Iron metabolism following intravenous transfusion with stored versus fresh autologous erythrocyte concentrate in healthy dogs

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OBJECTIVE
To determine effects of IV transfusion with fresh (3-day-old) or stored (35-day-old) autologous erythrocyte concentrate on serum labile iron concentration, iron-binding capacity, and protein interaction with iron in dogs.

ANIMALS
10 random-source healthy dogs.

PROCEDURES
Dogs were randomly assigned to receive autologous erythrocyte concentrate stored for 3 days (n = 5) or 35 days (5). One unit of whole blood was collected from each dog, and erythrocyte concentrates were prepared and stored as assigned. After erythrocyte storage, IV transfusion was performed, with dogs receiving their own erythrocyte concentrate. Blood samples were collected from each dog before and 5, 9, 24, 48, and 72 hours after transfusion. Serum was harvested for measurement of total iron, labile iron, transferrin, ferritin, hemoglobin, and haptoglobin concentrations.

RESULTS
For dogs that received fresh erythrocytes, serum concentrations of the various analytes largely remained unchanged after transfusion. For dogs that received stored erythrocytes, serum concentrations of total iron, labile iron, hemoglobin, and ferritin increased markedly and serum concentrations of transferrin and haptoglobin decreased after transfusion.

CONCLUSIONS AND CLINICAL RELEVANCE
Transfusion with autologous erythrocyte concentrate stored for 35 days resulted in evidence of intravascular hemolysis in healthy dogs. The associated marked increases in circulating concentrations of free iron and hemoglobin have the potential to adversely affect transfusion recipients. (Am J Vet Res 2015;76:996–1004)

Erythrocyte transfusion is helpful for the management of various disease processes. Even with pretransfusion screening of donors, crossmatching of donor and recipient blood types, and appropriate storage and administration of blood products, transfusion reactions can occur. Most reactions are mild and self-limiting, resulting in transient fever, vomiting, or facial swelling.1 However, more severe reactions such as acute hemolysis and anaphylactic shock can also occur.5–7

Attention has been increasing to the potential serious sequelae of febrile, nonhemolytic transfusion reactions and possible adverse consequences of transfusion on patient outcomes.3,8,9 Multiple studies8,10–12 have revealed potential associations between duration of erythrocyte storage and the likelihood of adverse outcomes in humans receiving transfusions, and clinical trials13–17 to address this issue are ongoing. Transfusion with erythrocytes stored for > 21 days has been associated with complications such as sepsis,11 pneumonia,11,18,19 multiorgan failure,20 acute renal failure,11 thrombosis,21 and death in humans.8,22,23 We previously identified a marked inflammatory response in dogs following transfusion with autologous erythrocyte concentrate stored for 21 days.24 In another study,25 an inflammatory response was identified in dogs that received 28-day-old autologous erythrocyte concentrate. Yet another study24 revealed a profound inflammatory response in dogs that received 35-day-old autologous erythrocyte concentrate, whereas no response was evident in the control group that received 3-day-old erythrocytes. In a large retrospective study,26 prolonged storage of erythrocyte concentrates (> 14 days vs < 14 days) was identified as a risk factor for nonsurvival in dogs with hemolysis.26 In dogs with experimentally induced bacterial pneumonia, transfusion with 42-day-old erythrocytes was associated with a higher likelihood of death and more severe lung damage than transfusion with 7-day-old erythrocytes.27,28

The biochemical and biomechanical changes that occur during storage of erythrocytes (ie, storage lesions) include cell shape changes, membrane vesiculation, hemolysis, and enzymatic and oxidative damage.29 Amounts of inflammatory cytokines (particular-
ly interleukin-8 
and microparticles can increase in canine erythrocyte concentrates with increasing storage duration. The sum effect of the storage lesions is a decrease in posttransfusion viability of erythrocytes. Precise mechanisms responsible for a decrease in the amount of surviving erythrocytes remain unclear, but an increase in storage duration has been associated with a decrease in amounts of erythrocytes surviving after transfusion in humans. 

Findings in humans and other animals indicate that transfusion with erythrocyte concentrate stored for long periods (eg, 21 to 42 days) is followed by an accelerated clearance of cells. Damaged transfused cells are removed by macrophages in the spleen and liver of recipients. Phagocytic digestion of the erythrocytes liberates iron, which then enters systemic circulation, eventually exceeding the transport capacity of plasma transferrin. Circulation of non–transferrin-bound iron is associated with various adverse effects, including an increase in the probability of infection and formation of hydroxyl radicals. Circulating concentrations of transferrin- and non–transferrin-bound iron increase in dogs with experimentally induced bacterial pneumonia in response to transfusion with stored erythrocytes. Blood iron concentration and iron binding capacity have yet to be determined for healthy dogs receiving stored erythrocyte concentrate.

The objectives of the study reported here were to measure serum concentrations of labile iron and proteins that interact with iron as well as blood iron-binding capacity in dogs receiving an IV transfusion with autologous erythrocyte concentrate stored for 3 days (fresh erythrocytes) or 35 days (stored erythrocytes). We hypothesized that transfusion with older erythrocytes would be associated with changes in blood iron-binding capacity because of the acute-phase inflammatory response, saturation of iron-binding proteins, and consequent increase in the amount of labile iron in circulation.

Materials and Methods

Animals

Ten mixed-breed dogs, each weighing approximately 20 kg (range, 18 to 22 kg), were used in the study. All dogs were assessed as healthy on the basis of unremarkable results of physical examination, CBC, and serum biochemical analysis. Dogs were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and cared for in accordance with requirements of the National Institutes of Health. On the first day of the study (day 0), dogs were randomly allocated by use of a random number generator to provide blood for and to receive autologous erythrocyte concentrate stored for either 3 days (fresh erythrocytes; n = 5) or 35 days (stored erythrocytes; 5).

The study was approved by the institutional animal care and use committee and was part of a larger research project involving 3 simultaneously conducted studies designed to investigate differences in the impact of transfusion of stored versus fresh autologous erythrocytes on recipient iron stores and iron metabolism (present study), inflammation (unpublished data), and hemostasis (unpublished data). The same dogs used in the present study were used in the other 2 studies. Although the overarching aim of the 3 studies was to evaluate the safety of current transfusion practices, specific hypotheses and diagnostic approaches in each investigation were distinct from one another.

Preparation of erythrocyte concentrates

One unit of whole blood was collected from each dog and processed for erythrocyte concentration on the first day of the study and again 32 days later. Only 1 of these 2 units (ie, the assigned concentrate) was used in the present study; the other was used in a different study. Immediately prior to collection, dogs were sedated by IM administration of atropine (0.01 mg/kg), dexmedetomidine hydrochloride (2 to 3 µg/kg), and butorphanol tartrate (0.05 mg/kg). A peripheral venous catheter was placed in a jugular vein, and a single unit of blood (420 g) was aseptically and atraumatically collected into a conventional triple-bag system, which contained 100 mL of cell preservative. This system contained a collection bag (for whole blood) and 2 additional satellite bags. After collection, the whole blood can be centrifuged to allow erythrocytes to be expressed into one satellite bag and plasma to be expressed into the other satellite bag. At that point, the satellite bags can be separated, clamped, and stored. As soon as blood collection was complete, sedation was reversed in each dog by IM administration of atipamezole (0.01 mg/kg). Lactated Ringer solution was infused through the catheter to partially replace the blood volume removed during collection.

Whole blood units were separated into components via centrifugation at 1,510 × g for 15 minutes at 10°C. The plasma component was then expressed into 1 satellite bag. For the second whole blood unit only, separated plasma was administered back to the donor (to replace the plasma proteins) via the peripheral catheter over a 2-hour period. Cell preservative (100 mL) was transferred from the satellite bag to the erythrocytes. Erythrocyte concentrates were stored upright at 4°C and gently mixed every 2 days.

Transfusions and blood sample collection

Three days after the second set of whole blood units was collected (35 days after the study began), dogs were prepared for transfusion with their particular erythrocyte concentrate. Prior to transfusion, a 21-gauge collection set was used to collect blood samples from each dog directly into evacuated tubes containing EDTA or no additive for hematologic analysis. An aliquot of erythrocyte concentrate was also...
aseptically collected from the erythrocyte concentrate and submitted to the Veterinary Diagnostic Laboratory for aerobic bacterial culture. For transfusion, a peripheral venous catheter was aseptically placed in a cephalic vein of each dog. Transfusion with the dog’s particular erythrocyte concentrate was then performed after a 2-hour period via gravity-assisted flow. Blood samples for hematologic analysis were again collected as described 5, 9, 24, 48, and 72 hours after transfusion was initiated.

Hematologic analysis
All EDTA-treated blood samples were submitted to the Veterinary Diagnostic Laboratory for CBCs. Blood samples in plain tubes were allowed to clot at room temperature (approx 23°C) for 1 hour, and then centrifuged at 3,000 X g for 10 minutes. Serum was harvested, and serum aliquots were then flash frozen and stored at –80°C until analysis. Serum aliquots were submitted to a different laboratory for measurement of analytes indicative of iron content and metabolism.

Serum transferrin concentration was measured by use of an ELISA in accordance with the manufacturer’s instructions. Serum labile iron concentration was measured via bleomycin assay, as described elsewhere. Briefly, all assay components (except bleomycin) were pretreated with chelating agent (30 mg/mL) to remove contaminant metals. Assay reagent was prepared to contain type 1 DNA from calf thymus (625 µg/mL), 1 mM ascorbic acid, 1 mM HCl (to adjust the pH to 7.4), 6.25 mM MgCl₂, and bleomycin (100 U/L). Serum samples were diluted 17-fold with assay reagent, then incubated at 37°C for 1 hour. Assay reaction was stopped by the addition of EDTA to achieve an EDTA concentration of 10 mM. Thiobarbituric acid (5 mg/mL) and 775 mM HCl were added, and the mixture was incubated at 80°C for 20 minutes. The mixture was then cooled, and isobutanol was added to achieve an isobutanol concentration of 60% and to elicit a color change indicative of the presence of iron (ie, extraction of the color created by the reaction into the isobutanol layer). Samples were added individually to wells of a polypropylene 96-well plate, and absorbance of the isobutanol layer at 535 nm was measured with a microplate reader.

Serum ferritin concentration was measured by use of an ELISA, as previously described. Serum cell-free hemoglobin concentration was measured by use of Drabkin reagents, with comparison to a hemoglobin standard solution. Serum haptoglobin concentration was measured by use of an ELISA in accordance with the manufacturer’s instructions.

Statistical analysis
All statistical analyses were performed by use of a commercial software program. Results of hematologic tests were analyzed for normality of distribution by means of the Shapiro-Wilk test. Non-normally distributed data are reported as median and range, and normally distributed data are reported as mean and SD. Two-way repeated-measures ANOVA was used to test for differences between the group that received fresh erythrocytes and the group that received stored erythrocytes and for differences among measurement points within groups. When a significant effect of group or time point was identified, Dunnett multiple pairwise comparisons were performed. Values of P < 0.05 were considered significant.

Results
For each of the 10 dogs in the study, 2 U of whole blood was collected for preparation of autologous erythrocyte concentrate and a complete set of pre- and posttransfusion blood samples was obtained for measurement of hematologic variables. No bacterial growth was obtained after bacterial culture of samples obtained from erythrocyte concentrates at the time of transfusion. Each dog was normocytic prior to transfusion with autologous erythrocyte concentrate, and Hct did not differ between dogs that received fresh (3-day-old) autologous erythrocyte concentrate and those that received stored (35-day-old) concentrate at any measurement point (Figure 1).

None of the variables used to characterize iron content and metabolism in serum samples differed significantly between the 2 groups at baseline (immediately prior to transfusion); however, after transfusion, significant differences became evident. Values for dogs that received stored erythrocytes changed significantly with time, whereas most values for dogs that received fresh erythrocytes did not change significantly. Specifically, after transfusion with fresh erythrocytes, serum total iron, trans-
ferrin, ferritin, and haptoglobin concentrations remained unchanged from baseline concentrations over the 72-hour follow-up period. On the other hand, after transfusion with stored erythrocytes, serum total iron concentration increased markedly with time, reaching a peak 5 hours after transfusion and returning to baseline values by 48 hours after transfusion (Figure 2). Serum transferrin concentrations were significantly lower than at baseline 5 and 9 hours after transfusion with stored erythrocytes, but increased to near baseline values by 48 hours after transfusion (Figure 3). Serum ferritin concentration was 43-times as high as at baseline 5 hours after transfusion with stored erythrocytes and 63 times as high 9 hours after transfusion, and values did not return to baseline at any point during the 72-hour follow-up period (Figure 4). Serum haptoglobin concentration was significantly lower than at baseline 5 and 9 hours after transfusion with stored erythrocytes (Figure 5).

For dogs that received fresh erythrocytes, serum labile iron concentration was mildly higher than at baseline 9 hours after transfusion (Figure 6). For dogs that received stored erythrocytes, serum labile iron concentration was markedly higher 5 hours after transfusion and peaked 9 hours after transfusion, when the magnitude of the increase was a median of 17 (range, 11 to 94) times as high as at baseline, then returned to baseline values 72 hours after transfusion.

For dogs that received fresh erythrocytes, serum cell-free hemoglobin concentration was slightly higher than at baseline 24 hours after transfusion (Figure 7). For dogs that received stored erythrocytes, serum hemoglobin concentration was markedly higher than at baseline at all measurement points through to 48 hours after transfusion.
hours after transfusion. Separate evaluations indicated that, at the time of transfusion, mean hemoglobin concentration in the supernatant of the stored erythrocyte concentrate was 481 mg/dL (SD, 258 mg/dL). Assuming that the volume of supernatant in each blood unit was 50 mL and that this volume was distributed into recipient dogs with a circulating blood volume of 90 mL/kg, transfused hemoglobin could have accounted for a maximum of 12 mg/dL of that identified in the serum of recipient dogs immediately after transfusion. Serum hemoglobin concentrations in dogs that received stored erythrocytes were significantly higher than that value, suggesting in vivo release of hemoglobin in the recipients caused by hemolysis of the transfused cells. Comparison of molar serum concentrations of hemoglobin and haptoglobin revealed that hemoglobin concentration exceeded haptoglobin binding capacity at all measurement points after transfusion with stored erythrocytes, suggesting the presence of free hemoglobin in circulation.

**Discussion**

The present study was performed to evaluate the impact of erythrocyte storage duration on indicators of iron metabolism in healthy dogs receiving an IV transfusion with autologous erythrocyte concentrate. Serum total iron concentration was measured by use of an assay that involves denaturation of associated proteins, causing release of Fe³⁺. Reduction of Fe³⁺ to Fe²⁺ then allows for colorimetric measurement of the amount of iron present in the original sample. This assay can be used to measure the combined amount of transferrin-bound iron and non–transferrin-bound iron, with little contribution from other iron pools. The amount of iron that is transferrin-bound typically far exceeds that associated with extracellular hemoglobin or ferritin, although most of the iron stored in the body is in the form of intracellular ferritin. When a considerable amount (>40 mg/dL) of heme iron exists in serum, assay results are heavily influenced by that heme iron. For dogs in the present study that received stored (35-day-old) erythrocyte concentrate, IV transfusion resulted in a marked increase in serum cell-free hemoglobin concentration (and consequently serum heme iron concentration) as well as an increase in serum ferritin concentration (and consequently serum sequestered-iron concentration). In serum samples from those dogs, denaturation of protein during the assay process resulted in the release of iron associated with transferrin, hemoglobin, and ferritin, rather than iron associated with just transferrin. Assay results, which reflected the total amount of iron from all of these sources, indicated a marked increase in serum total iron concentration for the dogs that received stored erythrocytes.

Total iron-binding capacity is often measured to estimate serum transferrin concentration and is calculated from values for the binding capacity of unsaturated iron (mostly a function of apotransferrin capable of binding iron) and serum total iron concentration. Binding capacity of unsaturated iron is measured by adding excess iron to a serum sample, then determining the maximum amount of that excess iron that becomes bound. When heme exists at high concentration (as was characteristic of some serum samples in the present study), measurement of serum total iron concentration fails to accurately reflect the relevant contribution of transferrin-bound iron to that value. Although serum samples were used to measure binding capacity of unsaturated iron so that total iron-binding capacity could be calculated (data not shown), the high amount of heme identified in the present study made the results poorly reflective of the true iron binding capacity of transferrin. We consequently measured serum transferrin concentration more directly by use of an ELISA.

Serum transferrin concentration decreased from pretreatment values for dogs that received stored erythrocyte concentrate, but not for dogs that received fresh concentrate. Transferrin is a known negative acute phase reactant, and its concentration is ex-
expected to decrease during inflammation. Because transfusion with stored erythrocytes in dogs results in an inflammatory response, the observed decrease in serum transferrin concentration was likely a result of this inflammatory process. The associated decrease in serum transferrin-dependent iron binding capacity further decreased the recipient’s ability to handle the iron load, contributing to the marked increase in serum labile iron concentration. In typical conditions, most iron in serum is bound to transferrin. Transferrin prevents labile iron from causing adverse consequences, including oxidative injury, enhanced bacterial proliferation, and systemic inflammatory responses. Approximately 30% of transferrin molecules in plasma carry iron, with 2 high-affinity binding sites/ transferrin molecule. Consequently, a reserve capacity exists for iron binding, minimizing the presence of non–transferrin-bound iron in circulation and its toxic effects. The dogs that received stored erythrocyte concentrate in the present study had a profound increase in serum labile iron concentration after transfusion. The amount of iron present far exceeded the binding capacity of transferrin in those dogs. It is possible that some of the labile iron in the recipient’s serum was already contained in the erythrocyte concentrate prior to transfusion and was transfused already free of protein, as the amount of non–transferrin-bound iron can increase mildly in both human and canine erythrocyte concentrates during storage.

Excessive labile iron promotes the production of reactive oxygen species as a result of Fenton reactions. These highly toxic radicals can lead to lipid peroxidation, DNA hydroxylation, and protein oxidation. Labile iron is also readily taken up by some tissues and cell types, including the liver, pancreas, heart, brain, and mature erythrocytes and erythroid precursors, in which it can accumulate, leading to considerable iron deposition and organ dysfunction. Labile iron suppresses colony formation by hematopoietic progenitor cells in vitro in a dose-dependent manner. Free iron is more readily available to pathogens and may impair host defense responses to infection.

In contrast to transferrin, ferritin is a positive acute-phase inflammatory reactant and its concentration is expected to increase during inflammation. This protein is the primary iron storage protein in tissues, and serum ferritin concentration is generally proportional to the volume of total body iron stores. Serum ferritin concentration can be influenced by inflammation, abnormal liver function, and ascorbate deficiency. Ferritin oxidizes excess ferrous ions and stores the nontoxic bioavailable ferric form, preventing iron from catalyzing Fenton-type redox reactions. Ferritin also suppresses bacterial growth by preventing bacterial use of the sequestered iron. Ferritin concentrations do not appear to change in erythrocyte concentrate units during storage, suggesting that the increase in serum ferritin concentration observed in response to transfusion was likely an in vivo response. The increase in serum ferritin concentration in the present study was similar to that in a study involving human blood recipients, in whom serum ferritin concentration peaked at 24 hours after transfusion. High serum ferritin concentration has also been reported for humans with sickle cell anemia who receive transfusions.

Serum hemoglobin concentrations increased profoundly in the dogs that received stored erythrocyte concentrate in the present study. The marked increase in serum total iron concentration in that same group was in large part a function of this heme iron. Initially, we postulated that the source of circulating hemoglobin could have been ex vivo lysis of erythrocytes during the storage period. However, we recognized that serum hemoglobin concentration would peak immediately following completion of the transfusion if hemoglobin were delivered to the recipients in noncellular form. The peak in serum hemoglobin concentration occurred somewhere between 5 and 24 hours after transfusion, which was not consistent with a pattern that could be expected with infusion of free hemoglobin. Furthermore, measurement of extracellular hemoglobin in supernatants from the erythrocyte concentrate units indicated concentrations far too low to account for the marked increase in the amount of cell-free hemoglobin in the recipients’ serum samples. For comparison, serum cell-free hemoglobin concentration reportedly increases gradually in canine erythrocyte concentrate stored for 42 days, to a mean concentration approximately 3 times as high as in the concentrates of the present study (900 mg/dL) at 35 days of storage. In the study reported here, most of the cell-free hemoglobin detected in serum samples must therefore have been delivered to the recipient within erythrocytes rather than free in solution, indicating that the hemoglobinemia observed was the result of in vivo post-transfusion hemolysis of the transfused erythrocytes. This finding suggested that storage-related changes in canine erythrocytes increased erythrocyte fragility, an explanation that is supported by a report that washing canine erythrocytes after storage for 35 days results in loss of approximately 25% of the cells. Large amounts of circulating toxic cell-free hemoglobin were detected in the recipients of stored blood in the present study. In addition to providing a source of labile iron (and its negative effects), cell-free hemoglobin acts as a nitric oxide scavenger, resulting in vasoconstriction and vascular injury. Hemoglobinemia can lead to immune dysfunction, smooth muscle dystonias, platelet activation, and endothelial cell dysfunction.

Serum haptoglobin concentration was measured in the present study to assess the magnitude of the effect of cell-free hemoglobin in the circulation of transfusion recipients. Haptoglobin binds to hemoglobin, accelerating its clearance through the reticuloendothelial system and limiting its toxic effects. Haptoglobin, like ferritin, is an acute-phase reactant. A study involving human recipients of erythrocyte
concentrate transfusions revealed no impact of transfusion on serum haptoglobin concentration, even when stored cells were transfused. In contrast, serum haptoglobin concentration in the dogs of the present study was lower than before transfusion and 5 and 9 hours after transfusion but was significantly greater by 24 hours after transfusion. This difference may have been attributable to the fact that in the comparable human study, hemolysis of the stored erythrocytes appeared to be primarily extravascular in nature, whereas in the dogs, intravascular hemolysis clearly occurred. Comparison of the molar serum concentrations of haptoglobin to those of haptoglobin revealed that despite the observed increase in serum haptoglobin concentration, the haptoglobin was 100% saturated with hemoglobin in dogs that received stored erythrocytes, leaving some hemoglobin free in the circulation.

Multiple studies have been conducted to investigate the possible benefits of haptoglobin in the prevention of adverse consequences of transfusion. In guinea pigs, IV infusion of haptoglobin prior to transfusion dramatically attenuates the adverse effects of transfusion with stored erythrocytes, including intravascular hemolysis, short-term hypertension, vascular injury, and renal dysfunction. Glucocorticoid stimulation of endogenous haptoglobin synthesis can prevent hemoglobin-induced hemodynamic responses in dogs. Haptoglobin may prevent bacterial use of heme iron by pathogenic bacteria, reducing the risk of infection. A decrease in serum haptoglobin concentration has also been associated with a decrease in survival rate for septic dogs receiving transfusions with erythrocyte concentrate.

In the study reported here, dogs that received stored erythrocyte concentrate had high serum concentrations of labile iron and cell-free hemoglobin, both of which may have considerable adverse effects in transfusion recipients. Although the increases in both toxic substances were most profound 9 hours after transfusion, concentrations of these toxic substances could have peaked anywhere between the 5- to 24-hour measurement points. Establishment of the exact time to peak concentrations for labile iron and cell-free hemoglobin in dogs that received stored erythrocytes, leaving some hemoglobin free in the circulation.

Hemoglobinemia was visually apparent during processing of blood samples obtained from dogs that received stored erythrocyte concentrates. The apparent degree of erythrocyte lysis in the recipients was more profound than would have been expected given our extensive clinical experience with administration of stored allogenic erythrocyte concentrate units to diseased anemic dogs. It is unlikely that the severity of hemolysis observed in the present study was attributable to the use of autologous cells because autologous erythrocytes would be expected to be better tolerated by a recipient than would allogenic cells. We elected to use autologous erythrocytes in the present study to eliminate a potential influence, if any, of cell destruction as a result of minor antigenic incompatibility. The profound hemolysis could likely be explained by the fact that, at the time of the transfusion, dogs were normocytic and immune competent, with healthy vascular function. On the other hand, in clinical practice, erythrocyte recipients are usually anemic and often have compromised immune function due to critical illness.

In the present study, transfusion with autologous erythrocyte concentrate that had been stored for 35 days resulted in evidence of intravascular hemolysis with concomitant marked increases in free iron and hemoglobin in the circulation of healthy dogs, both of which could have serious consequences for recipients. Additional studies are needed to evaluate the relevance of these findings to clinically ill dogs, in which oxidative stress, organ injury, infection, and lack of immune competence would be of concern.

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Footnotes
6. Kansas State Veterinary Diagnostic Laboratory, Manhattan, Kan.
8. Chelex 100, Bio-Rad Laboratories Inc, Hercules, Calif.
9. Sigma Chemical Co, St Louis, Mo.
10. Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
11. Spectramax M5 microplate reader, Molecular Devices, Sunnyvale, Calif.
15. Sigma Stat, version 2.03, SPSS Inc, Chicago, Ill.

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