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# Antiseptic solutions modulate the paracrine-like activity of bone chips: differential impact of chlorhexidine and sodium hypochlorite

Kosaku Sawada [1], Jordi Caballé-Serrano [2,3], Dieter D. Bosshardt [2,4], Benoit Schaller [1], Richard J. Miron [2], Daniel Buser [2], Reinhard Gruber [2,5,6]

[1] Department of Cranio Maxillofacial Surgery, Inselspital, University of Bern, Switzerland

[2] Department of Oral Surgery and Stomatology, School of Dental Medicine, University of Bern, Switzerland

[3] Department of Oral and Maxillofacial Surgery, College of Dentistry, Universitat Internacional de Catalunya,

Barcelona, Spain

[4] Robert K. Schenk Laboratory of Oral Histology, School of Dental Medicine, University of Bern, Switzerland

[5] Department of Preventive, Restorative and Pediatric Dentistry, University of Bern, Switzerland

[6] Department of Oral Biology, Medical University of Vienna, Vienna, Austria

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**Corresponding address:** Reinhard Gruber, School of Dental Medicine, University of Bern, Freiburgstrasse 7, 3010 Bern, Switzerland; Tel: +41 31 632 8622; Email: reinhard.gruber@zmk.unibe.ch

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**Abstract**

*Aim*: Chemical decontamination increases the availability of bone grafts; however, it is unclear whether antiseptic processing changes the biological activity of bone.

*Materials and Methods*: Bone chips were incubated with 4 different antiseptic solutions including (1) povidone-iodine (0.5%), (2) chlorhexidine diguluconate (0.2%), (3) hydrogen peroxide (1%) and (4) sodium hypochlorite (0.25%). After 10 minutes of incubation, changes in the capacity of the bone-conditioned medium to modulate gene expression of gingival fibroblasts was investigated.

*Results*: Conditioned medium obtained from freshly prepared bone chips increased the expression of TGF-β target genes interleukin 11 (IL11), proteoglycan4 (PRG4), NADPH oxidase 4 (NOX4), and decreased the expression of adrenomedullin (ADM), and pentraxin 3 (PTX3) in gingival fibroblasts. Incubation of bone chips with 0.2% chlorhexidine, followed by vigorously washing resulted in a bone-

conditioned medium with even higher expression of IL11, PRG4, and NOX4. These findings were also found with a decrease in cell viability and an activation of apoptosis signaling. Chlorhexidine alone, at low concentrations, increased IL11, PRG4 and NOX4 expression, independent of the TGF-β receptor I kinase activity. In contrast, 0.25% sodium hypochlorite almost entirely abolished the activity of bone-conditioned medium, while the other two antiseptic solutions, 1% hydrogen peroxide and 0.5% povidone-iodine, had relatively no impact, respectively.

*Conclusion*: These in vitro findings demonstrate that incubation of bone chips with chlorhexidine differentially affects the activity of the respective bone-conditioned medium compared to the other antiseptic solutions. The data further suggest that the main effects are caused by chlorhexidine remaining in the bone-conditioned medium after repeated washing of the bone chips.

**Clinical Relevance**

*Scientific rationale for study*: Antiseptic solutions are commonly used for a variety of procedures in the dental field including decontamination of bone grafts and irrigation of bone defects. The impact of different antiseptic solutions on the biological properties of bone remains unclear.

*Principal findings*: Conditioned media from bone exposed to chlorhexidine significantly increased the gene expression of periodontal fibroblasts TGF-β target genes when compared to bone exposed to povidone-iodine and hydrogen peroxide. Furthermore, it was found that sodium hypochlorite almost entirely abolished the activity of the bone-conditioned medium.

*Practical implication*: The findings from this in vitro study demonstrate that antiseptic solution might considerably affect bone activity by modulating gene expression following rinsing. Chlorhexidine performed significantly better than the other modalities tested.

**Conflict of Interest and Source of Funding**

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**Introduction**

Antiseptic solutions are widely used in preventive and regenerative dentistry for procedures such as mouth rinsing prior and after surgical interventions (Prag et al. 2014), irrigation of pockets around implants (Pedrazzi et al. 2014), and decontamination of extraction cavities (Haraji and Rakhshan 2014). Moreover, antiseptic solutions are also indicated in soft tissue dehiscence, particularly when bone is exposed (Melea et al. 2014). Autogenous bone may also be exposed to antiseptic solution in the rare case of contamination during bone harvesting (Pommer et al. 2014, Hantes et al. 2008, Presnal and Kimbrough 1993, Hooe and Steinberg 1996). For allografts, decontamination with antiseptic solutions is part of routine processing protocols (Coraca-Huber et al. 2013, Haimi et al. 2008). Taken together, there are numerous situations where human bone may be exposed to antiseptic solutions.

Antiseptic solution not only reduces bacterial load but they also have cytotoxic effects on eukaryotic cells. For example, povidone-iodine is toxic to epithelial cells (Sato et al. 2014), and chlorhexidine and sodium hypochlorite are toxic to mesenchymal cells (Abbaszadegan et al. 2015, Proksch et al. 2014). Furthermore, high concentrations of hydrogen peroxide may decrease survival rate of human gingiva fibroblast (Furukawa et al. 2015). Besides toxicity studies, antiseptic solutions may provoke functional changes as exemplified by the gene expression profiling of the peritoneum in response to chlorhexidine in mice (Yokoi et al. 2012). Also data on the impact of antiseptic solution on bone grafts are available. For instance, rabbit bone has previously been decontaminated with chlorhexidine and povidone-iodine (Yazdi et al. 2012). Human bone has been exposed to hydrogen

peroxide for decontamination (Coraca-Huber et al. 2013). In vitro explant cultures showed that bone grafts that were previously exposed to chlorhexidine or sodium hypochlorite, but not povidone-iodine, considerably reduced or even abolished cell viability (Bauer et al. 2011, Verdugo et al. 2011). Still, the question remains to be answered whether antiseptic solutions are capable of changing the paracrine-like properties of bone grafts.

Recently, an in vitro bioassay was proposed to determine the paracrine-like activity of explant cultures of bone grafts (Peng et al. 2014). The bioassay measures the response of oral fibroblasts or other mesenchymal cells exposed to supernatants of native bone chips (Zimmermann et al. 2014), but also bone chips after demineralization (Schuldt Filho et al. 2015), or thermal processing (Sawada et al. 2015, Schuldt Filho et al. 2015). The response of oral fibroblasts to bone conditioned medium is characterized by the expression of TGF-β target genes, including interleukin 11 (IL11), proteoglycan 4 (PRG4), and NADPH oxidase 4 (NOX4), adrenomedullin (ADM), and pentraxin 3 (PTX3) (Zimmermann et al. 2014, Peng et al. 2014). Bone conditioned medium remains active even after repeated washing of the bone chips (Peng et al. 2014). This property provided the basis to prepare bone-conditioned medium after repeatedly washing of bone chips that have been exposed to antiseptic solutions.

As already cited (Zimmermann et al. 2014), IL11 is a member of the IL-6 family of cytokines and together with BMP-2, can accelerate bone regeneration (Suga et al. 2004). PRG4 is expressed in the superficial zone of articular cartilage (Rhee et al. 2005) and supports endochondral bone formation (Novince et al. 2012). NOX4 generates intracellular superoxide which also modulates osteoblasts BMP-2 activity (Mandal et al. 2011). ADM is a vasodilator also capable of affecting osteoblasts viability (Lausson and Cressent 2011). PTX3 is an multipotent inflammatory mediator that stimulating osteoclastogenesis (Lee et al. 2014). Overall, the genes that are regulated by BCM can target bone

cells and theoretically can translate into a cellular response that might be relevant in graft consolidation.

The bioassay was used to investigate the activity of conditioned medium obtained from bone chips exposed to antiseptic solutions. This in vitro basic research should add to the functional understanding of how antiseptic solutions change the biological properties of bone grafts.

## Material and Methods

### *Antiseptic solutions*

The following antiseptic solutions were selected: (1) povidone-iodine (PI, 11%) (Mundipharma Medical Company, Basel, Switzerland); (2) chlorhexidine diguluconate (CHX, 0.2%) (Glaxo Smith Kline, Consumer Healthcare, Bern, Switzerland); (3) hydrogen peroxide ($H_2O_2$, 2%) (Inselspital, Bern, Switzerland); (4) sodium hypochlorite (HYP, 5.25%) (Dr. Speier, Münster, Germany). Antiseptic solutions were diluted in sterilized water to reach a final concentration of 0.5% PI, 1% $H_2O_2$, and 0.25% HYP. Chlorhexidine diguluconate was not further diluted. All antiseptic solutions were filtered sterile (0.22µm pore diameter; Merck Millipore, Billerica, MA, USA).

### *Bone-conditioned medium (BCM)*

Bone was obtained from adult pigs within six hours postmortem (Metzgerei Balsiger, Wattenwil, Switzerland). Bone chips were harvested from the buccal-sided mandibular cortical bone with a bone scraper (Hu-Friedy, Rotterdam, Netherlands) and placed into sterile plastic dishes containing Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics and antimycotics (all from Invitrogen Corporation, Carlsbad, CA, USA). The culture medium was removed and 5g of wet

bone chips were exposed to 10ml antiseptic solutions for ten minutes at room temperature. Antiseptic solutions was removed and 5g of bone chips were washed with at least 10 ml phosphate-buffered saline three times consecutively for 30 seconds under continuously shaking. Phosphate-buffered saline was removed from the bone chips. Five-gram bone chips were incubated with 10 ml serum-free culture medium (50% weight/volume) (Peng et al. 2014). BCM was harvested after 24 hours of incubation at 37°C in a humidified atmosphere at 5% carbon dioxide. BCM was filtered sterile and kept frozen at −80°C. The BCMs were termed "PI BCM", "CHX BCM", "$H_2O_2$ BCM", and "HYP BCM". Also the respective "non-treatment BCM" as originally described by Peng et al (Peng et al. 2014) , which did not underwent washing with phosphate-buffered saline, was included in the experiments. The stocks were thawed immediately before each experiment.

*Primary gingival fibroblast*

Gingival fibroblasts serve as bioassay for the activity of BCM. As previously reported (Peng et al. 2014, Zimmermann et al. 2014), human gingival fibroblasts were prepared from tissue grafts of six anonymous adult donors. The Kantonale Ethikkommission of Bern approved this protocol. Cells were cultured in a humidified atmosphere at 37°C in growth medium consisting of DMEM, 10% fetal calf serum (FCS; Invitrogen), and antibiotics. Cells were plated in growth medium at 30,000 cells/$cm^2$ into culture dishes and exposed to 20% BCM diluted in serum-free medium for 24 hours, before gene expression analysis was performed.

*qRT-PCR analysis*

Total RNA was isolated with the High Pure RNA Isolation Kit (Hoffmann-La Roche, Basel Switzerland). Reverse transcription was performed with Transcriptor Universal cDNA Master (Hoffmann-La Roche). Polymerase chain reaction (PCR) was done with the FastStart Universal SYBR Green Master

(Hoffmann-La Roche) on a 7500 Real-Time PCR System (both Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Probes are indicated in Table 1. The mRNA levels were calculated by normalizing to the housekeeping gene beta actin using the ΔΔCt method.

*Cell viability*

After incubation of cells with BCM for 24 hours, gene expression of pro-apoptotic genes (BAD, BAX) and anti-apoptotic (BCL-XL, BCL-2) was evaluated and live-dead staining was performed (Enzo Life Sciences AG; Lausen, Switzerland). For formazan formation, cells were exposed to the various concentrations of chlorhexidine and sodium hypochlorite for two minutes. Cells were washed with phosphate-buffered saline and the conversion of MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 0.5 mg/mL) into formazan crystals was determined.

*Chlorhexidine and sodium hypochlorite exposure to gingival fibroblast*

To investigate the effect of chlorhexidine to change gene expression in gingival fibroblasts, cells were exposed to 0.00156% chlorhexidine in serum-free medium, with and without SB431542 (TGF-β receptor I kinase inhibitor), for 24 hours. Besides IL11, PRG4, and NOX4, also expression of inflammatory genes (IL6, CXCL1, CCL7, TNFα, IL1) and TGF-β1 was evaluated. Culture supernatant was used for IL6 immunoassay (R&D Systems, Minneapolis, USA). In a similar setting, the effects of sodium hypochlorite on the expression of IL11, PRG4, and NOX4 were investigated.

*Statistical analysis*

Experiments were repeated at least twice and data reported as the mean and standard deviation. Friedman-test was used for analysis for differences between five BCM. Paired T-test was used for post hoc testing to compare non-treatment BCM vs. other BCM respectively (Figure 1, Table 2 and 3). Paired T-test was used for comparison of without and with SB431452 (Figure 4). Statistical significance was established at $p<0.05$.

**Results**

*Chlorhexidine and sodium hypochlorite changed the activity of BCM*

To determine the impact of antiseptic solutions on the capacity of bone chips to produce an active bone-conditioned medium, the response of gingival fibroblasts towards their respective target genes was evaluated. It should be stated that gingival fibroblasts served as a bioassay for the activity of BCM, knowing that also bone-derived cells show a similar response to BCM (Sawada et al. 2015). The most striking finding was that BCM prepared from bone chips exposed to chlorhexidine was more potent than non-treatment BCM to increase IL11, PRG4, and NOX4 expression (Figure 1). However, chlorhexidine reversed the inhibitory activity of BCM on ADM and PTX3 gene expression. Thus, chlorhexidine has a diametric effect on modulating the activity of bone chips and their respective BCM. Also striking was that sodium hypochlorite almost completely inhibited the activity of BCM. Exposure of bone chips with povidone-iodine and hydrogen peroxide gave no consistent picture, only a weak trend towards a lowering of the activity of BCM (Figure 1). Taken together, while the inhibition of BCM activity by sodium hypochlorite was to be expected because of the strong protein denaturation activity (Winter et al. 2008), the increased expression of IL11, PRG4, and NOX4 by exposure of bone chips with chlorhexidine was remarkable.

*Chlorhexidine but not sodium hypochlorite BCM negatively affects cell viability*

The authors next focused on the role of chlorhexidine and sodium hypochlorite in this in vitro setting. First hints that BCM contains traces of chlorhexidine came from observations that gingival fibroblast exposed to "CHX BCM" showed a lowering of formazan formation (Table 2) and a rounding of the cells (not shown), indicating changes in viability and morphology, respectively. Moreover, CHX BCM caused more gingival fibroblasts to turn red in the live/dead cell assay demonstrating that CHX BCM had a negative impact on cell viability (Figure 2). CHX BCM also changed the expression of apoptosis genes. Compared to HYP BCM preparations, CHX BCM increased not only expression of pro-apoptotic genes (BAD, BAX) by around 3-fold and also the expression of anti-apoptotic (BCL-XL, BCL2) genes were increased around 5-fold (Table 3). Taken together, these findings demonstrate that in particular CHX BCM, but not HYP BCM and the other preparations, negatively affected cell viability.

*Chlorhexidine caused changes in gene expression in gingival fibroblast*

To investigate if the observations were caused by chlorhexidine or sodium hypochlorite acting as a contamination of BCM, further experiments were performed with chlorhexidine and sodium hypochlorite alone. Viability assays based on formazan formation revealed chlorhexidine and sodium hypochlorite to be non-cytotoxic for gingival fibroblast at 0.002% (data not shown). Interestingly, chlorhexidine at this low concentration caused a considerable increase in the TGF-β target genes IL11, PRG4, and NOX4 (Figure 3). Sodium hypochlorite alone at 0.002% also had a weak positive effect on gene expression in a pilot experiment with two cell donors (IL11: 2.5/7.5x, PRG4: 3.4/4.8x, and NOX4: 1.1/3.4x). However, these effects were in contrast to the inhibitory effects of sodium hypochlorite on bone chips and the respective activity of BCM. Thus, the inhibitory effects of sodium hypochlorite on the activity of BCM cannot be explained by a potential contamination with the antiseptic solution.

*Chlorhexidine caused changes in gene expression independent of TGF-β receptor I kinase activity.*

To determine if the increase in gene expression by chlorhexidine is caused by endogenously produced TGF-β1, the expression of the respective growth factor was investigated, and blocking the TGF-β receptor I with SB431542 was performed. TGF-β1 expression remained almost unchanged, and SB431542 did not prevent the effects of chlorhexidine on IL11, PRG4, and NOX4 expression (Figure 3). Further support for a TGF-β1 independent gene regulation came from observations that chlorhexidine provoked a robust proinflammatory cellular response including IL1, IL6, TNFα, and CCL7 (Table 4). In support of the gene expression data, IL6 protein was increasingly released into the supernatant (Control 8.85 ± 1.97 ng/ml; CHX 15.60 ± 1.76 ng/ml). Thus, the increased gene expression upon exposure of fibroblast with CHX BCM could be reproduced with chlorhexidine, suggesting that the main effects are caused by chlorhexidine remaining in the conditioned medium after repeated washing of the bone chips.

**Discussion**

Antiseptic solutions are frequently used for irrigation therapy and decontamination of bone grafts but it is unclear whether antiseptic solutions change the biological properties of bone (Coraca-Huber et al. 2013, Haimi et al. 2008, Hantes et al. 2008, Haraji and Rakhshan 2014, Hooe and Steinberg 1996, Melea et al. 2014, Pedrazzi et al. 2014, Prag et al. 2014, Presnal and Kimbrough 1993). This research is a logical extension of previous studies describing the paracrine-like function of bone chips in vitro – bone chips that were freshly prepared (Peng et al. 2014, Zimmermann et al. 2014, Caballe-Serrano et al. 2014), bone chips that underwent thermal processing (Sawada et al. 2015, Schuldt Filho et al. 2015), and demineralized bone chips (Schuldt Filho et al. 2015). The main finding of the present study was that the incubation of bone chips with chlorhexidine resulted in a conditioned medium exceeding the activity of similar preparation obtained from untreated bone chips. It turned out that chlorhexidine alone was able to exert an increase of IL11, PRG4, and NOX4 expression,

suggesting that the effect observed with CHX BCM were presumably additive; chlorhexidine and BCM can both stimulate the expression of the three target genes.

Another main finding was that sodium hypochlorite, but not the other two antiseptic solutions iodine and hydrogen peroxide, considerably lowered the capacity of bone chips to release active BCM, without impairing cell viability. The observed decrease in BCM activity by incubation of bone chips with sodium hypochlorite cannot be explained by contaminations with antiseptics because sodium hypochlorite alone caused the opposite effect: a weak increase of IL11, PRG4, and NOX4 expression at non-toxic concentrations. Moreover, HYP BCM failed to significantly change the expression of apoptosis marker genes, indicating a neglectable amount of possible remaining sodium hypochlorite in BCM. The reason for the decreased BCM activity presumably lies in the capacity of sodium hypochlorite to misfold and thus damage proteins, apparently including those provoking the expression of IL11, PRG4, and NOX4 (Wyatt et al. 2014). It should be noted though, that the original BCM was prepared as originally described (Peng et al. 2014), while the bone chips exposed to antiseptic solutions underwent repeated washing before BCM was harvested. Thus, the original and the antiseptic preparations cannot be directly compared; the activity of the original BCM is possibly slightly higher than the antiseptic BCMs. Taken together the data suggest that antiseptic solutions behave differently to the ability to change paracrine-like effects of bone chips.

The data confirm previous observations that BCM, even when derived from bone chips that underwent thermal processing (Sawada et al. 2015) or demineralization (Schuldt Filho et al. 2015), caused robust changes in gene expression including an increase of IL11, PRG4 and NOX4; and a decrease of ADM and PTX3. Also in line with the existing knowledge is the observation that chlorhexidine, at concentrations below 0.001%, caused changes in cell viability parameters including formazan formation and expression of genes controlling apoptosis (Giannelli et al. 2008, Basha et al.

1996). The findings that chlorhexidine, independent from BCM, provoked changes in inflammatory gene expression is not new (Yokoi et al. 2012). New, however, is that chlorhexidine increased typical TGF-β target genes but without requiring activation of the TGF-β receptor I kinase or forcing the expression of the respective ligand. Thus, the present observations are partially new, particular findings that chlorhexidine remaining in the BCM after repeated washing provokes an almost similar increase in gene expression, but in contrast to BCM (Peng et al. 2014) not via the TGF-β receptor I kinase activity.

When focusing on outcomes that sodium hypochlorite greatly reduced the activity of BCM, the data support previous studies showing that hypochlorite induces misfolding and thus inactivation of proteins (Winter et al. 2008). Therefore, sodium hypochlorite might have denaturated the original activity of BCM. Studies on the impact of hypochlorite to change the activity of growth factors such as TGF-β1 were not found. Most research was performed on how antiseptics solutions change gene expression. For instance, hypochlorite suppressed IL-1α in epithelial cells (Mohri et al. 2002), povidone iodine increased expression PDGF-A, EGF, and FGF-2 during wound closure (Koca Kutlu et al. 2013), and hydrogen peroxide activated the p38 MAPK pathway (Nishi et al. 2015). It can thus not be ruled out that BCM obtained from bone chips treated with iodine and hydrogen peroxide also contains contaminations of the antiseptics; even if this is the case, iodine and hydrogen peroxide had no considerable impact on the expression of the selected gene panel and were thus not followed up in downstream experiments.

The present in vitro data provide a rational for preclinical investigations to better understand the contribution of BCM, representing the paracrine-like activity of bone grafts, during process of bone regeneration and graft consolidation. Assuming that BCM has a biological role, incubation of bone chips with sodium hypochlorite impasses the paracrine activity and may slow down graft

consolidation. The observation obtain with chlorhexidine are even harder to interpret; on the one hand chlorhexidine contaminations supported the TGF-β related gene expression, on the other hand, chlorhexidine contaminations provoked a massive expression of inflammatory cytokines. The integration of these two diametric observations into a conclusion leaves room for discussion. The present data thus raise the hypothesis that the clinical impact of hypochlorite on the paracrine-like activity of bone chips is negative, while the impact of chlorhexidine is positive under the premise that the TGF-β activity of BCM contributes to graft consolidation. Iodine and hydrogen peroxide might have neglectable effects on the on graft consolidation under the same premise that BCM activity translates into in vivo response.

This pioneering study can be the primer for future research: The exposure time of bone chips, which was chosen, is used for cleaning steps with antiseptics solution of bone graft before they are stored into bone bank (Coraca-Huber et al. 2013, Haimi et al. 2008). However, the effect of exposure time in relation to the size of the bone chips was not investigated in this study. Another challenge is the concentration of antiseptics solutions. Several concentrations should be investigated to determine the optimal concentration to maintain BCM activity while keeping the antiseptic activity. The concentration used was estimated to be present in the oral cavity (Slots 2002, Rosling et al. 2001, Haas et al. 2014, Gonzalez et al. 2015). Moreover, antiseptic solutions were diluted in aqua dest, thus producing a possible hypotonic condition. We cannot rule out a possible impact of transient osmolarity of the release of activity on the conditioned medium (Fening et al. 2011). Thus, numerous questions involving exposure time and concentration of antiseptics solutions are required to be answered in future studies.

Gingival fibroblasts serve as bioassays; they are not the primary target cells for a paracrine activity of bone chips. Our previous data showed that porcine bone-derived cells behave similar, considering the cell response is rather conserved among the mesenchymal cell-types (Sawada et al. 2015, Schuldt Filho et al. 2015). Future studies should focus on the response of cells toward the endothelial lineage or inflammatory cells, and also the impact of BCM on osteoclastogenesis might change with antiseptic solutions (Caballe-Serrano et al. 2015). One limitation might be the use of gingival fibroblasts as target cells since bone chips may find their application in bone regenerative surgery. Bone cells like osteoblasts in the recipient area may be the first addressee for any paracrine influences. However, knowing that bone and gingiva derived fibroblasts show a similar response to BCM, gingival fibroblasts were chosen as the appropriate cell-type for the bioassays presented in the current study.

It remains unclear how chlorhexidine affects the molecular mechanisms with respect to inflammatory cytokine expression. Chlorhexidine is a positively charged molecule that even at low concentrations affects the integrity of the cell wall, which in turn can provoke a cellular response. Higher concentrations inhibit expression of inflammatory cytokines in osteogenic cells (Rohner et al. 2015). Chlorhexidine can modulate gene expression in bacterial species, for example of transporters, explaining their resistance to this antiseptic agent (Hassan et al. 2013). How these changes in cell wall integrity translate into the expression of IL11, NOX4 and PRG4, all TGF-β target genes remains unclear. Interesting though, chlorhexidine is used to provoke peritoneal fibrosis in preclinical models, an effect that is at least partially mediated via TGF-β signaling (Xiong et al. 2014). Our data do not support a TGF-β dependent effect because chlorhexidine did not further regulate ADM and PTX3. This concept is supported by IL-11 being produced by cells in an oxidative stress-dependent manner (Nishina et al. 2012). In support of this model, blocking of TGF-β1 could not consistently reverse the effect of chlorhexidine on IL11, NOX4 and PRG4, and also not change TGF-β1 expression. The data together support a possible explanation of a TGF-β independent mechanism of low concentration chlorhexidine induced gene expression.

Mandibular cortical bone chips have been used in this study, but the data can presumably be extrapolated for other anatomical regions of cortical bone, as for example also human bone chips hold a paracrine-like activity (Brolese et al. 2014) and commercial preparation of demineralized human bone also provoke the expected changes of TGF-β related gene expression (Filho et al. 2015). Moreover, the biochemistry of bone is similar among the different anatomical regions in the skeleton, suggesting that also the effects of chlorhexidine and hypochlorite on the activity of the respective conditioned medium are impended of the source of bone grafts, unless it is cortical bone. Moreover, iliac crest bone, which the trabecular structure homing the bone marrow cells and the endosteal cells, cannot be compared with a cortical bone graft. Cortical bone only holds few cells that appear after weeks in vitro (Kuchler et al. 2014) so it is mainly the osteocytes transiently releasing signaling molecules (Brolese et al. 2014). The activity of BCM, and thus also the impact of sodium hypochlorite in the present model, is likely better explained by denaturation of the molecular debris holding the TGF-β like activity that is passively released into the culture medium.

Taken together the study showed that antiseptics solutions have a differential impact on the biological activity of the conditioned medium of decontaminated bone chips. While a decrease in the activity of bone chips exposed to sodium hypochlorite was expected, the synergistic activation of TGF-β target genes by BCM and the contaminating chlorhexidine is a novel finding with potential clinical implications.

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**Figures**

*Figure 1: Expression of TGF-β regulated genes in gingival fibroblasts upon exposure with BCM*

Relative mRNA levels of (A) IL11, (B) PRG4, (C) NOX4, (D) ADM, (E) PTX3 of human gingival fibroblast exposed with BCM obtained from different processed bone chips, was assessed by RT-PCR. BCM obtained from non-treatment bone chips (non-treatment), BCM obtained from bone chips exposed to povidone-iodine (PI), chlorhexidine (CHX), hydrogen peroxide ($H_2O_2$), and sodium hypochlorite

(HYP), respectively. Data represent three different preparations of BCM tested in six gingiva fibroblast donors (n=18). *P<0.05 compared to BCM non-treatment.

*Figure 2: Cell viability staining of human gingiva fibroblast exposed to BCM*

Cells were exposed to 20% non-treatment BCM (A) and 20% CHX BCM (B). For cell viability, Live-Dead staining was done with viable cell appearing in green and dead cells are red.

*Figure 3: Expression of TGF-β regulated genes in human gingiva fibroblasts upon exposure with CHX*

Relative mRNA levels of IL11 (A), PRG4 (B), NOX4 (C), ADM (D) and PTX3 (E) of gingiva fibroblast exposed to 0.00156% CHX in serum-containing medium, with and without SB431542 (TGF-β receptor I kinase inhibitor) were assessed by RT-PCR. Data represent tested in six fibroblast donors (n=6). Non-significant.

Table 1. Primer sequences for human cells.

| Genes | Forward primer | Reverse primer |
|---|---|---|
| hIL11 | TGCACCTGACACTTGACTGG | AGTCTTCAGCAGCAGCAGTC |
| hPRG4 | CGACGCCCAATGTAAGAAGT | GGTGATGTGGGATTATGCACT |
| hNOX4 | TCTTGGCTTACCTCCGAGGA | CTCCTGGTTCTCCTGCTTGG |
| hADM | GGACATGAAGGGTGCCTCTC | TGTTCATGCTCTGGCGGTAG |
| hPTX 3 | TGTATGTGAATTTGGACAACGAA | CATTCCGAGTGCTCCTGAC |
| hBAD | CGAGTTTGTGGACTCCTTTAAGA | CACCAGGACTGGAAGACTCG |
| hBAX | AGCAAACTGGTGCTCAAGG | TCTTGGATCCAGCCCAAC |
| hBCL-XL | AGCCTTGGATCCAGGAGAA | AGCGGTTGAAGCGTTCCT |
| hBCL2 | CAGGAGAATGGATAAGGCAAA | CCAGCCAGATTTAGGTTCAAA |
| hIL1 | GGTTGAGTTTAAGCCAATCCA | GGTGATGACCTAGGCTTGATG |
| hIL6 | GAAAGGAGACATGTAACAAGAGT | GATTTTCACCAGGCAAGTCT |
| hTNF-α | CAGCCTCTTCTCCTTCCTGAT | GCCAGAGGGCTGATTAGAGA |
| hCCL7 | GAAAGCCTCTGCAGCACTTC | AATCTGTAGCAGCAGGTAGTTGAA |
| hTGF-β1 | ACTACTACGCCAAGGAGGTCAC | TGCTTGAACTTGTCATAGATTTCG |
| hβ-actin | CCAACCGCGAGAAGATGA | CCAGAGGCGTACAGGGATAG |

Table 2. Formazan formation of gingiva fibroblasts exposed to BCM

| non-treatment BCM | PI BCM | CHX BCM | $H_2O_2$ BCM | HYP BCM |
|---|---|---|---|---|
| 0.99 ± 0.07 | 0.97 ± 0.13 | 0.73 ± 0.11* | 0.97 ± 0.06 | 0.93 ± 0.09 |

Formazan formation when untreated control cells are compared with cells exposed to respective BCM for 24 Hours. Data represent three different preparations of BCM tested in six fibroblast donors (n=18). *P<0.05 compared to non-treatment BCM

Table 3. Apoptosis genes expression of gingiva fibroblasts exposed to BCM

| | PI BCM | CHX BCM | $H_2O_2$ BCM | HYP BCM |
|---|---|---|---|---|
| BAD | 0.97 ± 0.28 | 3.82 ± 2.25* | 0.80 ± 0.12 | 1.21 ± 0.10 |
| BAX | 1.01 ± 0.17 | 2.73 ± 1.68* | 0.81 ± 0.07 | 1.24 ± 0.17 |
| BCL-XL | 0.99 ± 0.32 | 5.17 ± 2.44* | 0.83 ± 0.20 | 1.31 ± 0.46 |
| BCL-2 | 0.92 ± 0.23 | 6.67 ± 1.19* | 0.98 ± 0.50 | 1.21 ± 0.75 |

Relative expression of apoptosis genes of cells exposed to non-treatment BCM with cells exposed to the respective BCMs for 24 hours. Results were normalized with non-treatment BCM. Data represent three different preparations of BCM tested in six fibroblast donors (n=18). *P<0.05 compared to non-treatment BCM

Table 4. Inflammatory gene expression of gingiva fibroblasts exposed to low concentration of CHX

| IL1 | IL6 | TNFα | CCL7 | TGF-β1 |
|---|---|---|---|---|
| 189.53 ± 81.80 | 24.42 ± 11.48 | 169.64 ± 120.48 | 10.86 ± 8.54 | 2.55 ± 2.21 |

Relative expression of inflammatory gene, when untreated control cells are compared with cells exposed to 0.00156% CHX for 24 hours. Data represent mean and standard deviation of six fibroblast donors.

Fig.1

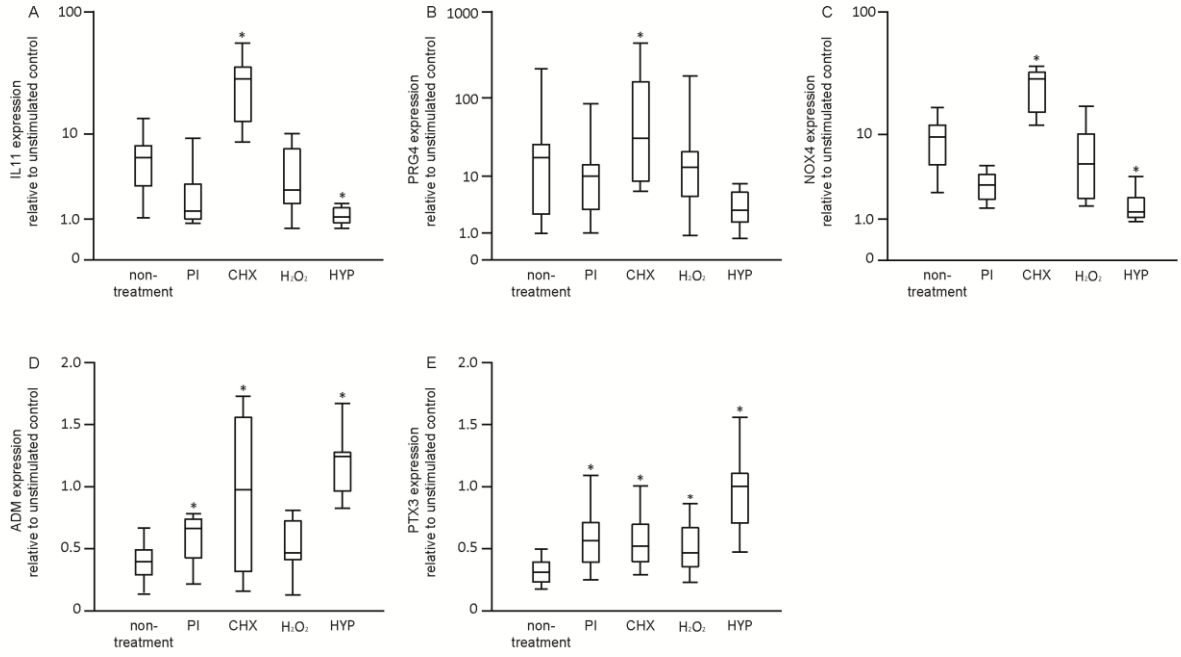

Fig.2

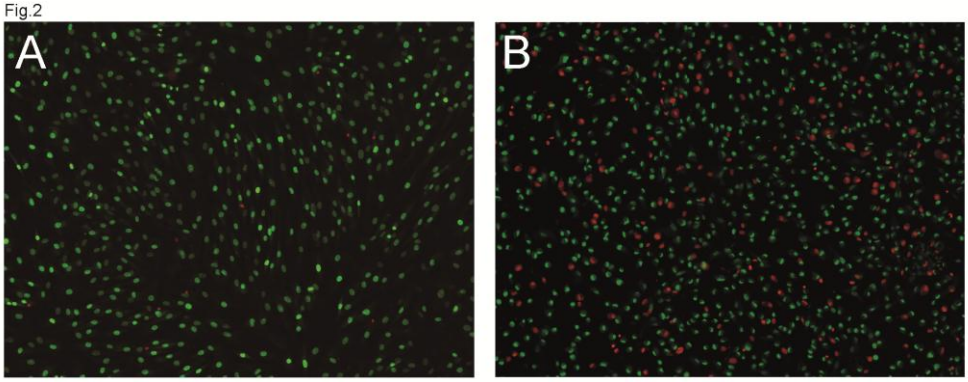


Fig.3