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The effect of Vancomycin and Gentamicin antibiotics on human osteoblast proliferation, metabolic function and bone mineralisation

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Structured Abstract

Study Design: This study investigates the effect of Vancomycin and Gentamicin antibiotics on primary human osteoblasts. Osteoblasts were incubated with vancomycin, gentamicin or with Povidone-Iodine (PVI), at concentrations advocated for wound irrigation. Osteoblast proliferation, metabolic function and bone mineralisation were measured.

Objective: To model Gentamicin and Vancomycin wound irrigation *in vitro* and examine the effect on osteoblast viability and cellular function in comparison to 0.35% PVI.

Summary of Background Data: Vancomycin, Gentamicin and dilute PVI are employed as wound irrigants in spinal surgery to reduce infection. However, we have recently demonstrated that 0.35% PVI has a detrimental effect on osteoblast cellular function and bone mineralisation. Studies to determine the effects of antibiotic wound irrigation solutions on osteoblasts and bone mineralisation are therefore warranted.

Methods: Primary human osteoblasts were exposed for 20 min to either PBS control, Vancomycin (35 mM or 3.5 mM), Gentamicin (34 mM or 3.4 mM) or 0.35% PVI for 3 min. Cellular proliferation was measured over 7 days by MTS assay. Osteoblast metabolic function was determined using a Seahorse XFe24 Bioanalyzer. Mineralised bone nodules were quantified using Alizarin red.

Results: At concentrations advocated for wound irrigation, both Gentamicin (3.4 mM) and Vancomycin (3.5 mM) induced a transient 15-20% reduction in osteoblast proliferation, which returned to control values within 72 h. This was in marked contrast to the effect of 0.35% PVI which resulted in a sustained reduction in osteoblast proliferation of between 40-50% over 7 days. Neither Gentamicin nor Vancomycin at concentrations up to 10x clinical dose had any effect on osteoblast oxygen consumption rate, or significantly affected mineralised bone nodule formation.

Conclusion: Vancomycin and Gentamicin solutions, at concentrations advocated for intrawound application in spinal surgery have a small but transient effect on osteoblast proliferation, and no effect on either osteoblast metabolic function or bone nodule mineralisation.

Key Words

Adolescent idiopathic scoliosis; osteoblasts; vancomycin; gentamicin; povidone iodine; PVI; bone; wound irrigation; antibiotics; bone mineralisation; cell proliferation, cell metabolism, surgical site infections

Level of Evidence: N/A

Key Points

- Acute exposure to clinically employed concentrations of Gentamicin and Vancomycin (3.4 mM and 3.5 mM respectively) induces a small but transient reduction in the proliferation of osteoblasts, in contrast to 0.35% PVI which induces a marked and sustained reduction in osteoblast proliferation
- Gentamicin or Vancomycin up to 10x clinical dose did not affect osteoblast metabolic capacity (OCR), or effect bone nodule mineralisation, in contrast to 0.35% PVI.

Mini abstract

This study modelled the effect of using Gentamicin and Vancomycin antibiotics during spinal surgery on human primary osteoblast cells. Osteoblasts treated with clinically employed concentrations of Gentamicin or Vancomycin, exhibited a small but transient reduction in proliferation, and no change in metabolic capacity or ability to form mineralised bone.

1 Introduction

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2 Surgical site infections (SSIs) following spinal surgery are a serious complication,
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4 which negatively impact on patient outcomes and can be fatal. Such incidences can
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6 require additional operations involving antibiotic irrigation of the wound and
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8 debridement ^{1,2}, and in certain cases, the spinal implants must be removed: an
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10 expenditure of both time and healthcare resources ³. Indeed, infection-related costs
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12 have been reported to be an additional \$30,000 per patient ⁴.
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19 In order to combat infection, several prophylactic measures are in use ⁵. Peri-
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21 operative broad spectrum antibiotic usage is standard in orthopaedic surgery and
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23 does significantly reduce risk of infection ⁶. However, in addition, in order to reduce
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25 rates further, and due to the rise of Methicillin-Resistant *Staphylococcus Aureus*
26
27 (MRSA) ⁷, intrawound broad-spectrum antibiotics including Vancomycin and
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29 Gentamicin ^{8,9,10} may be applied. Unfortunately, there are very few
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31 recommendations from historical literature with regards to the use of antibiotics to
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33 combat SSI rates in spinal surgery, and thus clinical practice varies considerably.
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41 In a systematic review, particularly high SSI rates (3.5% to 8.5%) were noted in
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43 spinal surgery in paediatric patients with cerebral palsy, spina bifida, patients with
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45 urinary tract infections, incontinence obesity and those patients undergoing pelvic
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47 fixation procedures ¹¹. This review was the basis for constructing the Best Practice
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49 Guidelines, where the intrawound application of Vancomycin is specifically
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51 recommended ¹² for spinal surgery in paediatric patients. Several studies, including
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53 large randomised control trials, have provided evidence that administration of
54
55 intrawound Vancomycin is safe ¹³ and efficacious in reducing the incidence of SSIs,
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26 when compared to prophylactic antibiotics alone ^{14,15,16,17,18}. Similarly, intrawound
27 application of Gentamicin has also been shown to reduce infection rates spinal
28 surgery in children with cerebral palsy, with a reduction from 15.2% to 3.9%
29 observed ¹⁹.

30
31 Some spinal units have reported that wound irrigation with a solution of Povidone-
32 Iodine (PVI;Betadine) ^{20,21} is safe and efficacious at reducing SSI rates in spinal
33 surgery. Furthermore, a protocol which combined PVI wound irrigation with
34 intrawound Vancomycin powder was recently advocated as being highly effective at
35 reducing SSI rates in spinal surgery, with a reduction of 50% reported ²². However,
36 we have recently reported that exposure of human osteoblasts to 0.35% PVI (the
37 concentration advocated for wound irrigation), is highly cytotoxic to osteoblasts, with
38 a marked and sustained reduction in osteoblast cellular proliferation and inhibition of
39 mineralised bone formation ²³. This particular study highlighted the importance for
40 spinal units to evaluate not only the SSI antibacterial efficacy of such intrawound
41 reagents, but to also to consider the effect of these reagents on the cellular
42 osteoblast function.

43
44 Critically, no studies have reported the effects of Vancomycin or Gentamicin
45 antibiotics on human primary osteoblast mineralised bone formation. Neither has
46 their effect on osteoblast proliferation been reported following an *in vitro* protocol that
47 mimics the transient high concentrations of antibiotics in the surgical wound following
48 intrawound application. Given that the goal of many spinal surgeries is to establish
49 solid bone union, it is important to know whether these reagents, like PVI, may
50 impair bone healing since this may help guide clinical practice. The aim of this study

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51 was therefore to model *in vitro* the effect of Vancomycin and Gentamicin antibiotic
52 solutions on osteoblast proliferation, osteoblast metabolism and mineralised bone
53 nodule formation.
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55 METHODS

56 3.1 Cell culture

57 Ethical approval was granted by the United Kingdom (UK) National Research Ethics
58 Service (National Health Authority, reference NRES 14-ES-1044), and institutionally
59 approved and sponsored by the University of Birmingham as required under the UK
60 Research Governance Framework. Study participants were provided in advance with
61 a participant information sheet, and a participant consent form.

62
63 Following patient's written consent, the femoral head was collected from a female
64 patient (aged 62 years) undergoing total joint replacement surgery for hip
65 osteoarthritis. The articular cartilage was removed from the femoral head and the
66 subchondral bone cut into small chips. The bone chips were then washed thoroughly
67 in serum-free primary osteoblast media to remove any excess blood, connective or
68 adipose tissue and then incubated in differentiation media (10% FCS, penicillin
69 (100units/ml), streptomycin (100µg/ml), L-glutamine (2mM), β-glycerophosphate
70 (2mM), Ascorbic acid (50µg/ml) and Dexamethasone (10nM)) in a culture flask at
71 37°C (5% CO₂). Differentiation media was replaced with fresh media 2x per week,
72 and the bone chips removed upon the appearance of osteoblast cells. Primary
73 human osteoblast cells were cultured in differentiation media.

74
75 The experimental concentrations of Vancomycin and Gentamicin utilised for this
76 study were based on the serial observation of average wound drain outputs following
77 turning patients from the table. From this it was estimated that an average scoliosis
78 wound held approximately 200 ml of fluid. Furthermore, it was considered that the
79 majority of Vancomycin and Gentamicin antibiotic would be washed out after closure

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80 following opening of the drain after 20 min. Therefore an experimental concentration
81 of 1g of Vancomycin or Gentamicin in 200ml of PBS solution (equating to 3.5 mM
82 and 3.4 mM respectively) exposed for 20 min would best mimic the clinical
83 concentration and exposure time. Higher concentrations at 10X the estimated
84 clinical concentration was utilised for the second experimental setting, equating to 35
85 mM and 34 mM for Vancomycin and Gentamicin respectively. The experimental
86 concentration of PVI utilised was 0.35%, which has previously been clinically
87 advocated. Cells were exposed for 3 min with 0.35% PVI, before washing with PBS
88 in order to mimic the clinical saline washout procedure.

91 **3.3 Osteoblast proliferation assay**

92 Primary human osteoblasts were seeded at 6×10^3 cells per well in a 96 well plate. At
93 confluency, osteoblasts were stimulated with either Vancomycin (3.5 mM or 35 mM),
94 or Gentamicin (3.4 mM or 34 mM) for 20 min, or with 0.35% PVI for 3 min. All wells
95 were then aspirated, washed 5x with PBS and then filled into osteoblast
96 differentiation media. After 0, 24, 48, 72 and 144 h incubation at 33°C, an MTS (Cell
97 Titer Aqueous One Solution Cell Proliferation Assay, Promega) assay was
98 performed as per manufacturer's instructions as a measure of osteoblast
99 proliferation.

101 **3.4 Osteoblast metabolic function**

102 Primary human osteoblasts were plated at 6×10^3 cells per well in a XF^e 24 Cell
103 Culture Microplate (Seahorse Bioscience, USA). At confluency, the cells were
104 stimulated, washed and incubated exactly as in the proliferation assay and then

105 placed back into media. After 24 h incubation at 33°C, XF Assay medium (XF base
106 medium with 2mM GlutaMAX™) was then added to the plate and incubated at 33°C
107 for 1 h. Osteoblast oxygen consumption rate (OCR) was determined using a XF^e
108 Extracellular Flux Analyzer (Seahorse Bioscience, USA), as a measure of osteoblast
109 metabolic function.

111 **3.7 Osteoblast bone nodule formation and mineralization assay**

112 Primary human osteoblasts were seeded at 6×10^3 cells per well in an 96-well plate
113 and stimulated, incubated and washed as previously described before being cultured
114 in differentiation media. After 28 days, cells were stained with Alizarin red solution in
115 order to quantify the degree of mineralisation following the formation of bone
116 nodules. Briefly, cells were incubated in 0.5% Alizarin Red staining solution (Sigma-
117 Aldrich, UK) in 1% ammonia solution at pH 4.5 for 10min at room temperature and
118 washed with PBS to remove excess stain. Cells were then incubated in 10% cetyl
119 pyridinium chloride (Sigma-Aldrich, UK) for 10 min at room temperature. The
120 supernatant was collected from each well and diluted 1:10 with the 10% cetyl
121 pyridinium chloride and read at OD_{550nm} on a SpectraMAX Microplate Reader
122 (Molecular Devices, USA).

124 **3.8 Statistical Analysis**

125 All statistical analyses were carried out using SPSS. Unless otherwise stated, all
126 data within figures represents Mean \pm SEM and were assessed by a two-way
127 analysis of variance (ANOVA) with a Bonferroni post-hoc test conducted where
128 appropriate. Significance was accepted as $P < 0.05$.

130 Results**131 3.1 The effect of acute exposure to Vancomycin and Gentamicin antibiotics on**
132 osteoblast proliferative activity

133 Relative to normal osteoblast proliferation (PBS control), 20 min exposure of human
134 osteoblasts to Gentamicin at the clinical concentration of 3.4 mM resulted in a
135 significant reduction in osteoblast proliferation of 12% after 24h ($P<0.05$), and 22%
136 after 48 h ($P<0.01$). However, at 3 days, osteoblast proliferation had returned to
137 control levels. At 10x clinical dose of 34 mM Gentamicin, a similar reduction in
138 osteoblast proliferation was observed, peaking at a 27% reduction after 48 h
139 ($P<0.001$). However, osteoblasts exposed to 34 mM Gentamicin exhibited a more
140 sustained reduction in osteoblast proliferation, with a significant 14% reduction
141 ($P<0.05$) observed at 7 days post exposure (Figure 1A).

142
143 Vancomycin at 3.5 mM had no significant effect on osteoblast proliferation at 24 h,
144 but there was a small 15% reduction in proliferation relative to control at 48 h.
145 However, proliferation levels returned to control levels at 3 days post exposure.
146 Similar to our findings with Gentamicin, the 10x clinical dose of 35 mM Vancomycin
147 induced a more pronounced inhibition of osteoblast proliferation of between 18-24%
148 and this effect was sustained up to 7 days post exposure (Figure 1B).

149
150 In contrast, osteoblasts exposed to 0.35% PVI for 3 min exhibited a much greater
151 reduction in proliferation of between 40-50% which was sustained between 24 h and
152 7 days ($P<0.001$) (Figure 1A & 1B).

153 **3.2 Acute exposure to Vancomycin and Gentamicin antibiotics does not affect**
154 **human osteoblast basal metabolic function.**

155 Given these differential findings between the effects of antibiotic reagents and PVI
156 on osteoblast proliferation, we next compared their effects on osteoblast metabolic
157 function. As we have previously reported, osteoblasts exposed for 3 min to 0.35%
158 exhibit a significant 94% reduction ($P < 0.001$) in basal oxygen consumption rate
159 (OCR)²³. However, in contrast neither Gentamicin nor Vancomycin, at either clinical
160 dose or 10x clinical dose, elicited any significant reduction in osteoblast OCR. In
161 osteoblasts that had been exposed 35 mM Vancomycin, OCR was on average lower
162 than PBS control, although this did not reach statistical significance (Figure 2A & 2B).

163

164 **3.4 Acute exposure of human osteoblasts to Vancomycin and Gentamicin does**
165 **not inhibit the formation of mineralised bone**

166 We next examined whether acute 20 min exposure of human osteoblasts to
167 Vancomycin or Gentamicin had a chronic effect on their ability to form mineralised
168 bone nodules. Following exposure to Vancomycin, Gentamicin or PBS control,
169 confluent osteoblasts were kept in culture for a further 2 weeks before mineralised
170 bone nodules were stained and quantified using Alizarin red (Figure 3A). Although,
171 on average the degree of bone mineralisation was lower in osteoblasts which had
172 been exposed to antibiotics, there was no significant reduction observed with either
173 Gentamicin or Vancomycin at either clinical dose or 10x clinical dose (Figure 3B and
174 3C).

Discussion

175 This study shows that, at concentrations advocated for intrawound application,
176 Vancomycin (3.5 mM) and Gentamicin (3.4 mM) elicit a small (15-20% reduction) in
177 osteoblast proliferation. Critically however, this effect was transient, with osteoblast
178 proliferation returning to normal within 72 h post-exposure. Furthermore, there was
179 no impact on either osteoblast metabolic capacity or the ability of osteoblasts to form
180 mineralised bone at these concentrations. These findings were in stark contrast to
181 our findings with PVI, where the detrimental effects on osteoblast proliferation were
182 significantly greater (40%-50% inhibition) and were sustained for up to 7 days.

184
185 These findings are clinically important since there are clear advantages to surgical
186 intrawound application of antibiotics compared to intravenous administration. Firstly,
187 in the Best Practice Guidelines, intrawound application of Vancomycin is favoured
188 over intravenous delivery due to concerns about its potential systemic toxicity ¹².
189 Critically, although intrawound vancomycin is absorbed systemically, the systemic
190 levels are low ¹⁴, thus higher concentrations can be applied where required without
191 concerns for systemic toxicity ¹⁴. Furthermore, wounds are complicated by seroma,
192 haematomas and devitalised tissue. Such tissues present barriers, which systemic
193 prophylactic antibiotics poorly penetrate ²⁴. Indeed, in a rabbit surgical wound
194 infection model, intrawound Vancomycin was significantly more effective at
195 eliminating SSIs than intravenous cefazolin alone ²⁵.

196
197 Despite our data showing that Vancomycin (at 3.5 mM) and Gentamicin (at 3.4 mM)
198 elicit only a small and transient effect on osteoblast proliferation, a previous study
199 using the human MG-63 osteosarcoma cell line ²⁶ found that Vancomycin at 1000

1 200 ug/ml (equivalent to 0.68 mM) had no effect on cell viability, but was toxic at a
2 201 concentration of 10,000 ug/ml (6.8 mM). Similarly, Gentamicin at concentrations up
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4 202 to 1000 ug/ml (2 mM) have previously been reported to reduce alkaline phosphatase
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6 203 activity in osteoblast-like cells from cancellous hip bone ²⁷. However, it is important
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9 204 to note that firstly in the study by Edin *et al.* ²⁶ the utilised cells were not primary cells
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11 205 and, although classed as “osteoblast-like”, their proliferation rate and alkaline
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13 206 phosphatase activity is not considered very representative of bone ²⁸. Secondly, in
14
15 207 the study by Edin *et al.* ²⁶ the cells were exposed to Vancomycin for 24-72 hours,
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17 208 and in the study by Isefuku *et al.* ²⁷ cells were exposed to Gentamicin for 4 days.
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20 209 Such lengthy *in vitro* protocols are likely to poorly mimic the transient high
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22 210 concentration of Vancomycin within the surgical site following intrawound clinical
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24 211 application ¹⁴. Our *in vitro* data supports clinical observations which report no
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27 212 indications of bone growth problems in patients where bone allografts have been
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30 213 supplemented with Vancomycin in hip revision surgery ²⁹.

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36 215 Importantly, despite the Best Practice Publication ¹² not including PVI irrigation
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38 216 amongst the recommendations, the use of PVI either alone or in combination with
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40 217 intrawound antibiotics is still being advocated by several spinal units ^{22,20,21}.
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43 218 Furthermore, there are currently no clinical studies which have demonstrated greater
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45 219 gains in reducing SSI rates using PVI irrigation compared to using intrawound
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47 220 antibiotics. Therefore, given the clear differential effects we report here on
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50 221 osteoblast viability, it would appear pertinent for clinicians to favour the practice of
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52 222 intrawound Vancomycin or Gentamicin application over 0.35% PVI wound irrigation.
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55 223 Furthermore, although the combination of Vancomycin intrawound with PVI wound
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57 224 irrigation was recently reported to provide a commendable 50% reduction in SSI
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1 225 rates in spinal surgery ²², our data suggests this practice should be approached
2 226 cautiously. Such caution may be particularly warranted for paediatric patients
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4 227 undergoing long fusion surgical procedures (4 levels of more), with high non-union
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6 228 rates ^{30,31,32}, where any gains in reducing SSI rates with PVI alone (or in combination
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9 229 with Vancomycin) may be offset by impaired bone healing.

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12 231 It is important to stress the limitations of this study. It is an *in vitro* study, and
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14 232 although the human osteoblasts are primary cells they do not originate from spinal
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16 233 bone tissue. Furthermore, such *in vitro* studies cannot fully replicate the complexity
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18 234 of the *in vivo* environment, where for example pluripotent stem cells may migrate to
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20 235 the site of surgery and aid bone healing and fusion. Nevertheless, our data suggests
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22 236 a clear differential between the effects of Vancomycin and Gentamicin antibiotics
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24 237 compared to PVI on osteoblast proliferation and function.
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33 239 In summary, at concentrations advocated for the reduction of SSIs in spinal surgery
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35 240 Vancomycin and Gentamicin had no sustained effect on osteoblast proliferation, or
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37 241 the formation of mineralised bone. We suggest therefore that in contrast to PVI
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39 242 wound irrigation, the intrawound application of the either Vancomycin or Gentamicin
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41 243 in spinal surgery would be unlikely to impair bone healing.
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244 **Abbreviations**

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246 **PVI** Povidone-Iodine

247 **PBS** Phosphate buffered saline

248 **MTS** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
249 sulfophenyl)-2H-tetrazolium, inner salt

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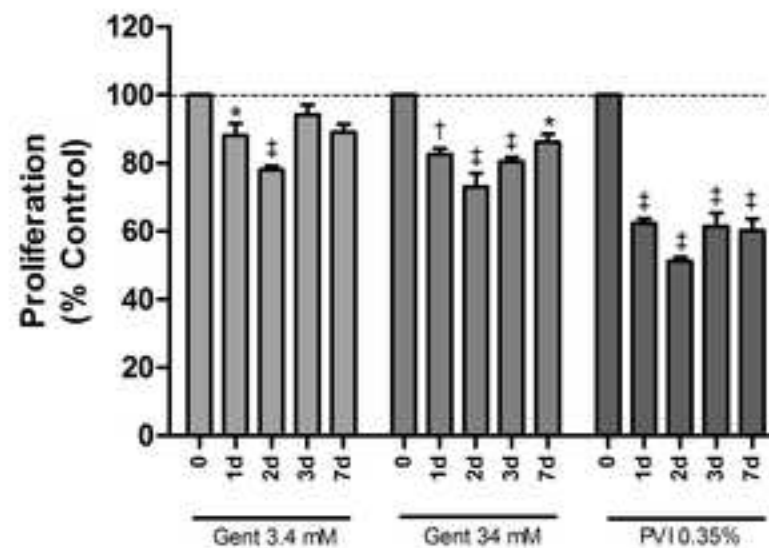
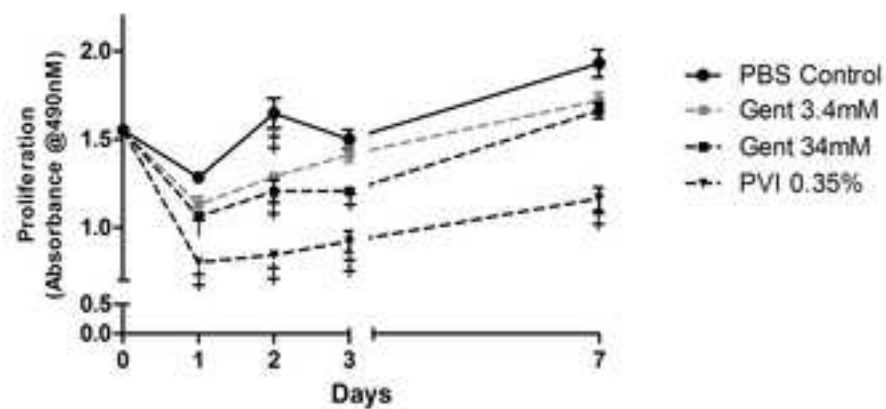
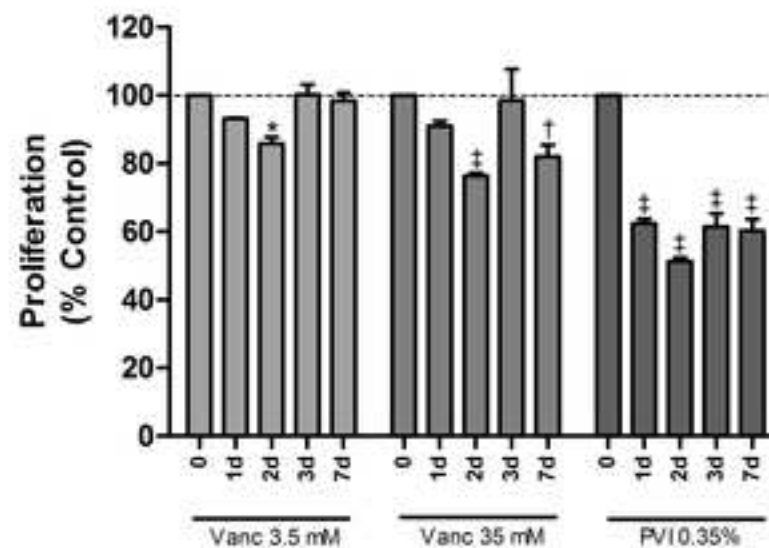
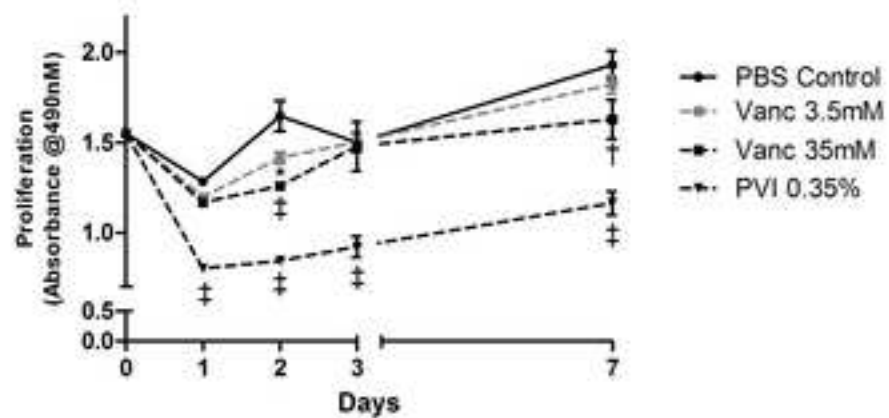
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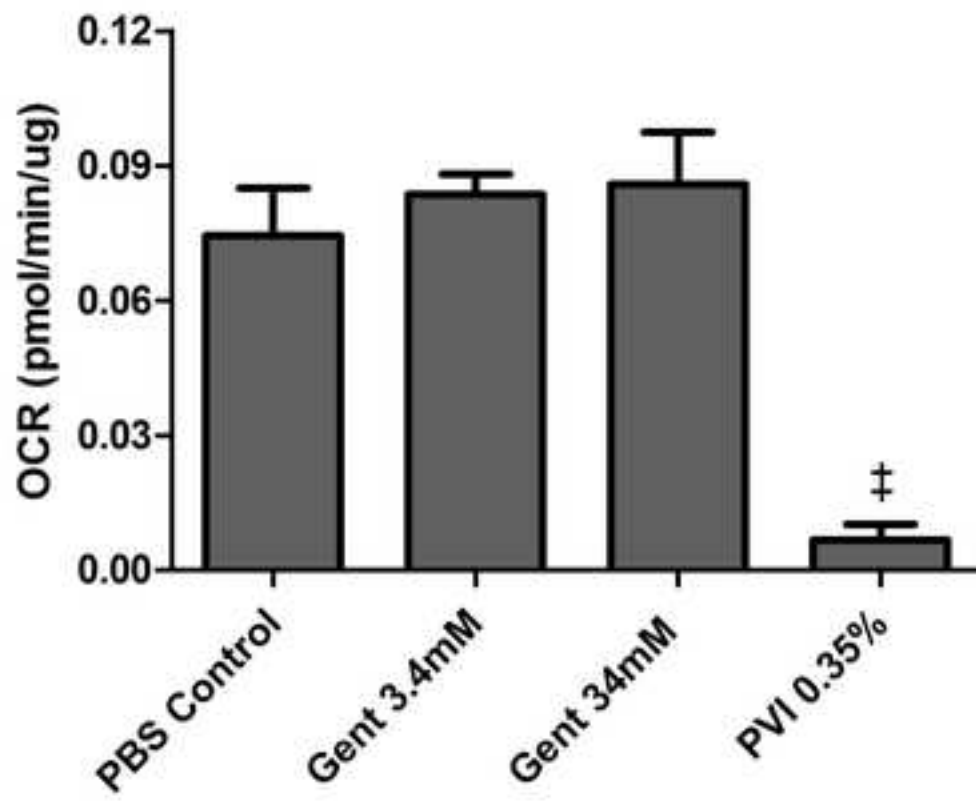
Figure 1. The effect of acute exposure to antibacterial intrawound reagents on primary human osteoblast proliferation. (A) Osteoblasts were stimulated for 20 min with either PBS control, Gentamicin (34 mM or 3.4 mM) or stimulated for 3 min with 0.35% PVI. (B) Osteoblasts were stimulated for 20 min with either PBS control, Vancomycin (35 mM or 3.5 mM) or stimulated for 3 min with 0.35% PVI. Osteoblast proliferation was measured by MTS assay (absorbance at 490 nm) at time 0, day 1, 3, 4 and day 7. Bars represent mean \pm SEM (n=3), with * $=P<0.05$, † $=P<0.01$, ‡ $=P<0.001$, representing values significantly different to PBS control.

Figure 2. Metabolic activity of primary human osteoblast following acute exposure to antibiotic intrawound reagents. (A) Oxygen consumption rate (OCR) of osteoblasts 24 h after 20 min exposure to either Gentamicin (34 mM or 3.4 mM) or 3 min exposure to 0.35% PVI. (B) OCR of osteoblasts after 20 min exposure to either Vancomycin (35 mM or 3.5 mM) or 3 min exposure to 0.35% PVI. Bars represent mean \pm SEM (n=3), with ‡ $=P<0.001$, representing values significantly different to PBS control.

Figure 3. Primary human osteoblast mineralisation following acute exposure to antibiotic intrawound reagents. (A) Representative microscopic images (x20 magnification) of phase contrast of primary human osteoblasts, and Alizarin red stained mineralised bony nodules after 28 days post fully confluent. Primary human osteoblasts were cultured out bone chips in differentiation media. (B) Quantification by absorptiometry (at 490 nm) of Alizarin red stained mineralised bone

nodules in osteoblast 14 days after acute 20 min exposure to either Gentamicin (34 mM or 3.4 mM), Vancomycin (35 mM or 3.5 mM) or PBS media control. Bars represent mean \pm SEM (n=3). (C) Representative microscopic images showing images of Alizarin red stained mineralised bone nodules 14 days after exposure to Gentamicin and Vancomycin antibiotics.

A**B**

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