

DOI 10.1515/pjvs-2015-0063

Original article

Cytomorphometry of serosal effusion in dogs

R. Przeździecki¹, M. Czopowicz², R. Sapieryński¹¹ Department of Pathology and Veterinary Diagnostics² Laboratory of Veterinary Epidemiology and Economics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW), Nowoursynowska 159c, 02-766 Warsaw, Poland

Abstract

Cytomorphometry made on cytological slides is the quantitative method of precise analysis of cellular structures, including both cytoplasm and nucleus. The aim of this study was to describe cytomorphometric parameters of mesothelial cells in the course of benign reactive and malignant proliferation and to compare them to carcinomas and adenocarcinomas located within serosal cavities in dogs. The second aim was to evaluate applicability of cytomorphometry to diagnostics of diseases causing accumulation of effusion in serosal cavities. Cytological samples of normal and non-malignant mesothelium, mesothelioma and various carcinomas were collected from dogs. Cytomorphometry was made on the smears stained with Giemsa solution. Mean nuclear and cellular perimeter, mean nuclear and cellular area, mean nuclear and cellular diameter, and mean nuclear and cellular roundness were determined. Moreover, nuclear to cytoplasmic ratio (N/C) was calculated. The data revealed statistically significant differences for all parameters, excluding mean nuclear perimeter, between compared groups. Normal mesothelium cells and their nuclei were significantly smaller and more elongated than cells and nuclei of both benign reactive and malignant neoplastic mesothelium. Only a few differences were observed between benign reactive mesothelium cells and mesothelioma cells – mean nuclear area and mean nuclear diameter of benign reactive mesothelium cells were significantly larger and N/C ratio was higher in comparison to mesothelioma cells. Even though some significant differences were observed, considerable overlap of these cytomorphometric parameters in animals with different diseases limited practical role of these observations. Cytomorphometric analysis of cellular samples collected from dogs with proliferative processes affecting serosal cavities can be only an auxiliary method increasing accuracy of preoperative diagnosis.

Key words: cytology, cytomorphometry, dog, mesothelioma, mesothelium, serosal effusion

Introduction

Pathologic processes taking place in the serosal cavities, especially in the pleural or abdominal cavity, are commonly encountered in small animal medicine (Charney et al. 2005, Bertazzolo et al. 2012). They usually manifest themselves with the serosal effusion accumulation and/or abnormal mass formation in the cavity. In many such cases presumptive or even definitive diagnosis can be based on microscopic examination of cellular samples (clinical cytology or cytopathology) collected during diagnostic procedures. However, in some cases cytology is insufficient and final diagnosis requires direct inspection of the affected cavity during laparotomy or thoracotomy, usually associated with collection of tissue samples for histopathological examination (Sisson et al. 1984, Stępien et al. 2000, Geninet et al. 2003, Brisson et al. 2006, Szczepulska-Wójcik et al. 2007).

An analysis of well-known and widely accepted microscopic criteria during histological and cytological examination of samples collected from tumours is still crucial in oncological diagnosis. Cytologists analyse size, shape and structure of cells as well as other characteristics, including cells arrangement, presence of features of malignancy but there is intra- and inter-observers variability between these parameters. Morphometry is the quantitative description of geometric figures of cellular structures in any dimension. This method of subjective description of cells morphology and this analysis allows to obtain important diagnostic information. It allows to make microscopic analysis more objective and to show differences that cannot be detected during direct observation by a cytologist. Morphometry (or cytomorphometry if made on cytological slides) is the quantitative method of precise analysis of cellular structures, including both cytoplasm and nucleus. The most important advantage of cytomorphometry is that this examination provides objective and reproducible results, contrary to direct microscopic analysis by cytologist that is burdened by some subjectivity. Microscopic analysis supported by cytomorphometry can detect changes not immediately apparent to the naked eye (Maiolino et al. 2002). Diagnostic and prognostic usefulness of morphometry, including cytomorphometry, has been pinpointed in studies on human tumours, especially epithelial malignant ones. There are also numerous studies on usefulness of cytomorphometry in veterinary oncology. These studies have shown that cytomorphometry is of diagnostic and prognostic usefulness in cases of different neoplasms such as feline and canine mammary tumours, canine anal gland sacs tumours, skin epithelial neoplasms as well as canine mast cell tumours and histiocytomas (De Vico et al. 2007,

Simeonov and Simeonova 2007a,b, Simeonov and Simeonova 2008, Simeonov and Simeonova 2009, Strefezi et al. 2009, Maiolino et al. 2012, Paździor-Czapuła et al. 2014). As it was recently shown on canine mast cell tumours quantitative analysis and obtained measurements are reproducible and they have no, or minimal, intra- and interobserver variation (Barbosa et al. 2014).

The aim of this study was to describe cytomorphometric parameters of normal mesothelial cells and mesothelial cells in the course of their benign reactive and malignant proliferation and to compare them to carcinomas and adenocarcinomas located within serosal cavities in dogs. The second aim was to evaluate applicability of cytomorphometry as diagnostic method in cases of diseases causing accumulation of effusion in serosal cavities.

Materials and Methods

This prospective study was conducted on dogs, patients of the Small Animal Clinic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW) in a period of 2007-2012. Final diagnosis in all these animals was based on cytology, immunocytochemistry, histopathology and immunocytochemistry results, as it was previously described (Przeździecki and Sapieryński 2014). The **first group of dogs** (n=10) involved animals after routine ovariohysterectomy. Samples of normal mesothelial cells were collected from these dogs by scraping the uterine serosal surface with a sterile surgical blade and then they were placed on microscopic slides. This procedure was done after surgical resection of uterus and normal morphology of mesothelial cells was confirmed by histopathological examination of uterine samples. The presence of normal mesothelial cells on cytologic slides was confirmed by examination of cell morphology and results of immunocytochemistry (positive reaction with anti-cytokeratin, anti-vimentin and anti-desmin antibodies). The **second group of dogs** (n=11) included animals with reactive process involving the mesothelium. Benign reactive mesothelial cells were collected by serosal fluid aspiration. Like above, cell morphology and immunocytochemistry (positive reaction with anti-cytokeratin, anti-vimentin and anti-desmin antibodies) confirmed presence of benign reactive mesothelial cells. The reactive process was recognized on the basis of lack of mass/masses detected during visualization techniques, follow-up observation, and autopsy made in some of these dogs. The **third group of dogs** (n=5) consisted of patients with mesothelioma diagnosed histopathologically and

Table 1. Comparison of the cytomorphometric cellular parameters of normal canine mesothelial cells, reactive mesothelial cells, mesothelioma cells and carcinoma/adenocarcinoma cells, presented as: mean \pm SD (range). MCD – mean cellular diameter, MCP – mean cellular perimeter, MCA – mean cellular area, MCR – mean cellular roundness. Groups denoted by at least one the same letter do not differ significantly ($p>0.05$).

Group	MCD (μm)	MCP (μm)	MCA (μm^2)	MCR
Normal mesothelium	13.4 \pm 2.6 ^a (7.9-22.6)	47.9 \pm 9.5 ^a (28.6-82.7)	139.7 \pm 55.9 ^a (46.7-420.4)	1.39 \pm 0.25 ^a (1.00-2.72)
Reactive mesothelium	17.6 \pm 4.6 ^b (8.1-35.6)	59.5 \pm 15.3 ^b (10.1-123.4)	255.4 \pm 135.0 ^b (51.0-917.0)	1.16 \pm 0.20 ^b (1.00-2.41)
Mesothelioma	17.2 \pm 4.2 ^{ab} (10.4-43.6)	60.1 \pm 15.3 ^{ab} (35.7-168.4)	233.4 \pm 150.3 ^{ab} (81.8-1336)	1.23 \pm 0.18 ^b (1.00-2.31)
Carcinoma/adenocarcinoma	18.6 \pm 4.9 ^b (8.5-66.1)	63.9 \pm 17.1 ^b (28.4-222.8)	279.2 \pm 193.4 ^b (49.8-3329)	1.22 \pm 0.17 ^b (1.00-2.26)
Nested ANOVA p-value: between groups/ between patients within a group	<0.001 <0.001	<0.001 <0.001	<0.001 <0.001	<0.001 <0.001

Table 2. Comparison of the cytomorphometric nuclear parameters of normal canine mesothelial cells, reactive mesothelial cells, mesothelioma cells and carcinoma/adenocarcinoma cells, presented as: mean \pm SD (range). MND – mean nuclear diameter, MNP – mean nuclear perimeter, MNA – mean nuclear area, MNR – mean nuclear roundness, N/C – nuclear/cytoplasmic ratio. Groups denoted by at least one the same letter do not differ significantly ($p>0.05$).

Group	MND (μm)	MNP (μm)	MNA (μm^2)	MNR	N/C
Normal mesothelium	9.3 \pm 1.7 ^a (5.9-15.2)	33.4 \pm 6.1 (5.6-52.8)	67.0 \pm 24.6 ^a (25.1-174.3)	1.37 \pm 0.23 ^a (1.00-2.40)	0.49 \pm 0.09 ^a (0.18-1.00)
Reactive mesothelium	10.6 \pm 1.8 ^{ab} (5.2-20.4)	36.3 \pm 6.2 (17.7-67.3)	90.4 \pm 31.2 ^{ab} (21.2-309.0)	1.24 \pm 0.23 ^b (1.00-2.69)	0.40 \pm 0.13 ^b (0.12-0.80)
Mesothelioma	10.1 \pm 2.7 ^{ab} (4.6-25.6)	35.5 \pm 9.2 (15.3-89.8)	80.4 \pm 46.0 ^b (16.2-505.5)	1.21 \pm 0.14 ^b (1.00-1.72)	0.38 \pm 0.14 ^b (0.05-0.67)
Carcinoma/adenocarcinoma	11.3 \pm 2.6 ^b (5.3-30.1)	38.8 \pm 9.1 (20.0-104.7)	99.2 \pm 53.1 ^b (22.1-699.1)	1.20 \pm 0.14 ^b (1.00-1.95)	0.38 \pm 0.11 ^b (0.10-0.69)
Nested ANOVA p-value between groups/ between patients within a group	0.015 <0.001	0.071* <0.001	0.013 <0.001	<0.001 <0.001	<0.001 <0.001

* no post-hoc analysis performed due to insignificant result of an overall nested ANOVA

confirmed by immunohistochemistry (cells revealed positive reaction with anti-cytokeratin, anti-vimentin and anti-desmin antibodies). Samples of malignant serosal effusions for cytomorphometry were collected by thoracocentesis or abdominocentesis, placed into EDTA tube, and then centrifuged. Sediment was used as a material for making smears. The **fourth group of the dogs** (n=19) comprised of patients with carcinomas or adenocarcinomas affecting serosal cavities diagnosed histopathologically and confirmed by immunohistochemistry (cells revealed positive reaction with anti-cytokeratin antibodies, positive or negative reaction with anti-vimentin antibodies and negative reaction with anti-desmin antibodies). Cellular samples for cytomorphometry were collected by fine-needle aspiration biopsy and/or malignant effu-

sions were collected by thoracocentesis or abdominocentesis. Samples from solid masses were collected during ultrasound-assisted fine-needle aspiration biopsy from lesion detected with imaging techniques (thoracic or abdominal radiography or abdominal ultrasonography). Serosal effusions were collected by thoracocentesis or abdominocentesis, placed into EDTA tube, and then centrifuged. Sediment was used as a material for making smears.

Cytomorphometry was made on the smears stained with Giemsa solution. At least 3 cytological smears of good quality were dried, fixed in 70% methanol, stained with Giemsa solution, and examined by the light microscope. Smears for cytomorphometry were selected based on the high quality (quantity and morphology of cells, quality of staining, absence of

artefacts, specimen thickness). Cytomorphometry was analysed using Olympus BX41 microscope coupled to a computer equipped with Cella[®] analysis system. Sufficient number of x100 objective fields containing non-ruptured, well-preserved cells (diagnosed as the normal mesothelium, benign reactive mesothelium, mesothelioma cells and malignant epithelial neoplastic cells) were randomly selected for each case. Images were captured and formatted as .TIFF files, and displayed on computer monitor. Mean nuclear perimeter (MNP; μm), mean nuclear area (MNA; μm^2), mean nuclear diameter (MND; μm ; estimated as arithmetical mean of nuclear length and nuclear width), and mean nuclear roundness (MNR; nuclear length/nuclear width) were determined. Similarly, mean cellular perimeter (MCP; μm), mean cellular area (MCA; μm^2), mean cellular diameter (MCD; μm ; estimated as arithmetical mean of cellular length and cellular width), and mean cellular roundness (MCR; cellular length/cellular width) were determined in each case. Moreover, nuclear to cytoplasmic ratio (N/C) was calculated. Cytomorphometry was conducted by two cytologists who were not aware of the lesion types. For each case, 60-100 cells and their nuclei were measured by outlining their profiles using computed tools. Overlapping, ruptured nuclei or cells, as well as nuclei or cells with unclear boundaries were rejected.

Statistical analysis

All values were presented as arithmetical mean, standard deviation and range. Normality of data distribution was assessed with Shapiro-Wilk test. Cell and nuclear parameters were compared using nested (hierarchical) analysis of variance (ANOVA) with the group as a fixed-effects factor and the animal as a random-effects factor. If an overall difference between groups was statistically significant multiple pairwise comparisons were carried out using nested ANOVA with Bonferroni correction. For all statistical tests a two-tailed p-value below 0.05 was considered to indicate statistical significance. Analysis was performed in IBM SPSS Statistics 21 and graphs were made in Statistica 10 (StatSoft Inc.).

Results

The values of cellular and nuclear cytomorphometric parameters and results of statistical analysis are presented in Table 1 and 2. At least one significant difference was revealed for all parameters, excluding MNP, however post-hoc analysis showed that significant differences were only between normal and

pathological mesothelium. Normal mesothelium cells and their nuclei were significantly smaller and more elongated than cells and nuclei of both benign reactive mesothelium and malignant neoplastic mesothelium. Moreover, the nucleus in normal mesothelium cells occupied significantly larger part of the cell (higher N/C ratio) in comparison to benign reactive and neoplastic mesothelium. No significant differences were observed either between benign reactive mesothelium and malignant neoplastic mesothelium or between mesothelioma and carcinoma/adenocarcinoma. Furthermore, significant differences were observed in all parameters between dogs within each group (for example Fig 1. and 2). This implies that investigated parameters are not a stable characteristic of a patient and may substantially vary between patients with the same diagnosis.

Discussion

Histo- or cytomorphometry using special computer programmes allow to objectively describe morphology of cells and tissues. It makes possible to avoid errors encountered during somewhat subjective direct observation by cytologist. Morphometry can be applied both in cytological and histological slides, but it seems that cytological application is more convenient for practical purposes (De Vico et al. 2002). Because of arrangement of cells in one plane and usually clear cellular and nuclear morphology on cytological slides, cytomorphometry and interpretation of obtained results are easier than in histological slides (Maiolino et al. 2002, Smieonov and Simeonova 2007b, Smieonov and Simeonova 2009). Contrary to other methods, cytomorphometry can be applied to microscopic slides stained with routine methods. No additional methods of samples handling (additional method of staining), except for specific computer application or programme, are necessary. It is especially important in cases when extra smears for performing additional staining are not available.

It is well known that cells of the same tissues usually change their morphology during various pathological processes. As it was shown in the present study many examined cytomorphometric parameters revealed statistically significant differences among animals with various pathologic lesions. However, two of these parameters seem to be especially useful for objective characterization of cellular morphology: the mean nuclear diameter (MND) and the mean nuclear area (MNA). In general, nuclear rather than cytoplasmic parameters have been shown to be more specific in the majority of the previous studies (Maiolino et al. 2002, Simeonov and Simeonova 2006, DeVico et al.

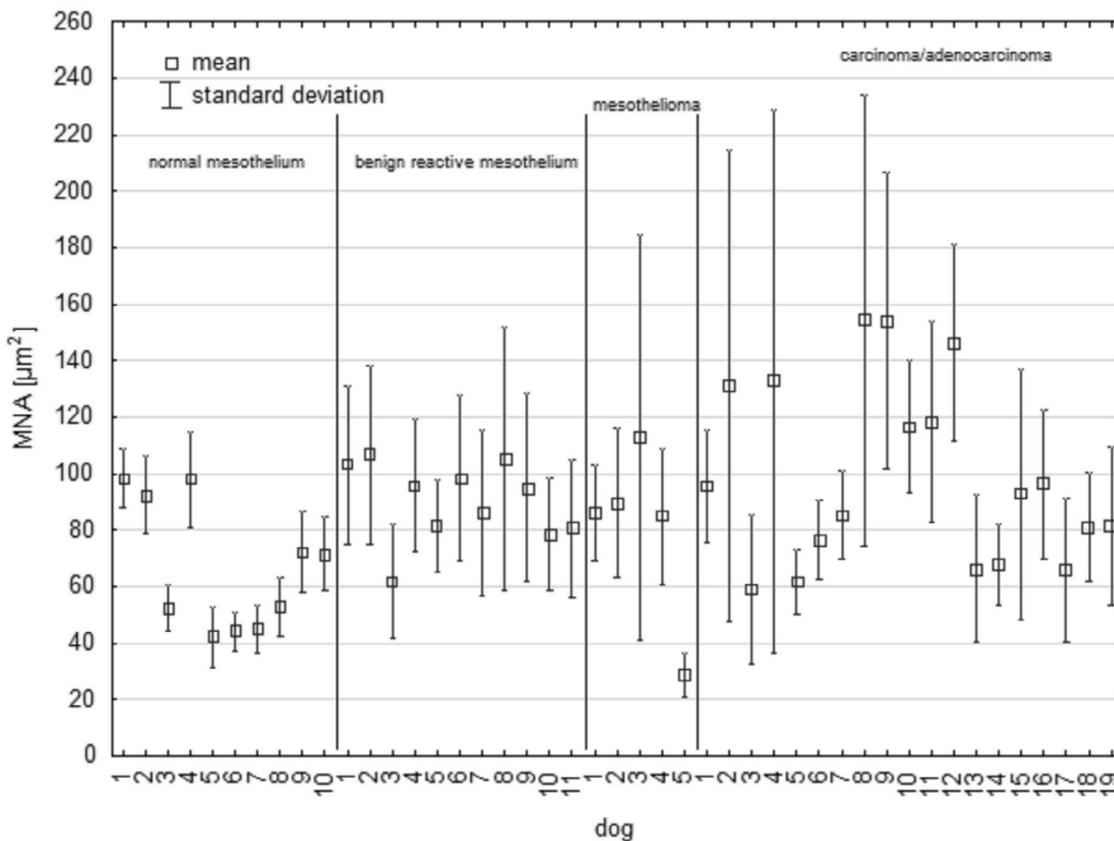


Fig. 1. Mean nuclear area (MNA; μm^2) of canine patients with different diagnoses enrolled in the study.

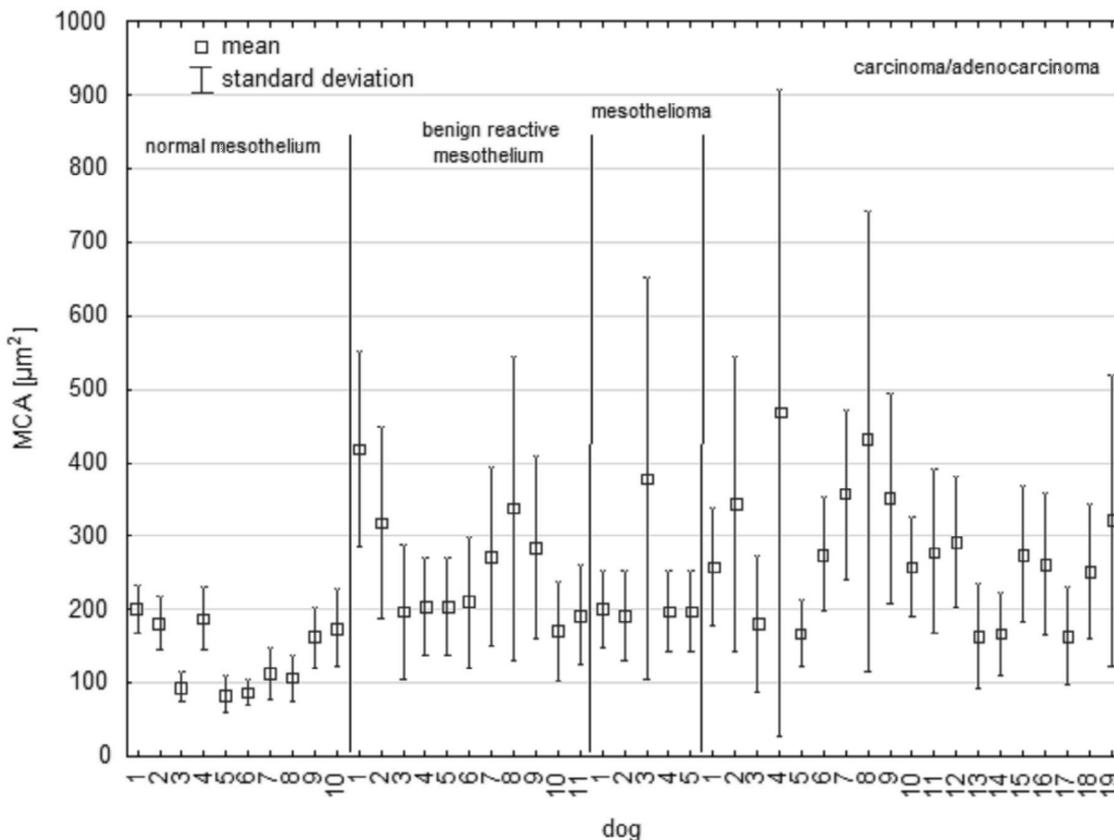


Fig. 2. Mean cellular area (MCA; μm^2) of canine patients with different diagnoses enrolled in the study.

2007, Simeonov and Simeonova 2007a,b, Simeonov and Simeonova 2008). As it is well known nuclear enlargement is a typical cytological feature of reactive non-neoplastic and malignant cells. Nuclear parameters including MNA and MND, were useful in differentiation between benign and malignant mammary gland tumours in bitches, benign and malignant tumours of anal sac in dogs, benign and malignant apocrine gland tumours in dogs (Simeonov and Simeonova 2006, Simeonov and Simeonova 2007a,b, Simeonov and Simeonova 2008). Moreover, MNA was also the best morphometric discriminant in cytological differentiation between canine mammary gland cancers with and without lymph node involvement (De Vico et al. 2007).

In the present study MNA and MND were highest in malignant epithelial tumours, yet they were higher in benign reactive mesothelium than in mesothelioma cells. As Simeonov and Simeonova (2007a) and De Vico et al. (2007) revealed, the values of the nuclear parameter investigated increased gradually along with tumour histologic malignancy. On the other hand, it is widely accepted that reactive non-neoplastic mesothelial cells are characterized by considerable cellular and nuclear pleomorphism, what was also confirmed in the present study. Similarly, cellular parameters were also highest in benign reactive mesothelium, and in malignant epithelial tumours. However, as it was mentioned above, nuclear cytomorphometry seems to be more reliable due to the fact that borders of nuclei are better preserved than cytoplasmic outlines. Although in our study only well preserved cells were examined, generally cytoplasm is more fragile, ruptures easily and the size can change during sample fixation.

Compared to benign reactive and neoplastic mesothelium, normal mesothelial cells and their nuclei are generally smaller, however N/C ratio of these cells is highest. This finding is also surprising, since high N/C ratio is considered as cytological feature of malignancy. However, this data is related to very small volume of cytoplasm in normal mesothelial cells, contrary to malignant cells in that high N/C ratio resulting from increased size of nuclei of neoplastic cells. Additionally, nuclei of normal mesothelium are more elongated than those of benign reactive mesothelium or neoplastic cells.

The preoperative diagnosis of hyperplastic processes involving serosal cavities is often challenging in the veterinary medicine. According to the results of numerous studies, differentiation between mesotheliomas, epithelial malignant tumours, and some sarcomas could be difficult or even impossible. It concerns not only cellular samples but also microscopic examination of tissue samples collected during more

or less invasive medical procedures (Smith and Hill 1989, Baker and Lumsden 2000, Brisson et al. 2006, Ordonez 2006, Gumber et al. 2011, Bertazollo et al. 2012, Przędziecki and Sapieryński 2014). Due to moderate to severe pleomorphism of benign hyperplastic mesothelium this reactive process could be confused with some cancers. In some cases, differentiation between benign reactive hyperplasia of mesothelium and malignant effusion associated with dissemination of some malignant epithelial tumours could be difficult as well. In the present study we used the cytomorphometric analysis of cells present in serosal effusion as an auxiliary method of preoperative cytodiagnosis.

As different fixation methods can affect cell morphology in both histological and cytological samples, in every case we standardized the method of slide preparation. In the present study each sample was air-dried and then methanol-fixed as it is routinely done in clinical cytopathology. If cytomorphometry is to be considered a reliable diagnostic method, procedure of sample collection, fixation and staining should be conducted in the similar or the same manner as in routine practice. Various methods of sample collection (fine-needle biopsy vs. aspiration of cell containing serosal effusion) in dogs with carcinomas and adenocarcinomas may be shortcoming of the study. However, there were no differences in MND and MNA values with regard to method of samples collection from malignant epithelial tumours.

As it was shown in the aforementioned studies (Maiolino et al. 2002, Simeonov and Simeonova 2006, De Vico et al. 2007, Simeonov and Simeonova 2007a,b, Simeonov and Simeonova 2008), in some types of lesions cytomorphometry can have diagnostic applicability, for that reason we wanted to estimate such possibility in the present study. Even though some significant differences were observed in the present study, considerable overlap of cytomorphometric parameters in animals with different diseases limited practical role of these observations. For example, some problems can be encountered in identification of carcinoma or mesothelioma cells in neoplastic effusion in which reactive mesothelial cells predominate. On the other hand, cytomorphometric analysis together with other diagnostic methods as routine cytopathology and immunocytochemistry may facilitate a diagnostic process and help veterinarians and owners of animals to make decision regarding future treatment methods (Przędziecki and Sapieryński 2014). Additional studies in this field are necessary, especially to establish minimum and maximum values of these parameters in specific pathologic processes affecting serosal cavities and to test cytomorphometry is a promising complementary diag-

nostic method in such cases. Moreover, the results of our study indicate that the simple diagnostic methods basing on subjective or objective estimation of cellular morphology are not sufficient in differentiation between various pathologic processes developing in serosal cavities. It seem that more invasive methods of the tissue samples collection (chest or abdominal surgery) or more complex staining methods (immunocytochemistry with panel of various antibodies) need to be applied in such cases.

In conclusion, objective cytomorphometric analysis of cells present in serosal effusion can characterize morphology of pathological mesothelial cells and carcinoma/adenocarcinoma cells as well. However, considerable overlap between animals with different diseases limits their applicability to clinical purposes. Cytomorphometric analysis of cellular samples collected from dogs with proliferative processes affecting serosal cavities may only be considered as an auxiliary method potentially increasing accuracy of pre-operative diagnosis.

References

- Baker R, Lumsden JH. Pleural and peritoneal fluids. In: Baker R, Lumsden JH (2000) Color atlas of cytology of the dog and cat. Mosby, St. Louis, pp 161-162.
- Barbosa DFL, Paraventi MD, Strefezzi RF (2014) Reproducibility of nuclear morphometry parameters from cytologic smears of canine cutaneous mast cell tumors – intra- and interobserver variability. *Vet Clin Pathol* 43: 469-472.
- Bertazzolo W, Bonfanti U, Mazzotti S, Gelain ME (2012) Cytologic features and diagnostic accuracy of analysis of effusions for detection of ovarian carcinoma in dogs. *Vet Clin Pathol* 41: 127-132.
- Brisson BA, Reggeti F, Bienzle D (2006) Portal site metastasis of invasive mesothelioma after diagnostic thoracoscopy in a dog. *J Am Vet Med Assoc* 229: 980-983.
- Charney SC, Bergman PJ, McKnight JA, Ferelly J, Novosad CA, Leibman NF, Camps-Palau MA (2005) Evaluation of intracavitary mitoxantrone and carboplatin for treatment of carcinomatosis, sarcomatosis and mesothelioma, with or without malignant effusions: A retrospective analysis of 12 cases (1997-2002). *Vet Comp Oncol* 3: 171-181.
- De Vico G, Sfacteria A, Maiolino P, Mazzullo G (2002) Comparison of nuclear morphometric parameters on cytologic smears and histologic sections of spontaneous canine tumors. *Vet Clin Pathol* 31: 16-18.
- De Vico G, Maiolino P, Cataldi M, Mazzullo G, Restucci B (2007) Nuclear morphometry in relation to lymph node status in canine mammary carcinomas. *Vet Res Commun* 31: 1005-1011.
- Geninet C, Bernex F, Rakotovo F, Crespeau FL, Parodi AL, Fontaine JJ (2003) Sclerosing peritoneal mesothelioma in a dog – a case report. *J Vet Med A* 50: 402-405.
- Glińska K (2009) Evaluation of the usefulness of abdominal fluid examination in diagnosing the etiology of ascites in dogs. *Med Weter* 65: 40-45.
- Gumber S, Fowlkes N, Cho DY (2011) Disseminated sclerosing peritoneal mesothelioma in a dog. *J Vet Diagn Invest* 23: 1046-1050.
- Maiolino P, Restucci B, Papparella S, De Vico G (2002) Nuclear morphometry in squamous cell carcinomas of canine skin. *J Comp Pathol* 127: 114-117.
- Ordóñez NG (2006) The diagnostic utility of immunohistochemistry and electron microscopy in distinguishing between peritoneal mesotheliomas and serous carcinomas: a comparative study. *Mod Pathol* 19: 34-48.
- Paździor-Czapula K, Otrocka-Domagala I, Rotkiewicz T, Gesek M (2014) Cytomorphometry of canine cutaneous histiocytoma. *Pol J Vet Sci* 17: 413-420.
- Przeździecki R, Sapieryński R (2014) Using of immunocytochemistry in differential diagnosis of neoplasms of serosal cavities in dogs. *Pol J Vet Sci* 17: 149-159.
- Sato T, Miyoshi T, Shibuya H, Fujikura J, Koie H, Miyazaki Y (2005) Peritoneal biphasic mesothelioma in a dog. *J Vet Med A* 52: 22-25.
- Simeonov R, Simeonova G (2006) Computerized morphometry of mean nuclear diameter and nuclear roundness in canine mammary gland tumors on cytologic smears. *Vet Clin Pathol* 35: 88-90.
- Simeonov R, Simeonova G (2007a) Computerized cytomorphometric analysis of nuclear area, nuclear perimeter and mean nuclear diameter in spontaneous canine mammary gland tumours. *Vet Res Commun* 31: 553-558.
- Simeonov RS, Simeonova GP (2007b) Use of quantitative analysis as a method for differentiation between canine cutaneous apocrine adenomas and carcinomas on cytologic smears. *J Vet Med A* 54: 542-544.
- Simeonov R, Simeonova G (2008) Quantitative analysis in spontaneous canine anal sac gland adenomas and carcinomas. *Res Vet Sci* 85: 559-562.
- Simeonov R, Simeonova G (2009) Nuclear cytomorphometry in feline mammary gland epithelial tumours. *Vet J* 179: 296-300.
- Simeonov R, Simeonova G (2010) Comparative morphometric analysis of recurrent and nonrecurrent canine basal cell carcinomas: a preliminary report. *Vet Clin Pathol* 39: 96-98.
- Smith DA, Hill FW (1989) Metastatic malignant mesothelioma in a dog. *J Comp Pathol* 100: 97-101.
- Strefezzi RF, Xavier JG, Kleeb SR, Catao-Dias JL (2009) Nuclear morphometry in cytopathology: a prognostic indicator for canine cutaneous mast cell tumors. *J Vet Diagn Invest* 21: 821-825.
- Szczepulska-Wójcik E, Langfort R, Roszkowski-Śliz K (2007) A comparative evaluation of immunohistochemical markers for the differential diagnosis between malignant mesothelioma, non-small cell carcinoma involving the pleura, and benign reactive mesothelial cell proliferation. *Pneumonol Alergol Pol* 75: 57-69.