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Alcohol based fixatives provide excellent tissue morphology, protein immunoreactivity and RNA integrity in paraffin embedded tissue specimens

Rositsa Milcheva[a,c], Pavol Janega[a], Peter Celec[b], Russy Russev[c], Pavel Babál[a,*]

[a] Department of Pathology, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 81372 Bratislava, Slovakia
[b] Department of Molecular Biomedicine, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 81372 Bratislava, Slovakia
[c] IEMPAM, Bulgarian Academy of Sciences, "Acad. G. Bonchev" Str. 25, 1113 Sofia, Bulgaria

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ABSTRACT

Fixation techniques preserving morphological fidelity, protein antigenicity and integrity of nucleic acids can have a high impact on both basic and applied biomedical sciences and diagnostic pathology. Different types of mouse tissues were fixed with neutral buffered formalin, ethanol supplemented with acetic acid and modified methacarn (methanol-Carnoy) fixative. The alcohol-fixed samples were processed in an Autotechnicon tissue processor or in an incubator. The preservation of tissue morphology was assessed in all specimens and the immunoreactivity was evaluated with antibodies specific for proteins with nuclear, membrane or cytoplasmic localization. RNA was extracted from all groups of fixed hind limb skeletal muscle specimens and was assessed versus unfixed tissue for preservation of its quantity and quality by amplification of gene-specific fragments of different lengths. Both alcohol-based fixatives preserved the tissue architecture and the specificity of immunoreactivity in excellent quality; the trimming approach did not result in detectable differences. Oligonucleotide fragments of length between 108 and 577 base pairs were amplified from all groups of alcohol-fixed skeletal muscle specimens in amounts comparative to the unfixed muscle tissue. We conclude that both alcohol-based fixatives are an excellent tool for storage of tissue samples designed for immunohistochemical and mRNA expression studies when the access to fresh samples is limited.

Introduction

Limited access to fresh experimental or diagnostic tissue samples for molecular analysis is a common problem for many researchers. A well-established approach providing long-term preservation of tissue samples involving fixation and embedding techniques in various media is essential in histotechnology and diagnostic pathology. Considering the progress in molecular biology in experimental research that has also become routine in diagnostic pathology (Babál, 2002), the adaptation of archival tissue specimens to molecular biological techniques would have a high impact on both basic and applied biomedical sciences.

For over a century, a 10% aqueous solution of formalin (4% formaldehyde) has been widely used as a fixative in histology and immunohistochemistry owing to the ability of the formaldehyde to create covalent bonds between the biological macromolecules, thus preserving the tissue architecture (Fox et al., 1985; Hayat, 2002). RNA extracted from formaldehyde-fixed paraffin-embedded specimens (FFPE) is chemically altered (Cox et al., 2006) and may be fragmented depending on the fixation time, embedding and storage conditions (von Ahlfen et al., 2007), which makes formaldehyde fixation less suitable for molecular studies. Several studies have evaluated the usefulness of alcohol-based fixatives for gene expression analysis (Shibutani et al., 2000; Gillespie et al., 2002; Cox et al., 2006; Dotti et al., 2010). In parallel, techniques for quantification of gene expression became optimized for routinely used formalin-fixed and paraffin embedded specimens (Wang et al., 2009; Mariastefania et al., 2010). Therefore, a contribution to the technical knowledge with a specification and popularization of a fixative that could be equally reliable for the needs of histomorphology, immunohistochemistry and RNA expression studies that require preservation of RNA in more intact form seems to be opportune.

Abbreviations: Ab, antibody; bp, base pairs; c(g)DNA, complementary (genomic) deoxyribonucleic acid; EtAc-A(M), ethanol with acetic acid and automated (manual) trimming; FFPE, formaldehyde fixed paraffin embedded; HE, hematoxylin and eosin; MetAc-A(M), methanol with acetic acid (modified methacarn fixative) and automated (manual) trimming; NBF, neutral buffered formalin; e(n)NOS, endothelial (neuronal) nitric oxide synthase; dNTP, deoxynucleoside triphosphate; OD, optical density; PCR, polymerase chain reaction; PCNA, proliferating cells nuclear antigen; PPIA, peptidyl prolyl isomerase A; RT, reverse transcription.

* Corresponding author at: Department of Pathology, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 81372 Bratislava, Slovakia.
E-mail address: pavel.babal@fmed.uniba.sk (P. Babál).

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Table 1
Fixatives used in the study and conditions of tissue processing. After fixation, the specimens were submitted to routine automated processing (A), or manual trimming (M). The abbreviations in this table are valid throughout the text and legends.

Fixative	Details	Trimming
Neutral 4% buffered formaldehyde (10% NBF)	10% aqueous solution of formalin (v/v) in phosphate buffer (PBS) with 33 mM NaH_2PO_4 and 48 mM Na_2HPO_4, pH 7.4	A
Modified methacarn (MetAc)	8:1 (v/v) methanol and glacial acetic acid (Cox et al., 2006)	A and M (2 × 100% ethanol 1 h, 60 °C, 2 × isopropanol 1 h, 56–58 °C, paraffin)
Acidic alcohol (EtAc)	8:1 (v/v) absolute alcohol and glacial acetic acid	A, M

In this study we evaluated the reliability of methanol- and ethanol-based fixatives, both supplemented with glacial acetic acid, for preservation of tissue morphology, protein immunoreactivity and integrity of RNA, in terms of quality suitable for molecular studies. Since this study was oriented more to research units, where the automated facilities for routine histology are not commonly used, we also compared two approaches of tissue processing: automated and manual. Considering the histological and immunohistochemical evaluation, both alcohol-based fixatives were investigated versus 10% neutral buffered formalin that is routinely used in histopathology laboratories. When the quality of RNA was assessed, the alcohol-based fixatives and the neutral buffered formalin were compared with unfixed skeletal muscle tissue.

Materials and methods

Experimental animals and tissue preparation

Three healthy BALB/C male, 6–8 weeks-old mice, 30 g in weight, housed under standard conditions, were sacrificed by intraperitoneal injection of an overdose of thiopental (0.02 mg/g). The mice were maintained in the experimental animal unit of the Department of Molecular Biomedicine at the Faculty of Medicine, Comenius University in Bratislava, and were treated in accordance to the local Ethical Committee requirements. All protocols for the study were approved by the institutional Ethical Committee. Specimens from brain, small intestine, kidney, liver, spleen and hind limb skeletal muscle were fixed for 48 h in freshly-prepared 10% neutral buffered formalin (NBF), ethanol and methanol, both supplemented with glacial acetic acid (EtAc and MetAc, respectively). Details regarding the preparation of the three fixatives are listed in Table 1. Fresh skeletal muscle specimens were stored at −80 °C. All fixed specimens were submitted to routine and automated processing (indexed as A) for 17 h, as follows: 70% ethanol for 30 min, 96% ethanol for 7 h (with six changes), xylene for 2.5 h (three changes), paraffin wax for 7 h (two changes). Specimens fixed in the alcohol-based fixatives were also processed manually (indexed as M), following a modified protocol of Cox et al. (2006). Briefly, specimens were immersed in absolute ethanol for 1 h (two changes), then isopropanol for 1 h (two changes) and infiltrated in molten paraffin wax for 10–12 h. All treatments were performed at 60 °C. The fixatives used in the study as well as the trimming procedures are summarized in Table 1. Differently fixed and trimmed specimens of each tissue were assembled into a tissue array in a single paraffin block and sections 5 μm thick were routinely stained with hematoxylin and eosin (H&E) for basic morphological evaluation (three sections per mouse) and for immunohistochemistry (four sections per mouse). Skeletal muscle specimens intended for molecular studies were embedded separately and the blocks were stored at room temperature and used for RNA isolation after a period of one week to six months.

Immunohistochemistry

The immunoreactivity of the normal tissue specimens was investigated with several antibodies specific for proteins with nuclear, membrane or cytoplasmic localization. Mouse monoclonal antibody (Ab) against proliferating cells nuclear antigen (PCNA, Dako, Glostrup, Denmark) was applied on small intestine specimens, rabbit monoclonal antibody against T-cell CD3 antigen (Thermo Scientific, Fremont, CA, USA) on spleen specimens and rabbit polyclonal antibodies against endothelial nitric oxide synthase (NOS3, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and neural nitric oxide synthase (nNOS, BD Transduction Laboratories™, San Jose, CA, USA) isoforms on kidney specimens. These tissues were chosen as recommended positive tissue specimens for the selected antibodies according to the on-line Knowledge Database of IHC World available at www.ihcworld.com. After optimization, each immunoreaction was performed on differently fixed and trimmed specimens of each tissue assembled in the tissue array, four sections per mouse.

Archival specimens of mouse skeletal muscle in early stages of myopathy induced by *Trichinella spiralis* from previous studies (unpublished) that were fixed with 10% NBF and MetAc-M, were treated with rabbit polyclonal Ab against caspase-3 (Cell Signaling Technology, Danvers, MA, USA). The significance of this contribution to the main study was comparison of the immunoreactivity under pathological conditions in specimens fixed with alcohol-based fixative and processed manually.

Parallel 5 μm thick sections from all specimens were stained with H&E and immunohistochemically with and without microwave antigen retrieval. The antigen retrieval step was performed in 10 mM citrate buffer, pH 6.2, at sub-boiling temperature (750 W microwave) for 1, 5 and 10 min. The duration of retrieval step for each antibody or material, the tested concentrations of the antibodies and the incubation times are listed in Table 2.

The primary antibodies were diluted in Dako Real antibody diluent (Dako, Glostrup, Denmark). Before application of the primary Ab, the endogenous peroxidases were extinguished by EnVision™ Peroxide-blocking reagent (Dako). The sections were subsequently incubated for 30 min with EnVision™ anti-rabbit (in case of CD3, nNOS, eNOS and caspase-3 Ab) or anti-mouse (in case of PCNA) polymer conjugated with horseradish peroxidase (Dako). Before application of an anti-mouse secondary Ab, the sections were incubated for 10 min with EnVision™ serum-free protein blocking solution (Dako) to diminish the non-specific reaction and background. The immunoreactivity was visualized with 3,3′-diaminobenzidine (Dako). In parallel, negative control specimens were incubated with Dako Real antibody diluents (Dako) instead of Ab and thereafter with the corresponding peroxidase conjugate. The immunohistochemical reactions were performed at least in triplicate on four sections per mice. The location and intensity of the PCNA, CD3, nNOS, eNOS and caspase-3 expressions were evaluated using a Nikon Eclipse 80i light microscope (Nikon, Kanagawa,

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