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# Estimation of Purkinje cell quantification and volumetry in the cerebellum using a stereological technique

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*Systematic sampling using the Cavalier's principle provides accurate, efficient, cheap, and simple quantitative estimates of objects within neuroanatomical structures like the cerebellum. We identified and isolated the Purkinje cell layer and used this information to extrapolate the stereological technique to estimate the total number of Purkinje cells and volume on light microscope in rabbits' cerebella. Volumes of the cell populations in the cerebellum have not been estimated previously. Using this method, we counted the Purkinje cells in the two right and three left hemicerebella of five male healthy rabbits and found the total number of Purkinje cells to have a mean of 671,597. The mean volume of the Purkinje cell was estimated at 2207 μm$^3$ for the entire cerebella. The contribution of the stereological method to cell quantification and volumetry was emphasised by the neuroanatomical experimental animal study. The method proved to be an excellent tool for evaluating the Purkinje cell numbers and volumes in the rabbits. The data may also support the significance of considering anatomical data when evaluating pathological changes in cerebella.* (Folia Morphol 2011; 70, 4: 240–244)

**Key words: Purkinje cell number, volume, estimation, stereological method**

## INTRODUCTION

Stereology was previously defined by Wiebel in 1970 as a body of mathematical techniques related with three-dimensional parameters determining the structure from two-dimensional measurements obtainable from consequent sections of a structure. The method is accurate and unbiased and precise results may be obtained [13]. Recent reports showed the role of the cerebellum in cognition and learning [14, 15]. It is important to quantify Purkinje cells and their volumes to understand pathological changes on neurodegenerative disorders, exposure to agents such as radiofrequency, microwave, etc., or to evaluate normal anatomical features. For that reason we aimed to evaluate calculating the volume of the cerebellar tissues using a new method for stereological sampling technique. Some studies were designed to represent Purkinje cell function in the rabbit cerebellar anterior lobe after eye-blink conditioning, and to assess whether Purkinje cells in re-

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cording locations may project to the interpositus nucleus. We identified and isolated the Purkinje cell layer and used this information to extrapolate nonbiased systematic sampling data to estimate the total number of Purkinje cells and volume in the tissues. The volumes of cell populations in the cerebellum have not been estimated previously.

## MATERIAL AND METHODS

### Animals

All protocols for routine housing and care, breeding and whelping, brain dissections, perfusion, and euthanasia were approved by the respective Institutional Animal Care and Use Committees of the institutions and were designed according to the principles described in the NIH Guide for the Care and Use of Laboratory Animals. Five specimens were studied. The study was performed using 5 healthy male rabbits' half cerebellar hemispheres (1–1.5 years, 3–4 kg) as two of them were right side, the others were left side. Each half of the vermis was included with the hemispheres. We also measured the wet weight of the rabbits' total cerebella to have a mean of 1.6 g. Euthanasia was performed by parenteral administration of an overdose of sodium pentobarbital. The Animal Experiments and Ethics committee of the University approved the experiments.

### Tissue preparation and sampling protocol

Cerebella were weighed (Kern, Balingen-Germany) and immersion fixed in neutral buffered 10% formalin for two weeks. For Purkinje cell counting the cerebella were sampled according to the rules of the optical fractionators [22, 23]. Each cerebellum was divided from the left and right side, selected randomly (1/2), the selected half of the cerebellum was again divided from the middle, and one part again selected randomly (1/2). Again that selected part was sliced from the middle and one part selected randomly (1/2). That selected part was embedded in paraffin and was totally sliced at 40 $\mu$m thicknesses by rotary microtome (Leica RM2155) and every 7$^{th}$ slab was put onto a glass slide and stained by Giemsa. For estimation of the Purkinje cell volume [11] on vertical sections the cerebella were sliced into 2 mm intervals (again the first cut at a random distance between the edge and 2 mm) and every second slab was chosen. The chosen slabs were cut into 2 mm bars, and with a random start every 4$^{th}$ bar was chosen. The vertical axis of the bars was defined and they rotated freely around their vertical axis, and they were embedded in paraffin. Only one slice, with 60 $\mu$m thickness, which passed through the centre of the tissue in the paraffin was held for the volume estimation [18, 19] of the cell and stained with Giemsa.

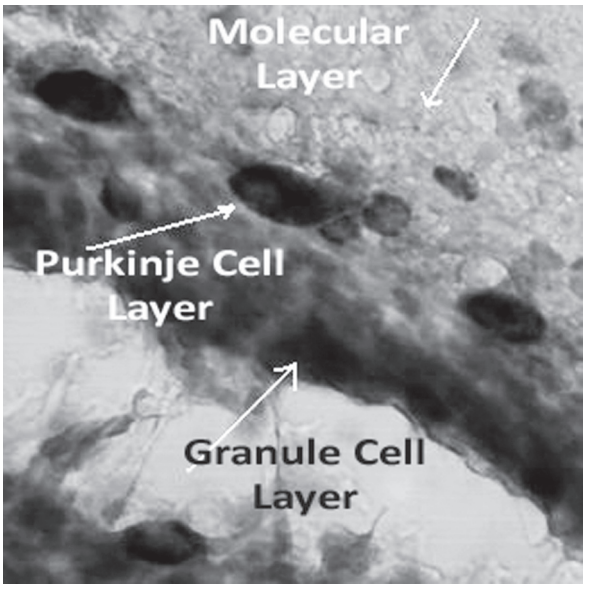


**Figure 1.** Purkinje cell layer of cerebellum was demonstrated under 100× magnification.

### Stereological analysis

We identified then isolated the Purkinje cell layer under 20× magnification and the 100× magnification of the Purkinje cell layer were showed in the Figure 1. The counting procedure was performed on Purkinje cell nuclei of the entire cerebellum. The number of the Purkinje cells was estimated using Shtereom computer software (Pamukkale University, Turkey), an Olympus BH2 light microscope with motorised stage (Lang MS 316) (for the step lengths on the X, Y axis) and a 3.2 MP Cmex camera (Euromex, Holland), under 20× (Fig. 2) and 100× magnification. The thickness of the tissue measured and the movements in the Z axis were controlled using a microcator (Heidenhain, Germany). For counting the nearly 100–200 Purkinje cell nuclei per half of the cerebellum, according to the dissector principle [8] the area of the counting frame was 20,449 $\mu m^2$ and the step length for the X and Y axis was 1250 $\mu$m. The height of the dissector was 10 $\mu$m. The Purkinje cell volume estimation was performed under 40× magnification (Fig. 3) which came into focus within the counting frame and in the dissector height on the vertical direction. The orientation frame and the transparent test probe were used for the Purkinje cell nuclei's volume measuring ruler.

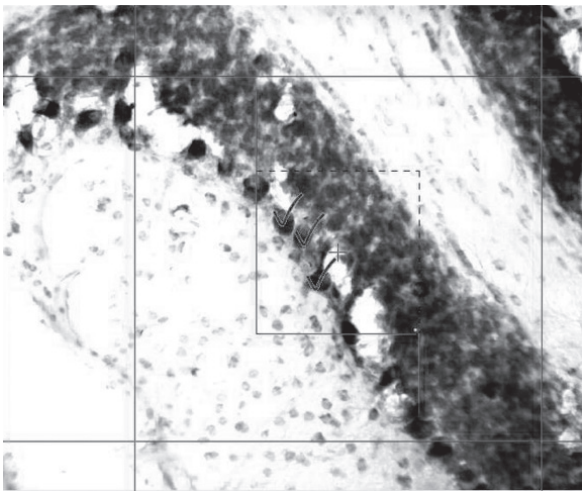

**Figure 2.** Calculating of the number of the Purkinje cells were demonstrated on light microscope under 20× magnification.

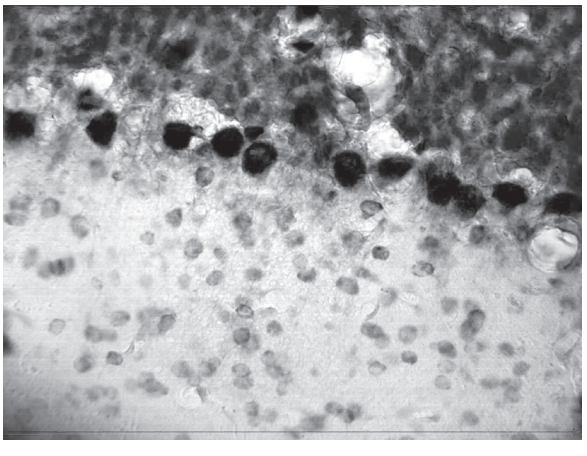

**Figure 3.** The Purkinje cell volume estimation was performed under 40× magnification.

**Table 1.** The coefficient of errors (CE) for the number of counted nuclei of Purkinje cells in the cerebella (mean) of five rabbits

| Cerebellum | Cell number | CE |
|---|---|---|
| 1 | 855456 | 0.10 |
| 2 | 493696 | 0.13 |
| 3 | 693728 | 0.10 |
| 4 | 749056 | 0.10 |
| 5 | 566048 | 0.11 |
| Mean | 671597 | 0.11 |

The area of the counting frame, the step lengths for the X and Y axes, the height of the dissector, the magnification of the Cmex, the monitor's final magnification, and the total final magnification were: 11,881 $\mu m^2$, 1250 $\mu m$, 10 $\mu m$, 0.4×, 49×, and 782×, respectively.

The total cell number was estimated according to the following formula [22, 23]:

$$N = F_{ssf} \times F_{asf} \times \Sigma Q^{-}$$

$F_{ssf}$ is the section sampling fraction (ssf = 56); $F_{asf}$ is the area sampling fraction; multiplying of the x-y step lengths together and divided to the frame area (asf = 76); $\Sigma Q^{-}$ is the number of counted nuclei.

The coefficient of error for the number of counted nuclei and the section thickness was estimated according to Gundersen's formula [8]:

$$CE = \frac{\sqrt{Var\,(noise) + Var\,(SRS)}}{\sum Q^{-}}$$

$$Var\,(noise) = \Sigma Q^{-}$$

$$Var\,(SRS) = \frac{3(A - Var\,(noise)) - 4B + C}{240}$$

$$CE(N) = \sqrt{CE^2(Disector) + CE^2(t)}$$

### Statistical analysis

Results have been expressed as number of observations (n) and mean ± standard deviation (SD). The mean coefficient of error for the cell number was also evaluated. All statistical analyses were performed with SPSS software as descriptive statistical analysis (Statistical Package for Social Sciences, version 15.0, SSPS Inc, and Chicago IL, USA).

## RESULTS

### Qualitative findings

All examined cerebella displayed a normal histology, and the Purkinje cells were clearly visualised at every depth in all the sections. The Giemsa stain was used to stain the nucleus and the cytoplasm together so that the volume of the cell could be evaluated.

### Quantitative findings

The mean weight of the cerebella was 1.6 ± 0.2. The number of Purkinje cells, presented in Table 1 as the mean number of Purkinje cells, was found to be 671,597. The mean coefficient of error for the cell number and tissue's final thickness together was 0.11.

The mean volume of the Purkinje cells was estimated at 2207 ± 425 $\mu m^3$.

## DISCUSSION

The cerebellum is involved in the control of motor functions, with Purkinje cells serving as the only output from the cerebellum. Clinically, cerebellar ataxia is characterised by the degeneration of the cerebellum, particularly the Purkinje cells, responsible for motor coordination and control of the motor functions in humans. Quantification of Purkinje cells and their volumes may be helpful for understanding the mechanisms of neurodegenerative diseases and the pathophysiological basis of cerebellar neuronal dysfunction [10, 20], or for evaluating normal anatomical substructures in the cere-bellum. Although there are a few reports about Purkinje cell numbering for rabbits, there is no data on the volume of the cells in the literature. In animal studies, normal or pathological cerebellar functional or morphological changes and neuronal numeral alterations, particularly on Purkinje cells, were evaluated [5, 6, 17, 21]. A recent demonstrative stereological animal study on the effects of exposure to mobile phones showed a significant decrease in the number of Purkinje cells of the rat cerebellum after microwave exposure of 2450 MHz [16]. Chen et al. [3] examined the long-term effects of early nicotine exposure on the number of pyramidal and granule cells in the Sprague Dawley rat hippocampus, and Purkinje cells in the cerebellar vermis, using a stereological method. The results showed that neither prenatal nor postnatal nicotine exposure reduces the numbers of pyramidal, granule, or Purkinje cells.

Estimating total cell numbers may give valuable information on cell population dynamics during development or specific pathological circumstances. Considering the clinical and laboratory experiences of the researchers, quantitative analysis may be required to detect more subtle morphological changes, such as small changes in cell numbers. When the detection of subtle morphological changes is critical to the decision-making process, sensitive quantitative methods are needed. Stereology provides the tools for obtaining accurate, precise quantitative structural data from tissue sections. These tools have the sensitivity necessary to detect small changes by combining statistical sampling principles with geometric analysis of the tissue microstructure. It differs from other morphometric methods based on tissue section analysis by providing estimates that are statistically valid, truly three dimensional, and referent to the entire organ. Furthermore, because the precision of the stereological analysis procedure can be predicted, studies can be designed and powered to detect subtle, potentially toxicologically significant changes [2]. Unbiased systematic sampling using Cavalier's principle provides accurate, efficient, cheap, and simple quantitative estimates of objects within neuroanatomical structures like the cerebellum. However, the simply principles of stereology are not optimally suited for counting objects that selectively exist within a limited but complex and convoluted portion of the sample, such as occurs when counting cerebellar Purkinje cells [1]. Mwamengele et al. [12] counted the number of Purkinje cells nucleoli as $0.623 \times 10^6$ for 3 rabbits. However, our samples from rabbits for cerebellar nucleus mean number was found $0.672 \times 10^6$ using the stereological method, and the mean volume of the Purkinje cell as 2207 $\mu m^3$ for 5 rabbits. The authors used physical sectioning technique, and nominal thicknesses of their serial wax sections were 4–8 $\mu$m. In their opinion it would be preferable to undertake counts of numbers of nucleoli per nucleus using thicker (over 25 $\mu$m) sections viewed at different focal planes by optical sectioning. In the present study, an optical fractionator was used to estimate the total number of cells in each of the cerebella. If optical sectioning facilities were available, this approach could be expected to be more straightforward and efficient than physical sectioning, permitting direct tracking of each individual nucleolus and nucleus [7, 22, 23]. Contrary to Mwamengele et al. [12], we preferred to apply the suggestion while determining the section thickness and the coefficient of error for the number of counted nuclei. In stereological studies a $0.25 < CE^2/CV^2 < 0.5$ ratio is preferred. In our study, we obtained a ratio of 0.26, which is in the acceptable range [9, 22, 23].

The question of sexual dimorphism in cerebellar size and cellularity has been investigated several times before [4]. As in our study on rabbit cerebella, the present study has failed to detect significant sex differences in comparison with right and left hemi-cerebellar asymmetry in nucleolar number. Consequently, further studies on larger samples are needed in order to obtain additional data.

## CONCLUSIONS

The stereological method proved to be an excellent tool for evaluating the Purkinje cell numbers and volumes in the rabbit cerebella. Our data also emphasises the significance of considering microscopic anatomical data when evaluating changes in the cerebellum in pathological conditions and following experimental procedures.

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