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Comparison between stereology methods for cell volume assessment: exemplified by estimation of neuronal nuclear volume in cirrhotic rats.

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ABSTRACT

The purpose of this paper is to compare the stereology methods to estimate cell volume. For particle volume estimates, a variety of local size estimators has been developed, beginning with assumption-based methods like planimetry. However, modern stereology provides new theoretically unbiased methods like rotator and nucleator ones. The planimetry, rotator and nucleator methods are illustrated by estimating neuronal nuclear volume in the mammillary bodies of control and cirrhotic rats. Cirrhotic rats show larger neuron nucleus volume than controls in all the mammillary nuclei. This difference was obtained with all the probes. Comparing the probes, we obtain smaller nuclear volume using planimetry than with the rotator and nucleator probes. Our results are discussed comparing the probes and the advantages and disadvantages of local size estimators to measure neuronal nuclear volume are suggested.

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Keywords: stereology, local size estimators, planimetry, rotator, nucleator.

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1.- Introduction

Hepatic encephalopathy (HE) is a nervous system functional disorder that occurs as a consequence of liver impairment (Ferenci, et al., 2002). When this organ fails, toxic substances that are not normally processed pass on to the brain producing disturbances in its functioning. This disorder concerns Psychobiology because the patients with HE present neuropsychological disturbances that affect attention, memory, visoespatial orientation, and motor and visoconstructive abilities (Weissenborn, et al., 2005; Ortiz, et al., 2006; Weissenborn, et al., 2003). The impairment of these processes affect the ability to do simple activities such as speaking clearly, getting one's bearings or making mental arithmetic calculations. As a result, studies on mechanisms that could explain these dysfunctions are necessary.

One of the main causes of HE is cirrhosis. This disease is one of the most important health alterations in the European public health system. It is among the 10 first causes of death in the western world (Stewart and Day, 2003). Consequently, it is normal to study HE using animal models which reproduce clinic features of cirrhosis. Animal models of HE allow to obtain interesting data for Psychobiology, which studies the behavioural alterations of HE and searches the brain systems involved. Psychobiology makes it possible to use experimental procedures which are not limited by the ethical considerations applied to human studies.

The cerebral changes described in HE involve neurotransmitter system dysfunctions (Haussinger, et al., 2000), and changes in the cellular morphology (Butterworth, 2002) characterized by "Alzheimer type II astrocytosis" wherein astrocytes exhibit nuclear enlargement and margination of their normal chromatin pattern (Noremberg, 1996). Related to this, our work assesses changes in neuronal nucleus morphology in mammillary bodies of cirrhotic and control rats by different stereology methods for volume estimation.

Mammillary bodies are located in the posterior hypothalamus and contain two nuclei: The medial mammillary nucleus (MM) and the lateral mammillary nucleus (LM). The MM is divided in two parts: a small medial part (MMm) located in the midline and the lateral part (MMl) and LM is located in the lateral regions of the mammillary complex. We have selected this brain region because it is part of the limbic system and it is involved in memory (Sziklas and Petrides, 1998; Vann and Aggleton, 2004; Santín, et al, 1999).

Stereology is a set of methods to obtain precise and efficient morphometric information from sections of an object of interest, in our case the brain. Stereological methods provide good tools to estimate bulk parameters, such as the total volume of a structure and also the number and size of cells or particles (Howard and Reed, 1998). Technological advances in microscopy led to the visualization of biological structures with unprecedented clarity and to the development of highly sensitive probes for cell visualization. Higher resolution of specific tissue constituents using sophisticated and powerful microscopes led to the inevitable questions about how many biological entities there are, what their size is or if there is a true difference in volumes. Volume is a parameter of significant biological importance. In anatomical regions and cell populations there are differences in volume that occur normally over time, e.g. in normal ageing, as well as differences from pathological,

immunological, and toxicological conditions (Finch, 1993; Lucas, et al, 2006; Belzunegui, et al, 1995). Understanding the significance of volume differences can provide insight into the underlying cellular processes and behaviour alterations. For cell volume estimates a variety of size estimators like planimetry, based on classical geometry, and the unbiased rotator (Jensen and Gundersen, 1993) and nucleator probes (Gundersen, 1988; Möller, 1990) has been developed.

Our aim is to assess neuronal nuclear volume in the mammillary bodies of two rat groups: cirrhotic and control group. Planimetry quantification and rotator and nucleator stereological methods will be applied in order to compare them.

2.- Method

We used a total of 12 adult male wistar rats (252,55 $\pm$ 3,66 g. weight). These rats were assigned to two different groups of 6 animals (cirrhotic and control groups). The animals were obtained from the University of Oviedo central vivarium. They were housed under standard conditions at constant room temperature of 21$\pm$2ºC (12-h light/dark cycle with lights on from 8:00-20:00 h). All experimental procedures carried out with these animals followed the 86/609 Directive from The Council of the European Communities and were approved by a local veterinary committee from the University of Oviedo.

The experimental group (TAA) received weekly thioacetamide (Sigma, Germany) concentrations administered in the drinking water during 14 weeks according to the method described by Li et al. (2002). The control group (CO) received normal water.

After 14 weeks, the animals were decapitated, brains were extracted and introduced in paraformaldehyde at 4% in phosphate buffer (0.1M pH 7.4) for 3 hours. Later, brains were embedded in paraffin and systematic cuts were performed with a microtome to obtain 8 μm thick serial sections of the mammillary nuclei (Leica, Germany). These sections were stained with cresil violet. The section thickness and the staining used made it possible to have a good visualization of the neuronal nuclear membranes that were submitted to image analysis to asses their volume in the medial part of medial mammillary nucleus (MMm), lateral part of the medial mammillary nucleus (MMl) and lateral mammillary nucleus (LM). In order to asses neuronal nuclear volume three methods were employed: planimetry, rotator and nucleator ones. 4 sections from each animal with a known distance between them and the nucleus were employed in each method. 25 measures were done in each section, so a total of 100 neurons were assessed.

Neuronal nuclear volume was calculated by planimetry drawing the contour of the nucleus membrane with the cursor. This was done by means of the image analysis program Leica DMR-XA with an immersion oil lens 100x1.25. This program permits to obtain the values to calculate a volume formula (see figure 1).

$$V = \frac{4}{3} \pi r^3$$

Figure 1. Formula to calculate the volume of a sphere.

We performed rotator and nucleator probes on a stereology automated system CAST2 System (Olympus, Denmark), installed on a Olympus-BX61 light microscope. With this system, we assessed 20% of MMm, 30% of MMl and 40% of LM. The cells were captured by an immersion oil lens 100x1.4 and neuronal nuclei were sampled using counting frames (frame sixe 755.8μm^2). We only used the rotator and nucleator probes in those nuclear profiles in focal plane whose midpoint of the nucleus was situated within the unbiased counting frame. Thus each midpoint of the nucleolus served as the sampling unit. We performed rotator and nucleator probes in each neuron. The program applied the volume formula for these probes automatically (see figure 2).

$$V = \frac{4\pi}{3} \cdot \ell^3$$

Figure 2. Formula to estimate the mean volume of the objects according to the nucleator and rotator estimator.

The rotator probe was done by marking a unique point inside the neuron nucleus, in our case the nucleolus. A line that crossed the nucleolus was automatically generated by the program. We moved the cursor to mark the shortest axis across the neuronal nucleus and we marked intersections between the line and the membrane nucleus. With the shortest axis marked, the program placed 3 parallel and perpendicular lines to the shortest axis onto the image. Then, we marked all intersections between the lines and the membrane nucleus (figure 3).

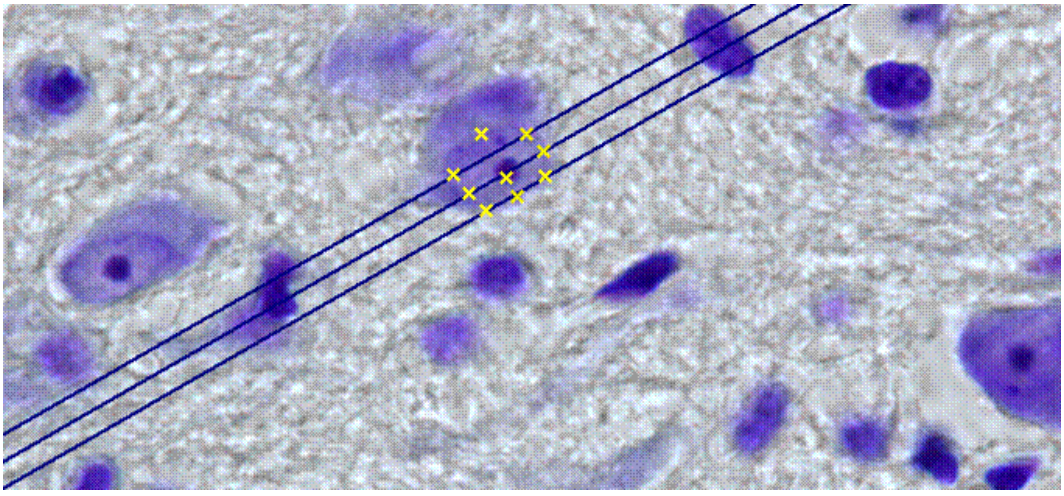


Figure 3. Rotator

The nucleator probe was done marking a unique point inside the neuron nucleus, the nucleolus. The system hereafter created 6 test lines intersecting the point. We marked the intersections of the test lines with the membrane (figure 4).

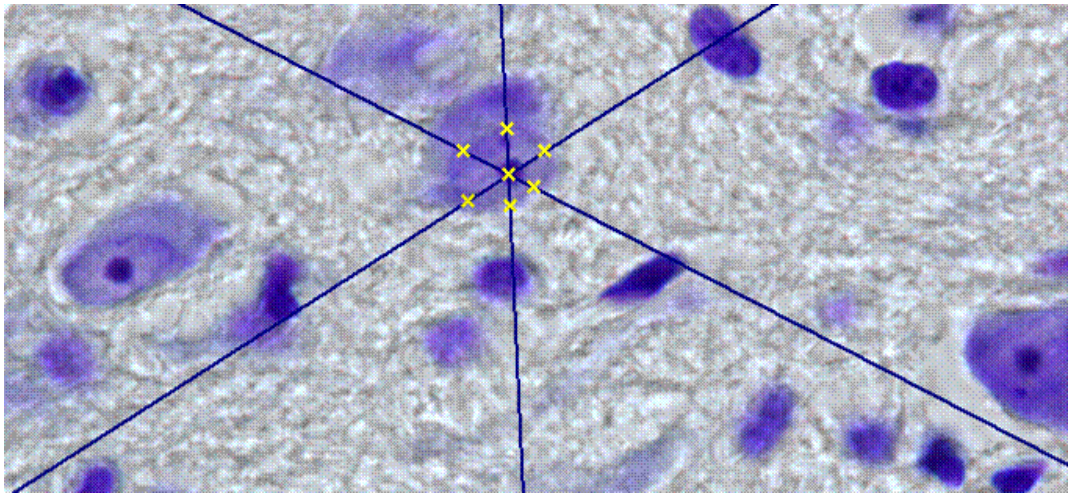


Figure 4. Nucleator

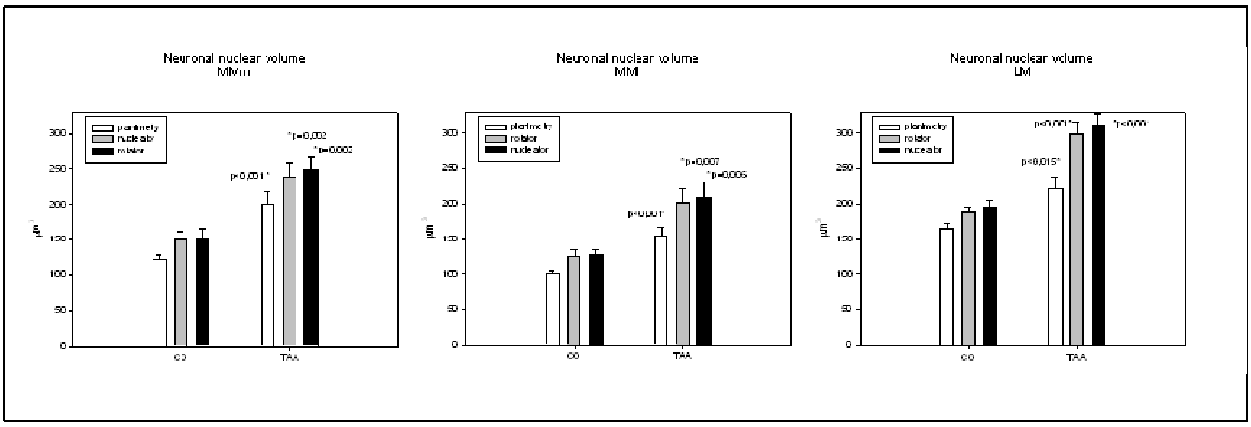
3.- Results

The results of each (planimetry, rotator, nucleator) probe in each mammillary nucleus were submitted to statistical ANOVA with group as independent variable. This was applied with a Sigma-Stat program. Results are shown on table 1 and Graph 1. To compare differences between probes a bifactorial ANOVA (group x probes) with a repeated factor (group) was done for each mammillary nucleus.

The neuronal nuclear volume difference between TAA and CO groups is shown in MMm ($F_{(1,10)}$ =22.176;p<0.001) with differences between planimetry, rotator and nucleator probes ($F_{(2,20)}$ =18.567; p<0.001). The neuronal nuclear volume in MMm is larger in TAA than in CO group (post hoc Tukey test p<0.001). Planimetry shows lower values than rotator and nucleator probes (post hoc Tukey test p<0.001). Nuclear volume difference is shown in MMl between TAA and CO groups ($F_{(1,10)}$ =15.429; p=0.003) with differences between planimetry, rotator and nucleator probes ($F_{(2,20)}$ =17.412; p<0.001). The neuronal nuclear volume in MMl is larger in TAA than in CO group (post hoc Tukey test p=0.003). Planimetry shows lower volume values than rotator and nucleator (post hoc Tukey test p<0.001).A nuclear volume difference is shown in LM between TAA and CO groups ($F_{(1,10)}$ =26.794; p<0.001) with differences between planimetry, rotator and nucleator probes ($F_{(2,20)}$ =97.223; p<0.001). An interaction effect is shown ($F_{(2,20)}$ =25.926; p<0.001). The neuronal nuclear volume in LM is larger in TAA group than in CO group (post hoc Tukey test p<0.001). Planimetry shows lower values than rotator and nucleator probes (post hoc Tukey test p<0.001).

NUCLEUS	PROBE	GROUP	RESULT μm^3 (Mean±SEM)	ANOVA
MMm	PLANIMETRY	TAA	201.015 ± 16.179	*F(1,10)=21.161; P<0,001
		CO	123.044 ± 5.054	
	ROTATOR	TAA	239.921 ± 18.191	*F(1,10)=17.86; P=0.002
		CO	149.532 ± 11.249	
	NUCLEATOR	TAA	248.698 ± 17.672	*F(1,10)=18.922; P=0.001
		CO	151.7± 13.599	
MMl	PLANIMETRY	TAA	155.76 ± 11.378	*F(1,10)=21.799; P<0.001
		CO	100.426 ± 3.316	
	ROTATOR	TAA	201.586 ± 19.947	*F(1,10)=11.915; P=0.006
		CO	125.902 ± 9.103	
	NUCLEATOR	TAA	209.272 ± 22.389	*F(1,10)=11.546; P=0.007
		CO	127.356 ± 8.94	
LM	PLANIMETRY	TAA	221.55 ± 17.795	*F(1,10)=8.566; P=0.015
		CO	165.518 ± 7.062	
	ROTATOR	TAA	298.187 ± 17.699	*F(1,10)=30.775; P<0.001
		CO	187.653 ± 9.151	
	NUCLEATOR	TAA	313.373 ± 15.894	*F(1,10)=42.262; P<0.001
		CO	196.195 ± 8.501	

Table 1. Neuronal nuclear volume estimated by planimetry, rotator and nucleator. ANOVA results show statistically significant differences (*) between groups TAA and CO.



Graph 1. Neuronal nuclear volume in mammillary bodies. * Significant differences between groups TAA and CO.

4.- Discussion

Egyptians and Greeks by 4000 B.C. were the first to quantify the basic stereological parameter: volume, surface area, length and number. Classical geometry provides the planimetry that is a model-based estimator for the volume of a sphere with radius. But this model is not generally appropriated for biological objects because it is only accurate for spheres. Classical geometry used inappropriate formulas and correction factors based on non-verifiable models and assumptions like sphere shape. This assumption-based method overestimated and underestimated the volume of non-classically-shaped objects like cells (Mouton, 2002).

Thirty decades ago, modern stereology emerged. It was characterized by theoretically unbiased principles that made it possible to develop techniques to obtain efficient, accurate and precise volume estimates in biological population (Cruz-Orive and Weibel, 1990). Modern stereolgy provides local size estimators for volume estimates of objects too small to be quantified using a plane through the object. A variety of unbiased size estimators has been developed in the past two decades, beginning with the nucleator and rotator principles. The nucleator uses the radius applied to biological objects and expands the classical formula to general relationships that permit theoretically unbiased area and volume estimates for objects of all shapes and dimensions, not only for those composed of circles and spheres. It is based on the idea that a mean length obtained from randomly oriented lines across profiles of a population of objects is an unbiased estimator of the mean volume for the objects (Gundersen, 1988). The rotator is a modification of the nucleator method. The rotator method is based on the Pappus-Guldinus theorem. This dictates that the solid of revolution generated by rotating a plane figure about an external axis to its plane is equal to the product of the figure area and the distance travelled by the centre of gravity of the figure. (Jensen and Gundersen, 1993). Currently, with computer-assisted stereology we can apply stereological approaches to estimate cell nuclear volume using planimetry, nucleator or rotator methods.

In the light of our results, it seems that volume probes are very useful to Psychobiology and Neuroscience research when it is necessary to assess the volume in nervous cells. All the probes show differences in the neuronal nucleus volume between the control and cirrhotic groups but we proved that planimetry shows shorter volume value against the rotator and nucleator methods. This could be because planimetry uses the classic formula to obtain sphere volume and loses information on the irregular contour of the neuronal nucleus. The other two probes have the advantage of allowing the assessment of volume in irregular particles due to the adaptation of the classic formula,.

In addition, modern stereolgy methods improve efficiency due to how the particle size is outlined. Whereas planimetry requires to draw the internal side of neuronal nucleus, rotator and nucleator only needs to mark intersection points between the lines and the membrane. Drawing all the membrane contour implies making more errors than marking a single point. Rotator and nucleator methods allow the researcher to be faster and more precise since he only has to focus on an intersection point. Therefore, rotator and nucleator methods are efficient to assess nuclear volume despite the advantages that the nucleator method has. The nucleator is precise both in sphere and ellipse nucleus, while the rotator method is imprecise when the nucleus to measure has an elliptical shape and the nucleolus is close to the membrane. In these cases the rotator method undervalues cell nucleus volume.

Considering all this, we conclude that nucleator probe is the most efficient method for neuronal nuclear volume estimation, as we proved when we assessed control against cirrhotic animals. Cirrhotic animals show larger neuronal nuclear volumes in mammillary bodies that could be correlated with behavioural disturbances reviewed by Weissenborn et al. (2001).

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