Mol Biotechnol
DOI 10.1007/s12033-011-9493-4

RESEARCH

# RNA Stability in Human Liver: Comparison of Different Processing Times, Temperatures and Methods

**Serene M. L. Lee · Celine Schelcher · Sevdije Gashi · Stefanie Schreiber · Reinhard M. K. Thasler · Karl-Walter Jauch · Wolfgang E. Thasler**



**Abstract** The accuracy of information garnered by real-time quantitative polymerase chain reaction (RT-qPCR), an important technology for elucidating molecular mechanisms of disease, is dependent on tissue quality. Thus, this study aimed to determine the effects of intra-operative manipulation, extended processing times, different temperatures or storage in RNAlater on RNA quality in liver samples for tissue banking. Liver samples, flash-frozen or in RNAlater, were collected over a time course (during surgery before blood arrest up to 1 day after surgery) with samples kept either at room temperature (RT) or on ice. This study showed that at the longest time-point at RT, the RNA quality decreased significantly by 20%. However, relative gene expressions of *FOS*, *GUSB*, *MYC*, *HIF1α* and *GFER* were in general not significantly different when the time-points were compared. In conclusion, samples should be kept on ice during processing, and either RNAlater or snap-freezing should be utilised for storage. Further, intra-operative manipulation and extended postoperative processing time generally does not change relative gene expression levels for the 5 genes studied, making such sampling suitable for RT-qPCR analysis. Thus, if relative gene expression of a gene of interest is stable, these guidelines will lead to increased accrual of samples to the tissue bank.

**Keywords** Bioanalyzer · Liver · RNAlater · RNA integrity · RNA quality · RNA stability · Real-time quantitative polymerase chain reaction (RT-qPCR) · Tissue bank

## Introduction

The Grosshadern Hospital Tissue Bank was established in the year 2003 and is regulated by the state-controlled Human Tissue and Cell Research (HTCR) Foundation. This human tissue bank serves as a repository of surgical remnant tissues, consists of tumour and surrounding "normal" tissue, and includes tissues such as liver, gut, skin, kidney, thyroid gland, pancreas and lung. The main purpose of this tissue bank is to disseminate tissues annotated with clinical information for basic and translational research. It is hoped that this resource will help boost research leading to the molecular characterisation of diseases.

An important technology for such investigations is real-time quantitative polymerase chain reaction (RT-qPCR), which is a powerful tool for quantitative determination of differential gene expression [1]. Presently, RT-qPCR is one of the most sensitive, accurate and reproducible methods for mRNA content quantification and can be adapted for use as a high-throughput method for examining gene expression for a limited set of genes [1, 2]. However, the accuracy of the information obtained from RT-qPCR is highly dependent on the quality of the tissue, which can be affected by

Serene M. L. Lee and Celine Schelcher are equal authors.

S. M. L. Lee · C. Schelcher · S. Gashi · S. Schreiber · W. E. Thasler
Grosshadern Hospital Tissue Bank, Department of Surgery, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany

R. M. K. Thasler
BioM BioTech Cluster Development GmbH, Martinsried, Germany

K.-W. Jauch · W. E. Thasler (✉)
Department of Surgery, Grosshadern Hospital, Ludwig Maximilians University, Munich, Germany
e-mail: Wolfgang.Thasler@med.uni-muenchen.de

Published online: 24 January 2012

intra-operative manipulation and postoperative processing of the tissues.

In an ideal situation for research, liver samples should be collected as soon as possible during surgery, without surgical manipulation, warm ischaemia, and processed for storage immediately, so that the samples collected are good representations of the in vivo situation. However, when working with human livers, resected liver pieces can generally only be available after the department of pathology is done with the tissue. The collection of human tissues is also sometimes subjected to delays in the cases of complications or if there is a requirement for transporting the sample to a different medical centre. In order to find out the effect of surgical duration and hence warm ischaemia on RNA quality, Almeida et al. [3] examined a 4-h time course in rat liver. For determining the effect of processing time, several articles have investigated the time course of RNA quality at room temperature as follows: in human lung for up to 5 h [4], in rat liver for up to 4 h [3], in human breast tissue for up to 3 h [5] and in human colon and tonsil tissue for up to 16 h [6]. Micke et al. [6] also examined the same time course in human colon and tonsil tissue placed on ice.

Further, when working with human tissues as compared to animal tissues, there are additional factors, such as intra-operative manipulation, warm ischaemia time during resection that could affect RNA stability. To our knowledge, the effects of intra-operative manipulation and postoperative processing time, temperature and storage method on RNA stability in human liver samples are not known at present. It is important to determine how the above conditions affect RNA stability in order to establish criteria for accrual of tissues to the tissue bank.

In general, surgical removal of the liver specimen in Grosshadern hospital does not exceed an hour after arterial cross-clamping, with the average time being 17 min for this study. After surgical removal, the sample is carried to the pathology department on ice, where a pathologist would examine the tissue at room temperature (25°C) and take the necessary samples. The residual tissue is then released to the tissue bank (in approximately 20 min) for further processing.

The effects of longer processing times on RNA stability are important as Grosshadern Hospital serves as a centralised tissue bank for the processing and storage of liver samples from other community hospitals in Munich. In this tissue collection network, it generally takes 1.5 h to transport the tissues back to the tissue bank at Grosshadern Hospital. At most, transport and processing of liver samples for storage will take a maximum of 3 h. Further, it is also of interest to determine whether tissues resected late at night are still suitable for banking if they are processed the next morning for long-term storage. To our knowledge, there have not been any investigations made on RNA stability in human liver subjected to extended processing times. Thus, this study aimed to address the above issues by investigating the effects of processing time, temperature and method on RNA quality. This is important for determining whether the liver samples collected would be appropriate to use for further molecular research and whether it is worthwhile to bank such tissues. This information can then be used to standardise and establish minimum criteria for banking liver samples in other tissue banks regulated by the HTCR foundation and in the newly formed Munich Biobank alliance, which consists of several de-centralised tissue banks in Munich.

In this study, RNA integrity was assessed by RNA integrity number (RIN) provided by the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The effects of processing times, temperatures and storage methods on the mRNA expression levels of 5 genes were measured by RT-qPCR. These genes included 2 reference genes (*HPRT1*, *GUSB*) [7, 8], 2 immediate early gene (*FOS* and *MYC*) [9], 1 hypoxia-induced gene (*HIF1α*) [10] and 1 hepatic growth factor (*GFER*) [11].

## Materials and Methods

### Human Liver Sample Collection

Remnant human liver samples were collected from surgical resections with informed consent in accordance with the guidelines of the state-controlled HTCR Foundation [12].

Figure 1 shows a schematic detailing the tissue collection procedure. Briefly, liver samples (approximately 100 mg) were collected from the margin of normal tissue that surrounds resected tumours over a time course for all donors ($N = 6$). These time-points were during surgery before the arrest of blood circulation and surgical resection ($T_0$) by means of a biopsy, immediately after surgery ($T_{OP}$), post sampling by the pathology department ($T_{PP}$), 3 h after $T_{PP}$ ($T_{PP+3h}$) and 1 day after $T_{PP}$ ($T_{PP+1d}$). For the last 2 time-points, half of the samples were kept for the duration in a container at room temperature (RT) and the other half of the samples were kept in a container left on ice. After the 5 time-points, individual liver samples were flash-frozen in liquid nitrogen for storage at −80°C or put in RNAlater (Ambion, Darmstadt, Germany) and processed for eventual storage at −80°C, according to the manufacturer's instructions. These frozen samples were then used for subsequent experiments.

### RNA Extraction

Pre-weighed liver samples (approximately 30 mg each) were homogenised in lysis buffer (from the RNeasy Mini Kit) using the Tissuelyser LT (Qiagen GmbH, Hilden,

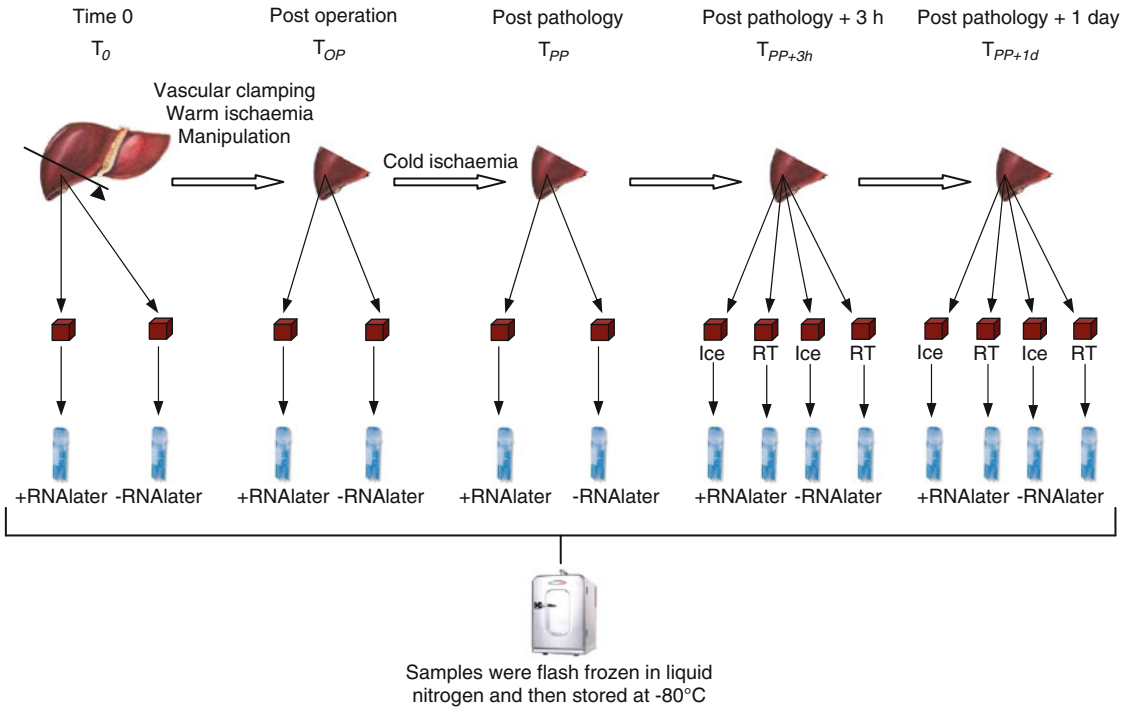


**Fig. 1** Experimental design for the collection of liver samples. The *red cubes* represent 100-mg liver samples. Room temperature is abbreviated as RT (Color figure online)

Germany), according to the manufacturer's instructions. Total RNA was then extracted from the homogenate using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), with DNA removed during the process by an on-column DNase digestion step using the RNase-free DNase Set (Qiagen GmbH, Hilden, Germany). All RNA extraction steps were carried out according to the manufacturer's instructions.

## Measurement of RNA Integrity

The Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) was used to analyse RNA and generate a RIN according to the manufacturer's instructions.

## RNA Quantification

RNA was quantified using the Quant-iT Ribogreen RNA Reagent and Kit (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions. Fluorescence was quantified using an Applied Biosystems Cytofluor 4000 Microplate Reader (Darmstadt, Germany).

## Complementary DNA Synthesis

Complementary DNA was synthesised from 2.0 µg of RNA in a final reaction volume of 20 µl using the Superscript Vilo cDNA Synthesis Kit (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions. This kit utilises random primers for cDNA synthesis. cDNA produced was stored at −20°C for subsequent use.

## Real-Time qPCR

Real-time qPCRs were performed in a StepOnePlus Real-time qPCR System (Applied Biosystems, Darmstadt, Germany) using Taqman Gene Expression Assays (Applied Biosystems, California, USA), with primer and amplicon information for the 6 genes studied given in Table 1. Multiplex quantitative polymerase chain reactions were performed in duplicate using a MicroAmp Fast Optical 96-well Reaction Plate with barcode, 0.1 ml (Applied Biosystem, Darmstadt, Germany). The final reaction volume was 10 µl, which contained 2 µl of the 10× diluted cDNA sample, 5 µl of Taqman Fast Universal PCR Mastermix (2X), No AmpErase UNG (Applied Biosystems, Darmstadt, Germany), 0.5 µl of Taqman Gene Expression Assay Mix linked to a FAM dye (Applied Biosystems, Darmstadt, Germany), 0.5 µl of Taqman endogenous control linked to a VIC dye and 2 µl RNase-free water. The final concentration of the forward and reverse PCR primers was 0.9 µM each and the concentration of the TaqMan probe was 0.25 µM. Samples were subjected to a 2-step cycling reaction; following an initial denaturation at 95°C for 20 s, samples were subjected to 40 cycles of (1)

**Table 1** Accession number, primer and amplicon information

| Gene | Exon boundary | Assay location | Amplicon (bp) | Accession number | PCR efficiency | Secondary structure | Assay identification number (Applied Biosystems) |
|---|---|---|---|---|---|---|---|
| *FOS* | 1–2 | 352 | 77 | NM_005252.3 | 1.8 | None | Hs00170630_m1 |
| *GFER* | 1–2 | 332 | 71 | NM_005262.2 | 1.9 | None | Hs00193365_m1 |
| *GUSB* | 8–9 | 1522 | 96 | NM_000181.3 | 2.0 | None | Hs00939627_m1 |
| *HIF1A* | 4–5 | 860 | 76 | NM_001530.3 | 1.9 | None | Hs00153153_m1 |
| *HPRT1* | 1–2 | 190 | 72 | NM_000194.2 | 1.9 | None | Hs01003267_m1 |
| *MYC* | 1–2 | 561 | 87 | NM_002467.4 | 2.0 | None | Hs00905030_m1 |

denaturation at 95°C for 1 s followed by (2) annealing and elongation at 60°C for 20 s. Negative controls without cDNA template were included for every gene of interest.

For reference genes *HPRT1* and *GUSB*, the effects of the experimental conditions on their gene expression were determined using the $2^{-\Delta C_T'}$ formula [13]. The PCR efficiency for each PCR was determined by using the program LinRegPCR (Heart Failure Research Centre, Amsterdam, the Netherlands) and average efficiency was found for each of the genes of interest (Table 1). For each gene, mRNA abundances expressed as quantification cycles (Cq) for each experimental condition were expressed relative to the reference gene *HPRT1* and normalised to the values at $T_0$ using the efficiency-corrected equation of Pfaffl [14].

### Statistical Analyses

Results are presented as means ± standard error of the mean (SEM), with $N = 6$. The data in Figs. 2, 3, 4 were analysed using repeated measures one-way analysis of variance (ANOVA), followed by Student–Newmen–Keuls test. In addition, paired *t* tests were carried out to evaluate the differences between means with or without RNAlater. Differences with $P < 0.05$ were regarded as statistically significant.

## Results

### Effects of Time, Temperature and RNAlater on RNA Integrity

RNA integrity as ascertained by RIN number was essentially stable for all time-points tested except for a significant decrease at $T_{PP+1d}$ at RT, with means of plus and minus RNAlater both 20% lower than corresponding $T_0$ values (Fig. 2). Although there were also significant differences between plus and minus RNAlater for $T_0$ and $T_{pp}$, the absolute change in RIN number is very small with only a 2.0 and 3.7% increase, respectively (Fig. 2).

### *HPRT1* and *GUSB* are Suitable for Use as Reference Genes

Although *HPRT1* and *GUSB* gene expressions were significantly decreased at $T_{pp+1d}$ at RT, there were no significant differences when their gene expressions were compared across the other time-points (Fig. 3). Further, the trends of *HPRT1* and *GUSB* mRNA levels with time were closely paralleled and as such, either gene would be suitable for use as a reference gene. Thus, for further calculations, *HPRT1* was chosen for use as a reference gene.

### Effects of Time, Temperature and RNAlater on Gene Expression

There was a significant decrease in gene expression of *GUSB* at $T_{PP+1d}$ (RT, +RNAlater) compared with that of $T_0$ and $T_{OP}$ by 26 and 22%, respectively (Fig. 4c). More importantly, there were generally no significant differences in relative gene expression levels for the 5 genes of interest (*FOS*, *GFER*, *GUSB*, *HIF1a* and *MYC*) when compared across the various time-points (Fig. 4). When comparing the effects of RNAlater, there was only a 18% decrease in gene expression level of *GFER* at $T_{PP+1d}$ (On ice) (Fig. 4).

## Discussion

In the past, RNA was commonly assumed to degrade rapidly after tissue excision or after death due to its highly unstable nature. However, while purified RNA is prone to degradation by the ubiquitous presence of RNases, RNA is surprisingly stable in fresh tissue possibly due to the preserved cellular structure [6]. This is evidenced by a number of studies, which have found that RNA is stable in rabbit hypocellular connective tissue, brain, lung and kidney for up to 96 h at 4°C [15], bovine muscle for up to 8 days at 4°C [16], human lung tissue for up to 5 h at room temperature [4], human breast tissue for up to 3 h at room temperature [5], human tonsil and colon tissue for up to

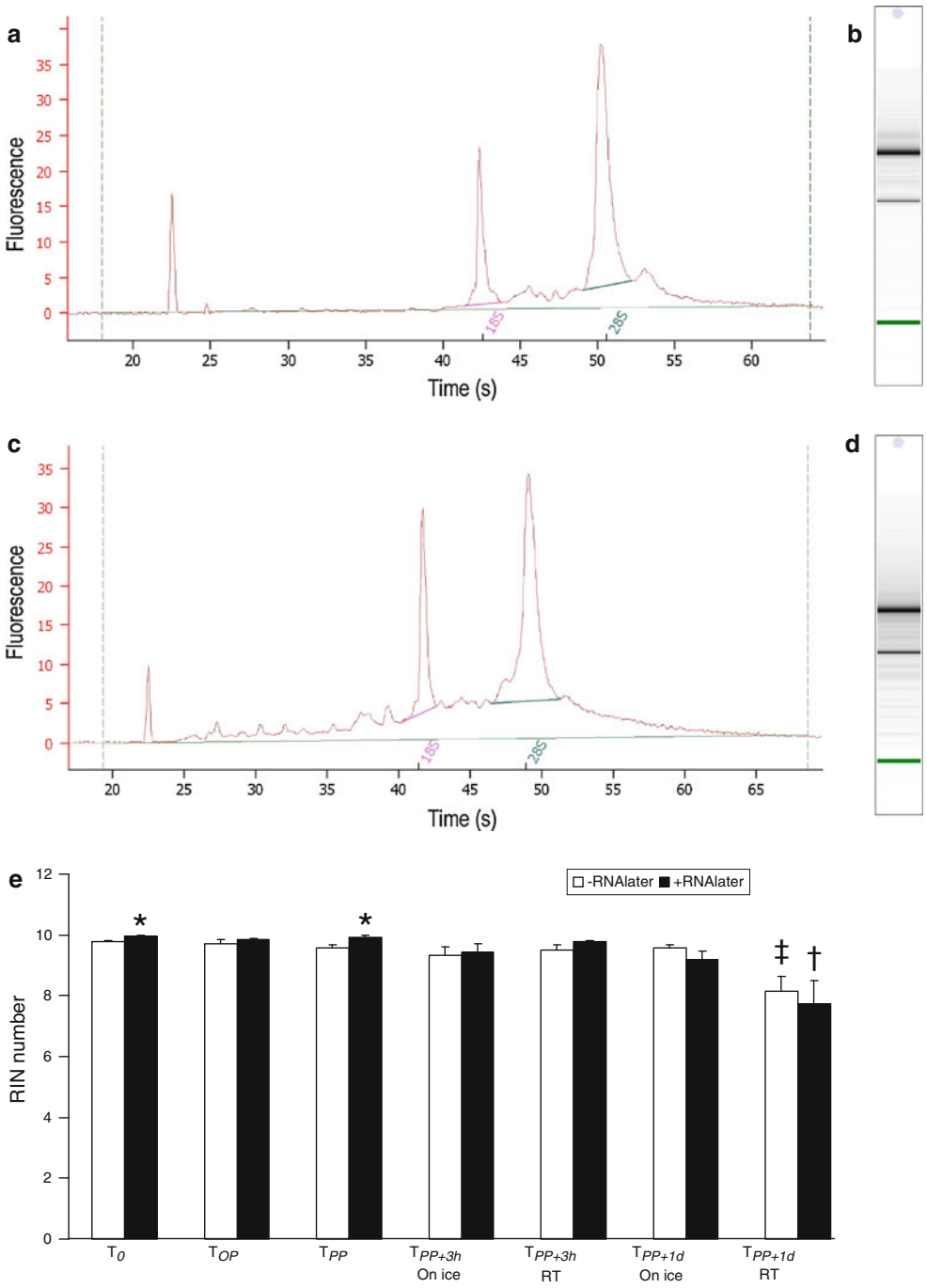


**Fig. 2** Electropherogram (**a**) and gel image (**b**) of a sample with a RIN number of 10 and electropherogram (**c**) and gel image (**d**) of a sample with a RIN number of 8.2. (**e**) RNA integrity number (RIN) as a result of processing time and temperature. Samples were either flash-frozen in liquid nitrogen (−RNAlater) or put in RNAlater (+RNAlater) for eventual storage at −80°C. Values represent means ± SEM with $N = 6$. ‡Significantly different from corresponding $T_0$, $T_{OP}$, $T_{PP}$, $T_{PP+3h}$ (on ice), $T_{PP+3h}$ (RT), $T_{PP+1d}$ (on ice) conditions, $P < 0.05$. † Significantly different from corresponding $T_0$, $T_{OP}$, $T_{PP}$, $T_{PP+3h}$ (on ice), $T_{PP+3h}$ (RT) conditions, $P < 0.05$. *Significantly different from corresponding −RNAlater condition, $P < 0.05$. Abbreviations are as follows: $T_0$ time 0 (at start of surgery before blood arrest), $T_{OP}$ immediately after liver resection, $T_{PP}$ after the Department of Pathology had taken liver samples, *RT* room temperature

16 h on ice or room temperature [6], and murine cutaneous tissues for up to 1 h at room temperature [17].

To our knowledge, most of the work done on RNA stability in liver was not done on human tissues [3, 15, 16, 18]. Finger et al. [19] did examine mRNA stability of human liver at 4°C for up to 16 h by determining the in vitro translation products using two-dimensional gel electrophoresis. However, in this study, we were interested in RNA stability, which is important for accurate RT-qPCR studies. Therefore, this study aimed to determine the effects of extended processing times, processing temperatures and storage methods on human liver RNA stability. Briefly, this was assessed by RNA integrity and relative mRNA levels of various genes. The genes measured in this study, *FOS*, *GFER*, *GUSB*, *HIF1a* and *MYC*, were chosen because their expression levels may be affected differently by the experimental conditions mentioned above.

Almeida et al. [3] showed that warm ischaemia at 37°C resulted in almost complete degradation of 18S and 28S rRNA by 4 h in mouse liver. However, the tissues obtained

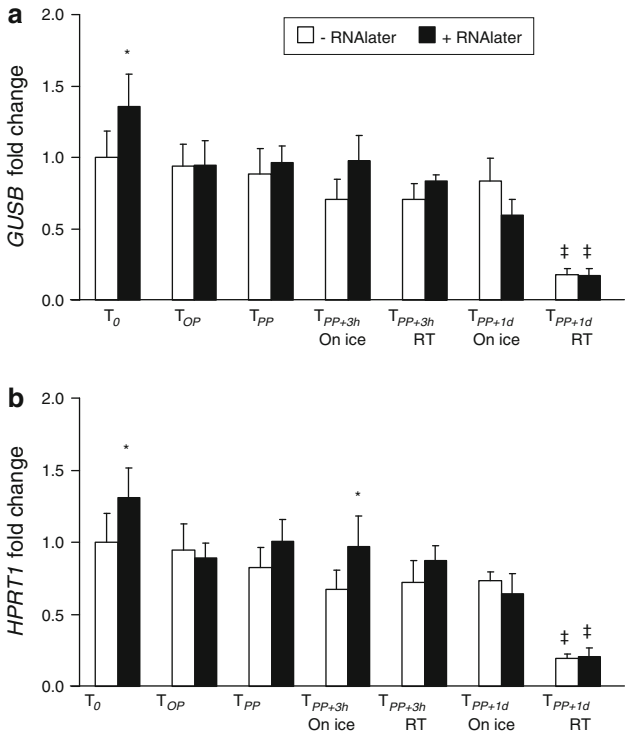


**Fig. 3** Fold change in gene expression as a result of processing time and temperature. The gene expression levels for **a** *GUSB* and **b** *HPRT1*, as quantified by RT-qPCR, were normalised with respect to the corresponding values at $T_0$ (−RNAlater). Samples were either flash-frozen in liquid nitrogen (−RNAlater) or put in RNAlater (+RNAlater) for eventual storage at −80°C. Values represent means ± SEM with $N = 6$. ‡Significantly different from corresponding $T_0$, $T_{OP}$, $T_{PP}$, $T_{PP+3h}$ (on ice), $T_{PP+3h}$ (RT), $T_{PP+1d}$ (on ice) conditions, $P < 0.05$. *Significantly different from corresponding −RNAlater condition, $P < 0.05$. Abbreviations are as follows: $T_0$ time 0 (at start of surgery before blood arrest), $T_{OP}$ immediately after liver resection, $T_{PP}$ after the Department of Pathology had taken liver samples, *RT* room temperature

in this study were subjected to relatively short warm ischaemia times (average time, 44 min) due to surgery. Thus, in agreement with Almeida's results, the RNA integrity of our samples as assessed by RIN number was not significantly decreased after surgery ($T_{OP}$). Furthermore, for the genes of interest (*FOS, GFER*, *GUSB*, *HIF1a* and *MYC*), there were no significant changes in relative mRNA expression of these genes after surgery. This is also in agreement with Almeida et al. [3], who found the relative mRNA expression levels for 6 genes (*Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf* and *Glut2*) unchanged by warm ischaemia, and Finger et al. [19], who found translation products of mRNA largely unchanged following incubation at 37°C for 2 h.

However, to our knowledge, the effect of longer processing times on RNA stability in human liver stored on ice and at room temperature is unknown. Marchuk et al. [15] found that although there is some decline in rabbit liver RNA stability at 48 and 96 h post-mortem, there does not appear to be a rapid activation of nuclease activity with subsequent degradation of mRNA or rRNA. Finger et al. [19] found that translation products of mRNA in human liver were mostly unchanged at 4°C for up to 16 h. This study has found in human liver that RNA integrity was significantly decreased by 20% only at $T_{PP+1d}$ at RT (Fig. 2). Thus, it is important to keep samples on ice to retain RNA integrity.

There is a concurrent down-regulation of reference gene expression with the decrease in RNA integrity at $T_{PP+1d}$ at RT (Fig. 3). This emphasises that liver tissue should not be left at RT for a day if an RT-qPCR experiment is planned. However, other than the above time-point, RNA expression of the reference genes was not significantly different and shows a very similar trend, as has been found by Port et al. [20].

When the relative mRNA levels of 5 genes were determined using *HPRT1* as a reference gene, it was found that there were generally no significant changes when the various time-points were compared (Fig. 4). The only significant difference that can be detected is at $T_{PP+1d}$ at RT for *GUSB*, which again is the time-point with decreased RNA integrity (Fig. 4a). Other than this time-point, it can be seen that gene expression of *GUSB* relative to *HPRT1* is very stable through the time-points, as expected for 2 reference genes.

Although, there were no other significant differences detected for the other genes of interest over the time course, it can be seen that certain genes, for example *GFER* and *HIF1α*, are more stably expressed. These genes have average relative gene expression levels close to $T_0$ and therefore are very reflective of the in vivo gene expression levels. In contrast, *FOS* and *MYC*, which do not show significant differences over the time course, have slightly more variation in their means. This may be as these two genes are immediate early genes and are more sensitive to stimuli during the surgical removal and collection process. It would be of interest to consider that in cases where comparative studies are carried out for such genes of interest, it would still be possible to detect significant differences, but the power to detect these differences would be diminished by the greater variance. Further, the stable gene expression levels obtained for these genes of interest could be due to the use of hexamer primers with short amplicons to transcribe cDNA for RT-qPCR. This method of reverse transcription would be better than using oligo-dTs in the case of RNA degradation allowing for representative relative mRNA levels to be determined. Another point that should be noted is that this study assumes that degradation rates of the various genes are similar. This assumption can only be made if amplicon sizes in RT-qPCR are similar as in this study. RT-qPCR with substantially different amplicon sizes result in different RNA degradation rates, since longer amplicons will be more likely to be nicked than a shorter amplicon.

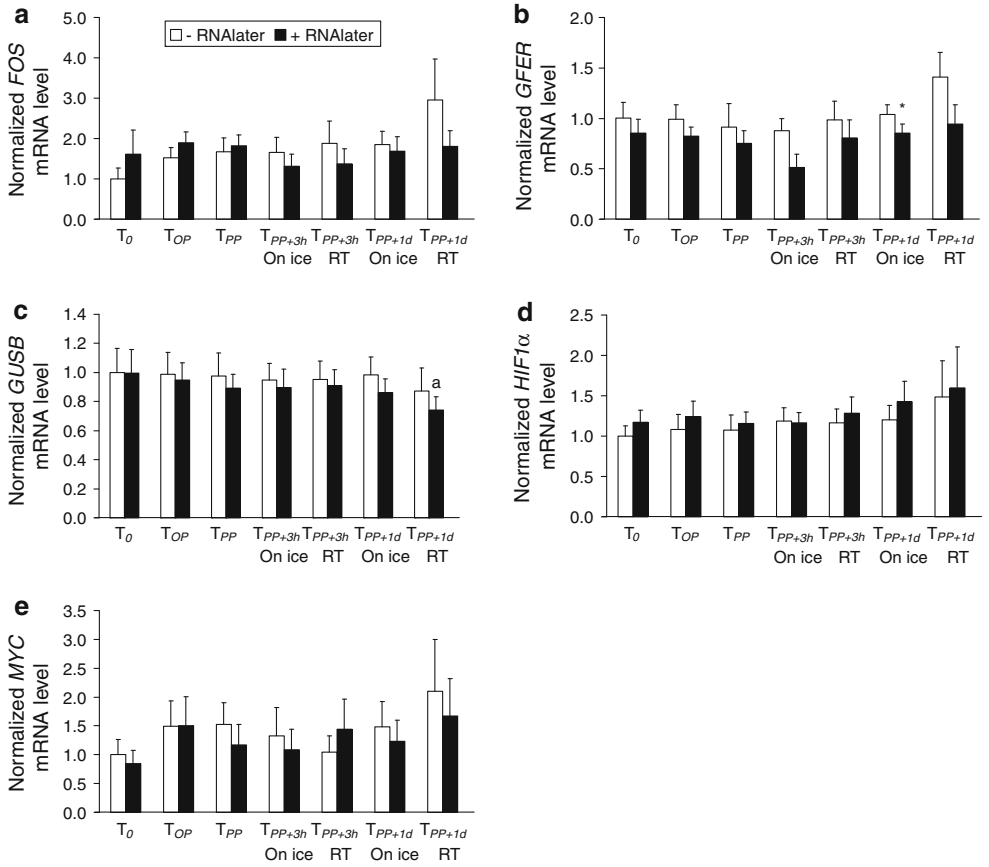


**Fig. 4** Normalised relative gene expression as a result of processing time and temperature. The mRNA levels for **a** *FOS*, **b** *GFER*, **c** *GUSB*, **d** *HIF1α* and **e** *MYC*, as quantified by RT-qPCR, were compared to values of *HPRT1* (reference gene) and then normalised with respect to the corresponding values at $T_0$ (−RNAlater). Samples were either flash-frozen in liquid nitrogen (−RNAlater) or put in RNAlater (+RNAlater) for eventual storage at −80°C. Values represent means ± SEM with $N = 6$. [a]Significantly different from corresponding $T_0$, $T_{OP}$ conditions, $P < 0.05$. *Significantly different from corresponding −RNAlater condition, $P < 0.05$. Abbreviations are as follows: $T_0$ time 0 (at start of surgery before blood arrest), $T_{OP}$ immediately after liver resection, $T_{PP}$ after the Department of Pathology had taken liver samples, *RT* room temperature

As for the use RNAlater, previous studies by other investigators have conflicting results, some advocate the use of RNAlater [21, 22], some prefer snap-freezing in liquid nitrogen [23], and the rest found no difference between the two methods [24–26]. This study found that there were generally very little differences between results obtained with snap-freezing or storage in RNAlater. Thus, based on this study, one can decide to use RNAlater or not, as long as the collection procedure is consistent, either always with RNAlater or without. However, in situations where it is not possible to freeze tissues expeditiously or efficiently homogenise the tissues for RNA isolation, then, the use of RNAlater would be recommended.

## Conclusions

It is recommended that whenever possible samples should be kept on ice during processing and that they should be processed as quickly as possible for storage at −80°C. This study also shows that the RNA stability of human liver was not decreased after surgery or after examination by the pathology department. Further, comparison of all time-points revealed generally no significant differences in relative gene expressions of the genes tested. Thus, if the relative expression of a gene of interest is proven not to be affected, it is possible to utilise liver samples that have been subjected to longer processing times for RT-qPCR. This will make it worthwhile to bank such samples, increasing accrual of valuable samples to the tissue bank.

**Acknowledgments** This work was made possible by the HTCR foundation, which makes human tissues available for research. We thank the technical assistants from the Grosshadern Hospital Tissue Bank for collecting the liver samples. Our thanks also go to Dr. Bauersachs from the Gene Center in Ludwig Maximilians University for kindly allowing us to use his Agilent Bioanalyzer. This work was carried out with financial support from the Bundesministerium für Bildung und Forschung (BMBF; grant name: $M^4$ Munich Biotech Excellence Cluster Biobank Alliance, grant number 01EX1020B).

**Conflict of interest** The authors declare that they have no conflict of interest.

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