Journal of Investigative Surgery, 28, 1–7, 2015

ISSN: 0894-1939 print / 1521-0553 online
DOI: 10.3109/08941939.2014.941445

informa
healthcare

ORIGINAL ARTICLE

Cytokine Expression in Human Osteoblasts After Antiseptic Treatment: A Comparative Study Between Polyhexanide and Chlorhexidine

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ABSTRACT

Purpose/Aim of the study: Chlorhexidine and polyhexanide are frequently used antiseptics in clinical practice and have a broad antimicrobial range. Both antiseptics are helpful medical agents for septic wound treatment with a high potential for defeating joint infections. Their effect on human osteoblasts has, so far, not been sufficiently evaluated. The aim of this study was to investigate the activating potential of polyhexanide and chlorhexidine on inflammatory cytokines/chemokines in human osteoblasts in vitro. *Materials and Methods*: Human osteoblasts were isolated and cultivated in vitro and then treated separately with 0.1% and 2% chlorhexidine and 0.04% polyhexanide as commonly applied concentrations in clinical practice. Detection of cell structure and cell morphology was performed by light microscopic inspection. Cytokine and chemokine secretion was determined by using a multiplex suspension array. *Results*: Cell shrinking, defective cell membrane, and the loss of cell adhesion indicated cell damage of human osteoblasts after treatment with both antiseptics was evaluated by using light microscopy. Polyhexanide, but not chlorhexidine, caused human osteoblasts to secrete various interleukins (1β, 6, and 7), interferon γ, tumor necrosis factor α, vascular endothelial growth factor, eotaxin, fibroblast growth factor basic, and granulocyte macrophage colony-stimulating factor as quantified by multiplex suspension array. *Conclusions*: Both antiseptics induced morphological cell damage at an optimum exposure between 1 and 10 min. But only polyhexanide mediated a pronounced secretion of inflammatory cytokines and chemokines in human osteoblasts. Therefore, we recommend a preferred usage of chlorhexidine in septic surgery to avoid the induction of an inflammatory reaction.

Keywords: human osteoblasts; polyhexanide; chlorhexidine; cytokines; chemokines

INTRODUCTION

The periprosthetic joint infection is a dreaded complication in orthopedic surgery [1–13]. Next to systemic therapy, a mechanical elimination of bacteria is usually supported by flushing with antiseptic solutions [14]. Unfortunately, most antiseptic solutions induce tissue toxicity [15–18]. Polyhexanide is a commonly used antiseptic and has a broad antimicrobial range. In previous studies, we showed the superiority of polyhexanide in terms of low toxicity in comparison to other antiseptics for the treatment of human chondrocytes [19–21]. But little is known about the effects of polyhexanide or other antiseptics in regard to their exposure on human osteoblasts. This may be of great interest due to the fact that bone tissue is also exposed to antiseptics during treatment of septic arthroplasty.

Still, chlorhexidine is rarely used in orthopedic surgery and thus the reasons for this limited application have, to our knowledge, been documented only

Received 1 April 2014; accepted 1 July 2014.

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scarcely. However, different case reports showed toxic effects of chlorhexidine on human cartilage after open or arthroscopic surgery [22–24]. Previously, we were able to demonstrate the induction of several cytokines and chemokines in human chondrocytes after the treatment with antiseptics. As chondrocytes and osteocytes are both mesenchymal cells, manifestation of a similar reaction here could be suggested.

Therefore, we compared effects of polyhexanide at a weight-to-weight concentration of 0.04% and two other concentrations of chlorhexidine on human osteoblasts. Chlorhexidine was used at the regularly employed low concentration of 0.1% as well as at the higher concentration of 2%. This is in our opinion of great interest because bone tissue is also exposed to antiseptics during revision of septic arthroplasty. The aim of this study was to investigate the impact of polyhexanide and chlorhexidine on the morphology of human osteoblasts and their ability to induce the secretion of inflammatory cytokines and chemokines.

MATERIALS AND METHODS

Tissue culture plasticware from TPP (Trasadingen, Switzerland) was used. Culture medium, phosphate buffer saline (PBS), trypsin, and fetal calf serum (FCS) from Biochrom (Berlin, Germany) were used. Other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) or other sources as specified below.

Osteoblast Isolation, Culture, and Treatment

Bone material was obtained from four donors with knee osteoarthritis undergoing a total knee arthroplasty (TKA). Prior to extraction, the patients under study showed no signs of infection in regard to clinical examination or blood values. Experimental protocols were approved by the local ethics committee.

Procedure: intraoperatively, after bone resection for the TKA, the remaining bone-cartilage tissue was collected in a container under aseptic conditions. Within 2–5 hr, the bone-cartilage tissue was prepared for the laboratory tests. Bone fragments were separated from cartilage and manually minced to fragments sized 1–4 mm^3 followed by washing bone fragments twice with PBS. Then they were suspended in an osteogenic medium consisting of DMEM Ham's F12 along with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, and a 1% vitamin solution, and cultured at 37°C, 95% air, and 5% CO_2. Experiments were performed after osteoblast colonies had reached subconfluency. Human osteoblasts were treated with either 0.04% polyhexanide (Serag Wiessner, Naila, Germany) or 0.1% and 2% chlorhexidine, which was freshly prepared from 20% chlorhexidine digluconate (Sigma Aldrich, Deisenhofen, Germany) and aqua ad injectabilia (B. Braun Melsungen AG, Melsungen, Germany). Osteoblasts were treated for either 1 or 10 min in the above-mentioned antiseptic solutions. As there were no differences in the outcome, the results were summarized.

Analysis of Cell Morphology: Light Microscopy

Human osteoblasts were cultured in 24-well plates and treated with 300 μl of 0.04% polyhexanide or 0.1% and 2% chlorhexidine for 10 min and then washed immediately with PBS. PBS-treated cells were used as a negative control, and 2% Triton X 100 as a positive control for the induction of cell damage. After treatment, osteoblasts were evaluated by phase contrast light microscopy (Axiovert 40 C Light Microscope, lens 10 × 0.25, ocular 10 × 18 Zeiss, Gottingen, Germany) and photographed using a digital single-lens reflex camera (Canon EOS 550D, 18 Megapixels, Japan). Digital photos were equalized using Adobe Photoshop Elements 9.

Multiplex Suspension Array

Supernatants of antiseptic solutions treated osteoblasts were harvested after 1 and 10 min. The collected samples were stored at −80°C until analysis. Supernatants of osteoblasts incubated in medium were used as a further control. The concentrations of various cytokines and chemokines (Interleukins 1β, 6, and 7, Interferon-γ, tumor necrosis factor-α, vascular endothelial growth factor, eotaxin, fibroblast growth factor basic, and granulocyte macrophage- colony stimulating factor) were quantified by multiplex suspension array (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions. Data acquisition was conducted using the Bio-Plex suspension system.

Statistical Analysis

A nonparametric Mann–Whitney test was used for statistical analysis. A p-value of $<.05$ was considered to be significant. Statistical analysis was performed with graph pad prism (version 5.0a).

RESULTS

Antiseptics Induced Microscopic Cell Damage

Light microscopy revealed increased cell structure defects of human osteoblasts incubated with polyhexanide or chlorhexidine when compared with osteoblasts treated with PBS (Figure 1). Osteoblasts

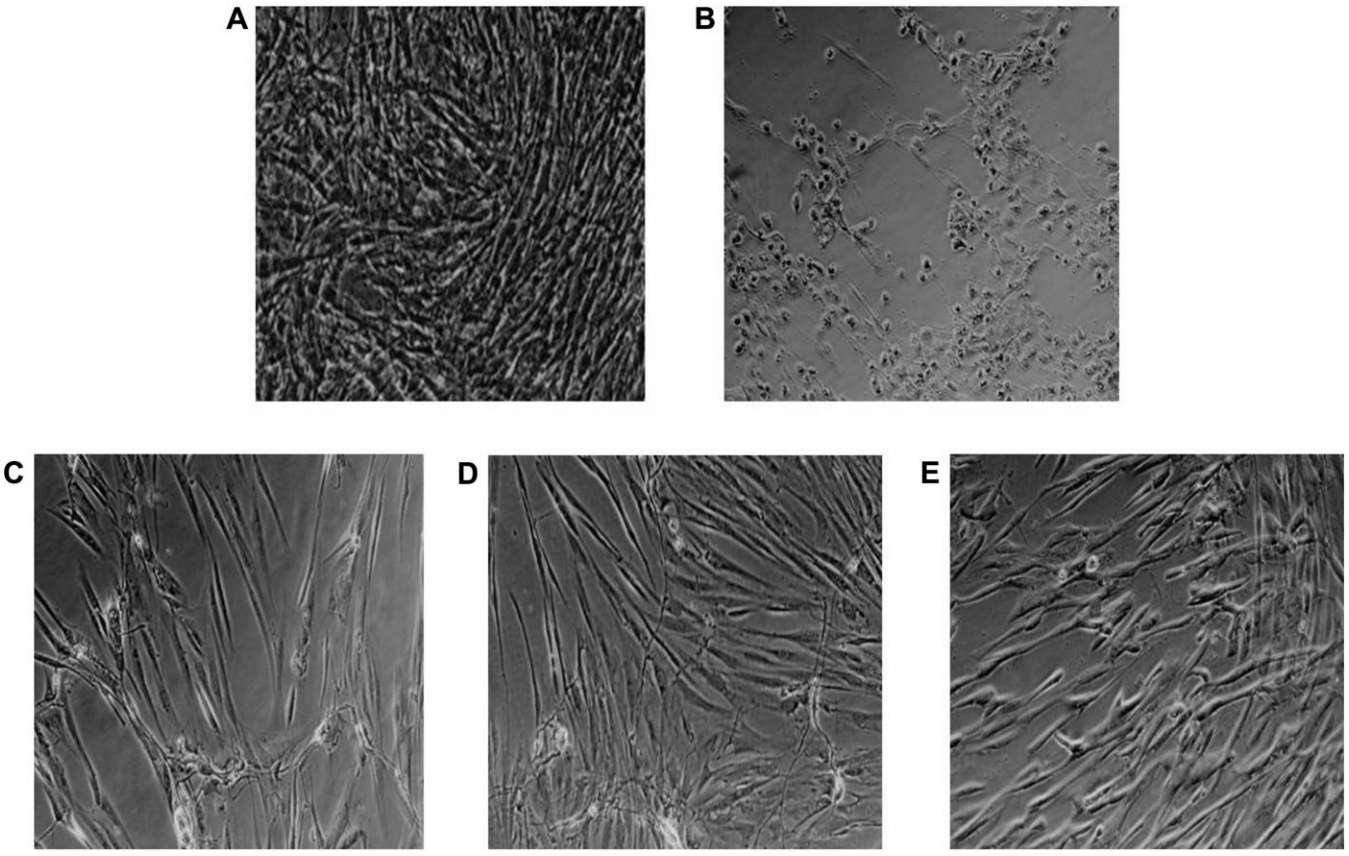

FIGURE 1 Polyhexanide and chlorhexidine induce cell damage. Osteoblasts treated with PBS did not show any cell damage (A). Osteoblasts treated with 2% Triton X 100 clearly showed signs of cell damage such as shrinking, loss of cell structure, and cell adhesion (B). Osteoblasts were treated with polyhexanide at 0.04% (C), chlorhexidine at a concentration of 0.1% (D), or chlorhexidine at 2% (E) for 10 min, each. Both antiseptics induced cell damage. One representative experiment of $n = 4$ is depicted.

treated with PBS showed a healthy and normal cell structure and regular cell morphology with intercellular contacts and a close cell adhesion (Figure 1A). Triton X 100—known to be a mediator of cell necrosis—induced total cell destruction in the form of shrinkage, complete loss of cell adhesion among osteoblasts, and served as a positive control for cell damage (Figure 1B). Osteoblasts treated with antiseptics showed a much darker and inhomogeneous cytoplasm. Cells showed partial structural defects with partial reduce of cell adhesion but at a significant lower level in comparison with the positive control Triton X 100. There were no clear macroscopic differences seen between polyhexanide and chlorhexidine. Polyhexanide-treated osteoblasts showed a consecutive shrinking, cell membrane blebbing, and reduction of cell adhesion (Figure 1C). Treatment with chlorhexidine rapidly caused a change in osteoblast morphology. An inhomogenous range of shrunk cells with membrane-bounded bodies and enlarged osteoblasts with inconsistent cell borders as well as a moderate amount of detached cells were detected in the case of treatment with 0.01% chlorhexidine (Figure 1D) and 2% chlorhexidine (Figure 1E). One representative result of $n = 4$ is shown.

Polyhexanide but not Chlorhexidine Induced a Pronounced Inflammatory Reaction in Osteoblasts

Osteoblasts were treated with either PBS (control), 0.04% polyhexanide, or 0.1% and 2% chlorhexidine. The supernatants were harvested and analyzed for the concentrations of (A) IL-1β, (B) IL-6, (C) IL-7, (D) IFN-γ, (E) TNF-α, (F) VEGF, (G) eotaxin, (H) fibroblast growth factor (FGF) basic, and (I) GM-CSF in a multiplex suspension array (Figure 2). Polyhexanide induced a pronounced secretion of all factors analyzed and this was significantly higher when compared with the treatment with 0.1% chlorhexidine ($p < .05$, Mann–Whitney test, all factors, $n = 4$) and also significantly higher when compared with the 2% chlorhexidine case ($p < .05$, Mann–Whitney test, for all factors except FGF basic, $n = 4$). FGF basic was the only factor that was also induced by 2% chlorhexidine. Undetectable values were replaced by the detection limit value of the test. Namely, the concentration of IL-1β after the treatment with polyhexanide was 43 $\pm$ 5.1 pg/ml, while under all other conditions the concentrations were below the detection limit of 15 pg/ml. The concentration of

IL-6 after the treatment with polyhexanide was 84 ± 3.7 pg/ml, after the treatment with chlorhexidine 0.1% was 10 ± 2.1 pg/ml, and after the treatment with chlorhexidine 2% was 9 ± 2.5 pg/ml. The concentration of IL-7 after the treatment with polyhexanide was 208 ± 19.8 pg/ml, while under all other conditions the concentrations were below the detection limit of 15 pg/ml. The concentration of IFN-γ after the treatment with polyhexanide was 1558 ± 176 pg/ml, after the treatment with chlorhexidine 0.1% was 9.1 ± 0.0003 pg/ml, and after the treatment with chlorhexidine 2% was 23 ± 9.3 pg/ml. The concentration of TNF-α after the treatment with polyhexanide was 4114 ± 469 pg/ml, while under all other conditions the concentrations were below 40 pg/ml. The concentration of VEGF after the treatment with polyhexanide was 297 ± 29 pg/ml, while under all other conditions the concentrations were below 13 pg/ml. The concentration of eotaxin after the treatment with polyhexanide was 139 ± 17.7 pg/ml, after the treatment with chlorhexidine 0.1% was 6 ± 0.3 pg/ml, and after the treatment with chlorhexidine 2% was 7 ± 0.7 pg/ml. The concentration of FGF basic after the treatment with polyhexanide was 219 ± 26.7 pg/ml, after the treatment with chlorhexidine 0.1% was 31 ± 13.6 pg/ml, and after the treatment with chlorhexidine 2% was 82 ± 40.6 pg/ml. The concentration of GM-CSF after the treatment with polyhexanide was 317 ± 77.6 pg/ml, after the treatment with chlorhexidine 0.1% was 13 ± 1.8 pg/ml, and after the treatment with chlorhexidine 2% was 9.4 ± 2.4 pg/ml.

DISCUSSION

In the present study, we were able to show morphological cell damage of osteoblasts after optimal exposure to polyhexanide, the most frequently used antiseptic in orthopedic surgery, and chlorhexidine, an antiseptic used in dental surgery. Additionally, polyhexanide but not chlorhexidine induced an inflammatory reaction in human osteoblasts.

We were able to confirm the toxic effects of polyhexanide on human osteoblasts as reported previously [25]. Polyhexanide-treated cells were shrunken and clearly showed less intercellular contacts. These morphological changes in human osteoblasts could indicate signs of apoptosis as we have demonstrated before [26, 27].

Chlorhexidine-treated osteoblasts also exhibited morphological signs of cell death. Blebbing cell membranes of shrunk cells most likely indicate cell apoptosis, whereas swollen cells with inconsistent cell borders suggest necrosis [26, 27]. Already in 2008, Giannelli et al. described both types of cell death under chlorhexidine incubation of the osteoblastic Saos-2 cell line [28]. Similar immediate toxic influences of chlorhexidine have been reported in further osteoblastic cell lines and in alveolar bone cells. Furthermore, a dose-dependent cytotoxic effect of chlorhexidine on primary osteoblasts has also been described [28–32].

In the present study, we detected increased cytokine/chemokine concentrations after treatment with polyhexanide. A study of Lachapelle et al. in 2014 showed a relevant importance of chemokine reaction in regard to allergic properties of antiseptic solutions [33]. Inflammatory cytokines and growth factors play a key role in the initiation and development of inflammatory diseases [34–36]. Increased level of VEGF, eotaxin, and FGF basic are highly involved in inflammatory processes. These factors modulate proliferation of immune cells, endothelial cells, and neo-angiogenesis as well as formation of capillaries from an existing vascular network [37, 38]. Additionally, we analyzed the pro-inflammatory cytokines IL-1β, -6, -7, IFN-γ, TNF-α. We consider it to be of interest that we were able to show increased levels of pro-inflammatory cytokines and chemokines after treatment with polyhexanide only. Activation of pro-inflammatory mediators could result in a reduced wound healing and osteoblasts' repair process in periprosthetic joint infection. Moreover, an imbalance of pro-inflammatory cytokines and anti-inflammatory cytokines results in an accelerated inflammation in joints [39–42]. Thus, the demonstrated induction of an inflammatory reaction could contribute to adverse effects like periprosthetic fractures. Furthermore, in patients with osteoporosis or with a disturbed inflammatory reaction such as that in patients with autoimmune diseases (e.g., RA), the increased risk of periprosthetic fractures already present due to poor bone quality could even be enhanced [43, 44].

Thus, in summary, we were able to clearly demonstrate the superiority of chlorhexidine at concentrations of 0.01% or 2% when compared with the use of polyhexanide. While chlorhexidine did not induce any cytokine/chemokine secretion, polyhexanide in fact did. Furthermore, chlorhexidine at 0.05% eliminates 99.8% of contaminating bacteria within 1 min in a tissue model [45]. In contrast, polyhexanide exhibits a late antiseptic onset after 5 min and therefore requires a prolonged exposure time [46]. When we designed our study, we hypothesized that treatment with low concentrations of chlorhexidine exhibits equally low toxic effects on human osteoblasts as those expected from polyhexanide. Now—in regard to effects on osteoblasts and comparison with the standard antiseptic polyhexanide—we would clearly recommend that chlorhexidine not only at the lower concentration of 0.01% but also at 2% is the more suitable antiseptic in orthopedic surgery.

We would like to discuss the following limitations: for the present study we used human osteoblasts to obtain results that match in vivo conditions as closely as possible. However, comparability can only be achieved to a limited extent. Isolated human tissue

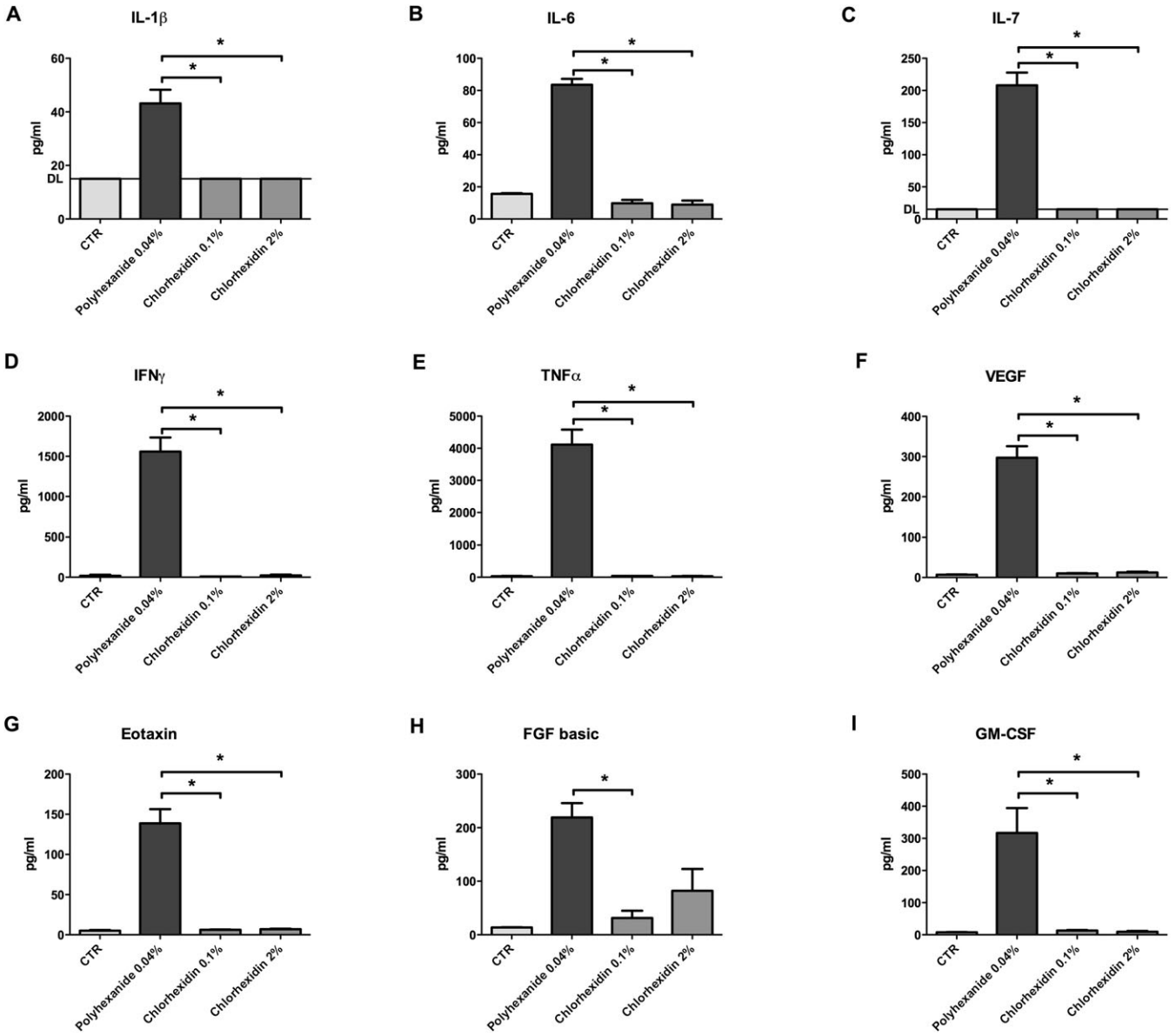

FIGURE 2 Polyhexanide but not chlorhexidine induces the secretion of cytokines/chemokines. Osteoblasts were treated with either PBS (control), polyhexanide at 0.04%, or chlorhexidine at 0.1%/2%. The concentrations of (A) IL-1β, (B) IL-6, (C) IL-7, (D) IFN-γ, (E) TNF-α, (F) VEGF, (G) eotaxin, (H) FGF basic, and (I) GM-CSF were examined in a multiplex suspension array. Polyhexanide induced all of the cytokines while chlorhexidine acted much more gently. Undetectable values were replaced by the detection limit value (DL). The results of four different donors of osteoblasts are summarized, $^*p < .05$, Mann–Whitney test.

is always subject to donor-related individual variations regarding age, gender, and hormone levels [46]. Additionally, the in vitro osteoblast cell culture does not offer all functions of a human bone tissue. Osteoblasts are exposed directly to the agent rather than passing different barriers of cell tissue in vivo. Also, antiseptic dilution through accumulating tissue fluid, regulation of body temperature, influences on immunologic effects, and the damage resulting from the joint infection itself were not considered within our in vitro analysis here. Additional investigations are therefore needed in order to better evaluate the full extent of antiseptic effects on the human bone tissue.

CONCLUSION

Taken together, both of the antiseptics—polyhexanide at a concentration of 0.04% and chlorhexidine at 0.1% and 2%—have cytotoxic effects on human osteoblasts in vitro. Of these two, only polyhexanide induced inflammatory cytokines/chemokines in osteoblasts. Increased levels of pro-inflammatory cytokines and chemokine in osteoblasts after incubation with polyhexanide resulted in disruption of osteoblast repair mechanisms and for this reason, osteoblast degradation has to be considered. We conclude that due to the osteoblast toxicity, both antiseptics should only be used

after careful consideration. But as antiseptics are useful agents in septic surgery, we would recommend from our results the preferential use of chlorhexidine. This could especially become important in patients with autoimmune diseases such as RA.

ACKNOWLEDGEMENT

The authors would like to thank Manuela Jakstadt for excellent technical assistance.

Declaration of interest: The authors have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the results and views presented in this article. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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