Evaluation of the ability of aqueous black walnut extracts to induce the production of reactive oxygen species

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Objective—To assess the in vitro capability of aqueous black walnut extracts (BWEs) to generate reactive oxygen species in water-based media ranging in makeup from a simple buffer solution to a complex solution containing serum.

Sample—3 BWEs.

Procedures—Production of reactive oxygen species by BWEs prepared in water or *N*-hexane was tested in PBS solution, PBS solution containing 0.5% bovine serum albumin and 5mM glucose (PBG), and RPMI–1640 medium (RPMI) containing 10% fetal bovine serum or 10% donor horse serum. Reactive oxygen species production was measured as conversion of nonfluorescent dihydrorhodamine 123 by reactive oxygen species to its fluorescent product, rhodamine-123. Hydrogen peroxide was used as a standard for reactive oxygen species activity.

Results—BWEs prepared in water generated reactive oxygen species in a dose-dependent manner over a 4-hour period, with peak activity detected when the BWEs were added as 10% (vol/vol) of the RPMI. The BWE prepared in *N*-hexane generated maximal reactive oxygen species activity after incubation for 3 to 4 hours when added at concentrations ranging from 0.3% to 0.5% (vol/vol) of the RPMI. The BWE prepared in water generated the highest fluorescent signal in PBS solution, whereas the BWE prepared in *N*-hexane generated the highest fluorescent signal in PBG.

Conclusions and Clinical Relevance—The BWEs prepared in water generated a dose-dependent induction of fluorescence in all the water-based solutions tested. These findings indicated that the BWEs, which are used to induce laminitis in horses, generate reactive oxygen species. (*Am J Vet Res* 2011;72:308–317)

Exposure of horses to BWE as a result of being housed in stalls bedded with black walnut shavings or being housed in the vicinity of black walnut trees has been linked with the development of laminitis in horses.[1,2] As a result of this, a research method for inducing laminitis in horses has been developed in which an aqueous extract of black walnut shavings is administered intragastrically.[3–5] In a study[6] conducted by our laboratory group, the administration of BWE in horses resulted in a rapid onset of clinical signs of acute laminitis that progressed to an Obel grade 1 lameness in approximately 8 to 12 hours, whereas the onset of clinical signs of acute laminitis progressed to an Obel grade 1 lameness far more slowly (approx 16 to 24 hours) after carbohydrate overload.

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Abbreviations

AFU	Arbitrary fluorescence unit
BWE	Black walnut extract
DHS	Donor horse serum
EC_{50}	Half-maximal effective concentration
FBS	Fetal bovine serum
LPS	Lipopolysaccharide
PBG	PBS solution containing 0.5% bovine serum albumin and 5mM glucose
RPMI	RPMI–1640 medium

Recently, results of several studies[6–11] have indicated a role of activated neutrophils early in the pathogenesis of laminitis. Results of 1 study[6] indicated that the production of reactive oxygen species by circulating leukocytes increases in horses administered BWE before the development of clinical signs associated with acute laminitis. In addition, the results of other studies[7,8] of plasma and tissues collected from horses administered BWE indicated that there were increases in the amounts of myeloperoxidase in the circulation, skin, and laminar tissue of horses that had lameness after being administered an extract of black walnut shavings, compared with values for horses not administered an extract of black walnut shavings; the assay

used in those studies identified functionally active myeloperoxidase. [9]

Analysis of the results of other studies indicates that neutrophils migrate into laminar tissue of the hoof after the administration of BWE[10,11] and that laminar tissues have extremely low amounts of superoxide dismutase, which is an enzyme critical for the neutralization of reactive oxygen species.[12] Therefore, it is hypothesized that reactive oxygen species generated by activated neutrophils could cause major damage in the laminar tissue because of the dearth of superoxide dismutase activity. Data supporting a role for reactive oxygen species in the development of laminitis were summarized in an editorial[13]; the authors of that editorial proposed that the infiltration of neutrophils into laminar tissue, including a concurrent release of reactive oxygen species, and the release of myeloperoxidase enzyme from degranulating neutrophils could be involved in the induction of local vascular damage through the reduced production of nitric oxide by vascular endothelial cells. This theory was supported by the results of another study[14] in horses in which interference with binding of myeloperoxidase to endothelial cells was correlated with a reduction in damage mediated by reactive oxygen species.

In addition, evidence exists that the in vitro exposure of epithelial cells to purified black walnut toxins increases cellular production of reactive oxygen species.[15] Moreover, the 2 most widely investigated toxins extracted from black walnut shavings (ie, juglone and plumbagin) cause damage to and, at high concentrations, kill epithelial cells via the induction of the cellular production of reactive oxygen species.[15] Toxins extracted from certain trees induce cells to produce these reactive oxygen species, but woody tissues of some trees also contain long-lived, immobilized radicals that help protect these trees against invaders, such as insect pests, fungi, and bacteria.[16] The long-lived, immobilized radicals appear to accumulate as trees age and can be eluted with water from the woody tissue.[16] Several families of compounds in these woody tissues, including those leading to the synthesis of juglone, contain ring structures that react with water molecules to generate free radicals.[17] Moreover, the principal toxins of black walnut trees (ie, juglone, plumbagin, and menadione) share naphthoquinone structures that react with water to yield superoxide radicals and hydrogen peroxide.[17] Because woody tissues lack free water (eg, water that is not tightly bound to other molecules), breaching of the outer protective layers of the plant allows water to enter these spaces, which results in activation of compounds that generate reactive oxygen species.[16,18,19] These reactive oxygen species damage invaders by attacking their cellular membranes, proteins, and nucleic acids, and they also modulate expression of specific genes in the tree that help to resist infestation.[19,20] Because of the need to preserve a homeostatic balance internally while responding to invaders and environmental stresses, plants produce and sequester antioxidant molecules in their tissues as well as enzymes that generate hydrogen peroxide.[19,21]

Because of our observation that the administration of BWE results in a more rapid development of signs of acute laminitis than does carbohydrate overload in horses, we hypothesized that reactive oxygen species are rapidly generated directly by BWEs prepared in water or *N*-hexane. The purpose of the study reported here was to assess the in vitro capability of aqueous BWEs to induce the production of reactive oxygen species in water-based media that range in makeup from a simple buffer solution to a complex solution containing serum, which would represent the more complex biological environment observed in a horse.

Materials and Methods

Aqueous BWE preparation—Three BWEs were prepared in water for use in a protocol to induce acute laminitis as described elsewhere.[4] Two of the 3 extracts had been used previously to successfully induce laminitis in horses, and the third extract was prepared but frozen prior to use when the horse to which the extract was intended to be administered proved unsuitable for use in that study. Briefly, the heartwood of a single black walnut tree was prepared as uniform 0.2 × 0.2 × 2-mm shavings, packaged in 1-kg units, and stored at –20°C until use. To prepare the BWE, 1 kg of shavings was placed in 7 L of distilled water and shaken continuously for 12 hours in an orbital shaker. The resulting solution was filtered through cheesecloth, and samples were transferred into 50-mL sterile centrifuge tubes[a] or 500-mL sterile bottles[b] and stored at –20°C until further processing.

At the beginning of the in vitro experiments, frozen BWEs were thawed and pooled for each preparation. A series of six 10-fold dilutions of each extract was prepared in sterile PBS solution for preliminary screening of each BWE for its capacity to generate radicals. In addition, samples of each extract were tested for microbial contamination by plating, in duplicate, 1 mL of each serial dilution on brain-heart infusion agar[c] contained in 100-mm Petri dishes. Because all the assays detect evidence of both the capacity to generate radicals and of bacterial contamination, each extract was centrifuged at 3,000 × *g* for 20 minutes and the supernatant was collected, pooled, and filtered through combination glass fiber and 0.2-µm membrane filters.[d] The filtrate was stored in 1-, 10-, or 25-mL aliquots at –20°C until used in the study. Furthermore, frozen aliquots were thawed no more than 3 times for use in the study.

Samples collected from 3 filtrates were evaluated for bacterial contamination by transferring a 200-µL aliquot of each filtrate into 3 replicate tubes that contained 10 mL of sterile brain-heart infusion broth,[c] and by directly plating 100 µL from each filtrate on plates that contained brain-heart infusion agar, and incubating the tubes and plates at 37°C for 48 hours. Furthermore, a 100-µL aliquot of each filtrate was analyzed for LPS by use of a colorimetric *Limulus* amebocyte lysate assay.[e] The limit of detection of the assay for serum was 200 pg/mL, and the functional limit for the colored BWE samples was 1 ng/mL.

Preparation of BWE in *N*-hexane—In preliminary experiments, lipid-binding proteins appeared to modulate the rate of reactive oxygen species generation. Therefore, an extract was prepared in *N*-hexane to de-

termine whether *N*-hexane would enhance the recovery of reactive oxygen species–generation activity. A BWE was prepared in *N*-hexane at another laboratory.[f] Briefly, 72 g of black walnut shavings was transferred into a 1-L beaker containing 500 mL of high-performance liquid chromatography–grade *N*-hexane. Shavings were mixed continuously for 12 hours at 4°C by use of an overhead stirrer. Then, the contents of the beaker were filtered through a filter paper[g] and the solvent was removed under reduced pressure. The resulting paste was stored at –20°C and then transported on dry ice to our laboratory[h] by overnight carrier. Then, the paste was thawed and a solution of 10 mg of paste/mL of sterile tissue culture–grade dimethyl sulfoxide[i] was prepared. The solution was centrifuged at 3,000 × *g* for 30 minutes. The supernatant was removed; aliquoted into 0.5-, 1-, or 2-mL portions; and stored at –20°C. The material was thawed only 1 or 2 times for each sample used in the experiments. Because dimethyl sulfoxide interferes with the colorimetric *Limulus* amebocyte lysate assay,[e] we were not able to determine whether this stock solution of the extract prepared in *N*-hexane was contaminated with LPS.

Dihydrorhodamine 123 preparation and measurement of reactive oxygen species—Dihydrorhodamine 123 is a sensitive probe used for the measurement of oxygen radicals and is colorless until it reacts with molecules containing chemical bonds featuring oxygen or nitrogen atoms with unpaired electrons (ie, radicals).[22] After reacting with oxygen or nitrogen radicals, dihydrorhodamine 123 relinquishes 2 protons and is transformed into a green fluorescent molecule (rhodamine-123).[23] The fluorescence intensity is directly proportional to the quantity of dihydrorhodamine 123 that is converted into rhodamine-123. In addition, rhodamine-123 accumulates in aqueous solutions; therefore, intensity of the maximal fluorescent signal is cumulative relative to the incubation time. Dihydrorhodamine 123 is most responsive to hydrogen peroxide but will react with many oxygen and nitrogen radicals to convert dihydrorhodamine 123 to rhodamine-123.[22] In the presence of high concentrations of radicals, particularly hydrogen peroxide, the ring structure of rhodamine-123 is broken to release nonfluorescent products, which results in loss of the maximal fluorescent signal.[23] Therefore, it is necessary to assess both the dose response and time course of signal generation.

A 10mM stock solution of dihydrorhodamine 123[j] was prepared by dissolving lyophilized powder in tissue culture–grade dimethyl sulfoxide. The solution was mixed gently and stored in 50-µL aliquots at –20°C. One 50-µL aliquot of stock solution was further diluted in 5 mL of each medium to be tested to yield a final 10^{-4}M working solution.

The total capacity and stability (resistance to quenching) of dihydrorhodamine 123 for the detection of radicals was assessed by measuring the maximal fluorescent signal generated by incubating the dye with hydrogen peroxide[b] prepared from a 30% stock solution in each medium. This assessment was performed beginning with a 1% hydrogen peroxide solution by use of a series of 2-fold dilutions and monitoring fluorescence (excitation, 485 nm; emission, 538 nm) before incubation at 37°C and at 30-minute intervals after incubation at 37°C. The assessment was accomplished by use of 100-µL aliquots transferred into clear polystyrene 96-well tissue culture plates. The capacity of hydrogen peroxide to convert dihydrorhodamine 123 to rhodamine-123 was assessed in PBS solution,[k] PBG,[i] RPMI[k] without phenol red containing 10% FBS,[l] or RPMI containing 10% DHS.[l] The lowest concentration of hydrogen peroxide yielding the maximal fluorescent signal in each medium was used as a positive control sample for fluorescence in that medium during the subsequent experiments. Choice of media for this experiment revealed 3 levels of chemical and biological complexity. The simple PBS solution contains no components that should directly modulate the interaction of radicals with dihydrorhodamine 123. Furthermore, the combination of media in the PBG was chosen for these experiments because preliminary attempts to measure the rate of radical interaction with dihydrorhodamine 123 indicated that albumin in the medium reduced the rate of dye conversion by BWEs prepared in water. Because bovine serum albumin binds lipids, interactions between bovine serum albumin and lipid-containing components of BWEs may affect the interaction of radicals that are within lipid-containing molecules and dihydrorhodamine 123. Furthermore, serum was added to the medium to more closely simulate the conditions in plasma. Finally, albumin and other components of bovine or equine serum in RPMI that bind to lipids or to features of the plant molecules containing radicals may alter the interactions between the radicals from BWEs with dihydrorhodamine 123.

Tissue culture plates were assessed for accumulated fluorescence at 37°C. In preliminary experiments, it was determined that the specific accumulated fluorescence signal peaked approximately 240 minutes after onset of incubation and that the nonspecific signal became prominent 300 to 360 minutes after onset of incubation. Therefore, the plates were incubated at 37°C and assessed again for accumulated fluorescence after incubation for 15, 30, 45, 60, 90, 120, 180, and 240 minutes. All assessments of accumulated fluorescence were performed by use of a fluorescent plate reader.[m]

Assessment of the influence of Ca^{2+} on accumulated fluorescence—Sensitivity of dihydrorhodamine 123 to radical production may be enhanced by Ca^{2+} ions. A 1M stock solution of $CaCl_2$[i] was prepared in sterile distilled water, and 0.1, 1, and 10mM Ca^{2+} aliquots were added to both of the balanced salt solution–based media. The accumulated fluorescence (production of rhodamine-123) was determined for each of these media as described.

Assessment of oxygen radical generation—Each of the black walnut stock solutions was prepared in PBS solution, PBG, and RPMI containing 10% FBS or 10% DHS. The initial concentration of all BWEs was 10%, then each was further diluted in a 2-fold series through 9 steps. Each of the 3 media without the inclusion of the BWEs was used as a negative control sample, and results were compared with results for optimal concentrations of hydrogen peroxide in the test medium as a maximum accumulated fluorescence standard. The

accumulated fluorescence was determined for each of these media as described.

Four replicates of 100-µL aliquots of each BWE dilution solution were transferred into individual wells of a standard 96-well tissue culture plate.[a] Then, a 10-µL aliquot of a dihydrorhodamine 123 working stock solution was added to each replicate-containing well. Plates were assessed for accumulated fluorescence and incubated at 37°C, and accumulated fluorescence was assessed again after incubation for 15, 30, 45, 60, 90, 120, 180, and 240 minutes. The accumulated fluorescence was determined for each of these media as described. Dose response to each BWE and the optimal incubation time for the conversion of dihydrorhodamine 123 to rhodamine-123 by radicals generated from each preparation in each medium were determined from results of accumulated fluorescence.

Effects of polymyxin B on generation of oxygen radicals—Polymyxin B binds to LPS and blocks the interaction of LPS with other molecules. When LPS-like activity was detected in samples of aqueous BWEs by use of the *Limulus* amebocyte lysate assay, 13, 130, and 1,300 U of polymyxin B[n]/mL of PBS solution was added to the extracts to determine whether LPS contributed to the generation of a maximal fluorescent signal. Briefly, a stock solution containing 13,000 U of polymyxin B/mL of PBS solution was prepared and then frozen at –20°C. An internal control sample was used to ensure that polymyxin B was capable of preventing the effects of LPS on the generation of a maximal fluorescent signal; furthermore, the internal control sample worked on the basis of the ability of 13 U of polymyxin B/mL of PBS solution to block dihydrorhodamine 123 conversion by equine neutrophils stimulated with 100 ng of *Escherichia coli* 0111:B4 LPS[o]/mL of RPMI without phenol red containing 10% FBS.

Effects of removal of LPS-like material with polymyxin B–coated beads—Beads covalently linked to polymyxin B[b] were prepared in accordance with the manufacturer's recommendations. Briefly, 25 mL of BWE was mixed with 5 mL of beads in a sterile 50-mL centrifuge tube. The mixture was placed on a hematology rocker and allowed to mix for 45 minutes at approximately 25°C. After the tube was mixed, it was centrifuged at 3,200 × *g* for 20 minutes at 4°C. The supernatant (fraction 1) was removed and retained. Then, the tubes containing the beads were washed by adding 10 mL of sterile PBS solution to the centrifuge tube, vortexing the tube 3 times for 30 seconds, and centrifuging the tube at 3,200 × *g* for 20 minutes at 4°C. The supernatant (fraction 2) was removed and retained. Next, the material bound to the beads was eluted by adding 25 mL of freshly prepared sodium deoxycholate[e] in accordance with the manufacturer's directions, mixing the beads and sodium deoxycholate on the hematology rocker for 45 minutes, and separating the beads from the elution solution by centrifugation as described. The elution procedure was performed twice, and the first and second elutions (fractions 3 and 4, respectively) were retained. The sodium deoxycholate solution from each elution was collected, and the sodium deoxycholate was removed from the eluate by use of a centrifugal filter[p] with a low–molecular-weight cutoff. Each of the 4 fractions was passed through a 0.2-µm sterile filter,[b] and the filtrate was divided into small aliquots and frozen at –20°C until tested. Samples (100 µL of each of the 4 fractions) were plated on 2 plates containing brain-heart infusion agar and on 2 plates containing trypticase soy agar,[c] incubated at 37°C, and examined after 24 and 48 hours of incubation. All the fractions were tested for their capacity to generate a maximal fluorescent signal and compared with results for the parent extracts.

Statistical analysis—Statistical analyses of the data were conducted by use of a software program.[q] The EC_{50} for hydrogen peroxide and BWEs was determined in each medium by use of the nonlinear curve-fitting routines (ie, sigmoidal dose response with variable slope). The rate of maximal fluorescent signal generation in each preparation was calculated via a linear best-fit slope comparison by use of a commercial spreadsheet software program.[r] In addition, comparisons of the production of maximal fluorescent signal among BWEs and between BWEs prepared in water and in *N*-hexane were performed by use of a 2-way ANOVA.[q] A value of $P < 0.05$ was used to indicate significance in all analyses.

Results

Bacterial contamination and LPS concentrations—The 3 preparations of thawed BWEs prepared in water contained 1,004, 736, and 121 bacterial colonies/mL before filtration. Evidence of bacterial growth after processing and filtration was not observed in any of the filtrates. However, on the basis of results obtained by use of a colorimetric *Limulus* amebocyte lysate assay, all BWEs tested were contaminated with LPS at concentrations between 50 and 200 ng/mL, and concentrations of LPS were not reduced by centrifugation or filtration of the extract.

Conversion of dihydrorhodamine 123 by hydrogen peroxide—Hydrogen peroxide maximally converted dihydrorhodamine 123 to rhodamine-123 at different concentrations and different durations of incubation in each the 4 media tested. The highest amounts of dihydrorhodamine 123 conversion and the lowest concentration of hydrogen peroxide that produced the maximal fluorescent signal were detected in RPMI containing FBS or DHS. In addition, the greatest amount of dye quenching was observed in samples tested in RPMI containing FBS or DHS. Dose response and the response time course for hydrogen peroxide conversion of dihydrorhodamine 123 to rhodamine-123 in RPMI containing FBS were determined (**Figure 1**); in addition, RPMI containing FBS yielded both the most sensitive and most efficient measure of hydrogen peroxide radicals tested.

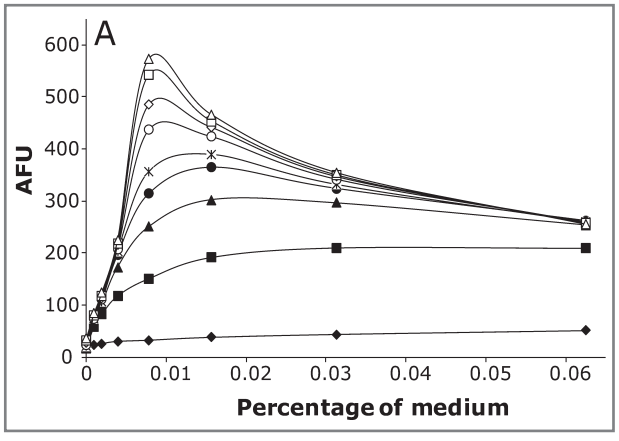
The maximal fluorescent signal induced by hydrogen peroxide accumulated progressively during the first 120 minutes of incubation and then remained stable through approximately 240 to 360 minutes of incubation in the RPMI prepared with FBS or DHS. High concentrations of hydrogen peroxide (ie, > 0.1% in RPMI) reduced the

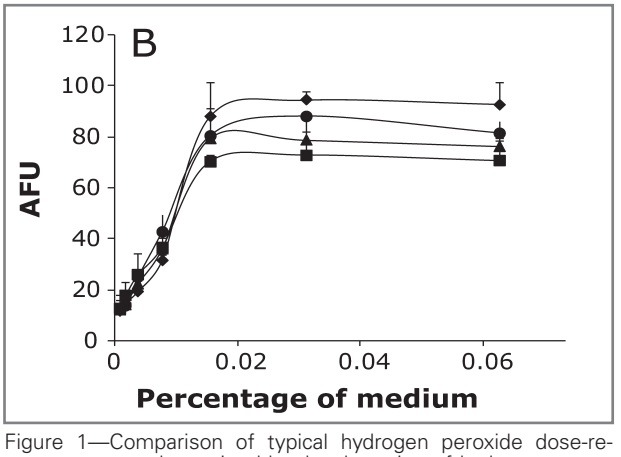
maximal fluorescent signal substantially, with the cumulative maximal fluorescent signal decreasing by ≥ 50% when hydrogen peroxide concentrations in serum containing RPMI were > 1% (data not shown); furthermore, there was evidence of quenching of the maximal fluorescent signal at concentrations of hydrogen peroxide > 0.02%. Significant quenching of the maximal fluorescent signal was detected when samples were incubated for ≥ 90 minutes.

Conversion of dihydrorhodamine 123 to rhodamine-123 by hydrogen peroxide in PBS solution or PBG was also assessed, and the maximal fluorescent signal was observed after incubation for 2 hours (**Figure 1**). Significant increases in the maximal fluorescent signal generated by 0.2%, 0.3%, and 0.6% hydrogen peroxide were observed when 10mM $CaCl_2$ was added to the PBS solution, compared with the maximal fluorescent signal generated by 0.2%, 0.3%, and 0.6% hydrogen peroxide in PBS solution alone. However, the addition of 0.1mM or 1mM $CaCl_2$ to PBS solution did not significantly enhance hydrogen peroxide–induced generation of the maximal fluorescent signal in PBS solution, and there was not a significant enhancement of hydrogen peroxide–induced generation of the maximal fluorescent signal when $CaCl_2$ was added to PBG.

Hydrogen peroxide generated a maximal fluorescence signal of 600 AFUs in RPMI containing FBS, 450 AFUs in RPMI containing DHS, 250 AFUs in PBG, and 60 AFUs in PBS solution. Peak maximal fluorescent signal was observed with concentrations of hydrogen peroxide at 0.008% (EC_{50}, 0.002%) in RPMI containing FBS or DHS, 0.06% (EC_{50}, 0.01%) in PBG, and 0.25% (EC_{50}, 0.15%) in PBS solution.

Assessment of radical production by BWEs prepared in water or *N*-hexane—Generation of fluorescence over time was assessed in each medium for the 3 BWEs prepared in water and the BWE prepared in *N*-hexane. Maximal fluorescent signal was observed after incubation for 240 minutes. Concentration response curves for 4 replicates of each of the 3 BWEs prepared in water and for 4 replicates of the BWE prepared in *N*-hexane were performed by use of each of the media tested (**Figure 2**). The BWEs prepared in water generated the strongest maximal fluorescent signal in PBS solution or in RPMI containing FBS and significantly weaker maximal fluorescent signals in PBG or RPMI containing DHS. In contrast, the BWE prepared in *N*-hexane generated the strongest maximal fluorescent signal in PBG, an intermediate maximal fluorescent signal in PBS solution or RPMI containing DHS, and the weakest maximal fluorescent signal in RPMI containing FBS. The signal generated by the BWE prepared in *N*-hexane in PBG was significantly greater than the signal generated in any other medium, and the signal gen-





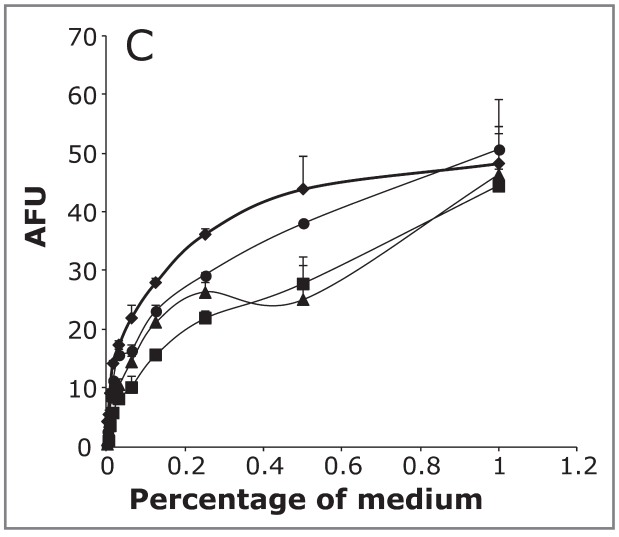


Figure 1—Comparison of typical hydrogen peroxide dose-response curves determined by the detection of hydrogen peroxide in aqueous solutions of BWE by the conversion of dihydrorhodamine 123 to rhodamine-123. A—A comparison of the typical hydrogen peroxide dose-response curves for 0.0001% to 0.06% hydrogen peroxide (vol/vol) in RPMI without phenol red[k] containing 10% FBS after incubation for 0 (black diamonds), 15 (black squares), 30 (black triangles), 45 (black circles), 60 (crosses), 90 (white circles), 120 (white diamonds), 180 (white squares), and 240 minutes (white triangles). Similar typical hydrogen peroxide dose-response curves for 0.0001% to 0.06% hydrogen peroxide (vol/vol) in RPMI containing DHS were constructed; hydrogen peroxide concentrations > 0.6% rapidly reduced the fluorescence of dihydrorhodamine 123 in RPMI containing DHS. B—A comparison of the typical hydrogen peroxide dose-response curves for 0.0001% to 0.625% hydrogen peroxide (vol/vol) in PBS solution after incubation with 0 (black diamonds), 0.1 (black triangles), 1 (black squares), and 10mM Ca^{2+} (black circles) for 120 minutes. C—A comparison of the typical hydrogen peroxide dose-response curves for 0.0001% to 0.625% hydrogen peroxide (vol/vol) in PBS solution containing 0.5% tissue culture–grade bovine serum albumin[i] and 5mM glucose[i] (PBG) after incubation with 0 (black diamonds), 0.1 (black triangles), 1 (black squares), and 10mM Ca^{2+} (black circles) for 120 minutes. Notice that each data point in each panel is representative of the mean value calculated from the results of 4 samples/preparation.

erated by an extract in PBS solution or RPMI with DHS was significantly greater than the signal generated by the same extract in RPMI with FBS.

The BWE prepared in *N*-hexane had EC_{50} values of 0.14%, 0.13%, 0.18%, and 0.25% in PBG, PBS solution, RPMI containing DHS, and RPMI containing FBS, respectively. The concentration of BWEs prepared in water (10%) required to generate a maximal fluorescent signal was approximately 20 times as high as the concentration of the BWE prepared in *N*-hexane (0.5%).

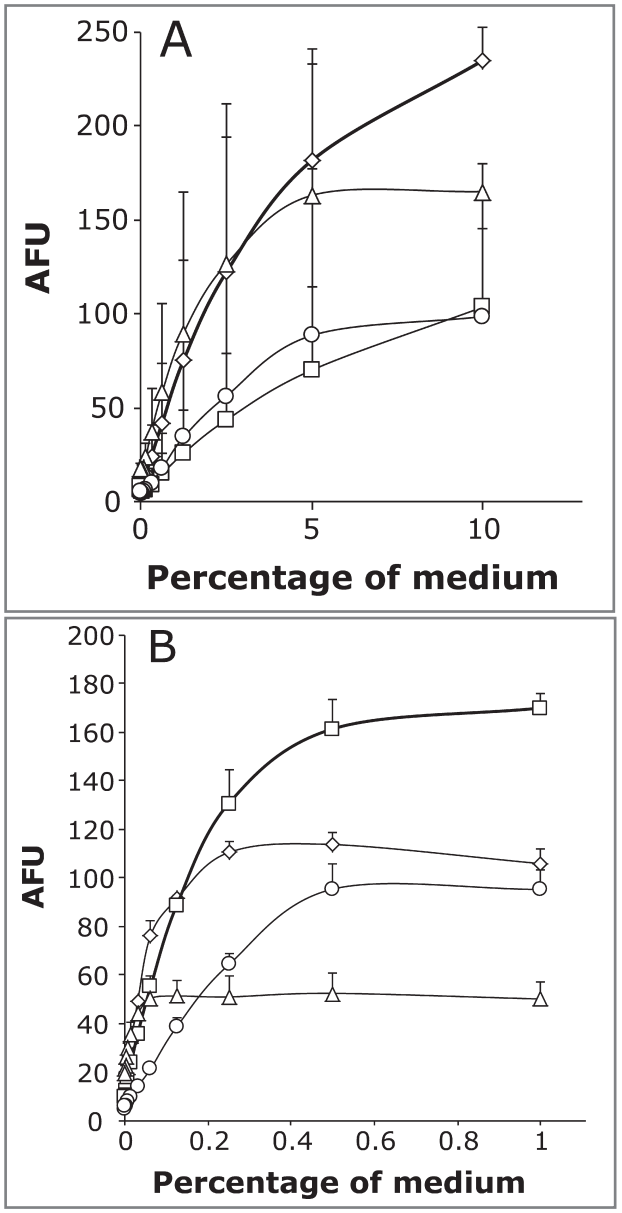


Figure 2—A comparison of the effects of the conversion of dihydrorhodamine 123 to rhodamine-123 by BWEs prepared in water (A) and *N*-hexane (B) and that were added to PBS solution (white diamonds), PBG (white squares), RPMI containing FBS (white triangles), and RPMI containing DHS (white circles). Fluorescence for the BWE prepared in *N*-hexane decreased 50% to 80% as the BWE concentration in the medium exceeded 1%. Values reported are the mean ± SD of maximal fluorescent signal values determined for 4 samples/preparation.

Assessment of radical production in PBS solution or PBG—Maximal fluorescent signals generated by all 4 BWEs in PBS solution were compared. The 3 BWEs prepared in water generated a stronger maximal fluorescent signal at a faster rate (approx 200 AFUs and 0.76 to 1.09 AFUs/min, respectively), compared with the maximal fluorescent signal and rate (100 AFUs and 0.46 AFUs/min, respectively) for the BWE prepared in *N*-hexane (**Figure 3**). The relationship between incubation time and generation of fluorescence was determined by use of linear regression analysis (R^2 = 0.99).

Generation of a maximal fluorescent signal by the BWEs prepared in water and in *N*-hexane was markedly different than when prepared in PBG. The BWE prepared in *N*-hexane generated a higher maximal fluorescent signal at a faster rate (180 AFUs and 0.76 AFUs/min, respectively), compared with the maximal fluorescent signal and rate (100 AFUs and 0.46 to 0.59 AFUs/min, respectively) of the BWEs prepared in water. The relationship between incubation time and generation of fluorescence was determined by use of linear regression analysis (R^2 = 0.93 to 0.99).

Assessment of oxygen radical production in RPMI containing FBS—All 3 BWEs prepared in water rapidly and progressively generated a maximal fluorescent signal in RPMI containing 10% FBS, and the generated signal increased with increasing incubation time (R^2 = 0.90 to 0.94; **Figure 4**). Furthermore, the rate (0.74 AFUs/min) at which fluorescence was generated in RPMI with FBS for these 3 BWEs exceeded the rate (1.2 AFUs/min) obtained in PBS solution.

Generation of maximum fluorescent signals by BWEs prepared in water progressed more slowly in RPMI containing 10% DHS (0.44 AFUs/min) than in RPMI containing 10% FBS (1.2 AFUs/min) and achieved a peak maximal fluorescent signal of only 100 AFUs. This pattern of radical production was similar to that observed in PBG.

Generation of maximal fluorescent signal by the BWE prepared in *N*-hexane in RPMI containing 10% FBS differed markedly from that observed in PBS solution or PBG. The maximal fluorescent signal increased rapidly for approximately 120 minutes to a plateau (approx 50 AFUs); in addition, the value of that signal was approximately 30% of the value achieved when the BWEs were incubated in PBG. Data from the replicates performed with BWE prepared in *N*-hexane were fitted (R^2 = 0.97) optimally to a second-order polynomial regression model as opposed to a linear model. Generation of a maximal fluorescent signal by BWE prepared in *N*-hexane in RPMI containing 10% DHS was similar to that observed in PBS solution. Generation of a maximal fluorescent signal relative to incubation time was fitted (R^2 = 0.90) optimally by use of a linear regression model and at a rate (0.48 AFUs/min) that was intermediate between the rates observed in PBS solution and RPMI containing FBS (0.46 and 1.20 AFUs/min, respectively); furthermore, the peak in maximal fluorescent signal was 95 AFUs.

Role of LPS in radical production—On the basis of the low amount of bacterial growth on brain-heart infusion agar (100 to 1,000 CFUs/mL) in samples of the BWEs prepared in water obtained before final process-

ing and detection of LPS in those extracts by use of the *Limulus* amebocyte lysate assay, the relative impact of LPS on oxygen radical production was measured in 2 ways. First, LPS, when tested at concentrations as high as 1 mg/mL, did not generate a maximal fluorescent signal during a 240-minute incubation period. Second, the addition of 13, 130, or 1,300 U of polymyxin B/mL directly to the medium of the assay did not cause a reduction in the generated maximal fluorescent signal by any of the 3 BWEs prepared in water when they were incubated in PBS solution, which was the most sensitive test for assessing radical production.

Because it was determined that LPS was not directly responsible for the production of the maximal fluorescent signal and that polymyxin B did not reduce the signal generated by the BWEs, experiments were performed to determine whether constituents of the BWEs that bound polymyxin B were associated with the generation of a maximal fluorescent signal. The 2 BWEs with the greatest activity for the *Limulus* amebocyte lysate assay were incubated with beads covalently linked to polymyxin B to determine whether the components of the BWEs responsible for generating the maximal fluorescent signal could be removed.

Bacterial growth was not observed in any of the fractions recovered from the bead processing after passing the fractions through the 0.2-µm membrane filter. Fraction 1 had a greater reduction in the amount of LPS detected by use of the *Limulus* amebocyte lysate assay,[e] in which the amount in fraction 1 was approximately 10% of that in the parent extract and was near the functional detection limit (1 ng/mL) of the assay as applied to the colored BWE for each of the BWEs assayed (n = 2). For each of these preparations, the amount of LPS detected in fraction 2 was between 50% and 60% of the amount of LPS detected in the parent extract assayed (approx 100 ng/mL). Fractions 3 and 4 had high amounts of LPS and were similar to or higher than that in the parent extract (220 and 370 ng/mL).

Nearly 100% of the capacity to generate a maximal fluorescent signal from these 2 BWEs was recovered in the supernatant, which is the nonbinding fraction (fraction 1), collected from the beads (**Figure 5**). Because the extracts stained the columns black and the bound material could not be completely removed during subsequent elutions, it was not possible to determine those components of the BWEs that remained bound to the beads or to the polymyxin B covalently linked to the beads. Because the component responsible for the capacity of the BWEs to generate a maximal fluorescent signal had been recovered in the nonbinding fraction, the bound components did not appear to contribute to the generation of a maximal fluorescent signal. Less than 10% of the activity of either BWE was recovered from the wash fraction (fraction 2) or from either of the detergent elutions (fractions 3 and 4) col-

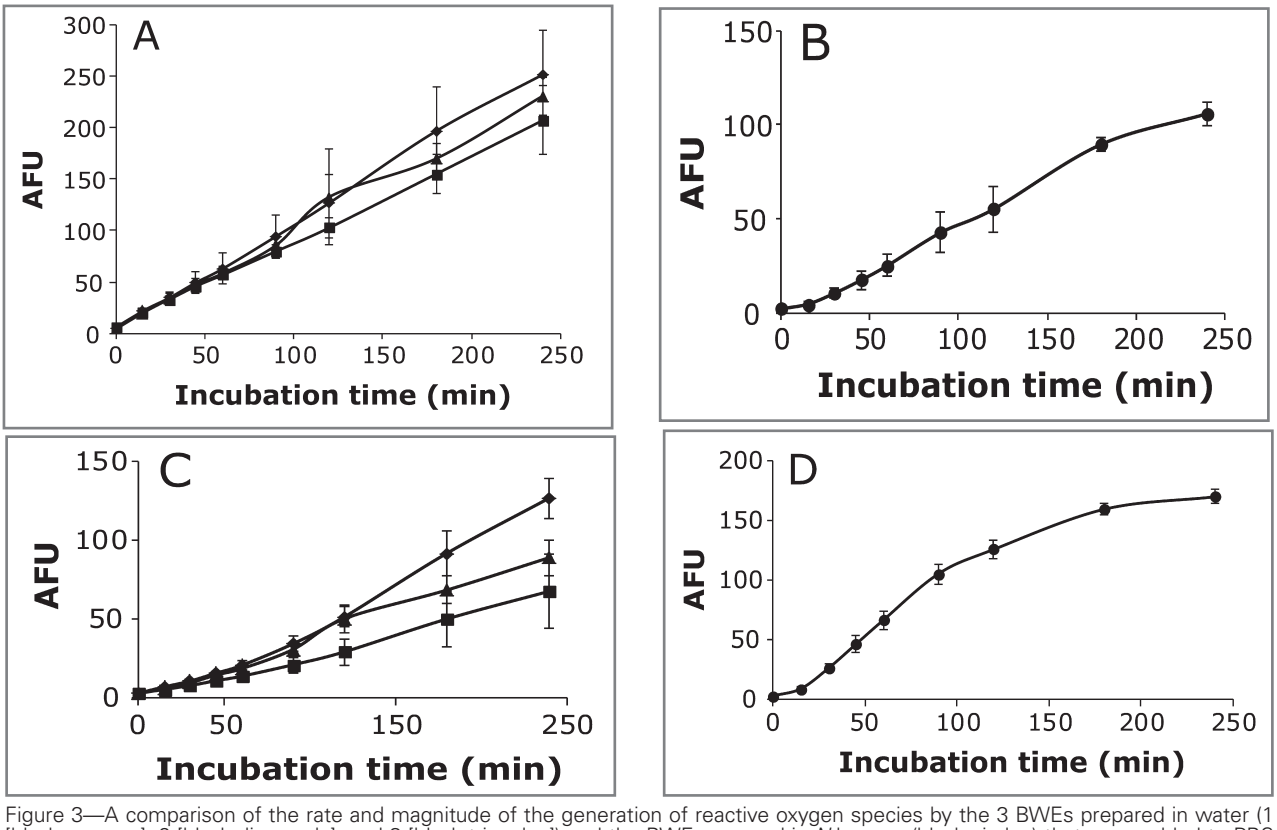


Figure 3—A comparison of the rate and magnitude of the generation of reactive oxygen species by the 3 BWEs prepared in water (1 [black squares], 2 [black diamonds], and 3 [black triangles]) and the BWE prepared in *N*-hexane (black circles) that were added to PBS solution (A and B) and PBG (C and D). Samples were incubated and collected throughout a 240-minute incubation period. Each BWE was tested at its optimal concentration in each medium (BWEs prepared in water, 10%; BWE prepared in *N*-hexane, 0.5%). Values reported are the mean ± SD of maximal fluorescent signal values determined for 4 samples/preparation. The rate of generation of the maximal fluorescent signal in each preparation was calculated via a linear best-fit slope comparison by use of a commercial spreadsheet software program[r]; data for all extracts were fitted to a linear model.

lected from the beads. These results suggested that the components of the BWEs responsible for generating a maximal fluorescent signal did not bind to the beads covalently linked to polymyxin B.

Discussion

To our knowledge, this is the first study in which investigators examined the capacity of BWEs to generate reactive oxygen species. In this study, we determined that BWEs prepared in water or *N*-hexane were potent producers of reactive oxygen species in a simple salt solution as well as in more complex media that contained albumin or serum. On the basis of the results of the study reported here, it appears that intragastric administration of BWE in horses could provide an external source of reactive oxygen species that may have the capacity to cause tissue damage directly or indirectly by entering the systemic circulation to participate in the pathogenesis of laminitis. Concentration response curves were generated by including hydrogen peroxide in the test media to provide a method for comparing the reactive oxygen species–generating capacities of these BWEs. In those experiments, production of reactive oxygen species by BWEs prepared in water or *N*-hexane, which were included as 10% or 0.5% of the medium, respectively, was equivalent to the ability of 0.01% to 0.1% hydrogen peroxide to convert dihydrorhodamine 123 to the fluorescent indicator rhodamine-123. Although we are not aware of any investigations in which the effects of reactive oxygen species on intestinal epithelial cells have been examined, results of studies[24] performed by use of U937 monocytes in culture indicate that hydrogen peroxide at concentrations similar to those used in the present investigation resulted in dose-dependent cell death. This suggests that the reactive oxygen species measured in the present study may contribute directly to cellular damage or death in the tissues of horses.

A second important finding of the present study was that the production of maximal fluorescent signal by BWEs prepared in water was highly efficient in the medium that simulated plasma, namely RPMI containing FBS. Furthermore, these BWEs generated a stronger maximal fluorescent signal when the medium contained FBS rather than DHS. This difference may reflect the differential diversity of lipids in DHS, compared with the diversity of lipids in FBS.[25] The differential distribution of lipids in DHS and FBS may account for differences in the generation of reactive oxygen species between the 2 serum sources in a study[24] performed by use of human and mouse cell lines. The FBS has a higher ratio of oleic (18:1) and docosahexaenoic (22:6) acids than does DHS, but DHS has a greater ratio of arachidonic (20:4), steric (18:0), and linolenic (18:2)

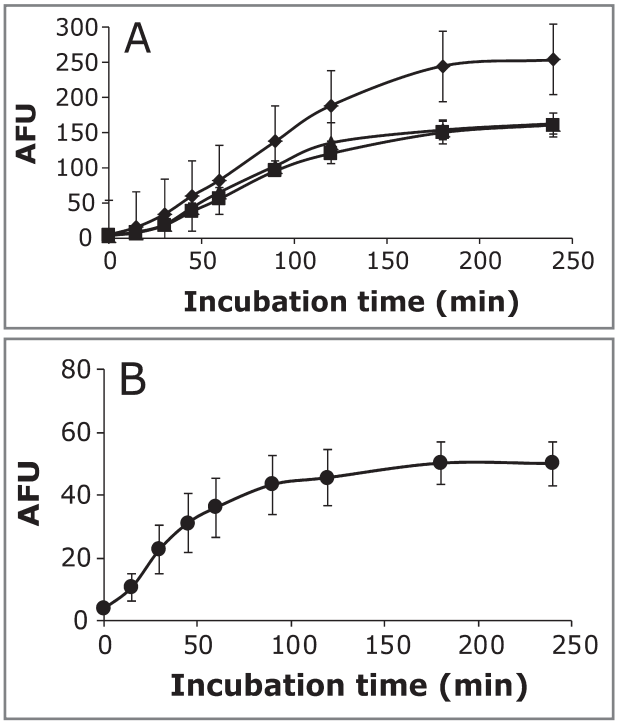


Figure 4—A comparison of the rate and magnitude of reactive oxygen species generation by the 3 BWEs prepared in water (1 [black squares], 2 [black diamonds], and 3 [black triangles]; A) and the BWE prepared in *N*-hexane (black circles; B) that was added to RPMI containing 10% FBS. Values reported are the mean ± SD of maximal fluorescent signal values determined for 4 samples/preparation. The rate of generation of the maximal fluorescent signal in each preparation was calculated via a linear best-fit slope comparison by use of a commercial spreadsheet software program[f]; data for all extracts prepared in water and *N*-hexane were fitted to a linear model (A) and a second-order polynomial model (B), respectively.

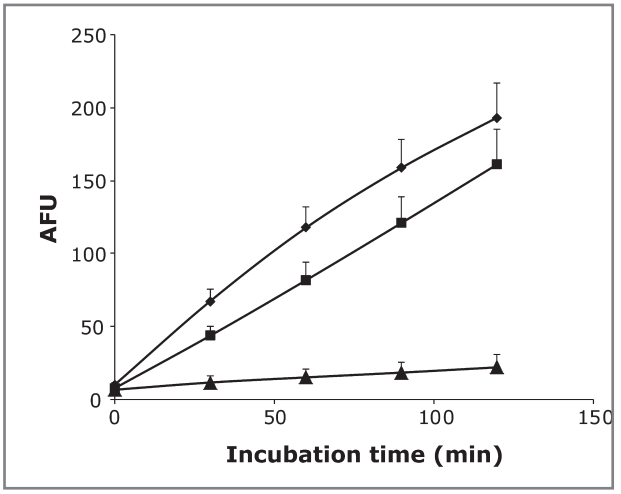


Figure 5—Separation of reactive oxygen species–generating activity and LPS-like activity by fractionation of 2 BWEs prepared in water, which had the greatest LPS-like activity as determined on the basis of results of a *Limulus* amebocyte lyaste assay,[e] with beads covalently linked with polymyxin B. The aqueous BWE preparation 2 (as designated in Figures 3 and 4; black diamonds) is referred to as the parent extract. Fraction 1 (black squares) is designated as the supernatant obtained after centrifugation of a mixture of 25 mL of BWE and 5 mL of beads. Fraction 2 (black triangles) is designated as the supernatant obtained after the subsequent washing of the beads with 10 mL of PBS solution and centrifugation. Fractions 3 and 4 (not shown) are designated as the first and second elutions collected after fraction 2 was collected from the beads; these fractions were eluted by the addition of sodium deoxycholate, agitation for 45 minutes, centrifugation (fraction 3), and repetition of these 3 steps (fraction 4). Fraction 1 contained 100% (when allowing for the measurement error) of the reactive oxygen species–generating activity of the unfractionated (parent) BWE, whereas the buffer wash fraction (2) and the LPS elution (with sodium deoxycholine) fractions (3 and 4) contained approximately 10% of the reactive oxygen species–generating activity of the original BWE preparations.

acids than does FBS.[25,26] Furthermore, FBS is much higher in monounsaturated fatty acids (34%) but lower in polyunsaturated fatty acids (28%), compared with the amounts of monounsaturated (19%) and polyunsaturated (46%) fatty acids in DHS.

In contrast, the maximal fluorescent signal produced by the BWE prepared in *N*-hexane was greater when the medium contained DHS than when it contained FBS. It is possible that the components of the radicals extracted from plant tissue in the organic extract responsible for generating reactive oxygen species were stabilized by interactions with specific lipid species or lipid-binding proteins in DHS that are not equivalently represented in FBS. Consequently, these components may have enhanced the ability of the BWE prepared in *N*-hexane to generate reactive oxygen species after interacting with water molecules. These observations suggest that the extraction of BWEs with water from black walnut shavings yields a different population of plant-derived radicals capable of generating reactive oxygen species than does the extraction of BWEs with *N*-hexane from black walnut shavings. The principal difference between the BWEs prepared in water and *N*-hexane appeared to be that the generation of reactive oxygen species by the BWEs prepared in water was not as strongly facilitated by interaction with the lipid-binding proteins, particularly bovine serum albumin, as was the BWE prepared in *N*-hexane. Thus, the BWE prepared in *N*-hexane may have contained lipid-associated, plant-derived radicals that were not equivalently extracted with water. A nonpolar family of naphthoquinone compounds is routinely extracted from black walnut seeds or woody samples as the starting material in the purification of toxin preparations.[27] It is possible that the differential elution of the naphthoquinone compounds by water and *N*-hexane accounted for much of the difference observed in the generation of reactive oxygen species by these BWEs in the present study.

Generation of reactive oxygen species is a hallmark of inflammatory activation of neutrophils, and excessive production by these cells is associated with tissue damage.[28] There is convincing evidence that circulating neutrophils are activated in horses during the development of laminitis secondary to administration of aqueous BWEs of black walnut shavings and that these activated cells migrate into the laminar tissues.[6–12] Furthermore, the nitrogen radical nitric oxide is produced by vascular endothelial cells as a mechanism for controlling vasoconstriction.[13] The combination of oxygen and nitrogen radicals leads to the production of nitrotyrosine adducts in vessels and is associated with impairment of vascular function.[13,29,30] Thus, it is possible that the combined production of radicals by the BWE, activated neutrophils, and vascular endothelial cells may contribute to the impairment of laminar microvascular function that is evident in this disease.[4,13]

We also determined that neither LPS nor other components of the BWEs prepared in water that bound to polymyxin B were a factor in the generation of reactive oxygen species in the media used for the experiments of the study reported here. Although it is not possible to discount a potential additive or synergistic role for LPS or other microbial cell wall products in the development of laminitis after administration of the aqueous BWEs collected from the black walnut shavings, it is likely that the condition is initiated and promoted by the direct generation of reactive oxygen species induced by interactions between BWE components and free water or compounds associated with feed located in the intestinal tract of a horse.

The results of the present study are consistent with a role of reactive oxygen species produced by components of BWEs in the pathogenesis of laminitis in horses. In the present study, all BWEs tested generated reactive oxygen species in solutions that mimic those found in horses. Production of reactive oxygen species in RPMI containing FBS or DHS persisted for 240 minutes, which suggested that sustained production of reactive oxygen species by components of the BWEs is possible in horses. Furthermore, a marked leukopenia is generally induced approximately 3 to 4 hours after the intragastric administration of aqueous BWE in horses that progress to Obel grade 1 laminitis.[6] Thus, it is possible that reactive oxygen species generated by components of the BWE within the aqueous milieu of the intestinal tract of horses may be a factor in the activation of leukocytes and can enhance their emigration into the tissues.

Notably, laminitis induced by exposure to black walnut shavings typically progresses to Obel grade 2 lameness, and the clinical signs generally subside with minimal treatment.[2] This may be related to the fact that the direct generation of reactive oxygen species by components of BWEs and their release into the body can be exhausted. After a finite period, the components of the extract that generate reactive oxygen species may no longer be functional, which would allow homeostasis to be restored in the affected tissues.

From the data provided in the study reported here, it appears that BWEs used to induce laminitis in horses contain components that produce reactive oxygen species when in an aqueous environment. When considered in light of information reported regarding the role of reactive oxygen species in the pathogenesis of laminitis,[13] it seems reasonable to conjecture that the direct production of reactive oxygen species described in the present study may be important in the initiation of acute laminitis induced by aqueous BWEs collected from black walnut shavings. Still, it is clear that additional studies are necessary to provide direct evidence of a role for reactive oxygen species generated from BWEs in the pathogenesis of laminitis in horses.

a. BD, Franklin Lakes, NJ.
b. Thermo-Fisher Scientific, Pittsburgh, Pa.
c. Difco, Detroit, Mich.
d. 0.2-µm Whatman GD/X glass fiber/membrane filters, Fisher, Pittsburgh, Pa.
e. Associates of Cape Cod, East Falmouth, Mass.
f. Organic Chemistry Laboratory, Department of Chemistry, Hamline University, Saint Paul, Minn.
g. Whatman fast flow No. 1 filter paper, Fisher, Pittsburgh, Pa.
h. The Comparative Inflammation Research Laboratory, College of Veterinary Medicine, University of Georgia, Athens, Ga.
i. Sigma, St Louis, Mo.
j. Molecular Probes, Eugene, Ore.
k. Media Tech, Herndon, Va.

l. Hyclone, Logan, Utah.
m. Thermo, Albertville, Minn.
n. Bedford Laboratories, Bedford, Ohio.
o. List Biologicals, Campbell, Calif.
p. Millipore/Amicon Ultra 5K MWCO centrifugal filter, Fisher, Pittsburgh, Pa.
q. GraphPad Prism, version 4, GraphPad Software Inc, San Diego, Calif.
r. Microsoft Excel, version 11.6.2, Microsoft Corp, Redmond, Wash.

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