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The effect of quercetin on iron overload and inflammation in β-thalassemia major patients: A double-blind randomized clinical trial

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ABSTRACT

Objectives: The aim of this study was to determine whether quercetin can reduce iron overload and inflammation in thalassemic patients.
Methods: Eighty four patients were recruited to this study and randomly assigned to two groups: 42 patients received a 500 mg/day quercetin tablet and 42 others took a 500 mg/day starch placebo for 12 weeks. Demographic, anthropometric and biochemical evaluation were performed.
Results: ANCOVA analysis revealed that compared to the control group, quercetin could reduce high sensitivity C-reactive protein (hs-CRP) ($P = 0.046$), iron ($p = 0.036$), ferritin ($p = 0.043$), and transferrin saturation (TS) ($p = 0.008$) and increase transferrin ($p = 0.045$) significantly, but it had no significant effect on total iron binding capacity (TIBC) ($p = 0.734$) and tumor necrosis factor α (TNF-α) ($p = 0.310$).
Conclusions: Quercetin could ameliorate the iron status in thalassemia major, but its effect on inflammation is indistinctive.

1. Introduction

Thalassemia is a group of genetic disorders resulting from defects in the rate of synthesis of globin chains. The β-thalassemia is characterized by the absence or inadequate β-globin chain production. This problem causes profound anemia that kills untreated affected children before the age of 3 years.[1] Blood transfusion therapy is a lifesaving treatment for these patients. However, iron overload caused by blood transfusion which transmits 200 to 250 mg of iron to the patient's body per unit, is this therapy's complication.[2] What makes the circumstances even worse, is that iron overload is linked to a distinct gene that favors excessive iron absorption if iron exists in the diet.[3] In other words, these patients experience severe iron toxicity.

Since the body has no means to deal with this large amount of excessive iron, it deposits iron into organs such as the heart, liver, and endocrine organs, which leads to the dysfunction of these organs and the implications outbreak as liver dysfunction, cardiomyopathy, diabetes, and even death.[4] On the other hand, free iron is a highly reactive element that can interact with oxygen to form intermediates and cause inflammation. It also helps generate excessive amounts of free radicals that attack cellular molecules.[5,6] Repeated exposure to foreign antigens due to blood transfusion may also induce deleterious effects on the immune system and deteriorate the inflammation.[7] Inflammatory biomarkers are found to be increased in thalassemia and have been found useful in studying this disease.[8–10]

Chelation therapy effectively removes excessive iron. desferrioxamine (DFO), deferiprone, and deferasirox are currently available chelating drugs which bind with Fe + molecules and eliminate them through urine or feces.[11] Recently though, biochemical studies have highlighted the possible iron-chelating properties of flavonoids and polyphenolic compounds with at least two iron binding sites.[12] Quercetin is a member of flavone family that mainly exists in apples, onions, tea, red wines, and berries.[13] The catechol moiety is the possible site on quercetin for the iron chelation and its high binding energy values indicate that quercetin is a powerful chelating agent that can sequester iron in such a way to prevent its involvement in oxidation reaction.[14] Animal studies have reported that quercetin is able to reduce the hepatic iron overload, decrease serum ferritin, increase the fecal excretion of iron, limit the rate of intestinal iron absorption and decreased subsequent basolateral iron efflux into the blood circulation.[15,16] It is also

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known to be able to reduce inflammation in both animal and human studies.[17]

Approximately 7% of the world's population is a carrier for hemoglobin disorders.[18] Even though the life expectancy of patients treated with regular blood transfusion and iron-chelation therapy is approaching normal,[19] according to the World Health Organization, about 12% of children born with hemoglobin disorders are transfusion-dependent β thalassemia patients, less than 40% of whom obtain adequate iron-chelation therapy.[20] To better these patients' quality of life, we decided that quercetin supplementation along with common iron-chelating treatment may be effective in reducing iron overload and inflammation in patients with β thalassemia major.

2. Materials and methods

This study was a randomized placebo-controlled clinical trial. The study protocol was approved by the Ethical Committee of Iran University of Medical Sciences (IR.IUMS.REC 1395.95-04-207-30254). This study was also registered in the Iranian Registry of Clinical Trials (IRCT20091114002709N46) and has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Eighty-four thalassemia major transfusion-dependent thalassemia patients were voluntarily recruited at Zafar Thalassemia Clinic (Tehran, Iran) from April 2017 to March 2018. All participants were 18–45 years of age, non-smoker, and were under exclusive treatment with DFO. Patients with no history of metabolic diseases (e.g. hypo- or hyperthyroidism, chronic liver disease, diabetes mellitus, renal dysfunction and cardiovascular disease) were enrolled only. Overtaking metabolic or infectious disease, change in sort or dose of medication, and compliance less than 80% were our exclusion criteria. Women were also excluded if pregnant or lactating. Participants kept taking vitamin C, Calcium, vitamin D, and multivitamin-mineral tablets during the trial, as their treatment routines. All the recruited patients were informed about the purposes of the study and completed a written informed consent. Patients were free to withdraw the trial at any time during the study.

Considering an effect size of 0.70, α equal to 0.05 and 20% loss of sample size, the total sample size was calculated as 84 using the data from Lehmann C. and Kalpravidh RW.'s studies.[21,22] For randomization, the permuted block randomization was used with quadruple blocks. According to the 84 participants sampled, 21 blocks were produced and in order to apply the concealment in the randomization process, unique codes were written on the pharmaceutical boxes, which were generated by the software. A university staff that was not aware of the purpose of the study and was provided with the codes, put the tablets in the boxes and gave each a code. By recruiting each individual into this study, the supplement boxes were assigned to the individuals and neither the researcher nor the patient was aware of the type of treatment. Patients were randomly allocated to two groups: The quercetin group (n = 42) was given 500 mg daily (Sigma-Aldrich, CAS Number: 117-39-5) in the form of a tablet after a main meal (lunch or dinner), and the placebo group (n = 42) was given a starch-containing tablet daily for 12 weeks. Pharmaceutical Research Center of Tehran University of Medical Sciences converted the quercetin powder into tablets and made the placebo tablets in the same color, shape and smell. We monitored the compliance of the participants by counting the returned tablets at the end of the trial. Participants were asked not to change their diet and physical activity during the study.

Height was measured with subjects not wearing shoes and to the nearest 0/01 cm. Weight in light clothes and without shoes was measured with a digital scale to the nearest 100 *g* (Seca, Germany). Body mass index (BMI) was calculated using weight (in kilograms) divided by squared height (in meters). Dietary information was collected using three 24-hour diet recalls for two typical days and one holiday. Information on physical activity was obtained using the International Physical Activity Questionnaire Baecke (IPAQ). Other information including age, sex, blood transfusion intervals, etc. were extracted from the patient's medical record or obtained from the patients themselves during the interview. Participants donated blood samples before and after the 12-week supplementation period. Blood samples (10 ml) were collected after 12 h of fasting and at least 10 days after the latest blood transfusion. Samples were then centrifuged at 2000 rpm for 10 min, and sera were stored at -80 °C until further analysis. All kits were obtained from Pars Azmoon (Iran) except for the Tumor Necrosis Factor α (TNF-α) which was measured using Zellbio kit (Germany). Hemoglobin (Hb), serum iron, ferritin, transferrin, total iron binding capacity (TIBC), transferrin saturation (TS), high sensitivity C-Reactive Protein (hs-CRP), and TNF-α were measured in this study.

For the quantitative variables mean (SD) or the median (interquartile range), and for categorical variables frequency (percentage) are reported. To compare the means of quantitative outcomes between the two groups, independent *t*-test or its non-parametric equivalent, Mann-Whitney test, were used. To compare the results before and after the intervention in each group, paired *t*-test or its nonparametric equivalent Wilcoxon test was used. Chi-square test or Fisher's exact test was used to compare categorical variables between the two groups. ANCOVA analysis was used to compare the two groups with adjustment for variables that were statistically different in the baseline. Non-parametric ANCOVA test was performed with R-3.4.1 software.[23] p-value < 0.05 was considered statistically significant. All analyses were performed using Statistical Package for Social Sciences (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

3. Results

Patient recruitment to this 12-week intervention began in April 2017 and lasted untill March 2018. From eighty four Patients, seventy one remained for the final analysis (40/42 in quercetin and 31/42 in placebo group). As is shown in Fig. 1, in the placebo group, six participants left the study because of gastro-intestinal complications, one patient reported hair loss, and two patients had less than 80% compliance. Four patients from both placebo and quercetin group withdrew the trial for personal reasons. Despite this huge loss of sample size, our results are still reliable as the post-hoc power measured was 0.83.

General characteristics of the two groups showed that all anthropometric and demographic features plus macro and micro nutrient intakes were similar in the two studied groups, except for physical activity, the age in which DFO treatment began, Fe, Folic acid, and Cu dietary intakes (Table 1). Body Mass Index (BMI), physical activity and dietary intakes remained unchanged after the trial.

The comparison of between and within quercetin and placebo groups are shown in Table 2. Quercetin could decrease hs-CRP, serum iron, ferritin, and TS and increase transferrin significantly, but it had no dramatic effect on TIBC and TNF-α. Serum iron and ferritin significantly decreased within the quercetin group, while no such changes were observed in placebo group. Regarding hs-CRP, there was no significant difference between the two groups in the baseline ($p = 0.51$) amount, but the difference between the two groups after the intervention was significant, with ($p = 0.046$) and without ($p = < 0.001$) adjustment for confounders. Similarly, TNF-α was not significantly different between the quercetin and placebo groups in both baseline ($p = 0.33$) and after the intervention ($p = 0.16$). This difference remained non-significant after adjustment for confounders as well ($p = 0.310$).

As is shown in Fig. 2, ferritin level of the placebo group participants slightly increased after the intervention, while there was a significant reduction in the ferritin level of the quercetin group. In other words, not only quercetin could prevent a rise in ferritin level, but it also plunged it.

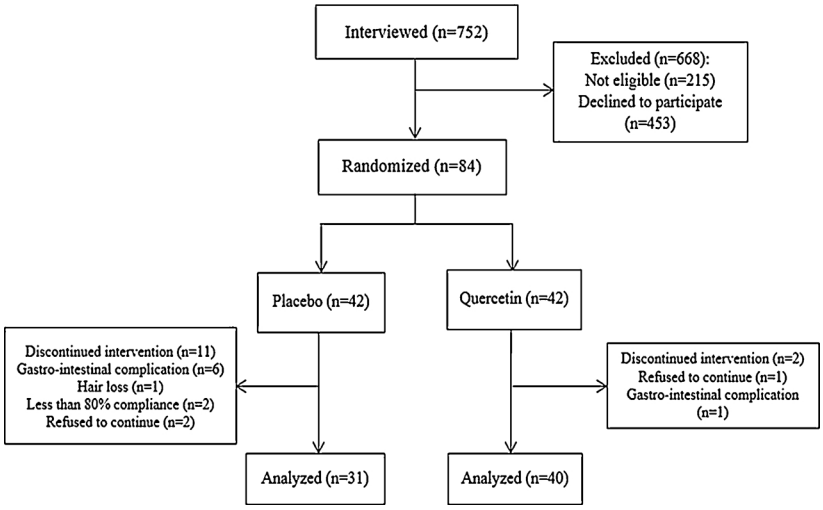

Fig. 1. The CONSORT flowchart.

Table 1
Baseline characteristics of patients according to treatment allocation: mean ± SD unless otherwise stated.

		Quercetin (n = 42)	Placebo (n = 42)	*p-value*
Age (years)		27.88 (4.73)	27.71 (5.49)	0.88
Height (cm)		161.59 (7.92)	164.91 (7.88)	0.05
Weight (kg)		57.35 (5.60)	58.28 (7.27)	0.51
BMI		22.00 (1.91)	21.44 (1.94)	0.19
Hb (g/dl)		8.53 (0.36)	8.55 (0.45)	0.60
Physical activity(MET-minute/week)		528.00 (609.18)	198.00 (222.75)	0.026¥
DFO dosage (Ampoules per week)*		24 (4)	28 (10)	0.06
Prime age of DFO treatment (months old)*		24 (36)	24 (12)	0.031¥
Energy (Kcal)*		1488.00 (975.06)	1646.26 (945.30)	0.17
Protein (g)*		58.93 (28.58)	60.35 (39.57)	0.56
Carbohydrate (g)		262.26 (95.07)	272.42 (104.79)	0.64
Fat (g)*		55.05 (72.27)	47.59 (56.60)	0.42
Vitamin C (mg)		89.34 (47.12)	92.95 (45.16)	0.72
Ca (mg)		639.83 (353.23)	644.75 (302.63)	0.94
Vitamin D (IU)*		1.57 (2.58)	2.37 (2.72)	0.13
Zinc (mg)		7.54 (4.10)	8.73 (3.93)	0.17
Fe (mg)*		10.62 (7.14)	12.02 (5.94)	0.006¥
Folic acid (micg)*		168.09 (180.90)	173.17 (207.54)	0.036¥
Cu (mg)*		0.84 (0.90)	1.20 (1.32)	0.041¥
sex£	Female	30 (71.4)	23 (54.8)	0.11
	Male	12 (28.6)	19 (45.2)	
splenectomy£	Yes	20 (47.6)	16 (38.1)	0.50
	No	22 (52.4)	26 (61.9)	

BMI: Body Mass Index, Hb: hemoglobin, DFO: desferrioxamine, Ca: calcium, Fe: iron, Cu: copper.
£ Frequency (percentage).
¥ Significant p-value.
* Median (interquartile range).

4. Discussion

In the present study, the effect of quercetin on thalassemia major patients has been investigated for the first time. According to the findings of this clinical trial, 12 weeks combined treatment with DFO and 500 mg quercetin in comparison to placebo could significantly ameliorate hs-CRP, serum iron, ferritin, TS and transferrin, but not TIBC and TNF-α. Quercetin was well tolerated and our participants experienced no serious complications.

The etiology of iron overload in thalassemia major roots back to blood transfusion therapy. Blood transfusion prevents from hypoxic anemia and helps the patient to continue on his normal social and physical activity.[24] However, 200–250 mg iron is transfused to the patient's body through this blood,[1,25] which is way too more than the body's capability to excrete iron.[26] Intestinal iron absorption on the other hand increases due to impaired hemoglobin production, and deteriorate the existing iron overload.[25,27] Therefore, serum iron increases and the excessive iron deposits in the end organs such as liver, pancreas and heart, which is a lethal complication.[5] To tackle this issue, liver increases the production of ferritin to reduce free serum iron.[28,29] Transferrin, another iron-binding protein, also increases to bind iron and prevent from its toxic effects. TIBC decreases and TS increases in this condition, which is a sign of iron overload.[30]

Our results indicate that quercetin could significantly reduce ferritin level in beta thalassemia patients, while an increase in ferritin level was observed in placebo group. This means that quercetin is not only able to prevent from ferritin's increscent, but it also reduces it. Quercetin could decrease free serum iron and TS, and increase transferrin too, all of which lead to a reduction in iron overload. The only insignificant result was observed for TIBC. This factor increased in both groups but its change was more dramatic in the quercetin group.

Studies in iron-dextran induced iron overloaded mice showed that injected quercetin could reduce the hepatic iron overload, decrease serum ferritin, and increase the fecal excretion of iron.[31,32] Tang Y. et al found that quercetin alleviated ethanol-mediated suppression of hepcidin expression in mice.[33] Lesjack M. et al also claims that quercetin can limit the rate of intestinal iron absorption and decreased subsequent basolateral iron efflux into the blood circulation in rats.[16] Human interventional studies reported various results. In a clinical trial, 6 month intervention with silymarin, a flavonoid similar to quercetin, in combination with DFO could significantly reduce ferritin and serum iron in comparison to the placebo group in thalassemia major children.[34] Another trial, in which thalassemia patients were subjected to silymarin supplementation for 9 months, showed that none of the iron overload indices changed significantly but ferritin.[35] In Gharagozloo M. study, 12 weeks supplementation with silymarin significantly reduced the level of transferrin in thalassemia patients, while the amount of transferrin increased in the placebo group.[36] Different results can be explained by the type and dose of antioxidant used, the age group studied, the duration of the study, and the sample size.

Inflammation is part of an immune response to infection or injury and is one of the well-known complications of thalassemia.[37–39] Iron has a considerable role in the production of Reactive Oxygen Species (ROS) through Fenton and Haber-Weiss reaction[40] which leads to an

Table 2
Results of within and between group comparison: mean ± SD unless otherwise stated.

Parameter	Quercetin (n = 40)			Placebo (n = 31)			*p-value*[2]
	Before	After	*p-value*[1]	Before	After	*p-value*[1]	
Transferrin (mg/dl)*	170.00 (32.00)	179.00 (51.00)	0.81	185.50 (51.00)	191.50 (54.00)	0.14	0.045¥
Serum iron (μg/dl)*	187.00 (43.00)	169.00 (88.00)	0.002¥	178.00 (27.75)	174.00 (69.50)	0.76	0.036¥
Ferritin (ng/ml)	2092.80 (513.36)	1834.12 (691.02)	0.001¥	1881.33 (451.63)	1921.43 (411.81)	0.41	0.043¥
TIBC (μg/dl)	265.28 (65.75)	276.25 (82.90)	0.38	246.26 (60.31)	249.83 (43.95)	0.06	0.734
TS (%)	69.19 (6.78)	63.65 (6.55)	< 0.001¥	70.72 (7.14)	68.48 (5.35)	0.003¥	0.008¥
hs-CRP (mg/L)*	0.30 (3.35)	0.00 (0.03)	< 0.001¥	1.50 (2.90)	0.65 (1.90)	< 0.001¥	0.046¥
TNF-α (Pg/ml)*	2.90 (3.60)	0.10 (1.25)	< 0.001¥	3.00 (2.60)	0.10 (0.60)	< 0.001¥	0.310

TIBC: total iron binding capacity, TS: Transferrin saturation, TNF-α = Tumor necrosis factor-α, hs-CRP = High-sensitivity C-reactive protein.
¥ Significant p-value.
* Median (interquartile range).
[1] Within group comparison.
[2] Between group comparison.

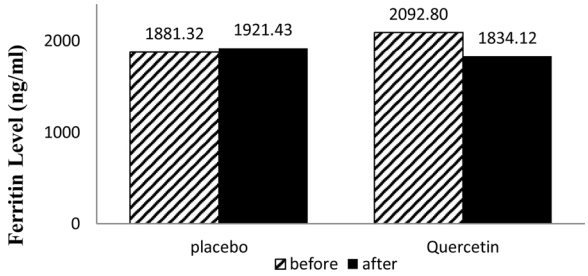

Fig. 2. The comparison of ferritin level before and after the treatment in the placebo and quercetin groups.

increase in inflammatory markers' level such as hs-CRP. Low antioxidant level which has been reported to exist in thalassemia patients worsens this inflammation.[41,42] Therefore, transfusion-dependent thalassemia patients develop chronic inflammation due to the generation of ROS.

ANCOVA analysis of our data indicated that quercetin along with DFO treatment could effectively reduce hs-CRP level, TNF-α remained unaltered though. According to previous reports, hs-CRP is the best indicator of inflammation in thalassemia major.[43,44] Nevertheless, insignificant result for TNF-α prevents us from making definitive comments. Owing to its unique chemical characteristics, quercetin has iron-chelating and ROS scavenging ability.[45] However, the inter-individual difference between the bioavailability of qurcetin which depends on genetic polymorphism, dietary history and variation in gut microbiota might be the reason for this indistinctive result we observed.[22,46,47] Chouhan S. et al investigated the effects of Quercetin supplement in iron-overloaded Rats. They reported that quercetin could reduce free radicals and oxidative stress.[48] In another animal study, ischemia-reperfusion rats were given quercetin supplement and significant reduction in TNF-α and hs-CRP were observed.[49] 12 weeks of supplementation with vitamin E in children and adolescents with β-thalassemia major could not significantly change serum hs-CRP and TNF-α.[50] Overall, as a meta-analysis stated, the effect of quercetin on inflammation seems to depend on many factors including its dosage.[51]

This was the first study assessing the effect of quercetin on thalassemia major, yet there were limitations to this study. First, the patients' diet was assessed using the 24-hour diet recall questionnaire which is based on self-reported dietary intake; therefore, information bias might have occurred. Secondly, we lost a significant amount of sample size in the placebo group due to gastro-intestinal complications. Although the placebo tablets were made of starch, cellulose and other excipients, we seem to have to come up with another placebo composition with less harmful effects for thalassemia patients, whose digestive system has been weakened by long-term treatment with iron chelation therapy. We also failed to measure and report other inflammatory markers such as Catalase, SOD, GPx, etc. due to the budget limitation. The strengths of this study include the adjustment for various confounders, using validated and reliable measurements, and sampling in a referral clinic. The results are not generalizable to the whole thalassemia major though, because of the many inclusion criteria considered for this study.

5. Conclusions

We conclude that quercetin is a useful supplement for transfusion-dependent thalassemia major patients and the efficacy of combined therapy with quercetin and DFO in removing excessive iron and suppressing inflammation is more impressive than DFO alone. These results need to be confirmed by studies with larger sample size, longer follow-up period and different doses of quercetin. It is also suggested that the liver iron content, urinary/fecal iron excretion, and hepcidin expression be investigated to figure out the mechanisms through which quercetin applies its positive effects.

Conflict of interest and sources of funding

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Authors' contribution

ZSH, FS designed research, ZSH, AA, SH conducted research, AA provided essential materials, LJ performed statistical analysis, ZSH wrote the paper, and FS had primary responsibility for final content. All authors have revised and approved the final manuscript.

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