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Title

Comparison between May Grünwald-Giemsa and rapid cytological stains in fine-needle aspirates of canine mast cell tumour: diagnostic and prognostic implications

Abstract

Mast cell tumours (MCTs) are often diagnosed by cytology based on the identification of purple intracytoplasmic granules with methanolic Romanowsky stains, including May-Grünwald-Giemsa (MGG). In clinical practice, aqueous rapid Romanowsky stains (RS) are commonly used, but mast cell granules may not stain properly. Aim of this prospective study was to investigate the frequency of MCT hypogranularity with RS and its potential implications in tumour identification, cytological grading assessment and recognition of nodal metastatic disease. Cytological preparations of canine primary MCTs and metastatic lymph nodes with subsequent histopathological confirmation were included. For each case, good-quality smears were stained with both MGG and RS and comparatively assessed. Eleven of 60 (18.3%) primary MCTs were hypogranular with RS; 9 of them were histologically high-grade tumours and in 3 cases (5%) a definitive MCT diagnosis could not be made. Accuracy in cytological grading assessment (85%) did not differ between RS and MGG. Thirteen of 28 (46.4%) metastatic lymph nodes were hypogranular with RS and three independent observers failed to identify nodal MCT metastases in 7-18% of RS-stained smears. This study confirms that, in limited cases, RS can be ineffective in staining MCT granules, particularly in high-grade tumours, thus making diagnosis more dependent on experience and quality of preparations. In dubious cases, methanolic stains should be applied. The use of RS is discouraged for the search of nodal metastases, as the identification of isolated mast cells can be more challenging.

Keywords

Canine; cytology; granules; Diff Quik; mast cell tumour; rapid stain; May-Grünwald-Giemsa

Introduction

The cytological diagnosis of mast cell tumour (MCT) is considered among the easiest and most consistent, being based on the identification of the characteristic purple intracytoplasmic granules.[1,2] Furthermore, cytological grading systems have been recently developed for canine MCTs, which can provide useful prognostic information on tumour biological behaviour at the

initial visit, thereby contributing to plan the most appropriate therapeutic strategy.[3-5] Additionally, MCT staging by cytological examination of regional lymph nodes, spleen, liver and bone marrow plays an essential role in the assessment of true disease extent; this procedure holds a well-acknowledged prognostic value, and again it is critical for therapy planning.[2,6]

Although toluidine blue is considered the best staining method for the demonstration of mast cell granules,[7,8] the cytological diagnosis of MCT is mostly performed on smears stained with methanolic Romanowsky stains (including May Grünwald-Giemsa [MGG] and Wright's), which over time have found widespread application in veterinary cytology due to their rapidity and easiness of use.[9]

The integration of cytology in daily clinical practice has raised the need to further simplify and reduce the time of staining procedures leading to the spread of rapid aqueous Romanowsky stains (RS). These methods allow to obtain cytological preparations in a few minutes and include a first dip in an alcoholic fixative (methanol) and two consecutive passages in two stains: Eosin Y, an acid dye, and Azure B, a metabolite of methylene blue. Since the introduction of the first RS (Diff Quik, Harleco, USA) in the mid-seventies, many other commercially-available versions have been developed, with equivalent chemical composition.[10]

The staining affinity of RS is similar to that of methanolic Romanowsky stains, although the majority of cytology textbooks report that, in a variable proportion of cases, RS may not adequately stain the granules of mast cells.[1,9,11,12] It has been hypothesized that this may be related to the water-soluble nature of the granule content and inability to form a stable precipitate.[12] Thus, depending on the poor differentiation of the neoplasm and inexperience of the cytologist, MCTs could be misdiagnosed with other tumour types, including histiocytoma, lymphoma, plasma cell tumour and melanoma, with detrimental clinical consequences.[1] However, this phenomenon remains anecdotic and has never been supported by scientific observations.

In the present study, the staining efficiency of MGG and RS was compared in fine-needle aspirates (FNAs) of primary and metastatic canine MCTs. The aim of this prospective study was to determine the frequency of mast cell hypogranularity using RS and the possible correlation with intrinsic tumour features, i.e. dermal or subcutaneous location and differentiation. Furthermore, the diagnostic and prognostic implications of RS hypogranularity were specifically investigated by evaluating accuracy in MCT identification, assessment of cytological grading and recognition of nodal metastatic disease.

Materials and Methods

Study design and cytological parameters

A prospective study was carried out on cytological preparations of primary canine MCTs and metastatic lymph nodes with subsequent histopathological confirmation. Cases were recruited at the pathology service of the Department of Veterinary Medical Sciences, University of Bologna, Italy and at the veterinary clinic "Centro Oncologico Veterinario", Sasso Marconi, Italy.

Surgical samples of cutaneous nodules with a diagnosis or a suspect of MCT and regional lymph nodes of MCT-bearing dogs were considered for inclusion in the study. For each case, 2-5 smears were obtained by FNA of the surgical sample, allowed to air-dry and stained with both MGG and RS. Smears with inadequate cellularity or with a high percentage of damaged cells were excluded.

Primary tumours were ultimately included upon histological confirmation of cutaneous or subcutaneous MCT; lymph nodes were included upon histopathological confirmation of early (HN2) or overt (HN3) metastasis according to Weishaar *et al*., 2014.[13]

Background information recorded for all cases included tumour site (cutaneous or subcutaneous), histological grading according to Patnaik *et al*. (1984) and Kiupel *et al*. (2011) for cutaneous MCTs and presence of histological negative prognostic factors according to Thompson *et al*. (2011) for subcutaneous MCTs.[14-16]

MCTs were classified as histologically malignant if high-grade according to Kiupel et al. (2011), grade III according to Patnaik et al. (1984) or, in the case of subcutaneous MCTs, in the presence of 2 out of 3 negative prognostic indices according to Thompson et al., 2011.[14-16]

The selected smears were microscopically examined by two of the authors (SS and AR) for the comparative assessment of granulation between MGG and RS, which was scored as follows:

- 0, absence of granules: no evidence of granules in more than 80% of mast cells;
- 1, poor granulation: grey cytoplasm with few granules in 50-80% of mast cells;
- 2, moderate granulation: cytoplasm of more than 50% of mast cells pink and dusty due to the presence of numerous fine granules or mixed granulation (presence of an equal proportion of mast cells with high and poor granulation);
- 3, high granulation: cytoplasm of more than 50% of mast cells homogeneous and dark blue/violet for the presence of numerous granules.

Based on this scoring system, cases were defined as hypogranular (RS smear with a lower granulation score compared with the corresponding MGG smear) or isogranular (no difference in the granulation score between RS and MGG).

In primary MCTs, further parameters assessed blindly for both stains were the certainty of MCT diagnosis (yes/no) and the cytological grade of malignancy (low/high) according to Camus *et al*. (2016).[5]

For metastatic lymph nodes, the proportion of mast cells on the total number of cells (defined as low: <10%; moderate: 10-50%; high: >50%) was evaluated. Moreover, for each case, two smears, stained with both MGG and RS, were randomly mixed with negative controls (FNA of non-metastatic lymph nodes obtained for the staging of other neoplasms) and shown to three independent observers routinely involved in diagnostic cytology (AR, GM and CA) with the request to express a judgment on the presence of nodal MCT metastasis (certain, suspected or negative) according to Krick *et al*. (2009).[6]

Statistical analysis

Descriptive statistics were used in the analysis of tumour characteristics.

For primary MCTs, differences in the distribution of hypogranular cases according to tumour site and histological malignancy were assessed by Fisher's exact test or Chi-square test. For nodal metastases, the same tests were applied to evaluate any relationship between hypogranularity and primary tumour site, histological malignancy of the primary tumour, presence of atypical mast cells in the cytological smear and histological degree of nodal infiltration according to Weishaar *et al*., 2014.[13] Chi-square test was also applied to compare the distribution of hypogranular cases between primary and metastatic MCTs.

Accuracy in cytological grading assessment was defined, for both staining, as the proportion of cases correctly graded, using histology as the gold standard.

Data were analysed by use of a commercial software program (SPSS Statistics v24, IBM, Armonk, NY, USA); *P* values ≤ 0.05 were considered significant.

Results

Primary mast cell tumours

Tumour characteristics

Sixty MCTs from 50 dogs fulfilled the inclusion criteria. Eight dogs (13.3%) had multiple MCTs, diagnosed simultaneously (*n* = 4) or at different times (*n* = 4).

There were 40 cutaneous (66.7%) and 20 subcutaneous MCTs (33.3%). On histological examination, 21 tumours out of 60 (35%) were judged malignant (including 16 cutaneous and 5 subcutaneous MCTs).

Granulation score

With MGG, 48 MCTs (80%) were highly granular, 10 (16.7%) were moderately granular, and 1 (1.7%) tumour each was poorly granular and not granular. With RS, 44 MCTs (73.3%) were highly granular, 8 (13.3%) moderately granular, 4 (6.7%) poorly granular, and 4 (6.7%) were not granular. All the 8 cases that were poorly granular or not granular with RS were histologically high-grade ($P < 0.001$).

With RS, 11 cases (18.3%) were hypogranular compared with the corresponding MGG smear, whereas in no case mast cells were more granular with RS (Figures 1-3). Hypogranularity was significantly correlated with a high tumour malignancy ($P < 0.001$; Table 1).

Diagnostic consistency and grading assessment

With RS, in 4 cases (6.7%) it was not possible to make a certain diagnosis of MCT but only of a poorly-differentiated round cell neoplasm. With MGG, the doubt persisted in only one of these cases.

With MGG, 42 low-grade (70%) and 18 high-grade MCTs (30%) were identified. The examination of the same cases stained with RS led to the identification of 46 low-grade (76.7%) and 14 high-grade tumours (23.3%). Using histology as the gold standard, the accuracy in grading assessment was exactly the same (85%) between MGG and RS.

Nodal metastases

Tumour characteristics

Twenty-eight cases of histologically-confirmed metastatic lymph nodes were included. The cases were retrieved from 24 dogs, and the corresponding primary tumours consisted of 22 cutaneous (78.6%) and 6 subcutaneous MCTs (21.4%); 11 of which (39.3%) had been judged as histologically aggressive. Histological examination of lymph nodes with toluidine blue staining allowed diagnosing 13 (46.4%) early metastases (HN2) and 15 (53.6%) overt metastases (HN3).

Granulation score

Overall, the percentage of mast cells in the smears was classified as less than 10% in 13 cases (46.4%), between 10% and 50% in 8 cases (28.6%) and more than 50% in other 7 cases (25%). Mast cells were equally distributed in the smears stained with both stains. Metastatic mast cells had evident cytological features of malignancy in 13 cases (46.4%).

With MGG, 12 MCTs (42.8%) were highly granular, 14 (50%) moderately granular and 2 (7,1%) poorly granular. With RS, 6 MCTs (21.4%) were highly granular, 10 (35.7%) moderately granular, 4 (14.3%) poorly granular, and 8 (28.6%) were not granular.

With RS, 13 cases (46.4%) were hypogranular compared with the corresponding MGG smear, whereas in no case mast cells were more granular with RS (Figures 4 and 5). Hypogranularity was not correlated with any of the considered clinicopathological variables (Table 2).

The proportion of hypogranular cases observed in nodal metastases was significantly higher than in primary MCTs ($P = 0.009$).

Diagnostic consistency

The evaluation of the smears stained with MGG resulted in a diagnosis of certain or suspected metastasis in the 100% of cases by all three observers. With RS, the same observers did not identify metastatic lesions in 2 (7.1%), 5 (17.8%) and 3 (10.7%) cases, respectively (Table 3).

Overall, with RS, 7 cases (25%) were erroneously diagnosed as non-metastatic by at least 1 of the 3 observers. All these cases were poorly ($n = 3$) or not granular ($n = 4$) with RS and had a proportion of mast cell under 10%. The 4 hypogranular cases with a higher percentage of mast cells were all correctly identified.

Discussion

These data confirm the anecdotal reports of a lower visibility of cytoplasmic granules with RS. In particular, 18% of primary MCTs were less granular with RS compared with the corresponding sample stained with MGG. In nodal metastases, this phenomenon was more frequent, with 46% of hypogranular cases. However, when evaluating only the cases completely devoid of granules with RS, the percentage decreased to 5% for primary MCTs and 29% for metastatic MCTs.

Hypogranularity was significantly correlated with the microscopic evidence of high tumour malignancy, with no significant difference between dermal or subcutaneous MCTs. In cytology as well as in histology, malignancy is often associated with a lesser differentiation and it is not surprising that the staining of granules tends to be proportional to their number, size and composition. Indeed, poorly differentiated mast cells have lower concentrations of heparin and other glycosaminoglycans (GAGs), which are the main molecules interacting with staining components; therefore, insufficient staining may reflect the lack of production or storage of these particular compounds.[17,18] However, apparently, this phenomenon was amplified by the use of RS.

The reasons why mast cell granules may not stain properly using RS are unclear. This phenomenon has been attributed to an inadequate fixation process. Indeed, while both RS and MGG incorporate an initial methanol fixation, the solutions used in RS are different for concentration, pH, and solvent mediums, and this may ultimately lead to the dissolution of granules.[12,19,20] It was suggested that more prolonged fixation (e.g., minutes) would result in a more effective staining.[20,21] However, this was not confirmed in a recent study.[22]

In the present study, an accurate MCT diagnosis could not be made in 5% cases with RS. While this number is not high enough to discourage the use of RS in canine skin tumours cytology, practitioners should be aware that, in a minority of cases, RS may lead to a wrong or inconclusive diagnosis. Therefore, if a staining deficiency is suspected during the examination of a round cell neoplasm, methanolic Romanowsky stains (*e.g.* MGG, Wright) should be applied on additional smears to ultimately exclude the presence of granules.

Hypogranularity may also potentially affect the assessment of cytological grading. According to a recent study by Camus *et al*. (2016), the cytological evidence of poorly granulated cells would be indicative of a high-grade tumour, without the need to further evaluate the presence of cellular atypia.[4] In that study, a modified Wright's stain comparable to MGG was applied. Notably, in the present study, the hypogranularity of RS does not appear to have influenced the determination of grading, probably due to the close relationship between hypogranularity and other cytologic criteria of malignancy. In turn, even the presence of massive granularity may limit the identification of high-grade tumours, by hindering the recognition of malignant nuclear features (*e.g*., mitosis, multinucleation, anisokaryosis), as recently suggested.[23,24]

The diagnostic limits of RS became more evident in staging, where three independent observers failed in the identification of MCT metastases in 7-18% of RS-stained smears. Diagnostic problems were limited to the smears with low numbers of infiltrating mast cells, whereas in cases with a larger amount of mast cells, hypogranularity did not impair their recognition. In the staging phase, the cytologist is already aware of MCT diagnosis and his task is to identify cells morphologically referable to mast cells in a foreign tissue. In this context, a moderate to high number of mast cells can be easily recognized even without granules, based on the evidence of a relatively monomorphic population of cells with a moderate amount of cytoplasm and a central nucleus, also forming aggregates. On the contrary, few isolated cells with this appearance, admixed with lymphocytes, can be misidentified as histiocytes, normally resident in the nodal parenchyma. Occasionally, the identification of isolated mast cells with RS was difficult despite a moderate granularity, because

the similar colour between granules and nucleus together with cytoplasmic shrinkage mimicked the morphology of large lymphocytes.

Beside the identification of mast cells, it must be considered that the diagnosis of MCT metastasis continues to have a high degree of uncertainty, at both a cytological and a histological level. This is because the percentage and distribution of mast cells in normal or reactive lymph nodes and spleen can be extremely variable and overlap that of metastatic organs.[6,13,25,26] Finally, it is not uncommon to observe nodal macrophages engorged with intracytoplasmic metachromatic granules that may mimic the presence of mast cells.[8]

As a whole, given the high percentage of nodal metastases not identified in this study, the undesirable consequences of this error in a clinical setting and the intrinsic uncertainty of the cytological diagnosis of MCT metastasis, the authors believe that the use of RS should be discouraged for MCT staging purposes.

In conclusion, this study confirms that, in a limited percentage of cases, RS can be ineffective in staining MCT granules, with a higher frequency in high-grade tumours. In these cases, the diagnosis may be more subjective and influenced by the cytologist's experience and quality of preparations. In case of doubt, methanolic Romanowsky stains should be applied. The use of RS is discouraged for the research of MCT metastases in fine-needle aspirates of lymph nodes, as the identification of isolated mast cells in the context of a heterogeneous cell population may be particularly challenging.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure legends

Figures 1-3. Dog. Fine-needle aspirates of primary cutaneous mast cell tumour (MCT) stained with both May Grünwald-Giemsa and rapid cytological stains. **(1)** The cytoplasm of mast cells is dark blue/violet for the presence of numerous granules with both May Grünwald-Giemsa (A) and rapid stain (B). **(2)** The cytoplasm of mast cells shows a variable amount of granules; overall, the staining intensity of granules is stronger with May Grünwald-Giemsa (A) compared with rapid stain (B). **(3)** The cytoplasm of mast cells is pale pink and dusty due to the presence of a moderate amount of granules with May Grünwald-Giemsa (A); with rapid stain (B) granules are not evident in the majority of cells, impairing the possibility of a definitive diagnosis; note prominent anisokaryosis and multinucleations, suggestive of a high-grade MCT. 400x magnification.

Figures 4-5. Dog. Fine-needle aspirates of lymph nodes with mast cell tumour metastasis stained with both May Grünwald-Giemsa and rapid cytological stains. **(4)** Presence of a moderate number of infiltrating mast cells with variable granulation in the smear stained with May Grünwald-Giemsa (A); with rapid stain (B) mast cells are almost completely devoid of granules but are still clearly identifiable. **(5)** Presence of scattered well-differentiated mast cells in the smear stained with May Grünwald-Giemsa (A) and lack of readily identifiable mast cells in the smear stained with rapid stain (B). 400x magnification.

Tables

Table 1. Difference in the staining of granules observed in fine-needle aspirates of 60 histologically-confirmed canine MCTs stained with both May Grünwald-Giemsa and rapid cytological stains, grouped according to tumour characteristics.

Variables	**Number of hypogranular cases with rapid stains†**	***P***
Tumour site		
cutaneous	10/40 (25%)	0.081
subcutaneous	1/20 (5%)	
Histological malignancy		
low	2/39 (5.1%)	<0.001
high	9/21 (42.8%)	
Total	11/60 (18.3%)	

† Compared with additional smears stained with May Grünwald-Giemsa.

Table 2. Difference in the staining of granules observed in fine-needle aspirates of 28 histologically-confirmed MCT metastatic lymph nodes stained with both May Grünwald-Giemsa and rapid cytological stains, grouped according to tumour characteristics.

Variables	**Number of hypogranular cases with rapid stains†**	***P***
Site of primary mast cell tumour		0.390
cutaneous	9/22 (40.9%)	
subcutaneous	4/6 (66.7%)	
Histological malignancy of primary mast cell tumour		0.389
low	9/17 (52.9%)	
high	4/11 (36.4%)	
cytological malignant features of nodal mast cells		0.708
present	6/14 (42.8%)	

absent	7/14 (50%)	
Histological degree of nodal infiltration‡ *HN2* *HN3*	 7/13 (53.8%) 6/15 (40%)	0.462
Total	13/28 (46.4%)	

† Compared with additional smears stained with May Grünwald-Giemsa.

‡ According to Weishaar *et al.*, 2014.

Table 3. Diagnoses of certain, suspected and negative metastatic involvement performed by 3 independent observers on fine-needle aspirates of 28 histologically-confirmed metastatic lymph nodes stained with both May Grünwald-Giemsa and rapid cytological stains.

	May Grünwald-Giemsa			**Rapid stains**		
	Certain	Suspected	Negative	Certain	Suspected	Negative
Observer No. 1	24	4	0	20	6	2
Observer No. 2	28	0	0	20	3	5
Observer No. 3	28	0	0	21	4	3
Mean percentage	95%	5%	0%	73%	15%	12%