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Treatment of chronic immunomediated thrombocytopenia with mesenquimal stem cells in dog – case report

[*Tratamento de trombocitopenia imunomediada crônica com células-tronco mesenquimais em cão – relato de caso*]

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ABSTRACT

Immunomediated thrombocytopenia is a systemic metabolic disorder in which the platelet count falls below reference values, as the patient's immune system destroys them. The main clinical signs in thrombocytopenia are petechial, hemorrhages, ecchymoses and suffusions. Hematomas can also occur in coagulation disorders. The diagnosis is based on clinical findings and hematological examinations. The treatment consists of the use of corticosteroids and immunosuppressants, delaying cell destruction, and may last for months, not always obtaining a cure for the disease. The present work reports the use of therapy with allogeneic mesenchymal stem cells, derived from the adipose tissue of dogs, for the treatment of chronic immunomediated thrombocytopenia, with an evolution of more than one year, in a Pinscher dog. The alternative treatment showed a good evolution, keeping platelets within the reference values during the treatment, giving the patient quality of life and removing the need for continuous medication for homeostasis after treatment.

Keywords: platelets, autoimmune, multipotent, stem cells

RESUMO

A trombocitopenia imunomediada é uma desordem metabólica sistêmica, na qual a contagem plaquetária fica abaixo dos valores de referência, pois o sistema imunológico do paciente a destrói. O principal sinal clínico na trombocitopenia são hemorragias, petequiais, equimoses e sufusões. Hematomas podem ocorrer também em alterações da coagulação. O diagnóstico baseia-se nos achados clínicos e nos exames hematológicos. O tratamento consiste na utilização de corticosteroides e imunossupressores, o que retarda a destruição celular, mas pode se prolongar por meses, nem sempre obtendo cura da doença. O presente trabalho relata a utilização da terapia com células-tronco mesenquimais alogênicas, oriundas do tecido adiposo de cães, para tratamento de trombocitopenia imunomediada crônica, com evolução de mais de um ano, em um cão da raça Pinscher. O tratamento alternativo revelou boa evolução, pois manteve as plaquetas dentro dos valores de referência durante o tratamento, o que proporcionou qualidade de vida ao paciente e tornou desnecessárias medicações de uso contínuo para a homeostase após o tratamento.

Palavras-chave: plaquetas, autoimune, multipotente, células-tronco

INTRODUCTION

Platelets are cells responsible for hemostasis, originated by the process of thrombocytopoiesis. They are derived from the cytoplasm of their precursor cells, the megakaryocytes, which in turn originate in the process of megakaryocytopoiesis. Megakaryocytopoiesis begins in the bone marrow endothelium, where hematopoietic stem cells generate progenitor cells, which develop, generating immature cells, which migrate to the perivascular niche and complete their maturation, releasing platelets into the circulation (Mazzarini *et al*., 2021).

Mesenchymal stem cells are responsible for the secretion of interleukins, cytokines, prostaglandins, leukemia inhibitory factors, generating the correct production of megakaryocytes and consequently keeping the

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number of platelets within the reference values, thus performing the correct homeostasis in the patient. Thrombocytopenia is a disorder characterized by low platelets in the bloodstream. The clinical signs presented by patients are hemorrhagic, among them petechiae can be observed throughout the body, which can progress to regions of cutaneous and mucous membrane hemorrhages (Marine and Bakchoul, 2019).

There may be several causes of thrombocytopenia, such as disorders with the precursor hormone, at the sites of cell formation, destruction of the precursor cells or destruction of the platelets themselves (Marine and Bakchoul, 2019). It is necessary to investigate causes such as infectious/parasitic, neoplastic, inflammatory, or toxic diseases (Shropshire *et al.*, 2020).

Immunomediated thrombocytopenia is a disorder in which the immune system destroys platelets. The pathophysiology of immune-mediated thrombocytopenia is not fully explained, and it can have several causes, such as the drop in immune tolerance; the production of antibodies to the specific glycoproteins of thrombocytes, thus generating phagocytosis through macrophages; cytotoxicity of T lymphocytes to autologous platelets; functional and T regulatory B cells (LeVine and Brooks, 2019; Marine and Bakchoul, 2019).

Treatment is usually carried out with corticosteroids and immunosuppressants, aiming to decrease the phagocytosis of macrophages on platelets, but it does not always produce results and / or achieve the cure of the disease (LeVine and Brooks, 2019). In some cases when platelet concentration is very low, in addition to the therapy already described, it is advisable to perform total blood transfusion to avoid complications (Shropshire *et al.*, 2020).

A complementary therapy that can be associated is the use of mesenchymal stem cells (MSCs). MSCs are progenitor cells that have a high capacity for renewal and can originate multiple cell lines (Li *et al*., 2020). MSCs are found more abundantly in adipose tissue, which facilitates their extraction and guarantees their greater number, when compared to other extraction sites (Santos, 2018).

The therapeutic potential of MSCs is directly linked to the immunosuppression capacity of thrombolytic macrophages (Marine and Bakchoul, 2019). They suppress the T cells that destroy platelets and induce the generation of new regulatory T cells (Li *et al*., 2020). In addition to performing immunomodulation, MSCs neutralize inflammation and support the regeneration of damaged tissue (Morigi *et al.*, 2016).

MSCs are mobilized to injured tissue by the homing process. This process is not fully understood, it involves the chemotaxis process, comprising the mobilization of MSCs, by the inflammation of the injured tissue, which causes the MSCs to migrate and remain adhered to the site where they develop (François, *et al.*, 2006).

Conventional therapy seeks to reduce the destruction of mature cells, generated by immunosuppression, while in treatment with MSCs, in addition, immunomodulation is observed and the production of platelets increases together (Marine and Bakchoul, 2019).

CASE REPORT

A 2-year-old Pinscher dog, weighing 4 kg, was received in private clinic in Curitiba/PR, Brazil. The patient had chronic thrombocytopenia, which had been treated with prednisolone-based corticosteroids for months (Corticorten, Prednisone, Neo Química, Brazil) 3.75mg/kg, BID, when the dose was reduced to 2.5mg/kg, BID, or lower dose, in order to withdraw the continuous use of the medication, the animal presented clinical signs such as: petechiae, hematomas, bleeding of the oral and vaginal mucosa.

The first clinical manifestation occurred 14 months earlier, when the animal presented areas of hematomas and petechiae on the body, gingival bleeding, apathy, and anorexia. On clinical examination, the animal had pale mucous membranes and tachycardia, the blood count revealed anemia, with a hematocrit value of 20% (reference value: 37% to 55%) and thrombocytopenia of 46 thousand / µL (reference value: 200 thousand/µL to 500 thousand/µL). Transfusion of whole blood was performed, after 24 hours, the tests that showed hematocrit values and platelets were increased to 33% and 276

thousand/μL, respectively. After 48 hours of transfusion, the exams showed hematocrit and platelet values of 22% and 60,000/μL, respectively. Among the suspicions were ehrlichiosis, immune-mediated hemolytic anemia and immune-mediated thrombocytopenia. The treatment instituted was prednisone 3.75mg/kg, BID, for 12 days and doxycycline (Doxifin Tabs, Doxycycline, Ourofino Saúde Animal, Brazil) 12.5mg/kg, BID, for 21 days.

After 12 days the exams revealed the hematocrit value within the reference values, the platelets had increased to 875 thousand/μL, for this reason the prednisone dose was reduced to 2.5mg/kg, BID. After 30 days there was a drop in platelets to 50 thousand/μL, the treatment consisted of increasing the dose of prednisone to 3.75mg/kg, BID. Thereafter, for 12 months, whenever the dose of prednisone was decreased, to wean and withdraw the drug completely, the platelet count was significantly reduced in the subsequent blood count, and the animal showed a clinical manifestation of hemorrhage in mucous membranes and cutaneous hematomas. During this time, immunosuppressants such as cyclosporine, mycophenolate and leflunomide were used, together with corticosteroids, showing no significant effect.

During treatment, follow-up blood counts were performed to find out the actual platelets count. Other tests were carried out, looking for a diagnosis, such as parasitological, which revealed negative results for *Ehrlichia canis, Borrelia burgodorferi, Anaplasma phagocytophilum, Dirofilaria immitis, Babesia spp., Leishmania infantum, Anaplasma spp., Canine hemotropic mycoplasma, Ehrlichia spp., Hepatozoon spp., Leishmania spp., Neoricketsia risticci, Rickettsia rickettsii, Heartworm antigen, Ehrlichia canis antibody, Anaplasma antibody, Lyme.* Elisa test for *Erlichia* showed negative result. The prothrombin activity time was within the reference values, whereas the activated partial thromboplastin time had a slightly increased result of 18.1 seconds (reference values: 10 to 17 seconds). Bone marrow cytology showed a large number of megakaryocytes, a large number of which are immature cells, indicating great demand.

After 14 months of treatment, and without a definitive cure, the option of treatment with MSCs was used, to immunomodulate, aiming at a regenerative therapy, withdrawal of continuous use of corticosteroids and immunosuppressants, aiming to cure the disease.

A complete blood count was performed to start treatment, which showed platelets at 24 thousand/μL, to raise counts, doses of prednisone 5mg/kg, BID and cyclosporine 30mg/kg, BID, were instituted. After 19 days, the animal showed a good response, with 338 thousand/μL platelets, enabling the start of treatment with stem cells. Table 1 summarizes, chronologically, the number of cells and therapy instituted.

The MSCs (Bio Cell Terapia Celular LTDA, Brasília, Brazil) used were from a bank of stem cells, originated from adipose tissue from other dogs, isolated and replicated in the laboratory of the company Bio Cell, frozen in cylinders with liquid nitrogen, separated by straws with ±1 million MSCs.

For the treatment, the straws containing the MSCs were removed from the liquid nitrogen, kept at room temperature for 10 seconds, after which they were immersed in a water bath at 37ºC for 30 seconds for thawing. After this process, the straws were cut, and the content was placed in a Falcon tube containing 5mL of PBS thawing medium, which was centrifuged at 1250 RPM for 3 minutes. The cell concentrate was deposited at the bottom of the tube, and the supernatant was discarded. The cell concentrate was resuspended in 5mL of washing medium with PBS medium, the tube was centrifuged at 1250 RPM for 3 minutes, the supernatant was discarded, and the process was repeated 2 more times. At the end of the process and disposal of the supernatant, the cell concentrate deposited at the bottom of the tube was resuspended in 2mL of Ringer Lactate solution, this solution containing the MSCs was stored in a syringe, where it was ready for administration (Franco, *et al.,* 2021).

The infusion can be performed in situ (intraosseous, intra-articular), or diluted in lactated Ringer's solution, and given intravenously, so that the MSCs reach the desired location by homing, which involves chemotaxis of the cells to the action location.

Table 1. Relationship of drug and cell therapy, related to the concentration of platelet used in dogs under treatment with msc

Day	-19	0	21	25	29	32	39	46	
Platelet (thousand cells/µL)	24	338	553		202		518	511	
Cyclosporin*	30mg/kg BID	30mg/kg BID	30mg/kg BID	30mg/kg BID	30mg/kg BID	30mg/kg BID	30mg/kg BID	30mg/kg BID	
Prodnisolone*	5mg/kg BID	5mg/kg SID	5mg/kg SID	2,5mg/kg BID	2,5mg/kg BID	1,25mg/kg BID	0,6mg/kg BID	0	
MSC**		$3x10^6$ I.O. $3x10^6$ I.V.		$6x10^6$ I.V.					
Day	52	53	63	73	82	84	103	122	142
Platelet (thousand cells/µL)		274	184	146	126		305	269	180
Cyclosporin*	30mg/kg BID	30mg/kg BID	0	0	0	0	0	0	0
Prodnisolone*	0	0	0	0	0	0	0	0	0
MSC**	6x106 I.V.					8x106 I.V.			

D0: Start of treatment with MSC. *Drug used, its dose and frequency. ** Number of MSCs used, and their infusion route.
I.V.: intravenous; I.O.: intraosseum; SID: once a day; BID: twice a day.
Source: Author, 2022

In the first infusion, day 0, were used $3x10^6$ of allogeneic MSCs derived from adipose tissue, infused intraosseous in humerus; and $3x10^6$ of allogeneic MSCs derived from adipose tissue, infused intravenously through the cephalic vein, with MSCs diluted in 30 mL of lactated ringer's solution (Fresenius Kabi Brasil Ltda, Aquiraz, Brazil). Treatment with cyclosporine was maintained and the dose of prednisone reduced to 5 mg/kg, SID. After 21 days of the beginning of the treatment, there was an increase of platelets to 553 thousand/µL.

The second infusion occurred 25 days from beginning of treatment, in which $6x10^6$ of allogeneic MSCs derived from adipose tissue were used, in intravenous infusion. The dose of prednisone was gradually reduced over 21 days, reaching zero, the treatment with cyclosporine was maintained. On day 29 of treatment the number of platelets reduced to 202 thousand/µL, on day 39, as the prednisone dose decreased, the number of platelets increased to 518 thousand/µL. On day 46, the platelet count was at 511 thousand/µL.

The third infusion took place on day 52 of the beginning of treatment, $6x10^6$ of allogeneic MSCs derived from adipose tissue were used, in intravenous infusion. Cyclosporine treatment continued for another 10 days, and then stopped. On day 53 the number of platelets was 274 thousand/µL, on day 63 it was 184 thousand/µL, on day 73 it was 146 thousand/µL and on day 82 it was 126 thousand/µL, with the presence of macro-platelets. As the number of platelets progressively reduced after the third infusion, a new infusion was chosen.

The fourth infusion occurred on day 84 of treatment, $8x10^6$ of allogeneic MSCs derived from adipose tissue were used, in intravenous infusion. The hemogram performed on day 103 revealed the number of platelets increasing to 305 thousand/µL, on day 122 showed the number of platelets in 269 thousand/µL, on day 142, there was a decline in the number of platelets to 180 thousand/µL. However, the patient remained stable, without clinical manifestations and without the need for immunosuppressive drugs.

DISCUSSION

The infusion of allogeneic MSCs, derived from the dog's adipose tissue, in three sessions of $6x10^6$ and one session of $8x10^6$, with a mean interval of 28 days between the infusions, proved to be efficient in the treatment of chronic immunomediated thrombocytopenia in dogs weighing up to 5Kg, removing the need to use immunosuppressants, and keeping the patient clinically stable, without clinical manifestation.

The patient with immunomediated thrombocytopenia is dependent on continuous doses of corticosteroids and immunosuppressants to keep the platelet counts within the reference values, to keep the concentration of antiplatelet antibodies low (Shropshire *et al.,* 2020). In the present case, both medications were used in the initial treatment, but when they were withdrawn or reduced, the platelet numbers consequently dropped, and the patient presented hemorrhagic clinical manifestations. After the initiation of treatment with MSCs, the doses of corticosteroids were gradually decreased, reaching zero after the second infusion, and the use of immunosuppressants was withdrawn after the third infusion, thus reestablishing the number of platelets within the reference values without the use of medications, as shown in Table 1.

After the fourth infusion of MSC, the patient underwent serial tests to monitor the number of platelets. Platelet values were continuously within the reference value, up to 40 days after the last infusion of MSC, after which the values dropped slightly from the reference, but without the need to use corticosteroids or immunosuppressants, and without clinical manifestations.

MSC infusions can be made in several ways. The peripheral intravenous route is the easiest to perform, less invasive and traumatic, allowing infusion without sedation (Xu and Liu, 2008). The percutaneous injection of MSCs, close to the fracture site, is an adjuvant technique, which accelerated tibial bone consolidation after osteosynthesis using the MIPO technique (Franco, *et al.*, 2021). A study carried out by Schaefer *et al.*, (2022), comparing intra-osseous and intravenous infusion in dogs, revealed little difference in the results between the administration routes, noting that both routes are safe and generated good results.

According to Garden (2022), the use of MSCs, applied intravenously in immunomediated cytopenias (immunomediated hemolytic anemia and/or immunomediated thrombocytopenia) showed a positive result in 17 of the 22 dogs followed up. The study showed that, of the 17 patients with a positive response, 12 had a complete response, with or without the help of immunosuppressants, and 5 had clinical and cell number improvement, but with a cell count below normal. In this study, the 2 cases of immunomediated thrombocytopenia responded positively to MSC therapy, removing the need for immunosuppressants to stabilize platelet numbers.

Immunosuppressive medications are the basis for the treatment of autoimmune diseases, but they can cause adverse effects in the patient. In other autoimmune diseases, such as systemic lupus erythematosus, in which the patient may suffer from several metabolic changes, the use of MSCs may be an option for the treatment of the disease (Ko, *et al.*, 2019).

According to Li *et al.* (2020), exogenous mesenchymal stem cells increase platelet concentration and reduce the inflammatory response and cytokines, which can be seen in the present case, in which the platelet values returned and remained within the reference range after the start of the treatment, and even decreasing after the fourth infusion, the clinical manifestations did not recur, and the use of immunosuppressive drugs was not necessary.

Before applying MSC, the treatment with corticosteroids and immunosuppressants reached 420 days, with no success in remission of drug therapy, and with hemorrhagic manifestations aiming to reduce medication doses. After the initiation of treatment with MSCs in 46 days, the patient maintained the reference values of platelets without the use of corticosteroids, after 62 days without the use of immunosuppressants, with the duration of treatment with MSCs being 84 days between the first and the fourth infusions. The time between infusions of stem cells can vary, being carried out in the present study with an interval of 25, 27 and 32 days.

CONCLUSION

The alternative treatment with MSCs is promising, as reported, because, through immunomodulation, it increased and maintained the platelet number in a regenerative way, generating clinical improvement in the patient and improved quality of life, when compared to the use of treatment with immunosuppressants. During and after treatment, the patient did not manifest bleeding, without the need for transfusion of blood components, with a progressive decrease and total withdrawal of immunosuppressants of continuous use, which can result in secondary metabolic alterations.

Serial exams are necessary to monitor the evolution of the disease, showing the real hematological result triggered by stem cells. These exams are essential to observe the metabolic journey of platelets, from their production in the bone marrow, to their development and systemic action.

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