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ORIGINAL PAPER

Inactivation of bacteria inoculated inside urinary stone-phantoms using intracorporeal lithotripters

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Abstract Intracorporeal lithotripsy is used to treat a high percentage of urinary calculi. Urinary calculi may contain bacteria, which might cause septicemia after lithotripsy; however, little is known about the effects of lithotripters on the viability of microorganisms inside renal calculi. The objectives of this study were to evaluate the bactericidal effect, and the potential effect on intra-bacterial protein release of four different intracorporeal lithotripters on *Escherichia coli* (*E. coli*) inoculated inside artificial kidney stones. An electrohydraulic, a pneumatic, an ultrasonic, and a holmium-laser lithotripter were used to pulverize a set of infected kidney stones inside a test tube containing a saline solution. Two different energy levels were tested per lithotripter. The stones were manufactured by mixing gypsum cement and Vel-mix-stone with a suspension containing *E. coli*. Results were analyzed by analysis of variance. The release of intracellular protein was measured with a spectrophotometer. Bacteria inactivation was observed with all lithotripters. The highest percentage of inactivated bacteria was obtained with the electrohydraulic lithotripter. The smallest effect was observed using the holmium-laser lithotripter. A relatively high amount of intracellular protein was released into the saline solution after stone pulverization. Intracorporeal lithotripters inactivate a high percentage of bacteria during stone comminution; however, intracellular protein is released, increasing the probability of septicemia.

Keywords Lithotripsy · *Escherichia coli* · Urinary calculi · Urinary tract infection · Septicemia

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Introduction

Percutaneous and endoscopic lithotripsy hold an important place in the treatment of urinary calculi. Devices in use include electrohydraulic, ultrasonic, holmium-laser and pneumatic lithotripters. Many patients with infection stones have a urinary tract infection [1, 2]. Bacteria living close to the surface of a stone can differ from microorganisms proliferating inside it. Furthermore, antibiotics may not penetrate into the stone, and bacteria inside it can produce an infection following percutaneous or endoscopic procedures. The incidence of sepsis caused by the entry of bacteria or their products into the bloodstream has been reported to occur in 1–2% of all patients, and the mortality rate is high when septic shock syndrome ensues [3–5]. Lipopolysaccharide existing in Gram-negative bacteria has a pivotal role in inducing septicemia and bacteremia [5, 6]. Even if reduction in renal infections after extracorporeal shock wave lithotripsy was reported [7, 8], little has been published on the bactericidal effect of shock waves [9–11]. As far as we know, a single article in the literature has reported the effect of intracorporeal lithotripters on struvite stones infected with *Proteus mirabilis* [12], and no articles dealing with the action of intracorporeal lithotripters on infected artificial stones have been published.

The aim of this research was to compare the bactericidal effect of four intracorporeal lithotripters on bacteria contained inside artificial stones. *Escherichia coli* (*E. coli*) was chosen for this study because it is a well-known non-spore-forming, Gram-negative bacterium, which is the first cause of urinary tract infection. A nonpathogenic strain was selected for security reasons, and well-standardized artificial kidney stones were manufactured. These stones provide reliable results and have been used for many years to examine the effectiveness of lithotripters.

Methods

Independent viability counts, using the plate count method, were performed on non-treated infected stones (control stones), shock-wave-treated infected stones and *E. coli* suspensions.

The lithotripters

Four intracorporeal lithotripters were used in this study: (a) a holmium 20 W Versa Pulse Power Suite laser lithotripter (Lumenis Ltd, Santa Clara, CA, USA) with a 520 μm fiber, (b) a USL-2000 ultrasonic lithotripter (Circon Acmi, Stamford, CT, USA), (c) a Calcutript, model 27080C electrohydraulic lithotripter (Karl Storz Endoscope, Tuttlingen, Germany) with a 1.6 Fr fiber, and (d) an EMS pneumatic lithotripter (Lithoclast, Le Sentier, Switzerland). Two energy settings (H = high; L = low) were chosen for each lithotripter. Direct comparisons between energy settings, frequency or "dose" should not be done, because each lithotripter operates with a different physical principle. The energy settings and exposure times used in this study were similar to those used in clinical practice.

Non-infected artificial kidney stones

Stones were manufactured at our laboratory by mixing 45% gypsum cement and 5% Vel-mix-stone (Kerr Division of Syborn Corp., Romulus, MI, USA) with 50% distilled water, using a hand mixer. The mixture was cast in cylindrical molds and dried for 4 h at 30°C. The desired compressive strength was obtained in previous experiments (not reported here) by varying the amount of gypsum, Velmix and water. The length and diameter of the stones was 10 ± 0.2 mm, and 10 ± 0.1 mm, respectively. Mean weight of non-infected stones was 0.785 ± 0.012 g.

Infected artificial kidney stones

A strain of *E. coli* ATCC 10536 was used to inoculate a set of stones. All infected stones were manufactured as explained in the previous section; however, in this case a saline solution containing *E. coli* was used instead of distilled water. Unless otherwise indicated, all microbiological media used were purchased from Bioxon (Becton Dickinson, Mexico City, Mexico). Microorganisms were stored on nutrient agar (Merck, Darmstadt, Germany) at about 5°C and transferred each month. The strain was activated by incubation on trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBY) at 35°C for 24 h. Cells were obtained in the stationary phase of growth. Bacteria were concentrated by centrifugation (3,000 rpm) for 10 min at room temperature and resuspended (0.85% w/v) in isotonic saline solution. The initial viable count was about 2.34×10^7 colony-forming units per milliliter (CFU/ml). A 0.01% (v/w) inoculum of the bacterial suspension was used. Stones were prepared, dried for 4 h close to two burners at about 30°C, and used immediately. A set of eight non-treated infected stones was crushed with a hand press to determine the variations in the amount of bacteria inoculated inside the stone phantoms.

Stone comminution

Infected stones were placed on a copper mesh with 1.8 mm by 1.8 mm openings, fastened to the bottom of a lucite test tube (Fig. 1). The lucite tube was placed inside a standard laboratory test tube, containing 10 ml of saline solution. The tip of the lithotripter was introduced inside the lucite tube in order to fragment the stone (Fig. 2). Energy and frequency settings are shown in Table 1. Previous experiments showed that 4 and 2 min were needed to completely fracture the stones using the laser, the ultrasonic, and the electrohydraulic lithotripter at L and H energy levels, respectively. With the pneumatic lithotripter 4 min were needed to comminute these stones to fragments smaller than 1.8 mm, at both energy levels. Five infected stones were fragmented with each lithotripter at each level. After treatment all remaining stone fragments were crushed with the hand press. The suspension containing stone powder and bacteria was centrifuged (3,000 rpm) at room temperature and incubated on agar plates for 36 h at 36 ± 0.5°C. Viable counts were made by plating on TSBY. The bactericidal action was defined as the logarithmic difference between control (untreated) and treated stones, i.e., $\log_{10}(N_0) - \log_{10}(N) = \log_{10}(N_0/N)$ CFU/ml. N is the number of viable bacteria obtained from treated, and N_0 the number of viable bacteria resulting from control (untreated) stones.

Bactericidal effect on *E. coli* suspensions

To study the effect on bacteria outside the stone, an *E. coli* suspension was prepared as described previously and

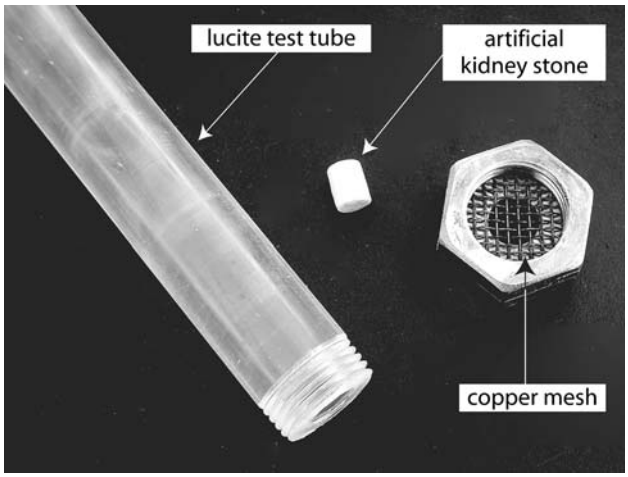

Fig. 1 Photograph of a special test tube, with copper-mesh bottom (disassembled), manufactured to fracture a urinary stone in vitro, using an intracorporeal lithotripter

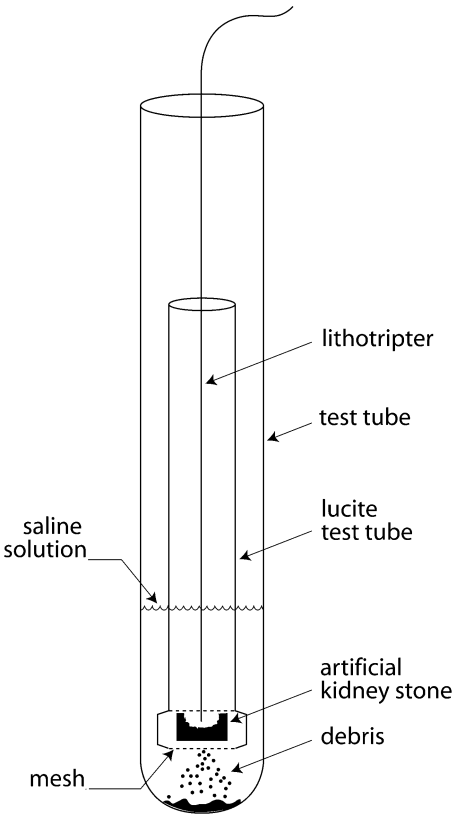

Fig. 2 Sketch of the experimental set-up to fragment urinary stones in vitro

subjected to the action of each lithotripter, using the same energy settings and exposure times as for *E.coli* inside infected stones (Table 1). The tip of each lithotripter was immersed in 20 ml of bacterial suspension inside a sterile test tube. The process was repeated five times for each lithotripter at each energy setting.

Table 1 Settings used with the laser, the ultrasonic, the pneumatic, and the electrohydraulic lithotripter

Level	Energy (J)	Rate (Hz)	Power (W)	Treatment time (min)
Laser lithotripter				
L	0.5	5	2.5	4
H	2.5	5	12.5	2
Level	Pulse rate setting (%)		Amplitude setting (%)	Treatment time (min)
Ultrasonic lithotripter				
L	55		55	4
H	100		100	2
Level		Air pressure (power) (bar)		Treatment time (min)
Pneumatic lithotripter				
L		1.2		4
H		3.0		4
Level	Energy setting (no units)		Frequency setting (no units)	Treatment time (min)
Electrohydraulic lithotripter				
L	1		C	4
H	3		C	2

L low, *H* high

Endotoxin release

Stone comminution of infected artificial kidney stones was repeated as described before, in order to measure the release of protein as a result of lithotripsy. After treatment, 5 ml of the suspension were centrifuged (3,000 rpm) at room temperature, and placed inside a spectrophotometer to measure absorbance at 280 nm [13].

Data analysis

Data were analyzed with the standard aov function of the R language (R Foundation for Statistical Computing, Vienna, Austria) [14]. Replicate influence was included as a blocking effect.

Results

Inactivation of bacteria inoculated inside artificial kidney stones

Mean initial viable count for infected stones, pulverized only with the hand press, was 7.37 $\log_{10}$ $(N_0/N) \pm 0.34$ $\log_{10}$ (N_0/N) CFU/ml. The variation in the amount of bacteria

inside these stones was not significant. Bacteria inactivation after lithotripsy to infected stones is given in Table 2. Figure 3 shows these results on two graphs. Complete inactivation (7.37 $\log_{10}$ (N_0/N) CFU/ml) resulted with the electrohydraulic lithotripter at both energy levels. No significant difference was obtained between inactivation obtained with the other lithotripters at their low energy settings. Increasing the energy resulted in higher bacteria inactivation for these three lithotripters (Fig. 3). The lowest inactivation was achieved with the laser lithotripter. The analysis of variance (ANOVA) revealed a statistical significant interaction between the energy level and the bactericidal effect of the laser, pneumatic and ultrasonic lithotripter, i.e., bacteria inactivation increased as the energy setting was increased.

Table 2 Mean logarithmic inactivation (CFU/ml) of *Escherichia coli* immobilized inside artificial renal calculi, after in vitro lithotripsy using four different intracorporeal lithotripters at two energy levels

Lithotripter	Level	Inactivation $\log_{10}$ (N_0/N) CFU/ml[a]	Standard deviation	*P* value
Laser	H	3.42	0.30	0.092
Ultrasonic	H	5.87	0.28	0.022
Pneumatic	H	6.24	1.59	0.080
Electrohydraulic	H	7.37	0.00	0.500
Laser	L	2.51	0.11	0.092
Ultrasonic	L	2.89	0.43	0.022
Pneumatic	L	3.29	1.07	0.080
Electrohydraulic	L	7.37	0.00	0.500

L low, *H* high

[a] Initial viable count = 7.37 $\log_{10}$ (N_0/N) CFU/ml

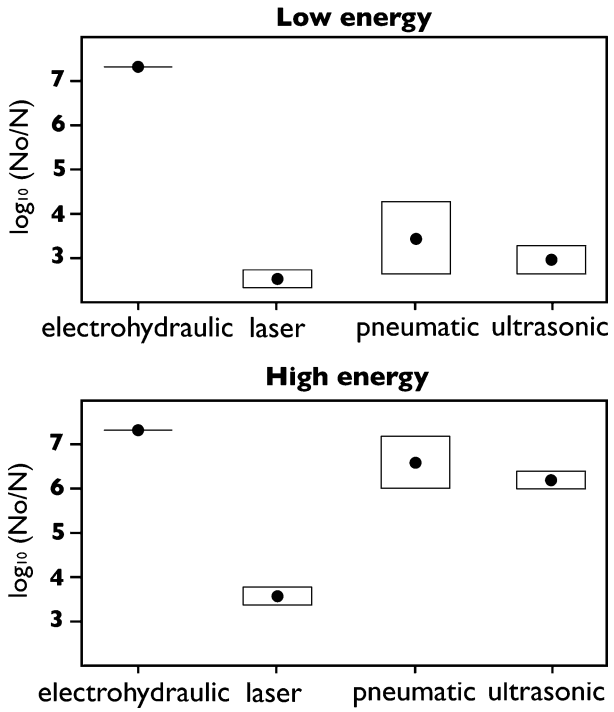

Fig. 3 Logarithmic inactivation of *Escherichia coli* immobilized inside artificial kidney stones, after in vitro lithotripsy with four different intracorporeal lithotripters at two energy levels

Inactivation of bacteria in suspension

No bacteria inactivation was observed using the pneumatic lithotripter in *E. coli* suspension without a stone to break. The other three lithotripters completely inactivated *E. coli* in suspension. This means that no viable bacteria could be detected after exposure to the laser, the electrohydraulic and the ultrasonic lithotripter. The temperature of the suspension containing *E. coli* increased up to about 60°C when using these lithotripters, except for the pneumatic lithotripter. This temperature increase was not observed during lithotripsy of infected stones.

Bacteria endotoxin release

The mean intracellular protein concentration, obtained for each lithotripter is shown in Fig. 4. The laser, the pneumatic and the ultrasonic lithotripters released a similar amount of protein at both energy levels; however, the electrohydraulic lithotripter showed a remarkable result. Maximum protein release occurred at low energy and almost no absorbance was measured for the bacteria suspension exposed to high energy. Considering that at this energy level all of the bacteria were inactivated (Table 2), it seems that proteins were denaturized by the treatment rendering them undetectable by the absorbance method. The ANOVA revealed that there is no significant difference between the amount of protein released with the laser, the pneumatic, and the ultrasonic lithotripters at both energy levels.

Discussion

One third of all stones located in the urinary tract have bacteria inside and may be responsible for systemic infections [3, 4]. Penetration of bacteria or their toxins into the bloodstream may occur after endoscopic manipulation. Patients with infected stones or urine infection in the proximity of the stones (renal pelvis), present a risk to develop urosepsis at least four times greater compared to patients with only positive midstream or bladder culture [4].

We are confident about the reliability of our results, because plate counting is a standard method in microbiology and enough independent replicas were done. Data capturing was triple checked. A well known software (R Software and Environment) [14] was used for mathematical calculations and analysis of variance (ANOVA).

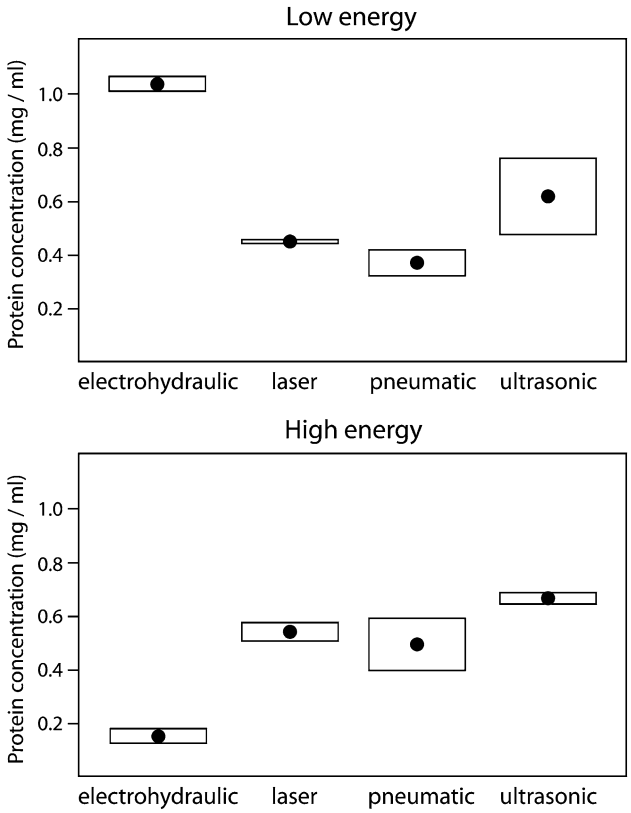

Fig. 4 Protein concentration, measured as protein absorbance at 280 nm, of *Escherichia coli* immobilized inside artificial renal calculi, after in vitro lithotripsy with four different intracorporeal lithotripters using two energy levels

Results show that all lithotripters inactivate more than 2.5 $\log_{10}$ (N_0/N) CFU/ml, i.e., more than 99% of the initial amount of bacteria. Analysis of variance yields valid results only if the analyzed data have a normal distribution. In microbiology it has been shown that data expressed as colony forming units per milliliter do not have a normal distribution; however, the logarithmic difference between N_0 and N, this is, $\log_{10} N_0 - \log_{10} N = \log_{10} (N_0/N)$, does follow a normal distribution [15]. Due to this, it has become common practice to express inactivation as $\log_{10}$ (N_0/N). Furthermore, if the logarithmic difference is used, the standard deviation is expressed in the same range of units, and data are easier to interpret.

The bactericidal action of a lithotripter depends on the energy setting, the dose, and its working mechanism. It has no sense to compare energy, power, and frequency settings between lithotripters of a different type. Because of this, no data on the energy delivered by each lithotripter is given. Treatment time was defined as the time needed to completely pulverize a stone.

The physical principle of a pneumatic lithotripter is analogous to that of pneumatic jackhammers. A rigid probe is connected to a hand piece within which a small projectile is contained. This projectile is accelerated by compressed air against the head of the probe 12 times per second. High stress is produced to any solid object in contact with the tip of the probe; however, no significant effect is produced when using this lithotripter only in fluid. It was expected that the pneumatic lithotripter, which does not produce significant acoustic cavitation or temperature increase, was not harmful to bacteria in suspension. It could only produce a bactericidal effect while the bacteria were inside the stone. Our hypothesis is that this lithotripter inactivates bacteria inside the stone as a result of mechanical stress.

Electrohydraulic lithotripters produce high-voltage discharges in liquid. This creates plasma bubbles that generate shock waves. The shock waves propagate through the liquid damaging any stone in the neighborhood of the tip. The fluid close to the tip will evaporate almost instantaneously. We believe that the whole amount of protein was denaturated after treatment of the suspension with the electrohydraulic lithotripter at the high-energy setting. Protein is undetectable by the absorbance method after denaturation. Treatment with the electrohydraulic lithotripter at low energy resulted in 100% inactivation and a high amount of protein released. In this case complete protein denaturalization did not occur.

Laser lithotripters create a plasma bubble close to the tip of the fiber, generating a pressure wave and a photothermal effect. Prabakharan et al. [12] reported viability reduction of *Proteus mirabilis* inoculated into struvite stones after holmium–YAG laser and electrohydraulic lithotripsy; however, sterilization of inoculated fragments was only observed with the laser. These authors did not report any significant bacteria reduction after ultrasonic or pneumatic lithotripsy. A limitation of their methodology was that they used reinfected struvite calculi and assumed that the reinfection colonized the stone surface with variable colonization of the interior. Our methodology was more realistic, because homogeneous colonization was achieved inside standardized artificial stones.

There are a few reports on the inactivation of microorganisms in suspension by extracorporeal shock waves [9–11]. These results are of little help to understand bacteria inactivation by intracorporeal lithotripters, because in our case, additional mechanisms like temperature increase, and direct laser or spark-gap radiation are involved. Shock waves produce transient holes in the membrane of eukaryotic cells [16, 17]. This increase in membrane permeability is due to shock wave-generated cavitation [18]. A similar effect is expected in bacteria subjected to the action of electrohydraulic, laser and ultrasonic lithotripters.

When no stone to pulverize was present, heating of the suspension occurred in all lithotripters, except in the pneumatic device, indicating that the stone absorbs most of the energy during lithotripsy. The strain of *E. coli* used in this

experiment dies in less than 2 min at a temperature over 60°C. If this happens, no cell rupture occurs and no protein is released. In our case, protein was detected. Nevertheless, we can not be certain if partial inactivation of bacteria in suspension was due to temperature increase.

Our purpose was to use protein absorbance at 280 nm as a quick and reliable method to show that bacterial lysis occurred. The only significant source of protein in our suspensions was *E. coli*. We conclude that main bacteria inactivation was due to lysis, because a large amount of intracellular protein was released into the saline solution after treatment with all lithotripters. Endotoxins or lipopolysacharide existing in the outer membrane are structural compounds which are released when bacteria are lysed; however, measurement of absorbance at 280 nm does not give information on whether complete lysis occurred in all microorganisms. The formation of transient microscopic pores in the membranes of bacteria, produced by microjets of fluid resulting after cavitation, may also release protein.

Clinically, intracorporeal lithotripsy of infected stones may be associated with postoperative urinary tract infection and risk to develop urinary sepsis. Our study confirms that all intracorporeal lithotripters inactivate bacteria. Nevertheless, the antibacterial effect does not necessarily indicate that intracorporeal lithotripters sterilize stone fragments; moreover, the presence of protein from bacteria after lithotripsy may increase the risk of bacteremia and potential urinary sepsis. Human blood monocytes are extremely sensitive to endotoxins producing IL1 and TNF in response to only 25–50 pg/ml of endotoxins; a concentration achieved in the circulation during septic shock [19]. Protein concentration obtained in our study ranges from 0.2 to 0.6 mg/ml. Therefore, it seems that the majority of these proteins are not absorbed into the bloodstream after stone manipulation. Further studies may be necessary to confirm the clinical relevance of our observations. Other bacteria to inoculate inside of artificial stones could be *Proteus*, *Staphalococcus*, and *Klebsiella*; however, it may well be that the activity against all of these organisms is similar.

In summary, electrohydraulic, laser, pneumatic and ultrasonic intracorporeal lithotripters inactivate most of the bacteria inoculated inside an infected urinary stone. A potential limitation of our study is that bacterial viability was only studied in *E. coli* suspension and in *E. coli* suspension with stone fragments, but not in stone fragments alone. Since fluid is needed for the laser and the electrohydraulic lithotripters to operate properly, stone fragmentation without fluid was not included. Since cell lysis occurs, a considerable amount of protein (endotoxin) is released, potentially increasing the probability of septicemia. Whether it is desirable for a lithotripter to efficiently inactivate instead to destroy bacteria or not, is still to be answered.

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