Clin. Lab. 2017;63:XXX-XXX

SHORT COMMUNICATION

Human Peripheral Blood Cells mRNA Levels are Highly Sensitive to Duration of *Ex Vivo* Post-Sampling Conditions Prior to RNA Isolation

Eva Bohackova and Pavlina Dankova

Department of Anthropology and Human Genetics, Faculty of Science, Charles University, Prague, Czech Republic

SUMMARY

Background: **This study aimed to evaluate an effect of the time period between drawing the peripheral blood and specimen processing on the stability of mRNA levels of 7 selected genes.**
Methods: **Blood samples derived from 15 healthy volunteers were always processed at five consecutive time points 0.5, 1.5, 2, 3, and 9 hours; mRNA was quantified by real-time PCR.**
Results: **Anti-inflammatory genes *CCL2* and *IL10* showed a significant rise of expression between the 3rd and 9th hour after blood collection ($p \leq 0.5$). Significant decrease of mRNA levels in relation to time lag was observed for *TLR4* and *MYC* genes ($p \leq 0.5$). Interestingly, the initial two hours after drawing the blood revealed a high interindividual variability in cellular response to stress connected with blood drawing and *ex vivo* post-sampling condition.**
Conclusions: **These results point out the need for a strict standardization of handling the blood specimen with regards to peripheral blood sample processing time between phlebotomy and RNA isolation.**
(Clin. Lab. 2017;63:xx-xx. DOI: 10.7754/Clin.Lab.2017.170606)

Correspondence:
Dr. Pavlina Dankova, Ph.D.
Department of Anthropology and Human Genetics
Faculty of Science
Charles University
Vinicna 7
128 43 Prague 2
Czech Republic
Phone: +421 221-951-622
Email: pavlina.dankova@natur.cuni.cz

KEY WORDS

phlebotomy, post-sampling time, PBMC, mRNA, real-time PCR, BAX, BCL2, CCL2, IL10, MYC, TLR4, TNFα

INTRODUCTION

Blood is commonly used for diagnostic testing in clinical studies and as a source of biological material in biomedical research. Messenger RNA (mRNA) serves as a tool for detection of a real-time activity of cells. However, different modes of blood collection, sample preparation, and type of RNA isolation technique may alter gene expression and RNA stability and consequently affect resulting mRNA expression profiles. *Ex vivo*, peripheral blood cells may be affected by different cellular mechanisms such as metabolic stress due to the lack of glucose and oxygen during storage of blood after phlebotomy [1]. Several investigations have shown that the addition of anticoagulant (ethylenediaminetetraacetic acid, sodium citrate or heparin) to blood specimen affects viability of cells and causes various *ex vivo*

Short Communication accepted July 30, 2017

changes in cytokine production [2-4]. Further, it has also been suggested that the type of RNA isolation method used may influence gene expression and mRNA profile [5,6]. Contrary to expectations, no differences have been found in mRNA profiles between peripheral blood mononuclear cells (PBMC) obtained by Ficoll-Paque density gradient centrifugation and PBMC obtained from a BD Vacutainer cell preparation tube that enables direct separation of PBMC in the primary blood collection tube without need for any other subsequent separation steps [7].

Time ranging from the phlebotomy to RNA isolation represents another factor that can influence levels of detected mRNA [8]. Delays in RNA extraction from PBMC after blood collection can activate expression of some genes with subsequent upregulation of their mRNA, whereas expression of other genes may be suppressed and their mRNA downregulated. Further, time-dependent and gene-specific degradation of already existing mRNA molecules may occur. In order to avoid this happening, the special system was designed to prevent or mitigate changes of *ex vivo* mRNA levels due to stabilization of intracellular RNA [9]. This system allows inhibition of RNases, reduces RNA degradation, and inhibits or eliminates gene induction [10]. The drawback of such a system, however, is its inability to perform immunoseparation of a desired type of the cell in case one needs to assess the mRNA expression only from a specific cell type.

Changes in gene expression and RNA degradation in the time period between phlebotomy and RNA isolation could be minimized if the RNA is extracted immediately after blood drawing. Nevertheless, the immediate processing of blood may not always be feasible; this occurs when the blood sample is collected in a remote location from the laboratory [11] or, when buffy coats derived from blood donors registered at transfusion units serve as a source of biological material for research. Because of testing their blood for infectious disease and because of plasma separation, it is actually impossible to obtain buffy coat specimens from transfusion units for research purposes earlier than 9 hours after phlebotomy. For that reason, using blood from transfusion bags cannot be recommended for gene expression testing, as shown previously [4].

Handbooks or protocols recommend performing RNA extraction within 2 hours after phlebotomy because the first two hours after blood collection are generally considered to be safe with regards to possible post-sampling changes in mRNA profiles. However, the cellular processes occurring within the very first two hours after phlebotomy have not been monitored so far in details. For these reasons, we designed the experiment consisting of the set of five time intervals for RNA extraction 0.5, 1.5, 2, 3, and 9 hours after phlebotomy and determined the mRNA levels of 7 genes that were selected in order to represent specific molecular or metabolic processes within a cell.

MATERIALS AND METHODS

Subjects

Fifteen healthy adult individuals were recruited, and peripheral blood was collected into 9 mL Vacuette® Coagulation tubes (3.2% sodium citrate). Each specimen was aliquoted and stored under sterile conditions at room temperature (18 - 21°C) for different periods of time. Volunteers were without inflammatory or infectious illness during the 2 weeks prior to blood drawing and without suspected or diagnosed autoimmune disease, genetic disorder or other chronic illness. Informed written consent was obtained from all participants, and the ethical committee approved the study.

Methods

Total RNA from whole blood was isolated by BiOstic Blood Total RNA Isolation Kit Sample (MO BIO, USA) at five different time points 0.5, 1.5, 2, 3, and 9 hours after phlebotomy according to the manufacturer's instructions with final elution volume of 50 μL. Seventy-five samples of total RNA were obtained and reverse-transcribed into cDNA by the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). This cDNA was analyzed by real-time PCR with human *PGK1* as an endogenous control to monitor mRNA expression of IL10, CCL2, TLR4, TNFα, MYC, BAX, and BCL2 by using LightCycler® 480 Probes Master (Roche Applied Science, USA) and TaqMan® Gene Expression Assays (Life Technologies-Applied Biosystems, USA) on LightCycler® 480 (Roche Applied Science, USA). Each of the genes was selected to represent a specific cellular process. Relative mRNA quantitation was performed using the 2^-dCt method [12]. Intra-assay variation was assessed using relative amount of PGK1 and IL10 mRNA in four different runs at different time points.

Statistical analysis

GraphPad Prism 5.03 software was used for statistical analysis. To assay for normality, we performed D'Agostino-Pearson normality test, Kolmogorov-Smirnov test, and the Shapiro-Wilk test. The statistical difference between and among gene expressions across multiple time points was analyzed by the non-parametric Wilcoxon signed-rank test or parametric Student's *t*-test. A value $p \leq 0.05$ was considered statistically significant. Intra-assay variation of 0.6205 was determined according to method by Perini and colleagues [13].

RESULTS

To evaluate how the age of the blood specimen at the time of RNA isolation affects the expression levels of selected genes, blood from volunteers was always processed at five different time intervals. Genes with significantly decreased or increased levels of mRNA over time in a healthy-donor cohort are illustrated in Table 1.

Table 1. Time-dependent changes in relative mRNA levels of 6 selected genes reflect the effect of different time periods between phlebotomy and processing of the blood specimen until RNA isolation.

Gene	Compared different time points [hours]	Difference	p-value	Test
IL10	9 > 3	1.7 x	0.0009	Wilcoxon test
CCL2	0.5 > 3	1.9 x	0.0003	Wilcoxon test
	9 > 0.5	7.3 x	0.0004	Wilcoxon test
	9 > 1.5	9.7 x	0.0004	Wilcoxon test
	9 > 2	10.1 x	0.0001	Wilcoxon test
	9 > 3	14.4 x	< 0.0001	Wilcoxon test
TLR4	0.5 > 3	1.6 x	< 0.0001	Wilcoxon test
	0.5 > 9	3.5 x	< 0.0001	Wilcoxon test
	1.5 > 9	3.4 x	< 0.0001	Wilcoxon test
	2 > 9	3 x	< 0.0001	Wilcoxon test
	3 > 9	2.2 x	< 0.0001	Wilcoxon test
TNFα	0.5 > 2	1.6 x	0.0009	Wilcoxon test
	0.5 > 3	1.6 x	0.0043	Student *t*-test
MYC	0.5 > 9	2.1 x	0.0006	Wilcoxon test
	1.5 > 9	2.7 x	< 0.0001	Wilcoxon test
	2 > 9	1.7 x	< 0.0001	Student *t*-test
	3 > 9	2.1 x	< 0.0001	Wilcoxon test
BAX	1.5 > 9	1.5 x	0.0022	Student *t*-test

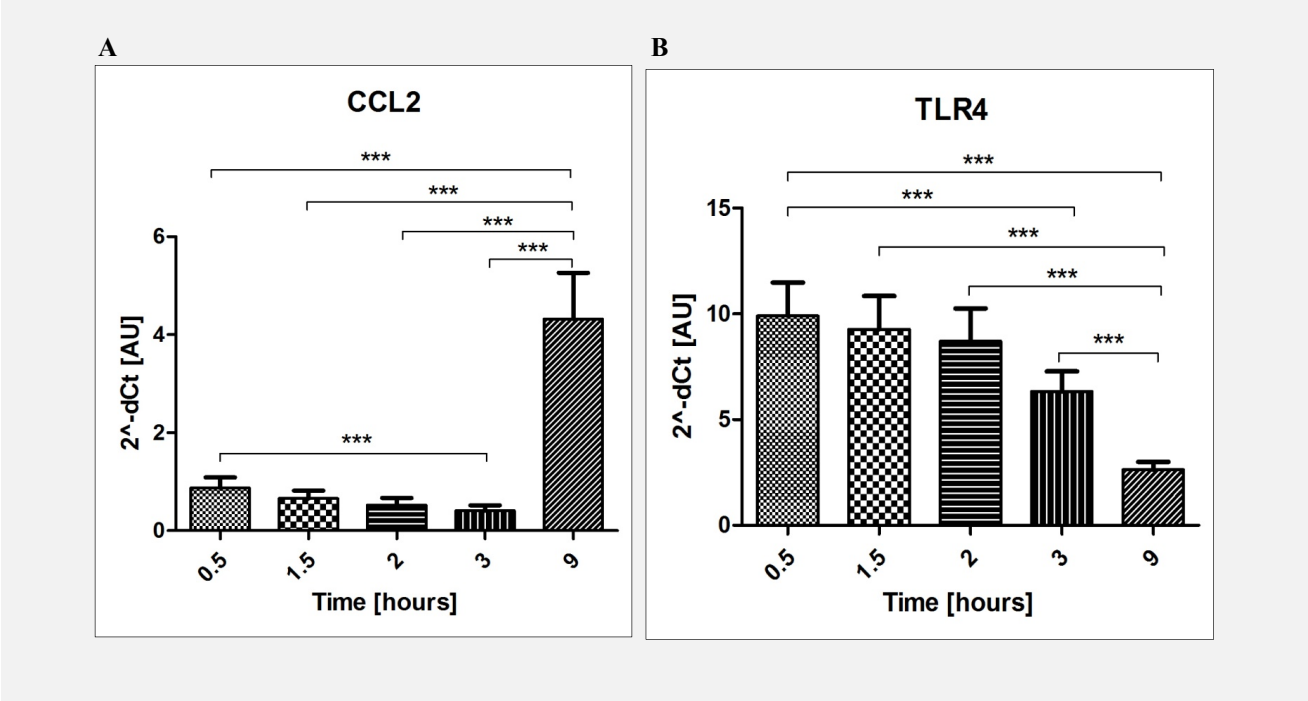

Figure 1. Effect of storage of blood samples on A) CCL2 and B) TLR4 mRNA levels.

A) Significant increase of CCL2 mRNA levels after 9 hours of blood storage and B) significant time-dependent gradual decrease of TLR4 mRNA levels expressed by 2^-dCt (AU = arbitrary unit; *** p < 0.001). The upper limit of the box indicates the mean; the upper error bar indicates standard error of mean. For better graphical representation, 2^-dCt values in CCL2 and TLR4 assays were multiplied by 10^3.

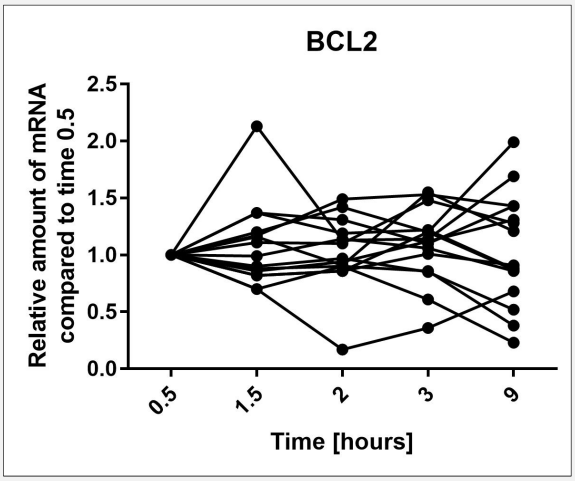

Figure 2. Specimen age-dependent changes of mRNA levels.

Considerably heterogeneous but statistically insignificant differences in BLC2 mRNA levels at different ages of specimens. Data are presented as a ratio to the mRNA amount at time 0.5 hour after phlebotomy.

DISCUSSION

Gene expression of PBMCs may be affected by anticoagulant agents presented in blood collection tubes [14], time, and temperature conditions applied during blood storage before RNA isolation [15], and overall specimen handling [16]. These changes are not well described. The presented study focused on the impact of time lag between the phlebotomy and process of RNA isolation. PBMCs are exposed *ex vivo* to a variety of extracellular agents, which influence their activation status and behavior. One may speculate that a change of the environment after phlebotomy and an exposure of cells to *ex vivo* conditions during and after blood collection are likely to affect gene expression [8] and, the longer the exposure, the greater effect.

RNA molecules are involved in the process of translation; their transport from the nucleus to the cytoplasm is crucial for gene expression [17]. During cellular stress, protein synthesis is severely reduced, mRNA is recruited to stress granules and its translation is inhibited [18]. RNA localized in different cellular compartments (nucleus, cytoplasm or stress granules) indicates different storylines of the cell; unfortunately, design of our study does not allow identification of these cellular processes. We found no statistically significant differences among the mRNA levels of IL10 in samples processed within first 3 hours of blood specimen storage at room temperature; further prolongation, however, slightly but significantly increased expression of the *IL10* gene in leukocytes. IL10, a key regulator of the immune system, is linked with anti-inflammatory reaction; its augmented expression, though, may be also responsible for a proinflammatory reaction during storage of blood sample [19].

A significant increase in CCL2 mRNA level between 3 and 9 hours after phlebotomy (Figure 1A) could be caused by strong gene expression of CCL2, suggesting an activation of signaling pathways leading to necrosis [20]. Lam and colleagues proposed that an increase in plasma cell-free DNA concentrations after 24-hour storage was probably a result of PBMC necrosis or apoptosis [21].

Time-dependent gradual reduction across all 5 time points in TLR4 (Figure 1B), across 3 time points (between 0.5 and 2 hours, followed by stable mRNA quantity) in TNFα, and overall decline with minor peaks at time points 1.5 and 3 hours in MYC mRNA levels might be explained by gradual selective degradation of some mRNA or by degradation after translation into the protein [22].

No significant differences over time were found in mRNA levels of the anti-apoptotic gene *BCL2*. Compared to that, mRNA levels of pro-apoptotic BAX slightly but significantly decreased from 1.5 up to 9 hours after blood collection. By detecting BAX and BCL2 mRNA, we noticed inter-individual variability in cellular response during blood storage (Figure 2); this can be attributed to the highly inter-individual immune response. Nevertheless, apoptosis-related signaling pathways other than those including BCL2/BAX may be employed as well. To clarify whether and how *BAX* and *BCL2* genes are involved in the apoptotic process during nine hours of blood storage, larger number of

samples need to be analyzed.
Messenger RNA levels of selected genes seem to be very unstable within the first three-hours of blood storage [23]. Cellular stress caused by phlebotomy, sample handling, storage and temperature conditions may dramatically affect levels of mRNA as these post-phlebotomy changes may lead to gene induction, gene silencing, cell death and/or enzymatic RNA degradation. All PBMC specimens intended for RNA extraction need to be processed at the same time and as soon as possible after phlebotomy; alternatively, where possible, specialized blood collection tubes that guarantee RNA stabilization within a cell need to be used [11]. It is therefore of critical importance to understand the amount of variability that can be introduced by using blood specimens of different age for gene expression analysis. It is crucial to make an effort to minimize this variability.

Acknowledgement:
This work was financial supported by SVV 260436/ 2017 and PROGRES Q43.

Declaration of Interest:
The authors declare that there is no conflict of interest.

References:

1. Härtel C, Bein G, Müller-Steinhardt M, Klüter H. Ex vivo induction of cytokine mRNA expression in human blood samples. J Immunol Methods 2001;249(1-2):63-71 (PMID: 11226464).

2. Brunialti MK, Kallás EG, Freudenberg M, Galanos C, Salomao R. Influence of EDTA and heparin on lipopolysaccharide binding and cell activation, evaluated at single cell level in whole blood. Cytometry 2002;50(1):14-8 (PMID: 11857593).

3. Engstad CS, Gutteberg TJ, Osterud B. Modulation of blood cell activation by four commonly used anticoagulants. Thromb Haemost 1997;(4):690-6 (PMID: 9134644).

4. Herzogova E, Dankova P. Ethylenediaminetetraacetic Acid, Sodium Citrate, Heparin and Citrate Phosphate Dextrose-Adenine Anticoagulants Differentially Affect Cytokine mRNA Expression in Blood Leukocytes. Clin Lab 2016;Jul 1;62(7):1371-4 (PMID: 28164647).

5. Vartanian K, Slottke R, Johnstone T, et al. Gene expression profiling of whole blood: comparison of target preparation methods for accurate and reproducible microarray analysis. BMC Genomics 2009;10:2 (PMID: 19123946).

6. Debey S, Schoenbeck U, Hellmich M, et al. Comparison of different isolation techniques prior gene expression profiling of blood derived cells: impact on physiological responses, on overall expression and the role of different cell types. Pharmacogenomics J 2004;4(3):193-207 (PMID: 15037859).

7. Corkum CP, Ings DP, Burgess C, Karwowska S, Kroll W, Michalak TI. Immune cell subsets and their gene expression profiles from human PBMC isolated by Vacutainer Cell Preparation Tube (CPT™) and standard density gradient. BMC Immunol 2015;26;16:48 (PMID: 26307036).

8. Pahl A, Brune K. Gene expression changes in blood after phlebotomy: implications for gene expression profiling. Blood 2002; 100(3):1094-5 (PMID: 12130480).

9. Feezor RJ, Baker HV, Mindrinos M, et al; Inflammation and Host Response to Injury, Large-Scale Collaborative Research Program. Whole blood and leukocyte RNA isolation for gene expression analyses. Physiol Genomics 2004;19(3):247-54 (PMID: 15548831).

10. Rainen L, Oelmueller U, Jurgensen S, et al. Stabilization of mRNA expression in whole blood samples. Clin Chem 2002; 48(11):1883-90 (PMID: 12406972).

11. Das K, Norton SE, Alt JR, Krzyzanowski GD, Williams TL, Fernando MR. Stabilization of cellular RNA in blood during storage at room temperature: a comparison of cell-free RNA BCT(®) with K3EDTA tubes. Mol Diagn Ther 2014;18(6):647-53 (PMID: 25178583).

12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402-8 (PMID: 11846609).

13. Perini TA, Oliveira GL, Ornellas JS, Oliveira FP. Technical error of measurement in anthropometry. Rev Bras Med Esporte 2005; 11(1):81-5. http://www.scielo.br/scielo.php?pid=S1517-86922005000100009&script=sci_arttext&tlng=en

14. Duvigneau JC, Hartl RT, Teinfalt M, Gemeiner M. Delay in processing porcine whole blood affects cytokine expression. J Immunol Methods 2003;272(1-2):11-21 (PMID: 12505708).

15. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. Cytokine 2000;12(11):1712-6 (PMID: 11052823).

16. Malentacchi F, Pizzamiglio S, Wyrich R, et al. Effects of Transport and Storage Conditions on Gene Expression in Blood Samples. Biopreserv Biobank 2016;14(2):122-8 (PMID: 26886447).

17. Köhler A, Hurt E. Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol 2007;8(10):761-73 (PMID: 17786152).

18. Kedersha N, Stoecklin G, Ayodele M, et al. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol 2005;169(6):871-84 (PMID: 15967811).

19. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr Opin Pharmacol 2009; 9(4):447-53 (PMID: 19481975).

20. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 2009;29(6):313-26 (PMID: 19441883).

21. Lam NY, Rainer TH, Chiu RW, Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clin Chem 2004;50(1):256-7 (PMID: 14709670).

22. Shyu AB, Wilkinson MF, van Hoof A. Messenger RNA regulation: to translate or to degrade. EMBO J 2008;27(3):471-81 (PMID: 18256698).

23. Baechler EC, Batliwalla FM, Karypis G, et al. Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation. Genes Immun 2004;5(5):347-53 (PMID: 15175644).