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Effect of polyhexanide and gentamicin on human osteoblasts and endothelial cells

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Summary

Questions under study: Infection of total joint replacements is painful, disabling and difficult to treat because of the increasing bacterial resistance against antibiotics. In view of this, antiseptics show limited bacterial tolerance and have a broad-spectrum antimicrobial activity. However, the application of antiseptics to bone is insufficiently studied in literature. Therefore, we investigated the biocompatibility of the antiseptic polyhexanide with bone related cells and asked whether supplementation to bone cement is appropiate in the management of total arthroplasty infections.

Methods: We performed an in vitro study with immortalised human foetal osteoblast cells (hFOB 1.19) and human endothelial cells (EAhy 926). The cultured cells were exposed to media containing various concentrations of gentamicin (12.5–800 µg/ml) and polyhexanide (0.0006–0.01%) for six hours. We measured the phase-contrast microscopy images, the cell viability, cell number and the alkaline phosphatase activity as a parameter for osteogenic function.

Results: The exposure of hFOB and endothelial cells to polyhexanide showed a severe reduction of viability and cell number. Gentamicin did not have negative effects on hFOB and endothelial cell number and viability. The alkaline phosphatase activity of hFOB showed a significant decrease after exposure to polyhexanide and gentamicin. The viability and the cell number of endothelial cells seem more negatively affected by polyhexanide than the parameters of the hFOB-cells.

Conclusions: The exposure of human osteoblasts and endothelial cells to polyhexanide at concentrations with questionable antibacterial activity resulted in severe cell damage whereas exposure to high dosed gentamicin did not. These results raise questions as to the feasibility of using antiseptics in bone cement for the treatment of total arthroplasty infections. Further in vivo studies are necessary to show the in vivo relevance of these in vitro findings.

Key words: antiseptics; gentamicin; polyhexanide; osteoblasts; endothelial cells; toxic effects

Introduction

Total joint replacements are very successful in the treatment of osteoarthritis since several decades. However, device-related infection is a serious problem in orthopaedic surgery, which can deteriorate the excellent outcome of total joint replacements. Improved infection control measures and systemic perioperative antibiotic prophylaxis, reduced the infection rate to 0.5–2% of patients who received joint replacements [1, 2]. The management of such infections includes the often performed two-stage exchange arthroplasty and an accurate debridement of the affected tissues [3, 4]. Then an antibiotic loaded bone cement spacer is placed into the joint cavity for infection treatment, for at least 6 weeks. After this period a new joint replacement is implanted provided the infection is cured before. However, the poor efficiency of antibiotics against commensals in biofilms is a serious cause of concern. Increasingly, pathogens are resistant to antimicrobial agents; current surveillance reveals steadily increasing rates of resistance to oxacillin among *Staphylococcus aureus* and to vancomycin among *Enterococcus species* [5]. The recent recovery of vancomycin-resistant *S. aureus* indicates the urgency to control antimicrobial resistance and to develop new approaches [6]. Therefore, the application of antiseptics could be a new approach in the battle against infection because of their lower bacterial tolerance and the broad spectrum of antimicrobial activity [7, 8]. They are originally agents which prevent or inhibit the growth or action of microorganisms on several surfaces. They are used currently for preoperative antisepsis of the oral cavity and conjunctives [9, 10].

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Cleansing the birth canal with antiseptics reduced neonatal and maternal postpartum infections [11].

However, the application of antiseptics to bone tissue and vascular tissue is insufficiently studied in the literature. Osteointegration of orthopaedic devices requires sufficient numbers of cells and high levels of biosynthetic activity to produce a protein matrix for mineralisation. Furthermore, neovascularisation at the bone implant interface is necessary for osteogenesis and therefore important for osteointegration of orthopaedic devices [12]. Therefore, we investigated the biocompatibility of the antiseptic polyhexanide with osteoblasts and endothelial cells and asked whether supplementation to bone cement is appropriate in the management of total arthroplasty infections.

In this attempt, we investigated the cellular response of human foetal osteoblasts (hFOB 1.19) [13] and endothelial cells (EAhy 926) [14] after exposure to a) the commonly used antibiotic gentamicin and b) the antiseptic polyhexanide. We documented phase contrast images and measured cell-viability, cell number and the synthesis of alkaline phosphatase.

Materials and methods

Human osteoblasts and endothelial cells were exposed to various concentrations of gentamycin (g) and polyhexanide (px). In order to measure the biocompatibility of these anti-infectives following parameters were examined: viability, cell number and microscopic images. The function of osteoblasts was investigated by means of alkaline phosphatase activity. All experiments were done twice with 6 samples for each concentration and parameter unless mentioned otherwise. A sample (control) without any drug exposition was considered as control.

Cell culture

We used the human foetal osteoblast cell line (hFOB) [13] and the EAhy 926 human endothelial cell line [14]. The cells were plated into 96/24 – well tissue culture plates at a density of 10 000/50 000 cells and cultured at 37 °C in 95: 5% air: CO_2 for 24 hours. The medium was changed every two days and consisted of a 1:1 solution of Ham's F12 and Dulbecco's Modified Eagle's Medium (DMEM's / Ham's F12), supplemented with 10% heat inactivated foetal calf serum (Biochrom, Berlin, Germany) without any supplemented antibiotics.

The controls consisted of cells without any drug exposure.

Antiseptics/antibiotics

The following commercially available substances were tested by incubation for six hours:

Lavasept® concentrate (Fresenius-Kabi AG, Bad Homburg, Germany) contains 20 g polyhexamethylene biguanide (polyhexanide) with an average molecular weight of 2800 and 1 g macrogolum 4000 in 100 ml aqueous solution. 0.01, 0.005, 0.0025, 0.00125, 0.0006 and 0.0003% (w/v) polyhexanide were prepared by dilution with DMEM's / Ham's F12 culture medium, supplemented with 10% heat inactivated foetal calf serum.

Gencin® (curasan, Kleinostheim, Germany) contains 0.04 g/ml gentamycinsulfate-solution. Gentamycin concentrations of 12.5 to 800 µg/ml were prepared by dilution with DMEM's / Ham's F12 culture medium, supplemented with 10% heat-treated foetal calf serum.

We used polyhexanide in concentrations (0.0006–0.01%) much lower than clinically (0.01–0.02%) recommended [15]. Polyhexanide is currently used as an irrigation solution for antisepsis of wounds [15, 16], ophthalmic mucosa [10] and treatment of acanthamoeba keratitis [17].

The concentrations of gentamicin ranged from 12.5 µg/ml to 800 µg/ml which reflect the concentrations measured in vivo after local application with bone-cement beads [18].

Cell viability

Cell viability were measured by staining with the colorimetric reagent WST-1 (Roche Diagnostics GmbH, Penzberg, Germany), which is designed for non-radioactive quantification of cellular metabolism as previously described [19]. The cells were rinsed with 1× phosphate buffered saline and incubated with 0.1 ml DMEM's / Ham's F12 culture medium, supplemented with 10% heat inactivated foetal calf serum and 10 µl WST-1 reagents at 37° C for 30 minutes. The absorbance of the resulting formazan in the supernatant was measured at 450 nm with a microplate reader (SLT, Crailsheim, Germany).

Cell number

The automatic cell analyzer system CASY 1 TTC (Schärfe System, Reutlingen, Germany) was used to measure the quantity and size distribution of viable cells as previously described [20]. The system works according to the resistance principle combined with an additional pulse area analysis and a signal scanning frequency of 1 MHz. Equipped with a multi-channel analyzer with 512,000 channels, the equipment is capable of sensitive, reproducible, and nearly error-free analysis of the concentration and size distribution of a whole cell population within one minute. Experimentally, 100-µl aliquots of the cell cultures were diluted in 10 ml phosphate buffered saline, and 400-µl aliquot samples were analysed three times by the system. Mean cell number and size distribution were calculated automatically with the CasyStat software (Schärfe System). The following concentrations of polyhexanide and gentamicin were evaluated: polyhexanide 0.0006, 0.0025 and 0.01%; gentamicin 25, 100 and 400 µg/ml. To prepare the cells for electronic counting, they were rinsed twice with phosphate buffered saline and detached by treatment with 0.2 ml trypsin (0.5 g/ml trypsin, Gibco BRL, Life Technologies). After five minutes, 0.6 ml DMEM's / Ham's F12 culture medium, supplemented with 10% heat-treated foetal calf serum was added to stop the reaction.

Alkaline phosphatase (ALP)

Osteoblasts are able to produce the enzyme alkaline phosphatase as part of their osteogenic function. The following concentrations of polyhexanide and gentamicin were evaluated: polyhexanide 0.0006, 0.0025 and 0.01% and gentamicin 25, 100 and 400 µg/ml. The hFOB-cells were plated and cultured until confluence as described before. The cells were then exposed to the drugs for six hours and ALP activity was measured.

The cells were rinsed twice with phosphate buffered saline and incubated with lysis buffer (0,75M 1 amino 2 methyl 2 propanol (Sigma A 65182)) supplemented with 20 mg p-nitrophenol phosphate substrate per ml lysis buffer for one hour at room temperature as previously described [13]. The absorbance of p-nitrophenol was measured at 405 nm with a microplate reader (SLT). Chemicals were purchased from Sigma-Aldrich, Munich, Germany.

Statistical methods

Data was evaluated using the software SPSS 12.0 for Windows (Lead Technologies, Chicago). We used the ANOVA test followed by the Dunnett test for statistical analysis; $p < 0.05$ was considered to be significant. The Dunnett's test is a test of all means against a control and the type I error rate applies to this set of comparisons, not to each individual comparison.

All experiments were done twice, usually with either 6 or 3 samples per concentration (see figures 2 to 4). Each point on a graph is an average of two replicate experiments.

Results

Cell morphology

The microscopic images (phase contrast) of the hFOB and endothelial cells appeared to be altered after 6 hours at any concentration of polyhexanide. The cells became spherical without the typical oblong dendritic morphology and they were partly detached from the well-ground. The morphology of the cells exposed to gentamicin did not change after six hours exposure and after 24 hours recovery (figure 1a–f).

Cell viability of hFOB

The viability of hFOB-cells exposed to various concentrations of gentamicin (12.5 to 800 µg/ml) for six hours showed no significant decrease compared to the control cells which were not exposed to any antibiotic. An increase of hFOB-cell viability after exposure to gentamycin was documented for concentrations >25 µg/ml (figure 2a). This result was reproducible (data not shown) and maybe due to changes in the pH of the medium after application of gentamycin. However, this is speculative.

The exposure of hFOB-cells to polyhexanide for six hours showed a significant decrease in viability at concentrations of 0.0025 (w/v) to 0.01% polyhexanide (figure 2b).

Cell number of hFOB

The cell number of hFOB after exposure to gentamicin was comparable with the control cells without drug exposure (figure 2c). The exposure of hFOB to polyhexanide ≥0.0025% showed a severe reduction of cell number (figure 2d).

Cell viability of endothelial cells

The endothelial cells showed no decrease of viability after exposition to gentamicin at concentrations of 25, 100, 400 µg/ml for six hours (figure 3a). After exposure to polyhexanide a clear decrease at any concentration was seen in the viability (figure 3b).

Cell number of endothelial cells

The cell number of endothelial cells was also not affected by exposure to gentamicin (figure 3c).

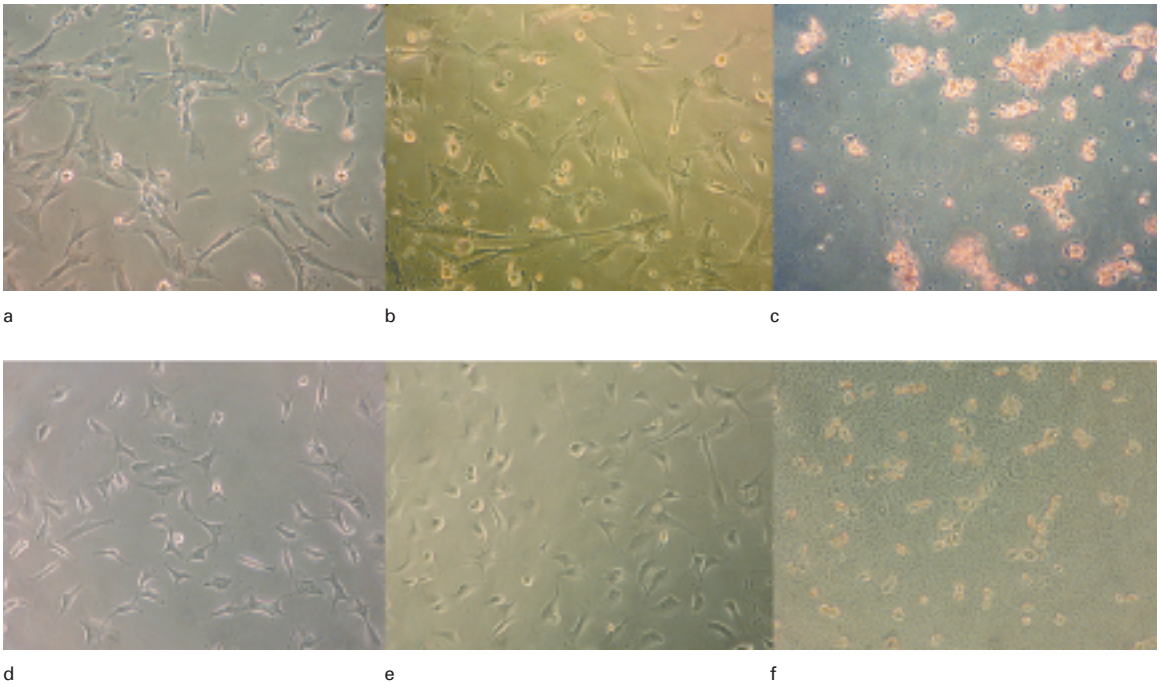

Figure 1

Phase contrast images of human foetal osteoblast a) control b) after exposure to gentamicin (400 µg/ml) and c) after exposure to polyhexanide (0.01%) for six hours; phase contrast images of human endothelial cells d) control e) after exposure to gentamicin (400 µg/ml) and f) after exposure to polyhexanide (0.01%) for six hours (magnification ×100).

The decrease of cell number was pronounced at any tested concentration of polyhexanide (figure 3d).

Alkaline phophatase activity of hFOB

The ALP activity of hFOB-cells was decreased after exposure to gentamicin (figure 4a) and polyhexanide, even at low concentrations of 25 µg/ml and 0.0006% respectively (figure 4b). The decrease of activity seems more pronounced after incubation with polyhexanide.

Discussion

Increasing bacterial resistance against antibiotics complicate the management of device-related infection in orthopaedic surgery. New approaches and anti-infectives are necessary to guide this development. In the light of this, antiseptics provide less bacterial tolerance and have a broad-spectrum antibacterial activity. Therefore, they seem to be preferable as a supplement to bone cement. More recently their use in the management of infection as a coating of orthopaedic devices has been investigated [7, 8, 21, 22].

The antiseptic polyhexanide was introduced in the 1980's in Europe [16]. It has been shown to be less irritative in the test with chorion allantoin membrane of hens [23]. Polyhexanide is the first known antiseptic which has a specific action against negatively charged cell layers of procaryontic cells and is less affecting eucaryontic neutral lipid membranes [24, 25]. This fact encourages a possible application of polyhexanide to bone cement for the therapy of arthroplasty infections.

The current study presents adverse effects of the antiseptic polyhexanide on human osteoblasts and human endothelial cells. We compared the results of the polyhexanide with the antibiotic gentamycin because it is actually and commonly used as an addition to bone cement in the treatment and prophylaxis of total arthroplasty infections [1, 26, 27].

Our data demonstrated that the viability and cell number were negatively affected even at very low concentrations of polyhexanide. The antibi-

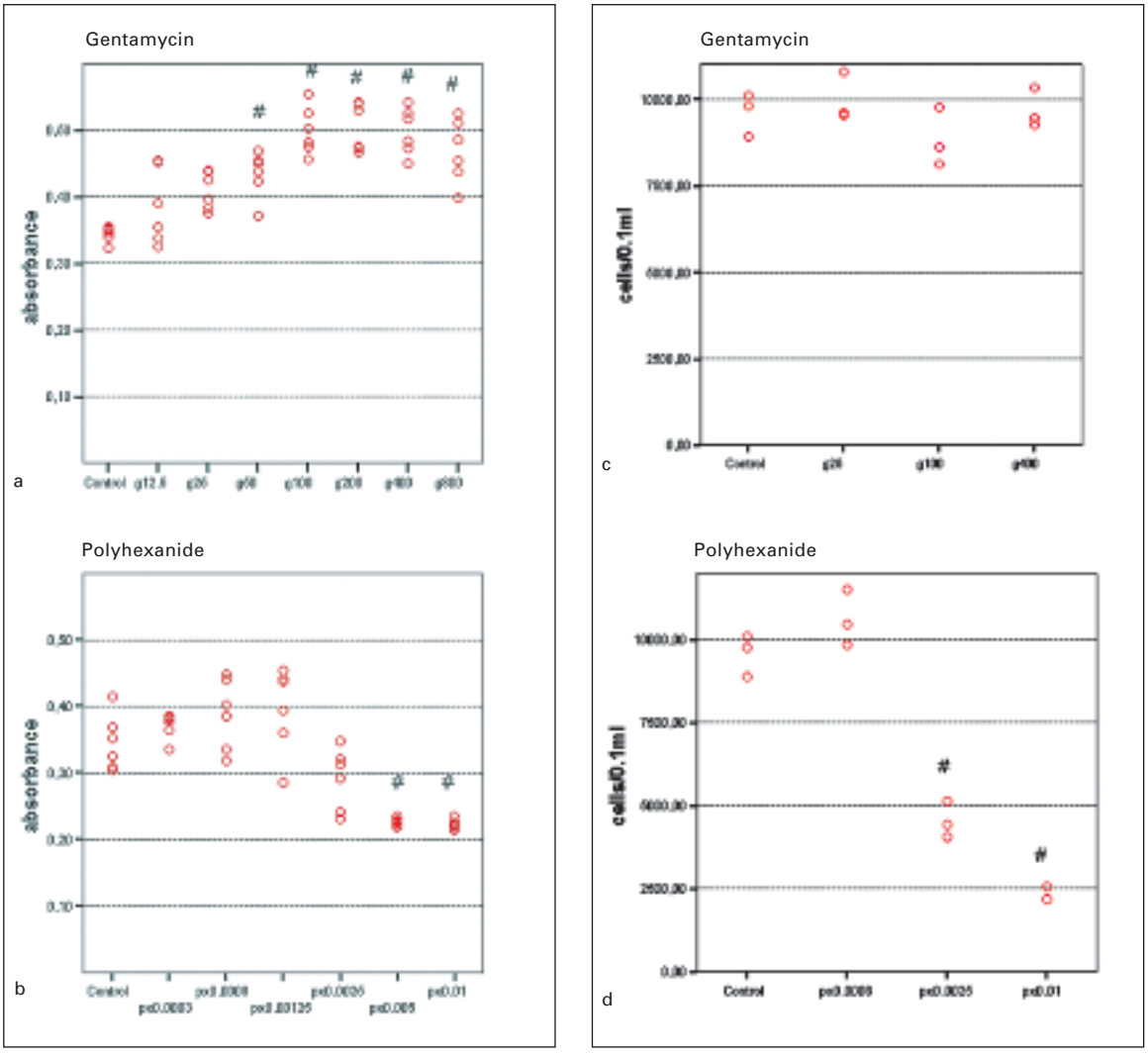

Figure 2

Effect of a) gentamicin and b) polyhexanide on human foetal osteoblasts *cell viability*. Effect of c) gentamicin and d) polyhexanide on human foetal osteoblast *cell number* (g = gentamicin, px = polyhexanide, # p <0.05 versus control).

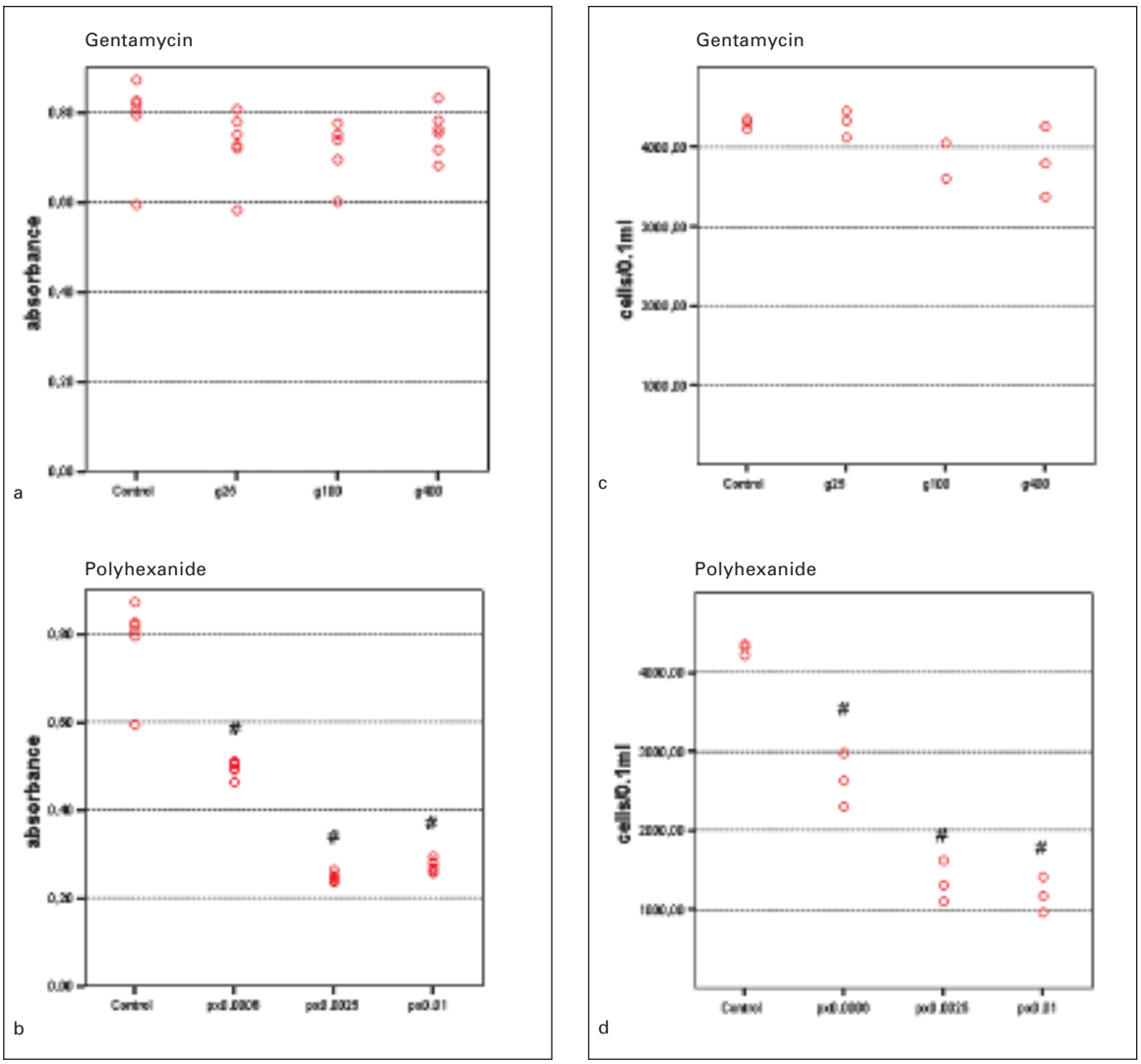

Figure 3

Effect of a) gentamicin and b) polyhexanide on human endothelial *cell viability*. Effect of c) gentamicin and d) polyhexanide on human endothelial *cell number*. (g = gentamicin, px = polyhexanide, # p <0.05 versus control).

otic gentamicin, in contrast, did not affect the viability and the cell number of hFOB and endothelial cells, even at high concentrations (400 µg/ml).

The synthesis of the osteogenic marker ALP (hFOB's) was negatively affected by both gentamicin and polyhexanide at any tested concentration of 25–400 µg/ml and 0.0006–0.01%, respectively. However, the viability and the cell number of endothelial cells seem more adversely influenced by polyhexanide than the parameters of the hFOB-cells.

In the literature, the impact of polyhexanide on bone tissue has not been sufficiently investigated.

To our knowledge, only two studies reported the biocompatibility of antiseptics with bone tissue. Kaysinger et al. [28] examined antiseptics on cultured chick tibiae specimens and osteoblast-like cells with an exposure time of two minutes. They found a severe reduction of cell number and metabolism after cell exposure to 5% povidone-iodine (Betadine-solution) and concluded that the use of antiseptics on exposed bone tissue should be considered with caution. The second study reported the results of murine osteoblast-like cells exposed to the antiseptic chlorhexidine. They documented toxic effects on cell viability and metabolism after exposure to chlorhexidine 1% and 10% for 2 minutes [29]. Both studies used high ranges of antiseptic concentrations which may address the requirements of oral cavities, wounds and fibrous tissue but not bone. The main weakness of the studies was the exposure time limited to a few minutes. In our study the exposure time was much longer, ie six hours. We are aware that a treatment of septic total joint replacements lasts longer, ie several months [18]. However, this time interval is not appropriate for in vitro investigations.

From the clinical point of view, only one study used polyhexanide in the treatment of total arthroplasty infections. Wagner [30] described 18 patients with infected total hip arthroplasties. A one-stage exchange arthroplasty was performed followed by an application of 0.025% px for 5 days, through a needle placed between bone and the acetabular component and also distal of the intramedullar stem. 14 patients showed no recurrence of infection after an average of 22 months. Four (22.2%) patients had a relapse of joint infection. One patient suffered a migration of the cup without evidence of infection. However, these early results are comparable with the results of other studies investigating one-stage exchange arthroplasty without this prolonged application of antiseptics [1, 31].

Our data indicated a severe toxic damage

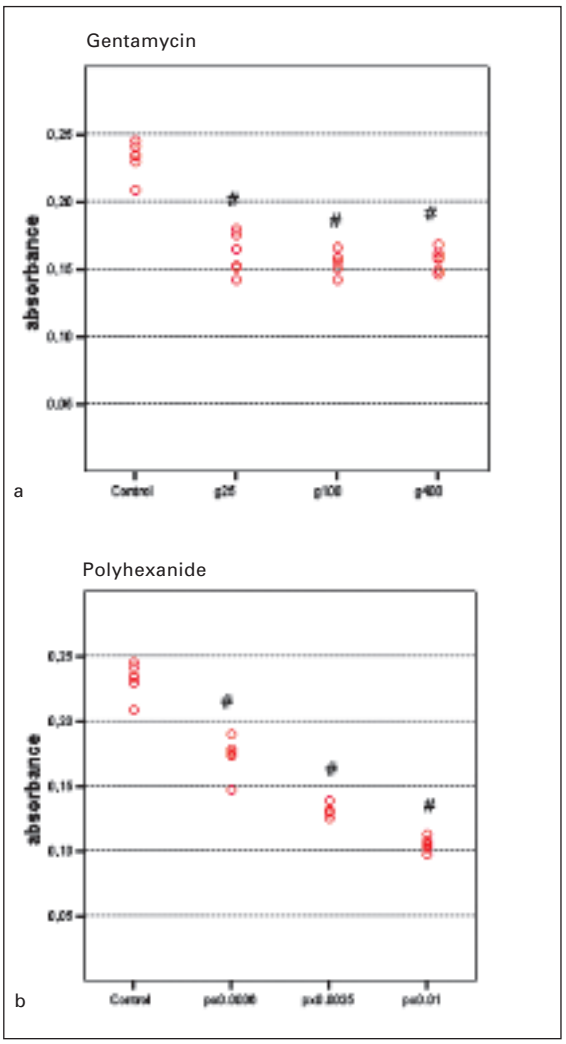

Figure 4

Effect of a) gentamicin and b) polyhexanide on *alkaline phosphatase activity* of osteoblasts after six hour exposure (g = gentamicin, px = polyhexanide, # p <0.05 versus control).

to bone and endothelial cells above 0.0025% polyhexanide. Unfortunately, concentrations ≤0.0025% polyhexanide are only effective in killing 10^2–10^3 colony forming units/ml of *Staphylococcus aureus* and 10^4–10^5 colony forming units/ml of *Escherichia coli* [32]. Therefore, polyhexanide seems not to be appropriate for supplementation to bone cement to cure total arthroplasty infection.

Gentamicin is a commonly used aminoglycoside-antibiotic as an addition to bone cement. Isefuku showed that gentamicin at a concentration above 100 µg/ml had detrimental effects on osteoblast-like cells. Similarly, Pedersen et al. [33] observed a dose dependent decrease in the release of previously incorporated calcium-45 and alkaline phosphatase activity in mouse calvaria exposed to various concentrations of gentamicin (20–320 µg/ml). Our data of the gentamicin samples is in line with these studies.

In conclusion, the exposure of human osteoblasts and endothelial cells to polyhexanide at concentrations with questionable antibacterial activity resulted in severe cell damage whereas exposure to high dosed gentamicin did not. These results raise questions as to the feasibility of using antiseptics in bone cement for the treatment of total arthroplasty infections. Further in vivo studies are necessary to show its relevance in vitro findings.

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