Veterinary Immunology and Immunopathology 146 (2012) 97–99

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Use of the nitroblue tetrazolium reduction test for the evaluation of Domperidone effects on the neutrophilic function of healthy dogs

Pablo Gómez-Ochoa[a,*], David Sabate[b], Josep Homedes[b], Lluis Ferrer[c]

[a] Animal Pathology Department, University of Zaragoza, Miguel Servet 177, CP 50013 Zaragoza, Spain
[b] Research and Development Department, Veterinary Division, Laboratorios Dr. Esteve, Barcelona, Spain
[c] Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Spain

ARTICLE INFO

Article history:
Received 3 August 2011
Received in revised form 5 January 2012
Accepted 23 January 2012

Keywords:
Nitroblue tetrazolium test
Domperidone
Immunity
Dog

ABSTRACT

The nitroblue tetrazolium reduction test (NBT) is an assay based on the activation percentage of neutrophils in peripheral blood, that has been proposed for the follow up of canine leishmaniosis owing to the narrow relationship between the molecules involved in the oxidative burst and the leishmanicidal activity of phagocytes. Domperidone is a drug used for the treatment of canine leishmaniosis having been claimed to stimulate the dogs' cell-mediated immune response. The aim of this study was to evaluate the degree and the lasting of phagocytic activation induced by a 30-day course treatment with Domperidone (0.5 mg/kg/day) in healthy dogs, by using the NBT. A statistically significant increase in the percentages of activated phagocytes was observed in the treated group during treatment, thereafter remaining high for up to one month after the end of treatment. In contrast, untreated dogs maintained the baseline percentage of activated neutrophils all along the study. It is concluded that the NBT is a useful tool for the follow up of the stimulating effects of Domperidone on the neutrophilic response of healthy dogs and that these effects persist for up to one month after treatment with this molecule.

1. Introduction

The Nitroblue tetrazolium reduction test (NBT) was introduced in 1968 (Baehner and Nathan, 1968) as the main diagnostic test for chronic infant granulomatous disease. Since then tetrazolium salts have become the most widely used tools in cell biology for measuring the metabolic activity of mammalian and microbial cells (Berridge et al., 2005). The basis of the test is the same in all mammals: nitroblue tetrazolium, soluble and colourless, is transformed into formazan, which is insoluble and blue-grey in colour, when it is reduced by the NADPH-oxidase of the activated neutrophils inside the phagocytic vacuole. The rate of NBT reduction is obtained calculating the percentage of neutrophils with formazan in the cytoplasm by conventional light microscopy. The amount of NBT reduced is directly proportional to the amount of oxygen radicals produced by phagocytes in the oxidative burst (Muniz-Junqueira and de Paula-Coelho, 2008). The NBT has remained as an efficient and simple screening test for canine phagocytosis (Nagahata et al., 1991), but in veterinary clinical practice there are very few references in the literature regarding the use of this test (Lechowski et al., 1991). Recently NBT test has been proposed for the follow up of canine leishmaniosis (Gomez-Ochoa et al., 2010) owing to the narrow relationship between the molecules of the oxidative burst and the leishmanicidal properties of phagocytes (Underhill and Ozinsky, 2002). Dogs with mild disease (Stages I showed a high NBT reduction rate, meanwhile dogs with severe leishmaniosis (Stage IV)

* Corresponding author at: Animal Pathology Department, Veterinary Faculty of Zaragoza, c/Miguel Servet 177, CP 50013 Zaragoza, Spain. Tel.: +34 976764288; fax: +34 976761612.
E-mail address: pablogomezochoa@gmail.com (P. Gómez-Ochoa).

0165-2427/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2012.01.018

exhibited a low NBT reduction rate (Gomez-Ochoa et al., 2010).

Domperidone, a dopamine D_2 receptor antagonist, has been included in the list of anti-*Leishmania* drugs in the current consensus guidelines for treatment of canine leishmaniosis (Oliva et al., 2010). Repeated administration of this drug has been claimed to activate dogs' cell-mediated immune response through an increase of prolactin blood levels (Berczi et al., 2000; Gomez-Ochoa et al., 2009). Prolactin has a central role in the immune reaction, being classified as a proinflammatory lymphocyte-derived cytokine (Hinterberger-Fischer et al., 2000). An increase in serum prolactin concentration generates an increment in CD4 + Th1 subsets, and in interleukin (IL)-2, IL-12, interferon (INF)-γ and tumour necrosis factor (TNF)-α release, leading to a natural killer (NK) cell and macrophage activation, followed by a decrease in CD4+ Th2 subsets and TNF-β (Di Carlo et al., 1993; Majumder et al., 2002; Richards et al., 1998). The goal of this study was to evaluate the degree and the duration of the activation of neutrophils in healthy dogs under a 30-day course treatment with Domperidone, by using the NBT reduction test.

2. Materials and methods

Twenty healthy dogs of different breeds that came into the Veterinary Faculty of Zaragoza, were entered in the study and divided into two groups. Group A ($n = 10$) consisted of 7 females and 3 males, with ages ranging from 2 to 7 years (mean ± SD = 4.7 ± 1.9), and weights from 7 to 32 kg (mean ± SD = 21.4 ± 7.5). Group B ($n = 10$) consisted of 6 females and 4 males, with ages ranging from 2 to 8 years (mean ± SD = 4.2 ± 2.1), and weights from 7 to 34 kg (mean ± SD = 17.2 ± 9.1). The females were not pregnant and all dogs were clinically healthy, with normal haematological and biochemical parameters. Dogs were negative against the most common infections in the region (erlichiosis, anaplasmosis, heart worm disease, Lyme disease) to rule out causes of chronic subclinical infections non related with leishmaniosis. Moreover all dogs had a negative serological test for leishmaniosis (Direct Agglutination Test, DAT < 1/400), and negative results for isolation of leishmania parasites from popliteal lymph node and bone marrow aspiration inoculated in Novy–MacNeal–Nicolle (NNN) culture media. All the procedures were performed in accordance with the University of Zaragoza Ethics Committee guidelines and the owners gave prior informed consent.

Dogs from group A did not receive any product while dogs from group B received 0.5 mg/kg of Domperidone PO every day during the morning for one month. On days 0, 15, 30, 60 and 90 all dogs underwent a clinical examination and blood sampling, drawing 5 ml of blood from a jugular venopuncture. At each examination a complete blood count, a biochemistry profile and serologic tests against (leishmaniosis, erlichiosis, anaplasmosis, heart worm disease, Lyme disease) were performed. The neutrophil phagocytic activity was assessed using the NBT reduction test. Three ml of blood were collected in tubes with EDTA (Aquifer S.L., Barcelona, Spain) and left at room temperature for 15 min. After mild agitation, two Win Trobe tubes were filled per sample and centrifuged at $4000 \times g$ for 10 min to obtain the leukocyte pellet which was extracted using a Pasteur pipette and placed in sterile plastic tubes. Incubation with NBT was performed depositing 0.05 ml of the leukocyte suspension in the same quantity of 0.1% concentration NBT (N6876, Sigma–Aldrich Co., St. Louis, USA) solution in a phosphate buffer in a heater at 38 °C for 15 min, with a partial CO_2 pressure of 5% and at saturation humidity, and then for another 15 min at room temperature. The blood smears were performed on glass slides and stained with Diff Quick (GmbH, Duding, Switzerland). Ordinary light microscopy was used to evaluate exclusively the neutrophils of unequivocal morphology, rejecting those aggregated or broken. At least 100 neutrophils were counted in every slide. The percentage of activated neutrophils, as defined by cells that present cytoplasmic formazan, the NBT reduction rate, is presented.

Statistical analysis of data was performed using the software packages SPSS 12.5 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov–Smirnov test was used to assess normality in the variable analysed (activation percentage in NBT). Values from every sampling were compared between groups using Student's *t*-test. Moreover a variance Analysis (ANOVA) and a Dunnett post hoc test were applied to determine significant differences comparing every value against the baseline activation percentage. Results were considered statistically significant when *p* value was <0.05.

3. Results and discussion

All the animals remained negative against (leishmaniosis, erlichiosis, anaplasmosis, heart worm disease, Lyme disease) during the entire experiment. The haematological values and the biochemistry profile were between the normal limits in all the dogs. The owners notified no clinical signs or any side effect during the study.

There was not significant association between NBT reduction rate and gender or age within any group. Table 1 show the mean ± SD values for the percentages of activated (NBT-positive) neutrophils and its statistical comparison in both groups along the study. As specified, statistic significant differences ($p < 0.01$) were retrieved between groups on days 15, 30 and 60. In day 90 NBT reduction rate levels had returned to baseline levels retrieving no significant differences between groups. Figs. 1 and 2 show the individual values and the evolution of the NBT reduction rate

Table 1
Mean ± SD activation percentages and its statistical comparison in both groups along the study.

	Group A		Group B (Domperidone)		*p* value
	n	Mean ± SD	*n*	Mean ± SD	
Day 0	10	5.900 ± 1.595	10	5.700 ± 2.541	0.8354
Day 15	10	5.700 ± 1.160	10	38.50 ± 9.192	<0.0001
Day 30	10	5.200 ± 1.549	10	40.00 ± 7.498	<0.0001
Day 60	10	4.600 ± 1.075	10	15.10 ± 3.985	<0.0001
Day 90	10	6.000 ± 2.108	10	5.900 ± 1.853	0.9115

Mean ± SD. NBT reduction rate (percentage of activated neutrophils) and its statistical comparison between both groups along the study.

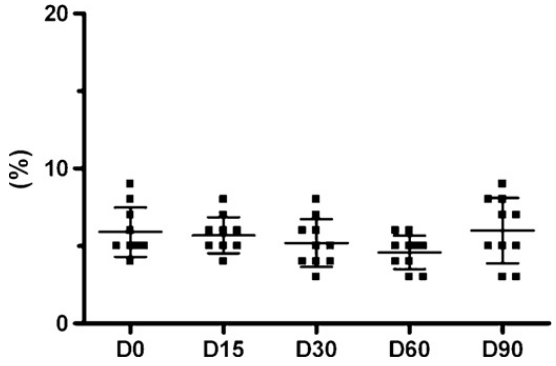

Fig. 1. Evolution of the NBT reduction rate during the study in group A (non-treated dogs).

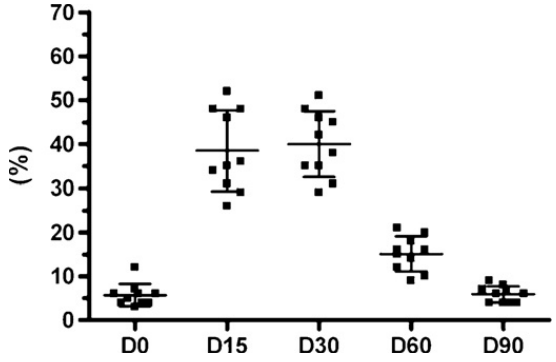

Fig. 2. Evolution of the NBT reduction rate during the study in group B (Domperidone treated dogs).

percentage in both groups. As depicted, the percentage of activated (NBT-positive) neutrophils in group A (untreated) remained without changes during the study. In contrast, percentage of activated neutrophils in group B (treated) showed a rapid increase. Statistically differences were retrieved in the treated group respect from the baseline on days 15, 30 and 60, but not in day 90. According to these results, the effects of a 30-day course treatment with Domperidone on neutrophilic function last at least up to one month after termination of treatment. This increase in the NBT reduction rate is clearly related to the amount of oxygen radicals produced by phagocytes in the oxidative burst (Muniz-Junqueira and de Paula-Coelho, 2008). Although further studies are needed to elucidate the cytokine environment related, we can conclude that according to the results of the present study, the NBT is a useful tool for the follow up of the stimulating effects of Domperidone on the neutrophilic function of healthy dogs and that these effects persist for up to one month after a 30-day course treatment.

References

Baehner, R.L., Nathan, D.G., 1968. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N. Engl. J. Med. 278, 971–976.

Berczi, I., Bertok, L., Chow, D.A., 2000. Natural immunity and neuroimmune host defense. Ann. N.Y. Acad. Sci. 917, 248–257.

Berridge, M.V., Herst, P.M., Tan, A.S., 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. 11, 127–152.

Di Carlo, R., Meli, R., Galdiero, M., Nuzzo, I., Bentivoglio, C., Carratelli, C.R., 1993. Prolactin protection against lethal effects of *Salmonella typhimurium*. Life Sci. 53, 981–989.

Gomez-Ochoa, P., Castillo, J.A., Gascon, M., Zarate, J.J., Alvarez, F., Couto, C.G., 2009. Use of domperidone in the treatment of canine visceral leishmaniasis: a clinical trial. Vet. J. 179, 259–263.

Gomez-Ochoa, P., Lara, A., Couto, G., Marcen, J.M., Peris, A., Gascon, M., Castillo, J.A., 2010. The nitroblue tetrazolium reduction test in canine leishmaniasis. Vet. Parasitol. 172, 135–138.

Hinterberger-Fischer, M., Ogris, E., Kier, P., Bauer, K., Kittl, E., Habertheuer, K.H., Ruckser, R., Schmid, A., Selleny, S., Fangl, M., Sebesta, C., Hinterberger, W., 2000. Elevation of plasma prolactin in patients undergoing autologous blood stem-cell transplantation for breast cancer: is its modulation a step toward posttransplant immunotherapy? Am. J. Clin. Oncol. 23, 325–329.

Lechowski, R., Lenarcik, M., Degorski, A., Winnicka, A., 1991. Serum lysozyme activity and nitroblue tetrazolium reduction test in dogs with diabetes mellitus. Zentralbl. Veterinarmed. A 38, 530–533.

Majumder, B., Biswas, R., Chattopadhyay, U., 2002. Prolactin regulates antitumor immune response through induction of tumoricidal macrophages and release of IL-12. Int. J. Cancer 97, 493–500.

Muniz-Junqueira, M.I., de Paula-Coelho, V.N., 2008. Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-alpha production, but only via TNF-alpha it indirectly increases nitric oxide production by phagocytes of healthy individuals, in vitro. Int. Immunopharmacol. 8, 1633–1638.

Nagahata, H., Sako, T., Reiter, J.A., DiBartola, S.P., Couto, C.G., Kociba, G.J., 1991. Neutrophil adherence, phagocytic-nitroblue tetrazolium reduction and chemiluminescence in canine whole blood. Vet. Res. Commun. 15, 181–188.

Oliva, G., Roura, X., Crotti, A., Maroli, M., Castagnaro, M., Gradoni, L., Lubas, G., Paltrinieri, S., Zatelli, A., Zini, E., 2010. Guidelines for treatment of leishmaniasis in dogs. J. Am. Vet. Med. Assoc. 236, 1192–1198.

Richards, S.M., Garman, R.D., Keyes, L., Kavanagh, B., McPherson, J.M., 1998. Prolactin is an antagonist of TGF-beta activity and promotes proliferation of murine B cell hybridomas. Cell Immunol. 184, 85–91.

Underhill, D.M., Ozinsky, A., 2002. Phagocytosis of microbes: complexity in action. Annu. Rev. Immunol. 20, 825–852.