

## BASIC RESEARCH

# Isolated total RNA and protein are preserved after thawing for more than twenty-four hours

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**OBJECTIVE:** The preservation of biological samples at a low temperature is important for later biochemical and/or histological analyses. However, the molecular viability of thawed samples has not been studied sufficiently in depth. The present study was undertaken to evaluate the viability of intact tissues, tissue homogenates, and isolated total RNA after defrosting for more than twenty-four hours.

**METHODS:** The molecular viability of the thawed samples (n = 82) was assessed using the A260/A280 ratio, the RNA concentration, the RNA integrity, the level of intact mRNA determined by reverse transcriptase polymerase chain reaction, the protein level determined by Western blotting, and an examination of the histological structure.

**RESULTS:** The integrity of the total RNA was not preserved in the thawed intact tissue, but the RNA integrity and level of mRNA were perfectly preserved in isolated defrosted samples of total RNA. Additionally, the level of  $\beta$ -actin protein was preserved in both thawed intact tissue and homogenates.

**CONCLUSION:** Isolated total RNA does not undergo degradation due to thawing for at least 24 hours, and it is recommended to isolate the total RNA as soon as possible after tissue collection. Moreover, the protein level is preserved in defrosted tissues.

**KEYWORDS:** Thawed tissue; Defrosted isolated total RNA; Ultra-low temperature; Molecular viability; Western blot.

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## INTRODUCTION

It is commonly recommended to store samples of plasma, tissues, and isolated total RNA in an ultra-low-temperature freezer to preserve the tissue structure and biochemical parameters by decreasing or inhibiting enzyme activity and other processes.

Due to a technical problem, an ultra-low-temperature freezer in our laboratory defrosted, and the samples were kept at room temperature for more than twenty-four hours. Therefore, we decided to determine if the samples could still be used for some analyses.

Kisand et al. (1) and Yokomuro et al. (2) observed that some but not all blood biomarkers are preserved after freeze/thaw cycles. In other studies, it was observed that freeze-thaw cycles induce biomechanical changes in arteries (3) and render sciatic nerve tissue inviable for grafting (4). Molecular analyses using cancer tissues have shown that storing the tissue or performing

the fixation with formalin at a low temperature can prevent the degradation of nucleic acids (5,6).

However, no information was found in the literature with regard to whether material that has experienced a prolonged thaw could be useful for either biochemical or molecular determinations. Because ultra-low-temperature freezers may defrost due to electrical or other problems, it is important to determine whether thawed biological material retains sufficient integrity for certain analyses.

Therefore, the present study was designed to evaluate samples that were defrosted for more than twenty-four hours with regard to the mRNA and protein levels, the histology of intact tissue and the integrity of isolated total RNA.

## MATERIALS AND METHODS

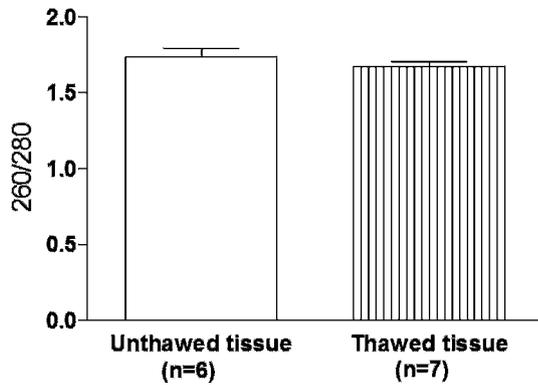
### Samples

The studied samples were thawed due an electric problem in ultra-low freezer (-80°C) and remained at room temperature for at least twenty-four hours. After that, they were transferred back to an ultra-low freezer (-80°C). Control, unthawed samples were also evaluated. The materials listed below were studied.

Thawed tissues: heart (n=12), pancreas (n=6), brown adipose tissue (BAT, n=9), white adipose tissue (n=9), liver

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No potential conflict of interest was reported.



**Figure 1** - The A260/A280 ratios of the total RNA extracted from the thawed and unthawed tissue samples.

(n=7), and kidney (n=12) obtained from twelve-week-old male Wistar rats.

Unthawed tissue: liver (n=7) and kidney (n=3).

Thawed (n=18) and unthawed (n=17) isolated total kidney RNA.

Thawed tissue homogenate: kidney (n=3), heart (n=3), and BAT (n=3).

Unthawed tissue homogenate: kidney (n=3).

**Total RNA extraction**

The total RNA was extracted from 50 mg of thawed samples of kidney (n=3), liver (n=4), heart (n=5), pancreas (n=3), white adipose tissue (n=5), BAT (n=3), and from unthawed samples (liver, n=7) using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA, <http://www.invitrogen.com>) according to the manufacturer’s instructions. The total RNA pellets were resuspended in 100 µL of diethyl pyrocarbonate-treated water and stored at -80°C until use.

The total RNA concentrations in thawed and unthawed tissue and in thawed and unthawed isolated total RNA samples were determined by measuring the absorbances at 260 nm and 280 nm, and the integrity was verified by ethidium bromide fluorescence.

Using reverse transcriptase, cDNA was synthesized from 1 µg of thawed isolated total RNA (kidney, n=18) or unthawed isolated total RNA (kidney, n=17) using an oligonucleotide dT primer (Promega Corporation, Madison, WI, USA, <http://www.promega.com>) and the ImProm II reverse transcriptase enzyme (Promega Corporation, Madison, WI, USA, <http://www.promega.com>).

A polymerase chain reaction (PCR) was performed to amplify the following specific cDNAs: renin (sense, 5'-CATTACCAGGGCAACTTTCAC-3', and antisense, 5'-TCATCGTTCCTGAAGGAATTC-3') and β-actin (sense, 5'-TATGCCAACACAGTGTCTGCTGG, and antisense, 5'-TACTCCTGCTTGCTGATCCACAT-3'). The sizes of the reaction products were estimated using a 100 bp DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA, <http://www.invitrogen.com>).

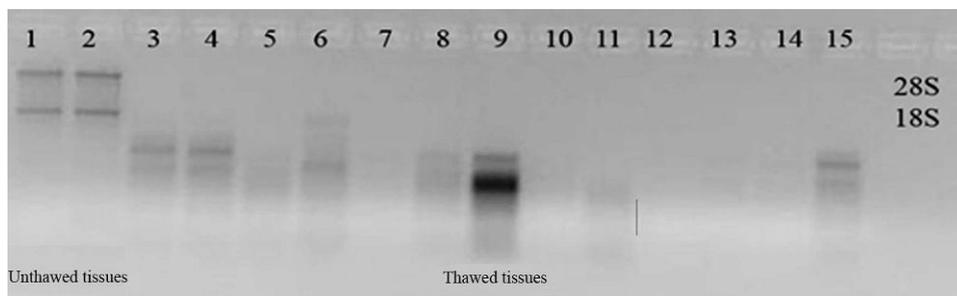
The renin and β-actin mRNA levels were determined using the isolated thawed and unthawed total RNA samples.

The PCR mixture contained 1 µl of cDNA, 2.5 µL of 10X buffer (100 mM Tris-HCl [pH 8.5] and 500 mM potassium chloride), 0.75 µl of 50 mM magnesium chloride, 15 pM of each primer, 2 U of Taq DNA polymerase (LGC Biotecnologia, Cotia, SP, Brazil, <http://www.lgcbio.com.br>), and diethyl pyrocarbonate-treated water in a total volume of 25 µl. The cDNAs were amplified under the following conditions: 94.0°C for 3 minutes; 35 cycles of 94.0°C for 1 minute, 51.5°C (for renin) or 57.8°C (for β-actin) for 1 minute, and 72.0°C for 1 minute; and 72.0°C for 10 minutes. Negative controls were routinely performed by omitting the cDNA from the PCR mixture. The PCR products were electrophoresed on 2.0% agarose gels with ethidium bromide, and the bands were semi-quantified using image analysis software (Alpha Imager™ 1220 version 5.5; Alpha Innotech Corporation, San Leandro, CA, USA, <http://www.alphainnotech.com>). All of the PCRs produced a single band of the predicted size.

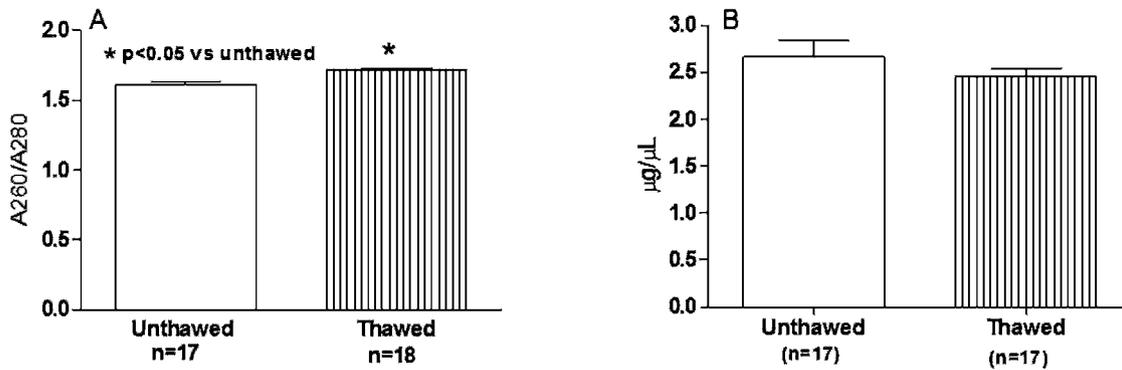
**Protein levels**

The protein level was measured by Western blotting according to the following protocol. Samples of thawed kidney (n=6), heart (n=3), brown adipose tissue (BAT, n=3), and unthawed kidney (n=3) were homogenized separately in lysis buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] [pH 7.6], 100 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 5 mmol/L Ethylenediamine tetraacetic acid [EDTA], 10% glycerol, 1 mmol/L dithiothreitol, 10% sodium dodecyl sulfate [SDS], and 1:10 protease inhibitor cocktail [Sigma-Aldrich Corporate Offices, MO, Canada, <http://www.sigmaaldrich.com>]) and centrifuged at 10,000 x g. The supernatant was recovered, and the protein concentration was determined using the Bradford method (7).

Aliquots of 100 µg of total protein from the homogenized thawed and unthawed tissue and thawed homogenate (kidney, n=3; heart, n=3; BAT, n=3) were separated on a 12% SDS polyacrylamide gel.



**Figure 2** - The 28S and 18S bands of the isolated total RNA from the control (unthawed) and thawed tissues. 1 and 2=control liver; 3 and 4=liver; 5 and 6=kidney; 7 and 8=heart; 9 and 10=white adipose tissue; 11=pancreas; 12, 13 and 14=brown adipose tissue; 15=neonate heart.



**Figure 3A** - The A260/A280 ratios of the total RNA in unthawed (control) and thawed isolated total RNA samples. **Figure 3B** - RNA concentration in unthawed (control) and thawed isolated total RNA samples.

The proteins were transferred onto a 0.20 µm pure nitrocellulose membrane (Bio-Rad Laboratories Inc, Hercules, CA, USA, <http://www.bio-rad.com>) for 1 h at 300 mA constant current using a semidry transfer cell (Bio-Rad Laboratories Inc, Hercules, CA, USA, <http://www.bio-rad.com>). The gel was stained with Coomassie blue to determine whether the proteins had been transferred to the membrane. To ensure an adequate protein transfer, the membranes were stained with Ponceau S. The blots were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline (1 mol/L Tris-HCl [pH 7.5] and 3 mol/L NaCl).

The membrane was washed with Tris-buffered saline plus Tween (TBST) to remove the Ponceau S and then incubated overnight at 4°C with 1:5000 anti-beta-actin primary antibody (Dako Denmark A/S, Glostrup, Denmark, <http://www.dako.com>).

After washing the blots in TBST, they were incubated with a secondary anti-mouse IgG-horseradish peroxidase-conjugated antibody (Dako Denmark A/S, Glostrup, Denmark, <http://www.dako.com>) at 1:5000 for 2 hours. The blots were then washed again in TBST. The proteins were visualized using a chemiluminescent agent (GE Healthcare, Piscataway, Uppsala, USA, <http://www.gehealthcare.com>) and autoradiography

with pre-flashed Kodak film (Eastman Kodak Company, Rochester, NY, USA, <http://www.kodak.com>).

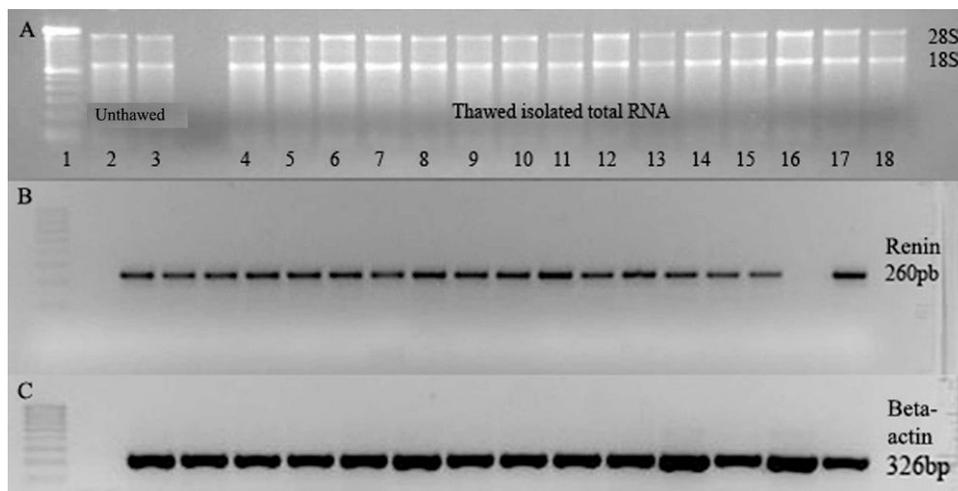
The intensity of the individual bands was quantified by optical densitometry using Scion Image for Windows (Scion Corporation, Maryland, USA, <http://scion-corporation.software.informer.com>). A pre-stained protein marker (Bio-Rad Laboratories Inc, Hercules, CA, USA, <http://www.bio-rad.com>) was used as the molecular weight standard.

**Histology**

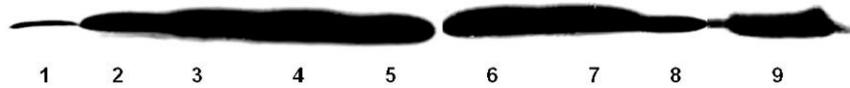
The thawed tissues [pancreas (n=3), white (n=4) and brown (n=3) adipose tissue, kidney (n=3), liver (n=3), and heart (n=4)] were fixed in 10% buffered formalin and embedded in paraffin blocks. The morphological analysis was performed as described (8). Briefly, 5-µm-thick sections were cut and stained with Masson’s trichrome and periodic acid Schiff for structural analyses. The tissue sections were examined using a magnification of 200X (Nikon Instruments Inc, Melville, NY, USA, <http://www.nikoninstruments.com>).

**Statistical procedures**

The data are expressed as the mean ± SEM. The normality of the distributions was tested before performing Student’s t



**Figure 4A** - Integrity of the thawed and unthawed isolated total RNA samples. 1 = ladder; 2 and 3 = unthawed kidney (control); 4 to 18 = thawed isolated total kidney RNA. **Figure 4B** - Renin mRNA level in thawed isolated total kidney RNA. **Figure 4C** - β-Actin mRNA level in thawed isolated total kidney RNA.



**Figure 5** - Protein level of  $\beta$ -actin in the thawed homogenates (samples: 1 = kidney, 2 = heart and 3 = BAT), thawed tissues (samples: 4 = brown adipose tissue; 5, 6 and 7 = kidney; 8 = brown adipose tissue) and unthawed tissues (samples: 9 = kidney).

test with Welch's correction analysis. Values of  $p < 0.05$  were considered statistically significant. The statistical analysis was performed using GraphPad 4.00 for Windows (9).

### Ethics

The experiments in this study were previously approved by the Ethics Committee of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

### RESULTS

#### A260/A280 ratio and the integrity of the total RNA from thawed tissues

The A260/A280 ratio of the total RNA isolated from the thawed tissues was not affected relative to that of the unthawed tissues (Figure 1). However, when verifying the integrity, the 28S and 18S bands were not observed, indicating that the total RNA was degraded (Figure 2).

#### A260/A280 ratio, total RNA concentration, RNA integrity and mRNA levels in thawed isolated total RNA samples

Although the concentrations were the same (Figure 3B), the A260/A280 ratio was higher ( $p < 0.05$ ) for the thawed isolated total RNA than for the unthawed isolated total RNA (Figure 3A). The integrity of the total RNA was evaluated by gel electrophoresis analysis of the 28S and 18S bands (Figure 4A), and this analysis confirmed the integrity of the isolated total RNA after defrosting. By RT-PCR, we observed that the levels of  $\beta$ -actin (Figure 4B) and renin (Figure 4C) mRNA were perfectly maintained in the thawed isolated total RNA. From these data, it could be concluded that thawed isolated total RNA can be analyzed and processed even after defrosting for up to 24 hours.

### Protein levels

The  $\beta$ -actin protein levels in the tissue and homogenate were analyzed by Western blotting, and these proteins were found not to have been degraded due to the defrosting (Figure 5).

### Histology

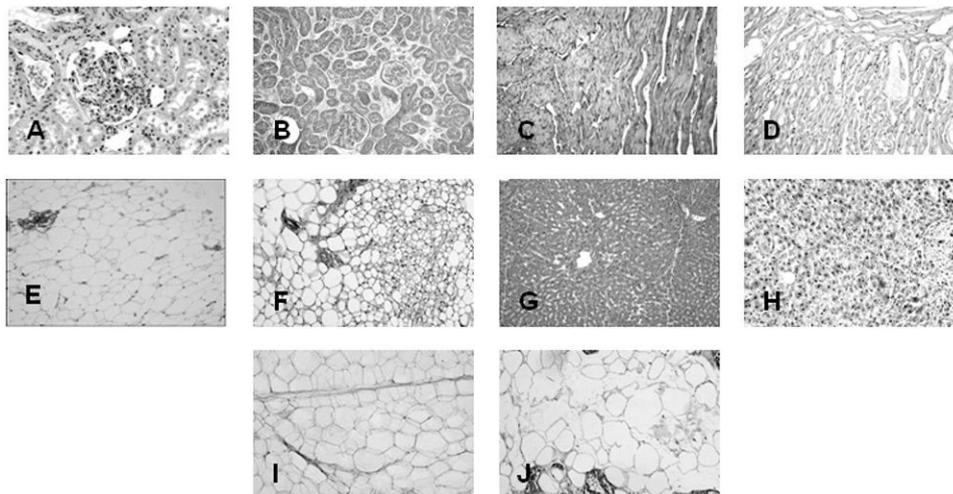
We found that the morphology of some of the thawed tissues was not preserved relative to that of the respective unthawed tissues (Figure 6).

### DISCUSSION

Some interesting and new observations were made in the present study. The samples used to determine the amount of intact mRNA in previously isolated total RNA and the protein levels in thawed intact tissues and homogenates are well preserved even after more than twenty-four hours at room temperature. These results suggest that, in the event of a technical problem with deep freezers that leads to defrosting, some material can still be used for some determinations.

However, the histological structure and RNA extracted from the thawed tissues were not preserved.

Okamoto et al. (10) have found that, during tissue freezing and thawing cycles, both the formation of ice crystals and the osmotic effect contribute to RNA degradation. In another study, it was found that RNA and DNA were stable only up to five hours after the tissue was excised when the tissue was maintained at room temperature (5). The results from the present study confirm that the RNA is not viable in defrosted tissues. The rapid degradation of RNA in tissues maintained at room temperature or submitted to freezing and thawing cycles may be due to RNAase digestion. The preservation of the thawed previously isolated RNA found in the present study supports this possible mechanism because RNAase is



**Figure 6** - Microphotographs of thawed and unthawed tissue. (A) kidney, (B) thawed kidney, (C) heart, (D) thawed heart, (E) BAT, (F) thawed BAT, (G) liver, (H) thawed liver, (I) white adipose tissue, (J) thawed white adipose tissue (400x).

absent from these samples. Therefore, isolated total RNA is protected from degradation due to thawing, maintaining the integrity of expression analyses. Accordingly, it is recommended that total RNA be isolated as soon as possible after tissue collection. The proteins in intact tissues are most likely denatured due to prolonged storage at room temperature. However, the protein level was preserved in the thawed intact tissues and homogenates. A possible explanation for this observation is that protein denaturation is inherent in the Western blot technique.

In conclusion, both gene and protein expression analyses may be performed using total isolated RNA and intact tissue or homogenates, respectively, that have been stored for many hours at room temperature.

## AUTHOR CONTRIBUTIONS

Oliveira IB performed the Western blotting and histology analysis and wrote the manuscript. Ramos DR, Lopes KL, and Souza RM performed the PCR-RT analysis and helped to write the manuscript. Heimann JC revised the manuscript. Furukawa LN conceived and coordinated the study and wrote the manuscript.

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