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Prevalence of ergot derivatives in natural ryegrass pastures: Detection and pathogenicity in the horse

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Abstract

In the present study, we determined the incidence and effects of season and weather on clinical manifestations of endophyte-infected ryegrass toxicity, performed chemical detection and pharmacological bioassays on ryegrass extracts, and conducted trials on: (i) effects of domperidone or metochlopramide on ovarian inactivity induced by endophyte-infected ryegrass; (ii) efficacy of buspirone or dihydrochloro phenyl piperazine (m-CPP) for preventing suppressed milk production induced by endophyte-infected ryegrass; and (iii) efficacy of domperidone to induce ovulation during winter anestrus. Mares with toxicosis had prolonged gestation, embryonic losses, dystocia, poor mammary gland development, low milk production, prolonged uterine involution, and suppressed ovarian activity. Foals had respiratory failure, abnormalities of the skin, umbilicus, bone, and muscle, failure to thrive, blindness, testicular atrophy, and decreased serum total immunoglobulin concentrations. Endophyte-infected ryegrass and the incidence of toxicosis were correlated ($r = 0.861$, $P = 0.03$). Ergot alkaloids were not detected in extracts of endophyte-infected ryegrass by either thin-layer chromatography or spectrophotometry, but their presence was inferred in bioassays of extracts (dose-related increases in the contractile response of rat uterus). Mares given metoclopropamide (0.6 mg/kg/d), given orally every 8 h for up to 7 d) ovulated earlier (4–7 d vs. 15–18 d, $P < 0.001$) than those given domperidone (1.1 mg/kg/d) orally for up to 18 d). Although both metoclopropamide and domperidone induced milk production, the latter did not induce ovarian cyclicity in healthy mares during seasonal anestrus. Based on these findings, we inferred that endophyte-infected ryegrass is associated with ergot alkaloid intoxication in horse.

Keywords: Ryegrass toxicity; Ergot alkaloids; Endophytes; Domperidone; Horse

1. Introduction

The toxic effects of feeding endophyte-infected grasses are well documented in several species [1–3]. Intoxicated mares had prolonged gestation, abnormal parturition, embryonic losses, ovarian inactivity, agalactia, placental abnormalities, and neonatal death [4–7]. Horses appeared to be particularly affected by infected

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fescue [5,6,8]. In cattle, the severity of the clinical intoxication increased during droughts, due to increased concentration of endophytes in the grass [9]. Conversely, few publications have addressed the toxic effects of feeding endophyte-infected ryegrass [10]. A field experiment in New Zealand studied the reproductive impact of endophyte-infected ryegrass in sheep and its relationship to a moist environment; there was a negative correlation between the severity of ryegrass staggers and live weight changes in sheep fed endophyte-infected pastures during drought conditions (the latter was attributed to pasture composition). The authors concluded that the endophyte had moderate negative effects on animal production in a cool, moist environment [11]. However, the relationship between weather patterns and severity of toxicity induced by ryegrass infected with endophytes found in cattle [9] and sheep [11] has not been studied in horses.

An additional study in New Zealand and Australia revealed symptoms similar to those of perennial ryegrass staggers in sheep fed *Echinopogon* spp., serologically related to *Neotyphodium lolii* (the endophyte of perennial ryegrass). Notably, these authors found no lolitrems (indole diterpenoids thought to cause perennial ryegrass staggers), peramine analogs, or ergot alkaloids in the infected specimens [12]. However, analogs of the indole–diterpenoid paxilline were detected [12].

Mares grazing endophyte-infected tall fescue did not improve when they were treated with selenium injections or corn supplements [6]. It has been suggested that the alkaloids of endophyte-infected tall fescue are acting as agonists of the D_2 dopamine receptor, explaining their prolactin-lowering effect. Domperidone, a dopamine receptor antagonist, prevented the signs of tall fescue toxicosis in horses, including reproductive and lactation problems, without neuroleptic side effects [6,13,14]. Conversely, agonists of the serotonin $5HT_{1A}$ receptor increased prolactin release through both direct and indirect (dopamine D_2 mediated) mechanisms in experimental animals and humans [15–17], and may be of benefit to reverse at least the lactation problems induced by endophyte-infested grass toxicity, if the same mechanism is present in the horse.

In the present study, we determined the incidence and effects of season and weather on clinical manifestations of endophyte-infected ryegrass toxicity, performed chemical detection and pharmacological bioassays on ryegrass extracts, and conducted randomized, placebo-controlled trials on: (i) the effects of the D_2 dopamine receptor antagonists, domperidone and metochlopramide, on ovarian inactivity induced by endophyte-infected ryegrass; (ii) the efficacy of two agonists of the serotonin receptor $5HT_{1A}$, buspirone and dihydrochloro phenyl piperazine (m-CPP), for preventing suppressed milk production induced by endophyte-infected ryegrass; and (iii) to assess its specificity, we determined the efficacy of the D_2 antagonist domperidone to induce ovulation during winter anestrus. We hypothesized that ryegrass toxicity in horses was due to an ergot derivative producing endophyte, and therefore that dopamine antagonist and/or serotonin agonists should be useful in preventing ryegrass toxicosis.

2. Materials and methods

2.1. Location

This study was conducted on a horse farm located in San Antonio de Areco, in the northern part of the Province of Buenos Aires, Argentina. The farm had 525 ha devoted to Thoroughbred breeding, with 200 resident mares and an additional 300 mares during the breeding season. In this latitude of the southern hemisphere, mares have several estrous cycles during long days (September to February).

2.2. Feeding

Forage content of pastures included 60% Lucerne (*Medicago sativa*), Red clover (*Trifolium pratense*), Prairie grass (*Bromus wildenowii*), Cocksfoot (*Dactilis glomerata*), and Falaris (*Phalaris aquatica*). The remaining 40% were annual pastures, including prairie grass and oats in winter, and corn and soybean in summer. Pastures were fertilized with phosphorus and sometimes with calcium carbonate, whereas annual pastures were fertilized with phosphorus and nitrogen. Winter annual pastures were sown in late February and perennial pastures in March and April. All pastures were contaminated with natural perennial ryegrass (that is frequently infected with endophytes).

Pregnant mares were fed oat rations and mineral supplements for 90 d prior to foaling, with supplements continued for several weeks after foaling. In winter, when the humidity content of annual pastures was high, horses were given access to lucerne blocks (to add fiber to their diet).

2.3. Rainfall

Rainfall was measured on three separate sites on the farm, using standard equipment. Daily registers were averaged monthly and yearly (Fig. 1).

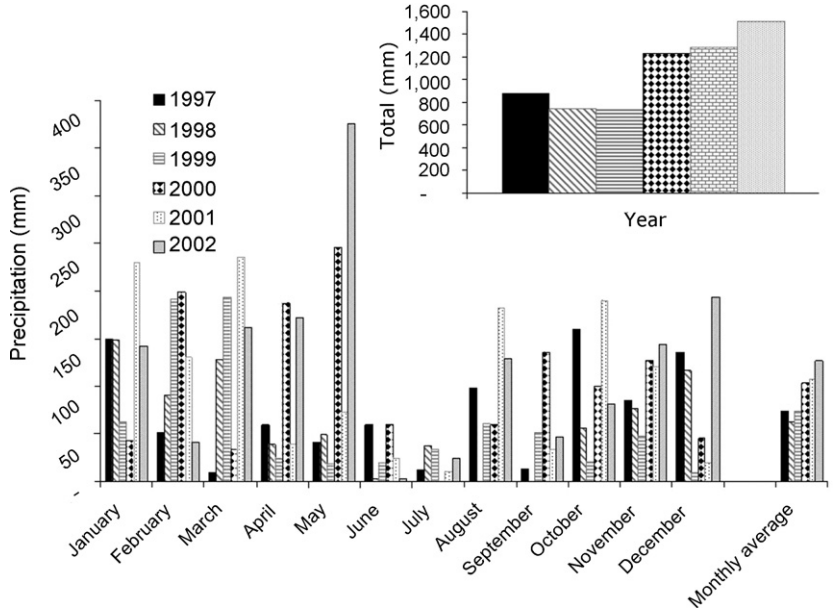

Fig. 1. Rainfall records for the entire study period. Total rain for each year is displayed in the inset.

2.4. Reproductive management

All mares in estrus or undergoing ovarian cyclic activity were inspected and examined daily; diagnostic procedures included ultrasonography, vaginoscopy, cytology or cultures, and were performed as needed. During the breeding season, mares with a presumptive preovulatory follicle were mated every second day. After confirmation of ovulation, transrectal palpation and ultrasonography were done 14, 17, 22, 28, and 35 d after ovulation.

2.5. Ryegrass sampling and sample processing

Samples were obtained randomly from affected batches, using the ring method [18]. Four rings (56 cm diameter, total surface 1 m^2) were thrown behind the operator in separate sectors of each field. A sample was taken from within each ring, containing a whole plant, and 10 samples were collected per batch. For endophyte detection, all samples were transplanted into plant pots and sent immediately to the laboratory. Endophyte infection was determined by peeling a strip of epidermis from the leaf sheath of each plant. This was mounted on a slide, stained with lactophenol–aniline blue, and examined by light microscopy for endophyte mycelium (Fig. 2), as described [12]. To detect ergot alkaloid derivatives, samples were dried at room temperature, packaged, and sent to the laboratory.

2.6. Statistical analysis of weather patterns and toxicosis incidence

Descriptive statistics, cumulative data, ANOVA with post hoc comparisons, and linear correlations were obtained using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Pearson linear correlations were used to predict rates of toxicosis and foal mortality based on endophyte infection rates on ryegrass. Group means for bioassay results were compared by one-way ANOVA (for treatments as the factor), and post hoc comparisons

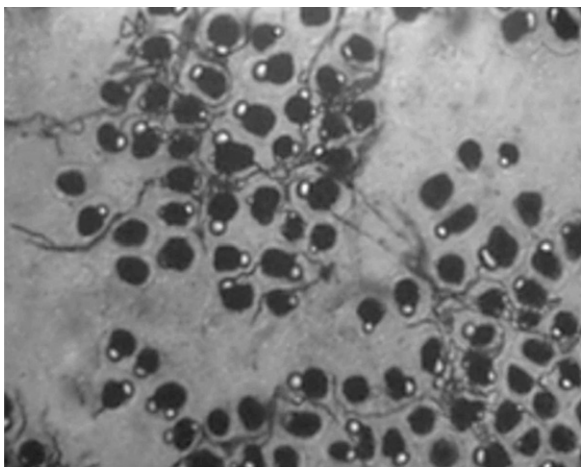

Fig. 2. Ryegrass sample stained with aniline blue. The appearance of the endophyte is typical of *Neotyphodium lolii*.

of means were carried out by Student–Newman–Keuls test. Number of days to ovulation was treated as survival time, and Kaplan–Meier survival statistics were used for comparisons between treatments.

2.7. Chemical analytical methods

Crude and purified alkaloids fractions were analyzed for ergot alkaloids by thin-layer chromatography (TLC) and spectrophotometry (UV–visible Shimadzu 2101 spectrophotometer, Shimadzu Scientific Instruments, Columbia, MO, USA).

2.8. Preparation of crude alkaloid extracts

2.8.1. Method 1

An aliquot (10 g) of powdered grass was extracted with 50 mL of 70% ethanol, mechanically stirred for 30 min, and filtered. The procedure was repeated twice, the filtrates combined, and the volume adjusted to 200 mL. This procedure was repeated with increasing amounts (25, 50, and 100 g) of plant material.

2.8.2. Method 2

An aliquot (10 g) of powdered grass was macerated with 50 mL of a mixture of ethanol and concentrated ammonia (9:1) for 24 h. The mixture was filtered and the volume of the extract adjusted to 50 mL with the same solvent. This procedure was repeated with increasing amounts (25, 50, and 100 g) of plant material.

2.9. Preparation of purified alkaloid extracts

2.9.1. Method 1

An aliquot (10 g) of powdered grass were extracted with 50 mL of 70% ethanol, stirred mechanically for 30 min, and filtered. The extract was concentrated under reduced pressure (7 psi), alkalinized (pH = 9) with concentrated ammonia, and extracted with diethyl ether (10 mL, done three times). The combined extracts were concentrated under reduced pressure (7 psi) and the volume adjusted to 20 mL.

2.9.2. Method 2

An aliquot (10 g) of powdered grass were extracted twice with 50 mL of a mixture of chloroform:methanol:concentrated ammonia (90:9:1). The extract was filtered, concentrated to 20 mL under reduced pressure (7 psi), and extracted with 0.1 M sulphuric acid (10 mL, done twice), and the extracts combined and adjusted to a volume of 25 mL.

2.10. Thin-layer chromatography

2.10.1. Preparation of sample solution

An aliquot (10 mL) of crude or purified extracts were dried under reduced pressure (7 psi), and the resulting residue was dissolved in 1 mL of ammonium hydroxide (9:1).

2.10.2. Preparation and dilution of standard solutions

A solution of 10 mg/mL of ergonovine maleate in alcohol:ammonium hydroxide (9:1) was prepared. Appropriate dilutions of the standard solution were made in the same solvent mixture to obtain concentrations of 0.2, 0.1, and 0.05 mg/mL. All dilutions were used immediately after preparation.

2.10.3. Chromatography

Aliquots (5 μL) of standard solution, standard dilutions and sample solutions were spotted on to a Silica gel 60 F254 plate (0.2 mm thick), and the plate developed with Solvent 1 (chloroform:methanol:water, 75:25:3) and Solvent 2 (ethyl acetate:*N*,*N* dimethylformamide:ethanol, 13:1.9:0.1), used sequentially (5 min each). After air drying, plates were examined under UV light at 366 nm (white blue spots) and sprayed with Ehrlich or Van Urk reagents (see below for composition) and heated at 80 °C until revealed for observation with daylight (blue spots).

2.11. Spectrophotometry of crude alkaloid extracts

A colorimetric reaction with PDAB (*p*-dimethylaminobenzaldehyde) and detection at 550 nm relative to standard of ergonovine maleate was used to quantify total ergot alkaloids in crude extracts [11,19,20].

2.11.1. Spectrophotometry of purified alkaloid extracts

Purified alkaloid extracts were assayed as previously described [20]. For preparation of standards, an ergonovine maleate solution (40 mg/mL) was prepared in water. For the assay, 40 mg of ergonovine maleate was transferred to a 100 mL volumetric flask and diluted with water; 10 mL of this solution was diluted to 100 mL with water. Thereafter, 5 mL each of the standard preparation, the assay preparation, and water to provide a blank, were transferred to separate conical flasks, and 10 mL of PDAB were added (with constant swirling) to each, and allowed to stand for 20 min. Concomitantly, the absorbances of the solutions in 1 cm cells were determined at the

wavelength of maximum absorbance 555 nm, against water as the blank.

2.12. Bioassay in isolated rat uterus

This assay was conducted as previously described [11]. Female Sprague–Dawley rats weighing between 120 and 150 g were given 100 μg of estradiol benzoate (im) 24 h prior to the experiments. Immediately before the assay, estrus or proestrus was confirmed by vaginal smear [21]. For each experiment, 1.5 cm sections were dissected from the central part of each uterine horn, freed from fat, and mounted in an organ bath containing 10 mL of nutritive solution (NaCl 113.3 mM, KCl 6.1 mM, $CaCl_2$ 0.5 mM, $MgCl_2$ 0.5 mM, $NaHCO_3H$ 30.5 mM, $Na_2HPO_4{\cdot}12H_2O$ 0.8 mM, $NaH_2PO_4{\cdot}2H_2O$ 0.2 mM, and glucose 2.8 mM) and aerated with a mixture of 5% of carbon dioxide and 95% oxygen. The organ bath temperature was maintained at 31 °C to inhibit spontaneous contractions of the uterus and ensure that the preparation maintained its sensitivity. The uterus was allowed to equilibrate for approximately 30 min. Oxytocin was used as a positive control (10^{-4} IU/mL, 2×10^{-4} IU/mL, 3×10^{-4} IU/mL). Extracts were administered cumulatively until a maximal response was reached. Antagonists (methysergide (10^{-5} M) or atropine (10^{-8} M) were added to the bath after the equilibration period and 30 min before adding the extract. Full dose response curves were obtained for each condition. Triplicates were performed in sections obtained from independent rats ($n = 3$).

2.13. Statistical analysis of bioassay in rat uterus

Data are expressed as mean ± S.E.M., and n refers to the number of samples. Group differences were analyzed using ANOVA and Student–Newman–Keuls test. Statistical calculations were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

2.14. Reagents

Ergonovine maleate, estradiol benzoate, oxytocin, methysergide, atropine sulphate were purchased from Sigma Chemical Co., St. Louis, MO, USA. *Ehrlich's reagent*: 1.0 g *p*-dimethylaminobenzaldehyde (PDAB) was dissolved in 25 mL concentrated hydrochloric acid and 75 mL methanol. *Van Urk reagent*: 0.125 g PDAB was dissolved in 100 mL of 65% sulphuric acid and 0.1 mL of 5% iron(III) chloride solution was added.

2.15. Clinical trials

2.15.1. Assessment of ovarian cycling on intoxicated mares treated with domperidone, metoclopramide or placebo

The spring–summer ovarian induction was determined by transrectal palpation and ovarian ultrasonography of affected mares that exhibited at least one clinical symptom of toxicosis. Ovulation was considered to be induced whenever follicles were detected by transrectal palpation and confirmed by ultrasonography; following disappearance of the follicles, they were replaced by a CL. Veterinarians assessing the mares were blind to treatments. In the 1998 season, 15 mares with ovarian inactivity were randomly assigned to one of three treatment groups receiving either vehicle (GR-1: honey, $n = 5$), domperidone (Dompergo, Laboratorio Fundación, Argentina; GR2, 1.1 mg/kg/d) BW orally in a single daily dose, $n = 5$) or metoclopramide (Sigma Chemical Co.; GR-3, 1.8 mg/kg/d) BW orally in three divided doses every 8 h, $n = 5$). Mares were matched by age. Treatments continued until the day of ovulation or for a total of 18 d. Rates of mares remaining inactive were compared by Kaplan–Meier survival statistics (SPSS 16.0, SPSS Inc.).

2.15.2. Assessment of milk production on barren mares treated with buspirone, mCPP or placebo

The production of milk was evaluated by visual inspection and milking of the gland, performed by evaluators blind to the treatments. In the 2001 breeding season, 10 non-lactating mares were randomly assigned to one of two treatment groups receiving a single dose of either vehicle (7.5 mL of saline solution given iv, $n = 5$) or buspirone (Sigma Chemical Co.; 0.1 mg/kg/d) given IV in a single daily dose, $n = 5$). This experimental design was repeated later in the same breeding season with another 10 non-lactating mares and substituting buspirone with a single dose of dichlorophenyl piperazine (Sigma Chemical Co.; m-CPP, GR-2, 0.1 mg/kg/d) BW orally in a single daily dose, $n = 5$).

2.16. Serum determinations

Progesterone was determined by chemoluminescence (0.5–4 ng/mL during estrus) and estrogen by radioimmunoassay (10–20 pg/mL during estrus). Immunoglobulin concentrations (total) were determined by electrophoresis.

2.17. Assessment of ovarian cycling on healthy inactive (winter) mares treated with metoclopramide or placebo

Physiological anestrus was determined by transrectal palpation and ultrasonography. Follicular development was assessed blindly by transrectal palpation and confirmed with ultrasonography. In the 1999 season, 10 mares were randomly assigned to one of two treatment groups receiving either vehicle (honey, $n = 5$) or metoclopramide (Sigma Chemical Co.; 1.8 mg/kg/d) orally, in three divided doses every 8 h, $n = 5$). The treatments continued until the day of ovulation, or for a total of 18 d. Rates of mares remaining inactive were compared by Kaplan–Meier survival statistics (SPSS 16.0, SPSS Inc.).

2.18. Ethics approval

All study protocols were approved by the Animal Studies Committee of the Instituto J.J. Naón, University of Buenos Aires, and were conducted in accordance with international guidelines [22].

3. Results

3.1. Clinical observations 1998–2002

The nosological picture of ryegrass intoxication in mares and foals involved several findings. Precipitation records are shown (Fig. 1). Mares had prolonged gestation, frequent embryonic losses, dystocia due to absence of relaxation of the soft and hard birth canal, or to foal abnormalities, premature expulsion of the placenta (with thickened necrotic areas and increased weights), pre-partum colic, and poor mammary gland development. After foaling, mares had low milk production, poor quality of the colostrum (resulting in low serum total immunoglobulin concentrations in foals), delayed uterine involution, and suppressed ovarian activity in postpartum and nonpregnant mares. There were low concentrations of progesterone (<4 ng/mL) and high concentrations of estrogens (>150 pg/mL) in blood. Foal abnormalities included respiratory failure in the initial inhalation reflex, abnormal skin, umbilical abnormalities, retraction of flexor tendons, angular deviation limbs, bone abnormalities, spinal deviation, failure to thrive, blindness, testicular atrophy, and decreased serum total immunoglobulin concentrations.

3.2. Prevalence of endophyte infection

Prevalence of endophyte infection, fescue toxicosis in mares and foal mortality over time are shown (Table 1). There was a significant linear correlation between the fraction of endophyte-infected ryegrass in the pasture and incidence of toxicosis (Pearson's $r = 0.861$, $P = 0.03$, Fig. 3), as well as a high correlation between tendency of infection and foal mortality (Pearson's $r = 0.928$, $P = 0.023$). All years had a minimum infection level >50%, with variations that in general mirrored average rainfall.

3.3. Detection of indolic alkaloids

3.3.1. Chemical detection

The limit of detection (LOD) of these assays was calculated as 1 ppm. No ergot alkaloids were detected by either thin-layer chromatography or spectrophotometry in extracts of contaminated ryegrass.

3.3.2. Bioassay

Both endophyte-free ryegrass (Endo−) and endophyte-infected ryegrass (Endophyte-infected ryegrass) stimulated smooth muscle activity on the uterus, but the latter had a much more powerful effect in increasing the force of contractions. The

Table 1
Percentile of *Neotyphodium lolii* infected ryegrass samples from the pastures where mares where feeding (values represent means for each year, $n = 30$), as well as prevalence of fescue toxicosis and foal mortality, and foal total immunoglobulin concentration over time

Year	Rainfall total (mm)	Rainfall monthly (mm)	Ryegrass fraction in pasture (%)	Endophyte infection (%)	Mares		Foals		
					Total	Affected	Total	$IG_t < 0.8$ g/100 mL	Died
1998	740	72	50	80	180	42	158	24	4
1999	733	73	50	50	200	35	140	32	3
2000	1232	102	60	55	202	39	137	25	3
2001	1289	107	70	60	189	72	122	27	3
2002	1513	126	85	80	178	75	118	50	8

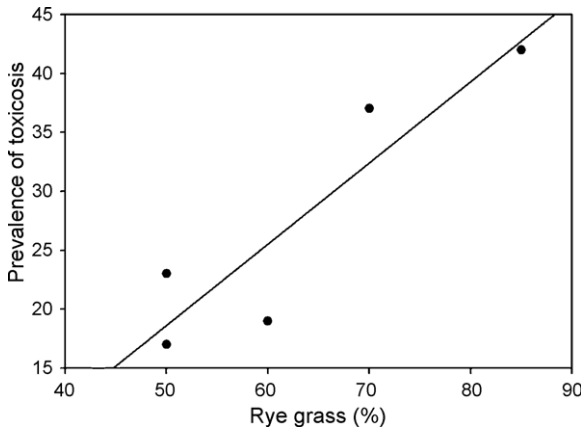

Fig. 3. Correlation between prevalence of endophyte-infected ryegrass on the pastures and prevalence (total number of affected horses per year) of clinical toxicosis among horses feeding on the grass (Pearson's $r = 0.861$, $P = 0.03$).

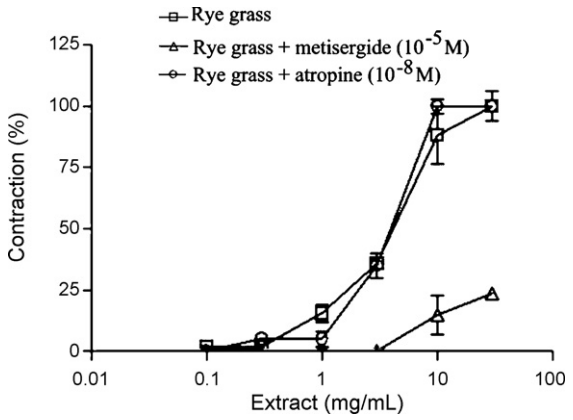

Fig. 5. Concentration–response curves of extracts of endophyte-infected ryegrass on isolated uterus with and without pretreatment with metisergide 10^{-5} M or atropine 10^{-8} M. Each point represents the mean ± S.E.M. of four experiments. $^{*}P < 0.01$ versus control.

percentage response of the uterus to the additions of 10 mg of each extract was calculated relative to the maximal response from control curves obtained by cumulative dosage of oxytocin. There was a dose-related increase in the contractile response of the isolated uterus to Endophyte-infected ryegrass (Fig. 4). The response of the uterus to the cumulative additions of extract was normalized to oxytocin response.

Pretreatment with 10^{-5} M methysergide followed by the cumulative addition of extract resulted in the displacement of the dose–response curve to the right and reduction of the maximal response. However, no change in the dose–response curves was observed with 10^{-8} M atropine (Fig. 5).

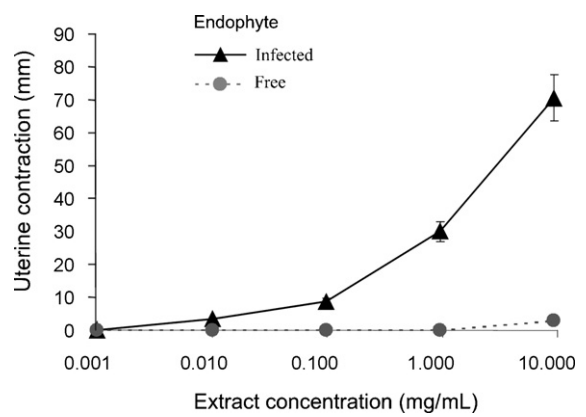

Fig. 4. Concentration–response curves for extracts of endophyte-infected ryegrass (Endo+) and endophyte-free ryegrass (Endo−) on isolated uterus. Each point represents the mean of five experiments. $^{**}P < 0.01$ compared to control.

3.4. Treatment of ryegrass intoxication

3.4.1. Trial 1. Assessment of ovarian cycling on intoxicated mares treated with domperidone, metoclopramide or placebo

A survival curve is shown (Fig. 6). Following the administration of vehicle, no ovulation was observed in the mares; by contrast, domperidone treated mares (GR-2, 1.1 mg/kg/d) began ovulating 15 d after the initiation of the treatment. By 18 d, all mares had ovulated (66% were double ovulations). In metoclopropamide-treated

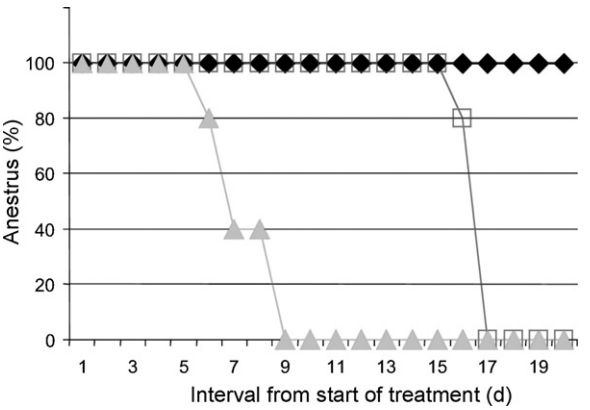

Fig. 6. Effect of domperidone, metoclopramide or vehicle on ovarian activity in mares with ryegrass toxicity. Survival curve of mares remaining in anestrus on each treatment group. Group one received vehicle (black diamonds, honey, $n = 5$), the second group received domperidone (white squares, 1.1 mg/kg BW, orally, every 24 h, $n = 5$) and the third received metoclopramide ((gray triangles, 0.6 mg/kg BW orally, every 8 h, $n = 5$). Treatments continued until ovulation occurred or for 18 d. Mean survival times were 20 weeks (vehicle), 16 weeks (domperidone, 99% CI = 15–17), and 7 weeks (metoclopramide, 99% CI = 4.9–9.1).

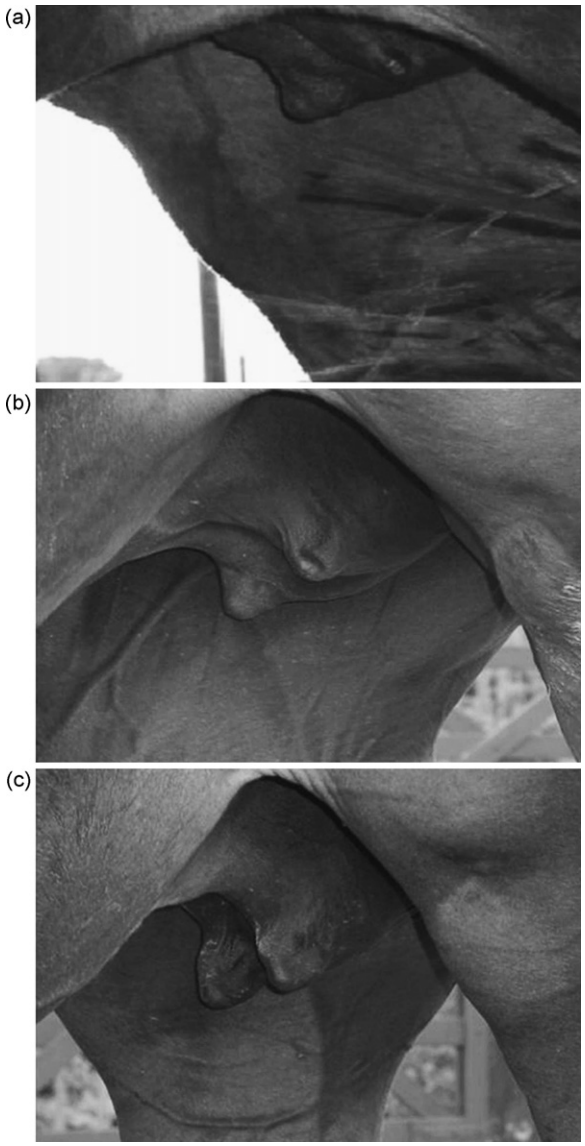

Fig. 7. Induction of lactation and development of the mammary gland in non-lactating mares with m-CPP (0.1 mg/kg). Panel a: the state of the gland before administration of the drug; Panel b: changes at 2 h; and Panel c: the result of milking (6 h after administration of the drug).

mares 1.8 mg/kg/d orally, given in three divided doses every 8 h), the first ovulation was detected 4 d after the initiation of the treatment, two ovulated on Day 5, and all remaining mares on Day 7. All ovulations were of single follicles.

3.4.2. Trial 2. Assessment of milk production on intoxicated mares treated with buspirone, mCPP or placebo

Milk production capacity of non-lactating mares was evaluated after administration of two serotonin agonists. Both drugs, but not vehicle, resulted in mammalian enlargement and induction of milk production within 6 h (Fig. 7). Initially the secretion had a waxy aspect, which gradually acquired the characteristics of colostrum.

3.4.3. Trial 3. Assessment of ovarian cycling on healthy inactive (winter) mares treated with domperidone or placebo

No ovarian activity was detected after 18 d of treatment in any of the groups.

4. Discussion

The prevalence, variation with time and clinical symptoms produced by the consumption of endophyte-infected ryegrass by mares and foals was studied between 1998 and 2002. The observed clinical picture was similar, but not identical, to that described for fescue toxicosis in horses and other species [1,6,12,23]. The most striking difference was that intoxicated mares in the prepartum, intrapartum and postpartum period had symptoms circumscribed to functions or organs involved in reproduction. In the case of foals, outcomes included perinatal death, respiratory failure, and osteoarticular alterations, but no neurological symptoms were observed, unlike previous reports in ryegrass-related toxicosis [10]. There was a significant linear correlation between the severity of toxicosis and the prevalence of Endophyte-infected ryegrass present in the fields of pasture (Fig. 3); field veterinarians observed that ryegrass was extremely palatable for the horses and therefore chosen by them. However, the first year of the study period (1998) had high prevalence of Endophyte-infected ryegrass, with less severe toxicosis. Conversely, low milk production, ovarian inactivity and embryo resorptions in subsequent years became alarming and led to the clinical trials. A possible reason for the absence of severe toxicosis in 1998 was the drought that occurred during the spring and summer months of that year (Fig. 1). The increase in rainfall observed during the rest of the study period correlated with increased severity of toxicosis, suggesting that endophyte metabolism and the production of toxic metabolites were influenced by water availability [24,25]. The degree of ryegrass contamination was determined using aniline blue staining on a large number of samples collected using the ring method, which allowed identification of the endophyte *N. lolii*.

Based on the clinical picture, it was suspected that the endophyte could produce alkaloids similar to the ergot of rye (*Claviceps purpurea*), which are usually

present as symbiotic products of the defense mechanism of plants [8,12,26–28]. Therefore, having proved the existence of the endophyte on ryegrass ingested by the mares, chemical and pharmacological evaluations of samples collected from the pastures were undertaken to determine if the assumption was correct.

Ryegrass samples were subjected to a process designed to extract and identify alkaloids if present. Two methods were attempted: thin-layer chromatography and spectrophotometry [10,11,20]. Under our experimental conditions, the limit of detection for these compounds was calculated at 1 ppm and no alkaloids were detected. Spectrophotometry enabled determination of total alkaloids content with a limit of detection similar to HPLC (1 ppm). Although HPLC would be a convenient method to identify the alkaloids present in the samples, since the concentration of alkaloids was below the limit of detection by spectrophotometry, HPLC analysis would be equally ineffective. However, the minimum toxic concentrations of ergot alkaloids are between 1×10^{-3} and 2×10^{-3} ppm in cows, and considerably lower in horses (5×10^{-5} and 1×10^{-4} ppm) [25]. Thus, our detection limit was far higher than the minimum concentrations required for toxicity. Moreover, other factors such as liver accumulation could increase toxicity [25]. Therefore, evaluation of pharmacological activity of the ryegrass samples used the effect of extracts on isolated rat uteri, a well characterized bioassay [8,25,26]. Ergot alkaloids act upon 5HT (serotonergic), α-adrenergic and dopaminergic receptors, all of which can mediate toxic effects. The uterus is a target of ergot alkaloid toxicity in most species. Endo− exhibited scarce uterotonic activity on the isolated uterus, whereas Endophyte-infected ryegrass (infected with *N. lolii*) extracts evoked concentration-dependent uterine contractions, suggesting that the presence of the endophyte on the ryegrass was necessary to trigger this effect (Fig. 4). To exclude a possible cholinergic effect, the uterus was pretreated with the muscarinic antagonist atropine, which failed to prevent the Endophyte-infected ryegrass evoked contraction. To determine if the endophyte produced indolic or ergot alkaloids normally present as symbiotic products of plant defense mechanisms [8,26–28], uteri were pretreated with methysergide, a powerful serotonergic antagonist, that prevented the effect of Endophyte-infected ryegrass extracts.

The same mechanism of action is applicable to the effect of ergot alkaloids on the nervous system, where a prominent sign is suppression of milk production in horses as well as in other species [6]. There are several subtypes of 5HT receptors involved in the regulation of the neuroendocrinological system, predominantly $5HT_{1A}$, $5HT_{2A}$ and $5HT_{2C}$, all of which have postsynaptic localization in the hypothalamus and induce prolactin, as well as other hormones [29]. Dopamine inhibits prolactin release acting on D_2 receptors [30], and the role of these receptors on ergot alkaloid intoxication in horses and other species has been evaluated using the antagonists metoclopramide and domperidone [13,14,31]. Although the mechanisms are not completely elucidated, metoclopramide and domperidone can trigger ovulation during the positive photoperiod; cycling under these conditions is associated with increased prolactin, but not with changes in FSH or LH [32]. We therefore designed clinical trials to test the efficacy of dopaminergic antagonists and serotonergic agonists as therapy for the toxic effects of the ryegrass. In the first group of experiments, we determined whether ovarian induction could be achieved by D_2 blockade in mares undergoing toxicosis in a positive photoperiod. Metoclopramide and domperidone induced ovulation occurred in intoxicated mares; the major difference between the two groups was the speed of activation/ovulation; that it was faster after metoclopramide treatment was attributed to its ability to cross the blood brain barrier with greater ease than domperidone. Conversely, domperidone induced frequent double ovulations. As a control of the specificity of the effect, we tested if D_2 antagonists could induce ovulation during winter anestrus and found that this was not the case. The last assay was a semi-quantitative determination of the milk production in non-lactating (non-foaling) mares by two different pathways: with the $5HT_{1A}$ agonist/D_2 antagonist buspirone, or with the $5HT_{1A}$, $5HT_C$ agonist m-CPP. Buspirone, an azapirone derivative, is used to increase plasma concentration of prolactin both in animals and humans [16,17,33–36], whereas m-CPP, among other neuroendocrinological effects, induces prolactin release [37,38]. It is known that m-CPP has relatively selective agonist activity on $5HT_{1C}$ and $5HT_{2C}$, but also antagonist activity on $5HT_{2B}$, and partial agonist activity on the $5HT_{2B}$ and $5HT_3$ serotonin receptors, whereas buspirone is selective for the $5HT_{1A}$ receptor but with some antagonism for the D2 receptor for dopamine. In the present study, both drugs induced the mammary gland to produce milk in mares, and may be useful to prevent failure to lactate in intoxicated mares.

In summary, our findings strongly suggested that endophyte-infected ryegrass is associated with ergot alkaloid intoxication in horse. Furthermore, ryegrass infected with *N. lolii* had uterotonic activity that was not

caused by Endo−. We inferred that this effect could be due to stimulation of serotonergic receptors because it was blocked by methysergide, but not by atropine. Furthermore, serotonergic agonists and dopaminergic antagonists prevented specific aspects of the endophyte-induced toxic syndrome.

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