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RAPID MICROWAVE-STIMULATED FIXATION OF ENTIRE PROSTATECTOMY SPECIMENS

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SUMMARY

Conventional fixation of large solid surgical specimens is a slow process. Consequently, autolytic damage to tissues may occur if the fixative does not reach the central part of the specimen in time. However, as there is also a time relationship between formalin fixation and antigen masking, fixation for too long can also be detrimental. In seeking the optimum balance for fixation, microwave irradiation might be of assistance. This study set out to evaluate methods for fixing entire prostate glands within a brief period of time, using microwave-stimulated formalin fixation. The results show that entire prostates can be optimally fixed if formalin is present throughout the tissue as the temperature is increased by microwave irradiation. This is achieved by injecting the fixative into the prostate at multiple sites immediately following prostatectomy. The technique described ensures standardization of a critical step during tissue processing, leading to uniform microscopic results with both routine and immunohistochemical stains. It is a simple, rapid method, suitable for routine diagnostic use. Using this modified approach, DNA of much larger sizes can be extracted from paraffin-embedded material, which could expand the possibilities for molecular analysis. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

One of the most widely used methods for tissue preservation is fixation with formalin. Besides important advantages, there are also drawbacks with formalin, such as the rather slow rate of fixation.[1] The long time involved in the fixation of large specimens is a major limitation in making a timely diagnosis. In addition, even after 24 h, solid specimens larger than 25 g are not always completely fixed.[2,3] Cutting the surgical specimen into smaller pieces facilitates fixation, but is undesirable for final anatomical orientation. Due to incomplete fixation, autolytic degradation of protein antigens and DNA may occur, but progressive formaldehyde fixation also has an adverse effect on many antigens.[4] The combination of prolonged exposure to formalin, especially at the periphery, with incomplete fixation in the centre of a large specimen may explain inconsistent immunohistochemical results.

The artefacts obtained with formalin fixation have encouraged the search for refinements of fixatives and techniques to preserve antigens. The best approach to preserve large specimens would be a short, intense fixation, to prevent formalin-induced and autolytic damage to protein antigens and DNA. For small tissue specimens, an evenly distributed fixation has been achieved by microwave treatment.[2,5] Similar results using microwave-stimulated fixation should be possible with large surgical specimens. We have therefore compared the results of fixation protocols using microwave irradiation with those of conventional fixation, with the prostate gland as a model.

MATERIALS AND METHODS

The initial development of the microwave-stimulated fixation method was carried out using 30 prostates from autopsy cases (maximum post-mortem interval 8 h). Each of the prostates weighed more than 50 g and was divided into two parts of equal size. These two parts were used to compare the degree of fixation between two protocols. Six different protocols were tested at least in duplicate. In all protocols, a 10 per cent

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Table I—The monoclonal antibodies used

Monoclonal antibody	Clone	Dilution	Source	Protocols (P) tested
E-cadherin	HECD-1	1:50	Takara, Berkeley, U.S.A.	P1: $N=42$, P4: $N=15$, P6: $N=95$
Ki-67	MIB-1	1:100	Immunotech SA, Marseille, France	P1: $N=20$, P4: $N=5$, P6: $N=50$
p53	DO1 and DO7	Both 1:100	Neomarkers, Fremont, U.S.A.	P1: $N=20$, P4: $N=5$, P6: $N=50$
Cytokeratin	CAM 5.2	1:20	Becton & Dickinson, San Jose, U.S.A.	P1: $N=10$, P4: $N=5$, P6: $N=10$

neutral-buffered formalin solution was used for fixation. There was no further exposure to formalin after the initial fixation.

Fixation protocols (P)

P1: Prostate parts were allowed to fix by formalin diffusion for 20–48 h at room temperature without microwave irradiation.

P2: The prostate parts were placed in a plastic container (diameter 10 cm, height 9 cm). Formalin was added to a total volume of approximately 400 ml (specimen and formalin volume together). The container was placed in a commercially available Miele M696 microwave oven. A temperature probe connected to the power control of the oven was inserted into the formalin solution. The microwave oven was programmed for a temperature of 50°C and a power level of 450 W for 6 min.

P3: These prostate parts were treated in a similar fashion to those in protocol 2. Immediately after the first microwave step, the machine was switched off for the subsequent 10 min. A second $3\frac{1}{2}$ min period of microwave irradiation followed, using the same temperature and power settings as those described above.

In protocols 4–6, prostate parts were perfused by injecting 100 ml of formalin into multiple sites (>50) of the gland using a fine needle (0·6 × 25 mm; Monoject, Sherwood Medical, West Sussex, U.K.).

The average time to perfuse the gland was 3 min. Most of the injected volume leaked immediately out of the gland. The mean increase in weight after perfusion of the gland was 3·1 g. After injections, a 15 min pre-soak period followed in formalin. After these treatments:

P4: conventional fixation followed according to protocol 1.

P5: microwave irradiation followed according to protocol 2.

P6: microwave irradiation followed according to protocol 3.

Macroscopic evaluation

After fixation, specimens were taken out of the formalin and immediately sliced at 4-mm intervals. Grey-white and firm areas were interpreted as completely fixed and red, soft tissue areas as incompletely fixed. Two patterns of incomplete fixation were recognized: a 'spotty' distribution for the admixture of both incompletely and completely fixed tissue areas and a 'crust effect' if the central part of the prostate slice was fixed incompletely. Paraffin tissue sections (4 μm) from two representative slices were stained with haematoxylin and eosin (H & E).

We continued the study with 152 radical prostatectomy specimens (mean weight 42 g, range 26–125 g), obtained from patients treated for clinically localized prostate cancer in the period from June 1993 to May 1997. These specimens were used to evaluate the quality of routine and immunohistochemical stains and of DNA preservation on tissue fixed with the microwave method (P6: $N=95$) compared with conventionally fixed specimens (P1: $N=42$ and P4: $N=15$). Directly following resection, the specimens were fixed uncut and completely submitted for histoprocessing. Routine stains included H & E, PAS, van Gieson's elastica stain, and Alcian blue.

Progressive formalin fixation

To investigate the time factor of formalin fixation on immunostaining, we used a fresh prostatectomy specimen. From the specimen, one piece was snap-frozen. Multiple other pieces of similar size were placed in formalin. After 1, 2, 3, 4, 8, 12, 18, 24, and 36 h, a piece was removed from the fixative and placed in physiological saline prior to histoprocessing.

Immunohistochemical staining

In prostatectomy specimens, all tumour blocks were used to evaluate the preservation of protein antigens. We evaluated the staining of monoclonal antibodies representing a selection of cytoplasmic (cytokeratin) and surface antigens (E-cadherin) in areas of benign epithelium present in the tumour blocks. Nuclear antigens were evaluated with Ki-67 and p53 in (tumour) cell nuclei (Table I).

The staining procedure for E-cadherin, Ki-67, and p53 was performed after microwave retrieval with two sequential 5-min intervals at 600 W in 0·1 M citrate buffer, pH 6·0. In addition, we compared the E-cadherin staining results with 20 specimens fixed according to P6 which were both treated and not treated with antigen retrieval. The staining procedure for cytokeratin was performed both with and without 10 min digestion in 0·1 per cent protease XIV at 37°C. Primary antibodies were incubated at 4°C overnight. A Vectastain Elite kit (Vector Laboratories, Burlingame, CA, U.S.A.) was used for peroxidase visualization.

Table II—Results of macroscopic inspection and H & E staining following the different protocols, using autopsy prostates

Protocol	Slicing of the specimen	Macroscopic inspection		H & E staining variability	
		Crust effect	Spotty	Crust effect	Spotty
1	Easy, no damage to tissue or capsule	+	+	+	+
2	Difficult, damage to tissue and capsule	+	+	+	+
3	As protocol 1	+	+	+	+
4	As protocol 1	−	−	−	−
5	As protocol 2	−	+	−	−
6	As protocol 1	−	−	−	−

+ = present; − = absent.

Table III—Results from autopsy prostates: range of changes induced by the variables of the fixation protocols (P)

Variables	Comparing protocols	Change induced
Microwave influence on fixation	Conventional vs. microwave irradiation (P1/2, P1/3, P4/5, P4/6)	Microwave irradiation intensified fixation significantly and consequently, less time was needed to fix the specimen
Formalin injections	Formalin injections vs. no injections (P1/4, P2/5, P3/6)	Injections ensured fixation of both the centre and the periphery of the specimens, resulting in absence of 'crust effects'
No. of microwave steps	One vs. two microwave steps (P2/3, P5/6)	Two steps ensured easy slicing of the specimen without damage to the capsule

Light microscopic evaluation

Stains were designated 'homogeneous' if the entire tissue section showed a similar staining pattern/intensity; 'variable', i.e. 'spotty' distribution, if different tissue areas differed in staining pattern/intensity; or a 'crust effect' if staining differences between the periphery and central part of the prostate were present.

Progressive formalin fixation

The reduction in staining intensity was expressed in percentages, with the frozen section staining result as a gold standard (100 per cent intensity).

DNA extraction

To evaluate the preservation of DNA, we randomly selected paraffin blocks from conventionally fixed specimens (P1, $N=4$) and from specimens that were fixed after formalin injections followed by microwave irradiation (P6, $N=4$). DNA extraction was carried out essentially as recently described.[6] DNA sizes were estimated by electrophoresis on a 1 per cent agarose gel with ethidium bromide staining and expressed in number of base pairs (bp). Relative amounts of DNA in regions of the agarose gels were estimated used a Biorad GelDoc 1000 system (Biorad, Hercules, CA, U.S.A.).

RESULTS

The results of macroscopic inspection of autopsy specimens processed with all protocols and their H & E staining results are shown in Table II. The range of changes induced by the variables of the fixation procedures in 30 autopsy prostates is summarized in Table III.

Prostate parts that were conventionally fixed (P1) usually showed macroscopically incomplete fixation, especially in the centre of the gland ('spotty' fixation with a 'crust effect'). The majority of the tissue sections showed intense eosinophilic H & E staining in the centre of the prostate, compared with the periphery of the gland ('crust effect'; Fig. 1a). These eosinophilic areas may be explained by ethanol-induced protein coagulation of the partially cross-linked tissue in subsequent histological processing steps.[1] The artefact, however, may also be attributed to improper processing or incomplete wax impregnation. With multiple injections into all sites of the intact gland (P4–6), no 'crust effects' were present. No tissue damage was present that could be attributed to the multiple formalin injections into the

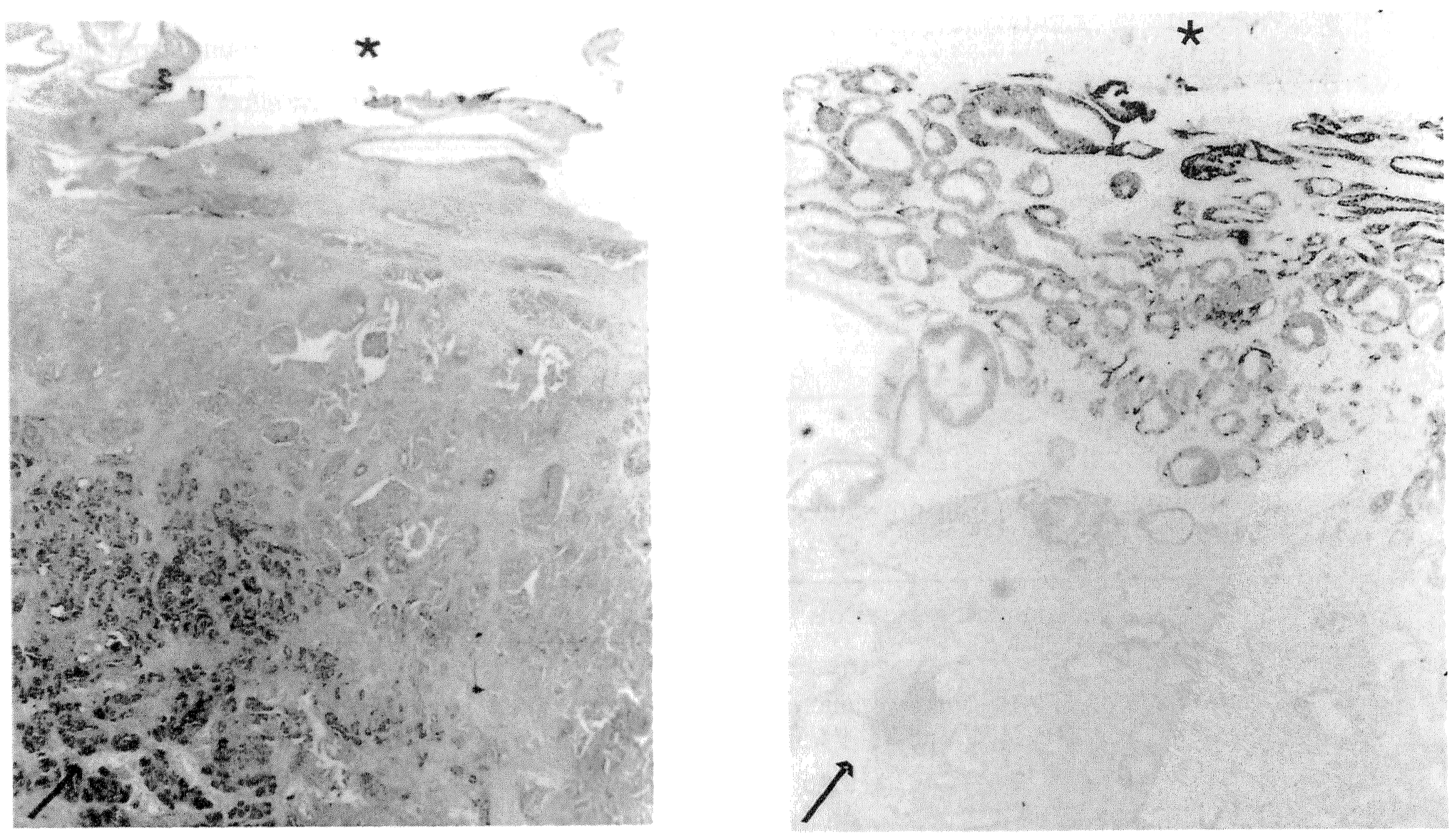

Fig. 1—Photomicrograph of an H & E-stained tissue section and an E-cadherin-stained tissue section. These specimens were fixed conventionally (protocol 1). The prostatic capsule is marked by an asterisk, and the centre of the gland with an arrow. (a) 10 × . Compared with the periphery of the gland, the H & E staining is more eosinophilic in the centre of the gland ('crust effect'). (b) 40 × . Only a small rim near the prostate capsule shows E-cadherin staining. The intensity decreases rapidly in the adjacent glands. The centre of the specimen does not show any immunoreactivity ('crust effect')

Table IV—Effects of different fixation protocols on E-cadherin staining

	Immunostaining			
	Homogeneous staining	Variable staining results		
Fixation protocol	Specific staining throughout the entire tissue section	'Spotty' distribution	'Crust effect'	Staining negative
Protocol 1 (N=42)	7 cases (16%)	4 cases (10%)	8 cases (19%)	23 cases (55%)
Protocol 4 (N=15)	8 cases (53%)	1 case (7%)	0 cases	6 cases (40%)
Protocol 6 (N=95)	88 cases (93%)	7 cases (7%)	0 cases	0 cases

gland. Microwave irradiation (P2, 3, 5, 6) significantly reduced the time necessary to fix the gland. Tissue architecture and cellular details were well preserved in all cases fixed according to P6 and results were independent of the prostate's prefixation weight.

Other routine stains

PAS, van Gieson's elastica, and Alcian blue staining patterns and intensities on tissue fixed by microwave irradiation (P6) could not be distinguished from those fixed by the conventional method (P1, 4).

Immunohistochemical staining

E-cadherin—Without any retrieval technique, expression of E-cadherin antigens was not detectable in any of the blocks. Table IV summarizes the effects of the fixation method on E-cadherin immunoreactivity. In 84 per cent of the conventionally fixed specimens (P1), for example, E-cadherin immunohistochemistry proved to be sub-optimal. A considerable number of these cases showed staining mainly localized as a small rim at the periphery of the gland (Fig. 1b). The number of cases with homogeneous immunostaining throughout the entire tissue section increased when specimens were fixed according to P4 (53 per cent) and even to 93 per cent with P6. Thus, the variables that improved the quality of immunostains are formalin injections and a shorter, intensified fixation, the latter illustrated in Fig. 2. Clearly, prolonged formalin fixation, as in P1 and P4, adversely influenced E-cadherin immunoreactivity.

Nucleus-specific staining (MIB-1 and p53)—p53 protein accumulation was present focally in tumour areas in a total of four cases. MIB-1 expression was found in all

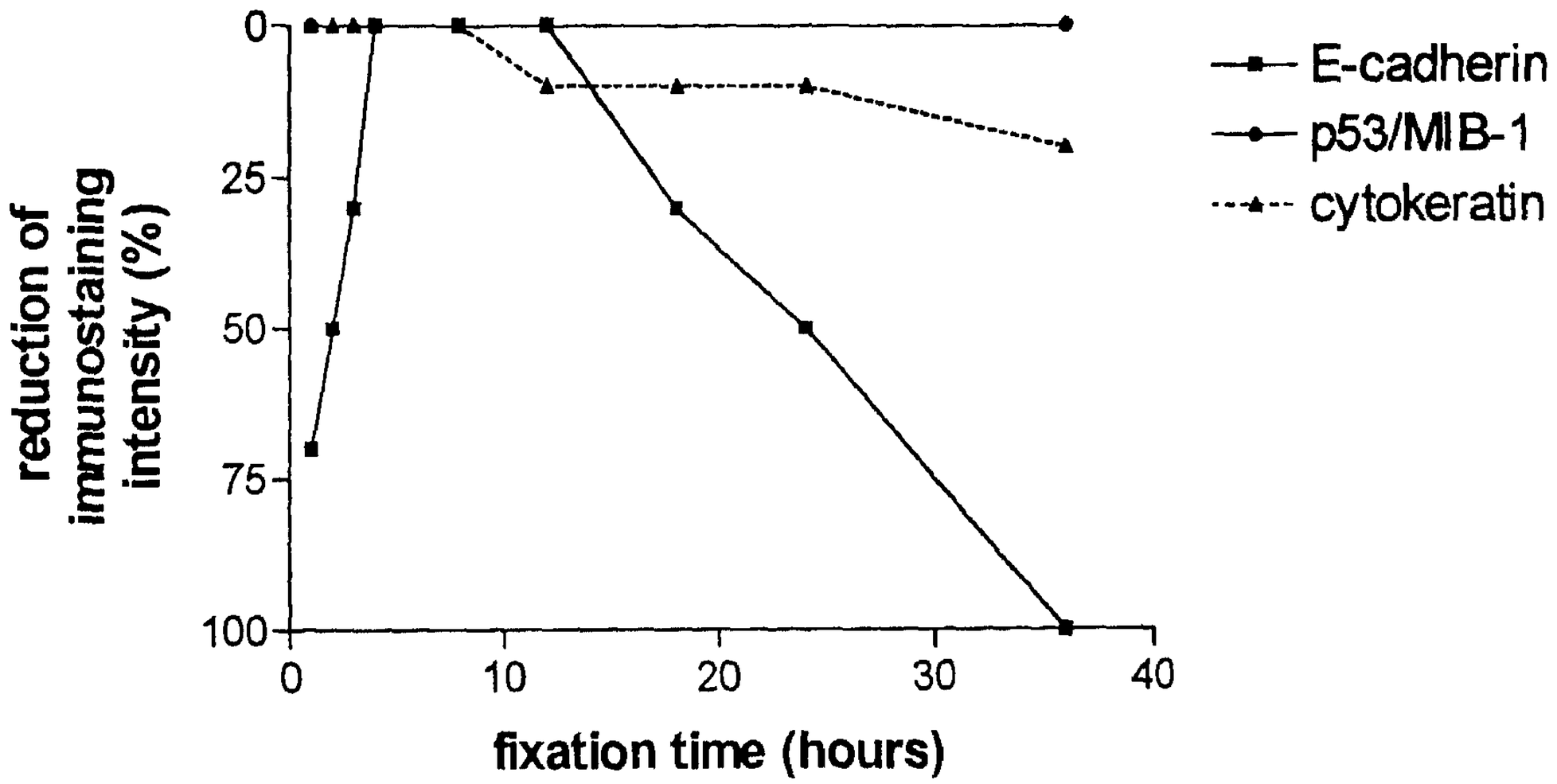

Fig. 2—Relationship between immunostaining and formalin exposure time during conventional fixation (P1)

tumours, and only focally in benign areas. In all cases, specific staining was present in nuclei only. The presence and distribution of stained nuclei appeared not to be dependent on the fixation time (Fig. 2) or on the choice of protocol.

Cytoskeleton-specific staining (cytokeratin)—In all prostates processed by P6, uniform staining was present throughout the sections. Pronase digestion did not significantly improve the staining intensity in P6, while the stains from P1 and P4 were always more intense after pronase digestion.

DNA extraction—Comparison of the DNA extracted from specimens fixed by P1 and P6 revealed that degradation was more pronounced when conventional fixation was used (Fig. 3). In the specimens fixed using microwave assistance (P6), over 75 per cent (mean 78 per cent, SD 5·4 per cent) of the DNA migrated at sizes over 1000 bp as estimated by agarose electrophoresis. By contrast, only 34 per cent (SD 9·5 per cent) of the DNA from conventionally fixed specimens (P1) was in this size range. The degradation was more apparent at larger sizes, since only 2·3 per cent of DNA from conventional fixation was larger than 4000 bp as opposed to 40 per cent of that extracted from microwave-fixed specimens.

DISCUSSION

In this study, the major goal has been to examine whether microwave irradiation can be used to fix large surgical specimens rapidly yet evenly, providing tissue which is morphologically, immunohistochemically, and molecularly well-preserved.

In protocols 1–3, macroscopic inspection revealed incomplete fixation. The 'crust effect' in protocols 1–3 was probably the result of formalin-fixed tissue in the periphery of the gland forming a barrier to further penetration of fixative. Microwave irradiation in protocols 2–3 reduced the time needed to fix the peripheral part of the gland, but left the centre unfixed. Only with multiple formalin injections into all parts of the intact gland (protocols 4–6) was this problem circumvented. The effect of formalin present throughout the prostate was further enhanced when the tissue underwent fixation using microwave irradiation (protocols 5 and 6). The second microwave step (protocol 6) allowed easy slicing of the specimen and consequently preservation of tissue morphology was then optimally achieved.

The additional effects of microwave irradiation during tissue fixation can be explained chemically. Aqueous solutions of formaldehyde used as fixatives contain mainly methylene glycol and its oligomers, but little formaldehyde.[7] The formaldehyde (HCOH) in the tissue is formed from methylene glycol [$CH_2(OH)_2$] as shown below:

$$CH_2(OH)_2 \rightleftharpoons HCOH + H_2O$$

The proportion of methylene glycol depends inversely on the temperature.[8] For fixation, the active component is formaldehyde, but it is mainly methylene glycol that rapidly penetrates tissues.[1] The formaldehyde binds very slowly to the tissue and hence disappears at a slow rate.

To maintain the chemical equilibrium, formaldehyde is formed by dehydrating methylene glycol. When microwave irradiation is started, this dehydration process is accelerated enormously due to the almost instantaneous and homogeneous increase in temperature.[3] In addition, heat-induced acceleration of diffusion of the fixative from the injection sites into the entire specimen allows acceleration of the reaction of formaldehyde with the tissue. Comparing tissue conventionally fixed after injections (protocol 4) with microwave fixation after injections (protocol 6), we presume that this accelerated process plays a crucial role in the preservation of E-cadherin antigens. It is not surprising, therefore, that superior fixation results are achieved by combining injection and microwave heating.

lane J, marker λ Hind III. Note the presence of larger-sized DNA fragments in lanes F–I

We believe that fixation is a 'balancing act'. The aim is to reach an optimal hybrid between time-progressive formalin damage of protein antigens, as clearly illustrated in Fig. 2, and autolytic degradation. The window of this optimal fixation is small. Relatively slow fixation for 20–48 h (protocol 4), for example, resulted in 'spotty' E-cadherin staining and sometimes in completely negative staining. The major advantage of protocol 6 is that the end-point of fixation is the same for all our specimens.

Variation in E-cadherin staining intensity, however, was still present in specimens fixed according to protocol 6, and this observation may be explained by incomplete diffusion of formalin after inadequate injections. Thus, heat exposure in the absence of fixative is probably not sufficient to stabilize protein antigens. This convinced us to allocate at least 3 min for the formalin injections followed by a 15-min immersion period, to ensure optimal distribution of the fixative throughout the prostate prior to microwaving. After this learning curve, there appeared to be still some variability in E-cadherin staining, even though these specimens were optimally and consistently handled. This could be explained by differences in tissue densities. Variability, however, was less distinct than in specimens fixed according to protocols 1 or 4.

Immunohistochemical stains for E-cadherin revealed another interesting finding. Cross-links, introduced by formaldehyde, can be responsible for antigen masking.[9] As early as 1948, it was reported that formaldehyde cross-links may be reversed, at least in part, by high temperature.[9–11] If formalin exposure is short (protocol 6; 34 min), it could be anticipated that retrieval techniques to unmask protein antigens would not be necessary. E-cadherin stains, however, were always completely negative if microwave retrieval was omitted. Our findings may illustrate that high temperatures during microwave retrieval not only hydrolyse formaldehyde-induced cross-links, but also change the three-dimensional structure of protein antigens, reducing steric hindrance. This hypothesis is further supported by data from ethanol-fixed tissue blocks. Although these tissue specimens lack any formaldehyde-induced cross-links, MIB-1 immunoreactivity is present only after microwave antigen retrieval.[12]

Another important advantage of microwave fixation was the possibility of omitting enzyme digestion in the cytokeratin staining procedure. Apparently, both MIB-1 and p53 immunoreactivity and the routine stains PAS, Alcian blue, and van Gieson's elastica were more resilient to different fixation protocols.

Finally, it appears that DNA degradation is reduced by microwave fixation. This might be explained by inhibition of endogenous DNase activity by immediate fixation. Using our modified approach, DNA of much larger sizes can be obtained from paraffin-embedded material, which could expand the possibilities for the use of this material in molecular analysis.

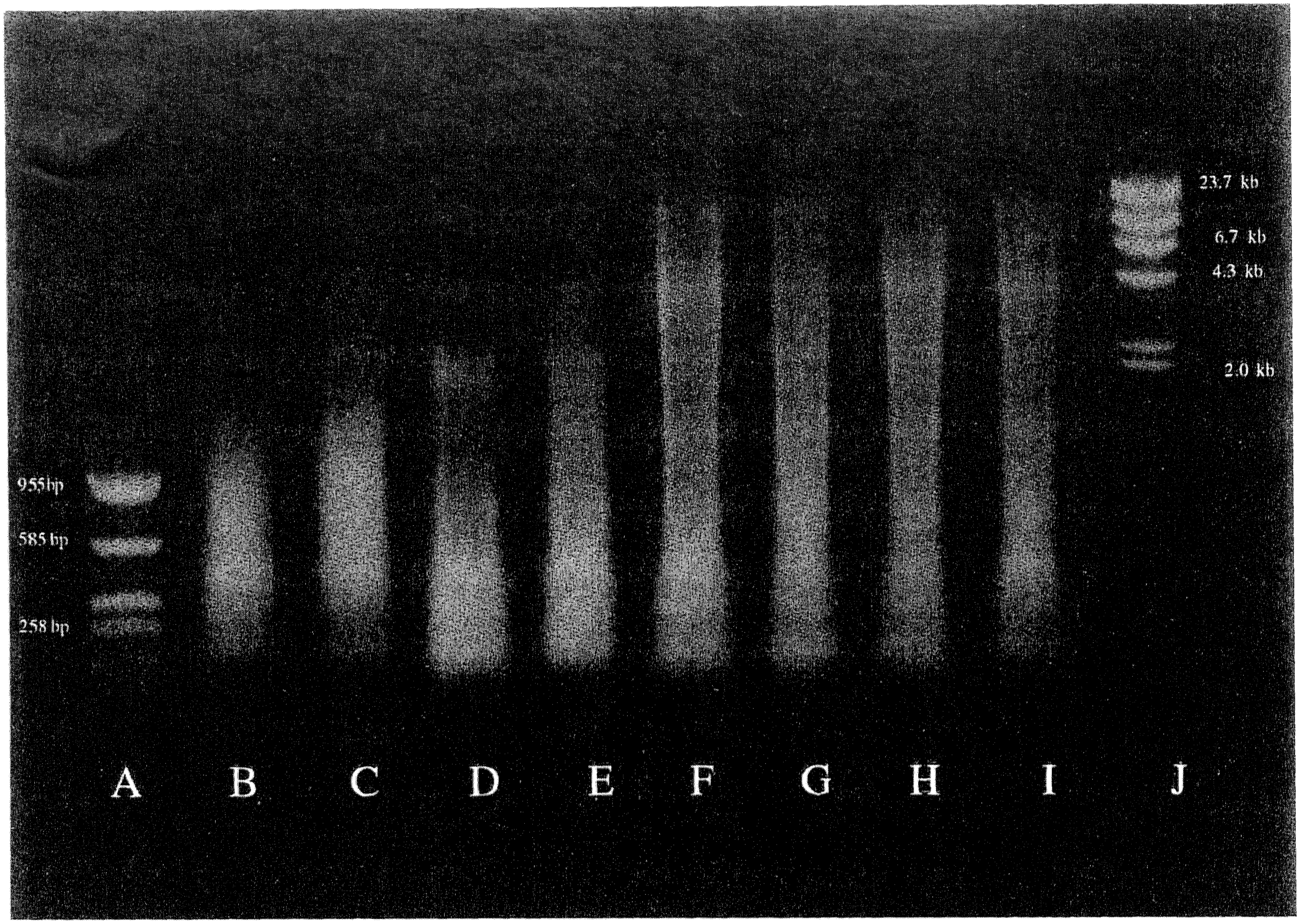

Fig. 3—Ethidium bromide-stained 1 per cent agarose gel showing the size distribution of double-stranded genomic DNA, extracted from the paraffin sections. The length of DNA fragments is expressed in the number of basepairs (bp). Lane A, marker Puc 18; lanes B–E, conventional fixation for 20 h (protocol 1); lanes F–I, microwave-stimulated fixation (protocol 6);

Although the practical aspects of the technique described are simple and straightforward, it should be noted that heated formalin vapours are potentially carcinogenic; the procedure should therefore be carried out only under an appropriate fumehood.

In conclusion, the advantages of microwave-stimulated formalin fixation of radical prostatectomy specimens are (1) rapid, standardized fixation, applicable for daily routine; (2) a thorough preservation of morphology; (3) improved immunoreactivity, resulting in reliable, almost homogeneous immunostaining in the majority of cases; and (4) decreased degradation of DNA. Standardization of the fixation process is important for the consistent interpretation of conventional and immunohistochemical stains and could be particularly helpful if different hospitals are providing specimens for central analysis. In addition to clinically orientated studies, the method described here has important applicability to studies of cancer pathogenesis, allowing the intensive investigation of genetic alterations through improved DNA quality in paraffin material.

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REFERENCES

1. Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. *J Histochem Cytochem* 1985; **33**: 845–853.
2. Boon ME, Kok LP. In: Kok LP, ed. Microwave Cookbook for Microscopists. Art and Science of Visualization. 3rd edn. Leyden: Coulomb Press, 1992.
3. Boon ME, Gerrits PO, Moorlag HE, Nieuwenhuis P, Kok LP. Formaldehyde fixation and microwave irradiation. *Histochem J* 1988; **20**: 313–322.
4. Leong ASY, Gilham PN. The effects of progressive formaldehyde fixation on the preservation of tissue antigens. *Pathology* 1989; **21**: 266–268.
5. Hopwood D, Coghill G, Ramsay J, Milne G, Kerr M. Microwave fixation: its potential for routine techniques, histochemistry, immunocytochemistry, and electron microscopy. *Histochem J* 1984; **16**: 1171–1191.
6. Isola J, De Vries S, Chu L, Ghazvini S, Waldman F. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. *Am J Pathol* 1994; **145**: 1301–1308.
7. Walker JF. Formaldehyde. 3rd edn. New York: Reinhold, 1964: 110–111.
8. Le Botlan DJ, Mechin BG, Martin GJ. Proton and carbon-13 nuclear magnetic resonance spectroscopy of formaldehyde in water. *Anal Chem* 1983; **55**: 587–591.
9. Taylor CR, Shi SR, Cote RJ. Antigen retrieval for immunohistochemistry. Status and need for greater standardization. *Appl Immunohistochem* 1996; **4**: 144–166.
10. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. *J Biol Chem* 1948; **174**: 827–843.
11. Shi SR, Imam A, Young L, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J Histochem Cytochem* 1995; **43**: 193–201.
12. Poel van der, Boon ME. Microwave-antigen retrieval for proliferation analysis (MIB-1) and quanticyt karyometry of bladder washings. *Eur J Morphol* 1994; **32**: 71–78.