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Nanoparticle-mediated delivery system alleviates the formation of infection stones by activating TRPV5

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Abstract. Infection stones constitute a small but intractable group of diseases of urinary system. In this study, we explored the potential therapeutic effect of a small activation RNA, ds-320, encapsulated in chitosan (320-chitosan). Western blot analysis verified the downregulation of TRPV5 in patients and rat model of infection stones, as well as the stimulation of ds-320 on TRPV5 expression. MTT assay showed that chitosan-mediated delivery was less cytotoxic to ds-320 compared with liposome delivery. Further a modified invasion assay revealed an inhibitory effect of 320-chitosan on bacterial invasion into normal rat kidney epithelial NRK-52E cells. The establishment of infection stone model was performed by intravesical injection of 1×10^8 CFU of *Proteus mirabilis*. In animal experiments, no visible stones were obtained. The number of live bacteria and white blood cells in urine showed no difference among all infected rats at the time of sacrifice. However, we observed a decline in urine calcium and pH, suggesting the effect of acidification. Overall, our study provides evidence for the protective effect of 320-chitosan, for its ability to down-regulate urinary calcium, acidify urine, and inhibit bacteria from invading renal epithelial cells. Thus, it can be served as an important complementary therapy for infection stones.

Key words: Infection stones — TRPV5 — saRNA — Chitosan nanoparticle

Introduction

Urolithiasis is clinically characterized by the formation of stones in the urethra, kidney or bladder, and the incidence in the human population is as high as 10% (Torzewska et al. 2014). Urolithiasis can be caused by various factors, such as metabolic disorders, bacterial infection, neurogenic diseases, congenital diseases, unhealthy life style and dietary structure (Dawson and Tomson 2012; Veser et al. 2018). Struvite stones, which account for 10–15% of all urinary stones, are mostly induced as the result of urinary tract infection (UTI) in the presence of urease-producing bacteria, such as *Proteus* species (Fowler 1984). Thusly, struvite stones are often referred to as infection stones. Moreover, recent studies shed light on the shifted paradigm in the bacteriology of infection stones, indicating that non-urease-producing organisms, such as *Escherichia coli*, are gradually taking a greater share to the development of struvite stones (Parkhomenko et al. 2017).

Struvite stones usually develop rapidly and can form staghorn calculi within 4 to 6 weeks. The composition of struvite stones is mostly heterogeneous, yet typically the stones contain magnesium ammonium phosphate, in the presence or absence of monoammonium urate or carbonate apatite. Struvite stones can be life-threatening without effective clinical intervention management. The optimal treatment regimen for infection stones includes the complete removal of stone fragments by using surgical techniques, followed by the acidification of the urine, inhibition of urease with acetohydroxamic acid (AHA), and the use of antibiotics (prior to and after stone removal) to combat infection (Flannigan et al. 2014; Marien and Miller 2015).

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In view of the high recurrence rate after stone removal, and the increasing proportion of aseptic infection stones in stone culture, traditional therapeutic approaches have become somewhat ineffective. Thus, it is urgent for the development of novel therapies to deal with struvite stones in the clinical practice (Hugosson et al. 1990; Parkhomenko et al. 2017).

The pathogenesis of infection stones requires an alkaline environment created by urease, urease-producing bacteria, water, ions (such as calcium, magnesium and phosphate), which together facilitate the formation of crystals in the vicinity of bacteria (Flannigan et al. 2014). The crystals then, in turn, provide a surface for the planktonic bacteria to adhere and to proliferate. This is part attributable to the intractability of infection stones. Crystals, or inorganic ions in the urine, play a critical role in the formation of all kinds of urinary stones. More than 75% of the stones are calcium-based, generally either calcium oxalate (70%) or calcium phosphate (10%) (Dawson and Tomson 2012; Xu et al. 2017). Infection stones are characterized by a mixture of struvite ($MgNH_4PO_4 \times 6\ H_2O$) and carbonate-apatite ($Ca_{10}(PO_4)_6 \times CO_3$) (Torzewska et al. 2014). Presumably, the declined calcium concentration in the urine might inhibit the formation of infection stone to a certain extent.

TRPV5 (transient receptor potential vanilloid 5) is a unique calcium-selective epithelial channel and the gate way to Ca^{2+} homeostasis. In the kidney, TRPV5 is mainly expressed in the distal convoluted tubule (DCT) and connecting tubule (CNT). It is the major regulating pathway for the reabsorption of Ca^{2+} in the placenta and the duodenum by regulating Ca^{2+} influx into the cells (Na and Peng 2014). Previous study found that some gene variants of TRPV5 were associated with the incidence of urolithiasis (Oddsson et al. 2015; van der Wijst et al. 2019). And, the knockout of TRPV5 gene in mice could cause severe hypercalciuria (Hoenderop et al. 2003).

Small activating RNAs (saRNAs), which belong to the family of non-coding RNA (ncRNA), were discovered in 2006 (Li et al. 2006). Unlike the extensively studied microRNAs, saRNAs were double-stranded and were found to stimulate the expression of target genes by targeting the specific promoters (Li et al. 2006). Nowadays, saRNAs have rapidly been widely explored and then applied in the intervention of various diseases, especially cancers (Zheng et al. 2014; Yoon and Rossi 2018; Zhou et al. 2018). Recently, nanoparticle-mediated delivery system was used to improve therapeutic effects. In this study, we intend to investigate the probable therapeutic value of TRPV5-targeted saRNA coated with chitosan for infection stones, and to clarity the underlying mechanism. When it comes to the selection of a suitable carrier, chitosan is a priority by virtue of its biodegradability and non-toxicity.

Table 1. The basic clinical information of 10 participants (PT)

Participant	Age/Gender	Diagnosis
PT-1	58/F	Infection stone
PT-2	57/M	Infection stone
PT-3	63/F	Infection stone
PT-4	65/F	Infection stone
PT-5	60/M	Infection stone
PT-6	62/F	RCC
PT-7	55/M	RCC
PT-8	68/F	RCC
PT-9	49/F	RPC
PT-10	57/M	RPC

RCC, renal cell carcinoma; RPC, renal pelvis carcinoma.

Materials and Methods

Patients

Five patients with infection stones and five controls with renal tumors that underwent nephrectomy were recruited in the study. The basic demographic and clinical information of the ten participants is listed in Table 1. Written informed consent was obtained from all the subjects. The renal tissues of the ten subjects were collected. The study was approved by the ethics committee of Zhongjiang People's Hospital.

Cell culture

Normal rat kidney epithelial cell line (NRK-52E cells) was cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies), and 1% penicillin/streptomycin (Life Technologies) in a humidified incubator with 5% CO_2 at the temperature of 37°C. Liposome-meditated transfection was performed by using Lipofectamine™ 2000 (Life Technologies), according to the manufacturers' instructions.

Preparation of chitosan-saRNA nanoparticles

saRNA sequence (ds-320) targeting TRPV5 promoter and negative control (ds-NC) were synthesized by using the following primers: 5'-AAGGGTCTCATGATTTCTCTA-3'; and 5'-TAGAGAAATCATGAGACCCT-3', respectively. As the saRNAs carrier, a chitosan derivative was produced using the polymer-analogous redox reaction technique by modifying chitosan (Yuhuan Ocean Biomaterial Co. Ltd, Yuhuan, China) with N-(2-hydroxyl) propyl-3-trimethyl ammonium (Sigma), according to the protocol described in previous study (Hu et al. 2010). The degree of deacetylation was found to be 95% by analysis, and the viscosity-average molecular weight (M_V) was determined to be 2.1×10^4. The

saRNAs and chitosan were separately dissolved in ultrapure water to obtain the solutions at the concentration of 1 μg/μl, which was then mixed at differential mass ratios at 4:1, 2:1, 1:1, 1:2 and 1:4, respectively. Subsequently, these mixtures were centrifuged to collect the supernatants for the analysis of non-encapsulated saRNAs.

The rat model of infection stones

A total of 30 male Sprague-Dawley rats (3 months old; 282.6 ± 30.6 g) were routinely fed and housed throughout the study. Rats experienced 12 h of light and 12 h of dark every day with free access to water and standard ordinary feed. All programs in this study were achieved in accordance with the Institutional Animal Care and Use Committee (IACUC), Zhejiang University School of Medicine center for animal experiment, Zhejiang, China (2020-951). After 1 week of acclimation, animals were thrown into experiment. The establishment of infection stone model was performed by intravesical injection of 1×10^8 colony-forming units (CFU) of *Proteus mirabilis*. Briefly, thirty animals were randomly divided into five groups: (1) Control, no invention group; (2) Saline treatment group; (3) *Proteus mirabilis* and saline treatment group; (4) *Proteus mirabilis* and chitosan treatment group, and (5) *Proteus mirabilis* and 320-chitosan treatment group. All the rats in the treatment groups were anesthetized with isoflurane, and then the bladders of the rats were exposed by performing suprapubic incision. 0.2 ml of saline containing 1×10^8 CFU of *Proteus mirabilis* bacteria were injected into the bladder of the rat. The incisions were closed with a polyglactin suture and covered with a dressing. Then the animals were injected with saline, chitosan (1 nmol) or 320-chitosan (1 nmol) *via* the tail vein once every 3 days for 21 days. Then the animals were sacrificed. Renal tissue and urine from bladder were collected for further analysis.

Western blot

Total proteins from renal tissues of patients and rats, and from the cultured NRK-52E cells, were extracted by lysing in RIPA lysis buffer containing protease inhibitor (Applygen, Beijing, China). SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed to separate the extracted proteins, which were then transferred onto PVDF membranes (Millipore, USA). After the PVDF membrane was blocked by Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature, the membranes were then incubated with anti-TRPV5 (1:10000, Abcam) primary antibody at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 25°C. β-actin (1:1000, Santa Cruz, sc-47778) was used as the internal control. The signal of interest protein was detected by using ECL chemiluminescence and the intensity was analyzed by using ImageJ software.

Cell viability assay by MTT

NRK-52E cells were cultured in a 96-well plate at a concentration of 5×10^4/ml. After cell adhesion, the cells were transfected with ds-320 carried packaged in different vehicles (1 μg/μl liposome or chitosan), followed by 72 h of incubation, and each treatment was repeated in five wells. Afterwards, 20 μl of 3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml, Solarbio, China) was added into each well for 4 h of incubation. After the reagents were removed, 150 ul of DMSO was added into each well. The plate was vortexed gently for 10 min, and the absorbance value at 490 nm was measured by a microplate reader (BioTek Instruments, Winooski, VT, USA). The cell viability of the untreated NRK-52E cells was taken as control, and the relative cell viability (arbitrary unit) of each group at indicated time was calculated as $OD_{treatment}/OD_{control}$.

Invasion assay

The ability of bacteria to invade urothelial cells was considered a driving factor for the recurrence of infection stones. Thus, we examined how the ability of *Proteus mirabilis* to invade NRK-52E cells would be affected when the cells were pretreated with 320-chitosan to create an extracellular environment of low calcium. NRK-52E cells were seeded into 24-well plates at a concentration of 5×10^4 cells *per* well and were routinely cultured until cell adhesion. Before the infection, the cells were grouped into five groups: (1) Control group; (2) Chitosan group; (3) 320-chitosan-24h, (4) 320-chitosan-48h, and (5) 320-chitosan-72h. Afterwards, all groups of cells were infected with *Proteus mirabilis* bacteria for 3 h, and were suspended in a mixture of DMEM medium (supplemented with 10% FBS) and synthetic urine (5:1) in the ratio of 10 bacteria *per* host cell. Then the suspension was discarded, and the cells were washed with PBS three times to remove the free bacteria, followed by incubation with amikacin (400 mg/ml, Solarbio, China) for 60 min to eliminate the extracellular bacteria. The numbers of intracellular bacteria in each group were determined after 48 h. A total of 10^4 cells were collected, and were lysed in 1% Triton X-100 to obtain the suspension. Then the suspension was seeded on LB agar plate containing 0.1% boric acid and incubated for 24 h at 37°C. The ability of *Proteus mirabilis* to invade NRK-52E cells was represented by bacterial CFU recovered *per* ml.

Statistical analysis

All experimental data were processed and analyzed by using SPSS 21.0 (IBM, Armonk, NY, USA) and GraphPad

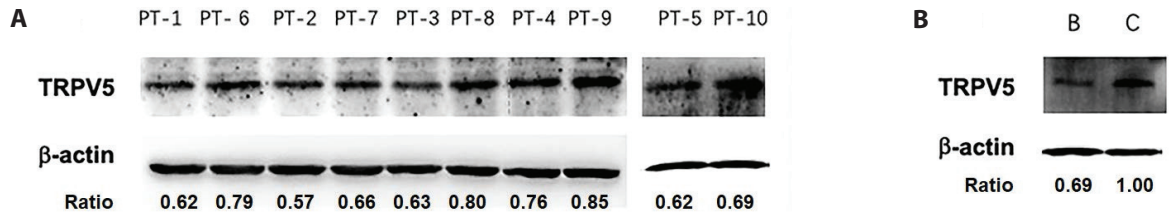

Figure 1. Differential protein expression of TRPV5 between the two groups of patients. **A.** TRPV5 expression in renal tissue of patients with infection stone (PT1–PT5) was notably lower than that of the controls (PT6–PT10). Para-carcinoma renal tissue of patients with tumors was used as control. **B**. TRPV5 expression in renal tissue of group C (*Proteus mirabilis* and saline treatment group) was lower than group B (Saline treatment).

Prism 8 (USA). Data were presented in the form of mean ± SD. The inter-group differences were analyzed by using Student *t*-test. A p value < 0.05 was considered to indicate statistical significance.

Results

TRPV5 expression was downregulated under infection

This study compared the protein expression level of TRPV5 in the kidney tissues with or without the complication of infection stones by conducting Western blot. As shown in Figure 1A, the expression level of TRPV5 in the kidney tissue collected from patients with infection stone (PT1–PT5) was notably lower than that collected from controls (PT6–PT10). Likewise, such pattern of down-regulated level of TRPV5 was consistent in the rat model of infection stones (Fig. 1B).

320-chitosan enhanced the expression of TRPV5 in NRK-52E cells

When the mass ratio of ds-320 and chitosan was reduced to 1:2, the concentration of free RNA in the supernatant was the lowest. Thusly, this ratio of 1:2 was chosen to produce ds-320/chitosan complexes (320-chitosan) and for further analysis. To test the effect of 320-chitosan on TRPV5 expression and transfection efficiency, 320-chitosan was added into NRK-52E cells reaching 70% confluency for 48 h. Simultaneously, an equal amount of 320-liposome and naked ds-320 were employed as positive and negative controls, respectively. 320-chitosan significantly promoted the expression of TRPV5 (Fig. 2A). The degree of promotion, however, was slightly lower than that of liposome-meditated transfection. The prolonged transfection of 320-chitosan to 72 h further elevates the level of TRPV5 (Fig. 2B).

Chitosan delivered ds-320 induced lesser cytotoxicity

We proceeded to evaluate transfection-related cytotoxicity caused by 320-chitosan. Liposome and chitosan delivered ds-320 were added into the NRK-52E cells for inculcation for 72 h, and MTT assay was performed at different time points, including 24, 48 and 72 h, respectively. As presented in Figure 3, both liposome-delivered and chitosan-delivered ds-320 showed cytotoxicity to a certain degree. When compared with liposome delivery, chitosan delivery did significantly less harm to NRK-52E cells at the point of 72 h.

TRPV5 activation alleviated the invasion of bacteria into kidney epithelial cells

The ability of bacteria to invade urothelial cells was considered as a driving factor for the recurrence of infection stones. Thus, we examined how the invasive ability of *Proteus mirabilis* to NRK-52E cells would be affected when the cells were pretreated with 320-chitosan, which aimed at creating an extracellular environment of low calcium. There was no significant difference in the CFUs between the control group and the chitosan groups (p > 0.05; Fig. 4). Compared with chitosan group, the CFUs in 320-chitosan treatment group did not change significantly at 24 h and 48 h. However, it is noteworthy that the number of intracellular bacteria began to diminish when the treatment was prolonged to 72 h.

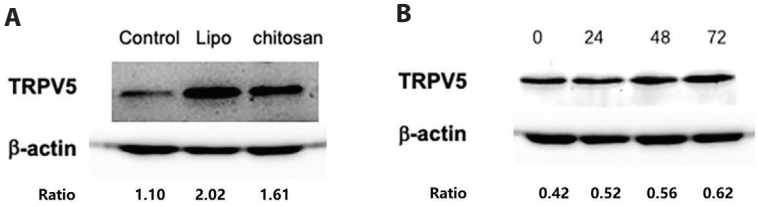

Figure 2. The effect of 320-chitosan on TRPV5 protein expression. **A.** 320-chitosan significantly elevated the expression of TRPV5 in NRK-52E cells. Transfection efficiency was slightly lower than that of liposome-mediated transfection (Lipo). **B.** Transfection of 320-chitosan for 72 h could further elevate the level of TRPV5.

Table 2. Urinary variables in rat model of infection stones

Parameter	Group				
	Control	Saline	*Proteus mirabilis* and saline	*Proteus mirabilis* and chitosan	*Proteus mirabilis and* 320-chitosan
Ca^{2+} (μmol)	26.8 ± 1.64	25.11 ± 3.06	23.41 ± 2.25	23.71 ± 1.71	20.94 ± 2.61[a]
pH	6.38 ± 0.31	6.5 ± 0.39	7.33 ± 0.51[b]	7.18 ± 0.53	7.07 ± 0.62[c]

[a] $p < 0.05$ *vs. Proteus mirabilis* and chitosan group, [b] $p < 0.05$ *vs.* Saline group, [c] $p < 0.05$ *vs. Proteus mirabilis* and chitosan group.

These results implied that the compromised invasive ability of *Proteus mirabilis*.

320-chitosan protected rat from infection to a certain degree

According to our results, no visible stones could be observed in the urinary system of rat models. Additionally, no significant difference could be determined in the numbers of bacterial CFU and the amount of WBC in urine when 320-chitosan was employed. However, the concentration of Ca^{2+} in the urine was obviously decreased in 320-chitosan-72h group. Moreover, it was found that 320-chitosan treatment downregulated the pH value of urine, which implies a prominent effect of acidification (Table 2).

Discussion

PCNL (percutaneous nephrolithotomy) is regarded as the gold-standard therapy for struvite staghorn calculi. However, a complete and effective therapeutic regimen for infection stones is yet to be developed. One prominent feature for the formation of infection stones is that when the urine pH is higher than 7.2, struvite is typically created by the crystallization of various naturally occurring cations in urine such as Mg^{2+}, $Ca^{2+}$and PO_4^{3-}.

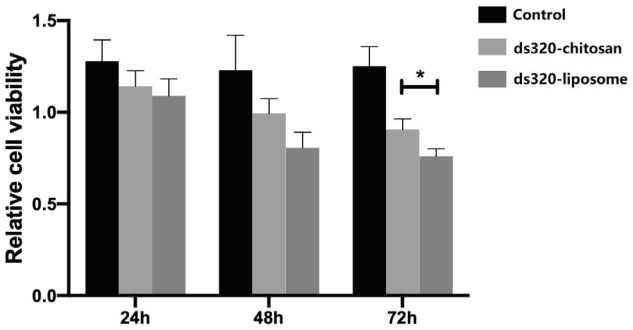

Figure 3. Different vehicles on the viability of NRK-52E cells were determined by MTT assay. Both liposome and chitosan showed toxicity to cells. At the time point of 72 h, chitosan induced less harm to cells compared with liposome. * $p < 0.05$.

Among the multiple genes involved in renal Ca^{2+} handling, such as *CASR*, *VDR*, *TRPV5* and *TRPV6*, *TRPV5* plays a prominent role in Ca^{2+} homeostasis (Na and Peng 2014). A genome-wide association study (GWAS) revealed that TRPV5 genetic code variants indicate a high risk of recurrent nephrolithiasis (Oddson et al. 2015).

saRNAs have exhibited promising effect on the intervention of various diseases, especially cancers after discovery (Zheng et al. 2014; Yoon et al. 2018; Zhou et al. 2018). MTL-CEBPA was the first human saRNA drug candidate for the treatment of liver cancer by elevating the expression of CEPBA (Reebye et al. 2018). Zeng et al. (2018) designed several pairs of small activating dsRNAs to target TRPV5, and determined the efficacy of one pair. Based on this, it is presumed that saRNA of ds-320 might be applicable in the treatment of infection stones. Previous study has indicated the down-regulation of TRPV5 in the infectious tissue (Cherng et al. 2019). To our knowledge, this study is the first one that verified the markedly reduced expression of TRPV5 during the formation of infection stone in human patients and animals.

Owing to the high biocompatibility, biodegradability and capability for the loading of DNA, RNA or proteins, chitosan

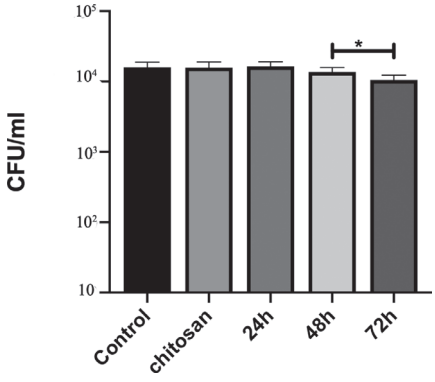

Figure 4. The ability of *Proteus mirabilis* to invade NRK-52E cells was evaluated by invasion assay. There is no significant difference in the CFUs (colony-forming units) between the Control group and the Chitosan groups ($p > 0.05$).When 320-chitosan treatment was maintained for 72 h, the number of intracellular bacteria as presented by CFU/ml was significantly reduced (* $p < 0.05$).

has attracted considerable attention in the field of nanomedicine in recent years (Zhang et al. 2019). The present study demonstrated that, both liposomes and chitosan of ds-320 have certain cytotoxicity. Compared with liposome, chitosan has significantly less damage to NRK-52E cells at the point of 72 h. These results indicated that chitosan-delivered of ds-320 has a lower promotion effect on TRPV5 expression in NRK-52E cells than liposome-delivery of ds-320 within 72 h, but has the advantage of less cytotoxicity.

Subsequently, we modified the protocol of invasion assay to assess whether chitosan-delivered ds-320 may exert any potent effect on the invasive ability of *Proteus mirabilis*. Before this assay, we speculated that CFUs would remain unchanged or increased since the concentration of intracellular calcium was elevated. This was not to overturn the anticipation of a protective effect of ds-320, but a frequent imperfection of experiment from a cellular level. Experiment results showed, when 320-chitosan was incubated with the cells for 72 h, the number of invading and proliferating bacteria was markedly reduced than the other groups. Therefore, it was speculated that a declined level of extracellular calcium had created an unfavorable environment for the bioactivity and concentration of bacteria. Consequently, this discovery is worth of further exploration.

In this study, we also conducted the animal experiments to ascertain whether stone formation could be inhibited by 320-chitosan treatment *in vivo*. Unfortunately, no visible stones were observed in the urinary system. Urine analysis revealed that calcium concentration was statistically decreased after the injection of 320-chitosan for 21 days. Besides, the pH value of urine was significantly lowered than that of rats in other groups. We speculated that such effect of acidification was probably attributable to the increased outflow of H^+ from the tubular epithelia of kidney due to the reabsorption of calcium.

In conclusion, our study provides concrete evidence to corroborate the potent inhibitive effect of chitosan delivered small activation RNA, ds-320 on the formation of infection stone *via* the following mechanism: (1) downregulation of Ca^{2+} in the urine, (2) a consequential inhibition of bacteria concentration to induce impaired invasiveness into the renal epithelial cells, and (3) an increased exchange of H^+ from the tubular epithelia to acidify the urine.

Limitation. The limitation with the study is the small sample size of human participants. To further validate the results of the study, large-scale research should be conducted in the future.

Conflict of interest. All authors declare that they have no conflicts of interest.

Authors contribution. HH and GQ designed the study. HX did the literature research. GQ conducted the experiments and acquired the data. BF performed the statistical analyses. HX drafted the manuscript. GQ reviewed the paper. All authors read and approved the final version.

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