LIMITATION OF HIF-1α WITH PENTOXIFYLLINE ON RENAL TUBULAR ISCHEMIA RESULT OF HIPEROXALURIA AND ESWL

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Summary.- OBJECTIVES: To evaluate hypoxia-inducible factor 1 subunit α (HIF-1α) expression during the performance of extracorporeal shock wave lithotripsy (ESWL) and to investigate the effects of pentoxifylline on HIF-1α expression.

METHODS: One hundred New Zealand Albino rabbit were used in the study divided in 5 groups. There were 20 rabbits in each group. The groups were divided in two parts: early (7 days) and late period (14 days) according to follow up duration. Immunohistochemical analyses were performed using nuclear staining to show HIF-1α expression in rabbit renal tissue sample.

RESULTS: HIF-1α expression was higher in rabbits undergoing ESWL (group 4). In the hyperoxaluria group taking pentoxifylline before ESWL (group 5), HIF-1α expression was lower in both early and late period subgroups (p < 0.05).

CONCLUSION: In this study we evaluated HIF-1α expression and showed that ESWL may cause renal cell injury. Our results suggest that pentoxifylline, as a circulatory regulator agent, may prevent renal cell injury induced by ESWL.

Keywords: Hyperoxaluria, Hypoxia-Inducible Factor 1 Subunit α, Extracorporeal Shock Wave Lithotripsy. Pentoxifylline.
Extracorporeal shock wave lithotripsy (ESWL) is one of the most important discoveries in the history of urology. Yet trials have shown that undesirable side effects could occur in the kidney tissue and the surrounding tissues in the short and long term. Histopathological trials have shown that high-energy shock waves could result in a reduced renal perfusion on the basis of vascular injury and thrombosis (1).

In clinical trials, using radionuclide investigations and Doppler ultrasonography, ESWL was shown to induce a reduction in the renal blood flow, particularly in the advanced age group (1). Accordingly, it was obvious that ESWL would add on to the renal tubular cell injury caused by hyperoxaluria (2). The mechanism underlying this injury involved two routes: lysosomal enzyme activation – inflammation and oxidative stress – free oxygen radicals (3).

The hypoxia-inducible factor 1 (HIF-1), a gene regulation protein, is significantly involved in the adaptation of the cells to the hypoxic environment. This protein induces the vascular endothelial growth factor (VEGF) gene expression and activates the endothelial cells, thereby providing neovascularization. In addition, by increasing the erythropoietin gene expression, it enables adaptation of the cells to the hypoxic environment. In other words, the increase in the HIF expression indicates the presence of hypoxic tissue (4).

Pentoxifylline is a methylxanthine-derived, phosphodiesterase enzyme (PDE) inhibitor agent. The primary mode of action involves the regulation of blood circulation and oxygenation of the tissues by increasing the flexibility of the erythrocytes, decreasing the blood viscosity, reducing the fibrinogen concentration and platelet aggregation, and increasing the microvascular circulation (5, 6).

At the initial stage of our trial, we investigated the potential presence of ischemia secondary to hyperoxaluria and/or ESWL. At the second stage, we tried to demonstrate the effect of pentoxifylline on ischemia with evaluating HIF-1α expression.

**MATERIALS AND METHODS**

The study was performed at Gaziantep University Medical Faculty Experimental Research and Animal Laboratory, after ethical committee approval. A total of 100 adult New Zealand Albino rabbits were used, and the mean weight was 1500±350 gr. The rabbits included in the trial were 10 to 12 months old. All the animals underwent thorough microbiological and biochemical assessments to investigate the presence of systemic infection and infestation.

We distributed the rabbits in different groups randomly. The rabbits were kept in cages (10 rabbits per cage), at the animal laboratory at a temperature of 20-25 ºC. The trial involved a control group and four investigational groups (n=20 in each group) and the investigational groups were divided into two subgroups (n=10 in each subgroup) as early (7 days) and late (14 days) (Table I). Five subgroups were designed as half of them (n=10) male and half of them (n=10) female.

**Study Groups**

- **Group I (n=20) (control group):** The rabbits fed with a standard diet and distilled water. No additional procedure was performed.

- **Group II (n=20):** The rabbits fed with a standard diet and distilled water. 0.75% ethylene glycol was administered in distilled water.

- **Group III (n=20):** The rabbits fed with a standard diet and distilled water. Extracorporeal shock wave lithotripsy was performed to rabbits by sixty shock waves/min, 1000 shock waves in total, with a voltage of 18 kV to the right kidney (Piezoelektrik, Stonelith lithotriptor PCK, Turkey) (7,8).

- **Group IV(n=20):** The rabbits fed with a standard diet and distilled water. 0.75% ethylene glycol was administered in distilled water. Extracorporeal shock wave lithotripsy was performed to rabbits same as group III.

- **Group V(n=20):** The rabbits fed with a standard diet and distilled water. 0.75% ethylene glycol and pentoxifylline (30 mg/kg) was administered in...
distilled water. Extracorporeal shock wave lithotripsy was performed to rabbits same as group III and IV.

In group II, IV and V, ethylene glycol was given to rabbits during 7 days at early period and 14 days at late period. Pentoxifylline was given during 7 days started from 3 days before ESWL. Ethylene glycol and/or pentoxifylline were administered every day until performed nephrectomy. Nephrectomy specimens sent for histopathological evaluation.

Assessment of Crystallization

The tissue histology was examined by light microscope. Olympus BX50 light microscope and Olympus PM10SP camera were used for slide imaging. The tissue samples were fixed in 10% formalin solution and then embedded in paraffin blocks; after obtaining 3-5 μm sections in the microtome, Hematoxylin-Eosin staining was performed. These tissue samples were examined under light microscopy and a grading system established according to the crystal deposition in the renal tubules observed in a field of 1 cm² (Table II).

Detection of HIF-1α Expression

The tissue samples were fixed in 10% formalin solution and then embedded in paraffin blocks; after obtaining 3-5 μm sections in the microtome, deparaffinization was performed in the incubator for 30 minutes. After boiling for 20 minutes in citrate buffer in a microwave, they were kept for 5 minutes in phosphate buffer solution (PBS). After treating with 3% hydrogen peroxide (H₂O₂) for 15 minutes, they were washed with PBS and HIF-1α antibody was dripped over the tissues (Rabbit anti hif-1 alpha polyclonal antibody) and left for 30 minutes. After washing with PBS, they were treated with Biotinylated Goat anti polyclonal for 15 minutes and rinsed with PBS; after 5 minutes of staining with Diaminobenzidine (DAB) chromogen, the sections were ready for examining under the microscope. Under light microscope, nuclear staining was assessed in a 10 x magnification field. Based on the nuclear staining rates, the HIF-1α expression grading system was established, as shown in Table III.

Statistical Analysis

“SPSS 11 for Windows” statistical packaged software was used for statistical assessment. The data were expressed as mean and standard errors. The Wilcoxon test for paired samples was used for the comparisons between two groups; when the number of the groups exceeded 2, Friedman variance analysis was used on repeated measurements.

RESULTS

Early and late period crystallization results

Significant crystal accumulation was observed in the early and late periods in group II,

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Administered only standard diet and distilled water (control group) (Early period)</th>
<th>Administered only standard diet and distilled water (control group) (Late period)</th>
<th>0.75% Ethylene glycol (Early period)</th>
<th>0.75% Ethylene glycol (Late period)</th>
<th>ESWL (Early period)</th>
<th>ESWL (Late period)</th>
<th>0.75% Ethylene glycol + ESWL (Early period)</th>
<th>0.75% Ethylene glycol + ESWL (Late period)</th>
<th>0.75% Ethylene glycol + ESWL + pentoxifylline (Early period)</th>
<th>0.75% Ethylene glycol + ESWL + pentoxifylline (Late period)</th>
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<tbody>
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<td>Group I a</td>
<td>Administered only standard diet and distilled water (control group) (Early period)</td>
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<td>Group I b</td>
<td>Administered only standard diet and distilled water (control group) (Late period)</td>
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<td>Group II a</td>
<td>0.75% Ethylene glycol (Early period)</td>
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<td>Group II b</td>
<td>0.75% Ethylene glycol (Late period)</td>
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<td>Group III a</td>
<td>ESWL (Early period)</td>
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<td>Group III b</td>
<td>ESWL (Late period)</td>
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<td>Group IVa</td>
<td>0.75% Ethylene glycol + ESWL (Early period)</td>
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<tr>
<td>Group IVb</td>
<td>0.75% Ethylene glycol + ESWL (Late period)</td>
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<td>Group Va</td>
<td>0.75% Ethylene glycol + ESWL + pentoxifylline (Early period)</td>
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<tr>
<td>Group Vb</td>
<td>0.75% Ethylene glycol + ESWL + pentoxifylline (Late period)</td>
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which was given a 0.75% ethylene glycol relative to the control group and group III. A comparison of group IV with group II and group III revealed that crystallization became even more severe (p values 0.046 and 0.004 respectively). However, group V receiving pentoxifylline and ESWL was detected to have a limited crystal deposition compared to group III and IV for the early (p values respectively 0.003 and 0.046) and late period (p values respectively 0.004 and 0.034). The crystallization rates for the early and late periods were presented in Table II.

**Hif-1α expression in the early period**

Group I, which was given only tap water, exhibited no nuclear staining. In group IIa receiving ethylene glycol, only one rabbit exhibited minimal nuclear staining, which was not significant relative to the control group and group IIIa (p values respectively 0.31 and 0.18). Similarly, a comparison of group IIa and IIIa were found to be statistically insignificant (p=0.41). Group IVa receiving ethylene glycol and ESWL without pentoxifylline exhibited a more intense nuclear staining relative to group Ia, group IIa and group IIIa in the early period (p values 0.001, 0.004 and 0.004, respectively). Group Va exhibited a nuclear involvement that was on the border in relation to group IVa (p=0.003). The differences between group IIa-IIIa and Va were not significant (p values respectively, 0.083 and 0.56). The HIF-1α expression occurring in the groups in the early period was shown in Table III.

**Hif-1α expression in the late period**

Group Ib, which was given only tap water, exhibited no nuclear staining or HIF-1α expression. In the late period, nuclear staining in group IIb and IIIb was not significant relative to the control group (p values respectively, 0.18 and 0.15). Group IVb receiving ethylene glycol and ESWL without pentoxifylline was exhibited moderate nuclear staining, which was significant relative to group IIb and group IIIb (p values 0.004 and 0.004, respectively). Group Vb receiving pentoxifylline exhibited a significantly low HIF-1α expression relative to group IVb (p=0.014). The HIF-1α expression occurring in the groups in the late period is shown in Table III.

As a result, in the histopathology investigation performed after ESWL, HIF-1α expression was thought to be evidence of hypoxia occurring in the kidneys secondary to ESWL and/or hyperoxaluria. Also when early and late-period HIF-1α expressions were assessed and compared to each other, pentoxifylline was observed to be effective in preventing the hypoxia-related changes occurring in the renal tubules.

**DISCUSSION**

The side effects of ESWL may increase depending on the features of the device, the emergent pressure, the number of shocks and the sessions. ESWL involves effects such as histologically detected neutrophil and fibrin accumulation and complete

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### Table II. Crystal deposition in the early and late period.

<table>
<thead>
<tr>
<th>Groups</th>
<th>(Early Period)</th>
<th>(Late Period)</th>
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<tbody>
<tr>
<td>Group Ia</td>
<td>10/10 (-)</td>
<td>10/10 (-)</td>
</tr>
<tr>
<td>Group IIa</td>
<td>8/10 (+) 2/10 (+)</td>
<td>2/10 (+) 3/10 (+) 5/10 (+)</td>
</tr>
<tr>
<td>Group IIIa</td>
<td>10/10 (-)</td>
<td>10/10 (-)</td>
</tr>
<tr>
<td>Group IVa</td>
<td>4/10 (+) 6/10 (+)</td>
<td>1/10 (+) 2/10 (+) 7/10 (+)</td>
</tr>
<tr>
<td>Group Va</td>
<td>8/10 (+) 2/10 (+)</td>
<td>4/10 (+) 2/10 (+) 4/10 (+)</td>
</tr>
</tbody>
</table>

(+) Minimal crystallization, crystallization in 1-3 tubules
(++): Moderate crystallization, crystallization in 4-7 tubules
(+++): Severe crystallization, crystallization in >7 tubules
tissue destruction in the middle of the focus (9). As indicators of post-ESWL, an increase in the secretion of small molecular proteins (β2 and α1-mikroglobulin) and a reduction in the excretion of Tamm-Horsfall protein are observed (10). In the study of Goktas et al, levels of urinary cytokines were measured to assess the inflammatory changes occurred after ESWL. They reported that level of IL-6 and IL-1α was increased 24 hours and 14 days after ESWL respectively, whereas there was no significant difference on level of TNF (11).

In addition renal changes may be considered as a direct effect of the shock waves. Two direct effects include the membrane injury caused by excessive free oxygen radicals released into the environment and secondary changes occurring on the basis of vascular injury – ischemia (1,9,10 ).

Suhr et al showed that ESWL could lead to permanent microvascular changes that might result in renal secretion and thus hypertension by impairing renal cortical circulation (12). Kaude et al reported morphological and functional changes such as subcapsular hemorrhage, loss of corticomedullary demarcation, perirenal fluid collection, hemorrhage of the existing renal cysts in 63% of the cases after ESWL. In the trial, they performed magnetic resonance imaging, which enabled a comprehensive assessment (13). However, Reis LO et al evaluated renal parenchymal changes analysed with 99mTc-DMSA after ESWL in children with kidney stones. Renal parenchymal changes were determined in the early follow-up period, but irreversible parenchymal changes and/or hypertension were not reported in long-term follow-up period(14).

Clinical and empirical trials were showed the prevention or reversing of the injury caused by ESWL secondary to free radicals by various antioxidants. In a trial using a rat model to investigate the renal injury secondary to high-energy shock waves, renal injury was decreased by using calcium antagonist verapamil (15). The protective effect of verapamil was attributed to its ability to decrease the renal ischemia by increasing renal blood flow and its protective effect on tubulus cells against FOR-mediated membrane injury. The protective effect of another calcium channel blocker, nifedipine, was also demonstrated in clinical trials (10, 16). The routine usage of this medication was controversial since the renal injury was moderate and recovered spontaneously in a majority of patients undergoing ESWL. Fegan et al observed a potentially significant reduction in renal injury in cases which were administered verapamil, mannitol and enalapril before ESWL. However, they demonstrated a significantly reduced fibrosis occurrence by using allopurinol (17). Sheng W et al investigated traditional Chinese Herbs for preventing shock wave induced renal damage in rabbits. Levels of plasma nitric oxide, endothelin-1, TNF-alpha and malondialdehyde were decreased statistically significantly compared with control group, after ESWL (18).

Strohmaier et al reported the protective effects of selenium on the oxidative stress caused by ESWL (19). In the other trial by the same group, the effect of

<table>
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<tr>
<th>Group</th>
<th>(Early Period)</th>
<th>Groups</th>
<th>(Late Period)</th>
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<tbody>
<tr>
<td>Group Ia</td>
<td>10/10</td>
<td>Group Ib</td>
<td>10/10</td>
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<tr>
<td>Group Ila</td>
<td>9/10</td>
<td>Group IIb</td>
<td>8/10 2/10</td>
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<tr>
<td>Group IIIa</td>
<td>8/10 1/10 1/10</td>
<td>Group IIIb</td>
<td>7/10 2/10 1/10</td>
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<tr>
<td>Group IVa</td>
<td>- - 4/10 6/10</td>
<td>Group IVb</td>
<td>4/10 4/10 2/10</td>
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<tr>
<td>Group Va</td>
<td>6/10 4/10 - -</td>
<td>Group Vb</td>
<td>8/10 2/10 - -</td>
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</table>

(+): Minimal intensity of nuclear staining, < 10% nuclear staining
(++): Moderate intensity of nuclear staining, 10-50% nuclear staining
(+++): Severe intensity of nuclear staining, 50-100% nuclear staining
nifedipine on the tubular injury was investigated and the results revealed the protective effect of nifedipine similar to verapamil (10).

ESWL in the subjects with induced hyperoxaluria group were found significantly higher HIF-1α expression than control group. This findings were confirmed that hyperoxaluria and ESWL caused ischemia in the renal tissue. Yencilek et al detected an increase in the HIF-1α expression as an indicator of rising ischemia in the renal tissue secondary to hyperoxaluria. They showed that the ischemic environment could be limited by verapamil in their rabbit model (20).

In our trial, we chosed pentoxifillyne, a circulation-regulating agent, to prevent or decrease the injury occurring in the kidneys resulting from the decreased intrarenal perfusion secondary to ESWL and hyperoxaluria. The primary mode of action for pentoxifillyne involves increasing the blood circulation and oxygenation of the tissues. It exhibits this effect through increasing the flexibility of the erythrocytes, decreasing blood viscosity and decreasing fibrinogen concentration and platelet aggregation (6, 21). As a matter of fact, HIF-1α expression was detected to be significantly decreased in pentoxifillyne-administered subjects relative to the group undergoing ESWL and/or induction of hyperoxaluria.

The limitations of our study was limited sample size and we might perform another subgroup "performed ESWL and receiving pentoxifillyne". In this way, we could compare this group with Group III and V to analyse the effect of pentoxifillyne for hyperoxaluria and ESWL seperately.

CONCLUSION

Based on the literature and the results we obtained, we can report that hyperoxaluria increases the HIF-1α expression, an indicator of tissue ischemia together with renal tubular cell injury and increased crystal deposition. ESWL further intensifies injury to the renal tissue, while pentoxifillyne can decrease the ischemic environment occurring in the renal tissue via specific modes of action and thus limit the HIF-1α expression. We believe that reduction of ESWL and hyperoxaluria-related renal injury occurring in the acute period by pentoxifillyne would enable protection of the renal functions.

REFERENCES AND RECOMMENDED READINGS
(*of special interest, **of outstanding interest)


