STUDIES ON EARLY FETAL LOSS IN MARES

BY

GIORGIA PODICO

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS - Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

Master’s Committee:

Assistant Professor Igor F. Canisso, Chair and Director of Research
Assistant Professor Fabio S. Lima
Clinical Associate Professor Clifford S. Shipley
Clinical Instructor Edgar F. Garrett
ABSTRACT

Early pregnancy loss (EPL) is an important multifactorial condition that affects up to 20% of equine pregnancies, and mare age, parity, previous endometritis and twin pregnancy have all been implicated as causes of early pregnancy loss. The timing of the abortion strongly influences the outcome on the fertility. The regression of the endometrial cups, which form from trophoblastic cells by 35 days of gestation, is a self-regulated mechanism that is independent from fetal death, therefore mares that abort after the endometrial cup formation will present abnormalities in the ovulations, follicular growth and cyclicity due to the concurrent presence of equine chorionic gonadotropin (eCG). Early pregnancy loss has short- and long-term consequences to the horse industry, therefore, the development of novel diagnostic and management tools for early pregnancy loss are warranted. Blood markers and ultrasonographic parameters were assessed in mares undergoing experimentally induced early fetal loss. It was hypothesized that intrauterine infusion of cloprostenol results in earlier fetal compromise than systemic administration. Ovarian structures (number and sizes of follicles and corpora lutea area), fetal heartbeat, and fetal mobility of thirteen singleton pregnancies were assessed daily by transrectal ultrasonography until induction of pregnancy termination (60±2 days of gestation). Mares received 500µg of cloprostenol intramuscularly every 12 hours (IM, n=7) or once transcervically (TC, n=6). After initial cloprostenol administration, ultrasonographic examinations were repeated at 6-hours-intervals until loss of fetal heartbeat. Plasma progesterone, estradiol-17β, and alpha-fetoprotein were assessed for five days before and after pregnancy loss. In addition, plasma PGFM concentrations were assessed immediately before cloprostenol administration (0 min), and then 15, 30, and 45 minutes, and 1, 2, 3, 4, 6, 12 hours after administration. Data were analyzed using the MIXED procedure with repeated measures in SAS. Significance was set at \( P<0.05 \). All mares lost their
pregnancies within forty-eight hours after initial cloprostenol administration, with no difference in time to pregnancy loss. There were significant effects of time starting by 12 h post-induction of pregnancy termination but there was no time by group interaction for progesterone concentrations. Estradiol-17β and alpha-fetoprotein concentrations were not altered upon impending abortion. Concentrations of PGFM increased significantly by two hours after cloprostenol administration, but there were no differences between groups. No time effects or time by group interaction for fetal mobility and heartbeat was detected. Expectedly, the number and area of corpora lutea decreased significantly after cloprostenol administration with no significant differences between groups. In conclusion, intrauterine administration of cloprostenol was not different than repeated systemic administration to terminate the pregnancy. Both models for early fetal loss were equivalent for the endpoints assessed herein. Next, to explore the use of infusions of kerosene to enhance the regression of endometrial cups, thirteen mares had intrauterine kerosene infusions at 21- and 35-days post-abortion. Anecdotally, intrauterine infusion with kerosene has been proposed as a method to treat endometrial cup retention, however there are no controlled studies evaluating kerosene’s usefulness for enhancing endometrial cup regression following abortion. We hypothesized that intrauterine kerosene infusions would hasten regression of endometrial cups without detrimental effects on the endometrium and the mare’s general health. The objectives of this study were to assess the uterine response, systemic side effects and efficacy of intrauterine kerosene infusions to enhance regression of endometrial cups. Nine light-breed mares were enrolled in the study after an experimentally-induced abortion with cloprostenol (n=12) by 60 (60±2 days) days of gestation. Mares were randomly allocated an intrauterine infusion with 500mL of kerosene (Ker. n=6) or 500mL saline (Cont. n=6) on day 21 and 35 after the pregnancy termination. Uterine biopsies were collected at day 7, 21, 35 and 49, stained with H&E and graded
according to the Kenney & Doig 1986 classification. Lymphocyte B CD20, lymphocyte T CD3 and macrophage IBA cell populations were characterized by immunohistochemistry in endometrial biopsies from a subset of mares (n=4/group). Physical examinations, complete blood cell counts, and serum biochemistry were performed before each infusion and then repeated for 2 days after each uterine infusion. Uterine lavage was performed 24 hours after each infusion. Serum samples were collected right before the abortion induction, then at 7, 21, 28, 35, 42, and 49 days after the pregnancy termination for assessment of serum eCG. Continuous data were analyzed with MIXED procedure with repeated measures in SAS, categorical data with LOGISTIC procedure in SAS. Significance was set at p<0.05. Kerosene infusion did not affect complete blood cell counts and serum chemistry parameters. There were no appreciable abnormalities on the physical examinations following kerosene infusions. Concentrations of eCG decreased over time (p<0.001), but there were no differences between groups or time by group interactions (p=0.7128). Histology samples from the uterus showed no signs of increased fibrosis or degeneration in the treatment group. In conclusion, while kerosene infusions did not appear to have detrimental effects on mare health, our findings suggest that the use of kerosene in the uterus does not enhance the regression of endometrial cups.
ACKNOWLEDGEMENTS

“Only those who dare may fly”

*L. Sepúlveda, The Story of a Seagull and the Cat Who Taught Her To Fly.*

I am the most grateful to Dr. Canisso for giving me the opportunity to dare and fly. Thank you for believing in me since we first met. I am grateful to the Koteska fellowship and Department of Veterinary Clinical Medicine for funding this research project.

Thank you to all the people I have met and worked with for the last two years, in particular to my master’s committee members (Drs. Fabio Lima, Edgar Garret, and Clifford Shipley); thank you for all your help, support, and smiles. I also would like to thank all co-authors (Robyn Ellerbrock, Scott Austin, Robert Douglas, Patrick Roady, Mariano Carossino, Vitor Mercadante, and Nicholas Dias), I could not have done this without all of you, as you imagine, and flying is sometimes hard!
# TABLE OF CONTENTS

## CHAPTER 1: LITERATURE REVIEW

1.1 Endocrinology of equine pregnancy ................................................. 1
1.2 Endometrial cups biology ................................................................. 4
1.3 Early pregnancy loss in mares ............................................................ 7
1.4 Termination of pregnancy in horses ..................................................... 10
1.5 Usage of kerosene in broodmare practice .......................................... 12
1.6 Figure and tables ............................................................................. 15
1.7 References ..................................................................................... 19

## CHAPTER 2: ASSESSMENT OF PERIPHERAL MARKERS AND ULTRASONOGRAPHIC PARAMETERS IN PREGNANT MARES RECEIVING SYSTEMICALLY OR INTRAUTERINE CLOPROSTENOL

2.1 Abstract ......................................................................................... 25
2.2 Introduction ................................................................................... 27
2.3 Material and methods ................................................................. 30
2.4 Results ......................................................................................... 33
2.5 Discussion .................................................................................... 35
2.6 Conclusion .................................................................................. 38
2.7 Figures and tables ....................................................................... 40
2.8 References ............................................................................... 49
CHAPTER 3: UTERINE RESPONSES AND eCG CONCENTRATIONS AFTER TWO INTRAUTERINE INFUSIONS WITH KEROSENE POST EARLY FETAL LOSS IN MARES

3.1 Abstract
3.2 Introduction
3.3 Material and methods
3.4 Results
3.5 Discussion
3.6 Figures and tables
3.7 References

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS
CHAPTER 1: LITERATURE REVIEW

1.1 Endocrinology of equine pregnancy

Pregnancy is one of the most hormonally active stages of an animal’s lifespan. Several classes of hormones (e.g., steroids and proteins), play a role in the development and maintenance of the pregnancy. Progesterone production in mares, for example, rely for the first 100 days of gestation only on the ovaries; but, starting by 80-90 days of pregnancy, the placenta becomes able to synthesize steroids from fetal precursors into 5α-dyhidroprogesterone, which is a progestogen with the same function of progesterone. Throughout pregnancy, several estrogens, progestogens and androgens are produced in high concentrations by the equine fetoplacental unit and are released into the maternal circulation. The hypertrophied fetal gonads of both male and female fetuses secrete large quantities of androgens, particularly dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one also known as 5-androsten-3β-ol-17-one) and 7-dehydro DHEA (3β-hydroxy-5,7-androstadien-17-one) [1]. Of interest, DHEA serves as a precursor for classic phenolic estrogens (i.e., estrone, estradiol-17β oestradiol-17α and their sulfoconjugates), whereas 7-dehydro-DHEA serves as a precursor for ring B unsaturated estrogens (i.e. equilin, equilenin and their hydroxyderivatives 17α-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilin and 17β-dihydroequilenin) by the equine placenta [1]. During early pregnancy, the corpora lutea have been shown to be an important source of estrogen and testosterone [2,3]. The following sections contains a description of key hormones involved in the equine pregnancy.

First evidence of estrogen presence in the equine conceptus appears by 10-12 days of gestation with the detection of yolk-sac fluid [4-7]. At this early stage, estrogens are produced by the trophoblastic cells of the yolk-sac wall and by 24-26 days of gestation, estrone sulfate (E₁S) is
the predominant estrogen [6] (Table 1.1). Estrone sulfate is also produced by the early bovine conceptus in small quantities (<10pg/mL) [8,9].

During the first four weeks of gestation, the equine embryo proper is capable of metabolizing the different isoforms of estrogens located in the yolk-sac fluid and the conversion of estrone (E₂) into E₁S is dramatically increased [6]. After 100 days of gestation, the conversion to estrogens from androgens produced by the newly developed fetal gonads occurs in the placenta [10-12]. The isolation of radioactive metabolites of the exogenous testosterone administered to a 6-month pregnant mare was the first evidence that testosterone was the progenitor of maternal estrogens [11]. In this process, fetus and placenta constitute an indissoluble pair, the fetoplacental unit, and cessation of the production of androgens due to fetal demise causes a rapid decrease in the maternal estrogen level both in the mare and cow [1,13-16]. Another source of estrogens in the equine pregnancy is the corpus luteum increases the production of precursors and enzymes for steroid production [17]. As a result of this enhanced metabolism, large quantities of estrogens are present in the mare’s urine, uterine and fetal fluids. Furthermore, concentration of estrogens in the uterine fluid of pregnant mares (range 8-20 days post ovulation) is higher than in non-pregnant mares [5,18]. The presence of estrogens in urine allowed the opportunity for extensive studies on characterization of those hormones more than 50 years ago. In addition, to classic phenolic estrogens, horse have two unique estrogens with an unsaturated ring B tailored to the equine species, β-dihydroequilin and α-dihydroequilenin are present in high concentrations in plasma and urine of pregnant mares during the second and third trimester of gestation [1,19].

It has been postulated that estrogens are involved with the feto-maternal communication, but in horses the role of estrogens is not completely elucidated [6,20]. In pigs estrogens play an important role in maternal recognition of pregnancy [21,22]. In horses, it has been speculated that
estrogens may play a role in the maternal recognition of pregnancy, but it has not been confirmed. The presence of high concentrations of estrogens and their receptors in reproductive tissues throughout pregnancy suggest potential function of estrogens in further stages of development [6,7,23,24].

The detection of estrogens is usually performed by immunoassays, but the use of other methods such as liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) could decrease the risk of cross-reactivity within the different types of estrogens. As the name suggests, progesterone is essential for the maintenance of the pregnancy in the mare and other domestic animal (i.e. cow, pig, dog, sheep, goat, and cat). The main functions imply the block of the uterine contractions, the inhibition of estrus behavior and the production of the uterine histotrophe. In the horse, progesterone produced by the ovary is required for the first 50 to 70 days of gestation [25-27]. During this stage of pregnancy, progesterone is produced first by the primary corpus luteum and subsequently by the accessory corpora lutea formed under the influence of eCG [28] (Fig. 1). The increasing production of other pregnanes by the placenta with progesterone activity such as 5α-dihydroprogesterone (DHP) coincides with reduction of eCG concentration and ovarian luteal tissue [28]. By 105-110 days of gestation, the levels of pregnanes reach a sufficient level to completely support the pregnancy [29].

The detection of steroids is usually performed by immunoassays, but the use of other methods such as liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) could decrease the risk of cross-reactivity within the different types of steroids [29,30].
1.2 Endometrial cups biology

The equine placenta is epitheliochorial, characterized by the presence of six layers (maternal and fetal endothelium, connective tissue and epithelium) between the maternal side and the conceptus. The diffuse location of the villi throughout the allantochorion allows the exchange of nutrients, catabolites, and blood gases in a less intimate manner than the carnivore and primate placenta.

Although the equine chorioallantois is lightly attached to the endometrium by 25 days of gestation a group of cells start to organize at the middle of the embryonic vesicle to form the chorionic girdle, by 35 days of gestation cells from this region begin proliferating, differentiating into binucleate cells, and acquiring invasive phenotype [31]. Invasive trophoblastic cells invade the endometrium and subsequently migrate into the stroma to form white plaques known as endometrial cups [31]. The morphology of the endometrial cups is variable and dependent on the stage of maturation of the structure itself [31,32]. Cells in the mature endometrial cups classically present with large dimension, columnar, binucleate, with a basophil cytoplasm enriched with small vacuolization containing eCG secretion [31,33]. The enhanced invasive phenotype of the chorionic girdle cells has been confirmed with the first attempts of transplantation under the albuginea of a testis and in the non-gravid horn of a mare [34]. Both cases resulted in the formation of cell aggregates with the typical morphology of those cells, which constitute the endometrial cup in vivo. In another study, transplantation of tissue from endometrial cups under the vulvar mucosa of non-pregnant recipients caused estrus suppression for more than three months after the procedure [35,36].

Typically, by 100 to 140 days of gestation the endometrial cups regress leaving small pouches of necrotic material and avillous areas in the chorioallantois [32]. The exact mechanism by which the maternal immune response contributes to endometrial cup regression is still unclear.
Additionally, the lifespan of the endometrial cups is not influenced by the MHC-compatible or -incompatible status or by a previous sensitization with an allograft from the conceptus of the same stallion [37,38]. With an average more than 38 million cells in less than ten grams of tissue, the endometrial cups could constitute a hazard towards the maternal immune system and ignite a rejection of the cups ending their essential role of inducing the formation of accessory corpora lutea [36].

The presence of lymphocyte CD4+ and CD8+ clusters around the cups and the systemic modulation of the maternal immune system have been partially characterized. The local immune response results in high concentrations of lymphocytes, especially at the beginning of the formation but then decreases and it is limited to the periphery when the cups are regressing [39]. The distribution of white blood cells gathers a larger amount of lymphocyte CD4+ compared to lymphocyte CD8+ and a scarce amount of B cells. This proportion is significantly different from the normal endometrial population, where the majority of the lymphocytes present are CD8+ [36,39]. *In vitro* experiments have shown profound modifications of the lymphocyte proliferation and activation due to the local factors (i.e. cytokine) produced by the trophoblastic cells itself [40].

Cytokine IL-22 is produced by chorionic girdle cells is to stimulate the repair of damaged epithelium after the invasion of the cells from the chorionic girdle [40]. The concept of split tolerance has been developed to describe the absence of rejection for the endometrial cups and the conceptus [36]. This concept suggests that the local tolerance of the immune cells towards the invading trophoblastic cells without affecting the peripheral immunological response or both compartments of the immune system. While the local concentration and distribution of white blood cells around the endometrial cups change throughout gestation, there is no difference between the peripheral levels of white blood cells between pregnant and non-pregnant mares [36,41].
Interesting that the presence of endometrial cups triggers a strong humoral response by 60 days of gestation while the cellular response is not activated [42]. Increased expression of MHC-I molecules on the surface of the invasive trophoblastic cells has been suggested as the trigger for humoral response [42]. The expression of those surface antigens is starts slightly before the invasion by 33-35 days of gestation and continues until 41-44 days of gestation [43,44]. Trophoblastic cells of the chorionic girdle failing to migrate also express MHC-I [43]. The MHC class I molecules present both paternal and maternal origin [37], but the pattern and expression features does not change with the compatibility of the antigens between the stallion and the broodmare [43]. The presence of natural-killer cells inside and around the endometrial cups has been reported but their role in the equine pregnancy is still unclear [44].

The role of the endometrial cups as source of eCG, previously called pregnant mare serum gonadotropin (PMSG), was not elucidated for more than 30 years after their first description [32,45,46]. Although the origin of the molecule was unclear, the gonadotropic effect of the serum collected from pregnant mare on other animals, like mice, was rapidly discovered and applied in the clinical and diagnostic field [47]. Equine chorionic gonadotropin is a glycoproteic hormone formed by an alpha subunit, which is homologous to equine LH and FSH, and a beta subunit, which is sequentially different than other gonadotropins. The high affinity for LH receptor and the lower affinity towards the FSH receptor makes eCG responsible for the production of secondary/accessory corpora lutea. The secondary/accessory corpora lutea aid progesterone and consequent pregnancy maintenance. Low luteal function and consequent low progesterone concentration are known causes of pregnancy loss in mares [27,48].

The production of eCG is variable within mares reaching a peak that ranges from 101-143 UI/mL by 60-70 days of gestation. The peak of eCG is followed by a steady decrease until
disappearing between 120 to 150 days of gestation [49,50]. Positive correlations have been demonstrated between peripheral eCG concentrations, nutrition and parity, while a negative impact is related to the level of exercise [51]. The sire’s genetics influences eCG production in mares [49]. For instance, mares carrying mule pregnancies show a reduced peripheral eCG concentration despite the presence of morphologically normal endometrial cups [49]. In addition, eCG is not detectable in plasma of mares carrying mule pregnancies by 90 days of gestation [49] (Table 1.2).

The presence of endometrial cells or progesterone is not necessary for growth of cells from the chorionic girdle in vitro or in vivo. In vitro culture of cells from the chorionic girdle resulted in an eCG production that could last up to 180 days [34]. Successful transplantation has been reported in the vulvar mucosa of non-pregnant animals (i.e. low levels of progesterone) [38]. The presence of circulating eCG after a considerable time from abortion have been reported. Crabtree et al. (2012) reported two Thoroughbred mares with persistent endometrial cups producing eCG after more than one year after abortion [52].

1.3 Early pregnancy loss in mares

Causes of EPL in all the species of domestic animal includes non-infectious causes (genetic, nutritional, degenerative, inflammatory) and infectious causes [53]. The classification reported in a review focused on the equine species and identified intrinsic and extrinsic factors [54]. Intrinsic factors included endocrine causes (i.e. estrogens or progesterone insufficiency), oviductal abnormalities, uterine environment (fibrosis, endometritis, embryo-uterine synchrony), and maternal age. Extrinsic factors involve stress, nutrition, season, external temperature, sire effects, and embryonic effects (i.e. chromosomal defect) [54]. Few dissimilarities were seen due to the specific physiological features of each species; however, this classification could be applied
to all the domestic mammals. The occurrence of EPL in domestic animals is higher in the first few weeks of gestation. In horses EPL occurs up to 18.6% by the first 60 days of gestation [48,55-57].

Identifying a single factor that could positively or negatively influence the incidence of pregnancy loss in domestic animal can be challenging. In mares, retrospective studies recognized significant effects of age (> 14yo), parity (>6 pregnancies), twin pregnancy, presence of endometritis, low quality protein diets (35% low vs 7.3% high quality diet), and chromosomal abnormalities [55-58], while no effect from the breed, stallion, reproductive status, or foal heat breeding has been described [55-58]. However, a retrospective study reported an incidence of 32% of embryonic losses occurring between 14 days and 21 days in in Arabian mares [59].

Mare aging is one of the main predisposing factors to early pregnancy loss (EPL), resulting in the degeneration of oocyte quality [60], lower uterine contractility, more severe degenerative changes of the endometrium [60-63], and lower lymphatic drainage and increasing number of endometrial cysts in mares [56,63]. In one report, mares > 15 years old had 62% of the embryonic losses [63]. In mares, breeding time in regard to can influence pregnancy loss in mares, in one classic study mares being bred on the day of ovulation had 34% of pregnancy loss while mares bred before ovulation had 14% [55]. Recent multivariate analyses revealed that the use of ovulatory agents such as deslorelin and hCG, and the administration of flunixin meglumine during manual twin reduction could decrease the odds for pregnancy loss [64].

Experimental evidence exists linking low peripheral progesterone concentrations and pregnancy loss in horses [48,25]. Progestin supplementation has also been reported to rescue one pregnancy in a mare suffering with premature luteal regression [65]. Prolonged luteal insufficiency results in limited embryo migration, delayed fixation, decreased sizes, and altered orientation of the
embryonic vesicle [27,66]. Modification of the biochemical composition of the capsule have been detected after PGF2α (dinoprost tromethamine, 5mg IM) administration as a result of an altered interaction with the endometrium [67]. Changes in the composition of the mucine glycoproteins, which are present in higher quantities in the embryo from the PGF2α -treated group, likely produced a delay in fixation and abnormal orientation of the embryonic vesicle [27,67]. Although, in mares the impact of the progesterone on the pregnancy before the fixation of the embryonic vesicle is not fully elucidated, a study reported negligible influence of progesterone for the development of the embryonic disc and embryo proper appeared at ~3 days [68]. Additionally, one study reported that administration of progestin up to 3 days after the onset of the low progesterone concentrations was able to rescue the embryonic vesicle and promote further embryonic development [27].

Premonitory signs (e.g., growth retardation, uterine edema, abnormal morphology) of an impending embryonic loss (before day 12) are not consistently present [27]. Ultrasonographic findings correlating with pregnancy loss include uterine edema, shift of the conceptus from the uterine horn to the body, smaller size, deformity of the vesicle, collapsed yolk sac, and lack of heart beat [25,69]. Abnormal looking embryos in the early phase of pregnancy are more likely to get reabsorbed [25,56].

One retrospective study reported increased serum amyloid A concentrations in prospectively sampled mares experiencing early embryonic death [59]. The increased in peripheral concentrations of serum amyloid A could be due to chronic endometritis, since this protein is highly expressed in the equine endometrium [70]. The pronounced hormone production capacity of the equine conceptus makes hormonal assessment a potentially useful approach to determine the pregnancy status. In fact, the equine conceptus produces estrogen by day 10 during its
development [6,7] and those estrogens could be potentially assessed in the mare’s blood and used as a prognostic marker. A steep rise of total estrogens has been reported by 70-75 days of gestation [50] and estrone sulfate could be detected in the serum of pregnant mares (Kasman et al., 1988). Evidence that a viable fetus is necessary for the production of estrone sulfate were reported [17,71]. A linear decrease in the total estrogen concentration 5 hours before fetal expulsion was reported in pregnant mares (average 92 days gestation) treated with cloprostenol sodium [17].

1.4 Termination of pregnancy in horses

Pregnancy termination in mares can be performed after mismating, legal disputes, undesired fetal sex, or twin pregnancy. The formation of the endometrial cups by 35 days of gestation constitutes as an important landmark in equine pregnancy. The presence of eCG stimulates the production of accessory corpora lutea that are fundamental as the progesterone source after the primary corpus luteum. Additionally, by 120-150 days of gestation, the endometrial cups regress spontaneously with a decrease in the peripheral level of eCG and the placenta increases the production of progesterone becoming the only and most important source of progesterone. Based on those events, it is paramount to classify the methods to terminate pregnancy in mares along three different time-period: before 35 days of gestation, between 35 days and 120 days, and from 120 days (mid-gestation) to term.

Compared to the other farm animals, there is no countercurrent transfer of PGF$_{2\alpha}$ from uterine vein to the ovarian artery to induce luteolysis in mares [72]. In mares, PGF$_{2\alpha}$ is released systemically and achieve the ovaries via systemic circulation [73]. Once in the systemic circulation, PGF$_{2\alpha}$ is rapidly metabolized (half-life <1min) into PGFM (13, 14-dihydro-15-keto-
The low amounts and short half-life of PGF$_{2\alpha}$ reaching the corpus luteum is thought to be compensated by higher affinity to the receptors present in the corpus luteum [73].

First evidence of the abortifacient effect of PGF$_{2\alpha}$ is reported when the administration of dinoprost tromethamine (5mg, IM) successfully induced abortion in pregnancy of 12, 21, and 30 days in mares [48].

A single administration of PGF$_{2\alpha}$ terminates approximately 50% of the pregnancies between 40 and 150 days, with higher percentage of success between 40 and 70 days [75]. Consecutive daily administration of 250 µg fluprostenol sodium terminated 70-day old pregnancies faster compared to twice daily administration [76]. Another study reported promising results in terminating 42-day pregnancies with daily administration of 5 mg dinoprost tromethamine with an average time between the first administration to the abortion of 3 days [77]. These results are similar to the ones reported with daily injections of 250 µg cloprostenol sodium between 98 and 150 days of gestation [17]. Side effects of multiple administrations (1.25-1.50 mg PGF$_{2\alpha}$ im) were reported to be transitory from 30 to 45 minutes and generally include sweating, soft manure, and mild colic-like signs [75]. More invasive methods include surgical removal of fetus and fetal membranes [13], the infusion of saline during a laparotomy procedure [71], manual dilation of the cervix, and digital puncture of the fetal membranes [78].

During mid-gestation, the use of prostaglandin analogues require multiple administrations, as single administrations are ineffective to induce abortion [79,80]. Two intramuscular administrations of 500 µg of fluprostenol were unsuccessful to induce the abortion in mares between 105 and 133 days of gestation [79]. Furthermore, the administration of intramuscular and intravaginal fluprostenol simultaneously caused abortion in only one of the five mares enrolled in one study at 141.5 days of gestation average [79]. In two studies when the systemic administration
of dinoprost failed to induce the abortion, the local administration of saline or dexamethasone inside the allantoic cavity successfully terminated the pregnancy in all mares [80,81]. However, the transcervical administration of drugs is thought to potentially expose the mare to greater risks for uterine infections, which could lead to metritis and laminitis [81]. Due to the size of the fetus at this stage of gestation, dystocia could occur as well as retained [81] (Table 1.3).

More recently, a single transcervical administration of 500 µg cloprostenol sodium to 60-day pregnant mares results in abortion within 48 hours in 84-94% of the mares treated [78,82].

1.5 Usage of kerosene in broodmare practice

In equine practice, endometritis is the third most common medical problem in horses [83]. Traditionally, administration of antibiotics, systemically or locally in the uterus, steroids, non-steroidal anti-inflammatory drugs, uterine lavage and ecbolic agents are used to manage endometritis in mares [84,85]. Despite all the efforts and possibilities for the treatment, chronic endometritis could be challenging, and conventional therapy usually fails; thus, alternative therapies have been proposed and variously explored [85]. To date, the beneficial effects of kerosene on mare’s fertility has not been explored in any controlled situations.

Kerosene has proposed as an alternative therapy to treat chronic endometritis and to reverse fibrosis [86]. The first anecdotal communications came from Australia where kerosene was reported as successful therapy to treat subfertile mares. Its use is in broodmare practice is not unanimously accepted, and almost considered a taboo by many practitioners. A group-of mares (n=28) presenting severe degenerative changes of the endometrium reached 45% of foaling rate after an intrauterine kerosene infusion [86]. No control group was used in the study [86], but the perceived results on the fertility was markedly positive since the expected foaling rate of similar
mare is about 10% according to the Kenney and Doig (1986) classification [61]. Similarly, Scoggin (2016) reported that 60% of mares, classified as subfertile and barren for more than two years, became pregnant after the infusion with kerosene [85]. It has been thought that kerosene induces a chemical curettage that would strip the superficial layer of epithelium, resulting in regeneration of the luminal epithelium and the removal of inspissated secretion from the uterine glands [86]. The hypothesis of an increased amount of ciliated cells after the kerosene infusion causing an increased uterine clearance was rejected [87].

The amount of kerosene used in the intra-uterine infusions varies from 50 to 500mL [85-88]. Due to the chemically irritant nature of kerosene, the tools used for the administration should be resistant to it. It has been recommended to perform a uterine lavage by 24 hours after kerosene infusion to remove the remaining potential debris.

The potential side effects of kerosene administration to mares and operators have been poorly documented. One case report described colic signs and pyometra in a mare with chronical endometritis treated with kerosene [88]. Other authors did not mention any remarkable side effects to mares or operators [52,85-87]. Dermatitis was reported in one case where the reflux in the vagina eroded the sleeve and attained direct contact with the operator’s skin [88].

The effects of kerosene on the uterus is usually mild and self-resolving. After 24 hours post kerosene infusion hysteroscopy, cytology, and histology examination revealed a mild to moderate inflammatory response with the presence of uterine edema, intrauterine fluid accumulation, and the presence of polymorphonuclear cells and decreased number of ciliated epithelial cells [85-88]. Rare presence of diphtheroid inflammation with focal areas with erosion were described [85-88]. The inflammation is usually resolved by 7-14 days and no signs of adhesion, fibrosis, or necrosis are reported afterwards [85,88].
Anecdotally, intrauterine infusion of kerosene has been used in clinical practice to treat retained endometrial cups. A case report with two mares described the successful regression of the endometrial cups in one of the two mares [52]. The underlying mechanism is the physical removal of the superficial layers that constitute the endometrial cups and the stimulation of a strong inflammatory and immune response that would improve the regression of the cups. No case control study examining the effects on the endometrial cups removal were performed.
Fig. 1.1. Peripheral concentrations of eCG, progesterone and dihydroprogesterone (DHP) in the first 210 days of the equine gestation. The shaded area represents the luteo-placental shift that take place (from Conley, 2016).
Table 1.1. Concentration of estrogens in yolk-sac fluid in equine conceptus at 24-26 days of gestation (Raeside 2009)

<table>
<thead>
<tr>
<th>Estrone sulfate (E$_1$S)</th>
<th>39.67 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (E$_1$)</td>
<td>7.74 ng/mL</td>
</tr>
<tr>
<td>Estradiol 2 sulfate (E$_2$S)</td>
<td>4.14 ng/mL</td>
</tr>
<tr>
<td>Estradiol 17-β (E$_2$)</td>
<td>2.01 ng/mL</td>
</tr>
</tbody>
</table>
Table 1.2. Peripheral concentration of eCG at the peak of the production in horse, donkey, and hybrid pregnancies (Allen 1969)

<table>
<thead>
<tr>
<th>Dam x Sire</th>
<th>Days of gestation</th>
<th>Peak (UI/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse x Horse</td>
<td>60</td>
<td>101-143</td>
</tr>
<tr>
<td>Donkey x Donkey</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>Horse x Donkey</td>
<td>47</td>
<td>17</td>
</tr>
<tr>
<td>Donkey x Horse</td>
<td>47</td>
<td>203</td>
</tr>
</tbody>
</table>
Table 1.3. Pregnancy termination in horse

<table>
<thead>
<tr>
<th>Technique</th>
<th>Dose</th>
<th>Stage of Gestation (days)</th>
<th>Efficiency</th>
<th>Time to fetal demise</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinoprost tromethamine</td>
<td>5mg IM</td>
<td>12</td>
<td>100% (n=4)</td>
<td>6.2 ± 0.2 d</td>
<td>Ginther 1985</td>
</tr>
<tr>
<td>Dinoprost tromethamine</td>
<td>5mg IM</td>
<td>21</td>
<td>100% (n=4)</td>
<td>1.8 ± 0.2 d</td>
<td>Ginther 1985</td>
</tr>
<tr>
<td>Dinoprost tromethamine</td>
<td>5mg IM</td>
<td>30</td>
<td>100% (n=4)</td>
<td>2.0 ± 0.4 d</td>
<td>Ginther 1985</td>
</tr>
<tr>
<td>Dinoprost tromethamine</td>
<td>5mg IM q24h x2</td>
<td>&lt;35</td>
<td>100% (n=11)</td>
<td>n/a</td>
<td>Ginther 1985</td>
</tr>
<tr>
<td>Dinoprost tromethamine</td>
<td>5mg IM q24h x4</td>
<td>&gt;35</td>
<td>n/a</td>
<td>6.2 ± 0.2 d</td>
<td>Ginther 1985</td>
</tr>
<tr>
<td>PGF2α</td>
<td>10mg IM x1-3</td>
<td>47-89</td>
<td>100% (n=7)</td>
<td>n/a</td>
<td>Kasman 1988</td>
</tr>
<tr>
<td>Saline</td>
<td>20mL in the allantoic cavity</td>
<td>45</td>
<td>100% (n=10)</td>
<td>3-24 h</td>
<td>Jeffcott 1987</td>
</tr>
<tr>
<td>Fluprostenol sodium</td>
<td>250µg IM Q12-24 h</td>
<td>70</td>
<td>100% (n=16)</td>
<td>5.2 ± 0.6 d</td>
<td>Squires 1980</td>
</tr>
<tr>
<td>Cloprostenol sodium</td>
<td>500µg added to 8mL saline, TC</td>
<td>60-76</td>
<td>94.8% (n=32)</td>
<td>&lt;48 h</td>
<td>Cuervo-Arango 2015</td>
</tr>
<tr>
<td>Cervical dilation</td>
<td>With digital puncture of fetal membranes</td>
<td>70-77</td>
<td>96% (n=22)</td>
<td>&lt;48 h</td>
<td>Estradé 2016</td>
</tr>
<tr>
<td>PGF2α</td>
<td>2.50mg SC q12h</td>
<td>80-90</td>
<td>100% (n=2)</td>
<td>54.5 ± 24/5 h</td>
<td>Douglas 1974</td>
</tr>
<tr>
<td>Cloprostenol sodium</td>
<td>250µg IM q24h</td>
<td>82-102</td>
<td>100% (n=6)</td>
<td>48.6 ± 5.6 h</td>
<td>Duels 1995</td>
</tr>
<tr>
<td>Fluprostenol</td>
<td>375µg IM q6-12 h</td>
<td>92-240</td>
<td>100% (n=6)</td>
<td>47 h ± 25</td>
<td>Van Leeuwen 1983</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>30mg in 15mL saline</td>
<td>167-174</td>
<td>100% (n=4)</td>
<td>44-72 h</td>
<td>Wichtel 1988</td>
</tr>
<tr>
<td>PGF2α</td>
<td>2.50mg SC q12h</td>
<td>160-180</td>
<td>100% (n=7)</td>
<td>41.3 ± 3.2 h</td>
<td>Douglas 1974</td>
</tr>
</tbody>
</table>
1.6. References


2.1. Abstract

Blood markers and ultrasonographic parameters were assessed in mares undergoing experimentally induced early fetal loss. It was hypothesized that intrauterine infusion of cloprostenol results in earlier fetal compromise than systemic administration. Ovarian structures (number and sizes of follicles and corpora lutea area), fetal heartbeat, and fetal mobility of thirteen singleton pregnancies were assessed daily by transrectal ultrasonography until induction of pregnancy termination (60±2 days of gestation). Mares received 500µg of cloprostenol intramuscularly every 12 hours (IM, n=7) or once transcervically (TC, n=6). After initial
cloprostenol administration, ultrasonographic examinations were repeated at 6-hours-intervals until loss of fetal heartbeat. Plasma progesterone, estradiol-17β, and alpha-fetoprotein were assessed for five days before and after pregnancy loss. In addition, plasma PGFM concentrations were assessed immediately before cloprostenol administration (0 min), and then 15, 30, and 45 minutes, and 1, 2, 3, 4, 6, 12 hours after administration. Data were analyzed using the MIXED procedure with repeated measures in SAS. Significance was set at $P<0.05$. All mares lost their pregnancies within forty-eight hours after initial cloprostenol administration, with no difference in time to pregnancy loss. There were significant effects of time starting by 12 h post-induction of pregnancy termination but there was no time by group interaction for progesterone concentrations. Estradiol-17β and alpha-fetoprotein concentrations were not altered upon impending abortion. Concentrations of PGFM increased significantly by two hours after cloprostenol administration, but there were no differences between groups. No time effects or time by group interaction for fetal mobility and heartbeat was detected. Expectedly, the number and area of corpora lutea decreased significantly after cloprostenol administration with no significant differences between groups. In conclusion, intrauterine administration of cloprostenol was not different than repeated systemic administration to terminate the pregnancy. Both models for early fetal loss were equivalent for the endpoints assessed herein.

Keywords: abortion, pregnancy termination, early pregnancy loss, progesterone, ultrasonography, PGF2α analogue.
2.2 Introduction

Pregnancy wastage results in considerable economic losses to the horse industry. Costs and consequences associated with early pregnancy loss include stud fees, veterinary services and supplies, loss of valuable offspring, feeding and boarding costs, mare and/or semen transportation, increased odds for uterine infections, and retention of endometrial cups [1]. Early pregnancy (embryonic or fetal) loss (EPL) occurring within 60 days of gestation represents the bulk of pregnancy wastage in broodmares [2-4]. The financial implications of EPL are amplified after 35 days gestation, when retained endometrial cups may result in irregular estrous cycles and the inability to rebreed the mare that season [5,6].

Many conditions have been linked with EPL in mares including maternal illness, stress, luteal insufficiency, endometrial disease, embryonic/fetal abnormalities, interspecific pregnancy, iatrogenic drug administration, and extreme weather conditions [2,7]. The incidence of early pregnancy loss ranges from 2.5% to 18.6% [3,4,8-10]. A recent multivariate analysis study involving Thoroughbred mares reported strong and positive associations between EPL and age of the broodmare, the number of previous live offspring and the presence of uterine cysts [12]. The incidence of EPL increases in older mares (>15 years old) [9-12]; aged mares may lose up to 60% of pregnancies, in comparison with younger mares (<15 years old) which may experience up to 11% of pregnancy loss [10].

In clinical practice, pregnant mares are primarily monitored by transrectal ultrasonography during the first 60 days of gestation [13]. Mares are typically checked by 15, 30 and 60 days of gestation and not rechecked until after parturition unless there is a suspicion of pregnancy loss. However, mares with a history of EPL or presenting abnormal clinical findings (e.g., endometrial edema) at 15, 30, or 60 days may be checked more frequently and may additionally have
reproductive hormones, particularly progesterone [14-18], evaluated. As progesterone is thought to maintain pregnancy in the first trimester of pregnancy, a reduction in its peripheral concentration is associated with pregnancy loss in some reports [14,17].

Estrone sulfate concentrations have been shown experimentally to be a useful marker for early fetal demise in mares [19-21], however, assay availability and time for results has limited its use in clinical practice. Estradiol-17β assays, such as chemiluminescence (Immulite 1000 or 2000 platforms, Siemens Medical Solutions), are more widely available, allowing for faster turnaround time for results and a wider application in clinical practice. Since the equine conceptus produces appreciable quantities of estrogen during early gestation [19,22,23], estradiol-17β has the potential to be a useful marker for early fetal demise.

Anecdotally, compromised equine fetuses experience increased mobility during acute stress, and reduced mobility during chronic stress, and a similar trend has been suggested for the fetal heartbeat. However, these findings have not been critically assessed. While fetal parameters are well documented in physiological conditions [24], experimental models for pregnancy loss in the first trimester of pregnancy did not include a detailed ultrasonographic evaluation of fetal heartbeat and mobility [25,26]. Ultrasound evaluation of fetal parameters in mares undergoing experimentally induced EPL would allow a detailed assessment of fetal parameters under controlled conditions.

Alpha-fetoprotein is an albuminoidal protein normally present in the fetal fluids and maternal plasma throughout gestation [27,28]. Concentration of alpha-fetoprotein increase in the plasma with of mares with placentitis, twin pregnancies, or imminent abortion [28-30]. While one study reported an increase in alpha-fetoprotein concentrations in plasma of mares undergoing spontaneous EPL [29], this protein has not been evaluated under controlled experimental
conditions. It is possible that administration of cloprostenol compromises the fetoplacental unit culminating with leakage of alpha-fetoprotein to peripheral plasma in mares, however, this hypothesis has not been studied.

Until recently, pregnancy termination was not commonly conducted in horses, except for resolution of twin pregnancies, embryonic/fetal/maternal abnormalities, mismatings, or legal disputes [31]. However, recently, pregnancy termination has become widely used in Polo ponies, where female horses are preferred during the training and competitions and for the potential reproductive career. In Argentina, embryo recipients mares carrying male fetuses have the pregnancy terminated after fetal sexing by 60 days of gestation [26,32]. It is estimated that thousands of pregnancies are terminated via a single transcervical administration of cloprostenol [26,32].

Single systemic administration of natural or synthetic forms of PGF$_{2\alpha}$ is effective to terminate equine pregnancy in the first four weeks of gestation [33]. By five weeks of gestation mares become “resistant” to PGF$_{2\alpha}$ [34] and may require repeated administration of PGF$_{2\alpha}$ to effectively abort [19,34-36]. The reduced efficacy of PGF$_{2\alpha}$ coincides with the appearance and lifespan of endometrial cups, thus authors have attributed this phenomenon to the presence of endometrial cups and its secretion of eCG [34,37]. Of interest, mares are reported to abort rapidly (84% in <48h) after single intrauterine administration of cloprostenol [26]. This study aimed to compare methods of prostaglandin-induced abortion and to assess blood markers and ultrasonographic parameters in mares undergoing experimentally induced early fetal loss. It was hypothesized that intrauterine infusion of cloprostenol would result in earlier fetal compromise than systemic administration.
2.3. Material and methods

The present study was performed from September 2017 to August 2018 at the College of Veterinary Medicine of the University of Illinois Urbana-Champaign. The Institutional Animal Care Unit Committee approved all procedures carried out in the present study under protocol # 16129.

2.3.1. Animals and breeding management

Nine light breed mares were enrolled in the study (13 ± 4.5 years). Mares were kept on pasture and supplemented with grass hay at the Veterinary Medical Research Farm of the University of Illinois in Urbana Illinois. Trans-rectal palpation and ultrasonography examination were performed three times a week to determine the stage of the estrous cycle. When a pre-ovulatory follicle was detected (≥35mm in diameter in the presence of moderate to high uterine edema), mares were artificially inseminated with fresh extended semen from a single fertile, 13 year-old, Quarter Horse stallion kept at the Illinois Veterinary Teaching Hospital. Semen was collected off a dummy mount using a Missouri artificial vagina, in the presence of a teaser mare in the breeding shed. Immediately after collection, gel-free volume was determined, sperm concentration assessed with an Equine Densimeter (Animal Reproduction Systems Chino, CA), and motility assessed with a computer-assisted sperm analysis (Sperm Vision Minitube of America, Vernon, WI). Raw semen (i.e., 25mL) was extended (1:1, v:v) with a commercially available extender (INRA 96, IMV Maple Grove, MN) and transported at room temperature in a Styrofoam box to the farm to breed mares within 2 hours post-collection. The stallion consistently yielded ejaculates with satisfactorily (≥ 70%) progressive sperm motility. Mares were bred with an overall average of 2 billion progressively motile sperm.
By 24 hours post-breeding, mares were examined for ovulation and post-breeding intrauterine fluid accumulation. Mares received uterine lavage (2-3L of Lactated Ringer’s Solution) and oxytocin (20 units, IM q8h) as needed. If the mare did not ovulate 48 hours post-breeding, the mare was re-inseminated. Ovulation was not hastened with ovulatory induction agents. Pregnancy diagnosis was carried out by 15, 30, 45, 55, and 60 days post-ovulation by transrectal palpation and ultrasonography.

2.3.2 Study design and sampling

Thirteen singleton pregnancies were assessed daily by transrectal palpation and ultrasonography from 55 days post-ovulation until 5 days after induction of pregnancy termination (60±2 days of gestation). Ultrasonographic parameters assessed included ovarian structures (number and sizes of follicles and corpora lutea area), and fetal heartbeat and mobility.

Fetal movements were observed for 5 minutes. Major movements were defined as changes of the longitudinal fetal axis and minor fetal movements defined as head and leg movements [13]. Fetal heart rate was determined by counting the number of beats in 15 seconds by two different operators. Fetal fluid appearance (normal/abnormal echogenicity, depth) was also assessed at each time point.

Plasma was collected in 10mL heparinized tubes (BD Vacutainer® #367874, sodium heparin 158 USP Units; BD, NJ, USA) for five days before and after cloprostenol administration for assessment of progesterone and estradiol-17β. Alpha-fetoprotein assessments were performed daily from two days before to two days after cloprostenol administration. Progesterone, estradiol 17-β, alpha-fetoprotein were assessed with a chemiluminescence assay (Immulite 2000 XPi Platform, Siemens Medical Solutions, Inc USA). Intra-assay coefficient of variations obtained for
progesterone and estradiol-17β were 3.78% and 0.59%, respectively. Plasma was harvested at -80°C until the assessments. The assessments were performed all in one assay (no inter-assay coefficient of variation).

Mares received 500µg of cloprostenol sodium (Estrumate, Merck Animal Health) intramuscularly every 12 hours (IM, n=7 pregnancies) or transcervically every 48 hours (TC, n=6 pregnancies). The intramuscular injections were carried out by alternating the side of the neck during each administration. The transcervical cloprostenol infusion was carried out as reported elsewhere [26,32]. Briefly, the mare was placed in stocks, the tail was wrapped, the rectum evacuated, and the perineum aseptically prepared. Cloprostenol 500 µg of cloprostenol diluted in 8 mL of sterile 0.9% saline solution) was deposited in the internal cervical os with the use of a standard AI pipette, with particular care to avoid rupturing the fetal membranes. Transrectal ultrasonographic evaluation was performed immediately after the transcervical cloprostenol administration to confirm that the chorioallantois was not ruptured.

After initial cloprostenol administration, ultrasonographic examinations were repeated at 6-hours-intervals until loss of fetal heartbeat. Mares were housed in stalls at the Illinois Veterinary Teaching Hospital for close monitoring until abortion. In addition, plasma was collected immediately before cloprostenol administration (0 min), and then repeated at 15, 30, and 45 min, and 1, 2, 3, 4, 6, 12 hours to assess PGFM concentrations. Peripheral concentrations of PGFM were determined as a proxy for PGF2α, with a horse-validated immunoassay (Cayman Chemical; Ann Arbor, MI, USA Cat. #516671) according to the manufacturer recommendations [38]. When ultrasonographic signs of fetal demise (absence of heartbeat and active movements) were observed, the fetus and the fetal membranes were manually recovered from the uterus or the
vagina. A uterine lavage was carried out with 2-4 liters of Lactate Ringer’s Solution to promote uterine clearance.

2.3.3 Statistical analyses

Data analyses were performed with SAS 9.4 (SAS Institute Cary NC, USA). Non-normally distributed data were log-transformed. Data were then analyzed with mixed models with repeated measures. Mare was accounted as a random effect, and time and group as fixed effects. To facilitate interpretation data are presented as means ± SEM. Significance was defined as $P \leq 0.05$; whereas, the statistical tendency was defined as $0.05 < P \leq 0.10$.

2.4. Results

Minor side effects (e.g., pawing, flank watch, sweating, and bowel movements with soft manure) were observed with systemic administration of cloprostenol in 6 of 6 mares. One mare developed severe adverse reaction (heart rate $> 60$ beats/min, respiratory rate $> 30$ breaths/min) starting two hours after the first intramuscular cloprostenol administration. This mare was administered a single dose of xylazine (0.3 mg/kg, IV) and flunixin meglumine (1 mg/kg, IV, q12h). Clinical examination did not reveal any overt signs of gastrointestinal abnormalities (e.g., no signs of nephrosplenic entrapment). By 12 hours post initial cloprostenol administration the mare recovered, did not receive any additional doses of cloprostenol and was excluded from the study. There were no side effects observed following the intrauterine administration of cloprostenol. None of the mares in TC group ruptured the fetal membranes immediately after intrauterine infusion (Fig. 2.1).
All mares lost their pregnancies by forty-eight hours after initial cloprostenol administration, with no difference in time to pregnancy loss (TC 41.5 ± 4.1 h, range 24-48.5 h, IM 33.0 ± 4.1 range 18-48 h) (p>0.17). On average, mares aborted after 3.3 (ranging from 2 to 4 systemic cloprostenol administrations. One mare in the TC group had not aborted at 48 h post-initial cloprostenol but passed her fetus 30 minutes after the second infusion. All fetal membranes and accompanying fetuses were fully recovered (Fig. 2.2). All but one fetus were manually retrieved from the mare. One fetus was recovered in the vagina, eleven were recovered in utero, and one fetus was recovered from the stall.

For progesterone concentrations, there was no effects of group (p=0.93). There was an effect of time (p<0.001) starting by 12 h post-induction of pregnancy termination but no time by group interaction (p>0.73) (Fig. 3). Estradiol-17β concentrations were numerically lower in the IM vs. TC group, but there were no differences (p=0.09) (Fig. 2.4). There were statistical tendencies for time (p=0.055) and time by group interaction (p=0.08) for estradiol-17β concentrations. For alpha-fetoprotein, there was no effects of group (p=0.90), time (p = 0.54), or time by group interaction (p=0.15) (Fig. 2.5). Interesting that alpha-fetoprotein was present in greater concentrations in the allantoic and amniotic fluids (Table 2.1).

There were effects of time (p=0.008), but no group (p=0.59) or time by group interactions (p=0.19) for PGFM concentrations (Fig. 2.6). Mares receiving cloprostenol intramuscularly peaked PGFM concentrations from 2-4 h post-cloprostenol but returned to baseline by 6 h post-initial cloprostenol administration, conversely, cloprostenol intracervically did not result in a distinct peak in PGFM concentrations.

There were no effects of group (p=0.56), time (p = 0.89), or time by group interaction (p=0.17) for fetal heart rate (Table 2.2). Similarly, there were no effects of group (p=0.70) there
was a tendency for time (p=0.07) or time by group interactions (p=0.90) for fetal mobility (Table 2.2). The luteal tissue area increased from 5d to the day of cloprostenol administration and then decreased after cloprostenol administration (p=0.05) with no differences between groups (p=0.59) or time by group interaction (p=0.17) (Fig. 2.7). Mares receiving cloprostenol transcervically had progesterone concentrations below 1 ng/mL at 48 h (n=1), at 72h (n=3), and at 96 h (n=1), whereas mares receiving cloprostenol systemically had progesterone below 1 ng/mL at 48 h (n=3), and at 72 h (n=1). Two mares receiving cloprostenol systemically had progesterone above 1 ng/mL by 120 h post-initial injection of cloprostenol, whereas, one mare in the TC group had progesterone above 1 ng/mL by 120 h.

2.5. Discussion

The present study was designed to compare blood markers (progesterone, estradiol-17β, alpha-fetoprotein, PGFM) and B-mode ultrasonographic parameters of mares undergoing experimentally induced early fetal loss. Previously, studies focused on assessing time to abortion and peripheral concentrations of progesterone, PGFM, or estrone sulfate in mares receiving PGF2α or its analog systemically [19,21,25,33-37]. This is the first study comparing time to fetal death for intramuscular and transcervical administration of PGF2α and assessing parameters that could be used as early indicators of fetal demise. Our findings demonstrated that administering repeated doses of cloprostenol systemically was equivalent to administering a single dose of cloprostenol transcervically.

Administration of cloprostenol sodium, a luteolytic agent, either transcervically or intramuscularly resulted in a rapid reduction in progesterone and area of luteal tissue. Expectedly, herein and elsewhere [39] administration of cloprostenol systemically is followed by a peak in
PGFM concentrations. Surprisingly, PGFM did not peak in plasma of mares receiving cloprostenol transcervically. This could be a true finding or a limitation of the study design as PGFM was only assessed for the first 12 hours post-cloprostenol administration. A previous study comparing intravenous and intrauterine administration of dinoprost, to diestrous mares concluded that PGFM peaked in plasma by 2 hours and returned to baseline by 3 hours [39]. While early diestrous mares and 60-d of gestation cannot be directly compared, previous reports assessing intrauterine infusion of cloprostenol to 60-d pregnant mares did not measure peripheral concentrations of PGFM [26,32]. The lack of an apparent increase in peripheral concentrations of PFGM in the TC group could simply suggest an improper endogenous response to exogenous synthetic PGF$_{2\alpha}$. Since progesterone concentrations were dramatically reduced in both groups and there was a similar time to abortion, it is suggestive that the pharmacological properties of cloprostenol administered via either route was effective in achieving luteolysis.

Single systemic prostaglandin administration results in rapid pregnancy termination in mares during the first 40 days of gestation [33], however, a higher proportion of mares fails to abort after 40-60 days due to the production of eCG by endometrial cups [19,34-36]. The efficacy of administering a single dose of cloprostenol transcervically was similar to previous reports [26,32]. Only one mare required a second administration, which she responded rapidly by passing the fetus in 30 minutes. In addition, transcervical administration appears to be a superior approach to systemic administration as none of the mares in the TC experienced any side effects, while all mares developed some minor side effects (e.g., pawing, bowel movement with soft manure) following IM administration and one mare appeared to have developed strong reaction to the prostaglandin administration. Under clinical settings, typical luteolytic cloprostenol dose is 250µg, herein we have used 500µg, while it is possible that the present mare could have been more
sensitive to PGF$_{2\alpha}$, her breed American Quarter Horse is not known to be sensitive to exogenous PGF$_{2\alpha}$ administration. While it is uncertain whether this mare had other pre-existing or underdiagnosed condition not associated with the cloprostenol administration it remains unknown.

Contrary to anecdotal experience, the equine fetuses did not alter their major mobility pattern or fetal heartbeat following administration of cloprostenol by either route. This could be implied that the fetuses were not compromised until immediately before delivery [35]. Our findings appear to support this hypothesis, as mares herein were checked every six hours, and all fetuses had no visible heartbeats at retrieval. In addition, none of the fetuses or fetal membranes looked autolyzed, suggesting that these structures were well-oxygenated preceding retrieval. In addition, it is possible that the two models for pregnancy loss used herein was too acute to detect changes in the ultrasonographic fetal parameters, however, this remains to be determined. However, since ultrasound is the most widely used and available tool to practitioners, evaluation of fetal parameters in mares undergoing experimentally-induced EPL was necessary to allow a detailed assessment of fetal parameters under controlled conditions. While B-mode ultrasonography can be used to determine whether the fetus is alive, it does not appear to be useful to determine impending abortion. Doppler ultrasonography has been extensively used in human medicine [40] and one study in warmblood mares documented changes in the uterine blood flow in mares with compromised pregnancies [41]. However, Doppler ultrasonography was not used herein to assess uterine and fetal blood flow, it is possible that changes in blood-flow could have been more suggestive of a compromised fetus than B-mode ultrasonography, but this remains to be determined.

Alpha-fetoprotein is an albuminoidal protein expressed in the fetal fluids, fetal and maternal plasma. Peripheral concentrations were reported to be increased in plasma of mares
suffering pregnancy loss [28-30]. However, in the present study, while alpha-fetoprotein was confirmed to be present in high concentrations in the amniotic and allantoic fluids, there were no appreciable changes detected in the plasma of mares underdoing to EPL. It is possible that our models were acute and there was not enough time for mares to experience changes in concentration of alpha-fetoprotein in peripheral circulation. It remains to be determined if models for pregnancy loss resulting in the slower passage of fetuses would result in more changes in plasma.

Conjugated estrogens such as estrone sulfate have been shown experimentally to be a useful marker for pregnancy loss in mares [17-19]. However, assay availability and turn out time for results has limited its widespread use in clinical practice. Estradiol-17β, a free non- conjugated estrogen, has been shown to be a useful marker for pregnancy loss in late pregnant mares [30,42,43], however, the usefulness of the assay to assess early fetal loss has not been reported until now. While there was a tendency for estradiol-17β to be reduced over time, particularly in the group of systemic cloprostenol, there were no significant differences. This could be due to the small number of mares used herein and because of contributions from follicles in the maternal ovaries could have masked the reduction in estradiol-17β during pregnancy loss.

2.6. Conclusion

In conclusion, mares lost their pregnancies within forty-eight hours after initial cloprostenol administration, with no difference in time to pregnancy loss between the route of delivery. There were significant effects of time starting by 12 h post-induction of pregnancy termination but there was no time by group interaction for progesterone concentrations. Estradiol-17β and alpha-fetoprotein concentrations were not altered upon impending abortion. Concentrations of PGFM significantly increased by two hours after cloprostenol administration,
but there were no differences between groups. There no time effects or time by group interaction for fetal mobility and heartbeat. Expectedly, the number and area of corpora lutea decreased significantly after cloprostenol administration with no significant differences between groups. The present findings demonstrated that a single intrauterine administration of cloprostenol was equivalent to repeated systemic administration to terminate the pregnancy. Both models for early fetal loss were equivalent for the different endpoints assessed herein.

Acknowledgments

The study was funded by the Department of Veterinary Clinical Medicine. The Koteska Endowment in Equine Reproduction is acknowledged for funding the stipends for the first author.

Disclosure Statement

None
Fig 2.1. Representative image of mare receiving cloprostenol intracervically. The transrectal ultrasonography image of a mare at 60 days of gestation shows an intact fetal membrane immediately after intrauterine infusion with cloprostenol. (}`). Allantoic cavity; (=>) fluid accumulation denoting the place of infusion of cloprostenol; (*) mare’s bladder.
Fig. 2.2. Representative images of two aborted fetuses from mares receiving cloprostenol transcervically (A) or intramuscularly (B) IM. (*) Fetuses; (=>) amnion; (X) (I) Yolk sac remnants.
Fig. 2.3. Progesterone concentrations in mares before and after cloprostenol administration (IM, n=6; TC, n=6). There were no effects of group (p=0.93). There were effects of time (p<0.001) starting by 12 h post-induction of pregnancy termination but no time by group interaction for progesterone concentrations (p>0.73). *Denotes differences in time for progesterone concentrations for both groups. IM: intramuscular and TC: Transcervical.
**Fig. 2.4.** Estradiol-17β concentrations in plasma of mares receiving cloprostenol intramuscularly (IM, n=6) or transcervically (TC, n=6).
Fig. 2.5. Alpha-fetoprotein concentrations in plasma of mares receiving cloprostenol intramuscularly (IM, n=6) or transcervically (TC, n=6).
Fig. 2.6. Concentrations of PGFM in plasma of mares receiving cloprostenol intramuscularly (IM, n=6) or transcervically (TC, n=6).
**Fig. 2.7.** Area of ovarian luteal tissue (mm²) before and after administration of cloprostenol sodium (Time 0h) transcervically (TC, n=6) or intramuscularly (IM, n=6). Luteal area considered corpora lutea and luteinized follicles.
Table 2.1. Alpha-fetoprotein concentrations in the fetal fluids of mares receiving cloprostenol transcervically (TC) or intramuscularly (IM).

<table>
<thead>
<tr>
<th></th>
<th>Allantoic fluid</th>
<th>Amniotic fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM (n)</td>
<td>Mean ± SEM (n)</td>
</tr>
<tr>
<td>TC</td>
<td>141.8 ± 65.6 (3)</td>
<td>266 ± 0 (1)</td>
</tr>
<tr>
<td>IM</td>
<td>165.15 ± 57 (4)</td>
<td>338.3 ± 100 (4)</td>
</tr>
</tbody>
</table>
Table 2.2. Mean ± SEM - Fetal heart rates and major fetal movements assessed via transrectal B-mode ultrasonography before and after administration of cloprostenol sodium (Time 0h) transcervically (TC, n=6) or intramuscularly (IM, n=6). Fetal heart rate was assessed in 15 seconds by two operators. The number of major fetal movements were assessed over 5 minutes of observation. There were no differences between groups (p>0.05).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Fetal heart rate (bpm)</th>
<th>Major fetal movements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC (n)</td>
<td>IM (n)</td>
</tr>
<tr>
<td>-120</td>
<td>137.0 ± 9.8 (6)</td>
<td>146.8 ± 9.8 (6)</td>
</tr>
<tr>
<td>-96</td>
<td>130.7 ± 10.3 (6)</td>
<td>135.5 ± 9.8 (6)</td>
</tr>
<tr>
<td>-72</td>
<td>141.9 ± 10.4 (6)</td>
<td>132.0 ± 9.8 (6)</td>
</tr>
<tr>
<td>-48</td>
<td>129.7 ± 10.4 (6)</td>
<td>145.8 ± 9.8 (6)</td>
</tr>
<tr>
<td>-24</td>
<td>145.3 ± 9.8 (6)</td>
<td>126.7 ± 9.8 (6)</td>
</tr>
<tr>
<td>0</td>
<td>146.6 ± 9.8 (6)</td>
<td>132.3 ± 9.8 (6)</td>
</tr>
<tr>
<td>6</td>
<td>145.8 ± 9.8 (6)</td>
<td>144.8 ± 9.8 (6)</td>
</tr>
<tr>
<td>12</td>
<td>154.2 ± 9.8 (6)</td>
<td>137.0 ± 9.8 (6)</td>
</tr>
<tr>
<td>18</td>
<td>158.5 ± 9.8 (6)</td>
<td>138.0 ± 10.5 (5)</td>
</tr>
<tr>
<td>24</td>
<td>150.9 ± 10.5 (5)</td>
<td>159.3 ± 10.7 (5)</td>
</tr>
<tr>
<td>30</td>
<td>149.9 ± 10.7 (5)</td>
<td>150.1 ± 13.0 (3)</td>
</tr>
<tr>
<td>36</td>
<td>154.1 ± 10.7 (5)</td>
<td>146.2 ± 16.0 (2)</td>
</tr>
<tr>
<td>42</td>
<td>136.2 ± 15.5 (2)</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>134.5 ± 22.0 (1)</td>
<td>0</td>
</tr>
</tbody>
</table>
2.8 References


CHAPTER 3: UTERINE RESPONSES AND eCG CONCENTRATIONS AFTER TWO INTRATUTERINE INFUSIONS WITH KEROSENE POST EARLY FETAL LOSS IN MARES

Giorgia Podico¹, Igor F. Canisso¹*, Patrick J. Roady¹, Scott M. Austin¹, Mariano Carossino², Udeni Balasuriya, Robyn E. Ellerbrock³, Robert H. Douglas⁴, Fabio S. Lima¹

¹Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois Urbana-Champaign, Urbana IL 61802, USA

²Louisiana Animal Disease Diagnostic Laboratory, Department of Pathobiological Sciences, Baton Rouge LA, USA.

³Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens GA 30605, USA.

⁴BET Laboratory, Lexington, KY, USA.

The present manuscript is formatted for submission to the journal Theriogenology

*correspondent author: canisso@illinois.edu

3.1. Abstract

Pregnancy during normal life-span of endometrial cups (~35-80 days of gestation) may affect the mare’s ability to become pregnant in the same breeding season due to abnormal ovulations, follicular growth and cyclicity concurrent eCG secretions. Anecdotally, intrauterine infusion with kerosene has been proposed as a method to treat endometrial cup retention, however there are no controlled studies evaluating kerosene’s usefulness for enhancing endometrial cup regression following abortion. We hypothesized that intrauterine kerosene infusions would hasten regression of endometrial cups without detrimental effects on the endometrium and the mare’s general health. The objectives of this study were to assess the uterine response, systemic side
effects and efficacy of intrauterine kerosene infusions to enhance regression of endometrial cups. Thirteen light-breed mares were enrolled in the study after an experimentally-induced abortion with cloprostenol (n=13) by 60 (60±2 days) days of gestation. Mares were randomly allocated an intrauterine infusion with 500mL of kerosene (Kerosene, n=6) or 500mL saline (Control n=6) on days 21 and 35 after the pregnancy termination. Uterine biopsies were collected at day 7, 21, 35 and 49, stained with H&E and graded according to the Kenney & Doig 1986 classification. Lymphocyte B CD20, lymphocyte T CD3 and macrophage IBA cell populations were characterized by immunohistochemistry in endometrial biopsies from a subset of mares (n=4/group). Physical examinations, complete blood cell counts, and serum biochemistry were performed before each infusion and then repeated for 2 days after each uterine infusion. Uterine lavage was performed 24 hours after each infusion. Serum samples were collected right before the abortion induction, then at 7, 21, 28, 35, 42, and 49 days after the pregnancy termination for assessment of serum eCG. Continuous data were analyzed with MIXED procedure with repeated measures in SAS, categorical data with LOGISTIC procedure in SAS. Significance was set at p<0.05. Kerosene infusion did not affect complete blood cell counts and serum chemistry parameters. There were no appreciable abnormalities on the physical examinations following kerosene infusions. Concentrations of eCG decreased over time (p<0.001), but there were no differences between groups or time by group interactions (p=0.7128). Histology samples from the uterus showed no signs of increased fibrosis or degeneration in the treatment group. In conclusion, while kerosene infusions did not appear to have detrimental effects on mare health or operator. Our findings suggest that the use of kerosene in the uterus does not enhance the regression of endometrial cups.

Key-words: abortion, intrauterine therapy, pregnancy loss,
3.2. Introduction

Pregnancy loss during the first sixty days of gestation is an important problem for the equine industry, with incidence of loss varying from 2 to 20%, [1-5]. Many factors have been associated with early pregnancy loss, including luteal insufficiency, maternal illness, aging, endometrial fibrosis, poor nutrition and stress of the mare, embryonic and fetal abnormalities, or the iatrogenic administration of certain medications [3,7,8]. The most valuable mares are often the older ones with proven offspring or a successful athletic career, and these mares are more prone to experiencing early pregnancy loss [6].

Mares experiencing pregnancy loss between 40-70 days of gestation typically are not re-bred in the same breeding season due to the effects of retained endometrial cups [9,10]. The presence of endometrial cups post-abortion is associated with abnormal estrous cycles and subfertility in mares [9,10,11]. Endometrial cups are specialized fetal-derived structures that form by 35 days of gestation from invasive trophoblastic cells located at the chorionic girdle [12,13]. The phenotype of these cells change, causing invasive cell migration in the endometrium and formation of the endometrial cups [12-14]. These cups secrete equine chorionic gonadotropin (eCG) into the systemic circulation between 35 and 120-150 days of gestation [15,16]. It is unknown why abortions between 40-70 days results in prolonged lifespan of the endometrial cups [10,17-19]. Due to the FSH and LH-like activity of eCG, mares that are affected by retained endometrial cups exhibit recurrent anovulatory luteinizing follicles, abnormal cyclicity, and subsequent subfertility [9].

Anecdotal clinical experience and two clinical cases suggest that kerosene intrauterine infusions can be used to treat retained endometrial cups [10]. In addition, 45% of mares that were classified as infertile due to degenerative endometrial changes were able to carry a foal to term
after intrauterine infusion with kerosene [20]. However, to date, the possible side effects of this treatment are poorly documented. A recent case report documented the occurrence of severe vaginitis causing colic-like signs in a mare treated for pyometra and skin irritation on the operator who performed the procedure [21].

This study aimed to i) to determine the effects of intrauterine kerosene administration on the mare’s health, the endometrium, and the operator, ii) to evaluate the effects of kerosene infusions on the removal of retained endometrial cups and iii) to assess the utility of hysteroscopy, transrectal ultrasound, and serum eCG concentrations to detect the presence of active endometrial cups. We hypothesized that the use of kerosene could enhance the regression of persistent endometrial cups without detrimental effects on the mare’s health, uterus, or person performing the intrauterine infusion.

3.3. Material and methods

The present study was performed from September 2017 to October 2018. The Institutional Animal Care Unit Committee under protocol #16129 approved all procedures carried out in the present study.

3.3.1. Animals and pregnancy termination

Twelve pregnancies from nine healthy light-breed mares from 5-14 years of age were used in the study. The animals were housed in pastures and in indoor stalls at the University of Illinois, Veterinary Teaching Hospital and fed with mixed grass and alfalfa hay. All mares were bred with fresh semen collected from a single Quarter Horse stallion housed at the same facility. Ovulation was confirmed with ultrasound. Four mares were bred again after the first year, and a total of
twelve pregnancies were included in the study. When the pregnancy reached 60 days of gestation (60±2 days), induction of abortion was performed with a uterine infusion (n=6) of 500µg cloprostenol sodium (Estrumate, Merck Animal Health) diluted in 8mL of sterile saline (Cuervo-Arango et al. 2015), or with 500µg cloprostenol sodium administered intramuscular (n=6) every 12 hours for another parallel study.

3.3.2. Uterine infusions

Two uterine infusions were performed on each mare enrolled in the study. Mares (n=12) were randomly assigned to treatment with kerosene (Kerosene) or saline (Control). Six mares were treated with uterine infusion of 500ml of Kerosene (1-K Heater Fuel, Klean-Strip) on day 21 and 35 after the abortion, while other six mares were infused with 500 ml of saline solution. Each mare was restrained in stocks with the tail wrapped and the perineal area cleaned. Mares were sedated with xylazine hydrochloride (0.5mg/kg IV, Xilamed, Bimeda) before each infusion. During kerosene infusions, the operator wore nitrile glove and two sterile sleeves on the arm used for the uterine infusion, while the other hand wore one nitrile glove. Safety glasses were worn by all the people involved in the procedure. (Fig. 3.1). The same operator conducted all the infusions, in both kerosene and control group.

A silicone tube and a funnel were used to infuse the uterus. After removing the tube, the vaginal portion of the cervix was held closed for two minutes, and the amount of the kerosene left in the tube was measured. After the procedure, the status of the sleeves and of the skin of the operator were checked for signs of damage or irritation, respectively. Right after the infusion, the mare was turned outside in a small corral and behavior was monitored. The six mares assigned to the control group were treated with the same procedure using 500mL of sterile saline.
Twenty-four hours after each intrauterine infusion, every mare was examined by transrectal ultrasound for signs of uterine inflammation and treated with a uterine lavage with Ringer’s Lactate Solution followed by 20 IU of oxytocin intramuscularly. Mares continued to receive oxytocin twice a day until there was no evidence of intraluminal fluid based on transrectal ultrasound examination of the uterus.

3.3.3. Assessment of systemic and local secondary effects

Immediately before each treatment, each mare was evaluated through a physical examination, complete blood count, and serum chemistry. Mare demeanor, body temperature, respiratory and heart rates, mucous membranes, gut movements, and digital pulses were recorded. Physical examinations, a complete blood count, and serum chemistry were repeated 24 and 48 hours after uterine infusions.

Twice (d7, d21) before the first treatment and fourteen days after every infusion, an endometrial sample was collected using a biopsy forceps (Jackson uterine biopsy forceps, 60 cm length, 4x28 mm cutting area, Jorgensen Laboratories Inc.) and the sample was fixed in Bouin fixation fluid (Harleco, Merck) for 24 hours. A total of 52 slides from the endometrium and 4 slides with endometrial cup (obtained using biopsy forceps during hysteroscopy) in two mares were evaluated blindly by an experienced pathologist. Using Kenney and Doig’s classification [22], every mare was assigned to a category (I, IIA, IIB, III), and a detailed description was also recorded.

Biopsy samples from eight mares (four in the control group, four in the treatment group) were processed using single immunohistochemistry to characterize the subpopulations of lymphocytes and macrophages. Sections of the endometrial biopsies were prepared as described elsewhere [23]. Specific monoclonal antibodies against CD3 (clone LN10, Leica Biosystem,
Germany #PA0554), rabbit polyclonal antibodies against CD20 (#PA5-16701, Invitrogen, Thermo Fisher Scientific) and IBA-1 (Fujifilm Wako Pure Chemical Corporation) were used to identify lymphocyte T, B and macrophage, respectively. Staining intensity and distribution were evaluated blindly by an experienced observer and an overall subjective description of the intensity and distribution across the groups was given.

3.3.4. Evaluation of the uterine endometrium and eCG production

Transrectal ultrasound examination, and hysteroscopy examination were performed every two weeks from seven to forty-nine days after abortion (Figure 3.2). An ultrasound was performed before every hysteroscopy examination, before the uterine infusion, and then daily up to 48 hours after the treatment. Ultrasound examination was performed to identify areas with an increased echogenic texture that could be presumably associated with the shape, location, and size of endometrial cups located on the surface of the uterus.

During hysteroscopy, the entire endometrial surface was visualized to assess the macroscopic characteristic of the endometrial cups and to evaluate lesions, adhesion or other abnormalities. For the procedure, each mare was restrained in a stock under a light sedation with xylazine hydrochloride (0.5mg/kg IV, Xilamed, Bimeda; after every endoscopy, a uterine lavage with Lactated Ringers Solution was performed and a single injection of 20 IU of oxytocin was administered intramuscularly immediately after lavage.

Blood samples were collected every seven days from the day of pregnancy termination to fourteen days after the second intrauterine infusion. Blood was collected by puncture of the jugular vein, centrifuged at 600 g for 10 minutes and then plasma was stored at -20°C until analysis. Analyses of the eCG concentration were conducted with commercial immunoassay (PMSG Elisa
#DE1298, Demeditec, Germany) at the BET Laboratory in Lexington, Kentucky, USA. Values above 1 U.I./mL were considered positive sign for the presence of active endometrial cups. According to the manufacturer, this assay have approximately 5% of false negative rate and 10% false positive rate.

### 3.3.5. Statistical analysis

Statistical analysis was performed with SAS 9.4 software. Data were analyzed with a mixed model with repeated measures, where mare were considered random effect and time and group fixed effect. Data that were not normally distributed were transformed with a logarithmic transformation. The sensitivity of ultrasound and hysteroscopy examination was compared to eCG serum concentrations. The comparison was done by estimating the number of times that each detection method gave a positive diagnosis for the presence of endometrial cups in relation to plasma concentration of eCG. Data are presented as mean±SEM. Significance was set at p < 0.05, a statistical tendency was reported with 0.05<p<0.1.

### 3.4. Results

Minor side effects (tail flagging, frequent urination posture, and pacing) were observed in mares receiving intrauterine kerosene infusion. After mares were turned back out in pasture normal demeanor such as grazing and interacting with other horses were restored. In several mares, a small amount of kerosene reflux into the vagina was noted at the moment when the tube was retracted from the cervix. This reflux caused damage to the first layer of the obstetrical sleeve, but not the second sleeve, leaving the operator’s arm unharmed by the kerosene (Fig. 3.3). There were no
skin lesions observed in the operator’s skin. The amount of kerosene that was left in the tube during the procedure was between 0 (n=4) and 100mL (n=3) (Mean, 66.5mL).

All physical examination parameters remained within normal limits and were not influenced by time or type of treatment. No time effect (p=0.86) or treatment by time effect (p=0.17) was noted in body temperature; similarly, for the respiratory rate, there was no time effect (p=0.35) or treatment by time (p=0.91) (Table 3.1). A tendency for an increase in heart rate was noted over time (p=0.06) in the treated group, however, values remained within normal ranges (<60 beats/minute). Blood cell counts and chemistry assessments were within the normal limits between the two groups of mares, and there was no effect of time or treatment by time interactions (p>0.05). (Tables 3.2, 3.3, 3.4). Secondary effects on the uterus were negligible, there were no signs of uterine inflammation (edema, intrauterine fluid accumulation) upon transrectal ultrasound 48 hours after intrauterine infusion.

Histologic changes in mares subjected to intrauterine infusions showed a variable population of inflammatory and immune cells. A considerable amount of siderocytes were evident seven days after the abortion, but the number decreased dramatically in subsequent samples. No signs of degenerative or fibrotic changes were noted in the endometrial samples of the treated mares (Figure 3.4). The overall distribution of histological score within the groups seemed were not affected by the treatment (Table 3.5). No drastic shift in classification was noted in any mare. Immunohistochemistry revealed dissimilar proportions of the different type of leukocytes overtime, but those modifications did not seem to be affected by the type of the treatment. (Fig. 3.5, 3.6, 3.7)

The reproductive tract was evaluated by hysteroscopic exam for the presence of ulcerations or adhesions due to kerosene infusions. None of the mares in the treatment group showed local
lesions in the endometrium. The uterine surface appeared pink and normal in appearance, and no scarring was noted. The gross morphology of the endometrial cups changed overtime; the extensive presence of white, thick plaque evolved to a thinner, pink, and more subtle form of endometrial cups at a different velocity in every mare (Fig. 3.8). In few cases, gross distinguishable endometrial cups were not noted in the ultrasound and hysteroscopy but the peripheral level of eCG was still increased.

Concentration of eCG decreased overtime (p<0.001) but the type of infusion did not influence speed of decrease up to 49 days after the abortion (p=0.7128) (Fig. 3.9). Endoscopic evaluation of the reproductive tract revealed that the macroscopic characteristics of the endometrial cups changes between mares with time after pregnancy loss. The sensitivity of hysteroscopy exam decreased with time and cups that were regressing, but still active, were not easily detected. As expected, ultrasound examination was less sensitive than hysteroscopy for detecting retained endometrial cups. (Table 3.6)

Negative effects on fertility were not noted, six out of nine mares were bred and become pregnant after the experiment for a parallel study. Of the three remaining three mares, one from the treatment group and two from the control group were adopted, and no follow-up reproductive information is available.

3.5. Discussion

The present experiment is the first controlled study to perform a detailed assessment of the effects of kerosene on the mare’s health, the mare’s endometrium and the operator performing the infusion. The use of intrauterine kerosene in broodmare practice is controversial due to the
potential side effects on the mare’s and operator’s health, and the mares fertility. However, authors have reported positive results on the fertility of mares after kerosene infusion [20,25].

Our results demonstrated that intrauterine kerosene infusions could be performed in the mare with negligible side effects at the dose used in this study. During the preliminary work for this study, two intrauterine infusions were performed in a mare at 7 and 21 days after abortion at 60 days gestation; the infusions of 250mL kerosene were carried out with no sedation or anesthesia. The size of the uterus, laxity of the external cervical os likely favored the reflux of kerosene in the vagina causing a transient mild vaginitis. The mare showed mild discomfort signs (pacing, tail flagging, posturing to urinate) that self-resolved in few hours after the treatment. This occurrence is similar to the one reported by Coyle et. al [21], but the side effects on the mare in that case report were more severe and required systemic and local administration of analgesic and anti-inflammatory (i.e. flunixin meglumine 1.1mg/kg IV, 2% lidocaine). The status of the uterine involution and cervix relaxation likely influence the likelihood of kerosene efflux, the degree of vaginitis and discomfort, and the outcome of the procedure.

White blood cell counts changes are the mostly used markers for the inflammatory response in horses; modification of the subpopulations of the neutrophils (i.e., left shift, increased of band neutrophils and decreased of segmented neutrophils) can be observed, and related as a sign of inflammation, coupled with a normal, increased or decreased leukocytes counts [26]. Our blood results were unremarkable, complete cell blood count and blood chemistry values were within normal limits in both groups at all time-points.

The effects on the uterus were more notable, even though not severe enough to cause signs of systemic illness; the presence of intrauterine fluid was noted in mares in both groups of treatment for a limited time. These results agreed with the observation of Bracher et al. [20]; an
inflammatory response have been documented in mares infused with kerosene up to 14 days after; despite the presuppositions due to potential detrimental nature of kerosene, the endometritis that intrauterine kerosene infusions cause does not evolve in a metritis thus it does not have any systemic implications.

Kerosene infusion has reportedly had positive results on the fertility of chronically subfertile mares, with pregnancy rates post-infusion up to 60% [24,20,10]. One studies have attempted to identify the beneficial consequences of treatment with kerosene on the uterus [26]. It has been hypothesized that the removal of the superficial layer of the endometrium also removes inspissated agglomerate of glands secretions and distended lymphatic vessel, and that the regenerated epithelium develops greater cilia that improve uterine clearance, and therefore the fertility [26]. While the histological evaluation in our study did not show an improvement in the overall distribution of scores across the mares; interestingly, the mare in the preliminary trial that was treated at 7 and 21 days post-abortion showed a remarkable increased in the biopsy score (IIB to I). It is possible that if mares were treated in different time points, treatments would have resulted in more pronounced changes in biopsy scores. The regenerate appearance of the luminal epithelium and the active glandular epithelium suggests the need of further histological evaluations.

The effect of infusion on the regression of the endometrial cups was not significant up to 49 days post-abortion; in both groups of mares, eCG production was still detectable. Production of eCG is affected by several variables including mare parity, age, nutrition, level of exercise, and most importantly, genetics [15,16,28,29]. The invasion of trophoblastic cells by 35-38 reach the stroma deeply and it is reasonable that the chemical curettage obtained with the kerosene infusion likely did not affect the endometrial cups cells deeply immersed in the endometrium. A case report
with two cases of persistent endometrial cups indicated the positive outcome obtained by the kerosene in one of the two cases described, in the second case the intrauterine infusion was performed when the regression was already taking place (i.e. eCG concentrations were decreasing) and therefore was not effective [10].

With our findings, we conclude that the regression of retained endometrial cups after an abortion is not enhanced by the use of intrauterine kerosene infusion, thus the effect on persistent endometrial cups have not being investigated in this study. At the same time, the use of intrauterine kerosene infusions in broodmares did not appear to negatively affect their fertility and welfare. If proper care is taken during kerosene intrauterine infusion no detrimental effects will be observed on the mare or operator.
3.6. Figures and tables

Fig. 3.1. Representative image of an intrauterine kerosene infusion. The mare is restrained in stocks under a light sedation, one operator is infusing the uterus with 500mL of kerosene. Both the operators wear personal protective equipment (gloves, plastic eyeglasses); the operator that is performing the infusion wears a nitrile examination glove with a double layer of obstetrical sleeves on top.
Fig. 3.2. Study design
Fig. 3.3. Detail of the obstetrical sleeves utilized during an intrauterine kerosene infusion. The first layer of sleeve usually present area of melting due to the direct contact with the solution, the skin of the operator is untouched from the kerosene.
Fig. 3.4. Representative images of histology images of the endometrium in mares in the control (A, C, E, G) and in the kerosene group (B, D, F, H) before (A, B, C, D) and after one (E, F) or two (G, H) intrauterine infusions.
H&E, 10X, Bar = 20 μm.
Fig. 3.5. Representative images of immunohistochemistry images for the identification of macrophage (IBA1 +). Endometrial biopsy in mares in the control (A, C, E) and in the kerosene group (B, D, F), before (A, B) and after one (C, D) or two (E, F) intrauterine infusions. H&E; 200X; Bar, 250 μm.
Fig. 3.6. Representative images of immunohistochemistry images for the identification of lymphocyte T (CD3 +). Endometrial biopsy in mares in the control (A, C, E) and in the kerosene group (B, D, F), before (A, B) and after one (C, D) or two (E, F) intrauterine infusions. H&E; 200X; Bar, 250 μm.
Fig. 3.7. Representative images of immunohistochemistry images for the identification of lymphocyte B (CD20 +). Endometrial biopsy in mares in the control (A, C, E) and in the kerosene group (B, D, F), before (A, B) and after one (C, D) or two (E, F) intrauterine infusions. H&E; 200X; Bar, 250 μm.
Fig. 3.8. Representative images of hysteroscopy visualization of the reproductive tract in mares in the control (A, C, E, G) and in the kerosene group (B, D, F, H), before (A, B, C, D) and after one (E, F) or two (G, H) intrauterine infusions.
Fig. 3.9. Plasma eCG concentration in mares undergoing intrauterine infusions with kerosene or control solution after an experimentally induced abortion. (Time p<0.001, Treatment*Time p=0.7128).
Table 3.1. Physical examination parameters of mares in the control and kerosene group, before and after (+24, +48 hours) from the intrauterine infusions. (Mean ± SEM) (p>0.05)

<table>
<thead>
<tr>
<th></th>
<th>First Infusion</th>
<th></th>
<th>Second Infusion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Kerosene</td>
<td>Control</td>
<td>Kerosene</td>
</tr>
<tr>
<td>T (°C)</td>
<td>0 h</td>
<td>37.0±0.4</td>
<td>37.2±0.4</td>
<td>37.4±0.4</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>36.9±0.4</td>
<td>37.6±0.4</td>
<td>37.38±0.3</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>37.0±0.4</td>
<td>37.6±0.4</td>
<td>36.7±0.6</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>0 h</td>
<td>45.3±3.7</td>
<td>40.2±3.8</td>
<td>36.0±3.7</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>40.2±3.7</td>
<td>46.8±3.8</td>
<td>38.0±3.7</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>36.5±3.7</td>
<td>42.7±3.8</td>
<td>38.0±3.7</td>
</tr>
<tr>
<td>RR (bpm)</td>
<td>0 h</td>
<td>17.7±2.8</td>
<td>17.0±2.8</td>
<td>16.3±2.8</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>13.2±2.8</td>
<td>12.7±2.8</td>
<td>17.3±2.8</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>12.0±2.8</td>
<td>13.3±2.8</td>
<td>15.6±2.8</td>
</tr>
</tbody>
</table>
Table 3.2. Complete blood cell counts and total protein concentrations of mares in the control and kerosene group, before and after (+24, +48 hours) from the intrauterine infusions. (Mean ± SEM) (p>0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>Red blood cells (Range 7-13x10⁶/µL)</th>
<th>Hematocrit (Range 32–53%)</th>
<th>White blood cells (Range 5.5-12x10⁹/µL)</th>
<th>Total Protein (Range 5.5-7.3 g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Kerosene</td>
<td>Control</td>
<td>Kerosene</td>
</tr>
<tr>
<td>1º Infusion</td>
<td>0 h</td>
<td>7.2±0.2</td>
<td>7.6±0.3</td>
<td>34.2±0.8</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>7.1±0.2</td>
<td>7.9±0.2</td>
<td>34.7±1.2</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>7.0±0.3</td>
<td>7.6±0.4</td>
<td>34.2±1.1</td>
</tr>
<tr>
<td>2º Infusion</td>
<td>0 h</td>
<td>7.4±0.2</td>
<td>7.5±0.3</td>
<td>35.8±1.2</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>7.2±0.3</td>
<td>7.7±0.3</td>
<td>35.3±1.5</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>7.0±0.4</td>
<td>7.6±0.3</td>
<td>34.0±1.9</td>
</tr>
</tbody>
</table>
Table 3.3. Creatinine, blood urea nitrogen (BUN), glucose and alkaline phosphatase. total concentrations of mares in control and kerosene group, before and after (+24, +48 hours) from the intrauterine infusions. (Mean ± SEM) (p>0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>Creatinine Control</th>
<th>Creatinine Kerosene</th>
<th>Bun Control</th>
<th>Bun Kerosene</th>
<th>Glucose Control</th>
<th>Glucose Kerosene</th>
<th>Alkaline phosphatase total Control</th>
<th>Alkaline phosphatase total Kerosene</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>1.0±0.1</td>
<td>0.9±0.09</td>
<td>20.5±0.6</td>
<td>20.5±0.7</td>
<td>91.5±2.8</td>
<td>91.8±3.7</td>
<td>91.1±3.2</td>
<td>92.1±5.3</td>
</tr>
<tr>
<td>24 h</td>
<td>1.0±0.1</td>
<td>0.9±0.08</td>
<td>20.5±0.5</td>
<td>19.8±1.3</td>
<td>91.8±3.7</td>
<td>92.1±5.3</td>
<td>106.0±10.2</td>
<td>113.9±11.3</td>
</tr>
<tr>
<td>48 h</td>
<td>0.9±0.1</td>
<td>0.9±0.10</td>
<td>19.7±1.1</td>
<td>18.9±1.6</td>
<td>86.7±2.1</td>
<td>82.2±1.5</td>
<td>103.2±9.95</td>
<td>114.0±14.1</td>
</tr>
<tr>
<td>0 h</td>
<td>1.0±0.1</td>
<td>0.9±0.08</td>
<td>22.2±0.5</td>
<td>20.5±1.4</td>
<td>95.3±9.2</td>
<td>89.7±1.2</td>
<td>106.2±13.1</td>
<td>108.3±14.4</td>
</tr>
<tr>
<td>24 h</td>
<td>1.0±0.1</td>
<td>1.0±0.07</td>
<td>21.5±0.6</td>
<td>20.6±1.6</td>
<td>92.8±5.6</td>
<td>91.0±3.3</td>
<td>103.2±11.5</td>
<td>113.7±15.2</td>
</tr>
<tr>
<td>48 h</td>
<td>0.9±0.1</td>
<td>0.9±0.08</td>
<td>21.2±1.0</td>
<td>20.4±1.2</td>
<td>90.9±2.8</td>
<td>89.1±5.7</td>
<td>101.5±11.3</td>
<td>111.1±15.6</td>
</tr>
</tbody>
</table>
Table 3.4. Aspartate Aminotransferase (AST), Gamma-glutamyl transferase (GGT), total bilirubin and creatine phosphokinase (CPK) concentrations of mares in control and kerosene group, before and after (+24, +48 hours) from the intrauterine infusions. (Mean ± SEM) (p>0.05)

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Kerosene</th>
<th>Control</th>
<th>Kerosene</th>
<th>Control</th>
<th>Kerosene</th>
<th>Control</th>
<th>Kerosene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST</td>
<td>GGT</td>
<td>Total bilirubin</td>
<td>CPK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Range 150-294 u/L)</td>
<td>(Range 4-20 u/L)</td>
<td>(Range 0.5-2.3 mg/dL)</td>
<td>(Range 71-300 u/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>239.3±10.3</td>
<td>231.1±15.4</td>
<td>14.3±0.7</td>
<td>13.9±1.0</td>
<td>1.3±0.4</td>
<td>0.9±0.1</td>
<td>213.3±33.1</td>
<td>244.1±33.4</td>
</tr>
<tr>
<td>24 h</td>
<td>238.9±10.6</td>
<td>233.5±14.3</td>
<td>14.2±0.7</td>
<td>13.9±0.9</td>
<td>1.4±0.5</td>
<td>1.0±0.1</td>
<td>205.5±29.2</td>
<td>230.3±33.3</td>
</tr>
<tr>
<td>48 h</td>
<td>230.2±7.0</td>
<td>227.8±13.3</td>
<td>13.8±0.9</td>
<td>14.7±0.8</td>
<td>1.0±0.3</td>
<td>0.9±0.1</td>
<td>198.3±30.5</td>
<td>232.7±17.6</td>
</tr>
<tr>
<td>0 h</td>
<td>229.8±6.3</td>
<td>233.6±14.9</td>
<td>13.7±1.1</td>
<td>12.7±1.0</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>213.5±28.9</td>
<td>252.7±40.7</td>
</tr>
<tr>
<td>24 h</td>
<td>224.7±9.11</td>
<td>242.3±15.8</td>
<td>13.5±1.2</td>
<td>13.3±1.0</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>208.8±24.9</td>
<td>248.3±26.0</td>
</tr>
<tr>
<td>48 h</td>
<td>222.5±0.0</td>
<td>235.3±14.9</td>
<td>13.2±1.0</td>
<td>13.0±1.0</td>
<td>1.0±0.2</td>
<td>0.9±0.1</td>
<td>206.7±28.3</td>
<td>220.9±31.8</td>
</tr>
</tbody>
</table>
Table 3.5. Histological classification of endometrial biopsy according to Kenney & Doig [22].

<table>
<thead>
<tr>
<th>Mare n.</th>
<th>Control</th>
<th>7 days</th>
<th>21 days</th>
<th>35 days</th>
<th>49 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IIIB</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
</tr>
<tr>
<td>2</td>
<td>IIIB</td>
<td>II A</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>3</td>
<td>II B</td>
<td>II A</td>
<td>II A</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>4</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>5</td>
<td>III</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>6</td>
<td>II B</td>
<td>III</td>
<td>III</td>
<td>III</td>
<td>II B</td>
</tr>
<tr>
<td>Kerosene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>II A</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>8</td>
<td>II A</td>
<td>II B</td>
<td>II A</td>
<td>II B</td>
<td>II B</td>
</tr>
<tr>
<td>9</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td>II A</td>
<td>II B</td>
<td>II A</td>
<td>II A</td>
</tr>
<tr>
<td>11</td>
<td>II A</td>
<td>II B</td>
<td>II A</td>
<td>II A</td>
<td>II A</td>
</tr>
<tr>
<td>12</td>
<td>II B</td>
<td>II A</td>
<td>II B</td>
<td>II A</td>
<td>II A</td>
</tr>
</tbody>
</table>
**Table 3.6.** Comparison of positive detection of endometrial cups in mares after an artificially induced abortion at 60 days with the use of transrectal ultrasound (US), hysteroscopy (Hyst) and eCG assessment.

<table>
<thead>
<tr>
<th>Days</th>
<th>US</th>
<th>Hyst</th>
<th>eCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>+7 d</td>
<td>66% (8/12)</td>
<td>100% (12/12)</td>
<td>100% (12/12)</td>
</tr>
<tr>
<td>+21 d</td>
<td>42% (5/12)</td>
<td>75% (9/12)</td>
<td>100% (12/12)</td>
</tr>
<tr>
<td>+35 d</td>
<td>42% (5/12)</td>
<td>66% (8/12)</td>
<td>100% (12/12)</td>
</tr>
<tr>
<td>+49 d</td>
<td>8% (1/12)</td>
<td>42% (5/12)</td>
<td>100% (12/12)</td>
</tr>
<tr>
<td>Overall sensitivity</td>
<td>30%</td>
<td>71%</td>
<td>100%</td>
</tr>
</tbody>
</table>
3.7. References


CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The work within this thesis investigated features and consequences of early fetal loss in mares. The first experiment (Chapter 2) aimed to compare blood markers (progesterone, estradiol-17β, alpha-fetoprotein, PGFM) and B-mode ultrasonographic parameters of mares undergoing to experimentally induced early fetal loss. Specifically, two different routes of PGF2α analog administration (i.e. intrauterine and intramuscular) were examined in regard to fetal demise. The two models were not different in time to fetal demise or appearance of signs of impending abortion; but, due to the efficacy of a single administration and the absence of side effects elected, intrauterine administration appeared superior to the intramuscular administrations. In our study, ultrasonographic fetal parameters (fetal heartbeat, fetal mobility) were not affected by the impending fetal demise, this finding highlighted the need to explore other imaging tools that allow a prompt detection of sign of fetal stress (i.e. Doppler ultrasound). The decline of progesterone, luteal area tissue, and fetal expulsion occurred rapidly within 48 hours in all the mares, this fast response to the cloprostenol administration may constitute a study-design limitation to detect changes in the fetal and hormonal parameters. Similarly, the absence of changes in alpha-fetoprotein could be related to the acute nature of models used herein. Particularly, due to the fact that one study reported increased AFP concentrations in plasma of mares suffering spontaneous pregnancy loss. Therefore, further studies aiming to develop models of pregnancy loss mimicking spontaneous pregnancy loss are warranted.

The second (Chapter 3) detailed the effects intrauterine kerosene infusions on mares, endometrium, and operator and its potential ability to enhance the regression of endometrial cups. Retained endometrial cups is one of the main problems for mares losing the pregnancy between 35-80 days of gestation. The presence of endometrium cups after abortion prevent the mare to
cycle normally and impede the mare to be rebred in the same season. Even though one study described the successful effect of intrauterine kerosene on the removal of persistence endometrial cups, its effects on their natural regression and on the mare itself were not yet explored. Our results revealed that kerosene infusions are not effective in enhancing the endometrium cup regression. Only negligible side effects on the mare’s demeanor were observed, while physical examination and blood work were not affected by two intrauterine kerosene infusion. With our findings, we conclude that the regression of retained endometrial cups after an abortion is not enhanced by the use of intrauterine kerosene infusion, thus the effect on persistent endometrial cups have not been investigated in this study. At the same time, the use of intrauterine kerosene infusions in broodmares did not appear to negatively affect their fertility and welfare. If proper care is taken during kerosene intrauterine infusion no detrimental effects will be observed on the mare or operator.