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Original Article

Assessment of immunological factors in COVID-19 patients treated by convalescent plasma

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Abstract

Following the outbreak of COVID-19, several immunotherapy methods were used to modulate the immune responses of patients. In this study, we aimed to evaluate the immune response to COVID-19 in patients receiving convalescent plasma. In this regard, this randomized controlled trial included 30 patients who were divided into two groups according to receiving convalescent plasma or normal control plasma. Samples from both groups were collected on days 0, 1, 3, 5 and 7 after plasma infusion. We measured the expression level of TLR7/8, IRF3/7, CTLA-4, PD-1 and T cell transcription factors by Real-time PCR in the mentioned groups. Thirteen cytokines were also evaluated using flow cytometry method. Results showed that compared to the normal control plasma group, the expression levels of TLR7, 8, IRF3, 7 and PD-1 and CTLA-4, on days 3, 5 and 7 after convalescent plasma infusion, were significantly decreased. On the other hand, Gene expression results showed that the expression levels of Tbet, RORγ3 and Foxp3 on days 3, 5 and 7 after convalescent plasma infusion were significantly increased compared to the normal control plasma group. After convalescent plasma infusion, the viral load was significantly decreased compared to the normal control plasma group. Convalescent plasma infusion also reduced the plasma cytokines levels, including IL-6, IL-10, and IL-4, and enhanced the level of IL-2, IFN- γ and perforin comparing the normal control plasma group. According to the results, the convalescent plasma infusion led to a decrease in the expression of innate immunity receptors and an increase in the expression of transcription factors of adaptive immunity. Therefore, it may be concluded that convalescent plasma infusion can modulate the immune response. To achieve a reliable consequence, further studies are required.

Keywords: COVID-19, Convalescent plasma, Plasma therapy, Immune response, Co-stimulatory molecules

1. Introduction

Since December 2019, Coronavirus 2019 (COVID-19), caused by SARS-CoV-2 (Acute Respiratory Syndrome of Coronavirus 2), has spread worldwide with significantly high rates of transmission and substantial mortality. The symptoms of COVID-19 vary from person to person. In patients, mild and self-limiting respiratory illnesses up to severe progressive pneumonia, multiple organ failure, and even death [1, 2] may be seen. Currently, there is no efficient treatment to control this disease.

Following the coronavirus disease 2019 (COVID-19) pandemic caused by the novel human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), convalescent plasma has been used globally to treat hospitalized patients and prohibit the progression of disease in non-hospitalized patients [3, 4]. Due to the lack of definitive treatment for COVID-19, great hope of antibody therapy usefulness has also resulted in the commercial production of other immunoglobulin therapies, such as monoclonal antibodies and hyperimmune products [5-7].

In general, convalescent plasma represents a form of passive antibody therapy based on the transfer of pathogen-specific antibodies from a recovered patient to stop the severity or treat the disease [8]. In contrast to vaccines, this method just requires the availability of disease survivors willing to donate plasma and standard blood collection infrastructure to collect and distribute convalescent plasma [9].

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With no vaccines or monoclonal antibodies available at the onset of the COVID-19 pandemic, convalescent plasma was an immediately deployable option. As variant SARS-CoV-2 strains continue to emerge, convalescent plasma donated by survivors of infections with variant strains represents an immediately deployable therapeutic for patients identified with a variant infection. In contrast, other immune therapies may require development to target new viral strains precisely [5, 10]. To effectively neutralize SARS-CoV-2 and confer clinical benefit, convalescent plasma must adhere to the three fundamental principles of passive antibody therapy [11]. Convalescent plasma must i) Contain specific antibodies against the pathogen, the SARS-CoV-2 virus ii) Contain a sufficient level of anti-SARS-CoV-2 antibody, and iii) Be transfused prophylactically or early in the disease course [12].

Recent studies have shown that not only humoral immunity but also cellular immunity is actively involved in the removal of SARS-CoV-2 infection. The cooperation of cellular immunity with antibodies to eliminate the infectious agent is often neglected. But, the recovery of two patients with agammaglobulinemia suffering from SARS-CoV-2 infection, led the immunologist to believe that cellular immunity plays a greater role in the treatment of infections than previously thought. As a result, it appears that antibodies may launch direct antiviral activity and stimulate adaptive immune cells via FcRs or complement receptors, and/or advance more effective priming of T cells. Although signatures of efficacy have emerged consistently from worldwide matched control studies [7], there remains a paucity of data from large randomized clinical trials (RCTs) demonstrating efficacy. Recent reviews and meta-analyses have also generated disharmonious conclusions regarding the effect of convalescent plasma on COVID-19 patient outcomes, such as mortality and clinical improvement. Furthermore, recent studies indicated that convalescent plasma-based therapy is crucially dependent on the quality of the plasma, the timing of administration, and the immunological status of the patient [11] which adds further complications to this approach. Thus, this study aimed to evaluate the effect of COVID-19 convalescent plasma (CCP) on patients with COVID-19 admitted to intensive care units (ICU). In this study, immunological factors involved in the development of an immune response against the coronavirus and the kinetics of virus clearance from the body are evaluated within 1 week after plasma infusion.

2. Materials and Methods

2.1. Participants

This Randomized controlled trials (RCT) study which included patients was recruited from 2 medical centers (Shahid Faghihi and Namazi hospital). The study recruitment was from June 1, 2021, to October 1, 2021. Follow-up was completed on November 1, 2021. The study included 30 patients divided into two groups: 20 patients, who received convalescent plasma (CP) as intervention groups and 10 patients who received normal control plasma (NCP) as a control group.

Samples were collected from both groups on days 0, 1, 3, 5, and 7 after plasma infusion as serum and buffy coats and stored at -70°C to perform subsequent tests.

All patients were diagnosed as having severe COVID-19 according to the WHO Interim Guidance [13].

2.2. Ethics statement

The present investigation was conducted in accordance with the recommendations of ethical guidelines. The research protocol was authorized by the Shiraz University of Medical Sciences (IR.SUMS.REC.1399.020).

The details of the study were carefully described to the participants. According to the Helsinki Declaration, informed written consent has been acquired from every patient or his legal relative.

2.3. Inclusion Criteria

Inclusion criteria were as follows: (1) informed consents were signed; (2) the patients had to be aged 18 years or older ; (3) COVID-19 contamination was confirmed based on polymerase chain reaction (PCR) test; (4) Pneumonia was confirmed by chest X-ray; (6) According to the definitions of COVID-19, clinical signs were severe or life-threatening;

Severe COVID-19 was determined as respiratory distress (≥30 breaths/min; oxygen saturation ≤93% in the resting state).

2.4. Exclusion Criteria

Exclusion criteria were as follows: (1) Past history of allergy to plasma or its associated compounds (sodium citrate); (2) cases with critical general conditions, such as severe organ dysfunction, who were not appropriate for CP transfusion.

2.5. Standard Treatment

Standard treatment included symptom control and supportive care for COVID-19; possible treatments consisted of antiviral medications, antibacterial medications, steroids and other medications.

2.6. Extraction of mRNA and Quantitative Real-time Polymerase Chain Reaction (RT-PCR)

mRNA was extracted from the whole blood using RiboEx (GeneALL, South Korea) according to the manufacturer's instructions. cDNA synthesis was done by NG dART RT kit (EURx, Poland). Based on the intron inclusion method, the primer sequences were designed using Allele ID software (PREMIER Biosoft, USA) (Table 1). Primers were synthesized by Eurofins Genomics (Eurofins Genomics, Germany).

PCR was performed using SYBR®Premix Ex Taq™ II (Takara, Japan) with the ABI 7500 real-time PCR detection system (Applied Biosystems, Foster City, CA). A reaction mixture was prepared in a final volume of 10 µl containing 2-µl template cDNA of each sample, appropriate amounts of forward and reverse primers, SYBR Premix Ex Taq, ROX Reference Dye II and dH2O. The human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was included as the housekeeping gene or internal control. The list of primers and thermal cycling conditions for all genes is summarized in Table 1.

2.7. Cytokine array

Plasma from all cases was collected and stored at −20°C for cytokine measurement. Human NK/CD8T

Table 1. The list of specific primers, thermocycling condition and annealing temperature used for amplification of TLR-7, TLR-8, PDCD1, CTLA-4, IRF3, IRF7, T-bet, GATA-3, RORγt, FOXP3 and GAPDH genes.

GENES	Primer sequence (5'-3')	Annealing temperature	Thermo cycling condition
TLR 7 F	CGTGTCATCCAGGGCCCCAT	59°C	95°C/30 s, 40 cycles of 95°C/15. s,59 °C/20s, and 72°C/30 s
TLR 7R	GGAACCCAGAAGCAGGCCCA		
TLR 8 F	CACGTGCCACCCAAACTGCC	59°C	95°C/30 s, 40 cycles of 95°C/15. s,59 °C/20s, and 72°C/30 s
TLR 8 R	CACCTCGGACAGTTCCCGCT		
PDCD1 F	GTGGACTATGGGGAGCTGG	60°C	95°C/30 s, 40 cycles of 95°C/15. s,60 °C/20s, and 72°C/30 s
PDCD1R	CGCTAGGAAAGACAATGGTGG		
CTLA-4 F	TGAGTTGACCTTCCTAGATGATTCC	60°C	95°C/30 s, 40 cycles of 95°C/15. s,60 °C/20s, and 72°C/30 s
CTLA-4 R	CAGATGTAGAGTCCCGTGTCC		
IRF3 F	TTGGGGACTTTTCCCAGCC	59°C	95°C/30 s, 40 cycles of 95°C/15. s,59 °C/20s, and 72°C/30 s
IRF3R	TCCAGAATGTCTTCCTGGGT		
IRF7 F	GTGAGGGTGTGTCTTCCCTG	60°C	95°C/30 s, 40 cycles of 95°C/15. s,60 °C/20s, and 72°C/30 s
IRF7 R	TCGTCATAGAGGCTGTTGGC		
GAPDH F	GGACTCATGACCACAGTCC	58°C	95°C/30 s, 40 cycles of 95°C/15. s,58 °C/20s, and 72°C/30 s
GAPDH R	CCAGTAGAGGCAGGGATGAT		
T-bet F	AACACAGGAGCGCACTGGAT	62°C	95°C/30 s, 40 cycles of 95°C/15. s,62 °C/20s, and 72°C/30 s
T-bet R	TGGAGGGACTGGAGCACAAT		
GATA 3 F	AGATGGCACGGGACACTACCT	63°C	95°C/30 s, 40 cycles of 95°C/15. s,63 °C/20s, and 72°C/30 s
GATA 3 R	GCCTTCGCTTGGGCTTAAT		
RORγt F	GCTGAGAAGGACAGGGAGCCA	62°C	95°C/30 s, 40 cycles of 95°C/15. s,62 °C/20s, and 72°C/30 s
RORγt R	CCCACAGATTTTGCAAGGGAT		
FOXP3 F	CACCTGGAAGAACGCCATCC	63°C	95°C/30 s, 40 cycles of 95°C/15. s,63 °C/20s, and 72°C/30 s
FOXP3 R	CTCATCCACGGTCCACACAG		

cells 13 Plex cytokine assay kit (Biolegend, USA, Cat No 740267) were used according to the manufacturer's instructions. Flow cytometer set (FACS Calibur, BD, USA) was done to assess 13 proteins, including IL-2, IL-4, IL-10, IL-17A, IL-6, IFN-γ, and TNF-cytokine levels, as well as Granzyme A, Granzyme B, Granulyzin, perforin, FAS, and soluble FAS in plasma. Finally, the data were analyzed by LEGEND plexTM data analysis software, version 7.0.

2.8. COVID-19 detection

RNA was extracted from the serum with the BehPrep viral nucleic acid extraction kit (Beh Gene, Iran) according to the manufacturer. PCR was performed using Bio COVID-19 RT-PCR kit (Biorexfars, Iran) with the ABI 7500 real-time PCR detection system (Applied Biosystems, Foster City, CA).

2.9. Procurement of Normal Control Plasma and Convalescent Plasma

In brief, patients with a laboratory-confirmed COVID-19 diagnosis, and have been fully recovered and discharged from the hospital more than 2 weeks before, were recruited. The patients were divided into two groups: normal control plasma (NCP) and convalescent plasma (CP). The application of normal control plasma as a comparator allowed us to evaluate the effect of convalescent plasma. Convalescent plasma-specific donor selection criteria for plasma donation were based on the following criteria: age of 18 through 55 years, suitable for blood donation, initially determined with COVID-19 but with 2 negative PCR test results by nasopharyngeal swabs (at least 24 hours apart) prior to hospital discharge, having been discharged for more than 2 weeks, and no persisting COVID-19 symptoms. Convalescent plasma collection was done according to the routine plasma collection procedures via plasmapheresis. The plasma products were prepared as fresh-frozen plasma. COVID-19 convalescent plasma was collected and processed at Fars Blood Transfusion Organization. Neutralizing and RBD–specific IgG antibody titer was measured for convalescent plasma products and reported as the following: less than 1:160, 1:160, 1:320, 1:640, or greater than 1:640.

Convalescent plasma has been tested for hepatitis B, hepatitis C, HIV and syphilis according to the routine plasma collection of the Iranian Blood Transfusion Organization.

2.10. Normal Control Plasma and Convalescent Plasma Transfusion

The dosage of normal control plasma and COVID-19 convalescent plasma transfusion was almost 4 to 13 ml/kg of recipient body weight. ABO type of the plasma transfused was compatible with the patient's ABO type. In addition, the plasma was cross-matched with the patient's red blood cells to ensure compatibility. Plasma was transfused at approximately 100 mL per hour with close monitoring. Adjustments in the infusion rates were allowed based on the patient's risk for volume overload and tolerance, at the discretion of the treating physicians. No pre-medication was given before convalescent plasma transfusion.

2.11. Statistical Analysis

The results of expression analysis were expressed as mean values ± standard deviation (SD). Moreover, differences were considered significant when the P-value was less than 0.05.

In this study, the statistical differences in the expression levels of genes and the fold changes in the intervention group and controls were compared via the Livak methods ($2^{-\Delta\Delta CT}$). Statistical analysis was performed using SPSS software (version 22.0; IBM Corporation, Armonk, NY, USA) including non-parametric tests (Mann–Whitney *U* test for studying the *p*-value between two study groups, and k-independent tests for studying the *p*-value between more than two study groups). GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) was used to design graphs and calculate the statistical tests.

3. Results

3.1. General Characteristics of the Patients in the Trial

From June 1, 2021, to Oct 1, 2021, 20 severe COVID-19 patients including 15 males and 5 females were enrolled and received CP transfusion. The median age was 55.00± 10.523y and 48.82±15.521 in female and male, respectively. The control group (5 males and 5 females) was enrolled and received NCP transfusion. The median age was 59.20 ±24.325and 48.33±19.439 in female and male, respectively.

Median length between symptom onset and hospitalization ranged between 6 days and 12.5 days, respectively (Table 2).

The most common symptoms at disease onset were fever (18 of 20 patients), cough (14 cases) and dyspnea (18 cases), while less common symptoms included chest pain (7 cases), headache (5 cases), body pain (7 cases) and weakness (10 cases). In the control group, the symptoms were fever (10 out of 10 patients), cough (6 cases), and dyspnea (6 cases), and less common symptoms included chest pain (5 cases), headache (6 cases), body pain (5 cases) and weakness (6 cases). Their underlying chronic diseases, included diabetes, hypertension, cardiovascular and chronic kidney disease. (Table 2).

The list of medications for patients is given in Table 2. Antibacterial or antifungal therapy was applied when patients had co-infection.

Table 2. Demographic and clinical data of COVID-19 in the intervention and the control group**.**

	Intervention	**Control**
	NUMBER (Mean of Age±SD)	**NUMBER (Mean of Age±SD)**
Sex		
Female	5 (55.00± 10.523)	5 (59.20 ±24.325)
Male	15 (48.82±15.521)	5 (48.33±19.439)
	NUMBER (%)	**NUMBER (%)**
Chronic comorbidity		
Diabetes	5(25)	2(20)
Hypertension	6(30)	5(50)
Cardiovascular	6(30)	3(30)
Chronic kidney disease	9(45)	4(40)
Symptoms		
Cough	14(70)	6(60)
Weakness	10(50)	6(60)
Body pain	7(35)	5(50)
Headache	5(25)	6(60)
Fever	18(90)	10(100)
Dyspnea	18(90)	6(60)
chest pain	7(35)	5(50)
Drugs		
Remedesvir	10(50)	4(40)
Favipiravir	14 (70)	6(60)
Antibiotic	14 (70)	6(60)

3.2. CP Transfusion influence

3.2.1. TLRs

Results from gene expression profiles showed that compared to the control group, the expression levels of TLR7 and TLR8 on days 3, 5 and 7 after CP infusion were significantly decreased (P=0.03, P=0.0001). Also, further analysis indicated that CP infusion can decrease TLR7 and TLR8 expression on day 5 in comparison with day 3, and on day 3 in comparison with day 1 in the same group. (P=0.0001) (P=0.02) (P=0.02) (P=0.0001). (Fig1A).

Statistical studies showed that on the first day after CP infusion, there is an inverse relationship between TLR-7 and Tbet expression (P=0.03, r = -0.05).

Also, statistical analysis showed that on the third day after CP injection, there is a direct relationship between expression TLR7 and CTLA-4 (P=0.02, r = 0.05).

3.2.2. IRFs

Results from gene expression profiles presented the expression levels of IRF3 and IRF7 on days 3, 5 and 7 after CP injection being significantly less than the NCP group on the same days (P=0.0001, P=0.0001).

Also, further analysis showed that CP injection decreased IRF3 expression on day 5 compared to day 3, and day 3 compared to day 1 in the same group. (P=0.0001, P=0.05)

Gene expression analysis displayed that IRF7 expression decreased after CP infusion on day 3 compared to day 1 in the same group (P=0.0001) (Fig1B).

Statistical studies on the fifth day after CP infusion indicated that there is a direct relationship between the expression of IRF3, IRF7, and FOXp3 (P=0.009 r = 0.59, P=0.005 r = 0.62).

3.3. T Cell Differentiation

Laboratory evidence from clinical patients demonstrated that specific T cell responses to SARS-CoV-2 are important for the diagnosis and killing of infected cells. Gene expression profiling results showed that the expression levels of Tbet, RORγ3, and Foxp3 on days 3, 5 and 7 after CP infusion were significantly increased compared to the NCP group on the same days (P= 0.03, P=0.0001, P=0.0001).

Also, further analysis showed that CP infusion increased Tbet, RORγ3, and Foxp3 expression on day 7 compared to day 5 and, on day 5 compared to day 3 in the same group (P=0.0001, P= 0.2, P= 0.02), (P= 0.0001, P= 0.07, P=0.02), while no significant changes were seen in the expression level of GATA-3 in all days after CP infusion compared to the NCP group. (Fig 2).

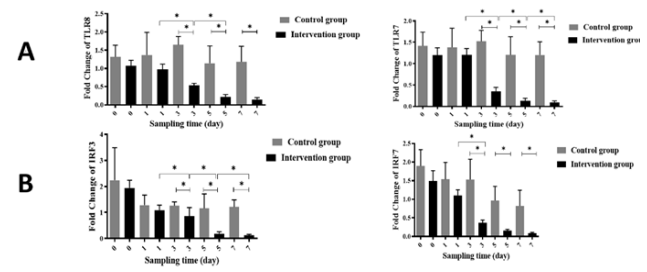

Fig. 1. A: Comparison of TLR7, and TLR8 expression levels between intervention and control groups on days 0, 1, 3, 5 and 7 after plasma infusion. B: Comparison of IRF3, and IRF7 expression levels between intervention and control groups on days 0, 1, 3, 5 and 7 after plasma infusion. *The significant differences between expression of genes intervention compared with the control groups are shown with *$p<0.05$.

3.4. Co-stimulatory molecules

Results from gene expression profiles showed that the expression levels of PD-1 and CTLA-4 on days 3, 5 and 7 after CP infusion significantly decreased compared to the NCP group on the same days (P=0.0001, P=0.0001). Also, further analysis showed that CP infusion decreased PD-1 expression on day 3 compared to day 1, and day 1 compared to day -1(the day before plasma injection) in the same group (P=0.0001 and P=0.0001, respectively). Gene expression analysis suggested that CP infusion decreased CTLA-4 expression on day 5 compared to day 3, and day 3 compared to day 1 in the same group (P=0.0001 and P=0.0001, respectively) (Fig 3). Statistical analysis indicated that on the fifth day, there was an inverse relationship between CTLA-4 and GATA-3 expression (P=0.049, r = -0.04).

3.5. Cytokine Production

As depicted in Figure 4, the level of cytokines including IL-2, IL-4, IL-6, IL-10, and IL-17A, and the proteins including granzyme A/ B and perforin were determined in the intervention and the control group.

Findings demonstrated that CP infusion reduced the plasma cytokines levels, including IL-6, IL-10, and IL-4 (Figure 4A) and enhanced the level of IL-2, IFN-y, and perforin on days 3, 5 and 7 comparing the control group (Figure 4B). Furthermore, CP infusion diminished the level of IL-17 on days 5 and 7 in the intervention group comparing the control group (Figure 4C) (P, 0.0001).

3.6. Disappearance of SARS-CoV-2 RNA

In order to evaluate the clearance of the viral load after

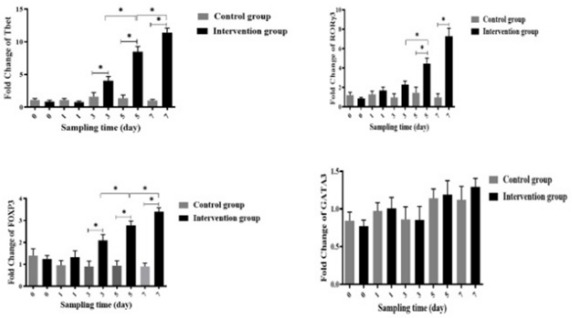

Fig. 2. Comparison of transcription factors (Tbet, RORγ3, Foxp3 and GATA3) expression levels between intervention and control groups. *The significant differences between expression of genes intervention compared with the control groups are shown with *p<0.05.

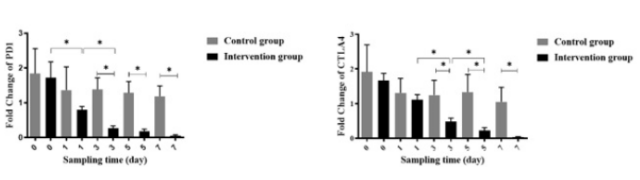

Fig. 3. Comparison of co-stimulatory molecules (PD-1 and CTLA-4) expression levels between intervention and control groups. *The significant differences between expression of genes intervention compared with the control groups are shown with *p<0.05.

plasma infusion, the expression of N and RDRP was examined. As the results showed, after CP infusion, the viral load decreased over time. This reduction was significant on days 3, 5 and 7 after CP infusion compared to the NCP group (Figure 5).

3.7. Adverse Effects of CP Transfusions

No serious adverse reactions or safety events have been reported after CP transfusion.

4. Discussion

In the context of the COVID-19 epidemic, the primary anticipated mechanism for the clinical benefits of plasma immunotherapy is the neutralization of SARS-CoV-2 virus [14]. Virus neutralization occurs when antibodies bind to spike proteins and prevent them from binding to host cellular receptors. Besides viral neutralization, convalescent plasma includes antibodies that mediate three other antiviral functions against SARS-CoV-2: (i) complement activation, (ii) antibody-dependent cellular cytotoxicity, and (iii) phagocytosis [15]. The antiviral outcome of convalescent plasma has been confirmed by RCTs and observational studies, which consistently display a decrease in viral load after transfusion [3, 15].

The pathogenicity of COVID-19 starts with an early viral stage that can progress to a life-threatening inflammatory stage [16]. The viral phase is determined by SARS-CoV-2 virus replication that is accompanied by variable symptoms that trigger an endogenous antibody response on days 10–12 of infection [17]. Some individuals may move to an inflammatory phase that may clear the virus but debilitates pulmonary gas exchange and sometimes leads to respiratory failure and death [17, 18]. As a result, early convalescent plasma transfusion during the viral phase is beneficial, since viral neutralization inhibits disease progression to the severe inflammatory phase. In accordance with this vision, convalescent plasma administration in COVID-19 leads to inflammation markers decrease [19].

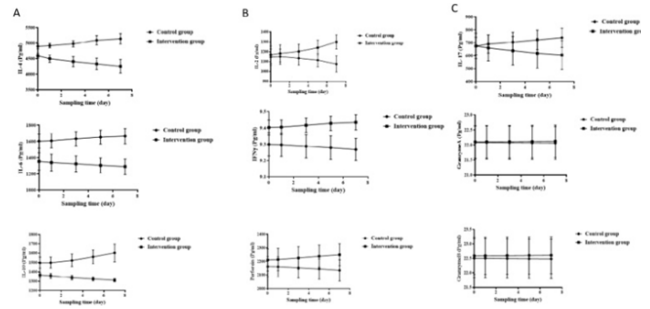

Fig. 4. A: Comparison of IL-4, IL-6 and IL-10 levels in intervention and control groups. B: Comparison of IL-2, IFN- γ and Perforin levels in intervention and control groups .C: Comparison of IL-17 and granzyme A/ B levels in intervention and control groups.

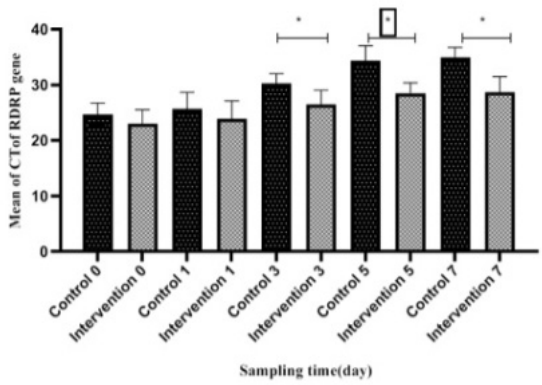

Fig. 5. SARS-CoV-2 RNA clearance in the intervention and the control group. *The significant differences between expression of genes intervention compared with the control groups are shown with *p<0.05.

Convalescent plasma therapy refers to the application of plasma-containing antibodies from a person recovered from an illness. All the collected information expressed the significant effect of CP therapy in multiple viral respiratory disorders. A multitude of studies have investigated the effectiveness and safety of CP injections in the management of clinical symptoms and treatment of COVID-19 in terms of viral clearance and longer survival times [20]. The hopeful consequence of CP therapy originates from the ameliorated survival rate and lower mortality rate of the patients with SARS-CoV-related pneumonia and influenza A (H1N1) through this approach [21, 22].

In the first COVID-19 research, by Shen et al., all five patients treated in China with convalescent plasma between days 10 and 22 of admission recovered clinically after receiving treatment [23]. In another study in China, ten patients with severe COVID-19 were treated earlier in their disease course, at a median time of 16.5 days after onset, describing considerable recovery in symptoms within the first days after CP therapy and lesser need for ventilator support [24].

Hopefully, more studies have been started in the USA, in which the safety and efficacy of CP therapy at the early stages of the expanded access program were investigated. For instance, 39 patients suffering from severe or immediately life-threatening diseases, who received CP, showed recuperation in supplementary oxygen requirements and more survival rate comparing the retrospectively matched controls [25].

Accordingly, the present study is the first report on the impact of CP therapy on innate and adaptive immune systems. We found that the expression levels of TLR7/8 and IRF3/7 on days 3, 5 and 7 after CP infusion were significantly decreased compared to the NCP group on the same days. Also, the expression levels of Tbet, RORγ3 and Foxp3 on days 3, 5 and 7 after CP infusion were significantly increased compared to the NCP group on the same days. On the other hand, the expression levels of PD-1 and CTLA-4 on days 3, 5 and 7 after CP infusion were significantly decreased compared to the control group on the same days.

As we know, in COVID-19 infection, the body's immune system confronts many challenges and there are still many questions about the interaction of body's immune response with this virus. Moreover, the function of each immune system component in this disease and its significance are also contested alongside other issues such as the duration of immunity, antibody response effectiveness, and the most effective and beneficial therapy. As a result, the efficacy of innate, cellular, and humoral immunity defines the consequence of viral infections. This means an appropriate immune response provides protection, while an overwhelming immune response is accompanied by immune-mediated pathogenesis in viral infections [26].

It appears that CP therapy could be applied in patients

with newly infected COVID-19 to ameliorate the immune response, probably through virus neutralization, viremia suppression, and viral clearance. No adverse effects were perceived in all the patients that were included in this research. However, some precautions should be taken, including evaluation of the neutralizing Ab activity titer and precise time for collecting the plasma and administering it [26]. In addition, previous studies demonstrated that CP treatment may be more effective if done in early stages of the disease [22, 27].

In most viral diseases, viremia peaks in the first week of the infection. The patient then develops a primary immune response by day 10–14, after which the virus clears. Hence, CP should be more helpful when administered in early stages of the disease. In SARS, viral load also reaches its highest point in the first week, and this might clarify the clinical uselessness of CP when given after day 16 [28].

As our results show, in the absence of CP infusion, we had a relative increase in the expression of CTLA-4 and PD-1. Although this increase was not significant, CP injection reduced the expression of CTLA-4 and PD-1 on days 3, 5 and 7 after infusion, which triggers a more effective immune response.

Like other coronaviruses, the N protein of SARSCoV-2 hinders IFN1 by regulating IFN-β synthesis and signaling. Conversely, the effectiveness of the innate immune response against viral infection depends mainly on IFN1 production and its downstream signaling, which leads to viral replication control and adequate adaptive immune response induction [29, 30]. However, the virus can avoid this innate immune attack due to the complex immune dysregulation by producing an infection. Chronic stimulation of T cells, leading to cytokine storm and T cell exhaustion, weakens the body's overall defenses and puts the patient in a dangerous situation. Chronic high-grade viral infections lead to the depletion of CD8+ T cells (Tex), resulting in diminished effector function and less proliferative capacity. Tex causes overexpression of inhibitory receptors such as CD279 (PD-1) which is a lymphoid cell surface protein of the Ig superfamily and a member of the extended CD28/CTLA-4 family of T cell regulator, and a mature T cell checkpoint for the modulation of apoptosis [31].

Angiotensin-converting enzyme 2 (ACE2), an entry receptor for SARS-CoV-2 interacts with the superficial S glycoprotein on the envelope of the virus. This binding appears to be (primarily) perceived by Toll-like-7 receptor (TLR-7), which is present in endosomes and then results in the secretion of the inflammatory cytokines. On the other hand, TLR7 can trigger various signaling pathways and transcription factors, including Janus kinase transducers (JAK/STAT), nuclear factor κB (NF-κB), activator protein 1 (AP-1), interferon response factor 3 (IRF3), and IRF7. The mentioned cascade results in a more frequent release of pro-inflammatory cytokines, like IL-1, IL-6, monocyte chemo attractant protein-1 (MCP-1), MIP-1A, tumor necrosis factor α (TNF-α) and ultimately interferon 1 (IFN1) [16].

In addition to the mentioned events, antigen presentation subsequently launches the body's specific adaptive immunity (both humoral and cellular immunity), which peaks in approximately 7–14 days after infection. After APC Antigen presentation to the CD4+ and CD8+ T-cells, pro-inflammatory cytokines are generated by the NF-κB signaling pathway. Activated B cells release virus-specific antibodies, whereas antigen-specific T cytotoxic cells destroy virus-infected cells [18].

As our results show, plasma infusion reduces the expression level of TLR7, TLR8, IRF3, and IRF7 on days 3, 5 and 7 after plasma infusion. However, due to the binding of ACE2 to TLR7, ACE2 increased the expression of TLR7 and inflammatory cytokines.

According to the recently published research, elevated release of certain plasma mediators, such as IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, IL-13, IL-17, TNF-α, MIP-1α, IP-10, IFN-γ, G-CSF, MCP-1, MCSF, and hepatocyte growth factor (HGF) result in the lung injury in several individuals suffering from COVID-19 [32]. The viral invasion arises when the virus particles bind to the respiratory mucosal tissue and infect other cells and launch a cascade of immune system responses and cytokine storm, which may be related to the severity of the disease in infected individuals [33]. Most research results clarify that severe pneumonia leading to failure and death, is due more to acute inflammation than the direct destructive effect of the virus itself [26, 34].

Once the viruses are recognized by the pattern recognition receptors such as Toll-like receptors 3, 7, 8, and 9 and viral-infection sensors RIG-I and MDA5, different Toll-Like Receptors (TLRs) prompt the transcription of the NLR family pyrin domain containing 3 (NLRP3) gene, and participate in the activation of the inflammasome complexes, like the secretion of key pro-inflammatory cytokines IL-1β and IL-18, IL-6, TNF-α and the caspase-1 activation. An augmentation in ferritin, transaminases, and certain cytokines (including IL-6, IL-10, G-CSF, and others) released by macrophages may be observed during infection, especially in severe pediatric and adult patients [35]. According to our results, CP infusion reduced the production of inflammatory cytokines such as IL-6, IL-1, TNF-α, and IL-4, and enhanced the level of cytokines IL-2, IFN-y and perforin in plasma on days 3, 5 and 7 in the intervention group compared to the control group. Our results are correlated with the others' results and proposed mechanism of COVID-19 triggering cytokine production. Due to the fact that the plasma collection process takes about 10 days from patients who have recovered from COVID-19 to infusion to patients who suffer from COVID-19, therefore it can be concluded that the level of cytokine detected in the patients has not been the donor's origin.

5. Conclusions

Understanding the pathophysiology of the disease and how the immune system affects the pathogen is of great importance. Our study findings suggest that CP therapy can be used to improve the immune response in patients with COVID-19 infection, possibly through neutralizing the virus, suppressing viremia, viral clearance, decreasing inflammation and shifting of immune response toward Th1.

CP therapy has demonstrated potential benefits for the treatment of coronaviruses in patients with severe illness who deteriorated even after prescribing other available treatments such as steroids and/or antiviral drugs. Although recovery therapy seems to be a safe treatment option both in general and in relation to COVID-19, it should be re-evaluated in future trials.

Abbreviation
Coronavirus 2019 (COVID-19), syndrome coronavirus 2 (SARS-CoV-2), randomized clinical trials (RCTs), COVID-19 convalescent plasma (CCP), convalescent plasma (CP), normal control plasma (NCP), polymerase chain reaction (PCR).

Conflict of interest
There is no conflict of interest to declare.

Consent for publications
All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
The present investigation was conducted in accordance with the recommendations of ethical guidelines. The research protocol was authorized by the Shiraz University of Medical Sciences (IR.SUMS.REC.1399.020).

Protection of Human Subjects and Animals in Research
All procedures followed were in accordance with the ethical standards of the Shiraz University of Medical Sciences on human experimentation. According to the Helsinki Declaration, informed written consent has been acquired from every patient or his legal relative.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Mozhde Heidari: Methodology, Validation, Formal analysis, Investigation Writing – original draft; **Ramin Yaghobi:** basic study conception and design; **Mohsen Moghadami:** clinical study conception; **Farid Zand:** clinical study conception; **Mohammad Javad Fallahi**: clinical study conception; **Ali Akbar Poufathollah:** basic study conception and design, manuscript preparation; **Golnoush Zarnegar**: sample collection; **Alireza Salah:** sample collection; **Saeedeh Soleimanian**: Methodology; **Mehdi Golshan**: Methodology; **Ali Jangjoo**: sample collection; , **Mohammad Hossein Karimi:** basic study conception and design, Writing – original draft, review & editing.

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