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ORIGINAL PAPER

Curcumin prevents shock-wave lithotripsy-induced renal injury through inhibition of nuclear factor kappa-B and inducible nitric oxide synthase activity in rats

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Abstract Shock wave lithotripsy (SWL) is commonly used for treatment of renal stones. Free oxygen radicals are involved in the pathophysiology of renal injury due to SWL. We investigated the protective effects of curcumin, which is an antioxidant and nuclear factor kappa-B (NF-κB) inhibitor, against renal injury. Forty-eight rats were included and divided into four groups: group 1, control; group 2, SWL (15 kW-1,500 shocks); group 3, SWL + curcumin (curcumin orally 75 mg/kg/day dissolved in 10% ethyl alcohol, 1 day before and 5 days after SWL); and group 4, SWL + vehicle (10% ethyl alcohol). The kidneys were removed on days 7 and 35 after SWL. A sample was fixed in formaldehyde solution. Renal tissues were examined for proximal tubular injury under light microscope. iNOS activity and active subunit of NF-κB, p65, were evaluated immunohistochemically using rat monoclonal antibodies interpreting results semiquantitatively. There were significant differences between SWL and control groups on days 7 and 35, considering histological changes under light microscope ($P < 0.02$). There was a significant decrease in necrosis and fibrosis in the curcumin group as compared to the SWL group. Expressions of iNOS and p65 on days 7 and 35 were at basal levels with immunohistochemical staining. These parameters had high levels in the SWL group ($P < 0.02$). No significant difference was present between the control and the curcumin groups ($P > 0.02$). Curcumin, decreasing expressions of iNOS and p65 and serum nitric oxide levels prevented interstitial, glomerular, tubular epithelial and endothelial cellular injuries. We suggest that curcumin, could be used, especially in high-risk patients, as a protective agent to prevent renal injury due to SWL.

Keywords NF-κB · Curcumin · SWL · iNOS · Kidney stone · Antioxidant

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Introduction

Clinical shock wave lithotripsy (SWL) shock waves applied in sufficient volume to break kidney stones invariably injure the renal parenchyma and impair renal function [1]. Microangiographic changes indicative of shockwave trauma were demonstrated by cortical, subcapsular, and medullary extravasation of barium and patchy loss of efferent vessels. Microscopically, there was crowding of glomeruli and areas of avascularity [2].

The mechanism of SWL-induced cellular damage is still controversial. One of the mechanisms discussed for tissue damage is free radical formation during SWL [3–5].

Suhr et al. presented intra and extracellular in vitro measurements of free radicals and investigated cell viability after shock wave treatment. They demonstrated an elevated concentration of intracellular free radicals during such treatment in suspended cells in vitro. It has been suggested that reactive oxygen species (ROS) such as hydroxyl radicals and superoxide anions act as mediators of nuclear factor kappa-B (NF-κB) action [6, 7]. Excessive nitric oxide production due to elevated expression of iNOS may impose cytotoxic effects on various organs, including the kidney [8, 9]. The nitric oxide at high level can rapidly react with superoxide anion to yield a potent antioxidant, peroxynitrite (OONO–), that in turn causes extensive protein tyrosine nitration [10]. The expression of iNOS is mainly controlled by the activation of its transcriptional factors, including NF-κB [10]. Curcumin (diferuloymethane), a polyphenol derived from turmeric, *Curcuma longa*, is a pharmacologically safe and effective agent that can block NF-κB activation. Curcimin has been shown to suppress NF-κB activation induced by various inflammatory stimuli [11] through inhibition of the activation of IκBα kinase (IKK) activity needed for NF-κB activation [12, 13]. Our goal was to determine whether curcimin can suppress SWL-induced NF-κB activation and inducible nitric oxide synthase (iNOS) expression.

Materials and methods

Animals

Forty-eight adult male Sprague–Dawley rats (240–300 g) were acquired from the Experimental Animal Laboratory of Istanbul University, Cerrahpasa Medical Faculty vivarium sources and maintained in a 14-h light/10-h dark cycle with free access to food and water.

Experimental conditions

Rats were firstly divided into four major equal groups:

Group 1, control group; the rats received only anesthesia but were not exposed to SWL.

Group 2, SWL group; the rats were exposed to 1,500 shock waves at 15 kW in SWL machine (Stonelith-V5 lithotriptor, PCK, Ankara, Turkey).

Group 3, SWL + curcumin group; the rats were fed with curcumin (75 mg/kg/day, dissolved in 10% ethyl alcohol) via gavage and were exposed to 1,500 shock waves at 15 kW.

Group 4, SWL + vehicle group; the rats were fed with 10% ethyl alcohol via gavage and were exposed to 1,500 shock waves at 15 kW.

All the major groups were divided into two equal groups according to the experimental sampling periods, 7 and 35 days after SWL. Finally, groups were named, Group 1a: control 7, Group 1b: control 35, Group 2a: SWL 7, Group 2b: SWL 35, Group 3a: SWL + curcumin 7, Group 3b: SWL + curcumin 35, Group 4a: SWL + vehicle 7, and Group 4b: SWL + vehicle 35.

The animals were placed in cages 7 days before beginning the experiment, to acclimate them to the cages. During the experimental period, all groups had free access to regular rat chow. Curcumin was given starting 1 day before and continuing 5 days after SWL (totally 6 days).

The anesthetized rats (intraperitoneal sodium pentobarbital, 50 mg/kg body weight) were fixed in a supine position on the on the platform of the lithotriptor and fixed at the thorax and hip to allow direct entry of the shock waves through the abdominal wall into the left rat kidney. All the rats were injected with intravenous 0.7 ml nonionic contrast medium (iopromide, Ultravist® Injection 370 mgI/100 mL, Bayer Health Care Pharmaceuticals Inc., Wayne, Germany), the left kidney was treated after localization by means of a video fluoroscopic system (control groups received anesthesia but were not exposed to SWL). At the end of the experimental period (day 7 and 35), rats were killed under anesthesia (intraperitoneal sodium pentobarbital, 50 mg/kg body weight). The kidneys were quickly removed and decapsulated and the renal cortex carefully separated from the medulla, homogenized as described previously [14]. Small samples were fixed in formaldehyde solution for histopathological and immunohistochemical examination.

Histopathological examination

For the histopathological examination the tissues were prepared for routine examination by light microscopy (Nikon, Tokyo, Japan). The kidney sections were analyzed semiquantitatively using the technique of Houghton et al. [15].

Immunohistochemical evaluation

For immunohistochemical evaluation, specimens were processed for light microscopy and sections were incubated at 60°C overnight and then dewaxed in xylene for 30 min. After soaking in a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. The sections were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37°C for 15 min washed with PBS. The sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. Then the sections were incubated with NF-κB/P65 (Rel A) Ab-1 (Neomarkers R-B-1638-R7) and

inducible nitric oxide synthase (iNOS) Ab-1 (Neomarkers R-B-1605-R7) antibodies histochemically. The ultravision HRP-AEC staining protocol was used at this stage.

The sections prepared for each case were examined by light microscopy. The sections of rat lung were used for control of immunohistochemical staining specificity according to data provided by the antibody producing company.

The cases were evaluated for diffuseness and staining. According to staining diffuseness sections were graded as follows: 0 = no staining; 1 = staining less than 25%; 2 = staining between 25 and 50%; 3 = staining between 50 and 75%; 4 = staining more than 75%. According to staining intensity sections were graded as follows: 0 = no staining; 1 = weak but detectable above control; 2 = distinct; 3 = intense staining. Immunohistochemical values were obtained by adding diffuseness and intensity scores.

Biochemical determinations

All tissues were washed two times with cold saline solution and immediately stored at −80°C for the measurement of MDA, GSH and NO levels. Tissues were homogenized in four volumes of ice-cold buffer containing 20 mM Tris, 10 mM EDTA (pH 7.4) Total nitrite (NO_x) was quantified by the Griess reaction, after incubation of supernatant with *Escherichia coli* nitrate reductase to convert NO_3 to NO_2. Thiobarbituric acid (TBA) reacts with lipoperoxidation aldehydes, such as MDA, as the most common method to assess lipid peroxidation in biological samples. The procedure was modified from Buege and Aust [16].

The GSH level was determined by the spectrophotometric method of Ellman, based on the development of a yellow color when DTNB (5,5′ dithiobis-2-nitrobenzoic acid) is added to compounds containing sulfhydryl groups [14].

Statistical analysis

Statistical analyses of histopathologic and immunohistochemical evaluation of groups were compared by Chi-square test and biochemical values were compared by Mann–Whitney *U* test. Probability values of less than 0.05 were considered significant.

Results

Biochemical variables in urine, serum and tissue

There were significant lower MDA and NO levels ($P < 0.01$) and higher GSH levels ($P < 0.05$) in kidney cortex in the SWL + curcumin groups as compared to the SWL groups for days 7 and 35 (Table 1). There were significant differences in MDA and NO levels of SWL groups ($P < 0.01$). The mean tissue MDA and NO levels showed a statistically significant reduction in day 7 as compared with day 35.

There was significant difference between MDA, NO and GSH levels in controls and SWL + curcumin groups for day 7. The MDA and NO levels were higher and GSH levels were lower ($P < 0.05$) in kidney cortex in the control group as compared to SWL + curcumin group for day 7 (Table 1). There was no significant difference between controls and SWL + curcumin group for MDA, NO and GSH levels for day 35 ($P > 0.05$) (Table 1).

There were no statistically significant differences between group 1 and group 4 in any biochemical variables at any sampling time.

Histological examination

The conventional microscopic examination revealed a similar pattern of histological damage in SWL, SWL + curcumin and vehicle groups in day 7. Histological damages were more apparent in day 7 compared to day 35 (SWL and vehicle groups compared to SWL + curcumin) (Table 1).

Subcapsular and intraparenchymal hemorrhages were more severe at the corticomedullary borders and perivascular areas in SWL and vehicle groups, and were more apparent than SWL + curcumin group in day 7. Furthermore, changes in the tubulus system with blood and protein in the tubulus lumen, expanded tubuli, swollen tubuli and mononuclear inflammatory cells infiltrating into the interstitium were observed in all of SWL groups and rarely in SWL + curcumin groups for day 7 (Table 1).

Although some of the pathological changes seem to be recovered in day 35, hydropic degeneration, glomerular and tubular necrosis accompanied with fibrosis, were more apparent in SWL and vehicle day 35 group than the same groups in day 7 (Table 1) (Fig. 1a–c).

Immunohistochemical studies

In the immunohistochemical evaluation, there were more intensive positive expressions of iNOS and p65 staining within the renal cortex and the proximal tubules in the SWL groups (SWL groups in day 7 and 35) as compared with others (Fig. 2a–c, day 35).

Immunohistochemical scores of SWL groups in day 7 were higher than those of SWL groups in day 35 but there were no statistically significant differences between these two groups.

There were poor or mild positive expressions of iNOS and p65 staining in the SWL + curcumin groups (days 7 and 35) and control groups as compared with the SWL and

Table 1 Serum, urine and tissue variables and histological findings in SWL + curcumin, SWL, control and vehicle groups in day 7 and 35

	Day 7				Day 35			
	Group 1a Control 7	Group 2a SWL 7	Group 3a SWL + C7	Group 4a SWL + V7	Group 3b Control 35	Group 2b SWL 35	Group 1b SWL + C35	Group 4b SWL + V35
MDA (nM/g wet tissue)	49.6 ± 9.4	128.5 ± 11.4^{yab}	62.2 ± 8.6^{xa}	125 ± 9.8^{yab}	51.1 ± 6.9	94.6 ± 13.8^{yabc}	54.1 ± 7.9	99.1 ± 10.9^{yabc}
NO_x = (NO_2 + NO_3) (nM/g wet tissue)	48.3 ± 8.1	97.4 ± 10.6^{yab}	60.2 ± 8.4^{xa}	100.3 ± 7.7^{yab}	47.6 ± 7.8	85.8 ± 7.9^{yabc}	51.7 ± 9.3	88.6 ± 8.4^{yabc}
GSH (μM/g wet tissue)	1.7 ± 0.3	0.8 ± 0.3^{xab}	1.4 ± 0.3^{xa}	0.9 ± 0.3^{xab}	1.7 ± 0.3	1.1 ± 0.3^{xab}	1.6 ± 0.2	1.0 ± 0.3^{xab}
Glomerular bleeding	−	++	+	++	−	+	−	+
Tubular dilatation	−	++	+	++	−	+	−	+
Tubular atrophy	−	+	+	+	−	++	−	++
Tubular red blood cells	−	+++	+	+++	−	+	−	+
Interstitial necrosis	−	++	+	++	−	+++	+	++
Interstitial fibrosis	−	+	−	+	−	+++	+	+++
Venous dilatation	−	++	+	++	−	+++	−	+++
Neutrophilic infiltration	−	++	+	++	−	+	−	+

Histopathological findings: − none, + isolated, ++ frequent, +++ highly frequent

Values are mean ± SD: ^{x} $P<0.05$; ^{y} $P<0.01$

[a] Significant compared to control group

[b] Significant compared to SWL + curcumin group

[c] Significant compared to day 7

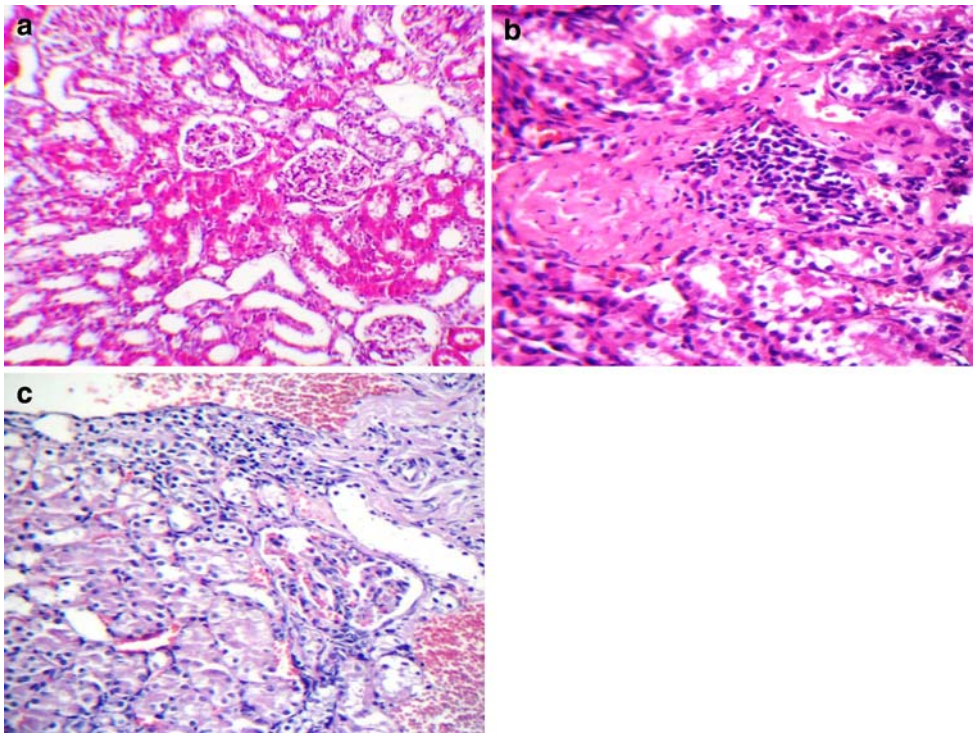

Fig. 1 **a** Normal glomerular and tubular structures in control day 35 (H&E ×100). **b** Highly intense parenchymal damage in tubulary and glomerulary areas with chronic inflammation and fibrosis in SWL day 35 group (H&E ×200). **c** Minimal parenchymal damage and fibrosis in day 35 in curcumin group. Minimal irregular appearance and bleeding areas are seen (H&E ×100)

vehicle groups at any sampling time. iNOS and p65 expressions in SWL + curcumin day 35 group were weak positive than SWL + curcumin day 7. It could be said that SWL-related damage appears especially in tubular structures and recovery was more apparent in late term.

Discussion

A recently published study showed the protective effect of curcumin against a well known renal carcinogen, ferric nitrilotriacetate, which generates ROS in vivo. Curcumin

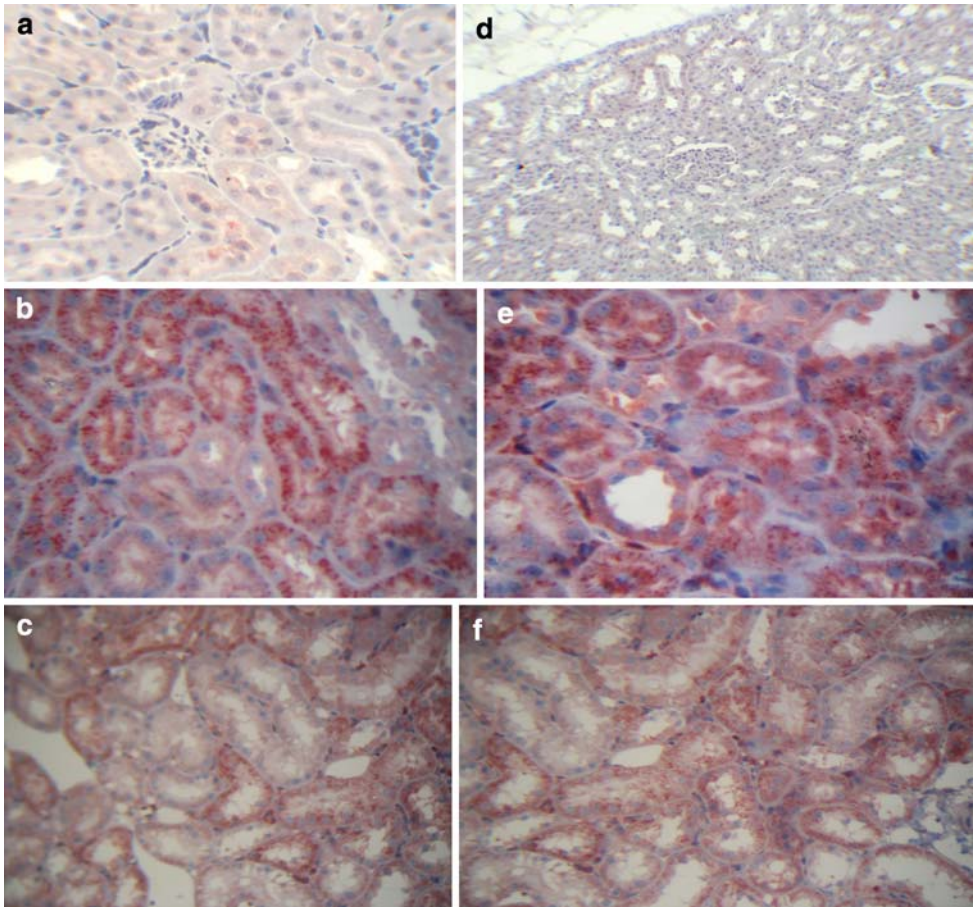

Fig. 2 **a** Little NF-κB/p65 expression in proximal and distal tubulus epithelium in control group in day 35 (IHC staining ×100). **b** Diffuse and dense intracytoplasmic NF-κB/p65 staining in SWL group in day 35 (IHC staining ×200). **c** Low intracytoplasmic NF-κB/ p65 staining in SWL + curcumin group in day 35 (IHC staining ×200). Treatment with anti-oxidant after SWL procedure lowers the p65 expression in glomeruli and tubulus epithelium. **d** Little iNOS staining in proximal tubules and glomeruli in control group in day 35 group (IHC staining ×100). **e** Diffuse and intense intracytoplasmic iNOS staining in proximal and distal tubules in SWL day 35 group (IHC staining ×200). **f** Mild intracytoplasmic iNOS staining in proximal tubules in SWL + curcumin day 35 group (IHC staining ×200)

counter-acted the ROS by increasing ornithine decarboxylase, glutathione, antioxidant enzymes and phase II metabolizing enzymes and therefore protected the kidney from oxidative damage [17].

Curcumin has been found to increase expression of conjugation enzymes and has been shown to be one of the most potent inhibitors of NF-κB, thereby exerting anti-inflammatory effects [18].

Curcumin inhibits the induction of NOS in activated macrophages and has been shown to be potent scavenger of free radicals like nitric oxide (NO) [19]. In RAW 264.7 macrophages activated with lipopolysaccharide and the interferon-gamma system, curcumin treatment showed antitumorigenic potential by significantly reducing the levels of iNOS [20]. NF-κB has been implicated in introduction of iNOS, which causes oxidative stress, one of the causes of tumor initiation. Curcumin prevents phosphorylation and degradation of inhibitor κBα, thereby blocking NF-κB activation, which results in downregulation of iNOS gene transcription [21].

In our study we have found immunohistochemically that iNOS and p65 expression were significantly reduced in rats given SWL + NF-κB inhibitor (curcumin) when compared with SWL application only.

Free radical formation is discussed as an important mechanism in shock wave induced renal injury [3, 4, 22, 23]. Serel et al. [24] demonstrated that melatonin significantly reduced the shock wave induced renal impairment resulting in increased MDA levels, urine NAG activity. Ozguner et al. [25] found that the novel free radical scavenger caffeic acid phenethyl ester (CAPE) treatment provided significant protection against SWL induced free radial damage. Following shock wave exposure there was a significant rise in MDA, NAG and uric acid and white cell counts. CAPE reduced the rise in MDA, NAG, uric acid and white cell counts. Our results demonstrated that curcumin significantly reduces the shock wave induced renal impairment resulting increased MDA and NO levels in renal tissue when compared with SWL groups.

GSH levels were significantly higher in kidney cortex tissue in SWL + curcumin groups as compared with only SWL applied groups.

In light microscopy, acute morphological changes such as glomerular bleeding, tubular dilatation, atrophy and partial necrosis occurred immediately after SWL through the kidney. In the long term groups, hematomas progressed to interstitial fibrosis with segmental retraction of renal convexity. There was no fibrosis in SWL + curcumin groups

for day 35. Consequently we propose that NF-κB inhibitor, curcumin is one of the most potent and specific antioxidants for hydroxyl radicals and it acts in the kidney as a potent scavenger of free radicals and it prevents the side effects of SWL applications. However, these results also reinforce the significant role of nephropathies induced by SWL applications and further studies are needed to be done.

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