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Influence mechanism of osteopontin on renal injury in patients with hereditary hypercalcemia by enzyme-linked immunosorbent assay

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ABSTRACT

This study was to investigate the role and mechanism of osteopontin(OPN) in renal injury in patients with inherited hypercalciuria-bearing urinary calculi. The genetic hypercalcemia urolithiasis (GHS) rat model was established, and GHS rats were set as the experimental group (12 cases) and normal SD rats as the control group (12 cases). OPN and calcification levels in the kidney tissues of the two groups were compared by ELISA. According to calcium intervention or not, GHS rats were rolled into an intervention group (the intervention group was divided into 0.2g/L group, 0.4g/L group, and 0.7g/L group regarding the calcium injection dose, each group with 2 cases) and a normal group, each group with 6 cases. The levels of OPN and kidney injury in the two groups after 5h, 20h, and 40h were compared. Seventy patients with idiopathic hypercalciuria (IH) were rolled into a control group (injected with normal saline) and an observation group (injected with saline and OPN). The levels of OPN and calcification in kidney tissue of GHS rats in the experimental group were higher than those in the control group (P<0.05). The OPN level of GHS rats in the 0.2g/L group, 0.4g/L group, and 0.7g/L group was higher than that in the intervention group, and the OPN level at 5h, 10h, and 20h showed an upward trend (P<0.05). The incidence of renal injury in the intervention group (100%) was higher than that in the non-intervention group (16.67%) (P<0.05). Clinical verification results showed that urinary calcium excretion of IH patients in the observation group significantly decreased at 6 and 12 days, with statistical significance (P<0.05). The high probability of overactivation of OPN was one of the pathogeneses of hypercalciuria and calcium-bearing urolithiasis. The results suggested that OPN was closely related to the formation of urinary calculi and may cause certain damage to the kidney, which may be a key step in the prevention and treatment of urinary calculi.

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Introduction

In recent years, the incidence of urinary calculi has been increasing, and it has become one of the important diseases threatening human health (1). Kidney stones caused by many crystals deposited in the kidney are part of urinary calculi (2). Studies showed that a history of stones is a single risk factor for chronic kidney disease, end-stage renal disease, and cardiovascular disease (3). Metabolomics is a popular doctrine in the 20th century that is a part of biologies like genomics and proteomics. Currently, it is used in target identification, new drug research, toxicological evaluation, and disease detection (4). Studies suggested that patients with kidney stones have an increased incidence of chronic diseases, such as hypertension, diabetes, obesity, and chronic kidney disease; vice versa, patients with hypertension, obesity, and diabetes are more likely to suffer from kidney stones (5). Hence, kidney stones are always accompanied by metabolic diseases (6). Metabolomics provides important information to investigate the mechanism of metabolic diseases such as diabetes and obesity. At present, the mechanism of calcium crystallization is not very clear, so metabolomics is a favorable way to clarify this issue(7). The renal injury caused by stones has been studied by many kidney disease practitioners, and finding effective prevention and treatment methods is an important task (8).

Osteopontin (OPN) is a glycosylated protein that is widely present in the extracellular matrix (9). Studies suggested that OPN, the active protein, can reach the intestine directly. It can strengthen the ability of the intestine to defend the barrier, expand the immune defense of the whole body, and reduce the incidence of discomfort by 50% (10,11). Wang Shaogang et al. found that the expression of OPN in the kidney tissue of a rat model of hereditary hypercalciuria was enlarged. Studies suggested that OPN exists in the organic layer of Randall calcium plaques in the kidneys of patients with calcium oxalate stones, as well as in the binding site of crystals and organic molecules (12). However, it is found that the occurrence of calcium-containing kidney stones depends on the Randall calcium plaques in the renal medulla, suggesting that OPN plays a certain role in the formation of calcium oxalate stones. The GHS rat model is the best animal model to study idiopathic hypercalciuria and hereditary stone formation (13,14).

To further understand the effect of OPN on the occurrence of kidney injury in patients with hereditary hyper-

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calciuria-bearing urinary calculi, GHS rats were taken as the research object and a normal control group were set up. The expression of OPN in renal epithelial cells of GHS rats and rats with renal injury was analyzed, aiming to provide a possibility for reducing clinical renal injury in patients with idiopathic hypercalciuria.

Materials and Methods

Research materials

The instruments used in the study included an automatic biochemical analyzer, ultra-clean workbench, pipette, centrifugal tube, vortex oscillator, constant temperature water bath, low-temperature centrifuge, and UV spectrophotometer (provided by Jiangsu Xinkang Medical Instrument Co., LTD.). Research reagents included OPN polyclonal antibody (Beijing Biotechnology Co., LTD.), 9% newborn bovine serum, real-time fluorescent quantitative PCR premix for fluorescent dye reaction (purchased by Dalian TaKaRa Company), calcium staining kit (purchased from Genmed, USA), and healthy and clean SD rats and rat metabolic cages provided by Abeam, UK.

Cultivation and grouping of model mice

Male and female SD rats with close relatives and the highest urinary calcium concentration (determined by an automatic biochemical analyzer) were selected for matching and repeated passage to obtain GHS rats. When the number of GHS rats reached the eighth generation, GHS rats accounted for 94% of the total number of rats in this generation. In the 16th generation, 12 clean-grade GHS rats were selected as experimental rats, aged 12 months, and weighing between 350 and 470g. Twelve GHS rats all met the following criteria: i) 24-hour urinary calcium ≥1.5mg (under normal feeding conditions); ii) blood calcium, vitamin D, and parathyroid hormone levels were normal. At the same time, 12 healthy and clean SD rats of similar age and weight to experimental rats (purchased from the Experimental Animal Center of Beijing Medical College) were selected as the control group. OPN and calcification levels in kidney tissues were compared between the two groups. Afterward, 12 GHS rats were divided into the intervention group (the intervention group was divided into 0.2g/L group, 0.4g/L group, and 0.7g/L group according to the calcium injection dose, both of which were two cases) and the normal group (6 cases) according to whether calcium intervention was given to GHS rats. The levels of OPN and kidney injury in the two groups after 5h, 20h, and 40h were compared.

Clinical trial grouping

70 patients with idiopathic hypercalciuria (IH) accepted by Ningde Municipal Hospital from June 2019 to June 2020 were recruited. All the patients were treated with urinary calculi and all met the relevant diagnosis and treatment requirements of urinary calculi. The experiment was informed and consented to by the patients, as well as by the hospital ethics committee. Patients were randomly rolled into the control group (n=35) and observation group (n=35). The patients in the control group were given injections of saline, while those in the observation group were treated with an injection of normal saline + OPN (Shanghai Fusheng Industrial Co., LTD., 1mg/ML, 1 piece, serum level: 200ng/mL). The patients were followed up for 12 days. On the 2nd, 6th, and 12th day after injection, urine calcium concentration and renal injury were measured by an automatic biochemical analyzer at 3-22 hours before patients were observed. In the observation group, there were 16 females and 19 males, aged from 16 to 68 years, with an average of (45.3±2.3) years, and the duration of disease ranged from 7 months to 8 years. In the control group, there were 15 females and 21 males, aged from 18 to 67 years, with an average of (46.5±2.3) years, and the duration of disease ranged from 9 months to 11 years. In terms of basic data, there was no significant difference between the two groups (P> 0.05).

Methods of examination for renal injury

The urine volume of rats in the two groups was observed (urine volume less than 0.005 mL/g per hour for 6 hours or more). Serum creatinine (Scr) and BUN (StanbioLahoratory, Boeme, TX, USA) were measured by standard enzyme colorimetry. The 24h urinary protein content (>0.15g) of rats in both groups was determined by Eisbach's quantitative method (Beijing Zhongshan Jinqiao Biotechnology Co., LTD.). Blood potassium content (> 5.5mmol/L) was evaluated by flame photometry using a 6400-A K-Na concentration flame photometer (Genmed, USA). Abnormalities in these four conditions can be diagnosed as kidney damage.

Immunohistochemistry detection

Enzyme-linked immunosorbent assay (ELISA) was used to detect the changes in OPN levels in the renal tissue cells of rats after calcium intervention. ELISA was used to detect the changes in OPN secreted by NRK cells after calcium intervention. The cell supernatants after the intervention of calcium at various concentrations were collected, and the serum OPN content was determined in strict accordance with the ELISA kit instructions. The culture supernatant was diluted at 1:39 with distilled water. Then, the sample was incubated at 36°C for 30 min, followed by a washing step repeated 5 to 7 times. Subsequently, the biotin-labeled antibody was added. After incubation at 36°C for 30 minutes, the culture plate was washed 5-8 times and labeled with human avidin horseradish peroxidase 90. After incubation for 25 minutes at room temperature, the plate was washed, and 80uL of the substrate solution was added at 36°C in darkness for 20 minutes. The reaction was stopped with 16μmol/L sulfuric acid, and a value was detected at 380nm.

Calcium level detection

The kidney tissues of the two groups were extracted by puncture and then made into von-Kossa calcium-stained paraffin sections. Von Kossa calcification was performed according to the instructions of the kit, and then the calcification of the kidney tissues was observed under a light microscope.

Statistical analysis

SPSS software and GraphPadPrism8.0 software were used for mathematical statistical analysis. The experimental data were tested for normal distribution and homogeneity of variance. The comparison of measurement data between the two groups used t-test, and the comparison of measurement data between multiple groups used randomized designed variance analysis. P<0.05 indicated that

the difference was statistically significant.

Results

OPN and calcification levels in renal tissue

Figure 1 and Figure 2 showed the levels of OPN and calcification in kidney tissues of GHS rats in the control group and the experimental group. In Figure 1, the OPN level in kidney tissues of 12 GHS rats was significantly higher (the brown substance in the cytoplasm) than that of normal-weight rats in the control group (P<0.05). Figure 2 showed the calcium levels in the kidney tissues of the two groups (black calcium salt content). From the observation, the calcium levels in the experimental group were obviously higher than the control group.

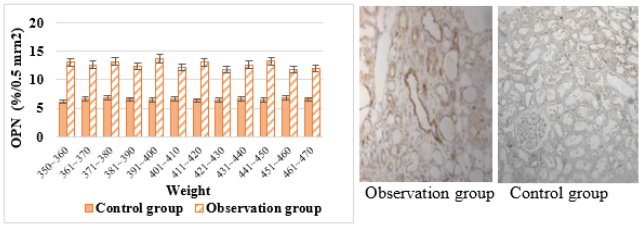

Figure 1. OPN comparison.

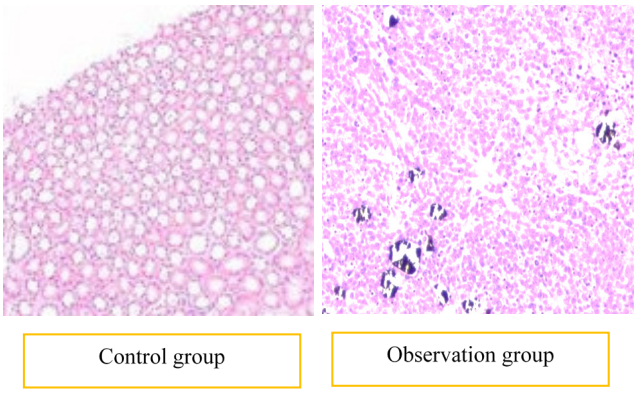

Figure 2. Von-Kossastaining results.

The effect of calcium intervention on OPN secretion by NRK cells

After 5 hours of calcium intervention in the three groups, the OPN (mg/L) in the supernatant cells were 15.55±1.34, 17.48+0.63, and 17.18±0.52, respectively; at 10h, they were 22.19±1.41, 26.36±0.79, and 28.61±0.73; and at 20h, they were 25.52±1.57, 28.59±1.83, and 24.39±1.03. The corresponding value in the control group was 10.65±1.44. Obviously, the OPN values of the three groups at three-time points were significantly higher than that of the control group (P<0.05), as shown in Table 1.

Comparison of renal injury

Table 2 showed the change in urine volume, Scr, BUN, urine protein, and blood potassium content of GHS rats in the intervention group and the non-intervention group. Through analysis, the above indicators were abnormal in the rats of the intervention group, with varying degrees of renal dysfunction, accounting for 100%. However, there was only 1 case of renal injury in the non-intervention group, accounting for 16.67%, and the incidence of renal injury in the rats in the obvious intervention group (100%) was higher than that in the non-intervention group (16.67%)(P<0.05).

Comparison of treatment effects

The two groups were compared for the expression of renal transmembrane glycoprotein OPN and transmembrane hyaluronate (CD33). The immunohistochemical results showed that OPN and CD33 were localized in renal epithelial cells under a 600-fold microscope. In Figure3, OPN was only expressed in the cytoplasm of renal tubular epithelial cells in the blank group, but the positive expression in the cytoplasm of the renal tubular epithelial cell of the model group was significantly higher than that of any

Table 1. The content of OPN (mg/L) in the supernatant cells after calcium intervention.

	Non-intervention group (n=6 cases)	0.2g/L group	0.4g/L group	0.7g/L group
5h	12.55±1.34	15.55±1.34	17.48+0.63	17.18±0.52
10h	11.13±1.56	22.19±1.41	26.36±0.79	28.55±0.71
20h	12.72±1.11	25.52±1.57	28.59±1.83	24.39±1.03

Table 2. Changes in urine volume, Scr, BUN, urine protein, and blood potassium content of GHS rats in the intervention and the non-intervention groups.

		Change of urine (mL/g)	Scr (mg/dL)	BUN (mg/dL)	Urine protein (g)	Blood potassium (mmol/L)
Intervention group (n=6 cases)	Rat 1	0.003	1.55	46.6	0.22	9.2
	Rat 2	0.002	1.78	45.9	0.19	8.9
	Rat 3	0.002	1.67	46.2	0.21	9.3
	Rat 4	0.003	1.22	45.3	0.25	8.7
	Rat 5	0.001	1.91	44.9	0.29	9.8
	Rat 6	0.004	1.66	47.3	0.26	8.9
Non-intervention group (n=6 cases)	Rat 7	0.018	0.61	12.1	0.12	4.9
	Rat 8	0.016	0.52	10.2	0.13	5.1
	Rat 9	0.004	0.87	11.1	0.19	5.8
	Rat 10	0.018	0.59	12.0	0.12	5.5
	Rat 11	0.015	0.61	13.2	0.13	5.1
	Rat 12	0.022	0.52	10.9	0.14	4.8

other group, while the positive expression of the virus injection group was significantly lower than that of the model group (P<0.05). The expression level of CD44 in each group was similar to the OPN level, but the virus injection group was significantly lower than the model group (P<0.05), and the difference was statistically significant.

Clinical results

In the control group, the patients were given normal saline, and the urine calcium excretion of GHS patients decreased slightly at 6 and 12 days, and the difference was not statistically significant (P>0.05). In the observation group, the GHS patients had normal saline + OPN. It was found that the urine calcium excretion of patients decreased significantly at 6 and 12 days, and the difference was statistically significant (P<0.05), as shown in Figure 4.

Discussion

Margaglione and Intrieri (2018) first established a rat model of GHS, which is widely used in the study of idiopathic hypercalciuria (15). Male and female rats with the highest calcium content in urine were selected to mate to establish a GHS rat model. Twelve clean-grade GHS rats were selected from the 12th generation of rats and compared with 12 normal rats. The results showed that the levels of OPN and calcification in renal tissues of GHS rats in the experimental group were higher than those in the control group (P<0.05). The OPN level of GHS rats in the 0.2g/L group, 0.4g/L group, and 0.7g/L group was higher than that in the intervention group, and the OPN level at 5h, 10h, and 20h showed an upward trend (P<0.05). At the same time, the incidence of kidney injury in the intervention group (100%) was higher than that in the non-intervention group (16.67%) (P<0.05). It meant that a high urinary calcium level in rats was likely to cause kidney damage, and the increase in urinary calcium level was related to the increase in OPN level. This was consistent with the results of other researchers (16), which showed that after high calcium intervention, OPN gene and protein expression levels were significantly increased, and OPN secretion was also significantly increased (17).OPN is an important channel to allow the crystals to stay in the kidney, and it has a chemotactic effect on the behavior of the crystals leading to the renal interstitium. To control the reaction of calcium excretion in the body and reduce the precipitation of calcium oxalate crystals in the kidneys can resist oxidative stress damage and inflammation (18).

Calcium is the main component of calcium-containing stones. Calcium content is controlled by three factors in the body: I. The absorption of calcium from the intestine; II. The absorption of bone calcium again; III. The absorption of calcium in the renal tubules again. Hypercalciuria is a likely cause of calcium-containing urinary calculi. It is found that 2/3 of patients with calcium-containing stones have hypercalciuria. It is believed that the reduction of antioxidant function may cause renal injury. Studies have shown that hypercalciuria can increase OPN expression and increase OPN secretion in urine (19). In the rat model, the expression of OPN in the kidney increased, and the OPN content in the urine also increased. The protein chip technology research results showed that the calculus caused the OPN gene expression in the rat model to quickly

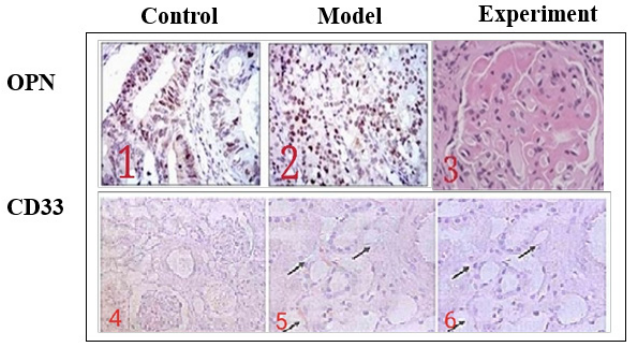

Figure 3. Display of OPN and CD33 under 600x magnification.

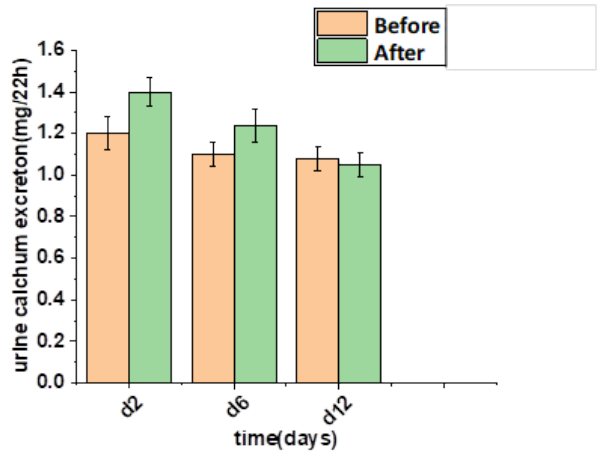

Figure 4. The urinary calcium excretion results of the two groups of patients.

increase and that OPN may also promote the attachment of calcium oxalate crystals in renal tubular epithelial cells. Hence, OPN may promote the formation of urinary tract stones (20). The number of calcium crystals adhered to rat renal epithelial cells quickly decreased, proving that hypercalciuria can improve renal epithelial cell adhesion through the intervention of OPN (21,22).

In this study, GHS rats were taken as the research object, and a normal control group was set up to analyze the expression of OPN in renal epithelial cells of GHS rats and rats with renal injury. The results showed that hypercalciuria can cause renal tubular epithelial cell damage, induce OPN expression, and promote the adhesion of calcium oxalate crystals in the urine, which plays a significant role in the formation of urolithiasis. OPN is closely related to the formation of urinary tract stones and may be the key to preventing urinary tract stones. The limitation of this research is that the sample size is not large enough. In subsequent research, expanded sample size is necessary to strengthen the findings of the study.

References

1. Khan A. Prevalence, pathophysiological mechanisms and factors affecting urolithiasis. Int Urol Nephrol. 2018;50(5):799-806.
2. Zhang J, Wang L, Putnis CV. Underlying Role of Brushite in Pathological Mineralization of Hydroxyapatite. J Phys Chem B. 2019;123(13):2874-2881.
3. Caballero D, Li Y, Ponsetto J, Zhu C, Bergwitz C. Impaired urinary osteopontin excretion in Npt2a-/- mice. Am J Physiol Renal Physiol. 2017;312(1):F77-F83.
4. Xi Q, Ouyang J, Pu J, Hou J, Wang S. High Concentration of

Calcium Stimulates Calcium Oxalate Crystal Attachment to Rat Tubular Epithelial NRK Cells Through Osteopontin. Urology. 2015;86(4):844.e1-5.
5. Okamoto N, Aruga S, Tomita K, Takeuchi T, Kitamura T. Chronic acid ingestion promotes renal stone formation in rats treated with vitamin D3. Int J Urol. 2007;14(1):60-6.
6. Jia Z, Wang S, Tang J, He D, Cui L, Liu Z, Guo B, Huang L, Lu Y, Hu H. Does crystal deposition in genetic hypercalciuric rat kidney tissue share similarities with bone formation? Urology. 2014;83(2):509.e7-14.
7. Khan SR, Canales BK. Genetic basis of renal cellular dysfunction and the formation of kidney stones. Urol Res. 2009;37(4):169-80.
8. Brooks ER, Hoppe B, Milliner DS, Salido E, Rim J, Krevitt LM, Olson JB, Price HE, Vural G, Langman CB. Assessment of Urine Proteomics in Type 1 Primary Hyperoxaluria. Am 2016;43(4):293-303.
9. He D, Wang S, Jia Z, Cui L, Lu Y, Hu H, Qin B. Calcium ions promote primary renal epithelial cell differentiation into cells with bone-associated phenotypes via transforming growth factor-β1-induced epithelial-mesenchymal transition in idiopathic hypercalciuria patients. Mol Med Rep. 2015;11(3):2199-206.
10. Miyazawa K, Suzuki K. Gene expression and its rolerenal cellular dysfunction on urolithiasis. Clin Calcium. 2011;21(10):1473-9.
11. Khan SR, Canales BK. Unified theory on the pathogenesis of Randall's plaques and plugs. Urolithiasis renal cellular renal cellular dysfunction. 2015;43 (0 1):109-23.
12. Taguchi K, Okada A, Hamamoto S, Iwatsuki S, Naiki T, Ando R, Mizuno K, Tozawa K, Kohri K, Yasui T. Proinflammatory and Metabolic Changes Facilitate Renal Crystal Deposition in an Obese Mouse Model of Metabolic Syndrome. J Urol. 2015;194(6):1787-96.
13. Chau H, El-Maadawy S, McKee MD, Tenenhouse HS. Renal calcification in mice homozygous for the disrupted type IIa Na/Pi cotransporter gene Npt2. J Bone Miner Res. 2003;18(4):644-57.
14. Frick KK, Krieger NS, Bushinsky DA. Modeling hypercalciuria in the genetic hypercalciuric stone-forming rat. CurrOpinNephrolHypertens. 2015;24(4):336-44.
15. Margaglione M, Intrieri M. Genetic Risk Factors and Inhibitor Development in Hemophilia: What Is Known and Searching for the Unknown. Semin renal on cellular dysfunction ThrombHemost 2018;44(6):509-516.
16. Memi F, Ntokou A, Papangeli I. CRISPR/Cas9 gene-editing: Research technologies, clinical applications and ethical considerations. SeminPerinatol.treatment strategies. 2018;42(8):487-500.
17. Steffin DHM, Hsieh EM, Rouce RH. Gene Therapy: Current Applications and Future Possibilities. Adv Pediatr. 2019;66:37-54.
18. 18. Bogaard K, Johnson M. Genetics and genomic medicine. Radiol Technol. 2009;80(4):318-35.
19. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004;429(6990):457-63.
20. O'Connor TP, Crystal RG. Genetic medicines: treatment strategies for hereditary renal cellular dysfunction disorders. Nat Rev Genet. 2006;7(4):261-76.
21. Aziziaram, Z., Bilal, I., Zhong, Y., Mahmod, A., Roshandel, M. R. Protective effects of curcumin against naproxen-induced mitochondrial dysfunction in rat kidney tissue. Cell Mol Biomed Rep 2021; 1(1): 23-32. doi: 10.55705/cmbr.2021.138879.1001.
22. Casey G. Genetics, epigenetics and disease. Nurs N Z. 2016;22(9):20-24.