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ORIGINAL ARTICLE

Effect of storage on the nitro blue tetrazolium reduction test in dog blood samples

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Abstract

Introduction: The nitro blue tetrazolium (NBT) reduction test (NBTT) has been used for measuring the metabolic activity of phagocytes of mammals. Activated neutrophils transform NBT into formazan in the cytoplasm. The NBTT can detect the activation of neutrophils in peripheral blood and is used to assess neutrophil function in dogs. However, the NBTT is not used frequently in the clinical setting, as samples should be processed after blood collection.

Objective: The aim of this study was to evaluate the effect of storage on NBTT in dog blood samples.

Materials and Methods: Residual EDTA blood samples from 22 dogs were included of different ages, breeds, and sex. The buffy coat layer was separated from the blood and incubated with 0.1% NBT. The NBTT was performed at 0, 24, 48, and 72 h after the collection of blood. Blood samples were stored at 4°C until the tests were performed. Blood smears were evaluated by light microscopy, and the NBT reduction rate was reported, which represents the percentage of activated neutrophils. The NBT reduction rate was calculated after counting 300 neutrophils in each slide.

Results: The means of NBTT in dog blood samples at 0, 24, 48, and 72 h were 8.3%, 8.5%, 8.7%, and 7.8%, respectively. No significant differences were observed between time points.

Conclusions: This study showed that the NBTT can be performed up to 72 h after the collection of canine blood if correctly refrigerated at 4°C. This finding supports the performance of the NBTT in the clinical setting.

KEYWORDS
activated neutrophils, canine, formazan, metabolic measurement, NBT

1 | INTRODUCTION

The nitro blue tetrazolium reduction test (NBTT) has been widely used for measuring the metabolic activity of mammals and microbial cells.[1–3] The NBT is a soluble and colorless ditetrazolium salt that can be transformed into insoluble and blue-colored formazan when reduced.[1] Consequently, the reduction of NBT has been used to measure reactive oxygen species (ROS) production in phagocytes.[1]

For example, activated neutrophils and monocytes can reduce NBT into formazan inside the phagocytic vacuole by the

NADPH-oxidase enzyme,[1–3] and the amount of reduced NBT is directly proportional to the amount of ROS produced in the oxidative burst.[4] Then, the NBT reduction rate can be obtained by calculating the percentage of neutrophils and monocytes containing formazan in their cytoplasm by ordinary light microscopy.[2] Thus, NBTT can detect the activation of neutrophils and monocytes in peripheral blood.

Furthermore, this test has been previously used to assess canine neutrophil function in leishmaniosis,[3] monocytic ehrlichiosis,[5] diabetes mellitus,[6] and transitory immunosuppression following immunization with polyvalent vaccines.[7] However, the NBTT is not used frequently in veterinary clinical settings, probably due to the assay's protocol limitations which dictate that the samples should be processed within 2–6 h after sample collection.[8]

Hence, the aim of this study was to evaluate the effect of storage at 4°C of the samples up to 72 h on the results of NBTT in dog blood samples.

2 | MATERIALS AND METHODS

2.1 | Dogs

Residual EDTA blood samples from 22 dogs were included in this prospective study. Between one to six milliliters of blood was collected from the dogs by jugular or metatarsal venipuncture for routine laboratory tests. The dogs were from Catalonia (Spain) and were sampled in 2019 for an annual health check-up. The dogs belonged to private owners, and written informed consent was obtained from the respective owners (including staff and student volunteers). This includes consent for the use of any remaining materials from those initially obtained for diagnostic purposes. Therefore, ethical study approval was not needed due to the use of residual blood samples. A physical examination was performed for all dogs included in the study. Four dogs were considered sick due to dermatological clinical signs compatible with clinical leishmaniosis,[9] and they were also seropositive in a quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies.[10] Briefly, serum samples were diluted to 1:800 in phosphate-buffered saline (PBS)-Tween with 1% dry milk and incubated for 1 h at 37°C. After performing three washes with PBS–Tween and one wash with PBS (pH = 7.3), samples were incubated for 1 h at 37°C with peroxidase-conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.16 ng/μL. After incubation, washes were repeated, and the o-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) was added to the plate. The reaction was stopped with 5 M H_2SO_4. Results were read at 492 nm in a spectrophotometer machine (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China) and were defined as ELISA units (EU) in relation to a positive canine sera sample used as a calibrator set at 100 EU as described elsewhere.[10] Dogs were seropositive when having a result equal to or higher than 35 EU. The other 18 dogs were classified as apparently healthy.

2.2 | Residual EDTA blood storage and nitro blue tetrazolium reduction test

After blood collection, residual EDTA blood samples were placed into separate Eppendorf tubes (around 100 μL in each Eppendorf) within 1 h from sampling. One of the tubes was kept at room temperature and immediately used for NBTT, while the other three were stored at 4°C until further use at 24, 48, and 72 h, respectively.

The NBTT was performed as described elsewhere[3] with some modifications. Blood was left at room temperature (between 20°C and 25°C) for 15 min before NBTT was performed. Afterward, blood was mildly mixed, and three 40 mm/20 μL hematocrit capillary microtubes (Servopax, Wiesel, Germany) were filled. Then, microtubes were centrifuged at 2910 *g* for 5 min (Fugevet+ GDC005, Nahita International LTD, London, UK) to obtain the buffy coat layer. After centrifugation, the buffy coat layer from the three microtubes was harvested after carefully breaking the microtubes in half (as near as possible to the buffy coat without producing any damage) and then pushing the buffy coat with a syringe and placing it in an Eppendorf. When all buffy coats were placed in an Eppendorf tube, a similar volume of 0.1% NBT (N6876, Sigma-Aldrich Co., St. Louis, USA) solution was added (proportion 1:1). The Eppendorf was mildly mixed, and incubated for 15 min in a heater at 37°C, and another 15 min at room temperature (between 20°C and 25°C). After incubation, three blood smears were obtained by placing 3 μL of NBT-stained blood on each slide. Each slide was subsequently stained with Diff-Quik (Sigma-Aldrich Co., ST. Louis, USA) and evaluated by ordinary light microscopy, first at 10× and 20× magnifications to scan the slide and at 40× (Figures 1 and 2) and 60× magnification for cell counting, and 100× (Figure 3) magnification during cell counting to confirm the deposition of formazan in those cells with low deposition that could be confused with artifacts. The test

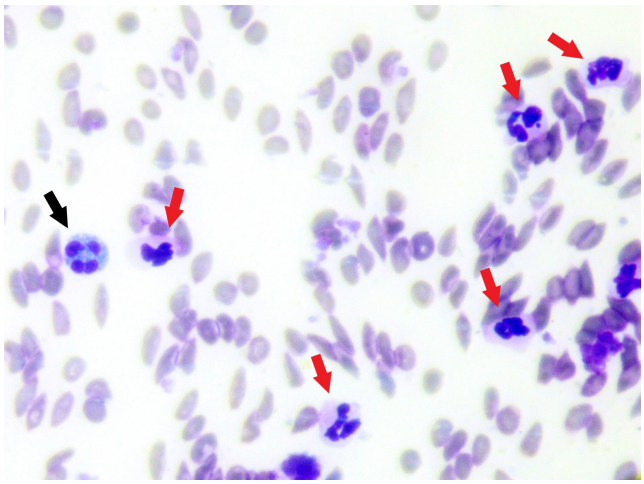

FIGURE 1 Several neutrophils without reduced formazan in the cytoplasm (red arrows) and another neutrophil/monocyte showing reduced formazan in the cytoplasm (black arrow) surrounded by red blood cells (×40 objective) stained with Diff-Quik. Formazan sometimes hinders differentiation between neutrophils and monocytes.

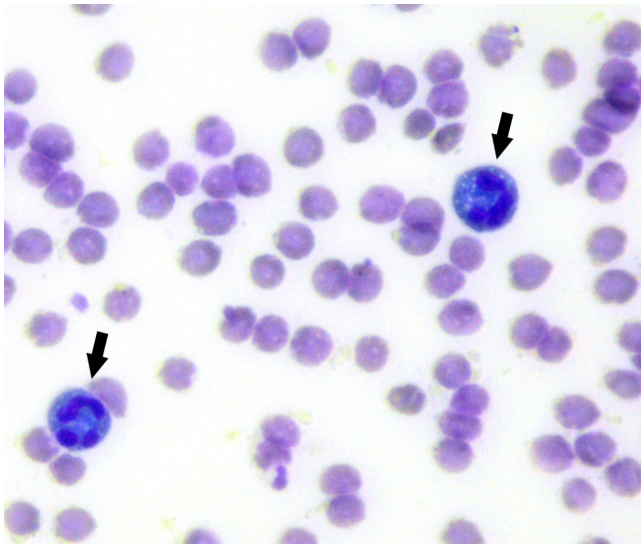

FIGURE 2 Two neutrophils/monocytes showing reduced formazan in the cytoplasm (black arrows) surrounded by red blood cells (×40 objective) stained with Diff-Quik. Formazan sometimes hinders differentiation between neutrophils and monocytes.

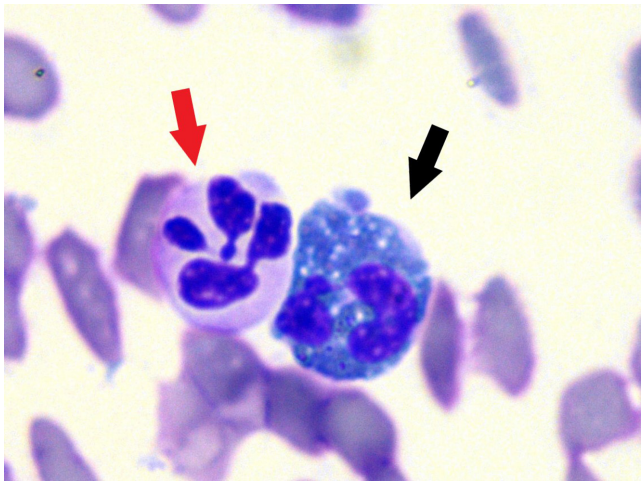

FIGURE 3 One neutrophil without reduced formazan in the cytoplasm (red arrow) and another neutrophil/monocyte showing reduced formazan in the cytoplasm (black arrow) surrounded by red blood cells (×100 objective) stained with Diff-Quik. Formazan sometimes hinders differentiation between neutrophils and monocytes.

results were reported as NBT reduction rate, which represents the percentage of activated neutrophils/monocytes. The NBT reduction rate was calculated after counting 300 phagocytes in each slide (which included cells with blue formazan deposits+non-activated neutrophils). Those cells aggregated or broken were rejected during cell counting. Thus, the percentage was the number of activated neutrophils/monocytes, as defined by those containing dark blue formazan deposits, divided by the total number of non-activated neutrophils and multiplied by 100. It is important to highlight that it is difficult to differentiate neutrophils from monocytes when there are blue formazan deposits.

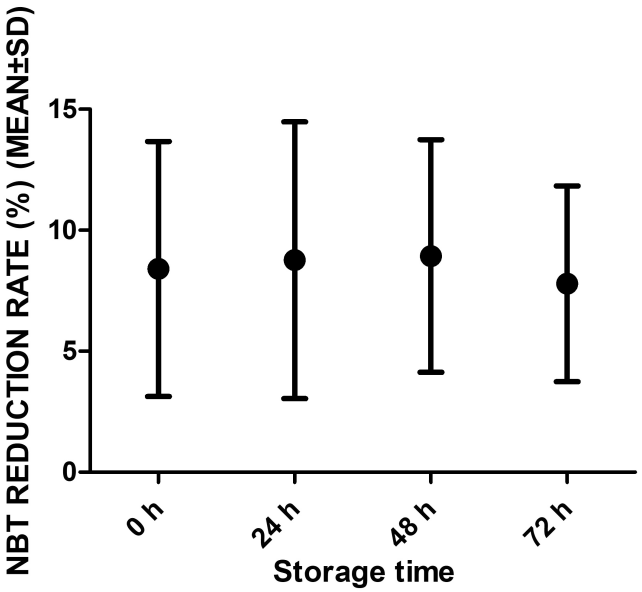

FIGURE 4 Change of NBT reduction rate (%) of peripheral blood neutrophils in each studied time point.

The procedure described above was performed at 0 h (fresh blood) and 24, 48, and 72 h (blood stored at 4°C) in 17 blood samples. In the other five cases, the procedure was not performed at 72 h due to a lack of samples.

2.3 | Statistical analysis

The statistical analysis was performed using the package Stats for the software R i386 3.6.1 for Windows, using an ANOVA for repeated measures with Dunnett's multiple comparison tests to detect differences between time points (0 h vs 24 h, 48 h, and 72 h) and *t*-test was used to compare between groups (crossbreed or purebred, female or male, and healthy or sick). The Shapiro–Wilk test was performed to detect the normal distribution of quantitative variables. NBT reduction rate, sex, and age data were normally distributed ($P > 0.05$). A *P*-value of <0.05 was considered statistically significant. Graphs were plotted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

Most of the dogs were classified as crossbred (72.7%, 16/22), while the purebred dogs included two Labrador retrievers, two Golden retrievers, one Spanish greyhound, and one Ibizan hound. The mean age was 6 years and ranged from 1 to 13 years. Both sexes were included, with 12 females (63.2%) and 7 males (36.8%). Information on age and sex was missing for three dogs.

The mean NBT reduction rate of neutrophils and monocytes at 0, 24, 48, and 72 h was 8.3%, 8.5%, 8.7%, and 7.8%, respectively (Figure 4). No significant differences were observed between time

TABLE 1 NBT reduction rate results.

Number of dogs		Mean NBT (%) reduction rate (±SD)			
		0 h	24 h	48 h	72 h*
Total (N = 22)		8.3 (±5.3)	8.5 (±5.4)	8.7 (±4.8)	7.8 (±4.0)
Breed	Crossbreed (n = 16)	7.7 (±4.7)	7.7 (±4.9)	8.2 (±4.4)	7.1 (±3.7)
	Purebred (n = 6)	10 (±6.7)	10.6 (±6.6)	10.1 (±6.1)	10.1 (±4.7)
Sex**	Female (n = 12)	8.2 (±5.6)	8.6 (±6.3)	8.8 (±5.5)	7.6 (±4.5)
	Male (n = 7)	8.6 (±4.2)	9.5 (±4.0)	9.6 (±3.6)	8.2 (±3.4)
Clinical status	Apparently healthy (n = 18)	8.5 (±5.6)	8.7 (±5.9)	8.9 (±5.2)	7.9 (±4.4)
	Sick*** (n = 4)	7.3 (±3.3)	7.4 (±2.7)	7.6 (±2.0)	7.4 (±2.6)

*The procedure was not performed at 72 h due to a lack of samples in five dogs.; **Information on sex was missing in three dogs.; ***Four dogs were considered sick due to dermatologic clinical signs compatible with clinical leishmaniosis, and they were also seropositive in a quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies.

Abbreviations: NBT, nitro blue tetrazolium; SD, standard deviation.

points (repeated measures ANOVA with Dunnett's multiple comparison tests: $F = 1.58$, $P = 0.2055$).

The mean NBT reduction rate depending on breed, sex, and clinical status at 0, 24, 48, and 72 h is shown in Table 1. No differences in NBT reduction rates were found when comparing crossbred and purebred, female and male, and healthy and sick dogs ($P > 0.05$).

4 | DISCUSSION

A previous study performed on human samples[11] described that storing blood samples for up to 8 h at 4°C or for up to 4 h at 23°C did not influence the test results. Additionally, when blood samples were stored at 23°C for 8 h or more, the test results increased, but the reasons were not further investigated.[11] In the present study, storage of dog blood samples at 4°C was longer, varying from 24 to 72 h, and the results were similarly not influenced by the time-lapse. We believe that this is the first time that such a result has been reported, as the NBTT protocol demands sample processing within 2–6 h after blood collection.[8] This finding might enable the NBTT to win a place among the standard laboratory tests available to the clinician to assess canine neutrophil and monocyte function in sick dogs.[3,5–7] For example, dogs with canine leishmaniosis presenting a mild disease have a higher NBT reduction rate than healthy dogs,[3] and thus, the NBTT could be used to detect improvement in these cases. In fact, any abnormalities or diseases that affect phagocytic activity in both inflammatory and bacterial processes could be studied using the NBT reduction rate. In human medicine, the NBT reduction rate has been used in numerous cases such as chronic granulomatous diseases,[12,13] tuberculosis,[14] viral meningoencephalitis,[15] and liver abscesses caused by amebiasis.[16] Recently, the NBTT has also been used in several other cases, such as to investigate the immune profile of human patients with fungus infection,[17] gingival fibromatosis,[18] chronic obstructive pulmonary disease,[19] and ROS production during HL-60 cell differentiation,[20] in biofilms,[21] and during heat-stressed whole blood cultures.[22]

Interestingly, other factors, such as the choice of anticoagulant and the type of blood sample (capillary or venous), have been previously investigated when performing the NBTT and have been proven to affect the results.[11,23–25] For example, using heparin as an anticoagulant could produce stimulation of oxidative metabolism and, thus, increase the percentage of activated neutrophils and monocytes in the sample.[11,23] Regarding the type of blood sample when comparing capillary and venous blood, Randall et al[24] reported no differences in the NBT reduction rate between capillary and venous blood in healthy patients. However, in another study,[25] the proportion of activated neutrophils and monocytes was significantly lower in capillary blood than in venous blood in sick patients with an increased proportion of NBT rate. Unfortunately, in the present study, only peripheral blood was collected from dogs and investigated with NBTT; thus, it was not possible to investigate the differences between peripheral and capillary blood in canine samples.

Furthermore, dog characteristics such as breed, sex, and clinical status do not seem to affect the NBT reduction rate during storage, although more information should be included to confirm these results.

Although the present study reports interesting results regarding NBTT in dogs, some limitations must be considered. Further studies should include a higher number of dog samples, specifically samples from dogs with diseases that could affect the number of activated neutrophils and monocytes, such as dogs with canine leishmaniosis,[3] monocytic ehrlichiosis,[5] or diabetes mellitus.[6] Moreover, further studies should evaluate if the NBTT can be performed in blood samples stored longer than 72 h. In addition, the use of imaging software could be helpful assessing the degree of the color change of activated neutrophils and monocytes in canine blood samples, as it has been used previously to assess the intensity of ROS production in mouse spermatozoa by NBTT.[26] Furthermore, non-activated monocytes should have also been included during the cell counting of each slide, even if their exclusion did not affect the results related to the effect of storage in canine blood samples. Not counting non-activated monocytes could affect the reported percentage,

even though the proportion of monocytes in canine blood samples is usually low, while the proportion of neutrophils is much higher.[27]

5 | CONCLUSION

This study showed that the NBTT could be performed up to 72 h after the collection of canine blood if correctly refrigerated at 4°C. This finding supports the performance of NBTT in the clinical setting.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

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