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Cherry Trees, Plant Cyanogens, Caterpillars, and Mare Reproductive Loss Syndrome: Toxicological Evaluation of a Working Hypothesis

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THE ONSET OF MARE REPRODUCTIVE LOSS SYNDROME (MRLS) in 2001 coincided with an exceptional abundance of eastern tent caterpillars (ETC), *Malacosoma americanum,* in Central Kentucky. Preliminary field studies by Henning and his co-workers also showed a high geographic correlation between ETC, black cherry trees, and MRLS. This correlation was strongly confirmed by a later epidemiological survey by R. Dwyer et al. (1). Further support for this association was provided by a toxicology report of high cyanide concentrations in the hearts of three late fetal loss (LFL) foals (L. Harrison, personal communication).

The MRLS 2001 syndrome was a completely new entity, having never been identified or described previously. It was also extremely transient, appearing and peaking in less than two weeks. As such, for the remainder of 2001, the syndrome was viewed, analyzed, and researched in an increasingly retrospective manner.

It was soon hypothesized that cyanide from black cherry tree leaves was the proximal cause of MRLS. Cyanide or cyanogens were thought to be transferred from the trees to the environment of the horse by ETC voraciously denuding these trees (Figure 1). The precise mechanism of the transfer was unclear. The source(s) of cyanogens could be the caterpillars themselves, caterpillar frass, leaf fragments, mandelonitrile ingestion, water contaminated by caterpillars, combinations of these factors, or by other sources such as pasture clover. The concentrations of cyanide involved were assumed to be sub-lethal for mares since mares did not show clinical signs, and it was assumed that the fetus was more sensitive than the mare to cyanide. If the hypothesis was correct, it should be possible to abort mares by exposing them to sub-clinical concentrations of cyanide.

Since there was virtually a complete absence of published literature about cyanide in the horse, let alone the pregnant mare, the objectives of this study were to define "normal" cyanide concentrations in equine blood in Central Kentucky, determine the "threshold" toxic level for cyanide toxicity in equine blood, and determine fetotoxicity of cyanide in the pregnant mare. The overall goal of this project was to reproduce MRLS in the laboratory.

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Figure 1. Cyanogenic cascade in black cherry (prunasin) and apricot (amygdalin). Both cascades proceed through prunasin, and mandelonitrile is the proximal cyanide donor.

(found in apricot and other fruit seeds)   (black cherry leaves)
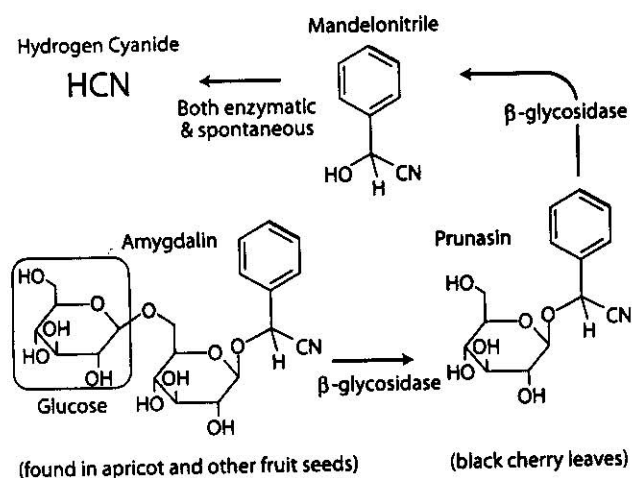
Materials and Methods

Horses

Mature Thoroughbred mares weighing 428 to 504 kg were used for this study. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and were de-wormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. Additionally, cardiac and ophthalmic evaluations were performed to ensure that those organs had no evidence of previous disease. All animals used in these experiments were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care Use Committee, which also approved the experimental protocol.

Cyanide Infusion

An intravenous catheter (Abbocath-T, 14g x 5½", North Chicago, IL) was inserted into the jugular vein and sutured in place. Sodium cyanide (NaCN) solutions were prepared by dissolving NaCN (J.T. Baker, Philipsburg, NJ) in saline and were infused at 3, 6, and 12 mg/minute for 1 hour and 1 mg/kg for 1 hour using an ambulatory withdrawal pump (Dakmed, Buffalo, NY). During infusion, the horses were monitored closely for signs of toxicity, which included restlessness, anxiety, flared nostrils, rapid respiration, sweating, and increased heart rate. Heart rates (HR) were recorded at 1-minute intervals by an onboard heart rate computer (Polar CIC Inc., Port Washington, NY). For the 3, 6, and 12 mg/minute infusions, blood samples were taken before and at various times during and after infusion for complete blood counts, chemistry panels, and blood lactates. For the 1 mg/kg for 1 hour infusion, blood samples were obtained for analyses before infusion (0 hours), during infusion (0.25, 0.5, and 1 hour) and after infusion (0.08, 0.17, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 hours) into Vacutainer® serum tubes (Becton Dickinson, Rutherford, NJ) and Vacutainer® plasma tubes (Becton Dickinson, Franklin Lakes, NJ) and analyzed immediately.

Mandelonitrile Administration

In a second series of experiments, four mature Thoroughbred mares were administered oral mandelonitrile (3 mg/kg). Blood samples for cyanide analysis were obtained before (0 hours) and after dosing at 0.05, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours into Vacutainer serum tubes and Vacutainer plasma tubes and analyzed immediately.

Safety Precautions

Two antidotes were prepared to counter any adverse effects from cyanide infusion. A 3% solution of sodium nitrite was prepared by adding 1.8 g of Na nitrite (J.T. Baker, Philipsburg, NJ) to 60 ml of saline. This mixture was to be administered intravenously at a rate of 10 to 20 ml/minute. A 25% solution of sodium thiosulfate (J.T. Baker, Philipsburg, NJ) was prepared by adding 100 g Na thiosulfate to 400 ml of saline. This mixture was to be administered immediately after the sodium nitrate at a rate of 200 ml/minute.

Analytical Detection of Cyanide

As detailed previously (1), an inexpensive, disposable alternative to the costly Warburg Distillation Flask was developed, which allowed simultaneous running of 100 cyanide analyses. Briefly, a 10-ml plastic cup was suspended by means of Scotch® tape inside a 120-ml plastic cup with a screw lid; 10 ml of 1 molar sulphuric acid (H_2SO_4) was pipetted into the larger cup. Exactly 2.5 ml of 0.25 normal sodium hydroxide (NaOH) was pipetted into the smaller cup. The cyanide-containing sample (e.g., 1 to 2 ml blood) was pipetted into the H_2SO_4, and the cup was immediately sealed with its lid and allowed to sit overnight at room temperature while cyanide as HCN gas was evolved from the acid solution and trapped in the NaOH. The small cup was then removed, and the NaOH solution was decanted into an autoanalyzer sample cup. In the presence of chloramine-T, the cyanide ion was converted to cyanogen chloride, which reacted with pyridine-barbituric acid to form a red-blue color, the intensity of which was measured spectrophotometrically at 578 nm. Use of an autoanalyzer ensured a precise and reproducible interval during which color developed and thus improved the detection limit to as low as 2 ng/ml in a 1-ml sample.

Standard curves were linear in the range of 2 to 300 ng/ml, with a regression coefficient $r^2 > 0.99$.

Toxicokinetic Analysis

The toxicokinetic parameters of cyanide were determined by compartmental analysis. Equations of a two-compartment model with zero-order input rate were fitted to the individual blood concentrations versus time by least squares nonlinear regression analysis using a nonlinear regression program (Winnonlin, version 3.1) (Pharsight Corporation, Cary, NC). The closeness of the fit was evaluated by the Akaike Information Criterion (AIC), residual plots, and visual inspection. The data were weighted as $1/(y_{pred})^2$, where y_{pred} was the model-predicted concentration at the actual time. Area under the curve (AUC) following intravenous administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (ß) of the log plasma drug concentrations versus time curve was determined by the method of least-squares regression (2). The rate constant of distribution α and distribution half-life ($t_{1/2}\alpha$) were determined using the method of residuals (2). Total body clearance (Cl_b) was calculated by use of Equation 1 (3).

$$Cl_S = IV\ Dose/AUC_{0-inf} \qquad \text{(Equation 1)}$$

The volume of distribution in central compartment (Vd_c) and volume of distribution at steady state (Vd_{ss}) were calculated according to Equations 2 and 3, respectively (4).

$$Vd_c = Dose\ (IV)/(A+B) \qquad \text{(Equation 2)}$$

$$Vd_{SS} = IV\ Dose/AUC_{0-inf} \times MRT \qquad \text{(Equation 3)}$$

A and B are the Y intercepts associated with distribution and elimination phase, respectively, and AUMC is area under the first moment curve and calculated by the trapezoidal method and extrapolated to infinity.

The mean residence time (MRT) (5) was determined according to Equation 4.

$$MRT = AUMC_{0-inf}/AUC_{0-inf} - (Infusion\ time/2) \qquad \text{(Equation 4)}$$

The pharmacokinetic variables (elimination half-life, area under the curve) of the cyanide following oral administration of mandelonitrile were determined using a noncompartmental approach (2). The maximum blood concentration of the cyanide (C_{max}) and the time to reach this concentration (T_{max}) were obtained directly from the blood-concentration versus time curves. The absolute bioavailability (F) was calculated from the AUC_{0-inf} ratio

obtained following oral and IV administration according to Equation 5 (4).

$$F = AUC_{0-inf}\ (Oral)/AUC_{0-inf}\ (IV) \times IV\ Dose/Oral\ Dose \qquad \text{(Equation 5)}$$

Total oral clearance (Cl_o) was calculated by use of Equation 6.

$$Cl_O = Dose\ (Oral)/AUC_{0-inf} \qquad \text{(Equation 6)}$$

Results and Discussion

Developing a Highly Sensitive Analytical Method

Most analytical methods for cyanide are used in the forensic detection of cyanide associated with lethal intoxication and have a limit of detection in the mg/ml range. However, it was obvious that if cyanide was involved in MRLS, the concentrations found in mares would be less than those associated with clinical signs of toxicity since no signs of intoxication were noted in any mares. Therefore, an analytical method was needed for cyanide detection in the subtoxic range (ng/ml). Such a method was developed in our laboratory and has been described previously (6). Figure 2 shows the standard curve of that method for the quantification of cyanide in biological fluids.

Defining "Normal" Blood Cyanide in Horses at Pasture in Kentucky

The first step in this project was to define what constitutes a "normal" blood cyanide for a mare at pasture in Central Kentucky. Figure 3 shows that the distribution of blood cyanide concentrations in horses at pasture in Kentucky averaged 8.5 ng/ml in the fall of 2001 (n = 48) and 3.2 ng/ml in the spring of 2002 (n = 100). The blood cyanide concentrations apparently follow log normal distributions, and the concentrations appeared to differ from field to field and also between fall and spring. These blood

Figure 2. Standard curve for the quantification of cyanide in biological fluids.
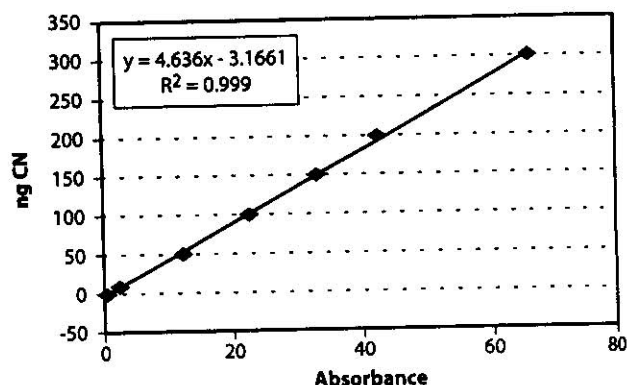
cyanide concentrations are very low, and it should be kept in mind that cyanide is substantially concentrated in blood. Free blood cyanide and presumably free tissue concentrations of cyanide in horses at pasture in Kentucky are likely about 1/50 of these concentrations (7).

Defining "Toxic" Blood Concentrations of Cyanide in the Horse

The next step was to define the blood concentrations of cyanide likely to be acutely toxic in the horse. To this end, horses were infused with increasing concentrations of cyanide, starting at 1 mg/minute and increasing to 12 mg/minute. Figure 4a shows the blood cyanide concentrations attained following infusion of 12 mg NaCN/minute, the dose that produced the first signs of toxicity. As shown in Figure 4b, there was a sharp increase in heart rate at the point of toxicity during the 12 mg/minute infusion, when the heart rate peaked at 150 beats per minute (bpm). Other signs of toxicity were sweating, rapid breathing, and apparent anxiety.

When clinical signs of toxicity appeared, the infusion was immediately stopped. Thereafter, the heart rate returned to normal, and the horse quickly returned to normal behavior without administration of an antidote. In earlier experiments, cyanide toxicity would sometimes cause the horse to weaken and drop to the ground. These clinical signs were always rapidly reversed following intravenous infusion of 3% sodium nitrite and 25% sodium thiosulfate.

These experiments suggested that if the experimental blood cyanide concentrations were held to less than 2,000 ng/ml, clinical signs of cyanide toxicity in the experimental horses were unlikely. Additionally, these results suggest that there is a large difference between the concentration of blood cyanide in normal horses at pasture and the concentration required for toxicity allowing for considerable experimental latitude to evaluate the fetotoxicity of cyanide in pregnant mares.

Defining the Toxicokinetics of Cyanide in the Horse

Based on the data of Figure 4, we infused four horses with NaCN at 1 mg/kg for 1 hour. Blood cyanide concentrations increased linearly to about 1,000 ng/ml at 1 hour, at which point the infusion was stopped. After infusion was stopped, there was rapid redistribution of cyanide with an alpha half-life of 0.74 hours, followed by a beta or terminal phase of metabolism, which was much slower, with a half-life of 16 hours (Figure 5). The insert shows the logarithmic plot of concentration versus time for the post-administration portion of the data. These data show clearly that the termination of action after a bolus administration of cyanide is by re-distribution and that cyanide is a classic agent for which its acute pharmacological/toxicological effects can be terminated by redistribution.

Figure 3. Distribution of blood cyanide concentrations in horses at pasture in Kentucky a) in the fall of 2001 (n = 48) and b) in the spring of 2002 (n = 100).
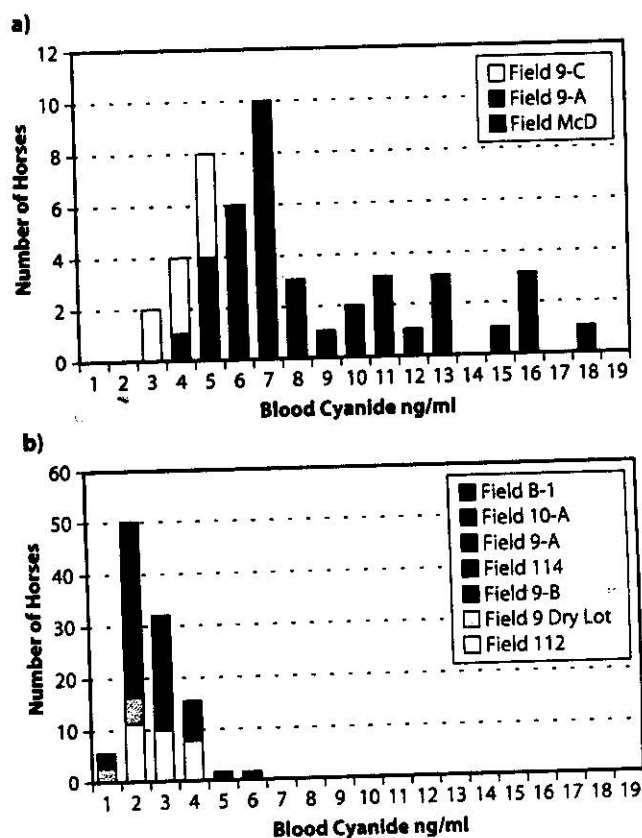

Figure 4. a) Mean blood cyanide concentrations attained following intravenous infusion of 12 mg NaCN/min, the dose that produced the first signs of toxicity; b) Heart rate of infused horse showing the point of toxicity, when the heart rate peaked at 150 bpm.
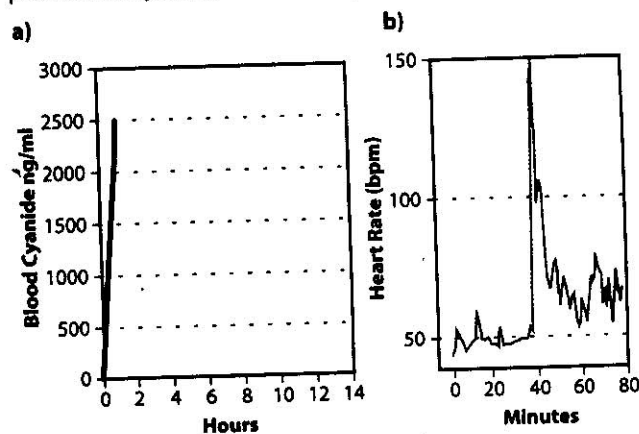
Figure 5. Blood cyanide concentrations following NaCN infusion at 1 mg/kg x 1 hour. Insert shows the logarithmic plot of concentration versus time for the post-administration portion of the data with an alpha half-life of 0.74 hours, followed by a terminal phase, with a half-life of 16 hours.
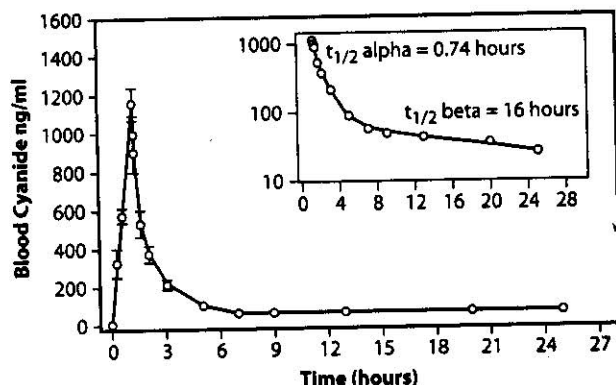
**Defining the Toxicokinetics
of Mandelonitrile in the Horse**

Cyanide is not present as such in black cherry tree leaves but rather as prunasin and mandelonitrile, as set forth in Figure 1. Mandelonitrile is the proximate precursor in the cherry tree system for cyanide release. Therefore, to more closely mimic the possible etiology of MRLS, horses were dosed with oral mandelonitrile, and the resulting blood cyanide concentrations were measured. Figure 6 shows the mean blood cyanide concentrations following dosing with 3 mg/kg mandelonitrile (n = 4). Mandelonitrile is about 20% cyanide; therefore, horses received about 600 mg HCN orally. There was immediate release and absorption of cyanide as indicated by the peak blood cyanide concentration at 3 minutes. In an earlier

Figure 6. Mean blood cyanide concentrations following bolus oral dose of 3 mg/kg mandelonitrile (n = 4). Insert shows semi-logarithmic plot of cyanide concentration versus time for the post-absorption portion of the data. Absorption was followed by rapid redistribution of cyanide, with an alpha half-life of 0.48 hours and a beta phase half-life of about 13 hours.
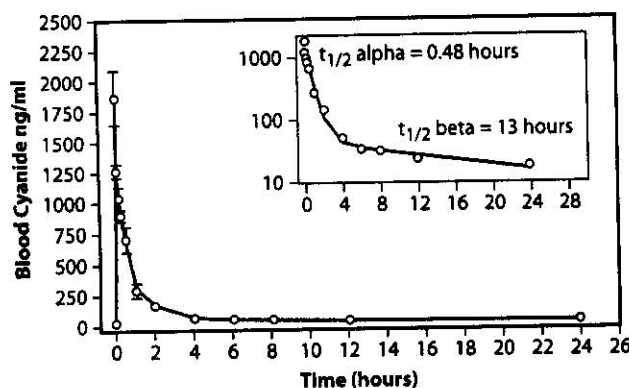
ranging experiment, in which a dose of 3 g/horse of mandelonitrile was used, the test horse became weak and uncoordinated for about 30 seconds.

The insert of Figure 6 shows a semi-logarithmic plot of cyanide concentration versus time for the post-absorption portion of the data. The apparent bioavailability of cyanide from mandelonitrile was about 57%; absorption was followed by rapid redistribution of cyanide, with an alpha half-life of 0.48 hours. This phase was followed by the beta or elimination phase with a much slower half-life of about 13 hours.

**Attempted Reproduction of MRLS by Administration
of Mandelonitrile to Pregnant Mares**

We next attempted to reproduce MRLS in pregnant mares by administration of mandelonitrile. With regard to developing a suitable dosing schedule for mandelonitrile, our pharmacokinetic results showed that it would be difficult to maintain a "steady-state" plasma concentration of cyanide following intermittent oral dosing with mandelonitrile. As a best experimental approach, seven pregnant mares were dosed with mandelonitrile (2 mg/kg twice a day in applesauce) for 14 days. Peak values (taken immediately after dosing) had a mean of 315 ng/ml and varied widely (range: 215 to 594 ng/ml) as indicated by the upper curve. The trough values (taken just before dosing) had a mean of 62 ng/ml and were more consistent than peak values (Figure 7).

Throughout this experiment, no fetal losses were recorded, and no clinical signs suggestive of fetal loss were observed. These results suggested that consistent blood concentrations of cyanide of about 60 ng/ml and/or considerably higher spikes of blood cyanide can occur without associated fetal losses. As such, these results are inconsistent with and do not support the original working hypothesis that cyanide from the black cherry tree is closely associated with or a proximal cause of MRLS.

**Other Factors Possibly Influencing
Blood Cyanide Concentrations**

As the 2002 MRLS season approached, we paid particular attention to the possible impact of pasture clover content and overnight freezing conditions on the cyanide content/bioavailability of clover cyanide and the blood concentrations of cyanide in horses grazing such pastures. Because damage from freezing temperatures has been reported to increase the cyanide content and/or its bioavailability from some clovers, James Crutchfield and his colleagues studied the cyanide content/yield of clover and the effects of morning temperature. As shown in Figure 8, morning temperature had little effect on Kentucky clover cyanide content/yield, in contrast with the reported effects of low temperatures on cyanide content/yield from

Figure 7. Peak and trough blood cyanide concentrations following oral dosing of mandelonitrile (2 mg/kg, twice a day, in applesauce) for 14 days.
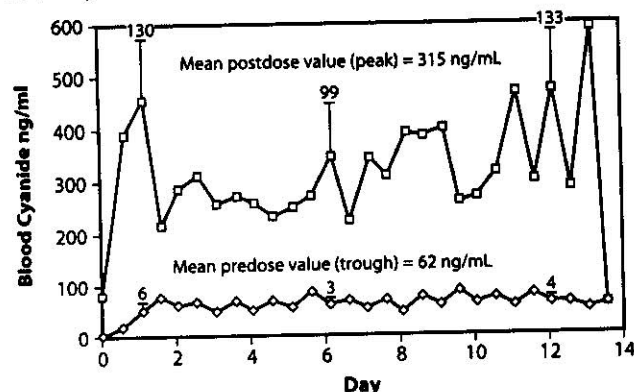
Figure 8. a) Blood cyanide concentrations in horses at pasture before a "freeze" (March 21); b) minimum, maximum, and average temperatures during mid-March; c) blood cyanide concentrations of same horses on March 22, after hard freeze.

a)

b)

c)
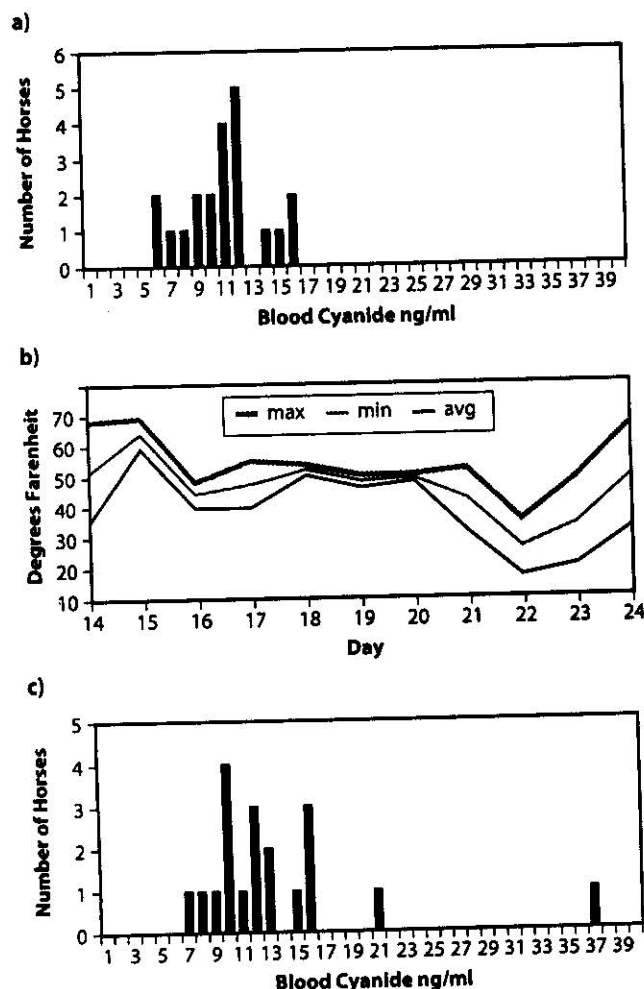
clovers in other geographic locations. There was no significant difference between the blood cyanide concentrations before and after freezing.

Consistent with these findings, blood cyanide concentrations in horses at pasture showed little change following an overnight frost. Figure 8a shows the blood cyanide concentrations in horses at pasture before a "freeze" on March 21, and Figure 8c shows the blood cyanide concentrations of the same horses on March 22 after the hard freeze of the night of March 21. Figure 8b shows the minimum, maximum, and average temperatures during mid-March. Consistent with the results of the Crutchfield group experiments, there was no apparent difference in blood cyanides between each group of horses prior to or after the freezing night of March 21, 2002.
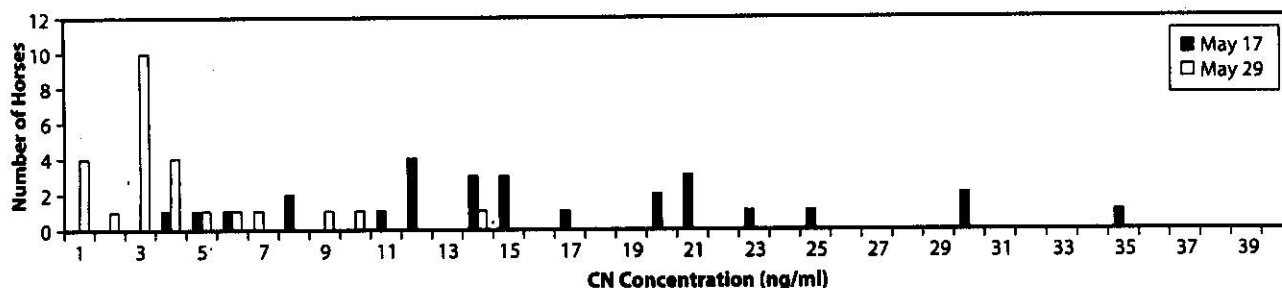
Field and Experimental Results from the 2002 MRLS Season

The interpretation of these experimental results is consistent with field and experimental data developed during the 2002 MRLS season. During the 2002 MRLS season, blood samples were drawn from a band of 20 pregnant mares grazing in proximity to black cherry trees. As set forth in Figure 9, the blood cyanide concentrations of these mares was not significantly different from "normal" mares shown in Figure 3. Furthermore, two of these mares (indicated with asterisks on Figure 9) had early fetal losses (EFL) consistent with MRLS, even though blood cyanide concentrations in these two mares were well within normal limits.

In cooperation with Drs. Webb and McDowell, we also monitored blood cyanide concentrations in mares undergoing ETC-induced EFL. Blood cyanide concentrations of mares after ETC exposure on May 29 were not significantly greater (p <0.05) than blood cyanide concentrations before exposure on May 17 to ETC (Figure 10). Again, the results do not support suggestions that increased blood cyanide concentrations are a factor in ETC-induced fetal losses.

Figure 9. Blood cyanide concentrations of mares grazing in proximity to wild cherry trees from April 28 - May 3, 2002. Asterisks denote blood cyanide concentrations of two mares that suffered EFL during this period.
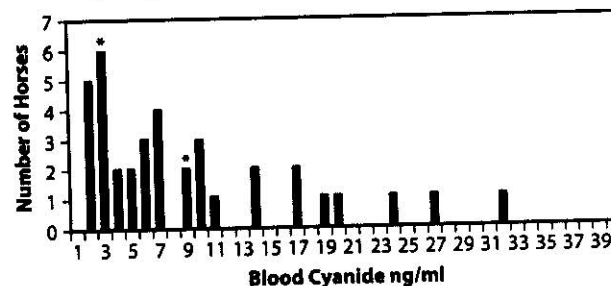
Figure 10. Blood cyanide concentrations of pregnant mares before exposure (May 17) and after exposure (May 29) to ETC.

Conclusions

Field and experimental events reported elsewhere in this workshop from the 2002 MRLS season have provided strong evidence in support of the close involvement of ETC in MRLS. On the other hand, the experimental and field work with cyanogens leading up to the 2002 MRLS season and results obtained during the 2002 MRLS season reported here make the involvement of cyanide in MRLS very much less likely.

Moreover, a study tracing the movement of cyanide from cherry trees to tent caterpillars and into the detritus pool sheds additional doubt on the possible involvement of caterpillar-borne cyanide in MRLS (8).

Simply put, we have been unable to reproduce MRLS by administration of mandelonitrile, a proximal cyanide donor, and no evidence whatsoever has been developed in support of suggestions that exposure to black cherry trees, ETC, or cyanogenic clovers was likely to increase the blood cyanide concentrations of mares to the point that such blood cyanide concentrations could be implicated in MRLS.

In further support of this conclusion, MRLS was seen to occur in mares in which blood cyanide concentrations were no different from or actually less than those seen in "normal" or "control" mares, consistent with suggestions that cyanide from ETC or any other source has not been a critical factor in MRLS as we know it.

Acknowledgments

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