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# The Effect of Silymarin on Serum Concentration of Soluble Apoptosis Markers in $\beta$ -Thalassemia Major Patients Receiving Desferrioxamine

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### Authors' contributions

This work was carried out in collaboration among all authors. Authors MG, NE and BM designed the study and wrote the protocol. Author NE collected all samples, and performed experiments. Authors NE and MG wrote the first draft of the manuscript. Authors MM and MGS did the literature search and also wrote part of the manuscript. All authors read and approved the final manuscript.

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## ABSTRACT

**Background:** Despite appropriate chelation therapy with desferrioxamine, iron deposition in visceral organs causes tissue damage in thalassemia major patients. Excess iron can generate reactive oxygen species (ROS) that may lead to cell death and apoptosis. Therefore, antioxidants such as plant flavonoids can be an effective treatment to reduce ROS in thalassemia patients.

**Aims:** In this study, we aimed to investigate the serum levels of apoptosis markers in  $\beta$ -thalassemia major patients treated with silymarin and desferrioxamine.

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**Study Design:** This study was a randomized, double-blind, placebo controlled 6-month clinical trial  
**Place and Duration of Study:** This clinical trial was carried out at Sayed-Al-Shohada clinic of thalassemia, Isfahan University of Medical Sciences, Iran, for 6-month between August 2009 and February 2010.

**Methodology:** The patients were randomized into two groups: one group (A) received desferrioxamine and placebo and the other group (B) received a combination of desferrioxamine and silymarin. Serum levels of soluble apoptosis markers including soluble Fas (sCD95,sFas), sCD95 ligand (sCD95L,sFasL), sTNF receptor type 1 (sTNFR1), sTRAIL and cytochrome C, were measured before and after the trial in two groups of 40 thalassemia major patients using ELISA kits. Statistical analysis was performed using SPSS 15 version for windows program; results were expressed as mean  $\pm$  standard deviation (SD) and significance set at  $P < 0.05$ .

**Results:** There was no statistically significant difference between desferrioxamine group and combined therapy group in serum concentration of apoptosis markers, except for sTNFR1 level (which decreased from  $0.16 \pm 0.12$  to  $0.11 \pm 0.10$  ng/ml) in the silymarin treatment group ( $P < 0.05$ ).

**Conclusion:** Our observation of decreased circulating concentrations of sTNFR1 in silymarin-treated patients may reflect anti-inflammatory activity of silymarin in  $\beta$ -thalassemia major. The finding that silymarin treatment had no effect on the level of soluble apoptosis marker could be an evidence for safety of silymarin treatment in thalassemia major patients. However, measuring the membrane levels of these markers is necessary to validate these results, as they are not expressed equally in different tissues.

*Keywords: Thalassemia; desferrioxamine; silymarin; sTNFR1; sCD95; sTRAIL; cytochrome C; sCD95L; Fas.*

## 1. INTRODUCTION

Iron overload is the most common clinical complication in thalassemia patients. Despite appropriate chelation therapy (e.g. desferrioxamine), iron deposition in visceral organs causes tissue damage [1]. The exact mechanism of tissue damage and iron deposition in visceral organs is not clear; however, it seems that generation of Reactive Oxygen Species (ROS) through the Fenton reaction is one of the possible mechanisms [2]. Antioxidants such as plant flavonoid can be an effective treatment to reduce ROS in thalassemia patients. Silymarin is a non-toxic flavonoid, which is widely consumed as a dietary supplement for its strong antioxidant and hepatoprotective activities. In a recent clinical trial, thalassemia patients with severe iron overload were safely treated with a combination of silymarin and desferrioxamine. Silymarin was generally well tolerated with no detectable abnormalities in complete blood count, differential cell counts, liver or renal functions [3,4].

Apoptosis is initiated by death-inducing transmembrane receptors. These receptors include TNF receptors and a variety of immune cell receptors and their corresponding ligands [5]. Also, apart from membrane-bound, receptors are detectable in soluble forms, due to shedding from

the cell surface. In this study, we investigated serum concentrations of soluble apoptosis markers which include: CD95 (sCD95, sFas), sCD95 ligand (sCD95L, sFasL), sTNF receptor type 1 (sTNFR1), sTRAIL and cytochrome C in  $\beta$ -thalassemia major patients following a combined therapy of silymarin and desferrioxamine.

## 2. PATIENTS AND METHODS

Homozygous  $\beta$ -thalassemia major patients were selected from referrals to Sayed-Al-Shohada clinic of thalassemia, Isfahan University of Medical Sciences. All patients were received regular transfusion by leucodepleted blood prophylactically matched for D, C, E, c, e, and K1 blood group to maintain the hemoglobin level above 9.5 g/dL and treated by subcutaneous desferrioxamine. Patients were excluded from the trial if they had one of the following conditions: under 12 years of age, chronic renal or heart failure, iron chelation therapy with iron 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 from all patients. The protocol for this study was approved by the Ethics Committee of the Isfahan University of Medical Sciences, Isfahan, Iran (Ethical approval number: 187050).

This study was a randomized, double-blind, placebo controlled, 6-month clinical trial of silymarin add-on therapy in  $\beta$ -thalassemia major patients on regular iron chelation therapy by desferrioxamine. Patients were randomized into a treated group and a placebo group based on the date of their first visit after enrollment. The treated 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. During the study period, no change was allowed in the dose of desferrioxamine. Compliance with Legalon was assessed by pill counts and diary cards that recorded the time and date of taking each pill. Patients received a new supply of capsules every month, and they returned all diary cards at the same time.

Fasting blood samples were taken from patients before a scheduled transfusion. Samples were drawn from all subjects and allowed to clot at room temperature and separated following centrifugation at 1000xg for 10 min. Serum was stored at  $-80^{\circ}\text{C}$  until analyzed. Serum soluble apoptosis markers in both placebo and silymarin groups were measured using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria). The sensitivity of the ELISA kits was 13.2 pg/ml for sFas, 0.7 ng/ml for sFasL, 0.05 ng/ml for sTNFR1, 0.05 ng/ml for cytochrome C, and 5 pg/ml for sTRAIL.

### 2.1 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 t-test of two independent samples. The mean  $\pm$  standard deviation (SD) was calculated for all data and significance was set at  $P$  value  $< 0.05$ .

### 3. RESULTS

Clinical characteristics of  $\beta$ -thalassemia major patients are summarized in Table 1. A total of 80 thalassemia patients in two groups were successfully recruited into 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 were no statistically significant differences in the mean levels of sFas, sFasL, TNFR1 and cytochrome C between placebo group and silymarin group at the baseline and after treatment. Moreover, the concentration of these serum soluble markers was slightly higher or below the kits detection limits in both groups. The sTRAIL was not detectable in two groups. Results are expressed as the mean of soluble apoptosis markers  $\pm$  SD in Table 2.

**Table 1. Clinical and biological features of patients with  $\beta$ -thalassemia major**

Features	Values
Age (year)	21.8 $\pm$ 5.4
Hemoglobin (g/dl)	9.5 $\pm$ 0.9
Body mass index (kg/m <sup>2</sup> )	20.26 $\pm$ 2.6
Desferrioxamine dosage (mg/kg)	40–50
Desferrioxamine treatment (nights/week)	5 $\pm$ 1
Transfusion interval (day)	16.8 $\pm$ 3.2
Transfusion volume (ml) <sup>a</sup>	535 $\pm$ 123
Splenectomy (%)	45.71

<sup>a</sup> Transfusion volume is the volume of blood that is transfused to patients in each transfusion referral

Table 2 shows in significant differences in serum concentration of apoptosis markers ( $P > 0.05$ ) except for sTNFR1 where a significant decrease was observed ( $P < 0.05$ ).

### 4. DISCUSSION

In the present study, we found that the serum levels of sFas, sFasL, sTRAIL and cytochrome C showed no significant difference between silymarin and placebo groups; however, serum level of sTNFR1 was significantly reduced after silymarin treatment, while no change was observed in sTNFR1 levels in placebo treated patients. In various pathologic conditions including chronic inflammatory diseases and infections, the serum concentrations of TNF $\alpha$  and sTNFR increase, which their balance may appear to reflect the activation state of the TNF $\alpha$ /TNFR system. Soluble forms of TNFR are the only natural molecules known to interfere with TNF activity by competing for TNF binding with receptors on target cells. Elevated levels of serum TNF $\alpha$  was reported previously in  $\beta$ -thalassemia major patients, which was decreased significantly by silymarin treatment in this study. Therefore, our observation of decreased circulating concentrations of sTNFR1 in silymarin-treated patients correlates with decreased TNF $\alpha$  levels, which may reflect anti-inflammatory activity of silymarin in  $\beta$ -thalassemia major patients.

**Table 2. Mean  $\pm$  SD serum concentrations of sFas, sFasL, TNFR1, and cytochrome C in thalassemia patients treated with placebo (group A) or silymarin (group B)**

Apoptosis markers	Placebo (Group A; n=40)			Silymarin (Group B; n=40)		
	Before	After	P-value	Before	After	P-value
sFas (pg/ml)	22.15 $\pm$ 15.82	19.52 $\pm$ 12.08	0.142	24.68 $\pm$ 24.22	26.17 $\pm$ 29.84	0.516
sFasL (pg/ml)	117.41 $\pm$ 95.34	114.87 $\pm$ 62.44	0.896	172.18 $\pm$ 155.13	126.89 $\pm$ 113.56	0.150
sTNFR1 (ng/ml)	0.12 $\pm$ 0.09	0.11 $\pm$ 0.11	0.747	0.16 $\pm$ 0.12	0.11 $\pm$ 0.10	0.029*
Cytochrom C (ng/ml)	0.15 $\pm$ 0.02	0.15 $\pm$ 0.02	0.595	0.17 $\pm$ 0.16	0.21 $\pm$ 0.29	0.204

*P-value is significant at P<0.05, \*= significant*

Although we do not yet completely understand the underlying mechanism of apoptosis in thalassemia patients, the production of ROS through Fenton reaction and iron-mediated oxidations as well as death receptor mediated pathways such as Fas-FasL ligand interactions could be possible explanations for inducing apoptosis in thalassemia patients [1,6]. Previous studies revealed that murine sFasL is a non-apoptotic ligand and can act as an antagonist of membrane-bound FasL (mFasL) activity [7]. Considering the high percentage of CD3<sup>+</sup>Fas<sup>+</sup> T lymphocytes in thalassemia major patients previously reported by Gharagozloo et al. [8], the low serum levels of sFas and sFasL markers may correspond to high levels of Fas<sup>+</sup> T lymphocytes in  $\beta$ -thalassemia major patients.

## 5. CONCLUSION

This study provides further evidence of anti-inflammatory effect of silymarin treatment in  $\beta$ -thalassemia major patients. The finding that silymarin had no effect on the level of soluble apoptosis marker could be an evidence that supports silymarin as a safe treatment for  $\beta$ -thalassemia major patients. Since apoptosis markers are not expressed equally in different tissues and affected by several factors, evaluation of membrane apoptosis markers is necessary to verify the result of current study on serum soluble markers. In this study we compared the levels of apoptotic factors in their soluble forms, further trials assessing the membrane levels of such markers and related parameters can help us to have a better understanding of the underlying mechanism of apoptosis and tissue damage in thalassemia patients and how silymarin can affect this phenomenon.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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