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Analysis of blood processing conditions to obtain high-quality total RNA from human leukocyte concentrate

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ABSTRACT. Blood samples are used as a biological source to discover biomarkers of hematological and non-hematological disorders. The present study shows the impact of different experimental conditions associated with cell lysis buffer, TRI-reagent protocol and blood cell storage buffer and their correlation with the quantity, quality and *Adrenomedullin* gene expression levels of total RNA when RT-PCR technique is used. A leukocyte cell bank protocol is also proposed for further mRNA expression analysis using RNAlater as storage buffer. There is evidence that total RNA isolated from leukocyte concentrate stored for 1 month at -70°C did not show significant differences concerning quality, purity and *Adrenomedullin* gene expression compared with the freshly processed leukocyte sample.

Key words: Leukocyte, RNA, RT-PCR, *Adrenomedullin*

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INTRODUCTION

Peripheral blood has been regarded as an attractive tissue for biomedical and clinical research due to its rich genetic information and the feasibility of sample collection. It plays a crucial role in immune-related diseases, including infection, cancer and metabolism in humans and animals (Kephart and Shenoi, 1998). Blood is used in the diagnosis of hematological diseases, and it is also being explored to discover surrogate markers for a wide range of non-hematological disorders. Therefore, the transcriptome of blood cells may be used as a health sensor, the so-called "sentinel principle" (Liew, 2004; Marteau et al., 2005; Burczynski and Dorner, 2006).

Gene expression profile studies with whole blood RNA samples have been difficult due to the heterogeneous cellular nature of blood and the high-globin mRNA concentration (Ambion, Inc., has estimated that globin transcripts represent as much as 70% of the total mRNA population). Many different techniques are used to separate fractions of blood cells prior to RNA isolation, but the impact of these different approaches on the whole genome is still being studied (GeneChip®).

RNA extraction methods from blood cells must be effective and efficient in order to guarantee high quality of the purified RNA, which is required for many of the current quantitative gene expression studies using real-time polymerase chain reaction (RT-PCR) or microarrays. RNA quality ensures accuracy and sensitivity in these techniques, which are a critical parameter for the interpretation of results.

The present report shows the impact of different experimental conditions associated with cell lysis buffer, TRI-reagent protocol and blood cell storage buffer as well as their correlation with the quantity, quality and *Adrenomedullin* (ADM) gene expression of total RNA when RT-PCR technique is used.

Here, a leukocyte bank protocol is proposed for future mRNA expression analysis. This protocol describes procedures for isolating RNA from leukocyte populations obtained from whole blood samples collected from human volunteers. The present results will help those who use gene expression techniques by providing an optimized protocol for obtaining total RNA of high integrity from leukocytes with the quality and quantity required.

Previous results have been influenced by other variables associated with individuals, such as gender, age, and health status (Fan and Hegde, 2005). In this study, samples from the same individual were used per protocol carried out by the same investigator, in order to minimize the effect of these variables.

MATERIAL AND METHODS

Erythrocyte lysis and isolation of leukocyte populations

After voluntary, written and informed consent, whole blood samples were taken by venipuncture and collected using EDTA tubes (EDTA, 1.5 mg/mL of blood). After gentle mixing, the whole blood was stored at 2-8°C for no more than 2 h. Leukocyte populations were obtained by standardized erythrocyte lysis for 5 min using an NH_4Cl lysis solution (containing 3.7 g disodium EDTA salt, 80.2 g NH_4Cl and 8.4 g $NaHCO_3$ per liter, and prepared with distilled H_2O treated with diethyl pyrocarbonate) (Klevezas et al., 2000) or

using a commercial cell lysis buffer (Promega Corporation, USA). Samples were centrifuged at 2500 rpm for 5 min. Lysis and centrifugation were repeated three times to obtain a blank pellet of cells.

RNA isolation from whole leukocytes

Leukocytes were processed immediately to obtain total RNA (time 0) or were stored at -70°C for 1 month in 1 mL RNAlater buffer (Ambion Inc., Austin, TX, USA) (Kraev et al., 2003) (time 1). The advantages and disadvantages of the RNAlater as storage buffer were evaluated in order to achieve an adequate equilibrium between RNA quality and gene expression during cell storage.

Cells were treated using TRI-reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, cells were lysed directly in the Eppendorf tube passing them several times through a pipette to form a homogenous lysate, and the samples were allowed to stand for 5 min at room temperature. Afterward, 0.2 mL chloroform per mL TRI-reagent was added and shaken vigorously for 15 s. Later, samples were left for 15 min at room temperature and were centrifuged at 12,000 rpm for 15 min at 4°C. The centrifugation separated the mixture into 3 phases; afterward, the aqueous phase was transferred to a fresh tube and then 0.5 mL isopropanol per mL TRI-reagent used was added. The sample was incubated for 8 min at room temperature and was then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed, adding 1 mL 75% ethanol per mL TRI-reagent, and then centrifuged at 12,000 rpm for 5 min at 4°C. The RNA pellet was air-dried for 5 min and an appropriate volume of water treated with diethyl pyrocarbonate was added.

Erythrocyte lysis, cell separation procedure and total RNA purification steps required at least 2 h for each sample processed (Table 1). The time needed for sample processing increased in accordance to the total number of samples processed at the same time.

Table 1. Time and equipment to obtain total RNA from peripheral blood.

Equipment for total blood processing during red cell lyses	Equipment for total RNA isolation from leukocyte pellets	Duration of the procedure of red cell lyses/number of processed samples	Duration of the procedure for total RNA isolation with TRI-reagent/number of processed samples	Total time delay
a) Centrifuge (Eppendorff 5804, USA) b) Shaker Heidolph, Reax 2, Schwabach, Germany)	a) Microcentrifuge (Eppendorf 5415, Hamburg, Germany) b) Vortex (Janke & Kunkel, Staufen, Germany)	≈40 min/1 sample	≈1 h, 20 min/1 sample	≈2 h
		≈1 h/6 samples	≈2 h/6 samples	≈3 h
		≈1 h, 10 min/ 14 samples	≈3 h/14 samples	≈4 h, 10 min

Four protocols for RNA isolation from leukocyte populations were tested. The conditions examined varied in the volume of blood per sample, in the TRI-reagent volume per volume of blood processed, and in the number of samples processed at the same time (Table 2).

Table 2. Experimental conditions.

Protocol	Blood volume per sample (mL)	TRI-reagent volume/ blood volume (mL)	Samples processed at the same time
1	10	0.1	14
2	5	0.6	14
3	1	0.6	6
4	3.2	1.25	6

RNA yield and quality were assessed by spectrophotometry at A260 and A280 nm and using agarose RNA electrophoresis. Absorbance 260/280 nm ratios of isolated RNA should be between 1.7 and 2.1 (Wilfinger et al., 1997). This means that RNA is free from most of the contaminating proteins, including RNases.

RT-PCR

Before RT-PCR technique, previously obtained whole RNA was treated with DNAase (Promega Biotech, Madison, WI, USA) as recommended by the supplier. RT-PCR assay was performed using a human 23-kDa highly basic protein as housekeeping gene (Zhumabayeva et al., 2004; Rogler et al., 2004; Mirza et al., 2005), and the ADM as sensor gene. ADM gene (a potent, long-lasting vasodilator peptide, originally isolated from human pheochromocytoma) was discovered in 1993. Its signaling has a particular significance in the biology of endothelial cells, protecting these from the apoptosis, promoting angiogenesis, and affecting vascular tone and permeability (Nagaya et al., 2005). It is known that this gene shows a rapid change in expression due to some technical influences such as temperature (Marteau et al., 2005).

cDNA synthesis was performed at 37°C for 60 min using 1 µg RNA, 0.5 µg hexamer primer, and 200 U Moloney murine leukemia virus (MoMLV) reverse transcriptase (Promega, USA). After reverse transcription, cDNA was amplified by a standard PCR protocol in the Mastercycler gradient instrument (Pharmacia, USA). The 30-µL PCR mixture for the 23-kDa highly basic protein and the ADM genes contained 1X PCR buffer, 4 mM $MgCl_2$, 5 µL template cDNA and 50 pM of each pair of oligonucleotides (Heber Biotec, Havana, Cuba). Oligonucleotide sequences were: 5'-TAAACAGGTACTGCTGGGCCGGAAGGTG-3' (forward primer) and 5'-CACGTTCTTCTCGGCCTGTTTCCGTAGC-3' (reverse primer) for 23KD gene (*X56932*), 5'-CTCTGAGTCGTGGGAAGAGG-3' (forward primer) and 5'-CGTGTGCTTGTGG CTTAGAA-3' (reverse primer) for ADM gene (*NM_001124*).

Cycling steps were an initial denaturation at 95°C for 5 min and 30 cycles for each of the following steps: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR products were analyzed by electrophoresis on 2% agarose gels to ensure amplification of the appropriate size fragment. Negative PCR control did not show detectable amplification products.

Statistical analysis

The assumption of normality was verified using the Shapiro-Wilk test, and Spearman's correlation coefficient was calculated as a measure of linear association between the total blood volume/sample and six variables included in the assay. The two-sample Student *t*-test was performed to compare ADM gene expression using two lysis buffers, and the RNA quality and stability to the different storage times were evaluated using the Wilcoxon signed-rank test. The level of significance selected was 0.05.

Image analysis

RNA and DNA image detection and quantification were obtained using UV light (Fisher Scientific, Leicestershire, UK) and digital camera (Kodak, Rochester, NY, USA). The optical density of the nucleic acid bands was quantified using the Image J 1.33µ software (Wayne Rasband, USA).

RESULTS

Quality, purity and ADM gene expression for the total RNA obtained from peripheral blood using two different cell lysis buffers (NH_4Cl lysis solution and a commercial cell lysis buffer) were compared in order to have a source of lysis buffer to use in gene expression studies. The results are presented in Table 3 and Figure 1. There were no significant differences between ADM gene expression with commercial buffer and NH_4Cl cell lysis solution (P = 0.213). Despite the fact that the two buffers showed significant differences according to quality and quantity of the total RNA isolated, these results are not biologically significant since they were within the appropriate range of A260/A280 and 28S/18S ratios and they had similar ADM gene levels.

Table 3. Total RNA quality and integrity using two lysis buffers.

Samples	A260/A280	rRNA 28S/18S ratio
NH_4Cl lysis solution (N = 6)	2.15 ± 0.34	1.27 ± 0.01
Commercial cell lysis buffer (N = 6)	1.77 ± 0.06	1.86 ± 0.47
Student *t*-test: two-sample assuming unequal variances	0.044	0.048

Data are reported as means ± SD.

Subsequently, total RNA extraction from concentrated leukocytes containing platelet cells, granulocytes (neutrophils and basophils) and mononuclear cells (lymphocytes and monocytes) was performed using NH_4Cl lysis solution. For this purpose, four RNA extraction protocols were tested using different conditions according to various amounts of blood per sample and different TRI-reagent volumes per volume of blood processed. In each protocol, different numbers of samples were tested by the same person simultaneously. The Spearman correlation test demonstrated a positive correlation among most of the variables examined (total RNA

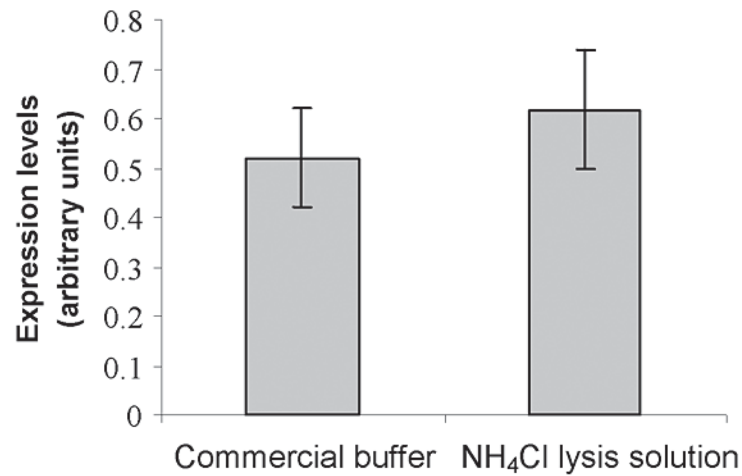

Figure 1. *Adrenomedullin* gene expression in total RNA isolated using NH_4Cl lysis solution or commercial lysis buffer. N = 12 (6 samples per group); P = 0.213.

yield/sample, A260/A280 nm ratio, time delay, number of samples processed at the same time, and optical density of 28S/18S ribosomal RNA bands (rRNA)), when total blood volume per sample was considered as the independent variable (Figure 2, Table 4).

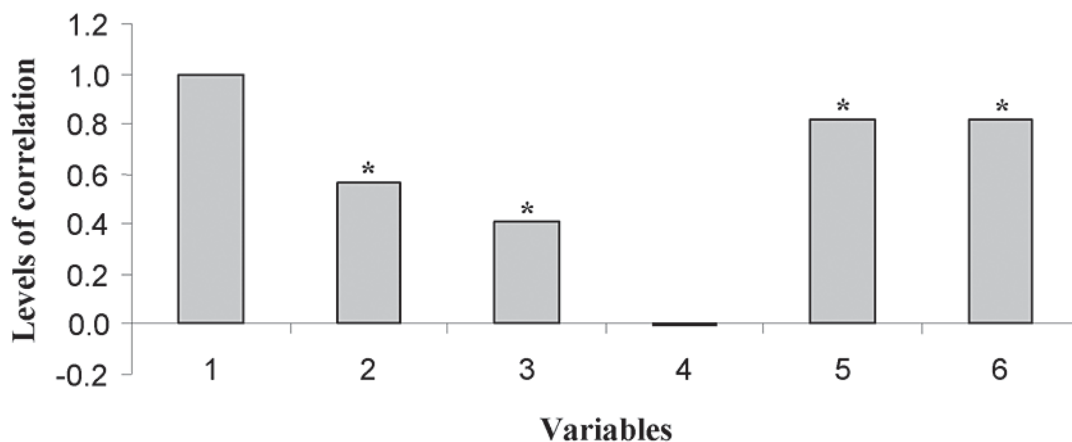

Figure 2. Spearman rank order correlations (r) between six variables included in the experimental protocols. Considering total blood volume per sample (number 1) as independent variable, a positive correlation for dependent variables studied was observed. Total RNA yield/sample (r = 0.56) (number 2), relation A260/A280 nm (r = 0.40) (number 3), time delay (r = 0.82) (number 5), number of processed samples at the same time (r = 0.82) (number 6). Optical density 28S/18S ribosomal RNA bands (r = -0.004) (number 4) showed a negative correlation with the independent variable. *P ≤ 0.05.

Table 4. Quantitative results of the variables evaluated in each protocol.

Protocol	Total RNA yield (µg)	A260/A280	rRNA 28S/18 ratio
1	31.36 ± 12.75	4.83 ± 4.78	1.208
2	11.13 ± 8.72	1.89 ± 0.07	0 (RNA degraded)
3	7.17 ± 2.86	2.15 ± 0.30	1.276
4	19.65 ± 3.01	2.01 ± 0.03	1.218

Data are reported as means ± SD.

To test the effect of temperature and time delay on RNA quality and ADM gene expression levels, leukocyte populations were stored at -70°C for a month. Table 5 and Figure 3 show the quantity and quality of total RNA and the ADM gene expression levels, respectively, in samples processed immediately (time 0) and one month later (time 1). Similar results were found for both conditions. Only the RNA yield of samples processed at time 1 was significantly higher.

Table 5. Quality, quantity and integrity of total RNA obtained from two times of conservation of leukocyte concentrate.

Samples (N = 5)	A260/A280	Total RNA yield	rRNA 28S/18S ratio
Time 0	2.15 ± 0.33	7.16 ± 2.86	1.27 ± 0.21
Time 1	1.66 ± 0.06	11.52 ± 6.22	1.86 ± 0.09
Wilcoxon (paired test)	0.345	0.0046	0.345

Data are reported as means ± SD.

DISCUSSION

Inappropriate processing, handling and storage of blood samples are common problems that cause undesired results in nucleic acid isolation and its subsequent analysis. RNA is particularly labile in the complex mixture of cells and proteins present in blood samples, and several considerations are critical for RNA isolation. Avoiding RNA degradation by RNase activity in blood lysates is a difficult task, due to the higher RNase activity in blood compared to other tissues. Samples should be processed as soon as possible after blood collection. College of American Pathologists (http://www.cap.org) guidelines recommend storing blood samples at 4°C and processing them within 2 h after their extraction. Allowing blood samples to stand at room temperature before being used for RNA isolation, leads to diminished quality of the isolated nucleic acids (Kephart and Shenoi, 1998).

The evaluation of purity, quality and expression levels of sensor genes in total RNA samples is an indispensable tool for gene expression profile studies because it offers a higher confidence in the interpretation of results. In this case, an RT-PCR analysis showed that ADM gene expression levels in RNA obtained using two different lysis buffers were similar. In addi-

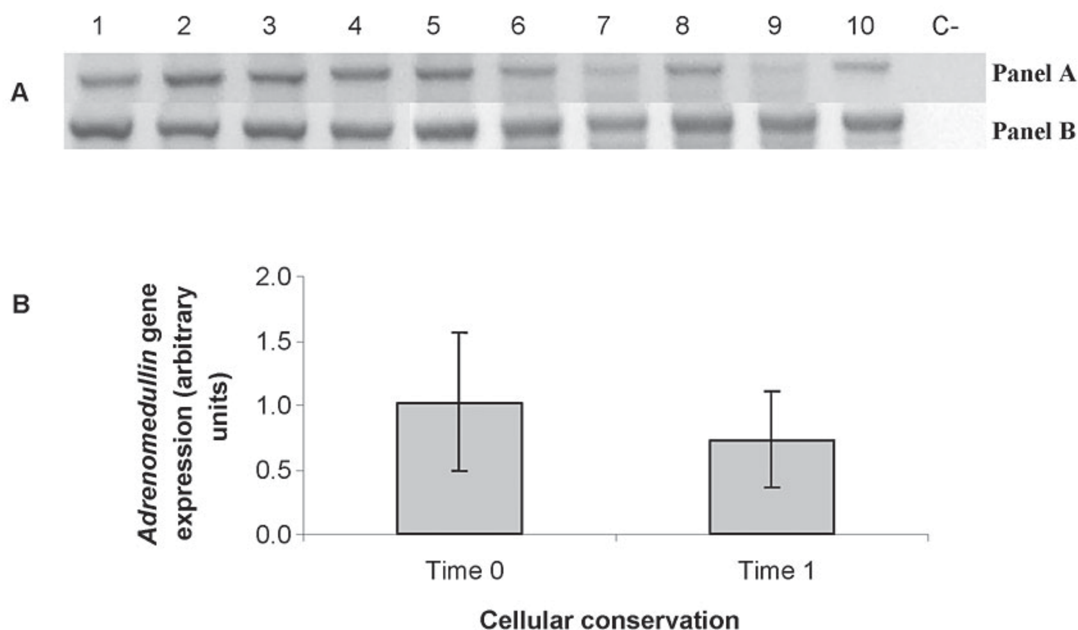

Figure 3. *Adrenomedullin* gene expression. **A.** RT-PCR results. *Panel A*: *Adrenomedullin* gene expression (422 bp). *Panel B*: 23KD gene expression (483 bp). *Lines 1-5*: samples obtained in time 0. *Lines 6-10*: samples obtained in time 1. C-: negative control. **B.** Quantification and normalization of the *Adrenomedullin* gene using the 23KD housekeeping gene. Wilcoxon matched pair test; N = 5; P = 0.50.

tion, similar results according to RNA quality and integrity were obtained. In this case, statistical significance was not in accordance with the biological interpretation, due to the mean values being within normal limits (rRNA samples with a 28S/18S ratio >1 indicate high-quality RNA) (Folgueira et al., 2006). These results demonstrate that NH_4Cl lysis solution for total RNA extraction could be used for gene expression studies.

On the other hand, different technical variables can produce alterations in the quantity, quality and purity of blood-purified RNA. The time delay between blood drawing and processing is one of these variables, because longer processing times and additional equipment may induce *ex-vivo* changes in the expression of certain transcripts (GeneChip®). Other variables are the amount of blood processed at the same time, and the proportion between blood volume processed and TRI-reagent.

The correlation study demonstrated when blood volume increases and it is processed by one person, the dependent variables increase significantly. That is the case of A260/A280 ratio, which should be within a recommended range, but it increased to very high levels (>2.1). An increment in RNA yields, in the number of samples processed, and in the time delay was also observed. However, rRNA 28S/18S ratio showed a tendency to decrease, which should be considered as an indication of total RNA degradation. With appropriate handling, reasonable amounts of high-quality total RNA were isolated using protocols 3 and 4. Additionally, the A260/A280 ratio (1.7-2.1) for RNA quality assessment offers an important criterion in RNA isolation.

In conclusion, a rapid and effective handling of blood samples is crucial for isolation of high-quality RNA, and the same person at the same time may process not more than 10 samples because it may affect the quality of RNA and gene expression (GeneChip®).

Sometimes, the immediate processing of blood samples may be inconvenient or impossible. However, several strategies can be used to improve RNA isolation (Kephart and Shenoi, 1998). Removal of red blood cells is not possible after freezing whole blood samples due to lysis of cells during the freezing process. Under our conditions, red cells were removed from the total blood and the concentrated leukocytes were stored in RNAlater buffer at -70°C for a month to stabilize blood cells prior to RNA extraction. In this sense, this study illustrates that the concentrate of leukocyte cells stored for a month at -70°C (time 1), gave similar results in quality and purity to the samples of RNA purified in time 0.

ADM gene expression showed a tendency to diminish, but the difference was not statistically significant when compared to the samples processed at time 0. The decrease was possibly due to the effect of temperature on cellular homeostasis during storage. For this reason, it is recommended to use other "sentinel" genes to examine the *ex vivo* effect on cellular gene expression in samples stored for months. Moreover, we recommend knowing the quantitative effect that blood sample manipulation will have on the cellular transcriptome.

CONCLUSIONS

The validation of blood samples for their use in differential gene expression studies should be rigorous. The inclusion of new techniques and variables to evaluate blood samples before their use in comparative gene expression studies must be useful to ensure the credibility of the results when sensitive, precise and expensive techniques such as RT-PCR and DNA microarrays are used.

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