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Study on HIF-PHI combined with iron supplement in treatment of renal anemia in rats

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Abstract

Background Roxadustat is a novel hypoxia- inducible factor-prolyl hydroxylase inhibitor(HIF-PHI) used to treat anemia in chronic kidney disease (CKD) patients. It has been reported that roxadustat can slow down kidney damage and delay the development of kidney fibrosis. Anemia and iron deficiency are often associated with the vast majority CKD patients, and insufficient available iron or total iron storage is often the most common cause of anemia and ESAs resistance in CKD patients. The role of iron availability in the pathogenesis of anemia in chronic kidney disease has received increasing attention.

Objectives To explore whether combined roxadustat and polysaccharide-iron complex (PIC) is more successful than standalone roxadustat, the appropriate iron supplement dosage and mechanism of roxadustat in the treatment of CKD.

Materials and methods Healthy male Sprague Dawley rats were randomly divided into two groups: the control (NC) group which were sham-operated and the CKD group. The CKD group was given an adenine diet for three weeks after right unilateral nephrectomy and further divided into 6 groups: the CKD only, CKD + PIC, CKD + Roxa, CKD + PIC (25 mg/kg) + Roxa, CKD + PIC (50 mg/kg) + Roxa, and CKD + PIC (75 mg/kg) + Roxa groups. The sham-operated rats receiving only standard diet served as the control group. Roxadustat were administrated intragastrically at 10 mg/kg thrice per week in groups with Roxa. The hemoglobin (Hb), reticulocyte hemoglobin equivalent (RET-He), reticulocyte % (RET%), plasma urea nitrogen (BUN), plasma creatinine (Cr), serum iron (SI), Total iron binding capacity (TIBC), serum hepcidin-25, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1β (IL- 1β), and High mobility group protein B1 (HMGB1) levels of each group of rats were assessed. Masson staining was used to evaluate renal fibrosis, and quantitative real-time Polymerase Chain Reaction (RT-PCR) was used to detect the mRNA expression of alpha-smooth muscle actin (α-SMA) and Fibronectin (Fn) in rat renal tissues to further evaluate renal fibrosis.

Results Level of Hb in the CKD + PIC (75 mg/kg) + Roxa group increased the fastest, roxadustat combined with PIC in the treatment of renal anemia was significantly more effective than Roxadustat or PIC alone. On day 105, in the CKD + PIC (75 mg/kg) + Roxa group, there was a significant decrease in BUN and Cr levels compared to the CKD

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only group ($p < 0.05$). Roxadustat reduces the level of hepcidin, IL-6, TNF-α, IL-1β and HMGB1in CKD rats. ($p < 0.05$). Roxadustat alleviates renal fibrosis in CKD rats ($p < 0.05$).

Conclusions HIF-PHI combined with iron supplement (Roxadustat combined with PIC) has an improved effect on the treatment of renal anemia, and early administration of sufficient iron enables the Hb to rise rapidly. Early administration of adequate dose of PIC is necessary for renal anemia. HIF-PHI can improve iron metabolism, alleviate the microinflammatory state, alleviate renal fibrosis and plays a beneficial role in the treatment of renal fibrosis in CKD rats.

Keywords Hypoxia- inducible factor-prolyl hydroxylase inhibitor, Roxadustat, Polysaccharide iron complex, Chronic kidney disease, Renal fibrosis

Introduction

Anemia is one of the most important complications in patients with CKD and is closely related to their morbidity and mortality. Anemia occurs at all stages of CKD and increases in prevalence and severity as the disease progresses [1, 2]. Inactive treatment of anemia results in recurrence of clinical symptoms, rapid deterioration of renal function, increased risk of cardiovascular events, and significant impact on patients' quality of life [3]. Decreased production of erythropoietin (EPO) due to renal dysfunction and iron deficiency are the most common causes of renal anemia [4]. Serum ferritin (SF) and transferrin saturation (TSAT) are routine biochemical examinations used to diagnose iron deficiency that reflect the storage condition of iron. Recent studies have shown that the Hb content of reticulocytes (CHr) and RET-He reflects the iron content of reticulocytes and evaluate the availability of iron for erythropoiesis [5–9]. Compared with SF and TSAT, RET-He is less affected by inflammation and can be used as an early predictor of response to anemia therapy [10]. It can accurately assess iron deficiency [11] and is recommended by NICE [12]. RET-He can be regarded as an important effective marker for assessing iron status in patients with CKD based on the recommendation of National Kidney Foundation- Kidney Disease Outcomes Quality Initiative in 2006 [13].

Renal anemia is a chronic inflammatory disorder that leads to elevated levels of many inflammatory factors, such as high-sensitivity C-reactive protein(hs-CRP), interleukin (IL)-1, IL-6, TNF-α, and interferon-γ(IFN-γ) [14, 15]. HMGB1, a highly conserved member of high mobility histone, is present in all cell types and has received some attention as a proinflammatory cytokine, which plays a role in the maintenance and amplification of inflammation [16].HMGB1 is a necessary and sufficient mediator of inflammatory response, has a wide range of immune activities, and is involved in a variety of chronic inflammatory reactions and the pathogenesis of autoimmune diseases during aging and is crucial in regulating the process of cell death and survival [17, 18]. Recent studies have shown that HMGB1 plays a key role in the progression and prognosis of CKD [19]. Hepcidin is an essential regulator of iron homeostasis and iron metabolism in the body and links inflammation and iron metabolism disorders in CKD [20, 21]. Studies have shown that inflammation mainly increases the expression of hepcidin through IL-6 [22, 23]. Microinflammation significantly impacts the efficacy of anemia treatment in CKD patients, leading to diminished response to ESA therapy. Furthermore, there is a notable absence of effective clinical interventions.

Roxadustat is the first oral small-molecule HIF-PHI used for the treatment of renal anemia [24]. Roxadustat can inhibit the ubiquitination degradation and stabilize HIF, promote the expression of erythropoietin and erythropoietin receptor, improve iron absorption, utilization, and transport, and comprehensively regulate erythropoiesis [25]. Some authors have proposed that the HIF system indirectly interacts with hepcidin synthesis through soluble factors released during erythropoiesis [26]. The efficacy of roxadustat in treating renal anemia is not only associated with the stimulation of EPO production, but also closely linked to iron metabolism. Research has demonstrated that roxadustat can modulate HIF expression in vivo, enhance iron absorption and utilization, inhibit hepcidin expression through various mechanisms, and elevate circulating iron levels for the treatment of renal anemia [27–30].

In addition to its significant achievements in the treatment of renal anemia, an increasing number of researches have demonstrated that roxadustat can alleviate the micro-inflammatory state of CKD and delay renal fibrosis [31]. This study simulated human renal anemia in an animal model of CKD and measured Hb, RET-He, RET%, BUN, Cr, SI, TIBC, TSAT, hepcidin-25, TNF-α, IL-1β and HMGB1 to investigate the efficacy of combining roxadustat with PIC in treating renal anemia, determine the optimal iron dosage for treating renal anemia, and elucidate the effect and mechanism of roxadustat in managing CKD.

Methods

Animals

Healthy adult male Sprague Dawley (SD) rats (7–8 weeks of age), weighing 200 ± 20 g, were provided by Pengyue Laboratory Animal Breeding Co Ltd., Jinan, China. Animal experiments were approved by the Ethical Committee of Qilu Hospital of Shandong University (Qingdao), the approval number: KYDWLL-201,906. All methods were carried out in accordance with relevant guidelines and regulations, and all methods are reported in accordance with ARRIVE guidelines. Animals were maintained under standard environmental conditions (22 ± 3 °C, 12/12 h light and dark cycle, and approximately 55% humidity).

Healthy adult male SD rats were randomly divided into two groups: the control (NC) ($N = 8$) and the CKD group. After one week of acclimatization, only rats in the CKD group underwent a right unilateral nephrectomy (UNx). Two weeks later, the CKD group was given an adenine diet (AD, adenine at 0.75% of the diet) for three weeks, which was then changed to a standard diet. All rats in the NC group were sham-operated and were fed a standard diet. After the end of the AD feeding, all animals had blood taken from the inner canthus. An overview is given in Fig. 1.

Fifty-two CKD rats survived, BUN and Cr increased significantly in the CKD group, and then the CKD group was randomly divided into six groups: the CKD only (intragastric administration of sterilized water for injection, $N = 12$), CKD + PIC(intragastric administration of PIC (25 mg/kg), $N = 8$), CKD + Roxa(intragastric administration of roxadustat (10 mg/kg), $N = 8$), CKD + PIC (25 mg/kg) + Roxa (intragastric administration of PIC (25 mg/kg) and roxadustat (10 mg/kg), $N = 8$), CKD + PIC (50 mg/kg) + Roxa (intragastric administration of PIC (50 mg/kg) and roxadustat (10 mg/kg), $N = 8$), and CKD + PIC (75 mg/kg) + Roxa (intragastric administration of PIC (75 mg/kg) and roxadustat (10 mg/kg), $N = 8$) groups. Roxadustat were administrated intragastrically at 10 mg/kg thrice per week in groups with Roxa. During the experiment, blood samples were collected through the inner canthus about every two weeks to measure levels of Hb, RET-He, RET%, BUN, Cr, and SI in each group. Rats that sacrificed during this period were excluded from the experiment. The rats were sacrificed at day 105. Rats were anesthetized with pentobarbital sodium (60 mg/kg body weight) by intraperitoneal injection to relieve painfulness. At the end of the experiment, all 8 rats survived in the control group, seven rats survived in the CKD only group, five in the CKD + PIC group, four in the CKD + Roxa group, CKD + PIC (25 mg/kg) + Roxa, CKD + PIC (50 mg/kg) + Roxa, and CKD + PIC (75 mg/kg) + Roxa group(In the PIC combined Roxa group, one rat in each group died dued to intragastric administration). Blood was collected from the heart before sacrificed, part of the kidney samples were frozen in EP tube at -80℃ and then detected by RT-PCR, part of the kidney samples were fixed in 4% paraformaldehyde at 4°C and embedded in paraffin for histopathologic observation using hematoxylin-eosin stain. Histomorphometric analyses of the renal tissue were performed in 3-mm-thick section. Masson staining was used to observe renal fibrosis.

Surgical interventions

Rats underwent standard sterile surgery and sodium pentobarbitone anesthesia (35 mg/kg body weight). After the rats are anesthetized, the body hair of the right kidney area was cut off and a 1–2 cm incision was made layer by layer. Following laparotomy, the perirenal capsule was peeled off cautiously without injuring adrenals. Renal vessels were ligated, and kidneys were removed. An abdominal suture was performed in two layers. After the wound was disinfected, the rat was returned to the cage. Sham-operated animals underwent the same procedure without right nephrectomy.

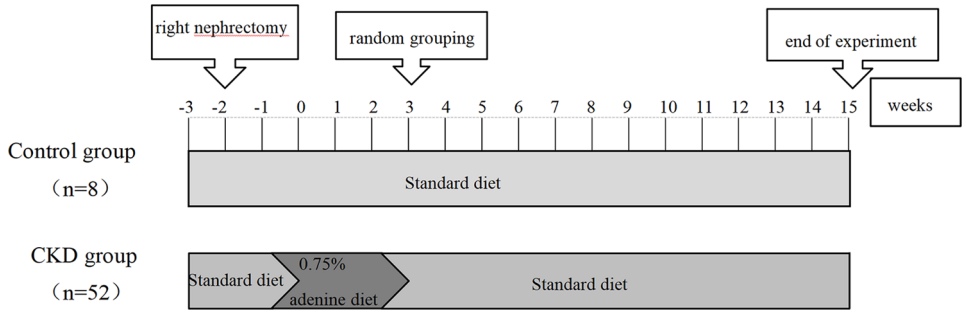

Fig. 1 Establishment of animal models. Sixty healthy male SD rats were randomly divided into two groups at first, the control group ($N = 8$) and the CKD group ($N = 52$). After one week of acclimatization only rats in the CKD group underwent right nephrectomy. Two weeks after right nephrectomy, the CKD group was given adenine diet (adenine at 0.75% in the diet) for 3 weeks and then changed to standard diet, and the CKD group randomly divided into six groups. All rats in control group which were sham-operated were given standard diet throughout the experiment. At the end of the experiments (15 weeks after adenine feeding) the animals were sacrificed

Table 1 Primers for real-time PCR

Gene	Forward5′-3′	Reverse5′-3′
Rat β-actin	CACGATGGAGGGGCCGGACTCATC	TAAAGACCTCTATGCCAACACAGT
Rat FN	TGACTCGCTTTGACTTCACCAC	GCTCATCTCCTTCCTCGCTC
Rat α- SMA	ACCCACAATGTCCCCATCTA	TCTCCAGGGAAGAAGACGAA

Reagents

We measured the levels of Hb, RET-He, RET% (Blood cell analyzer Sysmex XN-3000), BUN, Cr (Roche c701 biochemical analyzer), SI, (autobio TBA-FX8) in the laboratory department. TIBC was measured using the TIBC test kit (wuhan Elabscience Biotechnology Co., Ltd.). Serum IL-6, hepcidin-25, IL-1β, TNF-α and HMGB1 were measured using the rat IL-6, hepcidin-25, IL-1β, TNF-α and HMGB1enzyme-linked immunosorbent Kit (wuhan Elabscience Biotechnology Co., Ltd.). RT-PCR was used to detect α-SMA and Fn mRNA in rat kidney tissue to evaluate for renal fibrosis.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

TRIzol reagent (Ambion) was used to extract total RNA, and the RNA was reverse transcribed into cDNA using HiScript® II Q Select RT SuperMix for qPCR (VAZYME. Bio.inc). Realtime PCR was conducted using 2*Q3 SYBR qPCR Master Mix (TOLOBIO.Bio.inc).The primers used are shown in Table 1. β-actin served as the internal control. All PCR products were determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

Statistical analysis of the data was performed using the SPSS software (version 21.0, IBM Corp., Armonk, NY) and GraphPad Prism 8.0.1 (GraphPad, San Diego, CA, USA). Data are presented as means ± SEM, A p value of less than 0.05 was considered to indicate statistical significance. The normality test has been conducted in the study, differences among groups were subjected to one-way analysis of variance (ANOVA). LSD method was used for pairwise comparison between groups.

Results

Kidney function

BUN and Cr levels were used as indirect measures of glomerular filtration rate throughout the study. AD feeding (0.75%) combined with UNx resulted in a significant increase in BUN and Cr in the CKD groups compared with the NC group($p<0.05$). The kidney function of rats recovered gradually after the removal of adenine. On day 105, compared with the NC group, BUN and Cr levels in the CKD groups significantly increased ($p<0.05$), and the CKD + PIC (75 mg/kg) + Roxa significantly decreased compared with the CKD only group ($p<0.05$) (shown in Fig. 2a and b).

Body weight

Body weight of each group of rats during the experiment were measured. AD feeding resulted in a decrease in body weight. After resuming standard diet, the body weight of rats in CKD groups gradually recovered; On day 105, compared with the NC group, the body weight in the CKD groups was significantly lower ($p<0.05$), and the CKD + PIC (75 mg/kg) + Roxa significantly increased compared with the CKD only group ($p<0.05$) (shown in Fig. 3).

The level of Hb, RET-He, RET% and SI in each group

Hb, RET%, RET-He and SI were monitored in each group as the indicators of anemia and iron deficiency. Figure 4 shows the level of Hb, RET%, RET-He and SI in each group.

On day 21, Hb levels between the CKD and NC groups did not significantly vary($p>0.05$). On day 39, Hb levels were significantly lower in the CKD groups than in the NC group($p<0.05$), and then CKD + PIC (75 mg/

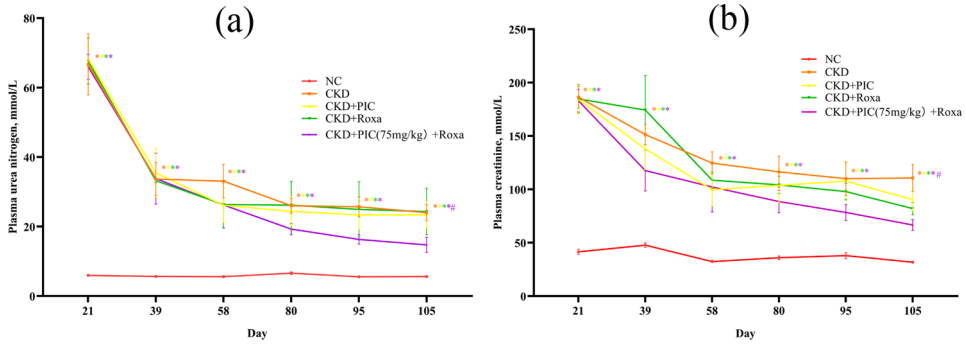

Fig. 2 The levels of BUN, Cr in different periods of rats in each group. Statistical analysis of the data was performed using the SPSS software (version 21.0, IBM Corp., Armonk, NY) and GraphPad Prism 8.0.1 (GraphPad, San Diego, CA, USA). *$p<0.05$ vs. NC group, # $p<0.05$ vs. the only CKD group. Each group is represented by a corresponding color

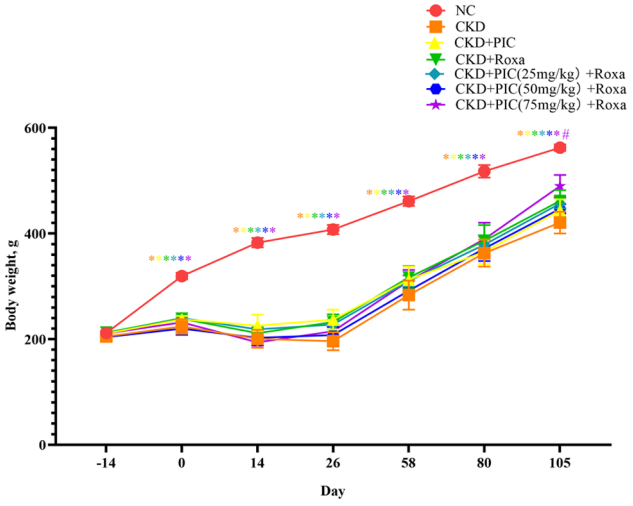

Fig. 3 The body weight in different periods of rats in each group. Statistical analysis of the data was performed using the SPSS software (version 21.0, IBM Corp., Armonk, NY) and GraphPad Prism 8.0.1 (GraphPad, San Diego, CA, USA). *$p < 0.05$ vs. NC group, # $p < 0.05$ vs. the only CKD group. Each group is represented by a corresponding color

kg) + Roxa exhibited the fastest increase in Hb. By the end of the experiment, Hb levels in all CKD groups were significantly lower than those in the NC group($p < 0.05$). Hb levels in the CKD groups which were administrated with PIC + Roxa were significantly higher than those in the CKD + Roxa, CKD + PIC, and CKD only group($p < 0.05$) (shown in Fig. 4a).

On day 21, the rats in the CKD groups showed a significant decrease in SI compared with the NC group, and an increase in RET-He; however, Hb levels did not decline until day 39. After intragastric treatment with PIC, SI levels increased while RET-He levels decreased, and these changes occurred earlier than the changes in Hb levels.

The level of TIBC, TSAT and hepcidin-25 on day 105 in each group

On day 105, compared with the NC group, TIBC levels were significantly decreased in the CKD only, CKD + PIC groups($p < 0.05$). There were no significant differences in the level of TSAT among the groups ($p > 0.05$) (shown in Table 2). Compared with the NC group, hepcidin-25 level was significantly increased in the CKD only and CKD + PIC groups($p < 0.05$). Compared with the CKD

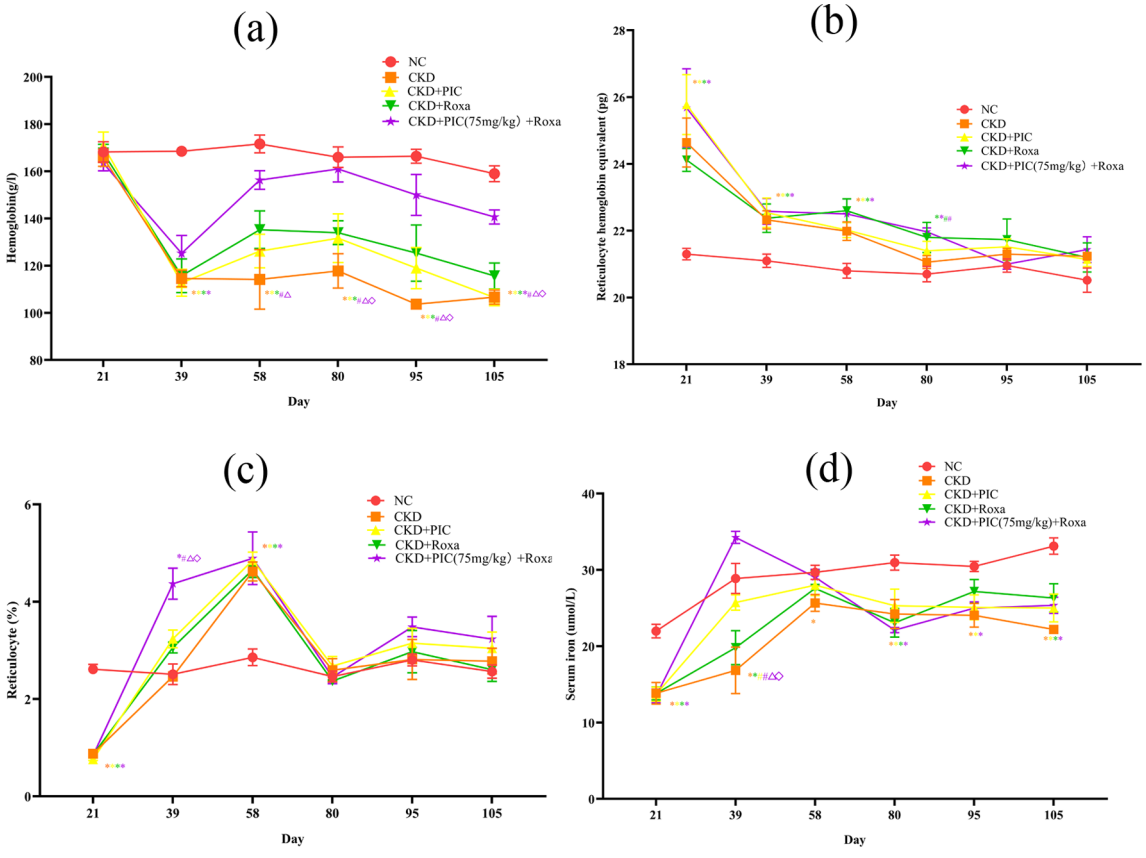

Fig. 4 The levels of Hb, RET-He, RET%, and SI of rats in each group. Statistical analysis of the data was performed using the SPSS software (version 21.0, IBM Corp., Armonk, NY) and GraphPad Prism 8.0.1 (GraphPad, San Diego, CA, USA). *$p < 0.05$ vs. NC group, # $p < 0.05$ vs. the only CKD group, Δ$p < 0.05$ vs. CKD + PIC group, ◇$p < 0.05$ vs. CKD + Roxa group. Each group is represented by a corresponding color

Table 2 The levels of TIBC, TSAT, and Hepcidin-25 in each group on day 105

Group	TIBC (umol/l)	TSAT (%)	Hepcidin-25(ng/ml)
NC	47.73 ± 1.70	69.51 ± 1.77	57.94 ± 3.60
CKD	34.58 ± 2.78*	65.20 ± 5.99	73.27 ± 5.85*
CKD + PIC	35.16 ± 5.33*	77.05 ± 12.26	72.06 ± 5.32*
CKD + Roxa	42.08 ± 2.86	63.64 ± 8.19	58.24 ± 1.80#
CKD + PIC (25 mg/kg) + Roxa	45.09 ± 2.62	64.16 ± 3.76	65.53 ± 3.46
CKD + PIC (50 mg/kg) + Roxa	39.71 ± 3.14	69.20 ± 4.65	64.92 ± 3.86
CKD + PIC (75 mg/kg) + Roxa	39.93 ± 6.38	66.00 ± 7.82	57.64 ± 2.65#

Data are presented as means ± SEM.* Statistically significant difference compared with the NC group($p < 0.05$), # statistically significant difference compared with the CKD only group ($p < 0.05$). * $p < 0.05$ vs. NC group, # $p < 0.05$ vs. the CKD only group, Δp < 0.05 vs. CKD + PIC group

TIBC: total iron binding capacity, TSAT: transferrin saturation

only group, hepcidin-25 level was significantly decreased in the CKD + Roxa and the CKD + PIC (75 mg/kg) + Roxa groups($p < 0.05$) (shown in Table 2). The findings indicated that roxadustat can reduce the level of hepcidin and improve iron metabolism.

The levels of IL-6, TNF-α, IL-1β and HMGB1 in each group

ELISA results showed that compared with the CKD only group, the levels of IL-6, TNF-α, IL-1β and HMGB1 in the roxadustat treatment groups were significantly decreased ($p < 0.05$) (shown in Fig. 5). The findings indicated that roxadustat was effective in reducing microinflammatory factors and alleviating the microinflammatory state.

Histopathology

The kidneys of rats in sham operation control group were fava-like in appearance, regular in shape, normal in volume without swelling, and the color was red-brown of normal renal tissue, with a firm and shiny texture; the cortex in section was normal red-brown, the medulla was light red, with a clear separation between the cortex and the medulla; the renal capsule was tightly bound not easy to peel off, and the pathological structure of renal tissue was clear. All kidneys of rats with CKD had varying degrees of volume enlargement with fibrosis, with a grayish-white granular appearance, an uneven surface, and no luster. The pathological manifestations were partial glomerular fibrosis, glomerular capillary loop ischemia shrinkage, balloon cavity expansion, diffuse tubular atrophy, partial renal tubules cystic expansion, epithelial cell flattening, renal interstitial fibrosis and lymphocyte infiltration. These features were completely absent in the kidneys of rats in sham operation control group. There were no statistically significant difference in pathology among CKD groups ($p > 0.05$).

The severity of tubular injury in the kidney was assessed and scored semi-quantitatively (0 = absent, 1 = minimal, < 10%; 2 = slight, 11–25%; 3 = moderate, 26–50%; 4 = obvious, 51–75%; 5 = severe >76%) by a pathologist, who was unaware of the treatment groups, and performed a blind microscopic score on renal sections. Sections stained with HE from the NC group showed normal kidney architecture and histology. They were given a score of 0 for tubular injury. Sections stained with HE in the CKD groups showed obvious tubular injury (shown in Fig. 6).

Masson staining

Masson staining showed that collagen fibers were dyed blue, indicating fibrosis, while no obvious collagen fibers were found in the control group. A large number of collagen fibers appeared in the kidney interstitial of CKD rats, manifested as increased blue fiber tissue, indicating successful modeling and renal interstitial fibrosis in all CKD groups. Compared with the NC group, there was significant collagen fiber deposition in each CKD group ($p < 0.05$). Compared with CKD + PIC and the CKD only group, the collagen fiber deposition of rats in CKD + PIC (75 mg/kg) + Roxa, CKD + PIC (50 mg/kg) + Roxa, CKD + PIC (25 mg/kg) + Roxa and CKD + Roxa groups was significantly decreased ($p < 0.05$) (as shown in Fig. 7). It is suggested that roxadustat can alleviate renal fibrosis in rats with chronic kidney disease.

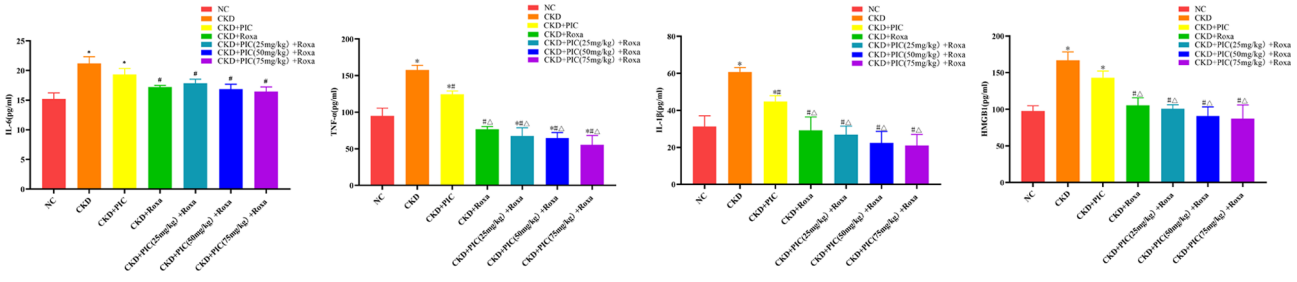

Fig. 5 The levels of IL-6, TNF-α, IL-1β and HMGB1 in each group. Statistical analysis of the data was performed using the SPSS software (version 21.0, IBM Corp., Armonk, NY) and GraphPad Prism 8.0.1 (GraphPad, San Diego, CA, USA). *$p < 0.05$ vs. NC group, # $p < 0.05$ vs. the only CKD group, Δp < 0.05 vs. CKD + PIC group

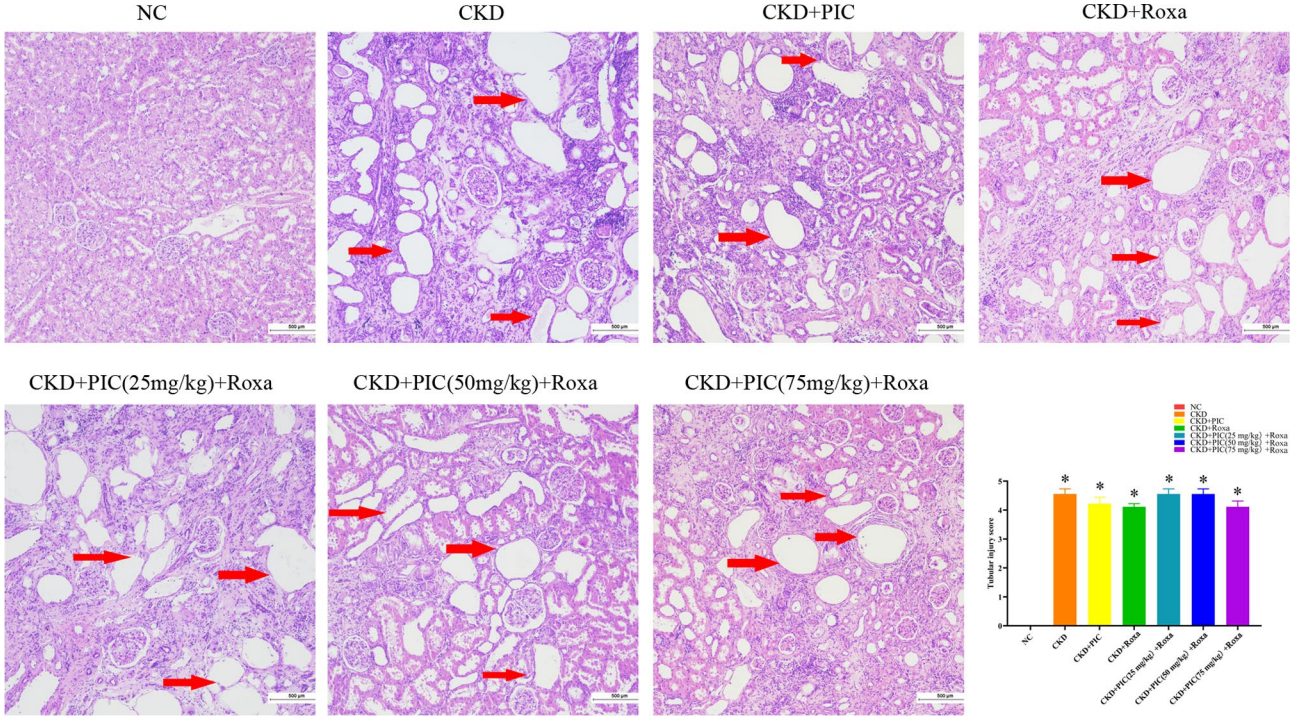

Fig. 6 HE staining of rats in each group using (×100). The pathological manifestations were partial glomerular fibrosis, glomerular capillary loop ischemia shrinkage, balloon cavity expansion, diffuse tubular atrophy, partial renal tubules cystic expansion, epithelial cell flattening, renal interstitial fibrosis and lymphocyte infiltration. These features were completely absent in the kidneys of rats in sham operation control group. The arrows in the figure indicate lesions.There were no statistically significant difference in pathology among CKD groups ($p > 0.05$). The tubular injury semiquantitative mean severity score of in CKD groups were 4.56 ± 0.18, 4.22 ± 0.22, 4.11 ± 0.11, 4.56 ± 0.18, 4.56 ± 0.18,4.11 ± 0.20. * $p < 0.05$ vs. NC group

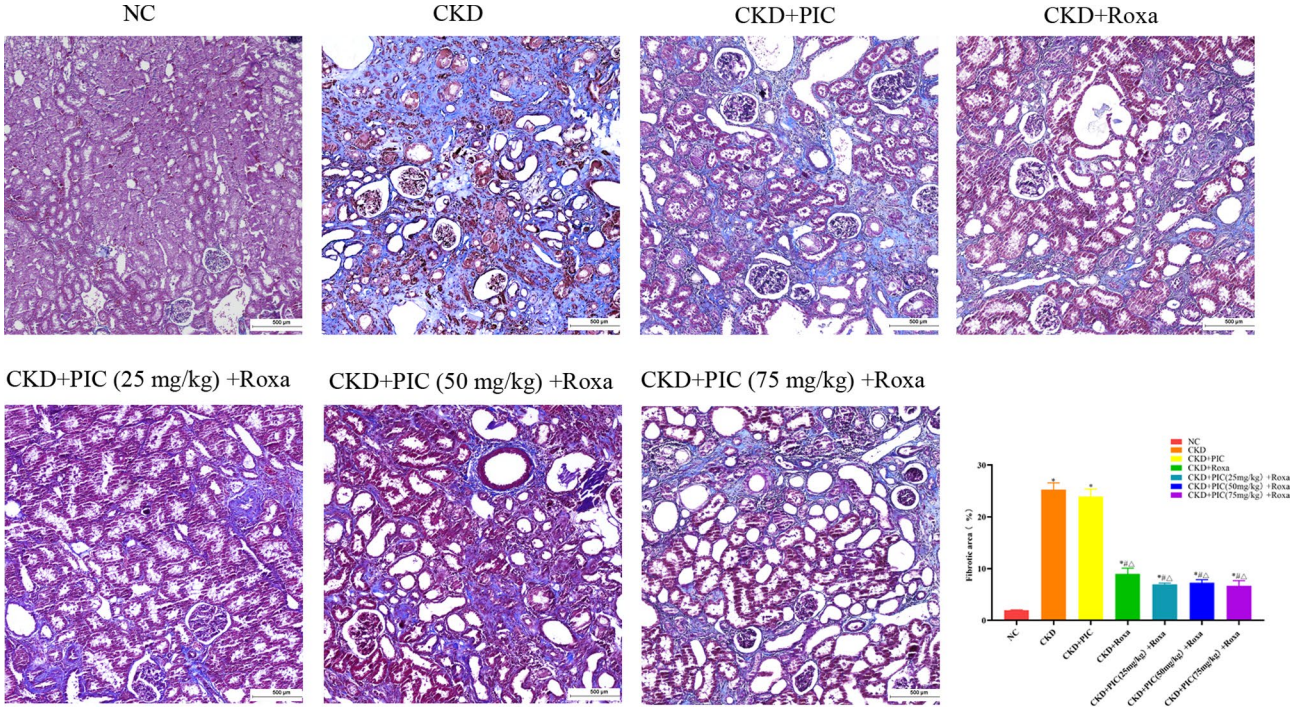

Fig. 7 Masson staining of rats in each group using (×100). Masson staining showed that collagen fibers were dyed blue, indicating fibrosis.* $p < 0.05$ vs. NC group,# $p < 0.05$ vs. the only CKD group, Δp < 0.05 vs. CKD + PIC group

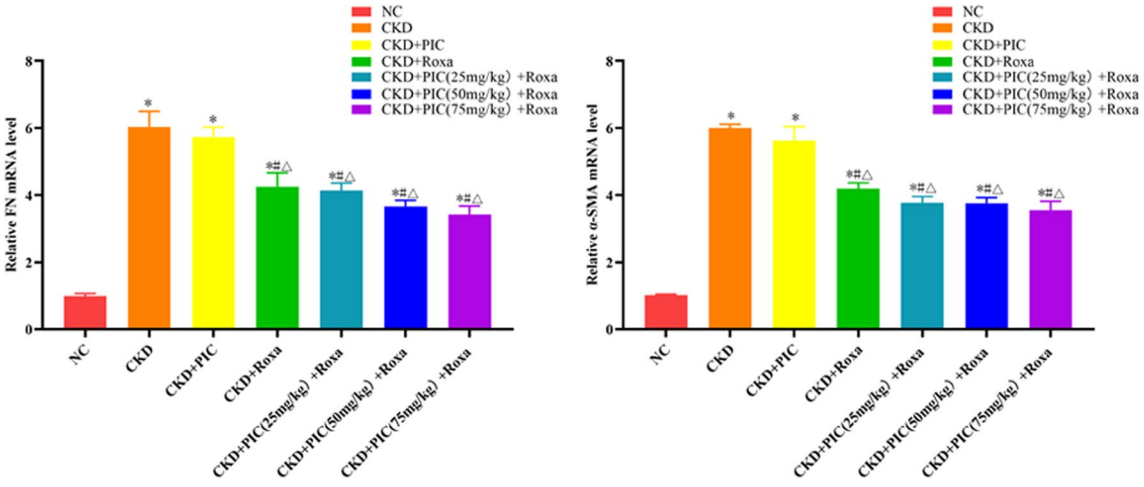

Fig. 8 FN and α-SMA mRNA expression in renal tissues in each group. *$p < 0.05$ vs. NC group, # $p < 0.05$ vs. the only CKD group, Δp < 0.05 vs. CKD + PIC group

FN and α-SMA mRNA expression in renal tissues in each group

In order to observe the renal fibrosis levels in each group, mRNA expressions of related genes FN and α-SMA were detected by RT-PCR. As shown in Fig. 8, compared with the NC group, the mRNA expressions of FN and α-SMA in kidney tissues of rats in CKD groups were significantly increased ($p < 0.05$). Compared with CKD + PIC and the CKD only group, the mRNA expressions of FN and α-SMA in CKD + Roxa, CKD + PIC (25 mg/kg) + Roxa, CKD + PIC (50 mg/kg) + Roxa, CKD + PIC (75 mg/kg) + Roxa groups were significantly down-regulated ($p < 0.05$) (as shown in Fig. 8), suggesting that roxadustat can alleviate renal fibrosis of rats with CKD and delay the progression of kidney disease.

Discussion

Numerous animal models have been established for the investigation of etiology and therapeutic interventions for CKD. Frequently, rat CKD models are induced by subtotal 5/6 nephrectomy, ureteral obstruction, ischemia/reperfusion injury, or adenine feeding. However, all models have certain limitations. The choice of the modeling method is related to the research direction. Adenine treatment-associated renal impairment results in profound renal anemia. Adenine (0.75%) in the diet (AD) triggers renal impairment in rats. This model of kidney disease is largely reversible when AD feeding is stopped. Research indicates that adenine feeding in combination with UNx restricts the adenine feeding period, while the animals still exhibit characteristics of a progressive state of CKD [32].

In this study, we used adenine feeding (0.75%) combined with UNx to establish CKD rat model, aiming to study the therapeutic effect of roxadustat combined with PIC in renal anemia in rats and the impact and mechanism of HIF-PHI in the treatment of CKD. On day 21 after adenine feeding, BUN and Cr levels in the CKD groups were significantly increased compared with the NC group. These results indicate the success of the CKD animal models. The results of BUN, Cr, HE, masson staining and RT-PCR demonstrated that unilateral nephrectomy combined with adenine diet leads to irreversible kidney damage and renal fibrosis in rats. This result was consistent with the previous study [32](Figs. 2, 6, 7 and 8).

Anemia is a major complication in patients with CKD. EPO is effective if iron is available; however, unnecessary iron supplementation results in iron overload. In recent years, RET-He, whose values are equivalent to reticulocyte hemoglobin content, has emerged as an additional parameter that is helpful in identifying iron deficient erythropoiesis [33]. The research found that RET-He is a more relevant marker of iron status than ferritin and TSAT [34]. RET-He is advantageous in that it has no interference with inflammatory conditions and can be easily measured with a widely available and popular blood cell counter [35]. It has also been found that changes in RET-He after 1 week of iron treatment are a strong predictor of hemoglobin response [35]. In the present study, we measured SI, RET-He, and TSAT to assess iron status in rats, and RET-He was detected using a blood cell analyzer Sysmex XN-3000. In this study, the RET-He level decreased initially after iron supplementation. On the 21st day, there was no abnormality in Hb, but the RET% was low and the RET-He was elevated. This might be related to the animal model. The specific mechanism needs further study. The results showed that both RET-He and SI levels changed earlier than Hb levels (Fig. 4b and d), RET-He can be used as an early iron evaluation indicator.

Roxadustat is a new type of HIF-PHI used to treat anemia in CKD patients. Regardless of whether the mechanism of action of roxadustat is to promote the production of EPO or increase the absorption and utilization of iron, adjuvant treatment with exogenous iron is still required in the case of insufficient iron reserves in the body. In our research, the results showed that in the treatment of renal anemia with PIC combined with Roxa, sufficient iron supplementation rapidly increased the Hb, and that the Hb reached a final stable level in each PIC combined with Roxa group. PIC combined with Roxa is more effective than either Roxa or PIC alone in the treatment of renal anemia. With the withdrawal of adenine, the renal function of the CKD group gradually recovered, and the CKD + PIC (75 mg/kg) + Roxa group recovered the fastest (Fig. 2), it may be related to the following reasons:1) Early administration of sufficient iron enables the Hb to rise rapidly, following a rapid increase in Hb levels, the animals' diet was improved, leading to a marked improvement in renal function compared to the CKD only group. 2) PIC has no direct effect on renal fibrosis, roxadusta can alleviate renal fibrosis, coupled with the rapid increase of hemoglobin, and then promotes the recovery of kidney function.

Reticulocyte count (reported as RET%) is an important index that reflects the hematopoietic function of bone marrow and is used to assess the curative effect of anemia. As shown in Fig. 4C, after PIC treatment, the increase in RET% was earlier than the increase in Hb, especially in the CKD + PIC (75 mg/kg) + Roxa group, which is an indicator for evaluating and predicting the effect of iron treatment. This result suggests that early administration of adequate iron supplementation can achieve better results.

Hepcidin is an important regulator of iron homeostasis and its main function is to regulate the balance of iron metabolism by reducing the level of iron transport. It is a negative regulatory hormone of iron metabolism that can downregulate the level of SI and play an essential role in anemia in CKD [20]. Some studies have shown that hepcidin expression is regulated by inflammation and iron load [36, 37]. Hepcidin-25 (2.5 kDa), which is made up of 25 amino acids, exhibits physiological activity. HIF stabilizers are involved in iron metabolism and reduce levels of hepcidin, which leads to improved intestinal absorption, as well as increased release of iron from macrophages to transferrin [38]. Research has indicated that HIF-PHI can stabilize HIF-2 by inhibiting PHD, leading to increased expression of Dcytb, DMT1 and FPN in intestinal cells. so as to antagonize the effect of hepcidin and enhances intestinal iron absorption [39].The same results were obtained in the present study. In our research, the results demonstrated a significant increase in hepcidin-25 levels in both the CKD only and CKD + PIC groups compared to the NC group ($p < 0.05$). Furthermore, there was a significant decrease in hepcidin-25 levels in the CKD + Roxa and CKD + PIC (75 mg/kg) + Roxa groups compared to the CKD only group ($p < 0.05$) (Table 2). These findings suggest that roxadustat may attenuate hepcidin levels and improve iron metabolism in CKD rats.

Inflammation is the key factor that causes the upregulation of hepcidin. CKD is in a microinflammatory state, which can lead to increased levels of various inflammatory factors, such as CRP, IL-1, IL-6, TNF-α, IFN-γ [14, 15]. In our research, the results indicated that the levels of IL-6, TNF-α and IL-1β increased significantly in rats with CKD induced by adenine feeding (0.75%) combined with UNx. Studies have also shown that in cisplatin induced kidney injury, inflammatory cytokines such as TNF-α, IL-1β and IL-6 are significantly increased, and roxadustat can significantly reduce these inflammatory factors [40]. Our results indicated that roxadustat can reduce the levels of IL-6, TNF-α, IL-1β in CKD rats. HMGB1 exists in all cell types and has received certain attention as a relatively new proinflammatory cytokine. It can induce the release of inflammatory factors such as IL-1, IL-6 and TNF-a, and at the same time, inflammatory factors can also reverse promote the further expression of HMGB1, thus promoting the expansion of inflammation in vivo and playing a powerful pro-inflammatory role outside the cell [41]. In our study, we also detected the expression of HMGB1 level in rats in each group by ELISA (Fig. 5). The results showed that roxadustat can reduce HMGB1 level in CKD rats. In conclusion, roxadustat can reduce the levels of IL-6, TNF-α, IL-1β and HMGB1, and alleviate the microinflammatory state of the body, which is consistent with the results of previous studies.

TSAT is an indicator of the effective utilization of iron stored in the body. HIF has been shown to increase TIBC and reduce hepcidin and TSAT in dialysis independent CKD [42–44], in our study, due to the combination with PIC, the TIBC level of the roxadustat treatment group was higher than that of the CKD only group, but there was no significant clinical significance, and the TSAT was not significantly significant among all groups, suggesting that roxadustat can increase iron utilization and decrease iron storage, and additional iron therapy is required.

Renal fibrosis is a common consequence of most progressive renal diseases and is closely associated with deterioration of renal function [45]. Abnormal and excessive deposition of extracellularmatrix (ECM) proteins in the glomerular and interstitial regions is a typical marker of renal fibrosis, α-SMA and FN are typical markers of renal fibrosis [45, 46]. At present, there are also studies reporting that roxadustat can slow folate-induced acute kidney injury (AKI) and delay the progression of renal fibrosis [31, 47, 48], but there are few studies on roxadustat in CKD models. In this study, adenine feeding

(0.75%) combined with UNx was used to establish an animal model of CKD, and the antifibrotic effect of roxadustat in CKD model was studied. At the same time, mRNA expression of FN and α-SMA in renal tissues were detected by RT-PCR. The results indicated that roxadusta can alleviate renal fibrosis and delay renal progression in CKD rats, which is consistent with previous research results.

The limitation of this study is that the limited amount of blood taken each time, EPO levels, hepcidin-25 and TIBC tests were not taken each time to observe the dynamic changes of indicators. The specific mechanism has not been thoroughly studied of the antifibrotic effect of roxadusta in CKD, and further studies on its possible mechanism may be considered in the future.

Conclusions

HIF-PHI combined with iron supplement (Roxadustat combined with PIC) has an improved effect on the treatment of renal anemia, and early administration of sufficient iron enables the Hb to rise rapidly, early administration of adequate dose of PIC is necessary for renal anemia. RET-He reflects the iron content of reticulocytes, compared with SF and TSAT, RET-He is less affected by inflammation, and it is easy to detect. RET-He can be used as an early iron evaluation indicator in CKD, which is conducive to early assessment of iron status. It is conducive to early correction of anemia in CKD. HIF-PHI reduces the level of hepcidin in CKD rats and improves iron metabolism in CKD rats. HIF-PHI can reduce the levels of IL-6, TNF-α, IL-1β and HMGB1, and alleviate the microinflammatory state of CKD rats. HIF-PHI alleviates renal fibrosis in CKD rats and plays a beneficial role in the treatment of renal fibrosis.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
Zhao Hu and Zhaoli Gao were involved in the conception and design; Zhaoli Gao, Yanxia Gao, Qiang Wang, Qi Wang and Peng Lu participated in animal experiments; Zhaoli Gao, Hailin Lv, Haoran Xue, Xiaotian Ma, and Shuen Li were involved in the analysis and interpretation of the data; Zhaoli Gao was involved in the drafting of the paper; Zhao Hu and Yanxia Gao were involved in revising it critically for intellectual content and the final approval of the version to be published; All authors read and approved the final manuscript.

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Data availability
The datasets supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate
This study protocol was reviewed and approved by the Ethical Committee of Qilu Hospital of Shandong University (Qingdao), the approval number: KYDWLL-201906.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Inker LA, Grams ME, Levey AS, et al. Relationship of estimated GFR and albuminuria to concurrent laboratory abnormalities: an individual participant data Meta-analysis in a global consortium. Am J Kidney Dis. 2019;73(2):206–17.
2. Dinh NH, Cheanh Beaupha SM, Tran LTA. The validity of reticulocyte hemoglobin content and percentage of hypochromic red blood cells for screening iron-deficiency anemia among patients with end-stage renal disease: a retrospective analysis. BMC Nephrol. 2020;21(1):142.
3. van Haalen H, Jackson J, Spinowitz B, Milligan G, Moon R. Impact of chronic kidney disease and anemia on health-related quality of life and work productivity: analysis of multinational real-world data. BMC Nephrol. 2020;21(1):88.
4. Portoles J, Martin L, Broseta JJ, Cases A. Anemia in chronic kidney disease: from pathophysiology and current treatments, to future agents. Front Med (Lausanne). 2021;8:642296.
5. Ogawa C, Tsuchiya K, Maeda K. Reticulocyte hemoglobin content. Clin Chim Acta. 2020;504:138–45.
6. Urrechaga E, Borque L, Escanero JF. Erythrocyte and reticulocyte indices in the assessment of erythropoiesis activity and iron availability. Int J Lab Hematol. 2013;35(2):144–9.
7. Mast AE, Blinder MA, Dietzen DJ. Reticulocyte hemoglobin content. Am J Hematol. 2008;83(4):307–10.
8. Brugnara C, Schiller B, Moran J. Reticulocyte hemoglobin equivalent (Ret He) and assessment of iron-deficient States. Clin Lab Haematol. 2006;28(5):303–8.
9. Kim JM, Ihm CH, Kim HJ. Evaluation of reticulocyte haemoglobin content as marker of iron deficiency and predictor of response to intravenous iron in haemodialysis patients. Int J Lab Hematol. 2008;30(1):46–52.
10. Gelaw Y, Woldu B, Melku M. The role of reticulocyte hemoglobin content for diagnosis of Iron deficiency and Iron deficiency anemia, and monitoring of Iron therapy: a literature review. Clin Lab. 2019;65(12).
11. Sany D, El Shahawi Y, Taha J. Diagnosis of iron deficiency in Hemodialysis patients: usefulness of measuring reticulocyte hemoglobin equivalent. Saudi J Kidney Dis Transpl. 2020;31(6):1263–72.
12. Ratcliffe LE, Thomas W, Glen J, et al. Diagnosis and management of Iron deficiency in CKD: A summary of the NICE guideline recommendations and their rationale. Am J Kidney Dis. 2016;67(4):548–58.

13. Wirawan R, Tedja AT, Henrika F, Lydia A. Concordance between reticulocyte hemoglobin equivalent and reticulocyte hemoglobin content in CKD patients undergoing Hemodialysis. Acta Med Indones. 2017;49(1):34–40.
14. Zhong H, Zhou T, Li H, Zhong Z. The role of hypoxia-inducible factor stabilizers in the treatment of anemia in patients with chronic kidney disease. Drug Des Devel Ther. 2018;12:3003–11.
15. Yilmaz MI, Solak Y, Covic A, Goldsmith D, Kanbay M. Renal anemia of inflammation: the name is self-explanatory. Blood Purif. 2011;32(3):220–5.
16. Zhao Z, Hu Z, Zeng R, Yao Y. HMGB1 in kidney diseases. Life Sci. 2020;259:118203.
17. Huang J, Chen X, Lv Y. HMGB1 mediated inflammation and autophagy contribute to endometriosis. Front Endocrinol (Lausanne). 2021;12:616696.
18. Nogueira-Machado JA, de Oliveira Volpe CM. HMGB-1 as a target for inflammation controlling. Recent Pat Endocr Metab Immune Drug Discov. 2012;6(3):201–9.
19. Liu T, Li Q, Jin Q, et al. Targeting HMGB1: A potential therapeutic strategy for chronic kidney disease. Int J Biol Sci. 2023;19(15):5020–35.
20. Atkinson MA, Warady BA. Anemia in chronic kidney disease. Pediatr Nephrol. 2018;33(2):227–38.
21. Antunes SA, Canziani ME. Hepcidin: an important iron metabolism regulator in chronic kidney disease. J Bras Nefrol. 2016;38(3):351–55.
22. Santos-Silva A, Ribeiro S, Reis F, Belo L. Hepcidin in chronic kidney disease anemia. Vitam Horm. 2019;110:243–64.
23. Agarwal AK, Yee J, Hepcidin. Adv Chronic Kidney Dis. 2019;26(4):298–305.
24. Locatelli F, Fishbane S, Block GA, Macdougall IC. Targeting Hypoxia-Inducible factors for the treatment of Anemia in chronic kidney disease patients. Am J Nephrol. 2017;45(3):187–99.
25. Besarab A, Provenzano R, Hertel J, et al. Randomized placebo-controlled dose-ranging and pharmacodynamics study of Roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients. Nephrol Dial Transpl. 2015;30(10):1665–73.
26. Ravasi G, Pelucchi S, Greni F, et al. Circulating factors are involved in hypoxia-induced Hepcidin suppression. Blood Cells Mol Dis. 2014;53(4):204–10.
27. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide Hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest. 2002;110(7):1037–44.
28. Song H, Yin D, Liu Z. GDF-15 promotes angiogenesis through modulating p53/HIF-1alpha signaling pathway in hypoxic human umbilical vein endothelial cells. Mol Biol Rep. 2012;39(4):4017–22.
29. Silvestri L, Pagani A, Camaschella C. Furin-mediated release of soluble Hemojuvelin: a new link between hypoxia and iron homeostasis. Blood. 2008;111(2):924–31.
30. Peyssonnaux C, Zinkernagel AS, Schuepbach RA, et al. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J Clin Invest. 2007;117(7):1926–32.
31. Naito Y, Yasumura S, Okuno K, et al. Hypoxia-inducible factor-prolyl hydroxylase inhibitor Roxadustat (FG-4592) reduces renal fibrosis in Dahl salt-sensitive rats. J Hypertens. 2024;42(3):497–505.
32. Munoz Abellan C, Mangold-Gehring S, Micus S, et al. A novel model of chronic kidney disease in rats: dietary adenine in combination with unilateral nephrectomy. Kidney Dis (Basel). 2019;5(3):135–43.
33. Auerbach M, Staffa SJ, Brugnara C. Using reticulocyte hemoglobin equivalent as a marker for Iron deficiency and responsiveness to Iron therapy. Mayo Clin Proc. 2021;96(6):1510–19.
34. Davidkova S, Prestidge TD, Reed PW, Kara T, Wong W, Prestidge C. Comparison of reticulocyte hemoglobin equivalent with traditional markers of iron and erythropoiesis in pediatric dialysis. Pediatr Nephrol. 2016;31(5):819–26.
35. Pei LX, Kroeun H, Karakochuk CD. Reticulocyte haemoglobin equivalent (RET-He) as an early marker of responsiveness to oral iron supplementation. J Clin Pathol. 2023;76(6):407–12.
36. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone Hepcidin. J Clin Invest. 2004;113(9):1271–6.
37. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide Hepcidin, is overexpressed during iron overload. J Biol Chem. 2001;276(11):7811–9.
38. Hazin MAA. Anemia in chronic kidney disease. Rev Assoc Med Bras (1992). 2020;66Suppl 1(Suppl 1):s55–8.
39. Yan Z, Xu G. A novel choice to correct Inflammation-Induced Anemia in CKD: oral Hypoxia-Inducible factor Prolyl hydroxylase inhibitor Roxadustat. Front Med (Lausanne). 2020;7:393.
40. Yang Y, Yu X, Zhang Y, et al. Hypoxia-inducible factor Prolyl hydroxylase inhibitor Roxadustat (FG-4592) protects against cisplatin-induced acute kidney injury. Clin Sci (Lond). 2018;132(7):825–38.
41. Pisetsky DS. The expression of HMGB1 on microparticles released during cell activation and cell death in vitro and in vivo. Mol Med. 2014;20(1):158–63.
42. Chen N, Hao C, Liu BC, et al. Roxadustat treatment for Anemia in patients undergoing Long-Term Dialysis. N Engl J Med. 2019;381(11):1011–22.
43. Chen N, Hao C, Peng X, et al. Roxadustat for Anemia in patients with kidney disease not receiving Dialysis. N Engl J Med. 2019;381(11):1001–10.
44. Liu J, Zhang A, Hayden JC, et al. Roxadustat (FG-4592) treatment for anemia in dialysis-dependent (DD) and not dialysis-dependent (NDD) chronic kidney disease patients: A systematic review and meta-analysis. Pharmacol Res. 2020;155:104747.
45. Li R, Shi C, Wei C, et al. Fufang Shenhua tablet inhibits renal fibrosis by inhibiting PI3K/AKT. Phytomedicine. 2023;116:154873.
46. Djudjaj S, Boor P. Cellular and molecular mechanisms of kidney fibrosis. Mol Aspects Med. 2019;65:16–36.
47. Li X, Jiang B, Zou Y, Zhang J, Fu YY, Zhai XY. Roxadustat (FG-4592) facilitates recovery from renal damage by ameliorating mitochondrial dysfunction induced by folic acid. Front Pharmacol. 2021;12:788977.
48. Li X, Zou Y, Xing J, et al. Pretreatment with Roxadustat (FG-4592) attenuates folic Acid-Induced kidney injury through antiferroptosis via Akt/GSK-3beta/Nrf2 pathway. Oxid Med Cell Longev. 2020;2020:6286984.

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