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Determining the optimal dose of cytochalasine B in the MN assay for the domestic cat

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Summary

Cytochalasine B is an indicator in the CBMN assay, but can also be geno- or cytotoxic. The aim of the study was to determine the optimal dose of cytochalasine B for the Cytokinesis-Block Micronucleus (CBMN) assay for the domestic cat. The results revealed a surprisingly high number of micronuclei (MN) in relation to the doses used. It was found that a concentration of 2.5 µg/ml is enough to obtain binuclear cells, with a minimal toxic effect on the lymphocytes of the domestic cat. In the material analysed, three forms of chromatin structure abnormalities were found. The most prevalent abnormalities were isolated micronuclei, with the mean number of binucleated cells with one micronucleus (BNCs + 1 MN) equal to 39.96 ± 9.02. Moreover, the numbers of binucleated cells with one micronucleus, nuclear buds and nucleoplasmic bridges did not differ significantly according to the sex or age of European cats.

Keywords: cytochalasine B dose, cats, micronucleus, nuclear buds, nucleoplasmic bridges

The domestic cat (*Felis catus*) is characterised by its ability to adapt quickly to various environmental conditions, which is why it can be found on almost every continent (12). Due to the close coexistence of cats and humans, both species are exposed to the same environmental risks and factors (e.g. pesticides, herbicides, fungicides, heavy metals, dust, certain antibiotics, as well as ionizing radiation, gases and small particles in smog). In addition, the domestic cat is used as a model species in studies on the aetiology of selected diseases affecting humans, both infectious and hereditary, including cardiomyopathy, dominant polycystic kidney disease, leukaemia, sarcoma, SARS and AIDS (11, 16). While examining various concentrations of bromodeoxyuridine (BrdU) used in the sister chromatid exchange (SCE) test, Szeleszczuk et al. (20) found an increased genomic sensitivity of cats to this substance, even in low concentrations. These authors also found that higher concentrations of BrdU are genotoxic, damaging the DNA of chromosomes and inducting additional SCE in the chromosomes of domestic cats.

One indicator of the harmful effects of chemical compounds of various origins on chromatin is the micronucleus (MN) assay. Micronuclei are small oval-shaped bodies found in some eukaryotic cells and located next to the nucleus of daughter cells after mitotic division. Micronuclei contain both acentric and centric chromosome fragments or whole chromosomes (2). The determination of the number of micronuclei in cells is a highly effective indicator of chromosome sensitivity to genotoxic substances. Micronucleus induction is widely regarded as a biomarker of diseases and processes associated with damage to genetic material (1, 2, 13, 19). One variant of the micronucleus assay, the so-called cytochalasin block micronucleus (CBMN) assay, relies on cytochalasin B (Cyt-B) to block the process of cell division at the stage of binucleated cells. Cytochalasins are organic mycotoxin compounds which exert pleiotropic effects on cells and affect the actin cytoskeleton. So far, approximately 60 different cytochalasins derived from several species of fungi have been classified (18). In addition to actin-binding properties, a number of cytochalasins also have other biological activities, such as inhibition of HIV-1 polymerase (L-696, 474), plant growth regulation (cytochalasin H derivatives), or inhibition of angiogenesis (cytochalasine E) (6). Cyt-B is used to block cell division immediately after nuclear division. It blocks cytokinesis by inhibiting the

polymerisation of actin filaments and their fragmentation without interfering with karyokinetic division (3). This leads to the presence of two nuclei in cells, with the resulting structures referred to as binucleated cells. Cytochalasine B is added to cell cultures after a complete cycle of mitosis and after the possible induction of DNA damage (2). The development of a technique relying on cytochalasin B makes it possible to assess the extent of chromatin damage across populations of cells varying in terms of the kinetics of division (2).

The addition of Cyt-B interferes with the structure and normal function of nuclear chromatin, inducing micronuclei and other cellular abnormalities, including apoptosis. The research hypothesis was that the addition of cytochalasine B may disrupt the structure and proper functioning of nuclear chromatin, inducing additional damage in the form of micronuclei, as well as generating cytoplasmic bridges and buds. The objective of the present study was to determine the optimal dose of cytochalasine B used in the CBMN test for a model species, such as the domestic cat. We also studied the influence of the sex and age of animals on the numbers of binucleated cells (BNCs) with one micronucleus (MN), nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs) for the optimal concentration of Cyt-B.

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Material and methods

Material. The study was conducted on eight European domestic cats: 4 males and 4 females. The animals were brought to the Salamandra Veterinary Clinic (Kraków, Poland) by private breeders from southern Poland during an occasional visit. The material for analysis was collected from five young cats (≤ 3 years old, 3 males and 2 females) and 3 older cats (> 3 years old, 1 male and 2 females). Blood samples were taken from the *vena cephalica antebrachi* during routine veterinary examinations. The study material consisted of peripheral whole blood lymphocytes.

Micronucleus (MN) assay. MN assays were performed using a fraction of lymphocytes isolated on a Histopaque-1077 (SIGMA ALDRICH). Cell cultures were grown on LymphoGrow Medium (CYTOGEN) under standard conditions (72 h, 37.5°C, 5% CO_2, constant humidity). A CBMN assay was carried out by a method described by Słonina & Gasińska (16). According to that methodology, cytochalasin B (SIGMA) was added at four different concentrations (2.5/5.0/7.5/10.0 µg/ml) for 44 h to lymphocyte culture, including negative control. The protocol of the MN assay makes it possible to identify cells that have completed one nuclear division as binucleated cells (BNCs) and to evaluate micronuclei only in BNCs (2). After 72 h of culture, the experimental material was fixed in Carnoy's solution (3 : 1 mixture of methanol and acetic acid, POCH). In the next step, microscope slides were stained with Giemsa (25%), in Sorensen's buffer, at pH 6.8 (SIGMA ALDRICH). For each cat, the percentage of BNCs in 1000 lymphocytes and micronucleus (MN) induction in 500 BNCs per animal were evaluated. In addition, each cat was assessed for the presence of other types of damage to the nuclear chromatin.

Microscopic analysis. Microscopic analysis and photographic documentation were made with a Zeiss Imager A2 epifluorescence microscope coupled with a Zeiss AxioCam MRc5 digital camera and the NIS-Elements image analysis software ver. F2.31. For each cat, 500 binucleated cells (BNCs) that met the criteria of the micronucleus (MN) assay (2) were evaluated in the slides.

Statistical analysis. The numbers of binucleated cells (BNCs) with one micronucleus (MN), nuclear buds (NBUDs), and nucleoplasmic bridges (NPBs) were analysed. The data was checked for normal distribution with the Shapiro-Wilk test using the SAS software (15). Square root transformations were used for all traits, but the actual mean values are listed in the tables. After the transformation, the distribution of all traits analysed did not differ from normal distribution.

In the first step, the effect of cytochalasin B concentration on the numbers of binucleated cells with one micronucleus (BNCs with 1 MN), NBUDs and NPBs was analysed by one-way analysis of variance (PROC ANOVA; SAS, 2014). The significance of differences in means was determined by the Tukey test. It was found that the increase in the numbers of BNCs with 1 MN, NBUDs and NPBs depended on the dose of Cyt-B used in the cultures. We decided to select a Cyt-B concentration with the lowest variation of BNCs with 1 MN, NBUDs and NPBs between individuals. For different cytochalasine B concentrations, the variability between animals was analysed by the chi-square test.

Next, for the optimal concentration of Cyt-B, the effect of sex and age was analysed using the MIXED procedure (SAS, 2014) and the following linear model:

$$Y_{ijk} = \mu + AGE_i + SEX_j + e_{ijk}$$

where

Y_{ijk} – number of binucleated cells with one micronucleus (BNCs with 1 MN) or nuclear buds (NBUDs) or nucleoplasmic bridges (NPBs),

μ – overall mean,

AGE_i – the effect of i-th age class (≤ 3 or > 3 years),

SEX_j – the effect of j-th sex (male or female),

e_{ijk} – residual effect.

The significance of differences in means was determined by the Tukey-Kramer test. Additionally, Spearman's correlation coefficients for all traits analysed, i.e. BNCs with 1 MN, NBUDs and NPBs, were calculated by the CORR procedure of SAS (SAS, 18).

Results and discussion

Three types of damage were observed in all experimental groups: micronuclei presence in binucleated cells with one micronucleus (BNCs + 1 MN), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs), as shown in Figure 1. Various forms of chromatin instability were determined per 500 BNCs for each cat and Cyt-B concentration. Table 1 presents the effect of various Cyt-B concentrations on the stability of nuclear chromatin in the lymphocytes of the European

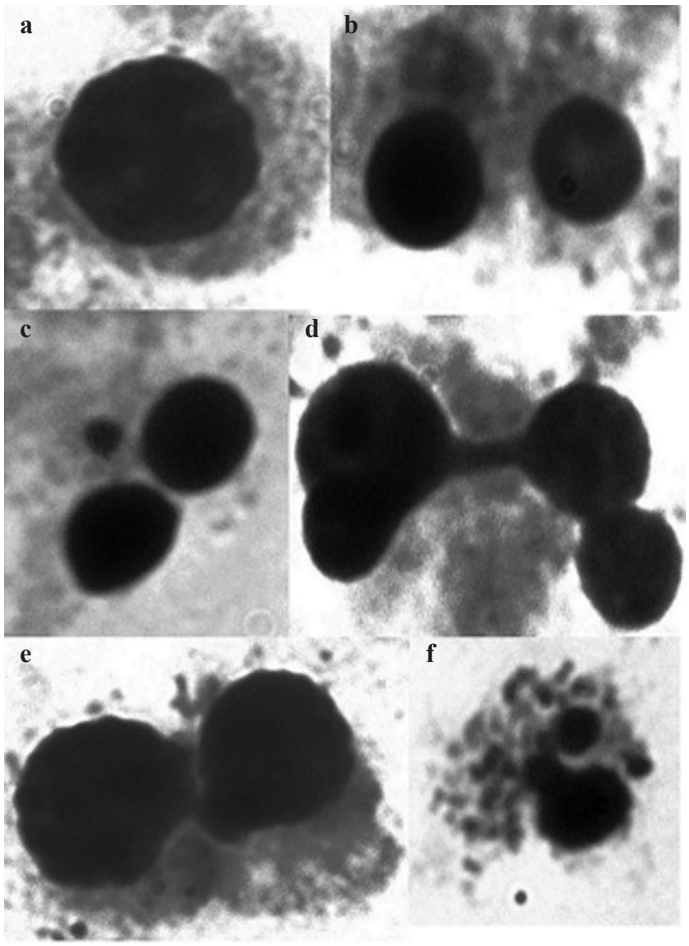

Fig. 1. Graphical representation of MN assay results: a) mononucleated cell; b) binucleated cell; c) binucleated cell with 1 micronucleus; d) nucleoplasmic bridge with an uneven division of the cell nucleus (NPB); e) NBUD; f) apoptosis cell. Giemsa staining. Scale bar equals 10 µm

cat. Differences between Cyt-B concentrations for all traits, i.e. BNCs with 1 MN, NPBs and NBUDs, were highly significant ($P < 0.01$). Moreover, the numbers of BNCs with 1 MN, NPBs and NBUDs increased with rising cytochalasine B concentrations. What is interesting, when cytochalasine B at a concentration of 2.5 µg/ml was used, the number of BNCs with 1 MN increased almost fourfold compared to the control group (0.0 µg/ml Cyt-B).

The lowest variability between cats was observed when the 2.5 µg/ml Cyt-B concentration was used. Additionally, no significant differences ($P > 0.05$) between the cats were observed for any of the traits analysed when the 2.5 µg/ml concentration of Cyt-B was used. For example, in the control group, in which Cyt-B was not used (0.0 µg/ml), the variability between individuals was highly significant ($P < 0.01$). Based on all these results, Cyt-B at a concentration of 2.5 µg/ml was found to be representative as an indicator in the CBMN assay for the domestic cat. The considerable and highly significant ($P < 0.01$) differences and the increase in chromatin instability at a higher dose of Cyt-B suggest a potential negative influence of Cyt-B. The concentration selected as an indicator should make it possible to observe binucleated cells.

Generally, the most common finding in the BNCs was damage in the form of single micronuclei BNCs + 1 MN, and the mean number of BNCs + 1 MN was 39.96 ± 9.02. The presence of 2 MN was found only in isolated cells. In addition, a significant proportion of NBUDs was recorded in the study material, with their mean number calculated for approximately 500 BNCs being 5.26 ± 5.64. Another type of damage observed in a significant number of cells were nucleoplasmic bridges (NPBs). The mean number of NPBs in the cats was 9.65 (with standard deviation: 4.28).

Further analysis aimed at determining the effects of the sex and age of the study animals was based on data obtained for the selected concentration of Cyt-B (2.5 µg/ml) as an indicator of CBMN. Compared with males, female cats were more susceptible to chromatin damage of two types: BNCs with 1 MN and NBUDs. Males, on the other hand, had a higher number of NPBs than females (Tab. 2). There were no significant differences in BNCs with 1 MN, NPBs or NBUDs between the sexes when Cyt-B was used at a concentration of 2.5 µg/ml (Tab. 2; $P > 0.05$).

In addition, the effect of age on the amount and type of chromatin damage was checked and evaluated. The animals were divided into two age groups: young

Tab. 1. Number and types of nuclear chromatin damage caused by various cytochalasine B concentrations in the European cat

Cyt-B [µg/ml]	BNCs	BNCs with 1 MN*	NBUDs*	NPBs*
0.0	500	$10.25^{A} \pm 11.30$	$2.28^{A} \pm 4.36$	$6.27^{A} \pm 8.63$
2.5	500	$39.96^{B} \pm 9.02$	$5.26^{A} \pm 5.64$	$9.65^{A} \pm 4.28$
5.0	500	$102.68^{C} \pm 23.92$	$23.63^{B} \pm 9.24$	$15.00^{B} \pm 3.21$
7.5	500	$189.46^{D} \pm 87.77$	$37.72^{B} \pm 29.30$	$25.49^{B} \pm 12.39$
10.0	500	$268.38^{E} \pm 41.98$	$41.69^{B} \pm 18.60$	$35.53^{B} \pm 11.73$

Explanations: * means and standard deviations (mean ± SD) of binucleated cells with one micronucleus (BNCs with 1 MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs), by cytochalasine B (Cyt-B) concentration; $^{A, B, C, D, E}$ values in the same column marked with different letters differ highly significantly ($P < 0.01$)

Tab. 2. Influence of the sex of the European cat on BNCs with 1 MN, NBUD, and NPB numbers

Sex	BNCs	BNCs with 1 MN*	NBUDs*	NPBs*
Female	500	$44.77^{a} \pm 6.96$	$6.22^{a} \pm 7.54$	$9.10^{a} \pm 4.23$
Male	500	$35.14^{a} \pm 8.91$	$4.29^{a} \pm 3.85$	$10.20^{a} \pm 4.91$
Total	500	39.96 ± 9.02	5.26 ± 5.64	9.65 ± 4.28

Explanations: * means and standard deviations (mean ± SD) of binucleated cells with one micronucleus (BNCs with 1 MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) for 2.5 µg/ml concentration of cytochalasin B (Cyt-B); a values in the same column marked with the same letter do not differ significantly ($P > 0.05$).

Tab. 3. Influence of the age of the European cat on BNCs with 1 MN, NBUD, and NPB numbers

Age [years]	BNCs	BNCs with 1 MN*	NBUDs*	NPBs*
≤ 3	500	41.62[a] ± 9.77	5.18[a] ± 7.00	9.08[a] ± 3.66
> 3	500	37.19[a] ± 8.66	5.39[a] ± 3.60	10.61[a] ± 5.93
Total	500	39.96 ± 9.02	5.26 ± 5.64	9.65 ± 4.28

Explanations: as in Tab. 2.

(≤ 3 years) and old (> 3 years). The results revealed a higher number of BNCs with 1 MN in the younger cats. In contrast, other types of damage, i.e. NBUDs and NPBs, were more prevalent in the older animals (> 3). Also, there were no significant differences in any traits between the two age classed when the 2.5 µg/ml concentration of Cyt-B was used (Tab. 3; $P > 0.05$).

In addition, correlations between all traits (BNCs with 1 MN, NBUDs, NPBs) were determined. The correlations were positive or negative. The correlation between BNCs with 1 MN and NBUDs was the lowest (0.02) and positive, whereas BNCs with 1 MN and NPBs showed the highest correlation (0.77, absolute value). The correlation between NBUDs and NPBs was moderate (0.24) and positive. Only the correlation between BNCs with 1 MN and NPBs was significant ($P < 0.05$).

One of the key elements in the CBMN assay is determining the optimal concentration of cytochalasin B in order to maximise the number of BNCs with blocked cytokinesis. The optimal cytochalasin B concentration for lymphocytes in human whole peripheral blood is 5 µg/ml (2). The same concentration is also routinely used for other mammalian cells. It is recommended, however, that the concentration of the Cyt-B indicator be optimised for each species or study material, such as cell lines, peripheral blood lymphocytes, oral mucosal epithelial cells, bone marrow cells or peripheral blood erythrocytes (2). Maisanaba et al. (10), who investigated the potential genotoxicity of thymol and carvarol (substances with antimicrobial properties, derived from essential oils) used in the production of food packaging, conducted a micronucleus assay on L5178Y/Tk mouse lymphoma cells. In that case, the optimal dose of cytochalasin B was 6 µg/ml. On the other hand, studies by Liu et al. (9), aimed at determining the role of miRNA in hepatotoxicity and genotoxicity induced by AFB1, were conducted on F344 rat hepatocytes treated with Cyt-B at a concentration of 4 µg/ml. Jacociunas et al. (8), in their experiment conducted on Chinese hamster ovary cells, used cytochalasin B at a concentration of 5 µg/ml. Other studies, conducted by Georgieva et al. (5), focused on evaluating the role of an artichoke extract in eliminating radiation-induced cellular damage. Experiments were performed on rabbit lymphocytes and Cyt-B at a concentration of 6 µg/ml. In addition, Szeleszczuk et al. (17), studying the stability of genetic material in lymphocytes sampled from blue and silver foxes and their interspecific hybrids, used cytochalasin B at a concentration of 5 µg/ml. In an experiment by Gauthier et al. (4), who sought to evaluate the effect of water pollution on marine mammals, cytochalasin B at a concentration of 7 µg/ml was used for analyses of various species, including the Arctic beluga whale, bottlenose dolphin, grey seals and harp seals. The analysis described in the present paper was aimed at selecting an appropriate concentration of Cyt-B by studying its effects on lymphocytes sampled from the European cat. Four doses of cytochalasin B were investigated: 2.5/5.0/7.5/10.0 µg/ml. The study demonstrated that the 2.5 µg/ml dose was sufficient to halt cytokinesis in an appropriate number of cells to carry out the CBMN in domestic cats. However, a dose of 2.5 µg/ml of Cyt-B should generate a minimum number of MN (low genotoxicity), so additional tests with lower doses of this mycotoxin should be performed.

Zúñiga-González et al. (20) studied the number of spontaneous nuclei in various species of mammals, birds, and reptiles. The species with the highest prevalence of micronuclei (micronucleus erythrocytes) per 10000 cells and belonging to the order Carnivora were ocelot (*Felis pardalis*) – 13.5 MN, lynx (*Lynx ruffus*) – 10.8 MN, and lion (*Panthera leo*) – 4.1 MN. On the other hand, the number of spontaneous micronuclei in the raccoon (*Procyon lotor*), bear (*Ursus americanus*), and coyote (*Canis latrans*) was found to be zero. Gauthier et al. (4), who compared the extent of damage to genetic material in marine mammals, found that the number of micronuclei ranged from 2 to 14 per 1000 BNCs. The micronucleus assay has also found applications in studies of the *Canidae* species. Harper et al. (7) investigated the effects of the cytotoxic drug cyclophosphamide on the reticulocytes of beagle dogs and found that the number of micronuclei was approximately 8. Szeleszczuk et al. (17) reported that the MN number was 15 in interspecific hybrids of blue and silver foxes, 3 in blue foxes and approximately 4 in silver foxes (per 500 BNCs). In the present study, the MN number in cats was 39.96 ± 9.02. The variation observed in the number of micronuclei in BNC cells among the above species of animals belonging to the order Carnivora might be due to the large variation in the number and structure of chromosomes, which affects the stability of their karyotype.

In another study, Zúñiga-González et al. (20), showed differences in the number of micronuclei between young and old cats (*Felis catus*). The mean number of micronuclei determined for young specimens was 23.2 per 1000 cells, whereas in older animals it was 8.4. A similar relationship for the difference in the MN number between age groups was also observed in our study. These findings indicate that micronuclei are more common in young cats, whereas in adult animals the number of micronuclei in peripheral blood cells decreases. Young cats are also known to have an

undeveloped immune system, which may contribute to increased levels of chromatin instability. In addition, our study demonstrated a greater prevalence of NPBs and NBUDs in older animals, which may suggest disorders occurring in the course of mitosis.

Quero et al. (14) conducted studies in the field of environmental monitoring. The authors assumed that domestic cats (*Felis catus*) could be exposed to the same harmful factors as humans, as they share the same (home) environment. Based on their analysis of the number of MN in cats (4.28 MN per 1000 cells (erythrocytes) in young and 4.00 MN in old cats), they suggested that the cat may be a good candidate species for biomonitoring studies in the home environment.

Our results suggest that in the case of cats, even a high concentration of cytochalasin B (10.0 µg/ml) can be used as an indicator in MN studies. It is necessary, however, to select the optimal concentration that will not cause additional chromatin damage in the form of NPB or NBUDs. Data from our studies also show that MN is a reliable method and can be employed as an instrument of environmental field monitoring.

The CBMN assay used in our study is a useful cytogenetic tool suitable for the detection of nuclear chromatin damage resulting from contact with mutagens of various nature. The CBMN assay with an appropriately selected Cyt-B concentration as an indicator can be used to estimate the effects of genotoxic factors on somatic cells expressed as the MN number in both humans and animals. Furthermore, it may have broader applications making it possible to identify MN and other forms of chromatin damage, such as NPBs and NBUDs, which improves the accuracy of analysis determining disorders in chromatin ultrastructure.

Due to the statistically significant increase in the number of MN in the study groups with low MN numbers, more experimental animals should be tested using even lower doses of Cyt-B than those used in our preliminary studies in order to conclusively determine the correct dose for the domestic cat in the CBMN assay. As demonstrated above, different species show a wide range of sensitivity to Cyt-B, even within closely related taxa (e.g. carnivores). This strongly suggests that optimal doses of Cyt-B should be established individually for each species.

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