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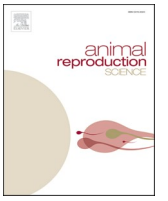

In mares resistant to endometrial infection, periovulatory treatment with ecbolic drugs does not influence uterine clearance or luteal development

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ABSTRACT

We aimed to determine associations between experimentally impaired uterine clearance or treatment with ecbolic drugs on luteal development in estrous mares after insemination. In a crossover design, eight mares were treated with saline (CON), clenbuterol (CLEN), oxytocin (OXY) and carbetocin (CARB) from the day of first insemination until 2 days after ovulation. Between treatments, the mares rested for one cycle. Estrous mares were examined for the presence of free intrauterine fluid by transrectal ultrasound. Endometrial swabs for cytology and bacteriology were collected on days 1 and 14. Blood samples were collected daily before AI until day 14 after ovulation for determination of progesterone and $PGF_{2\alpha}$ metabolites (PGFM). Differences between treatments were compared by a general linear model for repeated measures (SPSS 29). One mare was excluded because of a uterine infection in the control cycle. In all other mares, only minor amounts of free intrauterine fluid were present after insemination and decreased over time ($P<0.05$) with no treatment x time interaction. There was no effect of treatment on polymorphonucleated cells (PMN) in endometrial cytology after ovulation or PGFM secretion. Progesterone release from day 1–14 as well as pregnancy rate and conceptus size on day 14 was not influenced by treatment. In conclusion, treatment with clenbuterol does not impair uterine clearance in estrous mares resistant to endometritis. Repeated injection of the oxytocin analogue carbetocin during the early postovulatory period is not detrimental to corpus luteum function and can be recommended to enhance uterine clearance.

1. Introduction

The concentration of plasma progesterone during the first days after ovulation has a substantial influence on endometrial function regarding fetal nutrient supply before placentation and placentation itself in many species (Bazer et al., 2012). The progesterone-induced endometrial progesterone receptor downregulation prepares the endometrium for early pregnancy and

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placentation (Bazer et al., 2009; Okumu et al., 2010). In cattle, conceptus development and the embryonic signal for maternal recognition of pregnancy are directly correlated to plasma progesterone concentration (Mann and Lamming, 2001; Mann et al., 2006). The horse conceptus depends solely on nutrient supply from endometrium-derived histotroph before placentation that occurs from day 35 of pregnancy onwards and thus later than in many other species (Allen and Wilsher, 2009). Experimental reduction in early postovulatory plasma progesterone concentration changed the composition of endometrial secretions (Beyer et al., 2019), was associated with impaired embryo and placental development on day 34 after ovulation (Okada et al., 2020, 2022) and increased pregnancy loss beyond placentation (Wagner et al., 2023). In horses, there is thus a clear relationship between early luteal progesterone concentration and embryo development but the reasons for impaired luteal function in this species are still unclear.

In mares, breeding induces a physiologic inflammatory response of the endometrium that ceases within 12–24 hours (Watson, 2000). This endometrial response is characterized by a rapid increase in pro-inflammatory cytokines followed by the influx of inflammatory cells, mainly polymorphonuclear neutrophils (PMN; LeBlanc, 2009; Christoffersen et al., 2012). This reaction serves to eliminate bacteria, debris, seminal plasma, and excess spermatozoa from the uterine lumen (Kotilainen et al., 1994; Katila, 1995; LeBlanc and Causey, 2009; Christoffersen and Troedsson, 2017). In mares with impaired physical uterine clearance, however, the inflammation may persist and develop into a bacterial endometrial infection (Hughes and Loy, 1969; Morris et al., 2020). This so-called persistent breeding induced endometritis (PBIE; Causey, 2006) is an important cause for reduced pregnancy rates (LeBlanc and Causey, 2009). To the best of our knowledge, there is no information if endometrial prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release in mares with PBIE (Nikolakopoulos et al., 2000) impairs corpus luteum development when the inflammation persists beyond ovulation.

To prevent the development of PBIE and its detrimental effects on pregnancy, treatment of estrous mares with ecbolic drugs, such as oxytocin or $PGF_{2\alpha}$ and its analogues after breeding is routine (Morris et al., 2020; Pascottini et al., 2023). Although it has been suggested previously that the mare corpus luteum is resistant to the luteolytic effect of $PGF_{2\alpha}$ and its analogues until day 5 after ovulation (Oxender et al., 1975), it has been demonstrated that injection of cloprostenol in the early postovulatory period impairs luteal development and decreases progesterone secretion (Troedsson et al., 2001; Nie et al., 2003; LeBlanc and Causey, 2009) rendering it unsuitable for treatment of mares after ovulation. The ecbolic action of oxytocin is shorter than that of cloprostenol. The oxytocin analogue carbetocin which has an increased half-life has, therefore, been suggested an attractive alternative in broodmare management (Schramme et al., 2008). According to its molecular pharmacological properties, however, carbetocin is substantially different from endogenous oxytocin and does for example not mimic oxytocin functions in the brain (Passoni et al., 2016). Because more recent publications in luteal phase mares reported luteolytic effects of carbetocin if injected repeatedly (Bare et al., 2013, Diel de Amorim et al., 2023), we were interested to investigate its effects on corpus luteum development reflected in progesterone secretion following treatment of mares in the periovulatory period.

It was the aim of this study to investigate associations between experimentally impaired uterine clearance, treatment with ecbolic drugs and endometrial $PGF_{2\alpha}$ secretion in estrous mares after insemination. We hypothesized that in mares where uterine clearance is experimentally inhibited, there will be an increased endometrial $PGF_{2\alpha}$ release that decreases postovulatory progesterone concentration. In addition, we hypothesized similar detrimental effects of carbetocin on luteal development in mares after ovulation.

2. Material and methods

2.1. Animals

Eight Haflinger mares, aged 8.0 ± 2.3 years and weighing 492 ± 21 kg were enclosed in this study. Mares were kept as one herd in a large outdoor paddock with free access to a covered shed. All mares were fed hay twice daily and water was available *ad libitum*. Before start of experiments, mares underwent breeding soundness evaluation that included transrectal ultrasonography of the genital tract, collection of an endometrial swab for bacteriological analysis (Spergser et al., 2002) and a cytology brush for PMN cell counts (Rink et al., 2018; Card, 2005). Only genitally healthy mares with previously proven fertility were included in the experiments.

2.2. Experimental design

The study was approved by the Austrian Federal Ministry for Science and Research (experimentation license number 2020–0.547.780) and was conducted in Vienna, Austria (longitude 16.4°, northern latitude 48.3°) between April and October 2022.

Mares were examined three times a week by transrectal ultrasonography. Once a follicle with a diameter $\geq$35 mm together with endometrial edema was detected, the mare was considered to be in estrus and examinations were performed daily. Estrous mares were inseminated at 48-hour intervals until detection of ovulation which was indicated by disappearance of the preovulatory follicle.

The mares were randomly allocated to one of four different treatments (two mares per treatment) in a Latin square design: i) control (CON; 2 ml saline intravenously twice daily), ii) clenbuterol (CLEN; Planipart, Boehringer Ingelheim, Ingelheim, Germany: 0.8 μg per kg body weight intravenously twice daily), iii) oxytocin (OXY; Oxytocin, Vana, Vienna, Austria; 20 IU per animal subcutaneous four times daily), iv) carbetocin (CARB; LongActon Vetoquinol; Berne, Switzerland; 0.175 mg per animal intramuscular twice daily). All treatments were applied from the day of first insemination until 2 days after ovulation. On day 14, mares received the $PGF_{2\alpha}$ analogue cloprostenol for termination of pregnancy, the following cycle was a rest cycle. Each mare served as her control and received all treatments in four estrous cycles with always one rest cycle between treatment cycles. During estrus, mares were examined for the presence of free intrauterine fluid by transrectal ultrasound once daily in the morning before administration of any drug. Endometrial swabs for assessment of endometrial cytology and bacteriology were collected on days 1 and 14 after ovulation. From day 10 to day 14 after ovulation, transrectal ultrasound of the uterus was performed for detection and size determination of a conceptus. Blood samples

were collected by venipuncture of the Vena jugularis externa twice daily from the first insemination until day 5 after ovulation, thereafter once daily.

2.3. Breeding management

The reproductive tract of the mares was examined by transrectal palpation and ultrasonography (DP-6600Vet; Mindray, Shenzhen, China). When estrus was detected, mares were examined daily to monitor follicular growth, uterine edema, uterine fluid accumulation and ovulation. When a preovulatory follicle (diameter $\geq$35 mm) and uterine edema was detected for the first time, mares were inseminated with 500 million progressive motile spermatozoa of raw semen from a stallion of proven fertility at 48-hour intervals until detection of ovulation. The day of ovulation (day 0) was defined as the day when the preovulatory follicle could no longer be detected. Examination for pregnancy was performed by transrectal ultrasonographic of the whole uterus from day 10 after ovulation until day 14. On day 14, mares received the $PGF_{2\alpha}$ analogue cloprostenol (Estrumate, MSD, Vienna, Austria; 250 μg) intramuscularly for termination of pregnancy and induction of estrus.

2.4. Determination of intrauterine fluid accumulation

The uterus was monitored for the presence of free intrauterine fluid daily by transrectal ultrasonography from the day of insemination until no fluid was detectable after ovulation. The amount of free intrauterine fluid was determined by measuring the area of luminal fluid at the largest diameter in both uterine horns and the uterine corpus using the electronic calipers of the ultrasound machine and then calculating the total maximal area (Freccero et al., 2023).

2.5. Determination of conceptus size

The diameter of the embryonic vesicle was measured in two dimensions at a 90° angle once a day always at the same time using the electronic calipers of the ultrasound machine. The mean of the two measurements was calculated as the mean diameter of the embryonic vesicle.

2.6. Blood sampling

Blood samples were always collected before any intervention or treatment by jugular venipuncture into an evacuated tube containing EDTA as an anticoagulant (Vacuette 9 ml K3EDTA; Greiner bio-one, Kremsmünster, Austria). Samples were collected at 12-hour intervals from the first day of insemination until 5 days after ovulation, thereafter at 24-hour intervals until day 14. Samples were immediately transferred into iced water, plasma was separated by centrifugation at 5 ℃ (1200 x *g*, 10 minutes), and instantly transferred into aliquots and stored at −20 ℃ until progesterone and PGFM analysis.

2.7. Hormone analysis

Progesterone concentration in plasma was analysed by enzyme-linked immunosorbent assay (Enzo Progesterone ELISA; Enzo Life Sciences) as described (Beyer et al., 2019). The intra-assay coefficient of variation was 4.9 %, the inter-assay coefficient of variation was 7.9 % and the minimal detectable concentration 11.1 pg/ml.

The 13,14-Dihydro-15-keto-PGF2α (PGFM) in plasma was determined using a commercial ELISA kit (PGFM Enzyme Competitive ELISA Kit, Invitrogen, Thermo Fisher Scientific, Winsford, UK) as described previously. The assay was validated for horse plasma in the authors' laboratory. The standard curve ranged from 50 to 3200 pg/ml. The sensitivity of the PGFM assay was 5.6 pg/ml. The intra- and inter-assay coefficients of variation were 45.2 and 29.4 %, respectively.

2.8. Statistical analysis

Statistical analysis was done with the IBM SPSS statistics software (version 29.0, IBM-SPSS, Armonk, NY, USA). Data were analysed for normal distribution by Shapiro-Wilk test and homogeneity of variance by Levene test. If data were not normally distributed, log-transformation was performed. Differences between treatments over time were compared by the general linear model for repeated measures with time (intrauterine fluid day −2 to day 2; % PMN day 1 vs. day 14; progesterone concentration days 1–14; PGFM concentration 24 h before to 120 h after ovulation) as intra subject factor, and treatment (CON, CLEN, OXY and CARB) as between subject factor. Differences in embryo size on day 14 among treatments were compared by Wilcoxon test. Differences in the size of the preovulatory follicle on the day before detection of ovulation, the number of inseminations per estrus and the interval from the last insemination until ovulation among treatments were analysed by Friedman test. The pregnancy rate among treatments was compared by chi^2 analysis. All values are given as mean ± SEM. A *P*-value <0.05 was considered significant.

3. Results

In one mare, insemination in the control cycle resulted in endometritis that was detected on day 12 after ovulation because of increased free intrauterine fluid accumulation. An endometrial swab was taken and demonstrated the growth of *Streptococcus equi*

subspecies zooepidemicus. Because of the experimental design of this study, data from this mare had to be excluded from further analysis.

In the remaining 7 mares, the overall amount of free intrauterine fluid detected at transrectal ultrasonography performed from two days before to two days after ovulation was small (Fig. 1). The amount of fluid decreased over time ($P<0.05$) and was affected by treatment with more fluid detected in CON and CLEN cycles ($P<0.001$), but no treatment x day interaction. Treatment of inseminated mares during the periovulatory period did not significantly influence the percentage of PMN in endometrial cytology swabs collected on days 1 and 14 after ovulation (Fig. 2). The endometrial culture from the swab collected simultaneously was always negative.

The size of the preovulatory follicle on the day before detection of ovulation, the number of inseminations per estrus and the interval from the last insemination until ovulation did not differ among treatments (Table 1).

Progesterone concentration in plasma changed from day 1 to day 14 after ovulation ($P<0.001$), but no effect of treatment (n.s.) or time x treatment interaction (n.s.) was detected (Fig. 3). In only one mare ("Messalina"), the concentration of PGFM determined in blood samples collected at 12 h intervals from day 1 before to 5 days after ovulation was continuously above the lower detection limit of the assay system. The concentrations of PGFM in the four experimental cycles of this mare are shown in Fig. 4. In all other mares, PGFM was detectable in only a few samples and no relevant data analysis was therefore possible (data not shown).

Conceptuses were detected in all seven mares after CON, CLEN and OXY treatment and in six of the seven mares after CARB treatment (n.s.). The size of the conceptuses on day 14 after ovulation was not influenced by treatment (Fig. 5).

4. Discussion

In the present study, associations between uterine clearance in estrous mares, their treatment with ecbolic drugs, periovulatory endometrial $PGF_{2\alpha}$ secretion and subsequent corpus luteum function were investigated. We hypothesized that experimental impairment of uterine clearance by treatment with the β_2-adrenergic substance clenbuterol in estrous mares would contribute to an increased endometrial inflammatory response in the periovulatory period, thus increase peripheral $PGF_{2\alpha}$ concentration and eventually impair early luteal development. Treatment with clenbuterol has been effective in inhibiting uterine contractions under *in vivo* (Kolm et al., 2005) and *in vitro* conditions particularly in tissue preparations collected from estrous mares (Coruzzi et al., 1989). In other studies, the inhibitory effect was increased in luteal phase mares (Jones et al., 1991) or anovulatory mares primed with progesterone (Gastal et al., 1998). We therefore expected that clenbuterol would inhibit physical clearance of the uterus of mares during the periovulatory phase, i.e., under an increasing influence of progesterone. Although there was an increase in free intrauterine fluid in response to clenbuterol treatment, differences between treatments remained small, and accumulation of intrauterine fluid after insemination was accompanied by only minor increases in PMN in endometrial cytology, the absence of opportunistic bacteria on the day after ovulation and a very good conception rate. Taking into account that among β_2- adrenoreceptor selective agonists, clenbuterol is still the most frequently administered to horses with asthma (Pozzoli et al., 2020), it can be concluded that treatment with clenbuterol is unlikely to impair the fertility of estrous broodmares.

The mares included into our study apparently had an excellent uterine clearance, and, therefore with the chosen group of mares the experimental design turned out to be unable to address our first hypothesis. It remains therefore unclear if a PBIE that persists into the early postovulatory period impairs corpus luteum development in horses. When an acute inflammatory response was induced by embryo transfer in mares on day 7 after ovulation, there was a detectable release of $PGF_{2\alpha}$ as indicated by changes in peripheral PGFM concentration. This was followed by luteolysis in 30 % of mares (Koblischke et al., 2008). In that study, the PGFM concentrations determined in the mares in the first hour before and four hours after embryo transfer were similar as in the present study between 12 hours before to 5 days after ovulation in the absence of an acute inflammatory response. The acute endometritis after embryo

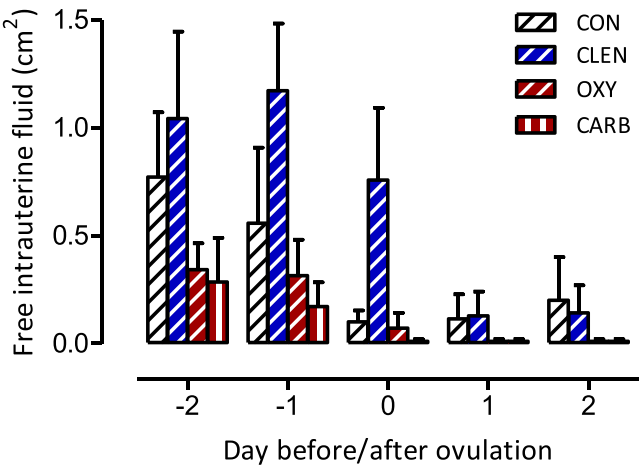

Fig. 1. The amount of free intrauterine fluid (cm^2) from two days before to two days after ovulation in mares (n=7) that were inseminated during estrus and were left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination to two days after ovulation (treatment $P<0.001$, day $P<0.01$, day x treatment n.s., data are presented as means + SEM).

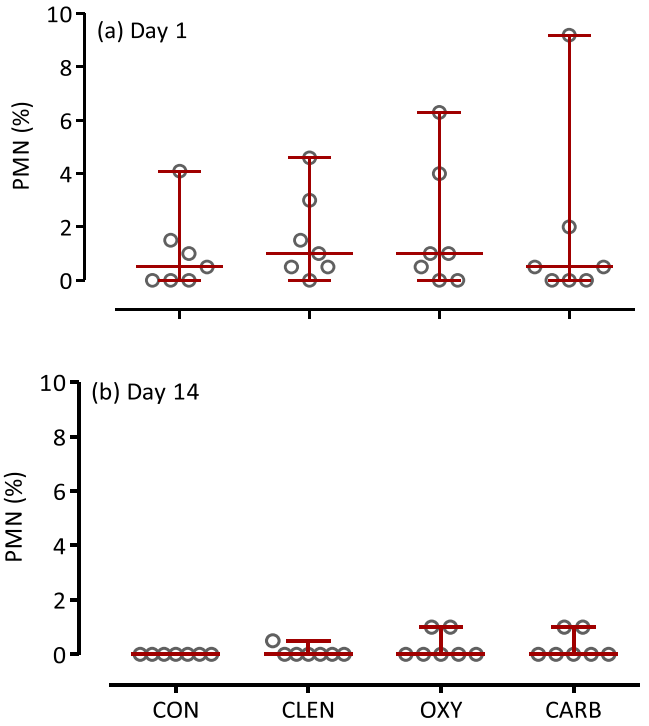

Fig. 2. Percentage of PMN (%) in endometrial cytology samples collected on (a) day 1 and (b) day 14 after ovulation in mares (n=7) that were inseminated during estrus and left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination after two days after ovulation (no significant differences among treatments or day 1 vs. day 14). Data are presented as scatter-plots of values from individual mares (open circles), and median with total range (red).

Table 1
Size of the preovulatory follicle on the day before detection of ovulation, the number of inseminations per estrus and the interval from the last insemination until ovulation in mares (n=7) that were inseminated during estrus and were left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination to two days after ovulation. Values are means ± SEM. No differences among treatments.

	CON	CLEN	OXY	CARB
Size of preovulatory follicle (cm)	4.7 ± 0.1	4.9 ± 0.2	4.9 ± 0.1	4.6 ± 0.1
Inseminations per estrus (n)	2.1 ± 0.3	2.4 ± 0.3	2.0 ± 0.2	2.4 ± 0.2
Interval from last insemination to ovulation (days)	1.6 ± 0.2	1.4 ± 0.2	1.9 ± 0.1	1.6 ± 0.1

transfer became apparent two to four days thereafter and was associated with a mean increase of PGFM to approximately ten times the concentration before (Koblischke et al., 2008). The low concentrations of PGFM determined in the present study irrespective of treatment are thus to be interpreted as baseline concentrations of PGFM in healthy estrous mares and not indicative of a pronounced endometrial inflammatory response. The present investigation can therefore not exclude that $PGF_{2\alpha}$ of endometrial origin (Nikolakopoulos et al., 2000) is contributing to impairment of luteal development in mares with PBIE. A follow up study with mares susceptible to endometritis is thus clearly indicated.

Although carbetocin treatment did not have the hypothesized effect, this investigation presents some interesting findings regarding possible effects of the long-acting oxytocin analogue carbetocin on uterine clearance and corpus luteum development. To prevent the development of persistent breeding induced endometritis, injection of ecbolic drugs is a routine treatment after breeding with the aim to improve uterine mechanical clearance (Morris et al., 2020; Pascottini et al., 2023). Injection of 20 IU oxytocin results in high-amplitude myometrial contractions for a period of approximately 30 min in most mares. The effect is greatest during estrus, but still present for 48 hours after ovulation (LeBlanc et al., 1994; LeBlanc and Causey, 2009). For effective enhancement of uterine clearance in mares susceptible to endometritis, repeated injections are thus required. Although more recent studies (Bare et al., 2013, Diel de Amorim et al., 2023) demonstrated that repeated treatment of luteal phase mares with carbetocin induced premature luteolysis whereas oxytocin increased the luteal lifespan (Bare et al., 2013), no detrimental effects of carbetocin treatment at 12-hour intervals until two days after ovulation were detectable in the present investigation. The effect of carbetocin treatment on luteal development and regression, thus, differs depending on the estrous cycle stage. In mares of the present study treated with carbetocin, neither progesterone secretion nor conceptus development until day 14 of the luteal phase were impaired. Peri- and postovulatory treatment

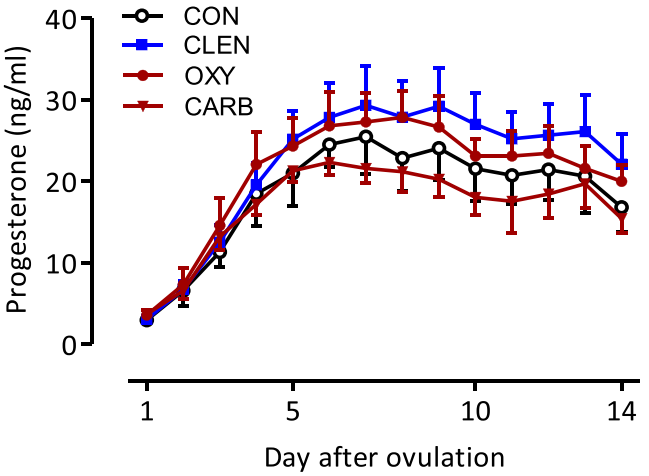

Fig. 3. Concentration of progesterone from day 1 to day 14 after ovulation in mares (n=7) that had been inseminated during estrus and were left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination to two days after ovulation (statistical analysis: time $P<0.001$, treatment n.s., time x treatment n.s.). Data are presented as means ± SEM.

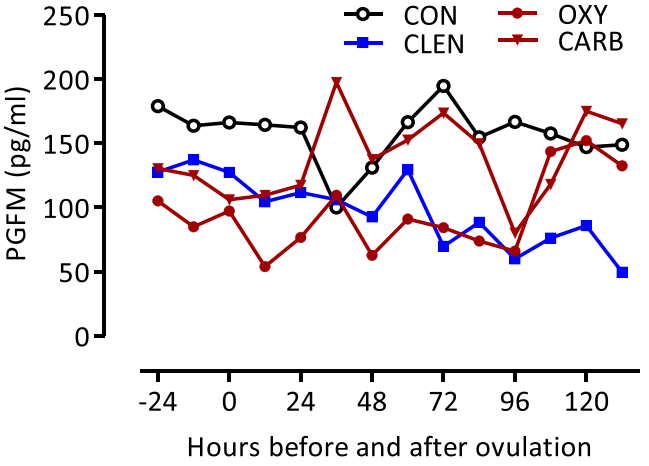

Fig. 4. Concentration of PGFM (pg/ml) in the mare "Messalina" from 24 h before to 120 h after ovulation. The mare was inseminated during estrus and left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination to two days after ovulation.

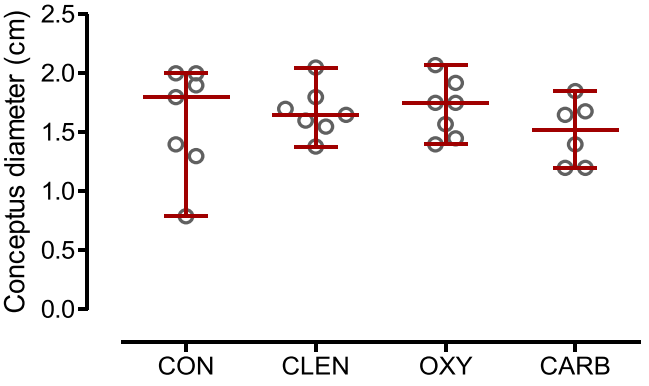

Fig. 5. Conceptus diameter (cm) on day 14 after ovulation determined by transrectal ultrasound in mares (n=7) that had been inseminated and were left untreated (CON) or treated with clenbuterol (CLEN), oxytocin (OXY) or carbetocin (CARB) from the first insemination until two days after ovulation (n.s.). Data presented as scatter-plot of individual mares in each group (open circles), with median and total range (red).

twice daily with carbetocin is thus safe in mares with impaired uterine clearance that have been inseminated.

5. Conclusions

Treatment with clenbuterol does not delay uterine clearance in estrous mares resistant to endometritis. There is thus still no evidence that persistence of PBIE during the early postovulatory phase is detrimental to corpus luteum development in horses. Respective research in mares susceptible to endometritis is thus required. Nevertheless, the results of this study demonstrate that repeated injection of carbetocin in mares during the early postovulatory period does not impair corpus luteum development and can be recommended to enhance uterine clearance.

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CRediT authorship contribution statement

Camille Gautier: Writing – review & editing, Methodology, Data curation. **Martim Kaps:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Amr El-Shalofy:** Validation, Methodology, Investigation, Formal analysis. **Younis Khan:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christine Aurich:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they hold no financial or personal affiliations with anyone or organizations capable of improperly influencing or biasing this article.

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