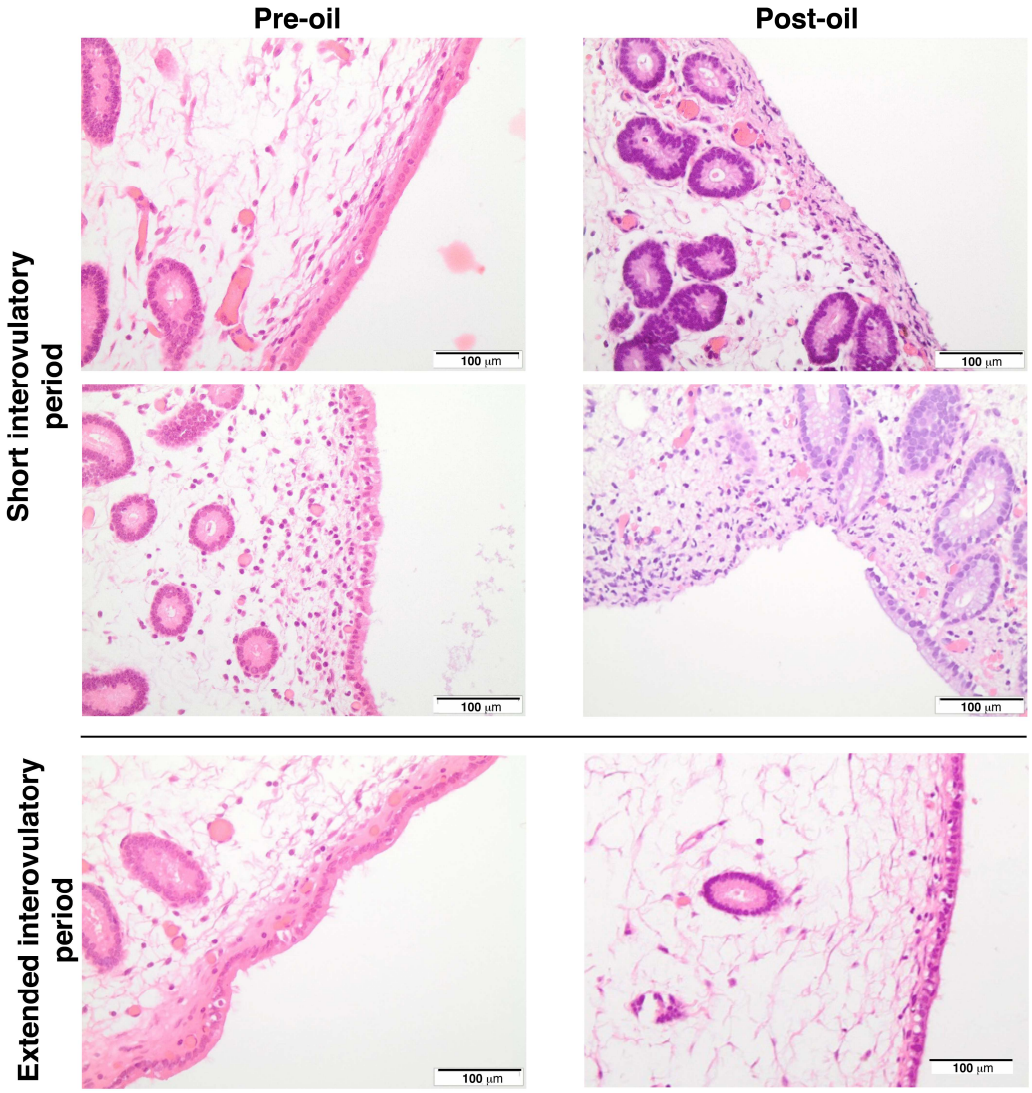
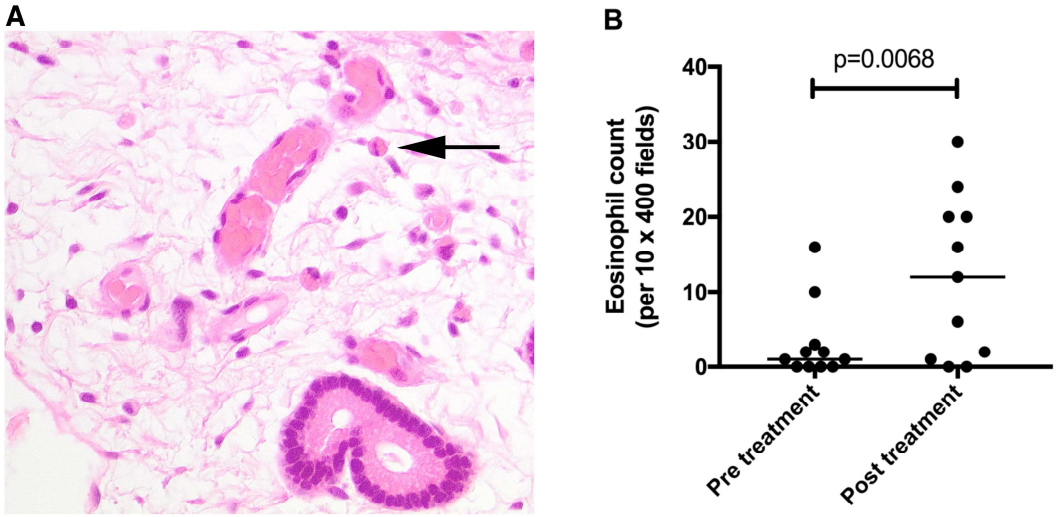

Pre-oil
Post-oil
Short interovulatory period
Extended interovulatory period
100 µm

Figure 4


A
B
p=0.0068
Eosinophil count
(per 10 x 400 fields)
40
30
20
10
0
Pre treatment
Post treatment

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Highlights:

Campbell et al, The effects of intrauterine infusion of peanut oil on endometrial health, salivary cortisol, and interovulatory period in mares

- The response to intrauterine infusion of peanut oil in dioestrus mares is variable
- Intrauterine peanut oil does not statistically prolong the luteal phase in mares
- Intrauterine peanut oil causes superficial erosion of endometrial surface epithelium
- Intrauterine peanut oil causes an increase in endometrial eosinophil numbers
- Rectal examination but not intrauterine peanut oil causes a rise in salivary cortisol

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