APPLICATION OF MICROWAVE IRRADIATION IN SURGICAL PATHOLOGY; IMPROVEMENT OF MICROSCOPIC-IMAGE OF CRYOSTAT SECTIONS AND EXPLORATION IN RAPID METALLIC STAININGS

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Application of microwave irradiation has been explored both in fresh and fixed frozen sections and in metallic stainings. The results showed a combination of irradiation alone followed by further irradiation immersed in any reagent containing about 50% alcohol made better microscopic-image quality in fresh frozen sections, but lesions of lymphoid tissues and lipid-rich brain tissues required further technical consideration for making better nuclear details and reducing spongy appearance. Fixed frozen sections had disappointing results with insufficient fixation and various degrees of freezing and mechanical damage. Standardization of fixed frozen sections may be a future problem. Metallic staining was time-saving and produced excellent results. A microwave oven can be a useful tool in surgical pathology.

Microwave irradiation accelerates fixation (3) and staining reactions (5, 6, 13-16). More rapid and accurate routine diagnostic surgical pathology can be expected with microwave irradiation, but with a domestic oven the results seem to be as yet unpredictable (4, 16). To obtain reproducible results we need further exploration. In this article we showed microwave stimulated cryostat procedures in fresh and in fixed frozen sections. Rapid metallic stainings are also explored.

MATERIALS AND METHODS

Spleens and kidneys of adult rats (Wistar, 12 week-old) and various surgical specimens for frozen section diagnosis were studied. We used a domestic oven of 2.45 GHz (Sharp R-5800) at the power level of 500 W and 200 W. Temperature was measured by digital thermometer (Yokogawa TX-560 with a TX-12D probe). Procedures are as follows;

1) Fresh frozen sections

For a handy and sure covering of sections with reagents, we used coplin jars in microwave irradiation. Sections of 4 to 5 micron in thickness were made in a cryostat (Bright microtome 5030) after CO₂ jet freezing of fresh materials embedded in O.C.T. compound. The sections are mounted on warm slides. Then the slides were put in a coplin jar with or without fixatives and set on a selected place of polystyrene platform.

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of 2 cm height in an oven. An ice-water bath (300/500 ml) was placed in the rear corner of the oven. We planned three different irradiation protocols.

(i) Irradiation alone in an empty coplin jar.
(ii) Irradiation immersed in various fixatives in a coplin jar.
(iii) Irradiation followed by fixation in various reagents with or without further microwave stimulation.

Irradiation times were 10, 15, 30 sec at the power level of 500 W and 15, 30, 60 sec at 200 W. Fixatives were ethanol in 100% and 50%, pure acetone, mixture of pure acetone and 100% ethanol in equal quantity, 10% buffered formalin, 4% paraformaldehyde in 0.1 M phosphate buffer, mixture of 4% paraformaldehyde solution and pure ethanol in equal quantity, Leiden fixative (3) (0.5: 100% ethanol, 0.43: distilled water, 0.07: polyethylene glycol-300), and physiological saline (17). Volume of the reagents was 40 ml (The final temperature of the solutions by 15 sec irradiation at 500 W were lower than and up to 40°C.). The sections were stained with hematoxylin and eosin (H. E.) in 30-60 and 15-30 sec, respectively. Routine permanent sections from frozen tissues were also made for detecting freezing damages.

2) Fixed frozen sections

Fixatives are cooled physiological saline (12) and 10% buffered formalin. Six pieces of tissue slices (5 x 5 x 3 mm) of the spleen and the kidney of an adult rat, immersed in a cylindrical glass vial (2.5 cm in diameter x 6.5 cm in height) were irradiated in the same oven system. The final height of the solution in the vials was 1.5 cm (11). Irradiation times were designed to reach final solution temperature of about 35°C and 60°C (about 40 and 120 seconds at 500 W, respectively). Each 3 pieces were removed at about 35°C and 60°C. Then cooling of the specimens in a saline or formalin solution at room temperature in 10 min followed. These 3 were sampled separately for an immediate frozen section, for a delayed frozen section after overnight substitution by 30% sucrose in phosphate buffered (PBS) solution, and for a routine permanent section. The specimens for permanent sections were left overnight in saline (saline-fixed) or PBS (formalin-fixed) of 4°C to avoid the influence of graded alcohol process. Again routine permanent sections from these frozen tissue blocks, re-fixed in 10% buffered formalin, were made for controls.

3) Metallic stainings (Periodic acid methenamine silver and Grimelius stains)

Procedures according to Brinn (5) were explored without modification. Microwave stimulation with methenamine silver working solution and silver nitrate solution is the essence.

RESULTS

a) Fresh frozen section

Table 1 shows summary of microscopic-image quality.

1) Air-dried sections showed almost the similar images to those of irradiation alone except for more swollen blurred cytological appearance. Sections of irradiation alone revealed a blurred reticular chromatin pattern, good cytoplasmic preservation, and relatively distinct cellular borders. Detection of renal tubular resorption granules suggested both morphological and functional preservation (12, 13).

2) On the whole microscopic image quality of sections under protocol-(iii) followed that of sections without microwave stimulation. All the sections immersed in
3) In cases of acetone reticular chromatin patterns and frequent overstimulated images with cracked cytoplasm, vacuolated nuclei and severely basophilic color-balance on H. E. were obtained.

4) Over-shrinkage in 100% ethanol and moderately crisp chromatinins in 50% ethanol suggested microwave-stimulated alcoholic reactions. Cytoplasmic preservation was moderate in 50% ethanol.

5) Sections of irradiation alone, acetone and formaldehyde revealed basophilic color-balance.

6) Apparent nuclear details from recticular aldehyde pattern to crisp alcoholic were considerably controlled by mixing ethanol and other reagents.

7) Irradiation at power level of continuous 500 W and intermittent 200 W revealed no significant difference in image quality.

8) Leiden fixative on the whole revealed better nuclear details than those of 50% ethanol. No obvious difference in cytoplasmic preservation was seen between these fixatives.

9) Protocol-(iii) (10 to 15 seconds irradiation at the power level of 500 W followed by immersing in Leiden fixative with further microwave-stimulation in 15 seconds) showed the best microscopic-image quality (Figs. 1 & 2). Further microwave-stimulation shortened the immersion step.

b) Fixed frozen sections

1) Permanent sections fixed with formaldehyde showed occasionally appreciable but insufficient morphological preservation. We found no influence of graded alcohol

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<th>Fixatives</th>
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TABLE 1. Summary of microscopic-image quality of fresh frozen sections irradiated in various fixatives.

reagents showed cytoplasmic loss in more or less. Within the reagents examined, acetone revealed best cytoplasmic preservation.
Fig. 1. Fresh frozen section of the rat spleen processed with irradiation alone followed by further irradiation immersed in Leiden fixative. H. E. stain. ×200

Fig. 2. Fresh frozen section of the rat kidney with process similar to Fig. 1. H. E. stain. ×200
FIG. 3. Human pancreas Grimelius stain. ×200

FIG. 4. Human kidney, Periodic acid methenamine silver stain. ×200
Fig. 5. Well-differentiated adenocarcinoma of the lung. H. E. stain. ×200

Fig. 6. Well-differentiated squamous cell carcinoma of the tongue. H. E. stain. ×200
Fig. 7. Malignant lymphoma of the pharynx. Note insufficient nuclear details. H.E. stain. × 200

Fig. 8. Scirrhous carcinoma of the breast. Note preservation of cytoplasmic mucin. H.E. stain. × 20
process in permanent sections. No significant difference was seen in cases of 35°C and 60°C.

2) Mildly (60°C) to moderately (35°C) spongy architecture was observed in saline fixed specimens.

3) All the sections of fixed and immediately frozen specimens revealed disappointing image quality. We observed too much artifactual damages, though the sections showed resemblance to compact image of permanent sections.

4) Well-fixed routine surgical specimens in 10% buffered formalin revealed better image quality than that of the microwave stimulated ones. Also true control specimens revealed better morphological preservation after freezing. These results suggested insufficient microwave-stimulated fixation in our experience.

5) Fixed frozen sections revealed more prominent freezing and mechanical damage than those of fresh frozen sections.

6) The fixed and substituted sections showed fairly good image quality.

7) Some kinds of skills were needed in sectioning the firm specimens fixed in saline in 60°C.

8) The substituted sections showed excellent plasticity for sectioning.

c) Metallic stainings

(1) Periodic acid methenamine silver

1) Overall staining time was within 20 min.

2) Excellent stainings were observed under 80 sec irradiation at the power level of 500 W (Fig. 3). The solution temperature was about 80 to 90°C. The silver impregnation was instantaneous. Neither irradiation time nor solution temperature was fully reliable for a reproducible result.

3) Cold spot was found in the lower portion of a wide section.

4) We found occasional detachment of sections.

5) Heating method with hot water bath revealed less acceleration of silver impregnation in contrast to the microwave stimulated one.

(2) Grimelius stain with Pascual's modification

1) Again the result was excellent (Fig. 4).

2) Irradiation time and solution temperature were 60 sec (70°C) in silver solution and 40 sec (65°C) in reducing solution. We needed no re-heating.

3) Reduced concentration of the silver nitrate enhanced background staining.

DISCUSSION

a) Fresh frozen sections

The major difference of our procedure to that of Kok (10) is the way to apply fixatives. The fact that a fluid-filled test-vial in a coplin jar has shown higher solution temperature than that of an exposed vial by irradiation revealed an antenna effect (20) rather than an energy loss in case of applying a coplin jar. Our preliminary trials have shown almost the similar results by "covering" a slide glass with a reagent and by "immersing" in a coplin jar containing a reagent. Various roles of microwave irradiation in fixation (20) have been reported, but we need several different considerations in fixing fresh frozen sections in contrast to fixing tissue blocks. Our results (Table 1) suggest that the loss of cytoplasm in various degrees into any applied reagents was a major problem in fresh frozen sections. Instantaneous fixing is important (1) in fixing
"cells" with bared and discontinous cytoplasmic membranes. Preservation of cytoplasmic content and volume without further swelling as seen in air-dried sections could be achieved by irradiation alone. The major roles of irradiation in fresh frozen sections seem to be heat delivery and protein denaturing in more or less, leading to reduced solubility of unravelled molecules (7). Acceleration of diffusion (3) or chemical reaction like cross-linking (4) and changes in permeability of the cytoplasmic membrane (2, 20) may not play a major role in our experiments. Apparent microscopic images of the chromatins could be handled considerably by mixing proper reagents in accentuating the reticular aldehyde pattern to an crisp alcoholic pattern. Fixation of nuclear materials may be secondary to cytoplasmic fixation (9). Chemical reaction of ethanol was accelerated with microwave stimulation as seen in sections in 50% ethanol. We therefore recommend a procedure consisting of rapid partial "fixation" or immobilization of the cytoplasm with irradiation alone and the following microwave-stimulated post fixation with any reagents of about 50% alcoholic content in order to obtain chromatin patterns in a moderately crisp type. Irradiation and postfixation with acetone without further microwave stimulation may be useful for functional preservation (14). Over-irradiation makes increased nuclear vacuolation and incorrectable blurred reticular chromatin pattern. Specimens of routine surgical pathology showed reproducible but occasionally insufficient microscopic-image quality (Figs. 5-8). Almost invariably, nuclear details were better preserved in sections irradiated in Leiden fixative or those under protocol-(iii). But cytoplasmic preservation did not always contribute to getting exact findings of a lesion. Routine alcoholic artificial shrinkage occasionally produced better understanding of a global orientation particularly in a fibrous tissue. A mild basophilic color-balance in microwaved section is not a significant compromization. A shorter period of microwave fixing seems to be better in cases of a cellular tissue or a loose tissue such as the lymph node or the lung. Lipid-rich brain tissues also had technical problems. Detachment of specimens was occasionally observed in cartilaginous or bony samples. Further evaluation of the two procedures above, microwave irradiation alone followed by postfixation with Leiden fixative or 50% ethanol and microwaving in Leiden fixative should be followed.

b) Fixed frozen sections

We have various problems in standardization of fixed frozen sections. Pathologists had received markedly insufficient frozen sections before introduction of cryostat. Frozen sections of even well-fixed specimens show severe damage without substitution. Reproducibility of microwave-stimulated fixation can be accomplished, but more rapid procedures are needed to get rid of freezing damages without loosing total balance of plasticity in heterogenous tissues involved in sections. We had unpredictable results on microwave-stimulated permanent sections even immersed in formaldehyde, and their frozen sections showed no improvement in image quality, though cellular boundaries were more compact than those of fresh frozen sections. Longer time (over 10 min), though not appropriate for rapid diagnosis, may be indispensable to accomplish stable chemical reactions and consequent good morphological preservation. Sucrose substitution (19) is time-consuming but sections treated with it revealed fairly good microscopic-image quality. There were no prominent freezing damages and no problem in sectioning. Compact cellular boundaries and improved color-balance could be achieved. We explored whether or not
microwave irradiation accelerates diffusion of sucrose solution leading to rapid substitution. The solutions were frequently exchanged below 20°C to avoid thermal damage. However we have not yet obtained significant results in terms of shortening of the time for substitution.

c) Metallic stainings

The procedures according to Brinn (5) showed excellent stainability. It is considerably time-saving in comparison to ordinary heating procedures with a hot water bath (within 20 min versus 40 min). The high concentration of the silver nitrate solution was appropriate for sparing times and for reducing background-staining. Since optimization of irradiation time is difficult, continuous observation of color changes in reagents or of sections is necessary to getting a good staining. Temperature difference in a coplin jar made focal insufficient staining in cases of larger sections. Occasional detachment of sections was given.

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