

Short Research Article

Investigation into the effect of silymarin on serum concentration of soluble apoptosis markers in β -thalassemia major patients receiving desferrioxamine treatment in an Isfahan clinic, Iran

Comment [O1]: There is no need to mention that

Abstract

Background: Despite appropriate chelation therapy with desferrioxamine, iron deposition in visceral organs causes tissue damage in ~~thalassemia~~ ~~thalassemia~~ ~~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~~ ~~thalassemia~~ patients.

Comment [O2]: Thank you so much for editing our manuscript. I'd prefer using "thalassemia patient" instead of "thalassemia patient".

Aim: In this study, we aimed to investigate the serum levels of apoptosis markers in ~~β -thalassemia major~~ ~~patients~~ ~~thalassemia~~ ~~thalassemia~~ patients treated with silymarin and desferrioxamine.

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Study Design: This study is a randomized, double-blind, placebo controlled 6-month clinical trial

Place and Duration of Study: This ~~study-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.~~ ~~and~~ ~~2010.~~

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Methodology: The patients were randomized into two groups: one group (~~BA~~) received desferrioxamine and placebo and the other group (~~AB~~) 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 expressed as mean $\pm$ standard deviation (SD) and significance set at $P < 0.05$.

Result: There was no statistically significant difference between desferrioxamine group and combined therapy group in serum concentration of apoptosis markers ($P > 0.05$), except for sTNFR1 level (which decreased from 0.16 ± 0.12 to 0.11 ± 0.10 ng/ml) in the ~~silymain treatment treated~~ 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 β -thalassemia major patients. 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. In conclusion, However,~~ measuring the membrane levels of these markers is necessary to validate these results, as they are not expressed equally in different tissues.

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

INTRODUCTION

41 Iron overload is the most common clinical complication in thalassemia patients. Despite appropriate
42 chelation therapy (e.g. desferrioxamine), iron deposition in visceral organs causes tissue damage(1). The
43 exact mechanism of tissue damage and iron deposition in visceral organs is not clear; however, it seems
44 that generation of Reactive Oxygen Species (ROS) through the Fenton reaction is one of the possible
45 mechanisms(2). Antioxidants such as plant flavonoid can be an effective treatment to reduce ROS in
46 thalassemia patients. Silymarin is a non-toxic flavonoid, which is widely consumed as a dietary supplement
47 for its strong antioxidant and hepatoprotective activities. In a recent clinical trial, ~~thalassemic~~thalassemia
48 patients with severe iron overload were safely treated with a combination of silymarin and
49 desferrioxamine. Silymarin was generally well tolerated with no detectable abnormalities in complete
50 blood count, differential cell counts, liver or renal functions(3, 4).
51 Apoptosis initiated by death-inducingtransmembrane receptors. These receptors includeTNF receptors
52 and a variety of immune cell receptors and their corresponding ligands(5). Also, apart from membrane-
53 bound, receptors are detectable in soluble forms, due to shedding from the cell surface.In this study, we
54 investigatedserum concentrations of soluble apoptosis markers whichinclude:CD95 (sCD95, sFas),
55 sCD95 ligand (sCD95L,sFasL), sTNF receptor type 1 (sTNFR1), sTRAIL and cytochrome C
56 in~~thalassemic~~~~β-thalassemia~~major patientsfollowing a combined therapy of silymarin and desferrioxamine.

57 PATIENTS AND METHODS

58 Homozygous β-thalassemia majorpatients were selected from referrals to Sayed-Al-Shohada clinic of
59 thalassemia, Isfahan University of Medical Sciences. All patients were received regular transfusion by
60 leucodepleted blood prophylactically matched for D, C, E, c, e, and K1 blood group to maintainthe
61 hemoglobin level above 9.5 g/dL and treated by subcutaneousdesferrioxamine.Patients were excluded
62 from the trial if they had one of the following conditions: under 12 years of age, chronic renal or heart
63 failure, iron chelation therapy with iron chelators other than desferrioxamine, hepatitis B or C infection, a
64 history of a positive HIV test, pregnancy, and antioxidant or herbal medicine consumption .Oral and
65 written informed consents were obtained from all patients. The protocol for this study was approved by
66 the Ethics Committee of the Isfahan University of Medical Sciences, Isfahan, Iran (Ethical approval
67 number:.....→187050)

68 This study was a randomized, double-blind, placebo controlled, 6-month clinical trial of silymarin add-on
69 therapy in β-thalassemia major patients on regular iron chelation therapy by desferrioxamine. Patients
70 were randomized into a treated group and a placebo group based on the date of their first visit after
71 enrollment. The treated group received 140 mg Legalon®, MadausPharma orally, at least an hour before
72 food, three times a day, 7 days a week with their routine desferrioxamine (Novartis Pharma AG, Basel,
73 Switzerland) dose of 4–6 days per week. The placebo groupreceived tablets, identical to Legalon®, that
74 were specially prepared for this study. During the study period, no change was allowed in the dose of
75 desferrioxamine. Compliance with Legalonwas assessed by pill counts and diary cards that recorded the
76 time and date of taking each pill. Patients received a new supply of capsules every month, and they
77 returned all diary cards at the same time.

78 Fasting blood samples were taken from patients before a scheduled transfusion. Samples were drawn
79 from all subjects and allowed to clot at room temperatureand separatedfollowing centrifugation at1000xg
80 for 10 min. g-. Serum was stored at–80°C until analyzed.Serum soluble apoptosis markers in both
81 placebo and silymarin groupswere measured using enzyme-linkedimmunosorbent assay (ELISA)
82 kits,according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria).The sensitivity of
83 the ELISA kitswas13.2 pg/ml for sFas, 0.7 ng/ml for sFasL, 0.05 ng/ml for sTNFR1, 0.05 ng/ml for
84 cytochrome C, and5 pg/ml for sTRAIL.

85 Statistical analysis

86 Statistical analysis was performed using SPSS 15 version for windows program. Statistical significance of
87 the differential circulating levels of apoptotic markers in thalassemia patients was determined using t-test
88 of two independent samples. The mean ± standard deviation (SD)was calculated for all data and
89 significance was set at P value< 0.05.

90 RESULTS

91 Clinical characteristics of β-thalassemia major patients are summarized in Table 1. A total of
92 80~~thalassemic~~thalassemia patients in two groups were successfully recruitedinto the study. In the
93 placebo group, 40 patients continued receiving desferrioxamine, and in the silymarin group, 40 patients

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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 β -thalassemia Major

Features	Values
Age (year)	21.8 $\pm$ 5.4
Hemoglobin (g/dl)	9.5 $\pm$ 0.9
Body mass index (kg/m ²)	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) ^a	535 $\pm$ 123
Splenectomy (%)	45.71

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

Table 2: Mean $\pm$ SD serum concentrations of sFas, sFasL, TNFR1, and cytochrome C in β -thalassemia patients treated with silymarin (group B) and 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*
Cytochrome 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*

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

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 α and sTNFR increase, which their balance may appear to reflect the activation state of the TNF α /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 α was reported previously in β -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 α levels, which may reflect anti-inflammatory activity of silymarin in β -thalassemia major patients.

Although we do not yet completely understand the underlying mechanism of apoptosis in β -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 β -thalassemia patients (1, 6). Previous studies

128 revealed that murine sFasL is a non-apoptotic ligand and can act as an antagonist of membrane- bound
129 FasL (mFasL) activity.(7) Considering the high percentage of CD3+Fas+ T lymphocytes in thalassemia
130 major patients previously reported by Gharagozloo et al.(8), the low serum levels of sFasand sFasL
131 markers may correspond to high levels of Fas+ T lymphocytes in β -thalassemia major patients.
132

133 CONCLUSION:

134 This study provides further evidence of anti-inflammatory effect of silymarin treatment in β -thalassemia
135 major patients. The finding that silymarin had no effect on the level of soluble apoptosis marker could be
136 an evidence that supports silymarin as a safe treatment for β -thalassemia major patients.

137 Since apoptosis markers are not expressed equally in different tissues and affected by several factors,
138 evaluation of membrane apoptosis markers is necessary to verify the result of current study on serum
139 soluble markers. In this study we compared the levels of apoptotic factors in their soluble forms, further
140 trials assessing the membrane levels of such markers and related parameters can help us to have a
141 better understanding of the underlying mechanism of apoptosis and tissue damage in
142 ~~thalassemic~~ thalassemia patients and how silymarin can affect this phenomenon.

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