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Effect of Feeding Fescue Seed Containing Ergot Alkaloid Toxins on Stallion Spermatogenesis and Sperm Cells

R Fayer-Hosken¹, A Stanley², N Hill³, G Heusner², M Christian¹, R De La Fuente⁴, C Baumann⁴, and L Jones²

¹Departments of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

²Animal Science, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

³Crop and Soil Sciences, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

⁴Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

Contents

The cellular effects of tall fescue grass-associated toxic ergot alkaloids on stallion sperm and colt testicular tissue were evaluated. This was a continuation of an initial experiment where the effects of toxic ergot alkaloids on the stallion spermiogram were investigated. The only spermiogram parameter in exposed stallions that was affected by the toxic ergot alkaloids was a decreased gel-free volume of the ejaculate. This study examined the effect of toxic ergot alkaloids on chilling and freezing of the stallion sperm cells. The effect of toxic ergot alkaloids on chilled extended sperm cells for 48 h at 5 °C was to make the sperm cells less likely to undergo a calcium ionophore-induced acrosome reaction. The toxic ergot alkaloids had no effect on the freezability of sperm cells. However, if yearling colts were fed toxic ergot alkaloids, then the cytological analysis of meiotic chromosome synapsis revealed a significant increase in the proportion of pachytene spermatocytes showing unpaired sex chromosomes compared to control spermatocytes. There was little effect of ergot alkaloids on adult stallions, but there might be a significant effect on yearling colts.

Introduction

Tall fescue in the southeastern United States is one of the most important pasture grasses, and it is an extremely successful grass that is protected by a commensal fungal endophyte *Neotyphodium coenophialum*. The endophyte secretes ergot alkaloids, and these bestow a variable degree of protection to the grass from insect infestation (Fayer-Hosken et al. 2008; Fayer-Hosken R, Personal communication). Generally, fescue (toxic or non-toxic) is grazed by multiple species without any problems. Ruminants and horses that graze toxic fescue grass do have clinical problems. Practically, the diseases caused by fescue-associated ergot

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Author's address (for correspondence): R Fayer-Hosken, Department of Large Animal Medicine, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA. rfh@uga.edu.

Conflict of interest None of the authors have any conflict of interest to declare.

Author contributions The research was conceived and planned by Drs. Fayer-Hosken, Hill and Heusner. The actual research was majorly performed by Drs. Fayer-Hosken, Hill and Heusner. The able research skills of Drs. De La Fuente and Baumann performed the chromosomal analysis. The masters student was Ashley Stanley, and the technical support was from Ms. Christian and Jones. Dr Fayer-Hosken wrote the paper, and all authors have edited the paper.

alkaloids have significant financial impact on the cattle and horse industries in the states with affected grasses (Hoveland 1993). The reported fiscal impact of ergot alkaloids is in the tens and hundreds of millions of dollars. The greatest clinical and financial impact is in the beef industry, with cattle being affected with a number of pathologies (Burfening 1973; Peters et al. 1992; Porter and Thompson 1992; Aldrich et al. 1993; Dyer 1993; Porter 1993; Hill et al. 1994, 2000; Oliver et al. 1994; Cheeke 1995; Schneider et al. 1996; Stuedemann et al. 1998; Schultze et al. 1999; Schnitzius et al. 2001; Parish et al. 2003; Matthews et al. 2005; Realini et al. 2005; Wang et al. 2009). The symptoms in cattle include increased respiration rates, increased rectal temperatures, salivation, nervousness and rough hair coats, decreased weight gains and decreased overall performance in beef steers (Hoveland 1993).

Studies in the mare have demonstrated (Marek and Roth 1980; Porter and Thompson 1992; Cross et al. 1995; Brendemuehl et al. 1996; Fiserova et al. 1997; Blodgett 2001; Ryan et al. 2001; Copetti et al. 2002; Evans 2002; Youngblood et al. 2004; Lehner et al. 2008) that consuming toxic endophyte-infected grasses during the last 3 months of gestation leads to a high incidence of foal loss and other associated medical problems. The clinical symptoms include spontaneous abortions, thickened placenta and agalactia. In 2009 for the State of Georgia, the 230 000 horse population generated \$300 million revenue, and Georgia's horse breeding industry generated \$750 million. This is a significant portion of the state's economy whose budget in 2009 was \$31.9 billion (Georgia 2009). The profound effects of ergot alkaloids in mares prompted the research to investigate the impact of ergot alkaloids on stallion reproduction and spermiogram (Fayrer-Hosken R, Personal communication). This was a two-stage research project with the initial stage examining the general physiological effects of fescue in stallions (Fayrer-Hosken R, Personal communication). The initial study (Fayrer-Hosken R, Personal communication) examined the general physiological parameters and showed there were no statistical changes in sperm motility, sperm number (concentration), sperm cell morphology, number of breeding doses produced, testicular size and volume, degree of bactospermia, diurnal testosterone levels and inducible steroidogenic capability of the Leydig cells. Also, there were no changes in core body temperature and superficial scrotal temperature. The only statistically altered parameter in the stallions consuming toxic endophyte-infected tall fescue seed was that they had significantly ($p < 0.01$) lower gel-free volumes than stallions consuming non-toxic endophyte tall fescue seed without affecting the numbers of ejaculated sperm cells.

Spermatogenesis in the stallion (Gebauer et al. 1974; Swierstra et al. 1975; Amann et al. 1979; Thompson et al. 1979; Freidman et al. 1991; Johnson et al. 1991, 1997; Hurtgen 1992; Rodriguez-Martinez 1992; Roser 2001, 2008; Einarsson et al. 2009) requires 62 days for the testicular phase and then 13 days for the epididymal phase. This provides a spermatogenic cycle for sperm production of 75 days. The stallion sperm cell is sensitive to cooling and freezing, similar to other mammalian sperm cells (Pommer et al. 2003; Thomas et al. 2006) in that they exhibit cryopacitization during chilling or freezing. During spermatogenesis do these ergot alkaloids have an effect on the sperm cells at the cellular level? In the bull, spermatozoal motility is reduced by ergonovine (Gallagher and Senger 1989), and the ergot alkaloids induce motility changes in bovine sperm cells by interacting with alpha-adrenergic receptors (Wang et al. 2009). However, there are no reports of cellular effects on equine sperm cells.

Cryopacitization (Amann et al. 1979; Cormier and Bailey 2003; Abad et al. 2007a,b; Einarsson et al. 2009) induces a series of complex biochemical changes in the plasma and outer acrosomal membranes of the cryopreserved sperm cell. A range of cryopacitization states predispose the sperm cells to a premature inducible acrosome reaction (AR). We reasoned that ingested ergot alkaloids could induce biochemical or structural defects in sperm cells during spermatogenesis and/or epididymal maturation. If the sperm cells were

affected, then one postulate is that cryocapacitated sperm cells would be more likely to undergo an induced AR. Clinically, chilled and frozen sperm cells would be more fragile and less fertile. Toxic ergot alkaloids can induce motility changes in bull sperm cells through alpha-adrenergic receptors (Wang et al. 2009). Ergot alkaloids have also been shown to induce abnormal proliferation in bovine vascular smooth muscle cells resulting in diminished blood flow to organs. Altered blood flow could potentially disrupt testis thermoregulatory mechanisms (Strickland et al. 1996), and this might affect critical stages of spermatogenesis such as the meiotic prophase I. The hypothesis for this research was that if stallions ingest toxic levels of ergot alkaloids, the sperm cells might be more fragile. In stallions fed ergot alkaloids, their sperm cells would be more sensitive to an induced acrosomal reaction induction than in control stallions. In colts, we theorized that there probably would be effects on the peri-pubertal spermatogenesis, and these might be chromosomal defects.

The induction of the AR provides a sensitive and comparative assay of membrane/sperm cell viability (McPartlin et al. 2008, 2009) as well as the status of the spermatozoal capacitation. Acrosome reaction induction agents include the zona pellucida glycoproteins, calcium ionophores, lysophospholipids and progesterone. However, the mechanism of induction varies with the specific agent and the capacitational status of the sperm cells. The zona pellucida glycoproteins are the principal physiological inducer of the AR in the capacitated sperm cell, and this leads to the classic biochemical cascade (Fig. 1). Progesterone is also a physiological inducer of the AR as it utilizes a specific sperm plasma membrane progesterone receptor (Cheng et al. 1998) and the same cascade (Fig. 1). The progesterone receptor (Contreras and Llanos 2001) was localized in the plasma membrane and was localized in the acrosomal region (Wu et al. 2005). Progesterone induces the AR and changes in motility; even if progesterone is bound to albumin (1.0 lg/ml of progesterone), it can induce the AR in the capacitated equine sperm cells (Rathi et al. 2003). Ligand-binding studies suggest that progesterone addition to capacitated human sperm cells was followed by a calcium ion (Ca^{2+}) wave spreading throughout the sperm head. The greatest increase in intracellular Ca^{2+} was near the equatorial segment (Flesch and Gadella 2000). Progesterone is a physiological inducer of the AR, but Ca^{2+} ionophores introduce calcium to stimulate the AR by bypassing normal regulatory mechanisms.

Lipids are critical to capacitation, and the cholesterol/phospholipid ratio changes over time in the female reproductive tract. The molar amounts of cholesterol and phospholipids (Brinsko et al. 2007) do not differ between sperm cells of fertile and subfertile stallions. But the cholesterol/phospholipid ratio was higher in sperm of subfertile stallions compared to the fertile stallions (Brinsko et al. 2007), emphasizing the importance of the cholesterol/phospholipid ratio in sperm functionality. For stallions with the subfertile sperm, a possible explanation (Brinsko et al. 2007) is that the altered cholesterol/phospholipid ratio resulted in a decreased rate of cholesterol depletion from the sperm cells. The decreased rate of cholesterol efflux could reduce time of capacitation. Ergot alkaloids might affect sperm cell lipids and thus perturb capacitation. Such membrane problems might manifest as changes in fertility.

The research hypothesis was to investigate whether ergot alkaloids derived from tall fescue seed had any effects on stallion sperm cells. A crossover design for two breeding seasons and six stallions was used. In season one, three stallions were fed enough infected fescue seed to produce high circulating levels, and three stallions were fed uninfected fescue seed for 70 days. After a clearance period of several months, the initial three control stallions were fed infected fescue seed, and the other three stallions were now fed uninfected fescue to become the controls for 70 days. During each research period, the stallions were collected weekly for semen freezing and production of chilled semen doses. For these semen samples,

their sensitivity to the induction of the AR was evaluated using fluorescence (pea-lectin) (McPartlin et al. 2009). This is an elegant assay where the control treatment was DMSO, the physiological AR inducer was progesterone, and the non-specific AR inducer was a calcium ionophore.

For our interpretation of the data, if progesterone induced more ergot alkaloid-affected sperm cells' ability to acrosome-react compared to control sperm cells, this would suggest that these sperm cells are more cryocapacitated compared to control stallion sperm cells. The greater the calcium ionophore induction of the AR compared to progesterone and DMSO controls this represented a greater membrane (acrosomal) fragility. The use of fluorescence to assess the AR induction was also important as the various response patterns that could be visualized represented a range of capacitational states. For this report, the numbers of sperm cells that are fully acrosome-reacted plus the numbers of partially acrosome-reacted sperm cells were used to represent the total AR.

Materials and Methods

Animals

For this study, 6-quarter horse stallions (*Equus caballus*) in excellent health and reproductive ability were used. During the research, the stallions were also used as breeding studs in the animal science breeding and research programme. For the juvenile feeding trial, 8-quarter-horse-year-old colts were utilized. To assess the effect on spermatogenesis, eight colts were produced from mares bred to the six experimental stallions during the study. A year and a half later, the yearlings were fed infected fescue seed for 75 days prior to castration. The testes were examined for cellular changes with light microscopy as well as chromosomal analysis (Baumann et al. 2011).

Extender optimization for each stallion

Prior to the study, an ejaculate from each stallion was collected, and the sperm cells were placed in three dilutions (1 : 3, 1 : 5 and 1 : 7) of INRA 96 (Exodus Breeders Corporation, York, PA 17406, USA) to determine the best extender ratio for optimum semen survival rate (96 h) post-collection. The best extender dilution was evaluated for per cent motile, total motility, progressive motility and incidence of hyperactivated motility daily for 4 days. The best survival defined the best dilution, and this was used for all subsequent semen extensions.

Experimental design

The stallions were divided up into matched pairs by age, for day of collection and type of feed (experimental or control). Only two investigators knew the actual composition of the feed with toxic fescue seed, and the experimental identity was only revealed after completion of the research. For the first cycle, three stallions were fed a feed with toxic endophyte-infected fescue seed, and three stallions were fed the non-infected endophyte fescue seed (control). The stallions were on the feed for at least 70 days, one spermatogenic cycle, and the stallions were then given at least a 70-day rest period. The rest period was to ensure that the infected stallions had excreted all ergot alkaloids from the fescue. If there had been any ergot-induced pathology of the reproductive system, these changes had been resolved before starting the next trial. This rest period was during the winter. The third cycle was a crossover repeat of the first cycle for at least 70 days. The stallions remained in their collection day pairing and thus served as their own controls. The amount of feed that contained the fescue seed fed to each stallion was determined by the stallion's body weight before and regularly during the study. The fescue seed content was compounded at 45% of a grain diet, and the stallions were fed the grain diet at 1% of their body weight. Regular diet

corrections were necessary as the weights of the stallions increased or decreased during the study.

Semen collection and preparation

The precise dilutions had been determined for optimum semen survival before the study, and for the current study, each stallion was collected weekly. Each stallion was collected with a Missouri Model artificial vagina, and the semen was immediately processed after collection. The gel-free volume of ejaculate remained after filtering in a sterile warmed graduated cylinder. The gel-free volume, sperm cell concentration and per cent alive (subjective evaluation by two experienced evaluators and Spermvision®) were recorded. The concentration of the ejaculate was measured with a densimeter (Animal Reproduction Services, Chino, CA 91710, USA). The per cent alive was determined with light microscopy at 200× magnification.

Immediately after collection, half of the ejaculate was prepared at a 1 : 1 (Fayrer-Hosken et al. 2008) dilution with warmed INRA 96 (Exodus Breeders). This sample was used for semen freezing experiment. The other half of the semen sample was processed immediately for the 0-h (baseline) AR sample. The rest of this half of the ejaculate was then diluted at the predetermined dilution ratio for the chilled semen experiment. This was kept in an Equitainer® and was evaluated after 48 h for motility and the per cent AR with fluorescent staining. The initial 10 ml sample of the chilled semen was used for computer-assisted motility analysis.

Acrosome reaction induction assay

For the AR induction assay (McPartlin et al. 2008, 2009), 10 ml of sample of the semen-extender mixture was centrifuged at $100 \times g$ for 1 min to remove large particulate material. After the initial centrifugation, the sample supernatant was decanted into a conical test tube and re-centrifuged at $650 \times g$ for 5 min. The supernatant was removed, and the sperm cell pellet was re-suspended with 1 ml of INRA 96.

Two hundred milligrams of paraformaldehyde (PF) was gently mixed with the 5 ml of PBS in a glass test tube and placed in a boiling water bath in a fume hood for 30 min. After boiling, the glass test tube was set aside to cool to room temperature for the 2% PF/PBS mixture (5 ml of PBS with 5 ml PF/PBS boiled mixture). As the subsequent assay is light sensitive, all subsequent manipulations were completed using only a red light. The calcium ionophore stock solution was 1 mM and was prepared by adding 1 mg of calcium ionophore to 2 ml of DMSO. Mixing 1 mg of progesterone with 4 ml of DMSO made the progesterone stock solution.

Five hundred microlitres of the semen-extender mixture was added to three test tubes. The tubes contained either 2.5 µl of calcium ionophore (Ca), 2.5 µl of dimethyl sulfoxide (DMSO) or 2.0 µl of progesterone (Pro). The semen mixtures were gently mixed and placed in a 37 °C incubator for 30 min. After 30 min of incubation, the semen motility was evaluated. One millilitre of PBS was added to each Eppendorf tube, mixed and centrifuged for 2 min using a TOMY Capsulefuge PMC-060 at a setting of $860 \times g$. The supernatant was removed, and the pellet was resuspended with 125 µl of PBS and vortexed. Three microlitre of PNA peanut lectin [*Arachis hypogaea*, Alexa Fluor® 488 conjugate (L21409; Invitrogen, Carlsbad, CA 92008, USA)] was added to each of the three Eppendorf tubes. The tubes were inverted to mix and placed in a 37 °C water bath for five and a half minutes. The tubes were then removed from the water bath, and the entire contents were carefully pipetted onto the appropriately labelled slides Ca (ionophore), DMSO and Pro. The slides were placed on a warmer for 6 min. The slides were very gently washed twice with 1 ml of PBS, and then the

excess surface PBS was removed by tapping the slide on paper towels. The slides were then fixed with the 2% PF/PBS mixture and then placed on the slide warmer for 11 min. After drying, the slides were gently washed three times with 1 ml PBS and gently tapped to remove excess PBS. A cover slip was mounted on each slide with 8 μ l of Vectashield (H1400; Vector Laboratories Inc, Burlingame, CA 94010, USA). Each slide was wrapped in aluminium foil to protect the slides from light and placed individually into polystyrene culture dishes (50809255, Tissue Culture Dish, Polystyrene; Fisher Scientific, Waltham, MA 02454, USA). The dishes were then placed in a refrigerator to be evaluated within 24 h. The same procedure was applied to the chilled semen sample at 48 h and to all frozen-thawed semen samples.

Evaluation of acrosome reaction

For evaluation of the AR, the patterns of fluorescence of the sperm cells were divided into three categories: non-reacted, partially reacted and completely reacted (McPartlin et al. 2008). Non-reacted sperm cells did not have any fluorescence; completely reacted sperm cells had defined, even fluorescence over the anterior half sperm cell (acrosome). Partially reacted sperm cells had either a fluorescent halo indicating the initiation of the AR or some slides had reaction patterns such as speckled, barred or only the mid-piece staining. All the patterns of incomplete staining of the acrosome were scored as partially reacted. Each slide was examined, and 200 sperm cells were observed and scored.

Testis tissue collection and preparation of meiotic spreads for chromosomal analysis of colts fed toxic fescue seed

To evaluate the possible effect of ergot alkaloids on developing testes and spermatogenesis, colts were fed toxic fescue seed at 1% of body weight using the previously described feed. The eight colts were randomly assigned into two groups of four. The colts were then fed toxic or control feed for 80 days. Seven of the eight colts were castrated for the testicular tissue. One was examined under anaesthesia and found to be a cryptorchid and was removed from the study. Additionally, spermatogenic cells obtained from seminiferous tubules were examined to compare chromatin configurations and the degree of meiotic chromosome synapsis during meiotic prophase I of spermatogenesis by high-resolution microscopy. For these experiments, equine testicular tissue was obtained from 1-year-old colts exposed to endophyte (E+) fescue grass (n = 3) and control non-exposed males (n = 4) following surgical castration. After removal of the tunica albuginea, individual seminiferous tubules were dissected in PBS supplemented with 10% foetal calf serum and immediately processed for standard analysis of marker proteins of meiotic sex chromosome pairing and chromosome synapsis on surface spread meiocytes as described below and previously for equine spermatocytes in (Baumann et al. 2011). Specifically, seminiferous tubules were subjected to a mild hypotonic treatment by exposure to a sodium citrate solution (30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate, 5 mM EDTA, pH 8.2) for 20–25 min at room temperature to dissociate germ cells from the tubular environment and to optimize meiotic chromosome spreading and subsequent protein marker analysis by chromatin decondensation. The germ cell suspension was subsequently applied to glass slides containing 1% PF and 0.15% Triton X 100 in H₂O to facilitate nuclear protein cross-linking. Slides were allowed to air-dry and then stored at -80 °C until immunochemical analysis.

Immunocytochemistry of chromosomal spreads

The stage of prophase I of meiosis in equine spermatocytes was determined according to the extent of chromosomal synapsis visualized by immunochemical detection of the condensing subunit structural maintenance of chromosomes 3 (SMC3) protein. Localization of SMC3 on meiotic chromosomes was detected using a 1 : 400 dilution of a polyclonal rabbit anti-SMC3 antibody (Abcam, Cambridge, MA, USA). Human anti-CREST antiserum (1 : 50 000

dilution; a generous gift from Dr W. Earnshaw) in combination with an anti-SMC3 antibody was used to detect kinetochore domains within centromeric heterochromatin. The subcellular localization of histone H2A phosphorylated at serine 139 (γ H2AX) was determined using a mouse anti- γ H2AX antibody (Upstate, Charlottesville, VA, USA) at a 1 : 500 dilution. Secondary antibodies were purchased from Molecular Probes (Eugene, OR, 97402-9132, USA). Alexa-fluor 488 goat-anti-rabbit and Alexa-fluor 555 goat-anti-mouse were used at 1 : 1000 or 1 : 500 dilution, respectively. After immunostaining, slides were counterstained with 8 μ l of anti-fading medium supplemented with DAPI (Vectashield; Vector Laboratories Inc) to visualize spermatocyte nuclei at different stages of meiotic prophase I, and cover slips were mounted with Vectashield anti-fading medium (Vector). Immunofluorescence was visualized using a Leica (Buffalo Grove, IL, USA) DMRE fluorescence microscope, and images were captured using a Leica DFC 350F CCD camera.

Statistical analysis

Statistical analysis of data was performed using the mixed procedure of SAS (SAS Institute Inc, Cary, NC, USA). The model included independent variables with the GLM procedure non-reacted sperm vs fully reacted sperm, which were statistically compared. Within these two major categories, the subcategories were treatment, trial, incubation time and medium; these were compared statistically to determine their effects on sperm's ability to AR. Time represented the date of sampling through each trial. The design of the statistical analysis in SAS was a crossover design with repeated measures. SAS was used to determine the least squares means and the treatment differences (significance at $p < 0.05$).

Results

Chilled semen

For semen assayed immediately after collection (0 h), there were no statistical differences in the per cent AR for the treatments of progesterone or calcium ionophore compared to controls of DMSO (Figs 2 and 3). However, the sperm cells from the stallions not fed ergot alkaloids were significantly ($p < 0.05$) more sensitive to calcium ionophore after 48 h than control (DMSO) and progesterone (P4)-treated sperm cells (Fig. 4). This was not the case for the sperm cells at 48 h from the stallions fed ergot alkaloids (Fig. 5).

Frozen semen

There was no statistical difference between the ability to induce AR in frozen sperm cells for stallions fed control (non-toxic-infected endophyte) or toxic ergot alkaloids (Figs 6 and 7). If the induction of the AR of frozen semen was then compared to the initial samples of collected semen, there were no statistical differences.

Cytological analysis of meiotic chromosome configurations

Consistent with our previous studies (Baumann et al. 2011), the SMC3 protein (green) exhibited a uniform staining throughout the length of the paired chromosomal axes (Fig. 8a). In control, non-exposed spermatocytes ($n = 131$), the majority of cells exhibited 31 synapsed autosomal bivalents and a partially synapsed sex chromosome (XY) bivalent, which could be easily recognized by its specific labelling with phosphorylated H2AX (γ -H2AX; red) as well as a characteristic enrichment with SMC3 protein (green). In contrast, spermatocytes obtained from colts exposed to toxic endophyte fescue grass ($n = 87$) exhibited a significant increase ($p < 0.01$) in the proportion of abnormal meiotic figures including unpaired X and Y chromosomes at the pachytene stage (Fig. 8b,c). In these cells, the completely asynapsed X (arrow) and Y (arrowhead) chromosomes can be recognized by persistence of γ H2AX staining (red) in correlation with the position of the acrocentric (Y) and submetacentric (X)

centromeres in sex chromosomes as detected by CREST (red) immunochemistry (Fig. 8d). Analysis of the proportion of abnormal meiotic figures in control and toxic ergot endophyte-exposed colts indicated that dietary alkaloids have a significant, deleterious and previously unrecognized effect in the establishment and/or maintenance of partial synapsis of the sex chromosomes.

Discussion

The impetus for the study was to examine the effect of fescue alkaloids in stallions and possible financial implications. Fescue is an extremely ubiquitous and successful pasture grass in the southeastern USA. From our initial examination (Fayrer-Hosken R, Personal communication) of six stallions, a double-blind crossover study, stallions consuming endophyte-infected fescue seed, had higher ($p < 0.01$) urinary alkaloid (lysergic acid derivatives) levels than stallions consuming the non-toxic endophyte tall fescue MaxQ seed. The mean time to maximal systemic toxicity was 8.33 h after starting toxic seed ingestion, and at that time, a mean toxicity level of 49.98 ng alkaloid/mg creatinine was reached. After cessation of feeding toxic seed, the systemic levels fell to control (baseline) levels within 48 h. For the reproductive parameters measured in the stallions, there were no statistical changes in sperm motility, sperm number (concentration), sperm cell morphology, number of breeding doses produced, testicular size and volume, degree of bactospermia, diurnal testosterone levels and inducible steroidogenic capability of the Leydig cells. Also, there were no changes in core body temperature and superficial scrotal temperature. The only statistically affected parameter in stallions consuming toxic endophyte-infected tall fescue seed was that they had significantly ($p < 0.01$) lower gel-free volumes than stallions consuming non-toxic endophyte-infected tall fescue seed. The lack of definable effects supported the findings of a preliminary reproductive study that ergot alkaloids had minimal effects of stallion reproduction.

In this study, the primary difference between toxic ergot-exposed and non-toxic ergot-chilled semen was that the ergot-exposed sperm cells are less likely to undergo a calcium ionophore-stimulated AR after storage for 48 h at 5 °C. A possible explanation is that ergot-exposed sperm cells were slower to cryocapacitate than control sperm cells. Or it is possible that the intracellular messenger systems associated with the AR (Fig. 1) induction had not been upregulated. Progesterone is a more physiological inducer of the AR than calcium ionophore. In the chilled semen at 48 h, progesterone was less effective than calcium ionophore in inducing the AR in control and treatment sperm cells. This suggests that the control and toxic ergot alkaloid-exposed sperm cells were still capable of undergoing the induced AR. The decreased ability to acrosome-react after 48 h might actually increase the survivability of chilled sperm. There are no comparative fertility data or conception rates reports to demonstrate that ergot alkaloids have a positive or negative impact on shipped semen. It is possible to argue that the decreased propensity of toxic ergot alkaloid-exposed sperm to undergo a prematurely induced AR might actually enhance fertility of chilled stallion semen.

The fluorescent stain evaluation of the control or treatment frozen sperm cells showed that there was no statistical difference in the acrosomal inducing sensitivity. This demonstrates that sperm cell membranes are not destabilized during freezing after exposure to ergot alkaloids compared to control sperm cells. The chilled and frozen studies combined clearly show that there was no detrimental effect on sperm physiology by ergot alkaloids. However, this study did not address the fertility of semen from stallions ingesting ergot alkaloids. Also as a note of caution, the effects of environmental temperature, amount of rainfall and type of fertilizer used might affect the dynamics of the ergot alkaloids within a pasture and their toxicity on stallions and colts. The lack of definable effects supported the findings of a

preliminary reproductive study (unpublished data) that ergot alkaloids have minimal effects on stallion reproduction.

The cohesin subunit SMC3 is known to co-localize with the synaptonemal complex proteins SYCP2/-SYCP3 during the pachytene stage of meiosis in the mouse (Eijpe et al. 2000; Prieto et al. 2004; Revenkova and Jessberger 2006). Importantly, we have recently demonstrated that SMC3 decorates the axial elements of the synaptonemal complex during meiotic chromosome synapsis in horse spermatocytes, therefore providing a reliable marker to determine the extent of chromosome synapsis during prophase I of meiosis in the equine species (Baumann et al. 2011). Cytological analysis of meiotic chromosome synapsis revealed a significant increase in the proportion of pachytene spermatocytes showing unpaired sex chromosomes following ergot alkaloid exposure compared to control spermatocytes. The number of autosomal homologues only showed tendency to chromosome asynapsis that remained statistically non-significant. Importantly, gonosomal univalency has been shown to have a profound effect on meiotic prophase I progression leading to meiotic arrest and apoptotic cell death upon checkpoint activation and is a common cause of sterility in murine interspecific hybrids (Matsuda et al. 1992). Notably, heat stress has been previously associated with synaptic failure of the X and Y chromosome bivalents in mice and rats following exposure to increased environmental as well as testicular temperatures (van Zelst et al. 1995). However, previous studies indicate that dietary alkaloids may potentiate the adverse effects of heat stress on reproductive fitness (Bhusari et al. 2007). Failure to partially synapse the XY chromosome bivalent was detected in approximately 32% of equine pachytene spermatocytes following ergot alkaloid consumption, suggesting a potential effect on meiotic progression in a subpopulation of meiocytes in this model. The presence of mature sperm in cytological preparations together with physiological fertility parameters investigated indicates that, although ergot alkaloid exposure might impair spermatogenesis in a proportion of cells, stallion reproduction is minimally affected. The mechanisms involved in this process require further investigation. In the present study, no changes in superficial scrotal temperature were found in endophyte-treated colts. However, diminished blood flow associated with fescue toxicosis (Strickland et al. 1996) may potentially disrupt critical intra-testicular oxygen radical-scavenging mechanisms (Bhusari et al. 2007), resulting in the generation of oxidative stress at the cellular level during the meiotic prophase I. Meiocytes that exhibit chromosomal abnormalities frequently arrest at the pachytene stage and undergo apoptosis shortly thereafter (Handel and Sun 2005). Elimination of this cell population before spermiogenesis is in line with our results indicating normal motility and morphology in mature sperm cells. In addition, our results indicate that the proportion of meiocytes affected did not have a significant effect on sperm cell numbers (concentration). It is also possible that additional cellular and/or molecular defects exist that are beyond the level of resolution of the parameters evaluated, and hence, further studies will be required to determine any potential long-term effects in the male germ line.

Horse owners should keep their current fescue pastures, as there is little or no impact on the lactating mare and foal or grazing stallions or fillies. Current established fescue pastures provide quality nutrition for these horses, and the cost of change the grass type in the pastures would not be sound financial wisdom. Further studies are required to determine the functional implications of the chromosomal effects observed in a subpopulation of meiocytes of colts, but the ergot alkaloids have little to no effect on stallions. The lack of definable effects supported the findings of a preliminary reproductive study that ergot alkaloids have minimal effects on stallion reproduction.

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Fig. 2.
Fluorescence of sperm cells at 0 h at 5 °C from stallions not fed ergot alkaloids

Fig. 3.
Fluorescence of sperm cells at 0 h at 5 °C from stallions fed ergot alkaloids

Fig. 4.
Fluorescence of sperm cells after 48 h at 5 °C from stallions not fed ergot alkaloids

Fig. 5.
Fluorescence of sperm cells after 48 h at 5 °C from stallions fed ergot alkaloids

Fig. 6.
Fluorescence of frozen sperm cells thawed from stallions not fed ergot alkaloids

Fig. 7.
Fluorescence of frozen sperm cells thawed from stallions fed ergot alkaloids

Fig. 8.
Chromosomal analysis of equine testicular cells from foals fed toxic ergot alkaloids compared to controls