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The Differences of 10% Neutral Buffered Formalin, 4% Paraformaldehyde, and Bouin Solution in Newborn Rat Brain

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Abstract

Rats are widely used in the study of embryological development of the central nervous system and its congenital disorders. Therefore, tissue fixation is very important in obtaining a good picture of the central nervous system, especially in newborn rats. This study aims to determine the difference in fixation using a solution of 10% neutral buffered formalin, 4% paraformaldehyde, and Bouin solution in the brain tissue of newborn rats. This experimental study used a post-test only controlled-group design with 18 newborn rats aged zero day. After randomization, the pups were grouped into 3 treatment groups, namely: fixation with 10% neutral buffered formalin solution (P1, n = 6), and fixation with 4% paraformaldehyde solution (P2, n = 6), and fixation with Bouin solution (P3, n = 6). Assessment using Hematoxylin Eosin staining, and analyzed by blinding based on the histology of the cerebral tissue (cell nucleus staining, cytoplasmic staining, staining clarity, uniformity of staining). Five cerebrum fields were examined. Data analysis used Kruskall Wallis test and post hoc Mann Whitney test using SPSS. The result was a significant difference in the histology of the brain tissue of newborn rats with the fixation of a solution of 10% neutral buffered formalin, 4% paraformaldehyde and Bouin solution ($p = 0.001$). Brain tissue fixation using 10% neutral buffered formalin solution showed better quality of histological pictures compared to 4% solution of paraformaldehyde solution and Bouin solution ($p < 0.05$).

Keywords: fixation, hematoxylin eosin, brain, rat

INTRODUCTION

Congenital malformations, deformities, and chromosomal abnormalities have caused infant mortality worldwide. Based on World Health Organization (WHO) data, there were 303,000 infant deaths caused by congenital abnormalities in 2015. The prevalence of babies with congenital disorders in Indonesia (1980-2001) was 59.3 cases per 1000 live births. Neural tube defects are the third leading cause of congenital abnormalities that occur frequently in Indonesia according to the 2014-2018 survey.(1) In many cases, the cause of this neural tube defect is still not clearly known. Although genetic and environmental risk factors have implications for this disorder in both humans and experimental animals.(2) Knowledge of human genes is still limited regarding the cause of neural defects, but various environmental factors such as folic acid deficiency, diabetes, and hyperglycemia during pregnancy can cause impaired development of the central nervous system of in the fetus.(3,4)

Rats are widely used in the study of embryological development of the central nervous system and its congenital disorders. To be able to identify lesions or abnormalities in tissues in experimental mice, it is necessary to have knowledge regarding the normal appearance of the organs and cellular architecture of each development stage. (5–7) A good tissue processing process will provide satisfactory quality of histopathological images to be examined. Therefore, tissue fixation is very important in obtaining a good picture of the central nervous system, especially in newborn rats. The brain tissue fixation of newborn rats differs in both quality and quantity compared to the fixation of brain tissue in adult rats. The shrinkage imparted by routine formalin fixation also occurs with other cross-linking fixatives, such as paraformaldehyde. Longer fixation may produce more shrinkage,

although most of the size reduction occurs during the initial stages of preservation. The death of neurons and neuroglia can also increase in cases where the brain shrinks during fixation. This will affect the number of neurons and neuroglia in the rat brain. (8,9) Research related to the method and type of fluid for fixation of brain tissue, especially newborn rats, is still limited. Thus, researchers are interested in testing an effective fixation fluid for the brain tissue of newborn rat.

METHODS

This study has ethical clearance form Faculty of Medicine, Universitas Udayana, Denpasar, Indonesia (no. 1728/ UN14.2.2.VII.14/LT/2020). This experimental study used a post-test only controlled group design. The sample was calculated using Federer formula which resulted in 27 newborn rats aged zero day. After randomization, the pups were grouped into 3 treatment groups, namely: fixation with 10% neutral buffered formalin solution (P1, n = 9), and fixation with 4% paraformaldehyde solution (P2, n = 9), and fixation with Bouin solution (P3, n = 9). The methods of fixation were immersion for a duration of 12-24 hours (P1 and P2) and 4-8 hours (P3). The fixation procedure was preceded by euthanasia by placing the newborn pups in ice for 10 minutes.(10) Both hemispheres were paraffinated and sliced at 6 µm parasagittaly. Slides were stained with hematoxylin-eosin and observed under a light microscope (Olympus, Japan). Assessment using Hematoxylin Eosin staining, and analyzed by blinding based on the histology of the cerebral tissue (cell nucleus staining, cytoplasmic staining, staining clarity, uniformity of staining, tissue degeneration, and tissue artifacts). Data were grouped based on 1=good, 2=intermediate, and 3=bad. The good category is categorized by open face typed neuron nuclei, the neuron cytoplasm contains basophilic colored granules, the coloring is clear and uniform, no tissue degeneration, and no tissue artifacts. The unfavorable category is dense face typed neuron nuclei, the coloring appears faded but uniform, no tissue degeneration, and no tissue artifacts. The bad category is categorized by the degeneration of neuron, the coloring is not clear and uniform, and there are tissue artifacts.(9) Five cerebrum fields were examined and the data were categorical. Data analysis was done using the Kruskall Wallis test and post hoc Mann Whitney test using SPSS. The level of significance was set at $p < 0,05$.

RESULTS

After the female rat gave birth, researchers found that the rat pups were dead due to being eaten by their mother. A total of 6 rat pups per group were then processed for research. When compared to the other groups, the assessment of cerebral histological picture in P1 was better quality ($p = 0,001$). In figure 1, the representative pictures of cerebrum of P1, P2, and P3 are shown.

Table 1. The results of assessment of cerebral histological picture

No	Groups	n	Median (Maximum-Minimum)	p
1	P1	6	1.0 (1.2 – 1.0)	0.0001
2	P2	6	1.7 (2.0 – 1.0)	
3	P3	6	2.0 (2.7 – 2.0)	

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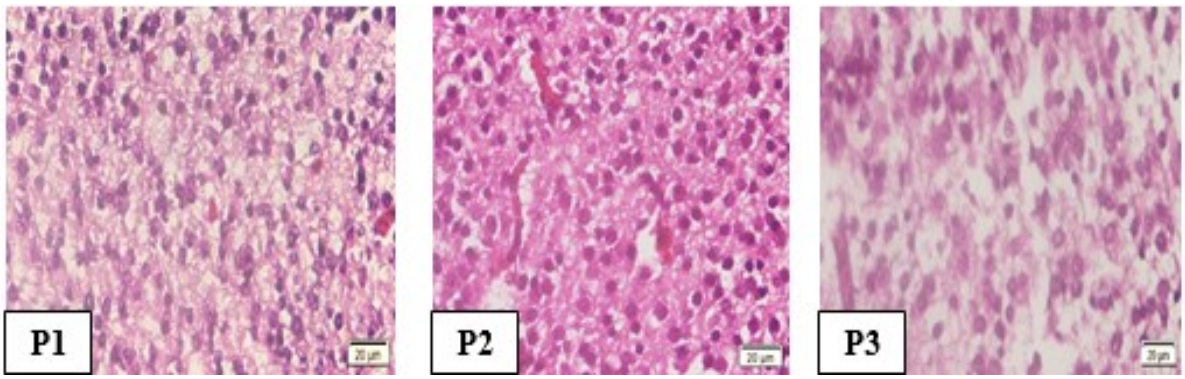

Figure 1. Histological picture of the newborn rat cerebrum with hematoxylin-eosin staining. Image at 400x magnification, 20µm scale. In the P3, there was a decrease in the number of neurons, tissue artifacts, and tissue degeneration compared to the P1 and P2 groups ($p < 0.05$).

DISCUSSION

The tissue fixation of P1 using 10% neutral buffered formalin showed open face type neuron cell nuclei, cytoplasm contained basophilic colored granules, clear and uniform staining, no tissue degeneration, and few tissue artifacts when compared to P2 and P3. This shows a significant difference ($p < 0.05$). Immersion fixation with 10% neutral buffered formalin can penetrate immature skin (in embryos).(5) Perfusion fixation had equal and occasionally superior histologic sections compared with traditional immersion fixation in terms of technical preparation of section, quality and intensity of staining with both hematoxylin-eosin and silver, and immunoreactivity localization with a variety of immunohistochemical reactions.(11) Chen et al reported that perfusion fixation with 10% neutral buffered formalin and 4% paraformaldehyde was generally selected for embryonic and neonatal rat brain tissue. This can speed up the entry of fixation solutions into the brain parenchyma tissue and reduce tissue artifacts.(5) This is due to the small size of the tissue, more fluid levels, and less myelination in the brain tissue of the pups.(10,12,13)

The histological picture of P3 tissue fixation using Bouin's solution showed that the neuron cells were degenerate, the staining was not clear and uniform, and there were tissue artifacts when compared to P1 and P2 groups. This can be seen in Figure 1 which shows a significant difference ($p < 0.05$). Immersion fixation with Bouin's solution can cause brain tissue to shrink and impact quantitative calculations.(10) Immersion fixation of Bouin's solution is preferred for bone or cartilage fixation because it penetrates thicker skin and thick soft tissue in mouse embryos and neonates.(5) New mouse brain tissue birth is still immature (soft and easily damaged) so it is very easy to form tissue artifacts when fortified with Bouin solution.(10)

CONCLUSION

In this study, the cerebrum tissue fixation of newborn Wistar rats using 10% neutral buffered formalin solution showed better histological picture compared to the fixation using 4% solution of paraformaldehyde and Bouin solution, with significant differences

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