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ORIGINAL ARTICLE

Rehmannia glutinosa Polysaccharide Increases the Expression of Erythropoietin and Vascular Endothelial Growth Factor in Rats with Chronic Renal Failure by Activating Hypoxia-Inducible Factor-2α

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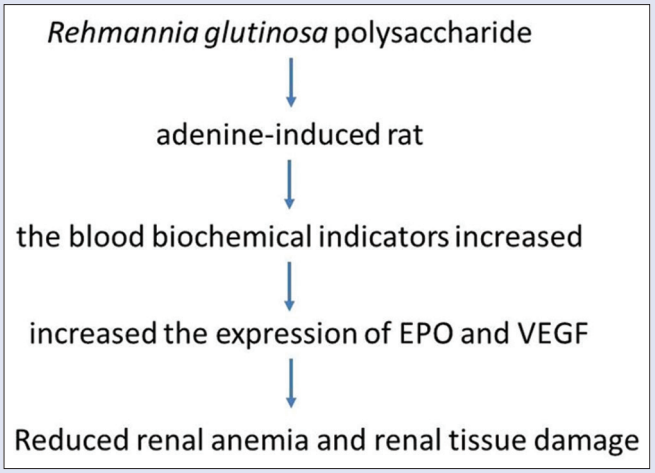
ABSTRACT

Background: To investigate the effect of *Rehmannia glutinosa* polysaccharide (RGP) in rats with chronic renal failure. **Materials and Methods:** In this study, we established a rat model of adenine-induced chronic renal failure. Hemoglobin (Hb), red blood cells (RBCs), total protein (TP), serum albumin (ALB), blood urea nitrogen (BUN), cystatin C (Cys C), and serum creatinine anhydride (SCA) were detected in the blood samples of rats. Hematoxylin and eosin staining was used to observe the pathological changes. Immunohistochemical was used to detect the protein expression of hypoxia-inducible factor (HIF)-2α, erythropoietin (EPO), and vascular endothelial growth factor (VEGF) and calculate the value of microvessel density (MVD) in renal tissues. M1001 and GPRRP + PT2385 groups were added to further validate the mechanism of RGP. **Results:** In the case of rats with chronic renal failure, GPRRP and EPO contributed to the repairment of renal tissue. The level of Hb, TP, and ALB, as well as RBC counts was significantly increased, and the renal function indexes, including BUN, SCA, and Cys C, were significantly decreased, and HIF-2α, EPO, VEGF, and MVD were significantly increased. The effects observed in M1001 group was similar to those in GPRRP group, but compared with GPRRP + PT2385 group, the biochemical indicators and renal function indexes were significantly decreased, which means HIF-2α inhibitor antagonized the protective effect of RGP. **Conclusion:** RGP played an essential role in repairing the renal injury in rats with chronic renal failure. The therapeutic mechanism of action may be related to the activation of HIF-2α, thereby increasing the expression of EPO and VEGF and then reducing renal anemia and renal tissue damage.

Key words: Chronic kidney failure, erythropoietin, hypoxia-inducible factor-2α, *Rehmannia glutinosa* polysaccharide, vascular endothelial growth factor

SUMMARY

- In this study, we validated the model of rats with chronic renal failure and affirmed the medicinal effect of *Rehmannia glutinosa* polysaccharide (RGP). The repairment of renal failure after the administration of RGP was better than erythropoietin (EPO) injections. RGP activates hypoxia-inducible factor-2α to mediate EPO/vascular endothelial growth factor gene expression suggesting its potential in inducing preconditioning-like effects under anemia.



Abbreviations used: RGP: *Rehmannia glutinosa* polysaccharide; HIF-2α: Hypoxia-inducible factor 2α; Hb: Hemoglobin; RBC: Red blood cells; TP: Total protein; ALB: Serum albumin; Bun: Blood urea nitrogen; Cys C: Cystatin C; and SCA: Serum creatinine anhydride; EPO: erythropoietin; VEGF: Vascular endothelial growth factor; MVD: Microvessel density; CKD: Chronic kidney disease.

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INTRODUCTION

Chronic renal failure, also called chronic kidney disease (CKD),[1] results in the gradual loss of kidney function.[2] It severely damages the function of the kidneys, thereby leading to other disorders, such as cardiovascular disease and pernicious anemia.[1,3] Worldwide, the millions of people across different countries such as America, Japan, and China are at great risk of kidney failure.[4] Anemia is the major complication in CKD and is more prevalent in the end stage of renal disease. In view of the perniciousness of renal anemia in daily life, it is usually corrected in the early stages of CKD. The early detection of CKD is mostly depended on blood pressure, urine albumin, and serum creatinine tests.[5,6] For an adult, if the levels of serum creatinine exceed 3 mg/dL,[7] then the renal damage cannot be reverted, and total loss of renal function is imminent. High blood pressure is the most common cause of CKD in people

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worldwide, which reduces the blood supply to the kidneys. Albuminuria is a sign of kidney disease, which is a protein exist in the blood rather than urine. The albumin transfers into urine from blood if the kidney damaged.

Rehmannia glutinosa polysaccharide (RGP) shows strong immunomodulatory effects.[8,9] Rehmannia has been widely used as a blood and yin tonic in the traditional Chinese medicine.[10] According to the history of traditional Chinese medicine, the kidneys are considered as the flame of life,[11] and kidney failure or dysfunction of the kidney meridian is known to cause an imbalance in the yin and yang.[12] Previous studies have revealed that the pharmacological efficacy of RGP, for example, in bone metabolism, hypoglycemic effect, anti-inflammatory effect, anti-allergic effects, neuroprotective effects, and diuretic effects.[8-10,13] Recently, it has been reported that RGP alleviates renal dysfunction in rats with ischemia/reperfusion-induced acute renal failure.[14]

Hypoxia inducible factor (HIF) participants in anaerobic metabolism,[15,16] which also induces the expression of hypoxia-related genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO). HIF has α and β subunits. HIF-α consists of an inducible α subunit: HIF-1α, HIF-2α, and HIF-3α, whereas the HIF-β subunit has two types of inducible subunits: ARNT1 and ARNT2.[17,18] HIF-2α is the major transcription factor that regulates the expression of other related genes.[19] Increasing research has shown that RGP plays an essential role in chronic renal failure.[18,20] Previous clinical data indicate that RGP has definite therapeutic effects on renal diseases.[21]

VEGF plays an important role during the pulmonary development and growth.[22] It is central to the regulation of the angiogenic process.[23,24] Abnormal blood pressure and blood-related diseases are the main factors of chronic renal failure.[11] Chronic, low-grade inflammation in the kidney tissue results in irreversible damage.[25] HIF-2α increases the production of EPO resulting from tissue hypoxia caused due to anemia.[4,21]

In this study, we established an adenine-induced rat model with chronic renal failure to reveal the effect and mechanism of action of RGP on the renal function. To the best of our knowledge, this is the first study to explore the beneficial effects of RGP in chronic renal failure. Furthermore, we show that RGP increases the expression of EPO and VEGF in rats with chronic renal failure through the activation of HIF-2α.

MATERIALS AND METHODS

Experimental animals

A total of 54 healthy male Sprague–Dawley rats (250–280 g, aged between 8 and 9 months) were purchased from Jinan Peng Yue Experimental Animal Breeding Co., Ltd. (China) (permission number: SCXK (Shandong Province) 2014-0007). Rats were fed in a clean and quiet-specific pathogen-free laboratory maintained at 22°C–24°C and 50%–60% humidity for 1 week. The animal study protocol was in-line with the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Weihai Rongcheng Center Hospital (No. 20181203-002).

Establishment and grouping of adenine-induced chronic kidney disease rat model

In this study, we established an adenine-induced rat model with chronic renal failure.[26] Rats were continuously administered with 0.75% adenine, 200 mg/(kg•d) solution of (0.3 g adenine dissolved in 40 mL of physiological saline) for 8 weeks.

The experimental rats were grouped as follows ($n = 6$):

1. Control group: Normal feeding with no special treatment
2. Model group: Modeling simultaneously administered with saline from the 3rd week onward
3. GPRRP group: Modeling simultaneously administered with 400 mg/kg RGP solution from the 3rd week onward[27]
4. EPO group: Modeling administered with 500 U/kg EPO from the 3rd week onward[28]
5. M1001 group (HIF-2 activator group): modeling, administered with 10 mg/kg M1001 (HY-111 547, the MCE, China) from the 3rd week onward
6. GPRRP + PT2385 group (RGP + HIF-2α inhibitor group): modeling administered with 400 mg/kg RGP and 10 mg/kg PT2385 (HY-12867, the MCE, China) from the 3rd week onward.

Measurement of indicators

Rats were weighed every week to assess the body weight. The abdominal aorta blood specimens of rats were collected after 8 weeks. Hemoglobin (Hb), red blood cell (RBC), total protein (TP), and serum albumin (ALB) were measured in the abdominal aorta blood samples after 8 weeks. Blood urea nitrogen (BUN), cystatin C (Cys C), and serum creatinine anhydride (SCA) were measured in the blood samples after 8 weeks.[29] After anesthetizing with 1% sodium pentobarbital (40 mg/kg), rats were sacrificed through the cervical dislocation method. The kidneys were harvested and a part of it was fixed in 4% phosphate-buffered saline (PBS) paraformaldehyde and then embedded in paraffin for routine hematoxylin and eosin (H and E) staining and immunohistochemical staining.

Assessing anemia in rats

After anesthetizing the rats with 1% sodium pentobarbital (40 mg/kg), their blood samples from the abdominal aorta were collected in heparin anticoagulation (1 mL). The percentage of Hb and RBC count in the samples was measured by using a blood cell analyzer. Another 8–10 mL of blood was withdrawn, centrifuged at 3000 r/min for 10 min, and the supernatant was collected. The serum TP and serum ALB content were detected by automatic biochemical analyzer.

Renal function indexes

The content of BUN, Cys C, and SCA, were analyzed according to the manufacturer's instructions by using an automatic biochemical analyzer.

Hematoxylin and eosin staining

The renal tissue specimens of rats were fixed in 4% paraformaldehyde solution, dehydrated in a graded series of alcohol, cleared in xylene and embedded in paraffin. The blocks were cut into 5 μm sections, dried, dewaxed, and dehydrated in an ascending series of alcohol. Subsequently, the sections were stained with hematoxylin (Solarbio, Beijing, China) for 5 min and rinsed with tap water. Finally, the sections were differentiated in the ethanoic acid for 30 s, soaked in tap water for 15 min, stained with eosin (Solarbio, Beijing, China) for 2 min, routinely dehydrated, and finally, transparent and mounted. Renal histopathological changes were studied under a 400 × optical microscope (Olympus Model BX51, Japan).

Immunohistochemistry

HIF-2α (rabbit anti-rat anti-HIF-2α Antibody, 1: 2500, ab20654, Abeam, UK), EPO (rabbit anti-rat anti-EPO Antibody, 1:1000, ab226956, Abeam, UK), and VEGF (rabbit anti-rat anti-VEGF Antibody, 1:500, ab53465, Abcam, UK) were used in immunohistochemistry.

Renal tissue samples were fixed in 4% paraformaldehyde (in PBS), rinsed with tap water, stained with SABC immunohistochemistry kit, developed with diaminobenzidine, dehydrated with a graded series of ethanol, transparent with xylene and sealed with neutral gum. Finally, the renal sections were observed under a 400 × optical microscope (Olympus, Japan).

CD141 (rabbit anti-rat anti-CD141 Antibody, 1:50, ab28364, Abcam, UK) immunohistochemistry was used to calculate microvessel density (MVD). The value of MVD refers to the Weidner notation. In 100 × perspective, select a region with high blood vessel density (perspective hot-SPOT). The average of five fields was counted as MVD value in 400 × perspective.

Statistical analysis

All statistics of in this study were counted by the SPSS 19.0. Mean ± standard deviation was used to measure the categorical data. Pairwise comparisons were performed with the analysis of variance test and least significant difference analysis used to further analysis. $P < 0.05$ at least is considered statistically significant.

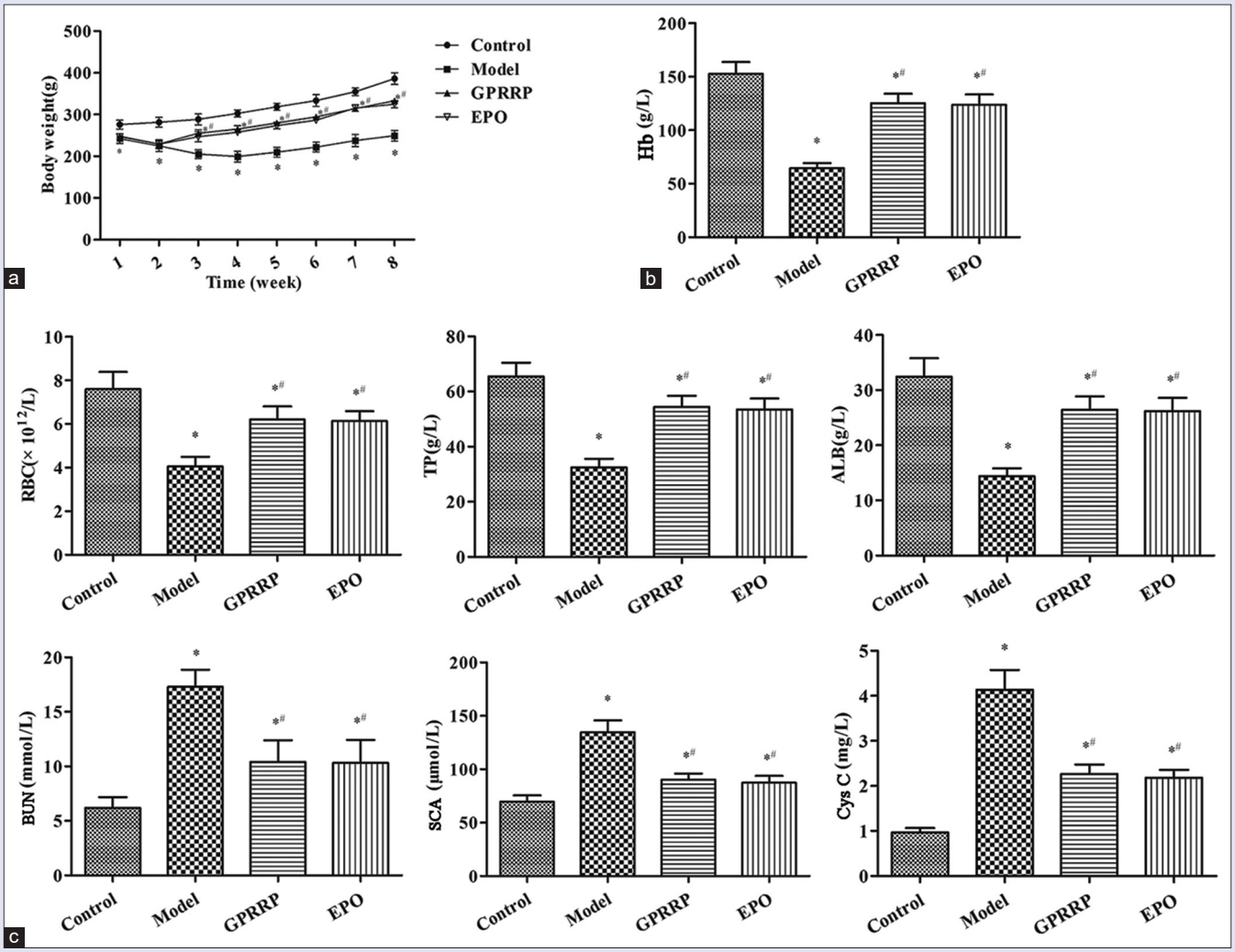

Figure 1: Effects of *Rehmannia glutinosa* polysaccharide and erythropoietin on renal anemia and renal failure in rats. (a) Body weight changes; (b) Blood routine and blood biochemical indicators; (c) Renal function indexes. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with the model group; $^{\&}P < 0.05$, compared with the GPRRP group

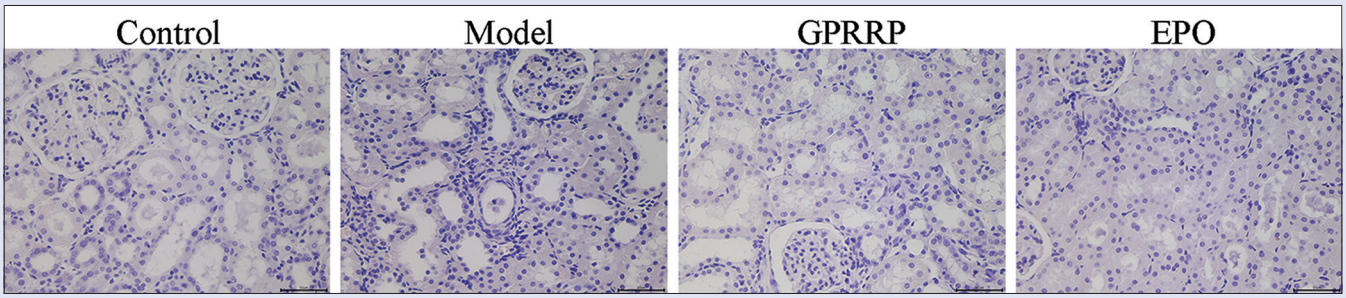

Figure 2: Effect of *Rehmannia glutinosa* polysaccharide on renal tissue pathology in rats (×400)

RESULTS

Effect of *Rehmannia glutinosa* polysaccharide treatment on anemia and renal function in rats

In order to accurately measure the changes, we recorded the body weight of each rat every week regularly. Figure 1 shows the observed value in the first 8 weeks. According to the results, the body weight of normal rats in the control group gradually increased with the prolonged feeding, whereas body weight of the model group rats decreased in the premodeling period. This shows that renal failure had a great effect the normal growth of rats. However, when the administration of adenine stopped, there was a partial recurrence in the body weight, which was slow but significant when compared with the control group ($P < 0.05$). Similarly, the body weight of RGP group decreased significantly in the first 2 weeks but increased significantly from the 3rd week onward ($P < 0.05$). The therapeutic effect in EPO group was also similar to GPRRP group, that is, compared with the model group, the body weight of rats in GPRRP and EPO group was statistically significantly increased ($P < 0.05$). These results show that RGP and EPO treatments favorably improved the growth of rats with renal failure.

The blood biochemical indexes and renal function indexes were detected at 8 weeks. Compared with the control group, model rats developed more severe anemia and showed significantly decreased levels of Hb, RBC, TP, and ALB. Other indicators, including BUN, SCA, and Cys C, were significantly increased. These results show that the renal tissue functions were significantly declined ($P < 0.05$). Compared with the model group, the level of Hb, RBC, TP, and ALB in GPRRP group and EPO group increased and the level of BUN, SCA, and Cys C significantly decreased ($P < 0.05$). Based on the results, we can say that RGP and EPO treatment in CKD rats effective improved the growth.

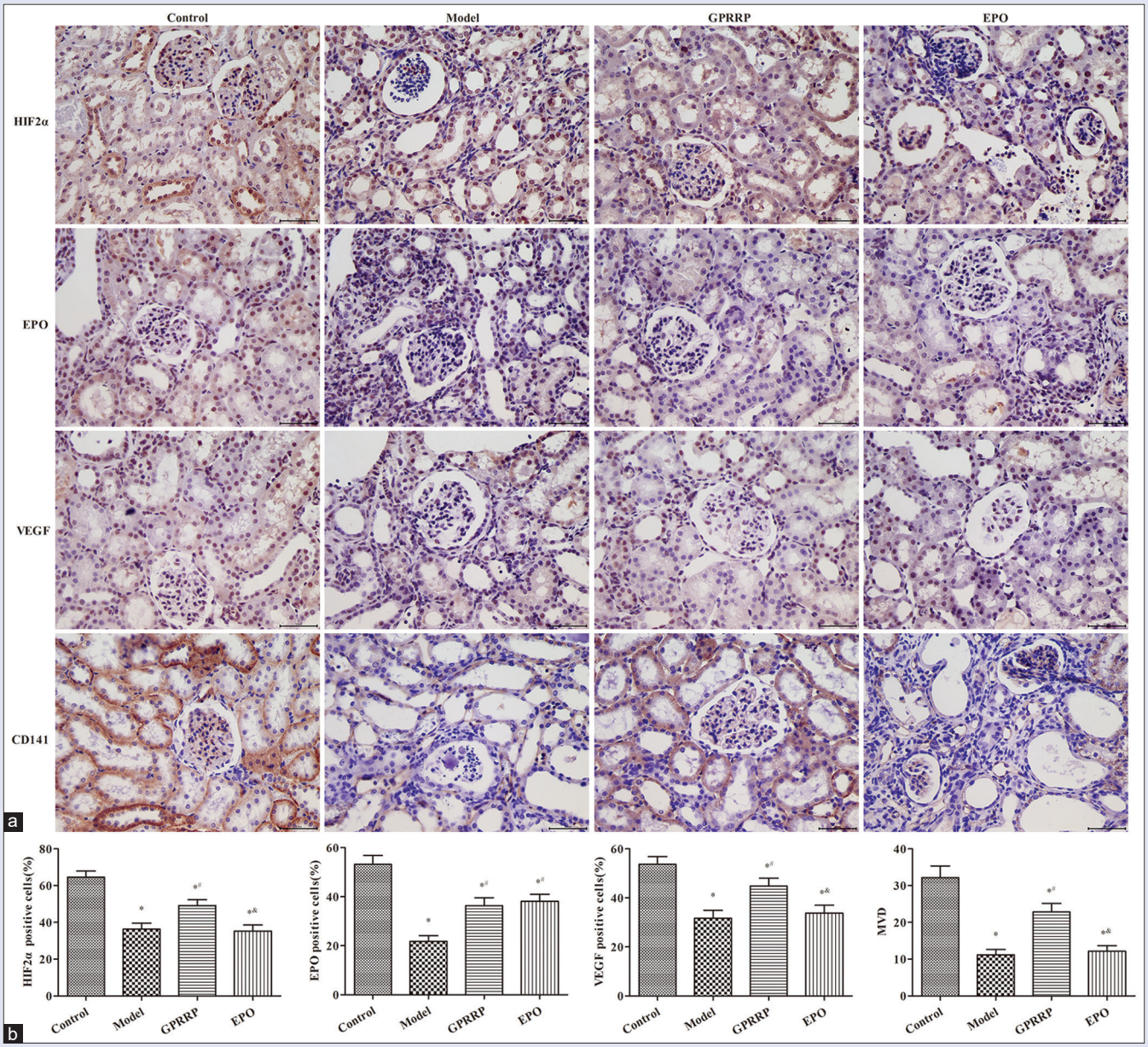

Figure 3: Effects of *Rehmannia glutinosa* polysaccharide treatment of hypoxia-inducible factor-2α, erythropoietin, vascular endothelial growth factor and microvessel density. (a) Immunohistochemistry; (b) Percentage of positive cells. *$P < 0.05$, compared with control group; #$P < 0.05$, compared with model group; &$P < 0.05$, compared with GPRRP group

Effects of *Rehmannia glutinosa* polysaccharide on the pathology of rat kidney

H and E staining is routinely used in the identification of tissue types and morphologic changes, for example, in the liver and kidneys. Figure 2 shows the images of H and E staining. According to the results, rats in the control group have normal morphology of the glomerulus, cortex, and medulla with clear boundaries. On the contrary, the renal tissue of model rats was seriously damaged and showed a decrease in the glomeruli and the presence of infiltrated inflammatory cells. In comparison to the model group, the degree of lesions in the renal tissue in the GPRRP and EPO groups was significantly improved, and inflammation was alleviated.

Immunohistochemistry

In this study, we examined the expression levels of kidney-specific proteins, such as HIF-2α, EPO, VEGF, and MVD in control, model, GPRRP, and EPO groups. Compared with the control group, the contents of HIF-2α, EPO, VEGF, and MVD statistically significantly decreased in the model group and had a weak positive expression ($P < 0.05$). To further investigate the therapeutic effects of RGP, we compared the expression levels of HIF-2α, EPO, and VEGF protein in GPRRP group with that of the model group and found that the values were all significantly increased. The expression of EPO protein in the EPO group was significantly increased ($P < 0.05$), but the other indicators did not show any significant change ($P > 0.05$) when compared with the model group [Figure 3]. These results show that the therapeutic effects of EPO were less than that of RGP.

With the help of the aforementioned tests, we found that RGP is effective in reverting the effects of chronic renal failure in rats. It also upregulated the expression of HIF-2α EPO and VEGF. To further verify whether the mechanism of RGP activation is related to HIF-2α, we attempted to use the HIF-2α activators and inhibitors alone or in combination with RGP.

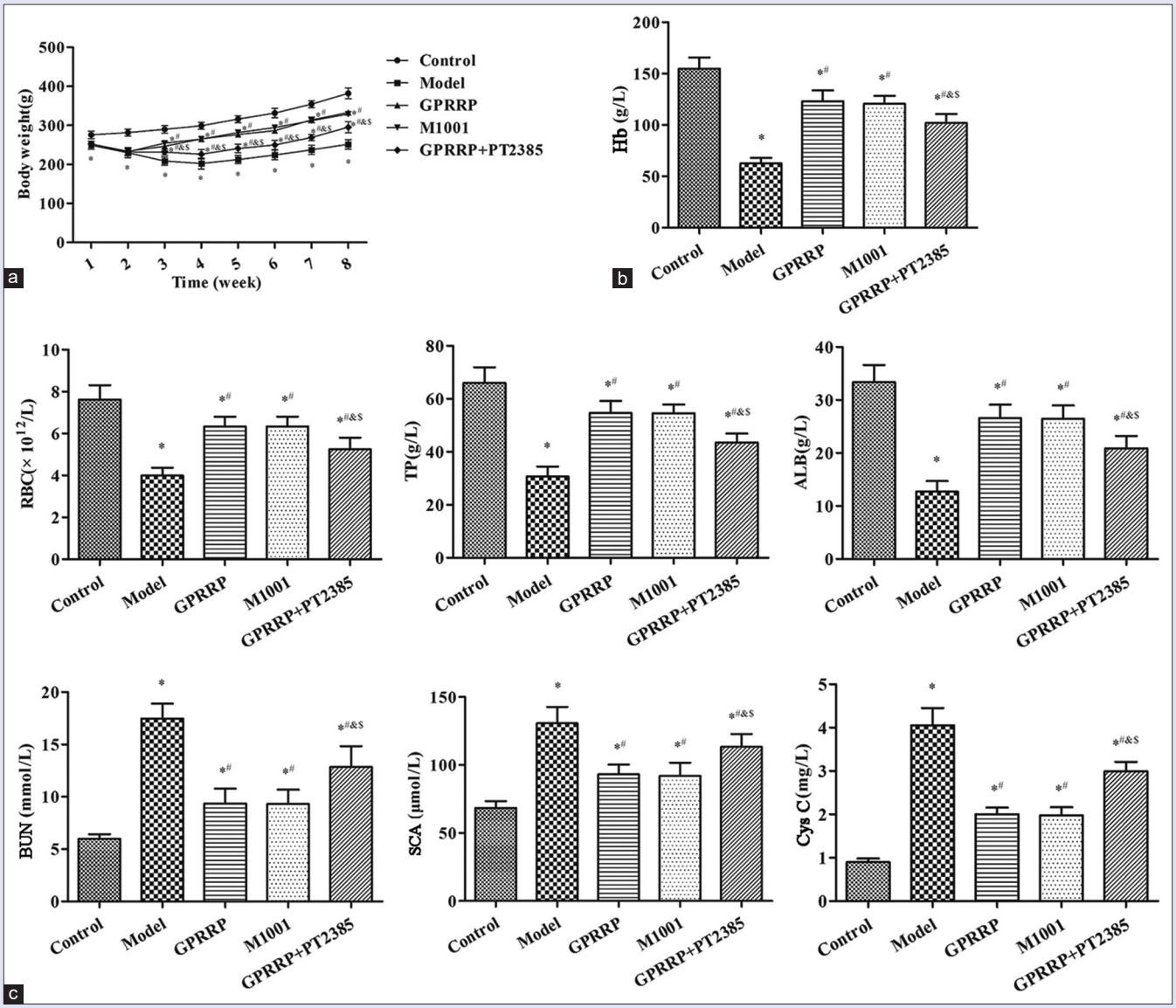

Figure 4: *Rehmannia glutinosa* polysaccharide improves renal anemia and renal function in rats by activating hypoxia-inducible factor-2α. (a) body weight changes; (b) Blood routine and blood biochemical indicators; (c) Renal function indexes. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with model group; $^{\&}P < 0.05$, compared with control group; $^{\$}P < 0.05$, compared with the control group

Rehmannia glutinosa polysaccharide improves renal anemia and renal function in rats by activating hypoxia-inducible factor-2α

To further investigate the function of HIF-2α, we added M1001 and GPRRP + PT2385 groups. After adding the activators and inhibitors of HIF-2α in these groups, the body weight, blood biochemistry indicators, and renal functional parameters of rats were studied. Figure 4 shows the results of these analyses. Compared with the model group, the body weight of rats was significantly increased in M1001 group after 3 weeks. After 8 weeks, the levels of Hb, RBC, TP, and ALB in blood were significantly increased, whereas the levels of BUN, SCA, and Cys C were significantly decreased ($P < 0.05$), which was similar to GPRRP group. However, compared with the GPRRP and M1001 groups, the body weight of rats in GPRRP + PT2385 group decreased. The decreased levels of blood biochemistry indicators and increased levels of renal functional indexes suggested RGP improved the renal function through activating HIF-2α.

Rehmannia glutinosa polysaccharide improves the pathology of rat kidney tissue by activating hypoxia-inducible factor-2α

Figure 5 shows the microscopic examination of tissue structure from the validation group. Compared with the model group, the severity of the pathological damage was reduced in the M1001 group, the inflammatory cell infiltration was reduced, and the renal interstitial edema was alleviated. Compared with the M1001 group and the GPRRP group, the renal tissue lesions in the GPRRP + PT2385 group were aggravated. These results showed that RGP alleviated the renal pathological injury in rats with chronic renal failure through activating HIF-2α.

Rehmannia glutinosa polysaccharide increases the expression of vascular endothelial growth factor and erythropoietin by activating hypoxia-inducible factor-2α

From the above results, we can say that RGP effectively alleviates the condition of rats with chronic renal failure. The mechanism of action of RGP is related to the activation of HIF-2α. To explore the related pathways, we examined the expression of proteins of VEGF, EPO, and HIF-2α after 8 weeks in M1001 and GPRRP + PT2385 groups [Figure 6]. Compared with the model group, the expression of HIF-2α, EPO, and VEGF protein and the MVD value in M1001 group was significantly increased ($P < 0.05$), and its effect was similar to GPRRP group. Compared with M1001 group and GPRRP group, the expression of these proteins and the MVD value in GPRRP + PT2385 group was decreased ($P < 0.05$).

DISCUSSION

In the recent years, the number of patients with CKD around the world has increased.[30] Detecting the pathogenesis of CKD, recognizing the potential makers of CKD, developing effective methods for CKD, and identifying the mechanism of action are all highly warranted.[31] Patients with CKD often have comorbid anemia, and the levels of EPO and erythropoiesis are always lower than that of normal people.[32] Previous studies have demonstrated that renal anemia and renal failure severely hamper the normal growth of rats.[33,34] Animal models of chronic renal failure with severe anemia offer some of the useful methods to detect the underlying-guided treatment. Recent reports have demonstrated the effectiveness of the traditional Chinese medicine in rats with chronic renal failure.[35] Adenine-induced CKD rat model has become popular because it does not require surgery and does not cause infection to the animal.[36]

RGP has been known to cure diabetic nephropathy and ameliorates progressive renal failure.[36] Previous studies have focused their research on the protective efficacy of RGP on the immune system, nervous system, cardiovascular system, endocrine system, and the circulatory system.[37] In this study, we evaluated the effects of rats with chronic renal failure and confirmed the beneficial effects of RGP in chronic renal failure.

According to a previous study, Sprague–Dawley rats quickly recovered from the effects of chronic renal failure after receiving EPO injections.[38] According to our results, RGP repaired the effect of renal failure better than EPO injections. Our study examined the therapeutic effects of RGP in rats with chronic renal failure and found that RGP facilitated positive effects in terms of improvements of renal function. The mechanism of action by which RGP protected the rats against adenine-induced CKD is not exactly known. Therefore, to investigate the functional pathway

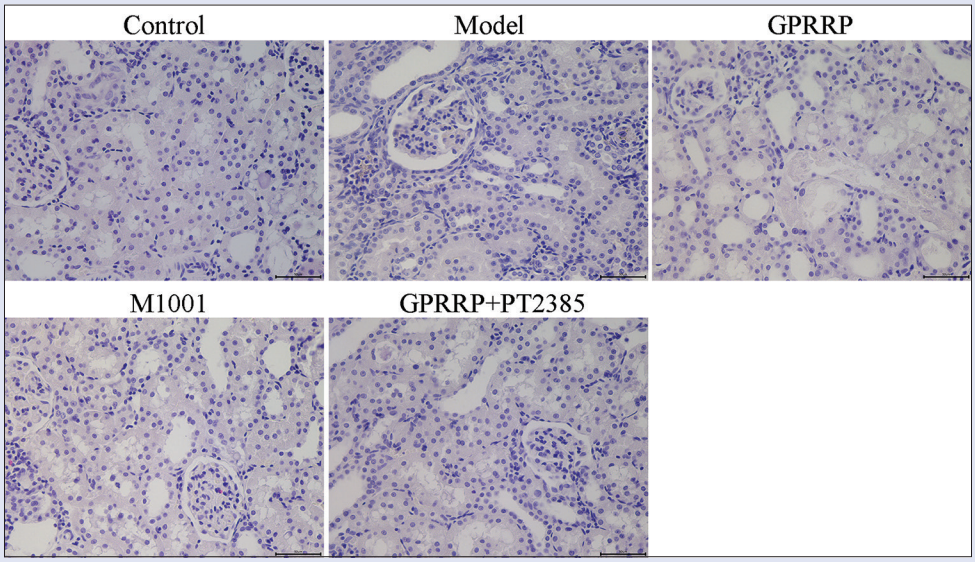

Figure 5: *Rehmannia glutinosa* polysaccharide improves the pathological structure of rat kidney tissue by activating hypoxia-inducible factor-2α (×400)

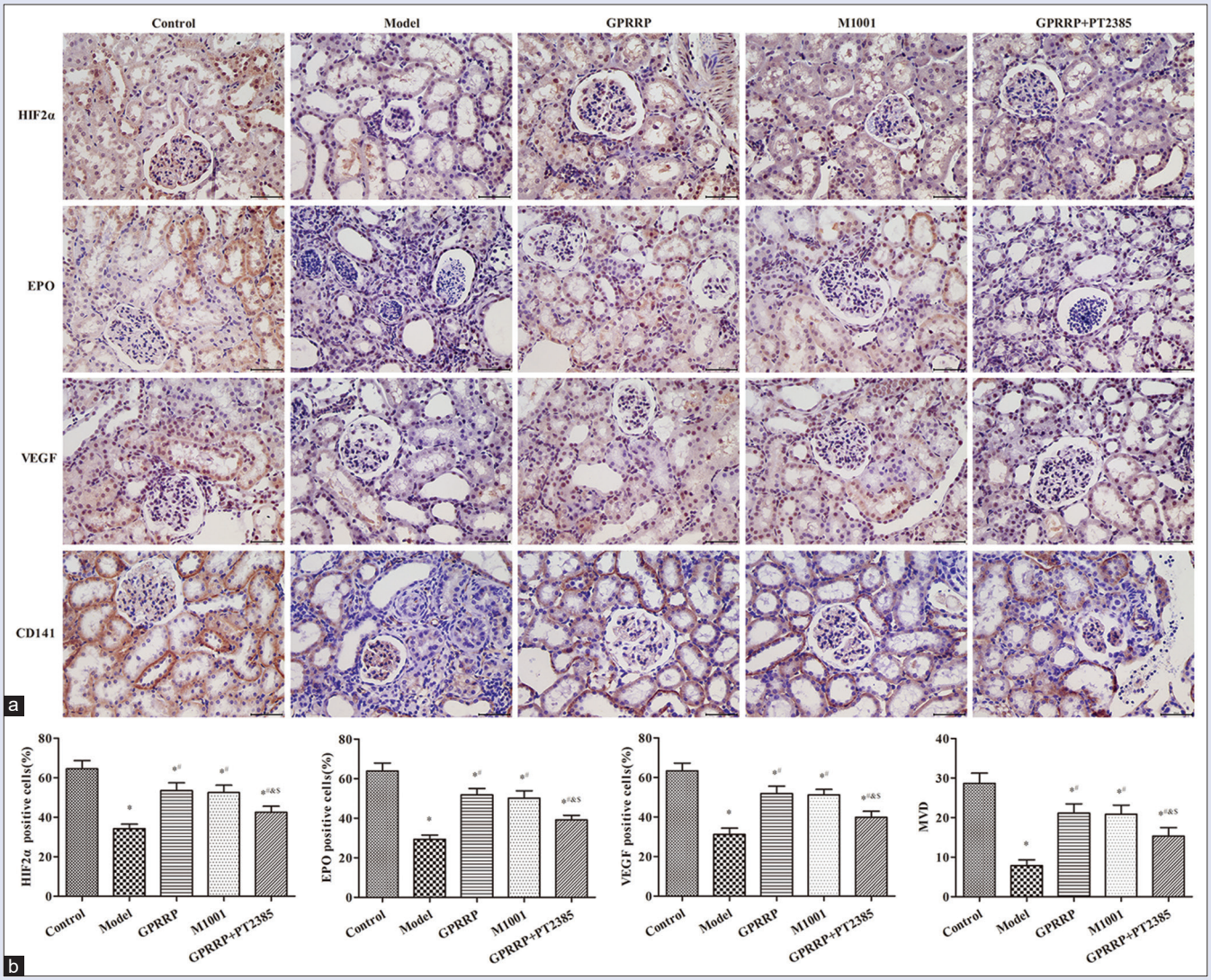

Figure 6: *Rehmannia glutinosa* polysaccharide increases the expression of vascular endothelial growth factor and erythropoietin by activating hypoxia-inducible factor-2α. (a) Immunohistochemistry; (b) Percentage of positive cells. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with the model group; $^{\&}P < 0.05$, compared with control group; $^{\$}P < 0.05$, compared with the control group

of RGP, we designed many model groups including GPRRP and EPO and added two validated groups, namely M1001 and GPRRP + PT2385 groups. Our results showed that RGP plays pivotal roles in the pathogenesis of chronic renal failure.

VEGF is one of the key molecules regulating the development of blood vessel, which was highly expressed during early kidney development. HIFs, especially HIF-2α, are a key factor regulating the expression of VEGF.[39] Kidneys are the major source of EPO during the development of mammals. In this study, the administration of RGP upregulated the expression of HIF-2α-regulated genes, such as VEGF and EPO and prevented the kidneys against the damage caused due to anemia. EPO and VEGF are neuroprotective cytokines with their gene transcription mainly mediated by HIF-1α or HIF-2α.

CONCLUSION

RGP activates HIF-2α to mediate EPO/VEGF gene expression suggesting its potential in inducing preconditioning-like effects under anemia. These results suggest that the usefulness of the RGP in rats with chronic renal failure. However, future studies should focus on the usefulness of RGP in other diseases.

Statement of ethics

The animal study protocol was in line with the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Weihai Rongcheng Center Hospital (No. 20181203-002).

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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