

Short Research Article**The effects of silymarin on serum soluble apoptosis markers in β -thalassemia major patients receiving desferrioxamine****Abstract**

Iron deposition in visceral organs causes tissue damage in thalassemia patients, despite appropriate chelation therapy e.g. desferrioxamine. Excess iron can generate reactive oxygen species (ROS) that may lead to cell death and apoptosis. Thereby, antioxidants such as plant flavonoid can be an effective treatment to reduce ROS in thalassemia patients. In this study, we measured the serum levels of apoptosis markers in thalassemia patients treated by silymarin and desferrioxamine. Serum levels of soluble apoptosis markers including soluble CD95 (sCD95), sCD95L, sTNF receptor type 1 (sTNFR1), sTRAIL and cytochrome C, were measured in two groups of 40 thalassemia major patients with ELISA kits. The patients were randomized into two groups: one group was received desferrioxamine and placebo and the other group received the combination of desferrioxamine and silymarin. There were no statistically significant differences in serum concentration of apoptosis markers between the desferrioxamine group and combined therapy of desferrioxamine and silymarin groups. In conclusion, assessing the membrane levels of these markers is necessary to validate these results, as they are not expressed equally in different tissues.

Keywords: thalassemia, iron overload, desferrioxamine, silymarin, sTNFR1, sCD95, sTRAIL, cytochrome C, sCD95L

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30 **Introduction**

31 Iron overload is the most common clinical complication in thalassemia patients, and iron
32 deposition in visceral organs causes tissue damage despite appropriate chelation therapy (e.g.
33 desferrioxamine). [1]

34 The exact mechanism of tissue damage and iron deposition in visceral organs is not clear;
35 however, it seems that generation of Reactive Oxygen Species (ROS) through the Fenton
36 reaction is one of the possible mechanisms.[2]

37 On the other hand, antioxidants such as plant flavonoid can be an effective treatment to
38 reduce ROS in thalassemia patients. Silymarin is a non-toxic flavonoid, which widely consumed
39 as a dietary supplement for its strong antioxidant and anti hepatotoxic efficacy. In a recent
40 clinical trial, thalassemia patients with severe iron overload were safely treated with a
41 combination of silymarin and desferrioxamine. Silymarin was generally well tolerated with no
42 detectable abnormalities in complete blood count, cell differential, liver or renal functions. [3]

43 Apoptosis is initiated by death-inducing transmembrane receptors. These receptors include,
44 TNF receptors and a variety of immune cell receptors and their corresponding ligands. [4] Also,
45 apart from membrane-bound, receptors are detectable in soluble forms, due to shedding from the
46 cell surface. In the present study, we measured the concentration of soluble apoptosis markers,
47 including: CD95 (sCD95) and sCD95L, s TNF receptor type 1 (sTNFR1), sTRAIL and
48 cytochrome C, in the serum of thalassemia patients and determined the changes in these
49 markers after a combined therapy with silymarin and desferrioxamine.

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51 **MATERIALS AND METHODS**

52 β -thalassemia patients (No=80) were selected from referrals to Sayed-o-shohada clinic of
53 thalassemia, Isfahan University of Medical Sciences. All patients had homozygous β -thalassemia
54 major, were regularly transfused to maintain the hemoglobin level above 9.5 g/dL and chelated
55 with s.c.desferrioxamine. Patients were excluded from the trial if they had one of the following
56 conditions: under 13 years of age, chronic renal or heart failure, iron chelation therapy with iron
57 chelators other than desferrioxamine, hepatitis B or C infection, a history of a positive HIV test,

pregnancy, and antioxidant or herbal medicine consumption . Oral and written informed consents were obtained. Blood was taken from patients before a scheduled transfusion.

The protocol for this study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (Isfahan, Iran).

The trial was a randomized, double-blind, placebo controlled, 6-month study of silymarin add-on therapy in β -thalassemia major patients on desferrioxamine monotherapy.

Patients were randomized into a treatment group and a placebo group based on the date of their first visit after enrollment. The treatment group received 140 mg Legalon®, Madaus Pharma orally, at least an hour before food, three times a day, 7 days a week with their routine desferrioxamine (Novartis Pharma AG, Basel, Switzerland) dose of 4–6 days per week. The placebo group received tablets identical to Legalon® that were specially prepared for this study. Fasting blood samples were drawn from all subjects and allowed to clot at room temperature. Serum was stored at -80°C until analysis.

Detection of soluble apoptosis markers in the sera was performed using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria). The sensitivity of the ELISA kits was 13.2 pg/ml for sCD95, 0.7 ng/ml for sCD95L, 0.05 ng/ml for sTNFR1, 0.05 ng/ml for cytochrome C and, 5 pg/ml for sTRAIL.

Statistical analysis

Statistical analysis was performed using SPSS 15 version for windows program. Statistical significance of the differential circulating levels of apoptotic markers in thalassemia patients was determined using the non-parametric Sample T test. The median and mean $\pm$ SD of the data were calculated.

Results

Clinical characteristics of β -thalassemia major patients are summarized in Table 1. A total of 80 thalassemia patients in two groups were successfully recruited to the study. In the placebo group,

40 patients continued receiving desferrioxamine, and in the silymarin group, 40 patients received a combined regimen of desferrioxamine and Legalon. There was no statistically significant difference in the mean levels of sFas, sFasL, TNFR1 and cytochrome C between the placebo group vs silymarin group. Also concentration of these markers in serum of thalassemia patients was near or below the lower limit of detection in two groups. sTRAIL was not detectable in two groups. (Table2)

Discussion

In the present study, we found that serum concentrations of sFas, sFasL, sTNFR1, sTRAIL and cytochrome C were near or below the lower limit of detection in thalassemia patients. Also no significant differences were observed between two groups in serum concentration of sFas, sFasL, sTNFR1, sTRAIL and cytochrome C.

Although we do not yet fully understand the underlying mechanism of apoptosis in thalassemia patients, possible explanations for this phenomenon include: reactive oxygen species (ROS) through Fenton reaction and iron-mediated oxidation and death receptor mediated pathways, such as Fas-Fas ligand interactions. [1, 5]

There is many data that show murine sFasL is non-apoptotic and, sFasL has even been shown that act to antagonize of mFasL activity. [6] Thus, the concentration of sFas and sFasL, that was near or below the lower limit of detection in thalassemia patients, may correspond to higher levels of CD3+CD95+ T lymphocytes, as previously demonstrated by Gharagozloo et al.[7] Also studies have shown that silymarin induces apoptosis by caspase-independent and caspase dependent manner. [8] So assessment of membrane markers is necessary to corroborate these results, as they are not expressed equally in different tissues.

Mitochondria have a key role in apoptosis by integration in different proapoptotic pathways and releasing of cytochrome c in to the cytosol. To determine of cytochrome c as an apoptotic marker, there is need to do accurate and precise laboratory techniques such as immunohistochemical staining and immunoblotting.[8]

Also a comparatively low level of sTNFR1 and TRAIL in this study can be viewed as evidence against an inflammatory condition in thalassemia patients.

In this study we compared the levels of apoptotic factors in their soluble form, therefore further trials assessing the membrane levels of such markers can help us have a better understanding of the underlying mechanism for apoptosis and tissue damage in thalassemia patients and how silymarin can affect this phenomenon.

References

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Table 1 Clinical and biological features of patients with β -thalassemia
Major

Features	Values
Age (year)	21.8±5.4
Hemoglobin (g/dl)	9.5±0.9
Body mass index (kg/m2)	20.26±2.6
Desferrioxamine dosage (mg/kg)	40–50
Desferrioxamine treatment(nights/week)	5±1
Transfusion interval (day)	16.8±3.2
Transfusion volume (ml) ^a	535±123
Splenectomy (%)	45.71

^a Transfusion volume is the volume of blood that is transfused to patients in each transfusion referral.

Table2: Concentrations of soluble Fas, Fas ligand ,TNFR1,cytochrome C in serum
Concentrations

Apoptosis markers	Placebo (n=40)	Silymarin (n=40)
sFas (pg/ml)	21.52±18.2	23.4±20.3
sFasL (ng/ml)	0.09±0.06	0.08±0.07
sTNFR1 (ng/ml)	0.12±0.1	0.11±0.1
Cytochrom C (ng/ml)	0.11±0.1	0.20±0.29