

ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol

February 2024; 23(1):107-114.

DOI: 10.18502/ijaai.v23i1.14958

Unraveling the Impact of Blood Transfusion on Transcription Factors Regulating T Helper 1, 2, 17 and Regulatory T cells

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Received: 12 July 2023; Received in revised form: 10 October 2023; Accepted: 12 November 2023

ABSTRACT

T helper 1 (TH1) and TH2 lymphocytes are the most important components of the immune system affected by blood transfusion. This study aimed to evaluate the effect of blood transfusion on gene expression of transcription factors related to the development of TH1, TH2, TH17 and regulatory T cells (Tregs).

In this cross-sectional study, 20 patients diagnosed with abdominal aortic aneurysms requiring surgical repair were studied from January 2018 to August 2020. We utilized real-time PCR to evaluate the expression of transcription factor genes associated with TH1, TH2, TH17, and Treg, namely T-box-expressed-in-T-cells (T-bet), GATA-binding protein 3 (GATA-3), retinoid-related orphan receptor (ROR γ t), and fork head box protein 3 (Foxp3), respectively. The sampling occurred before anesthesia, 24- and 72 hours post-transfusion, and at the time of discharge.

The results showed that the T-bet gene expression, compared to the time before transfusion, was significantly decreased 24 hours after blood transfusion and upon discharge while GATA3 genes exhibited a significant reduction both 24 and 72 hours after the transfusion, as compared to the pre-transfusion levels and the time of patient discharge. The Foxp3 gene demonstrated an increase at all study stages, with a notable surge, particularly 72 hours after red blood cell (RBC) transfusion. Conversely, the expression of ROR γ t gene, consistently decreased throughout all stages of the study.

RBC transfusion in abdominal aortic aneurysm patients altered the balance of transcription gene expression of TH1, TH2, TH17, and Treg cells.

Keywords: Abdominal aortic aneurysm; Blood transfusion; Helper-inducer T-Lymphocyte; Regulatory T-Cells; T helper 1 cells; T helper 2 cells; T helper 17 cells; Transcription factors

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INTRODUCTION

Abdominal aortic aneurysm (AAA) is a local enlargement of the abdominal aorta where the diameter is greater than 3 cm or more than 50% larger than

normal,¹ in the event of AAA rupture, there is 85-90% estimated likelihood of mortality.² Open surgical repair or Endovascular Aneurysm Repair (EVAR) is recommended when the aneurysm diameter increases to more than 5.5 cm or 1 cm per year. Abdominal aortic aneurysm repair surgery is associated with extensive bleeding, and the patients require blood transfusions.³

However, blood transfusions are associated with changes in the patient's immune system.⁴ Studies have shown that allogeneic blood transfusion, as a transplant, delivers large amounts of external antigens, including residual white Blood cells (WBC), apoptotic cells, cytokines, soluble human leukocyte antigens (HLA), WBC-derived soluble mediators, and free hemoglobin into the patient's blood. These antigens elicit an immune response that may enhance or modulate the immune system.⁵ Red blood cell transfusions are also associated with organ failure, infection, and cancer recurrence, indicating the inhibitory effect of blood transfusions on the immune system. Inhibition of the immune system is a function of mediators induced by white blood cells (WBC), hemolysis-induced compounds (heme, iron), platelet-derived agents, extracellular vesicles, TCD4 + T- cell changes, increased CD8 +T- cells, decreased lymphocyte proliferation in response to mitogens, a decrease and or increase in specific cytokines, and reduced phagocytic activity of monocytes and macrophages.^{6,7} CD4+ T-cell, T-Lymphocyte Helper 1 (TH1) and T-Lymphocyte Helper 2 (TH2) lymphocytes are the most significant parts of the immune system that are affected by blood transfusion.⁸ In the immune system of transfused patients, a decreased helper to suppressor T-lymphocyte ratio (T-Lymphocyte Helper/Suppressor ratio, T4:T8 ratio), decreased Natural Killer (NK) Cells function, defective antigen presentation, and reduction in cell-mediated immunity have been observed.⁹

T lymphocytes are divided into several categories in terms of their immunological role. A group causes the initiation of the immune response. Some regulate the quantity and quality of the immune response, and some respond by secreting lymphokine or by direct cellular action with the antigen. In contrast, some T lymphocytes suppress the immune response. A group also has intrinsic lethal activity due to surface receptors and specific antibodies, and some cause cytotoxicity by targeting virus-infected cells or cancer antigens.^{10,11} Inducing interferon (IFN) γ and Interleukin 12 (IL12) cytokines stimulate TH1 differentiation by activating transcription factors T-box-expressed-in-T-cells (T-bet),

Signal transducer and activator of transcription 1 (STAT1), and Signal transducer and activator of transcription 4 (STAT4).¹² The TH2 sub-category mediates phagocyte-independent defense in which eosinophils and mast cells play important roles. Cytokine IL4 stimulates TH2 differentiation. TH2 evolution by activating the transcription factor of STAT6, which, along with the T cell antigen receptor (TCR) signal, induces GATA-binding protein 3 (GATA-3) expression. GATA-3 is a transcription factor that is the significant regulator of TH2 differentiation.¹³ Th1 cells play an essential role in regulating this orchestra. Adaptive immune responses TH1 cells are immune mediators, that trigger responses to intracellular pathogens and cancer, and TH1 cells are also responsible for inducing some autoimmune diseases.¹⁴

The expression of the nuclear transcription factor Forkhead box P3 (FoxP3) has a determinant property that determines the normal functioning of the Regulatory T cells (Treg). Treg inhibits the activation, proliferation, and production of cytokines in CD4+ T cells and CD8+ T cells, and also suppresses B cells and dendritic cells.¹⁵ As well as it has the activity of the signal transducer and activator of transcription 1 (STAT1) and the Signal transducer and activator of transcription 4 (STAT4).¹⁶ Some transcription factors such as T-box-expressed-in-T-cells (T-bet), GATA-binding protein 3 (GATA-3), retinoid-related orphan receptor (ROR γ t), and forkhead box protein 3 (FOXP3) are specific for different subtypes of T cells. By carefully selecting the specific transcription factors and determining the exact quantity of gene expression patterns by polymerase chain reaction (RT-PCR) method for T cell subgroups, the activity of specific inflammatory pathways related to T-helper (TH) cell development can be determined.¹⁷⁻¹⁹

The TH17 subtype is also primarily involved in leukocyte recall and induction of inflammation. The evolution of TH17 cells depends on ROR γ t and STAT3 transcription factors.²⁰⁻²¹ Real-time PCR is used to study the expression of the desired genes, which is an accurate and sensitive method.²²

This study aimed to evaluate the effect of blood transfusion on gene expression and transcription factors of T-bet, GATA3, ROR γ t, FOXP3, and the Transcription factors as indicators of lymphocytes T-helper 1, T-helper2, T-helper17, and Treg, respectively. The mentioned factors were assessed after transfusion, 24 and 72 hours after it, and upon discharge time

T-helper 1, 2, 17 Gene Expression in Patients

compared to before transfusion in the abdominal aortic aneurysm surgery patients.

MATERIALS AND METHODS

The study protocol was approved by the ethical committee (Ethical Committee N° 007) of the High Institute for Research and Education in Transfusion Medicine, Tehran, Iran, with ethics code (IR.TMI.REC.1397.007) on May 22, 2018, and all patients filled out and signed the consent form in person before sampling.

Twenty abdominal aortic aneurysm patients transfused with RBCs were studied as cross-sectional from January 2018 to August 2020 at Shohada Tajrish Hospital. Inclusion criteria were hemoglobin levels higher than 9 gr/dL and normal patient coagulation profile tests.

Exclusion criteria were a history of blood transfusion and its products, use of medications, or history of diseases affecting the immune system, including immunodeficiency and connective tissue diseases such as Marfan syndrome, previous malignancy and surgery, and history of diabetes. In addition, patients with Ejection Fraction (EF) below 50-55% who should be tolerated under anesthesia following the appropriate principles by the guidelines prepared in the hospital were excluded.

Intraoperative monitoring included arterial line and central venous blood pressure monitoring, pulse oximetry, electrocardiography, and bi-spectral index (BIS). Midazolam (0.01 mg/kg), fentanyl (0.2 µg/kg), lidocaine (1.5 mg/kg), etomidate (0.2 mg/kg) and cisatracurium (0.2 mg/kg) were used to induce anesthesia in all patients. We then used 1% sevoflurane and cisatracurium for intraoperative anesthesia maintenance, and fentanyl was administered every 45 minutes. After intubation, a central venous catheter (CVC) and arterial line were placed for all patients. All patients had a similar aortic cross-clamping time of about 40 minutes.

The blood ordering schedule was according to the relevant guidelines (<http://shmc.sbm.ac.ir/index.jsp?siteid=90&fkeyid=&siteid=90&pageid=69087>).²³

Sampling was taken four times, before anesthesia, 24 and 72 hours after transfusion, and upon discharge. Then, Gene expression of the specific transcription factors of TH1(T-bet), TH2 (GATA3), TH17 (ROR γ t), and Treg (Foxp3) lymphocytes were evaluated both before and after transfusion.

Sample Collection and Transfer

Patients with abdominal aortic aneurysms from Shohada-e-Tajrish Medical Center were recruited for this study. Samples were collected according to the following schedule: before anesthesia, 24 and 72 h after packed red blood cell transfusion (PBRC), in the intensive care unit, in the surgery room and upon discharge. Blood samples were collected in K2 EDTA tubes, which were transferred in 2-8°C conditions within one hour to the laboratory. samples were immediately centrifuged at 3000 g for 10 minutes, Subsequently, 200 µL of buffy coat was homogenized with 1 mL of QIAzol lysis Reagent (Qiagen Germany).

RNA Isolation and Quantitative Real-time PCR

Homogenized buffy coat was extracted according to its manual with slight modifications. RNA was extracted by double chloroform and precipitated with isopropanol after a 5-minute incubation at room temperature. RNA was washed twice with 75% nuclease-free ethanol and dissolved in nuclease-free water. Its quality and quantity were further evaluated by nanodrop spectrophotometry and 1% agarose gel electrophoresis. qualified RNA was reverse transcribed by RevertAid First strand cDNA Synthesis kit (thermofisher. USA). Primers for Tcells subtype transcriptional factor (T-bet, GATA3, FOXP3 and ROR γ) designated by allele ID software (primer bio soft co. USA) and further Insilco evaluation by mfold, ncbi-blast and snap gene program. The specifications of primers are shown in the Supplementary Table. we choose relative quantification for gene expression analysis of transcriptional factors by Real-time PCR Sybr green method. Briefly Quantifast Sybr Green master mix (Qiagen, Germany) was prepared at a volume of 20 µL, including 10 µL SYBR Green, 1 µL primer, 7 µL double distilled water and 2µL cDNA. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control to normalize the data. The PCR program included an activation phase of 95°C for 5 minutes of one cycle, an amplification phase of 45 cycles at 95°C for 15 seconds and 60°C for 35 seconds.

Statistical Analysis

Graph Pad Prism 9.0.0 (121) software was used for statistical analysis, Kolmogorov-Smirnov test indicated the normal distribution of data. Fold changes of expressed genes were further evaluated by t-test statistical analysis. A *p*-value less than 0.05 was statistically significant.

RESULTS

The results (Figure 1A) showed that the T-bet gene expression, compared to the time before transfusion, was significantly decreased 24 hours after blood transfusion ($p=0.009$) and upon patient discharge ($p<0.0001$). The expression of the GATA3 gene showed a significant decrease at 24 ($p=0.013$) and 72 hours ($p=0.003$) after transfusion, at the time of patient discharge ($p<0.0001$) in comparison with before transfusion (Figure 1B). Also, the change in the expression ratio of the T-bet gene to the GATA3 gene increased significantly only 72 hours after blood transfusion ($p<0.0001$).

The results of Foxp3 gene expression, which is a T-reg specific transcription factor, increased in 24h and 72h after RBCs transfusion. This increase in expression was statistically significant 24 ($p=0.273$) and 72 hours ($p=0.0313$) after RBC transfusion. However, it was not statistically significant upon discharge (RE). ($p>0.05$) (Figure 1C).

The results of ROR γ t gene expression of TH17 specific transcription factor showed that the expression was decreased in all stages Although this expression was statistically significant only at 24 hours after blood transfusion (24 h) ($p=0.005$) (Figure 1D).

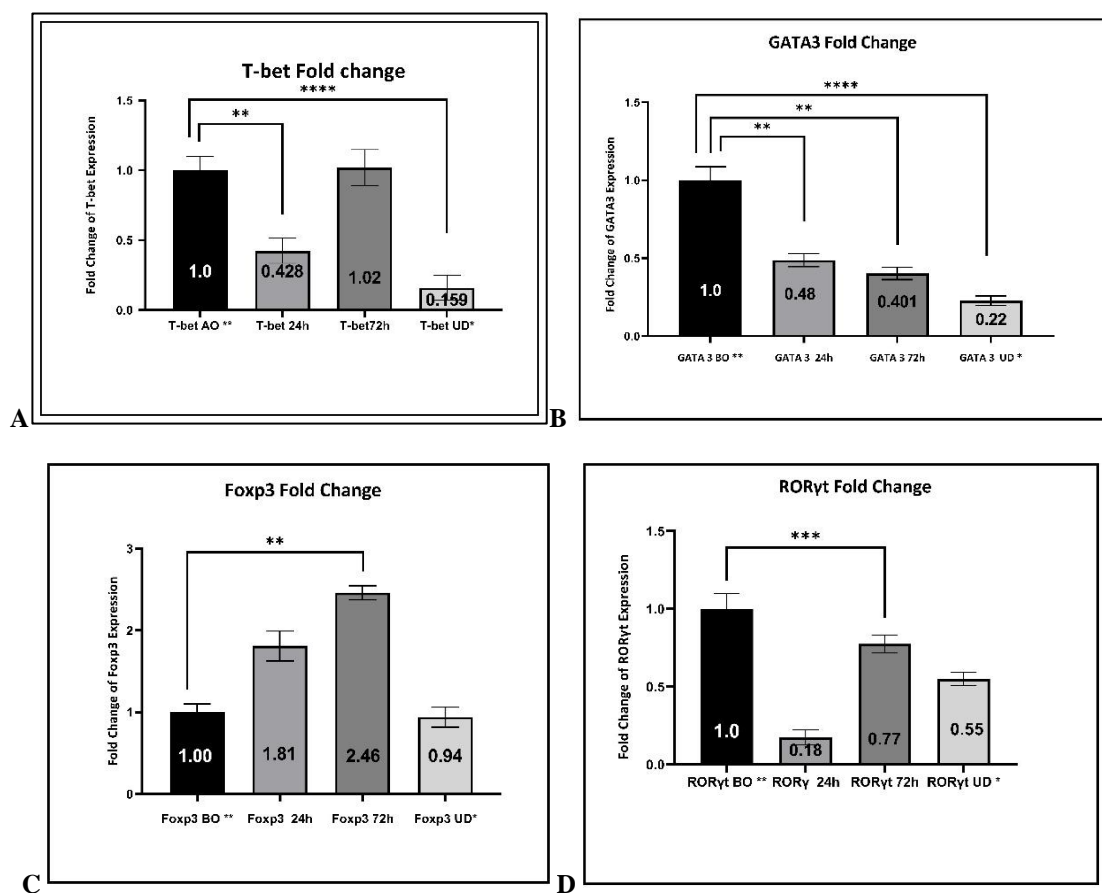


Figure 1. Comparison of fold changes in the expression of transcription factors regulating T helper 1, 2, 17 and regulatory T cells before operation, 24 and 48 hours after RBCs transfusion and upon discharge. A) T-bet gene expression was significantly decreased compared to the time before transfusion, 24 hours after blood transfusion and upon patient discharge. B) The expression of GATA3 gene showed a significant decrease in 24 and 72 hours after transfusion and at the time of patient discharge in comparison with before operation. C) The results of Foxp3 gene expression, which is a T-reg specific transcription factor, increased in 24, 72 h after RBCs transfusion. D) The results of ROR γ t gene expression showed that its expression was decreased in all stages, but this expression was statistically significant only 24 hours after RBCs transfusion, but not at the remaining times of sampling. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Bo: Before operation; 24 h: 24 hours after RBCs transfusion; 72 h: 72 hours after RBCs transfusion; UD: upon discharge

T-helper 1, 2, 17 Gene Expression in Patients

TH lymphocyte transcription factor gene expression and Treg results in 24 hours after RBC transfusion compared to the results before surgery, and showed a decrease in T-bet, GATA3 and ROR γ t gene expression, while an increase in Foxp3 gene expression (Foxp3> GATA3> T-bet> ROR γ t) (Figure 2A).

The results of the expression changes of T lymphocyte transcription factor genes 72 hours after blood transfusion compared to before surgery showed

that Foxp3 increased 2.46 folds, but T-bet did not change with a fold change of 1.02. However, the expression of GATA3 (fold change of 0.401) and ROR γ t (fold change of 0.77) decreased, which showed Foxp3> T-bet> ROR γ t> GATA3 (Figure 2B). Findings from the T lymphocyte transcription factor gene expression in patients at the time of discharge showed that the expression of T-bet, GATA3, Foxp3 and ROR γ t genes decreased (Figure 2C).

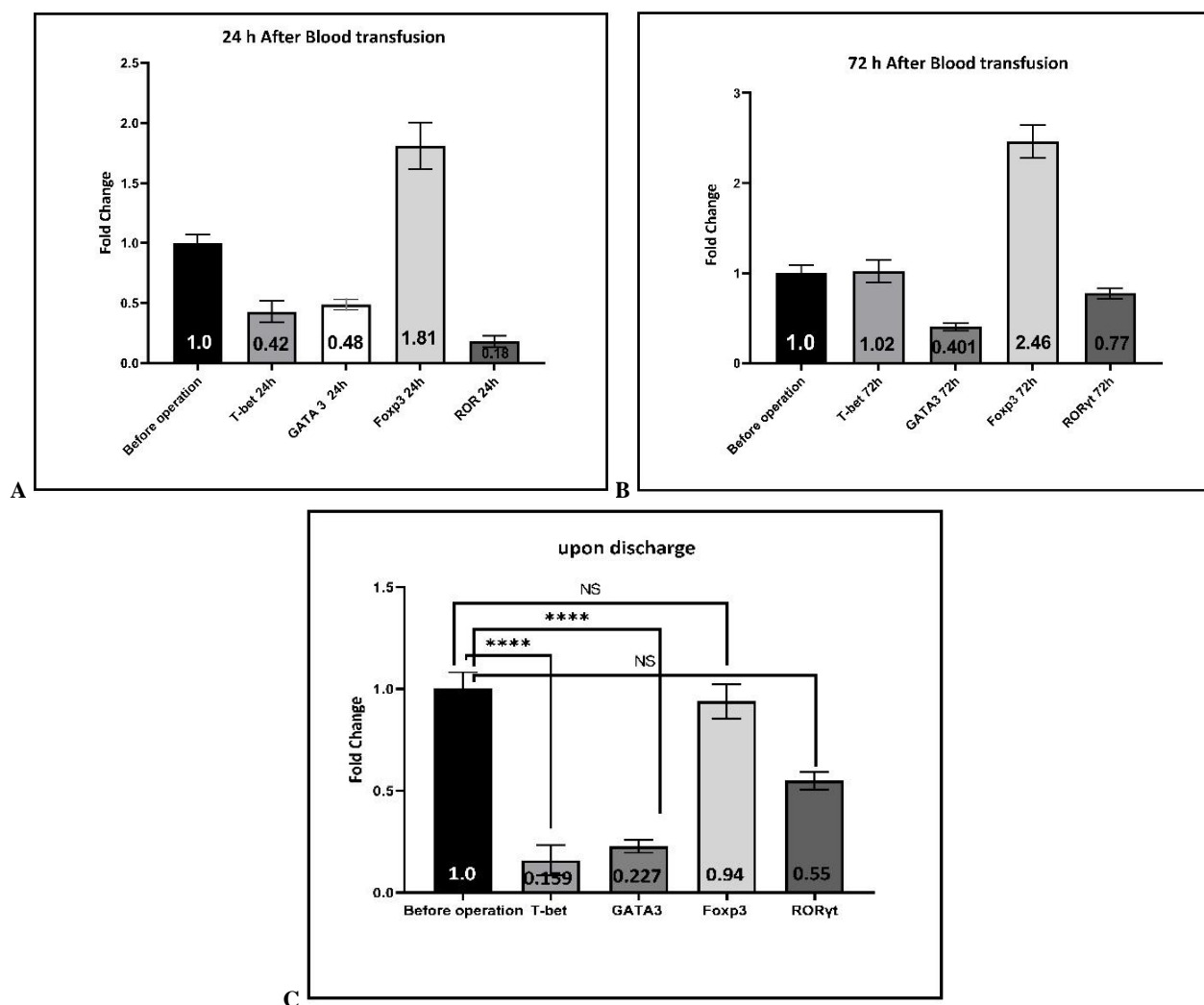


Figure 2. Comparing the results of the expression of transcription factors of Th1, 2, 17 and regulatory T cells before operation, 24 and 72 hours after RBCs transfusion and upon discharge A) The results showed a decrease in the expression of T-bet, GATA3 and ROR γ t genes and an increase in the expression of Foxp3 gene (Foxp3> GATA3> T-bet> ROR γ t) 24 h after blood transfusion. B) The results of changes in the expression of T lymphocyte transcription factor genes 72 hours after blood transfusion showed that Foxp3 increased with fold change of 2.46 but T-bet did not change anymore. However, the expression of GATA3 and ROR γ t decreased, which showed Foxp3> T-bet> ROR γ t> GATA3. C) The results indicated that the expression of T-bet, GATA3, Foxp3 and ROR γ t genes decreased compared to the before operation situation. ** p <0.01, *** p <0.001, **** p <0.0001

DISCUSSION

Surgical trauma and anesthetics can affect the functioning of the immune system in some patients, including those with lung cancer.²⁴ The effect of Etomidate as an anesthetic maintenance drug on immune function has been investigated. It was observed that such anesthetics have a transient suppression of the immune system function that heals quickly. Therefore, it does not have a significant inhibitory effect on patients' immune function.²⁵

The results of this study on the effect of RBC transfusion on the expression rate of TH1 lymphocyte-specific T-bet transcription factor genes revealed a decrease. Such a finding was in line with Torrance et al. study, which found a significant decrease in trauma patients in 24 hours after blood transfusion ($p=0.03$).²⁶ Findings obtained by Mei Zhu et al, also reported a significant reduction 48 hours after blood transfusion ($p<0.001$) in patients with gastrointestinal cancer.²⁷ In contrast, Fragkou PC et al, found no relationship between blood transfusion and T-bet gene expression rate in patients with gastrointestinal surgery.²⁸

The results of the present study on the expression of GATA3 gene were in line with the results obtained by Fragkou PC et al, in patients with gastrointestinal surgery²⁸ and Torrance in blood-receiving patients with trauma.²⁶ However, zhu M. et al, have shown an increase in such gene expression.²⁷ The difference in the reports was due to the selection of various patients, the surgery's duration, the amount of the presence of allogeneic leukocytes in the blood bag, and the storage time of the blood product.²⁷

Despite the decrease in the expression of both T-bet and GATA3 genes, this decrease in the expression level of T-bet was more than GATA3, which was in line with the findings of Mei Zhu et al. and Qian Y et al.²⁷⁻²⁹

Following the introduction of antigens through the blood unit and their delivery by the antigen-presenting cells to naive T-cells, the produced IL-4 is activated by the activated T-cell itself or by mast cells and eosinophils in response to GATA3 and STAT6 transcription factor antigens. These factors stimulate the differentiation of naive CD4+ T-cells into TH2 subcategory, IL-4 produced by TH2 cells enhances this response and inhibits the evolution of TH1 and TH17. However, more research is needed to effectively evaluate the effect of blood transfusion on the

expression level of T-bet and GATA3 genes, especially on specific cytokines and the type of antigen-stimulating cells to express them.²⁵

The results of changes in FOXP3 gene expression, namely a Treg-specific transcription factor with a regulatory role in the immune system, showed increased expression rates 24 and 72 hours after blood transfusion. This increase was significant 72 hours after blood transfusion ($p=0.0313$), and this gene did not change upon discharge. A study by Mei Zhu et al. on patients with gastrointestinal cancer within 24 hours of receiving blood ($p<0.0001$) showed similar findings.²⁷

The results of this study on the ROR γ t gene, a specific transcription factor of TH17 lymphocytes, showed a decrease in its expression. The findings of Fragkou PC et al. on patients with gastrointestinal surgery 24 and 72 hours after transfusion were consistent with our findings.²⁸ Also, the findings of Torrance et al, on patients with severe trauma showed a decrease in 24 and 72 hours after receiving blood, which is in line with our study.²⁶

The most remarkable point of all these findings is that the highest rate of change was related to FOXP3, which showed an increase in all times after blood transfusion.

Leal-Noval SR has shown that blood transfusions led to a shift of Th1/Th2 balance toward Th2 dominance, which might increase the risk of perioperative complications.⁸

The limitations of our study include the small number of patients and not considering various factors, such as aortic clamp during surgery, affecting the expression of genes and inflammatory responses. If we could compare our patients with those who had the same condition but did not receive blood, we might have a more definitive view of postoperative complications.

RBC transfusion altered the balance in the expression rate of TH1, TH2, TH17, and Treg cell genes. T-bet gene (TH1) significantly decreased 24 hours after blood transfusion and at the time of patient discharge compared to the time before transfusion. GATA3 gene (TH2) significantly reduced 24 and 72 hours after blood transfusion and at the time of patient discharge. ROR γ t gene (TH17) showed a decrease in its expression, which was statistically significant 24 hours after blood transfusion, and the Foxp3 gene (T reg) increased in all stages, which was statistically significant 72 hours after blood transfusion.

T-helper 1, 2, 17 Gene Expression in Patients

STATEMENT OF ETHICS

The study protocol was approved by ethical committee (Ethical Committee 007) of High Institute for Research and Education in Transfusion Medicine; Tehran, Iran with ethics code (IR.TMI.REC.1397.007) on 22 May, 2018.

FUNDING

This article is the result of a master's thesis of a student that was supported by Blood transfusion research center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

We are grateful to the patients and staff of Shohada Tajrish Hospital for their cooperation in this project.

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