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TECHNICAL NOTE

Ultrasound-accelerated formalin fixation improves the preservation of nucleic acids extraction in histological sections

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Abstract The aim of the present study was to examine an ultrasound-accelerated fixation technique that reduces the exposure time of the tissue to formaldehyde with respect to the analysis of nucleic acids. We extracted and analysed DNA and RNA from three series of autopsy specimens from five routine cases. Two series were shortly fixed in 4% buffered formalin (15 and 30 min, respectively) whilst being irradiated with high-frequency, high-intensity ultrasound. The last series (control) was routinely fixed in 4% buffered formalin for 24–48 h without irradiation. Although sufficient amounts of DNA of good quality could be extracted and amplified from all three series, the peak heights obtained from conventional fixation were smaller and allele dropout occurred more often, especially for the longer amplicons. RNA yield depended on the fixation procedure, i.e. the shortest fixation time led to the highest RNA yield and quality. No differences were observed with regard to the quality of the histological slides both with conventional and immunohistochemical staining methods. Keeping in mind the increasing need for molecular diagnosis, this fixation technique can be useful to ensure stable quality of nucleic acids in archived autopsy specimens.

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Introduction

Formalin fixation and paraffin embedding are the standard techniques to preserve biological material for storage and histological analysis [1, 2] as the high reactivity makes formaldehyde ideal for preserving tissue structures. Nucleic acids extracted from formalin-fixed archival samples are known to be of poor quality [3, 4]. Nevertheless, these samples are often needed for DNA [5] as well as gene expression analysis (e.g. [6, 7]). The effects of formalin on nucleic acids have been analysed [4, 8, 9] and consist of various chemical modifications rather than degradation (e.g. [1]). Upon storage, these modifications can lead to further fragmentation. The effects of formalin are also associated with the spread of diffusion. The quantity and quality of DNA extracted decreases with increasing fixation time [10], and a strong correlation between fixation time and chemical modifications of RNA seems to exist [1]. Additionally, formaldehyde treatment can be an obstacle for RNA extraction [11].

Thus, there is a rising need for fixation techniques that improve the quality and quantity of the extracted nucleic acids [12]. Ultrasound promotes the speed of tissue penetration [13]. A technique of ultrasound-accelerated formalin fixation (UAFF) that considerably shortens the fixation process and preserves antigens and nucleic acids has been proposed [14]. The aim of the present study was to investigate whether this technique increases the quality of extracted nucleic acids and whether the reduction of fixation time affects the quality of histological analyses.

Materials and methods

Three series of autopsy specimens from five routine forensic cases were collected within 48 h postmortem, i.e. samples of heart, lung, brain, pancreas, kidney, spleen and liver.

Fixation

One of the series (reference) was fixed for 24–48 h in 4% buffered formalin (Formaldehyd-Lösung 3.5–3.7%, Otto Fischer GmbH, Saarbrücken, Germany) and then processed according to standard protocols [1]. In this group, we provide the minimal and maximal fixation time; the exact time depending on the charge of work of the laboratory was not registered. The other two series were fixed in the same formalin for 15 and 30 min, respectively, whilst being treated with high-frequency, high-intensity (1.63 MHz, 30 W) ultrasound using a Project 38080 Ultrasound Nebulizer (Artsana S.p.A. Como, Italy). This device is usually used for medical aerosol therapy. A small plastic bowl which usually contains the drug to be aerosolised was filled with formalin. The specimens with a dimension of approximately 1 cm^2 and a thickness of 2 mm were placed in the bowl which was covered with Parafilms (Lab Depot, Dawsonville, GA, USA) to avoid nebulisation. Irradiation was performed in a water bath equipped with temperature control (starting temperature was 20°C; after 15 and 30 min, the temperature was 40°C and 56°C, respectively). After immersion in 75% (*v*/*v*) ethanol for 22 h, the specimens were dehydrated, cleared in xylene and embedded in paraffin in a Shandon Citadel™ 2000 Tissue Processor (Thermo Electron Corporation, Waltham, MA, USA) for 22 h overnight.

DNA extraction and analysis

DNA was extracted from heart and liver from three cases, from lung and kidney from two cases and from spleen from one case (Table 1), using 10 µm sections (n=4). In one sample (338/05 spleen 15 min), material was exhausted after RNA extraction and histological examination so that DNA extraction could not be performed. The specimens were deparaffinised by xylene and ethanol washes; Chelex and Proteinase K extraction with subsequent ultra-filtration (Microcon-30) were performed. The extracted DNA was quantified using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, Darmstadt, Germany). The DNA quality was checked by amplification with the Mentype Nonaplex^{QS} kit (Biotype, Dresden, Germany); 1 ng of human nuclear DNA was amplified. For fragment length analysis, an ABI PRISM 310 Genetic Analyzer with Genotyper software version 2.5 (Applied Biosystems) was used. The peak heights were normalised to the conventional size standard added to the polymerase chain reaction (PCR) product. The normalised values at each locus, for each group, were then compared using the Student's t test (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html).

RNA extraction and analysis

RNA was extracted from heart and liver from three cases and brain, kidney and spleen from two cases. Formalin-fixed, paraffin-embedded (FFPE) samples were deparaffinised as described and RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA). RNA was reverse-transcribed using the cDNA Archive kit (ABI). Complementary DNA (cDNA) was stored at −20°C prior to quantification with a commercially available TaqMan real-time PCR assay for the transcript of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID: NM 002046.3, Applied Biosystems) with a standard curve. In order to check the quality of the extracted RNA, three PCR products varying in length (F1=114 bp, F2=233 bp and F3=453 bp) from GAPDH cDNA were generated as described previously [15].

Histological staining

Paraffin sections were cut at 4 µm in all three series and stained by hematoxylin and eosin (HE). Furthermore, immunohistochemical investigations were carried out using antibodies against actin and CD68 (both antibodies by Dako, Glostrup, Denmark) for the lung [16].

Results

DNA extraction and analysis

Sufficient amounts of DNA could be extracted in every sample (Table 1). The lowest DNA concentration (2.6 ng/µl) was obtained from a sample of liver fixed for 15 min, whilst the highest (297 ng/µl) from a sample of lung fixed for 15 min.

Allele drop out occurred only once in samples fixed for 15 min and was rarely observed at D18, D21 and SE33 with both conventionally and 30-min fixed samples (Table 1). No differences in the peak height of short amplicons could be observed amongst the three groups. Significant differences showing a better amplification with shorter fixation time were observed with longer amplicons (Table S1). No differences could be observed between 15 and 30 min with the exception of the locus D18, where the shortest fixation time led to better results (p<0.05). A table with the normalised peaks, statistical analysis and allelic

Table 1 DNA yield and results of the amplification

Case	Sample n.	Tissue	Fixation time	DNA (ng/μl)	Nonaplex QS
338/05	1	Lung	Conventional	28.20	Single allele drop out D21, D18
	2	Lung	15 min	297.00	Full profile
	3	Lung	30 min	23.20	Full profile
	4	Kidney	Conventional	23.90	Drop out D18
	5	Kidney	15 min	272.00	Full profile
	6	Kidney	30 min	30.10	Full profile
	7	Spleen	Conventional	51,00	Drop out D18
	8	Spleen	30 min	13.60	Full profile, D18 weak
372/05	9	Lung	Conventional	17.80	Full profile
	10	Lung	15 min	79.50	Full profile
	11	Lung	30 min	14.40	Full profile
	12	Kidney	Conventional	28.00	Single allele drop out D18
	13	Kidney	15 min	33.90	Full profile
	14	Kidney	30 min	13.50	Single allele drop out D18, SE33 weak
303/05	15	Heart	Conventional	43.90	Single allele drop out SE33, drop out VWA, FGA
	16	Heart	15 min	86.90	Full profile
	17	Heart	30 min	23.30	Full profile
	18	Liver	Conventional	80.90	Full profile
	19	Liver	15 min	185.70	Single allele drop out D18
	20	Liver	30 min	3.40	Full profile
334/05	21	Heart	Conventional	24.80	Full profile
	22	Heart	15 min	2.80	Full profile
	23	Heart	30 min	40.20	Full profile
	24	Liver	Conventional	42.70	Full profile
	25	Liver	15 min	2.60	Full profile
	26	Liver	30 min	62.90	Single allele drop out D18
18/06	27	Heart	Conventional	22.40	Full profile
	28	Heart	15 min	19.50	Full profile
	30	Heart	30 min	15.30	Full profile
	31	Liver	Conventional	102.70	Full profile
	32	Liver	15 min	25.90	Full profile
	33	Liver	30 min	15.30	Full profile

profile in the five investigated cases is given as ESM (Table S2a, S2b, S2c).

RNA extraction and analysis

The highest RNA yield was constantly obtained from the first series with the shortest fixation time (15 min), whilst the differences between the 30 UAFF and the 48-h standard formalin fixation procedures were minor (Table 2).

The shortest fixation time was also associated with the best RNA quality. PCR was successful in the samples with the shortest fixation time (15 min), i.e. with all three amplicons (F1–F3). In the other series, only the short products could be amplified (Fig. 1). Again, only slight differences between the 30-min fixation and the standard fixation were visible.

Histological staining

The histological slides obtained from the short fixed series showed a good quality both in the classical HE staining and in the immunohistochemical investigations. Loss of quality towards shorter fixation times could not be observed (Fig. S1a, b).

Discussion

The UAFF method examined has shown to be more efficient for subsequent DNA extraction and analysis as conventional fixation. The peak heights of the long PCR products obtained from UAFF are highly indicative of a

Table 2 Example of RNA yields obtained from postmortem autopsy specimens preserved with various fixation techniques

Individual/tissue	Fixation method, time	RNA yield (ng/mg)
303/05/heart	UAFF, 15 min	2.1
	UAFF, 30 min	0.02
	Conventional	0.12
334/05/heart	UAFF, 15 min	5.75
	UAFF, 30 min	0.49
	Conventional	0.15
18/06/heart	UAFF, 15 min	7.62
	UAFF, 30 min	0.17
	Conventional	Undetermined

The highest yields were obtained from the specimen with the shortest fixation time

UAFF ultrasound-accelerated formalin fixation for 15 and 30 min, *Conventional* non-accelerated formalin fixation for 24–48 h

better quality of the extracted DNA. The evaluation of the differences between 15 and 30 min fixation time cannot be addressed here because we do not have intermediate time points to assess whether the observed relation is linear or exponential; this might be addressed in a subsequent study.

The findings of RNA yields have to be considered as preliminary, for data normalisation was performed against tissue amount only. Nevertheless, in RNA recovery and amplification, the differences are much more pronounced, with an obvious advantage for short time UAFF.

Ultrasound triggers the speed of fixation by opening micro-fissures, thus allowing formalin to penetrate more rapidly, the process of fixation being completed in ethanol after 22 h. This second step has no detrimental effect on the nucleic acids [16]. On the other hand, prior fixation in formalin prevents dehydration artefacts typical of ethanol-fixed tissues, thus resulting in a good quality of the microscope preparations.

Recent trends in the field of forensic pathology include an increasing interest in gene expression and RNA analysis [15, 17]. Haller et al. [18] stated that archived FFPE samples as well as frozen forensic autopsy material represent a wealth of material yet under-utilised by researchers. Some basic recent studies promote gene expression analysis on forensic material [15, 19]. With the fixation method presented here, forensic autopsy specimen can be preserved in a way that allows the availability of high quality nucleic acids for molecular genetics research. The device we used can be useful for research purposes if a limited number of specimens have to be fixed but it is not suitable for routine applications. The development of a dedicated ultra-sonic device capable of containing a sufficient number of samples would facilitate the introduction of this technique in those laboratories where gene expression analysis plays an important role.

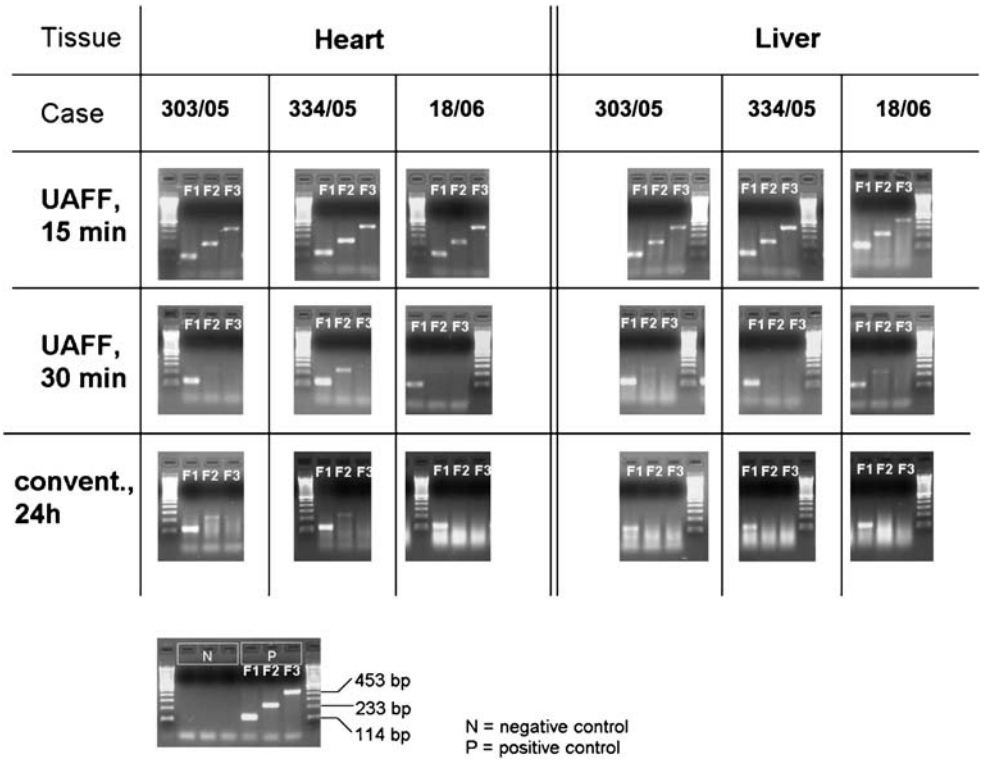

Fig. 1 Amplification of GAPDH fragments. In all three cases analysed (**a**–**c**), the shortest ultrasound accelerated fixation technique (*UAFF*; 15 min) showed the best results by amplification of all three fragments of GAPDH (F1=114 bp, F2=233 bp, F3=453 bp). Only minor differences were observed between the longer ultrasound accelerated fixation (UAFF, 30 min) and the conventional 24 h fixation without ultrasound

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