Microwave irradiation exposure enhancing melanin staining quality and act as time reducing factor in the Masson-Fontana method

Emad Eldin Abdullah Ahmeda, Magdi Mansour Salib, Asaad Ma. Babkerb, Osman Mohammed Elmahic, Lienda Bashier Eltyebd and Hisham Ali Waggiallah

aInstitute of Endemic Diseases, University of Khartoum, Khartoum, Sudan; bDepartment of Medical Laboratory Science, Taif University, Altaif, Saudia Arabia; cDepartment of Medical Laboratory Sciences, College of Health Sciences, Gulf Medical University, Ajman, UAE; dDepartment of Histopathology, College of Medical Laboratory Sciences, Karar University, Sudan; eDepartment of Medical Laboratory Science, Prince Sattam Bin Abdul Aziz, Alkhair, Saudia Arabia

ABSTRACT
This study was to evaluate the effects of microwave device in melanin pigment staining procedures and to compare between the classical Masson-Fontana staining technique at room temperature and the Microwave technique with Masson-Fontana stain. A total of 50 skin tissue samples were taken for processing. After processing, two histochemical staining techniques were carried out; the classical Masson-Fontana method at room temperature and the microwave device Masson-Fontana staining method at temperature 25°C. Both methods were compared. This study indicated that the use of a microwave device in staining of melanin pigment reduced the time significantly (P ≤ 0.001) from overnight (24 hours) to only 2 minutes with excellent staining quality as compared to the classical Masson-Fontana method (P ≤ 0.001). In conclusion, the use of a microwave device is a vital tool for staining of melanin with the Masson-Fontana method as it is a time reducer and staining quality enhancer.

1. Introduction

Microwaves are energy oscillations, not heat waves. The volume of microwave energy captured by the sample (or ‘load’) depends on several factors. These include the size of the load, its direction with respect to waves, and the dielectrics and thermal characteristics of the material (Kumar et al., 2014). Microwaves move directly to the solution. In a microwave device, a magnetron transforms normal electrical energy into microwaves, which are electromagnetic waves, much like radio and television waves, but which have shorter wavelengths and higher frequency. The microwaves join the oven via a wave guide. A fan-like apparatus called a field stirrer or a microwave stirrer allows dispersing microwaves equally in the oven chamber (Kumar et al., 2014). Based on the material, the microwave may be reflected, transmitted through, or absorbed. For instance, metal reflects microwaves. That is why the frames of microwave devices are usually made of metal, enclosing the microwave within the chamber. The see-through panel in the door of the microwave device includes a metal screen (Moravec & Mares, 2017).

The metal screen reflects the microwave and allows the inside of the cavity to be viewed while the visible light wavelengths will also travel through. The microwaves could not pass the screen, since the gaps in the screen are much smaller than the microwaves (Babu et al., 2011). So-called ‘transparent microwave’ materials, including some glass, pottery, paper, and many plastics, enable waves to move through. When used as containers, these materials do not absorb microwave energy; however, permit the solutions within them to absorb microwave energy (Kumar et al., 2014). The absorbed microwaves allow dipolar molecules (such as water) to move at a rate of 2.45 billion cycles per second. The reaction between rotating dipolar molecules, ions, and nonmoving molecules cause friction, which in turn generates heat that warms the solution. It is sort of like the way heat is produced when you rub your hands together. Microwaves warm solutions from outside, like the normal heating sources, but much quicker (Leong, 2004).

Heat is generally believed to be the main factor responsible for many of the impacts of microwaves on tissue staining. The quick movement of molecules with electromagnetic flux will also play a direct role in accelerating the diffusion of histological reagents. While heat or thermal energy can enhance molecular kinetics and increase molecular interactions, the accelerated movement of molecules directly caused by the oscillating electromagnetic field can lead to increased colliding particles and, in turn, accelerate chemical reactions (Singla et al., 2017).

Melanin is produced by a cell called melanocytes, through a process known as melanization. A variety
of factors affect melanization, genetics usually account for the variation in the number and kinds of melanosomes, rather than the number of melanocytes. Also hormones can affect the melanization, including the melanin-stimulating hormone (MSH) synthesized within the adenohypophysis, estrogen, and progesterone can also increase melanization during pregnancy. Age and a variety of pathologic conditions are also affecting the degree of melanization (Carriel et al., 2011). Exposure to ultraviolet light increases melanization primarily through two mechanisms; the first involves the increased synthetic and transfer activities of melanocytes, the second involves the multiplication of melanocytes by mitotic activity. The degree to which these events occur depends upon the genetic characteristics of the individual and in addition to the amount and duration of exposure (Bancroft & Gamble, 2008).

Tissue staining is dependent on two factors: the spread of stain to the cells and the attachment of stain to the substrate (Carriel et al., 2011). There are two simple techniques for staining in a microwave device; the first procedure requires staining carried out in a plastic coupling jar or a staining rack, with a temperature probe placed into the fluid in the jar at a very accurate temperature. The second procedure is to fill the slide with a few amounts of staining solution. The second procedure is to fill the slide with a few amounts of staining solution. Putting the slide on a plate form and microwaving for 20–30 seconds (Carriel et al., 2011).

Microwave penetration has many applications in the histopathology laboratory, such as improving the penetration of binding reagents to tissues and speeding the chemical reaction whereby fixatives cross-link to tissue proteins. The most common histological fixative, formalin, is a solution containing methylene glycol and formaldehyde (Katoh, 2016). In the literature, little work has been done regarding the use of a microwave device in melanin staining. To our knowledge, this is the first study on the application of microwave device in Masson-Fontana melanin staining in routinely processed paraffin-embedded specimens performed. The main objective of this study to evaluate microwave irradiation exposure in enhancing melanin staining quality and time reducing factor in the Masson-Fontana method.

2. Materials and methods

2.1. Study design

The study is a retrospective study aimed to evaluate the use of microwave oven technology in the Masson-Fontana staining method for the demonstration of melanin pigment.

2.2. Study population

A total of 50 histological skin specimens were taken from patients aging between 45 and 75 years. Twenty samples (40%) of patient belonged to age group 45–55 years, while 18 samples (36%) between 56 and 65 years, and last group there were 12 (24%) belonged to 66–75 years. Samples were collected from the records of the histopathology and cytology department in Soba University Hospital, National Health laboratory (NHL) and Ibn Sina Hospital. The selection criteria of samples in the study group were based on age and sex.

2.3. Ethical consideration

This study was approved by the ethical committees at the Faculty of Laboratory Sciences, Sudan University of Science and Technology. Skin specimens were taken with written consent from the patient after a good explanation of study objectives and aims.

2.4. Sample preparation processing

Skin specimens were fixed in 10% formalin and the Processing of specimens, which includes dehydration, clearing, and wax impregnation was made using an automated tissue processing machine. Thin sections of 4 microns in thickness were cut using Rotary microtome; sections were then taken onto clean, dry, labeled slides, and fixed in an oven at 60°C. Two sections from each block were obtained (Bancroft & Gamble, 2008).

2.5. Staining procedures

The Skin sectioned samples were dewaxed by two changes of xylene for 5 minutes for each one, then rehydrated in descending concentrations of alcohol ranging from 100%, 90%, and 70% for 3 minutes in each step, and finally to distilled water to remove the alcohol (Bancroft & Gamble, 2008).

2.5.1. Classical Masson-Fontana staining method

Skin sections with 4 microns in thickness were transferred to Masson-Fontana staining solution and stored in a covered coupling jar in the dark overnight at room temperature. Then the sections were well washed in distilled water and fixed in 5% sodium thiosulfate ‘hypo’ for 5 minutes, washed well in water, then the nuclei were stained by 1% aqueous neutral red solution for 3 minutes, and then washed quickly in distilled water. Finally, the slides were then dehydrated in ascending concentrations of alcohol ranging from 70%, through 90%, then to absolute alcohol for 2 minutes in each step, then cleared by two changes of xylene for 2 minutes each, and mounted with Distrene plasticizer Xylene (DPX) (Bancroft & Gamble, 2008).
2.5.2. Microwave device staining procedures
Skin sections were deparaffinized and hydrated similar to steps of the classical Masson-Fontana method. They were placed in working silver solution in an uncover Coupling jar in a microwave oven (Toshiba EC042ASC-B5 Countertop) at power 25 for 2 minutes, and the slides were checked microscopically for the adequate intensity of the stain. Sections were rinsed in distilled water. Then the sections were treated with 5% sodium thiosulfate (hypo) for 5 minutes, and then were rinsed in distilled water. Finally, sections were stained with 0.5% neutral red for 5 minutes, then washed in tap water, dehydrated, cleared, and mounted with DPX (Charles & Churukain, 2009).

2.6. Evaluation of slides
Sections were evaluated with investigators and the results were confirmed by a histopathologist who has more than 10 years’ experience in histopathology, the quality of staining procedures was also assessed and evaluated by the rule of thumb. According to the quality of the section, each slide was given a mark in the following order; 10–8 excellent and section quality, 8–6 very good quality section, 6–4 good quality section, and less than 4 is bad quality section.

2.7. Data analysis
Data were analyzed using SPSS programs to calculate the frequencies, student , and Chi-square.

3. Results
According to the data shown in Table 1, in the first age group (45–55), males constituted 32%, while females make up 8%, and in the second age group, we found the percentage of males is also the highest 22%, and females form 14%, while the third age group matters differently with males forming the lowest 4% While females make up 20%. When comparing the quality of staining between microwave and classical methods there was a highly significant (P ≤ 0.001) result because microwave boosting the quality of stain by reducing the time so as prevent the precipitation of stain in the tissue (Table 2).

Table 3 displays the correlation between the times was taken by microwave and classical Fontana stain at room temperature (25°C) and quality of staining, which resulted in a highly significant correlation (P ≤ 0.001) that means microwave irradiation has a potent impact on the staining process in both sides time and quality.

In Figure 1 Masson-Fontana stain with the classical method (×40) appeared slightly pale in comparison with Figure 2 in which microwave increases the intensity and resolution of the dye.

4. Discussion
Microwave irradiation throughout tissue processing greatly decreases the time needed for fixation, decalciﬁcation, staining with chemical reagents, and incubation with antibodies. Microwave irradiation has progressively been used in histological preparation. Microwave irradiation causes fast oscillation of water molecules (2.45 GHz) and thereby raises the temperature of the tissue. Microwave devices irradiate tissues both quickly and systematically, and microwave irradiation procedures vary depending on the particular microwave devices used. Microwave irradiation is usually used for special staining purposes (Temel et al., 2005). Microwave irradiation was also used for fixation and subsequent staining procedures, like enzyme-based staining and immunofluorescence staining (Avci et al., 2006).

Table 1. Shows distribution of demographic data.

| Age   | Number of specimens | Gender | Total percentage % |
|-------|---------------------|--------|---------------------|
| 45–55 | 16                  | M      | 32%                 |
|       |                     | F      | 8%                  |
| 56–65 | 11                  | M      | 22%                 |
|       |                     | F      | 14%                 |
| 66–75 | 2                   | M      | 4%                  |
|       |                     | F      | 20%                 |
| Total | 50                  | -      | 100%                |

Table 2. Comparison of Masson-Fontana results in classical and microwave device method.

| Parameter | Staining enhanced by microwave | Classical staining method | Total | P value |
|-----------|--------------------------------|---------------------------|-------|---------|
| Bad (-)   | 12 (24%)                       | 12 (24%)                  | 24 (48%) | 0.001** |
| Good (+)  | 2 (4%)                         | 17 (34%)                  | 19 (38%) |         |
| Very good (++) | 13 (26%)             | 12 (24%)                  | 25 (50%) |         |
| Excellent (++++) | 23 (46%)       | 9 (18%)                   | 32 (64%) |         |
| Total     | 50 (100%)                      | 50 (100%)                 | 100 (100%) |         |

**P ≤ 0.001

Table 3. Correlation between quality of staining and the time in the microwave and classical methods at room temperature (25°C).

| Quality of stain | Time in minutes/ No. of samples (n) | Microwave staining | Classical staining | P value |
|------------------|-------------------------------------|--------------------|--------------------|---------|
| Bad (-)          | Time                                | 0.25               | 180                | 0.000** |
|                  | n                                   | 3                  | 10                 |         |
|                  | Time                                | 0.50               | 360                | 0.000** |
|                  | n                                   | 9                  | 2                  |         |
| Good (+)         | Time                                | 0.75               | 540                | 0.000** |
|                  | n                                   | 1                  | 10                 |         |
|                  | Time                                | 1.25               | 900                | 0.000** |
|                  | n                                   | 9                  | 2                  |         |
| Very good (++)   | Time                                | 1.50               | 1080               | 0.000** |
|                  | n                                   | 4                  | 11                 |         |
| Excellent (++++)  | Time                                | 1.75               | 1260               | 0.000** |
|                  | n                                   | 9                  | 4                  |         |
|                  | Time                                | 2.00               | 1440               | 0.000** |
|                  | n                                   | 14                 | 5                  |         |

** P ≤ 0.001
In this result there was a significant outcome when comparing the degree of staining quality between classical and microwave methods. This means that microwave might boost the quality of staining besides shortening the time. Currently, heat is considered to be the key factor responsible for several of the impacts of tissue staining on microwaves. The quick acceleration of electromagnetic-flowing molecules can itself play a direct role in speeding up the diffusion of histological reagents.

Figure 1. Photomicrograph of Masson-Fontana stain with classical method at 25°C (×40) excellent grade (+++), the stain appeared slightly pale color (orange arrow).

Figure 2. Photomicrograph of Masson-Fontana stain with microwave method (undefined×undefined40) excellent grade (+++), the stain shows intensive stain color (yellow arrow).
Although heat or thermal energy induces molecular kinetics and speeds up molecular reactions, the accelerated motion of molecules immediately triggered by the oscillating electromagnetic field can lead to accelerated molecular collisions which, in turn, can speed up chemical reactions (Singla et al., 2017).

The present study showed that the use of a microwave device in the Masson-Fontana staining technique greatly reduces the time required to perform the method without compromising the quality of staining. The study results also showed that the staining time was reduced from overnight to only 2 minutes, which is the significant result with preserving the staining quality.

These results indicate that the staining obtained by using the microwave device technique was slightly better than the classical technique, because the microwave accelerates the diffusion and binding of the stain. This was observed in the short time that had been consumed and in the intensity of staining. These findings agree with (Abreu et al., 2012; Bond & Kinnamon, 2013; Shi et al., 2013), who believed that; staining of tissue depends on two factors. The first is the diffusion of the dye into the cells, and the second is the attachment of the stain to the substrate.

These findings have been long established by Mayer’s who assumed that; The staining techniques that usually take minutes can be achieved in a microwave device in seconds; those that take hours could be done in minutes and those that take days or even weeks can be accomplished in a matter of hours utilizing microwave methods (Gu et al., 2016).

Application of the microwave in some routine histopathological staining procedures was done by using a domestic microwave device to accelerate the following staining methods; Hematoxylin-Eosin stain on frozen sections, and many other stains on buffered formalin-fixed sections or cytological smears. It has been found that the microwave-assisted staining techniques are equivalent to or even superior to those of the standard techniques. Staining times can be up to 70% faster than classical staining methods. These trials and observations enhanced the researchers to improve the microwave device technique in other staining procedures (Shruthi et al., 2013).

It is recommended that further studies are needed by increasing sample size to improve the accuracy and precision of the microwave oven technique for melanin pigment staining.

5. Conclusion

We conclude that using microwave device is rapid, sensitive, and reliable in the Masson-Fontana staining technique and at the same time, compatible with classical methods. Therefore, staining required time was reduced from 24 hours to 2 minutes, besides that, a high quality of stain was obtained when compared to the classical Masson-Fontana staining.

Disclosure statement

Authors declare that they have no conflict of interest.

References

Abreu, C. C., Nakayama, P. A., Nogueira, C. I., Mesquita, L. P., Lopes, P. F. R., Varaschin, M. S., Seixas, J. D. N., Ferreira, E., & Bezerra, P. S. (2012). Domestic microwave processing for rapid immunohistochemical diagnosis of bovine rabies. *Histology and Histochemistry*, 27(9), 1227–1230. https://doi.org/10.14670/hh-27.1227

Avci, B., Kahveci, N., Kahveci, Z., & Sirmali, S. A. (2006). Using microwave irradiation in Marchí’s method for demonstrating degenerated myelin. *Biotechnic & Histochemistry*, 81, (2–3), 63-69. https://doi.org/10.1080/105209600783044

Babu, T. M., Malathi, N., & Magesh, K. T. (2011). A comparative study on microwave and routine tissue processing. *Indian Journal of Dental Research*, 22(1), 50–55. https://doi.org/10.4103/0970-9290.79975

Bancroft, J. D., & Gamble, M. (2008). *Theory and practice of histological techniques* (6th ed.). Churchill Livingstone.

Bond, A., & Kinnamon, J. K. (2013). Microwave processing of gustatory tissues for immunohistochemistry. *Journal of Neuroscience Methods*, 215(1), 132–138. https://doi.org/10.1016/j.jneumeth.2013.02.014

Carriel, V. S., Aneiros-Fernandez, J., Arias-Santiago, S., Garzón, I. J., Alaminos, M., & Campos, A. A. (2011). Novel histochemical method for a simultaneous staining of melanin and collagen fibers. *Journal of Histochemistry & Cytochemistry*, 59(3), 270–277. https://doi.org/10.1369/002155410398001

Charles, J., & Churukain, B. (2009). *Method of histochemical stains and diagnostic application* (2nd ed., pp. 122–126). Corey Walker & Yuehui Mao.

Gu, L., Cong, J., Zhang, J., Tian, Y., & Zhai, X. (2016). A microwave antigen retrieval method using two heating steps for enhanced immunostaining on aldehyde-fixed paraffin-embedded tissue sections. *Histochemistry and Cell Biology*, 145(6), 675–680. https://doi.org/10.1007/s00418-016-1426-7

Katoh, K. (2016). Microwave-assisted tissue preparation for rapid fixation, decalcification, antigen retrieval, cryosectioning, and immunostaining. *International Journal of Cell Biology*, 2016, 7076910. https://doi.org/10.1155/2016/7076910

Kumar, H., Kalkal, P., Buch, A., Chandanwale, S. S., Bamanikar, S., & Jain, A. (2014). Role of microwaves in rapid processing of tissue for histopathology. *Medical Journal of Dr D Y Patil Vidyapeeth*, 7(4), 458–462. https://www.mjdrdypu.org/article.asp?issn=09752870;year=2014;volume=7;issue=4;spage=458;epage=462aulast=Kumar

Leong, A. S. (2004). Microwaves and turnaround times in histoprocessing: Is this a new era in histotechnology? *American Journal of Clinical Pathology*, 121(4), 460–462. https://doi.org/10.1309/plq523dennh8r00q

Moravec, J., & Mares, J. (2017). A simple, time-saving, microwave-assisted periodic acid-Schiff’s staining of glycoproteins on 1D electrophoretic gels. *Electrophoresis*, 38(24), 3100–3103. https://doi.org/10.1002/elps.201700189
Shi, S., Cheng, Q., Zhang, P., Wang, N., Zheng, Y., Bai, X., & Chen, X. (2013). Immunofluorescence with dual microwave retrieval of paraffin-embedded sections in the assessment of human renal biopsy specimens. American Journal of Clinical Pathology, 139(1), 71–78. https://doi.org/10.1309/AJCPRZG8EXN7BAID

Shruthi, B. S., Vinodhkumar, P., Kashyap, B., & Reddy, P. S. (2013). Use of microwave in diagnostic pathology. Journal of Cancer Research and Therapeutics, 9(3), 351–355. https://doi.org/10.4103/0973-1482.119301

Singla, K., Sandhu, S. V., Pal, R. G., Bansal, H., Bhullar, R. K., & Preetinder Kaur, P. (2017). Comparative evaluation of different histoprocessing methods. International Journal of Health Sciences, 11(2), 28–34. PMID:28539860; PMCID:PMC5426407.

Temel, S. G., Noyan, S., Cavusoglu, I., & Kahveci, Z. (2005). A simple and rapid microwave-assisted hematoxylin and eosin staining method using 1,1,1 trichloroethane as a dewaxing and a clearing agent. Biotechnic and Histochemistry, 80(3–4), 123–132. https://doi.org/10.1080/10520290500303190