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Effects of reproductive status and management on cortisol secretion and fertility of oestrous horse mares

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Abstract

Stressful events may contribute to low reproductive efficiency due to glucocorticoid-mediated inhibition of hormone secretion in a variety of species. We therefore investigated effects of stress related to management of mares around artificial insemination on secretion of cortisol and fertility parameters. To avoid further disturbance of mares by frequent blood sampling, faecal cortisol metabolites (fCM) were determined instead (sample collection at 8-h intervals). A total of 50 mares (16 maiden, 17 barren, 12 foaling, 5 teaching mares) were included in the study. Mares were brought to the AI centre in vans or trailers (driving time between 30 min and 5 h). Teaching mares were housed in the clinic and had therefore not to be transported. Mares were inseminated either with fresh/cooled-shipped or frozen semen. Rectal palpations and ultrasound examinations were performed at 24- to 48-h intervals, in animals inseminated with frozen semen at 6-h intervals during the last 48 h before ovulation. In maiden, barren and foaling mares, fCM concentrations in faeces tended to be higher than in teaching mares at all times after arrival at the AI centre. At 24 and 48 h after arrival, fCM concentrations in maiden mares were significantly higher than in teaching mares (24 h: maiden mares 12.3 ± 3.1 ng/g, barren mares 8.5 ± 1.2 ng/g, foaling mares 11.0 ± 2.4 ng/g, teaching mares 3.8 ± 0.6 ng/g, $p < 0.05$). The time from arrival at the AI centre to detection of ovulation did not differ among the different groups of mares and was 4.5 ± 0.4, 5.0 ± 0.5, 3.8 ± 0.5 and 5.6 ± 0.9 days in maiden, barren, foaling and teaching mares, respectively (n.s.). Pregnancy rates were 53, 53, 55 and 60%, respectively (n.s.). The time from arrival at the AI centre to detection of ovulation was 4.4 ± 0.3 days and 4.9 ± 0.3 days in mares inseminated with fresh/shipped ($n = 39$) or frozen semen ($n = 11$; n.s.), respectively. The frequency of follicular checks influenced fCM secretion and was statistically significant at 16 h before

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ovulation (fresh/shipped semen: fCM 6.9 ± 0.7 ng/g faeces, frozen semen: fCM 16.9 ± 5.2 ng/g faeces, $p < 0.01$).

In the mare, gynaecological examinations seem to act as stressors and may increase cortisol secretion. However, this does not negatively influence fertility and in animals familiar with that procedure fCM concentrations are not elevated.

Keywords: Horse; Stress; Fertility; Cortisol

1. Introduction

Interactions between the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axis are numerous, however, glucocorticoids have frequently been indicated as one major factor mediating the suppressive effects of stress on reproductive functions. In the horse, the dominant glucocorticoid in plasma is cortisol (Bottoms et al., 1972). Glucocorticoids suppress reproductive functions at the hypothalamic, pituitary, ovarian and also uterine level (reviewed by Kalantaridou et al., 2004) in women (Sakakura et al., 1975; Rabin et al., 1990), rodents (Smith et al., 1971; Baldwin and Sawyer, 1974), sheep (Daley et al., 1999; Macfarlane et al., 2000) and cattle (Mann, 2001). Therefore, stressful events may contribute to low reproductive efficiency due to glucocorticoid-mediated inhibition of reproduction in a variety of species.

In many horse breeds, artificial insemination (AI) has more or less substituted natural breeding due to numerous advantages (Aurich and Aurich, 2006). However, the use of this biotechnique requires a more intensive management of the mare compared to natural breeding in order to detect the optimal time point for insemination. Because of the shorter examination interval, this is even more true when mares are inseminated with frozen–thawed semen. Management of the mare for AI is possible in the home stable or studfarm, however, the owner of the mare may prefer to bring the mare to an AI centre or clinic where the complete management is performed by trained personel. In this case, the mare is not only exposed to repeated restraint and gynaecological examinations, but also to other stressful events such as transportation to the AI centre and loss of the normal environment and social companions. Altogether, this has to be considered as disruption to homeostasis or stress and may cause increased secretion of glucocorticoids as is known to be caused by exercise (Gordon et al., 2006; Marc et al., 2000), transportation (Baucus et al., 1990a,b), pain (Merl et al., 2000) or social stress (Alexander and Irvine, 1998) in this species. Repeated gynaecological examination of teaching animals by veterinary students is also questioned occasionally for animal welfare reasons. Even the question how to define animal welfare and how to determine it are still under discussion. A potential indicator of animal welfare is the absence of stress, but to date there is no standard definition of stress and no single biochemical assay system to measure stress (Hofer and East, 1998).

In cattle, repeated restraint and blood sampling related to experimental procedures resulted in decreased fertility (Mann, 2001). In mares it has been shown that stress caused by transportation alone does not lead to fertility problems (Baucus et al., 1990a,b), however it has not been investigated whether stress related to the complex management of mares around AI may contribute to fertility losses. We have therefore investigated whether management of mares for artificial insemination in an AI centre affects cortisol secretion and reproductive parameters. The determination of blood cortisol is an established method to quantify reactions of animals to certain stressors (Terlouw et al., 1997). However, this requires repeated venipuncture and may cause stress by

itself and thus interfere with the results (Hopster et al., 1999). To avoid further disturbance of mares by frequent blood sampling, we therefore decided to determine faecal cortisol metabolites (fCM) instead.

2. Material and methods

2.1. Animals

Mares belonged to different breeds (Thoroughbred, Warmblood, Standardbred, Quarter Horse, Haflinger) and were referred to the Centre for Artificial Insemination and Embryo Transfer (AI Centre) for insemination with fresh, cooled-shipped or frozen semen. Stallion and semen were chosen by the owner. The mares had been examined for breeding soundness before and were brought to the AI Centre when signs of oestrus had been observed by the owner or they had been pre-checked by the referring veterinarian. When animals arrived in the clinic, they were brought into stocks and a gynaecological examination (rectal palpation, ultrasound examination, vaginal inspection) was performed. Only mares found to be in oestrus (uterine oedema, at least one follicle of >30 mm on one ovary) were included in the study. After examination, mares were brought to the clinic's stable where they were kept in individual boxes on straw. Hay and oats were fed three times daily, water and mineral supplements were freely available.

A total of 50 mares was included in the study. The history of the animals was requested from the owner or was available from the AI Centre's case log of previous years. Mares were assigned to the following categories:

1. Maiden mares which had not been bred before and were not used to gynecological examination irrespective of their age ($n = 16$)
2. Barren mares which had been used as broodmares for at least one season where repeated gynecological examinations during oestrus were performed for at least two consecutive cycles. They had not been bred or had not conceived the previous year ($n = 17$).
3. Foaling mares which had conceived the year before and where suckling a foal ($n = 12$).
4. Teaching mares which belonged to the teaching and research herd of the AI Centre for at least 1 year. They were used for gynaecological examinations by students during their clinical year on a regular basis i.e. at least during every oestrus. They were housed in a stable group in a group barn or spacious paddock and were fed hay two times daily, water and mineral supplements were freely available ($n = 5$).

2.2. Transportation to the AI centre

Mares were brought to the AI centre in vans or trailers by their owners. The driving time ranged between approximately 30 min and 5 h (details see Table 1). Teaching mares were housed in the clinic and had therefore not to be transported.

2.3. Management of mares

In the first days of oestrus, examination of the ovaries for follicular development and the uterus was always performed in the morning between 8:00 a.m. and 12:00 p.m. In early oestrus, these examinations were performed at 48-h-intervals, in late oestrus and inseminated mares at 24-h-intervals. In mares chosen for insemination with fresh or cooled-shipped semen, insemination

Table 1
Number, age, type of semen and duration of transportation to the clinic in the different categories of mares included in the study

Mares' category	Number (*n*)	Age (years; mean ± S.E.M.)	Type of semen (*n*)		Duration of transportation to the clinic (*n* per interval)				
			Fresh/ shipped	Frozen	0 h	<1 h	1–2 h	2–3 h	>3 h
Maiden	16	8.1 ± 0.9	12	4	0	3	5	5	3
Barren	17	11.8 ± 1.0	13	4	0	2	9	3	3
Foaling	12	8.3 ± 0.8	9	3	0	2	8	2	0
Teaching	5	10.3 ± 1.0	5	0	5	0	0	0	0

was performed at 48 h-intervals until ovulation was detected. In mares chosen for insemination with frozen semen, follicular checks were performed at 6 h-intervals in late oestrus. In most of these mares, ovulation was induced with human chorionic gonadotropin (1500 I.E. intravenously, Chorulon, Intervet, NL-Boxmeer) as soon as a follicle >35 mm diameter was present. Mares were inseminated with frozen–thawed semen as soon as ovulation could be detected. In client mares, pregnancy detection was performed by ultrasound examination between days 15 and 19 post ovulation. In teaching mares, uterine flushings for embryo collection were performed on day 7 after ovulation. All procedures were performed in accordance with Austrian legal requirements for animal experimentation (GZ 68.205/103-brGT/2003).

2.4. Sample collection

In all mares, faecal samples for determination of cortisol metabolites were collected daily at 0:00 a.m., 8:00 a.m. and 4:00 p.m. Samples were frozen at −18 °C until further analysis.

2.5. Determination of a group of cortisol-metabolites (11,17-dioxoandrostanes) in faeces

Extraction of samples was performed according to Merl et al. (2000) with methanol and dieethylether. Determination of 11,17-dioxoandrostanes (a group of faecal cortisol metabolites, fCM) was done by EIA with 11-oxoetiocholanolone as immunogen and a bioteniylated steroid as label as described by Palme and Möstl (1997).

2.6. Statistical analysis

Concentrations of fCM were calculated as the area under the curve (ng/kg × h) for the time periods 24 h after arrival at the AI centre, 72 h after arrival at the AI centre and 24 h before detection of ovulation. As not all data were normally distributed, non-parametric tests were used for statistical analysis. When data were compared in relation to the mare's category (maiden, barren, foaling, teaching mares), the Kruskal–Wallis test was used. When data were compared in relation to the type of semen used for insemination (fresh and shipped versus frozen semen) or in relation to pregnancy results (pregnant versus non pregnant), data were compared by Mann–Whitney *U* test. Correlations between different parameters were determined by Pearson correlation analysis. Determination of differences in pregnancy rates between different mares' groups were done by

Chi-square test. All data given are means ± standard error of mean (S.E.M.). A p-value < 0.05 was considered significant.

3. Results

3.1. Influence of the mare's category on cortisol secretion and fertility parameters

In maiden, barren and foaling mares, fCM concentration (fCM) tended to be higher than in teaching mares at all times after arrival at the AI centre (Fig. 1). At 24 and 48 h after arrival, concentration of fCM in maiden mares was significantly higher than in teaching mares (24 h: maiden mares 12.3 ± 3.1 ng/g, barren mares 8.5 ± 1.2 ng/g, foaling mares 11.0 ± 2.4 ng/g, teaching mares 3.8 ± 0.6 ng/g, $p < 0.05$; 48 h: maiden mares 12.7 ± 4.0 ng/g, barren mares 7.0 ± 1.1 ng/g, foaling mares 5.8 ± 1.0 ng/g, teaching mares 4.3 ± 0.8 ng/g, $p < 0.05$, Fig. 1). When the concentration of fCM was calculated as area under the curve for 24 respective 72 h after arrival at the AI centre, significant ($p < 0.05$) and nearly significant ($p = 0.058$) differences between the different mare's categories could be demonstrated (Fig. 2). Area under the curve values for 24 h were 256 ± 63 ng/g h in maiden, 188 ± 21 ng/g h in barren, 219 ± 53 ng/g h in foaling and 99 ± 5 ng/g h in teaching mares ($p < 0.05$). For 72 h after arrival at the AI centre, corresponding values were 779 ± 224, 549 ± 78, 517 ± 153 and 302 ± 26 ng/g h ($p = 0.058$). Concentrations of fCM were not affected by differences in the duration of transportation to the AI centre as given in Table 1.

In the last 24 h before ovulation, fCM did not significantly differ between the different mare's groups, area under the curve values for this time period were 188 ± 20 ng/g h in maiden, 179 ± 18 ng/g h in barren, 189 ± 45 ng/g h in foaling and 126 ± 13 ng/g h in teaching mares (n.s.). The area under the curve values for fCM concentrations 24 and 48 h after arrival at the AI centre did not significantly correlate with time to ovulation or age of the animals.

The time from arrival at the AI centre to detection of ovulation did not differ between the different mare's groups and was 4.5 ± 0.4, 5.0 ± 0.5, 3.8 ± 0.5 and 5.6 ± 0.9 days in maiden,

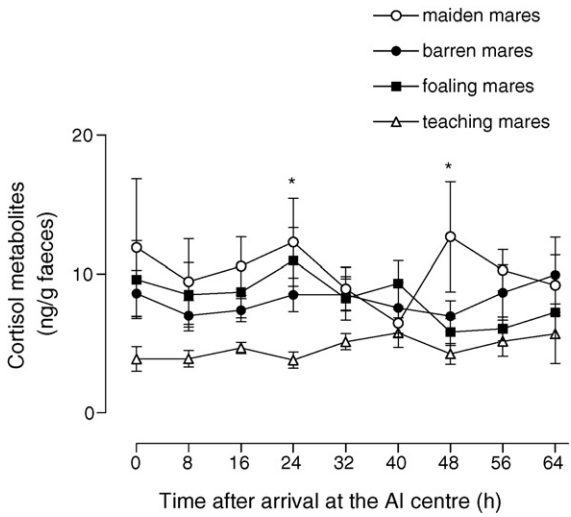

Fig. 1. Concentrations of faecal cortisol metabolites (ng/g faeces, mean ± S.E.M.) in maiden, barren, foaling and teaching mares after arrival at the AI centre. *Significant differences between groups ($p < 0.05$).

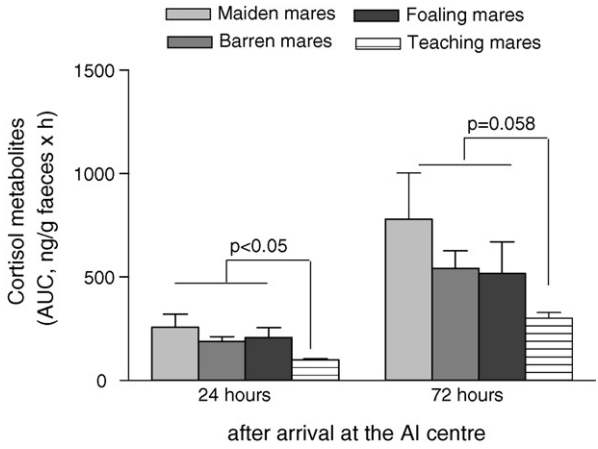

Fig. 2. Concentrations of faecal cortisol metabolites (ng/g h, mean ± S.E.M.) calculated as area under the curve (AUC) 24 and 72 h after arrival at the AI centre in maiden, barren, foaling and teaching mares.

barren, foaling and teaching mares (n.s.). Pregnancy rates were 53, 53, 55 und 60%, respectively and did not differ between the different groups (n.s.). The area under the curve values for fCM in the first 24 h after arrival at the AI centre were 206 ± 25 ng/g × h in mares that became pregnant and 204 ± 49 ng/g × h in mares that did not get pregnant (n.s.). Neither of the area under the curve values for fCM did correlate with time from arrival to ovulation or age of the animals.

3.2. Influence of management of the mare on concentration of fCM and fertility parameters

From the 50 mares, 39 were inseminated with fresh or cooled-shipped semen, while frozen semen was used in a total of 11 mares (Table 1). The time from arrival at the AI centre to detection of ovulation was not influenced by the management of mares in relation to semen used and was 4.4 ± 0.3 days in mares inseminated with fresh or shipped and 4.9 ± 0.3 days in mares inseminated with frozen semen (n.s.). Out of the 50 mares, 44 were checked for pregnancy by ultrasound at or around day 18 after ovulation. Pregnancy rate per cycle was 55% in mares inseminated with fresh or shipped and 45% in mares inseminated with frozen semen (n.s.). The management of mares (frequency of follicular checks) had an effect on fCM concentration that reached statistical significance in the time period 24–16 h before ovulation, that is approximately 24 h after start of the frequent follicular examinations in mares inseminated with frozen semen. At that time, fCM was 6.9 ± 0.7 ng/g faeces in mares inseminated with fresh or shipped semen and 16.9 ± 5.2 ng/g faeces in animals inseminated with frozen semen ($p < 0.01$, see Fig. 3). The area under the curve for fCM for the last 24 h before ovulation tended to differ between these groups, corresponding values were 166 ± 11 ng/g × h and 228 ± 49 ng/g × h for groups inseminated with fresh or shipped and with frozen semen, respectively ($p = 0.06$).

4. Discussion

In the present study, stress related to management of mares for artificial insemination resulted in increased secretion of cortisol determined as cortisol metabolites in the faeces. Among the

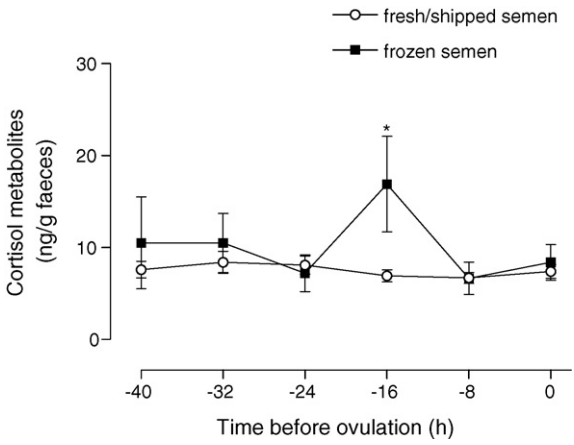

Fig. 3. Concentrations of faecal cortisol metabolites (ng/g faeces, mean ± S.E.M.) in mares with different management in relation to interval of rectal palpations due to insemination with fresh/shipped or frozen semen.

experimental groups, in maiden mares the highest and most variable cortisol metabolite secretion could be detected. This category of mares had not been bred before and was therefore not used to repeated gynaecological examinations in connection with AI. However, mares that are transported to an AI centre for the purpose of artificial insemination are not only exposed to stress connected with repeated gynaecological examinations and rectal palpations, but are also stressed by transportation and – in most cases – the loss of social partners in their home stable. These factors have been shown to substantially contribute to cortisol secretion in the equine species (Baucus et al., 1990a,b; Alexander and Irvine, 1998). A control group of teaching mares that was used to repeated restraint and gynaecological examination and that was not exposed to transportation and loss of social partners served as control. In this group, secretion of cortisol metabolites in the faeces was stable and lower than in the other groups tested. It is therefore feasible that the increased concentration of cortisol metabolites in the faeces was not only due to repeated rectal palpations but also to the other factors that contributed to disruption of homeostasis in these animals. However, the interpretation that repeated gynaecological examinations led to an increased cortisol secretion is supported by the fact that the higher frequency of gynaecological examinations in relation with management of mares for AI with frozen semen led to another rise in cortisol secretion independent of the category of mares: a significant increase in cortisol metabolites in faeces was seen approximately 24 h after that protocol of rectal palpations started. This seems to reflect a further stress connected to this procedure. The time point of that increase is in agreement with the detection of maximal concentrations of cortisol metabolites approximately 24 h after intravenous infusion of cortisol to pony mares (Palme et al., 1996).

In many species, e.g. in humans (Sakakura et al., 1975; Rabin et al., 1990), rodents (Smith et al., 1971; Baldwin and Sawyer, 1974) and sheep (Daley et al., 1999; Macfarlane et al., 2000; Breen et al., 2005), increased release of cortisol to concentrations found under stressful conditions leads to disruption of the oestrous cycle. Conflicting results exist for the pig (Barb et al., 1982; Turner et al., 1998a,b). Besides glucocorticoids, many other mediators such as CRF, AVP, endogenous opioid peptides, catecholamines and 5-hydroxytryptamine may be involved in the inhibitory effect of stress, and the significance of stress-induced secretion of cortisol varies with species (reviewed by Tilbrook et al., 2000). In ewes, management-related stress as induced by transportation can block

the expression of oestrous behaviour. This effect can be mimicked by long-term administration of glucocorticoids (Ehnert and Moberg, 1991). Moreover, stress-like infusions of cortisol inhibit follicular growth and development as well as the preovulatory LH surge and ovulation in female sheep (Macfarlane et al., 2000). In cattle, repeated blood-sampling during the oestrous cycle leads to a decrease in conception rate if cows not familiar to the procedure of blood-sampling are used for experiments. Acclimatization of cows to the experimental procedure resulted in a dramatic increase in pregnancy rates under similar conditions (Mann, 2001). In that study, stress-related hormones were not detected, but an involvement of impaired ovulation under stressful conditions is discussed as a possible reason.

In the current study in horses, no effect of an increased cortisol secretion most likely caused by transportation, management and loss of social partners on reproductive functions could be detected. Rate of ovulation and pregnancy did not differ between the different experimental groups and the control group where no variation in concentration of cortisol metabolites in the faeces occurred. This finding is in agreement with a previous study in the equine species where increased cortisol secretion induced via transportation of mares for 12 h did not result in disturbances of the oestrous cycle or changed secretion of reproductive hormones around ovulation (Baucus et al., 1990b). Similar experimental procedures were also not able to induce early embryonic death in mares (Baucus et al., 1990a). The horse thus seems to be less sensitive to effects of stress and/or cortisol on the hypothalamic–pituitary–ovarian axis than sheep and cattle, but the mare appears to be similar to the female pig. In gilts, reproduction was not impaired by stressful events in connection with increased cortisol secretion (Turner et al., 1998a,b). It can be concluded from the present study that even in maiden mares that are unfamiliar with the management related to AI, the related stress will not result in decreased pregnancy rates. In barren and even in foaling mares that are used to the situation in connection with AI, acclimatization to that kind of stress is likely to occur as has been shown in other species (reviewed by Tilbrook et al., 2000). A similar situation of acclimatization to stress has been found in stallions used as performance horses, where cortisol secretion was lower in stallions that regularly took part in competitions while it was increased in animals competing on an irregular basis (Lange et al., 1997). In the present study, a complete acclimatization to restraint and gynaecological examinations seemed to occur in teaching horses where repeated rectal palpations during oestrus did not affect concentration of faecal cortisol metabolites.

The increase in cortisol secretion determined as concentration of cortisol metabolites in the faeces seen in the present study is relatively low compared to the situation in horses after exposure to pain related to abdominal distress or castration (Merl et al., 2000). It can therefore not be excluded that more stressful situations leading to a more pronounced secretion of glucocorticoids could interfere with reproductive functions and fertility in the female horse. In that study, cortisol secretion was determined via metabolites in faeces with the same method as in the present study, therefore, values are directly comparable. This method offers the advantage of easy collection without any disturbances connected to blood-sampling that might lead to further secretion of glucocorticoids (Hopster et al., 1999). A diurnal secretion of cortisol that might have masked the potentials effects of stress was taken into account by the calculation of mean cortisol metabolite secretion as area under the curve values for the different time frames of interest.

As the lowest cortisol concentrations were found in the teaching mares, it can also be concluded that repeated gynaecological examinations in animals used to this procedure appear not to activate the hypothalamo-pituitary adrenal axis. This may indicate that no major disturbance of the animals' welfare occurred.

5. Conclusion

The results demonstrate that management of mares in relation to artificial insemination (gynecological examinations together with transportation and loss of social partners) may act as a stressor and induce greater concentrations of cortisol secretion as determined in faecal cortisol metabolites. However, long-term exposure as in teaching or research mares results in accustomation. In contrast to cattle and sheep, no effects of cortisol on fertility parameters (oestrus duration, pregnancy rates) could be found in the horse.

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