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Therapeutic effect of Halofuginone on ITP mice by regulating the differentiation of Th cell subsets

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ABSTRACT

The aim of the present study was to investigate the therapeutic effect of halofuginone (HF) in the treatment of idiopathic thrombocytopenic purpura (ITP) and explore the underlying mechanism. Sixty ITP mice were divided into four groups including control group, low dose group (25 mg/kg HF), medium dose group (50 mg/kg HF), and high dose group (100 mg/kg HF). Corresponding dose of HF was administrated by gavage daily in HF groups for 7 days, and the same volume of saline was given in control group. Platelet counts were $28.87 \pm 3.91 \times 10^9$/L, $57.13 \pm 2.75 \times 10^9$/L, $86.73 \pm 3.06 \times 10^9$/L and $89.73 \pm 2.84 \times 10^9$/L in control group, low dose group, medium dose group, and high dose group respectively, on day 7 after intragastrically administration of HF or saline. Compared with control group, three HF groups showed significantly increased levels of INF-γ and IL-2 (all $P < 0.05$), and significantly decreased concentrations of IL-4 and IL-10(all $P < 0.05$). The expression of T-bet mRNA increased and the expression of GATA-3 mRNA decreased (all $P < 0.05$) in ITP mice after intragastric administration with different dose of HF. HF significantly recovered peripheral platelet counts in ITP mice through promoting Th1 cell differentiation and attenuating Th2 differentiation in ITP mice.

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1. Introduction

Immune thrombocytopenic purpura (ITP), also known as primary immune thrombocytopenic purpura or idiopathic thrombocytopenic purpura, is defined as isolated low platelet count with normal bone marrow and undefined causes of thrombocytopenia [1]. ITP is an autoimmune disorder with anti-platelet circulatory antibodies against several platelet surface antigens such as platelet glycoprotein IIb/IIIa (GPIIb/IIIa) complex and GPIb [2,3]. It causes a typical purpuric rash and bleeding diathesis. Halofuginone(HF), the halogenated derivative of febrifugine, has been tested in multiple clinical trials for its potential applications in the treatment of various malignancies as well as fibrotic disease [4–7]. Recently, HF was reported to be able to inhibit Th17 cell differentiation by activating the amino acid response pathway [8] and the percentage of Th17 cells increased significantly in ITP [9]. The purpose of this study was to investigate whether HF administration could treat the ITP mice effectively and explore the effect of HF on two subtypes of $CD4^+$ T helper (Th) cells, designated as Th1 and Th2.

2. Materials and methods

2.1. Materials

This study was evaluated and approved by Ethics Committee of Affiliated Hospital of Jining Medical College. Sixty male and female BALB/c mice (3 months old and weight 22 ± 4 g) were purchased from the Animal Center of Guangdong Medical College. Halofuginone was purchased from Shanghai Biochempartner Co.,Ltd. Alzet micro-osmotic pump was purchased from Alza Corporation(Palo Alto, USA). $CD3^+$T cell enrichment Column was purchased from Becton Dickinson Company(New Jersey, USA).

2.2. Methods

Experimental mice, fed with standard diet, were maintained under a 12-h light–dark cycle at a temperature of 20 ± 2 °C and relative humidity of 55 ± 5%. A mixture solution composing of MWReg30 (anti-mouse CD41,165 mg/mL), human serum albumin (1.5 mg/mL) and an

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irrelevant mouse mAb against human CRP to check the infusion rate was continuously infused at a rate of 0.25 ml/h for 14 days using an osmotic pump implanted in the peritoneal cavity. Blood samples were collected on day 0, 4, 5 and 6 to determine platelet count in peripheral blood [10,11]. Once the ITP animal models were established, the mice were randomly divided into 4 groups including control group (normal saline, n = 15), low dose group (25 mg/kg HF,n = 15), medium dose group(50 mg/kg HF,n = 15),and high dose group(100 mg/kg HF, n = 15).25 $mg \cdot kg^{-1} \cdot d^{-1}$, 50 $mg \cdot kg^{-1} \cdot d^{-1}$, and 100 $mg \cdot kg^{-1} \cdot d^{-1}$ were orally administrated in low, medium and high dose groups respectively daily for 7 days. Mice in model control group were given the same volume of saline. On the last day, blood samples were collected by decapitating to measure platelet, IL-2, IL-4, IL-10, INF-γ, T-bet and GATA-3, while the platelet count was registered by a New Hematology Analyzer Sysmex XE-2100.

2.2.1. Measurement of cytokines (IL-2, IL-4, IL-10, INF-γ)

Enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich) was used to quantitatively measure serum interleukin (IL)-2. The kit consists of a 96-well microtiter plate precoated with anti-IL-2 antibody that binds to mice IL-2 in the samples. An IL-2 standard is simultaneously manipulated with the specimens. Bound IL-2 was detected by adding a secondary biotinylated anti-IL-2 antibody to all wells, which conjugated the IL-2/anti-IL2 complex. Unbound excessive antibodies were removed by washing. A streptavidin–horseradish peroxidase (HRP) conjugate that has a high affinity for biotin and the bound enzyme-labeled antibodies were then added, and the reactivity was measured via a chromogenic assay. The level of substrate conversion was measured colorimetrically at an absorbance that was proportional to the amount of IL-2. The results from the IL-2 standard were plotted and the cytokine concentration in the samples was determined. The same method was used to detect IL-4, IL-10,and INF-γ.

2.2.2. $CD3^+$T cells separation

Venous blood was collected from ITP mice in control group and different dose groups. Lymphocytes were segregated and incubated in a glass Petri dish at 37 °C to remove monocytes. Erythrocytes were lysed by incubating the sample with hemolysin (0.85% NH_4Cl) at 37 °C for 10 min. Finally, T cells were isolated using a mice $CD3^+$ T Cell Enrichment Column. The purity of T cells was analyzed by flow cytometry. The T cell viability was detected by using the Typan Blue Staining Cell Viability Assay Kit (Invitrogen, USA).

2.2.3. RNA extraction and reverse-transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was extracted from $CD3^+$ T cells using a Catrimox-14 RNA Isolation kit Ver.2.11 (Invitrogen, USA). The PCR products of T-bet, GATA-3 and β-actin were 317 bp, 350 bp, and 104 bp, respectively. β-actin mRNA was amplified with RT–PCR to calibrate the cDNA in different samples.

Primer sequences are as follows:

T-bet	Sense:	5′- TGACTGCCTACCAGAATGCC - 3′
	Antisense:	5′-TGCTCGAAACTCAGCCTCAT - 3′
GATA-3	Sense:	5′- AGATGGCACGGGACACTAC - 3′
	Antisense:	5′- TTCGGTTTCTGGTCTGGAT - 3′
β-actin	Sense:	5′- CCATCGTCCACCGCAAAT - 3′
	Antisense:	5′- CATGCCAATCTCATCTTG - 3′

The cycling parameters are as follows: for T-bet, 30 PCR cycles of 40 s for denaturing at 94 °C, 40 s for annealing at 56 °C, and 1 min for DNA synthesis at 72 °C; for GATA-3, 30 PCR cycles of 40 s for denaturing at 94 °C,40 s for annealing at 60 °C, and 1 min for DNA synthesis at 72 °C; for β-actin,30 PCR cycles of 40 s for denaturing at 94 °C,40 s for annealing at 55 °C, and 1 min for DNA synthesis at 72 °C.

RT–PCR reaction products (10 μl), 2 μl of sampling buffer, and 2 ul of STBRGreen were mixed and loaded for electrophoresis in 1.2% agarose gel. The results were viewed via UV transmission and photographed through a gel imaging system (Molecular Analysis, France). Expression level of T-bet and GATA-3 was represented as relative density normalized by actin using the following formula T-bet/β-actin and GATA-3/β-actin respectively.

2.3. Statistical analysis

Statistical analysis was performed using SPSS 18.0 (IBM, Chicago, USA). Quantitative values were presented as means ± SEM calculated with values from at least three independent experiments. Significant differences between control and test groups were assessed by one-way ANOVA analysis using student Newman–Keuls post-hoc test or independent-sample unpaired *t*-test, and the expression rates and proportions were compared using $\chi 2$ tests. Significance was set at $P < 0.05$.

3. Results

3.1. Platelet count in peripheral blood of mice in each group

Platelet count was 28.87 ± 3.91 × 10^9/L, 57.13 ± 2.75 × 10^9/L, 86.73 ± 3.06 × 10^9/L, and 89.73 ± 2.84 × 10^9/L in control group and low, medium and high dose groups respectively, as shown in Fig. 1. It is indicated that platelet count reached a steady plateau at a dose of 50 $mg \cdot kg^{-1}$ which is approximately 3 times higher than control group.

3.2. Test results of cytokines including INF-γ, IL-2, IL-4, and IL-10

Compared to control group, HF groups showed significantly increased levels of INF-γ and IL-2, as well as a significantly decreased concentrations of IL-4 and IL-10, as shown in Fig. 2. The levels of INF-γ, and IL-2 in medium dose group and high dose group were both significantly higher than that in low dose group (all $P < 0.05$),while the levels of IL-4, and IL-10 in medium dose group and high dose group were both significantly lower than that in low dose group (all $P < 0.05$).No difference was identified between medium dose group and high dose group regarding the levels of INF-γ, IL-2, IL-4, and IL-10(all $P > 0.05$) (Fig. 2).

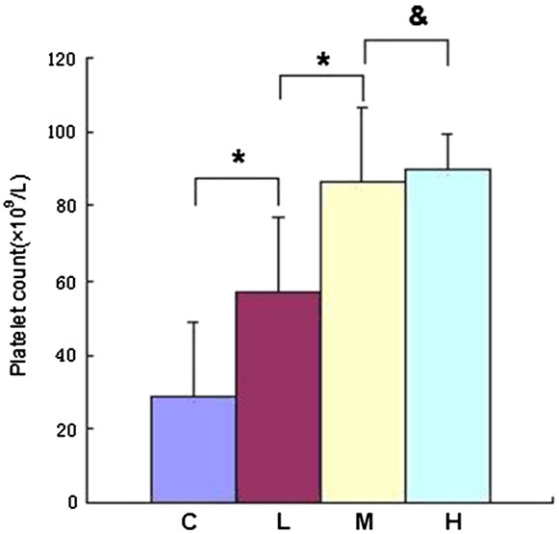

Fig. 1. Platelet count in peripheral blood of mice in each group. C-control group;L-low dose group; M-medium dose group; H-high dose group. Expression (mean ± SD) of the platelet count was studied in Control group and HF groups. *Significantly different ($P < 0.05$) and [&]Not significantly different ($P > 0.05$) using unpaired 2-tail *t* test.

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