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Baking Soda (Sodium Bicarbonate) As An Alternative To Lithium Carbonate *Blueing Agent* In Hematoxylin-Eosin Staining

Burhanuddin Burhanuddin[1*], Malik Abil Fadli[2], Neiny Prisy Foekh[3], Ayu Putri Utami[4]
[1-4]Poltekkes Kemenkes Jakarta III,Indonesia

Address Jl. Arteri JORR Jatiwarna Kec. Pondok Melati – Bekasi 17415 Telp. (021) 84978693 84978693
Author correspondence : burhannudin@poltekkesjakarta3.ac.id[*]

Abstract.*Hematoxylin-eosin staining is a crucial technique used in histopathology to differentiate tissue components. One of the steps in this staining is changing the color of the nucleus, which was previously reddish due to the acid solution, to bluish; this step is known as blueing. The bluing agent, lithium carbonate, is commonly used in laboratories but has side effects and low availability. Sodium bicarbonate, also known as baking soda, can be used as an alternative due to its optimal pH, lower toxicity, and availability. This study aims to find out whether baking soda is as effective as lithium carbonate when used as a blueing agent for hematoxylin-eosin staining. The study design used was a true experimental post-test only control group design with a sample size of 32 preparations. Results of preparations using baking soda microscopically had good color uniformity, the nucleus was visible, the cytoplasm was clear, so the boundaries between cells were visible, and no cell lysis was found. In percentage terms, 14 preparations received a score of two (87.5%), two preparations received a score of three (12.5%), followed by a P value of 0.051 after carrying out the Fisher's exact test in bivariate analysis. Based on the results, baking soda is as effective as lithium carbonate, so it can be used as an alternative to bluing agent of hematoxylin-eosin staining.*

Keywords: *Bluing, Lithium carbonate, Baking soda*

1. BACKGROUND

Tissue staining is one of the processes in making histological preparations that aims to show tissue morphology and its structure, the presence of certain cells in a tissue, and the prevalence of certain cells in a tissue. One of the routine stains that is often used is hematoxylin-eosin (HE) staining. This HE staining is based on a simple principle, namely the basic or acidic properties of a solution will bind to tissue components that have a tendency towards basic or acidic properties so that they can form a bond between tissue components and dye molecules. One of the stages in HE staining is the bluing process which functions to change the color of the cell nucleus (nucleus) which was originally reddish purple to blue/clear purple (Khristian & Inderiati, 2017) .

This bluing agent is basic with an acidity level ranging from 7.5 to 9.0 which works by neutralizing the pH, degrading H+ ions in the solution which then has an impact on the hematoxylin structure, and eliminating H+ ions from the ring structure. Commonly used bluing agents are lithium carbonate, ammonia water and Scott's Tap Water (Khristian & Inderiati, 2017) . In practice, lithium carbonate is more often used as a bluing solution compared to other bluing agents because lithium carbonate can produce optimal blue color and has an optimum

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pH so that it can accelerate the bluing process. (Mohandas *et al* , 2019). Excessive exposure to lithium salts can cause side effects such as nausea, vomiting, tremors and skin lesions while in the long term it causes cognitive impairment and sexual dysfunction. In terms of economic value, lithium has a relatively low level of availability with a higher price than other *bluing agents* (Gitlin , 2016; Salafudin , 2021).

In accordance with its basic characteristics, one of the chemical compounds that has the potential to be used as an alternative to lithium carbonate is sodium bicarbonate or more commonly known as baking soda. Baking soda is basic and also economical with a higher level of availability compared to lithium carbonate. (Pambudi, 2015). Therefore, researchers are interested in knowing the potential of baking soda (sodium bicarbonate) as an alternative to lithium carbonate in hematoxylin-eosin staining.

2. RESEARCH METHODS

The study was conducted at the Cytohistotechnology Laboratory of the Medical Laboratory Technology Study Program, Jakarta Health Polytechnic III. The research design used was *Post-test Only Control Group Design* with independent variables of lithium carbonate and baking soda while the dependent variable was the quality of histological preparations. The subjects of the study were the livers of male mice (Mus musculus) aged 8-10 months with a body weight of between 25-30 g which were then made into 32 preparations. A total of 16 preparations were used as the treatment group and 16 other preparations as the control group. The tools used in this study included *a hot plate* , *beaker glass* , microtome, *embedding set* , water bath, stirring rod, staining tub, *slide warmer* , microscope, tweezers, brush, *timer* , and *surgical blade* . The materials used are tissue preparations, 10% *neutral buffer formalin* , absolute alcohol, distilled water, 70% alcohol, 96% alcohol, xylol, entelan, slide glass, *cover glass* , filter paper, 0.05% lithium carbonate, 10% baking soda solution, hematoxylin, and eosin.

There are 2 stages in this study. The first stage is determining the optimum concentration of baking soda. The next stage is taking liver tissue from experimental animals, then fixed with *neutral buffer formalin* (NBF) solution in a ratio of 1:10. After the fixation process, it is continued with a dehydration process in graded alcohol from a concentration of 70%, 80%, 96% and absolute alcohol (each for 3 hours). Then clearing is carried out using xylol (each for 30 minutes) followed by impregnation in liquid paraffin in *a water bath* with a temperature range of 55-60°C for 2 hours. After impregnation, tissue printing is carried out on the embedding set. The frozen tissue block is removed from the mold for further cutting with

a microtome. The next stage is staining which begins with deparaffination using xylol 3 times (each for 5 minutes). Then rehydration is carried out with absolute alcohol (3 minutes), 96% alcohol (3 minutes) and 70% alcohol (3 minutes) then washing with running water (5 minutes). Then, Harris hematoxylin staining (7 minutes) and washing with running water (3 minutes) were carried out, then continued with *bluing* , with different *bluing agents*, namely lithium carbonate (3 dips) and baking soda (5 dips). After that, washing with running water (3 minutes) was carried out to be continued with staining using eosin Y (5 minutes). Furthermore, dehydration was carried out by dipping into absolute alcohol I, II and III (7 dips each) followed by clearing using xylol I, II and III (5 minutes each). The last stage is *mounting* using *mounting* reagents then covered with deck glass and the quality of the preparation results between lithium carbonate and baking soda were observed under a microscope.

3. RESULTS AND DISCUSSION

On study This done test introduction For determine the optimum concentration of baking soda that will used in the *bluing* process . The results obtained is as following :

Table 1 Concentration of baking soda to improvement pH level and solubility

No	Baking soda concentration	pH	Solubility
1	0.05%	7	Late
2	0.1%	7	Late
3	0.5%	7	Late
4	1%	7	Late
5	5%	7.5	Late
6	10%	8	Late
7	15%	8.5	No late
8	20%	9	No late

Based on pH and the solubility of baking soda in water then optimum concentration used is 10% baking soda solution where This in accordance with pH agent criteria *the blueing* that revolves around between 7.5 – 9 (Khristian & Inderiati, 2017) . Solution This 10% baking soda is what then used as lithium carbonate substitute in the process *of bluing* .

Samples used in study This is the liver organ of mice (*Mus musculus*) because the liver has relative structure simple And easy recognized so that allow analysis accurate histology And effective (Wang *et al* , 2022). Mice used is mouse 8-10 months old with male genitalia male Because No influenced by estrogen hormone , has more hormonal conditions stable If compared to with mouse female , and heavy body mouse male more heavy If compared to with mouse female (Ministry of Agriculture of the Republic of Indonesia, 2016; Sari, 2016, Yusuf *et al* , 2022). Results observation macroscopic stock can seen on Figure 1 and results scoring

coloring on group control And treatment can seen on Table 2.

Table 2 Percentage results between control and treatment groups

No	Description	Quality		Control		Baking soda	
		Scale Ordinal	Score	N	%	N	%
1	Color blue in the cell nucleus No clear , color red on cytoplasm No clear , color No uniform . Provision No Can diagnosed .	No Good	1	0	0%	0	0%
2	Color blue in the cell nucleus clear , color red on cytoplasm clear , uniformity color good . Provision can diagnosed .	Good	2	16	100%	14	87.5%
3	Color blue in the cell nucleus clear , color red on cytoplasm clear , uniformity color with high contrast . Preparation can diagnosed .	Very Good	3	0	0%	2	12.5%
Total				16	100%	16	100%

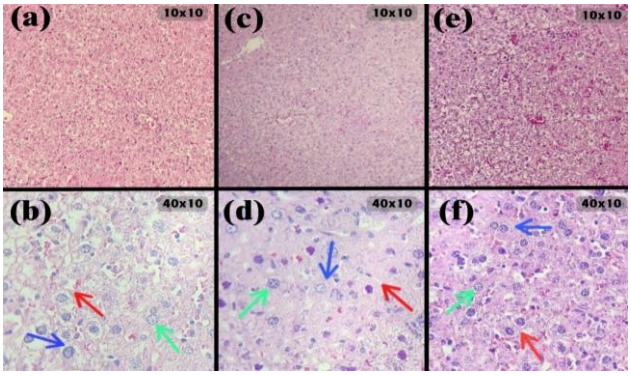

Figure 1. Microscopic results of treatment and control (a) Field of view of control preparation with 10X objective magnification (b) Field of view of control preparation with 40X objective magnification (c) Field of view of treatment preparation with 10X objective magnification (d) Field of view of treatment preparation with 40X objective magnification (e) Field of view of treatment preparation with 10X objective magnification (f) Field of view of treatment preparation with 40X objective magnification. Description: green arrows indicate cell nuclei, blue arrows indicate cytoplasm, and red arrows indicate cell boundaries.

The results of the microscopic images in Figure 1 (a and b) show that in the field of view of the color control preparation in the cell nucleus is clearly visible, the color in the cytoplasm is clear, the color uniformity is good, and the preparation can be diagnosed. In terms of the integrity of the cell shape, the cells can be seen that there is no cell lysis, chromatin in the cell nucleus is visible, the nucleolus is visible, and there is no damage to the cell shape, either shrinkage or enlargement of the cell so that the boundaries between one cell and another can be seen clearly.

In the field of view of the treatment preparation that received a score of two (Figure 1. c and d) can be described the same as the field of view of the control preparation. Meanwhile, in the field of view of the treatment preparation that received a score of three (Figure 1. e and f) it can be seen that the color in the cell nucleus is clearly visible, the color in the cytoplasm is clear, the color uniformity is very good, and the preparation can be diagnosed. In terms of the integrity of the cell shape, it can be seen that there is no lysis, chromatin in the cell nucleus is visible, the nucleolus is visible, and there is no damage to the cell shape either shrinkage or enlargement of the cell so that the boundary between one cell and another can be seen clearly. The difference between the field of view of the preparation that received a score of two (Figure 1. c and d) and a score of three (Figure 1. e and f) is the color intensity in each part of the cell and the contrast between the cell nucleus and its cytoplasm, where the preparation that received a score of three had a higher color intensity and contrast compared to the preparation that received a score of two.

Bivariate calculations were performed to see whether or not there was a difference between the control and treatment. Bivariate calculations in this study used the *Fisher's exact test* where the test is an alternative to the *chi square test* if the requirements of the *chi square test* are not met. Based on the results of *the Fisher's exact test* (Table 3), there was no difference between *bluing* using baking soda or lithium carbonate. This is because baking soda is a compound that is basic so that it can be in line with the principle of *bluing* where a basic agent is needed to stabilize tissue pH. Baking soda works as a *bluing agent* by preventing pH shifts that can result in detailed defects in the cell nucleus which result in the cell nucleus not being able to be diagnosed (Khristian *et al* ,2017 ; *et al* , 2020).

Table 3 Results of *Fisher's exact test*

No	**Group**	**Results**		**Total**	***P value***
		Good	**Very Good**		
1	Control	16	0	16	0.051
2	Treatment	14	2	16	
Total		30	2	32	

The significant comparison of concentration and solubility time is a constraint of this research. Baking soda requires a more concentrated concentration to be used as a *bluing agent* compared to lithium carbonate. Baking soda can be used as a *bluing agent* if the concentration is 10% where if made in 100 ml it requires 10 grams of baking soda so it takes longer to dissolve, while if using lithium carbonate only 0.05 grams are needed in 100 ml with a concentration of 0.05% to make it a *bluing agent* . However, when compared economically, a 10% baking soda solution is superior in terms of economy than 0.05% lithium carbonate.

4. CONCLUSION AND SUGGESTIONS

The results of this study indicate that baking soda has the potential to be used as a *bluing agent* to replace lithium carbonate. In addition to being more economical because the price is cheaper than lithium carbonate, its toxicity level is also lower.

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