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ORIGINAL PAPER

Effects of low-molecular-weight polyguluronate sulfate on experimental urolithiasis in rats

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Abstract Urinary macromolecules, especially glycosaminoglycans (GAGs), have attracted great interest as promising inhibitors of urinary stone formation. As an analogue of GAGs, low-molecular-weight polyguluronate sulfate (LPGS) with strong polyanionic nature was prepared by chemical modification of brown algae extract. The effects of LPGS both on ethylene glycol-induced nephrolithiasis and Zinc disc implant-induced urinary bladder stone formation in Wistar rats were evaluated, and its acute toxicity in Kunming mice and Wistar rats were also investigated. The contents of renal oxalate and calcium in ethylene glycol-induced nephrolithiasic rats were decreased significantly from 5.01 ± 0.96 to 3.26 ± 1.31 μmol/g kidney ($P < 0.01$) and 20.11 ± 4.60 to 11.83 ± 3.54 μmol/g kidney ($P < 0.01$), respectively, after oral administration of LPGS at dose-level of 100 mg/kg. The renal crystal depositions and histopathological changes were reduced also. The formation of zinc disc implant-induced urinary bladder stones in rats was inhibited considerably after oral administration of LPGS at dose-levels of 50 mg/kg ($P < 0.05$) and 100 mg/kg ($P < 0.01$). The intravenous LD_{50} and the oral maximum tolerance value of LPGS in mice are 6.29 and 25 g/kg, and in rats are 2.25 and 10 g/kg, respectively. These data show that LPGS has significant prevention effects both on nephrolithiasis and urinary bladder stone formation in rats, and negligible oral toxicity both in mice and rats. LPGS is a safe and promising drug candidate for the prevention of urolithiasis.

Keywords Polyguluronate sulfate · Urolithiasis · Nephrolithiasis · Urinary bladder stone · Acute toxicity

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Introduction

Urolithiasis is a common clinical disorder, with a reported incidence about 12% in the general population [1]. The formation of urinary stones is a multi-factorial and complicated process involving not only the supersaturation of urinary components and crystal retention in the urinary tract, but also reduction of urinary inhibitors [2]. Some urinary macromolecules with molecular weight greater than 10 kDa, such as Tamm-horsfall mucoprotein and glycosaminoglycans (GAGs), have attracted great interest as promising inhibitors of urinary stone formation [3]. It has been proved that GAGs and exogenous sulfated polysaccharides, at least in vitro, showed a beneficial effect on calcium oxalate (CaOx) crystallization and crystal-cell adhesion [4], due to their high-anionic nature. Previous studies also reported that sodium pentosan polysulfate (SPP) and dextran sulfate have potent inhibitory activities on CaOx stone formation in vivo [5, 6].

Alginate, which isolated from brown algae, is a linear polysaccharide containing β-D-mannuronic acid (M) and α- L-guluronic acid (G) residues, and it is the only polysaccharide that naturally contains carboxyl group in each residue [7]. Polyguluronate (PG), which separated from alginate, usually forms stiff twofold screw helical chains through intramolecular hydrogen bonding and has specific binding sites for calcium [8]. Epidemiologic data collected during several decades showed that the majority of stones are composed of

CaOx [9]. Therefore, low-molecular-weight polyguluronate sulfate (LPGS), which is an analogue of GAGs and has stronger anionic nature than SPP and dextran sulfate, was prepared by chemical sulfation of PG, and its potential effects on urolithiasis have attracted our attention. In the present work, the effects of LPGS on experimental urolithiasis in rats were evaluated, and its acute toxicity was also investigated. LPGS exhibits significant prevention effects both on ethylene glycol-induced nephrolithiasis and Zinc disc implant-induced urinary bladder stone formation in rats, and negligible oral toxicity in mice and rats.

Materials and methods

Animals

Male Wistar rats weighing 250–290 g were purchased from the Center for Laboratory Animal, Academy of Military Medical Science (Beijing, China). Kunming mice of either sex weighing 19–22 g, Wistar rats of either sex weighing 120–140 g, and standard diet (composed of corn, barley and rice containing 22% protein, 5% fat, 50% carbohydrate, 6% dietary fiber, 0.5% Ca, 0.4% P, 0.04% Mg, and 0.003% Zn) were provided by the Animal Center, Tianjin Institute of Pharmaceutical Research (Tianjin, China). All animals were housed in metabolic cages and had free access to pellet diet and tap water during the study. All experimental procedures were carried out in accordance with standard guidelines for the care of animals and were approved by the Ethics Committee for Animal Experiments of Tianjin Institute of Pharmaceutical Research.

Drugs and reagents

Low-molecular-weight polyguluronate sulfate was prepared as described previously [10]. Briefly, alginate was hydrolyzed in hydrochloric acid (HCl) and PG was isolated from the hydrolysate by pH fractionation. The sulfation of PG was carried out with formamide and chlorosulfonic acid. The degree of sulfate substitution of LPGS is 1.55 per monosaccharide residue, and the weight average molecular weight is 12.1 kDa (namely, the chain length of LPGS is around 34 monosaccharides). Uralyt-U granules were manufactured by Madaus AG (Koeln, Germany), and its active ingredient is potassium sodium hydrogen citrate, which is effective in preventing CaOx stone formation [11, 12]. All other reagents are of analytical reagent grade.

Prevention effects on nephrolithiasic rats

After acclimatization in metabolic cages for 1 week, male Wistar rats were divided randomly into six groups of 12 animals each as follows: Group 1—normal group rats fed with standard diet and saline; Group 2—control group rats fed with diet containing 1% ethylene glycol and 1% ammonium chloride [13] and distilled water; Group 3, 4, 5—three LPGS group rats fed with same diet as group 2 but LPGS at dose-levels of 25, 50, and 100 mg/kg, respectively; Group 6—Uralyt-U group rats fed with same diet as group 2 but Uralyt-U at the dose of 50 mg/kg. Distilled water, LPGS and Uralyt-U were administered by gastric gavage to rats at 0.5 ml per 100 g body weight once a day. After 6 weeks, all animals were killed. The kidneys on the right side were harvested and their weights were recorded following cleaning and drying. The renal oxalate and calcium contents of the right side kidneys were assayed by colorimetry [14] and atomic absorption spectrophotometry, respectively. The left side kidneys were analyzed histologically after they were fixed in formalin and embedded in paraffin.

Treatment effects on nephrolithiasic rats

Male Wistar rats were divided randomly into six groups of 12 animals each as described above. Group 1 rats were fed with standard diet and saline, and other group rats were fed with diet containing 1% ethylene glycol and 1% ammonium chloride and distilled water for 1 month. Then all groups of rats were fed with standard diet, and at the same time, Group 2 rats continued to receive distilled water, Group 3, 4, 5 rats were given LPGS at dose-levels of 25, 50, and 100 mg/kg, respectively, and Group 6 rats were given Uralyt-U at the dose of 50 mg/kg. Distilled water, LPGS and Uralyt-U were administered by gastric gavage to rats at 0.5 ml per 100 g body weight once a day. After 1 month, all animals were killed. The kidneys on both sides were treated in a similar way as in the prevention experiment described above.

Prevention effects on urinary bladder stone in rats

Male Wistar rats were anesthetized with sodium pentobarbitone (40 mg/kg, i.p.), and each urinary bladder of rats was incised and a zinc disc (38.1–43.4 mg) was inserted into the lumen under sterilization [15]. Then rats were divided randomly into five groups of 12 animals each as follows: Group 1—control group rats treated with distilled water; Group 2, 3, 4—three LPGS group rats treated with LPGS at dose-levels of 25, 50, and 100 mg/kg, respectively; Group 5—Uralyt-U group rats treated with Uralyt-U at the dose of 50 mg/kg. Distilled water, LPGS and Uralyt-U were administered by gastric gavage to rats at 0.5 ml per 100 g body weight once a day. After 6 weeks, all animals were killed and the stones were harvested and weighed.

Intravenous acute toxicity in mice and rats

Kunming mice of either sex were divided randomly into seven groups of ten animals each according to the dose ratio of 1:0.9 between non-lethal dose and 100% lethal dose, namely, Groups 1–7 are at dose-levels of 4,784, 5,315, 5,905, 6,561, 7,290, 8,100, and 9,000 mg/kg, respectively. About 0.25 ml of LPGS were injected intravenously into mice per 10 g body weight. Wistar rats of either sex were divided randomly into six groups of ten animals each according to the dose ratio of 1:0.85 between non-lethal dose and 100% lethal dose, namely, Groups 1–6 are at dose-levels of 1,331, 1,566, 1,842, 2,167, 2,550, and 3,000 mg/kg, respectively. About 0.75 ml of LPGS were injected intravenously into rats per 10 g body weight. After injection of LPGS, mice and rats were observed continuously for 14 days, and signs of toxicity and mortality were recorded. The intravenous 50% of the lethal dose (LD_{50})-values were calculated by Bliss method [16].

Oral acute toxicity in mice and rats

Twenty Kunming mice or Wistar rats of either sex were starved by withdrawing the diet for 6 h. LPGS solution (500 mg/ml) were orally administered by gastric gavage to mice 0.5 ml per 10 g body weight, and to rats 2 ml per 100 g body weight, respectively. Mice or rats were observed continuously for 14 days, and signs of toxicity and mortality were recorded. All the animals were weighed before drug administration and immediately after death, respectively. The surviving animals were weighed and killed at the end of the experimental period to inspect any changes in organs.

Statistical analysis

Student's *t*-test was used for statistical comparison of data between groups based on normality test. Results are expressed as mean ± standard deviation (SD). Differences between the data were considered significant at $P < 0.05$.

Results

Prevention and treatment effects on nephrolithiasic rats

The prevention and treatment effects of LPGS on ethylene glycol-induced nephrolithiasis in rats are showed in Tables 1 and 2, respectively. The contents of renal oxalate and calcium in LPGS groups of 50, 100 mg/kg and in Uralyt-U group were decreased significantly, compared with control groups. The effects of LPGS were dose-dependent. The renal pathological changes of LPGS on nephrolithiasic rats both in the prevention and treatment experiments are showed in Figs. 1 and 2, respectively. No crystal depositions were found in normal group animals, while remarkable and extensive crystal depositions were found in control group animals. The renal pathological changes of control group were obvious, and renal tubular atrophy and renal swelling were also observed in most of animals. The renal crystal deposition and pathological changes in all LPGS groups (25, 50, and 100 mg/kg) and in Uralyt-U group (50 mg/kg) rats were improved significantly in the prevention experiment. However, only in LPGS group of 100 mg/kg and Uralyt-U group of 50 mg/kg were seen considerable pathological improvements in the treatment experiment, compared with control group.

Table 1 Prevention effect of LPGS on nephrolithiasic rats

Groups	Dose (mg/kg)	Animals (number)	Weight of wet kidney (g)	Oxalate (μmol/g kidney)	Calcium (μmol/g kidney)
Normal		10	1.10 ± 0.19	1.28 ± 0.36	4.81 ± 1.47
Control		12	1.44 ± 0.25*	5.01 ± 0.96*	20.11 ± 4.60*
LPGS	25	11	1.34 ± 0.19	4.61 ± 0.90	16.40 ± 4.02
LPGS	50	11	1.28 ± 0.21	3.94 ± 0.85***	15.05 ± 4.56**
LPGS	100	12	1.21 ± 0.22**	3.26 ± 1.31***	11.83 ± 3.54***
Uralyt-U	50	11	1.22 ± 0.24**	3.93 ± 0.81***	13.85 ± 3.61***

* $P < 0.01$ (vs. normal)
** $P < 0.05$ (vs. control)
*** $P < 0.01$ (vs. control)

Table 2 Treatment effect of LPGS on nephrolithiasic rats

Groups	Dose (mg/kg)	Animals (number)	Weight of wet kidney (g)	Oxalate (μmol/g kidney)	Calcium (μmol/g kidney)
Normal		12	1.20 ± 0.24	1.24 ± 0.29	4.27 ± 2.51
Control		11	1.33 ± 0.13	4.37 ± 0.83*	17.48 ± 4.19*
LPGS	25	10	1.31 ± 0.15	4.25 ± 0.94	16.69 ± 5.26
LPGS	50	12	1.44 ± 0.15	3.49 ± 0.85**	10.62 ± 6.94**
LPGS	100	12	1.19 ± 0.12	3.01 ± 0.47***	9.75 ± 4.49***
Uralyt-U	50	11	1.29 ± 0.25	2.67 ± 0.86***	8.52 ± 4.14***

* $P < 0.01$ (vs. normal)
** $P < 0.05$ (vs. control)
*** $P < 0.01$ (vs. control)

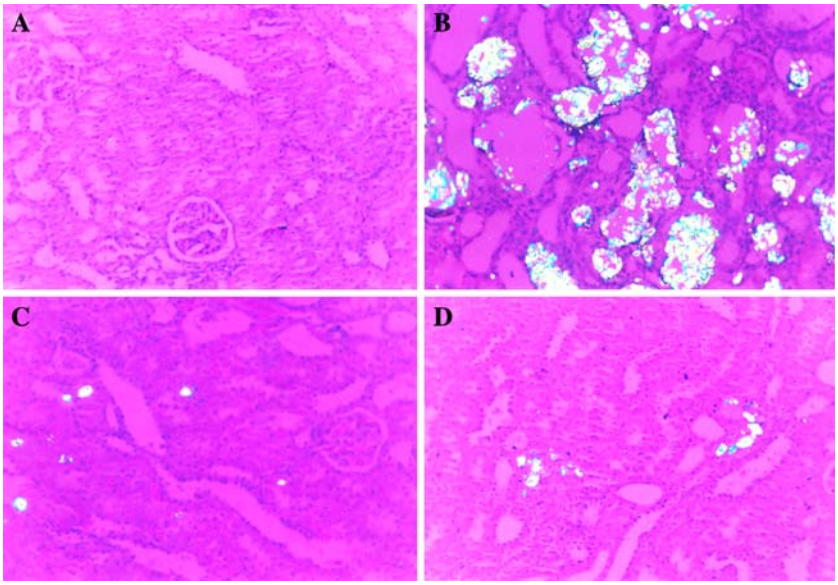

Fig. 1 Renal pathology (×100) of LPGS prevention effect on ethylene glycol-induced nephrolithiasis in rats for 6 weeks. **a** Normal group. **b** Control group. **c** LPGS group at the dose of 100 mg/kg. **d** Uralyt-U group at the dose of 50 mg/kg

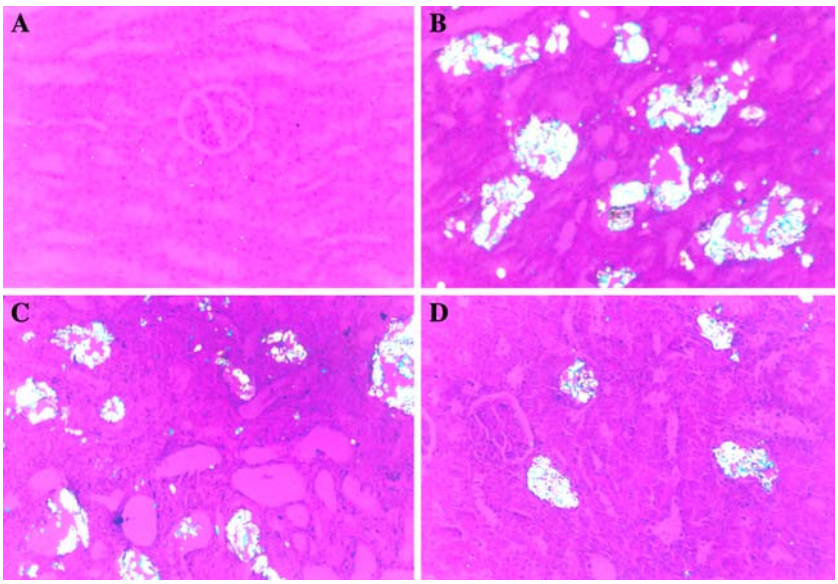

Fig. 2 Renal pathology (×100) of LPGS treatment effect on ethylene glycol-induced nephrolithiasis in rats for 1 month. **a** Normal group; **b** Control group; **c** LPGS group at the dose of 50 mg/kg. **d** Uralyt-U group at the dose of 50 mg/kg

Prevention effects on urinary bladder stone in rats

The prevention effects of LPGS on Zinc disc implant-induced urinary bladder stone in rats are showed in Fig. 3. After treatment for 6 weeks, the formation of urinary bladder stone was inhibited significantly in LPGS groups of 50, 100 mg/kg, and in Uralyt-U group of 50 mg/kg, compared with control groups. The effects of LPGS were dose-dependent.

Acute toxicity of LPGS

The intravenous LD_{50} of LPGS calculated by Bliss method was 6,293 mg/kg with 95% confidence limits between 4,144 and 9,554 mg/kg in mice, and 2,249 mg/kg with 95% confidence limits between 1,521 and 3,325 mg/kg in rats. Prostration, writhing, and clonic convulsions in mice and rats were noticed after injection, and these symptoms were aggravated with increasing doses of LPGS. Death occurred within 24 h in most injected animals, dyspnoea and jumping were observed before death but no macroscopic abnormalities were recorded at necropsy. All the surviving animals recovered within 2 days to an apparently normal state. The maximum tolerance value for oral LPGS was 25 g/kg in mice, and 10 g/kg in rats. Most mice and rats were prostrate and motionless after oral administration of LPGS, but no abnormal behaviors were noticed. All animals recovered within 24 h to an apparently normal state and no adverse effects were observed during the subsequent observation period. The water and dietary consumption of the animals were normal, and respiration had no significant changes. There was no mortality at the end of 14 days

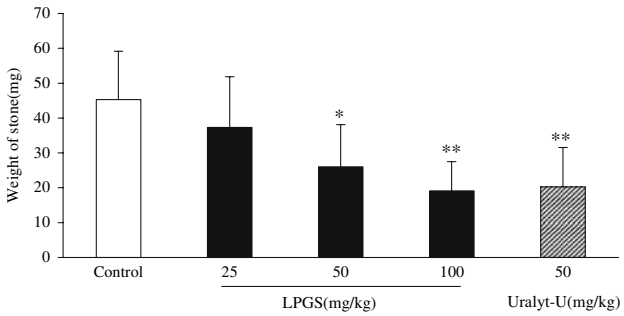

Fig. 3 Prevention effect of LPGS on urinary bladder stone in rats for 6 weeks (*$P < 0.05$, **$P < 0.01$, vs. control)

observation. When animals were killed at the end of the experimental period, no macroscopic abnormalities were recorded at necropsy.

Discussion

Urinary stone formation is based on supersaturation of urinary salts and crystal retention in the urinary tract [2]. Hyperoxaluria and hypercalciuria are important risk factors in the pathogenesis of urinary stone formation [17]. Several compounds, such as ethylene glycol, glycine, and glycolic acid, can form oxalic acid by metabolism in vivo [17]. CaOx crystalluria could be induced by ethylene glycol and ammonium chloride in rats without severe renal damage [13], and this animal model is always used to mimic the etiology of urinary stone formation in humans. Therefore, we evaluated both the prevention and treatment effects of LPGS on nephrolithiasis in rats using this model. The contents of renal oxalate and calcium in control groups (Tables 1, 2) were significantly higher than those in normal groups ($P < 0.01$), and remarkable pathological change and CaOx deposition in control group rats were observed, indicating that the experimental nephrolithiasis models were established successfully. The contents of oxalate and calcium were decreased significantly, after oral administration of LPGS and Uralyt-U (act as a positive control drug). We speculate that the decrease of renal oxalate and calcium might be relevant to the excretion reduction of oxalate and calcium, and then the supersaturation of urine may be decreased. Hyperoxaluria induced by ethylene glycol and ammonium chloride was accompanied by severe renal tubular injury [18]. CaOx crystals were found mainly in proximal and distal tubular cells in rats, and some crystals are also found in the interstitium. The renal crystal deposition and histopathological changes (Figs. 1, 2) were reduced after administration of LPGS, indicating that LPGS can prevent crystal adherence and renal tubular cell damage.

A dose-dependent inhibitory effect of LPGS on Zinc disc implant-induced urinary bladder stone formation (Fig. 3) was observed in this study. The implantation of Zn discs in urinary bladder induced a mild urinary tract infection and caused reliable stone formation as described by Satoh et al. [19]. The main composition of the stones was confirmed to be magnesium ammonium phosphate [20]. Previous studies have demonstrated that LPGS (previously known as G872) had a strong inhibitory effect on crystal growth and agglomeration of CaOx and calcium phosphate crystals in vitro for its high-negative charge [21, 22], and our recent investigation demonstrated that LPGS exhibits a considerable anti-inflammatory activity in cotton pellet-induced granuloma in rats [10]. Therefore, the significant inhibitory effect of LPGS on bladder stone formation may be relevant to its anti-inflammatory activity and strong polyanionic nature. In addition, in nephrolithiasic rats treated with ethylene glycol, crystal retention is probably preceded by crystal-induced tissue damage [23, 24]. Hence, LPGS could also exert its effect by inflammation inhibition.

It has been generally accepted that GAGs play important roles as inhibitors of urinary stone formation [4]. LPGS, with high-charge density and similar molecular weight as urinary macromolecules, was prepared from easily accessible marine brown algae. The significant inhibitory effects of LPGS on experimental urolithiasis may be ascribed to its structural similarity (Fig. 4) to GAGs. LPGS contains both sulfate and carboxyl groups, and has stronger anionic nature than SPP and dextran sulfate [5, 6]. The high contents of sulfate and carboxyl groups in LPGS, just like most GAGs, give it high affinity for calcium-containing crystal surfaces [4]. LPGS shares many characteristics with GAGs, it can reach the urine in an almost non-degraded form after oral administration. More importantly, LPGS can increase the excretion of GAGs in urine [25]. Clinical evidence has shown that a reduction of stone disease may also be associated with higher urinary GAG levels [26]. GAGs are also found as part of the layer covering the bladder urothelium and act as a permeability barrier, protecting against bacterial and crystal adherence [27]. Damaging this layer will result in a remarkable increase in crystal adhesion, and the application of heparan sulfate or SPP can restore the damaged GAGs layer and reduce crystal adhesion [28, 29].

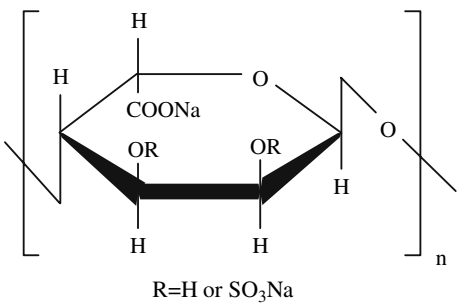

Fig. 4 The structure of LPGS

Histopathological examination indicated that the crystal deposition and histopathological changes were reduced after administration of LPGS. As these effects are similar to those obtained from heparan sulfate and SPP, we speculate that LPGS may work in a similar way as them, namely by protecting the GAG layer to reduce crystal adhesion.

As a low-molecular-weight sulfated polysaccharide, LPGS is devoid of several undesirable effects of highly sulfated polysaccharides, such as the potent anti-coagulation and bleeding caused by heparin [9]. The anti-coagulant potency of LPGS is about 25 IU/mg, which is only one-ninth potency of heparin [10]. The intravenous LD_{50} and the oral maximum tolerance values of LPGS in mice are 6.29 and 25 g/kg, and in rats are 2.25 and 10 g/kg, respectively. These data showed that LPGS has negligible oral toxicity, and has a higher safety than heparin.

To sum up, the present data indicate that LPGS has significant prevention effects both on ethylene glycol-induced nephrolithiasis and Zinc disc implant-induced urinary bladder stone in rats, and has a low toxicity both in mice and rats. We speculate that these effects are related to the structure and properties of LPGS closely resembling GAGs. Our results show that LPGS is a safe and promising drug candidate for the prevention of urolithiasis.

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