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The Effect of Lidan Huashi Pills on the Glutathione Peroxidase Activity and Growth of Calcium Oxalate Crystals in the Kidneys of Rats

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Abstract: *Objective:* To explore the effects of Lidan Huashi Pills on the glutathione peroxidase (GSH-Px) activity and growth of calcium oxalate crystals in the kidneys of rats. *Methods:* 30 rats were randomly divided into a blank group, a model group, and an experimental group, with 10 rats in each group. The blank group was free to eat and drink water for 8 weeks; the ethylene glycol method was used for the standard calcium oxalate crystal modeling in the control group and experimental group for 4 weeks. The model group was given free feeding and physiological saline (2 ml/d) by continuous gavage for 4 weeks; the experimental group was given free feeding and Lidan Huashi Pills (450 mg/kg, 2 ml/d) by continuous gavage for 4 weeks. After 4 weeks, all rats were euthanized, and the left kidney was taken for GSH-Px level detection. The right kidney was stained with hematoxylin and eosin (H&E) to observe the formation of calcium oxalate crystals. *Results:* After 4 weeks of modeling, the urinary calcium levels in the model group and experimental group significantly increased compared to the blank group (868.00 ± 39.29 vs 929.40 ± 33.61, $P < 0.05$), indicating successful modeling. The urine calcium ion concentration in the experimental group after modeling was significantly lower than that in the model group (929.40 ± 33.61 vs 888.60 ± 25.92, $P < 0.05$). The grading score of calcium oxalate crystals in the model group was significantly higher than that in the blank group ($P < 0.05$); the grading score of calcium oxalate crystals in the experimental group was lower than that in the model group, and the difference was not statistically significant ($P > 0.05$). The GSH-Px activity in the left kidney of the model group was significantly lower than that of the blank group [203.49 (208.21, 144.22) vs 494.91 (431.32, 538.18), $P < 0.05$]; the GSH-Px activity in the left kidney of the experimental group was significantly higher than that of the model group [433.60 (383.86, 504.49) vs 203.49 (208.21, 144.22), $P < 0.05$]. Morphological observation and H&E staining suggest that the formation of right kidney crystals and inflammation in the experimental group are between the blank group and the model group. *Conclusion:* Lidan Huashi Pills can enhance the serum GSH-Px activity in rats and inhibit the growth of calcium oxalate crystals and inflammatory response in the kidneys, thus playing a role in preventing and treating urinary tract stones.

Keywords: Calcium oxalate; Kidney stones; Oxidative stress; Lidan Huashi Pills; Rats

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1. Introduction

Urinary tract stones are a high-risk disease of the urinary system. However, the mechanism of stone formation is not yet clear [1]. Calcium oxalate stones are the most common component in urinary tract stones. Currently, there are numerous theories about explaining the formation of calcium oxalate stones, among which the oxidative stress theory is a research hotspot in this field [2]. Based on the theory of "pathogen and heat invading the kidneys" [3], traditional medicine has achieved ideal therapeutic effects in treating urinary tract stones using Lidan Huashi Pills [4]. Therefore, this study will explore the effects of Lidan Huashi Pills on calcium oxalate crystals and oxidative stress indicators in rat kidneys, elucidate their potential mechanisms of action, and provide a new theoretical basis for the prevention and treatment of urinary tract stones with Lidan Huashi Pills.

2. Materials and methods

2.1. Animal information

30 1-month-old specific-pathogen free (SPF) Sprague Dawley male rats with a body weight of 200 ± 20 g, sourced from Nanjing Kales Biotechnology Co., Ltd. [Production license number: SCXK (Zhejiang) 2019-0001]. The feeding environment provided was 12 hours of alternating light and dark, temperature maintained at 22 ± 2℃, humidity maintained at 45% to 55%, free diet, and adaptive feeding for one week.

2.2. Reagents and instruments

The materials used were 1% ethylene glycol and 2% ammonium chloride (analytical purity, provided by the low-value consumables procurement platform of Nanjing University of Traditional Chinese Medicine), Lidan Huashi Pills (LDHSP, internal preparation of Jiangsu Provincial Hospital of Traditional Chinese Medicine, provided by the pharmacy of Jiangsu Provincial Hospital of Traditional Chinese Medicine).

The instruments used included fully automated blood analyzer (SYSMEX pocH-100i, Nanjing Huaren Biotechnology Co., Ltd., Jiangsu Provincial Hospital of Traditional Chinese Medicine Laboratory), fully automated urine analyzer (iChemVelocity, Shanghai Haier Shi Medical Equipment Co., Ltd., Jiangsu Provincial Hospital of Traditional Chinese Medicine Laboratory).

Other reagents and instruments were glutathione peroxidase (GSH-Px) test kits, micropipette, vortex mixer, centrifuge, 37℃ constant temperature water bath, and visible spectrophotometer (wavelength 412 nm) (provided by Nanjing Jiancheng Biotechnology Research Institute).

2.3. Modeling methods

The ethylene glycol method was used for the standard calcium oxalate crystal modeling: free feeding, 1% ethylene glycol free drinking water, 2% ammonium chloride gavage every other day (2 ml/animal), and the modeling time was 4 weeks [5]. In the fourth week of modeling, the urine calcium ion levels of rats were detected and compared. The urine calcium ion levels of the model group were significantly higher than those of the blank group. In addition, the pathological examination of the kidney tissue in the model group showed crystalline substances in the renal tubules, indicating successful modeling [6].

2.4. Experimental plan and observation indicators

Experimental plan: All rats were adaptively raised for one week and randomly divided into groups based on body weight, with 10 rats in each of the blank group, control group, and experimental group. In the second week, the blank group had a free diet, while the control group and experimental group were modeled using the ethylene glycol method for a period of 4 weeks. In the 6th week, the blank group had a free diet, while the

control group had free feeding and was given physiological saline by gavage (2 ml/d); the experimental group was free to feed and administered Lidan Huashi Pills by gavage (450 mg/kg, 2 ml/d) for 4 weeks. In the 10th week, all rats were euthanized by excessive inhalation of isoflurane (8 ml/min, 25–30 min). Blood samples were taken from the neck to detect serum GSH-Px levels, and bilateral kidneys were dissected for hematoxylin and eosin (H&E) staining to observe the formation of calcium oxalate crystals.

Observation indicators: (1) Urine calcium ion level: 24-hour urine was collected from rats using the "metabolic cage method" and the urine calcium ion level was measured to evaluate the modeling effect; (2) Morphological observation of the right kidney; (3) Observation of pathological changes under the microscope after H&E staining of the right kidney; (4) Right kidney calcium oxalate crystal grading score: The calcium oxalate crystal in renal tubules was observed under light microscopy and scored [7]; 0 level: No calcium oxalate crystal; Grade I: There are small crystals but not piles; Grade II: Calcium oxalate crystals grow larger and pile up, but are not connected to each other; Grade III: Interconnection between calcium oxalate crystals; Grade IV: Calcium oxalate crystals are widely stacked and connected in sheets. (5) Left kidney GSH-Px activity: Blood collection was after anesthesia. After centrifugation, the supernatant was taken and stored in a -20°C freezer. The specimen was sent to the laboratory of Jiangsu Provincial Hospital of Traditional Chinese Medicine for testing the activity of GSH-Px in the left kidney (**Figure 1**).

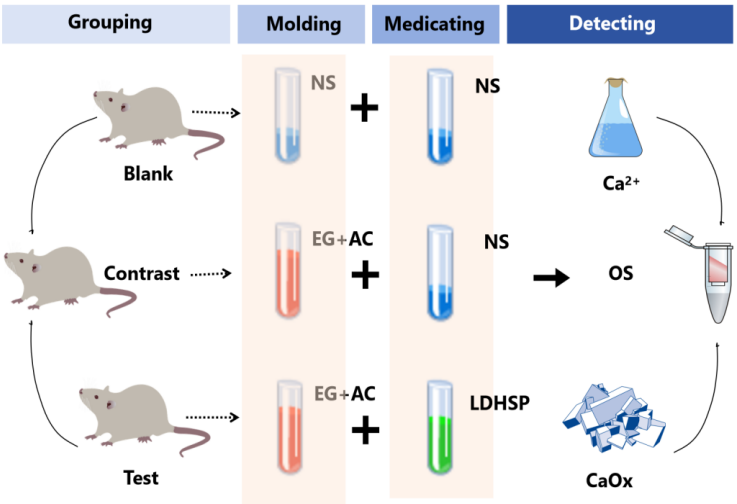

Figure 1. Experimental plan

2.5. Statistical processing

SPSS22.0 software was used to analyze the data. If the measurement data conformed to normality, they were represented by mean ± standard deviation (SD), non-normality was represented by M (P25~P75); count data were represented by [n (%)], when the distribution conforms to normality, independent sample *t*-test was used, and when deviating from normality, non-rank sum test was used. $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Comparison of urinary calcium ion levels among three groups of rats

After treatment, there was a statistically significant difference in urinary calcium ion concentration among the three groups of rats ($t = 3.476$, $P = 0.003 < 0.05$). Further pairwise comparison showed that the urine

calcium ion concentration in the model group was higher than that in the blank group after modeling, and the difference was statistically significant (t_a = 2.655, P_a = 0.019 < 0.05). The urine calcium ion concentration in the experimental group after modeling was higher than that in the blank group, but the difference was not statistically significant (t_b = 1.177, P_b = 0.260 > 0.05). The urine calcium ion concentration in the experimental group after modeling was lower than that in the model group, and the difference was statistically significant (t_c = 3.476, P_c = 0.003 < 0.05) (**Table 1**).

Table 1. Comparison of urinary calcium ion concentrations among three groups

Groups	Calcium concentration (Mmol/L)	χ^2	*P*
Blank group[ab]	868.00 ± 39.29[ab]		
Model group[ac]	929.40 ± 33.61[ac]	3.476	0.003
Experimental group[bc]	888.60 ± 25.92[bc]		

Note: Compared with the blank group, [a]$P < 0.05$, [b]$P > 0.05$; Compared with the model group, [c]$P < 0.05$

3.2. Comparison of right kidney calcium oxalate crystal grading scores among three groups of rats

After treatment, there was a statistically significant difference in the grading scores of calcium oxalate crystals among the three groups of rats (χ^2 = 17.843, P = 0.001 < 0.05). Further pairwise comparison showed that the grading scores of calcium oxalate crystals in the model group of rats were higher than those in the blank group, and the difference was statistically significant (χ^2_a = -3.773, P_a = 0.000 < 0.05), the grading score of calcium oxalate crystals in the experimental group rats was higher than that in the blank group, but the difference was not statistically significant（χ^2_b = -1.985, P_b = 0.141 > 0.05), the grading score of calcium oxalate crystals in the experimental group of rats was lower than that in the model group, but the difference was not statistically significant (χ^2_c = -1.609, P_c = 0.323 > 0.05) (**Table 2**).

Table 2. Comparison of grading scores for three groups of calcium oxalate crystals (n = 30)

Groups	0	I	II	III	IV	χ^2	P
Blank group[ab]	7	0	1	1	0		
Model group[ac]	0	0	0	6	2	17.843	0.001
Experimental group[bc]	1	1	1	4	0		

Note: Compared with the blank group, [a]$P < 0.05$, [b]$P > 0.05$; Compared with the model group, [c]$P > 0.05$

3.3. Comparison of GSH-Px activity in the left kidney of three groups of rats

After treatment, the activity of GSH-Px in the left kidney of the three groups of rats is detailed in **Figure 2**. The inter-group comparison showed a statistically significant difference in GSH-Px activity in the left kidney of the three groups of rats (U = 82.739, $P < 0.001$). Further pairwise comparison showed that the GSH-Px activity in the left kidney of the model group rats was lower than that of the blank group, and the difference was statistically significant (Z_a = 6.933, $P_a < 0.001$). The GSH-Px activity in the left kidney of the experimental group rats was lower than that of the blank group, but the difference was not statistically significant (Z_b = -1.282, P_b = 0.20 > 0.05). The GSH-Px activity in the left kidney of the experimental group rats was higher than that of the model group, and the difference was statistically significant (Z_c = 8.056, $P_c < 0.001$) (**Figure 2**).

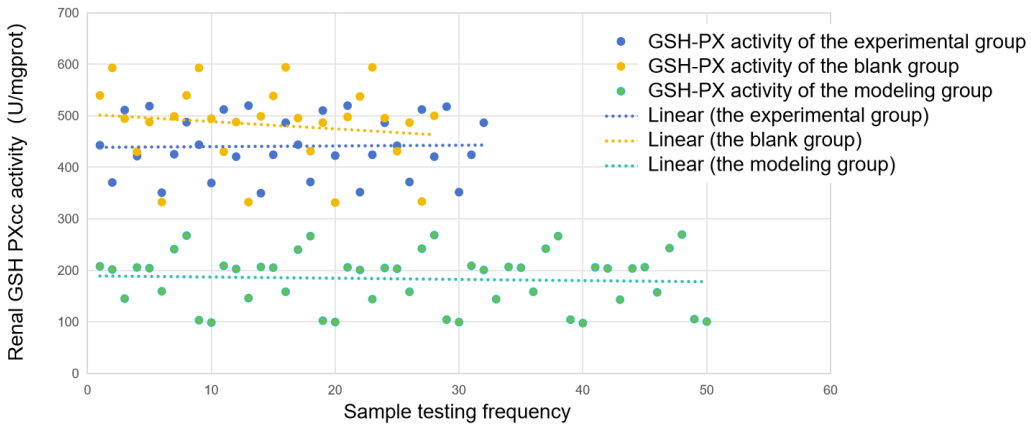

Figure 2. Comparison of GSH-Px activity in the left kidney of three groups of rats

3.4. Morphological observation and H&E staining comparison of the right kidney in three groups of rats

Morphological observation of the right kidney in the blank group of rats: The average weight of the right kidney was about 1.84 g, with a fava bean shape and a coordinated ratio of skin and marrow thickness. No stone and no friction sensation were found during the renal cortex and medulla cutting process (**Figure 3a**).

Pathological changes of H&E staining in the right kidney of blank group rats: The glomerulus volume was moderate, the renal tubules volume was moderate, the staining was light red, the epithelial cells of the proximal convoluted tubules were uniform and not protruding into the tubules, the lumen size was regular, and no crystals were seen. There were a small number of evenly distributed same-sized pink small particles in the cytoplasm, with clear nuclear structure (**Figure 3b**, **c**, **d**).

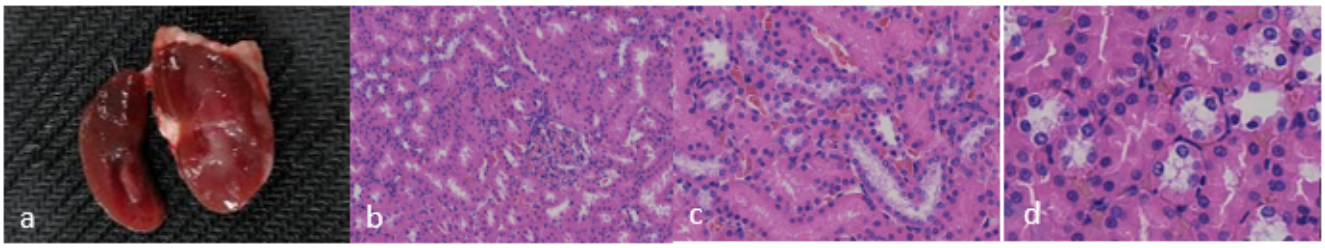

Figure 3. (a) Morphological observation of the blank group; (b) Blank group x20; (c) Blank group x40; (d) Blank group x80

Morphological observation of the right kidney in the model group of rats: The average weight of the right kidney was about 3.08 g, with a fava bean shape and granular protrusions on the surface. The thickness ratio of the cortex and medulla was not coordinated, and there was atrophy in the cortex. There was friction during the process of renal cortex and medulla cutting, and a large number of stones can be seen. Some large stones can be seen in the renal pelvis (**Figure 4a**).

Pathological changes of H&E staining in the right kidney of the model group rats: Renal tubules were large in volume, with light red staining, obvious edema of epithelial cells in the proximal tubules, protruding into the tubules, enlarged lumens, and a large number of crystals visible (5 or more/40x field of view). Inflammatory cells infiltrated the distal tubules, crystals were found in the papillary ducts, and a large number of detached cells were found in the renal calyces (**Figure 4b**, **c**, **d**).

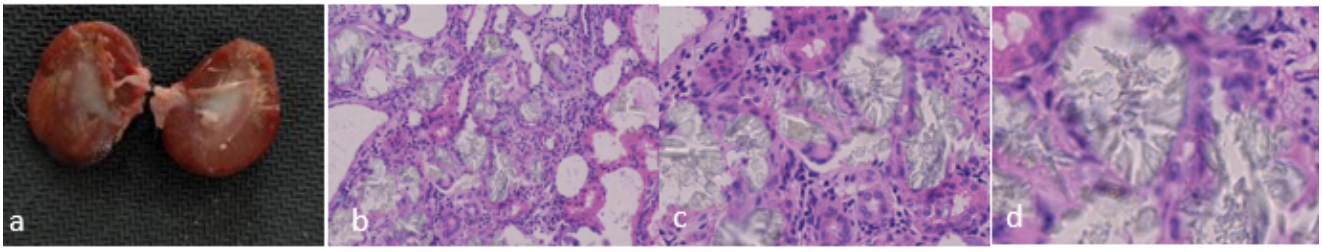

Figure 4. (a) Morphological observation of the model group; (b) Model group x20; (c) Model group x40; (d) Model group x80

Morphological observation of the right kidney of experimental group rats: The average weight of the right kidney was about 3.43 g, with a fava bean shape and granular protrusions on the surface. The thickness ratio of the cortex and medulla was not coordinated, and the cortex had atrophic changes. There was a mild friction sensation during the process of renal cortex and medulla cutting, and occasional stones were seen. Small stones are occasionally seen in the renal pelvis (**Figure 5a**).

Pathological changes of H&E staining in the right kidney of experimental group rats: Renal tubules were slightly larger in volume, with light red staining, mild edema of epithelial cells in the proximal tubules, not obvious protrusion into the tubules, enlargement of the lumen, and a small number of crystals visible (about 1/40x field of view). The amount of crystals was small, and there were more inflammatory cells infiltrating the distal tubules. A small number of crystals were found in the papillary ducts, and a small number of detached cells were found in the renal calyces (**Figure 5b**, **c**, **d**).

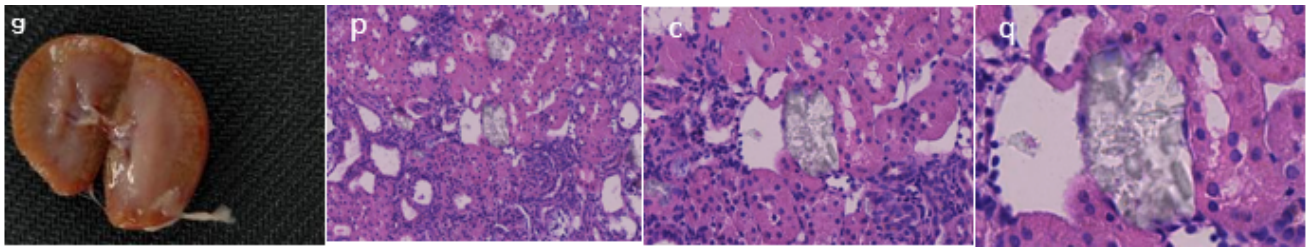

Figure 5. (a) Morphological observation of the experimental group; (b) Experimental group x20; (c) Experimental group x40; (d) Experimental group x80

4. Discussion

For thousands of years, traditional Chinese medicine has developed a unique understanding of the formation of stones through continuous clinical practice and exploration. According to *A Treatise on the Origins and Stages of Various Diseases*, "Pathogen and heat invading the kidneys and leading to gonorrhea syndrome as well as weak kidney cannot restrain the stones, so urinate profusely and drain the stones" [3]. It is pointed out that one of the mechanisms for the formation of kidney stones is "the pathogen and heat invading the kidneys." Based on this theory, this study verifies the anti-inflammatory and inhibitory effects of Lidan Huashi Pills on calcium oxalate kidney stones through animal experiments. It provides a new theoretical basis for the prevention and treatment of urinary tract stones with Lidan Huashi Pills.

Lidan Huashi Pills are mainly used to treat kidney stones and gallstones, and has achieved satisfactory therapeutic effects in clinical practice for many years. Lidan Huashi Pills are composed of *Lysimachia christinae*, *Plantago asiatica*, *Artemisia scoparia*, dandelion, *Verbena*, rhubarb, *Polygonum cuspidatum*, *Scutellaria baic*alensis, *Paeonia lactiflora*, *Corydalis yanhusuo*, elecampane, citron, *Rhizoma sparganii*, and

Curcuma zedoaria. The whole prescription has a cold and cool nature, fully reflecting the treatment method of "clearing heat." Among them, Lysimachia christinae and Plantago asiatica have a clear heat and diuretic effect; Dandelion, *Verbena*, rhubarb, *Artemisia scoparia*, *Scutellaria baicalensis* clear heat and dampness; *Paeonia lactiflora* softens liver and relieves pain; *Corydalis yanhusuo*, *Rhizoma sparganii*, citron, and *Curcuma zedoaria* all have the effects of relieving stones, promoting blood circulation and removing stasis, and promoting qi and relieving pain. In this study, compared with the model group, the H&E pathological staining of the kidneys in the experimental group showed a reduction in the degree of edema in the epithelial cells of the proximal tubules, and a decrease in the number of inflammatory cells and detached cells in the distal tubules, indicating that Lidan Huashi Pills have a good anti-inflammatory effect. The study by Zhang *et al.* shows that Lidan Huashi Pills can significantly inhibit egg white-induced plantar swelling and acute inflammatory response in the ear caused by xylene, and it has significant sustained analgesic and antibacterial activity [8]. In this article, the urine calcium ion concentration and renal calcium oxalate crystal grading score of the model group were higher than those of the blank group after modeling, indicating the successful modeling of calcium oxalate kidney stones in rats. After treatment, the urine calcium ion concentration in the experimental group was lower than that in the model group, indicating that Lidan Huashi Pills can inhibit the formation of calcium oxalate crystals in the urinary system by reducing the urine calcium ion concentration in rats with calcium oxalate kidney stones. Compared with the model group, the H&E pathological staining of the kidneys in the experimental group showed a decrease in crystal distribution density and crystal quantity, which was consistent with the results of urine metabolomics. The grading score of calcium oxalate crystals in the experimental group was lower than that in the model group, and the difference was not statistically significant. However, the experimental group did not show any advantages in improving the grading score of calcium oxalate crystals in rats, this is likely due to the short study time not reflecting the expected positive results and the lack of quantitative analysis of crystal fluorescence staining.

When the body's oxidative and antioxidant systems are imbalanced, it can lead to oxidative stress (OS), and the theory of oxidative stress is currently a hot topic in the study of the causes of urinary tract stones. This theory suggests that calcium oxalate crystals (CaOx) can induce the release of reactive oxygen species (ROS) in HK-2 cells, and the generated ROS can mediate related signaling pathways to induce the production of inflammatory factors in the kidneys again, and induce chemotactic inflammatory cells to enter the inflammatory tissue, thereby damaging HK-2 cells, promoting CaOx crystal aggregation, and ultimately leading to stone formation [9]. The endogenous or exogenous production of ROS in cells destroys HK-2 cells by attacking lipids, proteins, nucleic acids, or mitochondria. OS and inflammation are important factors in the formation of CaOx stones. The study by Cakir *et al.* [10] provided evidence that OS is related to CaOx formation, that is, the serum antioxidant enzyme levels in CaOx stone patients decrease. Oxalate-mediated red blood cell OS can activate free radical oxidation, which may lead to the interaction between CaOx crystals and HK-2 cells in patients with hyperoxaluria, ultimately leading to the accumulation of stones. Khan's research [11] found that renal tissue deposited with CaOx crystals has accumulated ROS. CaOx crystals stimulate HK-2 cells to produce ROS-mediated inflammatory responses, which release a large amount of inflammatory factors and interact with OS, triggering an inflammatory cascade reaction that promotes the aggregation, nucleation, and growth process of calcium salt crystals, ultimately leading to crystal aggregation and even the formation of stones [12].

In this article, the GSH-Px activity in the kidneys of rats in the model group was lower than that in the blank group after treatment, while the GSH-Px activity in the kidneys of rats in the experimental group was higher than that in the model group. This indicates that the antioxidant enzyme system of kidney stone model rats is inhibited and enters the OS state under the stimulation of CaOx, and Lidan Huashi Pills can effectively

counteract ROS production and oxidative stress response by upregulating GSH-Px activity. Glutathione peroxidase is a selenium-containing antioxidant enzyme that clears ROS during oxidative stress in mammalian cells. It has antioxidant effects and can eliminate oxygen free radicals in the body, reducing lipid oxidation [13]. Among them, glutathione peroxidase 1 (GPx1) can use glutathione (GSH) as a reducing substrate, not only catalyzing toxic H_2O_2 to non-toxic water, but also catalyzing other water-soluble hydroperoxides. Glutathione peroxidase 4 (GPx4) can not only catalyze H_2O_2, but also directly reduce phospholipid hydroperoxides on biofilms, thereby inhibiting cellular lipid peroxidation [14]. However. the sample size of this study is relatively small, and there is a lack of detection of changes in the activity of other urinary metabolomics and oxidative stress-related indicators for various reasons. Therefore, future prospective large-scale studies with more detailed data on Lidan Huashi Pills are necessary.

5. Conclusion

In summary, Lidan Huashi Pills can inhibit the growth of calcium oxalate crystals and inflammatory response in rat kidneys, thereby playing a role in preventing and treating urinary tract stones, and is worthy of clinical promotion and application.

Disclosure statement

The authors declare no conflict of interest.

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