Research Article

Radix Astragali Stimulates p38 MARK Phosphorylation in Pediatric Patients with β-Thalassemia

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A previous study conducted by our group demonstrated that *Radix Astragali* compounded with *Codonopsis pilosula* and *Plastrum testudinis* was effective in treating pediatric β-thalassemia in a randomized, controlled clinical trial. However, the mechanism of action that underpins this treatment remains to be elucidated. Blood was collected from patients participating in this clinical trial and nucleated red blood cell-enriched mononuclear cells were isolated to facilitate the extraction of RNA and protein. RT-PCR was used to monitor the expression of globin genes and p38 MAPK, and total and phosphorylated p38 MAPK expression was assessed using Western blot analysis. Expression of α-, β-, and Aγ-globin mRNAs was not significantly affected following treatment with *R. Astragali* or the compounded formulation. However, Gγ-globin mRNA levels increased significantly in both treatment groups (when compared with pretreatment levels) following 12 weeks of treatment. Moreover, posttreatment Gγ-globin expression was significantly higher in both treatment groups compared with the control group. Although neither p38 MAPK mRNA nor protein levels were affected by the treatments, posttreatment phosphorylation of p38 MAPK was significantly increased in the *R. Astragali* and compounded formulation groups compared with the control group. These data suggest that the molecular mechanisms that underpin the efficacious use of *R. Astragali* (and its compounded formulation) in pediatric β-thalassemia treatment facilitate the induction of Gγ-globin expression following activation of p38 MAPK.

1. Introduction

Hemoglobin (Hb) disorders, particularly β-thalassemia, are the most common single gene disorders caused by mutations in the β-globin locus. Resultant disorders cause abnormal or reduced adult hemoglobin (HbA) production and excess, unmatched α-chains in developing erythroblasts. Clinical symptoms include bone marrow expansion, splenomegaly, and a severe anemia that requires regular blood transfusions. Clinical management of β-thalassemia patients includes lifelong blood transfusions and chelation therapy to remove excess transfused iron [1] and, in some cases, bone marrow transplantation [2, 3]. β-Thalassemia syndromes are classified according to severity based on steady-state Hb and transfusion dependency. β-Thalassemia major and β-thalassemia intermedia are both caused by the inheritance of two β-globin gene mutations [4]. Furthermore, β-thalassemia results in moderate anemia in childhood, which often progresses to

transfusion dependency over time, iron loading, and unique complications related to expanded erythropoiesis and hemolysis.

Although allogeneic hematopoietic stem cell transplantation (HSCT) and gene transfer therapy can be used for patients with β-thalassemia, modern medicines and the requisite financial resources for these procedures are not available to most β-hemoglobinopathy patients [5–7]. The pharmacological induction of fetal hemoglobin (HbF) may be a promising alternative therapeutic option for treatment of these patients. HbF is comprised of two α-globin and two γ-globin chains ($\alpha_2\gamma_2$) and is highly expressed during fetal and early postnatal periods; however, HbF levels subsequently decline following concurrent increases in HbA ($\alpha_2\beta_2$) expression. An increase in HbF levels can functionally compensate for the shortfall in HbA synthesis in β-thalassemia patients and synthesized γ-chains can neutralize excess unbalanced α-chains to reduce erythrocyte damage and ameliorate anemia

and many of the complications associated with β-thalassemia [5]. Pharmacological induction of HbF also represents a potentially less expensive and more widely applicable means to treat β-hemoglobinopathies worldwide. Drugs that facilitate the induction of γ-globin include hydroxyurea (HU), 5-azacytidine, butyrate, and erythropoietin, among which HU is the only candidate that has been proven to improve the clinical course of β-thalassemia [8–11]. However, these agents are unsuitable in relation to efficacy, safety, and ease of use. For example, responses to HU are variable and weakened responses are often observed following long-term administration. In addition, γ-globin induction agents may cause myelotoxicity and are potential carcinogens. Furthermore, the cost of these treatment regimens is a significant limitation [7]. Thus, effective and safe alternative gene induction reagents are highly desirable [12–14].

Traditional Chinese medicine (TCM), alone or in combination with other treatment regimens, has been utilized to treat anemia [2, 15–17]. It has been suggested that Yisui shengxue granules [18] and *Plastrum testudinis* (tortoise plastron) [19] prolong erythrocyte lifespans via antilipid peroxidation and membrane stabilization. *Astragalus membranaceus* (*Radix Astragali* [AMW]; "Huang Qi" in Chinese) roots have previously been used in TCM to treat anemia [16, 20]. Our previous *in vitro* experiments indicated that *Plastrum testudinis*, AMW, and *Codonopsis pilosula* could enhance γ-globin mRNA expression and HbF production in both human erythroid K562 cell cultures and erythroid progenitors from patients with β-thalassemia [19, 21]. Subsequently, we conducted a randomized, controlled, double-blind clinical trial with AMW and its compounded formulation (AMW + *Codonopsis pilosula* + *Plastrum testudinis*) to treat β-thalassemia [14]. The resultant data indicated that, following 12 weeks of treatment, AMW and the compounded formulation increased the level of HbF, erythrocytes, mean corpuscular Hb, and reticulocytes in β-thalassemia, with no reported side effects [14]. Thus, AMW and the compounded formulation can ameliorate β-thalassemia symptoms by inducing HbF gene expression. Furthermore, an additional study performed by our group demonstrated that *Radix Astragali* and the compounded formulation can independently induce p38 phosphorylation in K562 cells and cultured human erythroid progenitor cells from the peripheral blood of β-thalassemia patients [22]. However, these results require further validation. Thus, we aim to elucidate the mechanism of action pertaining to AMW and its compounded formulation in β-thalassemia patients, with studies focusing on globin gene expression and downstream signal pathways.

2. Materials and Methods

Research in this study was performed in accordance with the Declaration of Helsinki and the study was approved by the Ethics Committee of Guangzhou Hospital of Traditional Chinese Medicine. All participating patients or their legal guardians signed informed consent forms before initiating the study.

2.1. Samples. The blood samples that were utilized in this study were from participants of a previous randomized, controlled, double-blinded clinical trial [14]. The trial included 35 patients, who were between two and 18 years of age, with confirmed β-thalassemia based on the *Criteria for Diagnosis and Treatment of Hematopathy* [23]. The subjects had not received a blood transfusion or treatment for anemia in the previous 12 weeks prior to enrollment and associated hemoglobin levels ranged from 4.5 to 10 g/dL at the time of enrollment. Thirteen of the subjects were treated with *R. Astragali* (AMW), 11 patients were treated with the compounded formulation (AMW + *Codonopsis pilosula* granule [CPG] + *Plastrum testudinis* granule [PTG]), and 11 patients were treated with a placebo. Pilot experiments performed in our laboratory (data not shown) indicated that a sample size greater than 10 subjects per group was required for this analysis.

Peripheral blood was drawn from the patients before treatment and 12 weeks after treatment as designated in the clinical trial [14]. All of the patients (or their legal guardians) permitted us to use the blood samples that were extracted in the present study to study the molecular mechanisms underlying the treatment effects. Blood samples were collected into whole-blood tubes containing Na-EDTA and aliquots from these samples were used for this study.

2.2. Treatment. The treatment procedure and information pertaining to the effectiveness of treatments with AMW and the compounded formulation were previously reported by Lu et al. (2012) [14]. Briefly, AMW and the compounded formulation (AMW + CPG + PTG) were produced by Guangdong Yifang Pharmaceutical Co. Ltd. (Guangdong Province, China). Herbs were extracted and granulated with 10 g of *R. Astragali* per g of AMW, 10 g of *Codonopsis pilosula* in 3 g of CPG, and 10 g of *Plastrum testudinis* in 0.7 g of PTG. The placebo was prepared using dextrin and an identical protocol to that used for herb granulation. All granules were administered orally with warm water in the fasted state and treatment was administered for 12 weeks.

2.3. Peripheral Blood Mononuclear Cell Isolation. A mononuclear cell population that consists mostly of nucleated red blood cells was isolated from peripheral blood using discontinuous Percoll density gradient centrifugation. Two milliliters of peripheral blood from each of the pediatric patients was placed in a centrifuge tube. Each blood aliquot was subsequently diluted with 2 mL of PBS (pH 7.4). A double-density Percoll column was prepared with one milliliter each of 1.090 g/mL and 1.075 g/mL diluted Percoll cell isolation media (Pharmacia). The 1.090 g/mL Percoll solution was layered at the bottom of a centrifuge tube, and the 1.075 g/mL Percoll solution was slowly and gently dispensed from a syringe over the denser solution without disturbing the interface. Four milliliters of diluted peripheral blood sample was slowly added to the top of the double-density Percoll column and centrifuged at room temperature at 400 rpm for 30 min. The cell layer between the 1.090 g/mL and 1.075 g/mL Percoll media was isolated. This cell population contained mostly nucleated red blood cells

and a small number of lymphocytes and granulocytes [24]. These cells were washed three times with two volumes of PBS. This was followed by a centrifugation step at 1,250 rpm for 10 min to discard the supernatant and keep the pellet. These cells were used for RNA and protein extraction.

2.4. Measurement of Globin mRNA.

RNA was isolated from nucleated red blood cell-enriched mononuclear cells using TRIzol® reagent (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. One hundred units of M-MLV (Invitrogen, Carlsbad, CA) were used to synthesize cDNA from $4\,\mu$L of total RNA. Quantitative real-time PCR was then performed using SYBR Green PCR Master Mix (Applied Biosystems, Norwalk, CT) and an ABI 3900 real-time system (Applied Biosystems). The following primer and probe sequences were used: α-globin forward primer ($5'$-GAGGCCCTGGAGAGGATGTT-$3'$) and α-globin reverse primer ($5'$-CGTGGCTCAGGTCGAAGTG-$3'$); β-globin forward primer ($5'$-GGCAACCCTAAGGTG-AAGGC-$3'$) and β-globin reverse primer ($5'$-GCAGCT-CACTCAGTGTGGCA-$3'$); Aγ-globin forward primer ($5'$-GAGCTCACTGCCCATGAAT-$3'$) and Aγ-globin reverse primer ($5'$-CTCTCAGCAGAATAGATTTATTATTTCT-$3'$); Gγ-globin forward primer ($5'$-AGCTCACTGCCCATG-AAG-$3'$) and Gγ-globin reverse primer ($5'$-CTCTTAGCA-GAATAGATTTATTATTTCA-$3'$); and p38 MAPK forward primer ($5'$-ACGTTCTACCGGCAGGAGCT-$3'$) and p38 MAPK reverse primer ($5'$-AAGCAGCACACACAGAGC-CA-$3'$). Human β-actin was used as an endogenous control; the forward primer sequence used for the amplification of this locus was $5'$-GCGCGGCTACAGCTT CA-$3'$ and the reverse primer sequence was $5'$-TCTCCTTAATGTCACGCACGA-T-$3'$. All data were analyzed after normalization to β-actin expression and each reaction was performed in triplicate. RT-PCR experiments were performed in triplicate.

2.5. Preparation of Standard cDNA and Standard Curve.

Three cDNA samples were randomly selected from each group and amplified using the corresponding primer pair to prepare standard control DNA. PCR products were separated and extracted from agarose gels. DNA concentration was measured using spectrophotometry (260 nm). DNA was considered to be of high quality if the $OD_{260\,nm}/OD_{280\,nm}$ ratio was between 1.8 and 2.0.

mRNA was measured following 10-fold serial dilution of standard DNA (10^8 to 10^4 copies in ultrapure water). This determination was performed in triplicate following the use of a SYBR Green real-time RT-PCR assay. Associated Ct values were plotted against DNA copy number to construct a standard curve.

2.6. SYBR Green Real-Time RT-PCR Assay.

A real-time RT-PCR assay was carried out using the SYBR Green Master Mix (Invitrogen) Kit. A concentration of $0.2\,\mu$M of each primer was used in each reaction. RT-PCR was performed in a final volume of $25\,\mu$L with each reaction containing $5\,\mu$L of $5\times$ SYBR Green Master Mix, $0.5\,\mu$L of each of $10\,\mu$M forward and reverse primers, $0.5\,\mu$L of dNTPs (10 mmol/L), $2\,\mu$L of cDNA, $0.5\,\mu$L of Taq (3 U/μL), and $16.5\,\mu$L of ultrapure water. The optimized thermal cycling conditions were as follows: 40 cycles of 93°C for 3 min, 93°C for 15 s, 55°C for 25 s, and 72°C for 25 s. Fluorescence was measured at the end of each cycle.

2.7. Western Blot and Antibodies.

For whole-cell extract preparation, nucleated red blood cell-enriched mononuclear cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing a complete protease inhibitor cocktail (Roche, Penzberg, Upper Bavaria, Germany) for 10 min at 4°C and the extract ($50\,\mu$g) was separated using SDS-PAGE and transferred onto polyvinyl difluoride (PVDF) membranes. Membranes were incubated with rabbit anti-p38 MAPK antibody ($1:500$), goat anti-mouse β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-phospho-p38 MAPK antibody ($1:500$, Bioworld Technology, St. Louis Park, MN). HRP-conjugated anti-goat or anti-rabbit secondary antibodies (both $1:5000$) were purchased from Sigma Aldrich (St. Louis, MO) and Santa Cruz Biotechnology, respectively. The sample sizes pertaining to the Western blot analyses for the three groups were similar to those described for previous experiments; that is, 13 patients treated with *R. Astragali*, 11 patients treated with the compounded formulation, and 11 patients treated with the placebo drug were analyzed during the Western blot analyses.

2.8. Statistical Methods.

Statistical analysis was conducted using SPSS software (Version 13.0). Results are presented as means ± standard deviation (SD). Greenhouse-Geisser corrections for nonsphericity were taken into account when determining significance. Student's t-test or one-way ANOVA was used to assess statistical significance among mean values for groups. Following ANOVA analysis, Fisher's Least Significant Difference (LSD) test was performed to confirm data and compare means ($p \leq 0.05$ was considered statistically significant).

3. Results and Discussion

3.1. Standard Curves and Assay Reliability.

A mononuclear cell population that consists mostly of nucleated red blood cells was harvested from patients and RNA was subsequently extracted. For each sample, γ- and β-globin mRNA levels were measured using a fluorescence-based RT-PCR assay to analyze the molecular mechanisms associated with 12 weeks of AMW and compounded formulation treatment. Ct values were plotted against diluted standard DNA copy number and the associated standard curve displayed a linear range across 3-4 log units of DNA copy number (10^8–10^4 copies/μL for Gγ-globin; 10^9–10^5 copies/μL for α-, β-, and Aγ-globin; and 10^9 to 10^6 copies/μL for p38 MAPK). Linear regression analysis was used to establish standard curves for α-globin ($y = -3.047x + 36.290$; $R^2 = 0.999$), β-globin ($y = -3.008x + 36.606$; $R^2 = 0.999$), Gγ-globin ($y = -3.128x + 35.796$; $R^2 = 0.999$), Aγ-globin ($y = -3.438x + 39.598$; $R^2 = 0.990$), p38MAPR ($y = -3.082x + 36.240$; $R^2 = 0.999$), and β-actin ($y = -3.394x + 39.996$; $R^2 = 1.000$).

3.2. Gγ-Globin mRNA before and after Treatment. Cells express α-, β-, and γ-globin genes during fetal and adult development in order to synthesize hemoglobin. Adult hemoglobin A is a tetramer containing two α-globin subunits and two β-globin subunits ($\alpha_2\beta_2$). Conversely, fetal hemoglobin is composed of two α-globin and two γ-globin subunits. Evaluation of globin gene expression in nucleated red blood cell-enriched mononuclear cells collected from patients before and after treatment using standard RT-qPCR and primer pairs specific to human α-, β-, and γ-globin revealed expression of mRNA transcripts specific to these globin genes. RT-PCR data indicate that Gγ-globin mRNA did not differ significantly between groups before treatment ($p = 0.923$) but was significantly different between groups after treatment ($p = 0.025$). Further multiple comparisons showed that Gγ-globin mRNAs from the compounded formulation group ($p = 0.010$) and the *Radix Astragali* treatment group ($p = 0.036$) were significantly higher than the control group; no significant difference was found between the compounded formulation group and the *Radix Astragali* treatment group ($p = 0.516$). Significant differences occurred between the placebo and treatment groups; however, the difference between the AMW and the compounded formulation group was not significant (Figure 1). The Gγ-globin mRNA levels in the compounded formulation group were 1.65-fold higher following treatment compared with levels before treatment ($p = 0.004$). Similarly, the Gγ-globin mRNA levels in the *Radix Astragali* treatment group were 1.53-fold higher following treatment compared with levels before treatment ($p = 0.002$). These differences were deemed significant. In contrast, no significant differences were observed in Gγ-globin levels in patients from the control group before and after placebo drug treatment ($p = 0.293$) (Figure 1), suggesting that AMW and its compounded formulation significantly increased Gγ-globin mRNA transcription. The latter phenomenon might explain the increased HbF levels that were observed in the clinical trial. However, neither AMW nor its compounded formulation affected α-, β-, or Aγ-globin mRNA levels after 12 weeks of treatment (Figure 2).

3.3. p38 MAPK Signaling Pathway Changes before and after Treatment. p38 MAPK was initially identified as a protein kinase that is activated by stress. This kinase has also been shown to coordinate cellular responses during erythropoiesis [25–27]. p38 MAPK is essential for hemoglobin synthesis and p38 mice exhibit severe anemia and die *in utero* due to defective angiogenesis and placental insufficiency [28]. In response to sodium butyrate and trichostatin A treatment of erythroid cells, p38 MAPK mediates HbF induction by activating cAMP response element binding protein 1 (CREB1). p38 signaling regulates Gγ-globin transcription during erythroid maturation via a downstream effector, CREB1, which binds to the Gγ-globin cAMP response element (G-CRE). Moreover, gain of p38 or CREB1 function augments γ-globin transcription and these regulatory effects were observed to be conserved under tested physiological conditions in primary erythroid cells [29].
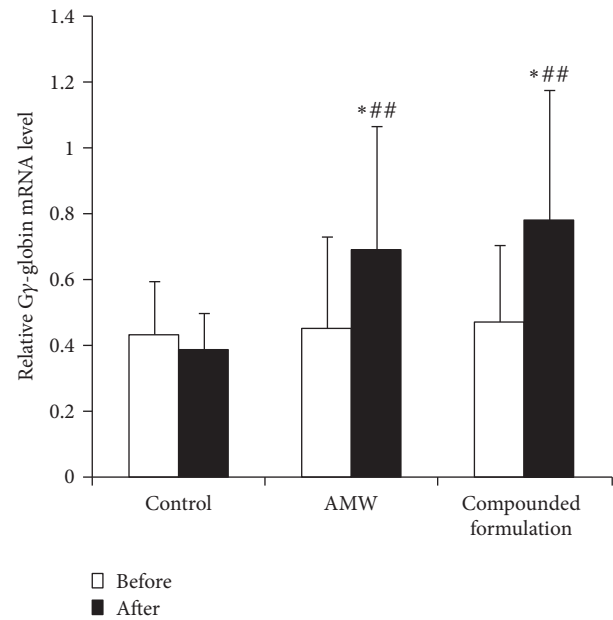

Figure 1: Relative Gγ-globin gene expression before and after treatment of pediatric β-thalassemia. $^* p < 0.05$ compared with the corresponding control; $^{\#\#} p < 0.01$ compared with before treatment of the same group.

Given that the p38 MAPK signaling pathway is the key mediator pertaining to stress induction of γ-globin mRNA [30], we proposed that the induction of Gγ-globin gene expression following AMW and compounded formulation treatment may increase p38 MAPK expression or activation. Indeed, a previous study performed by our group demonstrated that one of the compounded agents, *Plastrum testudinis*, facilitated γ-globin mRNA accumulation and HbF synthesis in K562 cells following activation of the p38 MAPK signaling pathway. These results were validated following the observation that the latter effects were suppressed after pretreatment of the K562 cells with the p38 MAPK inhibitor, SB203580 [30]. Furthermore, a separate study conducted by our group demonstrated that aqueous extracts of *Radix Astragali*, aqueous extracts of the compounded formulation, and 0.5 mmol/L sodium butyrate all independently induced p38 phosphorylation in K562 cells and cultured human erythroid progenitor cells from the peripheral blood of β-thalassemia. All of these reactions demonstrated a similar time-effect profile: phosphorylated p38 levels began to rise at 6–8 h, peaked at 48–60 h, and later began to decline. Once more, SB203580 inhibited the increases in p38 phosphorylation that were initially induced by the aqueous extracts [22].

Thus, we measured total p38 MAPK (t-p38) and phosphorylated p38 MAPK (p-p38) levels in nucleated red blood cell-enriched mononuclear cells collected in AMW-, compounded formulation-, or placebo-treated patients. We observed that t-p38 mRNA (Figure 3(a), $p = 0.999$ and 0.965 before and after treatment) and protein (Figures 3(b) and 3(c), $p = 0.554$ and 0.476 before and after treatment) levels were not significantly different between the groups. However, p-p38 protein levels were comparable in all three groups
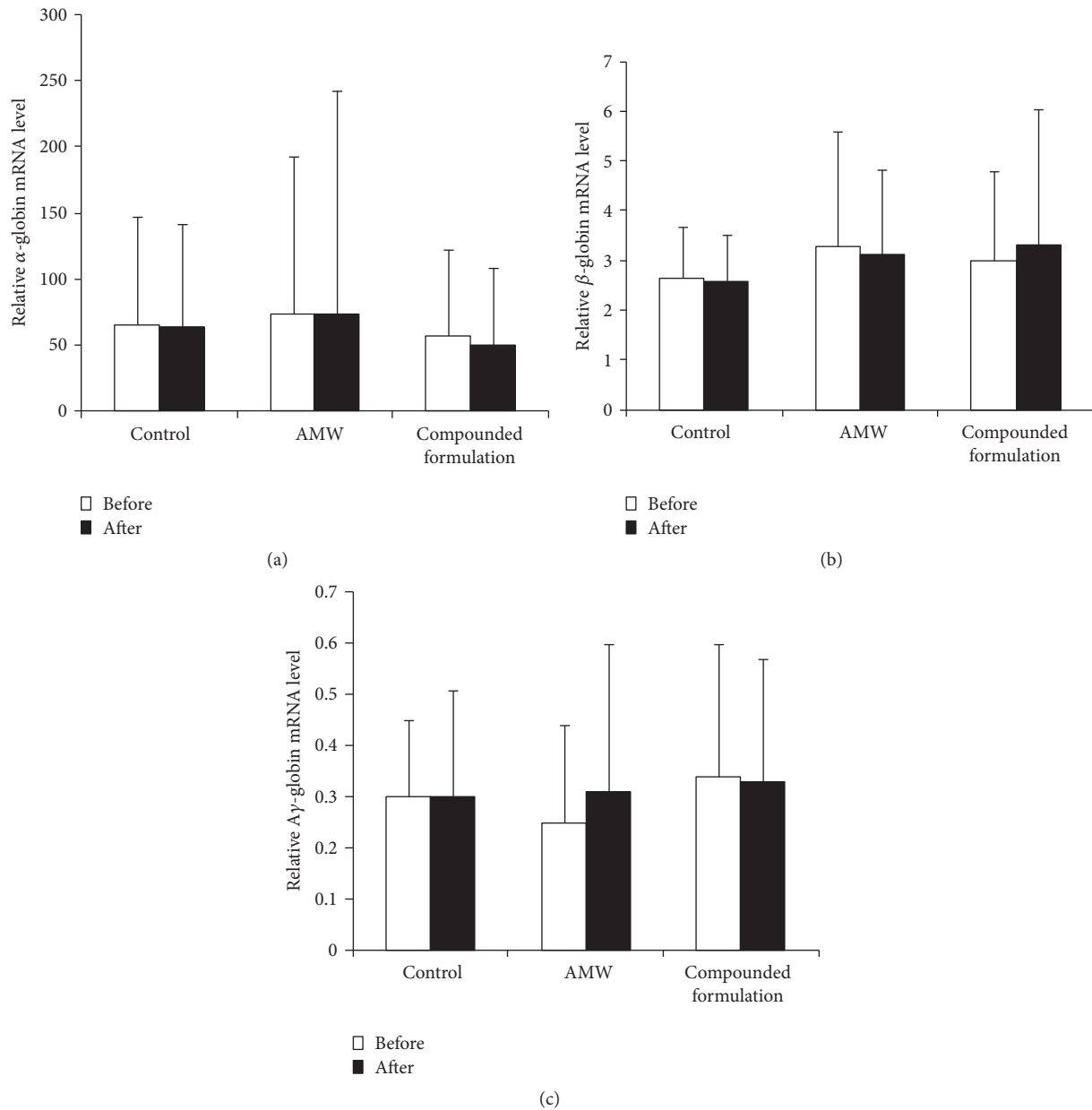
(a)

(b)

(c)

FIGURE 2: Relative globin gene expression before and after treatment of pediatric β-thalassemia: (a) α-globin; (b) β-globin; and (c) Aγ-globin.

(Figure 3(d); $p = 0.813$) prior to treatment, and protein levels were significantly different after treatment (Figure 3(d); $p = 0.013$). ANOVA analysis and subsequent LSD analysis indicated that p-p38 levels were significantly higher after AMW and compounded formulation treatments compared to placebo treatment. Compared with p-p38 protein levels that were measured before treatment, the compounded formulation and AMW groups expressed 2.3- and 2.2-fold more protein, respectively, after treatment (Figure 3). However, differences between these treatment groups were not significantly different.

We previously reported clinical outcomes pertaining to AMW and compounded formulation treatments [14]. Following 12 weeks of treatment, pediatric patients with

β-thalassemia had increased fetal Hb, erythrocytes, and mean corpuscular Hb and reticulocytes. Furthermore, no adverse effects were observed with respect to leucocytes, neutrophils, platelets, or hepatic and renal function. The treatment was effective in 64% and 62% of the children with β-thalassemia in the compounded formulation and AMW groups, respectively. These outcomes suggest that AMW and the compounded formulation are effective and safe. Moreover, increases in Hb and HbF levels were observed in AMW- and compounded formulation-treated patients, indicating that both treatments may be effective and safe in relation to HbF induction in patients.

Traditional Chinese herbal medicine (TCM) is believed to cause relatively few side effects. However, current reports
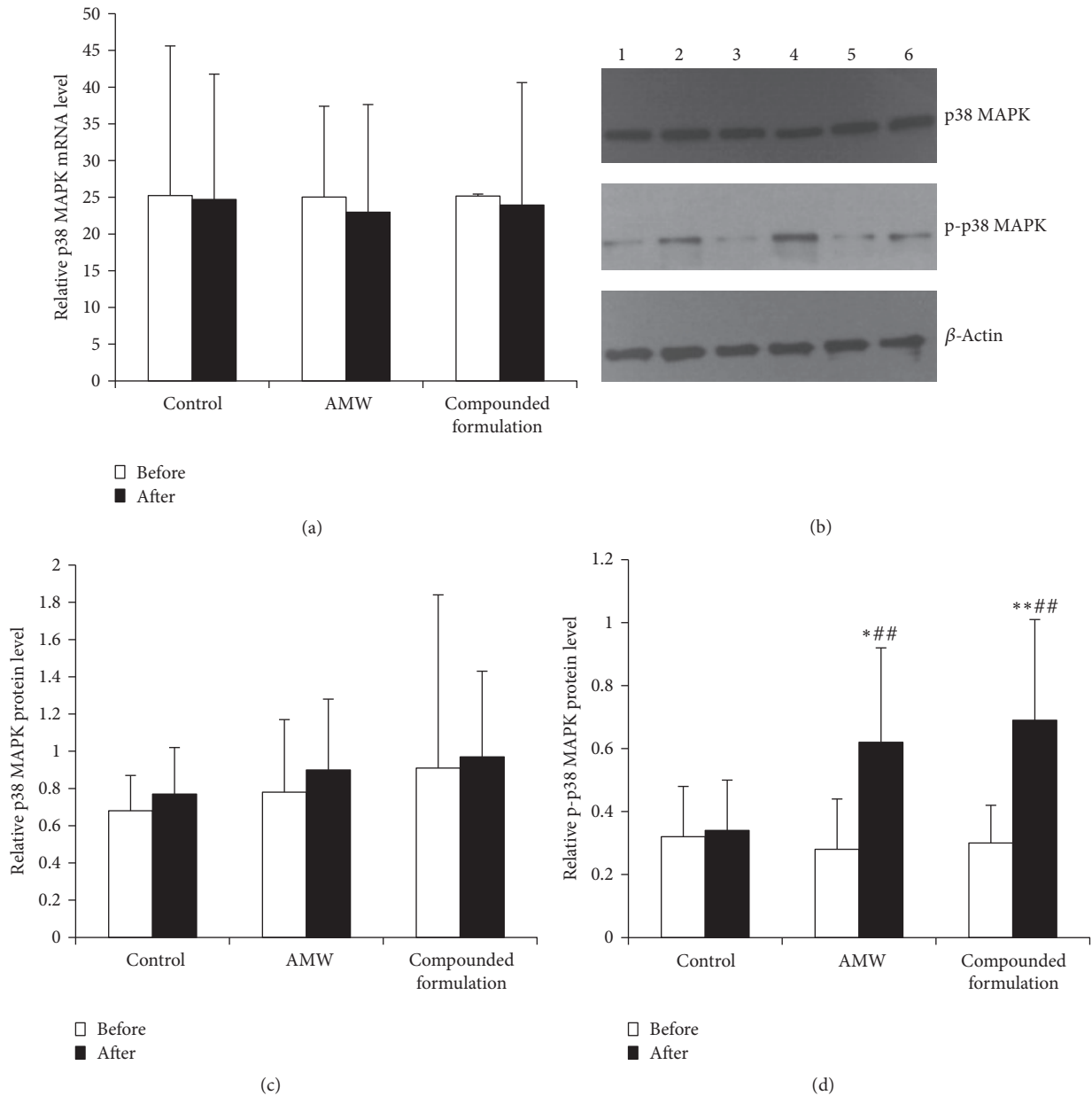
(a)

(b)

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(d)

FIGURE 3: Changes in p38 MAPK expression and activation before and after treatment of pediatric β-thalassemia. (a) Changes in mRNA levels. (b) Representative images of Western blot analyses. Graphs of blot quantification data pertaining to total p38 MAPK and phosphorylated p38 MAPK (p-p38 MAPK) levels are shown in (c) and (d), respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corresponding control; $^{\#\#}p < 0.01$ compared with before treatment of the same group.

pertaining to the use of TCM in relation to β-thalassemia are complicated and the identification of a single γ-globin inducer from TCM preparations is difficult. We previously reported that AMW and an associated compounded formulation for pediatric β-thalassemia can significantly improve hematological parameters [14, 16], resulting in elevated HbF levels in patients treated for twelve weeks with AMW or the compounded formulation. Compared with butyrate administration, induction was lasting longer and did not inhibit erythroid cells. Thalassemia is a hematological disorder and several TCM preparations have been reported to improve

hematological parameters in anemic patients [17]. More specifically, *Plastrum testudinis* has been shown to induce γ-globin expression in erythroid cells (unpublished data).

Based on TCM theory and effects observed *in vitro* and *in vivo*, we developed an AMW formulation and a combined AMW, CPG, and TPG formulation for β-thalassemia treatment. As part of this study, we report that treatment with AMW or the compounded formulation for 12 weeks significantly affected Gγ-globin mRNA levels and increased phosphorylated p38 MAPK levels with no concomitant changes to total p38 MAPK mRNA and protein levels. These

results suggest that AMW and the compounded formulation are γ-globin gene-inducing reagents. However, because TCM targets multiple receptors, definitive mechanisms of action are often difficult to identify. Larger studies are required to confirm our preliminary results regarding γ-globin gene induction and improvements in relation to the occurrence of anemia. In addition, we believe that additional factors including iron load, β-thalassemia complications, such as hepatosplenomegaly, heart failure, delayed growth and development, and long-term safety, dose optimization, and treatment regimens pertaining to TCM are also worth exploring. Thus, treatment with AMW and the compounded formulation can induce Gγ-globin gene expression *in vivo*. This induction is likely to stimulate HbF synthesis and enhance total hemoglobin levels in patients with β-thalassemia. Furthermore, independent studies performed by our group and another group demonstrated that the agents used in the treatment regimens used in the current study do not elicit these HbF stimulation phenomena through cytotoxic mechanisms [21, 31]. Thus, we believe that HbF synthesis stimulation may occur via the p38 MAPK signaling pathway.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Zhuoming Lu designed the study, recruited and treated the patients, performed the molecular biology experiments, analyzed the data, and authored the manuscript. Xinhua Qian assisted with study design, patient recruitment and treatment, and molecular biology experiments. Chunhong Zhang, Zhiwen Chen, and Guangliang Du assisted with the recruitment and treatment of patients and the execution of molecular biology experiments. All authors read and approved the final manuscript.

Acknowledgments

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