Comparison of Microwave and Convective Heating in Rapid Specimen Preparation Techniques for Electron Microscopy

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Abstract

Chemical fixatives preserve tissue for electron microscopy. Microwave heating reduces fixation time from hours to seconds. Comparison of microwave heating (915 MHz and 2.45 GHz) with convective heating suggests that acceleration of fixation is not unique to microwave exposure. Other heating modes appear suitable and may have advantages in cost and convenience.

Introduction

The use of microwave heating (MWH) to enhance glutaraldehyde fixation of specimens prepared for electron microscopy is well established [1]-[5]. In addition, previous studies have demonstrated the utility of MWH in related light and electron microscopic techniques such as antigen preservation [1], [12], [6]; enzyme activity [7]; and histochemical staining [8], [9].

A primary benefit of the use of MWH in all these techniques is the significant reduction in time needed to complete the fixation process. For example, typical glutaraldehyde fixation of mammalian cells can be reduced from 1-2 hours under conventional fixation conditions (room temperature or 4°C) to <10 seconds at 40-50°C [2].

There have been several mechanisms proposed for the increased speed of glutaraldehyde fixation when MWH is used. Microwave energy might participate directly in the fixation process. Dipole rotation caused by the oscillating electric field could create favorable molecular orientations resulting in faster protein cross-linking [4]. Alternatively, heat generated by resistive losses and dipole rotation may increase chemical reaction and diffusion rates thus reducing overall fixation times [4], [3].

The present study explores the role of MWH as a unique agent for reducing fixation times. We demonstrate that equally good fixation can be obtained by raising the temperature of specimens in glutaraldehyde to 40-45°C in a short time interval (10-60 s) via MWH or convective heating (CH). Our results suggest that heat is the primary cause of the reduced fixation times associated with MWH and that the heating mode is secondary.

Materials and Methods

Specimens.

Liver tissue was removed from euthanized female white mice weighing approximately 40g. Tissue was immersed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.05 M $CaCl_2$ and 5.0% sucrose and cut into $1mm^3$ blocks. For each heat treatment or control approximately 20 tissue blocks were processed in a glass vial, 4.5 cm (height) x 1.4 cm (diameter), containing 2.0 ml of solution as specified. The glutaraldehyde fixative solution consisted of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.05 M $CaCl_2$ and 5.0% sucrose.

Microwave Heating (MWH).

Specimens were heated in both a conventional home microwave oven and a specially designed resonant cavity.

Conventional home microwave. This procedure follows the method of Loggin and Dvorak [2]. Specimens were heated in the glutaraldehyde solution for 10-15 seconds in a microwave oven (Sears Model #88627, 650 W, 2.45 GHz). The initial solution temperature was room temperature (20-22°C) and final temperature was 40-43°C. Temperatures were measured immediately before and following exposure using a laboratory thermometer. The oven magnetron was warmed up for 2 minutes prior to specimen exposure and a 300 ml water load was placed in the oven during heating. Following heating, the fixative solution was immediately removed and replaced with room temperature buffer.

Microwave transmitter and resonant cavity. Specimens were also heated using a modified medical sterilizer unit (Microwave Medical Systems, Littlefield,

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MA). This system was used to avoid problems associated with microwave ovens - uneven and unpredictable field patterns, variation in power levels, and inefficient power transfer to specimens.

The sterilizer unit consisted of a solid state transmitter (25 W, 915 MHz) and dielectric-loaded halfwave resonant cavity. A hole bored in the dielectric positioned the specimen vial at the center of the cavity. A stub tuner was used to match the impedance of the cavity and specimen to the transmitter resulting in 75-80% power transfer. Although the transmitter has only a fraction of the output power of a microwave oven, the greater efficiency of coupling resulted in comparable exposure times.

Specimens were heated for 25-30 seconds from room temperature and to 41-42°C. Temperatures were measured immediately before and after heating with a laboratory thermometer. Following heating, the fixative solution was immediately removed and replaced with room temperature buffer.

Convective heating, (CH). Vials containing specimens in glutaraldehyde solution were placed in a gyrotary water bath shaker for 40-60 seconds and heated from room temperature to 40-43°C. Temperatures were measured immediately before and after heating with a laboratory thermometer. Following heating, the fixative solution was immediately removed and replaced with room temperature buffer.

Controls.

Conventional (long duration) fixation. Specimens were left in room temperature glutaraldehyde solution for 1.5 hours. The glutaraldehyde solution was subsequently replaced by room temperature buffer.

Short duration unheated fixation. Specimens were left in room temperature glutaraldehyde for 40 seconds. The glutaraldehyde was subsequently replaced by room temperature buffer.

Convective heating, no fixative. Vials containing specimens in buffer only were heated in a gyrotary water bath shaker for 40-60 seconds from room temperature to 40-42°C. Warm buffer was immediately replaced with room temperature buffer.

Microwave heating (MWH), no fixative. Vials containing specimens in buffer were heated using the 915 MHz transmitter for 30 seconds from room temperature to 41°C. Warm buffer was immediately replaced with room temperature buffer.

Further processing for electron microscopy. After the initial experimental and control steps, specimens were washed in three 10 minute buffer rinses, osmicated in 1.0% OsO_4 in buffer for 1 hour and further washed in buffer as previously described. Tissue was dehydrated in a graded series of ethanol and embedded in Epon-Araldite. All steps were at room temperature. Sections were cut on a RMC MT-6000 ultramicrotome, post-stained with uranyl acetate and lead citrate and viewed on a JEOL 100B6 electron microscope.

Results

Microwave Heating (915 MHz, 2.45GHz), Convective Heating, Conventional Fixation, all with Glutaraldehyde.

Liver tissue fixed by these four protocols showed comparable ultrastructural details characteristic of good fixation [11]: Rough ER consisted of long profiles of flattened cisternae with attached ribosomes and dense matrix. Free ribosomes were plentiful. Mitochondria had clearly discernable double membranes with inner cristae and were not swollen or shrunken. Lipids were round in shape and uniformly dense. Ground substance and chromatin were dense with no gaps in their matrix. The nuclear envelopes appeared as two distinct membranes with the outer one studded with ribosomes. Nuclear shape was generally round or oval. Glycogen appeared as dark clumps (Figures 1-3).

Short Duration Fixation; Convective Heating and Microwave Heating Without Fixative.

All of these protocols produced signs of poor fixation [11]. Tissue exposed to short fixation times at room temperature had ground substance of nonuniform matrix density and areas of extraction. Mitochondrial cristae were indistinct and the outer membrane sometimes distorted. Nuclei had irregular shapes (Figure 4). Specimens in buffer (no fixative) subjected to MWH or CH contained mitochondria with indistinct cristae and non-uniform matrix. Ground substance was extracted, especially in the MWH specimen. Rough ER was swollen and lacked dense contents in tissue with both MWH and CH and there was a high number of small vesicles present (Figure 5). There was a reduced number of free ribosomes in all three preparations.

Discussion

Previous studies of microwave exposure of specimens in glutaraldehyde revealed that MWH can reduce time needed for good fixation [1]-[4]. The present study using mouse liver confirms this phenomenon. Tissue in glutaraldehyde subjected to MWH for 25-30 seconds and heated to a final

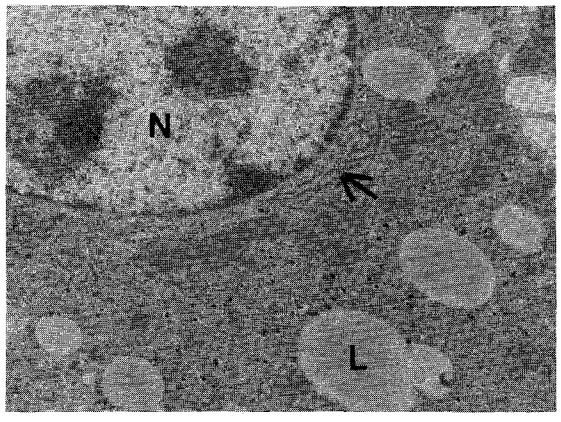

Figure 1. Microwave heating (915 MHz) and glutaraldehyde fixation. Tissue heated at 2.45 GHz in glutaraldehyde was comparable. N,nucleus; L,lipid; arrow,RER. X10,000

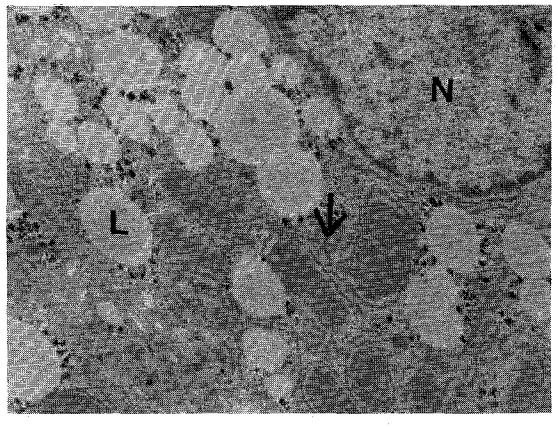

Figure 2. Convective heating and glutaraldehyde fixation. N,nucleus; L,lipid; arrow,RER. X10,000

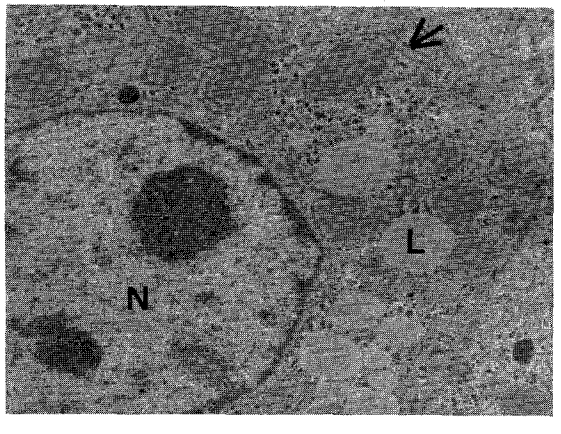

Figure 3. Conventional (long duration) glutaraldehyde fixation. N,nucleus; L,lipid; arrow,RER. X10,000

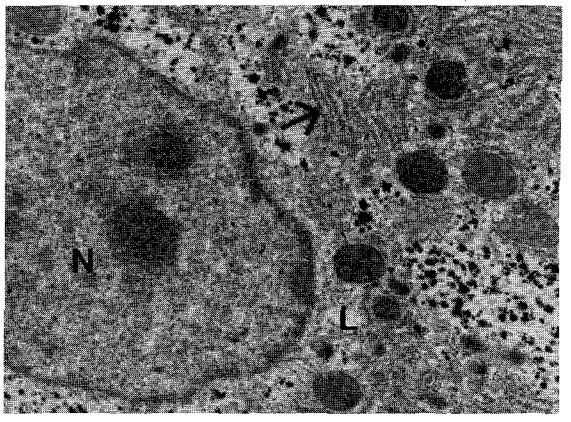

Figure 4. Short duration glutaraldehyde fixation. N,nucleus; L,lipid; arrow,RER. X10,000

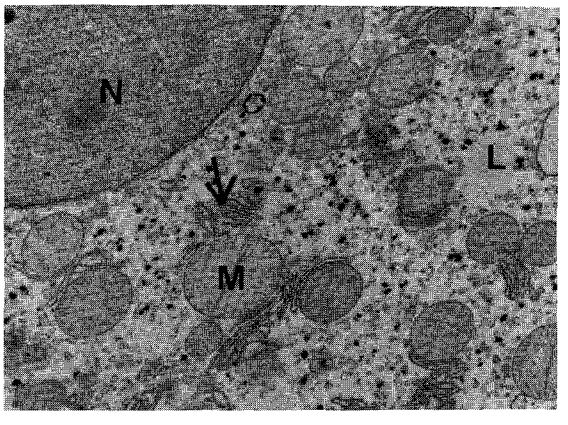

Figure 5. Microwave heating (915 MHz) without glutaraldehyde. Note vesicles and extracted ground substance. Convective heating without glutaraldehyde was comparable. N,nucleus; M,mitochondria; L,lipid; arrow,RER. X10,000

temperature of 41-43°C had ultrastructure comparable to tissue fixed in glutaraldehyde for 1.5 hours at room temperature. There was no apparent difference in fixation quality using either a microwave oven (2.45 GHz) or a resonant cavity (915 MHz).

Both thermal and non-thermal mechanisms have been proposed to explain the enhancement of fixation due to MWH. If heat is the primary agent, other delivery modes besides MWH should result in similar fixation effects. In this study, when convective heating (CH) was used to raise the fixative and specimens to the same temperatures (40-43°) at a rate similar to that obtained with MWH, comparable results were achieved. Fixation in glutaraldehyde with both CH and MWH protocols was

comparable in quality to tissue fixed using conventional long duration protocols (1-2 hours) at room temperature. These results confirm that MWH is not a unique mechanism for reducing fixation times.

Controls eliminated the possibility that 1) MWH or CH by itself (no fixative) or conversely 2) a short immersion in room temperature fixative (no MWH or CH) could produce good fixation. Specimens from the three controls showed some or all of the characteristic signs of poor fixation. These results are consistent with a thermal mechanism, common to both MWH and CH, in combination with glutaraldehyde imparting good fixation in a short time.

Previous studies have also concluded that neither glutaraldehyde used at room temperature for short times (1-30 sec) nor MW exposure of specimens in buffer without glutaraldehyde result in satisfactory fixation [2],[12]. Contrary to our results, Login and Dvorak[12] found that CH of fixative and specimens did not produce fixation equal in quality to MWH or long fixation times at room temperature. We attribute this difference to the specimen sizes used in the two studies. Login and Dvorak [12] used larger tissue blocks - 2 mm^3 versus 1 mm^3 in this study. When specimens are heated convectively, heat must diffuse from the tissue surface to its interior. This process requires more time for larger samples and may not be rapid enough for satisfactory fixation.

Login and Dvorak also observed variable fixation within tissue specimens in MWH experiments when larger block sizes were used. Unlike CH, microwave energy is deposited throughout the volume of the specimens and fixative solution. However non-uniformities occur due to variations in permittivity, penetration depth, and non-uniform field patterns in microwave ovens. These variations are more pronounced as specimen sizes increase. Variable fixation within blocks after MWH has been noted by others [3] and by ourselves in blocks larger than 1 mm^3 exposed to either MWH or CH.

Heat could influence the time needed for chemical fixation in several ways. First, it is known that heat by itself can preserve protein constituents by limited denaturation and creation of disulfide bonds, leading to decreased solubility [1]. This phenomenon was observed in this study and in another [2] when MWH and CH without fixatives present resulted in some limited cellular preservation. Second, chemical reaction rates should increase with temperature as the increased molecular motion and rotation caused by added thermal energy promotes collisions between fixative and cellular molecules. Third, temperatures in the range obtained with MWH and CH (40-60°C) are sufficient to induce monomeric forms of glutaraldehyde from polymeric forms present at room temperature. Glutaraldehyde monomers have more rapid diffusion rates through tissue and cross-link proteins effectively resulting in increased quality of fixation [13]. Significantly, heating glutaraldehyde by either MWH or CH produces comparable increases in the monomer [13]. Finally, the deleterious effects of too high a temperature (total protein denaturation, loss of antigenicity, collapse of structure) are well known [14]. The positive effects of MWH fixation are greatest when the temperature range achieved is 40-60°C [2], [10]. These temperatures do not cause excessive protein denaturation [14] and agree with the range of optimal temperatures for tissue fixation for light microscopy [1].

This study has shown that glutaraldehyde fixation time for liver tissue can be significantly reduced with either MWH or CH. We conclude that heat is the primary agent in reducing fixation time and the mode of delivery is secondary.

Acknowledgments

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References

[1] Hopwood,D., et al Histochem. J.16:1171-1191,1984.
[2] Login, G.R., Dvorak, A.M. Amer. J.Path. 170:230-243, 1985.
[3] Wild, P., et al Histochemistry 91:213-220, 1989.
[4] Leong, A., Gove, D.W. 1990 EMSA Bulletin 20:61-65, 1990.
[5] Login, G.R., et al J. Histo. Cytochem. 38:755-762, 1990.
[6] Hjerpe, A., et al Histochem. J. 20:388-396, 1988.
[7] Stavinoha,W. in Microwave Fixation of Labile Metabolites, C. Blank et al, eds. 1-13,1983.
[8] Marani,E., et al, Histochem. J. 19:658-664, 1987.
[9] Boon, M.E., et al Amer. J. Clinical Path. 91:137-143, 1989.
[10] Wild, P. Microwave Newsletter 4:67-70, 1991.
[11] Hayat, M.A. Fixation for Electron Microscopy, p. 379, 1981
[12] Login,G.R., Dvorak,A.M. Histochem. J. 20:373-387, 1988.
[13] Ruijgrok,J.M., et al Histochem. J. 22:389-393, 1990.
[14] Joly,M. A Physico Chemical Approach to Protein Denaturation 1965.