

# Brain Structure Morphology After Being Fixated With Ethanol on Electron Microscope

## Morfología de la Estructura Cerebral Después de la Fijación con Etanol en Microscopio Electrónico

Arni Kusuma Dewi<sup>1</sup>; Chairul Anwar<sup>2</sup> & Yoshihiro Komohara<sup>3</sup>

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**SUMMARY:** Fixation is one of the processes in preparing histology and pathology. The common material for fixation is buffered formalin including paraformaldehyde. However, the effect of the damaged cells, which is fixed for a long time, causes the research for other fixation materials to become necessary. In addition, paraformaldehyde is also harmful to human body and natural environment. Ethanol is one of the alternative fixation materials, which has been used for two hundred years. It has been used for many purposes, both in routine staining and immunohistochemistry. Nonetheless, no research confirms its effect on the electron microscope. The authors studied the effect of 50 % of ethanol on the cell membrane, organelles, and nucleus of Purkinje cells (Neuron purkinjense) observed on a light microscope and Transmitted Electron Microscope (TEM). Then it was compared to buffered formalin. In the light microscope, it shows that both of fixations have no different effects of the morphology of the cell membrane, cytoplasm, the nucleus of Purkinje cells and the neutrophils. We assume that our 50 % of ethanol concentration is almost the same as BF 10 % in the ability of hardening tissue and color absorption based on the previous study. In TEM, the structure of the cell membrane, organelles, and cytoplasm of Purkinje cell look broken in the cerebellum of 50 % of ethanol except for the nucleus. There was no significant difference diameter of the nucleus. It happened in general because of the shrinkage effect of ethanol. However, the authors recommend using 50 % of ethanol for routine staining.

**KEY WORDS:** Fixation, Brain, Organelle, Electron Microscope

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## INTRODUCTION

Fixation is one of the processes in preparing histology and pathology. The process in fixation includes the selection of the materials, the concentration used, the method, and the length of time required. It is an area of research that continues to grow because it determines the results as expected.

The use of buffered formalin (BF) as fixation material was found by F. Blum in 1893 (Freudenthal, 1947) and has been used in common practice until now. BF is widely used because of its capability to hardening tissue without considering the size, the solubility in salt, and the capability to be used for various research purposes (Fish, 1896). However, the use of this fixation material for an extended period will cause tissue damage and is not suitable for anatomy museum where which is stored in cold temperatures (Fish; Burke, 1933).

The alternative fixation material that can be used to store specimens for a long period is alcohol. Alcohol has been used as a fixation material since 1922 by Freudenthal. His research was re-summarized in 1947 (Freudenthal). In general, he recommended ethanol as a fixation material for all tissues and cells except for fat staining. The research continues to find the effect of alcohol on each tissue since 1957 (Jarrett & Hardy, 1957). Then, in 1986, ethanol began to be investigated as fixation material for histochemical staining (Battifora & Kopinski, 1986).

Ethanol is an alcohol group that is more widely used. Based on several studies, ethanol provides excellent protection for alkaline phosphatase enzymes, coir collagen, DNA, RNA, and lipases (Stafford & Atkinson, 1948; Jarrett & Hardy; Su *et al.*, 2004). Based on the research, some modifications of the technique to approve the continuous growth until now. However, none uses an electron microscope.

<sup>1</sup> Department of Health, Faculty of Vocational Studies, Universitas Airlangga Campus B, Surabaya, East Java, Indonesia.

<sup>2</sup> Department of Histology, Faculty of Veterinary Medicine, Universitas Airlangga (Campus C), Mulyorejo street, Surabaya, East Java, Indonesia.

<sup>3</sup> Department of Cell Pathology, Faculty of Live Science, Kumamoto University 1-chome-1-1 Honjo, Chuo-ku, Kumamoto, 860-0811, Japan.

**MATERIAL AND METHOD**

This study utilized healthy male balb/c mice (*Rattus norvegicus*) as the experimental animals, weighing 200-300 g. The maintenance and treatment of the experimental animals and the research process have received an ethical conduct certificate from the Ethics Commission of the Faculty of Medicine, Universitas Airlangga. Most of the research were conducted in the Department of Health, Faculty of Vocational Studies, Universitas Airlangga.

The two brain specimens were fixated by immersion for 24 hours at room temperature, each with 50 % of ethanol and 10 % BF. Then, one of the hemispheres was processed with routine coloring for the light microscope and the other for Transmitted Electron Microscope (TEM).

**The method by light microscope.** The cerebellum of the hemisphere fixated with 50 % of ethanol was cut into 1 cm<sup>3</sup> to get embedded in paraffin, cleared dan stained using Hematoxylin-Eosin (HE). The same process for the cerebellum of the hemisphere fixated in 10 % BF.

**Method and Analysis by TEM.** Both cerebellum of in 50 % of ethanol and 10 % BF were cut into one mm<sup>3</sup> to get fixation to Karnovsky 1/2 for 720 hours at 4 °C. This process took 720 hours because the laboratory of Electron Microscope in Eijkman Institute Universitas Indonesia-Cipto Mangunkusumo Hospitals for later steps must be removed at that time. The

Karnovsky solution was manufactured as a kit by Electron Microscope Science (EMS) in Philadelphia. The authors observed the structure of cell membranes, organelles, and nucleus of the neuron cells. The authors would like to prove if the denaturation effect of ethanol occurred in the nucleus by analyzing the diameter by image raster3 tool and comparing it with BF.

**RESULTS**

There is a different result seen by a light microscope and TEM. In the light microscope. It shows that both of fixation has no different effect of the morphology of the cell membrane, cytoplasm, the nucleus of Purkinje cells (Neuron purkinjense), and no neutrophils are seen, as seen in Figure 1. The cell membrane and cytoplasm stained red and nucleus blue. The neutrophils in both objects were sharp. In TEM, the structure of the cell membrane, organelles, and cytoplasm of Purkinje cells look broken in the cerebellum fixated with 50 % of ethanol, except for the nucleus (Fig. 2).

The authors analyzed the diameter of the nucleus of Purkinje cells in both fixations (Table I). The data were tested for normal distribution with the Shapiro-Wilk test. Because the data were not normal, the authors continued with the Mann-Whitney test with IBM Statistic 20. It showed that there was no significant difference in the diameter between 50 % of ethanol and 10 % BF treatments ( $p>0.05$ ).

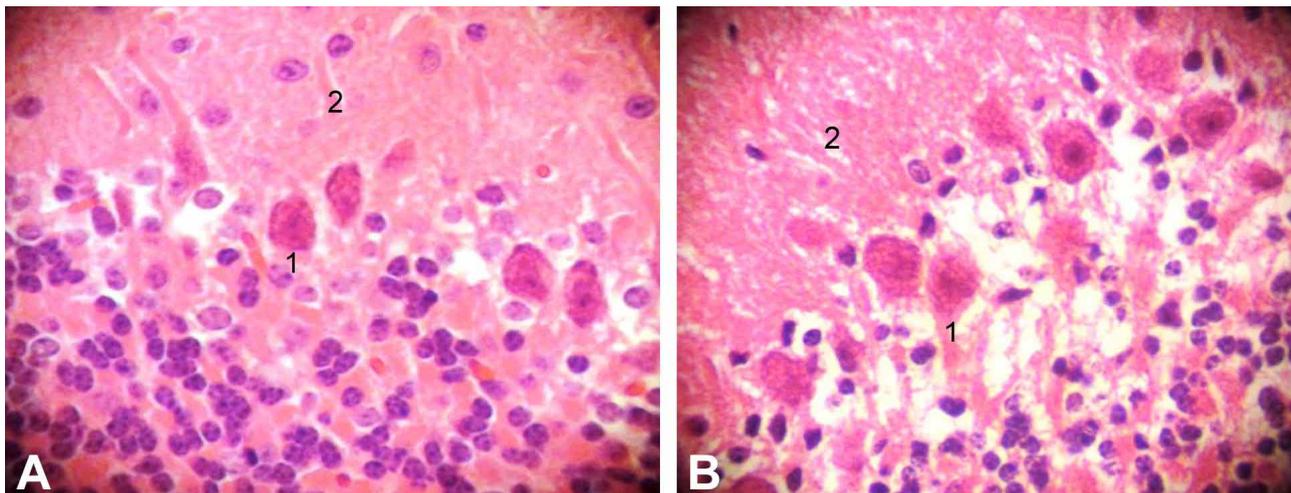


Fig. 1. Cerebellum observed in a light microscope using 400 magnification, HE staining. A. 10 % BF; B. 50 % of ethanol (1. Purkinje cell, considering to cell membrane and nucleus, 2. Neutrophil).

Table I. Description of the Effects of Fixation in TEM.

Variable	Mean ± SD (µm)	Mann-Whitney test
Nucleus diameter on BF fixation	10086.50±116.67 (n=2)	Asymt Sig = 0.121
Nucleus diameter on ethanol fixation	14808.52±176.27 (n=2)	

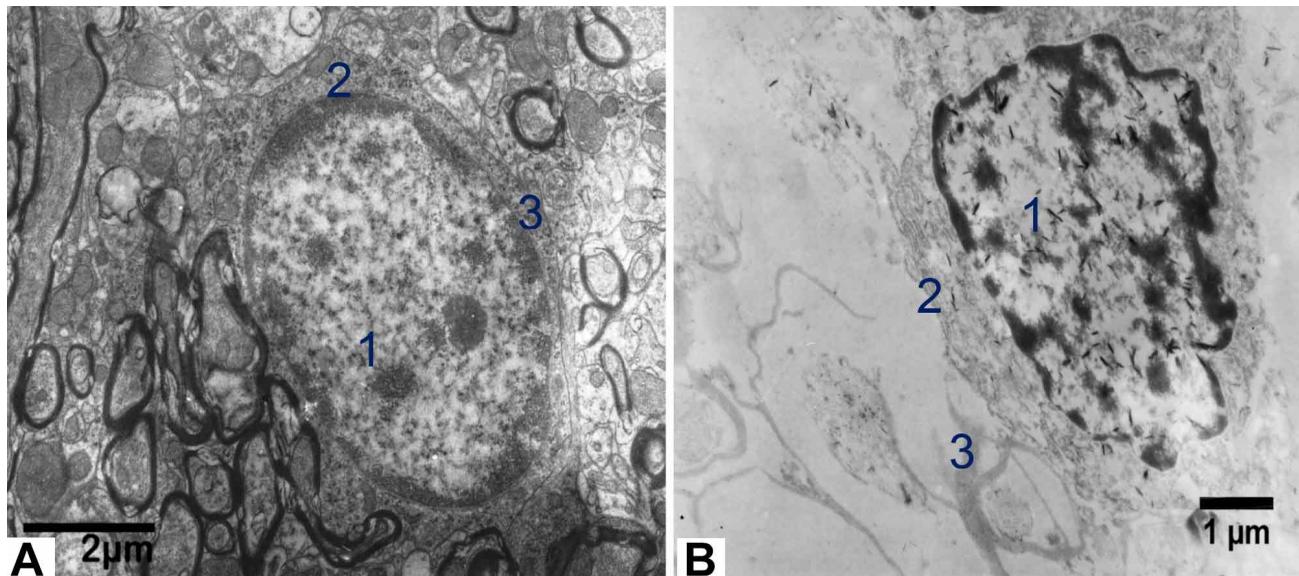


Fig. 2. Cerebellum observed in TEM. A. 10 % BF; B. 50 % of ethanol (1. Nucleus of Purkinje Cells, 2. Cytoplasm (consider to organelles), and 3. cell membrane)

## DISCUSSION

The results of the cerebellum preparation with HE staining between 50 % of ethanol and 10 % BF were not different. Recent research found that 50 % of ethanol had the capability to hardening tissue to increase contrast in micro-CT (Patzelt *et al.*, 2019). Based on these findings, the authors assume that 50 % of ethanol concentration is almost the same as BF 10 % in terms of their capability of hardening tissue and color absorption. Some studies prefer using ethanol with a concentration of 70 % for routine or immunohistochemical staining (Freudenthal; Jarrett & Hardy; Schutte *et al.*, 1987; Su *et al.*). However, there are also those who claim that ethanol shows better results (Battifora & Kopinski). Therefore, until now, research is still developing to determine the best protocol for ethanol concentration and duration of fixation.

Different results were indicated in the observation with TEM. The cell membrane and organelles in the Purkinje cell cytoplasm at 50 % of ethanol appeared to be degraded even though the storage applied Karnovsky 1/2 at 4 °C for 720 hours. Research showed that storing specimens in Karnovsky 1/2 more than six months did not cause any damage to the tissue and cell structures (Mount *et al.*, 1997). According to Patzelt *et al.*, the effect of 50 % of ethanol shrinkage is very low, compared to other ethanol fixation concentration. This notion had never been conformed with an electron microscope. However, the previous study showed that the effect of ethanol to damage

the morphology would not happen if the ethanol was used as a wash after fixation (Llewellyn-Smith & Minson, 1992).

The morphology and diameter of the nucleus did not seem to differ between 50 % of ethanol and BF. The authors tried measuring the diameter of the nucleus Purkinje cells and found no difference in the nucleus diameter based on the statistical results. However, preliminary research proved that the RNA yield from brain tissue fixated with BF could only be protected by 5 % on average, compared to 70 % ethanol (Su *et al.*). BF makes a cross-linking reaction between nucleotides and proteins so that less perfect separation and high temperatures can reduce the detected RNA yield (Su *et al.*).

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**DEWI, A. K.; ANWAR, C. & KOMOHARA, Y.** Morfología de la estructura cerebral después de la fijación con etanol en microscopio electrónico. *Int. J. Morphol.*, 38(2):305-308, 2020.

**RESUMEN:** La fijación es uno de los procesos en la preparación de muestras para histología y patología. El material más común para la fijación es la formalina tamponada. Sin embargo, el daño a las células que se mantienen en formalina durante mucho tiempo, hace necesario buscar otros materiales de fijación. Además, el paraformaldehído también es perjudicial para el cuerpo humano y el medio ambiente natural. El etanol es uno de los materiales de fijación alternativos que se ha utilizado durante muchos años, con diversos objetivos, tanto en la tinción de rutina como en la inmunohistoquímica. Sin embargo no se ha confirmado su efecto con microscopio electrónico. Los autores estudiaron el efecto del 50 % de etanol sobre la membrana celular, los orgánulos y el núcleo de las células de Purkinje observados en un microscopio óptico y un microscopio de transmisión electrónico (TEM). Luego se comparó con la formalina tamponada. En el microscopio óptico se observó que ambas fijaciones no tienen efectos diferentes a la morfología de la membrana celular, el citoplasma, el núcleo de las células de Purkinje y los neutrófilos. Suponemos que nuestra concentración de 50 % de etanol es casi la misma que BF 10 % en la capacidad de endurecer el tejido y la absorción de color según el estudio anterior. En TEM, la estructura de la membrana celular, los orgánulos y el citoplasma de la célula de Purkinje presentaban daño en el cerebelo con un 50 % de etanol, a excepción del núcleo. No hubo diferencia significativa en el diámetro del núcleo. En general lo anterior se debió al efecto de contracción del etanol. En conclusión los autores recomiendan usar 50% de etanol para la tinción de rutina.

**PALABRAS CLAVE:** Fijación; Cerebro; Organelo; Microscopio electrónico.

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Corresponding author:

Arni Kusuma Dewi  
Department of Health  
Faculty of Vocational Studies  
Universitas Airlangga (Campus B)  
Jalan Dharmawangsa Dalam No. 28-30  
Surabaya  
East Java  
INDONESIA

Email: arni-k-d@vokasi.unair.ac.id

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