Comparable Effects of Polyhexanide and Chlorhexidine on Cell Viability of Primary Human Chondrocytes

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Summary In septic orthopaedic surgery, one of the most common used antiseptics is polyhexanide, in contrast to chlorhexidine. Our hypothesis was that the use of low concentrated chlorhexidine has the same toxic effects on human chondrocytes as does treatment with polyhexanide. Human chondrocytes were isolated and cultured and 0.04% polyhexanide, 2% chlorhexidine or 0.1% chlorhexidine were added to the cultures. Toxicity analyses were performed by visualization of cell structure and quantification of LDH activity. Determination of vital chondrocytes was investigated by Cell-Counter and by fluorescence microscopy. Light microscopic data revealed cell structure defects and change of cell morphology after antiseptic treatment. A significant increase of LDH enzyme activity was shown after treatments with 0.04% polyhexanide and 0.1% chlorhexidine if compared to control. The determination of vital chondrocytes showed a significant decrease. Both antiseptics induce significant cell death of chondrocytes. No significant differences of toxic potential after the treatments with 0.04% polyhexanide and 0.1% chlorhexidine were found.

Key words Chlorhexidine, Polyhexanide, Toxicity, LDH activity.

One of the most threatening complications in orthopaedic and trauma surgery is joint infections, causing significant morbidity and mortality. Acute septic inflammations of knee, hip or shoulder joints are an orthopaedic case of emergency, and lead to immediate surgical treatment. It is evident that mechanical elimination of bacteria through lavage and surgical debridement can be supported by antiseptic substances. Nonetheless, all of the commonly used materials have shown considerable tissue toxicity (Atiyeh et al. 2009, Drosou et al. 2003, Hirsch et al. 2010). In contrast to antibiotic substances, antiseptics do not differ in eukaryotic and prokaryotic cells. Local treatment with antiseptics is still a standard procedure in septic joint surgery. One of the most commonly used antiseptics is polyhexanide. Literature reviews showed controversial data about the effects of polyhexanide on human chondrocytes. Its preferred use in septic joint surgery seems undisputed (Röhner et al. 2010a, Schaumburger et al. 2010), yet chlorhexidine has a broad activity against oral pathogens and has been successfully used in endodontics and periodontology. Moreover, the absence of developing resistance can be added to its benefits. Significant substantivity by the slow release from hard or soft oral tissues has been observed, too. It ensures antimicrobial activity for prolonged time intervals following application (Cecchin et al. 2011, Hitz Lindenmüller et al. 2011,

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Matthews 2011, Shen et al. 2011). Interestingly, the reason of the limited use of chlorhexidine in orthopaedic surgery is rarely documented in literature. Various case reports showed toxic effects of chlorhexidine on human cartilage after open or arthroscopic surgery. In all cases an unclear or high concentration of chlorhexidine was used for an unknown period of treatment (Bellen 1987, Douw et al. 1998, Rombouts et al. 1986, Tricoit et al. 1984).

It is known that 0.05% chlorhexidine delivered by jet lavage eliminates 99.8% of contaminating bacteria within 1 min in a tissue model (Stevenson et al. 1976, Taylor et al. 1999, Wilson et al. 1994). Chlorhexidine does not lose full antiseptic activity in the presence of blood in contrast to polyhexanide, povidone iodide or hydrogen peroxide (Stevenson et al. 1976, Taylor et al. 1999). Also, various studies have proved that the use of 0.05% chlorhexidine has no negative effects on wound healing. Compared to physiological saline, low level concentrations of chlorhexidine (0.001% till 0.05%) revealed the same toxic effects on tissue (Sanchez et al. 1988, Severyns et al. 1991). Recent studies have shown that non-osteoarthritic cartilage treated with 0.05% chlorhexidine followed by rinsing was not significantly affected (Best et al. 2007).

To our knowledge, up to date the cellular effect of chlorhexidine on primary human chondrocytes is not known. Therefore, we compared cellular effects of regularly used polyhexanide with low concentrated chlorhexidine on primary human chondrocytes. We hypothesized that treatment with low concentrated chlorhexidine would have equal toxic effects on human chondrocytes as a treatment with polyhexanide.

Materials and methods

Tissue culture plastic ware was obtained from TPP (Trasadingen, Switzerland). Culture medium, phosphate buffer saline (PBS), trypsin and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). All other reagents were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Chondrocyte isolation and culture

Chondrocyte isolation was performed as described before (Röhner et al. 2010b). Cartilage tissue was obtained from 6 human donors with knee osteoarthritis not presenting any kind of infectious signals. Experimental protocols were approved by the local ethics committee. Cartilage was minced and digested in medium containing 1 mg/ml pronase (Sigma-Aldrich) for 30 min at 37°C. Further, digestion medium was discarded and the tissue was digested with medium containing 1 mg/ml clostridial collagenase (Sigma-Aldrich) at 37°C over night. The digested solution was then filtered (70 μm Nylon; BD Falcon, Bedford, Germany) and centrifuged at 1200 rpm for 8 min. The supernatant was discarded and cell pellet was washed 3 times with phosphate buffered saline (PBS). Then chondrocytes were suspended in DMEM Hams-F12 media containing 10% FCS, 1% penicillin/streptomycin, and cultured at 37°C, 95% air and 5% CO₂. All experiments were performed immediately.

Chondrocytes treatment and detection of cell structure

Human chondrocytes were cultured and seeded on 24-well plates at a density of sub-confluence; then, 100 μl undiluted solution of concentrated 0.04% polyhexanide (Charité Pharmacy, Berlin, Germany), 2% chlorhexidine, or 0.1% chlorhexidine (Charité Pharmacy) were added for 15 min. PBS treated chondrocytes were used as control. Immediately after incubation time, the results were analyzed by light microscopy (Axiowert 40 C Light Microscope, lens 10x0.25, ocular 10x18; Zeiss, Göttingen, Germany) and documented digitally (Canon EOS 500D, 15.1 Megapixels, Japan).
Activity of lactate dehydrogenase

LDH activity is a marker of advanced cell death. Release of LDH indicates the loss of membrane plasma integrity as possible marker of increased cell necrosis. Chondrocyte cell monolayers were treated for 15 min with 0.04% polyhexanide and 0.1% chlorhexidine. To determine the LDH activity, the cyto-toxicity detection kit from Roche was used. LDH activity in the supernatant was determined by colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH and expressed as the percentage of total enzyme activity liberated from chondrocytes in the presence of the antiseptic. Then LDH-Reaction mixture (100μl) was added to 100μl of the supernatants, and was measured by the absorbance of 490 nm by using an electronic Elisa Reader.

Determination of vital cells by cell counting

Isolated human chondrocytes were counted by using a Casy Cell-Counter (Schärfe-System, Reutlingen, Germany) and re-seeded on 24-well plates at a density of 2×10⁴ cells per well. After one day, chondrocytes were treated with 100μl of 0.04% polyhexanide, 2% chlorhexidine, and 0.1% chlorhexidine, while PBS and Triton served as control. The treatment lasted 5 and 15 min, respectively. Afterwards, cells were washed 3 times with 100μl of 0.9% physiological sodium chloride. Subsequently, all cells were suspended in growth medium for 48 h. After removing of growth medium all cells were detached with 100μl Trypsin. Detection of living was determined by the Casy Cell-Counter and Analyser System.

Detection of vital cells by fluorescence microscopy

Propidium iodide/flouresceine diacetate (PI/FDA) staining was performed to assess cell viability. Living cells actively convert non-fluorescent fluoresceine diacetate (FDA) into the green fluorescent compound fluoresceine. Propidium iodide (PI) is a membrane impermeable dye, which is excluded from viable cells and stains dead cells. Chondrocytes, seeded on chamber slides at a density of 2×10⁴ cells/cm², were treated with 0.04% polyhexanide or 0.1% chlorhexidine each. Triton X100, as an inducer of cell necrosis, was used as positive control. Chondrocytes were washed with PBS and stained with FDA solution (3μg/ml) at 37°C for 15 min. Then cells were washed again with PBS and incubated with PI solution (0.1 mg/ml) at RT for 2 min, followed by another washing stage. Documentation was performed immediately by fluorescence microscopy (CKX 41; Olympus).

Statistical analysis

A nonparametric Wilcoxon matched-pairs test was used as indicated in the legends. A p value of <0.05 was considered to be significant.

Results

Human chondrocytes treated with polyhexanide or chlorhexidine showed defects in the cell structure after an incubation time of 15 min. Controls, cultured without antiseptic treatment, did not show any cell damage (Fig. 1A). The incubation with Triton X100 as a known mediator of cell necrosis revealed immediate induction of necrosis in human chondrocytes (Fig. 1B). Contrasting, chondrocytes treated with 0.1% chlorhexidine (Fig. 1C) or 0.04% polyhexanide (Fig. 1D) were swollen and showed a completely defect cell structure.

LDH activity was analyzed in the supernatant of each cell culture. Compared to control (chondrocytes treated with PBS), a significant increase of LDH activity after 15 min of treatment with 0.04% polyhexanide or 0.1% chlorhexidine), was noted p<0.05 (Fig. 2). There were no significant differences between 0.04% polyhexanide and 0.1% chlorhexidine. Triton X100 was used as a known mediator of cell necrosis and induced a high LDH activity. The determination of vital cells...
by Casy Cell Counter of polyhexanide and chlorhexidine treated chondrocytes showed a significant decrease of vital cells in comparison with control after 5 min (Fig. 3A) and after 15 min (Fig. 3B). When comparing 0.04% polyhexanide to 2% chlorhexidine, we detected significant differences of vital cell numbers after treatment times of 5 and 15 min (\(p<0.05\); Fig. 3A, B). There were no significant differences between 0.04% polyhexanide and 0.1% chlorhexidine after treatment times of 5 and 15 min (Fig. 3A, B).

Accordingly, fluoresceine diacetate and propidium iodide staining examined by fluorescence microscopy of chondrocytes treated with antiseptics showed no significant differences between 0.04% polyhexanide and 0.1% chlorhexidine after treatment times of 15 min (Fig. 4).
Discussion

The appropriate therapy of joint infections is still an unsolved problem (Stutz and Gächter 2001). Besides treatment using antibiotics, the mechanical elimination of bacteria through joint and tissue lavage as well as surgical debridement can be supported by antiseptic substances. In our ex-
Experiment a temporary irrigation of cartilage tissue was simulated by using antiseptics (Atiyeh et al. 2009, Drosou et al. 2003, Hirsch et al. 2010). We compared one of the most frequently used antiseptic (polyhexanide) with different concentrations of chlorhexidine which is an antiseptic still being discussed controversially in septic joint surgery. After treatment with polyhexanide or chlorhexidine, the qualitative microscopic analysis showed a considerable number of swollen chondrocytes with a defective cell structure in both treatment groups, indicating cell necrosis as the end-stage of toxic cell damage (Bühling et al. 2003, Simon 1997). Further, a significant increase of LDH activity already after an incubation time of 15 min with polyhexanide or 0.1% chlorhexidine if compared to control could be shown. Increased LDH activity after the treatment of human chondrocytes with 0.04% polyhexanide or 0.1% chlorhexidine indicates a loss of membrane plasma integrity as possible marker of cell necrosis (Simon 1997, Walker et al. 1988). The lower activity of LDH after treatment with 0.1% chlorhexidine in comparison to 0.04% polyhexanide may indicate that 0.1% chlorhexidine induces less cell necrosis than 0.04% polyhexanide.

Evaluation of cell viability showed that human chondrocytes were negatively affected by all antiseptics, if compared to the PBS treated control. A significantly reduced number of vital cells were seen after treatment with 2% chlorhexidine. No significant differences were observed when comparing 0.1% chlorhexidine and 0.04% polyhexanide if analyzed by Casy Cell-Counter as well as by fluorescence microscopy.

Chlorhexidine is antiseptic with a high antibacterial efficiency; it is very frequently used as an antiseptic in mouth rinsing, for bladder treatment as well as in periodontology, endodontics, and oral surgery (Rushton Schiott et al. 1976, Sheppard et al. 1997, Noetzel et al. 2009). Fewer previous studies have shown toxic (chondrolytic) effects of chlorhexidine on human cartilage after open or arthroscopic surgery. In a study by Huyssteen et al. (1999), 3 cases of chondrolysis knee joint tissue after arthroscopically-assisted reconstruction of the anterior cruciate ligament were reported (van Huyssteen et al. 1999). Rombouts et al. (1986) also reported about chondrolysis after knee arthrotomy with an additional rinsing with 0.5% chlorhexidine. In most cases, high concentrations of chlorhexidine were used for a prolonged irrigation time mostly on injured cartilage tissue (Douw et al. 1998, Tricoit et al. 1984).

However, recent studies were able to show that low concentrated chlorhexidine has no negative effects on rat or human articular cartilage metabolism. In an in vitro study by Best et al. (2007), the effects of 0.05% chlorhexidine on osteoarthritic and non-osteoarthritic human cartilage after a treatment time of 1 or 60 min were examined. 0.05% chlorhexidine did not significantly affect non-osteoarthritic cartilage metabolism. Yet, prolonged exposure of up to 1 h significantly affected cartilage metabolism of both cartilage types (Best et al. 2007). In a rat patella model, even a 1 min treatment with 0.05% chlorhexidine and chlorhexidine jet lavage did not significantly affect cartilage metabolic activity (Reading and Taylor 2000).

The results of our present study revealed that both 0.1% and 2% chlorhexidine affect human chondrocytes. Chlorhexidine damaged cell structure and reduced vital cell numbers of human chondrocytes. However, the comparison between 0.1% chlorhexidine and 0.04% polyhexanide did not show any significant differences in affected cell structures and vital cell numbers of human chondrocytes after a short treatment time (up to 15 min).

With in vitro studies using human cells uncontrollable influences may play an important role such as temperature, pressure, or contamination. Thus, the present cell culture model might have some limitations, and it is pertinent to note that our outcome does not represent in vivo situations, in particular since with in vitro simulations higher cell toxicity may occur (based on direct chondrocyte incubation). With antiseptic solutions used, in vitro barriers such as cartilage intercellular matrix do not prevent any passage; moreover, possible antiseptic effects after contact with proteins of tissue fluids such as blood will not be weakened.

In summary, chlorhexidine is an antiseptic with prepossessing tissue compatibility and a very
high anti-microbiological potential. In comparison with 0.04% polyhexanide, 0.1% chlorhexidine has the same toxic effects on human chondrocytes. Nevertheless, the use of chlorhexidine should be limited to a short exposure time and a low concentration of 0.1%. After this procedure an additional irrigation with sodium chloride or comparable solutions should be obligate to remove any antiseptic remnants.

Disclosure statement

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.

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