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Increased cortisol release and transport stress do not influence semen quality and testosterone release in pony stallions

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ABSTRACT

The use of breeding stallions for equestrian competitions requires that fertility is not negatively affected by competition or transport to the competition site. In this study, effects of cortisol release induced by road transport (600 km), adrenocorticotropic hormone (ACTH) administration (3 × 0.5 mg synthetic ACTH) and placebo treatment on semen quality and testosterone release were investigated in Shetland stallions (N = 13) using a crossover design. Saliva for cortisol and blood for testosterone analysis were collected for 10 weeks after treatments. Semen was collected daily for 5 days directly after treatments and twice weekly for another 9 weeks. Total sperm count, sperm morphology, motility, and membrane integrity were analyzed. We hypothesized that elevated cortisol decreases testosterone concentration and semen quality. Cortisol concentrations increased in response to transport and ACTH ($P < 0.001$) but not control treatments (peak concentration, transport: 7.6 ± 2.4, ACTH: 13.7 ± 1.5, control: 3.8 ± 0.9 ng/mL). No treatment effects on testosterone existed. Total sperm count decreased with daily semen collections in week 1 ($P < 0.01$) but did not differ between the treatments. The percentage of motile, progressively motile, membrane-intact, and morphologically defective spermatozoa did not change over time from Days 2 to 6, and there existed no differences between the treatments. In conclusion, road transport evoked a stress response which was mimicked by ACTH treatment. Both treatments had no effect on testosterone release and semen quality. Testicular function in stallions is apparently well protected against transiently elevated cortisol concentrations, and stallions can be transported over longer distances without negatively affecting their fertility.

1. Introduction

Sport horse stallions are often used for breeding and equestrian competitions in parallel. This requires that the stallions' fertility is not negatively affected by competition and training. Stallion owners and riders are often concerned that either sport or breeding activity could compromise the stallion's performance for the other purpose. In sexually experienced stallions, regular semen collection is not perceived as stressful [1]. Also, semen quality was not impaired in sport horse stallions entered in competitions either occasionally or on a regular basis [2]. However, equestrian sports require regular transport of the horses to the competition site. With an increase in

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international equestrian events by nearly 30% from 2009 to 2012 [3], both the number and the distance of horse transports have grown considerably. On the basis of analysis of cortisol release, road transport is far more stressful for horses [4–6] than equestrian training and competitions [7–10].

Species differences exist in the response to stress and the neuroendocrine regulation of reproduction. Glucocorticoid hormones are important mediators of the stress response. In some species, glucocorticoids suppress reproductive function at the hypothalamic, pituitary, ovarian, and also uterine level [11]. Thus, in male rats, boars, and humans, glucocorticoids inhibit the expression of proteins involved in testosterone biosynthesis [12–14].

Although stressful events may contribute to low reproductive efficiency in several species, effects of transport stress on semen quality in the horse have not been studied so far. It was thus the aim of the present study to investigate the influence of road transport and elevated cortisol concentrations on steroidogenesis and semen quality in the horse. We hypothesized that an increase in cortisol concentrations caused by 12 hours of road transport will lead to a temporary decrease in testosterone concentrations and a transient impairment of semen quality. Because the effects of transport are mediated *via* cortisol, they can be mimicked by repeated injection of adrenocorticotropic hormone (ACTH).

2. Materials and methods

2.1. Animals

A total of 13 fertile Shetland stallions were used in this study. All stallions were well accustomed to semen collection and transport. From the stallions, ejaculates are collected and examined on a regular basis (two to three times per week) either for breeding or teaching purposes throughout the year. At the start of the experiment, the animals were aged between 5 and 22 years (10.9 ± 1.9 years) and weighed between 118 and 199 kg (157 ± 8 kg). Stallions were kept in two groups in spacious stables. One group ($n = 5$) had access to an outdoor paddock from 7:00 AM to 6:00 PM, and the other group ($n = 8$) had access to a paddock at all times. The stallions were fed hay twice daily, and water was freely available.

2.2. Experimental procedures

Experiments were carried out from March to October. All stallions were transported over a distance of 600 km (treatment T; transport time approximately 12 hours) and received treatments of synthetic ACTH (Synacthen, 0.25 mg/mL; Defiante Farmaceutica, Funchal, Portugal) on 1 day (three treatments of 0.5 mg at 4-hour intervals; treatment A) or received 0.9% NaCl as the control (treatment C). All stallions received all three treatments in a triple crossover design and thus served as their own controls. The interval between the treatments was always 10 weeks.

The study was approved by the competent authority for animal experimentation (Austrian Federal Ministry for Science and Research, license number BMWF-68.205/0230-II/3b/2012).

2.3. Transport

Always four to five pony stallions were transported together in a standard horse trailer (floor size 3.4×1.7 m). They were not tied and allowed to move loosely in the trailer. Transport was started immediately after loading and followed two-lane national roads in Lower Austria State, Austria. The region is predominantly flat and the transport neither led through bigger cities nor through hilly or undulating terrain. Every 2nd hour, the transport was stopped for collection of saliva samples. The stallions received hay on the trailer and water every 4 hours. Immediately after the end of the transport, they were unloaded and returned to their stable or paddock.

2.4. Sample collection and hormone analysis

Saliva for cortisol determination was collected at 60-minute intervals starting 2 hours before experimental treatments. Further saliva samples were taken at 2-hour intervals until 16 hours after treatment. During the subsequent 5 days, saliva samples were taken three times per day (6:00 AM, 12:00 PM, 6:00 PM) and once a week during the following 9 weeks. Saliva was collected with cotton-based swabs (Salivette; Sarstedt, Nümbrecht-Rommelsdorf, Germany) as described [9]. The Salivette was placed onto the tongue of the horses with the help of a surgical arterial clamp for at least 1 minute until it was well soaked with saliva. The cotton roll was then returned to the Salivette polypropylene tube and stored at 4 °C until centrifugation at the end of the transport or centrifuged within 10 minutes at $1000 \times g$ for 10 minutes. At least 1-mL saliva per sample was obtained and frozen at −20 °C until analysis. The sampling procedure was well tolerated by all stallions and conducted by a single person without restraining the animal.

Cortisol was determined by direct enzyme immunoassay without extraction as described [9]. The antibody was raised in rabbits against cortisol 3-CMO-BSA in the authors' laboratory, and cortisol obtained from Steraloids (Wilton, NH, USA) was used for preparation of the standard curve. Because the antiserum cross-reacts with cortisone and several corticosterone metabolites, values obtained have to be interpreted as cortisol immunoreactivity. The intra-assay coefficient of variation determined from duplicates of a control saliva in each assay was 4.5%, the interassay variation was 11.7% ($n = 66$), and the minimal detectable concentration defined as two standard deviations from zero binding was 0.05 ng/mL.

For testosterone analysis, blood was collected from one jugular vein into polystyrene tubes containing lithium heparin (Vacuette; Becton Dickinson, Schwechat, Austria). On the experimental days, blood was taken one hour before start of individual treatment (5:00 AM) and again in the late afternoon (6:00 PM). Further samples were collected daily for 5 days and afterward once weekly for 9 weeks. Blood samples were centrifuged immediately after