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Optimisation of postmortem tissue preservation and alternative protocol for serotonin transporter gene polymorphisms amplification in SIDS and SIUD cases

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ABSTRACT

The major obstacle to genetic research in SIUD (sudden intrauterine unexplained death) and SIDS (sudden infant death syndrome) cases is the complex characteristics of the human anatomic samples available. In fact, in Italy autopsies are performed at least 24 h post-mortem and tissues can be left in formalin for long fixation times (>4/5 days), thus compromising nucleic acids integrity. In this study we compared the quality of DNA and RNA extracted from tissues differently preserved. As expected, the DNA and RNA from formalin-fixed and paraffin-embedded tissues, formalin-acetic acid-alcohol tissues and ethanol tissues were of poor quality and not adequate for subsequent molecular analysis. The best results were obtained with RNA*later* preserved tissues: this buffer was equivalent, if not superior, to freezing method for preservation of postmortem DNA and RNA.

In addition, we introduce a new protocol for the amplification of the serotonin transporter gene promoter region (*5-HTT*) ideal to obtain the increase of specific product, avoiding artifacts formation.

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Introduction

Molecular genetic investigations requires the extraction of DNA and RNA from a variety of tissue preparations, including paraffin wax embedded, formalin-acetic acid-alcohol fixed, ethanol-stored, frozen and fresh samples. In particular, routinely formalin-acetic acid-alcohol fixed (FAA) tissues or formalin fixed and paraffin wax embedded (FFPE) tissues are an important source for molecular studies because they are an endless source of material for research. DNA extraction techniques from several tissues have been amply described but extraction from paraffin wax embedded tissues requires special procedures. In addiction, the degradation of nucleic acids is more extensive in the FAA and FFPE postmortem tissues than in the biopsies or in the surgical specimens. In literature it has been reported that the average fragment length of DNA is 300–400 bases in biopsy tissues, but much shorter in FFPE tissues.

RNA is also fragmented and chemically modified in formaldehyde or paraformaldehyde-fixed, paraffin-embedded (FFPE) tissues.

Therefore, even if these techniques are the most commonly used in the histopathology laboratories, they compromise the quality and integrity of nucleic acids.

Ethanol fixation offers permanent preservation of the tissues, easy storage and histological quality. Unfortunately, ethanol fixation is inappropriate for DNA preservation because it gives rise to small segments, such as those recoverable from formalin-fixed, paraffin embedded tissues, and compromises analysis of others biomolecules, in particular mRNA and proteins (Catts et al., 2005; De Paepe et al., 2002; Heinrich et al., 2007; Heller et al., 1992; Jackson et al., 1990; Lehmann and Kreipe, 2001; Ninet et al., 1999).

It is a worldwide knowledge that the best DNA and RNA extraction results can be achieved from fresh and −80 °C frozen tissues. The major obstacle to genetic research in SIUD (sudden intrauterine unexplained death) and SIDS (sudden infant death syndrome) cases is the complex characteristics of the human anatomic samples available (Moon et al., 2007; Weese-Mayer et al., 2007). In fact, autoptic specimens are performed in Italy at least 24 h post-mortem and tissues can be left in formalin for long fixation times. Nevertheless commercial kits for nucleic acids extraction from FFPE tissues are available but they are not the ideal thing because they give best results in tissues fixed for few hours. In fact, longer fixation times may result in two effects: a higher degree of cross-links between biomolecules, and a higher degree of DNA fragmentation, resulting in small DNA fragments (Fig. 1). Fragmentation of DNA causes a reduction of genome equivalents that can be detected by PCR (polymerase chain reaction) and therefore has a large effect on downstream assays.

To solve this problem, the use of a novel and innovative buffer, called RNA*later*® (Ambion, Austin, TX), has been introduced in our Research Centre to preserve the nucleic acids integrity. This buffer is

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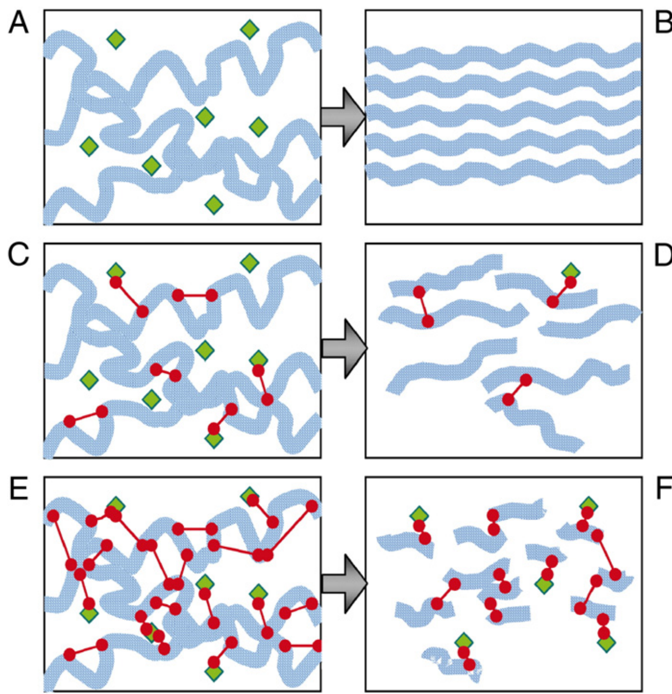

Fig. 1. Effects of formalin fixation on DNA. When started with fresh/frozen tissues (A) DNA sizes will be in the high molecular weight range (B); when started with FFPE material (C), DNA sizes will vary due to formalin fixation that cross-links DNA to other biomolecules (green boxes) and causes fragmentation (D). Prolonged formalin fixation time (E) increases cross-linking in the DNA and subsequent fragmentation (F).

also mentioned in the guidelines imposed by the recent Italian Law.31 (Italian Law n. 31, 2006).

Samples treated with this buffer have comparable quality to fresh frozen tissue samples (Florell et al., 2001). RNA*later*® is an aqueous, non-toxic, tissue storage reagent that rapidly permeates tissues to stabilize and protect nucleic acids in fresh specimens. It eliminates the need to immediately process or freeze samples; the specimen can simply be submerged in RNA*later*® and stored for analysis at a later date. Samples in RNA*later*® can be stored for extended periods under conditions where RNA degradation would normally take place rapidly. Tissues can be stored indefinitely in RNA*later*® at −20 °C or below.

In this study, we have analyzed the results obtained by the extraction of nucleic acids from autoptic specimens derived from SIUD and SIDS cases. We compared the efficiency of purification in tissues differently preserved in order to optimise nucleic acids extraction in these sensitive samples in order to generate DNA and RNA suitable for further experiments in molecular genetics.

DNA extracted from different autoptic specimens was also used for polymorphisms identification in the serotonin transporter gene promoter region (*5-HTT*) commonly studied in sudden infant death syndrome cases (Heils et al., 1996; Weese-Mayer et al., 2003). In fact this gene was the first one linked to SIDS vulnerability and was studied on the basis of reports of decreased serotonergic receptor binding in brain stems of SIDS victims in the United States (Panigrahy et al., 2000) and Japan (Ozawa and Okado, 2002). An association between the more effective promoter long (L) allele of *5-HTT* gene and SIDS risk has been demonstrated (Narita et al., 2001).

Materials and methods

Speciments of brain and liver were obtained from 30 fetal (from the 22nd to the 40th week of gestation) and 18 infant (within the first year of age) autopsies performed (24 h post-mortem) in the last 3 years. All the fetuses were fresh.

DNA: pre-digestion treatments

The amount of the starting material was: for FFPE (10% buffered formalin) tissues one or two 20 µm sections; for all the other tissues, 40 mg (brain) or 20 mg (when using liver) of tissue sample.

-from formalin-fixed and paraffin- embedded; the samples were initially heated and spun, the supernatant removed and then washed with fresh xylene, followed by 100% ethanol washes (7);
-from formalin-acetic acid-alcohol and ethanol; the samples were washed and rehydrated in RNA*later*, drained and cut in a Petri dish;
-from RNAlater; the samples were drained and cut in a Petri dish;
-from frozen; the samples were allowed to thaw and then cut in a Petri dish.

DNA: digestion

The tissue pellets were digested with proteinase K (final concentration 200 µg/ml) in Lysis Buffer G (Invisorb Spin Tissue Mini Kit, Invitek GmbH) for at least 3 h at 52°C, with gentle agitation every hour. To increase the lysis efficiency, tissue samples have been mechanically ground with a pestle.

DNA: extraction

The DNA of each tissue sample was obtained by elution according to the manufacturer's protocol (Invisorb Spin Tissue Mini Kit, Invitek GmbH). The eluted DNA (30–100 µl) was stored at −20 °C for later PCR analysis.

RNA: isolation

The samples used for RNA isolation derived from tissues conserved in ethanol, RNA*later* , frozen tissues or frozen tissues + RNAlater ®-ICE (the frozen tissue samples were put to soak overnight in RNA*later* ®-ICE (Ambion, Austin, TX) at −20 °C; once permeated with RNA*later* ®-ICE, tissue samples can be processed using the sample techniques as for fresh tissue).

To obtain high quality RNA from tissue samples we used the RiboPure™ Kit (Ambion) following the producer instructions (the amount of the starting material was 20 mg). To avoid degradation of RNA only RNase-free materials have been employed.

RNA was stored at −80 °C.

The integrity and quality of both nucleic acids were assessed by 1% agarose gel electrophoresis and spectrophotometry.

PCR amplification

The extracted DNA was used to genotype the 5-HTTLPR (5-HTT-linked polymorphic region) polymorphism as follows: oligonucleotide primers flanking the 5-HTTLPR and corresponding to the nucleotide positions ranging from −1416 to −1397 and from −910 to −889 (respectively, forward primer F5-HTT: GGCGTTGCCGCTCT-GAATGC; reverse primer R5-HTT: GAGGGACTGAGCTGGACAACCAC) (Invitrogen Corporation, California, USA) of the gene regulatory region were used to generate a 484/528 bp fragment.

PCR was carried out in a final volume of 50 µl consisting of approximately 50 ng genomic DNA. Amplification was performed using: 20 µl of genomic DNA, 1x PCR Gold buffer (Applied Biosystems, Foster City, USA), 2 mM $MgCl_2$, 0.4 mM dNTPs, 50 pmol each of the required primers and 2U Ampli*Taq* Gold (Applied Biosystems).

Temperature cycling was performed using an Applied Biosystems 2720 Thermal Cycler with the following new protocol which was performed in our laboratory (different from the commonly used method described for the first time by Heils et al. (1996): 10 min at

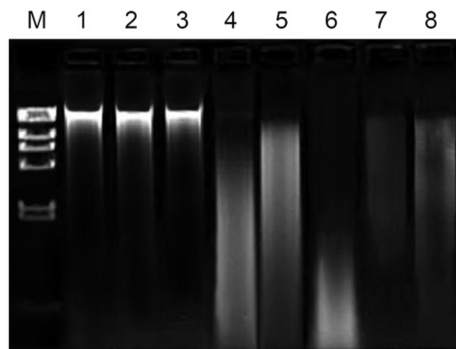

Fig. 2. 1% agarose electrophoresis gel. DNA extracted from tissues fixed in different fixative agents: (1) fresh frozen; (2–3) RNA*later*; (4–5) 70% ethanol; (6–7) FFPE; (8) FAA. (M) DNA marker.

95°C, followed by 40 cycles of 95 °C for 1 min and 61 °C for 10 min, with a final extension for 7 min at 72 °C.

PCR amplification products were electrophoresed through a 1.5% agarose gel and visualized by UV-light in presence of ethidium bromide.

Results

We isolated DNA from RNA*later* preserved tissues and, for comparative purposes, fresh frozen tissues, from formalin-fixed and paraffin-embedded tissues, formalin-acetic acid-alcohol tissues and tissues fixed in 70% ethanol. Multiple samples from the same case generally gave similar results, indicating that the method of extraction was uniform.

All the DNA derived from frozen and RNA*later* preserved tissues was successfully extracted, showing broad and definite bands of high molecular weight (Fig. 2). The amplification of serotonin transporter gene promoter region (*5-HTT*) using these extracted DNA samples was successful on all the cases (Fig. 3). Comparable results were obtained from fresh tissues (data not shown).

Indeed, the quality of the DNA from formalin-fixed and paraffin-embedded tissues and formalin-acetic acid-alcohol tissues was not equivalent to the above mentioned. Electrophoresis on an agarose gel showed either the absence of DNA or the presence of a vague DNA smear, which is consistent with degradation (Fig. 2). We had no amplification success with these samples fixed in formalin (Fig. 3).

The DNA from the ethanol-fixed samples migrates on an agarose gel as a vague band and a smear of fragments, ranging in size from several hundred base pairs (bp) to few kbp (Fig. 2). PCR of the extracted DNA was not successful (Fig. 3).

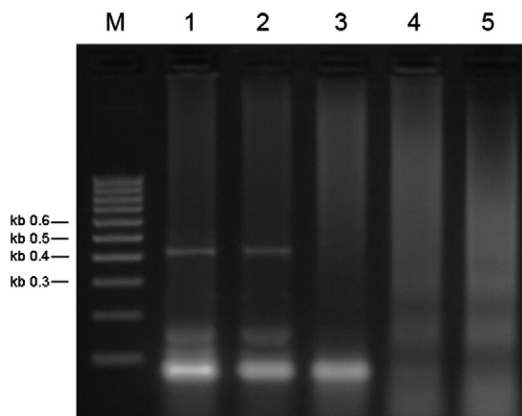

Fig. 3. PCR. Amplification products generated with primers flanking the serotonin transporter gene promoter region from genomic DNA derived from tissues preserved in different agents: (1) fresh frozen; (2) RNA*later*; (3) 70% ethanol; (4) FFPE; (5) FAA. (M) 100 bp ladder. The reaction products were electrophoresed through 1.5% agarose gel and visualized by UV illumination in the presence of ethidium bromide.

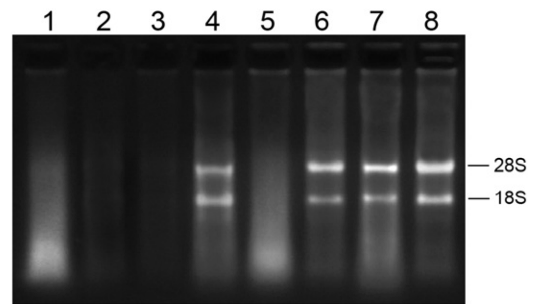

Fig. 4. 1% agarose electrophoresis gel. RNA (18S and 28S = ribosomal RNA bands) extracted from tissues fixed in different fixative agents: (1–2) FFPE; (3) ethanol; (4) fresh frozen treated with RNA*later* ICE ; (5) FAA; (6) fresh; (7–8) RNA*later*.

We compared the recovery of total RNA from the same tissues used for DNA extraction. As expected, the RNA sample derived from RNA-later preserved tissues show the distinct 18S and 28S ribosomal RNA (rRNA) bands. Frozen tissues treated with RNAlater ICE provided high-quality RNA as judged by intact 28S and 18S ribosomal RNA bands. In RNA isolated from formalin-fixed and paraffin-embedded tissues, formalin-acetic acid-alcohol tissues and ethanol-fixed tissues we observed no RNA or significant RNA hydrolysis and fragmentation, with loss of the 28S and 18S bands and the appearance of an RNA smear on the gel (Fig. 4).

Discussion

Formalin fixation and paraffin embedding is the standard processing method used in histopathology laboratories. This protocol allows the permanent preservation of the tissues, easy storage, and optimal histological quality. Unfortunately, formalin fixation severely compromises analysis of biomolecules, in particular DNA and RNA. Ethanol 70% is another commonly used solution for morphological sample preservation, but it degrades nucleic acids very fast.

In fact we showed that after tissues preservation in ethanol 70% it was impossible to obtain RNA (in some cases we saw only a vague smear), while the extracted DNA was of inferior quantity and quality and not adequate for further PCR analysis regarding the serotonin transporter gene (5-HTT).

While preservation techniques use formaldehyde to maintain tissue structure and prevent putrefaction, this makes it difficult to perform molecular analysis on the samples. DNA and RNA are trapped and modified through protein-protein and protein-nucleic acids crosslinks.

The major obstacle to our genetic research is the complex characteristics of the human anatomic samples available. In fact, in Italy autoptic specimens are performed 24 h post-mortem and samples are formalin-fixed. Usually the samples that arrive in our laboratory from different hospitals have been taken in formalin for a minimum of four/five days. DNA extracted from FFPE and formalin-acetic acid-alcohol tissues was scarce, degraded, and often contains remnants of substances that inhibit the amplification reaction, such as formalin, or inhibit proteinase K used in the extraction procedure, such as xylene. In our experiments we confirmed the amplification problems: no specific bands were detectable on agarose gel electrophoresis after PCR of the 5-HTT gene. As expected, no RNA was recoverable from this samples. Formalin reacts with the bio specimen by creating covalent bounds between biological macromolecules, thus stabilizing them and inhibiting enzymatic activities. The chemical action of formalin binds severely DNA, RNA and proteins and makes them difficult or even impossible to extract in adequate quantities and qualities.

It was necessary to find the best way to preserve both tissues histology and nucleic acids integrity in order to perform different

kinds of experiments. For this reason, in this Research Centre, a novel and innovative buffer, called RNA*later*, has been introduced to preserve nucleic acids: as shown, DNA and RNA extracted from samples treated with this buffer are comparable in quality and quantity with those obtained from fresh and frozen tissues (to obtain the maximum RNA yield from frozen tissues, we utilized a novel reagent called RNA*later*®-ICE, for transitioning frozen tissue to rapidly processed to obtain high quality RNA). In addition to obtaining high quality/quantity RNA, it is important to point out the practical advantages of tissue storage in RNA*later*, namely that tissues in RNA*later* can be stored at room temperature, allowing for easy integration of this collection procedure into complex autopsy protocols. The tissue is also easily transported, thereby facilitating interinstitutional collaborations.

We had 100% amplification success rate for the samples treated with in RNA*later* and RNA*later*-ICE. The new utilized protocol for the amplification of the serotonin transporter gene (5-HTT) is different from the commonly known: we noted that with the protocols present in literature it was sometimes difficult to obtain PCR products of good quality, because the bands were vague and there were a lot of non specific PCR products. To avoid this inconvenient, we introduced the AmpliTaq Gold® DNA Polymerase which is a chemical hot start enzyme that provides lower background and increased yield of specific product. This enzyme has been substituted for AmpliTaq DNA Polymerase because, by adding a 10 -min 95 °C pre-PCR incubation step, it provides the increase of specific product, avoiding reactants to be wasted in artifacts formation. Thus we prolonged the extension step from 1 to 10 min to further on increase the yield of the reaction.

In conclusion, we have shown that RNA*later* is a simple and handy buffer that can be the ideal solution to the problem of preservation of postmortem tissues. With this buffer it would be possible to perform parallel histopathological examination and genetic research, indeed besides to preserve the nucleic acids integrity, it is able to maintain the morphological structure of the tissues. This is a very important aspect for further studies of gene expression.

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