Per-unit-living tissue normalization of real-time RT-PCR data in ischemic rat hearts


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Quantification of gene expression is typically performed with real-time RT-PCR, a technique that provides sensitive and accurate analyses of mRNA abundance. However, real-time RT-PCR analyses are often hampered by the lack of accurate procedures for data normalization (4, 12), with prevailing strategies typically assuming stable expression of internal reference genes or stable abundance of total RNA (13). This assumption may lead to false conclusions (12), particularly in studies involving extreme physiological challenges such as oxygen deprivation (8) and cell death. Efforts have therefore been made to find alternative approaches (6, 8, 23). The geNorm procedure reported by Vandesompele et al. (23) has been a particularly valuable contribution, providing normalization based on geometric averaging of internal reference genes rather than on a single gene (23). However, this strategy is also vulnerable to changes in the expression of genes, and we recently demonstrated that in some instances it may be more suitable to use an external RNA control (external reference gene) (8).

For most studies, normalization of real-time RT-PCR data using an external reference gene would be appropriate. However, for experiments involving large-scale tissue remodeling such as acute cell death seen during ischemia, the approach has its limitations. More specifically, because the external reference gene is added to the tissue on a per-weight-unit basis (8), it does not allow per-living-unit analyses of gene expression in necrotic tissue. In such experiments, internal reference genes are left as the only alternative for real-time RT-PCR data normalization. To obtain proper analyses using these genes, they need to show stable expression in living cells as well as complete degradation in dead cells at the time of tissue sampling. In many studies of acute myocardial infarctions ~50% of the tissue is necrotic, and this could possibly contribute to errors in gene transcript evaluation. Overall, little is known about the expression and degradation of RNA in ischemic tissues, with the few existing reports focusing on microRNA (2), postmyocardial infarction and failing hearts (3, 10, 18), and the ischemic brain (11, 15). To our knowledge, no report has defined the need for per-unit-living tissue as opposed to per-unit-weight normalization in acute models in this large field of research. This simple but important insight is often ignored, at best leading to dubious interpretations and at worst leading to erroneous conclusions. Although our recently reported normalization approach using an external standard is of limited value for direct normalization of real-time RT-PCR data in acute ischemic tissue, the approach is the only way to study the relationship between per-unit-weight expression and per-unit-living tissue expression.

We hypothesized that mRNA abundance of the seven commonly utilized internal reference genes β-actin, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA (18S), 60S ribosomal protein L-32 (RPL-32), DNA-directed RNA polymerase II subunit A (PolR2A), and β2-microglobulin, as evaluated from a survey of the literature, would provide appropriate normalization strategies for data normalization. A marked difference in the expression of genes was found for cyclophilin A, GAPDH, RPL-32, and PolR2A mRNA, with GAPDH showing the highest degree of stability (R = 0.11), suggesting unchanged rates of mRNA transcription in live cells and complete degradation of mRNA from dead cells. The infarct size-dependent degradation of GAPDH was further supported by a close correlation between changes in GAPDH expression in acute ischemic tissue, internal reference genes need to show stable expression per-unit-living tissue to hinder dead cells from biasing real-time RT-PCR data. Until now, this important issue has not been appropriately investigated.

It was further supported by a close correlation between changes in GAPDH expression in acute ischemic tissue, internal reference genes need to show stable expression per-unit-living tissue to hinder dead cells from biasing real-time RT-PCR data. Until now, this important issue has not been appropriately investigated. We hypothesized that the expression of seven internal reference genes would show stable per-unit-living tissue expression in Langendorff-perfused rat hearts subjected to ischemia-reperfusion. This was found for cyclophilin A, GAPDH, RPL-32, and PolR2A mRNA, with GAPDH showing the highest degree of stability (R = 0.11), suggesting unchanged rates of mRNA transcription in live cells and complete degradation of mRNA from dead cells. The infarct size-dependent degradation of GAPDH was further supported by a close correlation between changes in GAPDH mRNA and changes in RNA quality measured as RNA integrity number (R = 0.90, P < 0.05). In contrast, β-actin and 18S rRNA showed stable expression per-unit-weight tissue and a positive correlation with infarct size (R = 0.61 and R = 0.77, P < 0.05 for both analyses).

The amount of total RNA extracted per-unit-weight tissue did not differ between groups despite wide variation in infarct size (7.1–50.1%). When β-actin expression was assessed using four different normalization strategies, GAPDH and geNorm provided appropriate per-unit-living expression, while 18S and total RNA resulted in marked underestimations. In studies of ischemic tissues, we recommend using geometric averaging of carefully selected reference genes for normalization of real-time RT-PCR data. A marked shift in the mRNA/rRNA ratio renders rRNA as useless for normalization purposes.

External RNA control; housekeeping gene; ischemia; necrosis; Langendorff
determined by adjusting for tetrazolium chloride (TTC)-measured infarct size, obtained from four neighboring slices (two on each side of the gene expression slice). Tissues were sampled from a study investigating the effects of H2S on ischemia-reperfusion injury (1). Finally, we examined the consequences of assessing β-actin expression using four different normalization strategies: 1) and 2) internal reference gene normalization (GAPDH and 18S), 3) geNorm, and 4) total RNA.

**MATERIAL AND METHODS**

**Animal handling and experimental design.** The experiments were evaluated and approved by and conducted in accordance with the Norwegian Animal Health Authority, and the animals received humane care in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Isolated rat hearts (male Wistar, 250–300 g; Scanbur, Nittedal, Norway) were retrogradely perfused in Langendorff mode. Three experimental protocols were performed: 1) Control, consisting of 30 min of stabilization (n = 6); 2) H2S, consisting of 30 min of stabilization followed by 40 min of global ischemia and 120 min reperfusion with H2S supplementation (40 μM NaHS added from 15 min of stabilization and throughout experiment) (n = 6); 3) PAG, applying a perfusion protocol similar to the H2S group but without H2S supplementation, endogenous H2S production was blocked using D,L-propargylglycine (PAG, 50 mg/kg injected intraperitoneally before heart was excised) (n = 8). For details see Blikken et al. (1). We have previously observed that long-lasting perfusion protocols may lead to the development of small necrotic areas in the myocardium (Ellefsen S, Rutkovskiy A, and Stensløkken KO; unpublished results). These patches of necrotic cells are difficult to identify with the TTC staining method and are likely to lead to the misinterpretation of necrotic tissue as being healthy. Accordingly, for the control group, we chose a shorter perfusion protocol, ensuring tissue stabilization while at the same time minimizing the occurrence of necrosis.

**TTC staining.** At the end of each protocol the ventricle was sectioned into 1 mm transverse slices from base to apex. For each heart, four slices from a central part of the ventricle was used to obtain TTC-based determination of infarct size. The TTC protocol was carried out as previously described (14). Infarct sizes are presented as means ± SD and were compared by ANOVA, followed by the Tukey-Kramer post hoc test. Analysis of the correlation between RIN value and infarct size was performed using a parametric correlation test (Pearson r). P < 0.05 was considered significant. Primers for all genes were designed with Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with exception of primers for 18S rRNA (Applied Biosystems). For each gene, three primer pairs were tested, and the pair that displayed distinct melting curves and the highest crossing point (Cp) values was chosen. cDNA syntheses, using 1 μg total RNA, and real-time RT-PCR were performed as previously described (8). See Table 1 for primer sequences, amplification efficiencies, and Cp values. Total RNA yields of each of the three experimental groups are presented as means ± SD, and differences were tested by ANOVA.

Per-unit-weight tissue expression of internal reference genes was calculated using the external reference gene mw2060, as described by Ellefsen et al. (8) and utilized in several recent studies (e.g., 5, 7, 21, 22). Per-unit-living tissue expression was calculated by combining mw2060 normalized data and infarct size data:

\[
gene\text{expression}_{\text{mw2060}} \times \frac{1}{100} = \text{gene expression}_{\text{per-unit-living tissue}}
\]

Correlation analyses between each of the two gene expression modes and infarct size/RIN were performed using a parametric correlation test (Pearson r). In these analyses, all tissue samples were treated as individual units rather than as belonging to one of the three experimental groups. Figures 1 and 2 give an overall significance and Pearson’s correlation coefficient.

The β-actin expression was evaluated using four different normalization strategies: 1) and 2) single gene normalization (GAPDH and 18S, respectively), 3) geNorm, and 4) total RNA. Data from the ischemic groups (H2S and PAG) were referenced to the perfused control group, which was set to one (see Fig. 4). The data are presented as means ± SD

**Table 1. Primer sequences used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primers For real-time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_031144</td>
<td>F: CTAAGGCCAACCGGTGAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGAAGTCTTAGGCAA</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>NM_017101</td>
<td>F: GGATTGTCGATATAGGTTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGTTGTCACAGTCGGAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>F: GAATGTTGGCTATCGGAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAGAAGGGCAGATGACTC</td>
</tr>
<tr>
<td>18S rRNA*</td>
<td></td>
<td>F: TCGGCCTCTGTTGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGACACATTGTGACGAAATC</td>
</tr>
<tr>
<td>PolR2A</td>
<td>XM_343922</td>
<td>F: TGATCTGCTACTGACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTAGAGGCTCCGATGAA</td>
</tr>
<tr>
<td>β2-Microglobulin</td>
<td>NM_012512</td>
<td>F: GAAGAGGCCCCAAACCTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTCCAGTATCCGACTTCCCAT</td>
</tr>
<tr>
<td>mw2060</td>
<td>DQ075244</td>
<td>F: GTCTGGACATCAGGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTCTGGACATCAGGAG</td>
</tr>
</tbody>
</table>

Values are means ± SD. F, forward primer; R, reverse primer. Priming efficiencies (E) and crossing point (Cp) values of real-time RT-PCR primers are presented as averages of all real-time RT-PCR reactions and are summarized in the 2 rightmost columns (n = 408). *Commercial primers from Applied Biosystems.
and were tested for statistically significant variation using ANOVA, followed by the Tukey-Kramer post hoc test.

RESULTS

In ischemic H2S- and PAG-treated hearts, the average infarct size was 21 ± 14 and 41 ± 6%, respectively (infarct sizes ranged from 7.1 to 50.1%). TTC staining of perfused control hearts did not reveal necrotic areas. RNA quality, measured as RIN, was lower in H2S (8.3 ± 0.5) and PAG hearts (7.7 ± 0.4) than in control hearts (9.1 ± 0.1) (P < 0.05) (Fig. 1A), indicating degradation of RNA from necrotic cells. Accordingly, a negative correlation was found between infarct size and RIN (R = −0.87, P < 0.05) (Fig. 1B), indicating that the degree of RNA degradation in individual hearts was directly dependent on infarct size. The slope of this correlation (y = −0.031t + 8.99) was similar, though inverse to that previously reported for the correlation between degrees of intact RNA and RIN (y = 0.031t + 5.40) (19). Yields of total RNA extracted from the tissue did not differ between groups, amounting to 1.21 ± 0.08, 1.20 ± 0.15, and 1.16 ± 0.12 µg/mg heart tissue in control and H2S- and PAG-treated tissues, respectively.

Of the seven internal reference genes investigated, four genes showed per-unit-weight mRNA expression that correlated negatively with infarct size (cyclophilin A, R = −0.63; GAPDH, R = −0.76; RPL-32, R = −0.50; and PolR2A, R = −0.68; P < 0.05 for all analyses) (Fig. 2A). This indicates that their abundance decreased progressively as the degree of living cells decreased. Of the remaining three genes (β-actin, 18S, and β2-microglobulin), none of which showed a significant correlation with infarct size (Fig. 2A), 18S rRNA stood out by showing the weakest correlation with infarct size (R = 0.13) (Fig. 2A). 18S thus showed remarkably stable abundance per-unit-weight tissue despite infarct sizes approaching 50%. In accordance with these analyses, GAPDH abundance showed a positive correlation with RNA quality (R = 0.90, P < 0.05).

Fig. 1. RNA quality in 3 experimental groups of isolated perfused rat hearts (Con, H2S, and PAG; see MATERIAL AND METHODS for group details). A: group-wise analysis of RNA integrity number (RIN). Values are means ± SD. Data were tested for treatment-dependent differences by ANOVA, followed by Tukey-Kramer’s posttest. *P < 0.05. B: RIN plotted against infarct size. Values are individual hearts. Data were tested for infarct size-dependent correlation using Pearson’s correlation.

Fig. 2. Expression level of internal reference genes in isolated perfused rat hearts subjected to 40 min ischemia and 120 min reperfusion plotted against infarct size. In A the per-unit-weight tissue expression is presented, achieved by normalization to the external reference gene, mw2060 (8), while in B the per unit living tissue expression is presented, achieved by tetrazolium chloride (TTC) infarct size adjustment of mw2060 normalized data. The data are referenced to the control hearts (no visible necrotic areas), which was set to 1. Data sets were tested for infarct size-dependent correlation using Pearson’s correlation. P < 0.05 was considered significant. Values are individual hearts.
control, H2S, and PAG. For geNorm normalization, cyclophilin for normalization of the outcome of utilizing GAPDH, 18S, geNorm, and total RNA tion strategies to analyze target gene expression, we examined R
In contrast, 18S showed a very strong positive correlation with suitable candidate for normalization of real-time RT-PCR data. However, it is not clear if the differences in gene expression profiles found for the different genes in the myocardium are due to differences in the rate of RNA degradation or to differences in the active regulation of RNA transcription. It is important to remember that only a fraction of the total RNA is mRNA and that by far the largest fraction is made up of rRNA. Our result of stable 18S expression could indicate that ribosomal RNA is not rapidly degraded. On the other hand, the close correlation observed between RNA quality and infarct size is highly similar to the one found between RNA quality and amounts of degraded RNA in Riedmaier et al. (19). This suggests degradation of RNA from dead cells in the ischemic myocardium. If so, the observed differences in mRNA abundance must be caused by differences in gene transcription regulation. In support of this, previous studies have shown that in dead tissue, different mRNA species degrade at similar rates, as investigated several hours postmortem (24). To the best of our knowledge, no previous study has assessed mRNA degradation in ischemic tissue within the short time frame used in our study (<3 h). It is important to note that the RIN values (Fig. 3), suggesting that GAPDH mRNA levels increased linearly with increasing RNA quality, whereas 18S rRNA abundance failed to show correlation with RNA quality ($R = 0.08$) (Fig. 3).

While the per-unit-living tissue expression of cyclophilin A, GAPDH, RPL-32, and PolR2A showed no correlation with infarct size (Fig. 2B), the expression of beta-actin, 18S, and beta2-microglobulin showed a positive correlation ($R = 0.61$, $R = 0.77$, and $R = 0.47$, respectively; $P < 0.05$ for all analyses) (Fig. 2B). As for the former, GAPDH showed a particularly weak correlation with infarct size ($R = 0.11$), suggesting a highly stable abundance per unit living tissue and making it a suitable candidate for normalization of real-time RT-PCR data. In contrast, 18S showed a very strong positive correlation with infarct size ($R = 0.77$), making it least suited for normalization purposes.

To illustrate the consequences of using different normalization strategies to analyze target gene expression, we examined the outcome of utilizing GAPDH, 18S, geNorm, and total RNA for normalization of beta-actin data in the three treatment groups; control, H2S, and PAG. For geNorm normalization, cyclophilin A, GAPDH, and RPL-32 were evaluated to be the three most stable internal reference genes, showing M values of 0.23, 0.26, and 0.23, respectively, all well <0.7, the upper limit set by Vandesompele et al. (23). Using GAPDH for normalization, we found beta-actin expression to be increased by 40 and 50% in H2S and PAG hearts compared with control hearts ($P < 0.05$) (Fig. 4). Similarly, using geNorm for normalization, beta-actin expression was found to be increased by 50% in PAG hearts ($P < 0.05$) (Fig. 4). In contrast, using 18S for normalization, beta-actin expression was found to be decreased by 20% in PAG hearts ($P < 0.05$) (Fig. 4), and using total RNA no alterations were found (Fig. 4).

**DISCUSSION**

In studies assessing tissues with high degrees of acute cell death, such as ischemic tissues, it is necessary to prevent the content of dead cells from affecting molecular analyses of living tissue. Traditionally this has been difficult to achieve and has often been ignored, either because of difficulties in the physical removal of necrotic tissue prior to analyses or because of difficulties in accounting for the necrotic tissue during analyses. In the present study we demonstrate the consequences of failing to account for dead cells in assessments of gene expression in infarcted heart tissue. We do this by combining our recently developed real-time RT-PCR approach (8) with TTC-based measurement of infarct size. The study provides the first surveillance of internal reference gene expression in infarcted heart tissue that accounts for the presence of dead cells.

One important finding is the absence of differences in amount of RNA extracted from the different experimental groups despite large variation in infarct size. In some of the investigated tissue up to 50% of the tissue was necrotic, yet RNA yield was not different. This indicates that partly degraded RNA contributes to the total amount of extracted RNA. However, it is not clear if the differences in gene expression profiles found for the different genes in the myocardium are due to differences in the rate of RNA degradation or to differences in the active regulation of RNA transcription. It is important to remember that only a fraction of the total RNA is mRNA and that by far the largest fraction is made up of rRNA. Our result of stable 18S expression could indicate that ribosomal RNA is not rapidly degraded. On the other hand, the close correlation observed between RNA quality and infarct size is highly similar to the one found between RNA quality and amounts of degraded RNA in Riedmaier et al. (19). This suggests degradation of RNA from dead cells in the ischemic myocardium. If so, the observed differences in mRNA abundance must be caused by differences in gene transcription regulation. In support of this, previous studies have shown that in dead tissue, different mRNA species degrade at similar rates, as investigated several hours postmortem (24). To the best of our knowledge, no previous study has assessed mRNA degradation in ischemic tissue within the short time frame used in our study (<3 h). It is important to note that the RIN values...
utilized to assess RNA degradation in our study are calculated from electrophoretic peaks of highly abundant rRNA species rather than mRNA species (20) and thus cannot be utilized to assess the degradation of mRNA directly. Although a correlational relation has been reported between RIN value and abundance of internal reference genes (20), the correlation seems to be much less pronounced in the higher range of RIN (RIN 6–9), suggesting that in this range mRNA species are largely unaffected by RNA degradation [as evaluated by visual inspection of the correlation plot presented in Schroeder et al. (20)]. This led the authors to define RNA samples showing RIN values >5.5 as being suitable for real-time RT-PCR analyses. The RIN values found in our study are well above this lower limit, being between 7 and 9. Ultimately, neither of the two possible explanations behind the observed intergene differences in expression profiles in the present study can be excluded, i.e., different rates of mRNA degradation or differential regulation of transcription.

Expression of internal reference genes. Some of the gene expression patterns observed in this study are similar to those reported in studies of acutely ischemic rat brain tissue. These include a stable expression of cyclophilin A and GAPDH (11, 15) and an increased expression of β2-microglobulin and β-actin (11, 15). In addition, ribosomes have been shown to accumulate in rat heart infarct border areas (16), which may explain the elevated abundance of total RNA and 18S rRNA in the ischemic tissue in this study. This could suggest increased rates of protein synthesis in the border area cells (16) and may be important for cell survival. The observed increase in total RNA and 18S rRNA per-unit-living tissue suggests that there is a distinct increase in the rRNA/mRNA ratio in infarcted hearts. Although we cannot determine the cause of this shift, the consequence should be to abandoned total RNA and 18S for normalization in experiments involving ischemic tissues in general.

GAPDH mRNA showed the most stable expression per-unit-living tissue, as indicated by the correlation plots in Figs. 1–3. This evidently makes it the best suited single reference gene for real-time RT-PCR data normalization. The decrease in GAPDH mRNA abundance observed in infarcted hearts either can be a passive consequence of the degradation of RNA in dead cells or may be the result of altered transcription in live cells, as previously discussed. GAPDH was ranked top three by geNorm, suggesting that it shows a preserved geometric position compared with the other internal reference genes (23). In contrast to our finding, Brattelid et al. (2) recently found the abundance of GAPDH mRNA to be highly unstable compared with small, noncoding RNAs (ncRNA) in ischemic rat hearts. Accordingly, the authors concluded that GAPDH should be avoided for normalization purposes in ischemic heart tissue. Based on our results, it is tempting to speculate that this might be caused by the stability of the ncRNAs per-unit-weight tissue rather than the instability of GAPDH. Speculatively, the small ncRNAs investigated by Brattelid et al. (2) might show less degradation or even altered expression, thereby being unsuitable representatives of the viable part of the tissue. As discussed by the authors, the observed differences between ncRNA and mRNA may be a consequence of the differences in biology between different RNA species. Indeed, our results indicate that there are large differences in degradation or transcription kinetics between different groups of RNA species such as rRNA and mRNA. Notably, we have previously demonstrated that coinciding changes in gene expression constitute a true hazard for geNorm analyses (8). Whatever the cause, the contradistinctive GAPDH expression profile reported by Brattelid et al. (2) underlines the need for careful assessment of reference gene expression. In ischemic tissues, target gene expression per-unit-living tissue should always be the aim. The need for proper validation of reference gene expression is also underlined by the discrepancy between our finding and the recent finding that GAPDH is unsuitable for normalization of target genes in models of failing heart tissue (10, 18), which arguably does not contain RNA from dead cells. Unfortunately, on a general basis, the suitability of reference genes are often erroneously generalized to be valid across similar albeit different experimental protocols, such as acute versus longitudinal studies of ischemic tissues.

The importance of per-unit-living normalization. The importance of per-unit-living tissue normalization of gene expression data is illustrated by the β-actin analyses shown in Fig. 4, which is greatly affected by the choice of normalization strategy. In accordance with this, random and uncritical choice of normalization strategy in studies of ischemic tissues is likely to result in false findings. For example, for the PAG-treated hearts, which showed the highest degrees of infarction, utilization of 18S for normalization resulted in a 94% underestimation of β-actin expression compared with geNorm normalization. A similar underestimation of gene expression was found using total RNA and would also have been found if β-actin itself were employed for normalization. This suggests that total RNA, ribosomal RNA, and β-actin are unsuitable for gene expression studies in isolated perfused hearts and possibly also for other ischemic tissues, at least when sampled after a relatively short reperfusion time. Furthermore, to avoid erroneous conclusions caused by normalization to single genes, it seems appropriate to recommend the use of geNorm for normalization of real-time RT-PCR data in studies involving degraded ischemic tissues. In this context, it is important to point out that geNorm analyses are prone to coinciding changes in expression of internal reference genes, as previously discussed. The ultimate guideline should always be to obtain per-unit-living tissue normalization. Moreover, careful evaluation of reference gene expression should be performed even when working with closely related experimental protocols.

We have only investigated per-living-tissue normalization in isolated perfused hearts. However, we suggest a wider application for this important normalization strategy. The approach can easily be adapted to suit acute investigation of ischemic trauma to the brain, and it is very likely that our findings will influence choices of reference genes in such studies. It should also be suitable for other experimental situations involving dying cells, such as strenuous exercise of skeletal muscle (17) and acute effects of cytotoxins and the likes on cancerous tissue, wherein partially degraded RNA could influence the results. In contrast, the approach does not seem to be needed in analyses of tissues with apoptotic events, typically affecting single cells or small clusters of cells (9), given that the apoptotic cell death is not large scale and that they are not highly concerted in time. The knowledge of RNA degradation under such conditions is limited but should be easy to investigate and account for with the use of the external standard in synergy with internal housekeeping genes.
The utilization of the substances H₂S and PAG during the experimental protocol did not affect the purpose of this study: to investigate the suitability of internal reference genes. These substances may of course themselves have led to changes in gene expression but merely represented additional risk factors. The use of H₂S and PAG does not change the important notion that internal reference genes need to show stable expression per-unit-living tissue.

In conclusion, when performing real-time RT-PCR analysis on RNA from tissues with necrotic areas it is necessary to employ a normalization strategy that accounts for the presence of RNA from dead cells. Failing to do so will lead to erroneous conclusions. Though we have investigated ischemic heart tissue, this notion is likely to have wide application. We suggest including an external standard when internal reference genes are evaluated, to better judge the relationship between per-unit-weight and per-unit-living tissue. We also suggest the utilization of a wide panel of internal reference genes and geNorm for normalization of real-time RT-PCR data rather than relying on singular mRNA species in studies involving investigation of samples showing substantial RNA degradation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES