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The effect of antibacterial acting extracorporeal shockwaves on bacterial cell integrity

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Antibacterial effects of extracorporeal shockwaves (ESWs) have been demonstrated *in vitro* against bacteria under static and dynamic growth conditions. This study assessed the effects of ESWs on the cell wall integrity of bacteria.

Material/Methods: Standardized suspensions of *Staphylococcus aureus* were exposed to various shockwave impulses (2000–12,000) of different energy flux densities (EFD, 0.38–0.96 mJ/mm²). Bacterial suspensions of equal concentration that had been permeabilized (to >99%) with isopropanol were used as positive controls. The bacteria of all groups were stained with Sytox Green nucleic acid stain. The fluorescence of the shockwave-treated, permeabilized, and untreated suspensions was measured and compared for bacterial survival, quantified by colony-forming units after plating.

Results: Although ESWs showed a significant energy-dependent antibacterial effect that reduced CFUs in the treated suspensions by between 56% and 99%, only maximum energies (4000 impulses at 0.96 mJ/mm² and 12,000 impulses at 0.59 mJ/mm²) were followed by a significant increase in fluorescence compared with the untreated control ($p<0.05$). However, the fluorescence of these treated groups was still far less than that of the alcohol-permeabilized positive control groups ($p<0.05$). Lower energies and impulse rates did not show increased intracellular uptake of the fluorescent dye ($p>0.05$).

Conclusions: This is the first study to assess bacterial cell wall permeability after ESW treatment. It was found that the permeabilization of bacterial cells after ESW treatment was far less than expected due to the corresponding antibacterial effect. Other mechanisms, such as intracellular effects, might be involved in bacterial killing after ESWs and still must be elucidated.

key words: **shockwave • infection • bacteria • cell integrity**

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Background

Extracorporeal shockwave (ESW) therapy is a well-established treatment in orthopedics. Enthesopathies such as epicondylitis, tendinosis calcarea, and plantar fasciitis are treated successfully in daily clinical practice. Considerable success has also been reported in the treatment of non-unions and impaired wound healing [1–3]. So far, ESWs are not applied to infected target areas since they might induce systemic spread of bacteria with bacteremia and the risk of secondary infection or parenchymal abscess formation. However, the risk of bacterial spread after the application of ESWs to an infected target area has not yet been adequately studied and only single case reports have been documented [4,5]. On the other hand, there is evidence that ESWs elicit a significant energy-dependent bactericidal effect. Bacterial killing by ESWs has been shown for bacteria suspended in normal saline, PBS (phosphate-buffered saline), and CAMHB (cation-adjusted Muller Hinton broth). So far, killing rates of more than 99% have been reported [6–8]. Additionally, positive effects of ESWs on bone infections have been observed in a rabbit model of chronic osteomyelitis without systemic bacterial spreading [9]. This could not only allow safe application of ESWs in infected cases, but might also provide a rationale for the treatment of chronic bone and soft tissue infections.

In spite of the various studies describing the antibacterial potential of shockwaves, their mode of action has never been elucidated. On the other hand, various studies investigated the effects of ESWs on eukaryotic cells. These effects include a change in membrane potential, DNA alterations, modulation of gene activity, and even altering the integrity of the cell itself. Transient permeabilization of the membrane has been reported, an effect allowing the transport of molecules such as drugs and dyes into the cell [10,11].

The aim of this study was therefore to assess the possible effects of ESWs on the integrity of bacterial cells by dye exclusion tests *in vitro*. In addition, treated and untreated bacteria were examined with a scanning electron microscope (SEM) for morphological alterations.

Material and Methods

Shockwave generator and experimental set-up

A Dornier Compact Alpha shockwave generator (Dornier MedTech, Weßling, Germany) was used. The energy flux density (EFD) was modified between 0.38 mJ/mm² and 0.96 mJ/mm² and the impulse rate was set to 120 impulses per minute.

Soft disposable plastic pipettes with a volume of 5 ml served as test vials (Micro bio Tec Brand, Siegen, Germany). Experimental data (not shown) provided by Dornier Medtech showed that the pipettes allowed a nearly complete transmission of the shockwave energy. The pipettes were filled completely with suspension to avoid air bubbles, thereby also minimizing the appearance of cavitation bubbles. The pipettes were fixed in a small water tank by a special device that allowed a reproducible location of the pipettes in the focus of the shockwaves. Acoustic coupling of the water tank to the shockwave generator was achieved by the use of a contact gel. The temperature of the water in the water tank was kept constant at 20°C.

Preparation of test bacteria

Staphylococcus aureus (*S. aureus* ATCC 25923) was used in all the presented experiments. First the bacteria where grown to visible colonies on blood agar at 37°C. Then two or three colonies were transferred to CAMHB (cation-adjusted Mueller Hinton broth; Becton Dickinson Labware, Le Pont De Claix, France) and grown for approximately 12 h at 37°C. Afterwards this suspension was washed and resuspended in normal saline. By diluting with normal saline, suspensions of defined bacterial concentration were adjusted by densitometry to 2.5×10^7 CFU. The treated and untreated control groups were taken from this suspension. A positive control-group of *S. aureus* in normal saline (taken from the above prepared suspension) was pelleted and resuspended in 70% isopropanol to produce a suspension of almost 100% permeabilized bacteria. The cells were incubated for 1 hour at 37°C, then pelleted and resuspended in normal saline. This resulted in a >99% permeabilization [13]. By dilution with normal saline and densiometric control, the bacterial concentration in all the groups (positive and negative control groups and sample groups) was adjusted to a final test concentration of 5×10^6 CFU/ml. The suspensions were stored at 4°C until use.

Staining of bacteria

By diluting Sytox Green stock solution with normal saline, a solution of 5 µM was prepared. All working steps containing Sytox Green were carried out in darkness, as the dye degrades in daylight or laboratory light. After ESW treatment, three samples of 160 µl were taken of each specimen. These samples were filled into a well on a cell culture plate (96-well plate; Nunc, Wiesbaden, Germany), which was filled with 40 µl of 5 µM Sytox. To avoid cross-fluorescence during the measurement, only every second well was used. The blank value of normal saline (carrier substance) was determined by mixing 160 µl of normal saline with 40 µl of Sytox. Fluorescence spectrometry was carried out with a plate reader (Viktor; PerkinElmer Life and Analytical Sciences Inc., Boston, MA, USA) after a delay of 10 minutes to assure complete binding of Sytox green to the available DNA. The samples were excited at 485 nm with transmitted light. Fluorescence was detected at 535 nm for 1 second.

Bacterial quantification

To quantify the amount of living bacteria before and after ESW treatment, 100 µl of the treated and untreated groups were taken and gradually diluted with normal saline. One hundred µl of each dilution were plated on Muller Hinton agar. The plates were inoculated for 48 hrs at 37°C. Plating, incubation, and quantification were carried out according to the guidelines of the CLSI (formerly NCCLS) [14].

Scanning electron microscopy (SEM)

Three samples were treated with 0.96 mJ/mm², three with 0.38 mJ/mm² (4000 impulses each), and three were untreated. All samples (approx. 3 ml with 5×10^6 CFU/ml)

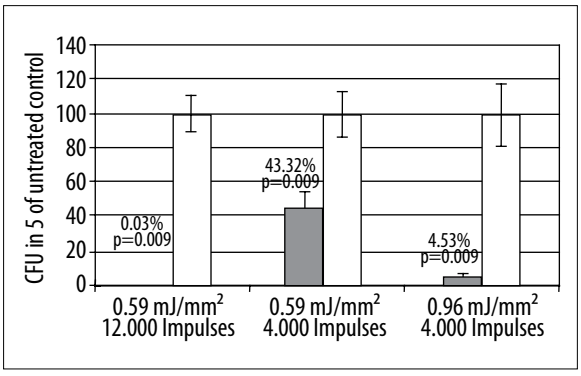

Figure 1. Decreases in CFU in a suspension of *S. aureus* treated with 4000 impulses at 0.59 mJ/mm² (middle) or 0.96 mJ/mm² (right) and 12,000 impulses at 0.59 mJ/mm² (left), each group compared with an untreated control group (n=5). Black bars represent treated samples, white bars the control group.

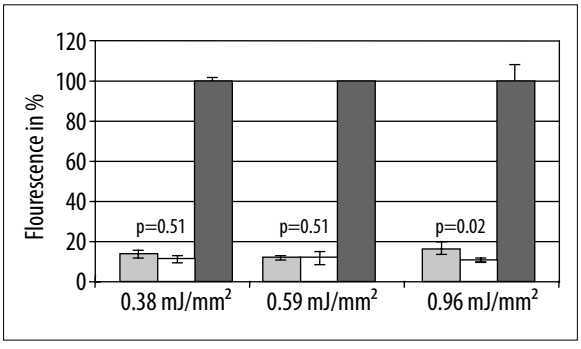

Figure 2. Fluorescence of ESW-treated suspensions of *S. aureus* (white) compared with both an untreated control group (gray) and a completely permeabilized group (100% control, black). The treated suspensions received 4000 impulses at various EFDs.

were completely rinsed through the bacteria filters. The bacteria on the filters were then fixed with glutaraldehyde (Sigma-Aldrich, Taufkirchen, Germany), dehydrated with alcohol, and sputtered with gold. SEM analysis was performed with a FE REM Leo 1530 (Zeiss, Oberkochen, Germany). All filters were scanned for morphologically altered cells or signs of cell debris. Sample pictures of representative areas were taken.

Statistical analysis

Each measurement returned three results for each sample, from which the mean was calculated for further analysis. Then the average blank value (normal saline and Sytox) was subtracted, resulting in the average fluorescence value of each sample. Again, the means of the various control and test samples were calculated and analyzed for statistical differences with the Mann-Whitney test. A p value of <0.05 was considered statistically significant. Quantification of the colonies on the agar plates was carried out as described in the guidelines of the CLSI. Colony quantities on the control and sample plates were also compared with the Mann-Whitney test with a p value of <0.05 considered statistically significant.

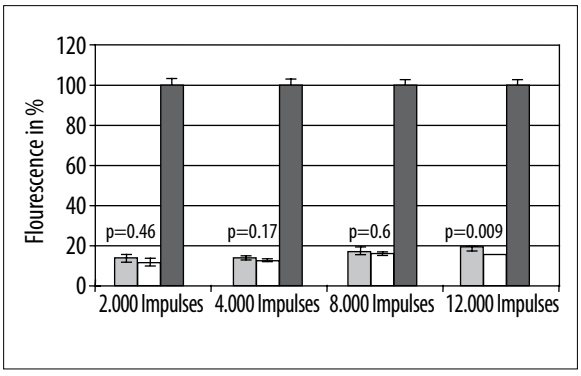

Figure 3. Fluorescence of various suspensions of *S. aureus* that received between 2000 and 12,000 impulses at 0.59 mJ/mm². Each group n=5. Black bars: control group, 100% permeabilized; gray bars: ESW-treated group; white bars: untreated control group

Results

Figure 1 shows the development of CFUs after ESW treatment with 0.59 mJ/mm² or 0.96 mJ/mm². The bacteria were in static growth conditions when the treatment was started. The number of CFUs in the suspension without any treatment (control) was counted and defined as 100%. After application of 4000 impulses (EFD=0.59 mJ/mm²), a significant decrease to 43.3% of the untreated control's CFU was observed. Application of 12,000 impulses at the same EFD led to a reduction to 0.03% and 4000 impulses at 0.96 mJ/mm² to a decrease to 4.53% ($p<0.09$ in all three tests). These results confirmed that the selected shockwave parameters elicited a significant reduction in growth of the suspended bacteria.

Figure 2 shows the fluorescence, and thus the amount of cell wall permeabilization, of the bacteria which were treated at 0.38 mJ/mm², 0.59 mJ/mm², or 0.96 mJ/mm² (4000 impulses each). A statistically significant increase in the fluorescence of the treated sample compared with the untreated control sample was seen only at 0.96 mJ/mm² ($p<0.05$). However, the fluorescence was only a small fraction of that of the 100% control. Furthermore, although the decrease in bacterial growth as determined by quantification of CFUs was more than 95% (cf. Figure 2), the increase in fluorescence as measured by intracellular binding of Sytox green was only 4.7%

Similarly, Figure 3 shows the fluorescence of suspensions of *S. aureus* treated with increasing impulse rates of 2000 to 12,000 impulses at a constant EFD of 0.59 mJ/mm². Only the highest total energy (12,000 impulses at 0.59 mJ/mm²) was followed by a significant increase in intracellular fluorescence ($p<0.05$). Low impulse rates at the same EFD level did not lead to a significant change in fluorescence compared with the untreated control suspension ($p>0.05$ for 2000, 4000, and 8000 impulses).

Finally, Figure 4 shows sample images of the SEM scan of ESW-treated and untreated bacterial cells on filter membranes (not all pictures taken are shown here). No morphological differences between the control group and the bacteria treated with 0.96 mJ/mm² are present, although

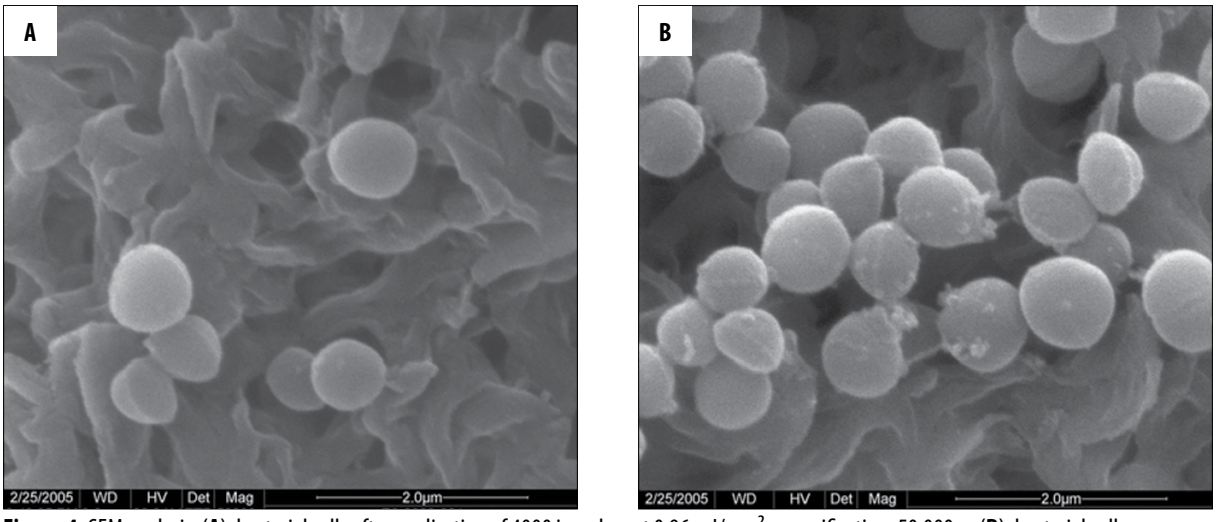

Figure 4. SEM analysis. (**A**): bacterial calls after application of 4000 impulses at 0.96 mJ/mm^2; magnification: 50,000×. (**B**): bacterial calls, untreated; magnification: 50,000×.

the EFD is capable of killing >95% of all cells. Furthermore, there is no cell debris or signs for disrupted bacteria cells.

Discussion

In various *in vitro* studies it has been demonstrated that ESWs have bactericidal effects if a suitable combination of EFD and impulse rate is chosen. However, it is unknown how this bactericidal effect is mediated. It is known that ESWs can alter the integrity of eukaryotic cells and they are used to destroy various stones (e.g. gall stones, salivary stones, kidney stones). The aim of this study was to show whether distinct destruction of cell integrity is the reason for the bactericidal effects of ESWs. We chose impulse rates and EFDs that are in regular use in the treatment of patients in our department. Furthermore, these parameters were chosen and assessed in previous studies [7,8,15] and allow relating our results to these findings. These studies showed that 0.38 mJ/mm^2 does not lead to a significant reduction of viable bacterial cells [7,8], but it is unknown whether this EFD can already lead to morphological alterations and was therefore assessed (mainly by SEM) in this study.

Concerning the bactericidal effect, we were able to confirm earlier findings of other authors [6–8] after treating suspended bacteria with 0.96 mJ/mm^2 and 0.59 mJ/mm^2. Thereby we also found the antibacterial effect to be energy dependent, with high impulse rates compensating for lower EFD (Figure 1). Preliminary studies with prolonged incubation times of ESW-treated bacterial suspensions demonstrated that the antibacterial shockwave effect is irreversible. More than 99% of all viable colonies were grown after 48 hrs and only very few and single colonies spread out after prolonged incubation of 10 days (data not shown). However, the metabolic condition of the bacteria plays a major role, as low EFDs can lead to an increase in CFU in a treated suspension of bacteria with optimal growth conditions, whereas the same EFD and impulse rate leads to a significant reduction of CFU in a suspension of resting bacteria. Furthermore, bacteria in a metabolically active state are less susceptible to high shockwave energies than those in a suspension of metabolic static bacteria. This was previously demonstrated by our group [15].

To use the bactericidal effect of shockwaves despite infections in the target area (which is still considered a contraindication), the antibacterial mode of action of ESWs against bacterial cells should be understood. So far, many papers describe extra- and intracellular changes in eukaryotic cells after being exposed to ESWs; different groups showed an increase in cell-membrane permeability after shockwave application [16,17]. Other authors observed a change in the membrane potential of cells [18]. Kodoma et al. reported that systematic transvection of dye molecules by means of ESWs was possible [12]. Lauer et al. assessed the possibility of ESW-supported gene transfer [19]. These results might explain the higher cytotoxicity of chemotherapeutic drugs such as bleomycin against tumor cells under the influence of ESWs [20].

Other investigations on shockwaves found that low and moderate EFDs resulted in a higher metabolic rate of osteoblasts [21,22]. Haake et al. could demonstrate on bone marrow cells that a relatively low EFD (0.25 mJ/mm^2) altered cellular morphology and they did not observe cytodestructive effects [23]. Gambihler, in contrast, found such effects on leukemia cells of mice at an EFD of 0.4 mJ/mm^2, reporting membrane damage, cell destruction, and lowered proliferation rates after shockwave application [10].

So far, all these cellular effects have only been studied and observed in eukaryotic cells, and no study is known to the authors investigating the molecular and cellular effects of shockwaves on bacteria. The basic principle for the present study was a publication by Roth et al., who showed that the membrane integrity of bacterial cells, which was altered by antibiotics, heat, or alcohol, could validly be assessed with the special dye Sytox Green (Molecular Probes, Leiden, the Netherlands). Sytox Green cannot penetrate the intact cell wall of viable bacteria. The dye is subject to neither any active or passive transmembrane transporters nor passive diffusion. Only damaged cell walls allow intracellular uptake of the dye, which then specifically binds to dsDNA, resulting in significantly increased fluorescence. On the other hand, unbound Sytox Green is virtually non-fluorescent. This causes a low background fluorescence which is dependent on the

concentration of dye and bacteria in the assessed suspension. Roth and coworkers also showed that the intensity of the fluorescence is linearly dependent on the amount of membrane-compromised bacteria in a suspension containing both living and compromised bacteria [13].

In our studies, a significant discrepancy was observed between the reduction of bacterial growth as determined by CFU and increased bacterial cell wall permeability as measured by intracellular fluorescence. A significant increase in fluorescence was only observed in the bacterial suspensions treated with the highest total energies (12,000 impulses at 0.59 mJ/mm² and 4000 impulses at 0.96 mJ/mm²), although bacterial killing was also obvious with lower energy levels or impulse rates. Quantification of the surviving bacterial cells showed a reduction of more than 99% and 94% for those treated with 12,000 impulses at 0.59 mJ/mm² and 4000 impulses at 0.96 mJ/mm², respectively, whereas less than 8% of bacterial cells were determined to be permeabilized after applying the same shockwave energies. Furthermore, the fluorescence level of the alcohol-permeabilized bacteria could never be reached. This suggests that many bacterial cells were killed by the ESWs but remained morphologically intact. This assumption is corroborated by the results of the SEM examination; the images of both the treated and untreated groups showed morphologically intact cells. Also, there was no cell debris visible on the filter paper. Cell debris could be a sign for completely destroyed bacterial cells. However, as complete destruction of the cells would lead to the release of DNA (and allow the binding of dye), significantly higher fluorescence values should have been detected.

We were not able to determine whether transient permeabilization plays a role, as Sytox Green is destroyed by shockwaves (already at low EFD) and therefore cannot be applied during shockwave admission. However, we regard a transient destruction of murein layers and cell membranes with consecutive repair as unlikely as we chose conditions that limit the metabolic activity of the cells.

In summary, the bactericidal effects of ESWs do not appear to be based on the permanent destruction of bacterial cell walls but rather on an intracellular shockwave effect, such as the generation of free radicals. Free radicals can lead to an unselected alteration (oxidation, reduction) of metabolites or the destruction of cellular organelles by cracking covalent bindings. Some of these effects have already been described in eukaryotic cells [24–26]. The assumption that the cell membrane remains intact after ESW treatment may also be explained by the stability of the murein layer compared with a lipid double layer; the former is composed of covalently bound macromolecules and the latter is held together only by van der Waals' forces, which are weaker than covalent bonds.

CONCLUSIONS

Shockwaves have a significant antibacterial effect against bacterial cells in static and dynamic growth phase. Different intra- and extracellular modes of action have been postulated. While extracellular mechanisms can alter membrane proteins and channels, intracellular effects can be, for example, the formation of free radicals and the destruction of cell organelles or dsDNA. However, in contrast to eukaryotic cells, enduring damage to the cell wall or cell membrane obviously does not play a major role. We could confirm that ESWs can lead to a significant reduction of CFUs in a solution, as was shown before. Furthermore, we could demonstrate that only a very small fraction of bacterial cells is permeabilized after application of either high EFDs or high impulse rates (or a combination of low EFD and high impulse rates). In all cases, the fraction of permeabilized cells was out of all proportion to the reduction of viable cells in the same solution. We therefore conclude that permeabilization, has only a minor effect, if any, in the bactericidal effect of shockwaves. On which part of the cell ESWs deploy their destructive effect, and whether therapeutic use can be made of it, remain unclear. If shockwaves become a therapy option also for infected target areas or in bone infection in general, the alteration of bacterial cells by shockwaves should be of further interest. Especially intracellular changes and gene activity must be aspects of further investigation.

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