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RESEARCH ARTICLE

First and second order stereology of hyaline cartilage: Application on mice femoral cartilage

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ABSTRACT

Stereological techniques could be considered in research on cartilage to obtain quantitative data. The present study aimed to explain application of the first- and second-order stereological methods on articular cartilage of mice and the methods applied on the mice exposed to cadmium (Cd).

The distal femoral articular cartilage of *BALB/c* mice (control and Cd-treated) was removed. Then, volume and surface area of the cartilage and number of chondrocytes were estimated using Cavalieri and optical dissector techniques on isotropic uniform random sections. Pair-correlation function [$g(r)$] and cross-correlation function were calculated to express the spatial arrangement of chondrocytes–chondrocytes and chondrocytes–matrix (chondrocyte clustering/dispersing), respectively.

The mean ± standard deviation of the cartilage volume, surface area, and thickness were $1.4 \pm 0.1\ mm^3$, $26.2 \pm 5.4\ mm^2$, and $52.8 \pm 6.7\ \mu m$, respectively. Besides, the mean number of chondrocytes was $680 \pm 200\ (\times 10^3)$. The cartilage volume, cartilage surface area, and number of chondrocytes were respectively reduced by 25%, 27%, and 27% in the Cd-treated mice in comparison to the control animals ($p < 0.03$). Estimates of $g(r)$ for the cells and matrix against the dipole distances, r, have been plotted. This plot showed that the chondrocytes and the matrix were neither dispersed nor clustered in the two study groups.

Application of design-based stereological methods and also evaluation of spatial arrangement of the cartilage components carried potential advantages for investigating the cartilage in different joint conditions. Chondrocyte clustering/dispersing and cellularity can be evaluated in cartilage assessment in normal or abnormal situations.

1. Introduction

Evaluation of cartilage has been repetitively mentioned in different experimental conditions in humans or animals. Numerous histological grading systems have been presented for morphometric evaluation of cartilage histology. However, assessment of renewal, healing, and histopathological changes needs a suitable and reliable evaluation system (Foldager et al., 2015). In fact, making conclusions from the data obtained by grading systems might give rise to difficulty in interpretation of the results. For example, direct morphometric measurement cannot provide comprehensive data about the cartilage tissue. In has also been stated that two histologically very dissimilar tissues might have identical grades (Foldager et al., 2015). In addition, morphometric scoring is commonly believed to have humble comparability and reproducibility compared to other robust methods, including stereological quantitative methods. For example, if it is necessary to achieve the lost/preserved number of chondrocytes, volume of an articular cartilage, articular surface area, and spatial arrangement of chondrocytes, stereological methods could result in reliable data.

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Therefore, unbiased, reproducible, swift, and genuinely quantitative assessment of the cartilaginous tissue based on stereological values is a necessary tool that should be added to the toolbox of cartilage research. First-order stereological techniques apply two-dimensional (2D) data to measure volume, surface area, length, and number of structural components in three-dimensional (3D) structures (Foldager et al., 2015; Hartlev et al., 2013). On the other hand, second-order stereological methods provide researchers with data regarding spatial arrangement of the tissue components (Hartlev et al., 2010). Although the spatial arrangement of cell–cell, matrix–matrix, and cell–matrix can be informative, it has received less attention in the study of cartilage. Besides, application of these methods on the articular femoral cartilage has been presented in control and cadmium (Cd)-treated mice. Cd is a toxic metal used in many products (Sherlock, 1984; Stohs et al., 1997; El-Sokkary and Awadalla, 2011). Cd can also accumulate in bones or cartilaginous tissues. Therefore, the effect of this metal on the hyaline cartilage of the mice has been presented as a practical example.

Thus, the present study aims to evaluate the articular femoral cartilage of the mice using modern stereological methods. The study hypothesized that the volume, surface area and thickness of the hyaline cartilage, the number of chondrocytes and their spatial arrangement can be changed in different conditions including Cd-exposure. In fact, this study aims to answer to the following questions: How much is the volume of the articular cartilage? How much is the articular surface area of the cartilage? How much is the thickness of the articular cartilage? How many chondrocytes exist in the cartilage? How are the chondrocytes positioned relative to each other (spatial arrangement)? Do these parameters change after Cd-exposure?

2. Materials and methods

2.1. Animals

This study was conducted on 12 healthy male *BALB/c* mice weighing 33 ± 5 g and aging 12 weeks. The study was approved by the ethical committee of the University (approval no.: 92-01-01-6901). The study animals were randomly divided into two experimental groups each including 6 mice. Normal saline (1 ml normal saline/day) and Cd chloride (1 mg/kg/day) were prescribed to the control and Cd groups, respectively for 4 weeks by intraperitoneal injection (Mollaoglu et al., 2006; Eybl et al., 2006). The dose of Cd was selected according to the previous studies. After considering the different lethal doses of Cd it was deducted that 1.0 mg/kg/body weight (sub-lethal dose) was suitable for the experimental studies (Paul et al., 2013).

2.2. Tissue preparation

At the end of the experiment, the animals were anesthetized with ether inhalation and the distal end of the right femur including articular cartilage was removed, immersed in neutral buffered formalin, decalcified, and processed. To obtain an isotropic uniform random section that is necessary for stereological quantification, the femur end was embedded in a spherical paraffin mold according to the dissector method (Nyengaard and Gundersen, 1992). The femur distal end was then cut into serial sections of 25 μm thickness. Afterwards, the sections were stained using Heidenhain's AZAN trichrome stain.

2.3. Estimation of the articular cartilage volume

The volume of the articular cartilage was estimated on 8–10 sections per mouse at the final magnification of 25× using Cavalieri's principle (Fig. 1) (Gundersen et al., 1988a,b). The images of the sections were evaluated using a microscope connected to a computer. The stereological probes were generated using the software designed at the University (Stereolith, Shiraz University of Medical Sciences, Shiraz, Iran). In doing so, a grid of points was superimposed on the images using the stereology software. The product of the areas and tissue thickness (T) between the sampled sections were calculated. The area was estimated using the point-counting method. Accordingly, the area per point (a/p) was 0.04 mm^2 and 120 points (P(cartilage)) were averagely counted per animal. Finally, the cartilage volume (V(cartilage)) was estimated using the following formula:

$$V\ (\text{cartilage}) = (a/p) \times \Sigma P\,(\text{cartilage}) \times T$$

2.4. Estimation of the articular surface area

In order to estimate the articular surface area of the cartilage "S (cartilage)" the intersections of the cartilage boundary on the section with straight test lines of a superimposed grid were counted at the final magnification of 25× (Fig. 1) (Gundersen et al., 1988a,b). The articular surface area on the sections was estimated as follows:

$$S\,(\text{cartilage}) = 2\Sigma I/[(l/p) \times \Sigma P\,(\text{cartilage})] \times V\,(\text{cartilage}).$$

where "$\sum I$" is the number of the intersections between the test lines and the articular surface, "l/p" is the length per test point on the counting grid, and P (cartilage) is the number of the points hitting the cartilaginous tissue. Overall, 250 intersections were counted per animal.

2.5. Estimation of the mean articular thickness

The following formula was used to achieve an approximation of the thickness without direct measurement of the cartilage:

$$T\,(\text{cartilage}) = V\,(\text{cartilage})/S\,(\text{cartilage})$$

2.6. Estimation of the chondrocytes number

Optical dissector method was used to estimate the total number of chondrocytes in the articular cartilage (Fig. 1) (Gundersen et al., 1988a,b). The counts were made at random locations of the microscopic slides selected through systematic random sampling. The area of the counting frame (a/f) was 35 μm × 35 μm. Besides, section thickness was used as the height of the dissector (h), excluding the 4 μm thick guard zones at the top and bottom of each section. To calculate the suitable guard zone, Z-axis distribution of the nuclei was plotted (Gardella et al., 2003). The counted neurons were scored and grouped in 10 histograms from the percentiles 0 to 100 through the tissue section from the upper (0%) to the lower surface (100%). The Z-axis distribution of the nuclei has been presented in Fig. 1. The upper and lower 20% of the histogram were considered to be the guard zones and the counting box was placed on the remaining 60% (h). According to the histogram, under-sampling was balanced out and corrected. Any nucleus coming into maximal focus within the next focal sampling plane was selected if it was located completely or partly inside the counting frame and did not touch the exclusion line (Fig. 1) (Gardella et al., 2003). Post-shrinkage thickness was measured during cell counting and was used to determine an average thickness of 20 μm (t) (Dorph-Petersen et al., 2001). In order to determine numerical density (Nv),