Microwave Processing and Ethanol-Based Fixation in Forensic Pathology

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Abstract: An ethanol-based fixative (FineFIX) has been used, together with rapid microwave-stimulated processing, in postmortem material, resulting in a rapid fixation and processing of the tissues with morphology, histochemical stains, and immunocytochemistry comparable to formalin-fixed material. Furthermore, this alternative fixation gives better DNA recovery in higher amounts if compared with DNA extracted from formalin-fixed tissue, particularly advantageous in forensic pathology.

Key Words: forensic pathology, FineFIX fixative, microwave processing, immunocytochemistry, DNA extraction

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Formalin fixation has been considered a "standard" for over 1 century as it gives a good preservation of morphology and consequently allows a standardized classification of diseases.

The problems derived from its action on proteins, namely, the demonstration of some tissue antigens, have been solved with the introduction of antigen retrieval protocols; however, until today one of the major problems connected with formalin-fixed material is represented by the recovery of nucleic acids with usually poor results.[1] In fact, although formalin-fixed and archived tissues are a huge source of DNA for molecular biologic studies, attempts to extract DNA from formalin-fixed tissues have been variably successful[2]

Another important problem relates to toxicity of formaldehyde and formalin solution. A very recent statement by the International Agency for Research on Cancer (IARC)[3] reports that there are enough evidences of carcinogenic action of this chemical. This suggests the need of an alternative fixation to overcome the negative aspects of formalin, without a loss in morphology.

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A group of alternatives is commercially available, some of them based on glyoxal (a dialdehyde), some based on ethanol, while in some others, both chemicals are present.

Ethanol is a well-known fixative, used even before formaldehyde, but the presence of nuclear pyknosis and intense tissue shrinkage has led to its dismissal, with the exception of cytologic specimens, where it is still used.

The available alternatives are mixtures of chemicals that try to prevent the negative aspects of pure ethanol fixation.

The introduction of microwave processors has greatly enhanced the speed of tissue-processing protocols currently used. In fact, biopsy material can be processed in roughly 30 minutes, while bigger specimens—surgical and autopsy (thickness 3–4 mm)—can be processed in 3–4 hours, largely depending on the fat content of the tissues.[4–6]

Microwaves can be used also to stimulate the fixation of specimens, with good, rapid results either using formalin or its alternatives.

MATERIALS AND METHODS

The present series of cases refer to postmortem material, with some cases taken from necropsies performed on forensic cases (Legal Medicine Section, Bologna University, Italy) and others from hospitalized patients (Borgo Trento Hospital, Verona and Monselice Hospital, Italy) (Table 1).

The delay from death and postmortem examination varies from 24–72 hours for hospitalized patients up to about 6 days for forensics.

The patients have been randomly selected, and the specimens collected were partly fixed in formalin solution (neutral buffered formalin) and partly in FineFIX (Milestone, Bergamo, Italy), an ethanol-based fixative, where its concentration is 72%, with the addition of water, glycol, and polyalcohols, creating mirror blocks of the same tissues.

The quantity of FineFIX used for fixation was roughly the same as formalin.

Part of the specimens were processed in a microwave processor (RHS1, Milestone), using the standard protocols consisting in 1 step of dehydration and fat extraction, followed by a "vaporization" step (under vacuum) and a last step of paraffin impregnation, also under vacuum.

The remaining specimens were processed in a conventional processor (ATP 1; Kaltek, Padova, Italy) through ascending alcohols, xylene, and paraffin cycle.

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TABLE 1. Causes of Death in Autopsies

Forensic Cases	Hospitalized Patients
Multiple injuries in car passenger	Myocardial infarction
Planned complex suicide by gunshot wound of the head and hanging	Thromboembolism
Drug-related fatalities	Bronchopneumonia
Suicidal shotgun wound to the chest	Neoplasm

All the specimens were stained with H&E in an automated histostainer for morphologic evaluation. In selected material, some histochemical and immunocytochemical reactions were performed using an automated immunostainer (Benchmark; Ventana, Tucson, AZ), without changing the protocols.

Finally, part of the material was selected for DNA extraction. Twelve mirror blocks, 6 taken from formalin-fixed and 6 from FineFIX-fixed material, were analyzed, representing 4 kinds of tissues: cerebellum, brain cortex, heart, and lung.

Two 10-mm-thick sections were cut with a microtome from each paraffin block and placed in a 1.5-mL microcentrifuge tube. The section*s* were deparaffinized at 37°C under agitation and further steps in ethanol, together with a negative DNA control.

All samples were digested overnight with proteinase K solution (20 mg/mL) at 56°C, followed by denaturation at 95°C for 10 minutes. The isolated DNA was treated according to a commercial Kit (Qiagen-Experteam Sas; Marghera, Venice, Italy) and diluted in 200 μL H_2O.

Tissue samples, together with negative control of extraction and amplification, were amplified by PCR. Twenty microliters of each sample was amplified by 25 cycles of PCR using primers for housekeeping gene β-globin (256 bp).[7] To evaluate the amount of the purified DNA, 10 μL of the DNA solution were run on agarose gel 2%.

DNA was visualized with ethidium bromide staining.

RESULTS

All the specimens fixed in FineFIX were diagnostic, with only minor differences versus formalin-fixed material. The differences were slightly more eosinophilia of cellular cytoplasm and background substance, with an accentuated clumping of chromatin. Red blood cells were usually evident, especially in the traditionally processed specimens, with well-evident external membrane expression and a decreased staining of the internal part (Figs. 1–5).

Detachment from basement membrane and epithelium was more accentuated in FineFIX-fixed material, especially in tissues where epithelium lies over a more dense connective tissue (eg, breast tissue and prostate).

In microwave-processed material, there was a slightly more evident lysis of red blood cells, probably due to the action of the electromagnetic waves themselves.

Histochemical stains revealed an equal expression in formalin and FineFIX-fixed material (Fig. 6).

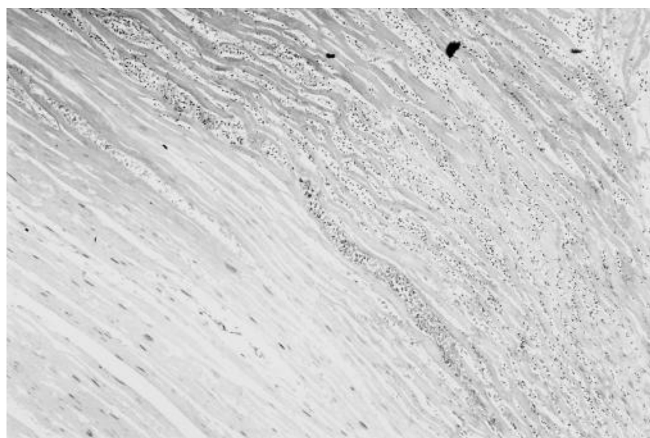

FIGURE 1. FineFIX, H&E stain. Myocardial infarction with coagulative changes and neutrophils infiltration between the fibers.

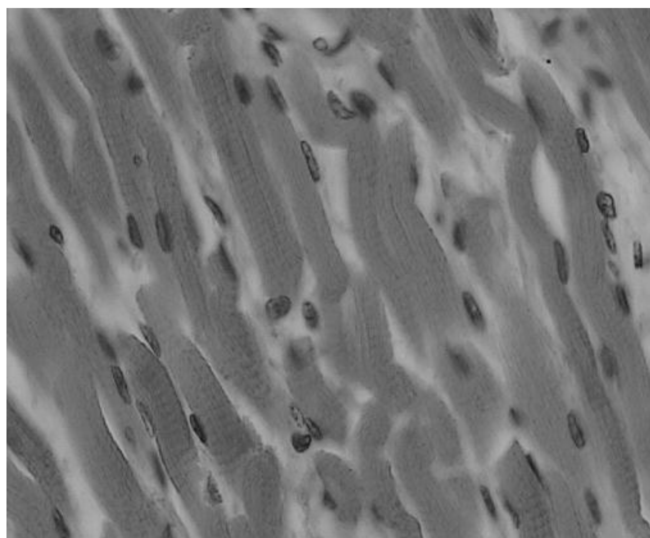

FIGURE 2. FineFIX, H&E × 600). "Wavy fibers" with evident cross-striation surrounding myocardial infarct.

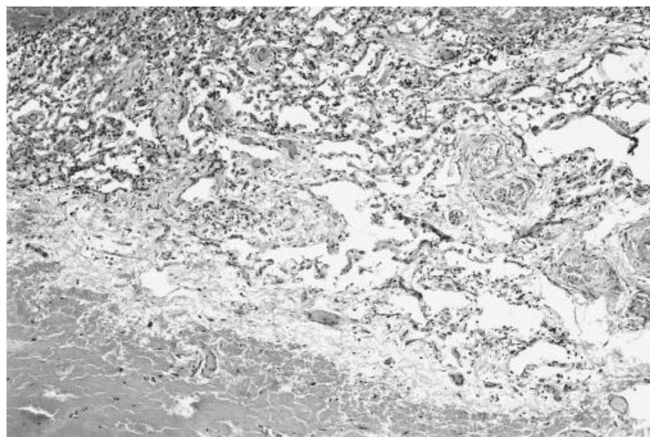

FIGURE 3. FineFIX, H&E stain. Lung hemorrhagic areas in traumatic injury.

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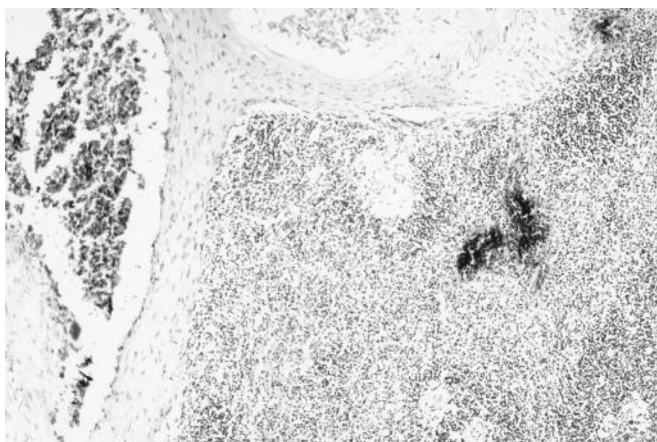

FIGURE 4. FineFIX, H&E stain. Spleen parenchyma.

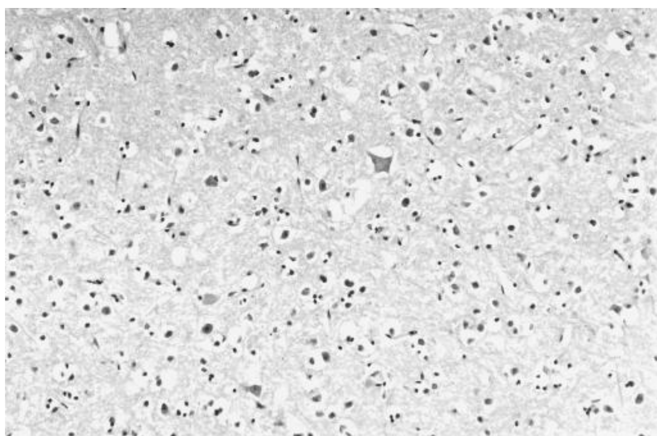

FIGURE 5. FineFIX, H&E stain. Cerebral tissue in drug-related fatality.

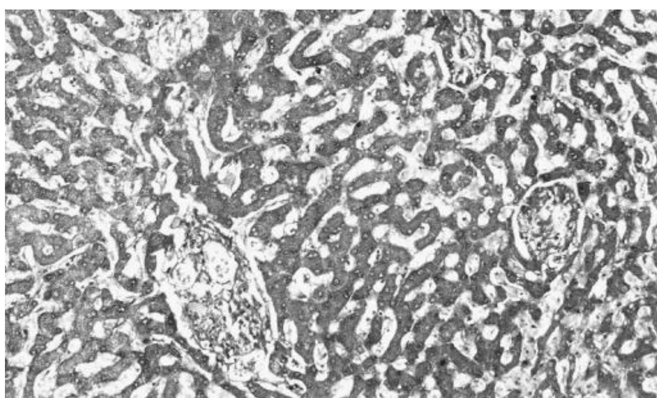

FIGURE 6. Azan Mallory, FineFIX. Connective tissue stain.

Also, the immunocytochemical reactions revealed good antigen preservation, without cross-reactivity (Figs. 7–11).

Regarding DNA extraction, FineFIX-fixed material revealed an improved and homogeneous recovery of it compared with a weak or absent extraction in the formalin-fixed material. Furthermore, the DNA seemed not as degraded as with formalin and in higher amounts.

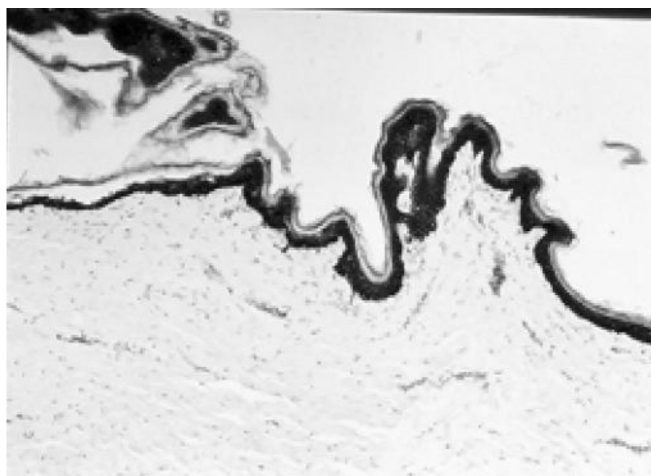

FIGURE 7. Immunohistochemical expression of cytokeratins AE1–AE3 in ligature mark (skin).

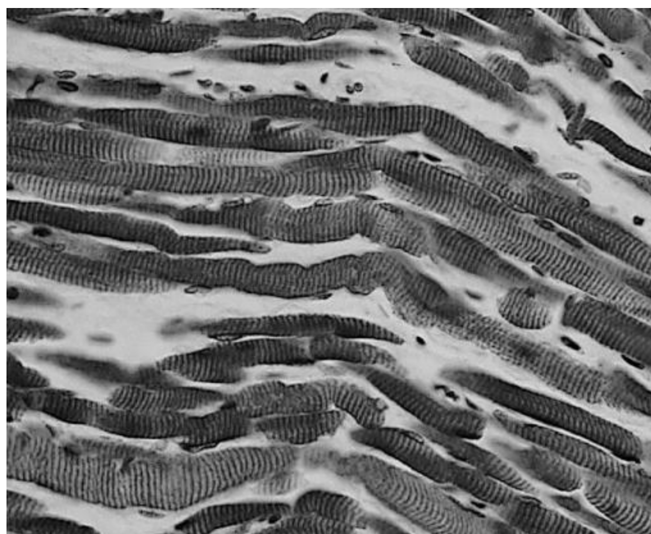

FIGURE 8. Immunostaining of cardiac muscle showing strong desmin expression (FineFIX).

DNA was extracted in all the FineFIX specimens, regardless of the time delay from death and fixation (Fig. 12).

DISCUSSION

Formalin is the most used fixative in routine surgical and forensic pathology, and the morphology resulting from that fixation is considered the "standard" one. From a safety point of view, nowadays it is considered carcinogenic, and thus its use should be discarded or greatly reduced.[3]

In recent years, a number of formalin alternatives have been developed but their use has not become widespread. In our experience, an ethanol-based fixative (FineFIX) revealed

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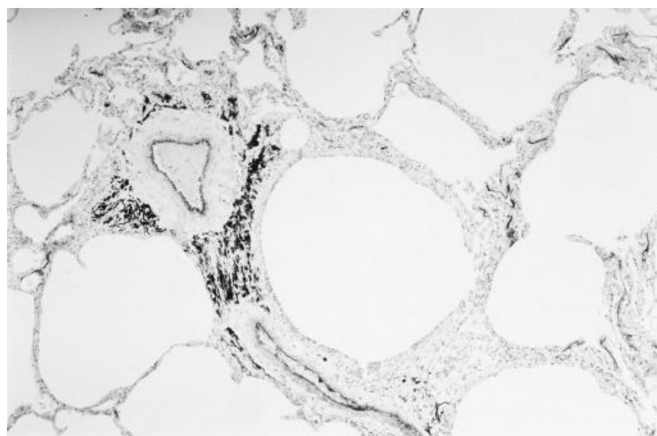

FIGURE 9. CD 34 positivity in lung parenchyma showing high vessel density (FineFIX).

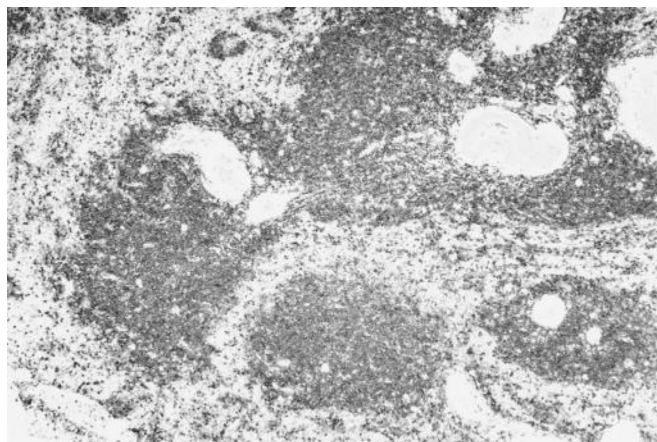

FIGURE 10. Leukocyte common antigen (LCA) expression in spleen parenchyma (FineFIX).

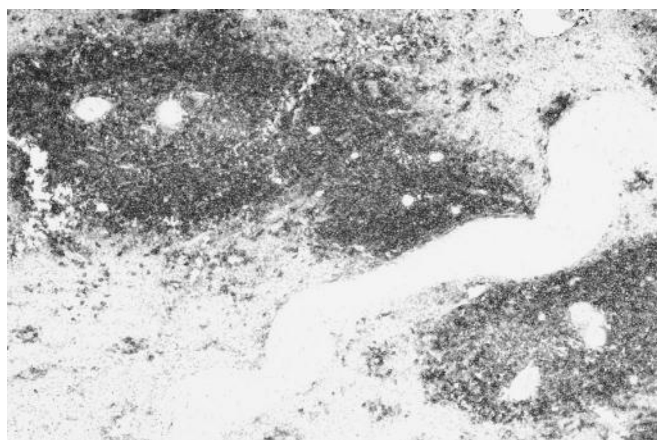

FIGURE 11. B marker CD20 positivity in spleen follicles (FineFIX).

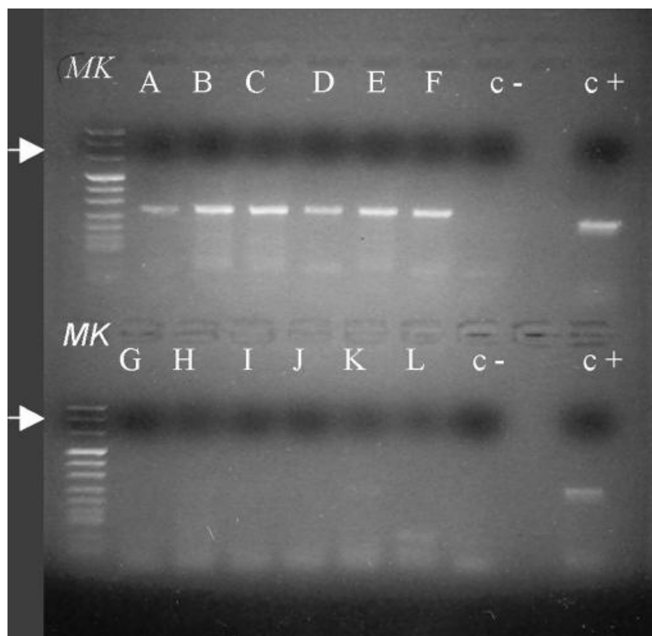

FIGURE 12. DNA analysis legend. MK: molecular weight marker VIII (Roche). A–F, FineFIX fixed samples. G–L, Formalin-fixed samples. c+, Positive control of the PCR reaction. c−, Negative control of the PCR reaction. A, E, G, L, Lung tissue. B, H, Cerebellar cortex. C, F, I, K, Cardiac tissue. D, J, Brain cortex.

a good preservation of morphologic details, histochemical and antigenic properties of the tissues. Regarding morphology, the differences were minor, and they did not interfere with the interpretation of the slides. It may be used in both conventional and microwave histoprocessors, the last allowing a rapid fixation and processing of the blocks, with a morphology comparable to that of conventional processed material.[8]

The rapid tissue stabilization operated by microwave irradiation may be particularly useful in forensic pathology, where phenomena of autolysis and putrefaction can greatly affect morphology and antigenic preservation of the specimens. The rapid action of microwaves reduces the time of fixation, allowing reduction of such artifacts.

But the real advantage of ethanol-based fixation is in the molecular biology field, where ethanol acts as a good preserving agent of nucleic acids and protein material. In fact, recent articles indicate that paraffin blocks may be used as a source of material for proteomic analysis when the tissue has been fixed in ethanol or ethanol-based fixatives.[1]

Furthermore, the risk ratings of FineFIX are only regarding its flammability, thus allowing a real reduction of hazards for pathologists and technicians, with creation of a safer working environment.

The increasing importance of molecular biology tests in both surgical and forensic pathology leads to the need of new protocols in tissues fixation that allow good recovery of genetic and protein material. The differences in morphologic

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expression of the tissues are not dramatic and can be overcome by the application of optimal fixation procedures and by a training period of morphologic comparison between formalin- and nonformalin-fixed specimens.

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