Arq. Bras. Med. Vet. Zootec., v.76, n.4, e13136, 2024

http://dx.doi.org/10.1590/1678-4162-13136
Case Report - Veterinary Medicine

Stem cell therapy as adjunctive in the management of anemia and thrombocytopenia partially responsive to corticosteroid in a dog treated with Phenobarbital – case report

[*Terapia com células-tronco como adjuvante no manejo de anemia e trombocitopenia parcialmente responsiva a corticosteroides em cão tratado com fenobarbital – relato de caso*]

L.G. *Santos*, J.A. *Hasan*, B.A. *Teixeira*, A.R. *Viana*, A. *Krause**

Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

ABSTRACT

This report describes a dog with anemia and ITP, associated with phenobarbital, treated with prednisolone with a good response after association with three intravenous transplantations of $2.5x10^6$ allogenic Mesenchymal Stem-Cells. After the first transplantation, the number of platelets had an improvement, but started to decrease after three weeks. However, the dose of corticosteroid could be reduced. After the second and third administrations, new increases were observed. The platelet count varied from 102,000 to 365,000 until the end of follow-up and without increase of prednisolone doses. Although phenobarbital therapy has been associated with thrombocytopenia, it was not possible to change the anti-convulsant therapy. The response to corticosteroid-based therapy was partial. Cell therapy was followed by an improvement in platelet count with no observed side effects and therefore could be considered safe. Effects on platelet count were transitory, but until the end of follow-up, no severe thrombocytopenia was observed. Based on the observation that platelet numbers dropped a few weeks after cell therapy, more applications would be beneficial in order to maintain higher platelet counts.

Keywords: bone marrow, canine, mesenchymal stem cells, Phenobarbital, platelet

RESUMO

Este relato descreve um cão com anemia e TIM associada ao fenobarbital, tratado com prednisolona, e boa resposta após associação com três transplantes de $2,5x10^6$ células-tronco mesenquimais alogênicas. Após o primeiro transplante, o número de plaquetas melhorou, mas começou a regredir após três semanas. Contudo, pode-se reduzir a dose de corticosteroide. Após a segunda e terceira administrações, observaram-se novos aumentos. A contagem de plaquetas variou de 102.000 a 365.000 até o final do acompanhamento, sem necessidade de aumentar as doses de prednisolona. Embora a terapia com fenobarbital seja associada à trombocitopenia, não foi possível alterar a terapia anticonvulsivante. A resposta à corticoterapia foi parcial. A terapia celular foi associada à melhora na contagem plaquetária, sem efeitos adversos relacionados e, dessa forma, pode ser considerada segura. Efeitos na contagem de plaquetas foram transitórios, mas, até o final do acompanhamento, não se observou trombocitopenia severa. Com base na observação de que o número de plaquetas caiu poucas semanas após a terapia celular, mais aplicações seriam benéficas, no intuito de manter a contagem de plaquetas mais alta.

Palavras-chave: canino, células-tronco mesenquimais, fenobarbital, medula óssea, plaquetas

INTRODUCTION

Primary immune thrombocytopenia (ITP) is a disorder characterized by the destruction of platelets by autoantibodies, where a clearance by the reticuloendothelial system happens and it results in thrombocytopenia. It is considered the most common acquired primary hemostatic disease in dogs and diagnosed after ruling out other etiologies (LeVine and Brooks, 2019). Immunosuppression with corticosteroids is the first choice for therapy. However, some

*Corresponding author: alexandrekrause@ufsm.br
Submitted: August 26, 2023. Accepted: January 17, 2024.

conditions are refractory to treatment and several adverse effects are reported for many immunosuppressors (Kristiansen and Nielsen, 2021; Olivares *et al*., 2023).

Mesenchymal stem cells (MSC) have immunomodulatory, antiapoptotic and anti-inflammatory properties and could be useful as a therapy for ITP, once they downregulate the expression of co-stimulatory molecules (He *et al*., 2022). MSC can go to distant targets under attraction by chemokines (Ullah *et al*., 2019). This report describes an allogeneic cell therapy approach in a dog with anemia and thrombocytopenia partially responsive to corticosteroids.

CASUISTRY

A four-year old female dog, poodle, non-spayed, 7.5 kg, was admitted to the hospital with apathy, ataxia, and nausea. Seven months before, the patient had a diagnosis of idiopathic epilepsy, and 2.5 mg/kg phenobarbital *per os* (PO) every (q) 12 hours (h), was prescribed. No alterations were found at the physical examination. Complete blood count (CBC) showed 15,000 platelets/µL (reference: 159,000-451,000/µL) and anemia (hematocrit: 32.6%; reference: 38.2-58.5%) (Bonamigo, 2022). Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were above reference values for the species – ALT: 240 UI/L (reference: 17-63 UI/L); ALP (892 UI/L; reference: 15.2-190.4 UI/L) (Cornell University, 2017). Ultrasonography revealed severe splenomegaly. The clinician tried to discontinue the phenobarbital, but it wasn't possible due to seizures.

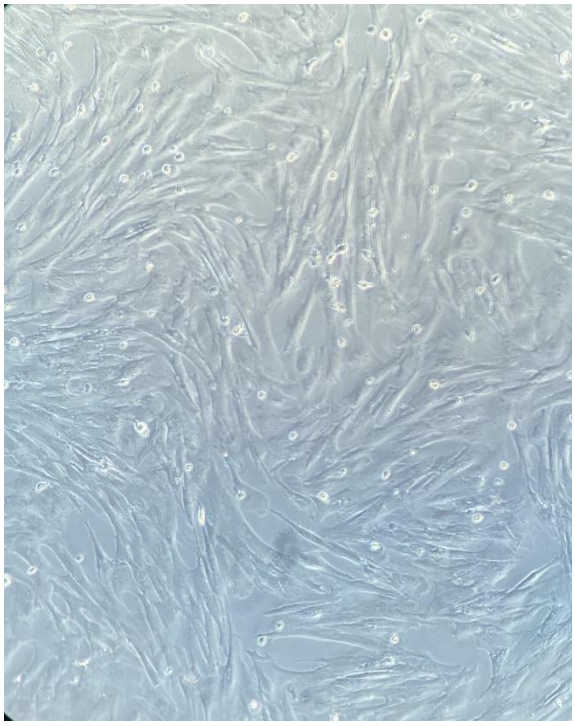
Rapid snap tests for *Ehrlichia canis, Anaplasma phagocytophylum, Dirofilaria immitis* and *Borrelia burgdorferi* (4Dx Plus®, IDEXX, Cotia, Brazil) and for *Leishmania* sp. (Leishmaniose Ac Test Kit®, Alere, Itajaí, Brazil) were negative. A therapy with 4 mg/kg prednisolone PO q 24 h was instituted for a therapeutic diagnosis of ITP. After three days, a CBC showed increase in platelet count (58,000/µL) and no more anemia (hematocrit 50.5%).

A spleen cytopathology was carried out to investigate possible alterations which could explain the thrombocytopenia, but the findings were normal. The dog underwent splenectomy to decrease the platelet destruction by the splenic mononuclear phagocytic system. The organ was sent to histopathological analysis, which found a mild multifocal myeloid metaplasia, a moderate-to-severe multifocal histiocytosis, and accentuated lymphoid depletion. Adjacent to trabeculae, there was a mild number of macrophages with an intracytoplasmic brownish material, interpreted as hemosiderin.

A myelogram of humerus bone marrow aspiration was carried out and showed hypocellularity, with a fat proportion higher than 50%; myeloid-to-erythroid ratio (M:E) 5.3:1 (reference 0.75-2.75) (Messick, 2023); mild megakaryocytic hyperplasia, with predominance of mature megakaryocytes, mild dysplasia (atypical nuclear lobulation); myeloid series was present and complete, with a very small amount of mature neutrophils. Some myeloid progenitors had abundant, hypogranular cytoplasm and extensions or vacuolization, presence of eosinophils with cytoplasmic vacuolization and pyknotic nucleus; erythroid series had no alterations; a great amount of reticular, endothelial, and stromal cells, as well as extracellular matrix suggested an increase of fibrotic tissue; the findings were interpreted as compatible with erythrophagocytosis, medullar fibrosis, and reactive megakaryopoiesis.

After three months the patient developed adverse signs of the corticosteroid-based therapy, like weight gain (reached 10 kg), ALT (497 UI/L) and ALP (4,073 UI/L) activity and lymphopenia (575/µL). Other drugs were added in an attempt to decrease the prednisolone dosage. Cyclosporine (5mg/kg q 24 h) and azathioprine (2mg/kg q 24 h) were instituted, both with no effectiveness.

A decision for cell-therapy approach was taken to decrease the inflammatory response and stimulate platelet production. MSC obtained from a healthy dog bone marrow – ethical approval from the Ethics Committee on Animal Use (CEUA) of the Federal University of Santa Maria (protocol number 6702160621) – were cultivated in Dulbecco Modified Eagle Medium enriched with 10% Fetal Bovine Serum in a chamber at 37°C with 5% CO_2 (fig.1). Cells were cryopreserved at a -80°C freezer.



Source: Santos *et al.* (2024).
Figure 1. Canine bone marrow mesenchymal cells.

For application, cells from two cryotubes, each with 1.25 x 10^6 cells, were thawed at 37^o in water bath, centrifuged twice for 10 minutes at 300x g and the pellet, resuspended with 0.9% saline. Cell viability was assessed with dye exclusion Trypan Blue assay, and viability was higher than 90% in all transplantations. The patient received three intravenous (IV) transfusions of 2.5 x 10^6 viable cells in cephalic vein. The sequence of events related to cell therapy is available in Fig. 2.

The transplantations happened in days 134 (T1), 163 (T2), and 225 (T3) after the detection of ITP. Six and nine days after T1, improvements (from 86,000 to 234,000 and then 259,000 platelets/µL) were observed. The dose of prednisolone was decreased from 4mg/kg to 2mg/kg q 24 h. A decrease (150,000 platelets/µL) was detected 21 days after T1.

The second transfusion (T2) happened after eight days, when another CBC showed 63,000 platelets/µL. An increase to 180,000 platelets/µL was observed after the application, and a decrease after 14 days (90,000 platelets/µL). One day before T3, the dog had 98,000 platelets/µL. Six days after T3, the platelet count was 213,000 platelets/µL. A decrease of platelet count was observed 21 days after T3 (102,000/µL). The number of platelets variated between 168,000 up to 365,000/µL until the end of follow-up at 121 days after T3 (day 346). The prednisolone doses were maintained at 2.0 mg/kg/day, lower than before the start of cell-based therapy.

The patient died due to a *status epilepticus* 289 days after T3. At that time, the dog's platelet count was 214,000/µL.

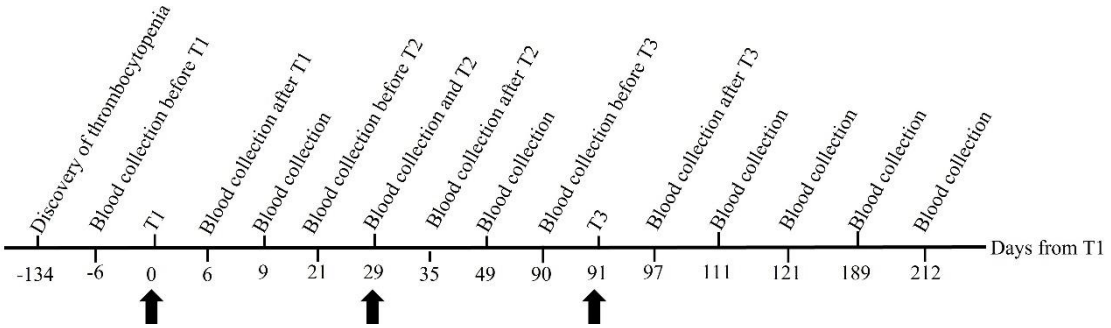


Figure 2. Sequence of events during stem cell therapy in a dog with immune thrombocytopenia. Arrows indicate the days of mesenchymal stem cell transplantation.

DISCUSSION

Dogs with ITP are under risk of spontaneous bleeding when platelet count is under 50,000/µL (Simpson *et al*., 2018). However, petechiae and an increase in bleeding time were not observed in our patient, even presenting severe thrombocytopenia (15,000 platelets/µL) at

admission, but immunosuppressive treatment was prescribed. The anemia may be due to the presence of a chronic inflammatory environment (anemia of inflammatory disease) (Chizakawa and Dunning, 2016), possibly associated with phenobarbital toxicity, which therapy was linked to aplastic anemia (Thrall, 2022).

Splenic myeloid metaplasia, histiocytosis and hypersplenism suggest a pattern which shows the cause and effects of a cytopenia due to sequestration, hyperactivation of splenic macrophages (Fighera and Graça, 2016). After splenectomy the platelet count remained low, and therefore, an increased platelet destruction by the spleen could be ruled out.

Blood dyscrasias can be secondary to exposure to phenobarbital (Khoutorsky and Bruchim, 2008). Dogs treated during more than three years seem to be more predisposed to develop them, with a median period of 100 days after the diagnosis of epilepsy (Bersan *et al*., 2014). The dog reported had a compatible age in the start of signs, but the thrombocytopenia was detected more than 200 days after the beginning of phenobarbital therapy. Some studies show an improvement if the drug is discontinued (Khoutorsky and Bruchim, 2008; Mathis *et al*., 2014). We couldn't stop the phenobarbital in our patient due to the severity of the epilepsy. Therefore, it is not possible to postulate that the single cause of thrombocytopenia was the use of phenobarbital. However, suggestive myelofibrosis was observed in the bone marrow aspirate, and this is associated with the use of phenobarbital (Weiss, 2005). The erythrophagocytosis is compatible with immune-mediated disorder and the megakaryocytic hyperplasia indicates a response against the thrombocytopenia. The presence of clear vacuoles in myeloid precursors are indicators of toxicity (Messick, 2023).

Corticotherapy was accompanied by an increase in platelet number in this case, but high doses were necessary, and associated adverse effects were observed, like signs of iatrogenic hypercortisolism. Other side events (hypercoagulability, gastrointestinal ulceration, myotonia) are also described. The side effects due to the high dosage are reasons for the use of adjunctive drugs, which may improve outcomes and decrease the adverse events (Kristiansen and Nielsen, 2021). As alternative drugs for immunosuppression, two were prescribed in this report: azathioprine and cyclosporine. Unfortunately, with unsatisfactory response.

LeVine and Brooks (2019) questioned whether patients with thrombocytopenia require a therapy, once the immunosuppression is linked to boke marrow suppression and predisposition to potentially fatal secondary infections, and the risk of spontaneous bleeding theoretically occurs with less than 50,000 platelets/µL. In this context, maybe the use of a cell therapy approach can be a useful tool to improve the platelet count. In this case report, three IV infusions of 2.5×10^6 cells helped to decrease the corticosteroid dose in a dog with immune thrombocytopenia. Stem cells have been considered a promising therapy for autoimmune disorders, as well as ITP, due to their capacity of immunomodulation (Zhang *et al*., 2019).

MSC have shown the ability to regulate the balance of immune cells and recover functional activity in refractory cases (He *et al*., 2022). Their use showed a capacity to improve the platelet count, possibly inhibiting the proinflammatory macrophages by the activation of an alternative phenotype with low phagocytic and regulatory activity. TGF-β1 is an inhibitory mediator with increased secretion after MSC transplantation. It is important in development of regulatory T cells (Treg), immunomodulators that are reduced in number and function in patients with ITP (Zhang *et al*., 2019). We could also observe quick improvements after transplantation. A reduction in glucocorticoid dose was prescribed and kept the dog far from risk of spontaneous bleeding.

Many questions remain unanswered, such as the optimal route of administration, cells origin and the ideal cell dosage. The literature shows a wide variation in these aspects (He *et al*., 2022; Zhang *et al*., 2019). In a case report of a dog with chronic immune-mediated thrombocytopenia, four transplantations were performed. The first involved 3.0×10^6 cells administered via intraosseous and 3.0×10^6 cells via intravenous; the second and third each used 6.0×10^6 cells intravenously; the fourth utilized 8.0×10^6 cell administered intravenously (Santi *et al*., 2023).

In this case, a dog with thrombocytopenia exhibited a partial response to corticosteroid-based therapy, which could be observed in the days before T1. A graphic with the variation in platelet counts is presented in fig. 3. Despite some improvement in platelet count, the severity of adverse events and the ineffectiveness of other drugs prompted the exploration of a new alternative to address thrombocytopenia. The increasing trend in platelet count observed after T1, T2 and T3 suggests that the transplantations had a beneficial impact on the patient, albeit in a limited magnitude. During the follow-up, platelet counts remained higher than before the transplantations even with the reduction in the dosage of prednisolone, which is uncommon in the therapy of immune thrombocytopenia when using corticoids alone. The procedure of cell transplantation was considered safe because no adverse effects were observed. The responses observed after each transplantation underscore the necessity of testing additional protocols with varying dosages, frequencies, and intervals.

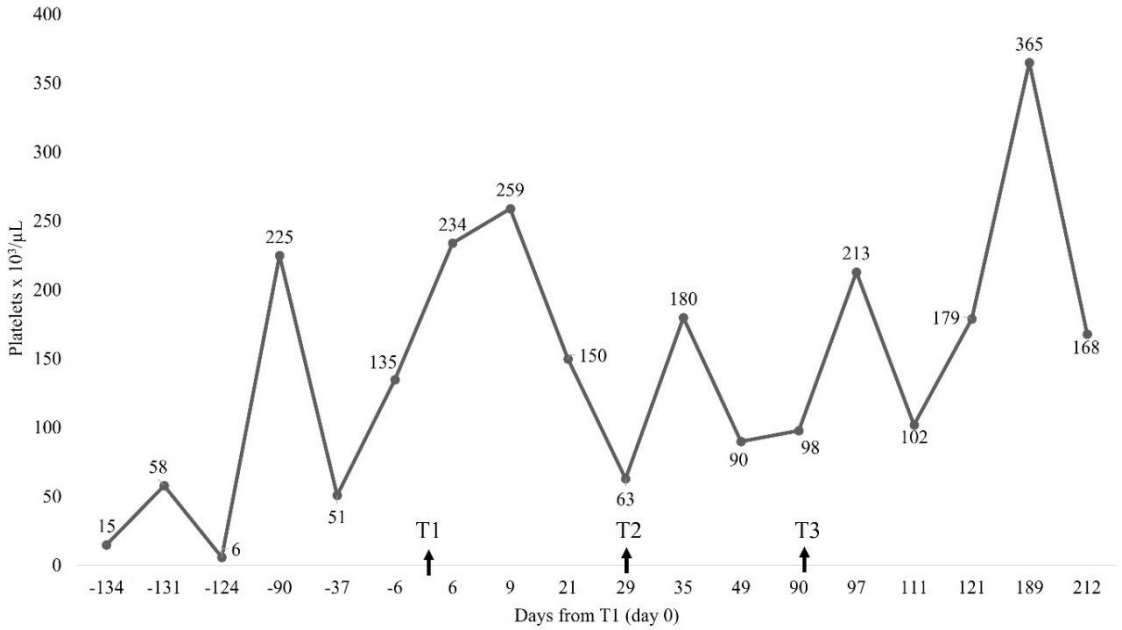


Figure 3. Variations in platelet count before, during, and after the cell therapy in a dog with immune thrombocytopenia. Arrows with T1, T2 and T3 indicate the moment of the cell transfusions.

The transitory effect of cell transplantations could be explained by many factors, such as the number of cells administered, the maintenance of the phenobarbital therapy or the primary condition of the patient. More studies are necessary to investigate all aspects of the use of MSCs in thrombocytopenic patients.

ACKNOWLEDGEMENTS

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).
.

REFERENCES

BERSAN, E.; VOLK, H.A.; ROS, C. *et al.* Phenobarbitone-induced haematological abnormalities in idiopathic epileptic dogs: prevalence, risk factors, clinical presentation and outcome. *Vet. Rec.*, v.175, p.247-247, 2014.

BONAMIGO, R. *Intervalos de referência para exames laboratoriais de cães da região de Santa Maria, Rio Grande do Sul, Brasil*. 2022. 44f. Dissertação (Mestrado em Medicina Veterinária) - Universidade Federal de Santa Maria, Santa Maria, RS.

CHIKAZAWA S.; DUNNING M.D. A review of anaemia of inflammatory disease in dogs and cats. *J. Small Anim. Pract*., v.57, p.348-53, 2016.

CORNELL UNIVERSITY, C.O.V.M. Chemistry (Cobas). 2017. Available in: https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/clinical-pathology/reference-intervals/chemistry. Accessed in: 8 Jul. 2023.

FIGHERA, R.A.; GRAÇA, D.L. Sistema hematopoiético. In: SANTOS, R.L.; ALESSI, A.C. (Eds.). *Patologia veterinária*. São Paulo: Roca, 2016. p.311-406.

HE, Y.; JI, D.; LU, W. *et al.* The mechanistic effects and clinical applications of various derived mesenchymal stem cells in immune thrombocytopenia. *Acta Haematol.*, v.145, p.9-17, 2022.

KHOUTORSKY, A.; BRUCHIM, Y. Transient leucopenia, thrombocytopenia and anaemia associated with severe acute phenobarbital intoxication in a dog. *J. Small Anim. Pract.*, v.49, p.367-369, 2008.

KRISTIANSEN, P.S.; NIELSEN, L.N. Immunomodulatory and immunosuppressive drug protocols in the treatment of canine primary immune thrombocytopenia, a scoping review. *Acta Vet. Scand.*, v.63, p.1-16, 2021.

LEVINE, D.N.; BROOKS, M.B. Immune thrombocytopenia (ITP): Pathophysiology update and diagnostic dilemmas. *Vet. Clin. Pathol.*, v.48, p.17-28, 2019.

MATHIS, J.C.; YOO, S.H.; SULLIVAN, L.A. Diagnosis of secondary dysmyelopoiesis via costochondral rib aspirate in a dog. *J. Vet. Emerg. Crit. Care*, v.24, p.739-744, 2014.

MESSICK, J.B. A primer for the evaluation of bone marrow. *Vet. Clin. North Am. Small Anim. Pract.*, v.53, p.241-263, 2023.

OLIVARES, G.; SHARMAN, M.; MILLER, R. *et al.* Use of tranexamic acid in dogs with primary immune thrombocytopenia: a feasibility study. *Front. Vet. Sci.*, v.10, p.946127, 2023.

SANTI, P.; FRANÇA, M.; MILISTETD, M. Treatment of chronic immunomediated thrombocytopenia with mesenquimal stem cells in dog – case report. *Arq. Bras. Med. Vet. Zootec.*, v.75, p.221-226, 2023.

SIMPSON, K.; CHAPMAN, P.; KLAG, A. Long-term outcome of primary immune-mediated thrombocytopenia in dogs. *J. Small Anim. Pract.*, v.59, p.674-680, 2018.

THRALL, M.A. *Veterinary hematology, clínical chemistry, and cytology*. New Jersey: Wiley-Blackwell, 2022. 1056p.

ULLAH, M.; LIU, D.D.; THAKOR, A.S. Mesenchymal Stromal Cell Homing: Mechanisms and Strategies for Improvement. *Iscience*, v.15, p.421-438, 2019.

WEISS, D.J. Bone marrow necrosis in dogs: 34 cases (1996-2004). *J. Am. Vet. Med. Assoc.*, v.227, p.263-267, 2005.

ZHANG, P.; ZHANG, G.; LIU, X. *et al.* Mesenchymal stem cells improve platelet counts in mice with immune thrombocytopenia. *J. Cell. Biochem.*, v.120, p.11274-11283, 2019.