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Investigation of the potential of leukoreduction filters in the creation of anti-inflammatory compound

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ABSTRACT

Background: Some viruses such as SARS, SARS-CoV-2, and MERS cause an imbalance in immune responses and leads to an acute inflammatory reaction named cytokine storm. In this situation, an anti-inflammatory component can modulate the immune system and decrease mortality. The aim of this study was investigate the potential of leukoreduction filters (LRFs) in creating an anti-inflammatory compound.

Materials and methods: In this experimental study, firstly optimal dose of the anti-inflammatory drug was obtained through LRFs treatment with 0.1 mg, 0.4 mg, 0.6 mg of Betamethasone. Then inflammatory and anti-inflammatory cytokine in gene and protein level was evaluated. In the next step, LRFs were categorized into treatment 1, treatment 2, control assay, and control groups and treated with the optimal dose of the drug. Finally, the obtained compound was investigated for the concentration of IL1, IL6, and TNF- α as inflammatory and IL4, IL1Ra, and IL10 as anti-inflammatory cytokines.

Results: The results of the current study showed that the concentration of 0.4 mg of Betamethasone lead to a significant increase of anti-inflammatory cytokine in gene and protein levels. The results also showed that the Betamethasone treated groups (treatment1) causes a significant increase in the secretion of anti-inflammatory cytokine compares to the control while inflammatory cytokine remained at the control level.

Conclusion: The results showed that under influence of anti-inflammatory drug treatments the production and secretion of anti-inflammatory cytokines can be induced in LRFs.

1. Introduction

The immune system in the encounter of microorganisms progresses the inflammatory and anti-inflammatory responses as parallel. At the peak of infection, inflammatory responses increase to control infection, and then anti-inflammatory responses are overcome to regulate the inflammatory response [1–4]. Some viruses such as SARS, SARS-CoV-2, and MERS cause an imbalance in immune responses and lead to cytokine storm [5–6] with high mortality in potentiating patients with abnormal inflammatory responses [7–11]. The use of anti-inflammatory drugs leads to complications in the treatment process due to the suppression of inflammatory responses needed to overcome infection. Nowadays immunomodulatory treatment is used to decrease cytokine

storm symptoms, in this regard; some studies focus on MSC cell products (cell, supernatant, and microvesicle) [28–29] and some Studies used inflammatory cytokine blockers to cope with cytokine storm in COVID-19 [12–13]. Various mediators and cytokines are involved in preventing this phenomenon [14–16], and the use of an anti-inflammatory cocktail is needed for more acceptable and repetitive results.

Studies show that activation of monocytes following adhesion secret anti-inflammatory cytokines. In this regard, products such as Orthokine, Regenokine, and Autokine have been commercialized the decrease inflammation in osteoarthritis [17–19]. In Leukocyte-reducing filters (LRFs) Monocytes are separated by the adhesion mechanism [20]. Therefore, LRFs potentially create a suitable space for monocyte

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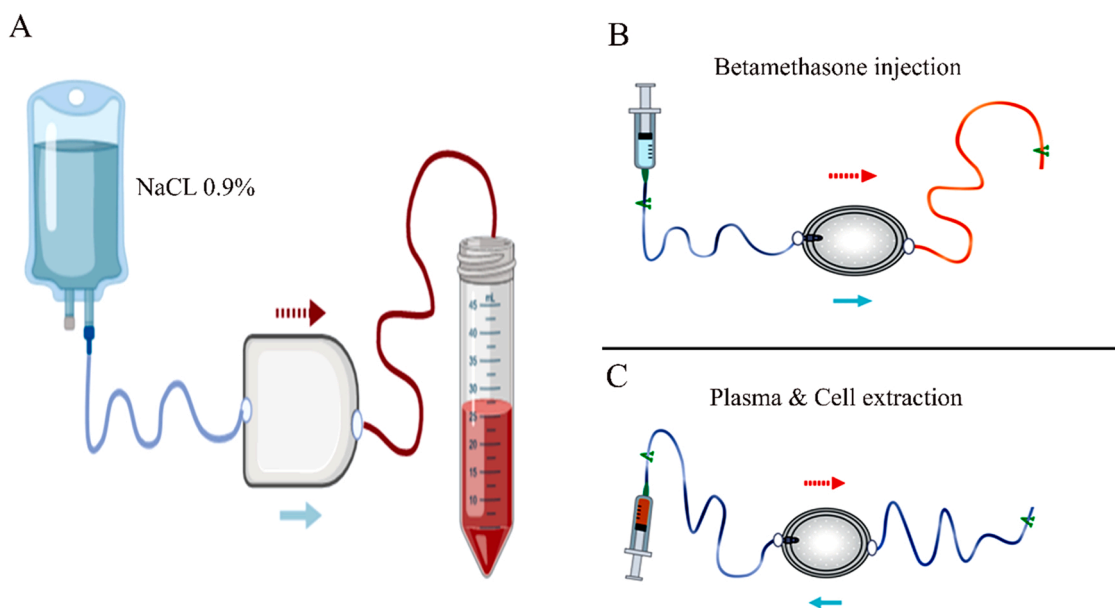


Fig. 1. Filter preparation and treatment. Direct washing for RBC removal (A), betamethasone injection in direction of blood filtration (B), and plasma and cell extraction in the opposite direction of blood filtration (C). (Red arrow: direction of blood filtration & Blue arrow: direction of filter manipulation).

activation and can be a cellular source for the preparation of various cytokines and mediators. Also, cells' direction to the anti-inflammatory phase can be useful for repeatability. Anti-inflammatory drugs such as betamethasone and dexamethasone increase the secretion of anti-inflammatory cytokines by inducing transcription of anti-inflammatory cytokines genes such as IL10, IL4, TFG- β , IL1RA as well as suppressing the expression of inflammatory cytokines genes such as IL1, IL6, TNF- α [21–27]. Since the leukocytes are trapped in the LRFs, Anti-inflammatory drugs can be removed from the filter after treatment without losing the cells and cells can be incubated for more time and secretion pure cytokines.

In this experimental study, we used Betamethasone as an anti-inflammatory drug to treat the adherent/activated monocytes in the LRFs and evaluated the final compound in terms of inflammatory and anti-inflammatory cytokines secretion profile.

2. Material and method

2.1. Filters preparation

41 BioR blood filters (FRESENIUS KABI, Austria) were collected from Tehran Blood Transfusion Center (TBTC, Iran) after leukodepleting 450 ml of whole blood from normal healthy donors. The exterior surfaces of the filters and tubing were wiped with 70% ethanol (Merck, Germany), and the following procedures were performed in a biosafety cabinet (Jal Tajhiz, Iran). Sterile scissors were used to cut the sealed ends of the tubing. For optimal treatment of the leukocytes in LRFs, the remaining RBCs were removed by rinsing the filter in the direction of blood filtration. One bottle of sterile injectable normal saline (NaCl 0.9%) was connected to the filter, and then pouches of normal saline to filter under influence of gravity. The amount of washing varies from 500 ml to 1000 ml in proportion to the number of trapped RBCs and the number of $< 2 \times 10^6$ RBC/ul was considered as the cutoff. (Fig. 1A).

2.2. Betamethasone dose optimization

9 prepared filters were obtained from the previous step selected to determine of optimal dose of Betamethasone. 3 filter was selected for each 0.1, 0.4, 0.6 mg concentrations. 10 ml of normal saline with a total concentration of 0.1, 0.4, and 0.6 mg of betamethasone was injected

into the filters for each dose in direction of blood filtration. Fig. 1B. The input and output filter cords were then locked and placed at 37 °C for 2 h. After this time, the filter was washed with 100 ml of normal saline, and the plasma source was injected into the filter and incubated for 6 h. Also, 3 filters with 30 min incubation without any treatment were considered as baseline control. Mononuclear cells and plasma were extracted from incubated filters with mechanical forces in the opposite direction of blood filtration. Fig. 1C then the final product was evaluated for IL1, IL6, TNF α , IL1Ra, IL4, and IL10 at gene and protein levels.

2.2.1. ELISA assay

The final product from the previous step was centrifuged at 2000 g for 15 min then the levels of inflammatory cytokines (IL-1 β , and IL-6, TNF α), and anti-inflammatory cytokines (IL10, IL1Ra, and IL4) in the obtained plasma were measured by ELISA and commercial kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Detection Sensitivity were 1 pg/ml, 0.7 pg/ml, 6.23 pg/ml 3.9 pg/ml 18.3 pg/ml, 10 pg/ml, for IL-1 β , and IL-6, TNF α , IL10, IL1Ra, and IL4 respectively. All assays were run in duplicate.

2.2.2. Gene expression assay

2.2.2.1. Leukocyte extraction and Density gradient cell separation. Cell precipitate obtained from the previous step was suspended and added to a density gradient medium (Inno-train, Germany) in a 2:1 vol ratio in a slow manner in order not to mix the layers. The tube was Centrifuged at 800 x g for 20 min with the brake OFF. The mononuclear cells were harvested carefully by inserting the pipette directly through the upper layer. The harvested cells were washed twice with PBS.

2.2.2.2. RNA extraction and Real-Time PCR. For molecular analyses, RNA extraction was initiated with mononuclear cells lysis induced by TRIzol reagent (Life Technologies Corporation; Carlsbad, CA, USA), followed by the addition of chloroform to extract RNA. Isopropanol was then added to precipitate RNA, followed by dissolution in nuclease-free water. The concentration and purity of the isolated RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific; Wilmington, DE, USA), and the integrity of RNA was verified by agarose gel electrophoresis. To perform quantitative real-time polymerase chain reaction (RT-PCR), oligo dT primers and the SuperScript reverse

Table 1
Primer sequences.

Sequence definition	Sense	Antisense
interleukin 1 beta (IL1B)	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
interleukin 6 (IL6)	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
interleukin 10 (IL10)	TCTCCGAGATGCCTTCAGCAGA	TCAGACAAGGCTTGGCAACCCA
tumor necrosis factor (TNF)	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
interleukin 1 receptor antagonist (IL1Ra)	ATGGAGGGAAGATGTGCCTGTC	GTCCCTGCTTCTGTTCTCGCTC
interleukin 4 (IL4)	CCGTAACAGACATCTTTGCTGCC	GAGTGTCTTCTCATGTTGGCT

Table 2
Treatment groups.

Condition	Treatment 1	Control assay	Treatment 2	Control
Betamethasone injection	✓	–	✓	–
Neutrophil removal	–	–	✓	–
Incubation time	8 h	8 h	8 h	30 min

transcriptase kit (Promega, USA) were used for reverse transcription. An EvaGreen QPCR master mix (Solis BioDyne, Estonia) was used for RT-PCR, with the following PCR parameters: initial denaturation (one cycle at 95_C for 15 min); denaturation, amplification, and quantification for 40 cycles at 95_C for 15 s, 56–64_C for 17 s, and 72_C for 25 s; melting curve at 65_C, with the temperature being gradually increased to 95_C. The mRNA expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase mRNA, and the changes were calculated according to the standard curve and efficiency (E) for each primer. Primer sequences used for RT-PCR are listed in Table 1.

2.3. Preparation of Treatment groups

8 prepared filters obtained from step 1 were selected for each group. For Treatment1 preparation, 10 ml of optimum dose of Betamethasone was injected into filters and incubated at 37 °C for 2 h. After this time, the filter was washed with 100 ml of normal saline and a plasma source injected into the filter and was incubated for 8 h. For Control assay preparation 10 ml of normal saline was injected into filters and incubated at 37 °C for 2 h. After this time, the filter was washed with 100 ml of normal saline and a plasma source injected into the filter and was incubated for 8 h. Treatment 2 preparations were conducted through injection of 10 ml of normal saline to the filter and kept overnight at 25 °C after this time the filter was washed with 100 ml of normal saline to remove the neutrophil debris then 10 ml of optimum dose of Betamethasone was injected to filters and incubated at 37 °C for 2 h. After this time, the filter was washed with 100 ml of normal saline and a plasma source injected into the filter and was incubated for 8 h. Finally, the Control group was created through plasma source injection to the filter and incubated for 30 min. After incubation time for each group, the plasma was extracted from the filter and centrifuge in 2000 g for 15 min then the incubated plasma was evaluated for inflammatory and anti-inflammatory cytokine concentration. Table 2.

2.4. Ethical consideration

The project was approved by the Ethics Committee of the High Institute for Research and Education in Transfusion Medicine. (IR.TMI.REC.1399.010).

2.5. Statistical analysis

Statistical analyses were carried out with SPSS software [SPSS 22, IBM]. Quantitative Data obtained from RT-PCR and ELISA was analyzed by a nonparametric test (Kruskal Wallis). A p-value of less than 0.05

Table 3
Gene expression profile.

		Gene Expression (% of Control)	P Value ^a
		Mean ± SD	
IL1	0.1 mg	2431.50 ± 1604.78	0.001
	0.4 mg	40.50 ± 14.34	0.021
	0.6 mg	74.50 ± 14.52	–
IL6	0.1 mg	99.50 ± 56.83	–
	0.4 mg	106.33 ± 23.31	–
	0.6 mg	86.67 ± 75.53	–
TNF-α	0.1 mg	1501.67 ± 200.14	0.086
	0.4 mg	157.67 ± 15.01	–
	0.6 mg	52 ± 5	–
IL1Ra	0.1 mg	1576.67 ± 1490.04	0.005
	0.4 mg	1281.50 ± 207.98	0.001
	0.6 mg	206.50 ± 78.93	–
IL10	0.1 mg	717.33 ± 345.58	0.004
	0.4 mg	562.50 ± 217.46	0.016
	0.6 mg	497.17 ± 345.83	–
IL4	0.1 mg	1899.17 ± 385.13	0.038
	0.4 mg	8386.00 ± 2380.01	0.001
	0.6 mg	1841.83 ± 435.20	–

^a Compare to Control.

Table 4
Cytokine secretion profile.

		Cytokine concentration pg/ml	P Value ^a
		Mean ± SD	
IL1	0.1 mg	199.00 ± 64.13	–
	0.4 mg	64.13 ± 11.82	–
	0.6 mg	7.07 ± 5.88	–
	Control	3.90 ± 1.81	–
IL6	0.1 mg	267.10 ± 43.83	–
	0.4 mg	106.33 ± 23.31	–
	0.6 mg	7.10 ± 5.85	–
	Control	3.44 ± 1.44	–
TNF-α	0.1 mg	18.09 ± 1.86	–
	0.4 mg	5.17 ± 5.25	–
	0.6 mg	8.37 ± 9.53	–
	Control	10.54 ± 5.83	–
IL1Ra	0.1 mg	778.24 ± 218.05	–
	0.4 mg	1582.91 ± 815.09	0.031
	0.6 mg	25.26 ± 24.42	–
	Control	57.67 ± 35.02	–
IL10	0.1 mg	31.35 ± 39.19	–
	0.4 mg	10.29 ± 7.87	–
	0.6 mg	29.22 ± 16.95	–
	Control	16.75 ± 20.35	–
IL4	0.1 mg	36.33 ± 16.65	–
	0.4 mg	47.67 ± 17.79	0.057
	0.6 mg	18.89 ± 17.58	–
	Control	5.22 ± 0.83	–

^a Compare to Control

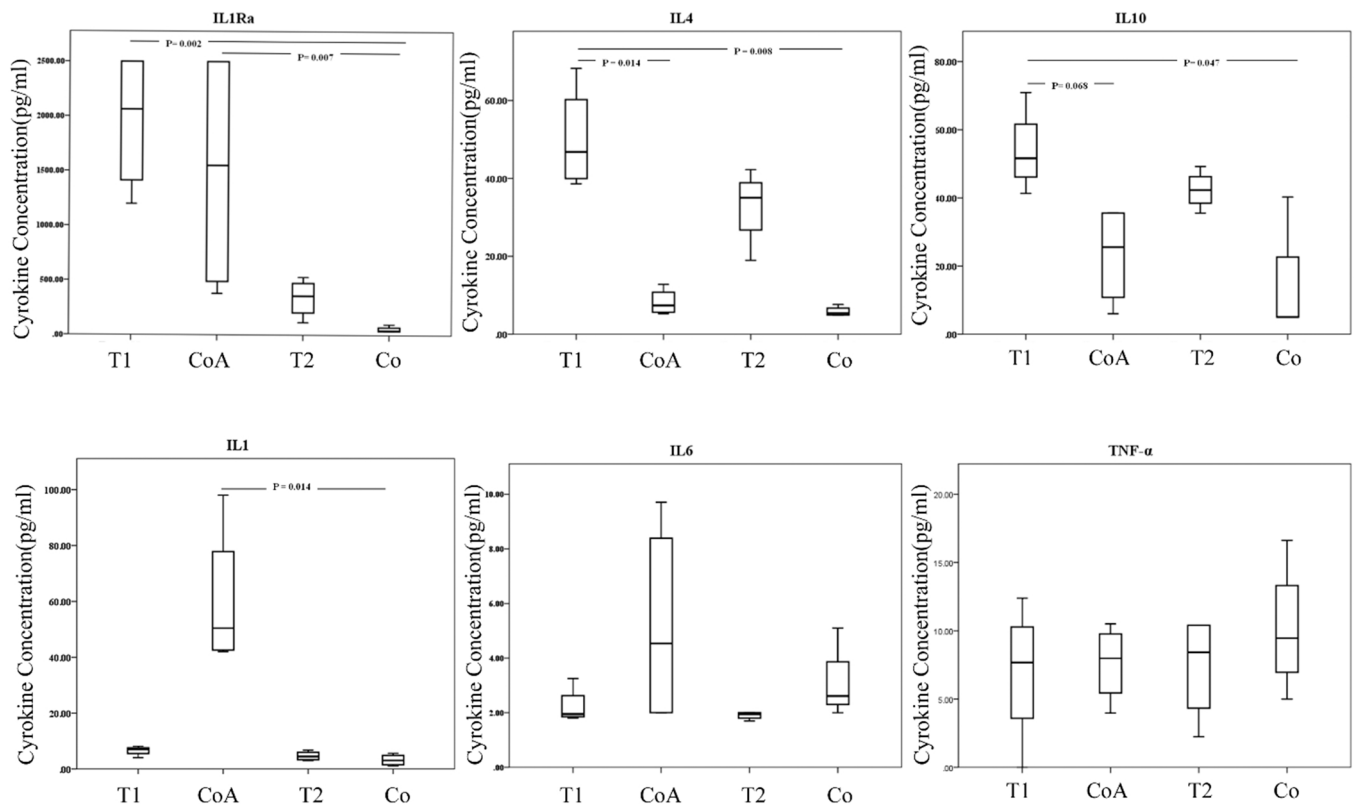


Fig. 2. Anti-inflammatory and inflammatory Cytokines profiles in different groups.

indicates the difference is significant.

3. Result

3.1. Determining the optimal dose of Betamethasone

3.1.1. Molecular result

Molecular result showed that 6 h incubation of filters with of 0.1 mg, 0.4 mg, and 0.6 mg of Betamethasone causes a significant increase in IL1 gene expression at a concentration of 0.1 mg ($P = 0.001$) and a significant decrease at a concentration of 0.4 mg ($P = 0.021$) compare to control. Also, the expression of TNF- α and IL6 genes did not show a significant difference compared to the control. In addition, IL1Ra gene expression showed a significant increase compared to controls at concentrations of 0.1 mg ($P = 0.005$) and 0.4 mg ($P = 0.001$). Also, an increase in IL10 gene expression was observed in all three concentrations, compare to control that was significant at concentrations of 0.1 mg ($P = 0.004$) and 0.4 mg ($P = 0.016$). The same increase was observed in IL4 gene, that was significant at concentrations of 0.1 mg ($P = 0.038$) and 0.4 mg ($P = 0.001$). (Table 3).

3.1.2. ELISA result

ELISA results showed that IL1Ra and IL4 concentrations were increased compared to the control at concentrations of 0.4 mg of Betamethasone which was significant for IL1Ra ($P = 0.031$) and borderline for IL4 ($P = 0.057$). Also, this Result showed that the concentration of inflammatory factors such as IL1, TNF- α , and IL6 at 0.4 mg of Betamethasone was lower than 0.1 mg concentration. (Table 4).

According to the result of dose determination the high expression of the IL10 gene was observed while cytokine level didn't change, so an increased secretion of this cytokine was predicted by increment incubation time, therefore the treatment time was raised from 6 h to 8 h for treatment groups.

3.2. Cytokine profile in different groups

The results showed anti-inflammatory factor (IL1Ra, IL10, IL4) had significant increase in the treatment 1 (Beta+/Neut+) group compared to the control (P Value = 0.008, 0.002, and 0.008 respectively). the results showed that in the control assay (Beta-/Neut+) group a significant increase in the secretion of IL1 and IL1Ra compare to control is visible ($P = 0.014$, 0.007 respectively), while other anti-inflammatory factors (IL4, IL10) in the Treatment1 compared to the control assay showed a significant and borderline decrease ($P = 0.014$, 0.068 respectively). On the other hand, the concentration of inflammatory factors in all groups did not show a significant difference compared to the control and Remained within the normal range. (Fig. 2).

4. Discussion

Studies have shown that monocytes' adhesion to plastic and glass surfaces leads to the creation of an anti-inflammatory cocktail named autologous conditioned serum [17–19]. but the efficiency of this product is controversial since the elevation of pro-inflammatory cytokine as well was observed [18,34]. The results of the current study showed that leukocytes trapped in LRFs can direct release the anti-inflammatory cytokines under influence of betamethasone treatment as an anti-inflammatory drug.

In this study, dose optimization results indicate that a 0.4 mg concentration of betamethasone at the level of the gene leads to an increase in the expression of genes related to anti-inflammatory cytokines. While, at the protein level, the level of IL10 cytokine did not increase. This result showed that more incubation time is needed to increase the protein levels of IL10 cytokine so the incubation time for the next steps was increased from 6 h to 8 h. The following treatment results showed that IL1Ra cytokines in the treatment1 group didn't significant difference compared to the treatment2 and control assay groups. In this regard, we interpret that the secretion of this cytokine is more affected by the

activation of monocytes due to their baseline condition (adhesion to LRFs fibers and incubation at 37 °C). However, a significant increase in cytokine levels of IL10, and IL4 in treatment1 compared to the control assay indicates the effect of betamethasone on stimulating the secretion of these anti-inflammatory cytokines. In this regard, Vladimir Riabov describes the development of an optimized cytokine cocktail consisting of IL4/IL10/TGFβ1 (M2Ct) that induces a long-term anti-inflammatory phenotype in human primary monocyte-derived macrophages and induces stable M2-like macrophage phenotype with a significantly decreased proinflammatory cytokine [30]. Accordingly, betamethasone probably progresses M2-like macrophage formation through secretion of IL10, and IL4 from LRFs leukocyte and finally leads to decreased pro-inflammatory cytokine and increased anti-inflammatory cytokine secretion profile. Ping Liu also reveals that macrophages have both pro-inflammatory (M1) and anti-inflammatory (M2) actions and M2 macrophages have four subtypes (M2a, M2b, M2c, and M2d) that stimulating factors for M2a formation is IL10 and IL4 [31].

In addition, the decrease in IL1Ra secretion in treatment2 (neutrophil depleted) compared to treatment1 showed that neutrophils are also involved in IL1Ra secretion. In this regard, McColl showed the ability of human neutrophils to produce the IL-1 receptor antagonist (IL-1Ra), a 17–23-kD protein to respond to GM-CSF and TNF-alpha [32] also Malyak demonstrate that IL-4 enhanced LPS-induced IL-1ra production by peripheral blood neutrophils and inhibited LPS-induced IL-1 beta production [33]. Moreover, the control assay result showed a significant increase in IL1 level compared to control which was according to Marijn Rutgers study that showed IL1 elevate in autologous conditioned serum compared to control [18,34]. This result showed that betamethasone in treatment 1 groups prevents the creation of proinflammatory conditions probably through M2a formation from adherent/activated monocytes.

In summary, this study creates modeling for the direction of LRF leukocytes to produce various cytokines and mediators under certain circumstances. This compound is more cost-effective than methods such as cloning. The main limitation of this study was the lack of an appropriate template for optimal anti-inflammatory compound concentration as a reference. Therefore animal studies are needed to evaluate the efficacy of the final product (cytokine cocktail or immune cells) through control of cytokine storms or septic shock. Also, it is suggested that the effect of other anti-inflammatory drugs such as methylprednisolone or dexamethasone is evaluated. Moreover, this study can be used as a model for the optimization of vaccines or the creation of a convalescent cocktail from the immune person.

CRedit authorship contribution statement

Sahar Balaghohi: Formal analysis, Methodology, Writing Original draft, Data Curation preparation, Investigation **Rasul Dabbaghi:** Conceptualization, Methodology, Investigation, Writing - Review & Editing **Saeed Mohammadi:** Validation, Methodology, Writing Review & Editing **Zahra Abbasi malati:** Data Curation, Investigation, Writing Review & Editing **Mohammad Reza Javan:** Investigation, Validation. **Pyman Eshghi:** Validation, Methodology, Writing Review & Editing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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