Urol Res (1997) 25:257–262

ORIGINAL PAPER

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Morphological correlates of urinary enzyme loss after extracorporeal lithotripsy

Received: 28 July 1996 / Accepted: 27 November 1996

Abstract Urinary loss of the tubular marker enzyme N-acetyl-β-D-glucosaminidase (NAG) immediately following extracorporeal lithotripsy suggests corresponding morphological changes in the kidney. To date, the morphological correlate of the enzymuria remains unclear. In this animal study with Wistar rats acute morphological changes in the tubulus cells beneath isolated tubulus necrosis were demonstrated. The mechanically induced lesions of the cell organelles included fragmentation of the lysosomes and severe alterations of the cell membrane. The tubulus damage was quantified. With the help of histochemical NAG staining and electron microscopic observations, a significant correlation between number and intensity of shock waves and tubular damage was found. The intracellular lesions described here are at least part of the morphological basis of shock-wave-induced enzymuria. The results show that enzymatic changes in urine reflect visible renal damage.

Key words Extracorporeal lithotripsy · Tubular lesions · Tubular marker enzyme

Urinary protein losses are regularly observed following extracorporeal lithotripsy (EL) [7, 10, 32]. Potential marker proteins for renal parenchymal damage are particularly interesting in this connection because the extent of renal damage from treatment with different lithotriptor generations within the therapeutic shock wave dose range in connection with imaging techniques varies widely and is nearly impossible to estimate [19]. Not all urine proteins are suitable for this purpose. In an experimental clinical study, it was shown that EL-induced haematuria was the main cause of raised urine concentrations of apolipoprotein A1, albumin, immunoglobulin G and transferrin [8]. These proteins are thus disqualified as markers. This dependence was not seen for α_1-microglobulin and *N*-acetyl-β-D-glucosaminidase (NAG). Whereas microglobulin elimination can also be influenced by glomerular filter damage, NAG enzymuria is a specific marker for tubulus damage [2, 13, 26, 27], especially in the proximal tubuli. Loss of this intracellular lysosomal enzyme immediately following EL [22, 29] suggests corresponding morphological changes in the tubulus system. Isolated fibrinoid tubulus necroses, tubular dilatation and atrophy [5, 21] and tubular dilatation with vacuolar and hydropic degeneration [9] have been described. Whether graduated lesions exist beneath these lesions, and whether these depend on the shock wave energy applied, is not known. We therefore also do not know how accurately enzymatic changes in urine reflect the extent of visible renal damage [3]. In an animal model, we pursued the question of which acute morphological changes occur in the tubulus cells as the basis of enzymuria and what dependencies exist between number of shock waves, intensity and the extent of the renal damage.

Material and methods

Four study series were used to delimit the therapeutic shock wave energy range:

1. In an animal preparation, confirmed ultrasonic location of rat kidneys with the piezolith inline scanner was established.
2. Under a decreasing number and intensity of shock waves, the point was determined at which macroscopically visible lesions were no longer recognizable in the rat preparation. These levels were defined as the upper limits of the therapeutic range.
3. To determine the minimum shock wave energy level required for a reproducible case of enzymuria, NAG elimination in 24 h urine (U/g creatinine) was determined according to the method of Maruhn [14, 24] [creatinine determination by the Jaffe method (Boehringer, Mannheim, Germany)] following application of an increasing number and intensity of shock waves to the left kidneys of Wistar rats. The treatment modalities that

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resulted in increases in enzymuria in all animals were defined as the lower equipment-specific therapeutic limit.

4. The constancy of the applied shock wave energy was checked indirectly by monitoring the disintegrative effect on standardized calcium sulphate stones [23] (High Medical Technologies, Kreuzlingen, Switzerland). Checking of the precise location of the focal point was done in accordance with the manufacturer's instructions for the lithotriptor (cross-hairs in a water bath).

In 20 male Wistar rats weighing 324 ± 17 g, the lower pole of the left kidney was subjected to an extracorporeal piezoelectric shock wave application (EPL) with a Piezolith 2500 (Wolf, Germany). Number of shock waves (SW) and intensity were varied (Table 1). Kidney removal was undertaken in the same anaesthetic session. The morphological changes were studied in the treated left kidney and the right kidneys were used for the control.

Shock wave application and kidney removal were carried out with the animals under intraperitoneal sodium phenobarbitol narcosis (6 g phenobarbitol/100 ml aqueous solution; 0.1 ml/100 g body weight). Kidney location was determined after shaving using the 3.5-MHz ultrasonic inline sector scanner of the Piezolith. We used degasified water (34°C) to obtain acoustic coupling, replacing the commercially available acoustic membrane with a thinner one to enhance the ultrasonic image.

Transperitoneal removal of both kidneys followed 30 min after EPL. The lower poles of both kidneys were halved longitudinally following macroscopic evaluation. One of the halves was fixed in formalin and embedded in paraffin. Sections 2 µm thick were then microtomed at five different cutting levels as transverse sections of the lower pole to ensure that all relevant parts of the renal parenchyma (e.g. capsule, cortex, medulla, papilla) were analysed. Following PAS, haematoxylin-eosin and Goldner staining, the sections were subjected to histological evaluation, and the extent of the renal parenchymal damage (for criteria see Table 2) was quantified by means of a cross code separately according to a dyeing method and left and right kidney (control). The allocation of the preparations to the treatment groups was unknown to the researcher. In the other half of the lower pole, tubulus damage was studied using histochemical NAG staining and a transmission electron microscope.

NAG staining consisted of pretreatment of the preparations with prefixation in 4% paraformaldehyde in citric acid phosphate buffer pH 7.2 (7 h) [12]. Then 8-µm cryostatic sections were made from the preparations, which were then stained for 60 min at 37°C in an incubation medium. For intracellular staining of NAG, we used simultaneous azo-coupling modified according to Hayashi [6]. We dissolved 20 mg naphthol-AS-Bl-β-*N*-acetyl-D-glucosamide (Sigma, Daisenhofen, Germany) in 1.25 ml *N*,*N*-dimethylformamide and mixed the solution with buffered hexazonium-*p*-rosaniline (buffer: citric acid phosphate buffer, pH 6.0) [1]. pH adjustment to 5.0 was done with 1 *N* NaOH. For aftertreatment the preparations were rinsed in distilled water and stained with haematoxylin-eosin. After covering with Kaiser's glycerin gelatin C (Merck, Darmstadt, Germany), the preparations were placed in 4% formaldehyde and rinsed in phosphate buffer, pH 7.3.

Evaluation of the tubulus damage in the histochemical preparation was at first descriptive. A quantitative assessment of the lesions was then undertaken with morphometric methods according to the size and number of NAG-positive lysosomes, lysosome position and number of NAG-positive fragments in the tubulus lumen. Eight sections were evaluated in each animal (100X): five tubulus cross-sections each. Only those tubuli were considered cross-sectioned that showed 6–11 cell nuclei and had comparable diameters and an intact tubulus geometry. Tubuli sections lengthwise or obliquely were not considered due to the difficulties involved in quantification. The data were recorded groupwise in tables as well as numerically. Statistical data preparation included the calculation of the mean, the standard deviation, variance analysis and the Tukey test.

The electron microscope preparations were fixed in 3% glutaraldehyde in 0.1 *M* cacodylate buffer, then placed in 2% OsO_4 for 2 h. The ultrathin sections (400 Å) made following dewatering (alcohol series) were assessed after contrasting with lead citrate and uranyl acetate in a Siemens 101 transmission electron microscope (Siemens, Erlangen, Germany).

Table 1 EPL treatment of rat kidneys (n = 20) under groupwise variation of number and intensity (*Int*) of shock waves (*SW*)

Group	Number of SW	Intensity of SW	Animals
1000 SW/Int 5	1000	5	5
2000 SW/Int 5	2000	5	5
3000 SW/Int 5	3000	5	5
3000 SW/Int 2	3000	2	5

Results

Preliminary experiments

1. The upper limits of the therapeutic range were 3000 SW at intensity level 5. At higher intensities, even low pulse rates resulted in visible renal haematomas in some cases; haematomas were frequent at higher pulse numbers.
2. A confirmed urine NAG increase in all animals treated was observed beginning at 1000 SW, intensity level 5. Mean NAG enzymuria was 7.47 U/g creatinine (SD 3.06) before EPL and 11.14 U/g creatinine (SD 3.31) after EPL. The differences were significant ($P < 0.05$ t-test).
3. Maximum deviation of disintegrative shock wave transducer capacity was 5%.

Table 2 Histopathologically detectable renal changes immediately following EPL according to number of shock waves (*SW*) and intensity (*Int*) applied

Shock wave parameters	1000 SW/Int 5	2000 SW/Int 5	3000 SW/Int 5	3000 SW/Int 2
Perirenal haemorrhage	0/5	1/5	0/5	0/5
Blood in collecting system	3/5	2/5	2/5	2/5
Subcapsular haemorrhage	1/5	1/5	1/5	1/5
Intraparenchymal haemorrhage	1/5	1/5	1/5	0/5
Perivascular haemorrhage	2/5	3/5	2/5	2/5
Vascular rupture	0/5	0/5	0/5	0/5
Isolated tubular necrosis	1/5	1/5	2/5	1/5
Protein in tubuli	1/5	2/5	4/5	2/5
Tubular dilatation	0/5	1/5	0/5	0/5
Tubular cell swelling	0/5	2/5	2/5	1/5
Glomerular change	2/5	4/5	3/5	2/5

Main experiments

Macroscopic abnormalities upon nephrectomy were found in only one animal (2000 SW, intensity 5). A small haematoma was found in the renal fat capsule. Conventional microscopic examination revealed a similar pattern of histological damage with normal control kidneys on the treatment side in all groups: minimum subcapsular and intraparenchymal haemorrhages, more severe at the corticomedullary borders and in perivascular areas (Fig. 1). Glomerular lesions in the form of loop collapse, haemorrhaging or protein precipitates in the glomerulus were rare. Changes in the tubulus system with blood and protein in the tubulus lumen, expanded tubuli, swollen tubuli and, rarely, cellular necrosis were observed in all of the kidneys treated. The type and severity typical for the pattern of damage showed no dependence on the number or intensity of shock waves (Table 2).

Tubuli of the control kidneys stained with histochemical NAG showed a clear cellular architecture. Staining of the lysosomes was most intensive in the proximal tubuli; the number and size of NAG-positive granula (lysosomes) per cell varied minimally (1.1–1.4 μm) and they were spherical, sharply defined and distributed solely in the basal cytoplasm. We saw no staining of the cytoplasm. An increasing number of shock waves caused the NAG-positive granula to lose their basal location stability, grow smaller and increase in number. At the same time, NAG-positive fragments appeared in the tubulus lumen (Fig. 2). These effects were most pronounced when 3000 SW were applied at intensity level 5. At 3000 SW and the lower intensity level 2, the changes were less pronounced than at intensity level 5.

Morphometric comparisons of control and treatment kidneys were only necessary for exact measurement of the lesions at 1000 and 2000 SW at intensity 5. The increased damage in the group with 3000 SW at intensity 5 and reduced tubulus damage at lower intensities was evident without morphometry. The morphometric results are shown here in tabular form (Table 3).

Typical artefacts in the electron microscopic preparations as described for immersion fixation by several authors [15, 16] were not detected either in the right-side control kidneys or in the treated kidneys. The control kidneys also served as reference findings for assessment of the treated kidneys.

Application of increasing numbers of shock waves led to increasing ultrastructural changes, characterized by the occurrence of endocytic vacuoles, swelling and – in some cases – fragmentation of mitochondria and alter-

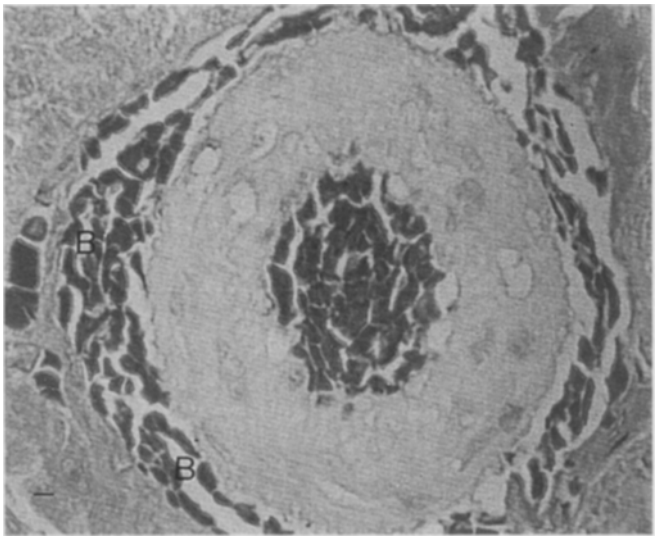

Fig. 1 Periarterial haemorrhage (*B*) following piezoelectric lithotripsy. Goldner staining, X160

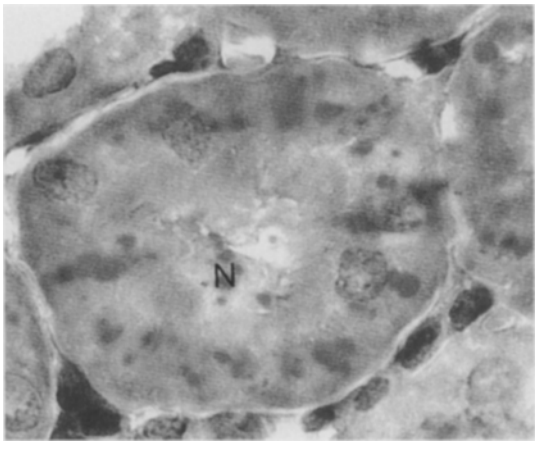

Fig. 2 Cross-section through proximal tubulus of rat following application of 1000 shock waves at intensity 5. Loss of basal position stability of NAG-positive granula (*N*) with dislocation into the lumen-side cell segment. Increased occurrence of small NAG-positive granula, which probably represent lysosome fragments. NAG staining, X1000

Table 3 Influence of number of shock waves on morphometrically detectable tubulus cell changes. * Variance analysis: differences according to number of shock waves significant at $P < 0.05$ (Tukey test)

Therapeutic modality	Control	1000 SW/Int 5	2000 SW/Int 5
Lysosome size ± SD(μm)*	1.264 ± 0.134	1.205 ± 0.03	1.096 ± 0.032
(range)	(1.111 – 1.406)	(1.150 – 1.237)	(1.069 – 1.131)
Lysosome count ± SD*	34.95 ± 3.12	37.55 ± 3.5	45.23 ± 7.46
(range)	(32.00 – 38.75)	(32.13 – 41.88)	(37.50 – 54.88)
Lysosomes in apical cell segment*	0.4 ± 0.35	1.58 ± 0.76	3.1 ± 0.97
(range)	(0 – 0.875)	(0.63 – 2.63)	(2.25 – 4.75)
Lysomes in tubulus lumen	1	2	15

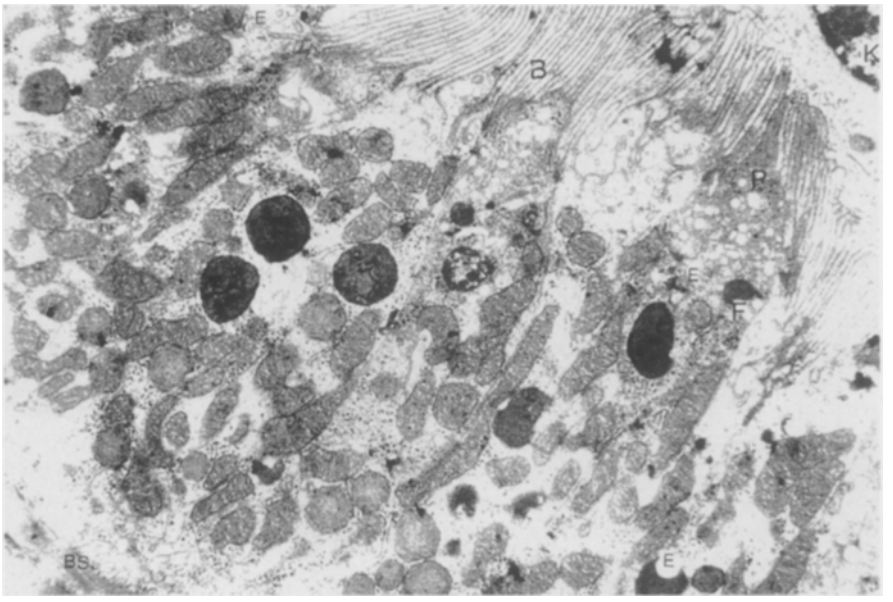

Fig. 3 Proximal tubulus cell of rat following application of 2000 shock waves at intensity 5. Fragmented lysosomes (*F*), cell contents with nucleus in tubulus lumen (*N*), incipient protrusion (*P*) through the flattened ciliated border, endocytic vacuoles (*E*). *BS* basilemma (basement membrane). Transmission electron microscope image, X7000

Table 4 Electron microscopic findings as dependent on number of shock waves (*SW*) and intensity (*Int*). Pathological findings: –none, + isolated, ++ frequent, +++ highly frequent

	Control	1000 SW/Int 5	2000 SW/Int 5	3000 SW/Int 5	3000 SW/Int 2
Endocytic vacuoles	–	++	++	+++	++
Reduction of lysosome size	–	+	++	++	++
Number of lysosomes	–	–	++	+++	++
Lysosome fragments	–	+	++	+++	++
Mitochondrial swelling	–	–	++	++	++
Mitochondrial rupture	–	–	+	++	+
Protrusions in brush border	–	–	+	++	+
Rupture of brush border	–	–	+	++	+
Cell organelles in tubulus lumen	–	+	++	+++	++

ations in the ciliated border with protrusion and even border rupture (Fig. 3, Table 4). Lysosomal changes were characterized by the occurrence of lysosome fragments and increased occurrence of small lysosomes. The numbers of cell organelles in the tubulus lumen increased with the pulse rate.

Discussion

The advantages of small animal models for quantifying morphological studies are the low cost and the potentially high case numbers per group [3]. Rats are particularly suitable for assessment of functional and anatomical tubulus lesions because their intrarenal enzyme distribution is similar to that in humans [20]. Objections to the principle of small animal studies with EL are based on the discrepancy between the size of the shock wave focus and the size of the kidney [19]. We therefore performed EL with the Piezolith, the small focus of which facilitates SW application to the partial volume of a kidney analogous to that in humans with an energy-dependent size of 3×5 to 4×12 mm. This lithotriptor also features real-time sonographic positioning without the potentially tubulotoxic effects [24] of contrast agents used in the X-ray positioning method frequently used in animal models [21]. To account for size-dependent tissue attenuation [4], we determined an equipment-related and species-related therapeutic range in preliminary experiments under standard conditions that is within the energy range used in humans.

The microscopically recorded early renal damage in our animals following EL corresponds to a large extent to the damage pattern known from other experimenters: subcapsular and intraparenchymal haemorrhaging, vascular alterations [18, 28], and minimum changes in the glomeruli and tubuli [9, 21]. Histopathologically, the EL-induced kidney lesions can be classified into three levels of severity [19]:

Grade 1: intrarenal petechial blood spotting and haemorrhages
Grade 2: subcapsula haematomas, haematopyelon, papillary necrosis
Grade 3: perirenal haematomas with renal capsule rupture

According to this classification, typical haemorrhage-associated lesions were not very pronounced. We found isolated animals with first-degree and second-degree renal lesions in all groups. On the other hand, we were unable to confirm a number of earlier reports of a dependence of these typical renal damage types on the therapeutic parameters number of SW and intensity [2, 17, 21]. As determined earlier by Neisius [19] in a dog model at levels within the energy range applied here, this dependence does not hold for extracorporeal piezoelectric lithotripsy. It is conceivable that the dose limit required to cause regular coarse histological renal lesions [11] is not reached by the Piezolith, even in the medium intensity range.

The possibility of assessment of the tubulus system of the kidneys following EL by conventional histological methods is limited. The tubuli are of particular interest, since they represent a major portion of the renal parenchyma and EL-induced enzymuria leads one to suspect tubulus lesions.

In our study of the morphological correlate to enzyme losses following EL, we used a histochemical NAG staining method for the tubular apparatus for the first time. This modified staining technique facilitates clearly defined imaging of NAG-positive lysosomes. Diffuse NAG activity in the cytoplasm was not detected. On the basis of the right-side control kidneys, we demonstrated that the NAG-positive lysosomes are localized in the basal cytoplasm of the tubulus cells, confirming the observations of Hayashi [6]. In agreement with the results obtained by Zeller [33], the size of these lysosomes varies only slightly. The form of the lysosomes was spherical and single lysosomes were readily delimited. Shock wave application within an energy range that would certainly lead to increased NAG enzymuria causes worsening tubulus cell lesions with increasing number and intensity of shock waves. We are dealing here with early damage immediately after shock wave exposure, so that a loss of basic position stability is more likely a mechanical result of the shock waves than a secondary functional state. The other histochemical findings can also be explained mechanically in the context of the electron microscopic observations. Occurrence of an increasing number of small NAG-positive lysosomes is the electron microscopic equivalent of the occurrence of lysosome fragments. As a result of fragmentation, the cytoplasm is NAG-positive. Serious alterations are seen parallel to this in other cell organelles as well, in particular mitochondria.

The increasing percentage of NAG-positive granula in the tubular lumina also has an electron microscopic correlate. With increasing shock wave energy, cell membrane damage becomes more frequent, at first in the form of protrusions, and finally as membrane ruptures into the lumina, whereby cell organelles enter the tubulus lumen. These effects are also probably related to mechanical causes due to the brief time span between EL and nephrectomy. It remains unclear why the numbers of small endocytic vacuoles increased. Vacuoles in the cytoplasm may develop from dilated apical vacuoles, dilated endoplasmic reticulum or the Golgi apparatus. We did not, however, detect any damage to these cell organelles in our preparations.

The observed tubulus damage can be quantified. With the help of histochemical NAG staining, we demonstrated a significant correlation between the shock wave parameters number of SW and intensity and the tubular damage. These factors are at least part of the morphological basis of shock-wave-induced enzymuria, the severity of which also depends on how much shock wave energy is applied [25, 30, 31]. Assessment of ultrastructural tubulus cell changes complements standardized determination of the morphology of the shock wave trauma and facilitates comparison of therapeutic efficacy with the degree of invasiveness involved.

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