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Effects of Blood Storage Time and Temperature on Döhle body and or Döhle body-like Inclusions in Feline Neutrophils

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Running Title

Time and temperature effects on Döhle body of cat neutrophils

Abstract

BACKGROUND: The clinical significance of detecting Döhle body inclusions in cat neutrophils as one of the most relevant toxic changes has necessitated the study of pre-analytical factors such as temperature and blood storage time on the formation of these changes. **OBJECTIVES:** The present study sought to

investigate the impact of blood storage time and temperature on Döhle or Döhle-like inclusions in cat neutrophils.

METHODS: EDTA blood samples were obtained from eight cats without evidence of Döhle inclusions on fresh blood smears (T0). Samples were stored at room temperature (RT) and 4°C, as routine storage temperatures of samples in the laboratory. Smears were prepared 2 (T2), 4 (T4), 8 (T8), and 24 (T24) hours following the blood draw for each storage condition. Döhle or Döhle-like inclusions were assessed on each smear randomly selected.

RESULTS: The percent of neutrophils with Döhle or Döhle-like inclusions in T8 and T24 increased significantly at RT and 4 °C, respectively (P< 0.001), in comparison with T0. The smears prepared from blood samples stored at RT contained more neutrophils with Döhle or Döhle-like inclusions than 4 ° C. A significant difference was not found in the percent of neutrophils with these inclusions between the two temperatures at any of the storage times.

CONCLUSIONS: The development of Döhle body-like in cat neutrophils occurs when the analysis is delayed, especially at higher storage temperatures, and may affect diagnosis and clinical decisions. Therefore, the blood smears should be prepared as soon as the blood is drawn to reduce pre-analytical changes.

KEYWORDS

Cat neutrophils, Döhle bodies, Döhle body-like inclusions, Temperature

Introduction

A toxic neutrophil is a neutrophil that exhibits certain specific morphologic abnormalities on Romanowsky-stained peripheral blood smears. The changes occur in bone marrow during the maturation process or in association with certain diseases. The most prominent cytoplasmic changes are Döhle bodies, basophilia, toxic granulation, and vacuolation. Nuclear changes and changes in cell size and shape also can occur. Döhle bodies are seen in neutrophils of healthy cats (Gori *et al.,* 2021). In other species, Döhle bodies are seen in animals that exhibit signs of illness and represent toxic change. In neutrophils and their precursors, Döhle bodies are bluish, angular inclusions in the cytoplasm. These structures are retained aggregates of the rough endoplasmic reticulum (Harvey, 2017).

Döhle bodies are more sensitive in human and feline hematology than cytoplasmic vacuolation in diagnosing bacterial infections (Lima *et al.,* 2010). Other researchers found that small and dotted blue Döhle body-like inclusions formed from the accumulation of a rough endoplasmic reticulum or ribosomes and were seen in neutrophils in cats that had no clinical signs of inflammation. These components appear to be a false secondary alteration due to prolonged or improper storage of the samples. Since toxic changes in the neutrophils of cats, including Döhle bodies, are clinically significant, it is necessary to know about pre-analytical factors such as storage time and temperature that may cause artificial changes and misdiagnosis of true toxic changes (Aroch *et al.,* 2005). This study aimed to determine the effects of time and temperature on the development of Döhle or Döhle-like cytoplasmic inclusions in the neutrophils of clinically healthy cats.

Materials and methods

The study protocol was approved by the local animal research ethical committee at Semnan University of Veterinary medicine. Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work for the procedure(s) undertaken.

Eight clinically healthy male cats (DSH and Persian, 4 of each breed with an age range of 2 to 4 years), without any clinical history of anemia and inflammation, were selected from cats referred to the Semnan University Veterinary Teaching Hospital (SUVTH) for this study. Two milliliters (2 ml) blood samples for a CBC were obtained in potassium EDTA-containing tubes (Non-vacuum K2EDTA, FARTEST, IRAN) and examinations were performed within 15 minutes of sample collection (to avoid EDTA storage artifacts) by Celltac Alpha MEK-6500K analyzers (Nihon Kohden) for the use of the most common animal types, including dog, cat, cow, and horse.

One blood smear for differential leukocyte counting and morphologic evaluation was prepared immediately after blood collection and was considered as baseline time (T0). Three Eppendorf tubes were considered for each temperature, and 150 µl of blood were added to each tube. Blood smears were prepared from tubes stored at RT (22±3 °C) and refrigerator temperature (4 °C) after 2, 8, and 24 hours, and they were stained with Giemsa. There were 56 blood smears (6 blood smears per cat for 2, 8, and 24 hours and one smear was also prepared for time zero). Each blood smear was evaluated to determine Döhle bodies or Döhle body-like inclusions.

Döhle body-like inclusions are defined as a small dotted, intra-cytoplasmic inclusion of light blue-gray (Boudrax *et al.,* 2010). Döhle bodies were considered larger intracytoplasmic inclusions, light blue-gray, elliptic to amorphous (Takeuchi *et al.,* 2010), counted as 100 neutrophils per slide, and the percentage of neutrophils with Döhle or Döhle-like cytoplasmic inclusions was recorded.

Statistical analysis was done by SPSS statistical software. First, to normalize the data distribution, their logarithm was calculated. Then the results of the percentage of neutrophils with Döhle body-like inclusions or Döhle bodies between two groups of temperature storage (RT and 4 °C) and for each storage period (2, 8, and 24 h) were compared by linear method for repeated measure. The Benjamini-Hochberg method was used to correct the 5 % alpha level for multiple pairwise comparisons, and *P*-values less than 0.001 following correction of 5% alpha level, were considered statistically significant.

Results

Neutrophils with Döhle (Figure 1) or Döhle-like cytoplasmic inclusions (Figure 2) were observed in most blood smears. The blood smear examination of all cases, with the exception of cases 1 and 4, prepared from blood stored at RT, showed that the percentage of neutrophils with Dohl or Dohl-like inclusions in 24 hours was more than 8 hours. (Figure 3), the percentage of neutrophils with Döhle body-like inclusions was higher than Döhle bodies at both temperatures. The percentage of neutrophils with Döhle bodies or Döhle body-like inclusions in T8 and T24 increased significantly for RT ($P< 0.001$) and 4 °C ($P< 0.001$) compared to T0, respectively (Table 1). The percentage of neutrophils with Döhle or Döhle-like cytoplasmic inclusions in blood samples stored at RT was more than 4 °C. No statistically significant difference was observed in the percentage of neutrophils containing Döhle bodies or Döhle body-like inclusions between the two storage temperatures in samples T2, T8, and T24 (Table 1).

Discussion

An integral part of a hematological evaluation is the accurate identification of neutrophil cytoplasmic inclusions. Among the most common cytoplasmic inclusions observed in cat neutrophils are Döhle or Döhle-like cytoplasmic inclusions. Though, other cytoplasmic inclusions of neutrophils in cats include

storage lysosomal granules includings mucopolysaccharidosis VI, VII, and gangliosidosis GM2, red granules in Birman cats (Gough *et al.,* 2018), and other cat breeds, pink-purple granules in Chediak-Higashi syndrome (Bauckley *et al*., 2020*)* large blue thread-like inclusions in May-Hegglin anomaly (Flatland *et al.,* 2011). Misdiagnosis of leukocyte inclusions is associated with serious clinical consequences, including misdiagnosis, incorrect treatment, and poor prognosis.

The manifestation of Döhle or Döhle-like cytoplasmic inclusions in cat neutrophils in the present study (Figure 1) increased after 8 hours of blood sampling relative to baseline time at both room and refrigerator temperatures. This increase with a steeper slope in most samples kept at room temperature, except for the first and fourth samples, reached its highest percentage in 24 hours. While at refrigerator temperature, the slope of increasing the percentage of neutrophils with Döhle-like bodies or inclusions was milder. It must be mentioned that the increase in the percentage of neutrophils with Döhle-bodies or Döhle body-like inclusions at room temperature was more significant than the temperature of the refrigerator. This finding was consistent with the results of a study by Bau-Gaudreault *et al*. (2019) which examined the impact of duration and maintenance on toxic or semi-toxic changes in neutrophils of dogs. As the results of Table 1 revealed, there was no statistically significant difference in the percentage of neutrophils with Döhle or Döhle-like cytoplasmic inclusions between the RT and refrigerator temperatures during storage times of 2, 8, and 24 hours.

Since the actual toxic change in bone marrow neutrophils occurs before diffusion into the peripheral blood (Takeuchi *et al.,* 2010), the nature of the Döhle body-like inclusions observed in the present study is uncertain. The inclusions are thought to result from accumulated rough endsoplasmic reticulum or ribosomes over time, in which case the cells become more permeable to staining, either as a result of the staining of previously invisible organs, or as a result of the destruction of existing organs. Evaluation of these smears by electron microscopy can significantly help to clarify their origin. Blood smears

assessment to evaluate toxic neutrophil changes is a cheap, fast, simple, and accessible process that indicates the infectious and metabolic diseases in cats. Observation of toxic neutrophils is a significant diagnostic finding and an aid in patient evaluation, disease course, length of hospital stay, and treatment planning. In cats, unlike dogs, toxic neutrophils were not associated with higher mortality (Gori *et al.,* 2021). Various reports have shown time- and temperature-dependent changes in erythrocytes, white blood cells, platelets, and automatic and manual CBC markers in cattle (Ihedioha *et al.,* 2007), laboratory animals, dogs, sheep, goats, horses, turkeys, and sea lions (Hadzimusic *et al.,* 2010). The time delay between sampling and sample analysis is when blood samples are sent to reference laboratories or when the analysis cannot be performed easily, affecting the quality of the analysis. Ameri *et al.* (2011) found that although most changes in the blood tests of monkeys, rabbits, mice, and rats when the sample was stored at 4 °C were clinically insignificant, the best way to test the blood of these animals is to process the blood promptly, preferably 1 hour after blood collection. In the present study, no other toxic changes such as basophilic cytoplasm and foamy vacuolation were observed in feline neurophiles. Perez-Ecija *et al.* (2020) found that increased basophilia and foamy vacuolation of the neutrophil cytoplasm in the smears within 1 hour of blood collection indicated inflammatory disease in donkey blood. Regarding the slight increase in foamy vacuolation that occurs over time in EDTA, the prominence of moderately vacuolated neutrophils in the smears that occur a few hours after blood collection is questionable. Though, moderate or severe foamy vacuolation should be considered clinically.

Conclusions

Based on the current study, morphological changes in neutrophils of healthy cats developed in vitro in addition to Döhle or Döhle -like cytoplasmic inclusions, including low foamy vacuolation, without

cytoplasm basophilia, which were different from neutrophils associated with severe inflammation. Therefore, determining the interpretation of toxic changes in cat neutrophils is affected by storage conditions and time. Hence, it is suggested that a freshly prepared blood sample be sent to the laboratory immediately after blood sampling with a blood sample stored at 4 °C. It is also recommended that the time and date of the blood sampling be clearly stated on the submitted samples.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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TABLE 1

A comparison of the percentage of neutrophils with Döhle bodies or Döhle body-like inclusions between T2, T8, and T24 with T0, and RT and RF.

Temperatures Times	RT	4 °C	
T0	5.00 ± 2.15		
T2	21.00 ± 3.07 ($P = 0.028^{a}$)	14.00 ± 2.45 ($P = 0.096^{a}$)	($P = 0.129^{b}$)
T8	24.87 ± 4.38 ($P < 0.001^{a*}$)	20.37 ± 2.92 ($P = 0.001^{a*}$)	($P = 0.060^{b}$)
T24	41.62 ± 5.39 ($P < 0.001^{a*}$)	30.62 ± 3.24 ($P < 0.001^{a*}$)	($P = 0.111^{b}$)

RT; Room temperature

[a] indicated that the comparison between T2, T8, T24 with T0.

[b] indicated that the comparison between RT and RF.

[*] indicated that significantly different following correction for multiple comparisons.

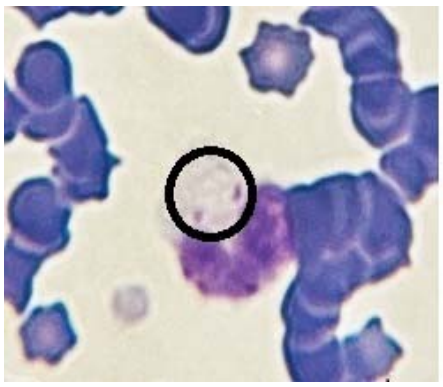

Figure 1. The Döhle bodies are circled in black in cat neutrophil

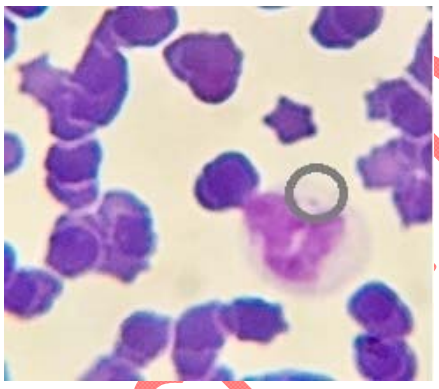

Figure 2. The Döhle body-like inclusion is circled in gray in cat neutrophil

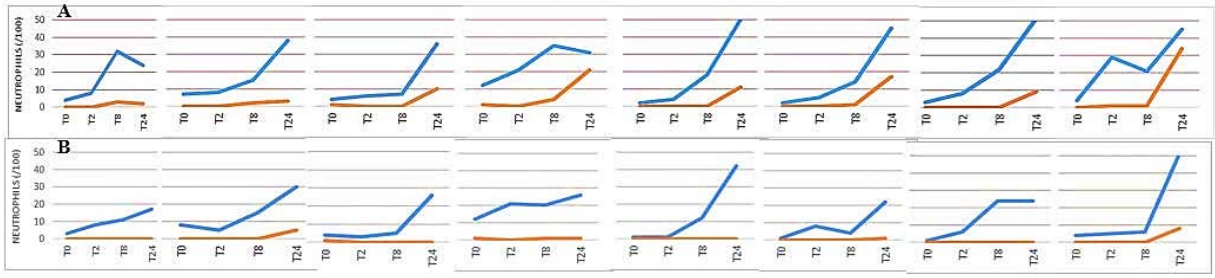

Figure 3. A histogram shows the progressive appearance of Döhle or Döhle-like cytoplasmic inclusions within each case for each temperature and time point. The Döhle-like cytoplasmic inclusions are displayed in orange, and the Döhle bodies are displayed in blue. A, Room temperature, B, 4 °C, T2: 2 h post blood collected, T8: 8 h post blood collected, T24: 24 h post blood collected

اثرات دما و زمان نگهداری خون بر گنجیدگی های دل و شبه دل در نوتروفیل های گربه

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خلاصه

زمینه مطالعه: اهمیت بالینی تشخیص گنجیدگی های دل در نوتروفیل های گربه به عنوان یکی از مرسوم ترین تغییرات توکسیک، مطالعه عوامل پیش تحلیلی مانند دما و زمان ذخیره سازی خون در شکل گیری این تغییرات را ضروری کرده است .

هدف: مطالعه حاضر به دنبال بررسی تأثیر زمان و دمای ذخیره سازی خون برگنجیدگی های دل یا شبه دل در نوتروفیل های گربه بود.

روش کار:

نمونه خون **EDTA** دار از هشت گربه بدون شواهدی از گنجیدگی های دل در گسترش های خون تازه **(T0)** بدست آمد. نمونه‌ها در درجه حرارت اتاق (**RT**) و 4 درجه سانتیگراد، به عنوان دماهای متداول نگهداری نمونه ها در آزمایشگاه نگهداری شدند. گسترش ها 2 ساعت **(T2)**، 8 ساعت **(T8)** و 24 ساعت **(T24)** پس از خونگیری برای هر دو دمای ذخیره سازی تهیه شدند. گنجیدگی های دل یا شبه دل در هر گسترش به صورت رندوم ارزیابی شدند.

نتایج: درصد نوتروفیل های دارای گنجیدگی های دل یا شبه دل در **T8** و **T24** به ترتیب در درجه حرارت اتاق و 4 درجه سانتی گراد در مقایسه با **T0** به طور معنی داری افزایش نشان داد (***P***<0/001). گسترش های خونی تهیه شده از نمونه های خون نگهداری شده در درجه حرارت اتاق دارای نوتروفیل‌های حاوی اجسام دل یا گنجیدگی های شبه دل بیشتری نسبت به نمونه‌های خون ذخیره شده ادر ۴ درجه سانتی‌گراد بود. اختلاف آماری معنی داری در درصد نوتروفیل های دارای این گنجیدگی ها بین دو دما در هیچ‌یک از زمان های ذخیره سازی یافت نشد.

نتیجه‌گیری: ایجاد گنجیدگی های شبه دل در نوتروفیل‌های گربه زمانی اتفاق می‌افتد که آرمایش خون به تاخیر بیفتد، به خصوص در دمای ذخیره‌سازی بالاتر، و ممکن است بر تشخیص و تصمیم‌گیری بالینی تأثیر بگذارد. بنابراین، یک گسترش خون باید به محض خونگیری تهیه شود تا تغییرات پیش از آزمایش کاهش یابد.

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کلمات کلیدی:

نوتروفیل گربه، اجسام دل، گنجیدگی های شبه دل، دما، زمان

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