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STRUCTURE OF RAT KIDNEYS FOLLOWING MICROWAVE ACCELERATED FIXATION

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Abstract

In contrast to fixation of tissue in externally heated fixative, microwave-irradiation can generate uniform internal heat, which is of utmost importance for successful fixation of biological tissue. To evaluate the effectiveness of microwave-accelerated chemical fixation, we compared the structure of rat kidney fixed by a conventional method and a microwave-accelerated method, by scanning and transmission electron microscopy. Following perfusion, rat kidney pieces 1-2 mm in size were irradiated in Karnovsky's fixative in a domestic Amana microwave oven, till the temperature of the fixative reached 45-50°C. For conventional fixation, tissue pieces were fixed overnight at room temperature in the same fixative. Both types of samples were processed further for electron microscopy using identical protocols. The microwave fixed samples showed excellent preservation of structure comparable to the samples fixed by the conventional method. Glomeruli and the renal tubules showed normal morphology with no cellular swelling. The cytoplasm and nuclear matrix of the epithelial cells was uniformly dense. Other fixation sensitive organelles like mitochondria and Golgi apparatus showed superior preservation with continuous membranes. These results demonstrate that microwave accelerated chemical fixation results in excellent preservation of tissue structure, reduces processing time significantly and is therefore a practical alternative to conventional protocols.

Key Words: Conventional fixation, kidney ultrastructure, Microwave-accelerated fixation, optimum temperature, electron microscopy, glomeruli, podocytes, proximal tubules, fixation-sensitive organelles.

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Introduction

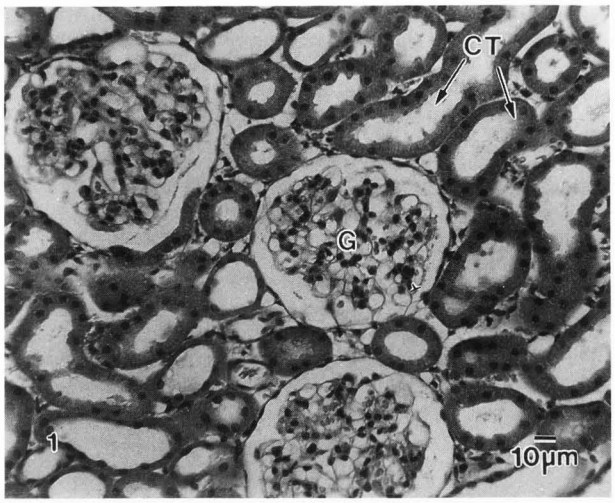
For the past century, chemical fixation has been used extensively for preparation of cells and tissues for microscopy. The processing protocols are designed to preserve the cellular components and macromolecules in a condition as close to their natural state as possible. The water content of the cells is gradually replaced with the fixative, dehydrating agents and finally the embedding medium. Since these exchanges are based on diffusion, heat can speed up these processes.

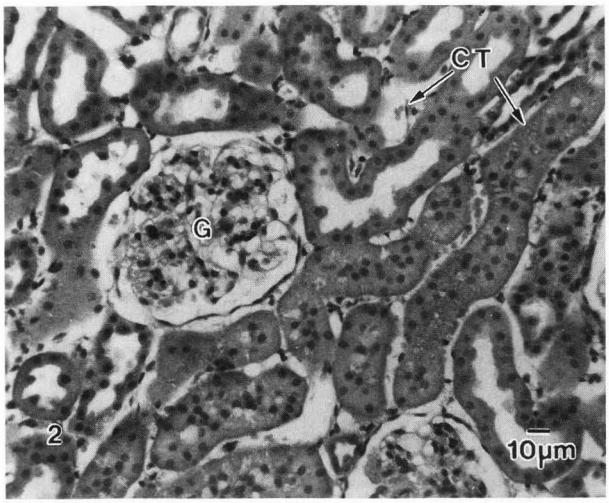
The application of heated formalin baths in tissue processing was first documented nearly a hundred years ago in 1898 by Ehrlich and Lazarus as cited in reference 1. Although external heat can speed up tissue processing, it is difficult to control the temperature distribution across the sample.

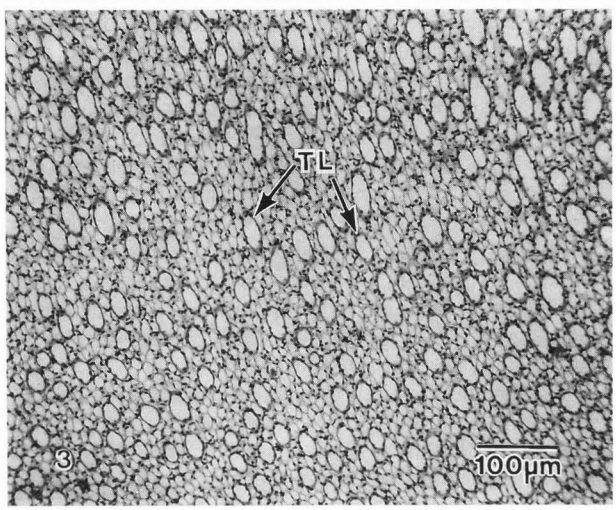
For successful processing of biological tissue, a uniform generation of heat across the sample is of utmost importance. Twenty years ago, Mayers (21) recognized that microwave based heating may satisfy this requirement. As an alternative to chemical fixation, he used direct irradiation of 1 cm cubes of human and mouse tissues for 90 seconds via a generator and claimed to have obtained satisfactory results. In contrast to conventional heating, microwave irradiation can generate uniform internal heat (1, pg 90) in a very short time period and can be used in combination with chemical fixation.

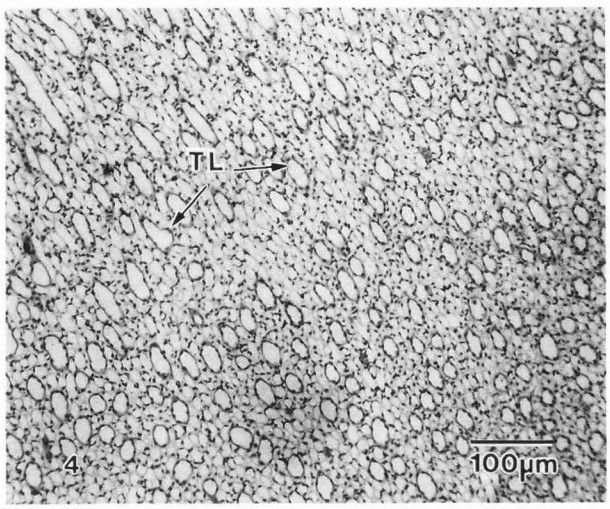
Despite its introduction twenty years ago, it is only in the last five years that the application of microwaves in the laboratory has made considerable advances. This technique has been used in a variety of procedures such as analysis of tissue structure by light microscopy (6,12,15,19), electron microscopy (3,6,7,8,12,16,18,19,29), preservation of antigens (12,19), embedding protocols (25), fixation and staining of cryostat sections (1,23), and immunogold-silver staining (26). It has also been used in diagnostic techniques like In Situ DNA hybridization (27).

Since application of microwave irradiation can significantly reduce the processing time, it is particularly valuable for diagnostic procedures. For example, as Boon et al. (2) have demonstrated, microwave application can reduce the dehydration, clearing and









impregnation time for tissues from a total of 720 seconds by conventional method to 15 seconds. Microwave irradiation can be used in one or more steps of a procedure and success of the technique depends on optimum irradiation temperature and specimen size. The protocol for microwave processing can be modified to suit the type of technique being used (1).

Although histoprocessing by microwave irradiation is used routinely in some laboratories (1), there are comparatively few reports which demonstrate structural preservation of a tissue by both scanning and transmission electron microscopy. In this study, to evaluate the effect of microwaves on preservation of tissue structure, we compared the structure of perfused rat kidney fixed by a conventional method and by a microwave-accelerated (MA) method, by light (LM), scanning (SEM) and transmission electron microscopy (TEM). Our results demonstrate that microwave irradiation of rat kidney tissue in a glutaraldehyde fixative accelerates the fixation process and results in excellent structural preservation, comparable to the conventional method.

Figure 1. Light micrograph of a section through the cortex from a microwaved specimen, showing the glomerulus (G) and the collecting tubules (CT).

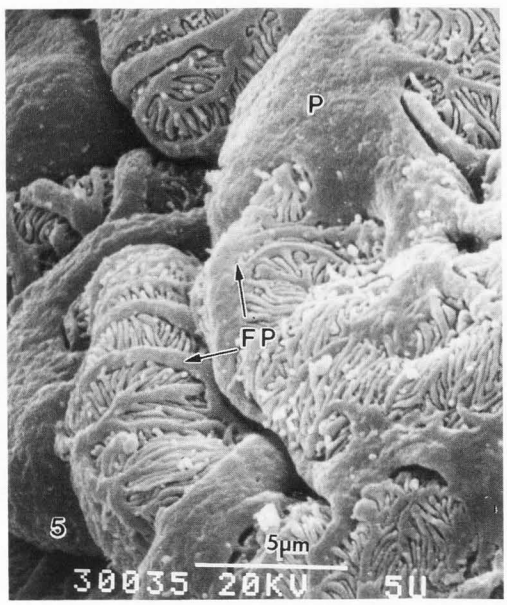
Figure 2. Control specimen, features same as in figure 1.

Figure 3. Light micrograph of a section through the inner medullary region of a microwaved specimen showing thin limbs of loop of Henle (TL).

Figure 4. A control specimen showing features same as in figure 3.

Materials and Methods

Male Sprague Dawley rats weighing approximately 200 gms were anesthetized by sodium pentobarbital injection (50 mgs/Kg body weight). Kidneys were perfused in situ, using a standard protocol (4). Briefly, kidneys were perfused with 50 ml of heparinized saline followed by half strength Karnovsky's



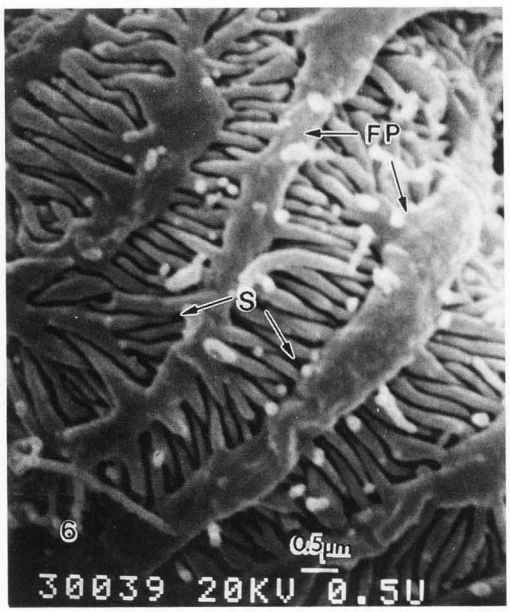


Figure 5. Scanning electron micrograph of a glomerulus from a microwaved sample, showing podocyte (P), with extended foot processes (FP) arising from the main cell body.

Figure 6. A higher magnification of the podocyte foot processes (FP) showing details of interdigitation and the filtration slits (S) between them.

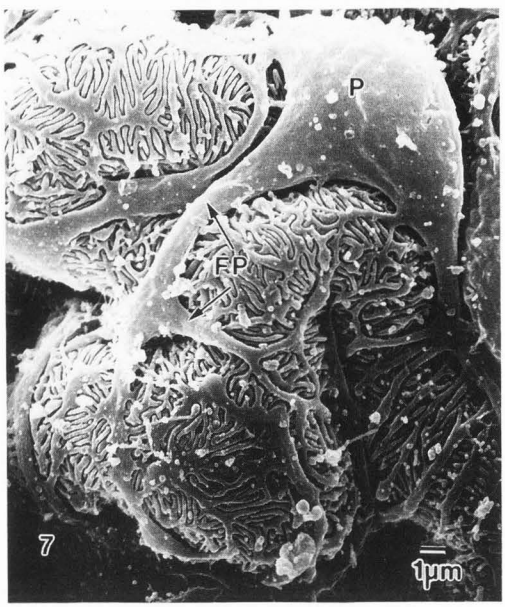


Figure 7. Control sample from a similar area as in figure 5.

fixative (10) for 10 minutes. After perfusion, kidneys were harvested, cut into 1-2 mm pieces and processed further.

Microwave processing

Based on current literature (1,16), it was decided that the optimum temperature range for processing 1-2 mm tissue pieces was 45-50°C. A domestic Amana microwave oven with a 700 watt output was used for irradiation. The microwave was calibrated for the optimum temperature as follows: A 25 ml beaker containing 10 ml of distilled water was placed in the exact center of the microwave. It was irradiated for different combinations of power levels and time intervals, at the end of which the beaker was removed and the temperature of the water was measured with a standard mercury thermometer. At the lowest power level, 30 seconds of irradiation was required for the water to be heated to a range of 45-50°C.

Based on this information and our experience with other types of tissues, the kidney tissue pieces were irradiated for approximately 30 seconds in 10 ml Karnovsky's fixative in a 25 ml beaker, at the lowest power level. Care was taken to ensure that each sample received identical irradiation.

Conventional processing

As a control, kidney pieces were fixed overnight in Karnovsky's fixative at room temperature.

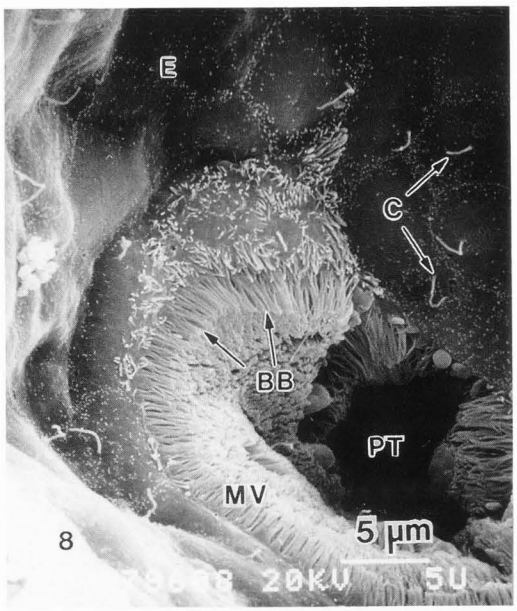
Further processing for LM, SEM and TEM was identical for both types of samples.

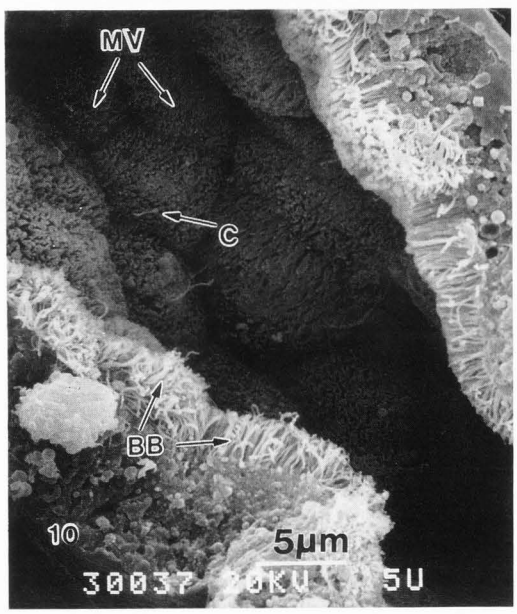
Light Microscopy

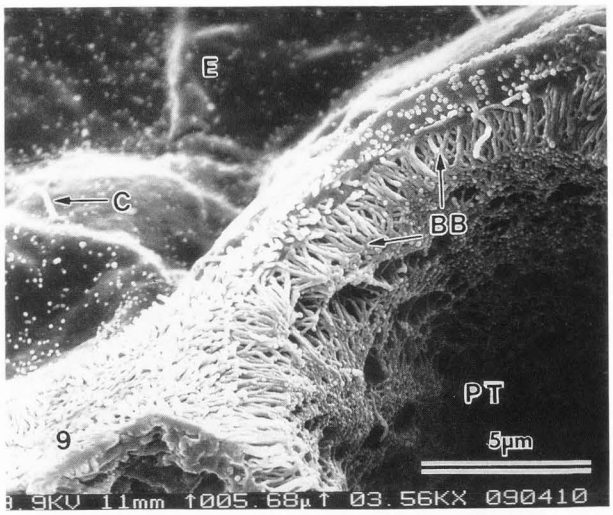
The fixed tissue was washed twice in phosphate buffered saline (PBS), dehydrated through a graded alcohol series followed by xylene and embedded in paraffin. The sections were stained with hematoxylin and eosin and examined under a Leitz microscope.

Scanning electron microscopy

The fixed tissue pieces were washed twice with cacodylate buffer (0.05 M, pH 7.2) and post fixed in 1% osmium tetroxide for one hour. They were then dehydrated through a graded alcohol series and critical







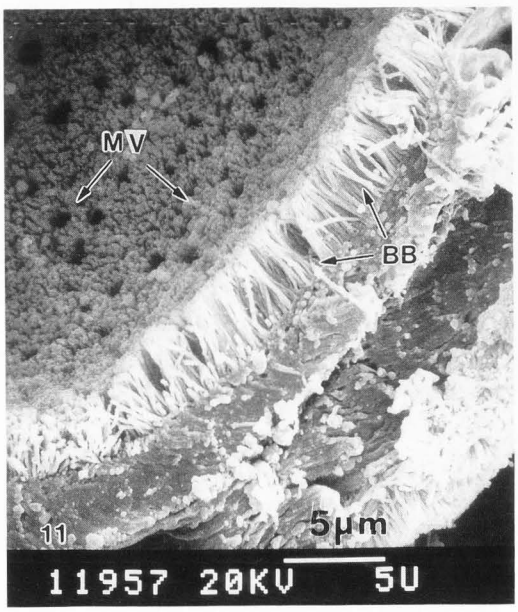


Figure 8. Scanning electron micrograph of a microwaved sample showing the parietal epithelium (E) continuous with the proximal tubule (PT). Single cilia (C) are prominent above the cell surface, longer microvilli (MV) form the brush border (BB).

Figure 9. Scanning electron micrograph of a conventionally fixed sample showing area similar to that in figure 8.

Figure 10. Scanning electron micrograph of a microwaved sample showing luminal surface of a collecting tubule showing microvilli (MV), single cilium (C) and brush border (BB).

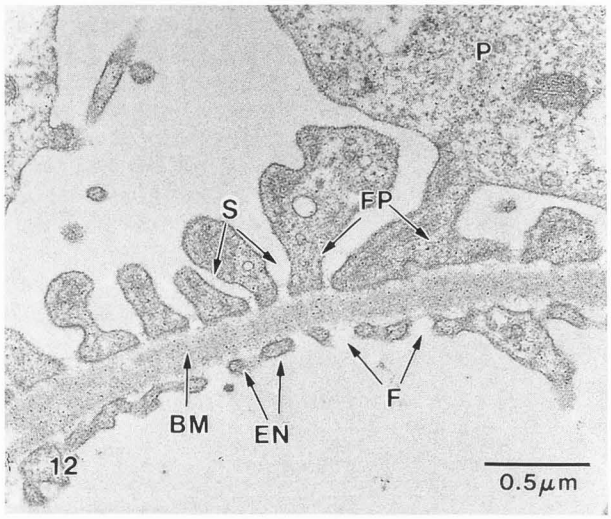
Figure 11. Scanning electron micrograph of a control sample, from a similar area as in figure 10.

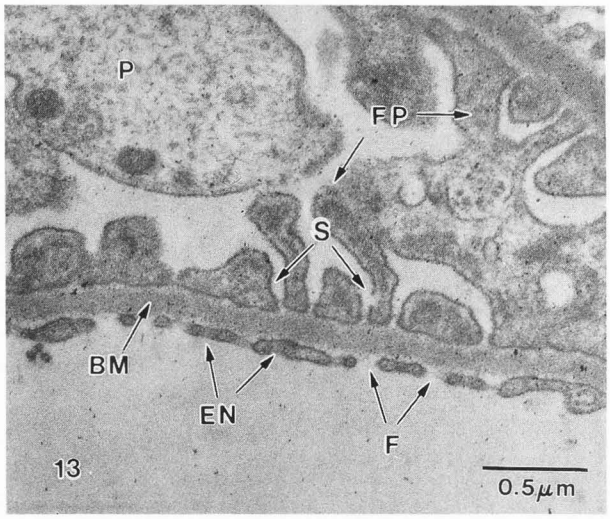
Bars = 5 μm.

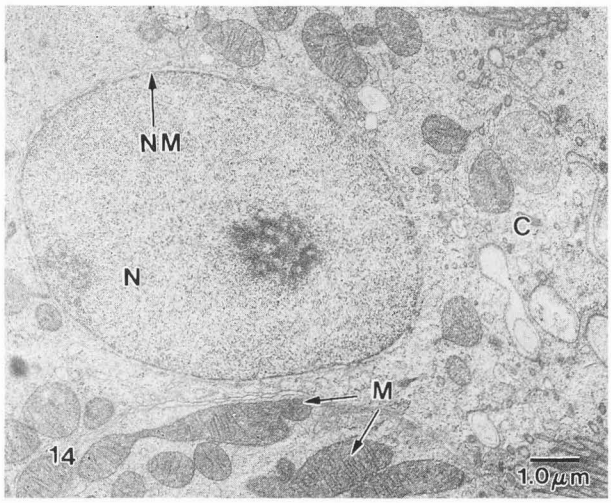
point dried in a Balzers critical point drier with absolute alcohol as the final transferring medium. The structure was analyzed under a Hitachi S-450 scanning electron microscope.

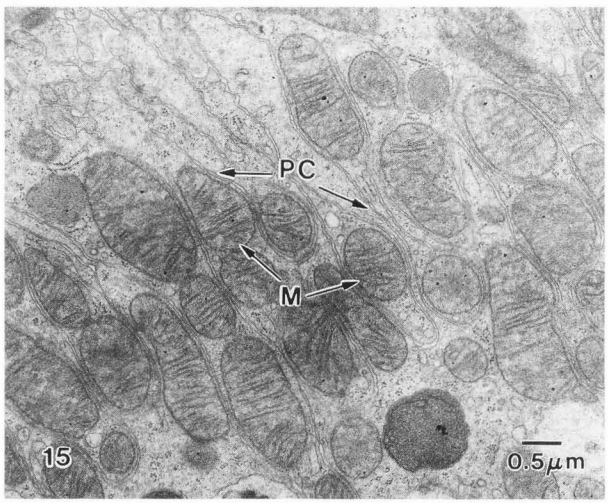
Transmission electron microscopy

The tissue was osmicated as in the case of SEM samples. It was dehydrated through a graded alcohol series followed by propylene oxide and embedded in Spurr's (22) resin in a vacuum oven at 60°C. Thin sections were cut with an LKB Ultrotome III, stained with uranyl acetate for 12 minutes, lead citrate for 5 minutes and observed using a JEOL 100 CX transmission electron microscope.









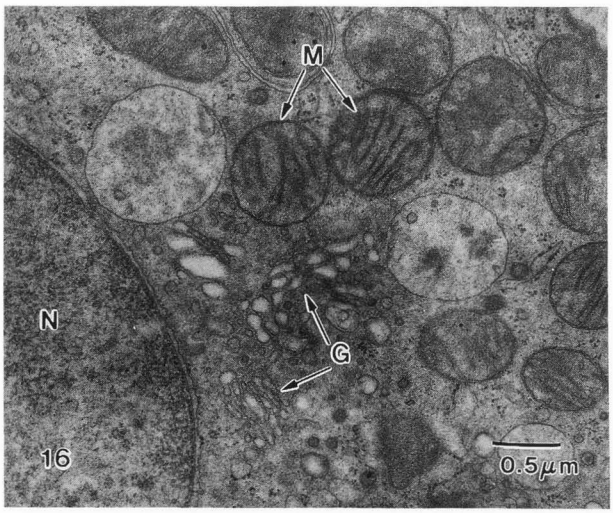


Figure 12. Transmission electron micrograph of a microwaved sample through the glomerulus, showing basement membrane (BM) between Endothelial cells (EN) and podocytes (P), fenestrae (F), filtration slits (S) and podocyte foot processes (FP).

Figure 13. Control sample showing similar features as in figure 12.

Figure 14. Transmission electron micrograph of a microwaved sample showing the cytoplasm (C), nucleus (N), nuclear membrane (NM) mitochondria (M).

Figure 15. Transmission electron micrograph of a microwaved sample showing mitochondria enclosed (M) in paramembranous cisternae (PC).

Figure 16. Transmission electron micrograph of a microwaved sample showing Golgi apparatus (G), nucleus (N) and mitochondria (M).

Results

In general, MA fixation of kidney at 45-50°C resulted in excellent preservation of the structure and showed optimum contrast. A comparison of the characteristic structural features demonstrated that MA fixation gave results comparable to or better than conventional fixation method.

Light Microscopy

A transverse section through the cortex region of a MA fixed specimen (Fig. 1) showed well preserved glomeruli and renal tubules with normal morphology. At this level of analysis, there was no evidence of any structural damage. The overall structure was comparable to the control (Fig. 2). Similarly, a typical section through the inner medullary region (Fig. 3) showed thin limbs of the loop of Henle with uniform outlines, similar to the control (Fig. 4).

Scanning electron microscopy

When examined at a higher magnification by scanning electron microscopy, no significant difference was seen in the MA fixed and conventionally fixed samples. Typical examples of the type of fixation obtained by the MA fixation protocol are illustrated in figures 5-11. Examination of the outer surface of the glomerulus (Fig. 5) showed normal looking components. Similar to the control sample (Fig. 7), the visceral epithelial cells (podocytes) were comprised of the main cell body with multiple processes extended and wrapped around the capillaries. The cells did not show any swelling. As seen at a higher magnification (Fig. 6) in the irradiated sample, interdigitation of the foot processes from different podocytes was not disrupted, indicating unaltered morphology. In both MA fixed (Fig. 8) and conventionally fixed samples (Fig. 9) the parietal epithelium of the Bowman's capsule showed squamous cells with single cilium extending from the cell surface to the capsular space. The surface of the cells was sparsely populated with short microvilli, which were denser at the border between the adjacent cells. The capsular space was continuous with the lumen of the proximal tubule at the urinary pole. The urinary tubules were open, as would be expected with good preservation and the brush border was normal looking. The luminal surface of the MA-fixed proximal tubules (Fig. 10) showed intact brush border with finger like microvilli. Single cilia projected from the epithelial cells into the tubular lumen. Details of a similar area from a conventionally fixed sample are shown in figure 11 for comparison.

Transmission electron microscopy

At the ultrastructural level, a section through the glomerulus showed identical images in MA-fixed sample (Fig. 12) and control sample (Fig. 13). Typically, the intact basal lamina was interposed between the fenestrated capillary endothelium on the inside and slit pores between pedicles (foot processes) of the visceral epithelial cells on the outer side.

Different cellular components were ideally preserved in MA-fixed samples (Figs. 14,15 and 16) and showed features similar to the control samples (not illustrated, 24). As shown in figure 14, the cytoplasm was uniformly dense. The nucleus showed uniform matrix and intact nuclear membrane without any breaks or swelling. Mitochondria (Fig. 15) showed well defined membranes and cristae. Their elongated profiles were enclosed in extensive invaginations of the plasma membrane, forming the paramembranous cisternae. The membranes were continuous and uniform. Other fixation sensitive organelles like the Golgi apparatus (Fig. 16) showed excellent preservation.

Discussion

Several parameters need to be considered for standardization of microwave processing for a given protocol. They are as follows: 1) temperature of irradiation, 2) specimen type and size, 3) type and volume of fixative.

Reports regarding the ideal temperature range for irradiation vary from 45-50°C (2) to 63-72°C (10) and may differ depending on the type and size of tissue. As cited in reference 14, optimum temperature may be different for each tissue and even for each individual tissue component. In our studies, we used a temperature range of 45-50°C for 1-2 mm tissue pieces and obtained satisfactory preservation.

Microwaves are electromagnetic waves with a limited depth of penetration (13). In addition to the conductive properties of the medium (tissue), uniform fixation of the tissue also depends on whether the waves span the whole sample. Therefore it is necessary to limit the size of the tissue pieces in order to obtain generation of uniform heat across the sample. From our own experience with different tissue types (unpublished) and from other reports (1), the tissue size should not exceed 1 cm in dimensions.

Login and Dvorak (17) have shown that with specialized equipment and an ultrafast method, 26 milliseconds of irradiation gave satisfactory results with tissue pieces up to 2 mm in size. In addition they obtained satisfactory results with a domestic microwave oven with 5-9 seconds irradiation of single specimens. In our experiments, we obtained excellent results with 1-2 mm tissue pieces irradiated for approximately 30 seconds, in 10 ml of fixative in a domestic microwave oven, confirming that specialized equipment was not necessary. However it must be emphasized that since microwave ovens differ in energy output characteristics, each one should be calibrated individually before use.

As discussed by Boon and Kok (1), the rise in temperature of a substance by microwave irradiation depends on its dielectric, antenna and thermal properties. As fluids react differently to irradiation, it is necessary to determine the time of irradiation in each fluid separately (2). We calibrated the microwave under conditions similar to those used for sample irradiation to ensure that each sample received identical treatment.

Microwave irradiation has two primary effects on the sample: 1) uniform distribution of temperature across the sample and 2) accelerated fluid diffusion. Although it is accepted that the heat itself may be responsible for some fixation, it has been speculated that the fixation is due to the rapid movement of the dipolar molecules of water and proteins during the diffusion process (2). In a study comparing the effects of heat and microwaves on proteins, Hopwood et al. (7) demonstrated that controlled delivery of heat was the most important element of microwave fixation.

During fixation of a tissue with aldehydes, the fixative diffuses into the cells and stabilizes them by crosslinking proteins. With microwave irradiation, the diffusion is accelerated (2) resulting in a reduction in the processing time (2). However, irradiation can introduce artifacts like abnormal cytoplasm which does not take up adequate stain and pyknotic nuclei (11). Therefore to determine the acceptability of the results obtained, we used tissue fixed by standard immersion method as a control to compare and evaluate the preservation of structure by microwave irradiation. At all microscopic levels of analysis, the preservation of structure was satisfactory. Our observations agree with the report of Mac-Moune-Lai et al. (20) who obtained similar structural preservation of human renal biopsy specimens using conventional and microwave fixation.

The light microscopic examination showed excellent preservation of general features of the cortex and medullary regions. There was no visible difference in the structure of the control samples.

To facilitate comparison of structure at a higher magnification, the tissues were processed and observed by scanning electron microscopy. When seen at a higher magnification, the interdigitation of the foot processes of the podocytes was typically like that seen in the control confirming that this characteristic structure was not disrupted. We concluded that the various elements of Bowman's capsule and the renal tubules were satisfactorily preserved. An evaluation of use of microwaves in stabilization and fixation of human and mouse tissues for scanning electron microscopy (6) showed that at a temperature of 55-60°C, results were comparable to conventional method. Our observations with SEM were similar.

The use of microwaves for preservation of ultrastructure and cytochemical reactivity has been documented (1,13,14,17,28). As discussed by Hayat (5), at the ultrastructural level, one of the major criteria for satisfactory fixation of tissue is continuity of membranes. In our study, the microwave fixed samples did not show any breaks in the membranes of cells, nuclei, mitochondria and Golgi apparatus.

Specifically in the case of kidney, the criteria for satisfactory fixation include open tubular lumen (not collapsed), and uniform cytoplasmic density without any swelling. On the other hand, mesangial cell swelling is an indicator of poorly fixed glomeruli and corresponds to rapid swelling of proximal tubular epithelial cells (9). In this study, irradiated samples did not show any cellular swelling, indicating excellent fixation. We obtained images identical to the control samples, in some cases with better contrast.

Other characteristics of optimum preservation include intact cristae and dense matrix of mitochondria, uniformly dense nuclear contents and finely granular cytoplasmic substance without any empty spaces (5). MA-fixation of rat kidney showed these features, similar to the conventionally fixed samples.

A primary advantage of using microwave irradiation is reduction in processing time compared to conventional methods. As shown in this study, the fixation time for rat kidney tissue was reduced from overnight to 30 seconds, without any compromise in the quality of preservation of structure. MA processing can also be used in dehydration, embedding and staining. Due to this time saving feature, this technique becomes very valuable in routine laboratory work, specifically for diagnostic procedures.

In summary, our results demonstrate that microwave-accelerated processing is a viable alternative to conventional processing. It is a versatile technique which offers the advantage of significant reduction in processing time and can be used in conjunction with a variety of procedures.

Acknowledgements

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Discussion with reviewers

D.B. Jones: Do you have any information as to what quality of fixation would you obtain if you immediately processed the tissue after perfusion ?

Authors: We can not answer this question directly since we have not tried processing perfused tissue which did not receive any additional fixation. Perfusion allows the fixative to reach the organ/tissue quickly. However the time for which the fixative is in contact with the cells is probably not sufficient for stabilization of the tissue. As discussed by Hayat (5), even when vascular perfusion is carried out under optimal conditions, relatively large organs such as brain and kidney show uneven fixation and various parts of the kidney exhibit different qualities of fixation.

J.R. Manaligod: The excellent results obtained with transmission and scanning microscopy were obtained with tissues of experimental animals that were, at the outset, ideally fixed by perfusion. In reference to clinical material, would the same results be expected if tissues are not initially perfused? We know that equally good results can be obtained using light microscopy (H & E and special stains).

Authors: Perfusion alone may not necessarily result in ideal fixation. With regards to the clinical samples, our own experience and reports from others (Text reference #1) demonstrate that unperfused clinical specimens should give satisfactory results when optimum specimen size and irradiation temperature are used.

J.R. Manaligod: Are there any conceivable situations in which microwave accelerated fixation is not possible?

Authors: We are not aware of situations where microwave accelerated fixation would not be possible. However, the results will depend on the type and size of specimens, the depth of penetration of the microwaves and the dielectric properties of the material, as discussed by Boon and Kok (Text reference #1). Microwave irradiation has been demonstrated to enhance the quality of fixation in extremely difficult to fix specimens such as fungal spores with thick coats (personal communication).

Reviewer #5: Wouldn't controls like a) perfusion with no additional fixation and b) immersion in the fixative for 30 seconds at 45-50°C be more appropriate?
Authors: a) This may be a good control to include in order to emphasize the acceleration of fixation resulting from microwave irradiation. We plan to use it in our future experiments. b) Although in theory this may be an adequate negative control, it does not serve our purpose. In this paper, we have evaluated the effect of microwave irradiation for acceleration of fixation, and compared it with results obtained by a conventional fixation protocol. From our own experience, good fixation of 1 mm deep soft animal tissue with glutaraldehyde (2%) requires a minimum of 1-2 hours at room temperature. In addition, based on the available literature (Text reference #1), it can be speculated that a short exposure of 30 seconds at 45-50°C in the fixative will result in very little diffusion into the tissue. Therefore, we think that using this control will not provide any new information.