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Keywords: *bone marrow, nucleated cells, nuclear volume, activated carbon, enterosorption, flow cytometry, confocal microscopy, acridine orange.*

DOI: https://doi.org/10.15407/oncology.2024.02.120

ANALYSIS OF THE VOLUME OF BONE MARROW CELL NUCLEI FOR ASSESSMENT OF CYTOSTATIC MYELOSUPPRESSION AND ITS PREVENTION BY ACTIVATED CARBON

Bone marrow suppression (myelosuppression) is a frequent complication of chemotherapy, and the need for monitoring and managing this side effect is still in great demand. ***Aim:*** *to study the changes in the volume of nuclei in bone marrow cells of chemotherapy drug-treated laboratory animals with or without enterosorption by activated carbon (AC).* ***Object and methods:*** *both flow cytometry and confocal microscopy were used to analyze acridine orange (AO)-stained bone marrow samples of intact, doxorubicin (DOX)-, and DOX+AC-treated rats. Confocal Z-series that represent sequential scans of cell nuclei directed from top to bottom at 0.5-μm step size were acquired at 40× magnification using argon laser (488 nm) for excitation of AO. Green fluorescence emitted by DNA-bound AO was detected through a 505–530 nm band-pass filter, allowing distinct visualization of nuclei and their boundaries. Z-series were further processed and analyzed with ImageJ software to quantify the values of nuclear volumes.* ***Results:*** *there were apparent differences between the nuclear volumes in the bone marrow samples of intact, DOX-treated, and DOX+AC-treated rats. A significant increase of the volume of nuclei in DOX+AC-treated rats, compared with those in DOX-treated (1.42-fold) and intact rats (1.14-fold), is likely due to an active DNA replication, suggesting an ongoing recovery of the pool of nucleated cells. Notably, in these three groups, the populations of bone marrow nucleated cells, as estimated by flow cytometry, correlated well with the aforementioned values of nuclear volumes. However, the volume of nuclei may not necessarily correlate with the height of Z-series representing the thickness of nuclei, providing a clue that can help to delineate the role of nuclear deformability.* ***Conclusion:*** *analysis of the volume of bone marrow cell nuclei proposed in this work is important in terms of obtaining supplementary information in the study of the course of induced myelosuppression and of the ways of its prevention.*

Rapid and reliable bone marrow cytological examinations are much needed to assess the chemotherapy side effects and to test the effectiveness of the means designed for tackling these effects. In fact, bone marrow that contains hematopoietic cells of various lineages is a sensitive target for a number of chemotherapy drugs. Among techniques that can rapidly and reliably examine bone marrow affected by chemotherapy, flow cytometry appears to be superior. Flow cytometry can precisely discriminate cell populations of interest and promptly quantify responses/effects in a large cohort of cells in sufficient numbers for precise statistical certainty. Bone marrow cytotoxicity induced by cyclophosphamide, melphalan, and doxorubicin (DOX), has been studied by staining bone marrow cells with metachromatic dye acridine orange (AO) followed by flow cytometric analysis [1–3]. This protocol based on determination of changes in the populations of total nucleated cells and polychromatic (immature) erythrocytes has been also applied for the study of myeloprotective modalities, e.g., such as administration of activated carbon (AC) preparations to tackle DOX-induced myelosuppression [3]. Exactly the same protocol has been used for the study of bone marrow cytotoxicity caused by implanted tumors and/or low-dose whole-body irradiations [4, 5].

In the current research, we propose to analyze the changes of the volume of bone marrow cell nuclei in a confocal Z-series (expressed in μm^3), as a supplemental approach aimed to shed more light on the data obtained by flow cytometry that is capable of determining the proportional change of the population of nucleated cells (expressed in %) in AO-stained bone marrow samples of DOX-treated animals with or without enterosorption by AC. In addition, it does seem reasonable to measure the heights of Z-series that

represent the thicknesses of nuclei (expressed in μm), providing with a potentially helpful information in terms of deformability of nuclei that theoretically may not depend on their volume. Supposedly, the nuclear deformability that depends on the state of cytoskeletal organization can be sensitive parameter drastically changing under the influence of various agents affecting bone marrow.

An increase of the population of nucleated cells, suggesting an active bone marrow recovery caused by AC, could hypothetically be accompanied by an increase of nuclear sizes due to DNA replication (to be tested in this work). In mammals, the volumes of nuclei and cells typically correlate, but there are situations when depending on physical and chemical characteristics of the extracellular microenvironment the nuclear volume and shape may readily and widely change, influencing transcriptional regulation and intra-nuclear protein concentration [6]. On the other hand, it is still unclear in terms of timing of the changes in cell and nuclear sizes during cell-cycle, assuming that they may not be changing simultaneously. In this regard, it is worthy to mention a curious finding that the increase of the nuclear volume begins 6 h before the onset of DNA synthesis [7], an event that is likely to precede the increase of cell volume.

OBJECT AND METHODS

Experimental design. Adult female Wistar rats (200–220 g) were used in the experiment approved by the Committee of Bioethics at IEPOR (protocol № 1a of February 16, 2017). The experiment was performed according to the rules and requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The animals were randomly assigned to three groups. In the first group ($n = 5$), DOX under the brand name Doxorubicin Ebewe (EBEWE Pharma Ges.m.b.H. Nfg.KG, Austria) was delivered intraperitoneally twice a week with single doses of 3.25 mg/kg, so the cumulative dose was 26 mg/kg. In the second group ($n = 4$), 2 cm^3 of AC beads (150–250 μm), that were prepared with very high surface area of 4490 m^2/g [3], were mixed with about 5.5 cm^3 of freshly cooked oatmeal and given to animals in the morning under fasting conditions next two days after DOX administrations, so the daily dose of AC was about 1 g per 1 kg of body weight. Two hours later, the animals were fed with a regular food provided by vivarium. The third group ($n = 5$) represented intact animals, who received placebo (isotonic saline without DOX). On the 3rd day after the last administration of AC, bone marrow cells were isolated from femurs of sacrificed animals followed by glutaraldehyde fixation and AO staining, as described [1].

Flow cytometry. Samples were analyzed with a BD FACSAria flow cytometer/cell sorter (BD Biosciences, San Jose, CA) using 100-mW blue laser (488 nm). Parameters and settings were largely the same as proposed for AO-stained bone marrow cells [1]. Forward light scatter (FSC) and side light scatter (SSC) signals were collected in a linear mode, whereas fluorescence signals of AO preferentially bound to DNA and RNA were collected in a logarithmic mode through 530/30 and 695/40 band-pass filters, respectively. The acquisition rate was not higher 1000 cells per second. At least 2.5×10^4 events were collected for each sample. Analysis of the data was performed using WinMDI software (version 2.8) developed by Dr. J. Trotter. Cells were gated on FSC-height vs. SSC-height plots to eliminate debris and aggregates from analysis, although their numbers were very low according to microscopic observations. According to this protocol, nucleated cells can be discriminated from enucleated cells representing polychromatic and normochromatic erythrocytes, while within enucleated cells polychromatic erythrocytes can be discriminated from normochromatic erythrocytes.

Confocal microscopy, image processing and analysis. Fifteen-μl aliquots of the samples of AO-stained cells were placed on the surface of microscope slides followed by careful covering them with 18×18 mm coverslips to ensure uniform spreading of cells with no air bubbles. Samples were analyzed with a Zeiss LSM 5 PASCAL confocal microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-NEOFLUAR 40×/0.75 dry objective. Argon laser (488 nm, 30 mW) was used for excitation of AO. A band-pass 505–530-nm filter was set to collect green fluorescence emitted by DNA-bound AO, allowing distinct visualization of cell nuclei with their boundaries. The images of randomly selected nuclei were acquired and analyzed with a Laser Scanning Microscope LSM 5 PASCAL software (Carl Zeiss, version 3.2). These images consisted of a confocal Z-series of sequential scans with 0.5-μm step size directed from top to bottom of cells. Scan speed and zoom were set on "7" and "2", respectively. To ensure the acquisition of high quality images, the pinhole size was set on 125 μm, providing a superior spatial resolution through the entire stack of optical sections. Amplifier, gain and other settings, including intensity of the laser, were kept unchanged at the time of image acquisition within each set of experiments.

The images in Z-stacks were processed and analyzed with ImageJ software (version 1.48v) [8]. First, they were filtered by "3D Gaussian blur" option with x, y, and z sigma values of 1.0 followed by setting threshold boundaries perfectly coinciding with nuclear boundaries. Next, by operating with "voxel counter" option, showing the voxel dimensions in Z-stacks as 0.22×0.22×0.50 μm, a thresholded volume of the object (cell nucleus) was calculated and expressed in cubic micrometers (μm^3).

Statistical analysis. Significance of the differences between cohorts of the data was assessed by t-test using Microsoft Excel 2019. Probability (P) values of

less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

The flow cytometric data clearly demonstrate that the course of DOX administration resulted in a significant and persistent myelosuppression, as evidenced by a substantial decrease of the population of bone marrow nucleated cells (*Fig. 1A*) accompanied by depletion of polychromatic erythrocytes (*Fig. 1B*). Notably, as a result of enterosorption with AC in DOX-treated animals (DOX+AC group), there was a remarkable recovery of the population of nucleated cells (*Fig. 1A*). Although the population of nucleated cells in DOX+AC group was 1.18-fold larger than that in CTR group, this difference was insignificant ($P > 0.05$). An obvious recovery of polychromatic erythrocytes was also observed in DOX+AC group, but the population of these cells was still much smaller than that in CTR group (*Fig. 1B*), which can be explained by delayed formation of erythrocytes.

The aforementioned changes in the populations of nucleated cells in bone marrow of these groups of animals were concordant with the changes of the volume of cell nuclei in the corresponding groups, as evidenced by calculated mean values (±SD): 179.9 ± 70.7 μm^3 (CTR group), 144.3 ± 49.3 μm^3 (DOX group), and 204.3 ± 77.5 μm^3 (DOX+AC group) (*Fig. 2A*). This finding is probably suggestive of the state of cell proliferation. For example, in a rapidly expanding population of cells (like in bone marrow of DOX+AC group), the average volume of cell nuclei become larger, assuming an active replication of DNA. In contrast, in a growth-arrested cell population (like in bone marrow of DOX group), the average volume of cell nuclei become smaller due to hindered DNA replication.

However, measurements of the height of Z-series representing thickness of the nuclei (*Fig. 2B*) revealed the lack of correlation with the corresponding nuclear volume data (*Fig. 2A*). Although, as shown in *Fig. 2B*, the average thickness of nuclei in DOX+AC group was higher than that of nuclei in DOX group (6.15 ± 1.11 vs 5.35 ± 0.86 μm, respectively), this value was obviously lower than that in CTR group (6.15 ± 1.11 vs 6.81 ± 0.83 μm, respectively). Perhaps, in spite of an active recovery of bone marrow in DOX+AC group, the state of cytoskeletal organization that could predetermine the nuclear deformability in this group is still far from being normal (like in CTR group). These data persuasively show that deformability of the nuclei is a parameter that does not depend on their volume. Undoubtedly, the nuclear volume represents the real (true) size of the object, thereby offering the obvious superiority over 2D-based analysis of nuclear dimensions, which is incapable of providing with an auxiliary information on the object's height. Although bone marrow cells can morphologically be differentiated, there are extremely scarce reports to date related to in depth dimensional characterization of their nuclei under normal and pathologic conditions. In accordance with 2D dimensional analysis of the normal bone marrow of rats, the diameter of myelocytes and the width of their nuclei are 12.45 ± 0.59 and 6.76 ± 1.63 μm, respectively, while the diameter of metamyelocytes and the width of their nuclei are 12.24 ± 0.55 and 4.22 ± 0.70 μm, respectively (presented as the mean ±SD) [9]. Notably, during this stage of maturation, the nuclei become significantly smaller ($P < 0.05$) with a little change in cell sizes.

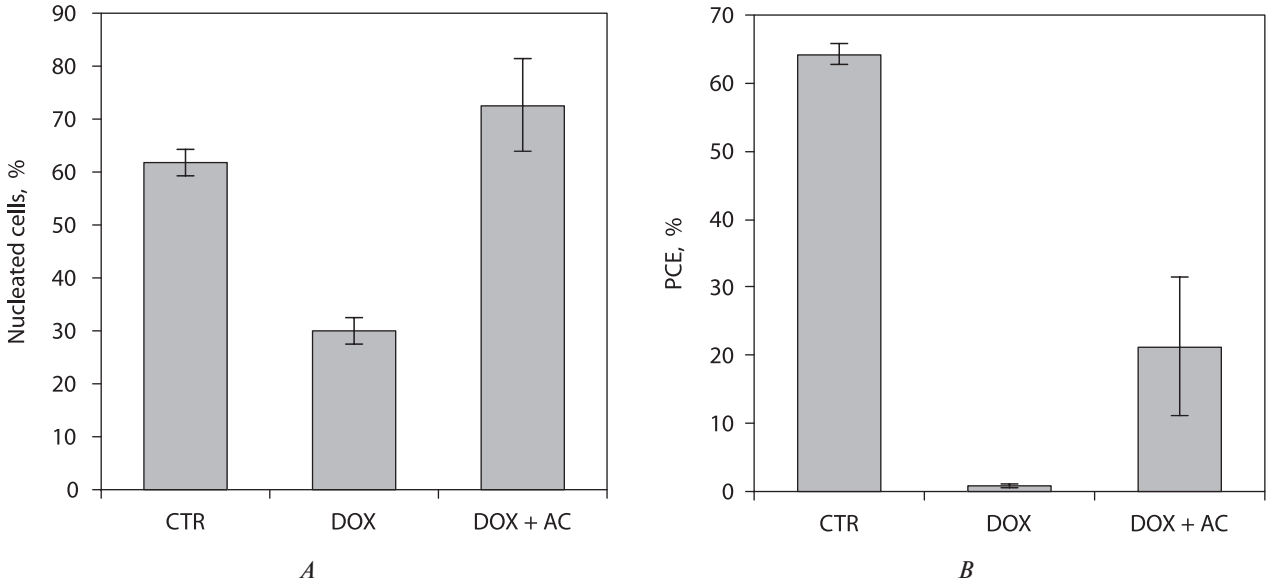

Fig. 1. Comparison of the populations of nucleated cells (*A*) and polychromatic erythrocytes — PCE (*B*) in bone marrow of different groups of animals: CTR — intact control ($n = 5$), DOX — animals treated with doxorubicin ($n = 5$), DOX+AC — doxorubicin-treated animals with enterosorption by activated carbon ($n = 4$). Date presented as the mean ± standard error of the mean

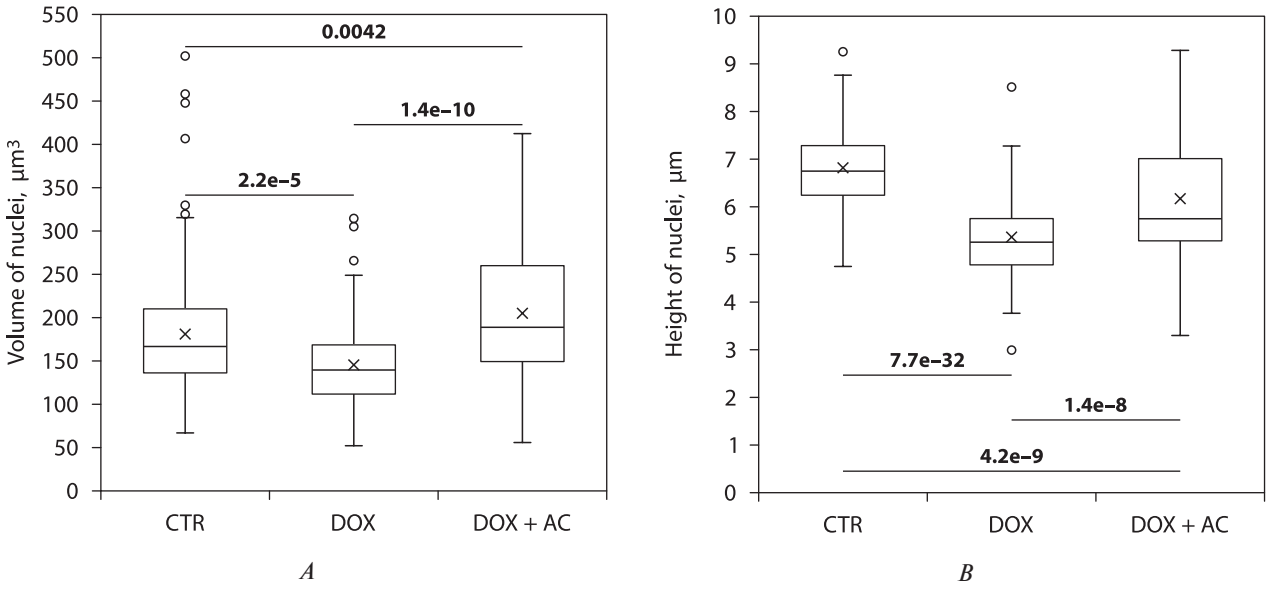

Fig. 2. Comparison of the volumes of bone marrow cell nuclei (*A*) and their deformability (*B*) in different groups of animals: CTR — intact control ($n = 161$), DOX — animals treated with doxorubicin ($n = 95$), DOX+AC — doxorubicin-treated animals with enterosorption by activated carbon ($n = 147$). The mean values are denoted by symbol "×" in the boxes. The inter-group differences were significant according to *P*-values shown in the plots.

As regards AO cell staining, metachromasia of this dye can still potentially be useful to gain an information on the "health status" of cells, based on the findings reported in the middle of the 1970s, demonstrating the possibility to simultaneously evaluate DNA and RNA contents in AO-stained cells by simultaneous measurements of green and red fluorescence intensities per cell, respectively [10]. Recently, the ratio of green to red fluorescence intensity signals from AO-stained bone marrow cells has been shown to firmly depend on cell life activity state that is readily affected by a cytostatic drug, thereby providing with an auxiliary data for monitoring the course of chemotherapy-induced myelosuppression with or without myeloprotective therapy [3].

CONCLUSION

Thus, 3D-based analysis of bone marrow cell nuclei employed in this study offers an excellent opportunity for obtaining the precise values of their volumes, which is helpful in terms of monitoring the course of chemotherapy-induced myelosuppression with or without myeloprotective therapy. Here, enterosorption with AC was shown to result in a distinct myeloprotective effect under ongoing treatment with DOX, as evidenced by recovery of bone marrow accompanied by an increase of the average volume of cell nuclei.

ACKNOWLEDGEMENTS

This work was carried out within the framework of the theme approved by the National Academy of Sciences of Ukraine (project 2.2.5.446).

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АНАЛІЗ ОБ'ЄМУ ЯДЕР КЛІТИН КІСТКОВОГО МОЗКУ ДЛЯ ОЦІНКИ ПЕРЕБІГУ ЦИТОСТАТИЧНОЇ МІЄЛОСУПРЕСІЇ ТА ЇЇ ЗАПОБІГАННЯ АКТИВОВАНИМ ВУГІЛЛЯМ

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Резюме. *Пригнічення функції кісткового мозку (мієлосупресія) є частим ускладненням хіміотерапії, і, відповідно, потреба в моніторингу та запобіганні цього побічного ефекту все ще користується великим попитом.* ***Мета:*** *дослідити зміни в об'ємі ядер клітин кісткового мозку лабораторних тварин внаслідок дії цитостатика при застосуванні або без застосування ентеросорбції активованим вугіллям (АС).* ***Об'єкт і методи:*** *проточну цитометрію і конфокальну мікроскопію використовували для аналізу забарвлених акридиновим оранжевим (АО) зразків кісткового мозку інтактних щурів і щурів, які отримували доксорубіцин (DOX) і DOX з ентеросорбцією (DOX+AC). Конфокальні Z-серії, отримані при послідовному скануванні клітинних ядер, спрямованого зверху вниз з розміром кроку 0,5 мкм, збирали при 40-кратному збільшенні за допомогою аргонового лазера (488 нм) для збудження АО. Зелену флуоресценцію, яка випромінювалася зв'язаним з ДНК АО, реєстрували з використанням смугового фільтра (505–530 нм), що дозволяло виразно візуалізувати ядра та їхні межі. Z-серії далі обробляли та аналізували за допомогою програмного забезпечення ImageJ для вирахування значень ядерних об'ємів.* ***Результати:*** *виявлено очевидні відмінності між ядерними об'ємами у зразках кісткового мозку інтактних щурів і щурів, які отримували DOX і DOX+AC. Значне збільшення об'єму ядер у кістковому мозку тварин, які отримували DOX+AC, порівняно з такими у кістковому мозку тварин DOX та інтактних груп (1,42- і 1,14-кратне, відповідно), ймовірно, пов'язане з активною реплікацією ДНК, що свідчить про стале відновлення пулу ядерних клітин. Примітно, що в цих трьох групах, популяції ядерних клітин кісткового мозку, які аналізували за допомогою проточної цитометрії, добре корелювали зі значеннями ядерних об'ємів. Однак, об'єм ядер не обов'язково може корелювати з висотою Z-серії, що представляє товщину ядер, тим самим надаючи корисну інформацію стосовно деформаційної властивості ядер.* ***Висновки:*** *запропонований в цій роботі аналіз об'єму ядер клітин кісткового мозку є важливим для отримання допоміжної інформації у вивченні перебігу індукованої мієлосупресії та шляхів її запобігання.*

Ключові слова: кістковий мозок, ядерні клітини, ядерний об'єм, активоване вугілля, ентеросорбція, проточна цитометрія, конфокальна мікроскопія, акридиновий оранжевий.

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Одержано: 07.05.2024