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Original Article

Effect of amoxicillin and clindamycin on the gene expression of markers involved in osteoblast physiology

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Abstract *Background/purpose:* Amoxicillin and clindamycin are the most effective decontaminants for intraoral bone grafts before their application in bone regeneration without cytotoxic effects on osteoblasts, but their effects on the gene expression of markers involved in osteoblast growth and differentiation remain unclear. The study objective was to determine the effects of amoxicillin and clindamycin on the gene expression of markers involved in osteoblast growth and differentiation.

Materials and methods: Real-time polymerase chain reaction (RT-PCR) was performed to explore the effect of 150 μg/mL clindamycin or 400 μg/mL amoxicillin on the gene expression by primary human osteoblasts (HOBs) of runt-related transcription factor 2 (Runx-2), osterix (OSX), alkaline phosphatase (ALP), osteocalcin (OSC), osteoprotegerin (OPG), receptor activator for nuclear factor κ B ligand (RANKL), type I collagen (Col-I), bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7), TGF-β1 and TGF-β receptors (TGF-βR1, TGF-βR2, and TGF-βR3), and vascular endothelial growth factor (VEGF).

Results: Treatment with 150 μg/mL clindamycin significantly increased the gene expression of

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TFG-β1, TGF-βR1, TGF-βR2, TGF-βR3, RUNX-2, Col-1, OSX, OSC, BMP-2, BMP-7, ALP, VEGF, and RANKL by HOBs. Treatment with 400 μg/mL amoxicillin significantly increased the gene expression of TGF-β R1, Col-I, OSC, RANKL, and OPG alone.

Conclusion: These findings suggest that 150 μg/mL clindamycin is the decontaminant of choice to treat intraoral bone grafts before their application in bone regeneration. The osteogenic and antibacterial properties of clindamycin can favor and accelerate the integration of bone grafts in the oral cavity.

Introduction

Osseointegrated implants are frequently used in oral cavity rehabilitation. Techniques to regenerate lost bone volume are sometimes necessary to reestablish function. Bone substitutes are often used for this purpose, but autologous bone tissue remains the gold standard due to its unique characteristics, including its cellularity and morphogenetic proteins, which increase the potential for growth and integration of the graft in receptor tissue. In most cases, rehabilitation require a relatively small amount of graft material, and particulate bone grafts from intraoral localizations are used to achieve small increases in bone tissue that yield a functional bed for implants. One possible complication of this procedure is contamination by oral cavity microbiota during handling of the graft.[1,2]

Studies comparing contamination among different methods of gathering intraoral particulate bone[3] reported a lower bacterial count with the low-speed biological drilling of medullar bone tissue without irrigation.[4,5] This technique has also been associated with the presence of viable osteoblasts with proliferative and mineralization capacity in the particulate bone.[6,7]

Various decontaminants have been used to develop a safe prophylactic antimicrobial protocol for bone grafts.[8] Our group previously compared the efficacy of two antibiotics (amoxicillin and clindamycin) and an antiseptic (chlorhexidine) on bone samples obtained by biological drilling and observed the greatest decontaminant effect with clindamycin, followed by chlorhexidine and amoxicillin.[9] The risk of cytotoxicity posed by these decontaminant agents has been tested in fibroblasts, myoblasts, osteoblasts, and epithelial cells, studying their impact on the growth and differentiation of these cells in both fresh and cultured explants.[10—14] Chlorhexidine was found to induce an abrupt decrease in proliferation and an increase in the percentage of apoptotic cells, whereas low-dose clindamycin increased cell proliferation, alkaline phosphatase activity, and calcification.[15,16] Other researchers have published similar observations on the impact of these decontaminants on bone cells.[17,18]

Amoxicillin and clindamycin are currently the two most effective decontaminants without cytotoxic effects on osteoblasts, and the objective of this study was to determine their effects on the gene expression of markers involved in osteoblast growth and differentiation.

Materials and methods

Tissues

To obtain the primary osteoblasts cell line, bone fragments were collected from volunteers during oral surgery. Volunteers were all university students aged 20—23 years from the School of Dentistry (University of Granada) requiring impacted third molar extraction at the School Clinic. The study was approved by the research ethics committee of the University (reference no. 3312/CEIH/2023). All participants signed their informed consent.

Isolation and culture of osteoblasts

The procedure described by Manzano-Moreno et al. (2013)[6] was used to process the bone fragments. Primary osteoblasts were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA). After adding 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 2.5 mg/mL amphotericin B (Sigma, St. Louis, MO, USA), 50 mg/mL gentamicin (Braum Medical SA, Jaen, Spain), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK), cultures were kept in a humidified atmosphere at 37 °C with 95% air and 5% CO_2. Next, cells were detached from the flask with 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid solution (EDTA; Sigma) and then rinsed and resuspended in culture medium with 10% FBS (Gibco).[19] Previously published methods were used for the identification and characterization of osteoblasts.[20]

Gene expression by real-time polymerase chain reaction (RT-PCR)

The methodology reported by Manzano-Moreno et al. (2018)[21] was used to evaluate the effect of clindamycin and amoxicillin on osteoblast gene expression.

RNA extraction and cDNA synthesis (reverse transcription)

After 24 h of osteoblast culture with 150 μg/mL clindamycin or 400 μg/mL amoxicillin in DMEM without penicillin and gentamicin (untreated cells served as controls), mRNA was

Table 1 Primer sequences for the amplification of osteoblasts' cDNA by real-time PCR. Abbreviations: TGF-β1, TGF-βR1, TGF-βR2, and TGF-βR3 (Transforming growth factor-β1 and TGF-β receptors); Runx-2 (Runt-related transcription factor 2); VEGF (Vascular endothelial growth factor); OSX (Osterix); BMP-2 and BMP (Bone morphogenetic proteins 2 and 7); ALP (Alkaline phosphatase); Col-I (Type I collagen); OSC (osteocalcin); OPG (Osteoprotegerin); RANKL (Receptor activator for nuclear factor κ B ligand); UBC (Ubiquitin C); PPIA (peptidylprolyl isomerase A); RPS13 (Ribosomal protein S13).

Gene	Sense Primer	Antisense Primer	Amplicon (bp)
TGF-β1	5′-TGAACCGGCCTTTCCTGCTTCTCATG-3′	5′-GCGGAAGTCAATGTACAGCTGCCGC-3′	152
TGF-β R1	5′-ACTGGCAGCTGTCATTGCTGGACCAG-3′	5′-CTGAGCCAGAACCTGACGTTGTCATATCA-3′	201
TGF-β R2	5′-GGCTCAACCACCAGGGCATCCAGAT-3′	5′-CTCCCCGAGAGCCTGTCCAGATGCT-3′	139
TGF-β R3	5′-ACCGTGATGGGCATTGCGTTTGCA-3′	5′-GTGCTCTGCGTGCTGCCGATGCTGT-3′	173
RUNX-2	5′-TGGTTAATCTCCGCAGGTCAC-3′	5′-ACTGTGCTGAAGAGGCTGTTTG-3′	143
VEGF	5′-CCTTGCTGCTCTACCTCCAC-3′	5′-CACACAGGATGGCTTGAAGA-3′	197
OSX	5′-TGCCTAGAAGCCCTGAGAAA-3′	5′-TTTAACTTGGGGCCTTGAGA-3′	205
BMP-2	5′-TCGAAATTCCCCGTGACCAG-3′	5′-CCACTTCCACCACGAATCCA-3′	142
BMP-7	5′-CTGGTCTTTGTCTGCAGTGG-3′	5′-GTACCCCTCAACAAGGCTTC-3′	202
ALP	5′-CCAACGTGGCTAAGAATGTCATC-3′	5′-TGGGCATTGGTGTTGTACGTC-3′	175
COL-I	5′-AGAACTGGTACATCAGCAAG-3′	5′-GAGTTTACAGGAAGCAGACA-3′	471
OSC	5′-CCATGAGAGCCCTCACACTCC-3′	5′-GGTCAGCCAACTCGTCACAGTC-3′	258
OPG	5′-ATGCAACACAGCACAACATA-3′	5′-GTTGCCGTTTTATCCTCTCT-3′	198
RANKL	5′-ATACCCTGATGAAAGGAGGA-3′	5′-GGGGCTCAATCTATATCTCG-3′	202
UBC	5′-TGGGATGCAAATCTTCGTGAAGACCCTGAC-3′	5′-ACCAAGTGCAGAGTGGACTCTTTCTGGATG-3′	213
PPIA	5′-CCATGGCAAATGCTGGACCCAACACAAATG-3′	5′-TCCTGAGCTACAGAAGGAATGATCTGGTGG-3′	256
RPS13	5′-GGTGTTGCACAAGTACGTTTTGTGACAGGC-3′	5′-TCATATTTCCAATTGGGAGGGAGGACTCGC-3′	251

extracted by a silicate gel technique included in the QiagenRNeasy extraction kit (Qiagen Inc., Hilden, Germany), which involves a DNAse digestion step. The amount of extracted mRNA was measured by UV spectrophotometry at 260 nm (Eppendorf AG, Hamburg, Germany), and contamination with proteins was determined according to the 260/280 ratio. An equal amount of RNA (1 μg of total RNA in 40 μL of total volume) was reverse-transcribed to cDNA and

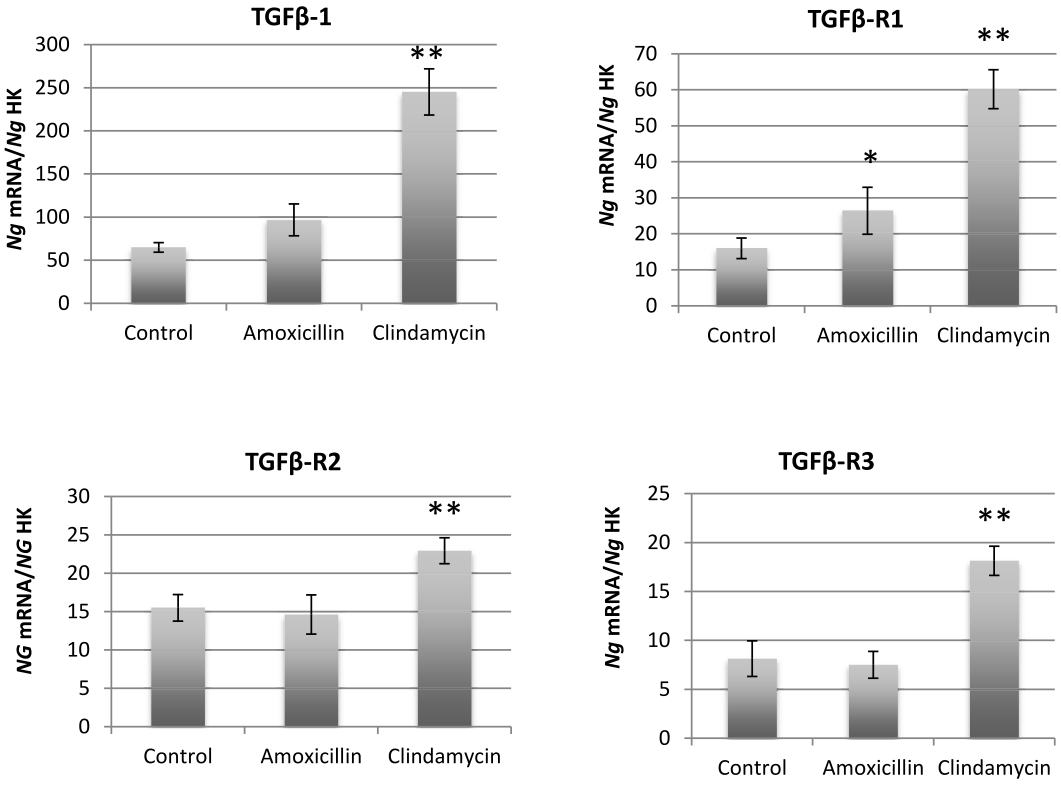

Figure 1 Expression of osteoblast genes (TFG-β1, TFGβR1, TFGβR2, and TFGβR3) treated for 24 h with 150 μg/mL clindamycin or 400 μg/mL amoxicillin. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. TGF-β1, TGF-βR1, TGF-βR2, and TGF-βR3 (Transforming growth factor-β1 and TGF-β receptors). *$P < 0.05$, **$P < 0.001$.

amplified by PCR using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) following the manufacturer's instructions.

Real-time polymerase chain reaction (RT-PCR)

The NCBI- nucleotide library and Primer3-design were used to design primers to detect mRNA of the following genes: runt-related transcription factor 2 (Runx-2), osterix (OSX), alkaline phosphatase (ALP), osteocalcin (OSC), osteoprotegerin (OPG), receptor activator for nuclear factor κ B ligand (RANKL), type I collagen (Col-I), bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7), TGF-β1 and TGF-β receptors (TGF-βR1, TGF-βR2, and TGF-βR3), and vascular endothelial growth factor (VEGF). According to different authors,[21–23] it is recommended to use a minimum number of three house-keeping genes. In the case of bone tissue and osteoblast, results were normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as housekeeping genes. Primer sequences are given in Table 1.

Quantitative RT-PCR (q-RT-PCR) was performed with the SsoFast™ EvaGreen® Supermix Kit (Bio-Rad laboratories) following the manufacturer's instructions. Samples were amplified in 96-well microplates in an IQ5-Cycler (Bio-Rad laboratories) at a specific annealing temperature for each gene (range, 60 to 65 °C) and an elongation temperature of 72 °C over 40 cycles. PCR reactions were carried out in a final volume of 20 μL, with 5 μL of cDNA sample and 2 μL of each primer. Ct values were plotted against log cDNA dilution to construct standard curves for each target gene. A melting profile was created after each RT-PCR, conducting agarose gel electrophoresis in samples to rule out nonspecific PCR products and primer dimers. The comparative Ct method was applied for the relative quantification of gene expression, and she mRNA concentration of each gene was expressed as ng of mRNA per average ng of housekeeping mRNAs. The cDNA from individual cell experiments was analyzed in triplicate RT-PCR studies.

Statistical analysis

SPSS 26.0 (IBM, Chicago, IL, USA) was used for data analyses. mRNA levels were expressed as means ± standard deviation (SD). A two-way repeated-measures analysis of variance (ANOVA) was performed, applying the Bonferroni correction when significant interactions were identified. A minimum of three experiments were performed for each assay. $P < 0.05$ was considered significant.

Results

Effect of clindamycin and amoxicillin on gene expression of TGF-β1 and its receptors (TGF-β R1, TGF-β R2, and TGF-β R3)

Fig. 1 depicts q-RT-PCR results for the gene expression of TGF-β1 and its receptors (TGF-β R1, TGF-β R2, and TGF-β R3). TGF-β1 expression was significantly increased *versus* controls after 24 h of treatment with 150 μg/mL clindamycin ($P < 0.001$) but not 400 μg/mL amoxicillin. Treatment with 150 μg/mL clindamycin significantly increased the expression of TGF-β R1, TGF-β R2, and TGF-β R3 *versus* controls ($P < 0.001$), whereas treatment with 400 μg/mL amoxicillin significantly increased the expression of TGF-β R1 alone ($P = 0.023$).

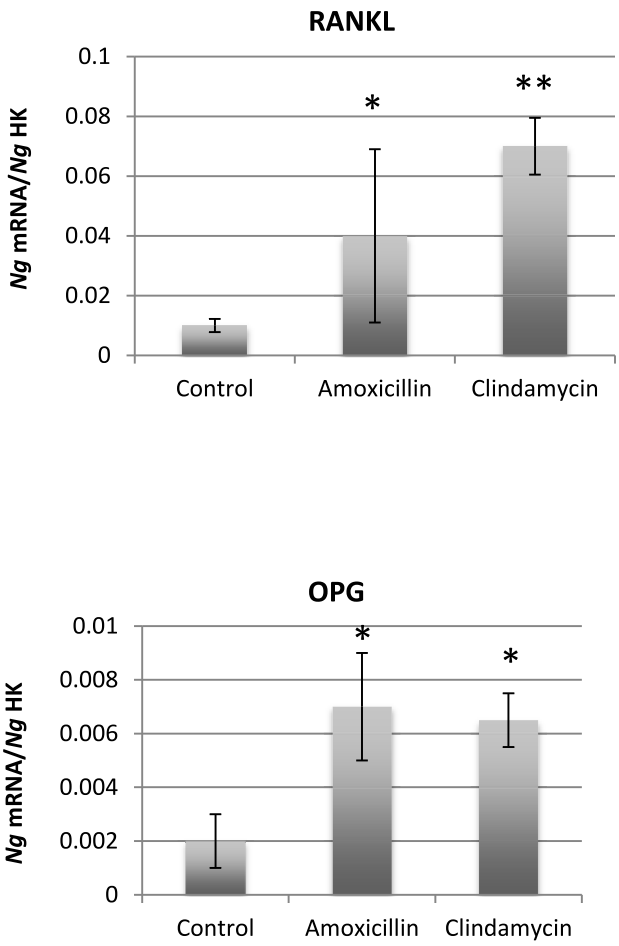

Figure 2 Expression of osteoblast genes (RANKL and OPG) treated for 24 h with 150 μg/mL clindamycin or 400 μg/mL amoxicillin. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. RANKL (Receptor activator for nuclear factor κ B ligand); OPG (Osteoprotegerin). *$P < 0.05$, **$P < 0.001$.

Effect of amoxicillin and clindamycin on gene expression of RANKL-OPG complex

Fig. 2 shows q-RT-PCR results for the gene expression of RANKL and OPG. Treatment for 24 h with 150 μg/mL clindamycin or 400 μg/mL amoxicillin significantly increased the expression of RANKL *versus* controls ($P < 0.001$ and 0.043, respectively). In addition, the expression of OPG was significantly increased by treatment with 150 μg/mL clindamycin and 400 μg/mL amoxicillin ($P = 0.024$ and $P = 0.004$, respectively).

Effect of amoxicillin and clindamycin on the gene expression of Runx2, ALP, OSX, Col-I and OSC

Fig. 3 displays q-RT-PCR results for the expression of osteoblast differentiation markers Runx2, ALP, OSX, Col-I, and OSC. Treatment with 150 μg/mL clindamycin for 24 h significantly increased the expression of Runx2, ALP, OSX, Col-I, and OSC *versus* controls ($P < 0.001$), and treatment with 400 μg/mL amoxicillin significantly increased the expression of Col-I and OSC ($P < 0.001$), whereas neither treatment significantly altered the expression of Runx2, ALP, or OSX.

Effect of amoxicillin and clindamycin on gene expression of BMP-2, BMP-7, and VEGF

Fig. 4 exhibits q-RT-PCR results for the gene expression of BMP-2, BMP-7, and VEGF. Expression of BMP-2, BMP-7, and VEGF was significantly increased *versus* controls after 24 h of treatment with 150 μg/mL clindamycin ($P < 0.001$) but not 400 μg/mL amoxicillin.

Discussion

Amoxicillin and clindamycin, widely used to decontaminate and avoid infectious complications after autologous bone grafting, were found to differ in their effects on the gene expression of osteoblasts. In this way, 150 μg/mL clindamycin significantly increased the expression of genes involved in osteoblast growth (TGF-β1, TGF-βR1, TGF-βR2, TGF-βR3, and VEGF) and differentiation (RUNX-2, Col-1, OSX, OSC, BMP-2, BMP-7, or ALP) *versus* controls, whereas 400 μg/mL amoxicillin only increased the expression of TGF-β R1, Col-1, and OSC. Both treatments modified gene expressions related to osteoblast-osteoclast interactions (RANKL-OPG). We selected these treatment doses/time because in a previous study by our group,[16] we demonstrated that treatment of osteoblasts with 150 μg/mL clindamycin and 400 μg/mL amoxicillin for 24 h did not have a toxic effect on osteoblasts.

The TGF-β superfamily has more than 40 members, including TGF-βs, Nodal, Activin, and BMPs TGF-β signaling is critical for the regulation of osteoblast differentiation and bone formation, and signaling relays at each stage leads to the final target gene expression. Low to moderately-elevated levels of TGF-β1 were found to stimulate early osteoblast proliferation, and this growth factor is involved in activating the MAPK cascade.[24,25] The MAPK signaling pathway is known to participate in the regulation of cell growth, development, and division through its influence on nuclear transcription factors[24,26] and in the activation and signaling of other osteogenic growth factors.[24,27] The expression of TGF-β1 and its receptors was significantly increased by treatment with 150 μg/mL clindamycin, which may be related to the increased proliferation of human osteoblasts observed by Olvera-Huertas et al. (2022)[16] after treatment with this antibiotic at the same dose. Other authors have reported that a low dose of clindamycin is effective to decontaminate different types of bone grafts or extraction sockets without impairing the viability, metabolic activity, or proliferation of osteoblasts. However, no evidence has been published on its impact on the gene expression of osteoblast growth and differentiation markers.

There are two main steps in the complex process of osteoblast differentiation: i) osteogenic commitment, characterized by upregulation of the transcription factor Runx2[28]; and ii) osteogenic differentiation, characterized by expression of the specific osteogenic proteins and transcription factors ALP, OPG, and OSX.[29,30] Among these, OSX is an essential transcription factor for osteoblast differentiation and bone formation, and its expression indicates the loss of bipotentiality from preosteoblast to osteoblast and chondrocyte. In the present study, treatment with 150 μg/mL clindamycin induced upregulation of the osteoblast expression of Runx-2, ALP, and OSX, which would be related to an increase in osteoblast differentiation, consistent with the by Olvera-Huertas et al. (2022)[16] of higher ALP expression and mineralization after the same treatment.

BMPs have multiple effects on osteoblast proliferation, differentiation, and general physiology. For instance, BMP-2 and BMP-7 have important roles in bone formation/remodeling and development and in osteoblast differentiation, inducing the expression of ALP and other osteoblastic markers and promoting calcium mineralization. In the present study, treatment with 150 μg/mL clindamycin significantly increased the gene expression of BMP-2 and BMP-7 ($P < 0.001$), indicating the activation of osteogenesis.[31] These findings suggest that stimulation of the BMP-Smad pathway may induce an osteogenic effect that is further sustained by Wnt signaling.[32,33]

Treatment with clindamycin also increased gene expressions of OSC and COL-I, osteogenic markers of the final stages of osteoblast differentiation that appear at the start of mineralization.

The expression and production of pro-angiogenic factors such as VEGF and angiopoietin (ANG) affect endothelial cell growth, migration, and vessel formation in many tissues and are important for regulating vascular growth in the skeleton. These factors are produced by osteoblasts and osteocytes, among other bone cells, and the present study shows an increased gene expression of VEGF by osteoblasts treated with clindamycin; which may therefore favor angiogenesis in bone tissue.

Treatment with 400 μg/mL amoxicillin was recently found to be an effective decontaminant of intraoral bone grafts and only increased the expression of TGF-β R1, Col-1, and OSC in the present study. Rathbone et al.[32] observed a moderate reduction of 26—49% in osteoblastic cell count and alkaline phosphatase activity after treatment with amoxicillin at a dose over 500 μg/mL and a reduction of >75% in cell count at a dose of 2000 g/mL or higher. Selection of the lower dose of 400 μg/mL is designed to maintain the decontaminating efficacy of amoxicillin without impairing the functional capacity of osteoblasts.

RANKL and OPG regulate bone remodeling by respectively stimulating and inhibiting osteoclast differentiation, and they have been found to participate in the complex set of molecular interactions leading to bone loss in peri-implantitis.[34] Local deregulation of the RANKL-OPG complex in favor of the pro-osteoclastic component may

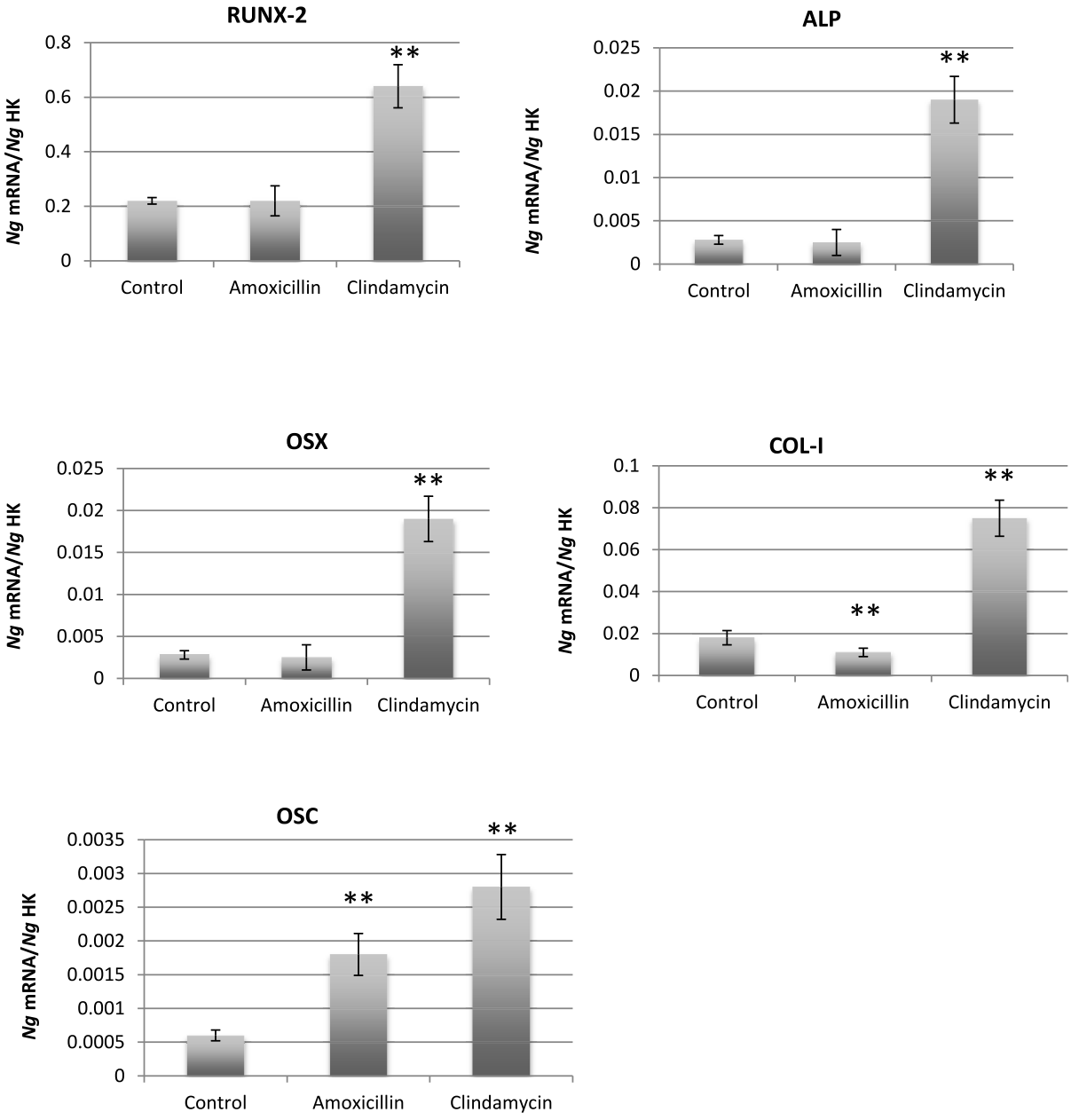

Figure 3 Expression of osteoblast genes (RUNX-2, ALP, Col-I, OSX and OSC) treated for 24 h with 150 μg/mL clindamycin or 400 μg/mL amoxicillin. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. Runx-2 (Runt-related transcription factor 2); ALP (Alkaline phosphatase); Col-I (Type I collagen); OSX (Osterix); OSC (osteocalcin). $^{**}P < 0.001$.

lead to bone resorption at the alveolar site[35] and increase the risk and severity of peri-implant disease.[36] In the present study, treatment with the two antibiotics (amoxicillin and clindamycin) produced significant changes in the gene expression of RANKL-OPG in osteoblasts, modulating the interaction of osteoblasts and osteoclasts and potentially altering the bone apposition-resorption process.

The antibiotic clindamycin appears to be a good option for the decontamination of intraoral bone grafts, offering a higher decontaminating capacity in comparison to other agents[9] as well as increasing the proliferation and differentiation of osteoblasts.[16] These effects are explained by analysis of its impact on the gene expression of markers with a key role in osteoblast physiology, as revealed in the present study.

According to these findings, low-dose clindamycin is the agent of choice to decontaminate intraoral bone grafts before their utilization in bone regeneration. Its osteogenic and antibacterial properties may favor and accelerate the integration of bone grafts in the oral cavity.

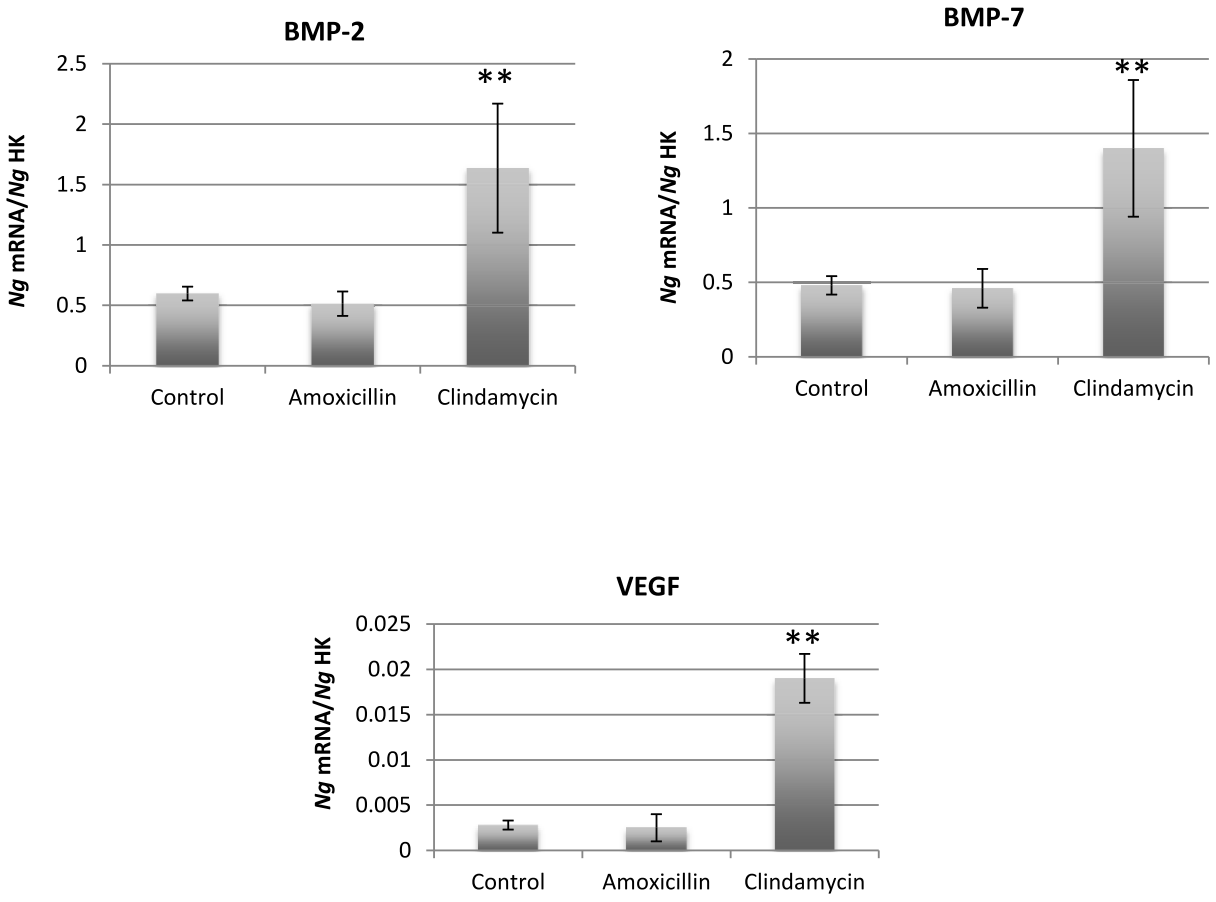

Figure 4 Expression of osteoblast genes (BMP-2, BMP-7, and VEGF) treated for 24 h with 150 μg/mL clindamycin or 400 μg/mL amoxicillin. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. BMP-2 and BMP (Bone morphogenetic proteins 2 and 7); VEGF (Vascular endothelial growth factor). $^{**}P < 0.001$.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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