Research Paper

Effects of post-mortem and physical degradation on RNA integrity and quality

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A R T I C L E   I N F O
Article history:
Received 25 May 2015
Received in revised form 10 August 2015
Accepted 11 August 2015
Available online 21 August 2015

Keywords:
Degradation
RNA integrity
RNA quality
RT-qPCR
RQI

A B S T R A C T
The precision and reliability of quantitative nucleic acid analysis depends on the quality of the sample analyzed and the integrity of the nucleic acids. The integrity of RNA is currently primarily assessed by the analysis of ribosomal RNA, which is the by far dominant species. The extrapolation of these results to mRNAs and microRNAs, which are structurally quite different, is questionable. Here we show that ribosomal and some nuclear and mitochondrial RNAs, are highly resistant to naturally occurring post-mortem degradation, while mRNAs, although showing substantial internal variability, are generally much more prone to nucleolytic degradation. In contrast, all types of RNA show the same sensitivity to heat. Using qPCR assays targeting different regions of mRNA molecules, we find no support for 5′ or 3′ preferentiality upon post-mortem degradation.

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1. Introduction

The quality of biological samples is very sensitive to the handling and treatment before the nucleic acids are extracted for analysis and degrading enzymes are inhibited or removed. Analyzing RNA is more challenging than analyzing DNA, because double-stranded DNA is more stable than single-stranded RNA, deoxyribonucleases (DNases) are readily denatured and inhibited compared to the highly stable ribonucleases (RNases). The post-mortem degradation of nucleic acids in biological samples has proven useful in forensic pathology, where the time of death can be estimated [1]. In diagnostic samples post-mortem nucleic acid degradation is only a nuisance that should be controlled and kept to a minimum [2–4]. Usually the confounding processing (technical) variation introduced by DNA degradation is small and can be ignored. For RNA analysis situation is quite different and several reports show expression data can be seriously biased and highly unreliable [5–11]. The main cause is poor RNA quality and integrity. This is particularly serious in medical molecular diagnostic, and has been thoroughly addressed by the SPIDIA consortium (www.spidia.eu), which ultimately led to the formulation of CEN and ISO guidelines for the preanalytical process in molecular diagnostics. The guidelines teach RNA quality/integrity should be tested in workflows aiming to quantify RNA biomarkers.

Currently, the quality of RNA in biological samples is determined by electrophoresis that separate the dominant RNA species by size. Those are ribosomal RNAs (rRNAs), which make up about 85% of total RNA in eukaryotes. These eukaryotic ribosomal RNAs are presented in four distinct sizes, referred to as small (55 and 5.8S) and long (18S and 28S), where the size is given in Svedberg units, reflecting the sedimentation coefficient [12]. The long rRNAs are usually produced in a 1:1 ratio and because of the roughly double size of the 28S species the electropherogram of fully intact RNA shows distinct bands for the 18S and 28S rRNAs, with the 28S band having approximately twice the intensity. A ratio deviating from 2 indicates RNA degradation [13,14]. The 28S:18S ratio shows correlation with RNA integrity [15], but can be affected by factors such as aging [16] and apoptosis [17]. Several companies have developed systems to measure RNA integrity based on the separation of the RNA molecules, such as the automated capillary electrophoresis systems such as Experion from Bio-Rad Laboratories, USA and Agilent Bioanalyzer 2100 from Agilent Technologies, USA. Those systems use chip-based technology for RNA quality and quantity measurements. The entire electropherogram is analyzed and then, using a complex algorithm trained to take into account all the features, the RNA quality/integrity is presented.

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http://dx.doi.org/10.1016/j.bdq.2015.08.002
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as a single quality indicator. The Bioanalyzer software uses RIN (RNA Integrity Number), while the Experion uses RQI (RNA Quality Indicator). Hence, the indicator is affected by several factors including the presence of small RNA fragments from degradation, presence of molecules longer than the 28S rRNA, and overall low signals of the rRNAs [14]. Recently, alternative instruments for large scale and sensitive RNA quality and integrity determination appeared such as Fragment Analyzer™ (Advanced Analytical Technologies), QIAxcel Advanced System (QIagen), ScreenTape (Agilent Technologies). These instruments also score RNA integrity using complex indicators such as RIS (RNA Integrity Score) for QIAxcel Advanced System and RIN® (RNA integrity number equivalent) for ScreenTape.

The indicators produced by the different instruments are not readily comparable, because each uses its own algorithm, but they all score RNA quality as a number between 1 and 10, where 1 indicates completely degraded RNA and 10 fully intact RNA [6,18]. In addition to the platform to platform variation, also the repeatability (repletion on the same instrument) and reproducibility (repetition on a different instrument of the same type) of the integrity index estimates has been questioned, particularly on extensively degraded samples. Furthermore, the assessment of the RNA integrity is based on properties of the rRNAs and does not necessarily reflect the state of the mRNA pool.

The quality of extracted RNA depends on the source tissue [8]. Tissues such as spleen and liver that are rich in nucleases degrade RNA faster and to greater extent than in tissues with less RNAse activity such as muscle and heart [19]. Common recommendation is to only analyse RNA from samples with RIN/RQI larger than five and microRNA from sample with RIN/RQI larger than seven [20]. RNA degradation is complex and three different types of mechanisms can be distinguished: enzymatic, physical and chemical. Enzymatic degradation occurs naturally in post-mortem tissue [21]. It initiates with either polyA tail/5' cap removal or endonucleolytic cleavage followed by exonuclease degradation [22,23]. Physical (e.g. UV light, high temperature) and chemical (e.g. aldehydes, paraffin) degradation is by quite different mechanisms. It may induce crosslinking, oxidation and modification of RNA molecules. In this work we study the effects of the main degradation mechanisms on different RNA molecules under defined conditions.

2. Materials and methods

2.1. Ethics statement

The study was carried out in accordance with the Act No 246/1992Coll., on the protection of animals against cruelty. Official permission was issued to Biotechnology institute AS CR, v.v.i. by the Central Commission for Animal Welfare under the Ministry of Agriculture of Czech Republic.

2.2. RNA samples preparation

Xenopus laevis females were injected with 500U of human chorionic gonadotrophin hormone (hCG, Sigma) to stimulate ovulation of oocytes. The females were kept overnight at 18 °C and oocytes were obtained by gentle squeezing. The oocytes were in vitro fertilized by sperm suspension prepared in L-15 Leibowtitz medium with 15% of fetal bovine serum. Fertilized oocytes were covered with 0.1x MBS medium (Modified Barthis Saline; 88 mM NaCl, 1 mM KCl, 0.7 mM CaCl2, 1 mM MgSO4, 5 mM HEPES, 2.5 mM NaHCO3, pH 7.7) for 20 min. Jelly coats were removed by 2% cysteine treatment (pH 7.7) followed by repeated washes with 0.1x MBS. The oocytes and tadpoles at stage 40 (3 biological replicates per condition) were collected and deep frozen at −80 °C, which cause their death. After thawing, the samples were incubated at room temperature for 0, 5, 10, 20 and 40 min. The total RNA was extracted using RNeasy Micro Kit (QIagen). Manufacturer’s instructions were followed during the extraction and elution was performed using 15 μl of water. Concentration of extracted total RNA was measured using the Nanodrop® ND1000 quantification system. Heat degradation was performed with total RNA extracted from tadpoles at stage 40 and diluted in RNase/DNase free water. Purified RNA was divided into separate tubes and heat treated for 0, 1, 2, 4 and 6 h at 80 °C. The RNA quality was evaluated using Experion capillary electrophoresis system (Bio-Rad) and RNA StdSens chip (manufacturer’s instructions were followed).

2.3. cDNA preparation

Isolated RNA from each sample was reverse transcribed into cDNA using SuperScript™ III Reverse transcriptase kit (Invitrogen). 50 ng and 300 ng of total RNA were reverse transcribed from oocytes and tadpoles (stage 40) samples, respectively. The RNA was mixed with 0.5 μl of oligo-dT and random hexamers (mixture 1:1, 50 μM each), 0.5 μl of dNTPs (10 mM each), 0.5 μl of spike (this RNA sequence is 1000 bases in length and includes a 5供电 Cap and a polyA tail of approximately 200 bases, prepared using in vitro transcription. Available as TATAA Universal RNA Spike, TATAA Biocenter) and DNase/RNase free water to a total volume of 6.5 μl. The spike was included to test for any unspecific bias in the processing of the degraded samples. The mixture was incubated for 5 min at 75 °C, 20 s at 25 °C followed by cooling to 4 °C for 1 min. 100 U of enzyme, 20 U of RNaseOUT™ (recombinant ribonuclease inhibitor, Invitrogen), 0.5 μl of 0.1 M DTT and 2 μl of 5供电 first strand synthesis buffer were added to a final volume of 10 μl. The mixture was then incubated at 25 °C for 5 min, 50 °C for 60 min, 55 °C for 15 min and 75 °C for 15 min. 50 μl of water was added to the cdNA and the samples were stored at −20 °C. qPCR assays for 16 transcripts (odc, imp3, RNA pol II, mamli1, atub, acta, eef1a1, mprl1, ube, antl, mdh2a, xkb1a1, scaRNA11, SS RNA, cycl1 and 18S rRNA) were designed using NCBI Primer–Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Several assays for small nucleolar RNAs and mitochondrial RNAs were designed and the most efficient genes for cycl1 and snRNA11 were selected for further experiments. Amplicon length was set to between 90–200 bp and Tm 60 °C. Specificity of all assays was confirmed by melting curve analysis measured from 65 °C to 95 °C in 0.5 °C intervals. qPCR mix contained 5 μl of JumpStart mastermix (Sigma), 0.5 μl of forward and reverse primers mix (mixture 1:1, 10 μM each), 2 μl of cdNA and water to final volume of 10 μl. qPCR was performed on a CFX384 cycler system from Bio-Rad. PCR conditions were: initial denaturation at 95 °C for 2 min, 40 repeats of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and elongation at 72 °C for 20 s.

2.4. Data analysis

Cq values of biological replicates were averaged and standard deviations were calculated (data not shown, SD < 0.6). Measured transcripts levels in Figs. 2, 4 and 5B are shown relative to those measured at time 0 as 2^−Cq; where Cq is the Cq value measured at time 0 and Cq is the Cq at the time _i_ of degradation. In Figs. 2 and 5B the ratios are expressed in logarithmic scale. The data were analysed with GenEx (MultiD, version 6). Briefly, data were pre-processed using a cut-off at 36 cycles and missing data were substituted by the average of valid Cq values at that stage and time. All data were converted to relative quantities (relative to the highest Cq for each gene, artificially
assigning an expression of 1 to the least expressed sample) and transformed to log2 scale. The data were mean centred (for each gene, subtracting the average expression across all samples) and two tests were applied to classify the profiles: the Kohonen self-organizing map (SOM, with two boxes) and hierarchical clustering presented as a dendrogram (Fig. 3). The SOM classification was repeated using independent seeds to validate the result.

3. Results

3.1. RNA degradation is faster in tadpole samples compared to oocytes

Total RNA was extracted from *Xenopus* oocytes and tadpoles at stage 40. The oocytes were selected because of their simple nature being a single cell, while the tadpoles at stage 40 were selected to
represent a complex biological sample. The tadpoles have already most of the body tissues, such as internal and sensory organs, differentiated. Samples were collected at 0, 5, 10, 20 and 40 min post-mortem. Total RNA was extracted and integrity was assessed by gel electrophoresis using Experion capillary electrophoresis system (Fig. 1). Oocyte RNA samples showed first signs of degradation, reflected by the presence of short fragments and the disappearing of the 28S rRNA band, 10 min post-mortem. The RNA quality was still quite high with RQI of 8 at 40 min post-mortem (Fig. 1A). In contrast, tadpole samples showed significant RNA degradation already 5 min post-mortem with RQI of 6.5 and after 40 min RQI was 3.4 (Fig. 1B).

3.2. Different rates of rRNA and mRNA post-mortem degradation

Next we tested if post-mortem RNA degradation depends on the type of RNA. As control for technical variation a RNA spike was added to all samples before the reverse transcription. The average standard deviation of the spike across all biological replicates was 0.37 cycles for the oocyte samples and 0.46 cycles for the tadpole at stage 40 samples. These low SDs reflect high reproducibility of the reverse transcription and the qPCR step.

We designed qPCR assays for the SS and 18S rRNA, the small nucleolar scaRNA11, the mitochondrial transcript cyc1, and for 12 mRNAs. Temporal degradation profiles were measured post-mortem (Fig. 2). Two distinct degradation profiles appeared, which we refer to as unstable and stable RNAs. The unstable RNAs include the genes: odc, imp3, RNA pol. II, mami1, atub, acta, eef1a1, mrt1, ubc, ant1, mdh2a, xba1a1. Several of these are so called housekeepers and often used as reference genes in expression studies. The degradation of unstable RNAs in the tadpole samples showed more than two orders of magnitude faster degradation than for the same RNAs prepared from the oocytes. In the oocyte samples, the fraction of unstable RNAs dropped from ~77% at 10 min to ~27% at 40 min post-mortem. For the tadpole samples the fraction of unstable RNAs dropped from about 16% at 10 min to 0.4% at 40 min post-mortem. These results are in concordance with the overall RNA degradation measured using gel electrophoresis (Fig. 1).

The stable RNAs include SS rRNA, 18S rRNA, scaRNA11 and cyc1, and exhibit minimal degradation. Their levels showed minimal changes during 40 min post-mortem in the oocyte samples (Fig. 2C) and only limited degradation (to 50–70%) with the tadpole samples. Notably, none of these RNAs is a cellular mRNA. The distinct difference in stability of stable and unstable RNAs was further supported by multiway analysis. Both SOM and hierarchical clustering clustered the stable RNAs with the RNA spike separately from the unstable RNAs (Fig. 3). The RNA spike was added after RNA extraction and was not degraded. Similarity of degradation pattern with spike RNA was used as a representation of stable RNA.

3.3. Differential sensitivity of RNA 5' and 3' ends to degradation

Three major pathways of enzymatic mRNA degradation have been described. Degradation is initiated by the removal of the 5' cap, deadenylation at the 3' end, or by endonucleolytic cleavage within the mRNA [23,24]. The deprotected mRNA is then rapidly degraded by exonucleases [25,26]. To test if degradation of mRNAs is preferential at either end we designed 5 separate qPCR assays covering essentially the entire length of xba1a1 (cytokeratin) and atub (alpha tubulin) mRNAs. All qPCR assays were designed with similar amplicon length (80–110 bp) and their efficiencies were higher than 90%. Uniformity of assay efficiencies, minimal biological variability of oocyte samples (standard deviation of biological replicates from one female is ~0.3 of Cq) and possibility to use samples at time 0 as a reference allow us precise normalization and comparison of all assays. All five qPCR assays per transcript showed similar decrease of mRNA content to ~60% at 10 min and to 20% at 20 min. Our data show no indications of statistically significant degradation preference for 5' or 3' ends for neither of studied genes (Fig. 4). The relationship among the assays was tested using Pearson correlation. The xba1a1 assays showed r-coefficients >0.96 and the atub assays showed r-coefficients >0.97 indicating high correlation among all five qPCR assays. In all cases the p-values were <0.05.

3.4. Physical degradation has the same effect on ribosomal and messenger RNA

Physical degradation was induced by heat treatment of purified tadpole at stage 40 RNA, because this RNA showed higher sensitivity to degradation and has higher complexity compared to oocyte. The tadpole RNA samples were incubated at 80 °C for 0, 1, 2, 4, and 6 h to induce degradation. Overall integrity of the RNA was assessed by gel electrophoresis (Fig. 5A) and degradation of the same transcripts as in the study of natural post-mortem degradation in Fig. 2 was measured with RT-qPCR (Fig. 5B).

Under the conditions used, the heat-induced degradation had much lower impact on the measured transcript levels compared to the natural post-mortem degradation. Heat induced degradation could be noticed first after one hour (Fig. 5A). The RQI values decreased to about 4.8 after one hour of heat treatment and
continued slowly decreasing for up to six hours. At earlier time points degradation was hardly noticed (5, 10, 20 and 30 min, Supplement Fig. 1). Neither did we see any significant degradation when using the lower temperature of 70 °C for up to even 6 h (Supplement Fig. 2). Notably, we found that also the impact of heat on the different RNA molecules varies, but differently from the effect of post-mortem degradation. The only RNA that showed resistance to the heat treatment was scaRNA11; its level remained close to 100% during the entire degradation. All the other RNAs, were degraded at roughly the same rate.

4. Discussion

Accurate quantification of gene expression with methods such as RT-qPCR, microarray profiling and next-generation sequencing requires integral RNA of high quality. It is well established that the pre-analytical steps in molecular analysis, comprising sample collection, transportation, storage, and extraction, may have profound effect on the RNA and, via reverse transcription, the cDNA quality and introduce substantial technical bias [2–4]. It is therefore critical to use highly optimized and validated pre-analytics, but also to test the quality and integrity of the extracted RNA. This is included in most workflows and also requested in the recent guidelines from the European Committee for Standardization. Our results presented here, suggests that testing of relevant RNA quality and integrity may be complicated.

We selected oocytes and tadpoles at stage 40 of frogs as model for our study of RNA degradation, because they are easily accessible, contain several micrograms of total RNA and, most importantly, sibling oocytes and tadpoles at stage 40 are synchronous and have nearly identical transcript levels, which makes the impact of individual variation that confounds the study negligible. Hence, we are neither limited by material nor biological reproducibility in those experiments. Routine assessment of RNA integrity and quality is based on the analysis of 18S and 28S rRNA, which are the far most abundant RNA molecules in most biological samples. Traditionally this was done by classical electrophoresis comparing their abundance, and is today done with more sensitive techniques, such as capillary electrophoresis, that analyze the entire electrophorogram with advanced multivariate algorithms in commercial software and calculate integrity indexes. Hence, ribosomal RNA molecules are used as prime indicator of RNA integrity.

Our main goal was to determine whether tRNA degradation reflects accurately mRNA degradation. The main conclusion of our manuscript is, that tRNA degradation is useful for artificial degradation, but it is the poor prediction tool for natural "post-mortem" degradation of mRNA fraction. While electrophoresis report indicated slight or even no degradation of tRNAs (Fig. 1), quantification
of RNA levels using RT-qPCR revealed majority of mRNA molecules to be degraded to several percent of their original concentrations. We can speculate that ribosomal RNA is more protected against enzymatic degradation than mRNA, because of its structure lacking cap and polyA tail. Localization of mRNA into ribosomes and covering of mRNA molecules with other proteins can also increase its protection against intercellular RNases. Physical and chemical degradation mechanism is independent on RNA structure, so mRNA fraction should degrade at the same rate as mRNA. Similar conclusion can be predicted for scRNA11 and mitochondrial cyc1. Those transcripts are located in nucleolus and mitochondria and this covering increases their protection during enzymatic post-mortem degradation. Several classes of small noncoding RNAs including scRNA11 has modified cap [27], which can increase their stability. High stability of scRNA11 to physical degradation can be explained by its short size, which prevents breaks and degradation during heat treatment.

Degradation rate is directly dependent on sample complexity. We compared oocyte samples as simple model versus tadpole at stage 40 samples as complex model. Oocyte showed minimal post-mortem degradation of mRNA pool and just slow degradation of mRNA pool. In contrast, tadpole samples showed gradual RNA degradation on a gel electrophoreogram, but quantification using RT-qPCR showed minimal decrease of RNA molecules. We suggest that RNAs are cleaved in complex samples faster than in simple samples, but the cleavage is not complete and short qPCR amplicons can still detect those fragments. Messenger RNA in tadpole samples were degraded two orders of magnitude faster than in oocyte samples. This can be explained by lack of protection factors in the tadpole samples. Oocyte as a single cell is full of material (RNA, protein, lipids etc.) and this matrix can surround and protect RNA molecules from post-mortem degradation. Further, oocyte mRNA fraction is not fully polyadenylated at 3’ ends and that could increase their stability too. On the other hand, tadpole mRNAs are mature with polyA tails and mRNAs are actively translated, so there would be minimal protection from surrounding molecules. In addition, tadpole samples probably contain much higher concentration of RNases. All these factors are probably behind the observed differences in degradation rates in our samples.

RNA molecules inside the cells are very sensitive to natural post-mortem degradation, but purified RNA samples (without RNases) can be degraded by other factors such as temperature, UV light, various chemicals etc. The mechanisms of physical and chemical degradation is assumed to differ from natural post-mortem degradation. In theory, physical and chemical degradation is not dependent on RNA structure including presence of cap and polyA tail. We used physical RNA degradation induced by heat treatment to demonstrate different mechanism. Overall the physical degradation using 80 degrees incubation showed much slower degradation rate compared to post-mortem degradation. As expected, the ribosomal RNAs showed the same trend of degradation as mRNAs during physical degradation. The only exception in our hypothesis was small nuclear RNA and we hypothesize that its short length makes it more stable to physical degradation than longer mRNA and rRNA molecules.

In conclusion, we performed several experiments to demonstrate problematic side of RNA integrity/quality estimation. We can summarize our finding: (1) ribosomal RNA is not useful indicator for natural degradation of mRNA in the post-mortem samples, but could be valuable indicator of physical and chemical degradation studies; (2) comparison of gene expression in samples with different degradation is problematic and could be overcome using proper references; and (3) post-mortem degradation of mRNA is not 5’ or 3’ end sensitive. Although RNA quality testing based on ribosomal RNAs has limits and problems, it is currently the most precise method for routine analysis and universal usage. It is known that better RNA quality and integrity estimate could be obtained using mRNA specific assays, but unfortunately no robust and universal method for routine laboratory work is available now.

Acknowledgements

This study was supported by Ministry of Youth, Education and Sports of the Czech Republic AV0Z50520701 and grant LK21305; BIOCEV CZ.1.05/1.1.00/02.0109 from the ERDF and by the Charles University grant SVV 260206.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bdq.2015.08.002.

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