ALLOGENIC MESENCHYMAL STEM CELL THERAPY FOR IMMUNE MEDIATED CYTOPENIAS – A 22 CASE SERIES

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ABSTRACT

Immune mediated cytopenias (IMCP) are common disease syndromes in veterinary practice (1) (2). It is one of the many diseases caused by autoantibodies that respond to mesenchymal stem cell therapy (3). Blood transfusions along with corticosteroids and other antimetabolites such as azathioprine, cyclosporine, meclofenoxate and leflunomide are the foundation of current therapy for immune mediated hemolytic anemia (3) (4). This current therapy is deemed effective as a cure in only about 1/3 of the cases, while another 1/3 of the cases become symptom free as long as immunosuppressive medications are given and the final 1/3 of the cases die from the effects of rapid uncontrollable hemolysis (2) (4). Mesenchymal stem cells represent great promise for the treatment of immune-related diseases due to their potent immunomodulatory properties (5). Twenty-two clinical cases of immune mediated cytopenia are described that have been treated with allogenic mesenchymal stem cells (AMSC) in addition to traditional therapy or as the only therapy. Of these 22 cases, 17 cases showed a positive response demonstrating the potential value of AMSC in modulating immune mediated cytopenia in the dog.

RESULTS

These are ongoing clinical cases occurring over a period of 20 months. Of the 22 cases 20 are immune mediated hemolytic anemia (IMHA) and 2 are immune mediated thrombocytopenia (ITP). Treatment of the 22 cases with intravenous infusion of AMSC’s resulted in 17 cases which demonstrated clinical improvement as documented by rising cell counts, improved clinical signs and reduction of immunosuppressive therapies and 5 cases that did not respond. 12 of 13 of the regenerative IMHA/ITP cases responded and 5 of 9 of the non-regenerative IMHA/ITP cases responded. Of the 17 responding cases, 12 were complete responses where the PCV and cell counts returned to normal values with or without additional immunosuppressive therapy and 5 of the 17 cases the pet returned to clinical normalcy with elevated cell counts, but the cell counts remained below normal. 2 cases of the 5 that did not respond had unresponsive bone marrow failure and the other 3 cases had poor response to or inadequate application of the stem cell therapy. One of these 5 dogs died and the other 4
INTRODUCTION

Immune mediated cytopenia is a disease that is commonly described as the overproduction of autoantibodies to cellular antigens causing the premature destruction or removal of the cellular elements of blood. Immune mediated cytopenia includes disease of the peripheral blood such as immune mediated hemolytic anemia (IMHA), immune mediated thrombocytopenia (ITP) and the combination of these two diseases or Evans syndrome. These diseases can be regenerative with normal bone marrow function or non-regenerative where the progenitor cells within the bone marrow are affected as well. Traditional therapy involves blood transfusions as needed to maintain homeostasis while immunosuppressive medications are used to halt the anti-blood cell antibody production and suppress the reticuloendothelial system. Current therapy, therefore, has been directed at reducing the antibody production using antimetabolite drugs that are aimed at the B-lymphocytes and their plasma cell clones. Immunosuppressive drug regimens, however, have a high incidence of adverse effects such as diabetes, Cushing’s disease, gastrointestinal ulceration, secondary infections and clinical ineffectiveness driving the need for alternative therapies. Anti-blood cell antibody production is ultimately the result of the breakdown of central and or peripheral immune tolerance mechanisms. These diseases result from a failure in the body’s ability to differentiate between cells from the body and foreign cells. Immune tolerance is mediated through a subset of T-lymphocytes with immune regulatory cytokine secretion (Treg cells).

Allogenic mesenchymal stem cell therapy has been documented as having anti-inflammatory properties to mediate autoimmune disease, and stimulate the production of Treg cells in the presence of inflammation. Mesenchymal stem cell therapy has been shown to both cancel the effects of the autoantibodies and to correct the errors in the immune tolerance mechanisms that mis-program the B-cells in the first place. Stem cells also produce Factor H which is the primary regulator of complement. Stem cell therapy seems to be an effective tool in stopping the immune destruction in immune mediated cytopenias. Allogenic mesenchymal stem cells are non-immunogenic, easily accessible, can be expanded to clinical scales in a short period of time, and can be biopreserved and shipped for point-of care delivery to the pet with minimal loss of potency enabling these donor cells to be used in clinical cases that are too sick or too time sensitive for autologous fat processing.


c Immune mediated neutropenia is another immune mediated cytopenia that has been treated by the author with AMSC but is not a part of this report as results are not yet available.
OVERVIEW

IMMUNE MEDIATED CYTOPENIAS IN DOGS

The bone marrow is the source of red blood cells, white blood cells and platelets. These cells come from the hematopoietic stem cell. Hematopoietic stem cells create the three lines of cells that form all three of the cellular blood components (white, red and platelets). Hematopoietic stem cells within the central marrow of the bones reside in a loose spongy matrix. The matrix is composed of calcified bony spicules which house osteoblasts and osteoclasts, fibroblasts, reticular cells, and adipose cells as well as bone marrow stromal cells (BMSC). These bone marrow stromal cells are stem cells and are essential in producing the chemical stimulus for hematopoietic stem cells to form the blood cells. Therefore, two essential stem cells lines are present in the bone marrow, one that produces the blood cells (hematopoietic) and another that provides the chemical instructions for this production (BMSC).

The hematopoietic stem cell produces daughter stem cells that become the source of the individual cell lines. The red cell line source is the erythroblast, the white cell line sources come from the myeloblast, lymphoblast and monoblast and the platelet cell line source cell is the megakaryocyte.

Immune-mediated hemolytic anemia (IMHA) is caused when antibodies are produced against the red blood cell antigens. This causes the red blood cell to be destroyed either within the blood stream (intravascular hemolysis) or within the spleen (extravascular hemolysis). This red cell destruction results in anemia which should stimulate the bone marrow to produce more red blood cells. This regenerative response is measured by identifying reticulocytes in the circulation. When there are not adequate numbers of red blood cells in the circulation it is called non-regenerative anemia (NRA). NRA occurs because the erythroblast has also been targeted in the immune attack. In some cases, the bone marrow failure is due to exhaustion of the erythroblast cells or lack of raw materials such as iron. In human medicine, a similar disease called aplastic anemia (AA) occurs where there is an immune attack on the hematopoietic stem cell itself so that all cell lines are affected and the person has low red blood cells, low white blood cells and low platelets as well. In these cases, human patients are usually given a bone marrow transplant or a hematopoietic stem cell transplant. This transplant must be given from a sibling donor or a suitable unrelated donor with matching tissue type.

Pure Red Cell Aplasia (PRA) is an infrequent hematologic complication from hematopoietic stem cell transplantation when the transplanted cells are mismatched with the recipient. Allogenic mesenchymal stem cells have also demonstrated efficacy in the treatment of refractory PRA in humans (20) (21).

Immune mediated thrombocytopenia (ITP) is characterized by immune-mediated destruction of normal platelets and suppression of platelet production that is associated with variable bleeding syndromes (22). The underlying immune dysregulation results in antiplatelet antibodies directed at the platelet and megakaryocyte surface glycoproteins. These antiplatelet antibodies develop secondary to a loss of tolerance to these surface-antigens of the platelets and megakaryocytes (23).

Recently it has been demonstrated that mesenchymal stem cells (MSC) from adipose tissue can support the hematopoietic matrix within the bone marrow (24). MSC transfusions have been shown to cure AA patients through the stimulation of the production of a new hematopoietic matrix. Allogenic Mesenchymal Stem Cell (AMSC) therapy can be used to improve the chances of recovery from these diseases through three paths: immunomodulation, increased hematopoiesis and the promotion of immune tolerance (25) (26).

IMMUNE TOLERANCE

There are natural balances in the controls of the immune system that we are just coming to understand. These balances involve the production of different types of T-Lymphocytes (T-cells) depending on the need for immunity
vs. tolerance. Immune tolerance is the need to tolerate the presence of a seemingly abnormal protein or substance. For example, our intestine houses many species of bacteria that are arguably foreign to our bodies, yet our immune system tolerates their presence. Mammals who maintain placental pregnancy within their bodies must tolerate the foreign tissue of the developing fetus with different DNA than the mother and not attack it as foreign. The surface of the skin and opening of the mucous membranes harbor “normal” bacterial and fungal flora that must be tolerated by the immune system. On the other hand, cells that are infected or damaged or that have turned cancerous should be attacked and removed from the body by the same immune system. It is this balance that is defective with IMCP.

Recent experiments involving the thymus gland have uncovered previously unknown elements of the T-cell populations that help control these systems. Mutant mice which do not have a thymus gland spontaneously develop fatal, widespread, early-onset autoimmunity (27). Removal of the thymus gland in three-day old mice led to the rapid development of autoimmunity that was corrected by the infusion of T-cells from adult mice. This research advanced our understanding of the creation and management of the T-cell lineage and how it controls the immune system. Certain T-cell types regulate or keep in check the aberrant or overactive activity of other T-cells. When functioning normally, the immune system produces a population of T-cells that have regulatory function (Tregs) that are specialized for immune suppression (18). In addition, it is becoming apparent that every immune reaction not only creates specific B-Cells and T-Cells but also Tregs and the balance between the different populations of cells is critical for the proper control of the immune response. IMHA is an example of an excessive attack on self-antigens as a result of improper immune tolerance resulting from dysregulation or deficiency of the Tregs (28) (18).

ALLOGENIC MESENCHYMAL STEM CELL THERAPY

Allogenic mesenchymal stem cell therapy is the intravenous use of adipose derived stem cells from the same species. In intravascular IMHA where red blood cells are destroyed by complement activation (3), stem cells block complement attacks by secreting Factor H (29) (30) (31) (32). Stem cells promote the creation of T-cell and the production of transforming growth factor beta (TGF-β) (19) (27) (32). Stem cells down-regulate the production of antibodies by B-cells and down-regulate macrophage consumption of red blood cells. The stem cells’ response is modulated by the relative need and will not affect the rest of the immune system’s ability to fight disease. AMSC’s are not allergenic or recognized by the immune system (33) (34). They have been shown safe when given intravenously in numerous human Phase I and Phase II Clinical Trials (35) (36) (19).

More general research has shown that stem cells possess anti-inflammatory and immunomodulatory properties; primarily by modulating the type and function of T-Lymphocytes (14) (15) (17) (38) (39) (40) (41) (42). There is a large body of research demonstrating the importance of subsets of T-cells in preventing disease. Th1 cells produce Interferon (IFN)-γ which stimulates cell mediated immunity against tumor cells, intracellular bacterial or viral infected cells. In contrast Th2 cells produce many cytokines including interleukin (IL)-4, IL-5, IL-9 and IL-13 which activate B-cells allowing them to transform into the plasma cell clones that produce antibodies. More recently, other subsets of cells such as the Th17 cells have been shown to play a role in the battle against infectious agents at mucosal barriers. And Th9 cells that are responsible for the response against helminths. These cells do not act, however, until they receive their orders in the form of chemical messages (cytokines). The cytokines tell the cells who to attack and with what weapons. Stem cells produce the cytokines that determine the type of T-cell and the “orders” for the action of these T-cells based on the needs of the animal. Stem cells, unlike conventional therapeutics, are alive and dynamically self-modulate the secretion of bioactive factors and signals at variable concentrations in response to local micro-environmental cues and actually offer a cure for IMCP’s as they reprogram the immune tolerance of the animal (19).
CASE DISCUSSION

Immune mediated cytopenias respond rapidly to the intravenous infusion of allogenic mesenchymal stem cells. Early administration of stem cells while there is a robust regenerative response is rewarded with the best clinical outcomes. Many of the cases in this report however are severe cases where stem cell therapy is the last resort only tried when all other therapies have failed. Even in these severe cases, however, stem cell therapy has demonstrated effectiveness. For example, some IMHA cases have very aggressive red blood cell destruction requiring more than one transfusion per day and this cycle can be stopped abruptly by the infusion of AMSC. See cases #15, #16 and #17 along these lines. Of the 9 non-regenerative anemia cases only 5 responded while 12 of 13 regenerative anemia cases responded to stem cell therapy. Some cases were not as successful because of poor timing of the stem cell therapy or because of abject failure of the bone marrow to respond to the stem cells as evidenced in cases #10 and #18. The therapeutic objective in some cases was not to increase the cell count but to reduce the immunosuppressive drugs. This is especially true with the two cases of ITP (Case #21 and #22) that were in remission but still on prednisolone that was weaned with the aid of stem cells. Finally, many pets in this series had concurrent disease such as vasculitis, dermatitis, transfusion related lung injury (TRALI), thromboembolism and seizure disorders that resolved after the stem cell therapy. See Table #2.
<table>
<thead>
<tr>
<th>Table #2 Clinical Case Details</th>
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<tr>
<td><strong>Signalment</strong></td>
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<tr>
<td><strong>CASE #1</strong></td>
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<tr>
<td>Non-Regenerative</td>
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<tr>
<td>13-year-old Dachshund with IMHA Non-regenerative. Diagnosed 3.5 months prior to stem cell therapy.</td>
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<td><strong>CASE #2</strong></td>
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<td>3.4-year-old dachshund/schnauzer mix that was referred for severe anemia over the past two weeks that had not responded to a steroid injection at referring vet.</td>
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<td><strong>CASE #3</strong></td>
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<td>3-year-old intact female Dachshund. She has had 3 transfusions before considering stem cell therapy.</td>
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<td><strong>CASE #4</strong></td>
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<td>Non-regenerative</td>
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<tr>
<td>15-year-old female Papillion which for two days she has been anorexic, has been having diarrhea, is very weak, and Pu/PD. Presumptive diagnosis is IMHA. She does not have adequate reticulocytes.</td>
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CASE #5
Non-Regenerative
10-years-old male dachshund/pit-bull mix that presented as a referral for anemia. Blood was given at the referral hospital. Stem cells were given three weeks later and again 6 weeks later. Responded Completely

CASE #6
4-year-old American Bulldog with IMHA diagnosed in March of 2018. The pet has a rash on its belly and his ear flaps are swollen, he is lethargic, anorexic, and has not needed any transfusions to date. One digit is swollen and is thought to have pythium. The pet has also been diagnosed with Bartonella. Pet is on Atopica and Mycophenolate, prednisone and Plavix. The pet had rare spherocytes noted after pathologist review but overall, his CBC suggests that his IMHA is in remission after the stem cells. Responded Completely

CASE #7
6-month-old Male Labrador Retriever with IMHA. The pet also has edema in the legs, and pneumonia as well as skin lesions. After the first round of stem cells the owner reports: "the pet has crazy amount of energy, pneumonia looking better, skin lesions clearing up, next to no swelling especially in the hock." The pet is currently on prednisolone. Responded Clinically

CASE #8
5-year-old Male dachshund mix with IMHA and a history of seizures. This pet has severe anemia and has had four transfusions and IVIG that has not stopped the cell destruction. The pet owner reports: “the pet has lots of energy, good mood, good appetite. There are tremors in hind legs. He has not had any seizures since his stem cells.” Splenectomy was performed at the referral center just after stem cells given. Responded Completely
CASE #9
Non-Regenerative
8-year-old male whippet with severe IMHA. The PCV was 14% which dropped to 12% before the first dose of stem cells arrived. Pet's lungs filled with fluid later the 4th night after the cells and the owners opted to euthanize. This pet likely had thromboembolism and was likely affected by rapid onset of intravascular hemolysis. Blood transfusions may have stabilized the pet long enough for the stem cells to be effective. Maybe add a synopsis statement?

CASE #10
3.5-year-old goldendoodle. This pet has had severe IMHA that has been managed with seven blood transfusions over the previous month as well as two doses of IVIG. The pet was on prednisone, cyclosporine and leflunomide. Only one dose of cells was given. Pet had vasculitis, swollen legs and anorexia before cells were given. The cells were given late in the disease course and toward the end of the client's ability to treat the pet. Euthanasia was elected.

CASE #11
Non-Regenerative
12-year-old beagle with IMHA who had her spleen removed in 2018 due to a benign mass. She has had IMHA for a month and had a transfusion one month ago. Since then she was treated with stem cells only. Two doses 6 days apart and another dose the next week and a final dose two months later. A blood smear one month after the stem cells showed no spherocytes, no mega platelets few target cells. These results indicated the immune destruction of the cells is now stopped but there may be an iron deficiency. Responded Clinically

CASE #12
Non-Regenerative
3.5-year-old female spayed terrier mix which presented with a PCV of 17. IMHA suspected. The pet was put on prednisolone for one month. One round of stem cells put the PCV back into the normal range. Responded Clinically
CASE #13

**EVANS**

11-year-old male dachshund with both IMHA and ITP. (Evans Syndrome) for the past three months that has been difficult to manage. The pet’s PVC was at 20% when cells were called for. A blood transfusion raised the PCV to 30%.

The first dose of cells was given 5 days after the transfusion. The second dose of stem cells was attempted three days later. The pet had an adverse reaction. He started drooling, got very white, became weak and his hind legs gave out, his heart was racing. The referring vet gave him Benadryl to which he responded, he “pinker” up and had his strength back. Since he had the reaction and was otherwise not responding the owner elected euthanasia.

CASE #14

**Non-Regenerative**

11.5-year-old male dachshund with severe IMHA. Five transfusions were given in the two weeks prior to stem cell therapy. The last transfusion was given the day before the stem cells were given. An additional cell therapy was sent but not given.

The pet collapsed at home with a PCV of 8% and was euthanized.

Add “euthanized” to the graph like the one above.

CASE #15

2-year-old, female spayed Shih Tzu who has severe IMHA. She was in the ICU for 10 days during which she received 7 blood transfusions.

She started out on prednisone, added mycophenolate day 3, Atopica day 7. Now she is doing well just on 5mg prednisone once every 3 days. Also, melatonin 3mg, salmon oil, and Plavix once daily and all blood values are normal.

Responded Completely

CASE #16a&b

**Pre-Regenerative**

8-year-old male Jack Russel Terrier with severe IMHA. This pet had 6 transfusions in 4 days as well as Therapeutic plasma exchange (TPE) or plasmapheresis. Two doses of stem cells at 2x10^6 cells/Kg were given one day apart followed by a third dose one week later.

TRALI (Transfusion Related Lung Injury) event after TPE. The lung issues resolved after the stem cell therapy.
The pet was in complete remission of the IMHA for 3 months then relapsed. The relapse was thought by the client to have correlated with the abrupt discontinuance of azathioprine. Two transfusions and 4 stem cell rounds were effective. The pet is now normal 6 months after the relapse.

**Responded Completely**

**CASE #17**
8-year-old female Rat Terrier with a one-week history of IMHA. She also has a seizure problem. This pet was treated with blood and cells in a rapid fashion resulting in a rapid reversal of the IMHA. In addition, she has not had a seizure since the stem cells were given.

**Responded Completely**

**CASE #18**
Non-Regenerative
8-year-old female dachshund with non-regenerative anemia or red blood cell aplasia. It has not had a bone marrow biopsy. Stem cells were given 5 times.

The stem cells did not restart the production of red blood cells, but the stem cells did increase the life of the transfused cells. She was on prednisolone the entire time. The pet did however develop an infection and had high glucose and elevated WBC. She was given Baytril and was to begin weaning off her prednisone the next day, when she took a turn for the worse. Owner opted to euthanize.

Aplastic Anemia or Bone Marrow Failure

**CASE #19**
EVANS
2-year-old male neutered Boston Terrier with IMHA and ITP. He was given two blood transfusions and IVIG therapy 7 months prior to giving stem cell therapy.

Today (18 months later) there are no spherocytes and 53% PCV. He is on Atopica and prednisolone both lowered.

**Responded Completely**
CASE #20
10.6-year-old male neutered mix breed dog with IMHA. He presented with white gums, was very lethargic and given a blood transfusion.

Two rounds of stem cells placed him on a stable PCV of about 30%. He is clinically normal but his PCV is still lower than the normal value. Additional cells should help this situation.

Responded Clinically

Case #21 – ITP
2 year old MN Chihuahua mix. Diagnosed two months prior with ITP. Previous Vet recommendations were to leave on prednisolone for life. Owner was not content with the prognosis that it always reoccurs.

He has had one round of cells and has reduced his prednisone by half and is waiting another round of stem cells before completely stopping the medications.

Responded Completely

Case #22 – ITP
4 Year old FS maltipoo diagnosed with ITP two years ago. Recently relapsed but responded to immunosuppressive therapies. The pet does not tolerate the drug protocol. Wants stem cells to wean off medications.

Two doses of cells given 30 days apart with discontinuation of the immunosuppressive medications. Close monitoring is continuing.

Responded Completely
MATERIALS AND METHODS

Allogenic mesenchymal stem cells are sourced from canine donors’ adipose tissues. The cells are processed, cultured and expanded following protocols described elsewhere. The cells are from a single donor and are “fresh” cells in that they come from actively growing cultures of less than 5 generations or passages. These cells are not reconstituted cryopreserved cells. Heparin is added to the culture medium (4U/ml) 12 hours prior to harvest to enhance therapeutic effectiveness and reduce the first pass effect (43)(44). The cells are then washed with PBS twice to remove residual heparin. Alternatively, the pet may be pretreated with heparin (200U/kg). The cells are then harvested from their cultures, counted with an automated cell counter, tested for viability and placed in a single use vial for the specific pet. The cells are dosed at 1 million cells per kg. Overnight shipping to the point-of-care facility results in minimal loss of potency, enabling these donor cells to be used in clinical cases that are too sick or too time sensitive for autologous fat processing. They are then diluted and administered intravenously over a 30-minute time period.

CONCLUSION

Stem cell therapy in the treatment of IMHA offers therapeutic advantages focused at the root of the IMHA disease. Research has shown that stem cells possess anti-inflammatory and immunomodulatory properties, primarily by modulating the type and function of T-Lymphocytes (14)(15)(37)(16)(38)(39)(40)(41)(42). In addition complement activation and subsequent hemolysis is a major factor in IMHA mortality (2)(15)(14)(4)(28) and is the mechanism for the rapid destruction of the transfused cells. Mesenchymal stem cells constitutively produce Factor H which is the primary regulator of complement activation. Factor H production from intravenous stem cell infusions effectively shuts down intravascular hemolysis secondary to complement activation thereby potentially saving the lives of the pets affected by this form of IMHA (45;46). Stopping production of the billions of anti-red blood cell antibodies produced per second is strategic for winning the battle in IMHA. Immunosuppressive drugs are the traditional linchpin functioning as anti-metabolites against antibody producing lymphocytes. Stem cells augment this strategy by producing Treg cells that produce a variety of cytokines that have the ability to kill antibody producing cells, effectively stopping the anti-red blood cell IgG antibody production responsible for the extravascular hemolysis (spherocytes) noted in Case #11 and case #19 of this report (47)(28). The processing of red cell surface proteins as “not-self” by macrophages and dendritic cells in the reticuloendothelial system is the underlying origin for anti-red blood cell antibody production in IMHA. Stem cells make Treg cells by producing the soluble protein Transforming Growth Factor Beta (TGF-β) (46)(49). TGF-β converts T-lymphocytes to Treg lymphocytes and Treg cells have the ability to direct the macrophages and dendritic cells to once again recognize the red blood cell antigens as “self” and not foreign (50)(28). In fact, these Treg cells also produce memory Treg cells that remember that the red cell antigens are “self” in the future, effectively having the potential to cure IMHA. Allogenic MSC are anti-inflammatory, immunomodulatory and regenerative. Intravenous infusion of these cells has been shown to be a safe way to decrease the antibody induced destruction of cells while increasing the production of red blood cells and platelets in animals suffering from various types of immune mediated cell and bone marrow damage. The therapeutic effect of stem cells on IMHA needs further evaluation for dose of cells, timing of administration and when to discontinue therapy.
COMPETING INTERESTS

The author, Dr. Garner owns and operates the stem cell laboratory at Safari Veterinary Care Centers in League City, Texas. As such he acknowledges a financial interest in stem cells provided for use in the cases in this report.

REFERENCES


