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No increase in pregnancy rate of mares after preovulatory deep uterine horn application of misoprostol

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ABSTRACT

A potential source of fertility loss in mares is oviductal dysfunction, potentially caused by masses or debris in the lumen, that may prevent either sperm from reaching the fertilization site or the embryo from reaching the uterus. Recently a novel therapeutic method leading to increased pregnancy results was described by infusing misoprostol, a synthetic prostaglandin E_1, in the uterus of mares with unexplained fertility problems. In this study, we aimed, after examining the compatibility of misoprostol with semen, to evaluate the pregnancy rate after routine preovulatory deep uterine horn application of misoprostol in clinically normal oestrous mares, which were inseminated in the same cycle. In experiment 1, ejaculates of 10 stallions diluted with INRA 96™ were mixed with different concentrations of misoprostol (0.01 mg/mL, 0.001 mg/mL, 0.0001 mg/mL, and 0.00001 mg/mL) and total semen motility was evaluated immediately, 12, 24, 48, and 72 h later, and compared with a control sample (mixed with NaCl 0.9%). In experiments 2 and 3, 33 privately-owned clinically normal oestrous mares were each allocated to a treatment or control group. Ovulation was then induced with intramuscularly 2.25 mg deslorelin acetate. At the moment of ovulation induction (experiment 2) and 24 h earlier (experiment 3), 0.2 mg misoprostol diluted in 2 mL NaCl 0.9% were applied deep in the uterine horn (treatment groups) and pure 2 mL NaCl 0.9% in the mares of the control groups. Mares were then inseminated 24 h after deslorelin administration and prior to ovulation with commercial chilled-warmed or frozen-thawed semen, as well as immediately after ovulation detection (both types of semen) maximally 48 h after ovulation induction. In experiment 1, regardless of time and compared with the control groups, all solutions with different concentrations of misoprostol had a negative effect on total motility of semen, which was significant for the highest concentrations (0.01 mg/mL: 18.0% reduction, CI = 22–13%, p = < 0.01). We found no beneficial effect of preovulatory uterine treatment with misoprostol on pregnancy rate (OR = 0.45, CI = 0.15–1.31, p = 0.14): in experiment 2, 2/11 (18.2%) mares of the treatment group became pregnant vs. 12/22 (54.5%) mares in the control group (OR = 0.19, CI = 0.03–1.06, p = 0.07), in experiment 3, 5/14 (35.7%) mares in the treatment group vs. 7/19 (36.8%) mares in the control group (OR = 0.95, CI = 0.23–4.02, p = 0.95), respectively. In conclusion, pregnancy rate was not increased in reproductively normal mares with routine preovulatory deep uterine horn application of misoprostol.

1. Introduction

Fertility problems in mares represent a major concern in the horse breeding industry and can be due to very different causes related to management factors [1–3], the quality of the stallion semen [2,4–8] or the reproductive system of the mare [9].

Regarding the latter, physiological factors such as age and reproductive status may affect the fertility of the mare [9–12], but also abnormal sexual development due to genetic disorders [13] and anatomical abnormalities [14]. However, the most common reasons for infertility are endometritis [15,16] and degenerative diseases like endometriosis [17], and, in addition, multifactorial diseases, like pneumovagina [18], traumatic injuries [19] or

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neoplastic disease [20].

Another potential source of reduced fertility in mares is the presence of masses in the oviduct that either may prevent sperm from reaching the fertilisation site or prevent the embryo from reaching the uterus. Because such masses were found in 42%–88% of mares [21–25] some investigators consider that the presence of oviductal masses may be "normal" and do not lead to subfertility in each case [23,24,26]. Aguilar et al. [23] showed that mature mares are significantly more affected than prepubertal mares (89,7% vs. 16.6%) and supposed that the incidence of oviductal masses increases with the number of ovulations and increasing age. Lantz et al. [24] histologically examined intraluminal masses of the oviduct 7–22 h after ovulation and frequently found fibroblasts producing type 1 collagen in association with the recent ovulation. It was supposed by the authors that collagen remains in place and eventually aggregates in the lumen so that oviductal transport would be impaired [24]. Based on all these findings, it was suspected that an intraoviductal mass can lead to tubal dysfunction and result in subfertility in older mares [24].

Diagnosis of oviductal obstruction is currently not easy to perform and techniques are invasive and could also cause issues themselves. To date, different tools have been developed [25] e.g. nonsurgical retrograde flushing of the oviduct from the uterotubal junction to the fimbria after inserting a catheter via the uterus to the uterotubal junction under endoscopic guidance [27,28], or infusing a dye into the ampulla and collecting the marker solution afterwards from the uterus using a laparoscopic lateral flank approach in the standing sedated horse with visualisation of the ovary, infundibulum and oviduct, and the possibility for catheterisation of the ampulla [29].

At the same time as diagnosis therapeutic flushing of the oviduct with saline solution becomes possible, e.g. in the study of Inoue and Sekiguchi [27], 26 of 28 mares with unexplained subfertility became pregnant after treatment. Another potentially effective therapeutical option is the local application of prostaglandins (PG) on the oviduct. These hormones are produced from arachidonic acid [30] and play an important role in conception and early gestation of the mare, including blastocyst growth and development, embryo transport, and embryo–maternal dialogue and also during the trophoblast invasion by increasing vascular permeability and angiogenesis at the implantation site [30,31]. Early uterine embryos secrete PGE_2 especially immediately before and during oviductal transport (on day 5 and 6 of gestation) [32], hereby relaxing the isthmic circular smooth muscle [33] and supporting the transport of the early embryo towards the uterus [32]. Local application of PGE_2 was tried in alternative ways, i.e. various authors laparoscopically applied 0.2 mg PGE_2 gel on the uterine tube surface of the mares [34,35]. Ortis et al. [34] carried out their study on 28 mares that were barren for an average of 1.9 years. Seventeen of 20 mares produced one or more embryos after their treatment and additionally seven of eight mares became pregnant (four in the first oestrus cycle after treatment and three in their second cycle).

Various researchers have tried to locally apply prostaglandins in the uterus to treat potential oviduct blockades and controversial results have been reported. Woods et al. [36] demonstrated an intrauterine application of 0.25 mg PGE_2 infusion 2 h prior to insemination to increase the pregnancy rate of reproductively healthy mares if semen with very good quality was used. Thirteen of 18 mares (72.2%) became pregnant, compared to seven of 16 mares (43.7%) in the control group. On the other hand, Brinsko et al. [37] conducted a very similar study and found no difference between the results of the PGE_2 group (0.25 mg PGE_2 in 1 mL 0.9% NaCl solution infused into the uterine horn 2 h before insemination) and those of the control group (1 mL 0.9% NaCl solution infused into the uterine horn 2 h before insemination), i.e. in each group 11 out of 18 mares (61.1%) became pregnant.

Recently, Alvarenga & Segabinazzi [38] described a new method to treat oviduct blockade by infusing 0.2 mg of a synthetic prostaglandins E_1 (misoprostol) in the deep uterine horn near the oviduct. Misoprostol has also been used in mares for promoting cervical relaxation, however with controversial results [39,40]. In their study, Alvarenga & Segabinazzi [38] treated mares that had been infertile for at least 2 years by an intrauterine application of 0.2 mg misoprostol diluted in 3 mL sterile water in each deep uterine horn during dioestrus. Mares with any uterine or ovarian pathology were excluded, and they all had negative uterine cultural and cytological results. Ten of the 22 mares (45.5%) became pregnant within the first cycle. After this first study another 20 mares were treated [41]: seven mares got pregnant or had an embryo after the following first cycle. In this 2nd part of the experiment, five mares were also treated during oestrus, and two of them became pregnant in the first cycle. Overall a pregnancy rate of 66.6% (28 mares out of 42) in the first three cycles after treatment was observed [41]. Based on these positive results, the authors concluded with the hypothesis that the intrauterine use of misoprostol in mares with unexplained infertility and suspected oviduct blockade could be a promising non-invasive, safe, and easy-handling treatment method in the future.

Considering these results, our investigations aimed to examine the compatibility of misoprostol with semen, as to the author's knowledge, no studies are available regarding this topic. In a second step, we evaluated the pregnancy rate after routine preovulatory deep-horn uterine application of misoprostol in oestrous mares during one season in a commercial insemination program. Intrauterine fluid accumulation as an indicator of potential side effects was also assessed.

2. Materials and methods

2.1. Horses and ethical note

The study took place at the Reproduction Centre of the Swiss Institute of Equine Medicine (ISME) in Avenches during the breeding season of 2020. Ten healthy, mature and sexually experienced Franches-Montagnes stallions (mean age 12 years, range 3–19 years) from the Swiss National Stud in Avenches from Agroscope/Switzerland and 97 clinically healthy privately-owned mares (all breeds, with and without foal, mean age 12.2 years, range 3–22 years) coming to the centre for the commercial insemination program were used for the experiment. The study was approved by the Animal Health and Welfare Commission of the Canton of Vaud (*permit number VD3564*) and strictly followed institutional guidelines for humane animal treatment.

2.2. Experiment 1: Semen analysis with different concentrations of misoprostol

To evaluate the influence of misoprostol on the semen quality, the ejaculates of ten stallions were mixed with different concentrations of misoprostol. Semen was collected using a dummy in presence of an ovariectomised teaser mare, and an artificial vagina (type Avenches, National Stud Avenches, Switzerland). The same collecting person and stallion handler performed the semen collection procedure in the same way for each stallion. The week before the experiment, extra-gonadal semen reserves were minimised by collecting semen from them on five consecutive days [42].

After collection, the gel fraction was removed, and the concentration of spermatozoa in the native semen was evaluated with the Nucleocounter SP-100 system (ChemMetec A/S, Allerod, Denmark).

Semen was diluted with INRA 96™ (IMV technologies, L'Aîgle, France) to a concentration of 30×10^6 spermatozoa/mL maintaining a temperature of 37 °C. 9 mL of diluted semen was then mixed with 1 mL of different concentrations of misoprostol (Cytotec, Pfizer AG, Zurich, Switzerland) dissolved in NaCl 0.9% (Bichsel AG, Interlaken, Switzerland) resulting in solutions with the following misoprostol concentrations: 0.01 mg/mL, 0.001 mg/mL, 0.0001 mg/mL, and 0.00001 mg/mL. Nine mL of a semen sample diluted with INRA 96™ and mixed with 1 mL NaCl 0.9% served as control. Samples were then stored at 5 °C in tubes (Polystyrene Conical Tube FALCON, Fisher Scientific GmbH, Schwerte, Germany). Spermatozoa motility analysis was performed immediately after dilution with misoprostol solution, and then after 12, 24, 48, and 72 h, respectively, using a computer-automated semen analyser (HTM-IVOS, Version 12, Beverly, MA, USA). For that, extended semen was placed in pre-heated 20 μL standard counting analysis chambers (SC 20-01-C, Leja, Nieuw-Vennep, Netherlands) and was analysed in ten fields, so that the total motility of sperm could be determined. Before analysis of the chilled semen, 0.5 mL of each sample were warmed up in a water-bath at 37 °C for 2 min before evaluation.

2.3. Experiment 2 and 3

After written consent from the private owners and recording of the reproductive history, the mares were allocated depending on their status regarding the national regulations on the use of veterinary medicinal products. Companion animals were included in the treatment group, treated with intrauterine misoprostol, and food-producing animals were assigned to a control group, treated at the same time with intrauterine NaCl 0.9%. Only clinically normal mares without history of infertility were included in the study. Exclusion criteria included mares with a history of severe post-breeding-induced endometritis, mares with intra-uterine fluid accumulation, and mares showing abnormal sexual behaviour.

To obtain the necessary number of mares to determine the pregnancy rates of the treatment group vs. the control group respecting potentially confounding factors, we aimed for a balanced distribution of maiden, barren and foaling mares 3–8 years old, 9–12 years old and older than 12 years, as well as for matching of mares inseminated with chilled-warmed or frozen-thawed semen.

2.3.1. Pre-insemination and insemination protocol

Uterus and ovarian activity of the mares were regularly assessed by transrectal ultrasonography using a 7.5 MHz ultrasound with a 50 mm linear probe (MyLab™OneVET, Esaote Spa, Florence, Italy), and behavioural signs of oestrus were evaluated by presenting the mare regularly to a teasing stallion.

Day −3 was defined as the day when transrectal ultrasonography revealed at least one follicle with a diameter of >35 mm, pronounced uterus oedema without intra-uterine fluid accumulation and when the mare showed oestrous behaviour. At 17:00 on day −2, 2.25 mg deslorelin acetate (Bova Specials UK Ltd, London, UK) was administered intramuscularly to induce ovulation, which was then expected to happen approximately 36.6–38.6 h later [43,44]. On the following day −1, the mares were examined at 8:00 and 17:00, i.e. 24 h after deslorelin application. Mares who had already ovulated at that point in time were excluded from the experiment for that cycle. All others were inseminated preovulatory with chilled-warmed or frozen-thawed semen (13 mares with minimum insemination doses of chilled-warmed semen: 10 mL and 500×10^6 motile spermatozoa; 53 mares with minimum insemination doses of frozen-thawed semen: 0.5 mL and 100×10^6 motile spermatozoids). Mares were prepared for insemination by cleaning the vulva with diluted iodine soap and water. For insemination with frozen-thawed semen, an insemination pipette (universal insemination pipette for equine, Minitüb GmbH, Tiefenbach, Germany) was placed deep into the uterine horn on the side of the dominant follicle using guided transrectal examination. If chilled-warmed semen was used, the pipette was placed in the uterine body. On day 0, mares were examined up to three times until ovulation was detected (at 7:00, 12:00, and 17:00), which was identified via ultrasonography by the presence of a normal corpus luteum. At the time of ovulation diagnosis, the mares were inseminated again with chilled-warmed or frozen-thawed semen (same volume and dose as in preovulatory insemination). If a mare had not ovulated 48 h after deslorelin application (i.e. 24 h after the first insemination), she was excluded from the experiment for that cycle. Pregnancy detection was performed by transrectal ultrasound 14–19 days after ovulation either at the reproduction centre or the breeder's farm.

At all examinations from the time of misoprostol application on, the presence and quantity of intrauterine fluid was recorded as the maximal depth of fluid observed in the uterus. If more than 20 mm of fluid was identified, mares received an intra-uterine lavage using 4 L or more of warm physiological saline solution (NaCl 0.9%) and three intramuscularly applications of 20 IU oxytocin (Oxytocin, Stricker AG, Zollikofen, Switzerland) each at an interval of 6 h.

Semen quantity and quality analysis were performed for every frozen-thawed and chilled-warmed semen sample immediately before insemination after dilution with INRA 96™ to a concentration of 30×10^6 sperm/mL. Mares inseminated with sperm having a total motility of less than 20.0% were excluded from the study.

2.3.2. Treatment protocols

2.3.2.1. Experiment 2. At the time of ovulation induction (day −2), 0.2 mg misoprostol, corresponding to one pill Cytotec, diluted in 2 mL NaCl 0.9% (and then packaged in four insemination straws of 0.5 mL each) were applied to mares of the treatment group with the same technique as mentioned above for insemination with frozen-thawed semen. The mares of the control group were also treated at the same time but with 2 mL pure NaCl 0.9% packaged in four insemination straws of each 0.5 mL. In the case where two follicles with a diameter >35 mm were each located on another ovary ($n = 7$ mares), the dosage of misoprostol for the treatment group or NaCl for the control group respectively was doubled and distributed into eight insemination straws, so that the same dosage could be applied into both uterine horns.

2.3.2.2. Experiment 3. In the third experiment, mares were treated 24 h before the administration of the ovulation induction agent (day −3) with 0.2 mg misoprostol (treatment group) or with 2 mL pure NaCl 0.9% (control group). The other procedures were performed in the same way as described above for experiment 2.

2.3.3. Post-insemination protocol

Transrectal ultrasonography was performed 24 h after ovulation detection to control for the absence of pathological uterine fluid excluding post-breeding endometritis. If more than 20 mm of fluid were identified, mares received an intra-uterine lavage using 4 L or more of warm physiological saline solution (NaCl 0.9%). In addition, 20 IU oxytocin (Oxytocin, Stricker AG, Zollikofen, Switzerland) were applied intramuscularly every 6 h over a 12 h period. If an intrauterine fluid accumulation of less than 20 mm was identified, only oxytocin was administered. These treatments were repeated if uterine fluid accumulation persisted in the following daily examinations for up to 72 h after ovulation. If intrauterine antibiotic treatment became necessary, the mares were not considered healthy and were excluded from the study.

2.4. Statistical analysis

Statistical analysis of experiment 1 was performed using R [45]. Linear mixed-effect models were run using the package "lme4" [46] to estimate the effect of treatment (NaCl, different concentrations of misoprostol), time and their interaction on total sperm motility. Different stallions were included as a random effect. Statistical analysis of experiment 2 and 3 was performed using the software NCSS 2020 [47] and only included the first cycle of each mare. Univariable logistic regression, chi-square test or fisher's exact test were applied depending on the nature of the explanatory variable potentially influencing the pregnancy rate. The level of significance was set at $p < 0.05$.

3. Results

3.1. Experiment 1

Results of experiment 1 are illustrated in Fig. 1. Regardless of time and compared with the control groups, all solutions with different concentrations of misoprostol had a negative effect on total motility of semen, which was significant for the highest concentrations (0.01 mg/mL: 18.0% reduction, CI = 22–13%, p = < 0.01).

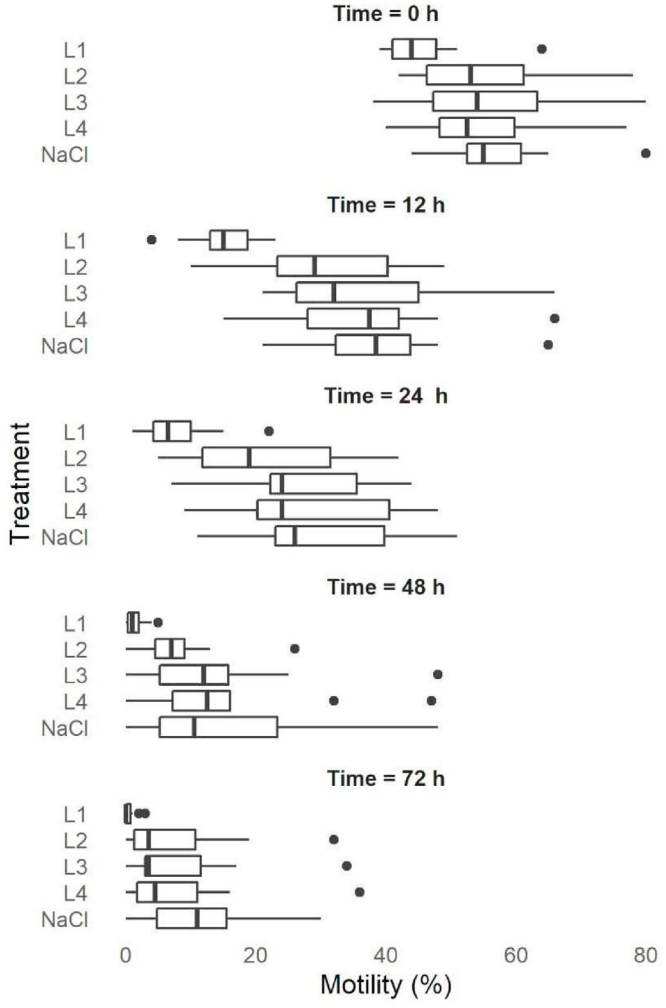

Fig. 1. Experiment 1: Effect of misoprostol treatment with 4 different concentrations (L1: 0.01 mg/mL, L2: 0.001 mg/mL, L3: 0.0001 mg/mL, and L4: 0.00001 mg/mL) on the total motility of semen after 0, 12, 24, 48 and 72 h *vs* the control solution (NaCl).

In addition, total motility decreased more rapidly over time when semen had been treated with higher misoprostol concentrations (0.01 mg/mL: 0.2% additional reduction per hour, CI = 0.1–0.3%, p < 0.01; 0.001 mg/mL: 0.1%, additional reduction per hour, CI: 0.0–0.3%, p = 0.02).

3.2. Experiment 2 and 3

From a total of 97 mares, 31 had to be excluded from the experiments due to ovulation before the preovulatory insemination (n = 10), no ovulation within 48 h after deslorelin application (n = 6), intrauterine antibiotic therapy (n = 4), and insemination with less than 20.0% motile sperm cells (n = 11).

In conclusion, 66 valid datasets of mares (26 maiden, 26 barren, and 14 foaling mares; mean age 12.2 years, 3 - 20 years old) could be analysed, 33 mares each allocated to experiment 2 and 3. The different reproductive statuses (maiden, foaling, barren) are illustrated in Fig. 2a and b.

The semen donor stallions were selected by the mare's owners and semen was available in chilled-warmed (n = 13) and frozen-thawed (n = 53) form: the type of semen (frozen-thawed or chilled-warmed semen) and total semen motility (20 - 78%) did not influence the pregnancy rate (chi-square test; p = 0.23 for the type of semen, and p = 0.56 for total semen motility). In the univariable logistic regression models, also age of the mare (p = 0.16), the reproductive status (barren: reference, maiden: p = 0.86, foaling: p = 0.79), the presence of double ovulation (p = 0.23) as well as the variables uterine flushing (preovulatory flushing: p = 0.34; postovulatory flushing: p = 0.45) and the administration of oxytocin (p = 0.63) had no influence on the pregnancy rate in both experimental groups. The detailed results of experiment 2 and 3 are shown in Table 1a and b.

3.2.1. Deep uterine horn treatment with misoprostol

There was no effect of the intrauterine treatment with misoprostol on pregnancy rate (chi-square test; p = 0.14). Overall, seven out of 25 mares treated with misoprostol (28.0%) and 19 out of 41 mares in the control group (46.3%) were diagnosed as pregnant.

In detail, in experiment 2, 2/11 mares of the treatment group became pregnant (18.2%) vs. 12/22 mares in the control group (54.5%) (Fig. 3a, Table 1a), in experiment 3, 5/14 mares in the treatment group (35.7%) vs. 7/19 mares (36.8%) in the control group (Fig. 3b, Table 1b), respectively.

3.2.2. Intrauterine fluid accumulation and treatments

Preovulatory flushing was performed in 5 mares (treatment groups: n = 1; control groups: n = 4). There was no significant difference in preovulatory fluid production between the treatment groups and the control groups in experiments 2 and 3 (p = 0.34).

Postovulatory oxytocin was administered in 31.8% (treatment groups: n = 9; control groups: n = 12) of the mares, and postovulatory flushing was performed in 47.6% of these mares (treatment groups: n = 5; control groups: n = 5). There was no significant difference in postovulatory fluid production between the treatment groups and the control groups in experiments 2 and 3 (p = 0.69).

4. Discussion

This study shows that the pregnancy rate was not increased by routine deep uterine horn application of misoprostol around the time of insemination in a study population of 66 reproductively normal mares participating in a commercial artificial insemination program.

Our results tend to support previous reports, which demonstrated intrauterine prostaglandin application not enhancing the

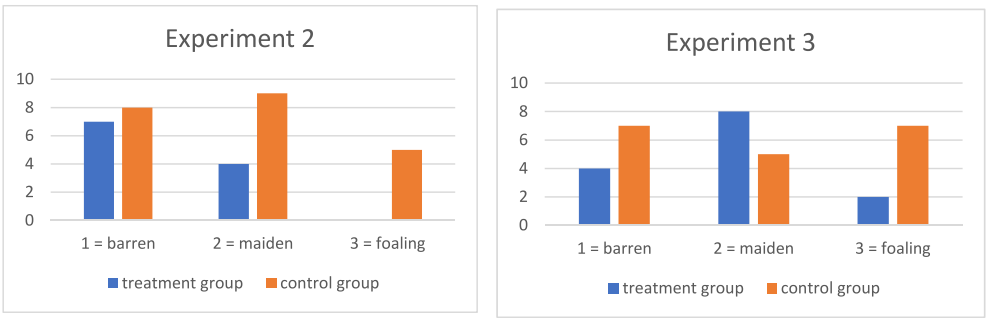

Fig. 2. a and 2b: Experiments 2 and 3: Distribution of reproductive status of mares (barren, maiden, foaling) in the treatment and control groups.

pregnancy rate of mares without history of unexplained fertility [37]. Indeed, Brinsko et al., who contrary to us used PGE_2, worked with a very similar study design (infusion of 0.25 mg PGE_2 in 1 ml 0.9% NaCl solution into the uterine horn 2 h before insemination) and could not demonstrate an increase in the pregnancy rate with this procedure [37]. Like in the present trial, the study population consisted of mares that were treated with PGE_2 or control NaCl, regardless of their reproductive history. These defined study populations could explain the different results compared to the results of Alvarenga and Segabinazzi [38,41] using exclusively mares with a history of unexplained fertility for at least 2 years with negative uterine cultural and cytological results, and excluding any further uterine or ovarian pathology [38,41]. Additionally, in the study presented here, misoprostol was administered during oestrus and not in dioestrus, as in the experiments of Alavarenga and Segabinazzi [38,41]. It should be mentioned that they did not include a control group in their study and aimed for successful insemination of mares in three oestrus cycles after intrauterine misoprostol administration. In our horse population, this was not feasible. However, it is not clear if the effect of misoprostol lasts over the whole period of three oestrus cycles. Further, the mares in the study of Alvarenga and Segabinazzi were standardly administered $PGF_{2\alpha}$ one day after misoprostol application to interrupt dioestrus. The authors did not specify the duration between $PGF_{2\alpha}$ injection and ovulation, i.e. earlier studies showed that a shorter duration between $PGF_{2\alpha}$ treatment and ovulation (4–6 days) results in a lower pregnancy rate and in general the pregnancy rate in the cycle after $PGF_{2\alpha}$ injection was shown to be slightly inferior to that after spontaneous ovulation [48]. This further underlines the positive effect of misoprostol in the research of Alvarenga and Segabinazzi. Notably, other authors were able to increase the pregnancy rate of subfertile mares with local application of PGE_2 [34,35] but using the method of laparoscopy.

The intrauterine application of misoprostol in oestrus would represent a much more practical procedure than in dioestrus, as breeding mares are presented for insemination in stud farm practice in the oestrous time of the cycle when the cervix is relaxed [49,50] and uterus clearance is active [51]. In human medicine, PGE_1 and PGE_2 are both used for labour induction [52,53,55], where one study has shown that PGE_1 has a strong effect on contractility of the myometrium in contrast to PGE_2 [54]. Furthermore, for practical use, misoprostol is less expensive and can be stored at room temperature, which represents an additional advantage of PGE_1 compared to PGE_2 products [52,53]. In horses, the influence of $PGF_{2\alpha}$ on myometrial activity is well described [56,57]. However, according to the authors' knowledge, any effects of PGE_1 and PGE_2 on the uterus have not yet been documented in this species.

According to our experiment 1, misoprostol has a negative effect on total sperm motility potentially contributing to decreased fertility [4–8,36]. To prevent any such influence in our study design, it was decided to apply the 0.2 mg intrauterine misoprostol 24 (experiment 2: around 36 - 39 h prior to ovulation) or 48 h (experiment 3: around 60 - 63 h prior to ovulation) before the preovulatory insemination instead of a simultaneous application. Nevertheless, the nearly significant difference ($p = 0.07$) of pregnancy results in the treatment group *vs.* the control group in experiment 2 suggest that misoprostol administration had more likely an adverse effect. However, it remains difficult to estimate the potentially predominant concentration of misoprostol in the uterus at the time of insemination in our experiments. The volume

Table 1a
Experiment 2: Influence of different variables on the pregnancy rates (%) of 33 mares after deep uterine horn treatment with misoprostol induction at the time of ovulation induction (n = 11 mares) *vs.* the control group (n = 22 mares).

Explanatory variable	Levels of categorical variables	Number of animals: pregnant/total	Pregnancy rate	P Value	Odds Ratio (Confidence Interval)
Deep uterine horn treatment	NaCl	12/22	54.5%	(ref.)	(ref.)
	Misoprostol	2/11	18.2%	0.07	0.19 (0.03–1.06)
Semen	Frozen-thawed	11/26	42.3%	(ref.)	(ref.)
	Chilled-warmed	3/7	42.9%	1.00	1.02 (0.19–5.53)
Reproductive status	Barren	7/15	46.7%	(ref.)	(ref.)
	Maiden	5/13	38.5%	0.68	0.90 (0.55–1.47)
	Foaling	2/5	40.0%	0.95	1.03 (0.36–2.97)
Double ovulations	No	12/30	40.0%	(ref.)	(ref.)
	Yes	2/3	66.7%	0.56	3.00 (0.38–12.57)
Age (years)				0.59	0.96 (0.81–1.13)
Semen quality (total motility in percent)				0.93	1.21 (0.02–87.75)
Uterine lavage preovulatory (number of days)				0.74	0.65 (0.05–8.02)
Uterine lavage postovulatory (number of days)				0.57	0.75 (0.27–2.05)
Oxytocin (number of days)				0.64	0.86 (0.45–1.63)

Table 1b
Experiment 3: Influence of different variables on the pregnancy rates (%) of 33 mares after deep uterine horn treatment with misoprostol infusion 24 h before ovulation induction (n = 14 mares) *vs.* the control group (n = 19 mares).

Explanatory variable	Levels of categorical variables	Number of animals: pregnant/total	Pregnancy rate	P Value	Odds Ratio (Confidence Interval)
Deep uterine horn treatment	NaCl	7/19	36.8%	(ref.)	(ref.)
	Misoprostol	5/14	35.7%	0.95	0.95 (0.23–4.01)
Semen	Frozen-thawed	8/27	29.6%	(ref.)	(ref.)
	Chilled-warmed	4/6	66.7%	0.16	4.75 (0.72–31.37)
Reproductive status	Barren	3/11	27.3%	(ref.)	(ref.)
	Maiden	5/13	38.5%	0.43	1.24 (0.73–2.1)
	Foaling	4/9	44.4%	0.78	1.13 (0.45–2.68)
Double ovulations	no	7/23	30.4%	(ref.)	(ref.)
	yes	5/10	50.0%	0.43	2.29 (0.54–8.41)
Age (years)				0.17	0.88 (0.73–1.06)
Semen quality (total motility in percent)				0.49	4.98 (0.05–481.82)
Uterine lavage preovulatory (number of days)				0.97	10000+ (0–10000+)
Uterine lavage postovulatory (number of days)				0.51	0.51 (0.07–3.82)
Oxytocin (number of days)				0.81	0.88 (0.32–2.48)

of the uterus varies markedly between mares and so far no studies exist regarding absorption of misoprostol by the uterus. We further suggest that the pre- and postovulatory uterine flushings did not interfere significantly in the outcome data of our study, as they were always performed before misoprostol application and after ovulation.

Interestingly only a low number of mares developed intrauterine fluid accumulation between misoprostol administration and preovulatory insemination, and no difference of indication for postovulatory oxytocin administration and uterine flushing was found in the treatment group vs. the control group. However, potential inflammatory reaction of the uterus provoked by misoprostol should be evaluated using cytological examination. In our study, other clinical reactions after the application of misoprostol could not be observed. In contrast, vaginal misoprostol application around the time of intrauterine insemination in humans can lead to severe pelvis pain, fever or vaginal bleeding [58]; if misoprostol was used at a dosage of up to 800 mg to induce abortion, further side effects like severe vaginal bleeding [59] nausea, vomiting, diarrhoea and fever [60] were observed. In horses, a recent case study of Kiviniemi-Moore [61] reports an anaphylactic reaction following intrauterine application of misoprostol in a Friesian mare.

Major limitations of this clinical field study were the availability of a relatively low number of mares and multiple variables including breed, age, reproductive history, as well as different management routines by the private owners. In addition, quite a high number of mares had to be eliminated from the experiments which further reduced the total number of mares. However, it is worth mentioning that age and reproductive status were fairly well distributed between the different treatment and control groups. The use of different types of commercial semen (chilled-warmed vs. frozen-thawed semen) suggested a high variance in semen quality (e.g. 20% to 78% total motility) and quantity. Another limitation is the fact that animals had to be allocated to the treatment and control groups depending on their status regarding the national regulations on the use of veterinary medicinal products. Misoprostol is not registered for use in food-producing animals, thus food-producing animals could not be allocated to the misoprostol treatment group. A potential bias due to this cannot be ruled out completely.

However, substantial efforts were undertaken to standardise test conditions and solid statistical methods were used.

Based on the findings of this study and previous reports, the deep uterine horn use of misoprostol in veterinary practice can be recommended only in selected cases of mares with a history of prolonged unexplained infertility and after exclusion of pathologies of the female reproductive tract using careful examination and

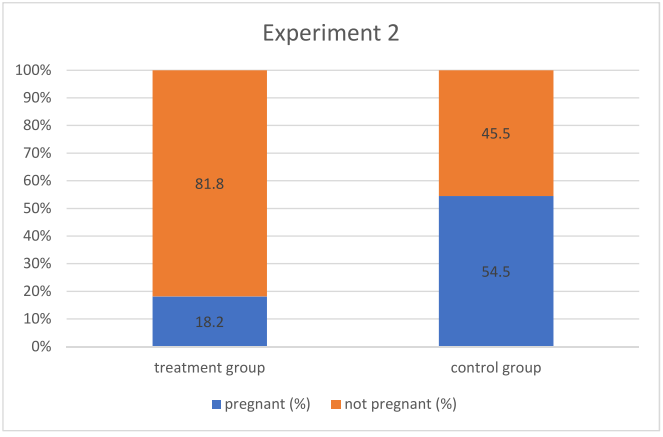

Fig. 3a. Experiment 2: Pregnancy rates (%) after deep uterine horn misoprostol infusion at the time of ovulation induction of the treatment group (2/11 mares pregnant) *vs.* the control group (12/22 mares pregnant). The difference was not significant (p = 0.07).

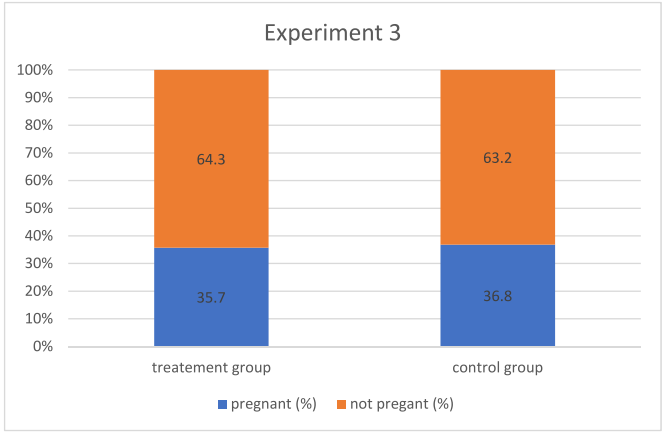

Fig. 3b. Experiment 3: Pregnancy rates (%) after deep uterine horn misoprostol infusion 24 h after ovulation induction of the treatment group (5/14 mares pregnant) *vs.* the control group (7/19 mares pregnant). The difference was not significant (p = 0.95).

current diagnostic procedures. The deep uterine horn application of misoprostol in mares is easy to perform, inexpensive and does not require anaesthesia nor surgical intervention. It remains open and would be of high practical interest, if the preovulatory administration of misoprostol in indicated mares or intra-uterine lavage in-between preovulatory misoprostol treatment and insemination would generate similar pregnancy results as the so far described use in dioestrous mares.

CRediT authorship contribution statement

L. Donatsch: Methodology, Investigation, Data curation, Writing – original draft. **B. Friker:** Validation, Formal analysis. **H. Sieme:** Conceptualization, Methodology. **R. Kaeser:** Conceptualization, Methodology, Supervision. **D. Burger:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

No conflicts of interest have been declared.

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