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Qizhufang (ZSF) Ameliorates Hepatic Iron Overload via Signal Transducer and Activator of Transcription 3 (STAT3) Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Iron overload is a prominent characteristic of liver injury, but there is no effective treatment at present. Qizhufang (ZSF) is a Chinese herbal formula showed anti-HBV activities, improved liver function, and anti-fibrosis effect. ZSF showed a series of liver-protection functions, but whether ZSF can relieve hepatic iron overload is still unclear.

Material/Methods: Ferric ammonium citrate (FAC) was used to construct iron-overloaded LO2 cells. The cell apoptosis and proliferation were measured by flow cytometry and CCK-8 assay, respectively. ROS level was analyzed by fluorescence probe. RNA and protein expressions were assessed by real-time PCR and Western blot.

Results: FAC upregulated apoptosis rate, ROS level, and expression of hepcidin and p-STAT3, but suppressed proliferation and expression of DMT1, FPN1, and CP in LO2 cells. However, Qizhufang (ZSF) reversed the effect of FAC. We also found that hepcidin overexpression suppressed the expressions of DMT1, FPN1, and CP, which were reversed by ZSF. Additionally, STAT3 inhibitor AG490 suppressed hepcidin expression. Moreover, exogenous IL-6 reversed the effect of ZSF on apoptosis rate, ROS level, and the expression of hepcidin, DMT1, FNP1, CP, and p-STAT3.

Conclusions: Qizhufang (ZSF) can ameliorate iron overload-induced injury by suppressing hepcidin via the STAT3 pathway in LO2 cells.

MeSH Keywords: Hepcidins • Iron Overload • Receptors, Interleukin-6 • STAT3 Transcription Factor

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Background

As the most important iron storage and metabolism organ, the liver is the main target of iron overload injury. Under normal conditions, iron ion, as an essential trace element, plays an important role in maintaining the normal physiological activities of the liver [1]. However, a many studies have shown that iron overload plays an important role in the occurrence and development of liver fibrosis, non-alcoholic fatty liver disease, liver cirrhosis, and other liver diseases [2].

Hepcidin, which is secreted by the liver, plays a key role in iron metabolism as a negative regulator of iron homeostasis [3,4]. Under physiological conditions, hepcidin interacts with ferroportin 1 (FPN1), an iron transporter, which help with iron release to maintain systemic iron homeostasis [5,6]. Divalent metal transporter 1 (DMT1) is another gene involved in iron transport, and it helps iron transfer into cytoplasm [7]. Ceruloplasmin (CP) regulates the transfer from Fe^{2+} into Fe^{3+} by oxidizing reaction and further reduces the formation of oxygen radical [2,8]. Previous studies showed that iron overload could induce hepcidin expression [9]. The increased hepcidin binds to ferroportin and blocks the export of iron [10].

Qizhufang(ZSF) is a Chinese herbal formula in which the main ingredients (Phyllanthus niruri, Polygonum cuspidatum, Salvia miltiorrhiza, Radix astragali, and Radix paeoniae rubra) showed anti-HBV activities, improved liver function, and antifibrosis effect [11–13]. Most liver diseases are usually accompanied by hepatic iron overload, which can cause the inflammatory reaction and cell apoptosis, and researches showed that this was caused by peroxidation of iron overload [14,15]. Qizhufang (ZSF) shows a series of liver-protection functions, but whether Qizhufang (ZSF) can relieve hepatic iron overload is still unclear. Therefore, in this research we explored the potential mechanism of Qizhufang (ZSF) in the treatment of hepatic iron overload.

Material and Methods

Cell culture and transfection

Human normal hepatocyte (LO2) cells and HEK293 cells were bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium (Solarbio, Beijing, China) or DMEM medium (Solarbio, Beijing, China) at 37°C with 5% CO_2.

A lentiviral plasmid which expresses hepcidin together with helper plasmids psPAX2 and pMD2G were used to transfect HEK293 cells. The viral supernatants were used to infect LO2 cells after 48 h of transfection. Real-time PCR and Western blot were used to check the transfection efficiency.

Cell proliferation assay Cell Counting Kit-8 (CCK-8) assay

The cell proliferation was analyzed by CCK-8 assay. Briefly, LO2 cells were seeded into 96-well plates with the density of 3×10^3 cells/well. After 24 h, FAC (100 μmol/L, Macklin, Shanghai, China), ZSF (0, 0.1, 0.2, and 0.4 μmol/L, pharmaceutical factory of Nanjing University of Chinese Medicine, Nanjing, China) or AG490 (10 μmol/L, MEC, USA) were used to treat the cells, and after 0, 24, 48, and 72 h later, the mixed liquids of 10% Cell Counting Kit -8 solution (SAB Biotech, MD, USA) and 90% RPMI 1640 medium were added. The optical density at 450 nm was measured after 1 h.

Real-time PCR

The mRNA expression levels in LO2 cells were analyzed by real-time PCR. Briefly, total RNA from LO2 cells was extracted with TriQuick Reagent (Solarbio) and then an equal amount of RNA was used for further inverse transcription using a Reverse Transcription kit (Fermentas, USA). Next, a PowerUp SYBR Green Master Mix kit (Thermo Fisher Scientific) was used to conduct the real-time PCR with these cDNAs. GAPDH was used as normalization. The primers used in this research were:
hepcidin (5'-TCCCACAACAGACGGGACAAC-3' and
5'-GTCTTGCAGCACATCCCACAC-3');
DMT1 (5'-GTTCTGACTCGCTCTATTG-3' and
5'-GCTCGTAAATGTGAGGATG-3');
FPN1 (5'-AGAGCAGCAGCAGCGATAG-3' and
5'-ACCGCAAAGTGCCACATCC-3');
CP (5'-GAGCCGATTGGGGTGAG-3' and
5'-GGTGAATGTTTCTGTGGGTG-3');
GAPDH (5'-AATCCCATCACCATCTTC-3' and
5'-AGGCTGTTGTCATACTTC-3').

Western blot

Target cells were isolated in radioimmunoprecipitation assay (RIPA) lysis buffer (JRDun, Shanghai) and the extracted protein was collected by centrifuging. The same amount of protein was separated by SDS-PAGE gel and then transferred onto a nitrocellulose membrane (NC membrane, Millipore, USA). After blocking with 5% skim milk, the NC membrane was probed with relevant primary antibodies (hepcidin, DMT1, FPN1, CP, p-STAT3, STAT3, and GAPDH, Abcam, Cambridge), followed by appropriate secondary antibodies (HRP-labeled Donkey Anti-Goat IgG (H+L), HRP-labeled Goat Anti-Rabbit IgG (H+L) and HRP-labeled Goat Anti-Mouse IgG (H+L), Beyotime, China). Finally, the target bands were assessed using an ECL detection kit (Millipore, USA) and quantified by ImageJ software.

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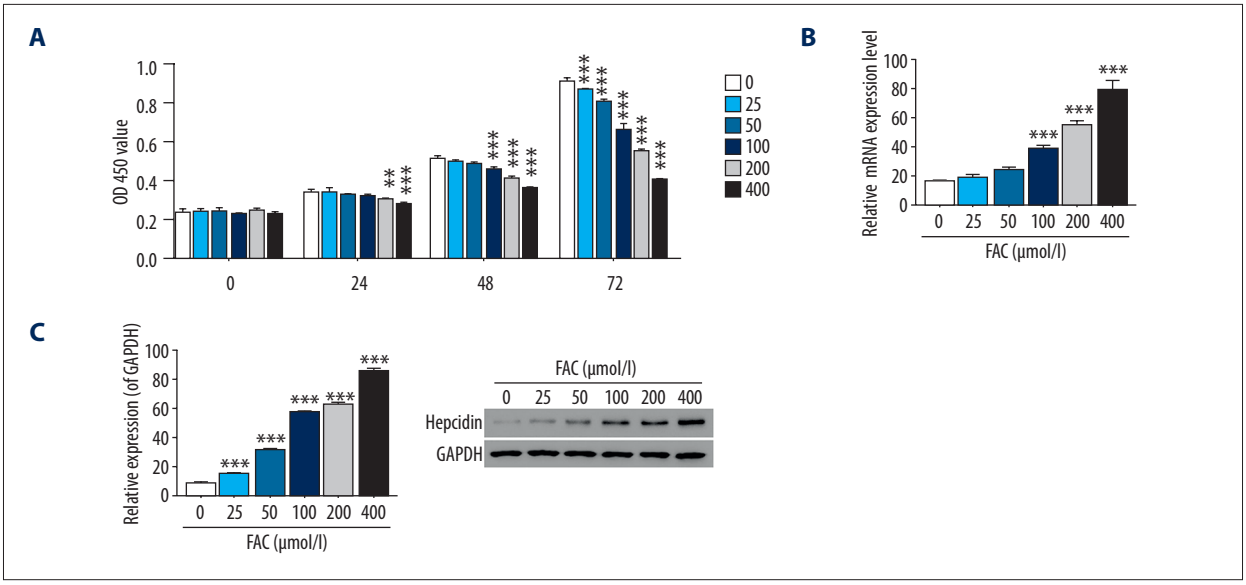

Figure 1. Ferric ammonium citrate (FAC) inhibited proliferation and upregulated hepcidin in LO2 cells. LO2 cells were cultured with a series concentration of FAC. (**A**) Cell proliferation was detected with CCK-8 assay at 0, 24, 48, and 72 hours after the treatment. The mRNA (**B**) and protein (**C**) expression level of Hepcidin were measured by real-time PCR or Western blot. LO2: control LO2 cells; 0: LO2 cells treated with 0 μmol/l FAC; 25: LO2 cells treated with 25 μmol/l FAC; 50: LO2 cells treated with 50 μmol/l FAC; 100: LO2 cells treated with 100 μmol/l FAC; 200: LO2 cells treated with 200 μmol/l FAC; 400: LO2 cells treated with 400 μmol/l FAC. ** p<0.01 *vs.* 0, *** p<0.001 *vs.* 0.

Cell apoptosis assay

Target cells were seed onto 6-well plates with a density of 3×10^5 cells/well and incubated overnight. Then, the cells were treated with FAC (100 μmol/l), ZSF (0, 0.1, 0.2, 0.4 μmol/l), AG490 (10 μmol/l), IL-6 (10 ng/ml), or vehicle (DMSO) for 48 h. The cells were prepared using an Annexin V-FITC Apoptosis Detection Kit (Beyotime, China) according to the manufacturer's instructions, and then cell apoptosis was evaluated with a flow cytometer.

Reactive oxygen species (ROS) detection

Reactive oxygen species (ROS) was measured by a Reactive Oxygen Species Assay Kit (Beyotime, China). Cells were collected and treated with 10 mM DCFH-DA for 20 min and then washed with culture medium 3 times. The ROS level was quantified by fluorescence intensity when excited at 488 nm and emitted at 525 nm.

Statistical analysis

Results are presented as mean ±SD. Difference significance analysis was carried out using GraphPad Prism software Version 7.0, and P<0.05 was considered as significant.

Results

Ferric ammonium citrate (FAC) inhibited proliferation and upregulated hepcidin in LO2 cells

To investigate the effect of iron overload on LO2 cells, we analyzed the cell proliferation and hepcidin expression level in LO2 cells after treatment with a series concentration of FAC (0, 25, 50, 100, 200, 400 μmol/l). As shown in Figure 1A, FAC inhibited LO2 cell proliferation in a time-dependent and dose-dependent way. However, as shown in Figure 1B and 1C, FAC increased hepcidin expression at mRNA and protein levels, showing a dose-dependent trend. These results show that FAC-induced iron overload affected LO2 cells, and these effects were significant at the concentration of 100 μmol/l; therefore, 100 μmol/L of FAC was chosen for the following experiments.

Qizhufang (ZSF) ameliorated the effect induced by FAC on LO2 cells

To test whether Qizhufang (ZSF) could ameliorate the injury caused by FAC treatment, we first analyzed the proliferation rate of LO2 cells after combined treatment of FAC (100 μmol/l) with ZSF (0, 0.05, 0.1, 0.2, 0.4, 0.8 mg/ml). As shown in Figure 2A, ZSF recovered the cell proliferative potential, and higher doses showed a larger effect. We further analyzed the apoptosis and ROS level of LO2 cells after combined treatment of FAC (100 μmol/l) with ZSF (0, 0.1, 0.2, 0.4, mg/ml). As shown in

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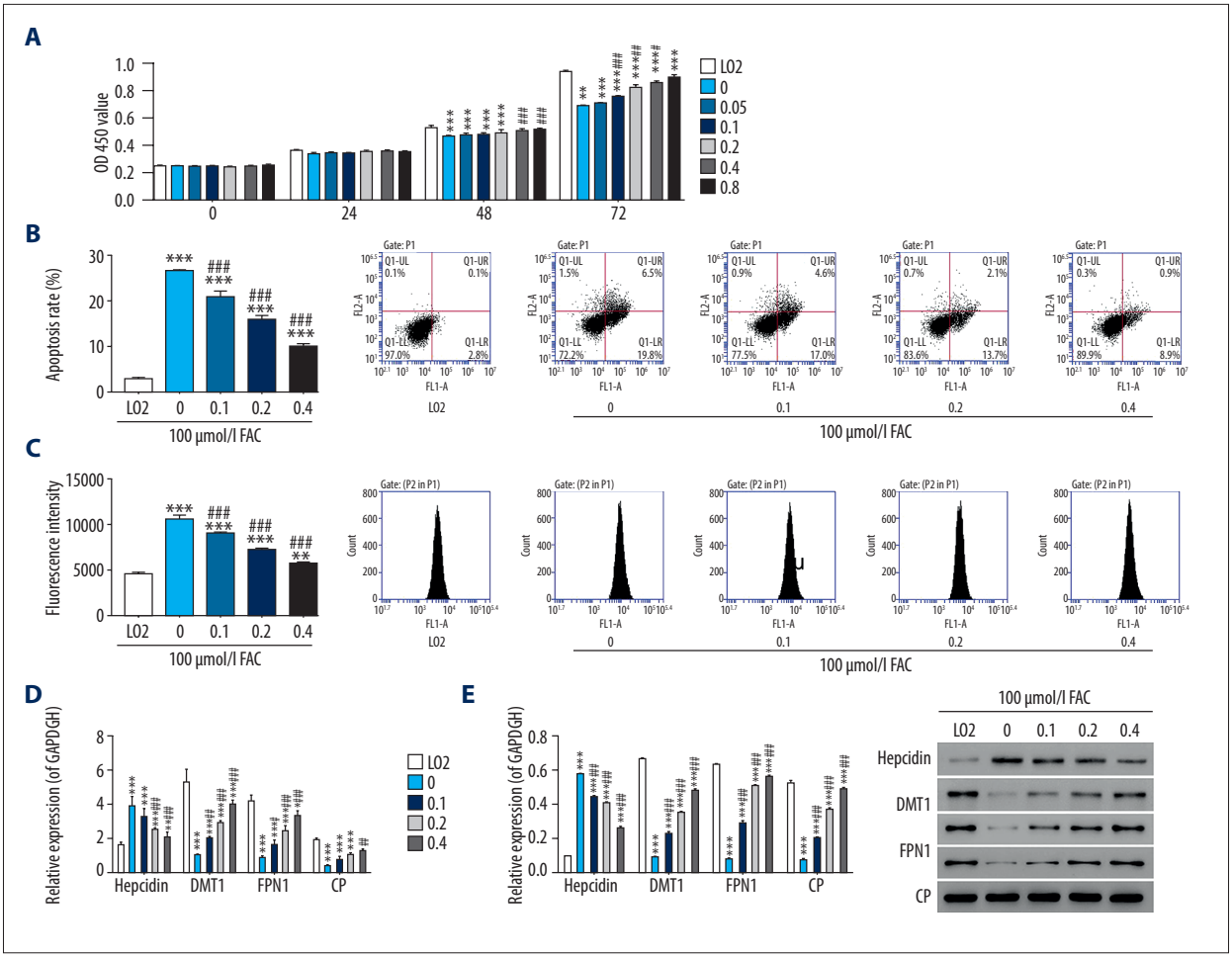

Figure 2. Qizhufang (ZSF) ameliorated the effect induced by FAC on LO2 cells. LO2 cells were cultured with FAC (100 μmol/l) and ZSF (0, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/ml). (**A**) The cell proliferation was analyzed by CCK-8 assay of the LO2 cells. The cell apoptosis rate (**B**) and ROS level (**C**) in LO2 cells were analyzed by flow cytometer and fluorescence probe. The expression levels of hepcidin, DMT1, FPN1, and CP were analyzed by real-time PCR (**D**) and Western blot (**E**). LO2: control LO2 cells; 0: LO2 cells cultured only with 100 μmol/l FAC; 0.05: LO2 cells cultured with 100 μmol/l FAC and 0.05 mg/ml ZSF; 0.1: LO2 cells cultured with 100 μmol/l FAC and 0.1 mg/ml ZSF; 0.2: LO2 cells cultured with 100 μmol/l FAC and 0.2 mg/ml ZSF; 0.4: LO2 cells cultured with 100 μmol/l FAC and 0.4 mg/ml ZSF; 0.8: LO2 cells cultured with 100 μmol/l FAC and 0.8 mg/ml ZSF. ** p<0.01 *vs.* LO2, *** p<0.001 *vs.* LO2, ## p<0.01 *vs.* 0, ### p<0.001 *vs.* 0.

Figure 2B and 2C, the apoptosis rate and ROS level in LO2 cells treated only with FAC (0) were higher than in control cells (LO2). However, the apoptosis rate and ROS level were reduced when ZSF was added. A similar trend was also shown for the expression of hepcidin. The expression of hepcidin at mRNA and protein levels was highest in the 0 group, but was downregulated by ZSF (0.1, 0.2, and 0.4) treatment (Figure 2D, 2E). However, the expressions of DMT1, FPN1, and CP showed the opposite trend. As shown in Figure 2D, the lowest mRNA expression levels of DMT1, FPN1, and CP were in the 0 group, but extra ZSF (0.1, 0.2, and 0.4) upregulated the expression level. Similar results were found in the protein expression levels of DMT1, FPN1, and CP (Figure 2E). These results suggested that ZSF could ameliorate the adverse effect of iron overload induced by FAC on LO2 cells.

Effect of Hepcidin overexpression in LO2 cells

To further analyze the effect of hepcidin during the iron overload process, we constructed hepcidin-overexpressing LO2 cells. As shown in Figure 3A and 3B, compared with the control group (Control) and vector control group (Vector), the mRNA and protein expression levels of hepcidin in LO2 cells infected with lentiviral plasmid expressing hepcidin (oeHepcidin) were higher. We further treated the hepcidin-overexpressing cells with 0.2 mg/ml ZSF. Figure 3C shows that the cell apoptosis rate was higher in hepcidin-overexpressing cells (Vehicle) than in control LO2 cells (Vector), but was reduced after ZSF treatment (ZSF). The ROS level showed a similar trend. As shown in Figure 3D, hepcidin-overexpressing cells (Vehicle) showed

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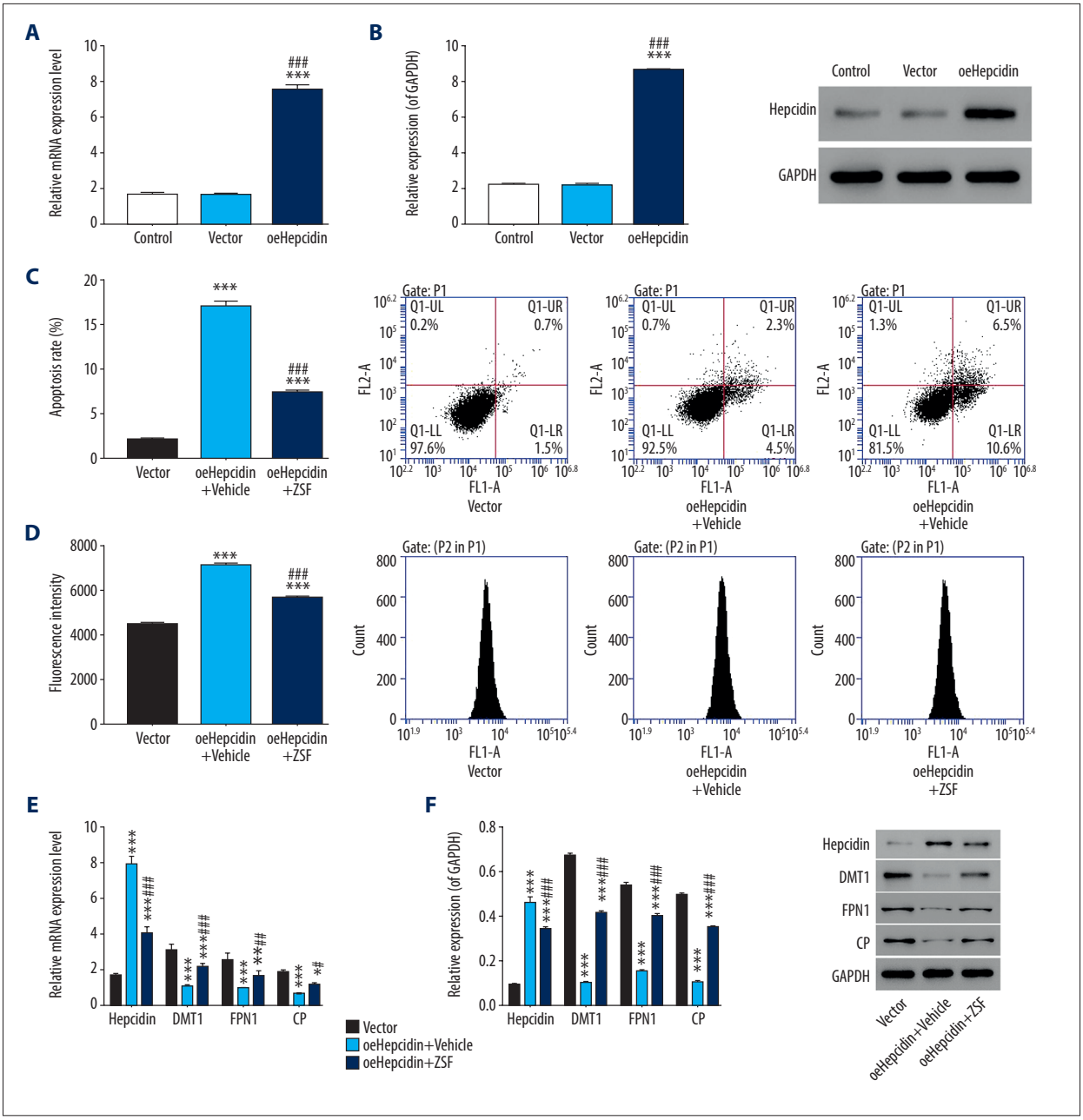

Figure 3. Hepcidin overexpression regulated cell activity and iron-related protein. Real-time PCR (**A**) and Western blot (**B**) were used to analyze the overexpression efficiency of hepcidin in LO2 cells. The cell apoptosis rate (**C**) and ROS level (**D**) was measured by flow cytometer and fluorescence probe. (**E**) mRNA and (**F**) protein expression levels of hepcidin, DMT1, FPN1 and CP were measured by real-time PCR and Western blot. Control: control LO2 cells; Vector: LO2 cells were infected with control lentiviral plasmid; oeHepcidin: LO2 cells were infected with lentiviral plasmid expressing hepcidin. * $p<0.05$ *vs.* Control or Vector, *** $p<0.001$ *vs.* Control or Vector, # $p<0.05$ *vs.* 0, ### $p<0.001$ *vs.* Vector or oeHepcidin+Vehicle.

a higher ROS level, and ZSF ameliorated this. We further analyzed the expression levels of hepcidin, DMT1, FPN1, and CP. As shown in Figure 3E and 3F, hepcidin-overexpressing cells (Vehicle) showed a relatively higher expression level of hepcidin, but relatively lower expression levels of DMT1, FPN1, and CP. ZSF treatment downregulated the expression level of hepcidin and upregulated the expression levels of DMT1, FPN1, and CP at mRNA and protein levels. These data suggest that hepcidin is an upstream regulator of iron overload.

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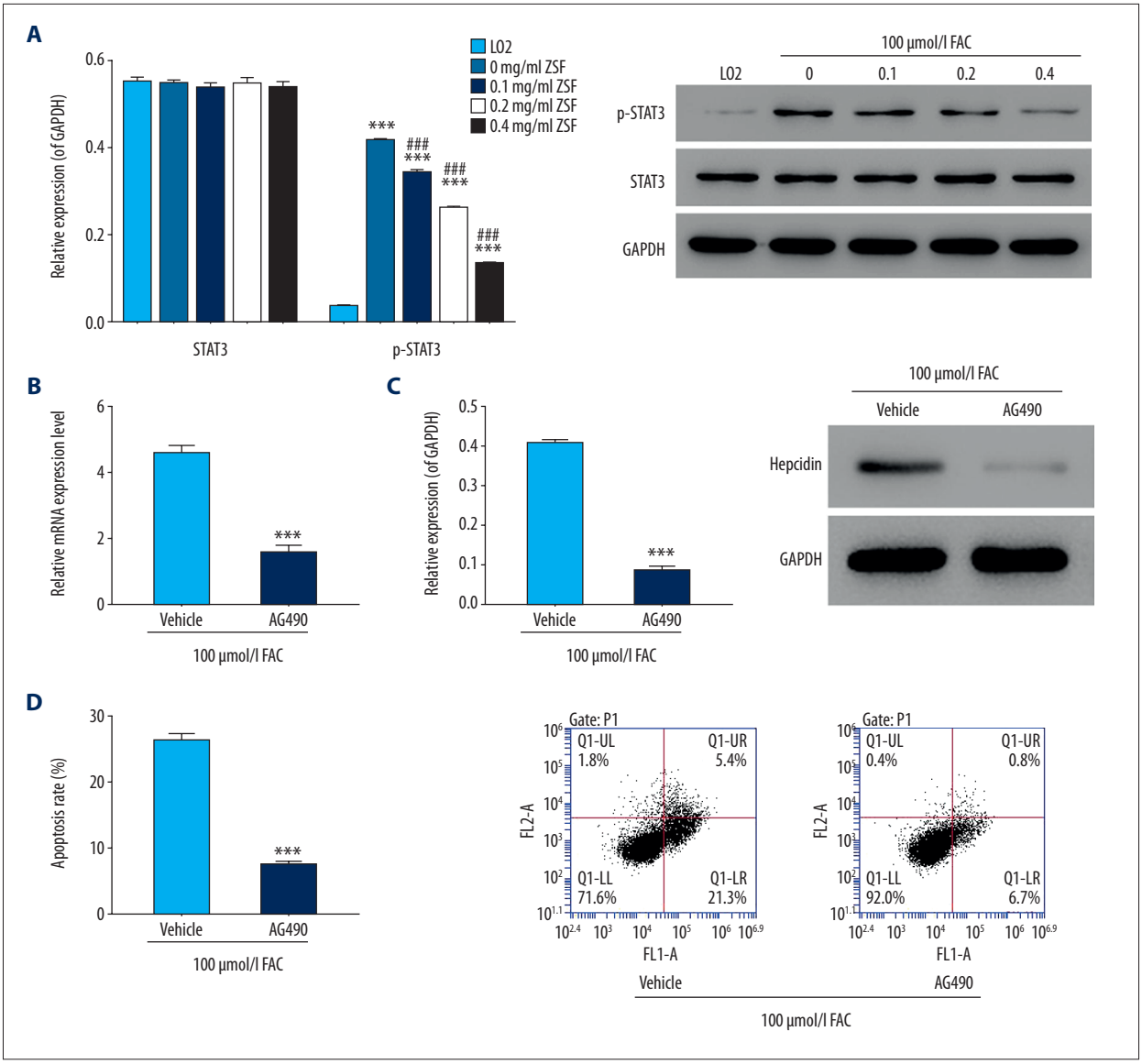

Figure 4. ZSF regulated hepcidin via STAT3. (**A**) The expression level of STAT3 and p-STAT3 were analyzed by Western blot. Hepcidin expression was analyzed by real-time PCR (**B**) and Western blot (**C**). (**D**) The cell apoptosis rate was detected with a flow cytometer. LO2: control LO2 cells; 0: LO2 cells cultured only with 100 μmol/l FAC; 0.1: LO2 cells cultured with 100 μmol/l FAC and 0.1 mg/ml ZSF for 48 hours; 0.2: LO2 cells cultured with 100 μmol/L FAC and 0.2 mg/ml ZSF for 48 hours; 0.4: LO2 cells cultured with 100 μmol/l FAC and 0.4 mg/ml ZSF for 48 hours; Vehicle: LO2 cells cultured with 100 μmol/l FAC and DMSO; AG490: Vehicle: LO2 cells cultured with 100 μmol/l FAC and AG490. *** p<0.001 *vs.* LO2/Vehicle, ### p<0.001 *vs.* 0.

Qizhufang (ZSF) suppressed hepcidin expression via STAT3

To further investigate the iron overload regulating pathway, we analyzed the effect of ZSF on the expression level of STAT3 and p-STAT3. As shown in Figure 4A, the expression level of STAT3 showed no significant difference among groups. However, results from Western blot showed that p-STAT3 was significantly increased by FAC treatment, but was reduced by ZSF treatment in a dose-dependent manner. However, the relationship between p-STAT3 and hepcidin was still unclear. Therefore, we analyzed hepcidin expression in LO2 cells after the combined treatment of FAC (100 μmol/l) and STAT3 inhibitor AG490 (10 μmol/l). As shown in Figure 4B and 4C, AG490 inhibited the expression level of hepcidin, and we also found that AG490 suppressed the apoptosis rate in LO2 cells (Figure 4D).

IL-6 abrogated Qizhufang (ZSF) effect

We evaluated the effect of IL-6 on the iron overload-regulating pathway. As shown in Figure 5A and 5B, IL-6 abrogated the

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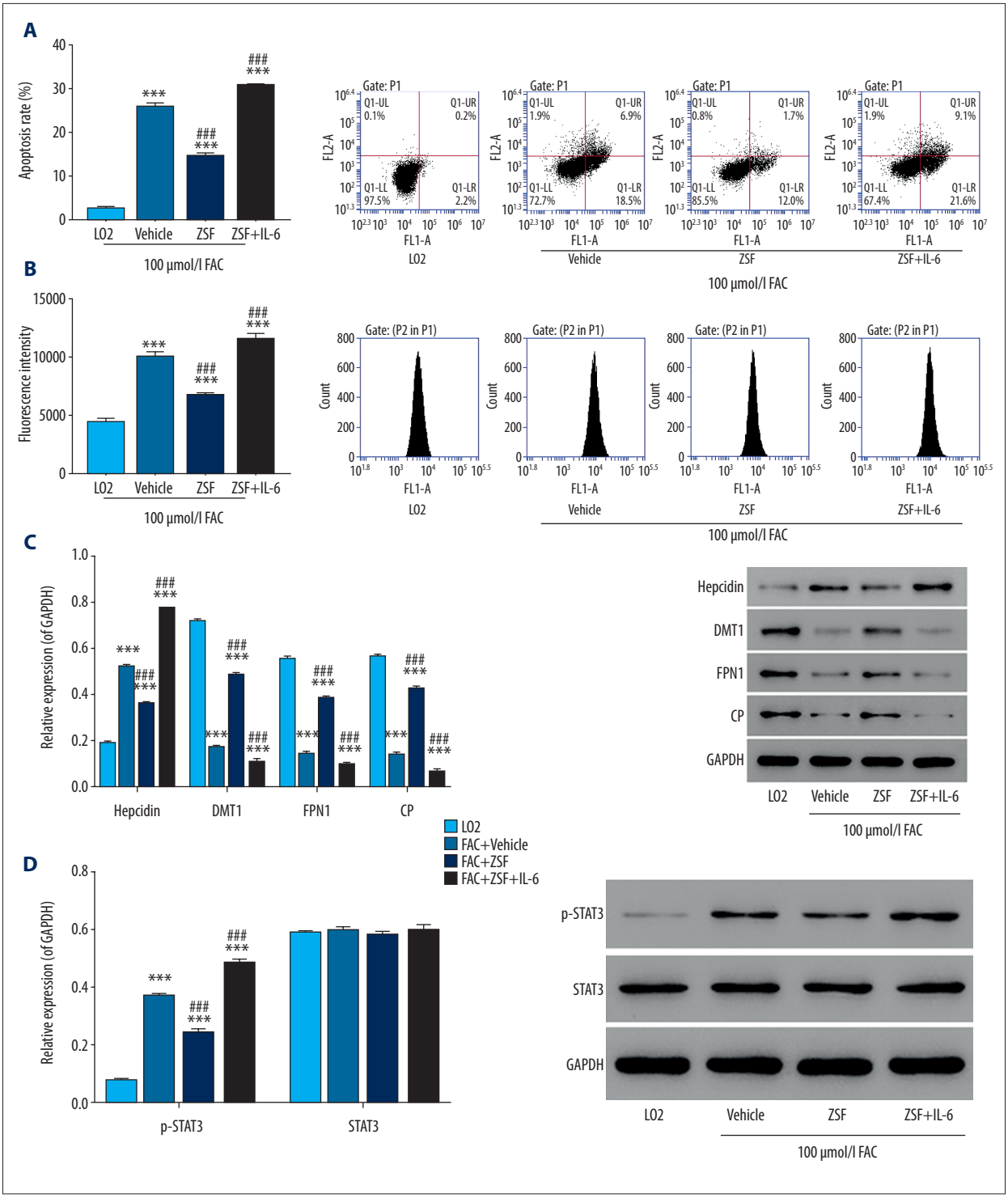

Figure 5. L-6 abrogated Qizhufang (ZSF) effect. (**A**) The cell apoptosis rate was measured with a flow cytometer. (**B**) ROS level was measured by fluorescence probe. (**C**) The expression levels of Hepcidin, DMT1, FPN1, and CP were analyzed by Western blot. (**D**) The expression levels of STAT3 and p-STAT3 were analyzed by Western blot. LO2: control LO2 cells; Vehicle: LO2 cells cultured with 100 μmol/L FAC and DMSO; ZSF: LO2 cells cultured with 100 μmol/L FAC and 0.2 mg/ml ZSF; ZSF+IL-6: LO2 cells cultured with 100 μmol/L FAC, 0.2 mg/ml ZSF, and 10 ng/ml IL-6. *** p<0.001 *vs.* LO2, ### p<0.001 *vs.* Vehicle.

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suppression of ZSF on cell apoptosis and ROS level induced by FAC. We also assessed the STAT3/p-STAT3 expression level by Western blot. Results showed that the expression of p-STAT3 was increased by FAC and suppressed by ZSF, but recovered when IL-6 was added (Figure 5D). The expression trend of hepcidin was consistent with expression of p-STAT3 (Figure 5C). We also evaluated the effect of IL-6 on the expression of DMT1, FPN1, and CP. We found that IL-6 reversed the increased expression of DMT1, FPN1, and CP, which was suppressed by FAC (Figure 5C). These data indicate that IL-6 abrogates the effect of ZSF on iron metabolism by activating the STAT3 pathway and modulating its downstream molecules.

Discussion

As one of the essential trace elements in the human body, iron helps hemoglobin to transport oxygen and maintains normal physical activity [16]. However, iron overload can also cause a variety of physiological diseases, especially liver injury. Previous studies reported that iron overload could cause liver fibrosis, non-alcoholic fatty liver disease, liver cirrhosis, and other liver diseases [15,17]. Although some investigations have assessed the potential mechanism of the occurrence of iron overload, there is still no effective treatment to avoid injury from iron overload. In this study, we first used ferric ammonium citrate (FAC) to treat LO2 liver cells to obtain iron-overloaded cells. As expected, our results showed that the proliferation of LO2 cells was inhibited and the expression of hepcidin in LO2 cells was increased after FAC treatment. Previous studies reported that hepcidin inhibits iron discharge from macrophages or absorption from the intestinal tract, so hepcidin is always overexpressed when iron overload occurs [4,18].

Qizhufang (ZSF) is a Chinese herbal formula and its main contents are usually used to treat liver disease [11]. Iron overload is an important cause of liver injury, so we proposed that Qizhufang (ZSF) would ameliorate iron overload in the liver. In the present study, we found that Qizhufang (ZSF) rescued the LO2 proliferative potential and reversed the apoptosis induced by FAC. Based on these results, we suggest that Qizhufang (ZSF) can rescue iron overload injury in liver cells.

A previous study also reported that iron overload increases ROS levels and affects lipids, proteins, DNA damage, and hepatotoxicity [19]. DMT1, FPN1, and CP are reported to be involved in regulating iron homeostasis [20]. Therefore, we also analyzed the changes in DMT1, FPN1, and CP expression in LO2 cells after ZSF treatment, finding that FAC suppressed the expression of DMT1, FPN1, and CP, but these levels were increased after ZSF was added. These data suggested that ZSF also regulates the expression of DMT1, FPN1, and CP. However, the regulatory relationship among hepcidin, DMT1, FPN1, and CP is unclear. It has been reported that hepcidin regulates the expression of DMT1, FPN1, and CP [21,22]. Therefore, we constructed hepcidin-overexpressing LO2 cells, finding that the expression of DMT1, FPN1, and CP was downregulated in hepcidin-overexpressing LO2 cells but was increased by ZSF treatment. As expected, ZSF suppressed the cell apoptosis and ROS level, which was increased in hepcidin-overexpressing LO2 cells.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which regulates a series of responses, including inflammatory reactions in cells, and it is also reported to mediate hepcidin expression [23,24]. Studies have reported that the activity of STAT3 phosphorylation is involved in a variety of diseases [25,26]. In this research, Western blot results showed that FAC upregulates the expression of p-STAT3 and is suppressed by ZSF. Therefore, phosphorylation of STAT3 is affected rather than STAT3 expression. We also found that STAT3 inhibitor AG490 suppresses cell apoptosis and the expression of hepcidin, which was induced by FAC. These results suggest that ZSF regulates iron metabolism by inactivation of the STAT3 pathway.

As a pro-inflammatory cytokine, interleukin-6 (IL-6) regulates a series of transduction pathways that contain STAT3. Previous research showed that IL-6 induced the expression of hepcidin via STAT3 [27]. Thus, we speculated that IL-6 regulates the phosphorylation of STAT3, which is involved in the regulation of ZSF in iron metabolism. As expected, IL-6 reversed the effect of ZSF on p-STAT3 expression, and the suppressed apoptosis rate and ROS level in FAC-treated LO2 cells were also reversed by exogenous IL-6. Western blot assay showed that the increased expression of DMT1, FPN1, and CP was also suppressed by IL-6 treatment. Overall, these results suggest that IL-6 abrogates the effect of ZSF on iron metabolism by activating the STAT3 pathway and modulating its downstream molecules.

Conclusions

We found that iron overload in the liver induces cell apoptosis and increases ROS production and expression of hepcidin, and decreases the expression of DMT1, FPN1, and CP in LO2 cells. Our results show that Qizhufang (ZSF) ameliorates this iron overload-induced effect by suppressing hepcidin via the STAT3 pathway in LO2 cells.

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