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Evaluating the Role of Silymarin in Coordinating the Relationship between Inflammation and Thrombosis by Affecting Endothelial Cells

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Abstract

Background: A widespread crosstalk between inflammation and coagulation has been shown in numerous studies. This suggests that coagulation can trigger an inflammatory response which ultimately leads to coagulation activation. Previous research has shown that polyphenols can affect blood pressure and endothelial dysfunction, resulting in reduced risk of cardiovascular diseases. This study aimed to investigate whether Silymarin, a flavonolignans, could play a role in the interaction between inflammation and coagulation by influencing endothelial cells.

Materials and Methods: In this experimental study**,** human umbilical vein endothelial cells (HUVECs) were seeded with and without various concentrations of silymarin. In vivo, treatment with silymarin was also carried out. Coagulative and fibrinolytic factors, including Von Willebrand factor (VWF) and Factor VIII (FVIII), tissue plasminogen activator-1 (TPA-1), and inflammatory factors, including interleukin 8 (IL-8) and tumor necrosis alpha-factor (TNF-α), were evaluated by flow cytometry, Real-Time Polymerase Chain Reaction (qPCR), Enzyme-Linked Immunosorbent Assay (ELISA) and Immunocytochemistry (ICC).

Results: Silymarin increased the gene expression, release, and storage of VWF while diminishing the gene expression, release, and storage of TPA-1 ($P < 0.05$). The activity of FVIII was dramatically increased, and IL-8 and TNF-α levels were augmented. The in vivo study also indicated an elevated plasma level of VWF and IL-8 by silymarin administration.

Conclusion: The results showed that, although silymarin reduces inflammatory factors, it can affect coagulation factors by increasing the levels of VWF and FVIII activity and inhibiting TPA-1 production, thereby making thrombosis probable. Consequently, it is advisable to prescribe this medication with caution for individuals who are susceptible to thrombotic events.

Keywords: Factor VIII (FVIII), Inflammation, Silymarin, Von Willebrand factor (VWF)

Introduction

Various degrees of coagulation activation are present in most patients with systemic inflammatory conditions. Recent studies have shown a great interaction between coagulation factors and inflammatory markers, resulting in coagulation activation and inflammatory activity (1). Proinflammatory cytokines, however, can both activate and downregulate the coagulation system.

Consistently, stimulation of endothelial cells involves the huge secretion of Von Willebrand factor (VWF), a coagulant glycoprotein mainly located in Weibel-Palade bodies (WPBs) associated with Factor VIII (FVIII) (2). Besides, studies have identified an important role for VWF in the sequestration of interleukin-8 (IL-8), an inflammatory cytokine, in WPBs (3). Moreover, a positive correlation of IL-8 secretion with both tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine,

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and VWF has been reported to be in the circulation (4, 5). Tissue-type plasminogen activator (TPA-1), a crucial factor in fibrinolysis bearing the cleavage of plasminogen to plasmin and subsequently fibrin degradation induction, is also up-regulated in endothelial cells in response to mediators such as interleukin-beta, TNF-α, thrombin, histamine, and vascular endothelial growth factor (6). Silymarin, a flavonolignans, extracted from milk thistle (*Silybum marianum*, Asteraceae), has extensively been used in the treatment of liver disorders, such as hepatitis, and cirrhosis, which is owing to its hepatoprotective and antioxidative properties (7-10). Recent studies have emphasized the anti-inflammatory effect of silymarin (11-17). Considering these traits of silymarin, this study aims to investigate the effect of silymarin on the endothelial cell release of VWF, FVIII, and TPA-1, the most important factors in clot formation and fibrinolysis. Besides, the levels of IL-8 and TNF-a are measured to check if silymarin can orchestrate a relationship between coagulative and inflammatory factors.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were received from the National Cell Bank (NCBI) belonging to the Pasteur Institute of Iran. The cells were cultured in a complete medium that contained Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM-F12, Gibco), 10% heat-inactivated Fetal Bovine Serum (FBS, Gibco, Invitrogen, UK), 50 U/ml penicillin G, and 50 µg/ml streptomycin sulfate (P/S, Gibco). Silymarin was purchased from Sigma-Aldrich (St. Louis, Mo), and a stock solution was prepared in Di Methyl SulfOxide (DMSO, Merck).

Vasopressin was acquired from Sigma-Aldrich, and the corresponding solution was prepared in a sterile medium and used as a positive control.

Measurement of the surface VWF attachment of HUVECs

The cells were split in a 6-well plate (200,000 cells per well) with and without silymarin at the concentrations of 25, 50, and 100 µg/mL or vasopressin (0.01 mM/mL) as determined by MTT. The cells were harvested after 24 hours of incubation, a quantitative study was performed using a flow cytometer ((Becton Dickinson, Oxford, UK), and the results were analyzed using the Graph pad Prism software.

Measurement of VWF, TPA-1, IL-8, TNF-α secretion, and FVIII activity by HUVECs

After 24 hours of cell treatment, the supernatants were separated to detect the secretion of VWF with an ELISA kit (AsserachromVWF, Stago, Paris, France). The results were reported as normal values in percentages. The minimum detectable concentration of VWF was 0.35 %. FVIII activity was also measured in the supernatant on STA-R automated coagulation analyzer (Diagnostica Stago, STA® analyzers, USA) using substrate plasma for Factor VIII assay. IL-8 and TNF-α detection was also performed using ELISA, based on the manufacturer's instructions (Abcam, UK).

Immunocytochemistry (ICC)

HUVECs were cultured in a 4-well plate at a density of 50,000 cells per well under the conditions of 37°C and 5% CO2 within a humidified environment for 24 hours, either in the presence or absence of silymarin at a concentration of 100 mg/ml. Following this, the cells were permeabilized with Triton X-100 and subsequently stained with DAPI and a sheep polyclonal anti-von Willebrand factor antibody which was conjugated to fluorescein isothiocyanate (FITC; Abcam) at a dilution of 1:200. The silymarin-treated HUVECs were also stained with

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DAPI and an anti-tissue plasminogen activator (TPA) antibody (LifeSpan BioSciences, 1:200), and incubated overnight at 4°C as previously specified. Lastly, the cells were exposed to a secondary antibody (anti-rabbit antibody, Alexa Fluor 594, Invitrogen™). Imaging was conducted through fluorescence microscopy, and the results were analyzed with the ImageJ software and compared to the control conditions (medium and DMSO) for each experimental setup.

Real-time Polymerase Chain Reaction (PCR)

The HUVECs were treated with different concentrations of silymarin. They were collected after 24 hours, and the total RNA was extracted using AccuZolTM (Bioneer) according to the manufacturer's instructions. The quantity and quality of the isolated RNA were checked through optical density measurements in 260 and 280 nm and agarose gel electrophoresis. Using 1µg of RNA, a reverse transcription reaction was conducted (cDNA Synthesis kit, Bioneer, AccuPower Cycle Script RT PreMix) according to the manufacturer's instructions. VWF, TPA-1, and GAPDH (housekeeping genes) expression levels of mRNA were detected using a Rotor-Gene_ 6000 (Corbett Research, Sydney, Australia) and by the ΔΔCT method. PCR reactions were performed in a volume of 10 µL (5 µl PCR Master Mix, AccuPower 2X Greenstar qPCR Master Mix, Bioneer, 1 µl cDNA, 50 ng, 2 µl PCR-grade water, and 10 pmol of specific primers, Invitrogen, UK). VWF primers (forward: 3′- GGGCTGTGTGGCAACTTTAAC -5'; reverse: 3'-CATAAGGGTCCGAGGTCAAGG -5′), TPA-1 primers (forward: 3′-GTGACTGCTACTTTGGGAATGG -5′; reverse 3′-TTCTGTGCTGTGTAAACCTTGC -5′), and GAPDH primers (forward: 3′-AACATCATCCCTGCCTCTACTGG-5′; reverse 3′-TCCGACGCCTGCTTCACC-5′) were designed. The thermal cycling conditions were as follows: 95ºC for 5 min, 40 cycles at 95ºC for 15 s, 60ºC for 45 s, and a melting curve program from 60 to 95ºC. The mRNA productivity was measured by the comparative threshold cycle (Ct) method so as to normalize it to the housekeeping gene (GAPDH) and compare it to the control by the 2-ΔΔCt method.

In vivo study

The Ethical Committee of Animal Experiments at Iran University of Medical Sciences approved the present study (approval number: 29807). Male WISTAR rats, aged 16-20 months and weighing 450–500 g at the time of the experiments, were purchased from Razi Institute, Karaj, Iran. A group of eight rats was studied in the present study. Before going through intraperitoneal (IP) administration of silymarin, tail blood sampling on EDTA was carried out from all the rats. Later, a dose of 100 mg/kg silymarin dissolved in polyethylene glycol was injected into them all. After 24 h, the tail blood of all the rats was collected. Then, the VWF and IL-8 levels in plasma were measured with the ELISA method.

Statistical analysis

The experiments were done at least three times. The expression levels of VWF and TPA-1 were compared to the normal ones using Student's t-tests and ANOVA. A paired sample t-test was used to analyze the effect of silymarin on the expression of VWF and TPA-1 in rats. The images were analyzed with the Image J software & FlowJo. The data were analyzed with Graph pad prism five and expressed as mean ± standard deviation (SD). The level of significance was set at $p < 0.05$. The data are available upon request from the authors.

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Results

Effect of silymarin on the Il-8, TNF-α and TPA-1 release, FVIII activity and VWF expression

The levels of the secreted VWF, TPA-1, IL-8 and TNF-α in the supernatant were measured after the treatment of HUVECs for 24 h with silymarin (Sigma-Aldrich) (50 µg/ml) and vasopressin (Sigma-Aldrich) (0.01mM). There was a significant increase in the levels of VWF, IL-8, TNF- α and FVIII activity ($P = 0.04$; $P = 0.0014$, $P = 0.0032$, $P = 0.0001$, respectively), whereas a reduced level of TPA-1 ($p = 0.003$) was found when compared with the control samples as evaluated by an ELISA reader. The result also showed a significantly increased level of VWF cell surface expression after the treatment of the endothelial cells with silymarin ($P < 0.001$, Figure 1).

Effect of silymarin on VWF and TPA-1 storage

The results demonstrated the significant enhancement of localized VWF and the decreasing level of TPA-1 storage following the endothelial cells treatment with silymarin, compared to the control samples ($p = 0.004$ and $p = 0.007$, respectively). In this regard, immunofluorescence staining, an Olympus IX71 inverted microscope and the ImageJ software were used (Figure 2).

Effects of silymarin on VWF and TPA-1 gene expression

As shown in Figure 3, the treatment of HUVECs with silymarin significantly increased the VWF mRNA expression ($p = 0.011$), while the TPA-1 mRNA expression was decreased ($P = 0.047$).

Effect of silymarin on the plasma levels of VWF and IL-8 in the rats

The plasma levels of VWF and IL-8 were detected before and after the intraperitoneal injection of silymarin. The results demonstrated an increase in the VWF and IL-8 plasma levels 24 h after the intraperitoneal injection ($p = 0.03$ and $p = 0.02$, respectively) (Figure 4).

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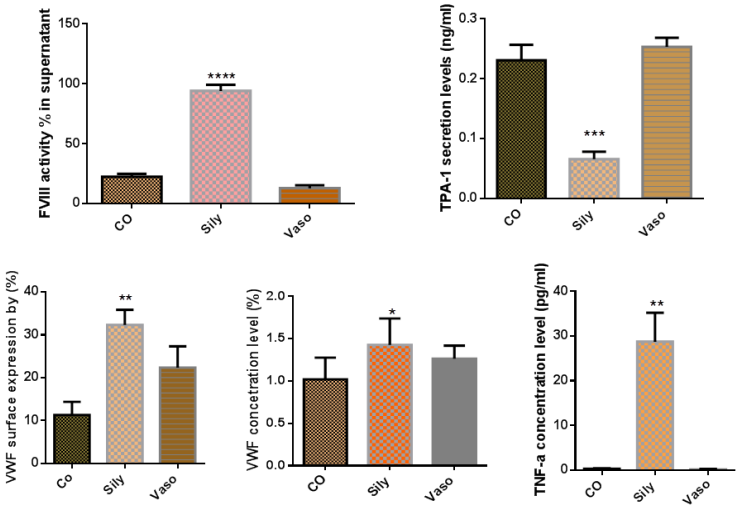

Figure 1. The effect of silymarin on the VWF, TPA-1, IL-8, TNF-α surface expression or secretion and FVIII activity by HUVECs: The histogram shows a significant diminished level of TPA-1 after treatment with silymarin for 24 h at 50µg/m while there are enhanced levels of IL-8, and TNF-α secretion and VWF surface expression and secretion, when compared with controls as measured by ELISA and flow cytometry. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-hoc test. They are presented as percentage and mean ± SD, where (*) denotes P < 0.05, (**) specifies P < 0.01, (***) point to P < 0.001 and (****) indicates P < 0.0001. Co: Control (DMSO), sily: Silymarin, Vaso: Vasopressin, VWF: von Willebrand factor, TPA-1: tissue-type plasminogen activator, IL-8: interleukin 8, and TNF-α: tumor necrosis factor alpha.

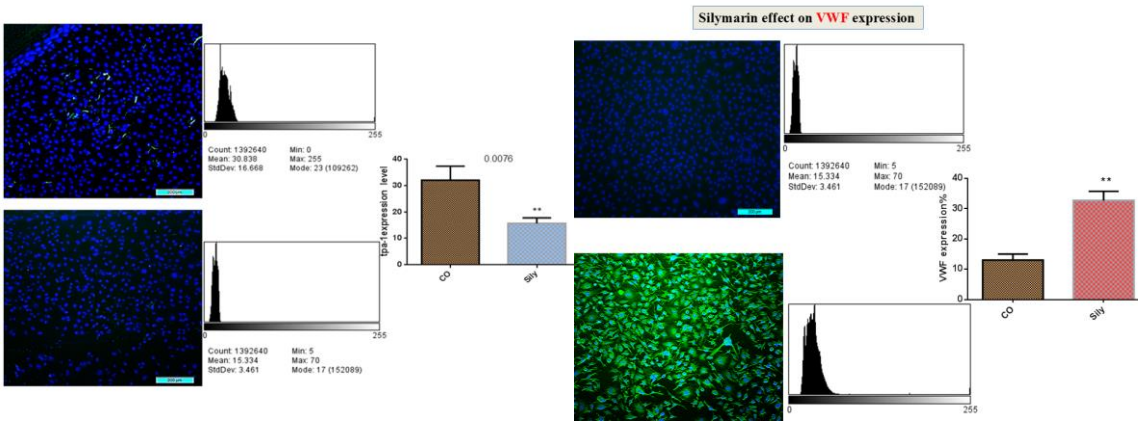

Figure 2. The effect of silymarin on VWF and TPA-1 storage: Images of the HUVECs treated with silymarin (50 µg/mL) stained with DAPI and anti-VWF-FITC antibody (down-left) and the control (medium, DMSO) treated cells stained with DAPI and anti-VWF-FITC antibody (top-left). The silymarin -treated cells stained with DAPI and anti-TPA-FITC antibody (down-right) and the control (medium, DMSO) treated cells stained with DAPI and anti- TPA-FITC antibody (top-right) where (**) indicates P < 0.01. An independent samples t-test was used to analyze the data. Sily: Silymarin, DAPI: 4'-6-diamidino-2-phenylindole, HUVEC: human umbilical vein endothelial cell culture, VWF: von Willebrand factor, TPA-1: tissue-type plasminogen activator

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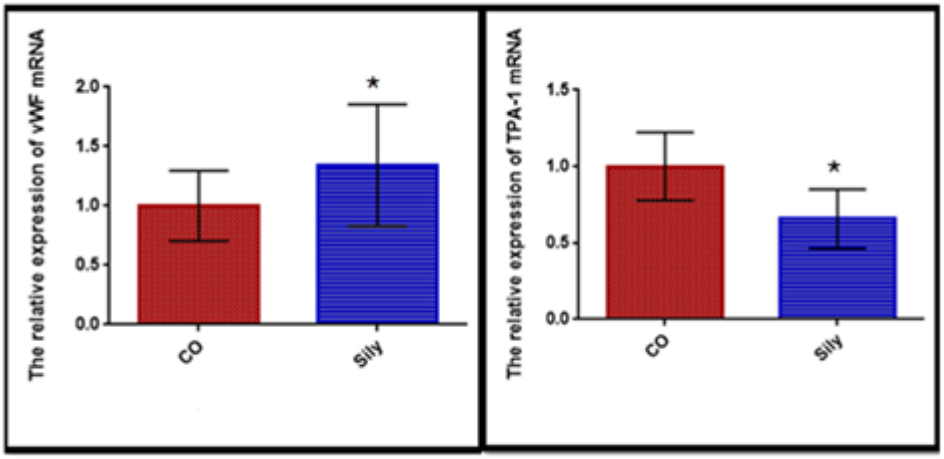

Figure 3. The effect of silymarin on VWF and TPA-1 gene expression in the endothelial cell: A significant increase in VWF and a decrease in gene expression were observed after treatment with silymarin at 50 µg/ml in comparison to control as normalized with the housekeeping GAPDH. An independent samples t-test was used to compare the means of the two groups. Co: Control, sily: Silymarin, vaso: vasopressin, VWF: von Willebrand factor, TPA-1: tissue-type plasminogen activator. The data are presented as percentages and mean ± SD, where (*) denotes $P < 0.05$.

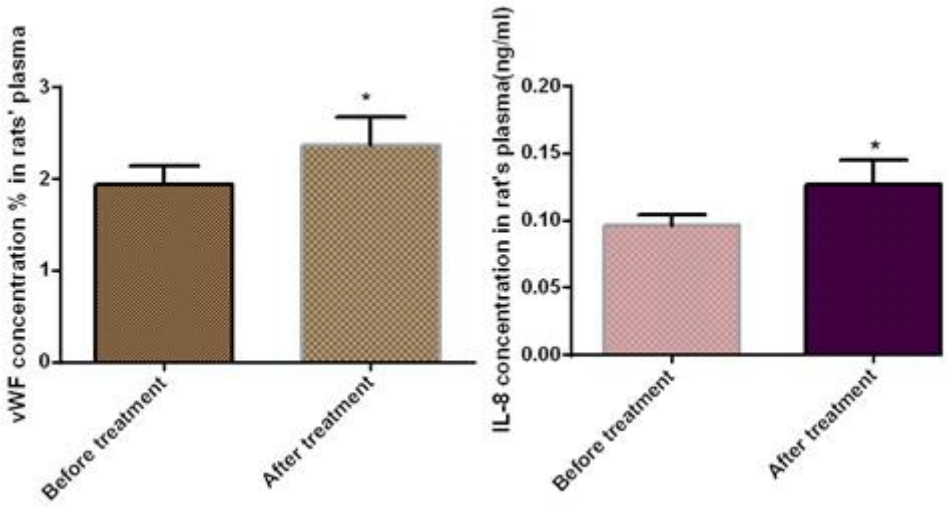

Figure 4. The effect of silymarin on the plasma levels of VWF and IL-8 in the studied rats: Intraperitoneal (IP) injection of silymarin in rats revealed a significant increase in the plasma levels of VWF and IL-8 after 24 h when compared with the case before the injection. A paired samples t-test was used to analyze the data. Sily: Silymarin, VWF: von Willebrand factor. The data are presented as percentages and mean ± SD, where (*) denotes $P < 0.05$.

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Discussion

As the results showed, although silymarin reduced inflammatory factors, including IL-8 and TNF-α, it could increase coagulation factors by increasing the levels of VWF and FVIII activity and inhibiting fibrinolysis through suppressing TPA-1 production. This may have caused the likelihood of thrombosis. Until now, there is no report of the effect of silymarin on VWF and TPA-1 expression at the level of gene or protein. Our novel findings, nevertheless, point to increased levels in VWF surface expression and secretion, which were confirmed by the PCR results. This indicates that the increased levels of VWF expression and secretion could be due to both being released from Wible-pulled bodies and elevating the mRNA expression. As VWF is the principal carrier of FVIII, the activity of this factor was also measured in the present project; there was a tremendous increase in the FVIII activity. Besides, the in vivo study showed an increased level of VWF, which was consistent with the in vitro findings. Concomitantly, a decreased plasma level of TPA-1 was found. Since coagulation and fibrinolytic systems are highly interconnected and they function through mechanisms that maintain hemostatic balance, the results of the present study may be of substantial relevance. To the best of our knowledge, there are no prior investigations about the impact of silymarin on clotting and fibrinolysis factors. Assuming that VWF, FVIII and TPA-1 play essential roles in hemostasis, any variations in their levels can be life-threatening and may lead to thrombosis or bleeding (3-5, 18). Desmopressin is known as a pharmaceutical agent that promotes the degranulation of Weibel-Palade bodies (WPB) and increases the circulation of VWF in patients with hemophilia and von Willebrand disease (vWD) (19). The current study specifies that silymarin has the potential to enhance the expression and secretion of VWF and FVIII activity while simultaneously reducing the expression and secretion of TPA. Accordingly, silymarin administration may be as useful as desmopressin or vasopressin for individuals suffering from vWD and hemophilia. To verify it, a comparison was performed of the effects of silymarin and vasopressin on the VWF release from HUVECs. Our in vitro results demonstrated silymarin with a greater pronounced effect than vasopressin; however, the study does not clarify which agent is more promotive in the early release of VWF. While no adverse effects of silymarin have been indicated so far, the present study suggests its likely effect as a novel therapeutic choice for dealing with deficiencies in FVIII and VWF. Moreover, the treatment of endothelial cells with silymarin caused a rise in TNF-α level. Previous research has shown that TNF-α can diminish endothelial fibrinolytic activity, which may explain the detected reduction in TPA-1 levels as a consequence of the increased TNF-α (4, 6, 18). Previous studies have highlighted the significant function of VWF in the sequestration of IL-8 within Weibel–Palade bodies (3). This shared storage mechanism is likely to facilitate a concurrent release of these molecules. Similarly, both IL-8 and TNF-α have been shown to stimulate the release of ultra-large VWF (ULVWF), resulting in a buildup of hyper-reactive ULVWF on the surfaces of endothelial cells and in plasma, as previously reported (3). In this context, our findings showed an increase in VWF binding at the surface of HUVECs, accompanied by elevated levels of IL-8 and TNF-α secretion following silymarin treatment. Further research on the bioactive components of silymarin may explain which specific substance is most dominant in these detected effects.

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Beyond its interaction with VWF and IL-8, this flavonoid may also increase the expression of FVIII activity in HUVECs while simultaneously reducing the secretion and expression of TPA-1, as evaluated in the present study.

Conclusion

The findings of this study suggest that silymarin may enhance the activity of the coagulation system while simultaneously diminishing the function of the fibrinolytic system. This calls for further in vivo investigations for justification. Upon such validation, silymarin could potentially be utilized to promote the release of VWF and FVIII in people with coagulation disorders, including von Willebrand disease, mild hemophilia A, and thrombocytopenia. However, more research is essential to prove these effects and clarify the mechanisms involved. It is also advisable to take caution when administering conventional silymarin therapy to patients with cardiovascular conditions who are at the risk of thrombotic events.

Altogether, these findings specify that, even though silymarin reduces inflammatory factors, it can promote coagulation by increasing VWF and FVIII activities and inhibit fibrinolysis by suppressing TPA-1 production, thereby making thrombosis probable. This drug should, therefore, be cautiously prescribed for patients prone to thrombosis.

Ethical considerations

All the animal experiments were performed based on the guidelines of NIH and the Ethics Committee of Iran University of Medical Sciences (ID: IR.IUMS.REC.1401.1031).

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Authors' contributions

Minoo Shahidi, the director of the project, conceived the manuscript and revised it. Mahmood Barati and Minoo Shahidi carried out the QPCR and flow cytometry. Roya Sharifi, Fereshteh Parhizkary and Fatemeh Sadeghi Shirazi performed the ELISA, ICC tests and provided the clinical data. Farhad Zaker and Kazem Mosavizadeh analyzed the data. Roya Sharifi and Minoo Shahidi wrote the manuscript.

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Conflict of interest

The authors declare no competing interests.

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