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Microwave-stimulated formaldehyde fixation of experimental renal biopsy tissues: computerised morphometric analysis of distortion artefacts

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Abstract

Although microwave irradiation has been used in the histopathology laboratory for several years, there has been minimal published experimental data on its effects on the technical and staining quality of histological sections. Furthermore, it has not been clear whether the advantages gained in reduction of fixation and staining duration has been at the expense of increasing architectural distortion to the tissues. We report here our experience with computerised morphometric analysis to investigate glomerular artifacts caused by microwave-stimulated fixation of renal tissues. 39 rat and 33 human autopsy kidney samples were subjected to (1) fixation in neutral buffered formaldehyde (control), (2) microwave-stimulated fixation followed by neutral buffered formaldehyde, and (3) neutral buffered formaldehyde followed by microwave irradiation. In addition, the effect of post-fixation in 70% ethanol was also investigated. Microwave irradiation was delivered through a dedicated laboratory microwave oven at 80% power and at 55°C for 3 minutes. The different fixation methods were compared with regards to shrinkage (distortion) to glomerular structures (glomeruli and Bowman's spaces) on H&E sections, as determined by morphometric image analysis using a temporary assembled-system consisting of a trinocular microscope, a digital video camera and personal computer. A FlashPoint VGA 3.3 film-grabber card was used to capture images for morphometric analysis by using a *Scion Image* program. Morphometric analysis of glomerular structures showed that microwaves caused more shrinkage to the area bounded by the Bowman's capsule than the glomerulus proper, but post-fixation with ethanol reduced this shrinkage. These findings have implications on the logistics of tissue preparation of renal biopsies in clinical practice.

Key words: renal biopsy, microwave-fixation, artefact, computerized morphometry

INTRODUCTION

The use of the microwave oven is well-established in histopathology practice, especially in bringing about better exposure of antigens in tissue sections for immunohistochemical staining.[1,2,3] Microwave-enhanced fixation of tissues have also been mooted for situations when rapid diagnosis is needed,[4,5] such as in the examination of renal transplant allografts for evidence of rejection. However, as with the adaptation of any new technique into a diagnostic repertoire, it is important to be aware of any artefacts introduced that may impact on the quality of the examination. We have observed that such changes may be very subtle and its interpretation may be subjective. In order to investigate for changes in an objective manner, we turned to the use of computers and bioinformatics in its documentation and analysis.[6] We report here our experience with computerised morphometric analysis to investigate glomerular artifacts caused by microwave-stimulated fixation of renal tissues.

MATERIALS AND METHODS

39 kidney core samples (2mm thick) from 9 *Sprague Dawley* rats and 33 kidney core samples taken at autopsy from two healthy human subjects who died acutely from traumatic injuries were accordingly subjected to: **Experiment A:** (1) fixation in 4% neutral buffered formaldehyde (control), (2) microwave-stimulated fixation followed by 4% neutral buffered formaldehyde, and (3) 4% neutral buffered formaldehyde followed by microwave irradiation. In addition to the design of Experiment A, the effect of post-fixation in 70% ethanol (15 hours) for rat samples (**Experiments B**) and human samples **(Experiment C)** were investigated. All fixed

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samples were subsequently subjected to conventional processing into H&E sections. The distribution of samples according to type and duration of fixation are summarised in Tables 1, 2 and 3.

Microwave irradiation was delivered through a dedicated laboratory microwave oven (Model H2500 manufactured by Energy Beam Sciences, USA) set at 80% power (480W) and a pre-set temperature of 55°C for 3 minutes.

Morphometric analysis: The different fixation methods were compared with regards to shrinkage (distortion) to glomerular structures (glomeruli and Bowman's spaces) on H&E sections, as determined by morphometric image analysis using a temporary assembled-system consisting of a trinocular microscope, a digital video camera and personal computer. A FlashPoint VGA 3.3 film-grabber card was used to capture images for morphometric analysis using a public domain *Scion Image* program developed by the National Institutes of Health, USA. About 100 complete glomeruli from each sample were analysed for the parameters indicated in Figure 1. It was assumed that in the normal healthy state, the glomerulus would normally fill up the area bounded by the Bowman's capsule, leaving a minimal urinary space. This would mean that Area 1 should approximate Area 2, and Area 3 should be a small numeric. It was assumed that Area 3 would be increased if there was greater shrinkage in Area 2 compared to Area 1. Hence, Area 3 can be an indicator of architectural distortion as a result of fixation and tissue processing.

Size Factor (SF) was obtained by comparing the mean areas of the microwave methods C2 and C3 against the control method C1. An increase in the mean area against the control method was indicated as "+" while a decrease in the mean area, i.e. shrinkage as "-".

Distortion (D) was calculated as Area 3/Area 1 x 100. The **distortion factor (DF)** of each sample was the mean of D for each sample, computed as the sum of D of all analysed glomeruli in the sample / number of glomeruli analysed. In the comparison of DF between samples, a decrease in DF compared to the control was indicated as "-" while an increase in DF is indicated as "+".

RESULTS

Comparison of shrinkages of Areas 1, 2 and 3 and distortions according to the various fixation methods are summarized in Table 4. Morphometric analysis of glomerular structures showed that microwaves caused more shrinkage to the area bounded by the Bowman's capsule than the glomerulus proper, but this did not impose any undue disadvantage compared to conventional formaldehyde fixation in terms of causing more architectural distortion. When used together with post-fixation ethanol, microwave irradiation may in fact result in less shrinkage and less architectural distortion. In this, method 2 (microwave-stimulated fixation followed by formaldehyde) appeared to be superior to method 3 (formaldehyde followed by microwave-stimulated fixation).

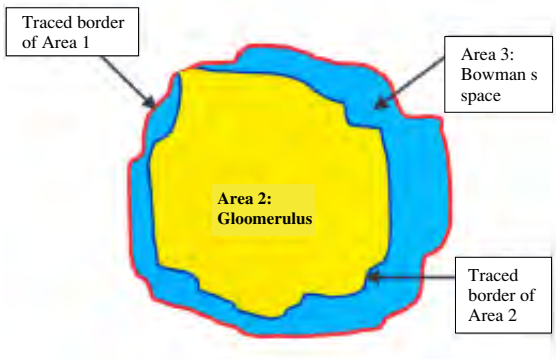

Figure 1: A diagrammatic representation of a glomerulus with areas of interest indicated.

TABLE 1: Experiment A - Distribution of rat samples according to the various methods of fixation (comparison of formaldehyde fixation and microwave-stimulated fixation)

	METHOD A1 (Fixed entirely in formaldehyde)		METHOD A2 (Microwaved prior to fixation in formaldehyde)		METHOD A3 (Fixed in formaldehyde prior to microwave treatment)	
Rat ID.	**Sample No.**	**Duration in formaldehyde**	**Sample No.**	**Duration in formaldehyde**	**Sample No.**	**Duration in formaldehyde**
1	1	10 min	2	10 min	3	10 min
1	4	20 min	5	20 min	6	20 min
1	7	30 min	8	30 min	9	30 min
2	10	1 hr	11	1 hr	12	1 hr
3	13	2 hr	14	2 hr	15	2 hr
3	16	4 hr	17	4 hr	18	4 hr

TABLE 2: Experiment B - Distribution of rat samples according to the various methods of fixation, followed by post-fixation in 70% ethanol.

	METHOD B1 (Fixed entirely in formaldehyde)		METHOD B2 (Microwaved prior to fixation in formaldehyde)		METHOD B3 (Fixed in formaldehyde prior to microwave treatment)	
Rat ID.	**Sample No.**	**Duration in**	**Sample No.**	**Duration in formaldehyde**	**Sample No.**	**Duration in formaldehyde**
4	19	30 min	20	30 min	21	30 min
5	22	1 hr	23	1 hr	24	1 hr
6	25	2 hr	26	2 hr	27	2 hr
7	28	4 hr	29	4 hr	30	4 hr
8	31	6 hr	32	6 hr	33	6 hr
9	34	8 hr	35	8 hr	36	8 hr
9	37	24 hr	38	24 hr	39	24 hr

DISCUSSION

Digital image analysis can provide fast, precise and objective quantitative results. A good correlation between results obtained by computed image analysis and classical morphometric analysis of renal tissue has been documented.[6] With rapid developments in computer hardware and the application of automatic image segmentation and object classification techniques in biomedical sciences, several automatic programs for routine quantification of histological renal samples have been made available.[7] For the renal system, there is scanty data reported in the literature on the morphometric study of the urinary space. Most research have focused on glomerular abnormalities in specific disease entities.[8]

Although microwave irradiation has been used in the histopathology laboratory for several years, there has been minimal published experimental data on its effects on the technical and staining quality of histological sections. Furthermore, it has not been clear whether the advantages gained in reduction of fixation and staining duration has been at the expense of increasing architectural distortion to the tissues. Our study has taken advantage of computerized image analysis to provide objective data to show that microwave-stimulated fixation *per se* while providing some advantage in shortening fixation time, does result in an increase in shrinkage to renal tissue structures. However, microwave-stimulated fixation together with post-fixation in ethanol resulted in both an improvement in technical quality and a reduction in shrinkage and

TABLE 3: Experiment C - Distribution of human samples according to the various methods of fixation, followed by post-fixation in 70% ethanol

	METHOD C1 (Fixed entirely in formaldehyde)		METHOD C2 (Microwaved prior to fixation in formaldehyde)		METHOD C3 (Fixed in formaldehyde prior to microwave treatment)	
Human ID.	**Sample No.**	**Duration in formaldehyde**	**Sample No.**	**Duration in formaldehyde**	**Sample No.**	**Duration in formaldehyde**
1	40	30 min	41	30 min	42	30 min
1	43	1 hr	44	1 hr	45	1 hr
1	46	2 hr	47	2 hr	48	2 hr
1	49	4 hr	50	4 hr	51	4 hr
1	52	6 hr	53	6 hr	54	6 hr
1	55	8 hr	56	8 hr	57	8 hr
1	58	24 hr	59	24 hr	60	24 hr
2	61	1 hr	62	1 hr	63	1 hr
2	64	2 hr	65	2 hr	66	2 hr
2	67	4 hr	68	4 hr	69	4 hr
2	70	8 hr	71	8 hr	72	8 hr

TABLE 4: Comparison of shrinkage and distortion effects from Methods 2 and 3 compared against Control (Method 1)

Comparison against Control (Method 1)			
	Method 2 (MW + F)	**Method 3** (F + MW)	**Comment**
Experiment A Rat renal tissues with fixation	• 67% show more shrinkage to Area 1 • 67% show more shrinkage to Area 2 • 83% show smaller Area 3 • 50% show reduction in distortion factor	• 100% show more shrinkage to Area 1 • 83% show more shrinkage to Area 2 • 67% show smaller Area 3 • 50% show reduction in distortion factor	Methods 2 & 3 cause more shrinkage to Bowman's capsule and glomerulus, but less differential shrinkage between the 2 structures. Method 2 is marginally superior to Method 3
Experiment B Rat renal tissues with fixation and ethanol	• 43% show more shrinkage to Area 1 • 29% show more shrinkage to Area 2 • 100% show smaller Area 3 • 100% show reduction in distortion factor	• 43% show more shrinkage to Area 1 • 43% show more shrinkage to Area 2 • 57% show smaller Area 3 • 71% show reduction in distortion factor	Methods 2 & 3 cause less shrinkage to Bowman's capsule and glomerulus and less architectural distortion. Method 2 is superior to Method 3
Experiment C Human renal tissues with fixation and ethanol	• 27% show more shrinkage to Area 1 • 36% show more shrinkage to Area 2 • 46% show smaller Area 3 • 46% show reduction in distortion factor	• 27% show more shrinkage to Area 1 • 27% show more shrinkage to Area 2 • 36% show smaller Area 3 • 46% show reduction in distortion factor	Methods 2 & 3 cause less shrinkage to Bowman's capsule and glomerulus, but more differential shrinkage and marginally more architectural distortion.

distortion artefacts compared with conventional formaldehyde fixation. These findings have implications on the logistics of tissue preparation of renal biopsies in clinical practice.

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