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ORIGINAL PAPER

**Fernando C. Delvecchio · Ricardo M. Brizuela
Saeed R. Khan · Karen Byer · Zaiquan Li · Pei Zhong
Glenn M. Preminger**

Citrate and vitamin E blunt the shock wave-induced free radical surge in an in vitro cell culture model

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Abstract Free radical formation plays a major role in shock wave lithotripsy induced renal damage. Moreover, previous studies suggest that free radicals may also promote de novo calcium oxalate crystallization of previously damaged urothelium. Citrate is a known inhibitor of renal stone formation and has also been used as a free radical scavenger. Using an in vitro model with Madin-Darby canine kidney (MDCK) cells, we investigated the influence of two free radical scavengers, citrate and vitamin E, on the prevention of the shock wave-induced free radical surge. Suspensions of MDCK cells were placed in containers for shock wave exposure. Six groups of six containers each were examined: (a) no scavengers 0 shocks, (b) no scavengers 100 shocks, (c) citrate 0 shocks, (d) citrate 100 shocks, (e) vitamin E 0 shocks, (f) vitamin E 100 shocks. An unmodified HM3 was used to deliver 100 shocks at 24 kV. The cell groups that were not shocked acted as the control group and were handled identically, except for the lack of shock wave exposure. After shock wave administration, the containers were emptied and cell suspensions were immediately centrifuged. The supernatant was examined for lactate dehydrogenase (LDH) and 8-isoprostane (8-IP), markers of cellular injury and free radical formation, respectively. Intracellular LDH uniformly increased in all groups exposed to shock wave energy. Similarly, 8-IP increased in all shocked groups. However, the 8-IP increase was significantly reduced when the free radical scavengers were employed. As citrate is a well-known inhibitor of calcium nephrolithiasis, its mechanism of action may be further enhanced, based on its ability to reduce free radical formation, by a protective effect on the urothelium. These data further support the use of citrate based medications during the peri-operative period of shock wave lithotripsy, not only to inhibit stone formation and facilitate fragment passage, but also to reduce the incidence of shock wave induced renal damage. Further studies are warranted to clinically test this hypothesis.

Keywords Free radicals · SWL · Shock wave lithotripsy cell culture · MDCK cells · Citrate · Vitamin E · Free radical scavengers · Cellular damage

F. C. Delvecchio · R. M. Brizuela · Z. Li · P. Zhong
G. M. Preminger (✉)
Comprehensive Kidney Stone Center, Division of Urology, Department of Surgery, Duke University Medical Center, 1572D White Zone DUMC 3167, Durham, NC 27710USA
E-mail: glenn.preminger@duke.edu
Tel.: +1-919-6815506
Fax: +1-919-6815507

S. R. Khan · K. Byer
Center for the Study of Lithiasis and Pathological Calcification, Division of Experimental Pathology, University of Florida, Gainesville, FL USA

Introduction

Free radicals are oxidants produced by the normal processes of cellular metabolism. The toxicity of free radicals is attributed to their ability to initiate lipid peroxidation of cellular membranes [1]. A free radical surge within renal tissue has been shown to occur after a variety of noxious stimuli, including shock wave lithotripsy (SWL) [2]. In fact, SWL induced free radical formation plays a major role in SWL induced renal damage. [3, 4].

Moreover, previous studies have suggested that free radical induced damage to the urothelium may predispose to de novo calcium oxalate stone formation [5–9]. Therefore, blunting free radical production, regardless of its predisposing origin, would be a therapeutic intervention both to prevent renal damage and nephrolithiasis in individuals at risk.

Potassium citrate has been proven to reduce renal stone formation by its inhibitory activities and to aid in the passage of residual fragments following SWL. As citrate is also a free radical scavenger, we elected to test the ability of two free radical scavengers, citrate and vitamin E in preventing the free radical rise in Madin-Darby canine kidney (MDCK) cells exposed to SWL.

Materials and methods

MDCK cells are a cell line originally derived from dog kidney cortex (American Type Culture Collection, Manassas, Va.). MDCK cells are also an established in vitro model to study SWL induced renal tubal injury and its potential prevention [10].

Cells were grown in culture flasks containing Dulbecco's modified Eagle's medium and fetal calf serum. Citrate or vitamin E were added at concentrations of 300 mg/dl and 30 μM, respectively, to two groups of cell cultures. Vitamin E and citrate concentrations were determined based on reference values from our institution for blood and urine, respectively. Other flasks underwent shock wave exposure without pretreatment with citrate or vitamin E. Cells were trypsinated after they had achieved a monolayer growth. Following this procedure, cell suspensions were obtained in a medium containing Dulbecco's modified Eagle's medium and fetal calf serum only, or in a medium with added citrate or vitamin E.

For SWL exposure, the cell concentration was adjusted to 1×10^6 cells/ml. The cells were then placed in specially designed tubes, which were filled completely to avoid fluid-air interfaces. An unmodified HM3 was employed for shock wave delivery. A specially constructed holder adjusted the tubes to the second focus of the ellipsoid. The impulse rate was 100 shock waves at a voltage of 24 kV. The cells which were not treated with shock waves (i.e. 0 shock group) were handled in same way as cells that receive shock wave energy, except for the absence of shock wave exposure.

Six groups of six containers each were examined: (a) no scavengers 0 shocks, (b) no scavengers 100 shocks, (c) citrate 0 shocks, (d) citrate 100 shocks, (e) vitamin E 0 shocks, (f) vitamin E 100 shocks.

After shock wave exposure, the containers were emptied and the cell suspensions immediately centrifuged. The supernatant was examined for lactate dehydrogenase (LDH) and 8-isoprostane (8-IP) as markers of cellular damage and of oxidative stress, respectively.

Cell viability—LDH

Media was aliquoted to designated wells of a 96 well plate (Fisher Scientific, Norcross, Ga.). The CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Fisher Scientific, Promega) was used to determine percent LDH release. Substrate (supplied with the kit) was added to all samples, positive control (MDCK cells lysed with lysis solution-supplied with kit), and blanks (acclimatization media). The plates were incubated at room temperature for 30 min in the dark. Stop solution (supplied with the kit) was added to all samples, positive control, and blanks. Optical density absorbency was read at 490 nm on a Bio-Rad 3550 microplate reader (Bio-Rad, Hercules, Calif.).

Lipid peroxidation—8-isoprostane

To determine lipid peroxidation, an 8-isoprostane kit (Cayman Chemical, Ann Arbor, Mich.) was used to determine the amount of 8-IP, an end-product of lipid breakdown. Media was aliquoted to a 96 well plate (supplied with kit). A standard curve was determined according to the kit's protocol (standard supplied with kit). A blank, non-specific binding (NSB), total activity (TA) and maximal binding control (B_0) were also set up to determine the background and proper function of the assay (according to the kit's protocol). To all samples, NSB, B_0, and standard tracer and anti-serum were added. The plate was incubated at room temperature for 18 h. After incubation, plates were washed with washing buffer (supplied with kit) five times. Ellman's reagent (supplied with the kit) was added and the plates were incubated at room temperature in the dark with gentle shacking for 75–90 min. Absorbency was read at 405 nm with a Bio-Rad 3550 microplate reader.

Statistical analysis

All statistical analysis was performed using ANOVA. $P < 0.05$ was considered significant.

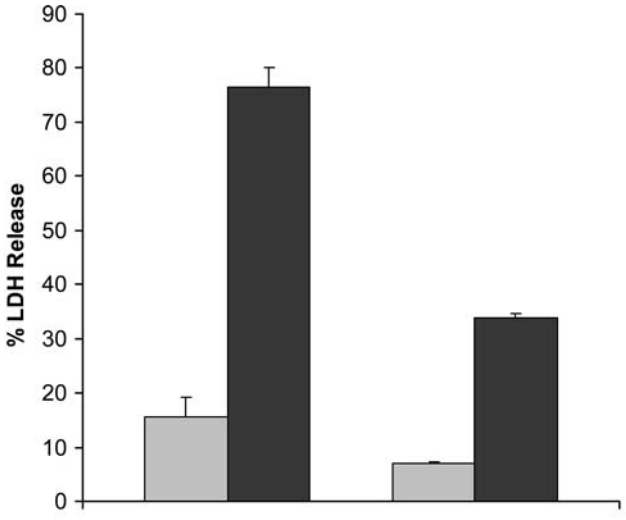
Results

Intracellular LDH increased significantly in all groups exposed to shock waves (Table 1). Similarly, 8-IP increased in all shocked groups, however, the 8-IP increase was significantly reduced when the scavengers were employed (Tables 2, 3).

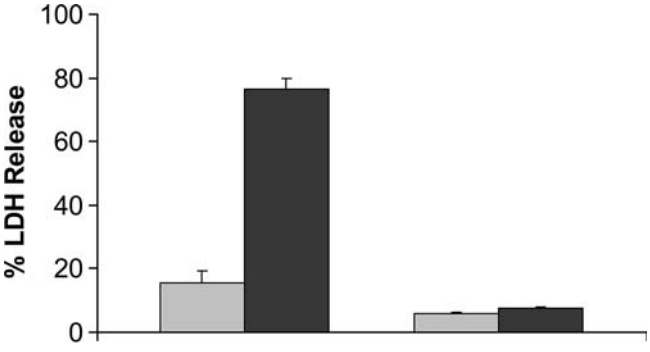
Discussion

Initially, the adverse tissue effects of SWL were attributed to renal damage resulting only from the direct action of shock wave energy, which was shown to produce gross areas of hematoma with subsequent cortical fibrosis. The mechanism of tissue injury was believed to be mechanical trauma to small vessels and tubules caused by cavitation forces or shear stress. Subsequently, a subtle and indirect mechanism of injury was proposed as an important contributing factor. In this mechanism, SWL was thought to induce renal capillary disruption. Resultant tissue edema causes relative ischemia and hypoxia with subsequent renal parenchymal damage. This hypothesis is supported by experimental animal studies showing evidence of a decreased glomerular filtration rate and renal plasma flow after SWL [11]. More recently, it has been proposed that SWL induced renal damage can occur as a consequence of local as well as regional vasoconstriction [12]. Tissue hypoxia leads to a reperfusion injury that can also generate free radicals [12–14]. Free

Table 1 Percentage LDH release from control cells and cells exposed to 100 shocks, without and with citrate and vitamin E



% LDH Release by Group	Mean	SD	SE	95% CI of Mean
0 shocks	15.48	5.251	3.713	-31.7 to 62.66
100 shocks	76.55	10.539	3.726	67.73 to 85.36
0 shocks cit	6.91	0.53	0.306	5.59 to 8.23
100 shocks cit	33.94	1.014	0.586	31.42 to 36.46

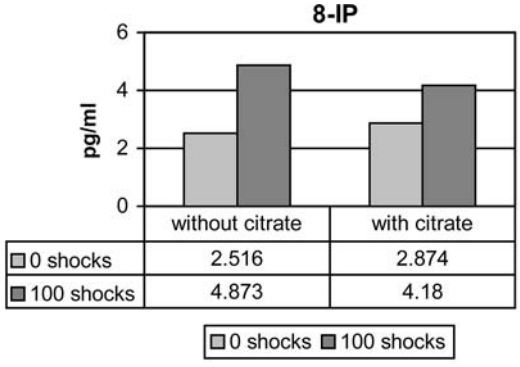


% LDH Release by Group	Mean	SD	SE	95% CI of Mean
0 shocks	15.48	5.251	3.713	-31.70 to 62.66
100 shocks	76.55	10.539	3.726	67.73 to 85.36
0 shocks Vit E	5.87	0.428	0.247	4.80 to 6.93
100 shocks Vit E	7.45	0.655	0.378	5.83 to 9.08

radicals are oxidants produced by the normal processes of cellular metabolism. The toxicity of free radicals is attributed to their ability to initiate lipid peroxidation of cellular membranes. After membrane integrity is altered, the cellular equilibrium is lost and cell death typically ensues. Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase function to protect against the toxic effect of free radicals since they inactivate these species. Nevertheless, extremely elevated free radical levels can overwhelm these natural protective mechanisms.

The current results support the multifactorial nature of SWL induced tissue injury. The observed uniform

Table 2 8-IP results without and with citrate



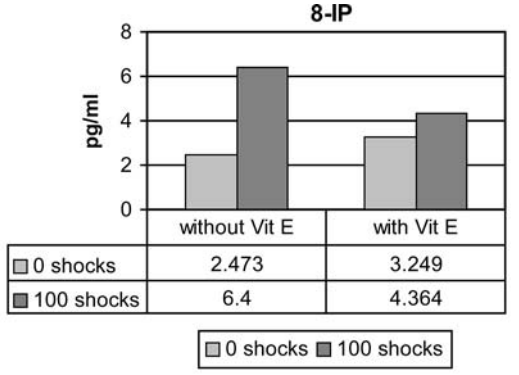
p=0.002 for cell group shocked in the presence of citrate.

8-IP by Group	Mean	SD	SE	95% CI of Mean
0 shocks	2.52	0.285	0.164	1.81 to 3.22
100 shocks	4.87	0.100	0.058	4.62 to 5.12
0 shocks Cit	2.87	0.633	0.366	1.30 to 4.45
100 shocks Cit	4.18	0.181	0.105	3.73 to 4.63

$P = 0.002$ for cell group shocked in the presence of citrate

leakage of intracellular LDH across all shock wave groups is best explained by a combination of direct mechanical trauma and free radical activity (which is not completely suppressed in the shock wave groups in which the scavengers were used). Previous studies have reported the damaging effects of SWL on cultured MDCK cells, manifested by the leakage in a shock wave dose dependent pattern of intracellular enzymes and the protective effect of fosfomycin or free radical scavengers like selenium [10, 24]. Moreover, the generation of free radical formation induced by SWL cavitation has been demonstrated using fluorescent dyes [4].

Table 3 8-IP results without and with vitamin E



p=0.003 for cell group shocked in the presence of Vitamin E.

8-IP by Group	Mean	SD	SE	95% CI of Mean
0 shocks	2.47	0.148	0.085	2.11 to 2.84
100 shocks	6.40	0.617	0.356	4.87 to 7.93
0 shocks Vit E	3.25	0.722	0.417	1.45 to 5.04
100 shocks Vit E	4.36	0.287	0.166	3.65 to 5.08

$P = 0.003$ for cell group shocked in the presence of vitamin E

It has recently been suggested that free radicals can be further detrimental to the kidney by promoting de novo calcium oxalate crystallization on the previously damaged urothelium. Animal studies have demonstrated that ethylene glycol induced hyperoxaluria in rats increases lipid peroxides in kidney tissue and urine, resulting in renal tubular cell damage and promoting calcium oxalate crystal deposition [15]. It therefore appears that hyperoxaluria (through the generation of free radicals) promotes stone formation in several ways: by providing a substrate for the formation of calcium oxalate stones and by inducing damage to renal epithelial cells [5, 6, 15, 16]. Damaged cells in turn would produce an environment favorable for crystal retention and provide membranous debris that promotes crystal nucleation, aggregation and adherence.

Although most SWL-induced renal damage is well tolerated with no untoward effects, there is a potential subset of patients with impaired renal function in whom diminishing SWL tissue damage could be beneficial. Animal studies have suggested that immature or juvenile renal tissue may be at increased risk for damage following SWL [17, 18]. These findings suggest that SWL should be used with caution in children as well as in patients with impaired renal function or a solitary kidney.

Previous work from our institution in an in vivo model has demonstrated that the free radical surge induced by SWL can effectively be blocked by the use of free radical scavengers, such as allopurinol [14]. We therefore sought to assess the potential beneficial effects of citrate, as both a medication to inhibit new stone formation, as well as a scavenger of free radicals. Our results suggest that the use of citrate, as well as a documented free radical scavenger, vitamin E, significantly reduces the free radical surge induced by shock waves in an in vitro MDCK cell culture model.

Citrate could be potentially beneficial via additional mechanisms. A significant number of SWL residual fragments have been shown to require intervention or produce symptomatic episodes if left untreated [19, 20]. Also, stone fragility to SWL can be increased depending on the composition of the surrounding fluid [21, 22]. Citrate therapy has been shown to significantly improve the stone clearance rate in sterile and infection stone patients, preventing residual fragment growth or reaggregation in subjects with residual stone fragments following SWL [23]. It would therefore seem useful to pre-treat patients undergoing SWL with citrate in order to protect the kidney from free radical induced injury, de novo stone formation and to facilitate fragment passage.

Conclusions

Citrate is a well known inhibitor of calcium nephrolithiasis, with very few side effects and contraindications to its use. Its known mechanism of action through the inhibition of heterogeneous nucleation and solubilization of calcium salts may be further enhanced, based on evidence of a protective effect on the urothelium. This direct tissue effect may not only inhibit stone formation due to free radical production, but also protect the renal parenchyma from shock wave induced free radical injury. Further animal and clinical studies are warranted to better define the clinical utility of the perioperative use of citrate.

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