Research article

Effects of storage temperature, storage time, and hemolysis on the RNA quality of blood specimens: A systematic quantitative assessment

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A R T I C L E I N F O

A B S T R A C T

Introduction: Blood samples are the most common biospecimen in biobanks, and RNA from such blood samples is an important material for biomedical research. High-quality RNA is essential for consistent, reliable results. Preanalytical environmental conditions can affect the quality of blood RNA. Here, we carried out a quantitative assessment of the influence of storage temperature, storage time, and hemolysis on the RNA quality of blood specimens in biobanks.

Methods: Before RNA purification, blood samples from volunteers were exposed to 4 °C for 2, 6, 12, 24, or 48 h, 3 days, or 1 week, or exposed to room temperature (22–30 °C) for 1, 2, 6, 12, or 24 h. Hemolyzed samples were collected from laboratory department and some of them were prepared using the freeze-thaw method. After exposure to different preanalytical environmental conditions, the RNA simple Total RNA Kit was used to purify the RNA, following which a NanoDrop™ One and Qsep 100 Bio-Fragment Analyzer were used to assess RNA concentration, purity, and integrity. In addition, a part of the RNA was immediately reverse transcribed into cDNA when it was purified, then the relative expression levels of *18S*, *ACTB*, *HIF1α*, *HMOX1*, and *MKI67* were determined by real-time quantitative PCR. Finally, 30 blood samples were collected from the surplus samples in our laboratory department to assess their RNA quality without knowledge of their storage conditions (duration/temperature).

Results: For blood samples stored at 4 °C, there was a significant difference between the RNA integrity after 1 week compared to after 2 h. For blood samples stored at room temperature (22–30 °C), the RNA integrity was also significantly different at 6 h and 0 h. Hemolysis caused by freeze-thawing severely affected RNA quality, whereas clinical hemolysis generally produced no significant effects. Moreover, expression of *18S*, *ACTB*, *HIF1α*, *HMOX1*, and *MKI67* in whole blood stored under different conditions showed irregular changes, suggesting that preservation conditions are also important for gene expression.

Conclusion: RNA integrity was qualified for blood samples stored at 4 °C for up to 72 h or at room temperature (22–30 °C) for up to 2 h. Hemolysis usually does not affect the RNA quality of blood samples unless the hemolysis method damages leukocytes.

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1. Introduction

In clinical biobank laboratory activities, the output is the biological samples themselves, which are banked for future use. Among the biospecimens stored, blood samples are the most common and useful for clinical research as blood not only reflects tissue oxidative stress but also metabolic status [1,2]. RNA is an important element of molecular biological research; high quality RNA can be used in reverse transcription polymerase chain reaction (RT-PCR), northern blotting, gene expression profiling, transcriptome sequencing, microarray analysis, and other experimental techniques. Further, research indicates that blood microRNA can be used to predict certain diseases [3,4]. However, blood RNA is easily degraded during collection and storage due to its structure, the strong presence of RNase in the environment, and endogenous RNase [5].

Blood RNA can be affected by many factors, such as specimen state, storage temperature, storage time, freeze-thaw processing, and various other processing methods. According to the Standard Preanalytical Code (SPREC), which was developed by the International Society for Biological and Environmental Repositories (ISBER) in 2009, the main preanalytical factors affecting clinical fluids consist of seven elements [6], including sample state, storage time, and temperature, which are difficult for biobanks to control. In the early stages of constructing biobanks, it is necessary to determine the environmental variables affecting samples within the laboratory itself. At present, there are some studies on the factors affecting blood RNA quality, but the results are inconsistent owing to variations in the specific environmental conditions. For example, Abraham et al. [7] found that creatinine, folate, and hemoglobin in blood samples were unstable after 12 h at 22–30 °C. Lee et al. [8] found that preanalytical variations contributed to variations in the output of omics analyses. Huang et al. [9] also observed considerable quality decline in blood RNA at a storage time of more than 24 h; furthermore, storage of whole blood samples in freezers would dramatically damage the RNA. However, the effect of hemolysis on total RNA quality has not yet been reported.

This study quantitatively assessed storage temperature, storage time, and hemolysis on the quality of blood RNA to establish the optimum conditions for maintaining high-quality blood RNA for clinical testing and experimental research. Furthermore, we investigated different storage methods for blood samples after centrifugation (data not shown) to provide a set of recommendations concerning collection, pre-processing procedures, and storage conditions. As a standard operating protocol, it will reduce undesirable variation in blood RNA quality.

2. Materials and methods

2.1. Sample collection

Peripheral blood samples were provided by three healthy, consenting adult volunteers from our Biobank and four pediatric patients whose legal guardians had given their written informed consent. Whole blood from healthy volunteers was drawn twice. The pediatric patients were from our Pediatric Heart Disease Treatment Center. The blood samples were collected into EDTA anticoagulant tubes (Gongdong Medical, Taizhou, China). All blood collection tubes were manually inverted ten times and thoroughly mixed. The hemolyzed samples were prepared using the freeze-thaw method or collected as fresh hemolyzed samples (in vitro, not meeting clinical laboratory test standards, 6 samples) from the receiver chamber of our laboratory department. In addition, 30 validation samples were collected from the surplus samples in our laboratory department to test the RNA qualification rate. This study was approved by the Ethics Committee of Jiangxi Provincial Children's Hospital (approval number: JXSETYY-YXKY-20220196). All samples collected from our laboratory department were approved by the Ethics Committee and exempt from informed consent.

2.2. Study design and sample treatment

Each blood sample was divided into aliquots of 250 μL, which were assigned to the following groups: 1) the room temperature storage group, in which the RNA was extracted from the samples after storage at room temperature (22–30 °C) for 1, 2, 6, 12, or 24 h; 2) the 4 °C storage group, in which the RNA was extracted after 2, 6, 12, 24, or 48 h, 3 days, or 1 week of storage at 4 °C; 3) the FT-hemolysis group, in which the hemolyzed samples were frozen at −80 °Cfor 30 min, then thawed at room temperature for 30 min. In addition, we collected blood specimens and routinely (in vitro) hemolyzed samples from the laboratory department. As there are many different hemolysis methods, it is worth noting that the hemolysis caused by freezing and thawing was not the same as that in the specimens collected from the laboratory department. In particular, the freezing and thawing process may compromise the structure of the white blood cells in the samples. Therefore, we established four experimental groups: normal whole blood samples (WB), frozen and thawed whole blood samples (FT-WB), white blood cells mixed with frozen and thawed red blood cells (WBC + FT-RBC), and red blood cells mixed with frozen and thawed white blood cells (RBC + FT-WBC). Cells were separated via centrifugation at 500g for 10 min at 4 °C.

2.3. Plasma free hemoglobin concentration

Hemolysis was evaluated through the concentration of plasma free hemoglobin and assessed using a SmartSpec™ Plus spectrophotometer (Bio-Rad, CA, USA) with the Free Hemoglobin Assay Kit (Jiancheng Bioengineering, Nanjing, China), which were operated according to the manufacturers' instructions.

2.4. Total RNA purification from whole human blood

RNA was extracted from 250 μL blood samples using the RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China), based on guanidine thiocyanate-phenol-chloroform extraction, followed by sorption of RNA onto a silicon membrane. Total RNA was isolated through usage of the kit according to the manufacturer's instructions.

2.5. Determination of RNA concentration, purity, and integrity

RNA concentration and purity were assessed with A_{260}, A_{260}/A_{280}, and A_{260}/A_{230} using a NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Fisher, Waltham, MA, USA). RNA integrity was evaluated with 28S/18S and RNA quality number (RQN) [10,11] using the Bioptic Qsep 100 Capillary Electrophoresis System (Bioptic, Taiwan, China) with an R1 RNA cartridge. Both instruments were operated according to the manufacturers' protocol.

2.6. Complementary DNA synthesis

Complementary DNA (cDNA) was reverse-transcribed from 500 ng of RNA from the whole blood using the Reverse Transcription System (Promega, Madison, Wisc. USA). To yield a total volume of 20 μl, Random Primers (0.5 μg/μl stock concentration), Reverse Transcription 10× Buffer, Recombinant Ribonuclease Inhibitor, dNTP Mixture (10 mM stock concentration), AMV Reverse Transcriptase, and water (PCR-grade) were added according to the manufacturer's instructions. The reaction mixture was at room temperature for 10 min, then incubate at 42 °C for 15 min to perform reverse transcription, then heated at 95 °C for 5 min, and incubated at 0–5 °C for 5 min using The C1000 Touch thermal cycler (Bio-Rad, Hercules, California, USA).

2.7. Real-time quantitative PCR

The RT-qPCR reactions were performed on a Gentier 96E real-time quantitative PCR system (Tianlong, Xi'an, China) using *TransStart® Green qPCR SuperMix UDG* (TransGen, Beijing, China). Five pairs of primers (Table 1) were designed and synthesized for five genes: *18S*, *ACTB*, *HIF1α*, *HMOX1*, and *MKI67* (Sunya, Hangzhou, China). All reactions contained 1 μl of cDNA, 0.4 μl of forward primer (10 mM), 0.4 μl of reverse primer (10 mM), 10 μl of *TransStart® Green qPCR SuperMix UDG* (2 ×) and 8.2 μl of nuclease-free water to yield a total volume of 20 μl. The PCR cycling program was: 50 °C for 2 min, 94 °C for 10 min, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. Each sample was run in technical triplicate, followed by a melt curve analysis to ensure primer specificity. Expression levels of each gene were calculated from the mean cycle threshold (Ct) values and were normalized to the expression of glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*).

2.8. Blood smear preparation and staining

A Pasteur pipette was used to drop a blood sample onto one end of the slide (HAILUN, Haimen, China). The slide was held in the left hand, and the blood was spread across the width of the slide using a spreader slide with the right hand. The spreader slide and the smear were made to form an angle of 30°, and then the blood was allowed to run across the slide to the edge at an even speed. The blood smear was laid down horizontally to dry. It was then treated with 2 mL of Wright-Giemsa Stain solution A (BaSO, Zhuhai, China) and stained for 1 min. Solution B was added next and gently mixed with a rubber suction bulb. After waiting for 3 min, distilled water was used to slowly rinse from one end of the slide. The slide was then placed on clean absorbent paper to dry and observed using a microscope (OLYMPUS, Tokyo, Japan).

Table 1
Primer sequences used for RT-qPCR.

| Gene ID	Primer	Forward	GenBank accession number	Length
		Reverse		
2597	*GAPDH*	GGTGTGAACCATGAGAAGTATGA	NG_007073	123 pb
		GAGTCCTTCCACGATACCAAAG		
124908489	*18S*	CACCAGACTTGCCCTCCAAT	NC_060946	173 bp
		CCTGAGAAACGGCTACCACAT		
60	*ACTB*	GAGAAAATCTGGCACCACACC	NC_000007	177 bp
		GGATAGCACAGCCTGGATAGCAA		
3091	*HIF1α*	GCAGCAACGACACAGAAACT	NG_029606	167 bp
		TGCAGGGTCAGCACTACTTC		
3162	*HMOX1*	CTGCTGACCCATGACACCAA	NG_023030	164 bp
		AGTGTAAGGACCCATCGGAGA		
4288	*MKI67*	ACTTGCCTCCTAATACGCCTC	NG_047061	168 bp
		AGCTTTGTGCCTTCACTTCCA		

2.9. Data processing

The experimental data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, USA). Data distribution was investigated using the Shapiro-Wilk normality test. For comparisons of multiple groups of data, one-way analysis of variance (ANOVA) test was used when the results were in line with normal distribution, and Kruskal-Wallis test was used when the samples did not meet the normality test. If significant differences were observed, then a Tukey's multiple comparison test, Duncan's multiple range test or Games–Howell test was used to assess pairwise comparisons. For analyzing two groups of data, the Mann–Whitney U test or independent samples t-test was applied for statistical analysis, according to data distribution. One-sample, one-tailed t-test was performed to examine if the RQN of RNA from blood samples stored at 4 °C for up to 72 h was significantly greater than RQN score cutoff of 7. Spearman correlation analysis was used to test associations between RQN and storage time of blood samples stored at room temperature or 4 °C. P values less than 0.05 were considered statistically significant. Significant marks were denoted by: ns, $P \geq 0.05$; *, $P < 0.05$; * *, $P < 0.01$; * * *, $P < 0.001$.

3. Results

Preanalytical environmental conditions are critical to sample quality in biobanks. In this study, we examined the influence of preanalytical storage temperature, storage time, and hemolysis on blood sample RNA quality. This was achieved by collecting blood samples from volunteers, then dividing them into three groups to assess their RNA quality.

3.1. Effect of storage time at room temperature on blood RNA quality

RNA concentration and purity (A_{260}/A_{280}) were determined using a NanoDrop One spectrophotometer, while RNA integrity was determined using a Qsep 100 bioanalyzer. As illustrated in Fig. 1, the mean concentration of RNA extracted from blood samples from adult volunteers (n = 6) exposed to room temperature for 0–24 h ranged from 29.20 to 33.57 ng/μL, with no obvious trend in the changes (Fig. 1A). There was no significant difference between each time group and the 0-h group. A_{260}/A_{280} changed slightly from 1.95 to 2.03, meaning there was no statistically significant difference in RNA purity between each group (Fig. 1B). RQN values ranged from 6.77 to 8.75, with significant differences between 6 and 0 h (P = 0.002; Fig. 1C). In addition, we observed that RQN was
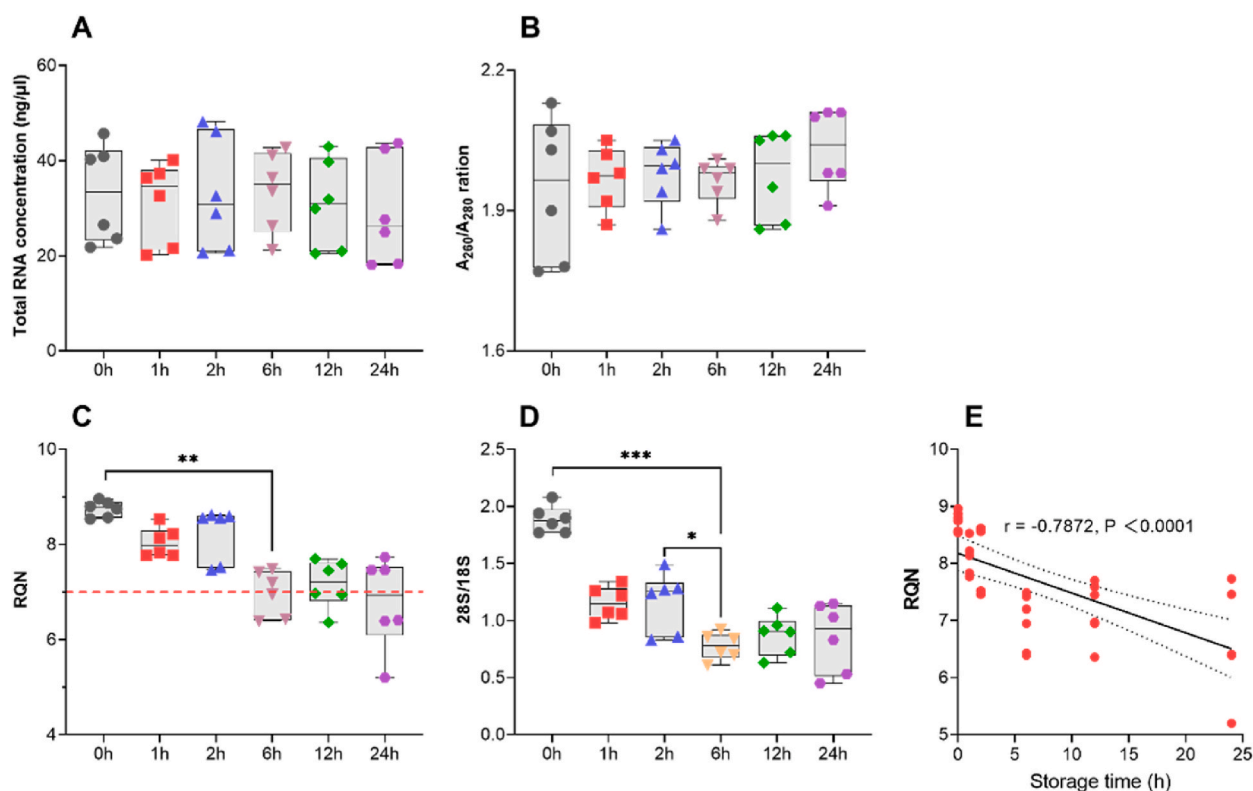
Fig. 1. (A) RNA concentration; (B) A260/A280; (C) RQN; and (D) 28S/18S ratio of blood samples from adult volunteers (n = 6) stored at room temperature for different periods of time. (E) Spearman correlation analysis between RQN with storage time of blood samples stored at room temperature. * Denotes statistical significance according to the Kruskal-Wallis test or one-way ANOVA test, followed by Tukey's multiple comparison test, Duncan's multiple range test or Games–Howell test. ANOVA, analysis of variance; RQN, RNA quality number.

significantly associated with the storage time of blood samples stored at room temperature (Spearman correlation analysis, r = −0.787, P < 0.0001; Fig. 1E). The 28S/18S ratio also differed significantly between 6 and 0 h (P < 0.001), whereas the 28S/18S ratio at 6 h was <1.0 (Fig. 1D), which is generally considered unqualified. These results indicate that storing blood at room temperature for >6 h affects RNA integrity without influencing the RNA yield or purity.
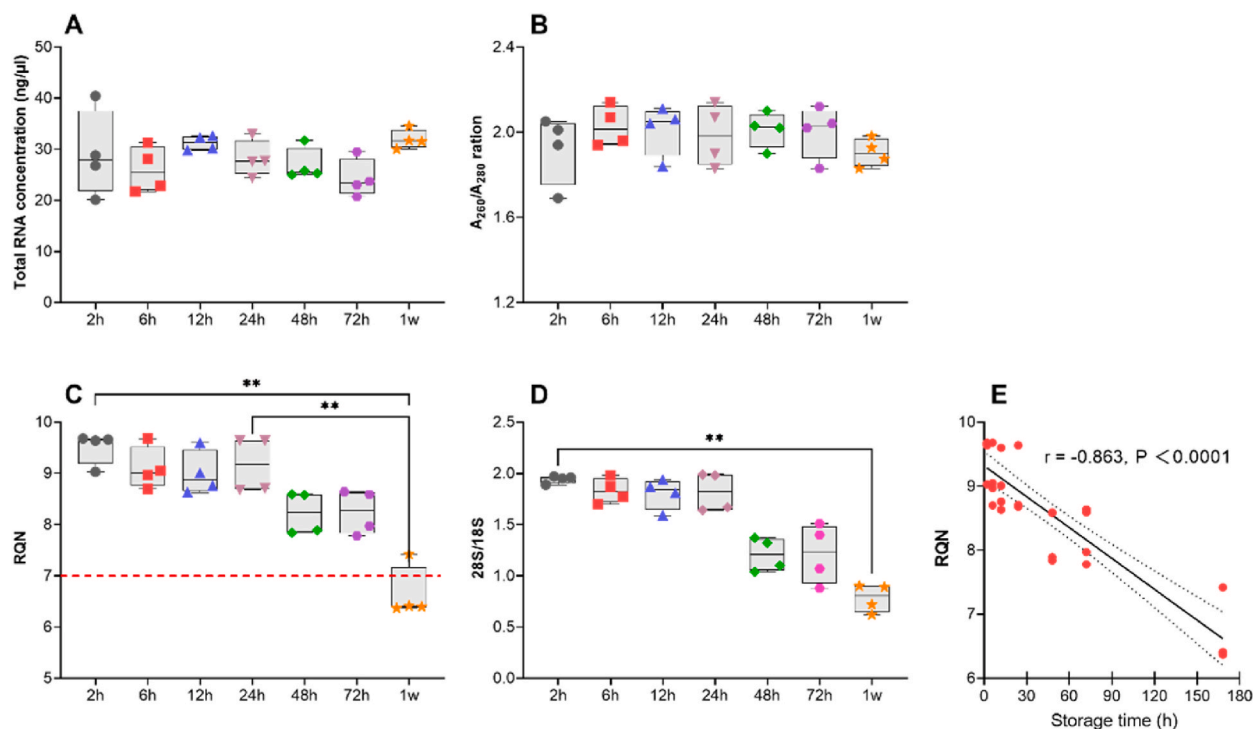
3.2. RNA quality of blood samples stored for different durations at 4 °C

The parameters related to the quality of RNA extracted from blood samples from pediatric patients (n = 4) stored at 4 °C for different periods of time are shown in Fig. 2. RNA concentrations ranged from 24.23 to 31.92 ng/μL and changed irregularly over time (Fig. 2A). The A260/A280 ratio changed slightly from 1.90 to 2.03 (Fig. 2B). No significant difference in concentration or A260/A280 were observed in any of the three groups. The RQN ranged from 6.65 to 9.50. The RNA integrity of blood stored at 4 °C for 48 h was significantly different from that for 2 h (P = 0.042), but both were within the normal range (Fig. 2C). The result of one-sample one-tailed *t*-test shows that the RNA RQN of blood stored at 4 °C for 72 h was significantly greater than RQN score cutoff of 7 (P = 0.0053). In addition, the RQN at 1 week was <7, which is the common quality criterion for RNA samples undergoing microarray analysis [9], and significantly different from that at 2 h (P = 0.001). Importantly, Spearman correlation analysis indicated a strong negative correlation between RQN and the storage time of blood samples stored at 4 °C (r = −0.863, P < 0.0001; Fig. 2E). Further, the 28S/18S ratio at 1 week was <1, and significantly different from that at 2 h (P = 0.001; Fig. 2D), which reflects the same trend. Based on these findings, blood RNA is best maintained when stored at 4 °C for 72 h.

3.3. Effect of hemolysis on RNA quality depends on the hemolysis method

Initially, we used the freeze-thaw method to prepare the hemolyzed samples [12], with the results depicting a severe reduction in RNA integrity. The RNA electrophoresis analysis results showed that 28S area and 18S area of FT-WB and RBC + FT-WBC group were almost completely degraded (Fig. 4B, D) compared with that of WB and WBC + FT-RBC group (Fig. 4A, C). There was only a slight change in concentration after freezing and thawing (Fig. 3A). The A260/A280 ratio of the RNA changed slightly from 1.84 to 1.95 (Fig. 3B). The RQN ranged from 4.55 to 8.42; the RQN after freezing and thawing was significantly different from the control (P = 0.005; Fig. 3C). The 28S/18S ratio after freezing and thawing also differed significantly from the control (P = 0.0001), whereas the 28S/18S ratio after freezing and thawing was <1.0 (Fig. 3D), which is generally considered unqualified.

However, different results were obtained from hemolyzed samples collected from the laboratory department. There were no

Fig. 2. (A) RNA concentration; (B) A260/A280; (C) RQN; and (D) 28S/18S ratio of blood samples from pediatric patients (n = 4) stored at 4 °C for different durations. (E) Spearman correlation analysis between RQN with storage time of blood samples stored at 4 °C. * Denotes statistical significance according to the Kruskal-Wallis test or one-way ANOVA test, followed by Tukey's multiple comparison test, Duncan's multiple range test or Games–Howell test. ANOVA, analysis of variance; RQN, RNA quality number.
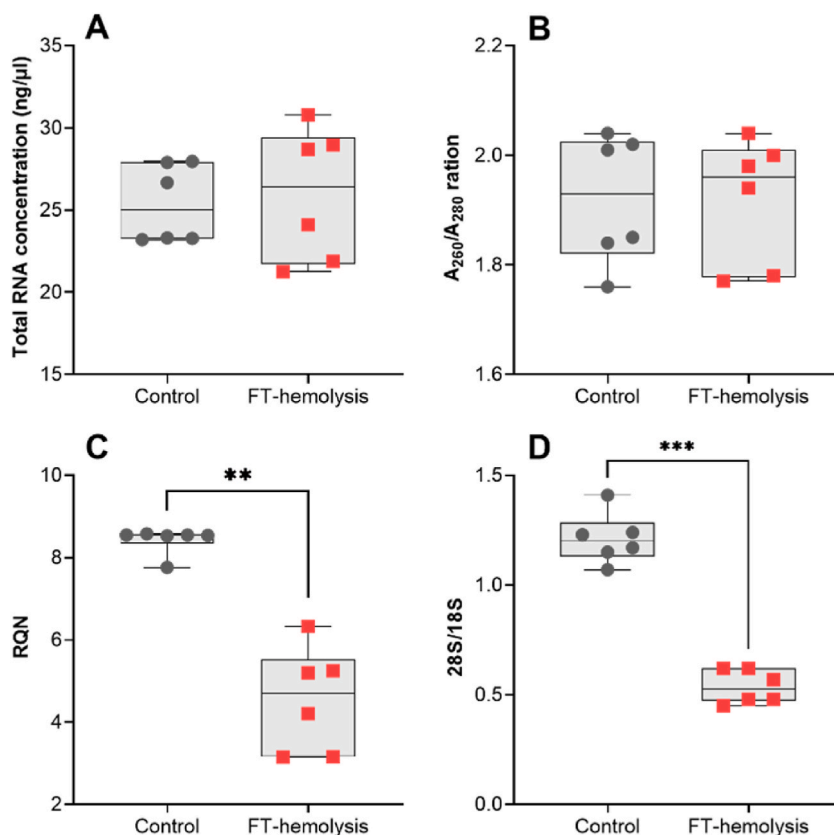
Fig. 3. (A) RNA concentration; (B) A260/A280; (C) RQN; and (D) 28S/18S ratio of RNA extracted from hemolyzed blood from adult volunteers (n = 6) after undergoing freeze-thawing. * Denotes statistical significance according to the Mann–Whitney *U* test or a *t*-test. RQN, RNA quality number.

significant differences in RQN between the hemolyzed samples and the controls, accompanied by a significant decrease in 28S/18S (P = 0.007) (Fig. 5A and B). The hemoglobin concentration of clinical hemolyzed samples (759.71 mg/L) was significantly different from that of normal controls (144.90 mg/L) (Fig. 5C), and the difference was statistically significant (P = 0.002). Following this, different combinations of hemolyzed blood components were compared. The results show that the RQN of the RBC + FT-WBC group was significantly different from that of the WBC + FT-RBC group (P = 0.013; Fig. 6A). Moreover, the 28S/18S of the RBC + FT-WBC group is far less than 1, and statistically different from any of the other three groups (Fig. 6B). A blood smear of the frozen-thawed whole blood showed that the leukocytes were shriveled but not ruptured (Fig. 7A and B), and the average diameter of lobulated cells decreased from 13.68 μm to 9.25 μm after freezing and thawing (Fig. 7C), which was statistically significant (P = 0.001). The results confirmed the previous hypothesis, that routine (in vitro) hemolysis of samples from the laboratory department does not interfere with RNA quality. However, if hemolysis destroys leukocytes as well as RBCs, for example, during the freeze-thawing process, then the RNA quality will be affected.

### 3.4. RNA quality of surplus blood specimens from the laboratory department

Thirty of the blood samples were surplus specimens from the laboratory department. We tested their RNA quality to assess the qualification of these samples. The results show that the unqualified rate of the RQN value (<7) and 28S/18S ratio (<1) were 33.33% and 23.33%, respectively (Fig. 8A and B). Therefore, it is important to record the storage temperature, duration, and status of the specimen (whether it is hemolyzed) when using surplus specimens from the laboratory department for scientific research or storage in biobanks.

### 3.5. Effect of storage temperature, duration, and hemolysis on gene expression levels of whole blood RNA

The gene expression level is an important aspect of RNA quality. To determine the effect of storage temperature, durations, and hemolysis of blood sample on gene expression levels of whole blood RNA, five genes (*18S, ACTB, HIF1α, HMOX1*, and *MKI67*) were selected to analyze gene expression of whole blood RNA. *18S* is a common internal control genes for protein-coding gene; *ACTB* (beta cytoskeletal actin) is a commonly used housekeeping genes; *HIF1α* and *HMOX1* are hypoxia-regulated genes; and *MKI67* is a marker of proliferation Ki-67. Fig. 9 shows the relative mRNA levels of the five specific genes, classified by different preservation conditions. As
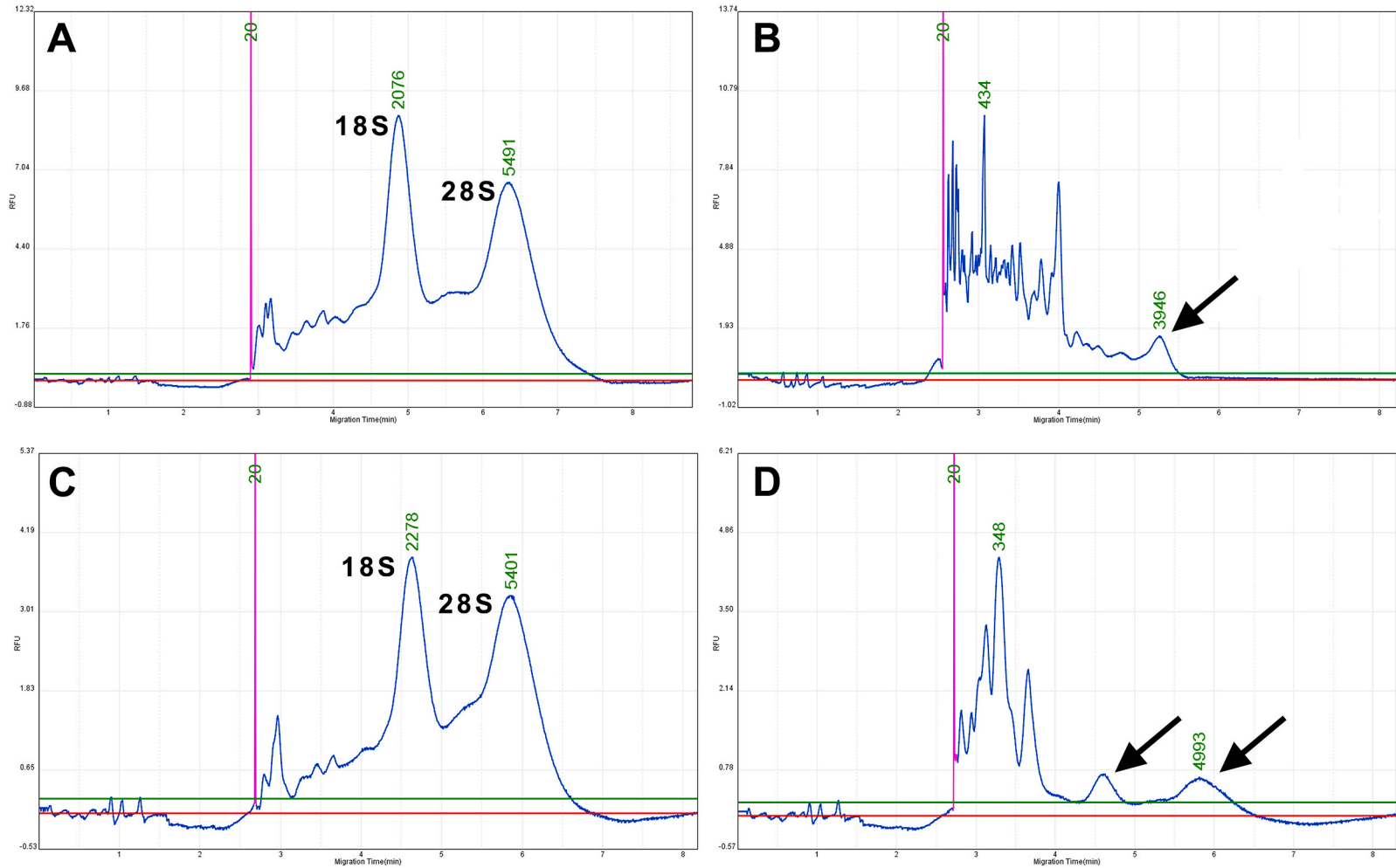
Fig. 4. RNA electrophoresis analysis of freeze-thawing hemolysis samples by Qsep100 system. (A) WB group; (B) FT-WB group; (C) WBC + FT-RBC group; (D) RBC + FT-WBC group. As shown by arrows in figure B and D, 28S area and 18S area were almost completely degraded.
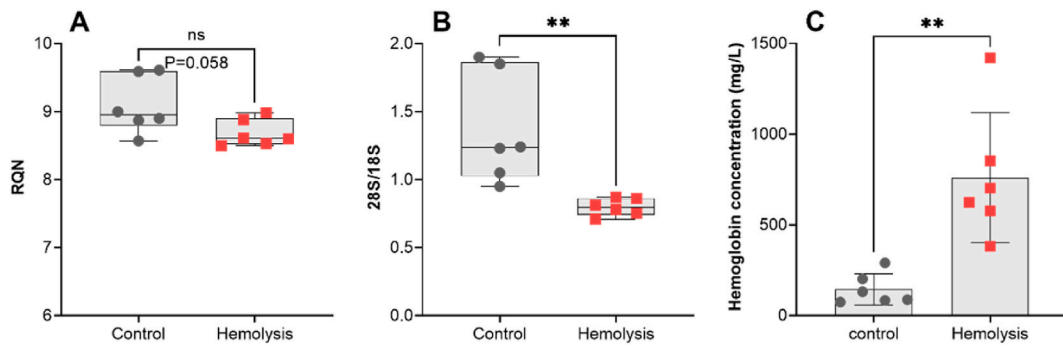
Fig. 5. (A) RQN; and (B) 28S/18S ratio of RNA extracted from routinely (in vitro) hemolyzed samples (n = 6) from the laboratory department. (C) Hemoglobin concentration of normal blood samples (n = 6), and routinely (in vitro) hemolyzed samples (n = 6) from the laboratory department. * Denotes statistical significance according to a *t*-test. RQN, RNA quality number.
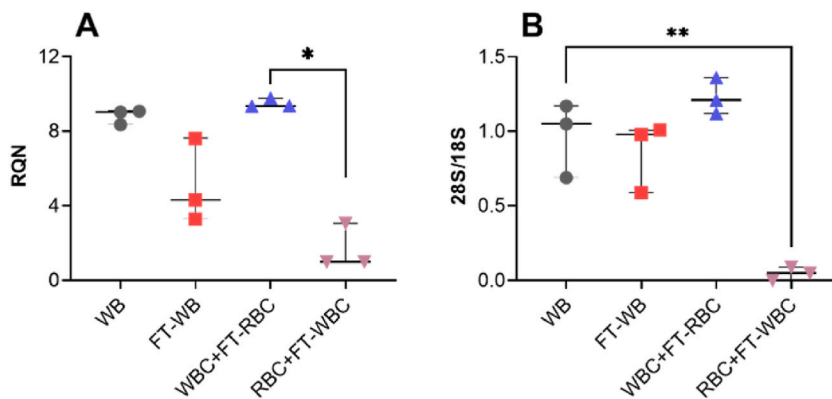

Fig. 6. (A) RQN; and (B) 28S/18S ratio of RNA in different combinations of hemolyzed blood components (n = 3) from laboratory department. WB: normal whole blood samples; FT-WB: frozen at −80 °C for 30 min, thawed for 30 min at room temperature; RBC + FT-WBC: red blood cells mixed with frozen and thawed white blood cells; WBC + FT-RBC: white blood cells mixed with frozen and thawed red blood cells. * Denotes statistical significance according to Kruskal-Wallis test or one-way ANOVA test, followed by Tukey's multiple comparison test or Duncan's multiple range test. ANOVA, analysis of variance; RQN, RNA quality number.
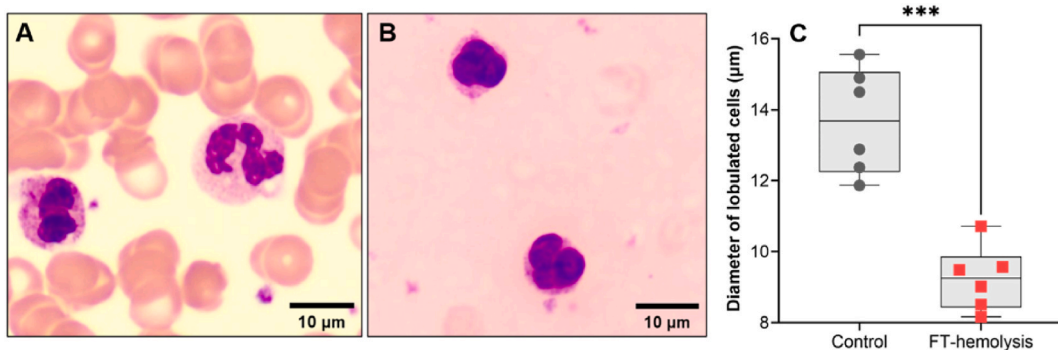
Fig. 7. Blood smears from normal blood samples and frozen-thawed blood samples. (A) Normal blood smear shows normal erythrocyte and lymphocyte morphology; (B) frozen-thawed blood smear shows that red blood cells were completely lysed, while lymphocytes were shriveled and shrunken. (C) diameter of lobulated cells from normal blood samples (n = 6) and frozen-thawed blood samples (n = 6). * Denotes statistical significance according to *t*-test.

shown in Fig. 9 A-C, there was no significant difference in the expression of *18S* (Ct = 8.24 ± 0.43) under the three conditions except under storage at 4 °C for 6 h and 12 h. The expression of *ACTB* (Ct = 18.24 ± 0.74) decreased significantly after storage at room temperature for 24 h and freezing (Fig. 9D, F), while the expression did not change after storage at 4 °C for 1 week (Fig. 9E). There was
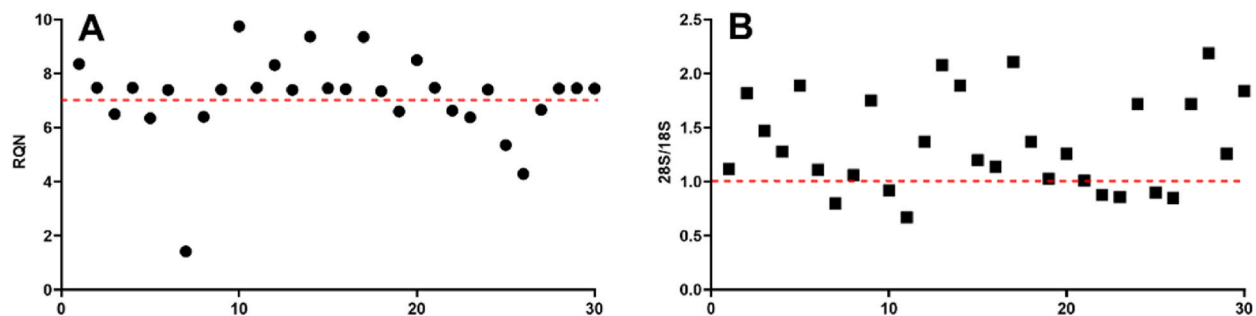
Fig. 8. (A) RQN; and (B) 28S/18S ratio of the RNA of surplus blood samples from the laboratory department. The RNA is of unstable quality, with no storage time or temperature records.

a significant decline in *HIF1α* (Ct = 23.76 ± 1.09) expression after freeze-thaw (Fig. 9I) and storage at room temperature for 6 h (Fig. 9G) or at 4 °C for 12 h (Fig. 9H), which recovered with an increase in storage time. The expression of *HMOX1* (Ct = 29.18 ± 1.03) did not change significantly after storage at 4 °C for 1 week (Fig. 9K) or after freeze-thaw (Fig. 9L), but it began to decrease significantly after storage at room temperature for 6 h (Fig. 9J). For *MKI67*, storage at 4 °C and cryopreservation resulted in a decreased expression tendency (Fig. 9N and O). In contrast, the expression of *MKI67* (Ct = 25.98 ± 0.84) at room temperature was increased at 2 h, which then returned to the initial level (Fig. 9M).

4. Discussion

Blood and tissue are the main materials used for clinical testing and scientific research in biobanks. Furthermore, blood RNA is the most commonly used material for studying gene expression and translation. Therefore, RNA quality is of great significance for ensuring the accuracy of scientific research. Due to its single-stranded nature, RNA is extremely easy to degrade, and many factors may affect its quality in blood. In 2009, ISBER developed a SPREC that identified the main preanalytical factors of clinical fluids and solid bio-specimens [6], as well as their simple derivatives. Using the instructions from SPREC along with standard biobank construction, we carried out a quantitative assessment of the influence of storage temperature, storage time, and hemolysis on blood RNA quality.

The quality parameters of RNA samples include concentration, purity, integrity, and RT-PCR amplification rate. RQN is one of the most important criteria for evaluating RNA quality. The correlation between RQN values and downstream experiments, such as next generation sequencing (NGS), microarrays, and RT-PCR, is prominent [13]. The expected RQN values of samples used in NGS and microarray analysis are ≥8 and ≥ 7, respectively [9,14]. RQN is also reported to have a relatively significant effect on quantitative analysis of gene expression, mainly affecting the cycle threshold (Ct) value, and having a small effect on PCR efficiency [15].

4.1. Storage temperature and time

Huang et al. [9] observed a considerable decline in RNA quality with a storage duration longer than 24 h. Song et al. [16] stored rabbit blood at 4 °C for different preservation durations. They found that the purity of blood RNA did not significantly change for different preservation durations (from 1 h to 7 days), and that the integrity significantly decreased after rabbit blood had been stored at 4 °C for over 3 days. Moreover, expression of *PCNA* of whole blood RNA is significantly decreased over 24 h. The results of Sarathkumara et al. [17] showed that RIN values declined over time and with temperature irrespective of the whole-blood collection tube.

Similar to previous research, our study found that none of the three factors (storage temperature, storage time, or hemolysis) affected the concentration or purity of RNA over the time frame of the study. Concentration and purity are usually affected by sample handling and extraction methods [18,19], as other substances may be introduced, or leukocyte numbers may be decreased in the process.

In this study, the RNA quality results revealed that in blood samples stored at 4 °C for 1 week or at room temperature for 24 h, the concentration and purity of RNA were not significantly different from those at 0 h or 2 h, indicating that the RNA concentration and purity is not affected in these conditions. The RQN value stored at 4 °C for 1 week < 7, and significantly different from that at 2 h, whereas the RQN value at 72 h was significantly greater than 7. The RQN value of blood samples stored at room temperature for 6 h was significantly different from that at 2 h and 0 h. Therefore, we recommend that blood samples be stored at 4 °C and room temperature for no more than 72 h and 2 h, respectively. The differences of recommended storage time in this study from the results of previous research may be caused by the environmental conditions in each laboratory; however, the general trends are similar. With increasing storage time, blood sample RNA content decreases due to the instability of its chemical structure and the enzymatic hydrolysis of endogenous and exogenous RNA enzymes.

4.2. Surplus specimens

Some researchers collect surplus blood samples from clinical laboratories. Therefore, we gathered 30 surplus blood samples from the laboratory department to assess their RNA quality without any records of their storage time or temperature. Approximately a third
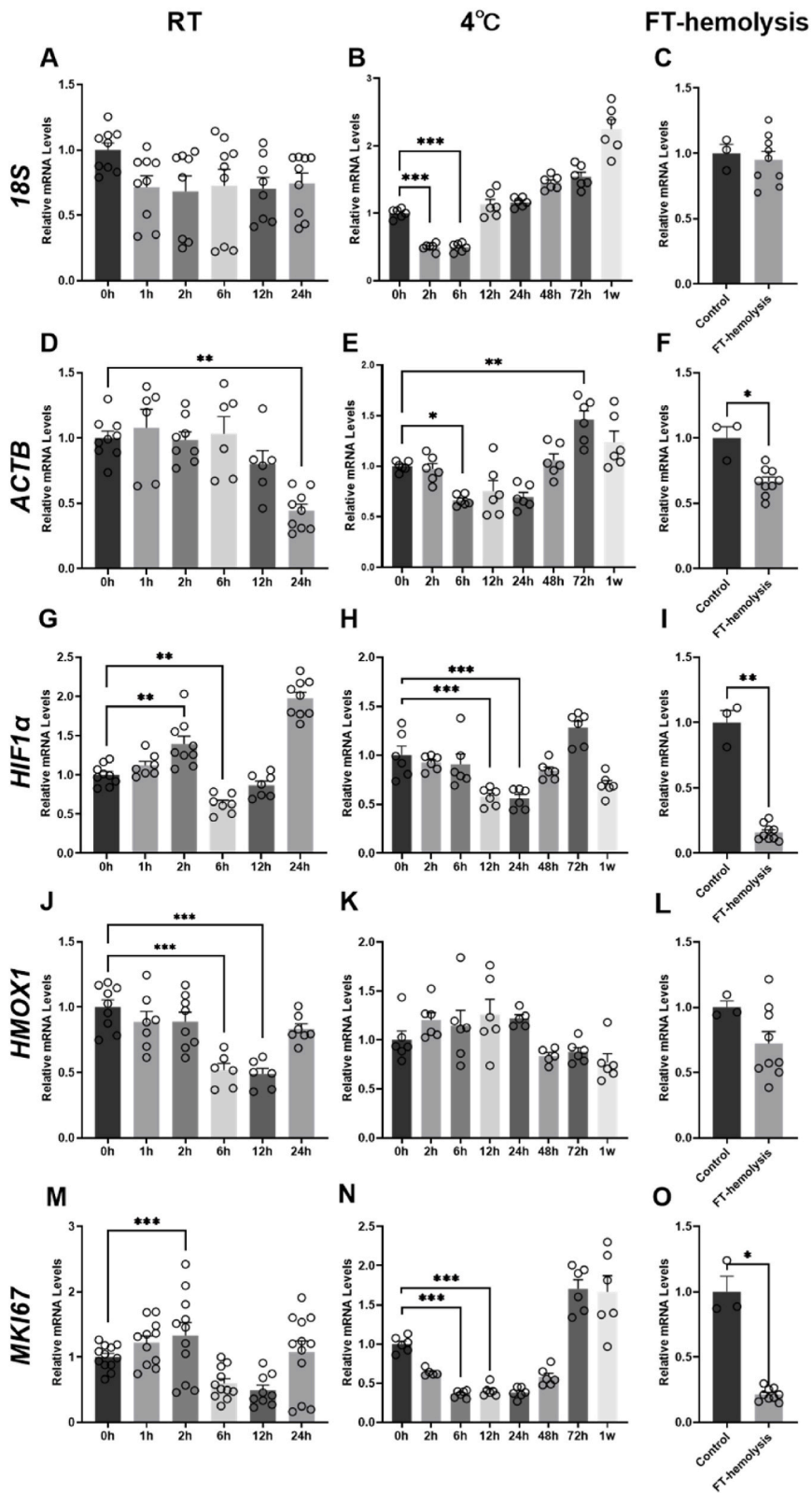
(caption on next page)

Fig. 9. Gene expression levels under different blood samples from adult volunteers (n = 6) preservation conditions. (A–C) The relative expression levels of *18S* gene of whole blood preserved at room temperature, 4 °C, and freezing-thawing condition, respectively. (D–F) The relative expression levels of *ACTB* gene of whole blood preserved at room temperature, 4 °C, and freezing-thawing condition, respectively. (G–I) The relative expression levels of *HIF1α* gene of whole blood preserved at room temperature, 4 °C, and freezing-thawing condition, respectively. (J–L) The relative expression levels of *HMOX1* gene of whole blood preserved at room temperature, 4 °C, and freezing-thawing condition, respectively. (M − O) The relative expression levels of *MKI67* gene of whole blood preserved at room temperature, 4 °C, and freezing-thawing condition, respectively. * Denotes statistical significance. Results at 4 °C and room temperature were analyzed according to Kruskal-Wallis test or one-way ANOVA test, followed by Tukey's multiple comparison test or Duncan's multiple range test, while results at freezing-thawing condition were analyzed according to independent-samples *t*-test, Mann-Whitney *U* test, or Welch t' test.

of the samples did not meet the requirements for RQN or 28S/18S ratio. Hence, it is not recommended to use surplus specimens from clinical laboratories, unless the storage time and temperature are recorded and fall within the reference ranges.

4.3. Hemolyzed specimen

One of the seven factors of the SPREC is sample type or sample status. Some samples used in this study had undergone hemolysis. As there are few studies on the effect of hemolysis on total RNA quality, we investigated this phenomenon. Even in the absence of a nucleus, RBCs contain approximately 600 RNA molecules and some microRNA. Some studies have analyzed the effect of hemolysis on microRNAs [20]. For example, Shkurnikov et al. [21] found that hemolysis of 0.05% of the RBCs in a sample significantly affected the concentration of 9 micro-RNA.

As clinical hemolyzed samples are difficult to obtain, we initially prepared hemolyzed samples using the freeze-thaw method [22], resulting in very poor RNA quality. We hypothesize that the freeze-thaw process not only induced hemolysis but also the destruction of leukocytes, which almost entirely degraded the RNA of the specimens. This is consistent with previous research [22,23]. We then collected hemolyzed samples from the laboratory department, from which we isolated the WBCs and RBCs, which were then frozen and thawed separately. As a result, the RNA quality of clinical hemolyzed specimens was almost entirely unaffected. After freezing and thawing RBCs, adding WBCs did not affect RNA quality. RNA was almost completely degraded in frozen-thawed WBCs, in the same manner as in whole blood samples. Therefore, it can be concluded that for hemolyzed specimens, the method of hemolysis has no effect on total RNA if it does not affect leukocytes. Kalaria et al. [24] also found that different hemolysis research protocols had different effects on a given parameter. They found conflicting effects of hemolysis on plasma sodium and chloride levels due to different hemolysis protocols. Therefore, when studying hemolytic interference, the hemolysis method is crucial.

4.4. Gene expression

Gene expression is an aspect of RNA quality because RQN value is mainly used to evaluate rRNA integrity and does not represent a specific quality of mRNA. mRNA quality can be evaluated by gene expression analysis. Several studies have compared the effect of blood collection tubes and blood collection methods on gene expression profiles [25–29]. Although RNA stabilizer containing tubes can represent a reliable option to avoid ex vivo RNA changes, traditional EDTA tubes are still widely used since they are less expensive and enable to study specific leukocyte populations [30]. In this study, we analyzed the expression profile of five specific genes, to observe the variation of whole blood RNA in response to different preservation conditions. Previous research has shown that pre-analytical variables can affect analytical RNA procedures, such as RT-qPCR [31]. In this research, the gene expression of *18S* remained relatively stable, which is supported by a previous study [32]. The expression of *ATCB* significantly decreased upon storage at room temperature for 24 h or freeze-thaw. Therefore, *ATCB* should not be preserved at room temperature for more than 24 h nor freeze-thawed when selected as a housekeeping gene. With respect to the other genes, the expression levels decreased irregularly during the storage period, which indicates that the storage conditions of blood samples affect the expression of multiple genes. Moreover, most of the changes began at 6 h. Therefore, it is recommended to use blood samples stored for no more than 6 h when expression of *HIF1α*, *HMOX1*, and *MKI67* needs to be examined, and the samples should be protected against freezing and thawing.

5. Limitations

Owing to the limitations of a small sample size, the number of samples in each group was inconsistent. The sample included both children and adult volunteers, they might be influenced by the RNA quality or inherent cellular physiological biological processes rather than reflecting pre-analytical differences. But Shen Y et al. showed that the influence of inherent cellular physiological processes on RNA-seq output could be negligible [33]. In addition, the 30 surplus samples selected in the laboratory department may not have accurately reflected the real conditions of whole blood samples. If the sample size is expanded and the collection range is widened, this would be a more accurate depiction of the real conditions involving clinical sample collection. In terms of gene expression analysis, *GAPDH* is not a stable gene under the influence of the pre-analytics, it seems like that *HIF1α* gene recovered with an increase in storage time, it's maybe not a recovering, but also degradation of the *GAPDH* reference gene, which evens out the ratio. Moreover, only three functional gene expression levels were analyzed, which could not adequately reflect the effects of different preservation conditions on the expression of a wide range of genes. Previous research indicates that RNA degradation and cellular biological processes seemed to have a significant influence only on a fairly small number of genes (27/37,000 genes) but not on the global gene expression profiles [33].

6. In conclusion

We quantitatively assessed the influence of storage temperature, storage time, and freeze-thaw hemolysis on the RNA quality of blood biospecimens in EDTA tubes. These three factors are the elements of SPREC that biobanks cannot conveniently control. Furthermore, we examined different storage methods for blood samples after centrifugation to establish a set of recommendations based on SPREC concerning collection, pre-processing procedures, and storage conditions that can guide and serve for the standardization of protocols and reduction of undesirable variation.

Ethics statement

This study was approved by the Ethics Committee of Jiangxi Provincial Children's Hospital (approval number: JXSETYY-YXKY-20220196).

Author contribution statement

Zhijun Jiang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yi Lu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Manying Shi: Conceived and designed the experiments; Performed the experiments.

Hong Li: Analyzed and interpreted the data.

Junkai Duan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Jiyi Huang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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