

## Effect of olmesartan on oxidative stress and klotho protein in rats with adriamycin nephropathy

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**Abstract:** To investigate the effect of olmesartan on oxidative stress and klotho protein in rats with adriamycin nephropathy. The male wister rats were randomly divided into three group. Healthy rats were served as normal control group (NC group, n=15), adriamycin nephropathic model were randomly divided into adriamycin nephropathy group (ADR group, n=15) and olmesartan group (OLM group, n =15). Adriamycin nephropathy was induced by a single tail vein injection of doxorubicin. OLM group were treated by olmesartan continuous lavage, NC group and ADR group were given the same dose of normal saline continuous lavage. After 8 weeks, 24-h urine protein excretion, MDA levels and SOD activities in renal cortex were measured. The histomorphology of kidney was observed by HE staining. The expression of klotho protein in renal cortex was determined by western blotting. 24-h urine protein excretion were significantly increased in ADR group than in NC group (P<0.05). MDA levels in renal cortex were significantly increased in ADR group than in NC group (P<0.05). SOD activities in renal cortex were significantly decreased in ADR group than in NC group (P<0.05). klotho protein levels were significantly decreased in ADR group than in NC group (P<0.05). All those parameters were significantly improved in OLM group (P<0.05). klotho decrease in rats with adriamycin nephropathy. The olmesartan can significantly increase the expression of klotho protein and reduce oxidative stress in rats with adriamycin nephropathy.

**Keywords:** Adriamycin nephropathy; oxidative stress; Klotho protein; Olmesartan

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### 1. Introduction

Nephrotic syndrome is an extremely prevalent and a relatively benign kidney disease that can progress to chronic kidney disease if not treated in time. Adriamycin is an anthracyclin antibiotic that belongs to a class of anticancer agents. It has a broad spectrum of anti-specific human tumors, including breast cancer, small cell carcinoma of the lung and acute leukaemia[1]. The use of adriamycin is restricted by a number of side-effects the most important is a dose-limiting nephrotoxicity[2].

Adriamycin-induced nephropathy in rats is a model of human renal microlesions. The exact mechanism of doxorubicin-induced nephrotoxicity is hard to establish. However, oxidative stress plays an important role in the pathogenesis of adriamycin-induced nephropathy. It has been suggested by many investigators that reactive oxygen species production has been clearly associated with the progression of CKD[3]. In previous studies found that oxidative stress in CKD patients enhances as the disease progresses[4,5].

The Klotho gene is mainly expressed in renal tubular cells[6]. previous studies have shown that Klotho protein has improving the ability of cells to remove reactive oxygen species (ROS) [7]. Studies has revealed that the levels of the Klotho protein were reduced in animal models of kidney disease[8]. Angiotensin-converting enzyme ACE activity plays an important role in hypertension control and hence

ACE inhibitors such as olmesartan are beneficial used as antihypertensive agents. Studies have shown that olmesartan have relieved endothelial cell damaged in diabetic mice by mitigating oxidative stress[9].

The purpose of this study was to investigate the relationship between oxidative stress and the level of klotho protein in the rat model of adriamycin-induced kidney, as well as the effect of olmesartan on oxidative stress and klotho protein. In view of the existing researches on the role of ACE inhibitors olmesartan on the oxidative stress. It is worthwhile to gain further insight into the role of olmesartan on the nephrotoxicity induced by doxorubicin.

### 2. Materials and Methods

#### 2.1. Animal Model

The male Wistar rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and raised in a specific pathogen free facility in a temperature-controlled room and had free access to food and water under a 12h light/dark cycle. The tests were conducted in compliance with the guidelines for the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals.

#### 2.2. Drugs and reagents

Adriamycin was obtained from MCE (USA) Olmesartan was purchased from First Three

Pharmaceutical Co., Ltd. (Shanghai). The rabbit anti-mouse polyclonal antibody was purchased from Abcam (UK).

## 2.3. Study Protocol

Rats were randomly divided into NC group, ADR group and OLM group, every group involved 15 rats. The NC group is the control group. The ADR group, OLM group were injected with a single dose of 5 mg/kg adriamycin via the caudal vein[6]. The NC group was injected with the comparable volume of normal saline. Compared with Proteinuria of rats before and after doxorubicin injection, the rats that proteinuria with significant statistical differences were included in this study. The OLM group was given olmesartan 10mg/(kg·d) after tail vein injection 1 week[10] and the NC and ADR groups were given same volumes of physiological saline until 8weeks. The 24-h urine of rats from each group was collected before the rats sacrificed. The rats were anaesthetized at 8 weeks after a single tail vein injection and their kidneys were removed. One half of each kidney was stored at -80°C. The remaining half was fixed in 10% buffered formaldehyde and then prepared as paraffin-embedded tissue blocks for haematoxylin and eosin stain.

## 2.4. Urinary protein sample analysis

The 24-hour urinary protein excretion was assessed that process and analysed in accordance with the procedure specified by the manufacturer.

## 2.5. Measurement of SOD and MDA levels

Total SOD activity and MDA levels in kidney tissue was respectively measured using a superoxide dismutase activity assay kit and thiobarbituric acid reactive substances assay kit in accordance with the procedure specified by the manufacturer.

## 2.6. Histochemistry

After kidneys were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin, 4µm thick sections were stained with HE stain according to the standard protocols. The morphological damage was independently scored by two experienced pathologists who were blinded to the experimental conditions.

## 2.7. Immunoblot analysis

The level of klotho protein was measured by immunoblotting of the renal tissues. A 30 mg total protein was used for immunoblot analysis according to our previous protocols[11]. Blots were probed with anti-Klotho (1:1000) overnight at 4°C. After washing, the membranes were incubated with the HRP-conjugated secondary antibody (1:2000). After washing, specific signals were determined using an ECL kit according to the directions of the

manufacturer. Gray values were analyzed with ImageJ software.

## 2.8. Statistical analyses

SPSS version 21.0 statistical software was used for statistical analysis. The results are expressed as the mean ± SEM. The data of groups were compared with analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test to identify significance among groups. P<0.05 was considered statistically significant. The experiments were performed at least three independent times.

## 3. Results

### 3.1. The level of 24hourly protein excretion

Proteinuria is a biochemical marker of adriamycin-induced nephropathy in rats. As shown in our results, after the rats administered with adriamycin, the ADR group exhibited significant increases in 24h urinary protein excretion compared with the NC group (P<0.05), and the levels of 24h urinary protein excretion was inhibited by the 8 weeks olmesartan treatment (P<0.05) (Figure. 1).

### 3.2. Oxidative stress is involved in adriamycin nephrosis

We examined the antioxidative mechanism of olmesartan in adriamycin nephrosis rats via measured the levels of MDA and SOD. As shown in our results, after the rats administered with adriamycin, the ADR group exhibited significant increases in MDA concentrations compared with NC group (P<0.05), and MDA concentrations was inhibited by the 8 weeks olmesartan treatment (P<0.05) (Figure. 2). In contrast, the activity of SOD in ADR group was downregulated compared to NC group (P<0.05), while by the 8 weeks olmesartan treatment the activity of SOD was improved (Figure. 3).

### 3.3. Histological examination of adriamycin nephrosis rats

As observed via light microscopy, HE staining further showed that compared with NC group, ADR group renal tubule exhibited unnormal morphological structures(epithelial hypertrophy, vacuolar degeneration, and infiltration of the interstitium by inflammatory cells).Whereas, damage to renal tubules in the OLM group was significantly alleviated compared to ADR group (Figure4). Tubulointerstitial injury index was assessed as previously described[12].

### 3.4. The expression of Klotho in adriamycin nephrosis rats

We studied the effect of olmesartan on klotho expression in adriamycin nephrosis rats. As shown in

our results, after the rats administered with adriamycin, klotho proteins had lower expressions in the ADR group compared with that in the NC group, after the 8 weeks olmesartan treatment, the protein expression of klotho was significantly improved (Figure 5).

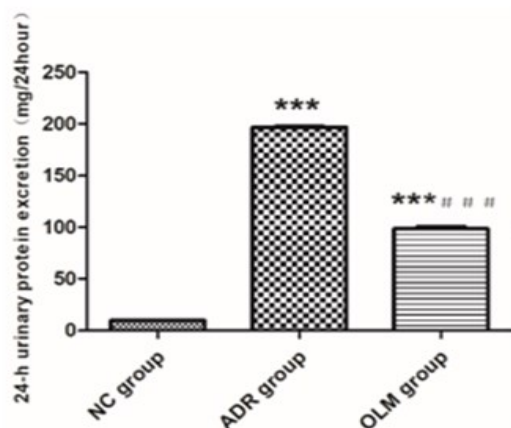


Figure 1. Representative differences in 24h urine protein excretion among three groups. olmesartan suppressed the elevated level of 24h urine protein excretion, ADR group showed a significant elevation in 24h urine protein excretion compared to the NC group, which can be improved by olmesartan. Bars represent the mean ± SEM (n=15 per group). P<0.05 vs. NC group (\*), P < 0.01 vs. NC group (\*\*), P < 0.0001 vs. NC group (\*\*\*), P < 0.05 vs. ADR group (#), P < 0.01 vs. ADR group (##), P < 0.0001 vs. ADR group (###).

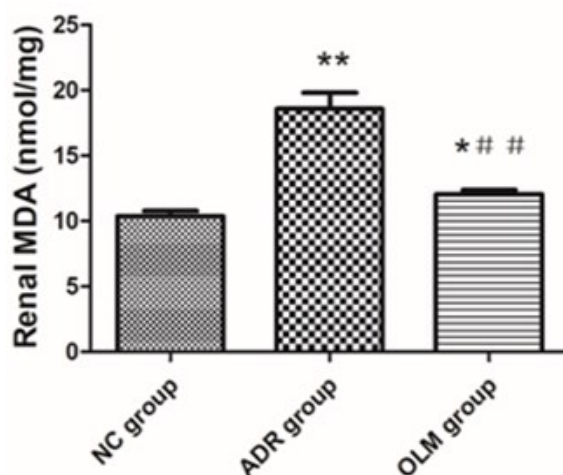


Figure 2. Oxidative stress is involved in adriamycin nephropathy. olmesartan suppressed the elevated level of MDA, ADR group showed a significant elevation in MDA content compared to the NC group, which can be improved by olmesartan. Bars represent the mean ± SEM (n = 15 per group). P<0.05 vs. NC group (\*), P<0.01 vs. NC group (\*\*), P < 0.0001 vs. NC group (\*\*\*), P < 0.05 vs. ADR group (#), P<0.01 vs. ADR group (##), P < 0.0001 vs. ADR group (###).

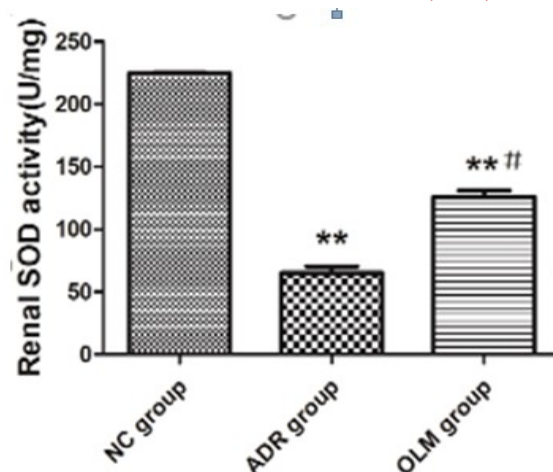


Figure 3. The olmesartan improved the weakened activity of SOD, ADR group showed a significant decrease of SOD activity compared to the NC group, which can be improved by olmesartan. Bars represent the mean ± SEM (n=15 per group). P<0.05 vs. NC group (\*), P<0.01 vs. NC group (\*\*), P<0.0001 vs. NC group (\*\*\*), P<0.05 vs. ADR group (#), P<0.01 vs. ADR group (##), P < 0.0001 vs. ADR group (###).

### 3.5. The relationship among MDA level, SOD activities, and the levels of Klotho protein

The Pearson’s correlation analysis was performed to investigate the associations among MDA levels, SOD activity, and Klotho protein. We demonstrated that klotho protein was negatively correlated with the levels of MDA (Figure 6) and was positively associated with total SOD activity (Figure 7). Therefore, our data demonstrate that oxidative stress suppresses the expression of klotho protein in the kidneys of rats administered adriamycin.

## 4. Discussion

Adriamycin has a chronic toxic effect on the kidneys, which causes kidney damage through a variety of mechanisms[13]. Oxidative stress plays an important role in the rats of adriamycin-induced renal injury. In our study, a single dose of adriamycin (5mg/kg) induced nephrotoxicity manifested biochemically by a significant increase in 24h urinary protein excretion and a significant decrease in klotho protein expression in kidney. Moreover, our researches are supported by a significant increase in MDA concentrations and a significant decrease in the activity of SOD in the kidneys of rats after treatment with adriamycin. Olmesartan have an effect on protecting the kidney of rat from damage induced by adriamycin. This protection was clearly indicated by a decrease of 24h urinary protein excretion and MDA concentrations. This protection was also clearly indicated by an increase in the activity of SOD and the level of klotho protein. This experiment also showed that klotho protein was negatively correlated

with the levels of MDA and was positively associated with total SOD activity. Therefore,

olmesartan may protect kidneys by preventing lipid peroxidation.

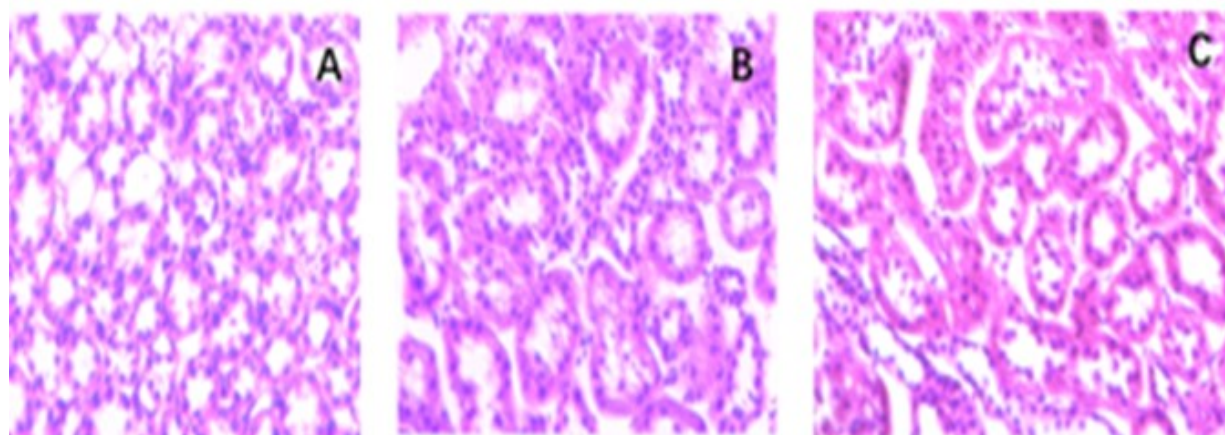


Figure 4. Representative kidney tubule sections stained with HE (x400). Three groups were included in this morphological observation (A. NC group, B. ADR group, C. OLM group). Compared with NC group, ADR group showed an obvious renal tubular injury. The olmesartan treatment improved the impaired of kidney tubules in adriamycin nephropathy rats.

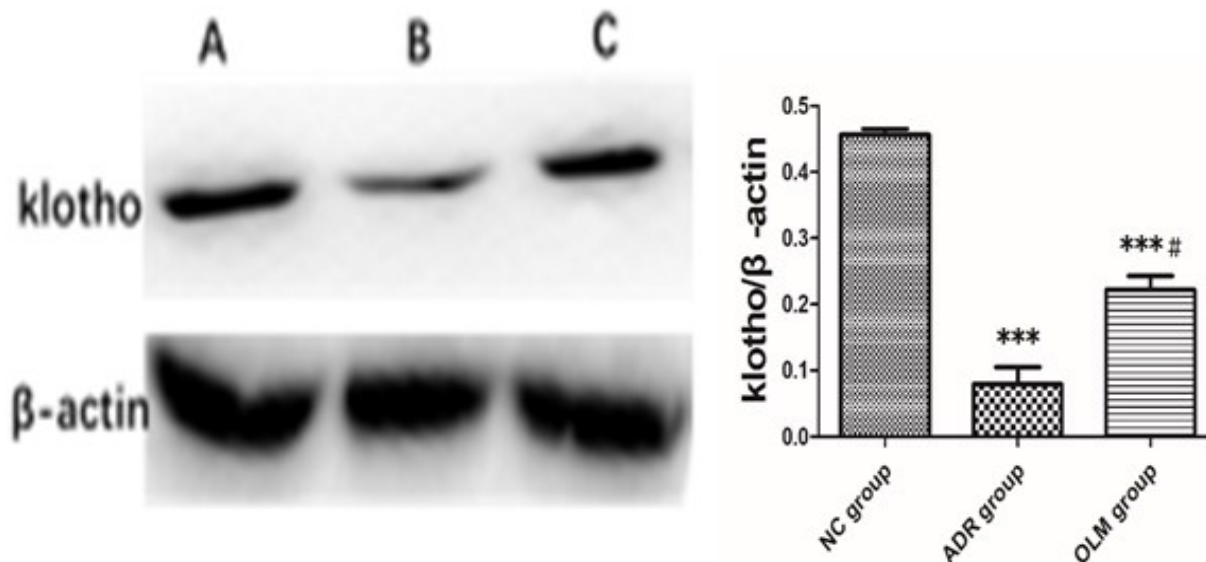


Figure 5. Representative immunoblot for klotho protein in three groups (A. NC group, B. ADR group, C. OLM group), olmesartan elevated the decreased level of klotho protein, ADR group showed a significant decrease of the MDA content compared to the NC group, which can be improved by olmesartan. Bars represent the mean ± SEM (n=15 per group). P<0.05 vs. NC group (\*), P<0.01 vs. NC group (\*\*), P<0.0001 vs. NC group (\*\*\*), P<0.05 vs. ADR group (#), P<0.01 vs. ADR group (##), P<0.0001 vs. ADR group (###).

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with adriamycin. Olmesartan have an effect on protecting the kidney of rat from damage induced by adriamycin. This protection was clearly indicated by a decrease of 24-h urinary protein excretion and MDA concentrations. This protection was also clearly indicated by an increase in the activity of SOD and the level of klotho protein. This experiment also showed that klotho protein was negatively correlated with the levels of MDA and was positively associated with total SOD activity. Therefore, olmesartan may protect kidneys by preventing lipid peroxidation.

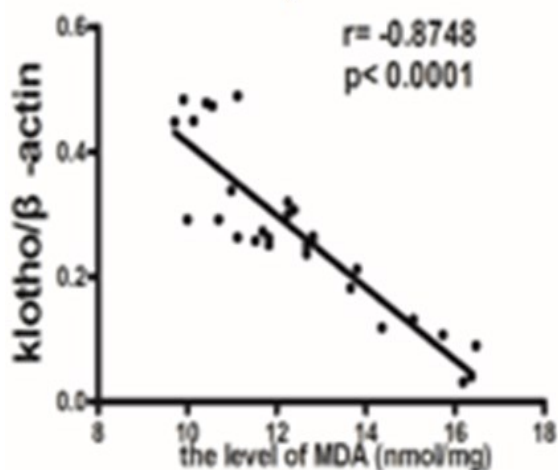


Figure 6. The correlation analysis between klotho/ $\beta$ -actin and MDA levels.

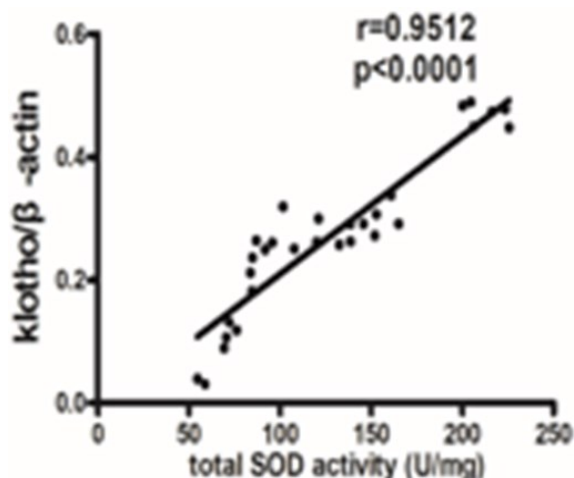


Figure 7. The correlation analysis between klotho/ $\beta$ -actin and SOD activity.

Oxidative stress is a process of damage caused by the imbalance between the production and elimination of oxygen free radicals, which leads to the accumulation of reactive oxygen species (ROS) and reactive nitrogen in physician. Studies have shown that ROS is an important factor causing podocyte injury[14]. Previous study found that the occurrence and development of chronic kidney disease are closely related to the increase of oxides and the decrease of antioxidants in vivo[15]. Klotho is an aging-related gene[16], and its protective effect on the body has been widely reported. Studies have shown that overexpression of Klotho protein in glomerulonephritis model mice can make glomerulonephritis model mice have the function of resisting oxidative stress and reducing kidney damage[17]. In this study, proteinuria levels were found to be higher in ADR group consistent with previous studies[18]. The levels of 24-h urinary protein excretion were inhibited by olmesartan treatment. The mechanism by which olmesartan reduces 24-h urine protein is mainly to block the

renin-angiotensin-aldosterone system. The study revealed that klotho decrease in rats with adriamycin nephropathy after the 8 weeks olmesartan treatment, the expression of klotho protein was significantly improved. previous studies have demonstrated that olmesartan protected the kidney by inhibiting oxidative stress induced by angiotensin II is consistent with our research[10]. Lim studies show that klotho protects the kidney by inhibiting oxidative stress, activating RAS system and improving mitochondrial function[19]. Our studies indicated that the levels of klotho protein were negatively correlated with the level of MDA ( $r = -0.8748$ ,  $P < 0.0001$ ) and positively correlated with SOD activity ( $r = 0.9512$ ,  $P = 0.0001$ ). Olmesartan play an important role in inhibiting oxidative stress in rats with adriamycin nephropathy, depending at least partly on promoting the level of klotho protein. Further investigations are needed to explore the possible mechanism of the protective actions of olmesartan.

## 5. Conclusion

In summary, the decrease of klotho expression in kidney of rats with adriamycin-induced nephropathy is closely related to oxidative stress. Olmesartan inhibits oxidative stress by increasing the expression of klotho in the kidney of rats with adriamycin-induced nephropathy. However, we did not investigate the exact mechanism of olmesartan in protecting kidneys of rats with adriamycin-induced nephropathy.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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