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Age-related reduction of chromatin fractal dimension in toluidine blue – stained hepatocytes

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ABSTRACT

In this study, we proposed a hypothesis that chromatin of mouse hepatocytes exhibits age-related reduction of fractal dimension. This hypothesis was based on previously published works demonstrating that complexity of biological systems such as tissues, decreases during the process of physiological aging. Liver tissue was obtained from 24 male mice divided into 3 age groups: 10-days-old (young, juvenile), 210-days-old (adult) and 390-days-old. The tissue was stained using a modification of toluidine blue (nucleic acid – specific) staining method. A total of 480 chromatin structures (20 for each animal) were analyzed. For each structure, the values of fractal dimension, lacunarity, textural angular second moment and inverse difference moment were calculated using ImageJ software and its plugins. The results indicated the age-related reduction in fractal dimension and increase in lacunarity ($p < 0.01$). Fractal dimension is a potentially good indicator of age associated changes in chromatin structure. To our knowledge, this is the first study to show that fractal complexity of hepatocyte chromatin decreases during the process of physiological aging. Fractal analysis as a method could be useful in detection of small age-related changes in chromatin distribution not otherwise visible with naked eye on conventional tissue micrographs.

1. Introduction

Fractal organization of natural phenomena is essentially based on the self-similarity concept, first described by Mandelbrot in 1975. Many biological structures exhibit fractal characteristics, meaning that smaller parts of their patterns resemble the larger ones, as well the system as a whole. Fractality is also a feature of many macromolecules and polymers, as well as other subcellular elements in human organism. Many recent studies have investigated this phenomenon in biosystems, as well as a potential application of fractal analysis and related mathematical algorithms in fundamental medical sciences (Lopes and Betrouni, 2009).

In recent years, structural and biophysical properties of chromatin have become the focus of many research endeavors. The concept of classical equilibrium globule, previously thought to best describe chromatin architecture, was challenged by the emergence of fractal globule model (Mirny, 2011). Fractal dimension of chromatin, both in optical and electron microscopy, was shown to be a sensitive parameter, capable of quantifying discrete structural or ultrastructural changes in chromatin distribution in the nucleus. This mathematical indicator of complexity, when applied to analysis of chromatin organization, can have potentially good predictive value for diagnosis of several diseases.

Several studies have recently suggested that complexity of biological structures and processes changes during the process of physiological aging (Goldberger et al., 2002; Pantic et al., 2013b; Zhang et al., 2007). In a number of tissues, aging is associated with significant morphological changes, many of which include structural degradation and deterioration, often quantifiable with fractal or other imaging methods. On the other hand, age-related changes in complexity in individual calls and their nuclei are not fully understood. This is particularly the case in cells which are during their life cycle exposed to toxins potentially affecting DNA and chromatin structure. Hepatocytes, the main cell population in

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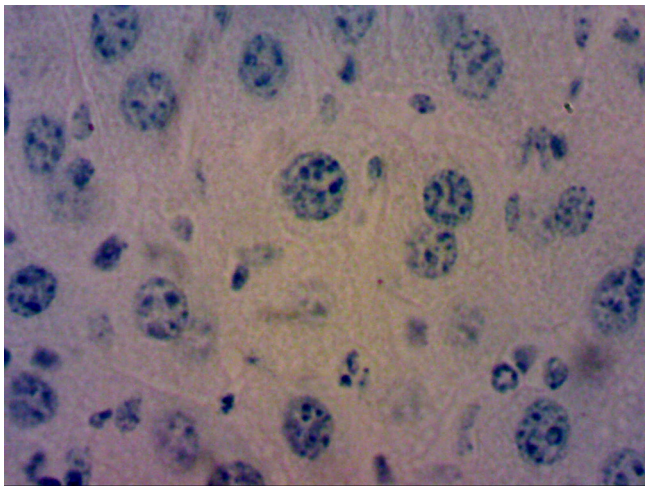

Fig. 1. Toluidine blue – stained chromatin structures of hepatocytes.

liver parenchym, responsible for detoxification processes, are typical example of the cells whose chromatin structure during aging may be influenced by these external factors.

In this study, we proposed a hypothesis that chromatin of mouse hepatocytes exhibits age-related reduction of fractal dimension. This hypothesis was based on previously published works demonstrating that complexity of biological systems such as tissues, decreases during the process of physiological aging. To our knowledge, this is the first study to confirm this hypothesis on a population of toluidine blue – stained cells.

2. Materials and methods

Liver tissue was obtained from 24 male mice (Swiss albino) previously kept at the breeding facility of the Institute of medical physiology, Belgrade. The animals were kept in controlled environmental conditions which included temperature of 22 °C, adequate diet, and day/night cycle. The animals were divided into 3 groups: 10-days-old (young, juvenile), 210-days-old (adult) and 390-days-old. The research followed the guidance for vertebrate animals use in research and training, created by the American Physiological Society (APS) in 1953 (revision on July 16, 2010). The research was approved by the Republic of Serbia, Ministry of Agriculture, as well as the Ethical Commission for welfare of experimental animals of the University of Belgrade, Faculty of Medicine. The lead investigator who handled the animals (IP) had the qualification/certificate for work with laboratory animals (No. PF/080001).

Liver tissue (right lobe) was fixed in Carnoy solution and kept in Paraplast®. Five-micrometer thick sections were created and stained using the modified toluidine blue technique. For details regarding the original toluidine blue method, the reader is referred to the work of Lilli (1965). Briefly, the following protocol was used: xylol (2 × 15 min) > 100% ethanol (2 × 2 min) > 96% ethanol (2 min) > 70% ethanol (5 min) > 0.5% toluidine blue in 0.01 N HCl (30 min) > 100% ethanol and xylol (15 min). Similar protocol was used for spleen tissue staining in our previous study (Pantic et al., 2013b).

Digital micrographs were created using Pro-MicroScan DEM 200 instrument (Oplenic Optronics, Hangzhou, CN) and American Optical Spencer 1036A microscope (Buffalo, NY, USA). Their dimension was 1600 × 1200 pixels, horizontal and vertical resolution 96 dpi, and bit depth equaled 24. Representative micrograph with visible hepatocyte nuclei is presented in Fig. 1.

A total of 480 chromatin structures (20 for each animal) were analyzed. They were analyzed directly from the micrograph

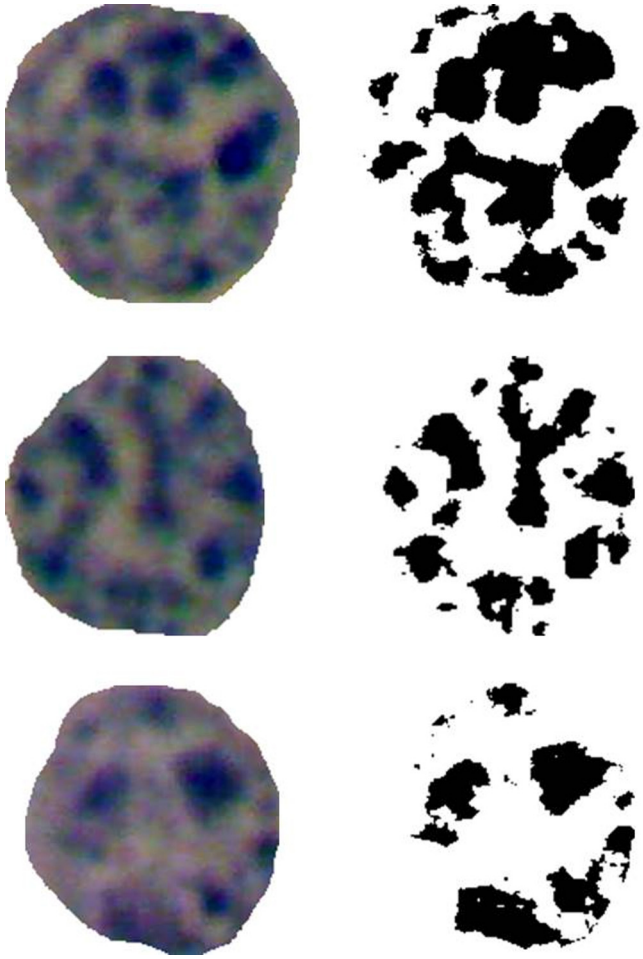

Fig. 2. In ImageJ FracLac plugin, nuclei were automatically converted to binary format for fractal analysis.

without cropping or saving in smaller scale formats. The analysis was done in ImageJ software of the National Institutes of Health (Bethesda, Maryland). The selection of the nuclei (Regions of interest, ROI) was done using the freehand selection option in ImageJ. Fractal analysis was done using the standard "box counting" method in plugin FracLac (Karperien, 1999–2007).

Box-counting method applies Richardson–Mandelbrot Plot after binarization of the structure (Figs. 2 and 3). A graph is created using the logarithmic values of box numbers (N) covering the area, and box scales (ε). From the slope of the estimated regression line, the software calculates the value of fractal dimension (D_B):

$$D_B = \text{regressionslope}[\ln(N)/\ln(\varepsilon)]$$

Fractal dimension reflects the level of complexity of the analyzed structure. Lacunarity (Λ), on the other hand, is an indirect measure of the degree of structural gappiness (level of structural gaps) in fractal architecture. It is calculated from the formula:

$$\Lambda = CV^2_{\varepsilon,g} = (\sigma/\mu)^2_{\varepsilon,g}$$

where μ, σ, ε and g represent the values of mean, the standard deviation, box size, and the orientation, respectively. Details on the fractal mathematical algorithm can be found in previously published studies.

Statistical analysis was don using ANOVA test in SPSS software (Chicago, IL)

Textural analysis was also performed using Grey level co-occurrence matrix (GLCM) method. For each chromatin structure