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Comparative Evaluation of RNAlater Solution and Snap Frozen Methods for Gene Expression Studies in Different Tissues

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Abstract

***Introduction**: Freezing of tissues with liquid nitrogen is the most common method in studies performed at the RNA level. However, the use of RNA stabilization solutions has become a popular alternative method. The aim of this study is to investigate the effectiveness of RNAlater on RNA stabilization in different tissues.* ***Material and Methods:** In this study, RNA were isolated from the lung, heart, liver and skeletal muscle tissues of rats that were frozen with liquid nitrogen (snap frozen, SF group) or stored in RNAlater solution (RL group), and the changes in concentration, purity, reference genes expression, and fold-change levels between groups were analyzed.* ***Results**: In the RL group, the concentration of RNA isolated from the liver tissues was higher (P<0.05), whereas the A260/280 ratio was lower in the heart and liver tissues (P<0.05). PPIA and SRP72 genes were found to have lower Ct values in the heart tissues of rats in the RL group (P<0.05 and P<0.001, respectively) than the SF group. Expression levels of PPIA, ACTB, and SRP72 genes across the tissues were found to be different between the groups (P<0.05). The gene expression level examined in terms of fold-change was significantly different in the RL group (upregulated up to 4 folds and downregulated about 0.5 fold) (P< 0.05).* ***Conclusions**: The results showed that RNAlater can maintain the RNA integrity and can also change the results of gene expression because it does not inhibit biological activity. The snap freezing method is more reliable because gene expression is more stable in tissues frozen with liquid nitrogen.*

***Keywords:** RNAlater, Snap Frozen, RNA Quality, Reference Gene, Gene Expression*

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Introduction

RNA is an important intermediary molecule for understanding the molecular mechanisms of living things. RNA activity has been investigated at the level of transcription and translation by using RT-qPCR technology, particularly in gene expression studies in which physiological and pathological conditions are evaluated. In addition, the quality of RNA isolated from various tissues and cells is one of the most significant parameters that can affect the study outputs (1).

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Therefore, particularly in studies performed at the mRNA level, the quality and integrity of RNA in target cells and/or tissues must be ensured and the tissues must be protected against temperature and various RNases (2).

To maintain the integrity of RNA in tissues, liquid nitrogen freezing immediately after the collection of tissues is commonly preferred. This method, which is also known as snap freezing method, is reliably used because it provides rapid RNase inactivation at a low temperature. However, there are some disadvantages in this process, such as the supply, transportation, and storage of liquid nitrogen (3, 4). As an alternative to the snap freezing method, various methods such as storage with formalin, paraffin, ethanol, and some storage solution for RNA stabilization like RNAlater RNAsafe have been reported. Some studies have shown that storage with these chemicals is not as efficient as snap freezing in terms of the maintenance of RNA quality (5-7). However, the use of RNAlater, an alternative method for preserving nucleic acids in tissues (particularly RNA), can maintain the quality of RNA (8). RNases are inactive when pH stabilization is ensured, owing to salts such as ammonium sulfate present in RNAlater-like chemicals; therefore, RNA quality can be maintained in stored tissues (9). In addition, RNAlater is recommended as an alternative to the snap freezing method because tissues stored with RNAlater can be kept at room temperature for up to 1 week, at 4°C for up to 1 month, and at −80°C for longer periods (4, 5).

RNA isolated from cells and tissues is evaluated in terms of quality, integrity, and purity parameters by electrophoretic and spectrophotometric methods. A260/280 ratio and 28S and 18S rRNA band integrity and ratios in total RNA are widely used, particularly in eukaryotic organisms (10, 11). In addition, there are various methods used for evaluating RNA integrity such as the calculation of total RNA amount, quantitation of tissues used in isolation, utilization of internal genes, and calculation of the RIN value. Expression levels of internal genes, particularly those used in relative gene expression studies, are more commonly preferred (12, 13).

Genes to be used as internal genes should be stable in the tissue and should not be affected by methodological procedures. Therefore, it is necessary to consider factors such as study hypothesis, organism, and tissue and cell type in the utilization of genes as an internal control that are called housekeeping or reference genes. However, numerous studies have reported that the reference gene to be internally used may be used as a reference in a particular tissue but may not be used in another tissue due to its tissue-dependent activity (12, 14). There are studies using standard methods (electrophoretic and spectrophotometric) to control the quality of RNA in tissues stored with RNAlater (15)ite></EndNote>. Although there are some studies comparing the quantity of genes through Ct values by means of RT-qPCR in addition to standard controls, studies performing their examinations at the level of gene expression are limited (15, 16).

In this study, possible RNA quality loss that may occur in the lung, heart, liver, and skeletal muscle tissues stored in RNAlater was studied in terms of Ct and gene expression through electrophoretic and spectrophotometric controls as well as *PPIA* (Peptidylprolyl Isomerase A), *ACTB* (Beta Actin), *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase), and *SRP72* (Signal Recognition Particle 72) genes, which are commonly used as reference genes (17-21).

Materials and Methods

Animal Materials

This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee with a decision number 2018/9-3. In this study, six male wistar albino rats (10-week-old), weighing approximately 200

g (202.22 ± 14.67 g), were used. The rats were maintained under standard conditions at Mustafa Kemal University Experimental Research and Application Center.

The ambient temperature and humidity were 21°C±2°C and 55% respectively. The lighting schedule was a 12–12 h light–dark cycle (light period: 07.00–19.00 h, dark period 19.00–07.00 h) during the study. The rats were maintained in polycarbonate transparent cages, each housing three rats, and were fed *ad libitum* with standard chow and water. At the end of the adaptation period, the rats were euthanized by exsanguination via cardiac puncture under anesthesia (80 mg/kg ketamine and 12 mg/kg xylazine, IP).

The lung (right), heart (left ventricle), liver (right lobe), and skeletal muscle (left leg, *m. gluteus*) tissues were immediately collected from the rats and divided into two parts. One part was rapidly frozen with liquid nitrogen (snap frozen), while the other part was stored in RNAlater® (Cat No: AM7021, ThermoFisher Scientific, USA). The samples treated with RNAlater were kept in the RNAlater solution at an amount 10 times their weight in accordance with the kit usage guidelines. Samples frozen in liquid nitrogen (snap frozen group, SF group) were stored at −86°C. Samples submerged in RNAlater (RL group) solution were kept at +4°C for one night to ensure the penetration of RNAlater in accordance with the kit usage guidelines and then stored at −86°C.

RNA isolation and cDNA synthesis

RNA was isolated using the Trizol method from samples stored at −86°C in the SF and RL groups (22). In accordance with the kit protocol, 1 ml Trizol (ThermoFisher Scientific, USA) was used per approximately 50 mg tissue. Total RNA was extracted from samples after homogenization in Trizol under cold conditions, followed by chloroform, isopropyl alcohol, and ethyl alcohol treatments, and the samples were diluted with 30–100 µL nuclease-free water based on the pellet sizes. RNA concentration of the samples and purity measurements were performed using nucleic acid meter (Merinton SMA 1000, USA), and the quality of RNA was checked in 1% agarose gel electrophoresis (100 V and 25 min).

After DNase treatment with DNase I (DNase I, RNase free, ThermoFisher Scientific, Cat no: EN0525, USA), the isolated RNAs were converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Cat no: 4368814). cDNA was synthesized using 1000 µg total RNA in Thermal cycler (Biorad T100, USA) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min, in accordance with the kit protocol. Following the reaction, the samples were made up to 200 µl with nuclease-free water and stored at −86°C until qPCR analysis.

Quantitative Real Time PCR Analysis

Amplification of the target genes was performed using 5 µl of cDNA sample using the kit containing SYBR Green I dye (Power SYBR® Green PCR Master, ThermoFisher Scientific, USA, Cat no: 4367659), in accordance with the kit protocol. Each sample was duplicated. The reaction in qPCR (CFX96 Touch, Biorad, USA) was arranged using the following parameters: 10 min at 95°C and 40 cycles of 15 s at 95°C, 60 s at 60°C. The *PPIA* primer sequences used in the study were obtained from the literature (23), and the primer sequences of *ACTB*, *GAPDH*, and *SRP72* genes were designed by the authors using Primer BLAST (NCBI) (Table 1). The area multiplied by each pair of primers was examined by melting curve analysis in qPCR, and the PCR product was run on the 2% agarose gel, confirming that the primers were multiplied by one area.

Statistical Analysis

The differences between the tissues stored by the snap freezing and RNAlater methods were calculated using the SPSS program (Version 22.0).

Table 1. Forward and Reverse Sequences of Genes Primers

Gene	Accession No	Forward and Reverse Sequences	Product Size (bp)
PPIA	NM_017101.1	F: 5'-CAGACAAAGTTCCAAAGACAGCA-3' R: 5'-CACCCTGGCACATGAATCCT-3'	117
GAPDH	NM_017008.4	F: 5'-AGTGCCAGCCTCGTCTCATA-3' R: 5'-TCCCGTTGATGACCAGCTTC-3'	234
ACTB	NM_031144.3	F: 5'-GCAGGAGTACGATGAGTCCG-3' R: 5'- ACGCAGCTCAGTAACAGTCC-3'	74
SRP-72	NM_001170601.1	F: 5'-ACCTGCCCTCATCGGATAGT-3' R: 5'-CCCTGTCCTTGTTCCTTTGGT-3'	133

The Ct levels differences between groups (SF vs RL) was analyzed by Student t-test. The difference among the tissues was determined with One Way Anova and later applicated by the Tukey test. The gene expression levels in the RL group relative to the SF group were calculated based on $2^{-\Delta\Delta Ct}$ calculation method and were demonstrated as fold-change (24).

Results

The concentration and purity values of total RNA isolated from the lung, heart, liver, and skeletal muscle tissues in the SF and RL groups were determined to be appropriate for cDNA and qPCR applications. RNA concentration in the liver tissues was found to be significantly higher in the RL group. The A260/280 ratios in the heart and liver tissues were found to be lower in the RL group than in the SF group (Table 2).

RNA integrities were checked in 1% agarose gel electrophoresis. RNA samples 28S and 18S rRNA subunits pictures were given in figure 1.

There was no significant difference between the groups in terms of Ct values of *PPIA*, *GAPDH*, and *SRP72* genes in the lung, liver, and skeletal muscle tissues (Table 3). However, the Ct values of *PPIA* and *SRP72* genes were found to be approximately 1.5 Ct higher in the heart tissues in the SF group. The Ct value of *ACTB* gene in the skeletal muscle tissues in the RL group was approximately 2 Ct higher than those in the SF group.

The highest Ct values for *PPIA* genes in both groups were found in the skeletal muscle tissues; in other words, this gene has the lowest expression level in the skeletal muscle among the other studied tissues. The highest *ACTB* gene expression was found in the lung tissue ($P<0.001$). There was no significant difference between the tissues in terms of *GAPDH* expression levels. The lowest SRP72 gene expression was determined in the skeletal muscle and heart tissue in the SF group and skeletal muscle in the RL group (Table 3).

The genes used in the study were used both as reference and target genes in all tissues, and

Table 2. Concentrations and A260/280 ratios of RNA samples in Tissues ($\overline{x} \pm s_{\overline{x}}$)

Tissue	Concentration (ng/µL)			A260/A280 ratio		
	SF Group	RL Group	P	SF Group	RL Group	P
Lung	246.52±39.61	356.01±63.66	-	1.74±0.02	1.77±0.02	-
Heart	767.34±72.98	291.73±85.92	-	1.86±0.08	1.77±0.02	<0.05
Liver	216.09±27.24	624.58±113.28	<0.05	1.86±0.75	1.77±0.19	<0.05
S. Muscle	300.06±38.43	334.87±88.94	-	1.95±0.09	1.84±0.05	-

SF: Snap frozen; RL: RNAlater; S. Muscle: Skeletal Muscle; -: P>0.05; X ±Sx: Average ± Standard Error

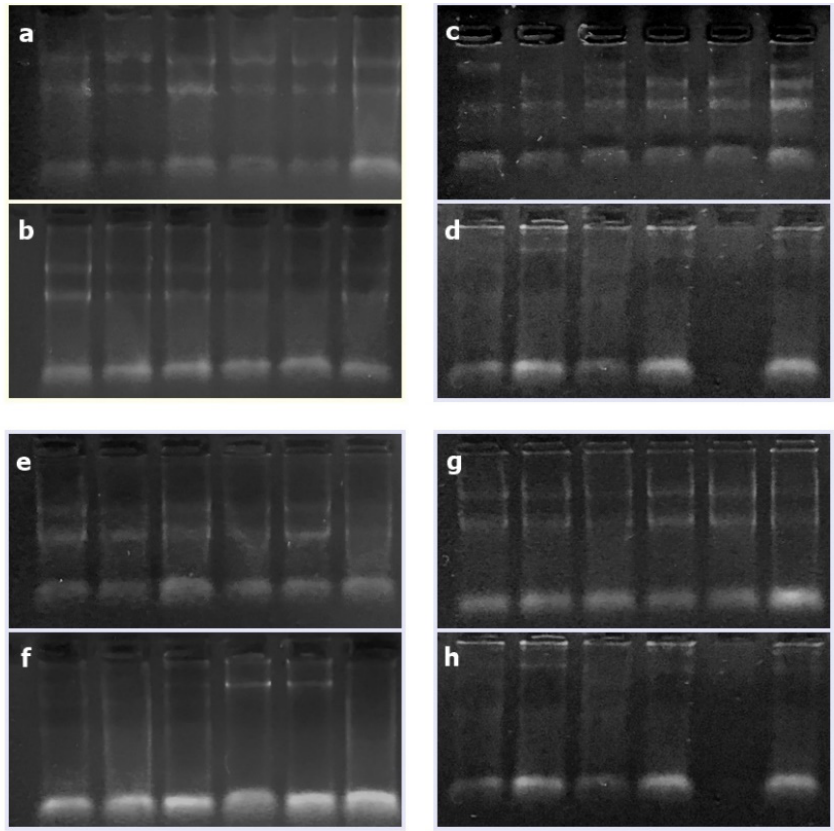

Fig. 1. rRNA bands of RNA samples in tissues in SF and RL groups. a) Lung tissue RNA samples in SF group, b) Lung tissue RNA samples in RL group, c) Heart tissue RNA samples in SF group, d) Heart tissue RNA samples in RL group, e) Liver tissue RNA samples in SF group, f) Liver tissue RNA samples in RL group, g) Skeletal Muscle RNA samples in SF group, h) Skeletal Muscle RNA samples in RL group.

Table 3. Averages of Ct Values in Different Tissues stored with different methods ($\overline{X} \pm S\overline{x}$)

Genes	Groups	Tissues				*P*
		Lung	Heart	Liver	S. Muscle	
PPIA	SF	17.37±0.28^{b}	19.89±0.13^{a}	16.85±0.15^{b}	20.66±1.07^{a}	<0.001
	RL	16.67±0.61^{b}	18.46±0.50^{b}	17.78±0.48^{b}	21.03±0.91^{a}	<0.001
	P	-	<0.05	-	-	
ACTB	SF	15.31±0.17^{c}	19.52±0.23^{a}	18.33±0.16^{b}	18.83±0.27^{a,b}	<0.001
	RL	15.77±0.70^{b}	19.84±0.89^{a}	19.35±0.43^{a}	21.11±0.28^{a}	<0.001
	P	-	-	-	<0.001	
GAPDH	SF	18.40±0.32	17.86±0.23	17.08±0.22	16.83±1.41	-
	RL	18.35±1.13	16.63±0.65	17.44±0.39	20.42±2.20	-
	P	-	-	-	-	
SRP72	SF	21.10±0.31^{b}	23.60±0.20^{a}	21.14±0.15^{b}	23.72±1.28^{a}	<0.05
	RL	21.41±0.76^{b}	21.91±0.15^{b}	21.50±0.45^{b}	25.41±1.17^{a}	<0.01
	P	-	<0.001	-	-	

SF: Snap Fozen; **RL**: RNAlater; -: *P*>0.05; $^{a, b, c}$: Means with different letters in rows differ significantly; X ±Sx: Average ± Standard Error

Table 4. Fold Changes of Genes in Lung, Heart, Liver, and Skeletal Muscle ($\bar{x} \pm s_{\bar{x}}$)

Genes		Lung	Heart	Liver	S. Muscle
REFERENCE	**TARGET**	**SF vs RL**	**SF vs RL**	**SF vs RL**	**SF vs RL**
PPIA	**ACTB**	2.36±0.97*	3.94±0.87*	1.14±0.27	3.30±0.83*
	GAPDH	2.38±1.07	1.27±0.25	1.05±0.36	53.97±40.13
	SRP72	2.06±0.24*	1.01±0.28	1.00±0.32	2.53±0.52*
ACTB	**PPIA**	0.48±0.08**	0.35±0.08***	0.99±0.16	0.15±0.07
	GAPDH	1.01±0.47	0.42±0.14**	0.72±0.16	13.94±11.69
	SRP72	0.91±0.12	0.44±0.16*	0.77±0.20	0.35±0.21
GAPDH	**ACTB**	1.66±0.29	3.41±0.66**	1.83±0.50	1.21±0.53
	PPIA	0.83±0.34	0.97±0.21	2.38±0.88	0.34±0.15*
	SRP72	1.43±0.240	0.94±0.30	1.03±0.14	0.62±0.25
SRP72	**ACTB**	1.11±0.15	7.20±2.66	1.90±0.48	1.59±0.53
	GAPDH	0.95±0.32	1.71±0.46	1.01±0.13	13.16±8.83
	PPIA	0.51±0.06**	1.44±0.36	2.30±0.83	0.40±0.09*

*: P<0.05; **:P<0.01; ***: P<0.001; X ±Sx: Average ± Standard Error

the fold-change values were calculated. When *PPIA* was considered as a reference gene, the expression level of *ACTB* and *SRP72* genes in the lung and skeletal muscle tissues in the RL group was 2 folds higher than that in the SF group (P<0.05). In addition, the expression level of *ACTB* gene in the heart tissues in the RL group was approximately 4 folds higher than that in the SF group. When *ACTB* is considered as a reference gene, the expression levels of PPIA, GAPDH, and SRP72 genes in the heart tissues and of only *PPIA* in the lung tissues were found to be downregulated (fold-change values <0.50; P<0.05). When *GAPDH* is considered as a reference gene, it was found that ACTB gene was approximately 4 folds upregulated in the heart tissues (P<0.01), whereas *PPIA* gene was downregulated (fold-change= 0.337 ± 0.148; P<0.05). When *SRP72* was considered as a reference gene, *PPIA* was found to be downregulated in the lung and skeletal muscle tissues (P<0.05) (Table 4).

Discussion

In quality control of RNA isolated from cells and tissues, the expression levels of reference genes used for internal control have been examined by methods in which various parameters such as RNA concentration and purity and 28S and 18S band integrity ratios (8, 12, 25, 26). There must be no contamination and degradation in RNA isolated from tissues to be studied under normal conditions. RNA quality is deteriorated due to storage conditions following the collection of tissues, and low-quality RNA leads to different results in gene expression studies performed with qPCR (13, 27).

In this study, RNA was examined in tissues stored with snap freezing and RNAlater methods in terms of purity, concentration, and integrity. The samples in both groups qualified for cDNA conversion based on the A260/280 ratio, concentration values, and 28S and 18S band ratio. In addition, the concentration levels in liver samples of the RL group were higher than those of the SF group, whereas the A260/280 ratios were lower (P<0.05). The A260/280 ratios in the heart tissues in the RL group were found to be lower than those in the SF group (P<0.05).

In addition, the concentration required for the various cDNA kits used in gene expression studies can be achieved with the samples obtained in both groups (such as High-Capacity cDNA Re-

verse Transcription Kit, RevertAid First Strand cDNA Synthesis Kit, ThermoFisher Scientific, and iScript cDNA synthesis kit, Biorad). If the A260/280 ratios, which give an idea about the purity of isolated RNAs, are ≥1.7, they are appropriate for gene expression studies (11, 28). However, some studies have reported that it is not possible to have sufficient knowledge about RNA quality by checking the integrity of rRNA bands (29-31). In this study, lung tissue rRNA bands qualities were the same in both groups (SF and RL groups). On the other hand, in RL group, heart, liver and skeletal muscle RNA samples were looking worse than SF group. RNA qualities in all tissues have enough quality with snap freezing method (Figure 1).

There was a statistically significant difference between the SF and RL groups in terms of Ct values of *PPIA* and *SRP72* genes in the heart tissues ($P<0.05$ and $P<0.001$, respectively). The Ct values of these genes were found to be lower in the heart tissues in the RL group (Table 3). In their study on the efficiency of RNAlater, Martin et al. (32) have examined the expression levels of some reference genes in placental tissues and reported that Ct values of these genes were significantly lower. Although in the light of these data, they reported that the storage with RNAlater could result in RNAs of better quality, protein activity varies across the tissues due to differences in the gene activity and perfusion rate (19, 33).

Higher Ct values in the tissues stored with different methods than those obtained via the snap freezing method have been associated with low RNA quality (34, 35). Although RNAlater contributes to the prevention of RNA degradation by limiting the RNase activity, its effect on gene activity is not exactly known when the tissues are stored in RNAlater at +4°C. A study has reported that when tissues are stored in the RNAlater, there were increases or decreases in the activity of about 3,000 genes (15). This may be caused by the fact that gene activity is not inhibited during fixation of tissues with RNAlater.

The lack of a difference between the groups in terms of Ct values of *ACTB* and *GAPDH* genes in the heart tissue in addition to the groups being similar in terms of other genes suggests that RNAlater does not exert similar effects in all tissues. The fact that the expression level of *ACTB* gene in the muscle tissue was higher in the SF group (approximately 2 Ct higher in the RL group, $P<0.001$) is an indication of this suggestion (Table 3).

In both groups, the highest expression level for the *ACTB* gene expression, which is of the reference genes, was observed in the lung tissue (Table 3). The lowest *SRP72* gene expression was observed in the skeletal muscle tissue (Figure 1). Although expression level of a particular gene provides an idea about its utility as a reference gene in the relevant tissue, Hruz et al. (36) and Sampaio-Silva et al. (37) have reported that *SRP72* gene could be reliably used as a reference gene in skeletal muscle tissues.

It is known that RNA quality is low if it is isolated from formalin-fixed and paraffin-embedded tissues as an alternative to the snap freezing method (38). In a study comparing formalin-fixed and snap frozen tissues, it was reported that Ct values were lower in snap frozen samples and increased in formalin-fixed samples (35). If this increase is up to 128 times higher as reported in the study by Viertler et al. (35), it can provide incorrect outputs in the gene expression studies since it can result in significant errors in the calculation of expression levels. On the contrary, in a study that emphasized the importance of RNA quality in microarray studies, RNAlater and snap freezing methods were compared using breast tumor tissues, and a high concentration of RNA was obtained from the tissues stored in RNAlater, similar to RNA concentration levels in the liver tissues. In the same study, the RNA integrity was also reported to be better (39). In

this study, although no significant differences were found in the concentration, purity and Ct values of different tissues, there were significant changes in the gene expression level (Table 3).

In a study performed on the storage of rat livers (5), the band integrity was reported to be better in liver samples stored in RNAlater solution for different time periods and at different temperatures than those stored at −80°C and those stored at −80°C after freezing with liquid nitrogen (5). Another study comparing snap freezing method and directly freezing at −80°C reported that snap freezing method allows isolation of higher quality RNAs from liver tissues (3).

In studies using RNAlater solution to examine Ct values of some reference genes by qPCR and to evaluate RNA quality using electrophoretic and spectrophotometric methods, this chemical has been reported to maintain the stability of RNA (4, 5, 9, 40). The use of internal gene is one of the important methods used to control the RNA quality (25). In this study, unlike other studies, the expression levels of *PPIA, ACTB, GAPDH,* and *SRP72* genes, which are used as internal genes, were calculated as fold-change and compared between the SF and RL groups. When the genes studied in terms of Ct levels were considered as reference gene and the fold-change in other genes is calculated based on the $2^{-\Delta\Delta Ct}$ method, it was observed that there were significant changes in the RL group. In lung, heart muscle and skeletal muscle tissues, upregulation up to 4 times or downregulation less than 0.5 times was observed in terms of *ACTB, PPIA* and *SRP72* (Table 4, $P<0.05$). This is one of the most significant indicators that the storage with snap freezing method is more reliable than the RNAlater method. In the examination of expression level, there was no statistically significant difference between both methods in terms of fold-change in the liver tissues. This shows the importance of tissue selection both in the selection of reference gene as well as in studies to be performed on the RNA quality. Liver is a tissue in which the metabolic activity is most intense. Considering this, the RNase activity is expected to be high (41). On the other hand, *GAPDH* gene expression levels showed that it is not a reliable gene for internal control in skleletal muscle which stored in RNAlater (Table 4). Although the RNAlater inactivates the RNases, a certain level of RNase activity causes degradation and leads to decreased gene expression.

In the study, the molecular effects of snap frozen and RNAlater methods were investigated in different tissues of rats. However, unlike similar studies, biological activity in more than one tissue was investigated by calculating at the level of gene expression. As a result of the findings and the literature searches, it is thought that RNAlater has changeable efficacies for maintaining RNA stabilization. In this study, only 4 different tissues in 6 animals were evaluated. Considering the material and methodology of this study, it can be said that Snap frozen method is better than RNAlater for gene expression studies.

Conclusion

With technological advances, experimental studies at the RNA level have become widespread in many areas. This leads to the emergence of alternative methods for the storage of tissues to be studied for gene expression calculations. With this study, it may be represented that the storage with RNAlater solution, which is one of these methods, is not as reliable as the snap freezing method. In conclusion, it is considered that more studies with more animal numbers is needed and the content of this chemical can be further improved, considering its unknown effects on mRNA fragments of different sizes in total RNA and on the inhibition of physiological activity.

Abbreviations

ACTB, Beta Actin
GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase
PPIA, Peptidylprolyl Isomerase A
RL, RNAlater
SF, Snap Frozen
SRP72, Signal Recognition Particle 72

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Authors' contribution

HO (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing)
EK (Methodology; Writing – original draft)

Conflict of Interest

The authors declare no conflict of interest.

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