1   **The Effects of Vancomycin on the Viability and Osteogenic Potential of Bone-**

2   **derived Mesenchymal Stem Cells**

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4   **Elzaan Booysen[a], Hanél Sadie-Van Gijsen[b,*], Shelly M. Deane[a], William Ferris[b], Leon M.T.**

5   **Dicks[a#]**

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7   [a]Department of Microbiology, Faculty of Natural Sciences, Stellenbosch University,

8   Stellenbosch, South Africa

9   [b]Division of Endocrinology, Department of Medicine, Faculty of Medicine and Health Sciences,

10   Stellenbosch University Tygerberg Campus, Parow, South Africa

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12   Running Head: Effect of vancomycin on mesenchymal stem cells

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14   [#]Address correspondence to: LMTD@sun.ac.za

15   *Present address: Division of Medical Physiology, Department of Medicine, Faculty of Medicine

16   and Health Sciences, Stellenbosch University Tygerberg Campus, Parow, South Africa

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19   **ABSTRACT** Periprosthetic joint infections (PJI), caused by methicillin-resistant *Staphylococcus*

20   *aureus* (MRSA), is the major cause of total hip arthroplasty (THA) failures. Traditionally,

21   MRSA is treated with vancomycin, administrated intravenously or applied directly onto infected

22   tissue. The effect of direct (as opposed to systemic) vancomycin treatment on bone formation

23   and remodelling is largely unknown. The minimal inhibitory concentration (MIC) of

24   vancomycin was determined by adding 200 µl of different concentrations (1 – 20 µg/ml) to

25   actively growing cultures of *S. aureus* Xen 31 (methicillin-resistant) and *S. aureus* Xen 36

26   (methicillin-sensitive), respectively, and recording changes in optical density over 24 h. Bone

27   marrow-derived and proximal femur-derived mesenchymal stem cells (bmMSCs and pfMSCs)

28   from rat femora were exposed to 1 x MIC (5 µg/ml) and 4 x MIC (20 µg/ml) of vancomycin for

29   7 days. Cell viability was determined by staining with crystal violet and MTT (3-(4,5-

30   dimethylthiazol-2-yl)-2,5-diphenyltetrazolium   bromide),   respectively,   and   osteogenic

31   differentiation by staining with Alizarin Red S. Vancomycin had no effect on the viability of

32   bmMSCs and pfMSCs, even at high levels (20 µg/ml). The osteogenic differentiation of

33   pfMSCs was partially inhibited, while osteogenesis in bmMSCs was not severely affected. The

34   direct application of vancomycin to infected bone tissue, even at excessive levels, may preserve

35   the viability of resident MSC populations. Short-term demineralization may thus be reversed

36   after cessation of vancomycin treatment, improving the outcome of THA surgery.

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INTRODUCTION

The three major load bearing joints in the human body are the ankle, knee and hip (1). The human hip joint has a complex anatomical structure and is responsible for carrying the weight of the upper body. During daily activity, the hip joint may experience loading forces up to 7 times the body weight of the individual, generating immense mechanical pressure which is most likely the main cause for osteoarthritis (OA) in this joint (2). OA effects more than 25% of elderly people and is therefore a major cause of disability in this population group (3, 4).

Currently the only treatment available for severe hip OA is total hip arthroplasty (THA) (5). A metal prosthesis is inserted into the proximal region of the femur, replacing the proximal femur head and neck (6). Although THA is considered the most successful orthopaedic procedure of the past 120 years, and although advances in the field have significantly improved the risk profile associated with this procedure over the past five decades, complications still do occur (7–9). Periprosthetic joint infection (PJI) is the main cause for THA revision surgeries and is defined as bacterial contamination of the joint prosthesis and infection of the adjacent tissue (10). Most, if not all, PJI are caused by bacteria capable of forming biofilms (5). Many strains of these bacteria are resistant to a number of antibiotics (11), and treating PJI is therefore extremely difficult. The most common bacterial infections after THA are Gram-positive cocci. *Staphylococcus aureus* was isolated in 38% of PJI cases. Fifty-three percent of the strains were resistant to methicillin (12).

Failed treatment of PJI with conventional antibiotic regimes places an immense burden on medical professionals. Conventional treatments include intravenous administration of antibiotics for a maximum of 2 weeks, while aggressive treatment includes removal of the infected prosthesis and flushing of the infected joint (one-step revision surgery), followed by another 12 weeks on antibiotics. Once the infection has been cleared, a new prosthesis is inserted (13). In

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66   severe cases a surgeon can opt for a two-step revision surgery. This entails initial removal of the

67   infected prosthesis and insertion of an antibiotic-augmented cement spacer, followed by the

68   implantation of a new prosthesis once the bacterial infection has been eradicated (13, 14). Both

69   one-step and two-step revision surgeries can result in prolonged immobility, hospitalization and

70   morbidity. The cost of treatment of a single episode of PJI can escalate to USD 50,000 or more

71   (9). The increase in antibiotic resistance among hospital-acquired infections like PJI has also

72   increased the risk of failing to successfully treat PJI (15, 16). It is therefore clear that PJI presents

73   a major challenge from both a health-care and economic perspective (17).

74   Methicillin-resistant *Staphylococcus* infections are usually treated with vancomycin (18).

75   Vancomycin is either administered intravenously or directly to the infected site, by means of

76   antibiotic-loaded cement spacers (19–21). However, the effect of direct (as opposed to systemic)

77   vancomycin treatment on bone formation and remodelling is largely unknown. Bone marrow-

78   derived mesenchymal stem cells (bmMSCs) have traditionally been used to study the effects of

79   pharmaceutical treatments on the viability, differentiation and function of osteoblasts (bone-

80   forming cells) *in vitro* (22–24). Recently Jacobs and co-authors (25) reported on a newly

81   characterized MSC, isolated from the proximal end of rat femora. Although these proximal

82   femur MSCs (pfMSCs) are phenotypically similar to bmMSCs, they are functionally distinct,

83   with increased glucocorticoid sensitivity and a reduced osteoblastic differentiation potential in

84   culture. *In vivo,* pfMSCs reside in the exact area of bone tissue that is in contact with the THA

85   prosthesis, and is therefore likely to participate in osseo-integration of the prosthesis.

86   Consequently, these cells can serve as a relevant *in vitro* model to assess the effects of directly

87   applied vancomycin on the MSC populations residing in the bone tissue, which could affect the

88   success of THA revision surgeries. In the present study we evaluate the effects on increasing

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89   doses of vancomycin in the viability and osteoblastic differentiation of cultured rat bmMSCs and

90   pfMSCs.

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92   **MATERIALS AND METHODS**

93   **Materials.** Bacterial growth medium was from Biolab Diagnostics (Midrand, South Africa),

94   unless stated otherwise. All constituents of osteogenic differentiation media were from Sigma-

95   Aldrich (Schndlldorf, Germany). Dulbecco's Modified Eagle Medium (DMEM: #BE12-604F;

96   4.5 g/L glucose), Penicillin/Streptomycin (Pen-Strep: #DE17-602E; 10000 units each in 100 ml),

97   trypsin (#BE17-161E) and Hanks' Balanced Salt Solution with calcium and magnesium (HBSS:

98   #BE10-508F) were from Lonza (Basel, Switzerland). Fetal bovine serum (FBS) was from

99   Biochrom (Berlin, Germany) and collagenase I (#CLS1) from Worthington Biochemical

100   Corporation (Lakewood, USA). Cell culture dishes were from NEST Biotechnology (New

101   Jersey, USA). Sodium pentobarbitone (Eutha-naze) was from Bayer (Kempton Park, Gauteng,

102   South Africa).

103   **Determination of MIC (minimum inhibitory concentration).** A micro-broth dilution

104   assay, described by Andrews (26), was used to determine the MIC of vancomycin.

105   *Staphylococcus aureus* Xen 31 (methicillin-resistant *S. aureus*: MRSA) and *S. aureus* Xen 36

106   (methicillin-sensitive) were used as indicator strains. Both strains were from Bioware™

107   Microorganisms (Caliper Life Sciences, Hopkinton, USA). Cells from an overnight culture in

108   Brain Heart infusion (BHI) broth, cultured at 37°C, were streaked onto BHI agar and incubated

109   for 24 h at 37°C. A single colony was inoculated into BHI broth and incubated for 18 h at 37°C.

110   Overnight cultures of the two strains were each adjusted to an OD of 0.1 (600 nm). Different

111   concentrations of vancomycin (1 – 20 µg/ml) were added to each liquid culture (final volume of

112   200 µl) and changes in growth observed by recording absorbance readings at 600 nm,

113   immediately after addition, and 6 h and 24 h later.

114   **Isolation and maintenance of MSC.** Ethical clearance to conduct experiments involving

115   animals was granted by the Research Ethics Committee: Animal Care and Use of Stellenbosch

116   University (reference number SU-ACUD15-00012). Male Wistar rats, 12 weeks old, with an

117   average body mass of 250 g, were housed at the Stellenbosch University Animal Facility and

118   kept according to the guidelines of the South African Medical Research Council. The animals

119   were fed *ad libitum* on standard laboratory feed and sacrificed with an intraperitoneal injection of

120   sodium pentobarbitone (12 mg/kg body mass).

121   Isolation of bmMSCs and pfMSCs was performed as described by Jacobs and co-workers

122   (25). Femora were surgically removed and cleaned from muscle tissue using sterile gauze.

123   Proximal regions of the femora were removed with sterile surgical side cutters, cut into 1 mm^3

124   fragments and digested in Hanks' Balanced Salt Solution containing 0.075% (w/v) collagenase I

125   and 1.5% (w/v) bovine serum albumin for 1 h at 37°C. Denuded bone fragments were washed

126   five times with DMEM, seeded into a culture dish with isolation media (DMEM containing 1%,

127   v/v, Pen-Strep, and 20%, v/v, FBS) and incubated at 37°C for 24h. Bone fragments were then

128   washed with sterile phosphate-buffered saline (PBS), retained in the dish and submerged in

129   standard growth media (SGM: DMEM containing 1%, v/v, Pen-Strep and 10%, v/v, FBS) for 7

130   to 10 days to allow migration of pfMSC from the fragments. bmMSCs were flushed from bone

131   marrow cavities with 9 ml (3 ml per flush) cell isolation media and collected in a cell culture dish

132   (100 mm diameter). pfMSCs and bmMSCs were cultured at 37°C for 24 h in 95% humidified air

133   and in the presence of 5% CO_2. Sterile PBS at 37°C was used to remove non-adherent tissue and

134   the media replaced with SGM.

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135   Both cell types (pfMSCs and bmMSCs) were cultured to 80% confluence and then

136   disaggregated with 1 ml 0.5% (w/v) trypsin and sub-cultured at a ratio of 1:4. All cell cultures

137   were expanded to passage 3 before being used for further experiments. Cell growth media,

138   including media containing supplements, were replaced every 2-3 days. Cells were maintained

139   at 37°C in 95% humidified air containing 5% CO_2.

140   **Cytotoxicity of vancomycin.** Isolated MSCs at passage 3 were seeded into 12-well plates

141   for crystal violet staining or 96-well plates for the MTT conversion assay and grown until post-

142   confluence in SGM. A combination of cycloheximide (10 µg/ml) and tumour necrosis factor-α

143   (TNF-α; 5 ng/ml) was used as the positive control for cytotoxicity in bmMSCs, while 1 µM

144   dexamethasone (Dex) was used as the positive control for cytotoxicity in pfMSCs, as reported

145   previously (25). Before crystal violet staining, cells were treated with 1 x MIC and 4 x MIC

146   vancomycin, respectively, in SGM for 7 days, fixed with 70% (v/v) ethanol, stained for 5 min

147   with 500 µl 0.01% (w/v) crystal violet solution and rinsed three times with PBS. In these

148   experiments MIC levels were calculated based on results obtained with MRSA strain Xen 31.

149   Bound crystal violet was extracted with 500 µl 75% (v/v) ethanol and absorbance measured at

150   570 nm. For the MTT conversion assays, cells were treated as described for crystal violet

151   staining, with a final volume of PBS being 100 µl per well. The methodology for the assay was

152   adapted from the protocol for the Sigma-Aldrich *in vitro* Toxicology Assay Kit (MTT-based,

153   #Tox1). After 7 days of treatment with 1 x MIC and 4 x MIC vancomycin, respectively, 10 µl of

154   5 mg/ml MTT stock solution (Sigma-Aldrich #M2128), dissolved in DMEM, was added to each

155   well. Plates were incubated for 2h at 37°C in the dark and the colour reaction stopped by adding

156   100 µl of solubilisation solution (10% Triton X-100 diluted with 0.1 N HCL in anhydrous

157   isopropanol) to each well. Solubilisation was aided by incubating the 96-well plate on a plate

158   shaker and repeated mixing with a pipette. Colour development was quantified

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159   spectrophotometrically at 690 nm (background absorbance) and 570 nm, respectively.

160   Background absorbance values were subtracted from the A_{570} values. All experiments were

161   performed with quadruplicate biological repeats and triplicate technical repeats.

162   **MSC differentiation.** Isolated MSCs at passage 3 were plated in 12-well culture plates and

163   cultures in SGM until post-confluence as described elsewhere. Osteoblastic differentiation was

164   induced with osteogenic media (OM: SGM supplemented with 50 μM ascorbic acid, 10 mM β-

165   glycerolphosphate and 10 nM dexamethasone), as described by Jacobs *et al*. (25). In parallel,

166   cells were differentiated in the presence of increasing concentrations of vancomycin.

167   Differentiation was evaluated by staining with Alizarin Red S stain (ARS: Amresco, USA) after

168   7 days (bmMSC) or 21 days (pfMSC). Before staining with 40mM ARS (pH 4.0 – 4.1), cells

169   were washed with PBS, fixed for 5 min at 25°C with 70% (v/v) ethanol and rinsed twice with

170   sterile water. BmMSCs were stained for 2 to 4 hours, after which the excess stain was removed,

171   and cells washed three times with sterile water, once with sterile PBS and an additional three

172   times with sterile water. Bound stain was extracted using 10% (w/v) cetylpyridinium chloride

173   (CPC) dissolved in 10 mM Na_2HPO_4 (pH 7.0) and quantified spectrophotometrically at 562 nm.

174   PfMSCs were stained overnight with ARS and washed, as described for bmMSC. Since pfMSCs

175   have reduced osteogenic potential, staining was quantified using image analysis. Images were

176   captured using an Olympus CKX41 microscope (CKX41, CachN 10x0.25 PhP objective), fitted

177   with a Canon EOS 600D camera at 10x magnification. Four random images (one in each

178   quadrant) were taken of each well, resulting in 12 images per treatment since experiments were

179   performed in triplicate. Images were analysed with ImageJ software (version 1.51 J8) and

180   converted into red-green-blue stacks. Analyses were performed in the green channel. A

181   threshold value of T=90 was used to exclude non-specific background staining and remained

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182 unchanged throughout. The percentage area stained was recorded and the average for the 12

183 images per condition was calculated.

184 **Statistical analysis.** GraphPad Prism (version 5.01) was used for all statistical analyses and

185 data were expressed as average ±SD. One-way ANOVA and Dunnett's *post hoc* test were used

186 to analyse the data. When $P < 0.05$, the difference was considered to be statistically significant

187 and indicated with an *.

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189 **RESULTS AND DISCUSSION**

190 The increase in antibiotic resistance among hospital-acquired infections such as PJI has

191 escalated the risk of PJI treatment failure (15, 16) and may force clinicians to employ ever more

192 aggressive treatment strategies with currently available antibiotics. In the case of PJI, these

193 strategies often involve direct application of antibiotics such as vancomycin to the infected

194 surgical site (13, 14), but the effects of such treatment on the surrounding bone tissue have not

195 been studied at a cellular level. In particular, detrimental effects of antibiotic treatment on the

196 stem cell population residing in bone may result in poor osseointegration of implanted

197 prostheses, leading to structural failure and impaired function of the reconstructed joint even

198 when the PJI has been successfully eradicated.

199 Based on micro-broth dilution assays, the MIC of vancomycin against *S. aureus* Xen 31

200 (MRSA) was 5 µg/ml and 1 µg/ml against Xen 36, suggesting that *S. aureus* Xen 31 has reduced

201 susceptibility for vancomycin (27, 28).

202 Crystal violet stained all cells (dead and alive), while the MTT conversion assay detected

203 only metabolically active cells. High doses of vancomycin, up to 20 µg/ml (4 x MIC for MRSA),

204 had no cytotoxic effects on bmMSCs (Fig. 1A and Fig. 2A) and pfMSC (Fig. 1B and Fig. 2B), as

205 shown with both assays.

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206   Based on ARS staining, bmMSCs were fully differentiated after 7 days of treatment with

207   OM, while pfMSCs formed individual mineralized nodules after 21 days of OM treatment.

208   Mineralization in bmMSCs increased slightly, but not significantly, when treated with 4 x MIC

209   vancomycin (Fig. 3A). In contrast, pfMSCs treated with vancomycin showed significantly

210   reduced mineralization, compared to OM-treated cells (Fig. 3B).

211   Vancomycin inhibitions bacterial cell wall synthesis by binding to the terminal D-Ala-D-Ala

212   dipeptide of peptidoglycan units (29). This may explain why vancomycin had no cytotoxic effect

213   on MSCs. However, the mechanisms involved in the reduction of mineralization, specifically in

214   pfMSCs, but not in bmMSCs, remains unclear. The two cell types react differently to external

215   factors, as also shown with exposure to glucocorticoids (25). In the latter study, the authors have

216   shown that pfMSCs are more sensitive to glucocorticoids than bmMSCs.

217   Our findings are of particular relevance for THA, as pfMSCs reside in the area directly

218   involved in THA (6). Our results suggest that direct administration of vancomycin to an infected

219   hip joint may not have a detrimental effect on the viability of the MSC population residing in the

220   affected bone tissue and may, therefore, spare the surrounding bone tissue, or at least preserve

221   the capacity for tissue regeneration. The mild anti-osteogenic effects of vancomycin in

222   differentiating pfMSCs may cause local short-term loss of bone mineral density at vancomycin-

223   treated sites. However, we hypothesize that the absence of cytotoxicity of vancomycin on

224   undifferentiated pfMSCs may result in the population of pfMSCs surrounding the affected area

225   remaining intact, which will contribute to repair of the bone tissue and successful

226   osseointegration of the prosthesis upon cessation of vancomycin treatment.

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228   **ACKNOWLEDGMENTS**

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305   **FIG 1**   The effect of vancomycin on the cell culture density of bmMSCs (left panel) and pfMSCs

306   (right panel).   Cells in SGM were treated for 7 days with 1 x MIC (5 µg/ml) and 4 x MIC (20

307   µg/ml) vancomycin.   Positive controls for cytotoxicity were 10 µg/ml cycloheximide together

308   with 5 ng/ml TNFα (CxT) for bmMSCs and 1 µM dexamethasone (Dex) for pfMSCs. The cells

309   were stained with crystal violet. The stain was then extracted and quantified

310   spectrophotometrically at 570 nm. Values for control (SGM-treated) wells were set as 100%. The

311   graph represents data of n = 4. A *post hoc* Dunnet test was done with SGM as control. Statistical

312   significance ($p < 0.05$) is indicated with *.

313

314   **FIG 2**   The effect of vancomycin on the metabolic activity of bmMSCs (left panel, A) and

315   pfMSCs (right panel, B).   Cells in SGM were treated for 7 days with 1 x MIC (5 µg/ml) and 4 x

316   MIC (20 µg/ml) vancomycin. Positive controls for cytotoxicity were 10 µg/ml cycloheximide

317   and 5 ng/ml TNFα (CxT) for bmMSCs and 1 µM dexamethasone (Dex) for pfMSCs. Cell

318   viability was measured via an MTT conversion assay and intensity of the colour product was

319   measured at 570 nm. Values for control (SGM-treated) cells were set as 100%. The graph

320   represents data of n = 4. A *post hoc* Dunnet test was done with SGM as control. Statistical

321   significance ($p < 0.05$) is indicated with *.

322

323   **FIG 3**   The effect of vancomycin on the osteoblastic differentiation of bmMSC (left panel, A)

324   and pfMSC (right panel, B).   Cells were treated with with 1 x MIC (5 µg/ml) and 4 x MIC (20

325   µg/ml) vancomycin. The formation of mineralized extracellular matrix was evaluated with ARS

326   staining after 7 days (bmMSCs) or 21 days (pfMSCs). Cells treated with control media (SGM

327   plus 0.1%, v/v, ethanol) did not form any mineralized deposits. The graph represents n = 7. All

328   data were compared to OM, which was set as 100%. Statistical significance is indicated with an
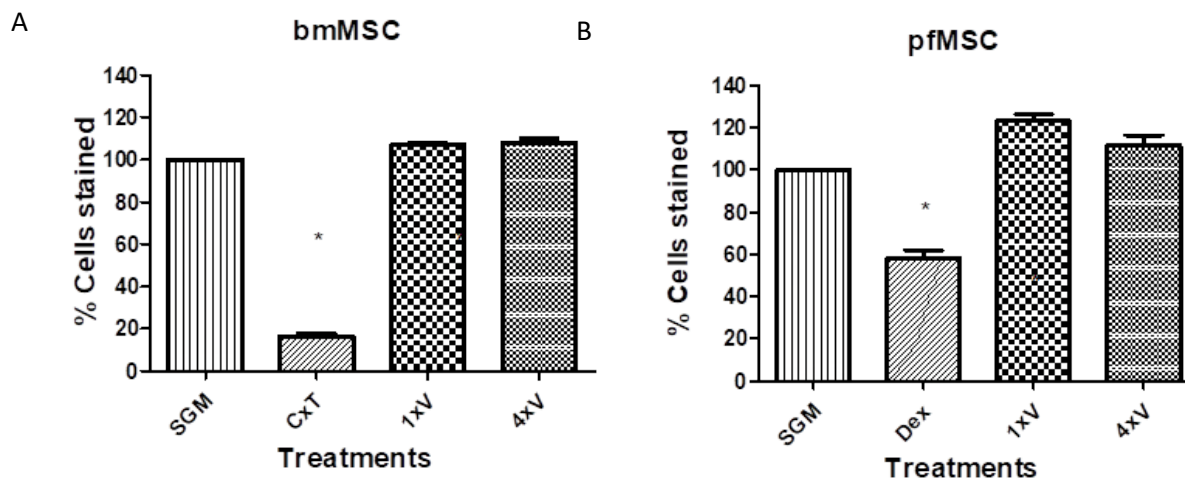
329   *.

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FIG 1 The effect of vancomycin on the cell culture density of bmMSCs (left panel) and pfMSCs (right panel). Cells in SGM were treated for 7 days with 1 x MIC (5 µg/ml) and 4 x MIC (20 µg/ml) vancomycin. Positive controls for cytotoxicity were 10 µg/ml cycloheximide together with 5 ng/ml TNFα (CxT) for bmMSCs and 1 µM dexamethasone (Dex) for pfMSCs. The cells were stained with crystal violet. The stain was then extracted and quantified spectrophotometrically at 570 nm. Values for control (SGM-treated) wells were set as 100%. The graph represents data of n = 4. A *post hoc* Dunnet test was done with SGM as control. Statistical significance (p<0.05) is indicated with *.
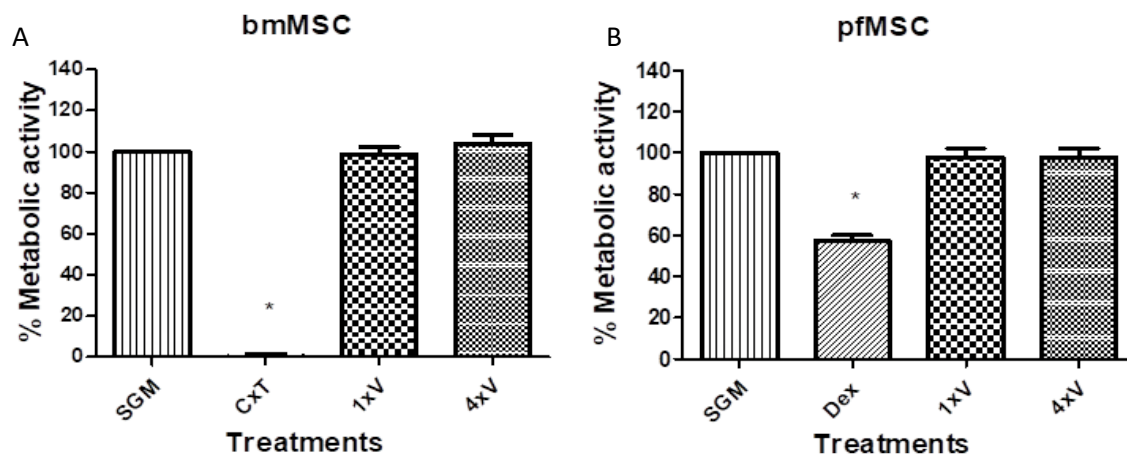
FIG 2 The effect of vancomycin on the metabolic activity of bmMSCs (left panel, A) and pfMSCs (right panel, B). Cells in SGM were treated for 7 days with 1 x MIC (5 µg/ml) and 4 x MIC (20 µg/ml) vancomycin. Positive controls for cytotoxicity were 10 µg/ml cycloheximide and 5 ng/ml TNFα (CxT) for bmMSCs and 1 µM dexamethasone (Dex) for pfMSCs. Cell viability was measured via an MTT conversion assay and intensity of the colour product was measured at 570 nm. Values for control (SGM-treated) cells were set as 100%. The graph represents data of n = 4. A *post hoc* Dunnet test was done with SGM as control. Statistical significance (p < 0.05) is indicated with *.
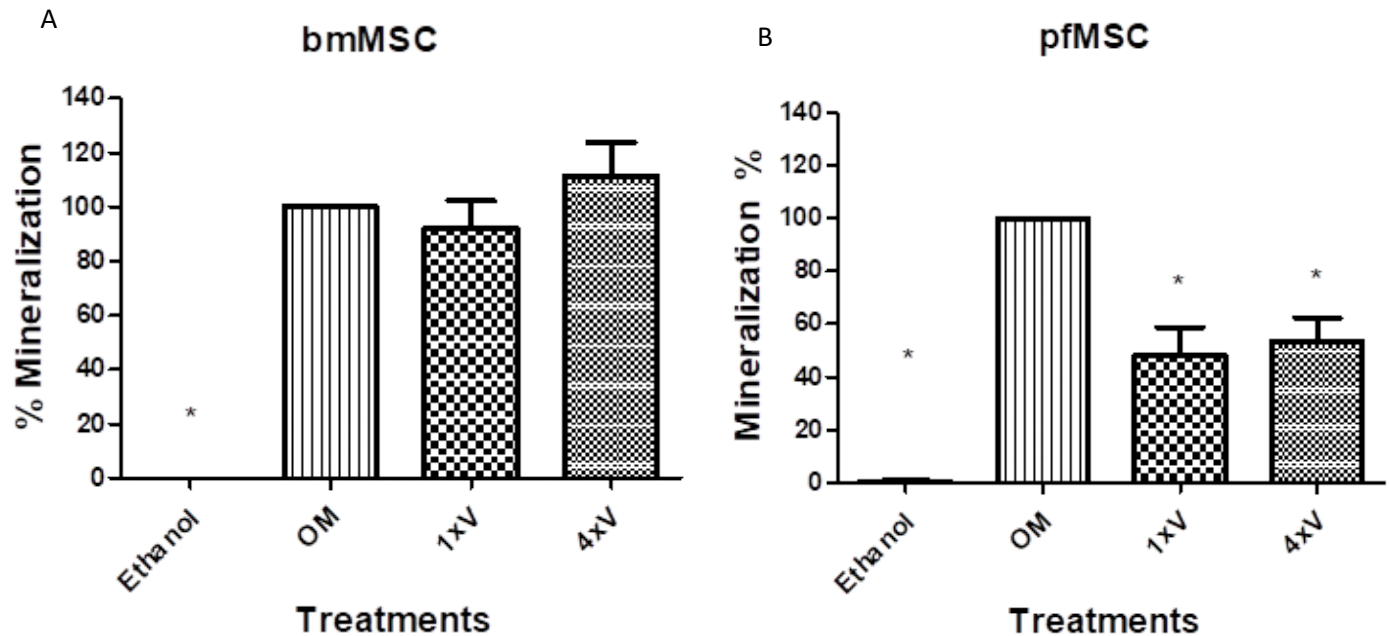
FIG 3 The effect of vancomycin on the osteoblastic differentiation of bmMSC (left panel, A) and pfMSC (right panel, B). Cells were treated with with 1 x MIC (5 µg/ml) and 4 x MIC (20 µg/ml) vancomycin. The formation of mineralized extracellular matrix was evaluated with ARS staining after 7 days (bmMSCs) or 21 days (pfMSCs). Cells treated with control media (SGM plus 0.1%, v/v, ethanol) did not form any mineralized deposits. The graph represents n = 7. All data were compared to OM, which was set as 100%. Statistical significance is indicated with an *.