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Application of Microwave Irradiation for the Phosphotungstic Acid Hematoxylin (PTAH) Stain Method

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The phosphotungstic acid hematoxylin (PTAH) stain method takes long time, usually over night, and the PTAH solution is recommended to be ripened for several months. In this study, the authors applied microwave irradiation (MWI) to shorten the time to complete the process. Among the steps of this method, the PTAH solution in which sections were immersed beforehand was exposed to the MWI, and then left for more than 10 minutes at room temperature. The best result was obtained when the MWI was intermittently applied at 100W for 14 minutes. The temperature of the PTAH solution reached about 60℃, and the skeletal muscle stained blue and the connective tissue, red. These results were compatible with staining by the conventional PTAH method. Owing to the MWI, the PTAH method could be completed around one hour. In addition, freshly prepared PTAH solution could be used for staining without ripening.

Key Words
Microwave irradiation,
Phosphotungstic acid hematoxylin stain,
Ripening,
Slow heating.

INTRODUCTION

Since Mallory published the phosphotungstic acid hematoxylin (PTAH) stain in 1897, this method have been applied for the staining of nerve fibers, striated muscle and fibrin (1-8). Several improvements of this method including the modification of the fixation, oxidation, mordanting and preparation of the staining solution were reported (3-8). Yet it still required a long time to complete this method. Recently, the authors applied MWI to the Grimerius silver impregnation (9), Kluver-barrera's method (10) and Groccot's methenamine silver staining (11), and reported that the application of MWI could produce results in staining as well as the conventional methods, while allowing us to shorten the time to complete these methods. In this study, the application of MWI to the PTAH method is described.

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MATERIALS AND METHODS

1. Materials.

Human psoas muscle was collected at autopsy and used for staining. The tissue was fixed coventionally in 10% formalin and embedded in paraffin. Sections were cut 4 μm thick.

2. Microwave irradiation (MWI).

A microwave oven, MR-A330 (Hitachi, Tokyo) was used. The level of the irradiation power was

changed from 100W to 500W. The irradiation was continuous at 500W, otherwise intermittent. The intervals of the intermittent irradiation were as following; 11 sec on and 6 sec off at 300W, 10 sec on and 7 sec off at 250W, 8 sec on and 9 sec off at 200W and 6 sec on and 20 sec off at 100W. Heat absorption water was not used during MWI.

3. Preparation of staining solution.

The PTAH solution was prepared according to a previous report (5), which consisted of hematoxylin 1 g, phosphotungstic acid 20 g, distilled water 1000 ml, and potassium permanganate 0.177g. This solution was used as stain soon after dissolution.

4. Conventional PTAH stain method.

The conventional method mainly followed Mallory's report (2) as written below.

1) Immerse sections into tap water after deparaffinization.
2) Place sections in 0.5% aqueous solution of potassium permanganate for 5 min.
3) Wash in running tap water.
4) Immerse in 1% aqueous solution of oxalic acid for 5 min.
5) Wash in running tap water.
6) Transfer to freshly prepared phosphotungstic acid hematoxylin solution overnight at room temperature.
7) Dehydrate rapidly in 100% alcohol, clear in xylene, and mount.

By this method, skeletal muscle was stained blue and connective tissue was weakly stained red (Fig.1)

5. Modification of the staining procedure by MWI.

Among the steps of the conventional method, the 6th step of the above conventional method was performed with MWI; Sections were immersed in 300 ml of the freshly prepared PTAH solution within a heat-resistant jar. After putting the lid on, the jar was placed on the center of the turn table in the microwave oven. Other steps were performed according to the conventional method.

1) Examination of the optimal temperature.

The MWI of sections in the PTAH solution was done for 1 min, 1.5 min, 2 min, 2.5 min, 3 min, at 500W, respectively. After each MWI, the solution was gently stirred in the jar by moving sections up and down in the solution and the jar was left at room temperature for more than 10 min. The temperature of around 60℃ was taken as standard by following the previous reports (5,7,8). In order to heat the solution evenly, the power level of MWI was set at 500W with continuous irradiation, which was thought to be more effective than intermittent.

2) Examination of MWI time and power level.

The MWI time sufficient to heat up the PTAH solution around 60℃ was set and applied for 14 min at 100W, 6 min at 200W, 4.5 min at 250W, 4 min at 300W, respectively. After each MWI, the solution was gently stirred in the jar and left at room temperature for more than 10 min as written above.

RESULTS

1. Determination of the optimal temperature. (Table 1, Fig.2)

The MWI was continuously performed at 500W. The temperature of the solution reached 43℃ by 1 min MWI, 52℃ by 1.5 min, 60℃ by 2 min, 66℃ by 2.5 min and 70℃ by 3 min, respectively. The section would not stain under 43℃. The higher the temperature of the solution, the more

Table 1. Determination of the optimal temperature for the PTAH staining

time	temperature (℃)		color pattern	
(min)	MWI*1	10min later*2	muscle	connective tissue
1	43	40	light pink	light pink
1.5	52	45	light blue(weaker)	light red
2	60	51	light blue ↓	red
2.5	66	53	light blue(deeper)	deep red
3	70	57	reddish blue	deep red

MWI was continuously performed at 500W.

*1. MWI: The temperature soon after the MWI.

*2. 10 min later: The temperature after leaving at room temperature for 10 min.

Table 2. Optimal conditions of MWI for the PTAH staining

power	time	temperature (℃)		color pattern	
(W)	(min)	MWI*1	10min later*2	muscle	connective tissue
100	14	62	52	blue	red
200	6	62	53	light blue	red
250	4.5	60	50	light blue	red
300	4	62	51	light blue	red

MWI was intermittently performed.

*1. MWI: The temperature soon after the MWI.

*2. 10 min later: The temperature after leaving at room temperature for 10 min.

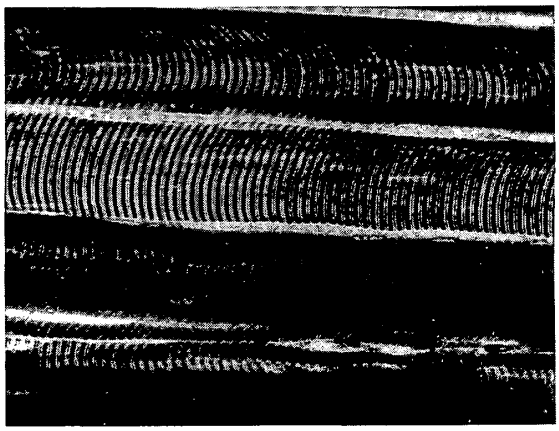

Figure 1. Conventional method. The striae of the skeletal muscle were well stained.

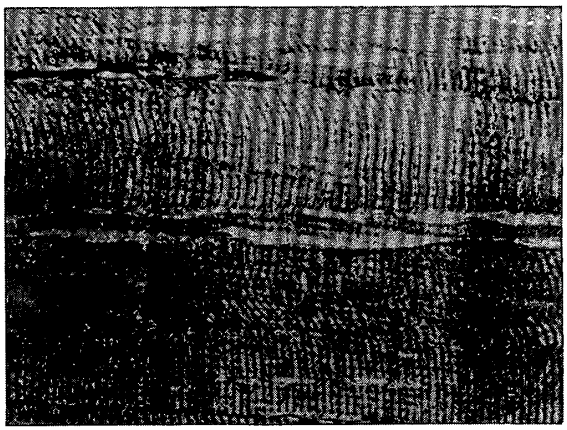

Figure 2. MWI for 2 min at 500W. The striae of the skeletal muscle were weakly stained.

deeply a section was stained. Although the staining color of the skeletal muscle was slightly different from that stained by conventional method in any case, the stained color at about 60℃ was rather close to that stained by conventional method. When the temperature of the PTAH solution was higher than 66℃, the sections tended to peel off, and higher than 70℃, the skeletal muscle stained reddish blue. Judging from these preliminary findings, the optimal temperature of the solution was found to be about 60℃. Accordingly, the following detailed examination was performed at 60℃.

2. Determination of optimal MWI time and power level. (Table2, Fig.3)

The MWI for 14 min at 100W heated the solution up to 62℃, 6 min at 200W to 62℃, 4.5 min at 250W to 60℃ and 4 min at 300W to 62℃, respectively. In the cases except for the MWI at 100W, the skeletal muscles stained light blue, and the connective tissue red. Only by the MWI at 100W did the skeletal muscle stain blue and the connective tissue red. This result was compatible with that by the conventional method. In addition, the connective tissue was stained red more deeply by the MWI than by the conventional method when the freshly prepared PTAH solution was used.

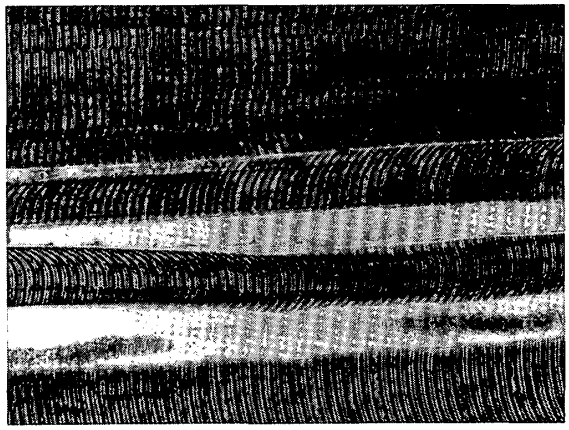

Figure 3. MWI for 14 min at 100W. The striae of the skeletal muscle were well stained, compatible with results by the conventional method.

DISCUSSION

Recently, the microwave oven has come to be widely used in many laboratories of histology not only for fixation but also for staining (9-13). However, it is still uncommon to apply MWI to the PTAH method because the PTAH solution has been thought to be unstable to heat (5,7). Thus this staining has usually been performed at room temperature, which required staining overnight to achieve proper results. In addition, the PTAH solution should be ripened for more than one month because the ripened solution produces much better results than the freshly prepared solution. With the latter, connective tissue is stained weakly, though skeletal muscle is properly stained blue. Several improvements of this method have been proposed to shorten the process (3-8). Among them, Puchtler (5) reported that the PTAH solution could be ripened quickly by using potassium permanganate, and the staining time could be shortened if the solution were warmed to about 60℃ in a paraffin oven.

In the present study, MWI was applied to the PTAH method to shorten completion time. Prior to the MWI, sections were placed in the PTAH solution at room temperature. According to puchtler's report (5), temperature of the staining solution might be critically important. Thus the optimal temperature for this staining was preliminarily examined under continuous MWI at 500W. At about 60℃ , sections were rather well stained, but the color of the skeletal muscle was slightly different from that after the conventional staining. Soon after the MWI, the skeletal muscle stained reddish blue, but if the sections were cooled for a while, the color turned blue.

To obtain better results, further experiments were performed at the various power levels. The best result was obtained when the sections were intermittently exposed to MWI at 100W for 14 min. This indicated that the sections should be slowly warmed in this staining method. In addition, the connective tissue stained red sufficiently, even if freshly prepared PTAH solution was used. This suggested that the staining solution might be ripened very quickly by MWI during the application.

With this improvement, the PTAH staining was completed in around one hour, using freshly prepared solution. The authors have also applied MWI to several other staining methods obtaining improved results in uniform and stable staining, suggesting that that MWI might be very useful for many other staining methods.

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