


## RESEARCH ARTICLE

# Functional mechanism of AMPK activation in mitochondrial regeneration of rat peritoneal macrophages mediated by uremic serum

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## Abstract

### Objective

To investigate the effects of AMPK activation on mitochondrial inhibition by uremic serum through the AMPK-activated rat peritoneal macrophages stimulated by uremic serum, thereby providing a reference for the clinical treatment of chronic kidney disease.

### Methods

Twenty-two male Sprague-Dawley (SD) rats were included as experimental subjects. Fifteen rats were constructed into chronic kidney disease models (the model group). The remaining seven rats only received renal capsule stripping instead of nephrectomy (the sham-operated group). Ten weeks after model construction, the bodyweight, blood biochemical indicators, and metabolic parameters of rats in groups were measured. Meanwhile, the expression of the M1 phenotype marker protein in peritoneal macrophages was determined.

### Results

Ten weeks after model construction, the bodyweight of rats in the model group was significantly lower than that in the sham-operated group. The values of urea nitrogen and serum creatinine were significantly higher than those in the sham-operated group ( $P < 0.01$ ). The levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and the monocyte chemoattractant protein 1 (MCP-1) of rats in the model group were significantly higher than those in the sham-operated group ( $P < 0.01$ ). After the lipopolysaccharide (LPS) stimulation, the expressions of M1 phenotype marker mRNA in the model group was significantly increased. The expression of mitochondrial structural protein mRNA in the peritoneal macrophages of rats in the model group was significantly lower than that in the sham-operated group. The expression of M1 phenotype marker mRNA was significantly decreased in the uremic serum group after AMPK agonist ( $P < 0.01$ ).

## OPEN ACCESS

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## Conclusion

In rats with chronic renal insufficiency, mitochondrial regeneration was dysfunctional in macrophages. By activating AMPK, the inhibitory effect of uremia serum on mitochondrial regeneration of macrophages was improved. Therefore, AMPK was a critical factor that could regulate mitochondrial regeneration of macrophages.

## 1. Introduction

Chronic kidney disease (CKD) includes most kidney diseases, such as glomerulonephritis, pyelonephritis, lupus erythematosus nephritis, IgA nephropathy, and nephrotic syndrome [1]. The disease is relatively benign that develops slowly. If it is not treated promptly and effectively, with the progression of the disease, the deterioration or delay will occur, which will develop into chronic renal insufficiency, renal failure, and eventually uremia [2]. According to the United States Renal Disease Data System (USRDS) in 2014, the incidence of CKD in adults in the United States is about 13.6%. The end-stage renal disease (ESRD) is the final development trend of kidney diseases of various causes, and the incidence of which accounts for 34–68% of CKD [3–5]. According to the 2016 epidemiological survey, nearly 1.2 million patients of CKD die every year in the world. The incidence of CKD in the Chinese adult population is 10.8%. There are more and more cases of uremia caused by CKD, but the pathogenesis is still unclear, so further research is needed.

Macrophages are white blood cells located in tissues. As an immune cell, they are vital for innate and adaptive immunity. Macrophages have the functions of ingesting and treating large foreign bodies, waste discharged by cells and end-of-life red blood cells [6]. Excess nutrients or systemic metabolic disorders in the body can activate the inflammatory signaling pathway of macrophages and make them in a chronic low-level inflammatory state. The infiltration degree of macrophages is closely related to the structure and function of the kidney, so macrophages play an important role in the occurrence and development of kidney diseases [7]. M1 phenotype is the pro-inflammatory macrophage, which can secrete many pro-inflammatory factors such as TNF- $\alpha$  and IL-6. Meanwhile, the M2 phenotype is an anti-inflammatory macrophage, which secretes anti-inflammatory factors such as IL-10 and IL-12. These different phenotypes of macrophages are critical for the occurrence and development of inflammation. M1-phenotype macrophages can impair the structure and function of the kidney, while M2-phenotype macrophages can relieve kidney damages [8]. Therefore, the pathogenesis of CKD may be related to macrophage-mediated inflammation. Chronic persistent low-grade metabolic inflammation is the major feature of CKD; however, its exact mechanism remains unclear.

Mitochondria is critical for the process of material metabolism and energy production. Mitochondrial regeneration is the key to maintain mitochondrial function. AMPK-PGC-1 $\alpha$  is an important indicator of mitochondrial regeneration [9]. The results show that activation of AMPK can increase the ratio of NAD<sup>+</sup>/NADH and directly phosphorylate PGC-1 $\alpha$ , thus enhancing the regeneration ability of mitochondria [10]. Mitochondrial regeneration and degradation are balanced in normal cells. When the balance is broken for some reason, oxidative stress will lead to the decrease of cell metabolism, which will lead to the disorder of the whole metabolic balance of the organism, leading to the occurrence of diseases [11].

To explore the effect of AMPK activation on the mitochondrial inhibition of uremia serum and provide a reference for the clinical treatment of CKD, in this study, based on rat model, uremic serum is used to stimulate AMPK-activated peritoneal macrophages, observe the

changes of mitochondrial regeneration and AMPK pathway, and further explore the effect of AMPK activation on uremic serum in inhibiting mitochondria, hoping to provide new ideas for clinical treatment or delaying the progress of CKD.

## 2. Materials and methods

### 2.1 Establishment of experimental animals and models

A total of 22 male 190~220g Sprague-Dawley (SD) rats from Laboratory Animal Center of Southern Medical University were selected as experimental subjects. They were kept in the laboratory at temperatures of 22~24°C, humidity 50%~65%, and circadian circulation of 12 h. Rats could eat and drink freely. The feed was standard high-pressure disinfection feed. After one week of adaptive feeding, 7 rats were randomly selected as the sham-operated group and 15 rats as the nephrectomy model group.

Construction of rat CKD model: 15 rats in the model group were anesthetized by intraperitoneal injection of 3% pentobarbital sodium. After successful anesthesia, rats were fixed on the operating table and their abdominal fur was removed. Routine disinfection and padding were needed on the lateral side of the left inferior rib rectus muscle. Under aseptic operation, a longitudinal incision of about 1 cm was made in the left subcostal region. Skin, subcutaneous tissue, and muscular layer were cut in turn to expose the left kidney. The kidney tissue was separated, and the renal capsule was dissected. Hemostatic forceps were used to gently clamp the pedicle of the kidney to block blood flow and avoid massive leakage of blood on the nephrectomy surface. The weight of both kidneys in rats accounts for about 0.72% of the total weight. The upper and lower poles of the kidney were excised, accounting for about 2/3 of the left kidney and they were weighed. The upper and lower poles were compressed by gelatin sponge to stop bleeding. The kidneys were returned immediately after hemostasis. The muscles and skin were sutured layer by layer after the upper and lower poles were observed without bleeding. Muscle hernia should be avoided during suture. Intramuscular injection of penicillin sodium was needed to prevent infection after the operation. The second operation was carried out after a week. All rats were anesthetized with the same anesthesia method one week after the operation. After the same anesthesia method, the right-back was cut to expose the right kidney of the rats. After ligating the pedicle of the kidney with hemostatic forceps, the whole right kidney was resected, and the chronic renal failure model of 5/6 nephrectomy of the rats was prepared. Seven rats in the sham-operated group were only peeled off the renal capsule without nephrectomy.

All procedures in this study were performed following the regulations of the Chinese Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health Publication No. 85-23, revised 1996, and was approved by the Ethics Committee of Gansu Provincial Maternity and Child-care Hospital (NO. GPMCH-8012). All sacrifices were performed under pentobarbitone anesthesia, and every effort was made to minimize animal suffering.

### 2.2 Detection indicators and methods

Measurement of body weight, blood biochemical and metabolic parameters of rats: 10 weeks after the establishment of the rat model of chronic renal failure, the rats were put to death by Bloodletting method. On the day before the execution, the rats were weighed, and 24-hour urine was collected. Intraperitoneal injection anesthesia rats: rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium. After successful anesthesia, supine rats were fixed on the operating table for routine disinfection of local skin. Blood samples were collected from the abdominal aorta of rats and the metabolic indexes were detected. The animal

metabolic cages measured the 24 h urine volume of rats in both groups. The levels of urea nitrogen (BUN) and serum creatinine (Scr) in rats were determined by the ELISA examination reagent kit (Shanghai Yuanmu Biotechnology, China). The effects of uremic serum on macrophage activation and reactivity were studied. Rats were put to death by bloodletting.

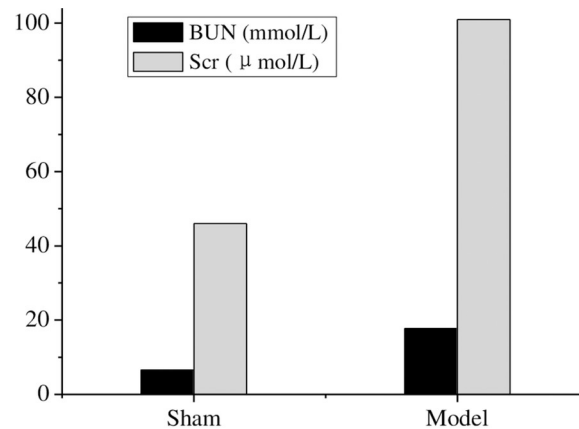
Second, rat peritoneal macrophages were extracted and identified to detect the expression of M1 marker protein. Rats were placed in closed containers. 2mL ether was poured into the anesthesia to kill the rats. Rats were immersed in 75% alcohol for 5 minutes to disinfect. Rats were lifted upside down and injected with DMEM medium (10% bovine serum + penicillin 100U/mL + streptomycin 100U/mL) as the basic culture medium. After gently massaging the abdomen of rats for 2 minutes, rats need to be placed for 8 minutes, and then they were laparotomies under sterile conditions. When the peritoneal fluid turns pale yellow, 8mL peritoneal fluid needs to be sucked and centrifuged at 800rpm for 5 minutes. DMEM medium was used to adjust the cell concentration to the desired level, and the medium was changed for 2 hours to remove a few non-adherent cells. The purity of rat macrophages was identified by flow cytometry. After collecting the cells, the PBS solution was used to suspend the cells and the cell concentration was adjusted to  $10^7$  cells/mL. Each tube was required to absorb 100 $\mu$ L cell suspension and add fixative to incubate at room temperature for 10 minutes. Each tube was added to 4mL of PB solution and centrifuged to remove the supernatant. Membrane breakers were used to suspend cells. Anti-rat antibodies of 10 $\mu$ L mice were added, and then mixed to avoid light and incubated at room temperature for 20 minutes. The antibodies were detected by flow cytometry. Peritoneal macrophages of rats in the sham-operated group and model group were planted on the cell culture plate. The concentration of M1 markers in the supernatant was detected by ELISA (Shanghai Yuanmu Biotechnology, China), including TNF- $\alpha$ , IL-6, and MCP-1. The total protein content was tested by ELISA. The expression of ML markers after 24 hours of stimulation by 1000ng/mL lipopolysaccharide (LPS) was detected by Real-time PCR. Peritoneal macrophages of normal rats were removed and cultured with 20% normal rat serum and 20% uremic rat serum for 24 hours, respectively. After stimulation with LPS, cells need to be collected to detect the expression of M1-type markers.

Thirdly, the effect of chronic renal failure on the expression of the mitochondrial structural protein (MSP) in rat peritoneal macrophages: Ice PBS solution was used to wash cells twice. 200 $\mu$ L cracking buffer was added. After incubation on ice for 20 minutes and centrifugation for 10 minutes, the supernatant was collected, and the total protein lysate was obtained. Real-time PCR was used to detect the expression of the mitochondrial structural protein (MSP) in peritoneal macrophages of both groups, including cytochrome C (CO), cytochrome C oxidase (COX) and ATP synthase.

Fourthly, the activation effect of AMPK agonist (AICAR) on rat peritoneal macrophages: normal rat peritoneal macrophages were removed, and the cells were treated with AMPK agonist (AICAR) for 4 h and cultured with 20% normal serum and 20% uremic serum for 24h. After collecting cells, RNA was extracted and the expression of M1-type marker in the rat was detected by Real-time PCR.

### 2.3 Statistical methods

In this experiment, SPSS20.0 statistical software was used to analyze the data, and the average values of three repeated experiments were used. The results of measurement data were expressed as mean  $\pm$  standard deviation. The T-test was used to assess the importance of experimental data. One-way ANOVA analysis was used, and the LSD method was used to compare the two methods to obtain significant difference analysis.  $P < 0.05$  shows significant difference.



**Fig 1. Comparison of blood biochemical indexes between two groups of rats.**

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### 3. Results

#### 3.1 Comparison of body weight and blood biochemistry between two groups of rats

Ten weeks after the establishment of the chronic renal failure rat model, weight, urea nitrogen (BUN) and serum creatinine (Scr) were measured in the two groups. The weight of the rats in the sham-operated group was ( $551.57 \pm 9.96$ ) g, which was significantly higher than that in the model group ( $442.19 \pm 10.33$ ) g, the difference was statistically significant ( $P < 0.05$ ). The specific values were shown in Fig 1. As can be seen from Fig 1, the weight of rats in the model group after 10 weeks was significantly lower than that in the sham-operated group ( $P < 0.01$ ). The urea nitrogen value and serum creatinine value of the model group were ( $100.98 \pm 7.92$ ) micromole/L and ( $17.68 \pm 0.31$ ) mmol/L respectively, which were significantly higher than those of the sham-operated group ( $P < 0.01$ ). It can be seen from this that chronic renal failure can cause a significant increase in urea nitrogen and serum creatinine in rats.

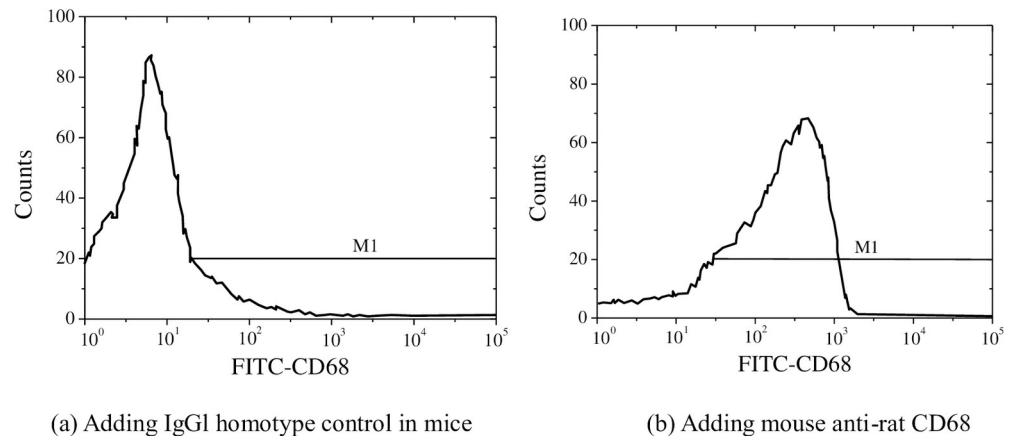
#### 3.2 Purity identification and M1 marker expression of peritoneal macrophages in rats

In this experiment, the purity of rat peritoneal macrophages was identified by flow cytometry, and the purity of the cells was 91.32% by incubation with mouse anti-rat FITC-CD68 direct labeled antibody. The purity of the cells was 91.32%, compared with non-immune FITC-IgG1, and the homologous control was 1.59%. The purity of rat peritoneal macrophages was identified as shown in Fig 2.

Real-time PCR was used to detect the expression of M1 markers in peritoneal macrophages of the two groups, and the expression of M1 markers after 24 hours of stimulation with 1000ng/mL lipopolysaccharide (LPS). The numerical results were shown in Table 1 and Fig 3. From the data in Table 1, it can be seen that the levels of TNF- $\alpha$ , IL-6, and MCP-1 in the model group were significantly higher than those in the sham-operated group ( $P < 0.01$ ). After 24 hours of stimulation with 1000ng/mL lipopolysaccharide (LPS), the expression of M1 markers in the two groups increases significantly, while that in the model group increases significantly ( $P < 0.01$ ).

#### 3.3 Activation of macrophages in normal rats by uremic serum

Real-time PCR was used to detect the effects of normal serum and uremic serum on normal rats. The expression of M1 markers in peritoneal macrophages was shown in Table 2. The



**Fig 2. Purity identification of rat peritoneal macrophages.**

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effect of uremic serum on the expression of the M1 marker in normal rats was shown in Fig 4. From the data in Table 2, it can be seen that the levels of TNF- $\alpha$ , IL-6, and MCP-1 in uremic serum group were significantly higher than those in normal serum group ( $P < 0.01$ ). After 24 hours of stimulation with 1000ng/mL lipopolysaccharide (LPS), the expression of M1 markers in rats increases significantly, while that in uremic serum group was significantly increased ( $P < 0.01$ ).

### 3.4 Comparison of expression of mitochondrial structural protein mRNA in macrophages between two groups

Real-time PCR was used to detect the expression of the mitochondrial structural protein in peritoneal macrophages of the two groups. The specific values were shown in Table 3. The comparison of mitochondrial structural protein mRNA of macrophages between the two groups was shown in Fig 5. From the data in Table 3, it can be seen that the expression of the mitochondrial structural protein in peritoneal macrophages of rats in the chronic renal failure model group was significantly lower than that of rats in sham-operated group ( $P < 0.01$ ).

### 3.5 Effects of AMPK agonists on the activation of peritoneal macrophages in rats

Real-time PCR was used to detect the expression of macrophage M1 markers in normal serum group, uremic serum group, normal serum + AICAR group and uremic serum + AICAR

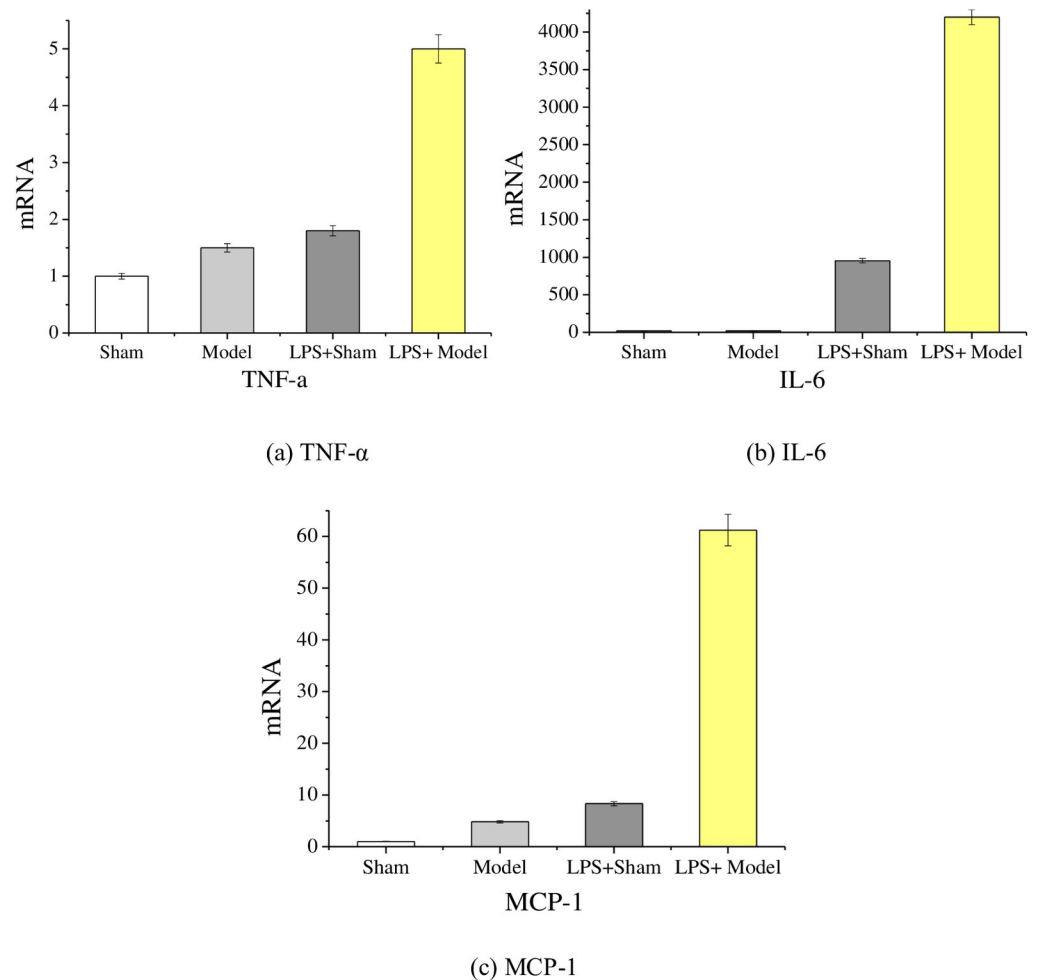
**Table 1. Expression of M1 markers in peritoneal macrophages of rats in two groups.**

| Group               | TNF- $\alpha$                  | IL-6                                | MCP-1                           |
|---------------------|--------------------------------|-------------------------------------|---------------------------------|
| Sham-operated       | 1.000 $\pm$ 0.001              | 1.000 $\pm$ 0.001                   | 1.000 $\pm$ 0.001               |
| Model               | 1.501 $\pm$ 0.059*             | 1.755 $\pm$ 0.089*                  | 4.812 $\pm$ 0.289*              |
| LPS + Sham-operated | 1.811 $\pm$ 0.205              | 954.279 $\pm$ 210.575               | 8.312 $\pm$ 0.912               |
| LPS+ Model          | 5.002 $\pm$ 0.312 <sup>#</sup> | 4199.208 $\pm$ 321.109 <sup>#</sup> | 61.254 $\pm$ 6.286 <sup>#</sup> |
| F                   | 79.457                         | 100.332                             | 105.862                         |
| P                   | <0.01                          | <0.01                               | <0.01                           |

Note

\* Compared with sham-operated rats,  $P < 0.01$ ; <sup>#</sup> Compared with sham-operated rats stimulated by LPS,  $P < 0.01$ .

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**Fig 3. Comparison of M1 marker expression in peritoneal macrophages of two groups of rats.**

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group. The comparison between groups was shown in Fig 6. From the data in the Fig 6, it can be seen that the expression of M1 marker mRNA in uremic serum group was significantly lower than that before the application of AMPK agonist (AICAR), and the difference was

**Table 2. Expression of M1 markers in peritoneal macrophages of rats in two groups after uremic serum treatment.**

| Group              | TNF- $\alpha$                  | IL-6                                | MCP-1                           |
|--------------------|--------------------------------|-------------------------------------|---------------------------------|
| Normal serum       | 1.000 $\pm$ 0.001              | 1.000 $\pm$ 0.001                   | 1.000 $\pm$ 0.001               |
| Uremic serum       | 1.722 $\pm$ 0.124*             | 2.128 $\pm$ 0.143*                  | 1.731 $\pm$ 0.154*              |
| LPS + Normal serum | 1.802 $\pm$ 0.286              | 850.073 $\pm$ 62.183                | 9.289 $\pm$ 1.002               |
| LPS + Uremic serum | 6.589 $\pm$ 0.703 <sup>#</sup> | 7987.355 $\pm$ 535.881 <sup>#</sup> | 38.267 $\pm$ 2.563 <sup>#</sup> |
| F                  | 47.657                         | 218.753                             | 172.367                         |
| P                  | <0.01                          | <0.01                               | <0.01                           |

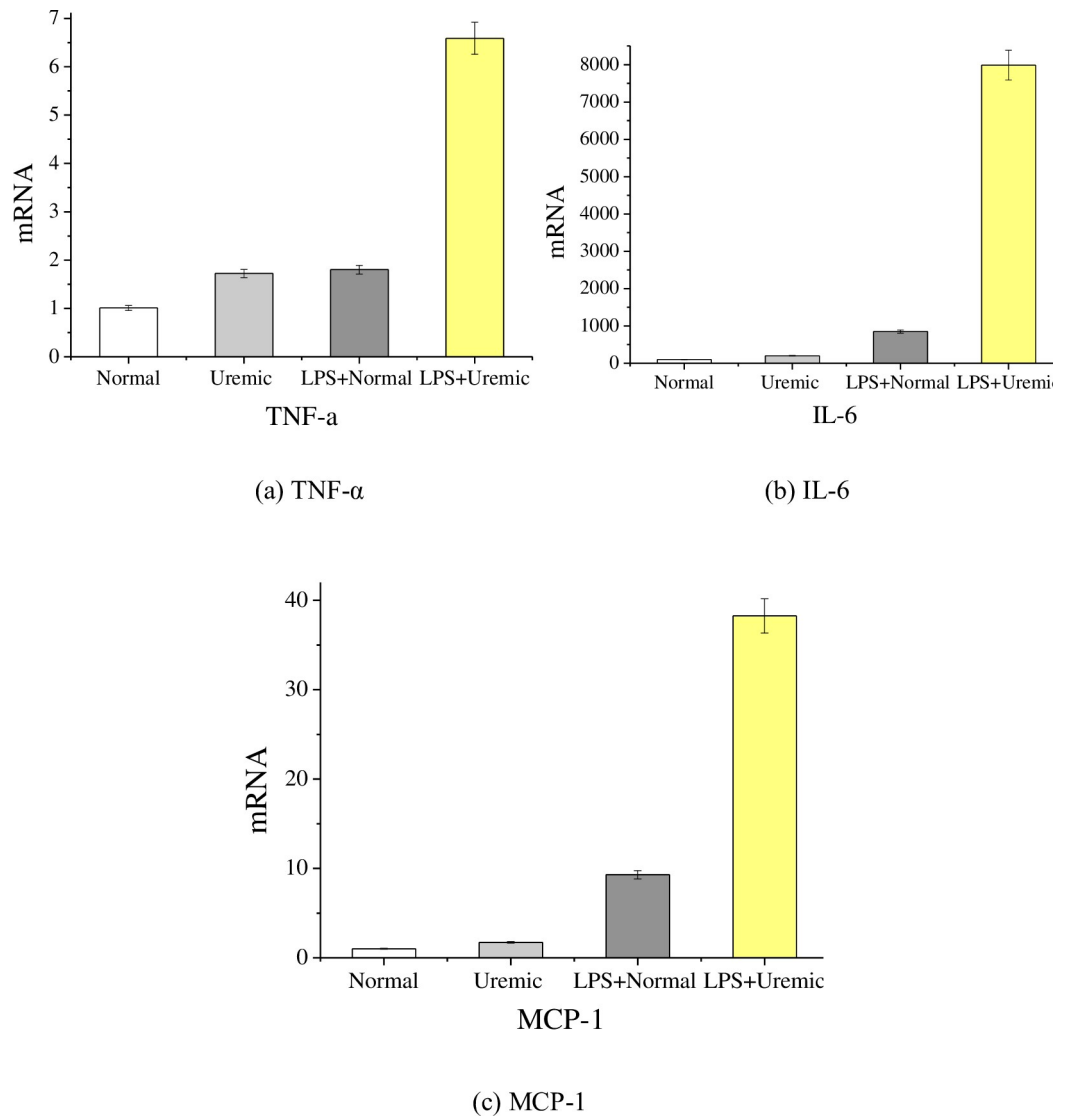
Note

\* Compared with normal serum group rats, P < 0.01

<sup>#</sup> Compared with normal serum group rats stimulated by LPS, P < 0.01.

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**Fig 4. Effect of uremic serum on the expression of M1 marker in normal rats.**

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statistically significant ( $P < 0.01$ ). It suggests that AMPK activation might promote the regeneration of mitochondria in rat peritoneal macrophages.

## 4. Discussion

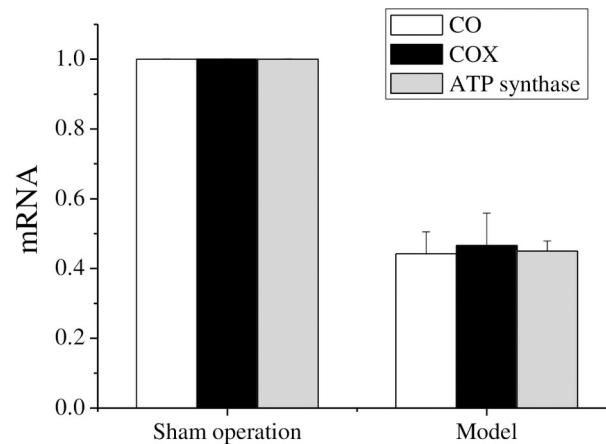
CKD was a chronic renal structural and functional disorder caused by various causes, including primary or secondary glomerulonephritis, renal tubular injury and renal vascular lesions,

**Table 3. Expression of mitochondrial structural protein mRNA in macrophages of two groups of rats.**

| Group         | CO          | COX         | ATP synthase |
|---------------|-------------|-------------|--------------|
| Sham-operated | 1.000±0.001 | 1.000±0.001 | 1.000±0.001  |
| Model         | 0.442±0.063 | 0.466±0.093 | 0.450±0.029  |
| t             | -9.753      | -11.322     | -21.035      |
| P             | <0.01       | <0.01       | <0.01        |

<https://doi.org/10.1371/journal.pone.0235960.t003>



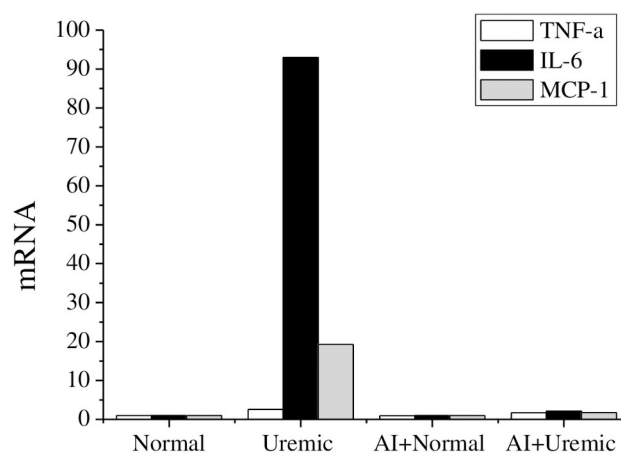


**Fig 5. Comparison of the expression of mitochondrial structural protein mRNA in rat macrophages between the two groups.**

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which seriously threaten human health [12–14]. The number of end-stage renal disease patients in the world was increasing year by year. In addition, in China, the incidence of CKD has exceeded the global average, accounting for about 11% [15] of the total number of patients worldwide. The pathogenesis of CKD was still unclear and needs further study. Many chronic diseases have microinflammation [16]. It has also been shown that with the decline of renal function in patients with CKD, the body will continue to show low levels of inflammation in the absence of systemic or local obvious clinical manifestations of infection, mainly with the persistent increase of tumor necrosis factor (TNF- $\alpha$ ), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) as the signal [17].

Chronic inflammatory diseases not only have a persistent pro-inflammatory response but also have the possibility of an uncontrolled inflammatory response [18–20]. It has been found that almost all kidney diseases are accompanied by secretion of inflammatory factors and infiltration of immune cells, while macrophages have strong plasticity in acute and chronic inflammation. Macrophage-mediated inflammation plays an important role in the occurrence and development of CKD by regulating its two phenotypes M1 and M2 to respond effectively to environmental signals [21]. Among them, M1 macrophages, as pro-inflammatory



**Fig 6. Comparison of M1 marker expression in macrophages of rats in each group.**

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macrophages, are activated by the classical phosphorylated immune pathway of AMPK, which can promote the production of inflammatory factors. M2 macrophages play an anti-inflammatory role by synthesizing anti-inflammatory cytokine receptors [22, 23]. The AMPK pathway is essential for mitochondrial regeneration and function. Experiments have shown that the mitochondrial structure and function of macrophages in rats with chronic renal failure are impaired. In addition, AMPK activation can alleviate macrophage-mediated inflammatory response. Furthermore, as an important indicator of mitochondrial regeneration, AMPK suggests that metabolic inflammation may be associated with macrophage mitochondrial dysplasia [24]. Based on the theoretical basis and previous experimental summary, the potential mechanism of CKD is analyzed from the perspective of macrophage mitochondrial aplasia by establishing a rat kidney failure model. By transfecting rat peritoneal macrophages with AMPK activator and stimulating AMPK activation with the relevant concentration of uremic serum, the intervention effect of AMPK activation on uremic serum inhibiting mitochondria is explored.

After 10 weeks of establishment, the bodyweight of rats with chronic renal failure was significantly lower than that of the sham-operated group. The urea nitrogen value and serum creatinine value were  $(100.98 \pm 7.92) \mu\text{mol/L}$  and  $(17.68 \pm 0.31) \text{mmol/L}$ , respectively, which were significantly higher than those of sham-operated group ( $P < 0.01$ ). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) levels in the model group were significantly higher than those in the sham-operated group. After 24 hours of stimulation with 1000ng/mL lipopolysaccharide (LPS), the expression of M1-type marker mRNA in the model group increases significantly. The levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in uremic serum group were significantly higher than those in normal serum group ( $P < 0.01$ ). After 24 hours of stimulation with 1000ng/mL LPS, the expression of M1-type marker mRNA in the uremic serum group increases significantly. The expression of mitochondrial structural protein mRNA in peritoneal macrophages in the model group was significantly lower than that in the sham-operated group. After using AMPK agonist in the uremic serum group, the expression of M1-type marker mRNA was significantly lower than that before using AMPK agonist. In summary, in rats with chronic renal insufficiency, macrophage mitochondrial regeneration dysfunction, through activation of AMPK, the uremic serum could improve the inhibition of macrophage mitochondrial regeneration. Therefore, AMPK was an important factor in regulating macrophage mitochondrial regeneration.

## 5. Conclusion

This study explored the mechanism of metabolic cells activating immune cells, which was significant for the clinical prevention and treatment of metabolic inflammation. CKD also belongs to metabolic inflammation. Its occurrence and development might be related to mitochondrial regeneration disorder. Therefore, through the treatment of activating the mitochondrial regeneration, it would be possible to delay the progression of CKD, thereby improving human health. The activation mechanism of immune cells by metabolic factors was explored, which was of great significance for the clinical prevention and treatment of metabolic inflammation. However, this experiment did not involve the analysis of CKD from the perspective of metabolic inflammation. Its occurrence and development might be related to mitochondrial aplasia. In subsequent explorations, the treatments of activating mitochondrial regeneration could be applied, thereby providing a reference for delaying the progression of CKD.

## Author Contributions

**Conceptualization:** De Chen, Kun Zuo, Xuan Liang, Mei Wang, Honghong Zhang, Rong Zhou, Xiaoli Liu.

**Data curation:** De Chen, Kun Zuo, Xuan Liang, Mei Wang.

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**Resources:** De Chen, Xuan Liang, Honghong Zhang, Rong Zhou, Xiaoli Liu.

**Software:** Kun Zuo, Xuan Liang.

**Supervision:** Kun Zuo, Xiaoli Liu.

**Validation:** De Chen, Xuan Liang, Mei Wang, Honghong Zhang, Rong Zhou, Xiaoli Liu.

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**Writing – original draft:** De Chen, Kun Zuo, Mei Wang.

**Writing – review & editing:** Honghong Zhang, Rong Zhou, Xiaoli Liu.

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