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Research

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Jian-Pi-Yi-Shen Regulates EPO and Iron Recycling Protein Expressions in Anemic Rats with Chronic Kidney Disease: Accumulation of Hypoxia Inducible Factor-2α via ERK Signaling

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Abstract:

Background: Jian-Pi-Yi-Shen (JPYS), the traditional Chinese medicine (TCM) decoction has been commonly used to treat chronic kidney disease (CKD) and its complications such as anemia. JPYS has been previously found to induce erythropoietin (EPO) production in HEK293T cells and CKD rats. However, the mechanism of JPYS in treating anemia of CKD rats has remained largely unknown. Here, we further extend our effort to investigate the translational control of hypoxia inducible factor (HIF)-α protein via ERK signaling and the effect on iron recycling related protein expression by JPYS, thus revealing the mechanism of JPYS in correcting anemia in CKD.

Methods: Experimental CKD rats with anemia were induced by 5/6 nephrectomy. Rats were administrated orally with high dose (6.0 g/kg/d) and low dose (1.5 g/kg/d) of JPYS for 90 days. Serum hepcidin level was determined to evaluate iron homeostasis. Protein expressions of HIF-2α, erythropoietin (EPO), ferritin and ferroportin (FPN), as well as the phosphorylation level of extracellular signal regulated kinase 1/2 (RK1/2) were detected by Western blot.

Results: Our data showed that JPYS treatment significantly ameliorated kidney function by reducing increased levels of blood urea nitrogen (BUN), serum creatinine (Scr) and the urine protein (UPRO). Periodic acid-Schiff (PAS) and Masson staining observation showed that the renal pathological damage was restored in JPYS-treated CKD rats. In parallel, JPYS markedly improved CKD anemia through up-regulation of red blood cell (RBC), hemoglobin (HGB) and haematocrit (HCT). JPYS stimulated EPO and HIF-2α protein expression in both kidney and liver of CKD rats. Furthermore, JPYS induced the phosphorylation of ERK1/2 protein. In addition, JPYS regulated protein expressions of ferritin and FPN in both liver and spleen of CKD rats, as well as serum level of hepcidin.

Conclusions: JPYS induces the expression of EPO through ERK-mediated HIF-2α protein accumulation, and regulates systemic iron recycling, supporting its role in promoting erythropoiesis and improvement of anemia in CKD.

Key words: Chronic kidney disease; anemia; HIF-2α; EPO; ERK1/2; Iron recycling

Background

Anemia is the most common complication of chronic kidney disease (CKD) [1, 2], and CKD anemia is associated with a poor quality of life and elevated cardiovascular mortality [3]. Inadequate production of erythropoietin (EPO) and iron metabolism disorders are considered as the two main causes that contribute to anemia in advanced CKD [4, 5]. At present, the treatment of CKD patients who experienced anemia with erythropoiesis-stimulating agents (ESAs) has greatly improved their quality of life in clinical practice. This treatment however has been associated with adverse effects, such as increased cardiovascular events, promotion progression or worse outcomes in several cancers and low or no response to ESAs in some patients [6]. In addition, for some CKD patients requiring dialysis, intravenous iron supplementation is needed for those who suffer from iron deficiency, yet its safety concerns also remain [7]. Therefore, it is still needed to find a more effective and safe approach for the treatment of CKD anemia.

Traditional Chinese medicine (TCM) plays a unique role in the treatment of many diseases, and a growing body of evidence has shown that TCM is increasingly being used for preventing CKD and anemia [8-10]. Jian-Pi-Yi-Shen (JPYS), a Chinese herbal formula, is composed of eight medicinal herbs, that is Astragali Radix, Atractylodis Macrocephalae Rhizoma, Amomi Fructus Rotundus, Dioscoreae Rhizoma, Cistanches Herba, Rhei Radix et Rhizoma, Salviae Miltiorrhizae Radix et Rhizoma and Glycyrrhizae Radix et Rhizoma Praeparata cum Melle. JPYS has been prescribed for treating CKD over 20 years in clinics. Previous cellular and animal experiments have revealed that JPYS stimulated the transcriptional expression of EPO in HEK293T cells, and improved the anemia symptoms by increasing the serum EPO level and hypoxia inducible factor (HIF)-α protein in 5/6 nephretomized rats [11, 12]. These findings present that activation of the HIF signaling occurs in JPYS-induced EPO expression in CKD anemia. However, the specific mechanism involved remains to be further investigated. Besides, regulation of JPYS in the utility of available iron stores in response to iron deficiency in anemia and CKD needs to be elucidated.

A glycoprotein hormone EPO is mainly produced by the kidneys in adults [13].

It critically affects the survival, proliferation and differentiation of early-stage erythroid progenitors in the bone marrow [14]. On one hand, HIF as a transcription factor regulates 500 to 1000 gene expressions including EPO [15]. Accumulation of HIF-α protein plays crucial roles in increasing transcriptional activity of HIF-regulated genes, resulting in induction of EPO expression. Stabilization and accumulation of HIF-α protein is required to be translocated to the nucleus, where HIF-α nuclear accumulation and transcriptional activity were found to be regulated by ERK1/2 [16]. More specifically, phosphorylation of ERK1/2 directly facilitates phosphorylation of the HIF-α isoforms, thereby inhibiting the ability of HIF-α to exit the nucleus and enhancing the activity of HIF-α binding to HIF-β. Notably, the kidneys of patients with CKD retain the ability to produce EPO [5]. Besides, liver-derived EPO synthesis could contribute to circulating EPO level as deficient kidney-derived EPO synthesis due to the loss of kidney function [17]. On the other hand, humans need iron every day to synthesize new red blood cells. The absorption of iron in the diet is rather limited and most of the iron is provided by macrophages recycling from senescent red blood cells [18]. However, functional iron deficiency due to inefficient utilization of iron stores can lead to the development of anemia in CKD patients [19]. Therefore, a key to the treatment of anemia is to improve the functional available iron and to eliminate the trapping that is iron sequestration in macrophages of tissues brought by CKD.

Based on the importance of EPO and iron homeostasis in the occurrence and development of anemia in CKD, the roles of JPYS on translational control of HIF-α protein via ERK signaling and iron recycling could be hypothesized. We therefore aim to further investigate the mechanism of JPYS in CKD anemia rat model. Specifically, the translational control of HIF-α protein via ERK signaling will be firstly revealed in JPYS-treated CKD rats. And then the effect on iron recycling related protein expression by JPYS will be investigated. In addition, the outcomes of EPO expression and anemia symptoms are also determined.

Materials and Methods

Drugs

JPYS was obtained from Pharmaceutical Department Shenzhen Traditional Chinese Medicine Hospital (Lot no.180813). JPYS consists of eight herbs and

the mixed proportion of respective herb is Astragali Radix (30 g, roots of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao), Atractylodis Macrocephalae Rhizoma (10 g, rhizomes of *Atractylodes macrocephala* Koidz.), Dioscoreae Rhizoma (30 g, rhizomes of *Dioscorea opposita* Thunb.), Cistanches Herba (10 g, fleshy stems with scaly leaves of *Cistanche deserticola* Y.C. Ma), Amomi Fructus Rotundus (10 g, fruits of *Amomum kravanh* Pierre ex Gagnep.), Salviae Miltiorrhizae Radix et Rhizoma (15 g, roots and rhizomes of *Salvia miltiorrhiza* Bunge.), Rhei Radix et Rhizoma (10 g, roots and rhizomes of *Rheum palmatum* L.), and Glycyrrhizae Radix et Rhizoma Praeparata cum Melle (6 g, roots and rhizomes of *Glycyrrhiza uralensis* Fisch.). JPYS was prepared as previously described [20]. Briefly, Amomi Fructus Rotundus, Atractylodis Macrocephalae Rhizoma, Glycyrrhizae Radix et Rhizoma Praeparata cum Melle, Rhei Radix et Rhizoma, and 1/3 of the Dioscoreae Rhizoma were crushed together into a superfine blend powder. The remaining three herbs and 2/3 of the Dioscoreae Rhizoma were extracted with boiling water twice for 1 h. After centrifugation, the supernatant was concentrated under reduced pressure, and which was further mixed with the prepared powder to form JPYS sample. The yield of the extract was ~30.8%. HPLC-MS/MS chromatograms of JPYS extract are shown in **Additional file 1**, which guarantee the quality of JPYS. The sample was freshly prepared and dissolved in ddH_2O before use.

Animals

10-week-old sprague-dawley (SD) rats (24 male and 24 female, 200 ± 20 g) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China). They were maintained in a specific pathogen-free (SPF) animal facility with free access to food and water. The rats were housed under a constant temperature (23 ± 2 ℃) and humidity (55 ± 15 %) with a 12-hour light/12-hour dark cycle. This animal study was approved by the Institutional Animal Care Use Committee of Guangzhou University of Chinese Medicine, and institutional guidelines for the care and use of laboratory animals were followed.

Establishment of CKD Anemia Model in Rats

Rats were conducted by a 5/6 nephrectomy to induce CKD anemia [21]. More specifically, the abdominal cavity was opened through an incision on the right back. After the renal pedicle was clamped by vein clip, the upper and lower pole of the right kidney was removed with electrocautery, and only left one third of the right kidney. A second operation was performed 2 weeks later to remove the left kidney. The sham operation group took the same steps to open the abdominal cavity and restore the kidney after exposure to avoid pulling the kidney. All rats were intraperitoneally injected under anesthesia with 10% chloral hydrate saline (3 ml/kg body weight). The rats were randomly assigned to 4 groups in average: (1) sham group (Sham), (2) renal anemia group (RA), (3) low dose JPYS treated group (LJPYS, 1.5 g/kg/d) and (4) high dose JPYS treated group (HJPYS, 6.0 g/kg/d). Rats in the treatment groups were administered through gastric gavages, while rats in sham and RA groups were treated with an equal volume of ddH_2O for totally 90 days. After the last administration, urine for 24 h was collected, then the rats were anesthetized and euthanized for sampling. Blood samples including whole blood and serum were obtained from the abdominal aorta. Kidneys, livers and spleens were collected and observed for pathological analysis and western blotting analysis.

Hematological Examination

Whole blood levels of white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB) and hematocrit (HCT) were detected by the automatic hematology analyzer (SIEMENS 2120i, Erlangen, Germany). Urine protein (UPRO), serum urea nitrogen (BUN) and creatinine (Scr) were measured by the detection kits according to the manufacturer's specifications (Nanjing jiancheng Institute of Biotechnology, Nanjing, China). Serum hepcidin was determined by enzyme‐linked immunosorbent assay (ELISA) according to the manufacturer's instruction (Shanghai Enzyme‐Linked Biotechnology, Shanghai, China).

Histopathological Examination

After fixation with 10% neutral formalin solution for 48 h, the rat kidney tissues were routinely dehydrated, paraffin-embedded and sectioned. Sections were then subjected to PAS staining and Masson staining, respectively. Pathological

examination and quantitative analysis were performed under an optical microscope (Zeiss, Oberkochen, Germany). The scoring method of tubular atrophy in PAS staining was described previously [12]. Image J software (NIH, Bethesda, USA) was used to evaluate renal interstitial fibrosis in Masson staining. In each section, 10 microscopic fields were randomly selected for observation at 200× microscope to observe glomerular changes and measure the extent of tubular atrophy and interstitial fibrosis area.

Western blot analysis

Tissue lysis was conducted in RIPA lysis buffer containing protease inhibitors (MCE, USA) and phosphatase inhibitors (Thermo, USA). Total protein extracts were determined by BCA Protein Assay kit (Beyotime Biotechnology, China), added 4× loading buffer (Bio-Rad, USA) and heated for 10 min at 100℃. Equal amounts of protein extracts were separated through 10% Sodium Dodecyl Sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose (NC) membranes (Millipore, USA). After the NC membrances were blocked at room temperature under 5% nonfat milk for 1 h, the membrances were washed by TBST for 10 min and incubated with primary antibodies at 4℃ overnight. The membranes were washed with TBST for 3 times, then incubated with the secondary antibodies (Life Technologies, USA) at room temperature for 1h, and washed repeatedly. Finally, the expression of proteins was determined by Tanon imaging system (Tanon, Shanghai, China). The primary antibodies included HIF-2α (Abcam, USA), EPO (Santa Cruz, USA), ERK1/2 (CST, USA), p-ERK1/2 (CST, USA), Ferritin (Abcam, USA), FPN (Abcam, USA), PHD1 (Abcam, USA), PHD2 (Novus, USA), PHD3 (Novus, USA), and GAPDH (Proteintech, USA).

Statistical Analysis

SPSS 22.0 software was used to analyze and process the data. The data of each group was expressed as mean ± standard deviation (SD). Statistical significance among groups was performed by one-way ANOVA and post hoc analysis with the Least Significant Difference (LSD) test or the Dunnett's T3 test. The value of P less than 0.05 was considered statistically significant.

Results

JPYS Attenuates Kidney Injury

To examine the effect of JPYS on kidney damage, the biochemical parameters related renal function was determined. After 90 days gavage, the levels of Scr, BUN and UPRO were significantly increased in RA group compared with sham group. And the increased Scr level was remarkably decreased in JPYS-treated groups, with a similar observed for BUN and UPRO levels (**Fig. 1a-c**). Data indicated that high dose of JPYS group showed better effect in the improvement of renal function.

Furthermore, the renal pathological changes of rats in various groups were detected by PAS and Masson staining. PAS staining outcomes showed that RA group had severe glomerular hypertrophy and tubular atrophy compared with sham group, while the JPYS treatment dose-dependently ameliorated the glomerular hypertrophy and tubular atrophy in kidney of rats (**Fig. 2a-b**). Massive EPO-producing cells were distributed in the renal interstitium, and interstitial fibrosis destroyed the production of EPO [22]. In the Masson staining, fibrotic area was robustly enlarged in RA group compared with sham group. Compared with the RA group, kidney fibrosis was significantly attenuated in JPYS treatment groups in concentration-dependent manner (**Fig. 2c-d**).

Effects of JPYS on Hematological Parameters

To analyze the effect of JPYS on anemia symptoms, the hematological parameters were investigated. As shown in **Fig. 3**, levels of RBC, HGB and HCT were significantly decreased in RA group compared with sham group, confirming anemia were successfully induced in 5/6 nephrectomized rats. After treatment with JPYS extract, the declined levels of RBC, HGB and HCT restored to near normal. These results showed that JPYS could improve the anemia symptoms of 5/6 nephrectomized rats.

JPYS Increases the Proteins Expression of HIF-2α and EPO

To explore the potential mechanism of JPYS, the protein expressions of HIF-2α and EPO in kidney and liver were detected by Western blot. As indicated in **Fig. 4**, the expression of HIF-2α in CKD rats was not statistically different from that

of sham rats, both in the kidney and liver. The kidney protein level of EPO was slightly declined in CKD rats compared with sham rats, but this difference in the liver was statistically significant. Both HIF-2α and EPO levels in CKD rats were significantly increased after JPYS administration. These results showed that JPYS increased the level of EPO protein in kidney and liver tissues, which might relate to the accumulation of HIF-2α signaling.

It has been reported that Raf/MEK/ERK signaling pathway directly affects the synthesis of HIF-α protein such that induces the expression of EPO gene [23]. We further investigated whether this regulatory pathway was involved in JPYS-treated rats. As presented in **Fig. 5**, compared with the sham group, the phosphorylation of ERK1/2 protein in the RA group was down-regulated, but the difference was not statistically significant. Administration of JPYS, the phosphorylation levels of ERK1 and ERK2 were significantly increased compared with the RA group, and the difference was in a dose-dependent manner.

Effects of JPYS on Iron Recycling

Hepcidin, ferroportin (FPN) and ferritin are important regulators for iron recycling. Serum hepcidin concentration in CKD rats was significantly higher than that of sham rats, while the hepcidin level of JPYS-treated groups was significantly lower than that of RA group (**Fig. 6a**). Compared with the sham rats, the expression of FPN protein in the liver and spleen of CKD rats was significantly decreased, while the protein expression of ferritin in CKD rats was significantly increased. In comparison with that in CKD rats, treatment with JPYS at two concentrations significantly enhanced the FPN expression, and the ferritin expression was dramatically decreased by JPYS in a dose-dependent fashion (**Fig. 6b-e**).

Discussion

The global prevalence of CKD is approximately 13%, amongst which 50% patients with stage 4 or 5 CKD have anemia complication [24]. Unlike anemia of iron deficiency, anemic patients with CKD suffer from various factors such as EPO resistance, functional iron deficiency and chronic inflammation [25]. In

consideration of the safety of recombinant EPO and iron treatment, some novel therapies such as HIF stabilizers, prolyl hydroxylase domain (PHD) inhibitors and hepcidin antagonists have emerged in recent years [26-29]. All these treatments are targeting HIF signaling that are associated with EPO expression and iron metabolism. HIF-α can be hydroxylated by PHD enzymes and then recognized by the von Hippel-Lindau protein (VHL)-E3 ubiquitin ligase complex for degradation through proteasome [15]. Therefore, inhibition of PHD enzyme activity is conducive to HIF-α stabilization. Hepcidin, a small molecular peptide produced by the liver, is a key regulator of iron metabolism that is stimulated by iron overload and inflammation [30]. Hepcidin is also regulated by HIF via EPO-induced erythropoiesis [31]. In this paper, the effect of JPYS on CKD anemia was studied based on both EPO expression and iron metabolism.

After 90 days of oral treatment, JPYS significantly improved renal function and blood routine levels in CKD rats. According to the pathological examination, renal structural damage and interstitial fibrosis in CKD rats were significantly inhibited by JPYS treatment. The occurrence of renal interstitial fibrosis is accompanied by the decrease of fibroblasts, resulting in the reduction of EPO expression. In this study, JPYS significantly reduced the fibrotic area in CKD rats, the effect of which might involve the activation of HIF-2α in the kidney. In support of this notion, a recent study has shown that long-term activation of HIF-2α can inhibit the progression of renal fibrosis and improve renal function [32].

Of note, activating the HIF pathway promotes endogenous EPO production [12]. It has been found that there are three isoforms of HIF-α, among which HIF-2α is the key regulator of endogenous EPO gene transcription [7, 12]. In this study, detecting the changes in the expression of HIF-2α and EPO proteins in the kidney, we found that these protein levels in CKD rats had no significant differences compared with the sham group. HIF-2α level in RA group was even slightly higher than that in sham group, while EPO level in RA group was slightly lower than that in sham group. We assumed this might be resulted from the

compensatory increase by residual kidney of rats. One clinical study showed that EPO level in patients with CKD anemia was generally normal or slightly elevated [33], which was consistent with our experimental results. After finding that JPYS significantly increased the expression of HIF-2α and EPO proteins in the kidney, we also detected the HIF-2α and EPO proteins in the liver. When the function of the kidney is limited and it is unable to synthesize enough EPO, hepatic HIF-2α is assumed as the major role in regulating serum EPO levels [34]. Consistent with this, the expression of hepatic EPO protein of CKD rats was significantly low, and the level of HIF-2α protein was slightly lower than that of the sham group. Notably, after JPYS intervention, the expression of hepatic HIF-2α and EPO proteins was significantly increased.

Moreover, in association with HIF and its upstream regulatory cascade, one vitro experiment found that Raf/MEK/ERK signaling pathway regulated the translation of HIF-α, while blocking the expression of p-ERK1/2 protein, the transcription of EPO was inhibited [23]. In this study, it was found that the phosphorylation level of ERK1/2 in both RA and sham group was low, while the phosphorylation level of ERK1/2 in the JPYS treatment groups was significantly increased. These results implicate that JPYS may promote the accumulation of HIF-2α protein by stimulating the phosphorylation of ERK1/2. In this experiment, we also detected the expressions of PHD1, PHD2, and PHD3 in the kidney and liver, and found no significant differences in protein expression among each group. Therefore, we speculate that JPYS-induced HIF signaling pathway activation is not mediated by inhibiting the PHD pathway but by activating the Raf/MEK/ERK pathway.

Recent studies have found that hypoxia signals correlate erythropoiesis with iron homeostasis [35], which brings new insights into the treatment of CKD anemia. FPN is the only known substance exporting inorganic iron in mammalian cells, while excess iron can be stored in ferritin protein, and their expression is closely monitored under normal physiological conditions [36]. The binding of hepcidin to FPN induces the internalization and inactivation of FPN

[37], which leads to the retention of iron in cells. Increased expression of ferritin protein and declined expression of FPN protein was observed in the liver and spleen of 5/6 nephrectomized rats, which indicated that excess iron in the spleen and liver was stored by ferritin and could not be released through FPN. These findings suggest that systemic iron recycling disorder is presented in CKD rats and further reduce iron utility for erythropoiesis. In this experiment, we found that JPYS significantly increased FPN protein expression and reduced ferritin protein expression, which indicated that an improvement of utilization of iron stores in the liver and spleen. Besides, the over-expression of serum hepcidin reverted to its original level by JPYS treatment, ensuring the systematic and intracellular iron homeostasis. In line with this notion, previous studies showed that JPYS could suppress pro-inflammatory cytokines production in 5/6 nephrectomized rats [38]. The hepcidin expression in the liver can be induced by the up-regulation of cytokines, most notably IL-6 and LPS [29], whereas increased systemic hepcidin level can act on macrophages, preventing the release of iron recovered from senescent red blood cells into plasma [39]. This could also explain the expression of iron recycling proteins in macrophages of liver and spleen of CKD rats. In addition, it has been reported that the expression of hepcidin, FPN and ferritin in the liver is involved in HIF signaling [40-42]. Stabilized HIF can regulate increased FPN and decreased hepcidin and ferritin, which implies that the expression of hepcidin and iron recycling proteins are associated with the activation of JPYS-induced HIF-2α signaling. Increasing evidences show that acquiring the essential iron for erythropoiesis is mainly targeting HIF signaling management [35, 43, 44].

Conclusions

In summary, our data indicates that JPYS can correct CKD anemia through induction of EPO production and regulation of iron metabolic targets, and the mechanism of which involves in translational control of HIF-2α protein accumulation via ERK signaling. These findings provide evidence for the use of JPYS as a novel therapy for CKD anemia.

Abbreviations

JPYS: Jian-Pi-Yi-Shen; HIF: Hypoxic inducible factor; ERK1/2: Extracellular signal regulated kinase 1/2; CKD: Chronic kidney disease; EPO: Erythropoietin; FPN: Ferroportin; IL-6: Interleukin‐6; LPS: Lipopolysaccharide; RBC: Red blood cell; HGB: hemoglobin; HCT: Hematocrit; WBC: White blood cell; UPRO: Urine protein; BUN: Blood urea nitrogen; Scr: Serum creatinine; RA: Renal anemia; TBST: Tris-buffered saline with Tween; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; NC: Nitrocellulose; PAS: Periodic acid Schiff.

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Authors' contributions

CJ and LS desighed the research. WF, YH, HS, ZL, ZP and ZS performed the experiments. WF wrote the manuscript. CJ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1: Effects of JPYSP on kidney function in rats. The levels of (**a**) Scr, (**b**) BUN and (**c**) UPRO from different groups. The results were presented as the means ± standard deviations (n = 6; $^{***}P < 0.001$ compared with the Sham group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the RA group).

Fig. 2: Effects of JPYSP on histopathological examination in rats. **a** Changes of renal atrophy among different experimental groups in PAS staining (×200, scale bar=100 μm). **b** Renal tubular atrophy score. **c** Changes of renal interstitial fibrosis among different experimental groups in Masson staining (×200, scale bar=100 μm). **d** Quantification of renal interstitial fibrosis. The results were presented as the means ± standard deviations (n = 6; $^{***}P < 0.001$ compared with the Sham group; $^{\#\#\#}P < 0.001$ compared with the RA group).

Fig. 3: Effects of JPYSP on the hematological parameters. The levels of (**a**) RBC, (**b**) HGB and (**c**) HCT in blood from different groups. The results were presented as the means ± standard deviations (n = 6; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Sham group; $^{\#\#\#}P < 0.001$ compared with the RA group).

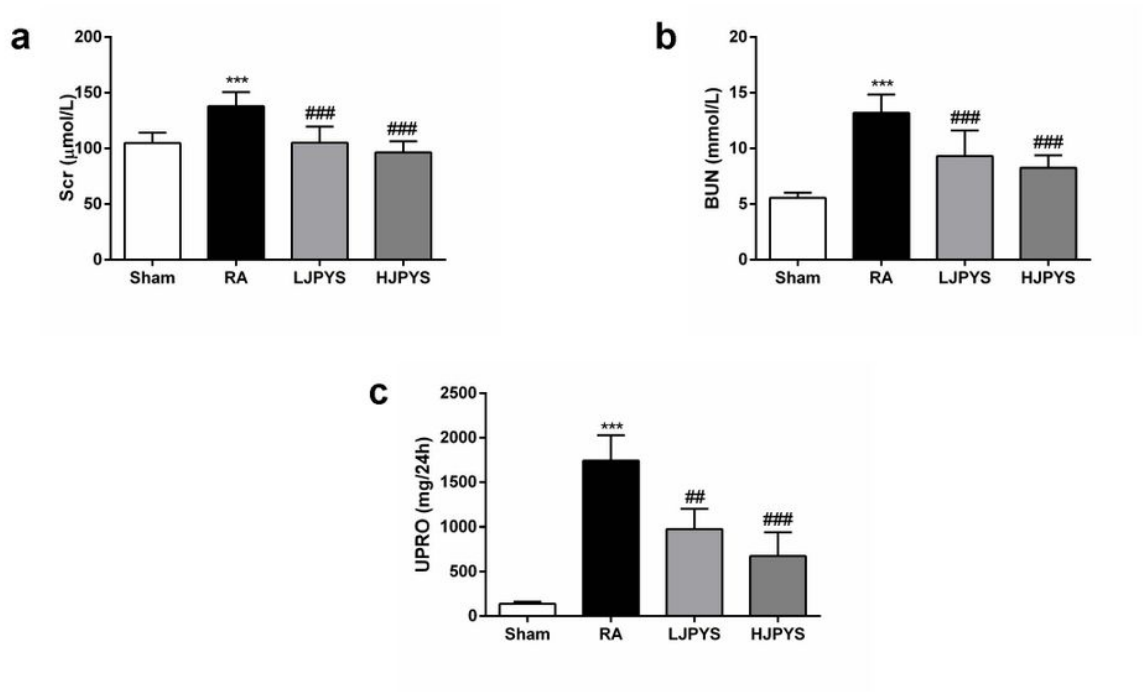
Fig. 4: Effects of JPYSP on the proteins expression of HIF-2α and EPO in rats. **a** Representative western blot images of HIF-2α and EPO in kidneys. **b** Kidney proteins expression of HIF-2α and EPO relative to GAPDH. **c** Representative western blot images of HIF-2α and EPO in livers. **d** Liver proteins expression of HIF-2α and EPO relative to GAPDH. The results were presented as the means ± standard deviations (n = 3; $^{**}P < 0.01$ compared with the Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the RA group).

Fig. 5: Effects of JPYSP on p-ERK1/2 protein expression in rats. **a** Representative western blot image of p-ERK1/2 in kidneys. **b** Kidney protein expression of p-ERK1/2 relative to t-ERK1/2. The results were presented as the means ± standard deviations (n = 3; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the RA group).

Fig. 6: Effects of JPYSP on the serum hepcidin level, and the proteins expression of FPN and ferritin in rats. **a** The level of hepcidin in serum from

different groups. **b** Representative western blot images of FPN and ferritin in liver. **c** Liver proteins expression of FPN and Ferritin relative to GAPDH. **d** Representative western blot images of FPN and ferritin in spleen. **e** Spleen proteins expression of FPN and ferritin relative to GAPDH. The results were presented as the means ± standard deviations (n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Sham group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the RA group).

Figures



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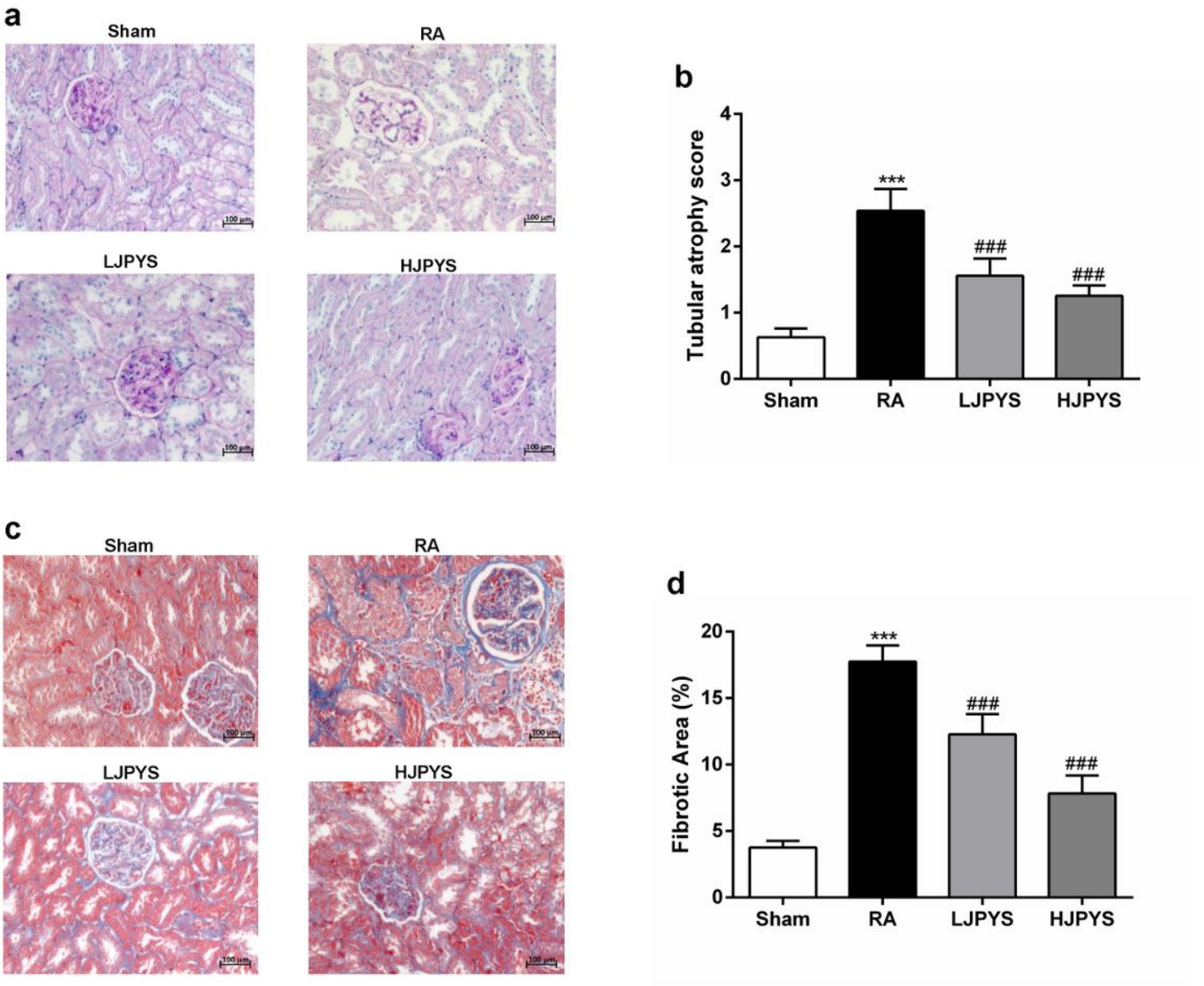

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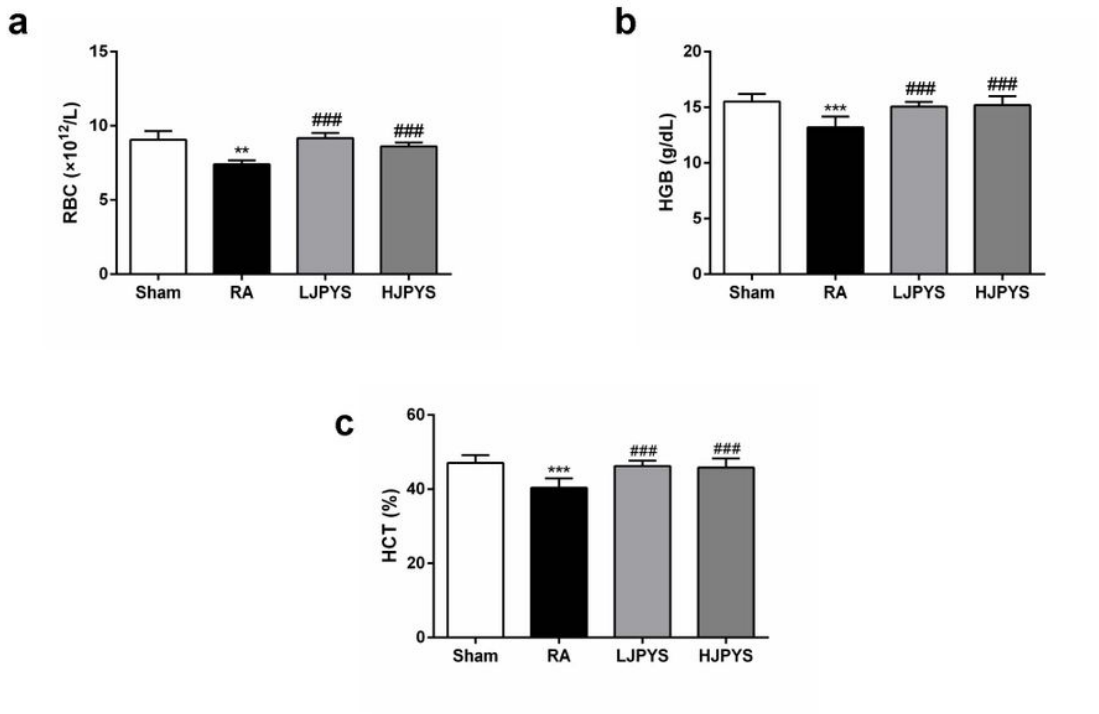

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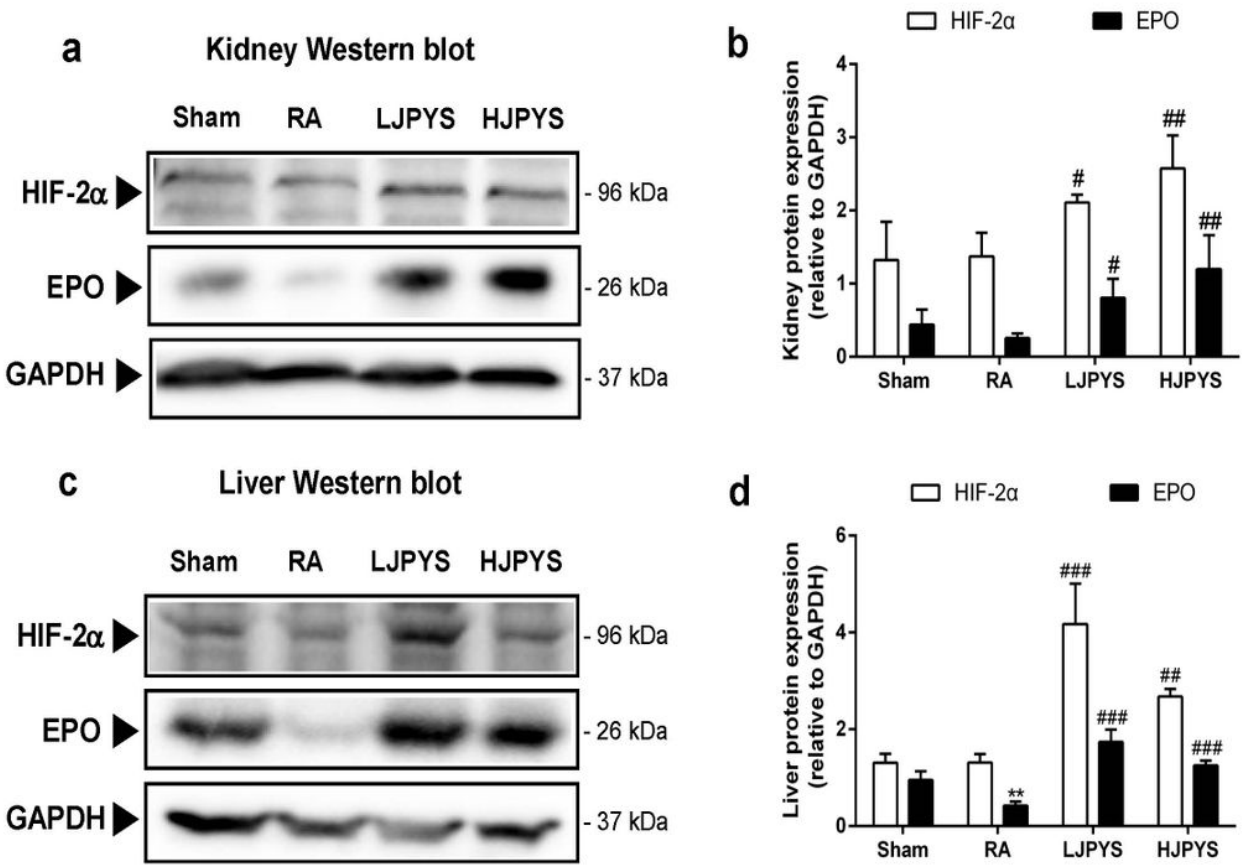

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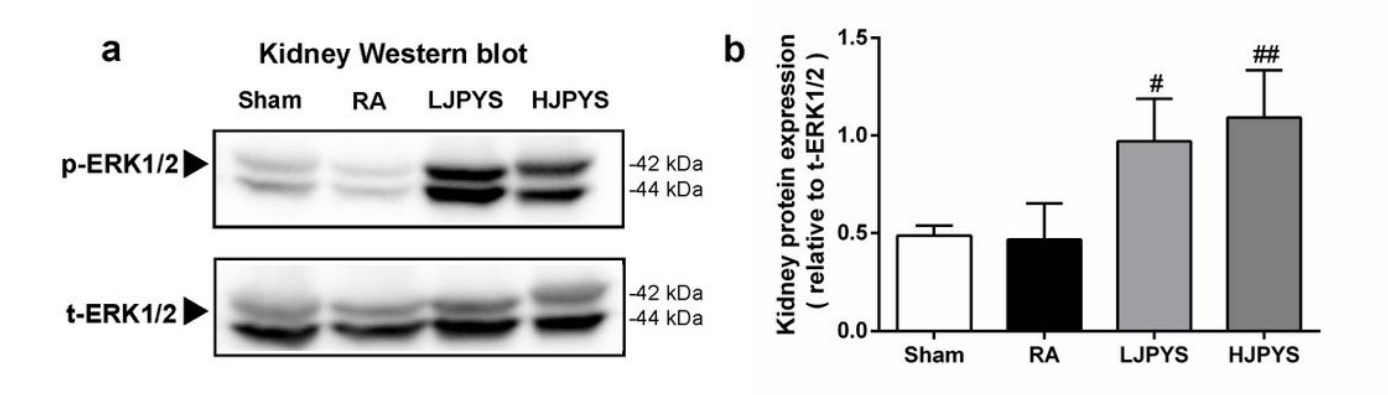

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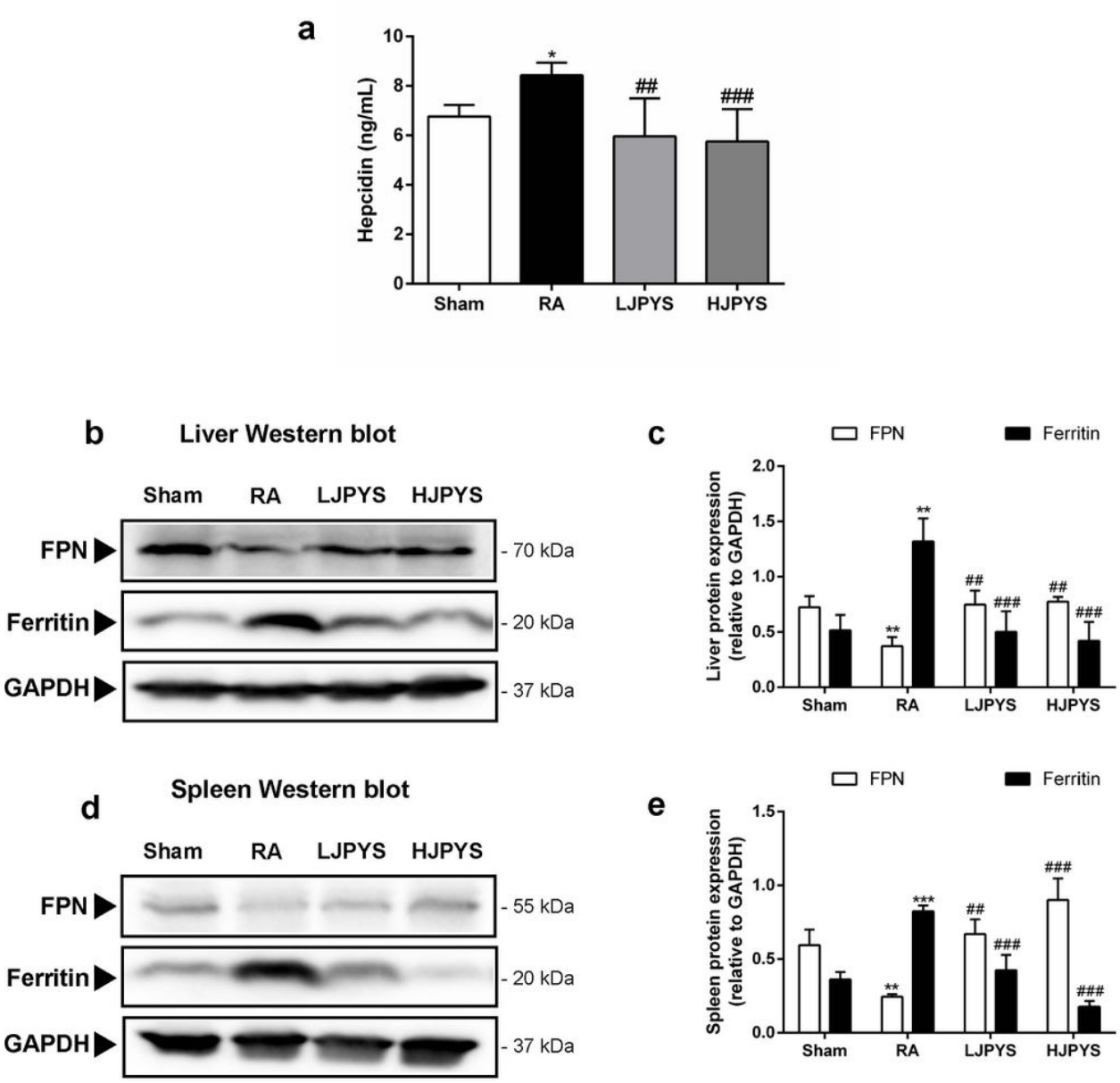

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Supplementary Files

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