



Original Research

The Effect of Different Irrigation Solutions on the Cytotoxicity and Recovery Potential of Human Osteoblast Cells In Vitro

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ABSTRACT

Background: Surgeons use various irrigation solutions to minimize the risk of prosthetic joint infection after total joint arthroplasty. The toxicity of these solutions is an important consideration in their use. This study investigates the effect of irrigation solutions Bacitracin, Clorpactin (sodium oxychlorosene), and Irrisept (chlorhexidine) on osteoblast cytotoxicity and proliferation.

Methods: Four replicates of 6 conditions at 3 time points (1, 2, and 4 min) were tested: control (normal saline), Bacitracin (33 IU/ml), Clorpactin (0.05%, 0.1%, 0.2%), and Irrisept (0.05% chlorhexidine gluconate). Human osteoblasts were cultured at 37°C and 5% CO₂ until confluent monolayers were obtained. The treatment solution was applied, and cells were washed 3x with warm phosphate-buffered saline and then supplemented with a fresh medium. Phase-contrast images were taken before and after treatment. The cytotoxicity and proliferation of the treated cells was measured for all conditions on day 3 and day 5 after treatment using the alamarBlue assay.

Results: All test conditions showed morphological changes to cells after treatment; controls did not. Cells demonstrated curling and detachment. This effect was the worst and permanent with Irrisept, whereas other treatments showed a return to normal morphology after 1 week. All treatments showed increased %alamarBlue reduction after 5 days except Irrisept, which showed decreased reduction. There was no statistically significant time or dose dependence with Clorpactin treatment.

Conclusions: Clorpactin and Bacitracin are damaging to human osteoblast cells in vitro as compared with normal saline. This damage is at least partially reversible as shown by morphology and cell viability assay. Irrisept caused more damage than either Clorpactin or Bacitracin, and the damage was not reversible.

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Introduction

Surgical site infections are a significant contributor to patient morbidity and mortality in orthopaedic surgery. Irrigation and debridement of surgical wounds is widely used in orthopaedic practice [1] to reduce the risk of infection by flushing out bacteria and removing devitalized tissue that serves as a medium for bacterial growth [2]. Irrigation solutions frequently use antibiotics or antiseptics that actively kill bacteria and prevent remaining

bacteria from reproducing [3,4]. Although all irrigants decrease bacterial load, they also have been shown to cause some amount of tissue damage [2]. The properties intended to minimize infections make irrigants potentially harmful to human bone cells.

Despite a wealth of research, the ideal irrigation solution remains unclear. As an example, several studies have demonstrated positive antibacterial properties of Bacitracin solutions [5–7], a polypeptide antibiotic with good gram-positive coverage, which functions by inhibiting bacterial cell wall synthesis and inhibiting proteases and other enzymes that participate in the cell membrane function [8,9]. Other studies have been unable to show a clear benefit of Bacitracin compared with normal saline (0.9%) or non-antibacterial detergents such as castile soap [10–12], with a

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potentially higher rate of wound healing complications in open fractures treated with Bacitracin [12].

Chlorhexidine gluconate (CHG) is a relatively inexpensive [13] chemical solution with well-established antimicrobial effects. It has particularly strong activity against both aerobic and anaerobic gram-positive organisms and functions by disrupting both the cell wall integrity and cell membrane integrity and inducing cytoplasmic congealing at higher concentrations [4,14,15]. The effect of chlorhexidine on human cells has been well studied, particularly in the field of dentistry, where it is commonly used as a mouthwash [16]. It has been shown to prevent growth and attachment of human fibroblasts in vitro, a widely accepted marker for cytotoxicity and inhibition of regeneration [17–19]. However, conflicting study results make it unclear whether this toxic effect is dose-dependent or time-dependent [16,18–20].

The effect of CHG on the bone is an area of current research with mixed results. Giannelli et al. showed that osteoblasts were even less resistant to CHG than fibroblasts and endothelial cells [21]. Other authors have described similar decreases in function alongside indicators of cell damage [22–24]. Some of these results have been challenged [25], whereas other investigators have found results to be insignificant vs control groups [26,27].

Irrisept (Irrimax Corporation, Gainesville, FL) is an irrigation solution that contains 0.05% CHG. According to the company's website, low-dose chlorhexidine is an effective irrigation solution that does not cause harm or irritation to surrounding tissues [28]. Irrisept is a popular irrigation solution among orthopaedic surgeons. Several studies have investigated the effect of CHG on the bone but used CHG at a much higher concentration than Irrisept [24,26,27]. Others have suggested that low-concentration CHG shows toxic effects on human cells [18,19,29]. These conflicting reports lend Irrisept as an interesting subject of further study.

Clorpactin (United-Guardian Inc., Hauppauge, NY) is a historical irrigation solution that has also been used to combat surgical site infections. Clorpactin (0.2% sodium oxychlorosene) is a modified buffered hypochlorous acid derivative [30] that is highly bactericidal to nearly all microorganisms via its mechanism of hypochlorination and oxidation of cell membranes and destruction of cytoplasmic contents [31,32]. It is a modified version of Dakin's solution (pH 9.3–10.2) with a more neutral pH of 6.5–6.9, which is less irritating to human tissues and improves germicidal activity [33,34]. Clorpactin has been studied in many fields including general, plastics, and anorectal surgery; otolaryngology; dermatology; and ophthalmology and demonstrated good antimicrobial effects with minimal damage to host tissues [30,35–40]. However, a literature review into the effects of Clorpactin on the health of bone cells yielded limited results, making Clorpactin another interesting subject of investigation.

The purpose of this study was to evaluate the effect of Clorpactin, Irrisept, and Bacitracin on osteoblast cytotoxicity and regeneration capacity and to compare those solutions with one another and with a normal saline control. It was hypothesized that all test conditions would show greater cytotoxicity than the control, with Irrisept demonstrating the greatest effect, but that all irrigation-treated osteoblast cells would show regeneration capacity.

Material and methods

Cell preparation for testing

Early-generation subcultivated (subculture passage 2 to 4) human osteoblast (HOB) cells (product number C-12720; PromoCell GmbH, Heidelberg, Germany) were seeded onto a 48-well plate at a density

of 3–5000 cells/cm² and supplemented with an osteoblast growth medium following the manufacturer's recommendations (0.3 ml/cm² of surface) [41]. The HOB cells were grown under standard culture conditions, that is, incubated under 5% CO₂ at 37°C for 72 hours without intervention to allow for complete attachment [41].

Testing conditions

When cells reached confluency, 200 µL of each irrigation solution was pipetted gently onto the walls of the designated wells without disturbing the cell monolayers. Six irrigation solutions were tested: normal (0.9%) saline solution (control), Bacitracin solution (33 IU/mL), Clorpactin (2g) in normal saline (0.05%, 0.1%, and 0.2% solution), and Irrisept (0.05% CHG solution). Three concentrations of Clorpactin were selected because it comes as a powder that can be mixed to a concentration as needed. We used the standard 0.2% and 2 less-concentrated solutions. Three different exposure times for each solution were tested: 1 min, 2 min, and 4 min. After exposure to the specific irrigant, the cells were washed 3 times with warm saline, the irrigation and wash solutions were collected, and the viability of the detached cells was tested using a dye that diffuses through the cell membrane of dead cells, Trypan blue (Thermo Fisher Scientific, Waltham, MA). After the saline wash, the fresh culture medium was then added. Each test condition was replicated 6 times for a total of 108 test conditions.

Each treatment culture was viewed via phase-contrast microscopy before, immediately after, at 24 hours, and at 1 week after treatment with irrigants, and images were taken to document qualitative morphological changes.

The alamarBlue assay

The alamarBlue assay (Invitrogen, Carlsbad, CA) is a sensitive nondestructive colorimetric assay that is based on the irreversible reduction of the fluorescent blue dye, resazurin, into a red fluorescent dye, resorufin, in response to intercellular metabolic activity [42,43]. It is often used as an oxidation-reduction indicator of cell viability in mammalian cell cultures [44,45] and is a popular way of measuring cell proliferation and cytotoxicity [42].

After treatment with the irrigation solutions and addition of the fresh culture medium, as described previously, HOB cells were allowed to rest for 48 hours. The culture medium was then replaced by the fresh medium supplemented with 10% vol of the alamarBlue solution and incubated for 24 hours. After incubation, the absorbance was measured at 570 nm (using 610 nm as a reference wavelength) using the Synergy HT Multi-detection Plate Reader (BioTek Instruments Inc., Winooski, VT). The results were collected using BioTek's KC4 software and exported into Microsoft Excel (Microsoft Corporation, Redmond, WA). The percent reduction of alamarBlue for each test condition was calculated as instructed by the manufacturer's guidelines. After the absorbance measurement, the alamarBlue-supplemented medium was replaced with a fresh medium, and the HOB cells were incubated for additional 48 hours. The procedure was then repeated to evaluate the recovery of the treated cells. The second alamarBlue reduction measurement was taken 5 days after the original irrigation treatment.

Statistical analysis

All statistical analyses were performed using Microsoft Excel. Single-factor analysis of variance was used to determine statistically significant differences in change in alamarBlue reduction between conditions. Four categories of conditions were tested: at

each time point, each test solution was compared with the control; at each time point, the test solutions used in practice (Bacitracin, Irrisept, Clorpactin 0.2%) were compared with one another; at each time point, Clorpactin was compared across concentrations; at each concentration, Clorpactin was compared across time points.

Results

Phase-contrast imaging

Examples of the phase-contrast imaging results can be seen in [Figure 1](#). When comparing the post-treatment phase-contrast imaging with the pretreatment imaging, there were clear qualitative changes to the osteoblast cells in all treatment lines but not the control. The post-treatment imaging showed decreased cell numbers and obvious morphological changes.

During treatment, the cells demonstrated a curling phenomenon in which the cells visibly curled and shriveled. Many of those cells then detached from the plate and died as confirmed by the Trypan blue exclusion test. This effect was visibly worse with Irrisept. There was no obvious difference between cells treated with Clorpactin (all concentrations) or Bacitracin, although the effect was present.

The phase-contrast imaging after 24 hours for cells treated with Clorpactin and Bacitracin indicated that the cells were beginning to return to normal. The curling phenomenon was still present in many cells, but it was not as drastic and not present throughout. When imaging was taken 1 week after the initial treatment, the cells appeared morphologically normal. The cells treated with Irrisept did not show return to normal morphology at either the 24-hour or 1-week time point, but rather shrunken abnormal cells.

The alamarBlue assay

[Figure 2](#) shows the average difference in the alamarBlue reduction over 5 days for each irrigation solution at each time point. The control group and the Bacitracin and Clorpactin test groups

showed increased alamarBlue reduction over the 5-day period. The Irrisept test groups showed decreased reduction after 5 days in culture.

[Table 1](#) shows the results of analysis of variance testing between each test solution and an NS control group at 1 min, 2 min, and 4 min exposure time. At 1 minute of exposure, there was a significant difference between only Clorpactin 0.2% and Irrisept and their respective control. Only Irrisept was significantly different than the control in the 2-minute exposure group. At 4 minutes of exposure time, Clorpactin 0.1% and 0.2% and Irrisept were significantly different than the control group.

The results of comparing the test solutions at the concentrations used in practice (Bacitracin 33 IU/mL, Clorpactin 0.2%, Irrisept) with one another individually can be found in [Table 2](#). All comparisons showed significant differences.

All 3 Clorpactin concentrations (0.05%, 0.1%, and 0.2%) were compared with one another at each time point as shown in [Table 3](#), and each Clorpactin concentration was compared across the 3 exposure times as shown in [Table 4](#). All differences across concentrations were significant, whereas only Clorpactin 0.1% showed significant difference across exposure times.

Discussion

The purpose of this study was to evaluate the effects of the irrigation solutions Bacitracin, Irrisept (0.05% CHG), and Clorpactin (sodium oxychlorosene) on HOB cells and the ability of the HOB cells to recover after irrigant exposure. Our analysis was qualitative using pre-exposure and postexposure phase-contrast imaging and quantitative using 2 measurements of the alamarBlue assay 2 days apart.

Bacitracin, Irrisept, and Clorpactin each achieve its well-accepted antibacterial properties via disruption of the bacterial cell wall, cell membranes, and cytoplasmic contents. Bacitracin does so via blocking the transfer of mucopeptides into the cell wall and inhibiting proteases and other enzymes that are necessary for cell membrane stability and cytoplasmic function [8,9]. CHG

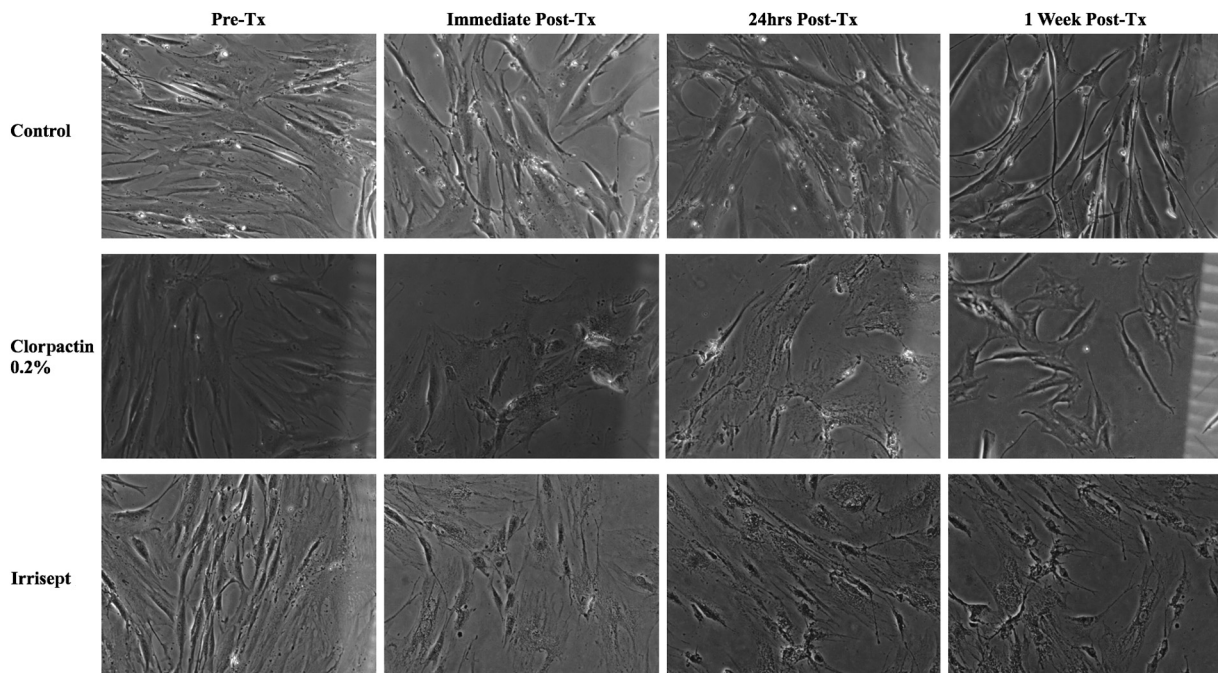


Figure 1. Comparison of phase-contrast imaging. All representative images are of the 2-minute exposure group at a 10× objective exposure.

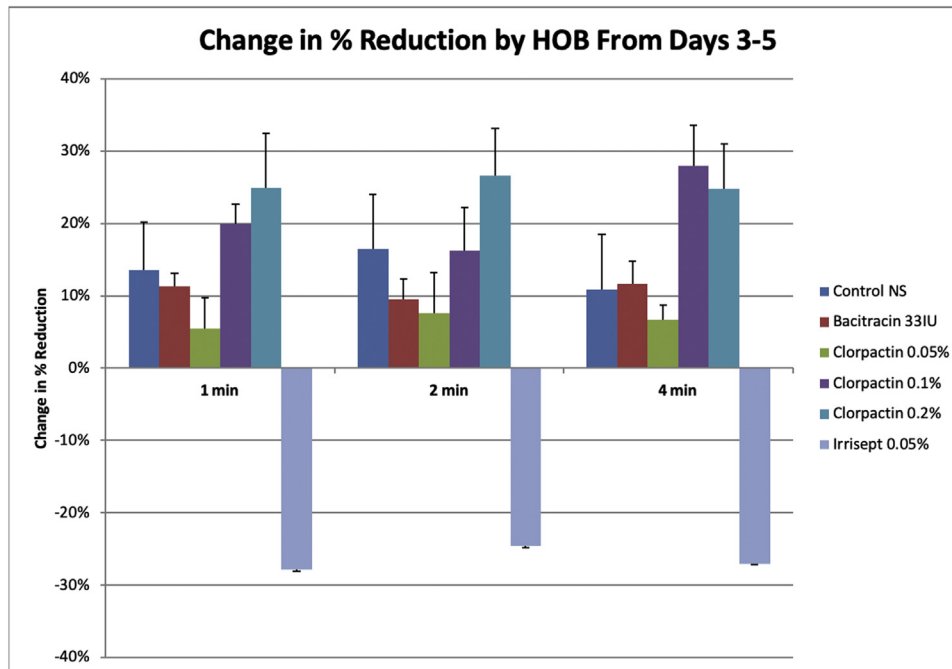


Figure 2. Change in alamarBlue reduction over 5 days.

achieves its mechanism of action by binding to the cell wall and cell membrane, causing a disruption in osmotic equilibrium and leakage of potassium and phosphorus, and at higher concentrations, it induces congealing of cytoplasmic contents [14,15]. Sodium oxychlorosene functions as a powerful hypochlorinating and oxidizing agent, which disrupts nearly all contents of the cell [30-32]. Scant primary research has focused on how HOB cells respond to exposure to these agents. It has been suggested that the CHG concentration in Irrisept remains low enough to be unharmed to human tissue [28], although the truth of that has been debated with mixed results [18,19,28,29].

The phase-contrast imaging of all test groups demonstrated clear numerical and morphological changes in the HOB cells with exposure to all test solutions. The cells showed curling and shrinking before detaching from the well plate. This negative effect has been demonstrated in other studies. Alleen et al. showed that 0.12% CHG solution inhibited cell attachment and therefore regeneration of human fibroblast cells [17]. Cline and Layman found a similar inhibition of fibroblast attachment caused by the CHG solution greater than 0.2% [19]. More recently, Rohner et al. observed cell shrinking as a result of changes in the cell membrane and loss of adhesion in HOB cells when exposed to 0.1% and 2% CHG solution [22]. Although we used a different concentration of CHG, our qualitative results with HOB cells reinforced what those authors described in fibroblasts and osteoblasts, that is, that exposure to CHG caused damage-induced morphological changes in human cells and detachment from

testing plates. Irrisept contains half or less of the concentration of CHG as the solutions used in the past studies. Our results imply that minimum concentration of CHG needed to induce such cell damage is much lower than previously described.

The observed cell damage was not unique to the CHG-based solution. The Bacitracin solution and all 3 Clorpectin solutions demonstrated similar but less-extensive cell morphological changes. Because there were no changes in control cells, the HOB cell changes were the result of the antiseptic solution and not from the washing process itself. Importantly, despite causing some initial HOB cell changes, after the 5-day waiting period, those cells treated with Bacitracin and Clorpectin appeared to return to normal, whereas the cells treated with Irrisept did not. Although Bacitracin and Clorpectin may induce damage-related changes to HOB cells, that damage appeared reversible. The damage caused by Irrisept appeared irreversible. These findings were supported by the alamarBlue assay results.

The CHG-induced damage was not limited to the cells directly treated with Irrisept. In the first trial of the experiment, the control cells were plated in very close proximity to the Irrisept group. As soon as Irrisept was added, we observed the same morphological changes and detachment in the previously normal postexposure control group (Fig. 3). This trial was repeated with more distance between cell groups, and the changes did not occur in the control group. Thus, it appears that cells in close proximity to CHG-treated cells were subject to the same damage as CHG-treated cells. The

Table 1
Test solutions vs control (P-value).

Irrigation solution	Exposure time		
	1 minute	2 minutes	4 minutes
Bacitracin	0.489	0.105	0.827
Clorpectin 0.05%	0.124	0.075	0.288
Clorpectin 0.1%	0.083	0.973	0.007
Clorpectin 0.2%	0.045	0.070	0.021
Irrisept	<0.001	<0.001	<0.001

Table 2
Test solutions as used in practice (P-value).

Irrigation solution	Exposure time		
	1 minute	2 minutes	4 minutes
Bacitracin vs Clorpectin 0.2%	0.007	<0.001	0.004
Bacitracin vs Irrisept	<0.001	<0.001	<0.001
Irrisept vs Clorpectin 0.2%	<0.001	<0.001	<0.001

Table 3
Comparison of Clorpactin—all concentrations (*P*-value).

Exposure time	<i>P</i> Value
1 minute	.001
2 minutes	.001
4 minutes	<.001

effect may have been from Irrisept “fumes” contaminating the neighboring wells.

The Irrisept company website states that as a low-dose CHG solution, Irrisept is an effective irrigation solution that does not cause harm or irritation to surrounding tissues [28]. This statement is not supported by much of the scientific literature. van Meurs et al. found that CHG 0.04% was completely cytotoxic to fibroblasts and mesenchymal cells and some toxicity persisted at as low as 0.02% CHG [46]. Mariotti and Rumpf investigated the toxic effects of CHG on gingival fibroblasts and found some level of toxicity down to 0.00009% CHG [18], whereas Cline and Layman described inhibition of fibroblast growth at 0.01% CHG [19]. Other investigators have examined CHG toxicity in the orthopaedic field. van Huyssteen and Bracey described marked chondrolysis and articular damage after arthroscopic anterior cruciate ligament repair using intra-articular CHG 0.02% [47]. More recently, Liu et al. tested the cytotoxicity of CHG on HOB cells [29]. They used a different cell viability assay to test for osteoblast survival 48 hours after exposure to CHG solution and found that cell survival at 0.02% CHG or a higher concentration approached 0% [29]. Our study sought to take the investigation a step further by examining if the apparent cytotoxicity could be overcome by the regenerative capacity of HOB cells by examining the HOB cells over a longer time period. Our results indicated (Figs. 1-3, Table 1) that the cytotoxic effect of CHG persists at a concentration of 0.05% and cells not only fail to recover but also worsen, over the course of 5 days. This suggests that Irrisept does cause harm to HOB cells and that harm cannot be overcome by their natural regenerative capacity.

The phenomenon of curling and detachment of the osteoblast cells from the plate presents a challenge to measuring cell recovery, as the detached cells in our samples were often washed away during rinsing. Although unlikely, it is theoretically possible that some of these cells maintained some regenerative capacity. Other authors [17,19] have hypothesized that the cell detachment is a harbinger of permanent damage or cell death. The Trypan blue exclusion test we performed confirmed that the cells that washed away were, in fact, dead.

The existing literature on the effects of Clorpactin on human cells is scarce. Although Gliedman et al. did not show evidence of damage to colonic tissue on exposure to Clorpactin [36], the tissue tested was canine. Clorpactin is no longer routinely used as a treatment for cystitis because of the advent of “less-painful” methods, implying that it causes irritation to human tissue [48]. The orthopaedic literature is especially lacking. Our results were mixed. The phase-contrast imaging showed evidence of morphological changes suggestive of cell damage at all concentrations of Clorpactin at every exposure time, but also the ability to recover. The results of the alamarBlue reduction supported this. The change

Table 4
Comparison of Clorpactin—all exposure times (*P*-value).

Clorpactin concentration	<i>P</i> Value
0.05%	.472
0.10%	.015
0.20%	.925

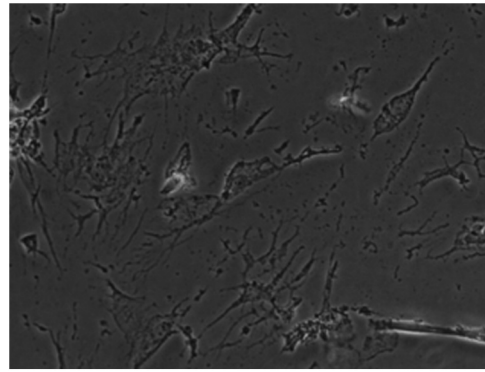


Figure 3. Example of untreated control cells in close proximity to Irrisept-treated cells.

in reduction of the test conditions was either not significantly different than the control or the change was significantly greater than the control (Table 1). We took this one step further by analyzing dose and time dependence for Clorpactin exposure. There were significant dose-dependent differences at all exposure times (Fig. 3), whereas Clorpactin 0.1% showed a time-dependent significant difference. This indicates that even at higher concentrations of Clorpactin, osteoblast cell damage was transient.

Limitations and areas for further study

As this was an in vitro study, there are limitations as to the extrapolation to the clinical realm and implications to in vivo bone cells. This and clinical trials assessing infection rates and union rates are interesting areas for further study. Mangum et al [49] recently failed to show reduction of bacterial load in in vivo animal models with Dakin's solution, likely because of a rapid rate of degradation on soft-tissue exposure. A randomized trial comparing the solutions in this study in open-fracture management would help address the clinical correlation.

Conclusions

The irrigation solutions Bacitracin, Clorpactin, and Irrisept cause morphological changes indicative of cell damage to HOB cells in vitro, whereas normal saline does not. This damage appears to be at least partially reversible in cells treated with Bacitracin and Clorpactin but not Irrisept, as measured qualitatively with phase-contrast microscopy and quantitatively using the alamarBlue assay. There does not appear to be a dose-related or time-related effect on this recovery potential. The clinical significance of our findings merits further investigation.

Conflict of interests

The authors declare there are no conflicts of interest.

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