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ORIGINAL ARTICLE

Therapeutic effect of *Colla corii asini* on improving anemia and hemoglobin compositions in pregnant women with thalassemia

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Abstract Currently there is no consensus on treating anemia in pregnant thalassemia patients. In China, *Colla corii asini* (CCA) has been widely used for treating anemia for more than 2000 years. However, its clinical application in the thalassemia population is limited by a lack of quantitative evidence. The present study aims to investigate the therapeutic effect of CCA in increasing hemoglobin (Hb) concentration and improving abnormal hemoglobin compositions in pregnant patients with β-thalassemia. Seventy-two pregnant patients who met inclusion criteria were randomly assigned to either the treatment group or control group. Patients in the treatment group were given 15 g of CCA, while the control group were observed and followed up without any treatment. Levels of Hb, serum iron (SI), serum ferritin (SF) and three types of Hb components [adult hemoglobin (HbA), fetal hemoglobin (HbF), minor adult hemoglobin (HbA2)] were measured before and after treatment. Treatment with CCA led to a significant increase of Hb. The major Hb component induced by CCA was HbA, while levels of both HbA2 and HbF dropped after treatment. CCA treatment significantly increased SI, while SF remained unaffected. Our data suggest that CCA can improve anemia and optimize Hb components in pregnant patients with thalassemia without affecting iron reserves.

Keywords Thalassemia · Anemia · Pregnancy · Hemoglobin · *Colla corii asini*

Introduction

Thalassemia is a type of hemolytic anemia disease caused by genetic defect of synthesis in one or more globin chains. Among all the single genetic disorders thalassemia has the highest incidence rate in the world and causes heavy burdens on public health system. In China, the southern provinces suffer from high incidence of thalassemia, which is particularly common in the population of Guangdong, Guangxi and Yunnan provinces. Epidemiological studies showed that in Guangdong alone about 17.83 % of the 14,332 pregnant women across 21 regions examined were diagnosed as carriers of thalassemia [1].

Recent studies showed that compared with healthy controls, women with thalassemia are associated with a wide range of abnormality and adverse pregnancy outcomes including cardiovascular disease, thrombotic disease, spontaneous miscarriage, premature delivery, oligohydramnios, fetal growth restriction and low birth weight. In addition, the severity of adverse pregnancy outcomes is closely linked to low level of hemoglobin (Hb) and high level of fetal hemoglobin (HbF) as well as iron-overloading related complications during pregnancy [2, 3]. Therefore, it is essential to improve the anemic state and optimize the compositions of Hb in pregnant thalassemia patients.

As the pregnancy progresses, women experience a series of physiological change, which can lead to physiological and nutritional anemia in thalassemia patients without prior

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or only mild anemia, further complicating the conditions of thalassemia patients during pregnancy. The risk of anemia becomes higher when the patients enter third trimester and many of them may need treatment for disease management [2]. Currently there is no consensus on treating anemia in pregnant thalassemia patients. Due to the absence of an effective treatment scheme with improved safety, many thalassemia patients are prone to develop low level of Hb, which can severely impact the fetal growth and maternal health.

In traditional Chinese medicine (TCM), *Colla corii asini* (CCA, E'jiao) is a gelatin-like preparation derived from donkey hide and has been widely used in clinical antanemic therapy for more than 2000 years. In the last decade, many studies had addressed the effect of CCA on the antanemia process using modern pharmacological approaches. The results indicated that collagen protein and polysaccharides which are the main components of CCA can promote hematopoiesis by a number of mechanisms which eventually increase the peripheral erythrocyte counts and Hb concentration [4]. Therefore, we proposed that these hematopoietic effects of CCA might also contribute to the treatment of thalassemia which is caused by insufficient or abnormal Hb concentration. However, its clinical application in the thalassemia population is limited by the lack of quantitative evidence. This study aims at investigating the therapeutic effect of CCA in treating mild anemia during pregnancy among β-thalassemia patients.

Materials and methods

Subjects

This is a randomized, open labelled, controlled prospective clinical trial. Subjects were pregnant women diagnosed of minor or intermediate β-thalassemia at the First Affiliated Hospital of Guangzhou University of Chinese Medicine between March 2015 and December 2015. The study was reviewed and approved by the institutional Medical Research Ethics Committee and patient recruitment was conducted in strict accordance to the inclusion and exclusion criteria. Informed consent was obtained from all subjects before enrollment.

Diagnosis, inclusion and exclusion criteria

Diagnosis criteria

Patients were diagnosed as carriers of thalassemia based on genetic test. Classification of mild and intermediate β-thalassemia was based on the guidelines published by the Thalassemia International Federation in 2008 [5]. In accordance with the diagnostic criteria of the World Health Organization, patients were classified as mild anemia (60 g/L $\leq$ Hb < 110 g/L) and anemia during pregnancy (Hb < 110 g/L) [6].

Inclusion criteria

(1) Pregnant women diagnosed as thalassemia carriers by genetic test with clinical presentation of minor or intermediate β-thalassemia; (2) patients with mild anemia (80 g/L $\leq$ Hb < 110 g/L) prior to study enrollment; (3) singleton pregnancy; (4) patients having not received blood transfusion or any forms of anti-anemia treatment in conventional medicine or TCM in the last 12 weeks; (5) informed consent obtained.

Exclusion criteria

(1) Patients with major thalassemia; (2) patients with severe anemia (Hb < 80 g/L) prior to study enrollment; (3) twin or multiple pregnancies; (4) patients with any of the following abnormalities: immunodeficiency, primary diseases involving cardiovascular system, liver, kidney, gastrointestinal tract, endocrine system and hematological system; (5) allergic to two or more drugs; (6) patients with mental illness or poor compliance to medical treatment; (7) patients having received blood transfusion or any forms of anti-anemia treatment in conventional medicine or TCM in the last 12 weeks; (8) no informed consent obtained.

Treatment scheme

Once diagnosed as thalassemia and enrolled in the study, patients would be required to cease taking medications that contain iron and folic acid. Patients in the treatment group received daily 15 g oral CCA (Shandong Dong-E E-Jiao Co., Ltd) in powder form for 4 consecutive weeks. The dosage was adjusted to 10 g per day for 6 consecutive weeks if patients encounter any of the following side effects: swollen gums, dry or sore throat, ulcers in oral cavity, local eczema (perioral or alinasal). Patients in control groups do not receive any intervention. Subjects had their blood drawing at baseline, 4 or 6 weeks after initiation of the study.

Evaluation

Evaluation of effectiveness

10 mL of blood were drawn after overnight fasting for at least 8 h next early morning before and after treatment for following analysis: (1) level of Hb was measured by

automated hematology analyzerXE5000 (Sysmex, Japan); (2) levels of adult hemoglobin (HbA), minor adult hemoglobin (HbA2) and fetal hemoglobin (HbF) in peripheral blood by automated capillary electrophoresis (Sebia, France); (3) level of serum iron (SI) by full automatic biochemistry analyzer cobas8000 (Roche, Germany); (4) level of serum ferritin (SF) by full automatic immune analyzer I 2000 SR (Abbott, USA).

Evaluation of safety

Any symptoms that may be caused by adverse effect were recorded throughout the treatment. Peripheral blood was drawn from subjects before and after treatment to monitor changes of blood cell compositions and function of liver and kidney: total white blood count [WBC ($\times 10^9$/L)]; platelet count ($\times 10^9$/L); the percentage of neutrophil (%); serum alanine aminotransferase (U/L); serum aspartate aminotransferase (U/L); serum urea nitrogen (mmol/L); serum creatinine (μmol/L).

Sample size calculation and patient randomization

Calculation of sample size to detect statistical significance was based on the study by Xu and colleagues where 3 month treatment of CCA led to increase of Hb level by 66.9 % [7]. The estimated change of Hb level after 4 weeks of CCA is 20 % and null hypothesis is rejected if α value <0.05 (power, 0.90; loss of follow-up rate, 0.2). In total 72 patients were included in the final study and patients were randomized to either treatment or the control group in a 2:1 ratio by statistical package for social sciences (SPSS) 21.0 random number generator.

Statistics

Statistical analysis was performed by SPSS 21.0. Continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as *n* (%). Homogeneity of variance analysis was performed. Group comparison was conducted with independent *t* test. Enumeration data was present as rates or proportions and analyzed with Chi-square test. $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics

Eighty-one patients were recruited and 76 were included in the final study (Fig. 1). Demographic characteristics were shown in Table 1. No significant differences were detected between the control and treatment groups in terms of pregnancy history, body mass index (BMI) and gestational week at the time of enrollment. Age of both groups ranged from 23 to 32 years, with an average age of 28.11 years. Gestational age ranged between 25 and 33 weeks with the average of 28.69 weeks. All subjects are married Chinese Han female without history of stillbirth.

Change of level and proportion in Hb before and after treatment

We examined the level of Hb and the proportion of each Hb component in subjects' peripheral blood before and after treatment. As shown in Table 2, before treatment, subjects from both groups had similar levels of Hb and Hb component. Therefore, the subjects did not differ in their severity of anemia and thalassemia. After receiving oral CCA for 4 weeks, patients experienced a significant increase of Hb concentration by an average 9.96 ± 2.75 g/L. On the other hand, the Hb concentrations in the control group dropped by 4.54 ± 2.14 g/L from baseline when no intervention was given. Level of HbA increased significantly after treatment while a simultaneous decrease of HbA2 and HbF was observed in subjects treated with CCA. The changes of different Hb components showed an opposite trend in control group where HbF and HbA2 increased while HbA decreased from baseline. Between treatment and control groups, the differences of three Hb components differed substantially after treatment ($P < 0.001$).

Effect on iron metabolism

As shown in Table 2, treatment of CCA increased SI while decreasing the level of SF in the subjects. In the control group, both SI and SF dropped slightly at the end of the study. Between treatment and control group, the change of SI was significant different after treatment of CCA while SF remained unaffected regardless of the treatment.

Adverse event

Among the treatment group, 78.0 % (39/50) of subjects completed the full course of 4-week treatment while the rest changed to 6-week treatment scheme due to inflammatory side effects including sore throat, swollen gums, ulcers in oral cavity and local eczema (perioral or alinasal). Two patients suffered from unbearable inflammatory side effects and discontinued the intervention. No subjects experienced any of the following severe adverse events: systemic rash and throat edema. No significant change was detected in WBC, platelet count, and liver function between control and treatment group (data not shown).

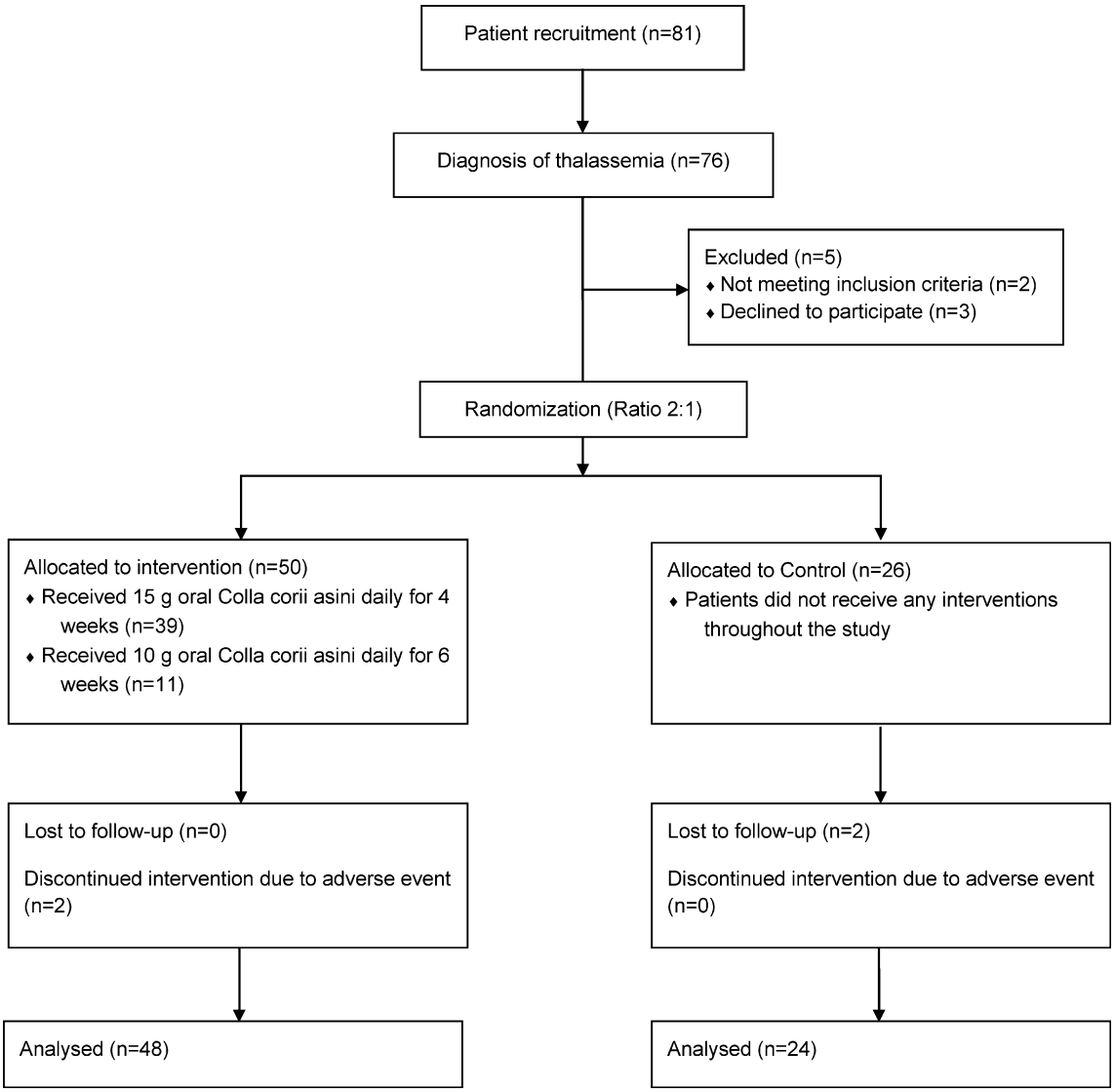

Fig. 1 Depiction of study procedure

Discussion

Thalassemia is a genetic abnormality that manifests hemoglobin, the major oxygen binding protein in red blood cells. The severity of the disease depends on the type and location of the mutation. Normal tetrameric Hb is consisted of two non-α chain (β, δ, γ) and two α chain. The compositions of the four globin chains determine the type of Hb. There are three major types of Hb: (1) adult hemoglobin (HbA), consisted of two α chains and two β chains (α2β2), which account for more than 95 % of total Hb; (2) minor adult hemoglobin (HbA2), consisted of two α chains and two δ chains (α2δ2), accounting for 2–3 % of total Hb; (3) fetal hemoglobin (HbF), consisted of two α chains and two γ chains (α2γ2), accounting for 1–2 % of total Hb [8].

β-Thalassemia is characterized by mutations in β-globin which result in reduced synthesis or complete loss of β chain. As a result, the relative amount of α chain as well as the compensatory synthesis of γ and δ chain increases, leading to elevated level of HbF and HbA2. The excess globin chain polymer further deposit on the membrane of red blood cell (RBC) and impair the mechanical stability and transformation ability by inducing immunological damage and reactive oxygen species. Eventually patients suffer from hemolysis and ineffective hematopoiesis [8, 9]. Studies in the last few decades have demonstrated that the imbalanced synthesis of Hb globin chain and abnormal Hb compositions are the major causes of thalassemia and therefore it is essential to target these mechanisms for better clinical management [8, 9].

Table 1 The demographic and clinical characteristics at baseline

	Treatment group ($n = 48$)	Control group ($n = 24$)	Statistic	P value
Age (years)	28.15 ± 2.44	28.04 ± 3.16	$t = 0.155$	0.878
BMI (kg/m^2)	20.04 ± 1.00	19.73 ± 0.97	$t = 1.248$	0.216
GW of treatment (weeks)	28.73 ± 2.10	28.62 ± 1.77	$t = 0.209$	0.835
Gravidity				
1	9 (18.8)	4 (16.7)	0.823	0.663
2	26 (54.2)	11 (45.8)		
3	13 (27.1)	9 (37.5)		
Parity				
0	45 (93.8)	22 (91.7)	Fisher	1.00[a]
1	3 (6.2)	2 (8.3)		
No. of spontaneous abortion				
0	33 (68.8)	18 (75.0)	0.303	0.582
1	15 (31.2)	6 (25.0)		
Hb (g/L)	89.54 ± 5.80	92.17 ± 4.46	$t = -1.945$	0.056
HbA (%)	91.76 ± 1.94	92.30 ± 1.63	$t = -1.003$	0.319
HbF (%)	3.30 ± 1.83	3.45 ± 1.65	$t = 0.343$	0.733
HbA2 (%)	4.95 ± 0.99	4.64 ± 0.57	$t = 1.394$	0.168
SI (μmol/L)	19.89 ± 4.94	22.04 ± 4.21	$t = -1.821$	0.073
SF (ng/mL)	58.93 ± 12.31	61.93 ± 10.27	$t = -1.028$	0.307

Continuous variables are expressed as mean ± standard deviation, categorical variables are expressed as *n* (%)

BMI body mass index, *GW* gestational week, *Hb* hemoglobin, *HbA* adult hemoglobin, *HbF* fetal hemoglobin, *HbA2* minor adult hemoglobin, *SI* serum iron, *SF* serum ferritin

[a] 2 cells (50 %) have expected count less than 5, Fisher's exact test is adopted

Table 2 The difference of concentration and component of Hb and iron metabolism indexes before and after treatment (standard deviation)

	Treatment group ($n = 48$)	Control group ($n = 24$)	95 % CI	Statistic	P value
ΔHb (g/L)	9.96 ± 2.75	−4.96 ± 2.14	13.637, 16.196	$t = 23.254$	<0.001
ΔHbA (%)	1.26 ± 0.78	−0.48 ± 0.23	1.480, 1.966	$t = 14.183$	<0.001
ΔHbF (%)	−0.57 ± 0.33	0.36 ± 0.20	−1.056, −0.812	$t = 15.120$	<0.001
ΔHbA2 (%)	−0.68 ± 0.67	0.10 ± 0.22	−1.002, −0.573	$t = 7.324$	<0.001
ΔSI (μmol/L)	1.38 ± 0.74	−1.16 ± 1.22	1.989, 3.090	$t = 9.406$	<0.001
ΔSF (ng/mL)	−2.66 ± 2.48	−2.83 ± 2.70	−1.110, 1.437	$t = 0.256$	0.798

Δ, the difference before and after treatment

Hb hemoglobin, *HbA* adult hemoglobin, *HbF* fetal hemoglobin, *HbA2* minor adult hemoglobin, *SI* serum iron, *SF* serum ferritin, *CI* confidence interval

Despite of the progress in thalassemia management, no consensus has been reached in treating pregnant thalassemia patients due to lack of safe and effective treatment, which present a major challenge to gynecologists and obstetricians. Therapies targeted at genetic mutations have captured much attention in the field of thalassemia. Early studies indicated that gene-targeting drugs can significant improve hemolytic anemia in patients with thalassemia. Currently the two major categories of drugs that regulate gene expression of globin chains are activators of γ chain and inhibitor of α chain. The former includes 5-azacytidine, hydroxyurea and other antineoplastic agents while the latter is consisted of anti-tuberculosis drugs like isoniazid. Both categories are potentially teratogenic and carcinogenic, rendering them unsuitable for pregnant patients. Besides, severe side effect like bone marrow suppression is another concern for clinicians to apply these drugs because fetus and newborns are at high risk of drug exposure through placenta and breast milk [10]. Transfusion remains the only effective means for treating severe anemia during pregnancy. However about 60–80 % of patients with thalassemia requires blood transfusion during pregnancy, with half of the populations never receiving any blood transfusion before. Therefore, an immune response may be elicited

and cause massive production of anti-RBC antibodies in thalassemia pregnant women, exposing them to higher risk of autoimmune hemolytic anemia. In severe cases, patients may suffer from a vicious cycle of hemolytic anemia and repeated transfusion in addition to the danger of contracting infections disease and iron overloading [2, 11].

Compared to conventional medicine where synthetic chemicals play an important role, TCM has the advantage of low toxicity and cost. Therefore, TCM is a lucrative resource for future exploration in treating thalassemia with broad prospects of clinical application. TCM practitioners classify thalassemia as a type of "blood deficiency" that is caused by both genetic and environmental factors like malnutrition and underdevelopment, the pathogenesis of which involves to the deficiency of *qi, blood, yin, yang*. The so-called "blood deficiency" in TCM is best defined as insufficient hematopoiesis in conventional medicine [12]. According to the theory of TCM, pregnant women are prone to 'blood deficiency' due to the physiological changes during pregnancy, similar to the aggravation of symptoms in pregnant thalassemia patients [13].

CCA, as one of the well-known TCM, has been used for treating insufficient hematopoiesis for more than 2000 years. Its therapeutic efficacy has been demonstrated in various hematological diseases including iron-deficiency anemia, aplastic anemia and thrombocytopenia. Clinical studies also showed that it can increase blood cell count and reduce bleeding from threatened abortion [4]. With the development of modern pharmacology, several key components have been purified from CCA including collagen protein, amino acids, polysaccharides, volatile substances and inorganic substances [4]. The hematopoietic effect and mechanism of CCA were investigated in anemic mice separately induced by 5-fluorouracil or cyclophosphamide [14, 15]. The results indicated that oral administration of CCA could activate the erythrocyte progenitor cells in bone marrow and subsequently increase the percentage of peripheral reticulocytes, eventually leading to the recovery of red blood cell counts and Hb concentration. By examining the expression level of granulocyte macrophage colony stimulating factor (GM-CSF) and erythropoietin (EPO) in the kidney and liver of the anemic mice, the investigators further confirmed that CCA treatment significantly increased serum GM-CSF and EPO level, which suggested that the hematopoietic effect of major components in CCA was partly due to promotion of the proliferation and differentiation of hematopoietic stem cells through stimulating GM-CSF and EPO secretion [4, 15]. These potential effect may explain the underlying mechanisms of CCA in the alleviation of thalassemia.

In the past, majority of the studies on treating thalassemia by TCM was restricted to mainly case report and observational studies. Quantitative studies in pregnant patients with systematic intervention are lacking, impeding greater usage of CCA in this particular population [16]. Our results suggest that powdered CCA alone can improve Hb levels and optimize Hb components without affecting iron reserves. SI is a common indicator of functional iron but can be easily influenced by diet and acute inflammation. Therefore, it is considered as an unreliable parameter for evaluating iron status. On the other hand, SF is an indicator of iron reserve and the most commonly used biomarker of iron status in pregnancy [17]. We observed an increase of SI in the treatment group which may be due to the rich iron contained in CCA [4]. It is of clinical importance to maintain a balanced iron metabolism because treatment-induced iron overloading is a severe issue in patients with thalassemia. A small fraction of patients experienced minor adverse events including sore throat and oral ulcers. All the side effects can be managed and alleviated via reducing the dosage and prolonging the treatment duration.

Recent studies have reported that imbalance of globin chain synthesis and abnormal compositions of Hb can largely affect the severity of thalassemia. Moreover the levels of HbF and HbA are closely linked to adverse pregnancy outcome in women. It has been demonstrated that elevated HbF level is associated with high rate of spontaneous abortion in thalassemia patients. One plausible explanation is that compared to the other two adult hemoglobins (HbA and HbA2), HbF has a higher affinity for oxygen, causing reduced dissociation of oxygen at local tissue and cells and subsequent local hypoxia. Thus it is speculated that by reducing the level of HbF, one can prevent adverse pregnancy outcome, particularly miscarriage by improving oxygen supply to the embryo [11]. Our results suggest that oral administration of CCA not only significantly increase the level of HbA, but also marginally decrease the level of HbF and HbA2. The total improvement in HbA is comparable to the total decline in HbA2 and HbF. However, the changes in HbA2 percentages and HbF percentages are only modest and likely within the range of laboratory variance, possibly caused by the insufficient sample size. The impact on HbF and HbA2 should be further verified in future study with larger sample size.

More importantly, our data demonstrated that CCA influences the level of HbA and HbF via mechanism that differs significantly from the conventional γ chain gene activator. The major role of γ chain gene activator is to promote synthesis of γ chain in order to replace the defective β chain so that the excess α chain can form HbF with the γ chain because the newly form HbF can partially compensate for the loss of HbA [10]. While this can heighten the total Hb, the increment in HbF also expose the patients to higher change of miscarriage. Other therapies including erythropoietin increased HbF substantially [10]. On the contrary, CCA improves anemia in β-thalassemia by

enhancing HbA but not HbF, although the exact molecular signaling pathway remains unclear. We suspect that CCA enhances total Hb mainly through induction of β-globin chain. As a result the compensatory synthesis of γ and δ chain is reduced, leading to higher level of HbA ($\alpha_2\beta_2$) but lower levels of HbF ($\alpha_2\gamma_2$) and HbA2 ($\alpha_2\delta_2$). This speculation should be confirmed in future study when more in-depth genetic study is coupled with biomarker analysis in order to provide a solid scientific basis for the treatment of anemia in pregnant women with thalassemia.

In summary, CCA significantly improves anemia symptoms without alternating iron reserves in β-thalassemia patients or inducing severe side effect. The therapeutic effect is mainly mediated through simultaneous increase of HbA in β-thalassemia patients respectively. It may also exert its effect via reducing HbF and HbA2 which requires further study to confirm. This study demonstrates the potential of a single ingredient from TCM for treating genetic disorder in pregnant patients. It brings insight to obstetricians and gynecologists to the potential of CCA in treating anemia in pregnant patients with β-thalassemia where optimization of Hb components holds the key for disease control.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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