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TECHNICAL NOTE

CRIMINALISTICS; PATHOLOGY/BIOLOGY

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Bone Marrow and Bone as a Source for Postmortem RNA*

ABSTRACT: The susceptibility of RNA to enzymatic degradation has been considered as a tool to estimate time-since-death in forensic samples, and it has previously been demonstrated that the choice of tissue is an important factor. In this study we have extracted RNA from decaying bone and bone marrow under the hypothesis that the delayed onset of putrefaction may render them a useful source in this context. In a preliminary study, total RNA was extracted from bone and bone marrow that had been sampled from six skeletally mature rabbits at time points between zero and 31 days after death. The levels of three specific RNA transcripts could be quantified using real-time polymerase chain reaction. Bioanalyzer results show rRNA bands in bone marrow samples up to 21 days postmortem. We hereby propose bone marrow as a potential source for postmortem RNA in forensic studies.

KEYWORDS: forensic science, ribose nucleic acid degradation, bone marrow, bone, reverse-transcription quantitative polymerase chain reaction, 18S ribosomal ribose nucleic acid, hemoglobin, hypoxanthine-guanine hosphoribosyltransferase

With recent developments in genetic analysis technology, molecular forensics has received increased interest over the last decades. For example, DNA is particularly useful for identification purposes and can determine the sex and genetic ancestry of an individual. It has also found its merit in archaeological sciences and has been demonstrated to be preserved in a variety of tissues, albeit in an often extremely fragmented manner (1). In addition, RNA content of a cell might also convey useful information about its source. However, in contrast to DNA, it has received much less attention, largely perhaps because of the fact that in many ways it is much less stable than DNA—its structure renders it relatively more available for the ubiquitous presence of RNAses that rapidly degrade RNA in many situations. Despite this, however, several recent studies have revealed that relatively intact RNA can be observed over longer postmortem periods than previously was expected (2,3). This finding has sparked a renewed interest in the potential of RNA for forensic sciences (4), and while this area is still in its infancy, with few standardized approaches advocated, a number of useful papers have been published. First, while in contrast to DNA, RNA holds little information of use to identify an individual, as an intermediate for protein synthesis, mRNA levels reflect tissue-specific gene expression, as different tissues require different levels of proteins or specific enhanced expression. This has led to the use of RNA in forensic science as a tool to identify tissues and body fluids, such as saliva and semen, and with the aid of such genetic markers, venous blood can be efficiently distinguished from menstrual blood (5) and saliva (6). For similar reasons, as differential gene expression is linked to certain pathologies, RNA could provide information about premortem conditions (2,4). As any change in functional activity in a cell is reflected in gene expression, it is in theory possible that cause and manner of death can be detected through mRNA analysis (4). In an additional use, it has been demonstrated that RNA markers can be used for wound age determination, showing differential gene expression through different stages of vitality (7). Lastly, RNA has also drawn attention as a potential tool for use in investigating tissue postmortem degradation. Several valuable papers have been published on this topic indicating useful sources for RNA (Table 1), although to date none on one particular tissue that might be of interest—bone marrow.

Bone and, especially, bone marrow may offer several possible advantages to forensic studies. First, a close relationship is expected in gene expression between bone marrow and blood, on which previous studies have been performed with success (Table 1). Second, putrefaction is known to be delayed in marrow even though it is, as is bone, a vascular tissue containing blood vessels and capillaries throughout, and marrow can be retrieved from rabbits after 2 weeks of burial (8). Third, various observations have hinted at potential relatively long-term RNA survival in marrow—in the early 1960s, it was found that cell motility in bone marrow was sustained up to 50 h postmortem (9) (indeed, this observation formed the basis for bone marrow transplantations carried out today). Such cell motility would suggest survival of RNA and therefore a suitability of this tissue as a source for postmortem RNA. From another study, it emerged that different cell types from bone marrow undergo postmortem autolysis in a predictable sequence, adding to the expectation that degradation patterns on a molecular level may still come forward (10). Fourth, as a part of the immune system, bone marrow

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TABLE 1—*Stability of RNA in postmortem tissues.*

Tissue Type	Stability of RNA	References
Brain	36 h–7 days	(2)*, (3)*,†, (13)‡, (17)†, (26)*, (20)†
Venous blood	3–4 days	(17)†
Dried blood	150 days–15 years	(27)†, (28)†, (29)†
Tendon	96 h	(13)‡
Ligament	96 h	(13)‡
Cartilage	96 h	(13)‡
Liver	24–48 h	(2)*, (13)‡, (20)†, (30)§
Heart	56–96 h	(2)*, (20)†
Lung	96 h	(2)*, (13)‡
Spleen	56 h	(20)†
Trabecular bone	48 h	(16)†
Retina / RPE	5–12 h	(31)¶
Kidney	56 h	(13)‡, (20)†
Hair roots	10 days	(22)†
Adipose tissue	22 days	(30)§
Skeletal muscle	118 h–8 days	(20)†, (30)§

Examined model: *Murine; †Human; ‡Rabbit; §Bovine; ¶Porcine.
RPE, retinal pigment epithelium.

may express an elevated level of specific genes and more variety in mRNA expression may be expected, as the immune system will keep up with slight changes to adapt to foreign antigens. Thereby, the likelihood of finding potential markers for either time-since-death indication, premortem condition or pathology markers is increased. In fact, B and T cells in bone marrow possess an RNA decay mechanism, referred to as the nonsense-mediated decay pathway (11) that clears any transcript with a premature termination codon to avoid the production of abundant and deleterious proteins. Finally, it has been suggested that bone creates a physical barrier for bone marrow that could prevent direct exogenous contamination or exposure, even following trauma (8).

Given these possible advantages, we have undertaken a preliminary analysis to isolate total RNA from compact bone and bone marrow, through the extraction of RNA from rabbit samples at various stages of decomposition. In addition, we subsequently characterized the levels of three different RNA transcripts using reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR). Two of the markers chosen were housekeeping genes verified in previous RT-qPCR studies: 18S ribosomal RNA (18S rRNA) and hypoxanthine-guanine phosphoribosyltransferase, while the third was hemoglobin, a gene expected to be highly abundant in bone marrow and known to be a stable transcript (12). The objective of this preliminary study was to establish, for the first time, up to which time postmortem RNA in bone could still be detected at a quantity and quality that was sufficient for RT-qPCR analysis. Rabbits were used as sample subjects to investigate trends of degradation of bone marrow while the marrow cavity still receives protection from surrounding body tissues. Furthermore, rabbit tissues have previously been investigated for postmortem RNA and could provide for comparative results (13).

Methods and Materials

Samples

Six skeletally mature wild rabbits (*Oryctolagus cuniculus*) were used as a model system for the experiment. These specimens were obtained from a game-keeper and originally destined for human consumption and were isolated at room temperature in glass tanks, within 24 h after death. Fine mesh was used to cover the tanks to allow aerobic decomposition and avoid oviposition by blowflies and other insects as experiments were conducted in Bradford (U.K.) during May and June. Bone and bone marrow samples were subsequently collected at the following times: 0, 4, 7, 15, 21, and 31 days postmortem from a single rabbit at each time point. The tibia and femur were removed after skinning the legs to avoid contamination through contact with any fur. Muscle tissue and ligaments were removed with aid of a sterile scalpel. A fresh hacksaw blade, cleaned prior to use with 70% ethanol, 1% SDS, and 3% H_2O_2, was used to saw the bone. Small samples of compact bone from the midshaft diaphysis of the femur (*c.*1 cm^2) were placed in 1.5-mL tubes in RNAlater (Ambion, Austin, TX). Bone marrow was collected using a sterile needle and stored in five volumes of RNAlater. All were subsequently stored at <−40°C.

RNA Extraction—Compact Bone

Prior to extraction, bone material was allowed to thaw and removed from the RNAlater stabilizing solution. The outside of the bone was carefully cleaned with a scalpel to remove the residual muscle, tendon, and ligament tissue. Eight hundred and fifty milligrams of bone was subsequently crushed and ground to powder using a Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany), plus crushing vessel cooled using dry ice (14). Powdered bone was recovered from the crushing vessel using 2 mL of 0.5 M EDTA (pH 8.0) and allowed to decalcify by incubation with agitation, overnight, at room temperature. The solid fraction was digested prior to purification in 1 mL of buffer containing 10 mM Tris–HCl pH 8, 10 mM NaCl, 5 mM $CaCl_2$, 2.5 mM EDTA pH 8, 1% SDS, 10% v/v Proteinase K solution, and DTT in excess and incubated overnight at 65°C. Two hundred microliters of this digest was then used for RNA purification.

Bone Marrow Samples

Bone marrow samples were allowed to thaw and centrifuged for 5 min at 9500 × *g*. The marrow separated into three phases during centrifugation: a white pellet on the bottom of the tube, most likely to contain leukocytes; a red pellet on the surface, most likely to contain erythroblasts and erythrocytes; and the supernatant consisting of RNAlater. The white pellet and solid red residue pellet were resuspended in 1 mL of digestion buffer and incubated overnight in 1 mL of digestion buffer at 65°C. As a control against RNA leaching from the bone or marrow while in storage, 100 μL of the storage RNAlater supernatant was also purified (the resulting data indicated no RNA leaching, data not shown). The remaining RNAlater supernatant was discarded. Two hundred microliters of the digest of all fractions was then used for RNA purification.

RNA Purification

We have previously demonstrated the Qiagen DNEasy Micro kit to be a suitable tool for the recovery of pure RNA (15). A pilot study performed here (data not shown) demonstrated equally good results using Qiagen's Qiaquick spin columns. Therefore, following digestion, RNA was purified from the lysates using the Qiaquick spin columns column protocol (Qiagen, Valencia, CA). RNA was eluted from the columns in final volumes of 100 μL of buffer EB, by centrifugation after 5-min incubation at room temperature (following the manufacturer's guidelines).

Assessment of RNA Integrity

RNA integrity was assessed using several methods. An initial overview of the state of RNA preservation was assayed using RNA

Pico Chips as implemented on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). This required pretreatment of the extracts to degrade any DNA, through incubation with DNase I (Invitrogen, Carlsbad, CA).

Subsequently, RNA quality was assayed for the three chosen transcripts of interest using SYBR-green-based quantitative real-time PCR (qPCR). A relative quantification was taken for all three markers, whereby the PCR amplifiable level of each transcript was assessed in comparison with the level of each transcript at time zero. These data in turn enabled two complementary analyses to be performed, relative quantification of each transcript through the degradation time series and the rate of differential degradation between the three transcripts. For the former, the level of each transcript RNA in each degraded sample was directly compared (following normalization for sample weight) to its level in the control (time = 0) samples. For the latter, the relative levels of each of the genetic markers within each sample was assessed and contrasted throughout the series. These two measures therefore enable the assessment of the rate of degradation of single markers and the relative rate of degradation of multiple markers, respectively.

Conventional and Quantitative PCR

The qPCR was directly performed on cDNA synthesized from 1 ng of total RNA template. DNA was initially removed from the purified nucleic acids using DNase I (Invitrogen) and then reverse-transcribed using random hexamers and the reverse transcriptase Superscript III (Invitrogen). Primer sets used in the subsequent qPCR were designed to target a number of genes (Tables 2 and 3), and where possible were located over exon/exon boundaries, to aid discrimination between RNA-derived cDNA and nuclear DNA. qPCR was performed in 25-μL reactions that contained 1 μL of cDNA, 1× PCR buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 0.4 μM forward and reverse primer, 0.1 μM equimolar dNTPs, 1 μM SYBR Green plus ROX reference agent (Invitrogen), and 0.5 U *Taq* Gold (Applied Biosystems). qPCR was performed using a Stratagene MXpro thermocycler (Stratagene, La Jolla, CA) in the presence of at least two negative template controls. All qPCR incorporated a triplicate dilution series of each sample to control for inhibition. Thermocycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, (30 s at 95°C, 45 s at 60°C) × 40, 1 min at 95°C, end. Amplified PCR products were checked for dimer formation by subsequent melting curve analysis. Amplicon length was checked by gel electrophoresis. Tests using conventional PCR were run under identical thermocycling conditions and reagents minus SYBR Green Dye.

Results

Decomposition of Rabbit Carcasses

Fresh muscle tissue was often tough to cut away from the bone, especially when rigor mortis was still present. Hair could be easily scraped away after 14 days postmortem. At 21 days postmortem, the epidermis had fully decomposed and muscle tissue had completely lost its structure. The rabbit left out for the longest period developed a manifestation of blowflies, evident on the 29th day of exposure postmortem. As the tanks were covered, oviposition likely took place before the rabbit was placed inside the tank. Liquefaction had not taken place as progressively as in the rabbit of 21 days, and muscle tissue was still relatively intact and had remnants of structure. The muscle tissue was desiccated along parts of the limbs.

TABLE 2—*Genes from rabbit used in quantitative RT-PCR for RNA degradation assessment.*

Gene	Accession Number	Function
HBB	AY034468	Metal ion homeostasis
HPRT	AF020294	Purine metabolism
18S rRNA	X00604	Ribosomal subunit

Bioanalyzer Results

Bioanalyzer analysis of RNA quality indicated that RNA degradation had occurred in most samples (Figs. 1 and 2), which can be attributed to the fact that even in the most fresh sample, some delay between somatic death and sample isolation had occurred. In the bone digest samples, the rRNA peaks could be observed up to 4 days. In the leukocyte (white pellet) fraction in bone marrow, peaks consistent with rRNA length were visible in samples from day 0, day 4, and even at 21 days postmortem. In the erythroblast (red pellet) samples, a high quantity of very short (ca. 30 nt) and discrete fragment lengths of RNA was detected in all samples, but no discrete rRNA bands were visible. A conventional reverse-transcription PCR (RT-PCR) run on the total RNA extract and the cDNA of the control sample (day 0) showed specific amplification of 18S rRNA of the cDNA template (Fig. 3).

Relative Quantification

For all tissue samples, the total amount of RNA was highest in the leukocyte fraction (Tables 4 and 5), consistent with what would be expected; leukocytes possess nuclei. The transcript level for the other fractions appeared to be no higher than the assay's background sensitivity. The relative quantification results for each transcript in the bone marrow fractions, and the bone digest, can be seen in Fig. 4. In this preliminary study, one tissue sample was collected at each time point. Each quantitative PCR experiment was run in triplicate. For all transcripts, in all samples in the leukocyte fraction, RNA degraded rapidly to the level of the assay's background sensitivity.

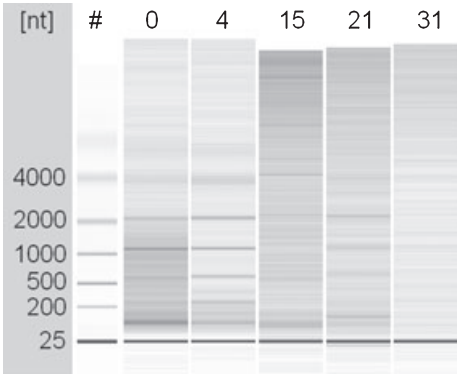

FIG. 1—*Virtual gel image of RNA in bone marrow leukocyte fraction at time points 0, 4, 15, 21, and 31 days postmortem. A ladder (#) is used as a standard size reference. Typical ribosomal RNA bands can be seen just over 2000 and 1000 nt, indicating the presence of 28S and 18S rRNA, respectively. The band at 25 nt is an internal standard. Intensity of the bands does not reflect RNA amount compared between samples, but nucleotide length depicted along the* y*-axis is the same in each image.*

TABLE 3—*Primer sequences, target position, and size of amplicons (in bp) for quantitative RT-PCR, designed in-house, based on rabbit sequences of the genes investigated.*

Gene	Forward	Start	Reverse	Start	Size
HBB	GAGGGTCTGAATCACCTGGA	210	GATCCACATGGAGCTTGTGA	272	82
HPRT	TGCTCGAGATGTGATGAAGG	144	AATCCAGCAGGTCAGCAAAG	221	95
18S rRNA	CCCGAAGCGTTTACTTTGAA	785	CCCTCTTAATCATGGCCTCA	901	136

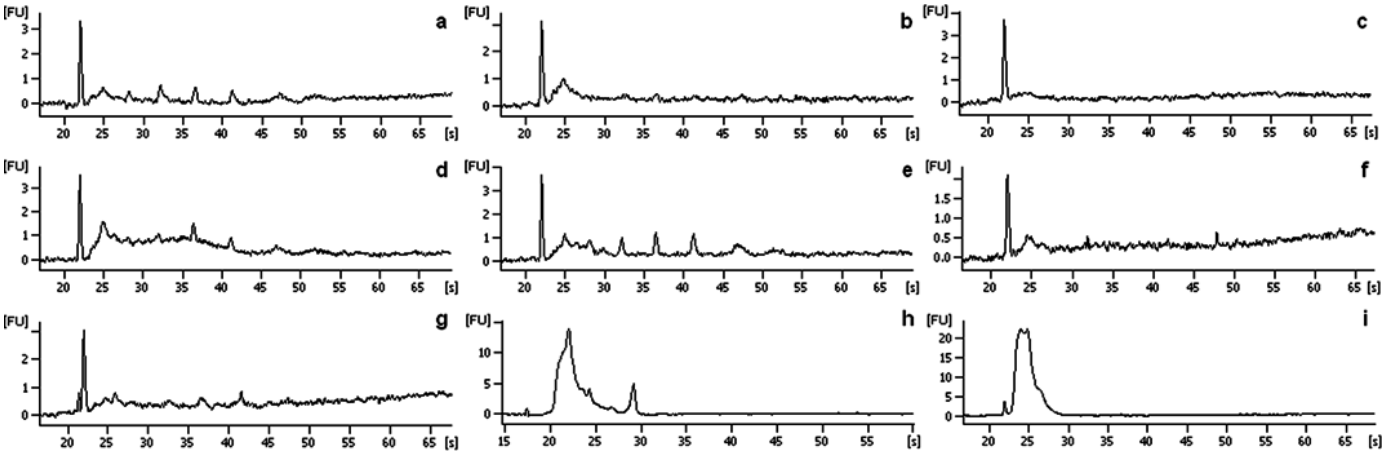

FIG. 2—*Electropherograms of RNA in bone digest, bone marrow leukocyte, and bone marrow erythroblast fraction. Top row: Bone digest, time points 0, 4, and 15 days. Middle row: Bone marrow leukocytes, time points 0, 4, and 15 days. Bottom row: Bone marrow leukocytes, time point 21 days, and bone marrow erythroblasts, time points 0 and 15 days. Fluorescence units along the* y*-axis are arbitrary and do not reflect a correlation between amount of fluorescence and amount of RNA present. The amount of RNA present can be deduced by integration under the curve and subsequent comparison to the known peaks of the standard. The retention time along the* x*-axis, however, corresponds with fragment size and is comparable between samples.*

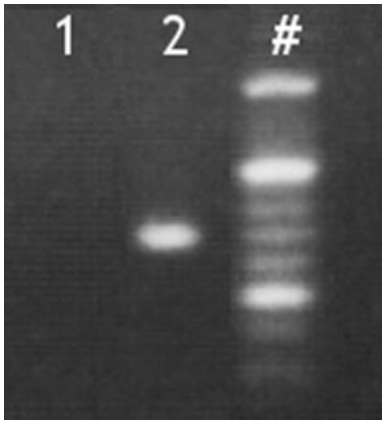

FIG. 3—*Analysis by electrophoresis to check for presence of nuDNA. Electrophoresis on a 2% agarose gel with a 20-bp ladder (#) as a reference conducted at endpoint after PCR. The product shown is the 136-bp 18S rRNA amplicon. Lanes: (1) Total RNA extract treated with DNase, (2) cDNA of total RNA extract.*

TABLE 4—*Level of gene amplification between tissue types at 0 days postmortem.*

Tissue type	18S rRNA (%)	HPRT (%)	HBB (%)
Leukocytes	100.00	100.00	100.00
Erythroblasts	1.43	0.00	4.19
Bone digest	0.033	0.23	0.11

Discussion

Degradation of RNA

Marchuk et al. (13) have reported that no significant degradation of RNA was detectable up to 96 h postmortem in hypocellular tissues of rabbits. In bone, no sustained RNA integrity was observed using either RT-PCR or Bioanalyzer results after 4 days postmortem. However, this is still in concert with Kuliwaba et al. (16) who determined RNA stability up to 48 h in trabecular bone. RNA stability in bone marrow itself has not been reported in this context, as far as we are aware. In terms of postmortem RNA stability, the most equivalent tissue to marrow leukocytes is arguably leukocytes from venous blood in which RNA integrity is maintained for at least 3–4 days postmortem (17), and our Bioanalyzer results indicate that in marrow leukocytes this may be up to 21 days postmortem. Rapid degradation of RNA in tissues for potential assay investigations may be disadvantageous, especially for quantitative studies. To say anything useful about time-dependent RNA degradation, the decay rate needs to be known and the mechanism or mechanisms well established. At this stage, current technology may only be sufficient to use RNA for qualitative studies and tissue identification.

Relative Quantification

The data indicate that changes occur in the relative rate of degradation of the three transcripts after initial decay of the transcripts (Fig. 4). It was expected that RNA levels would rapidly diminish after time point 0, and for most genes that were studied, this is indeed the case.

At day 15, RNA levels are remarkably low, whereas both Bioanalyzer data and qRT-PCR reveal higher signals for ribosomal RNA and the three genes investigated, respectively. With the nature of the experiment and the sample size, it is possible that this is because of a sample artifact or degradation after sampling. However, the sample from day 15 was obtained from the largest rabbit, and the most sample material was obtained and yet the RNA results are unexpectedly lower. These observed differences might be the result of several different factors. In the early stages of the timeline, metabolic levels of expression at the time of death could have a stronger influence than the amount of degradation. At later stages, around 7–15 days postmortem (as decay has found to be delayed in bone marrow [8]), chemical changes during degradation, such as the breakdown of proteins (18), may occur. As these may help to maintain the secondary structure of endogenous mRNA, the molecular environment can shift and mRNA becomes more easily unfolded, causing a better performance in cDNA synthesis. However, unfolded and exposed RNA templates are more vulnerable to enzymatic degradation, and primary structural features of RNA would then become the main determinant of the decay rate. The relationship between cDNA template quality and specific RNA degradation patterns has not yet been fully investigated, but there are indications that low quality of RNA template can produce astoichometric product ratios in cDNA synthesis (19).

TABLE 5—*Concentration of RNA samples in μg/g of tissue fraction starting material.*

Time Postmortem (days)	Leukocytes	Erythroblastss	Bone Digest
0	20.623	2.113	0.068
4	7.439	17.514	0.166
7	12.917	19.044	0.273
15	3.573	5.568	0.097
21	2.948	7.697	0.066
31	6.624	0.000	0.002

Choice of Tissue

The problem of obtaining bone and bone marrow samples poses the question whether there might be a more readily available tissue, which would allow for faster sampling and equal, or higher, potential in results. Brain has been demonstrated as a relatively stable tissue with regard to the preservation of RNA integrity, even after freezing and thawing of samples (2,17,20). However, mRNA quality from brain tissue has been known to be influenced by pre- and perimortem state of the deceased (21), involving factors such as pH, which may complicate matters. Alternatively, this may give rise to gene expression assays that concern pre- and perimortem conditions of the brain. For a time-since-death assay, tissue with a prolonged RNA integrity would be preferred. Hair roots may still provide an interesting noninvasive tissue to investigate, as King et al. (22) report that RNA could be successfully extracted from hair even after plucking and storage at room temperature for 10 days, suggesting that hair manages to protect RNA for a considerable time.

Conclusions

Although both this study and others (see Table 1) have observed that RNA degradation after death seems to be time dependent on the postmortem interval (PMI), and although RT-qPCR techniques can be applied to small sample sizes and can produce reproducible data and quantitative analysis, opinions do vary on the usefulness of RNA degradation assays for PMI indication. Bauer (4) is remarkably positive on many facets of RNA in forensic sciences and even proposes that theoretically a database could be constructed to encompass all environmental factors that are reflected in RNA degradation profiles.

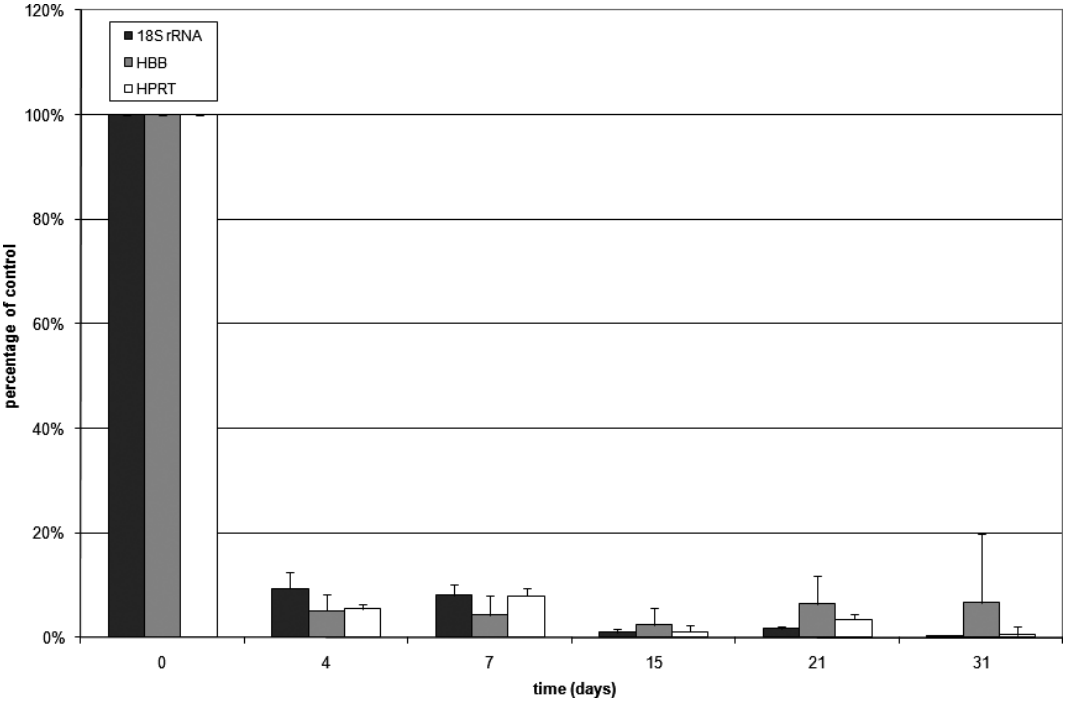

FIG. 4—*The percentage of PCR signal relative to the control is set out against postmortem time prior to sample collection. The control is the bone marrow leukocyte fraction at time point 0. All other samples were normalized against this standard set at 100% per definition and are averaged from triplicate reactions. All samples, including the control, were normalized against original sample weight.*

Henssge and Madea (23) are less enthusiastic about new methods that reflect merely postmortem changes, "since nearly all parameters are changing more or less with increasing postmortem interval," and saying that nearly all of those methods are easily influenced by ambient conditions. That noted, RNA degradation may be an indicator of molecular cell integrity and accessibility toward enzymatic degradation, rather than absolute time-since-death. Our results indicate that the leukocyte fraction from bone marrow is a good source of RNA: the ribosomal RNA peaks are visible for the oldest sample from the time series. It seems possible to extract RNA from bone up to 4 days in reasonable quality, although judging from the results RNA concentration in bone is low compared to original sample size and weight. However, estimation of time-since-death with the aid of RNA degradation remains of questionable value. A hiatus in literature on postmortem RNA decay mechanisms persists and the main determinants involved are still unclear.

It will be essential to not limit further research to simply refining the studies of timelines in postmortem tissues. An extensive hiatus regarding RNA in postmortem tissues still exists. Tomita et al. (24) have presented a first step toward clarifying the mechanisms of "nonprogrammed" cell death. However, regarding the degradation mechanisms in postmortem cells, very little is known about the biochemistry concerned with this type of decay. Almost every study dealing with RNA degradation in timelines poses interesting arguments for discussion, but there seems to be no consensus and sometimes arguments contradict (17,21). Different parameters may even be the determinant factors spread over the course of the postmortem interval, and these may alternate in their influence on enzymatic RNA degradation. Moreover, it has been found that a short half-life of mRNA *in vivo* does not necessarily mean that the postmortem susceptibility to enzymatic decay is higher (25). As every discussion regarding RNA in postmortem touches on this subject to explain the observed results, it is a critical point for understanding what happens with RNA and what its exact behavior is in laboratory processes and storage after sampling. Without exact knowledge of the truth about postmortem RNA, it will be difficult to confirm whether what is seen in degradation studies is in fact because of enzymatic turnover as it would occur in living cells. Investigating the mechanisms and occurrences on a molecular level in postmortem RNA is an inviting and interesting opportunity for any group aspiring to further investigate the forensic potential of RNA.

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