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Use of a Hotplate at 80°C for 3 minutes to help Fixation of Histological Preparations

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ABSTRAK

Fixation is a complex stage and requires a long time. The microwave heating process has been proven to accelerate the fixation rate, but the use of a hotplate in helping the fixation process has yet to be discovered. The study aims to determine the optimal time of 80°C hotplate heating in helping fixation. The study was conducted experimentally using a post-test only control group design. A total of 75 histological slides of hepatic tissue of male Wistar rats were divided into five groups with equal size: control group (conventional/K and microwave/M) and three treatments of 80°C hotplate for 3 minutes (H1), 5 minutes (H2) and 10 minutes (H3). Hematoxylin Eosin (HE) staining results of all histology slides were morphologically observed and scored based on previous studies. In addition, the staining scores were tested for differences between groups using the Kruskal-Wallis and Mann-Whitney tests, with p values <0.05 being significant. The results of morphological observations of HE stains stated that the median H2 group was worth a score of 3, while the other groups were worth a score of 2. The results of the Kruskal-Wallis and Mann-Whitney tests showed that the H2 group significantly differed from the control group (p<0.05). This shows that using a hotplate at 80 ° C for 5 minutes can help the fixation process in making histological preparations of rat hepatic tissue.

KEYWORDS:

Fixation, Heating, Hotplate, Duration, Histology

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INTRODUCTION

Histological specimens can be examined to assess tissue structure through routine Hematoxylin and Eosin (HE) staining (Mescher, 2017; Ellyawati, 2018). HE staining is widely acknowledged as the standard method for determining prognosis and treatment selection in cancer patients.

The process of tissue specimen staining in the Laboratory is a complex and time-consuming procedure (Khristian and Inderiati, 2017; Prasetyani, 2017; RSUD Dr. R. Koesma Tuban, 2020; RSUD Dr. Kanujoso Djatiwibowo, 2020). Therefore, refining tissue sample processing techniques in a research manner or clinical laboratory set-up still requires further development due to the intricacy of the stages involved and the time taken to complete them (Setiawan, 2016). Fixation is considered the crucial stage and key focus for the success of the processing technique (Khristian and Inderiati, 2017; Sriwahyunizah, 2018).

Microwave heating up to 80°C effectively expedites fixation (Tripathi et al., 2013; Ariyadi and Suryono, 2017; Bancroft et al., 2019). However, the effectiveness of hotplates for dry heating as an alternative to microwaves in enhancing fixation at 80°C has not been extensively evaluated (Alfita et al., 2021). Recently, fixation with hotplates has been limited to 60°C (Burhannudin, Warida and Puspita,

2023). Furthermore, Pratiwi (2018) emphasises that a hotplate is a more adept heating device than other options. Therefore, current research endeavours to determine the optimal duration for employing a hotplate at a temperature of 80°C to improve the fixation process while preparing histology slides.

METHOD

The study used an experimental design with a post-test-only control group. A single male Wistar rat (Rattus norvegicus) was used to prepare 75 histology slides. The slides were evenly divided into five groups: two control groups (conventional/K and microwave/M) and three hotplate groups heated to 80°C for 3 minutes (H1), 5 minutes (H2), and 10 minutes (H3). The conventional control group was treated without heating, and the microwave group was treated with heating at 50°C for 20 minutes. The tissue was immersed in fixation liquid when the precise temperature was reached. The thermometer was immersed within the fluid to observe the temperature stability.

Wistar rats were obtained from iRatco, Bogor, to ensure the purity of their strain. The rats were euthanised using chloroform, and liver tissue was then obtained through abdominal surgery. The liver tissue was subsequently immersed in FineFix solution (Milestone Medical) specific to their respective groups. To attain homogeneous temperature distribution, frequent stirring is carried out. This was followed by immersion in graded alcohol solutions consisting of 70%, 96% and absolute alcohol (dehydration process), after which it was cleared through immersion in xylene. The tissue was cleared and subsequently infiltrated and embedded in paraffin. After solidifying the tissue, it was sectioned using a microtome for about 4-5 µm. The resulting slide sections were mounted and stained with the routine Hematoxylin Eosin (HE) staining.

Histological sections were stained using haematoxylin and eosin. Following prior research by Ariyadi and Suryono (2017), morphological characteristics were examined and evaluated. Statistical analysis was conducted using Kruskal-Wallis and Mann-Whitney tests to compare scores of all groups and detect significant differences with a p-value of less than 0.05. The complete research process is presented in Figure 1. It has been granted ethical approval by the Non-Medical Health Research Ethics Committee at the University of Muhammadiyah Prof. Dr. Hamka (Uhamka), with reference number 03/25.05/02517.

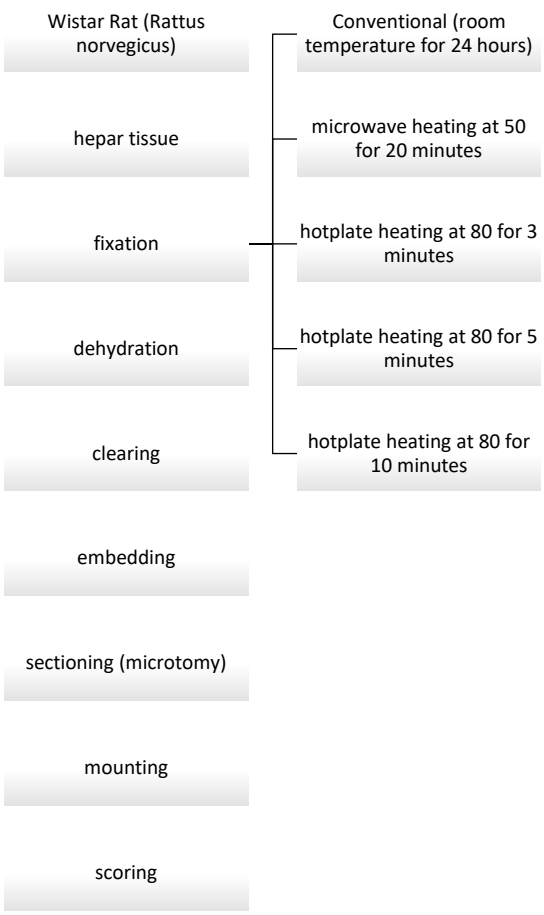

Figure 1. The research process is described as follows: Liver tissues were acquired and placed in tissue fixation solution based on their respective treatment groups. Following this, tissue underwent a maturation process involving dehydration, clearing, and infiltration, followed by embedding, microtomy (sectioning), staining, and observation of results.

RESULT AND DISCUSSION

Fixation is the most crucial step in the histological specimens' preparation, determining the entire process's success. Fixation is a complex stage, and efforts are ongoing to accelerate the process through heating. Recent research has employed heating with a hotplate at 80°C to aid the process.

The effective use of a hotplate for heating and assisting the fixation process of histology specimens was demonstrated by the favourable hematoxylin eosin (HE) staining outcomes. The HE staining results for all experimental groups are displayed in Figure 2.

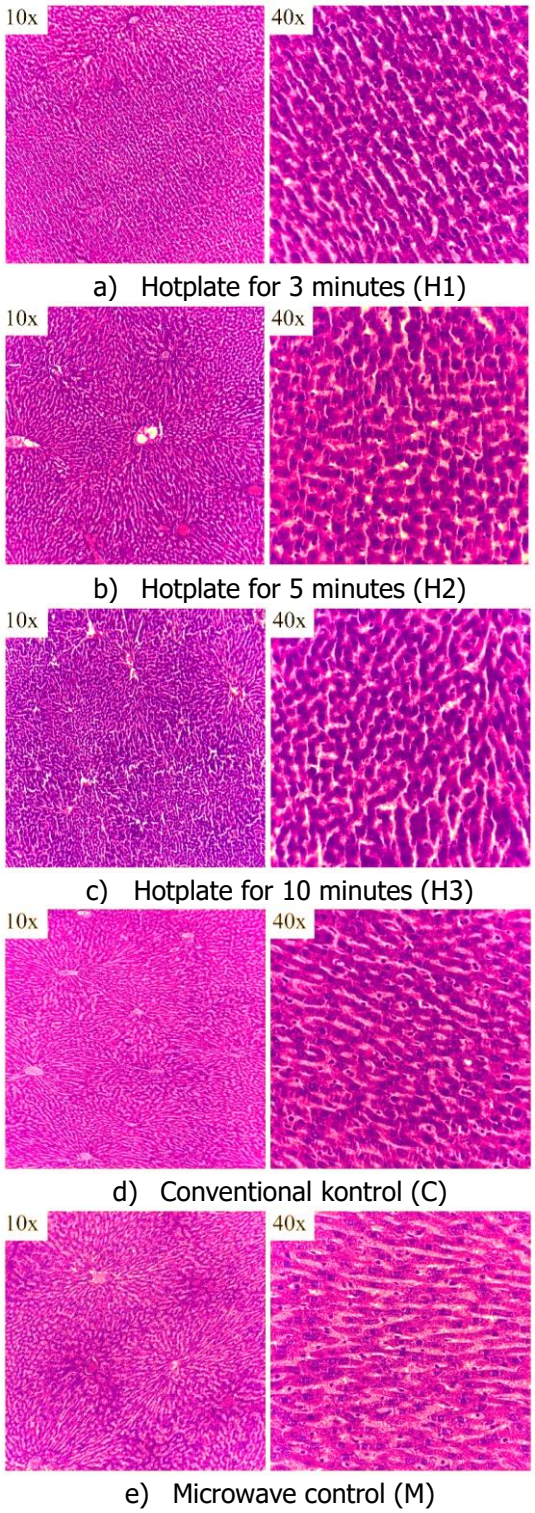

Figure 2. Illustrates the comparison of HE staining outcomes among groups a) hotplate 3 minutes (H1), b) hotplate 5 minutes (H2), and c) hotplate 10 minutes (H3), along with d) conventional control (K) and e) microwave (M). The results for group H1 demonstrated a score of 3, whereas 2 was the score for other groups. Furthermore, increased heating duration (H2 and H3) caused the tissues to be ripped.

Various factors, including temperature, duration, specimen size, the ratio of fixative solution to specimen, and pH influence the fixation process. Previous studies have shown that the use of heating can accelerate the fixation process in histological sample preparation, with the assistance of a hotplate set at 60°C (Burhannudin, Warida, & Puspita, 2023). Similar results were observed in recent research where using a hotplate at 80°C expedites the fixation process (figure 2). Figure 2 illustrates the scoring of the entire treatment and control groups. The results indicate a dominance of score 3 in the H1 group, while the other groups were dominated by score 2. The scoring results were analysed statistically to compare the treatment results with the control, as shown in Table 1.

Table 1. Comparison scoring of routine staining of heating treatment and control.

groups	N	Median (min – max)	p value
Conventional (C)	15	2 (1 – 3)^{d}	
Microwave (M)	15	2 (1 – 3)^{b}	
Hotplate 3 minutes (H1)	15	3 (2 – 3)^{c,d}	0,010
Hotplate 5 minutes (H2)	15	2 (1 – 3)^{a}	
Hotplate 10 minutes (H3)	15	1 (1 – 3)^{a,b,c}	

Data distribution test with Saphiro-Wilk, $p<0.05$. Data showed in median (minimum-maximum); Kruskal-Wallis tests significant, $p<0.05$. Mann-Whitney's test with the significant result is marked with a superscript notation; a,b,c different subscript value in the same column indicates significant difference ($p<0.05$).

The use of heating in the fixation process is directly proportional to the speed of penetration of the fixative solution into the tissue (Khristian and Inderiati, 2017). This was observed in group H1, where using a hotplate at 80°C for 3 minutes already showed good staining results (figure 2) and a score of 3 (table 1). High temperatures work by liquefying the fat in the tissue, thereby widening the pore spaces in the tissue. Furthermore, an increase in temperature can also hasten the chemical reaction rate between the fixative elements and the tissue, thus accelerating the fixation solution penetration into the tissue (Annisa and Sofyanita, 2023).

The timing selected for the fixation process greatly affects its success. Optimal timing produces superior staining outcomes, as evidenced by Group H1's results (Figure 2). Nonetheless, if the tissue is exposed to excessively high temperatures for a prolonged period, protein denaturation and the breakdown of phosphate and covalent bonds within the cells can damage the tissue (Hariyadi et al., 2018). This could be why the torn tissue scored 2 in Groups H2 and H3 (Table 1). This phenomenon denotes over-fixation, which leads to tissue hardening, contraction, and core and antigenic damage (Morgan, 2017). Laswati (2018) conveys similar information, suggesting that excessive heating can result in tissue becoming brittle and prone to cracking.

Proper maintenance of a stable heating temperature is crucial when using a hotplate. Environmental conditions significantly impact the hotplate's heating temperature (Sardjito and Yuningsih, 2020), and the instability of this temperature may affect the fixation process. Therefore, it is essential to strictly monitor the

hotplate's temperature stability to ensure the fixation process's success.

Heating treatment can be used in another fixative solution, such as Neutral Buffer Formalin 10% (NBF10%), which is considered the gold standard for fixative histology. Previous studies have shown the potential of heating treatment in fixation using NBF10% (Ariyadi & Suryono, 2017; Bauer et al., 2021). However, the heating was limited to 50°C due to toxicity if it exceeded this temperature. Finefix, an alcohol-based formalin-free fixative, is suitable for heating treatment to reduce the toxicity effects of heating. Although alcohol-based fixatives are not commonly used due to their tendency to cause water intake in tissue (Chafin et al., 2013), heating treatments can be used to minimize contact time.

CONCLUDING REMARK

The most effective option for assisting the fixation process in preparing histological specimens involves utilising a hotplate at 80°C for 3 minutes. Maintaining precise temperature control when operating a hotplate is of the utmost importance. Moreover, it is imperative to scrutinise the usefulness of lower temperatures for this purpose. Further investigations could focus on checking the efficiency of other heating apparatuses, including an oven.

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