EXPERIMENTAL STUDY

Protective effects of diltiazem and tadalafil on shock wave--induced kidney injury in rats

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ABSTRACT
BACKGROUND: We aimed to compare the protective effects of tadalafil and diltiazem on renal histology after ischemia and reperfusion injury in a rat model of shock wave lithotripsy.
METHODS: A total of 40 adult, male Sprague-Dawley rats were randomized into four groups as follows; control group (group C), group S (SWL + nephrectomy), group T (SWL + tadalafil given before nephrectomy) and group D (SWL + diltiazem given before nephrectomy). Both kidneys were evaluated regarding tubular damage, peritubular fibrosis and heat shock protein-70 (HSP-70) immune-expression of glomeruli, cortical and medullar collector tubules on light microscopy.
RESULTS: HSP-70 levels of cortical and medullar collector tubules, tubular damage and peritubular fibrosis scores were decreased in group T compared with group S. Similarly, HSP-70 immunostaining levels on cortical and medullar collector tubules, tubular damage and peritubular fibrosis scores were decreased in group D compared with group S. No significant difference was detected between group D and group T for all parameters.
CONCLUSION: As a result, shock waves induced renal cell damage due to increment of HSP-70 levels, morphological irregularity in tubules and increased peritubular fibrosis. Tadalafil and diltiazem had beneficial effects in decreasing renal tissue damage which was caused by SWL *(Tab. 2, Fig. 6, Ref. 29)*. Text in PDF *www.elis.sk*.
KEY WORDS: diltiazem, ischemia-reperfusion injury, heat shock protein, shock wave lithotripsy, renal tubules, tadalafil.

Introduction

Shock wave lithotripsy (SWL) has been acknowledged as an effective and non-invasive treatment modality for urolithiasis and is the first choice of treatment for most patients with urolithiasis as it is the least invasive method (1). However, it is associated with both short- and long-term risks of renal and extra renal complications (1–3). Several mechanisms, such as cavitation bubbles, temporary decrease of renal perfusion and ischemia-reperfusion (I/R) injury, are responsible for renal injury during SWL (4–6). New generation SWL devices are clinically used to decrease complication rates (1). Furthermore, several molecules including calcium channel blockers (CCBs), anti-inflammatory agents, phosphodiesterase type 5 (PDE5) inhibitors and antioxidants, have been shown to reduce I/R injury and SWL complications (7–16).

The use of CCBs in I/R injury decreases calcium influx through the cell membrane and reduces phospholipase activity, thereby protecting against tubular injury by reducing levels (12). A previous study reported the protective effect of diltiazem through the reduced calcium transportation and phospholipase activation in SWL-induced kidney damage (13).

Another group of molecules, PDE5 inhibitors, are currently in clinical use for erectile dysfunction and benign prostatic hyperplasia. These drugs are specific for the hydrolysis of cyclic guanosine monophosphate (cGMP) and play an important role in the regulation of nitric oxide (NO) release (14). Tadalafil, a PDE5 inhibitor exhibiting a maximum plasma concentration higher than other PDE5 inhibitors, has been shown to have protective effects in studies of renal I/R injury (15, 16).

In our study, we aimed to assess the histopathological findings of renal trauma and inflammation due to SWL exposure and compared the effects of tadalafil and diltiazem to prevent renal I/R injury in a rat model.

Materials and methods

All experimental studies were performed in compliance with ethical guidelines and confirmed by the local Ethics Committee of Uludag University (2015–02/05). Forty Sprague-Dawley rats were observed in climate controlled chambers. Surgical and SWL procedures were systematically and appropriately performed.

Surgical and SWL Procedure
The rats were anaesthetised via an intramuscular injection of 1mg/kg ketamine HCL and 10 mg/kg xylazine HCl. Laparotomy

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Tab. 1. Comparison of groups between each other regarding the tubular damage and peritubular fibrosis scores. Numbers indicate the p values of Mann-Whitney U test which was utilized to compare two individual groups.

	Tubular damage			Peritubular fibrosis		
	Group S	Group T	Group D	Group S	Group T	Group D
Group C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Group S	–	0.013	0.001	–	0.004	0.001
Group T	–	–	0.369	–	–	0.369

Tab. 2. Comparison of groups between each other regarding HSP-70 staining of glomeruli, cortical and medullar collector tubules. Numbers indicate the p values of Mann-Whitney U test which was utilized to compare two individual groups.

	Glomeruli			Cortical collector tubules			Medullar collector tubules		
	Group S	Group T	Group D	Group S	Group T	Group D	GroupS	Group T	Group D
Group C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Group S	–	0.314	0.512	–	0.002	0.001	–	0.005	0.001
Group T	–	–	0.678	–	–	0.799	–	–	0.718

was performed in all rats. Following abdominal midline incision, two haemoclips were attached to the perirenal fatty tissue. All animals except those in control group, were underwent SWL after laparotomy. Both haemoclips were targeted during the SWL procedure. In a single session, a total of 1500 shock waves were applied to each kidney using the Multimed Classic® (Elmed Inc., 2006, Ankara, Turkey) lithotripsy system at an energy setting of 14 kV.

Groups

A total of 40 male Sprague-Dawley rats were randomized into four groups of 10 rats. In the control group (group C), only laparotomy and metal clipping was performed. In group S, the rats received no treatment before the SWL procedure. In the tadalafil treatment group (group T), tadalafil (Cialis, Lilly, USA) was dissolved in saline solution and administrated as a single dose (1 mg/kg) through an orogastric tube 60 min before the SWL procedure. In the diltiazem treatment group (group D), diltiazem (Diltizem, Mustafa Nevzat Pharmacy, Turkey) was dissolved in saline solution and administrated as a single dose (10 mg/kg) through an orogastric tube 60 min before the SWL procedure. All subjects had bilateral nephrectomy 7 days after the procedure

Histopathological examination

The histopathological examination included a microscopic evaluation of glomeruli and tubular morphology. For this purpose 100 tubules and 20 glomeruli were evaluated randomly and were scored for each section. The morphology of tubules was classified into the four grades based on extent of tubular damage: grade 0 (normal tubule and glomeruli), 1 (mild abnormality in tubules, < 50 %), 2 (moderate abnormality in tubules, 50–90 %) and 3 (most tubules were losts, 90–100 %). Peritubular fibrosis score was also classified into flowing four grades: grade 0 (no fibrosis), 1 (< 50 % peritubular fibrosis), 2 (50–90 % peritubular fibrosis) and 3 (90–10 0% peritubular fibrosis).

Heat shock protein (HSP), a well-known intracellular stress protein, has been reported as an indicator of thermal and oxidative stress (17). First, the paraffinised tissues were deparaffinised with using xylene. Endogenous peroxidase in tissues was deactivated with 1% H_2O_2 solution for 5 min. The tissues were prepared with

10 μM sodium citrate solution and placed in a microwave, and primary antibody (hsp70Ab.2, Clone W27; Neo Markers) was applied to the tissues. HSP-70 staining was detected using the LSAB 2 kit and DAB chromogen (DAKO). Glomeruli and tubular staining was compared among all groups. HSP-70 staining level was recorded as 0 (non-staining), 1 (< 5 % stained), 2 (5–50 % stained) and 3 (> 50 % stained).

Statistical analysis

Statistical analysis was performed using the SPSS software ver. 22.0 (IBM Corporation, Chicago, IL). The non-parametric Kruskal–Wallis test was used to determine the statistically significant differences among the groups. The Mann–Whitney U test was used to compare the differences among the groups. A p value of < 0.05 was considered as statistically significant.

Results

Renal tubular damage and peritubular fibrosis were significantly increased in group S compared with those in the control group (p < 0.001). Tubular damage was significantly increased in group S compared with that in groups T and D (p = 0.013, p = 0.001, respectively). Peritubular fibrosis was significantly decreased in groups T and D compared with that in group S (p = 0.004, p = 0.001, respectively). No significantly difference was detected between the drug-receiving groups (Tab. 1).

The HSP-70 staining intensity in the glomeruli was higher in group S, T and D than in the control group; however differences in the HSP-70 staining intensities in the glomeruli among groups S, T and D were statistically insignificant (Table II). Proximal tubule cells were not stained with HSP-70 in any group.

The HSP-70 staining intensity in cortical and medullar collector tubules was significantly higher in group S than control group (p < 0.001). HSP-70 staining of medullar collector tubules was higher in group S than in groups T and D (p = 0.005 and p = 0.001 respectively). Similarly HSP-70 staining of cortical collector tubules was higher in group S than in groups T and D (p = 0.002 and p = 0.001 respectively). Moreover, no statistically significant difference was detected between groups D and T (Tab. 2).
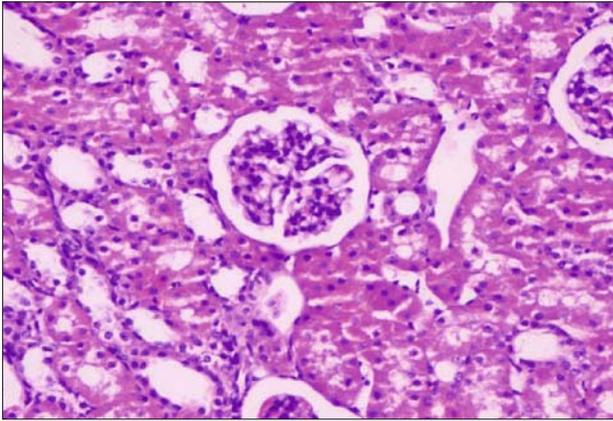
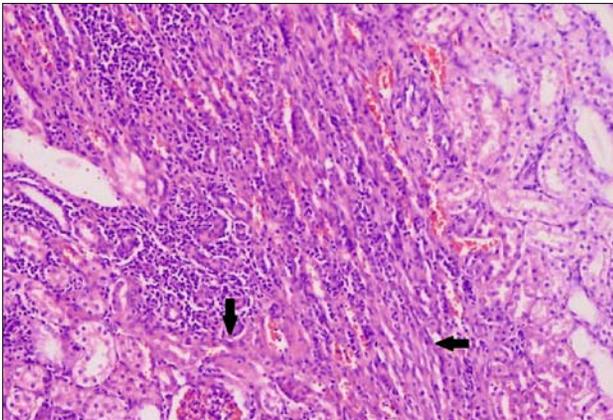**Fig. 1. Regular morphology of renal parenchyma in control group (Hematoxylin–Eosin x200).**

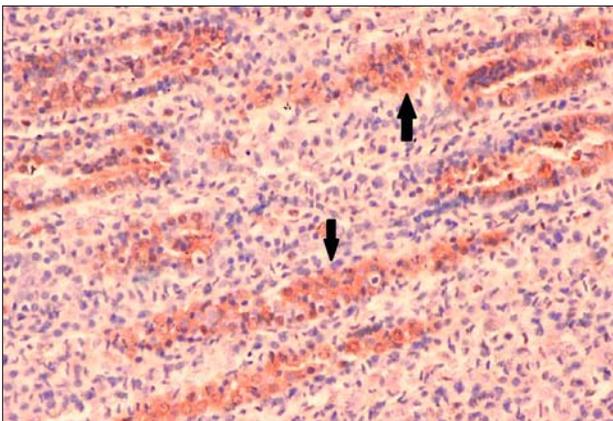**Fig. 2. Tubular necrosis and peritubular fibrosis in group S (Hematoxylin–Eosin 100).**

Fig. 3. HSP-70 staining of collector tubules in group S (HSP-70 x100).

Fig. 4. HSP-70 staining of renal glomerulus in group T (HSP-70 x40).

Fig. 5. HSP-70 staining of renal glomerulus in group D (HSP-70 x40).

Fig. 6. HSP-70 staining of cortical collector tubules in group D (HSP-70 x100).

Microscopic appearance was normal in the control group (Fig. 1). In group S, Bowman's space was enlarged, and most microvilli were lost at the epithelium of the proximal tubules. In cortical and medullar regions, tubular necrosis with hyaline degeneration was noticed. Interstitial space was enlarged, which was correlated with the presence of fibrosis (black arrows, Fig. 2). HSP-70 stain-
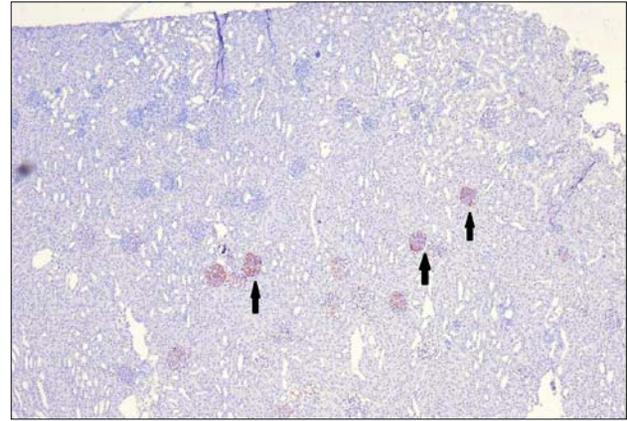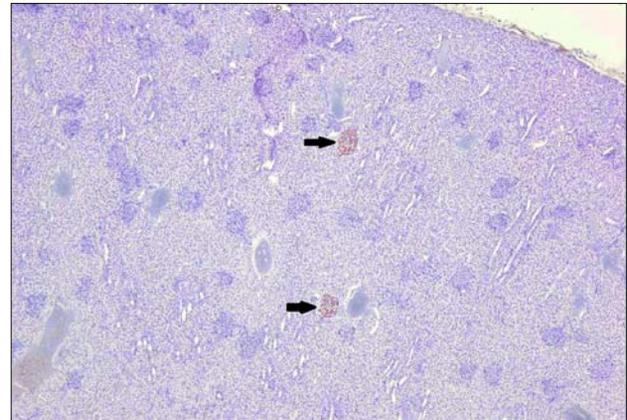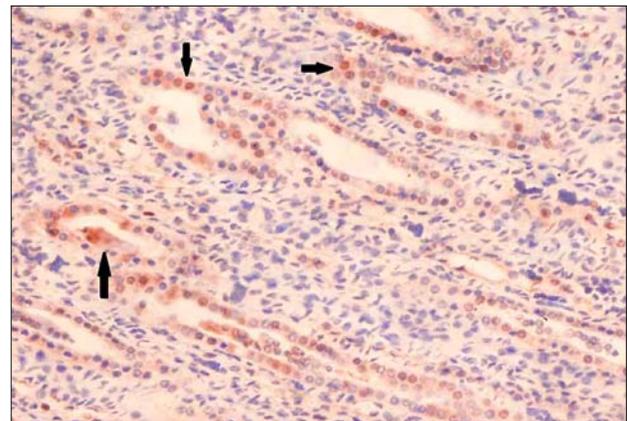
ing of tubular tissue was increased in group S (black arrows, Fig. 3). In group T, the morphology of capillaries in glomeruli and tubular cells was revealed to be normal. Areas with interstitial oedema were commonly observed near blood vessels. HSP-70 immunostaining of glomerular tissue was increased in group T (black arrows, Fig. 4). In group D, the kidney exhibited a pre-

served regular morphology of glomeruli and tubular cells. Microvilli structures were found to be preserved in many epithelial cells lining the proximal tubules. In group D, HSP-70 immunostaining of glomerular tissue was increased (black arrows, Fig. 5), and HSP-70 staining of cortical collector tubules was decreased (black arrows, Fig. 6).

Discussion

SWL is a non-invasive treatment that is commonly used for treating urolithiasis. Although the side effects of SWL can be changed by modifying the device properties, such as shock power and number of shocks. SWL mostly causes vascular trauma and I/R injury. These effects of SWL lead to intraparenchymal haemorrage, migration of inflammatory cells and cytokines, fibrosis and loss of microvilli on tubules (18–20). Additionally, the presence of renal tubular enzymes offer further evidence of tubular injury (20).

CCBs are used in decreasing blood pressure by vascular vasodilation and decreasing peripheral resistance (22). In studies, these drugs have been shown to have beneficial effects on ischemia induced tissues (22, 23). These drugs effectively decrease blood pressure mainly through vasodilation and the reduction of peripheral resistance. During an ischemia period, dysfunction of the Na^+-Ca^+ exchanger leads to an increase in calcium molecules. Subsequently, this intracellular calcium overload activates phospholipases, which occur membrane damage in I/R injury (23).

The effect of CCB on SWL-induced kidney damage was studied in literature. Li et al examined the beneficial effects of nifedipine (a CCB) and allopurinol (a xanthine oxidase inhibitor) on SWL-induced renal injury. The authors reported that patients treated with nifedipine had better results than those not receiving any drugs and there was no difference between the two drugs. They considered that these medicines reduced the effects of the reactive oxygen species on tissues (24). Strohmeier et al studied the effects of verapamil (a CCB) and nifedipine on SWL-treated patients and evaluated the tubular excretion markers after SWL treatment which indicated that these CCBs had protective effects on tubular injury and dysfunction of tubular excretion (5, 25).

In the present study, we preferred to use diltiazem for reducing SWL-induced renal injury, because of the observed beneficial effects of diltiazem in patients undergoing renal transplant surgery. In such cases, diltiazem is given intraoperativelly before removal of arterial clamp in our clinic. Before this study, the effect of diltiazem on HSP-70 levels on the SWL-induced kidney was not published in literature.

PDE5 inhibitors are currently in clinical use for erectile dysfunction, pulmonary hypertension and benign prostate hyperplasia (14, 26, 27). These drugs hydrolyse cGMP and increase NO. NO regulates intracellular calcium levels, induces vasodilatation and improves endothelial dysfunction in I/R injury (28). Gasanov et al demonstrated that a single dose of tadalafil administrated before I/R injury had protective effects against oxidative stress. Renal tubular damage and necrosis were found to be reduced in the microscopic examination of the tadalafil administration group (15).

HSP-70 has been correlated with cytoprotection in response to several injuries, including those due to oxidative stress and ischemia. When renal epithelial cells are injured, changes were observed in the HSP-70 protein structure and HSP-70 mRNA was increased (17). Danisoglu et al reported that tadalafil administration before SWL decreased HSP-70 levels in the SWL-induced kidney, however no difference was observed in HSP-70 staining between 3rd and 7th. days after SWL in the tadalafil treated rats. The authors suggested that renal damage from 3rd to 7th days was continuous and did not decrease (10). HSP-72, another heat shock protein, participates in the molecular protein mechanism and also prevents cellular damage against apoptosis molecules, such as cytochrome-c in mitochondrial membrane injury. In a study tubular damage and renal dysfunction were found to be reduced by HSP-72 in I/R injury of the kidney (29).

Conclusion

The present study revealed that SWL enhanced renal damage in rats. Tadalafil and diltiazem result in reduced HSP-70 staining with better preservation of the renal histology, as well as protective effects on the SWL induced-kidney. On the other hand, no significant differences were observed in treatment groups. The differences between these drugs are in terms of selectivity, side effects and pharmacokinetic features in the body. The choice of the treatment should be made considering the clinical findings and concomitant diseases in the patients.

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