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The combination of local infiltration analgesia reagents increases their detrimental effect on human hip OA patient osteoblast viability and function

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1 **Abstract**

2 The purpose was to evaluate the effect of Local infiltration analgesia (LIA) reagents in
3 monotherapy and in combinations at clinical doses, on the viability and function of
4 osteoblasts isolated from hip OA patients undergoing orthopaedic surgery. Human hip OA
5 osteoblasts were exposed to LIA reagents including Bupivacaine, Lidocaine, Ropivacaine,
6 Ketorolac and combinations with Adrenaline for 30 min. Osteoblast cellular viability and
7 function was determined at 24 h and 7 days post-exposure. In conclusion, our data shows
8 that LIA reagents, most notably Bupivacaine and its use in combination, are detrimental to
9 human hip OA osteoblasts at concentrations advocated for clinical use.

10

11 **Keywords**

12 Local infiltration analgesia, osteoblasts, anesthetics, lidocaine, bupivacaine

1. Introduction

With an aging society the number of orthopaedic surgeries to restore mobility is increasing. Over 220,000 surgical procedures, including total hip and knee replacement surgeries, were recorded by the National Joint Registry for England, Wales and Northern Ireland in the 2018 annual report [1]. Post-operative pain relief from these procedures is important not only for patient satisfaction, but also economically in reducing hospitalisation and in promoting a rapid recovery and a successful return to work for many patients.

To this end, the use of local infiltration analgesia (LIA), in which a dose of analgesia is delivered towards the end of a procedure, has become the new standard practice to facilitate rapid recovery from surgeries such as knee and hip arthroplasty [2] and is often compared to epidural analgesia in terms of efficacy [3]. This technique has been shown to enable near-immediate painless mobilisation of the joint [4] and therefore aids clinicians in being able to discharge patients from hospital earlier than otherwise possible. Studies into the use of LIA have reported a reduction in postoperative pain and reduced hospitalisation using a variety of different local anaesthetic reagents including Lidocaine, Ropivacaine or Bupivacaine, which are often combined with Ketorolac or with Adrenaline [5].

Critically, despite widespread use there is a growing concern over the use of LIA [6, 7]. Several studies have indicated that commonly used analgesia may have negative consequences on chondrocyte and articular cartilage health *in vitro*. Both Bupivacaine (0.5%) and Ropivacaine (0.5%) have been reported to reduce the viability of human chondrocytes, either when cultured in monolayer or in cartilage explants following a 30 min exposure to the reagents [8]. Similar results have been reported using either bovine [7, 9] or equine [10] chondrocytes. However, few studies have examined the effect of such reagents on primary human osteoblast health and function. Understanding the effect of LIA on human osteoblast health is critically important given the exposure of bone tissue to these reagents *in vivo* in

38 patients undergoing orthopaedic surgery, and the central functional role of osteoblasts in bone
39 remodelling and repair. Therefore, the aim of this study was to determine the comparative
40 effect of commonly used LIA reagents (Lidocaine, Bupivacaine and Ropivacaine) and their
41 combinations with Ketorolac and Adrenaline, at clinically relevant concentrations, on human
42 primary osteoblasts viability and function utilising osteoblasts isolated from patients
43 undergoing total hip replacement surgeries.
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47 **2. Methods**

48 **2.1 Cell culture**

49 Ethical approval was granted by the UK National Research Ethics Service (NRES 14-ES-
50 1044) in accordance with the Declaration of Helsinki (World Medical Association), and was
51 institutionally approved and sponsored by the University of Birmingham as required under
52 the UK Research Governance Framework. Study participants were provided in advance with
53 a participant information sheet, and a participant consent form.

54 Following patient's written consent, femoral heads were collected from patients undergoing
55 total joint replacement surgery for hip osteoarthritis. Osteoblasts were cultured out from
56 bone chips as previously described [11]. In brief, the articular cartilage was removed from the
57 femoral head subchondral bone cut into small chips. The bone chips were then washed
58 thoroughly in serum-free primary osteoblast media to remove any excess blood, connective
59 or adipose tissue and then incubated in differentiation media (10% FCS, penicillin
60 (100units/ml), streptomycin (100μg/ml), L-glutamine (2mM), β-glycerophosphate (2mM),
61 Ascorbic acid (50μg/ml) and Dexamethasone (10nM)) in a culture flask at 37°C (5% CO_2).
62 Differentiation media was replaced with fresh media 2x per week, and the bone chips
63 removed upon the appearance of osteoblast cells. Primary human osteoblast cells were
64 cultured in differentiation media.

65

66 **2.2 Osteoblast proliferation and morphology**

67 Primary human osteoblasts were seeded at a density of 10,000 cells per well in a 96 well-
68 plate. At confluency the cells were exposed for 30 min to either PBS control or to a clinically
69 relevant LIA treatment (Table 1). After 30 min of treatment, wells were aspirated, cells
70 washed with PBS and then placed back into 200ul of cell culture media and cultured at 37°C.
71 After 24 hours and 7 days of culture, cellular proliferation was determined by MTS assay

72 (Cell Titer Aqueous One Solution Cell Proliferation Assay, Promega, Southampton, UK)
73 performed as per manufacturer's instructions as a measure of osteoblast proliferation. To
74 assess osteoblast morphology, primary human osteoblasts were seeded at a density of 50,000
75 cells per well in a 24 well-plate. Following PBS or LIA treatment cells were visualised using
76 a confocal microscope and images of the cells captured.

77

78 **2.3 Osteoprotegerin and RANKL ELISAs**

79 Primary human osteoblasts cultured in a 96-well plate at 10,000 cells/well were treated with
80 either PBS or LIA treatment for 30 min as previously described., After 24 h and 7 days, OPG
81 and RANKL in the cell supernatants were determined using commercially available ELISAs
82 (DuoSet ELISA Development Series) performed as per the manufacturer's instructions.

83

84 **2.4 Alkaline phosphatase assay**

85 Primary human osteoblasts were seeded at a density of 10,000 cells per well in a 96 well-
86 plate. Cells were allowed to reach confluence, before being exposed to 100ul of either PBS or
87 to a LIA treatment (Table 1) for 30 min. Wells were then aspirated, washed with PBS and
88 placed in 200ul of culture media and cultured as before at 37°C. After 24 h and 7 days at
89 37°C, cells were lysed in 100ul of RIPA buffer containing protease inhibitors. Alkaline
90 phosphatase activity in the cell lysates was determined as previously described [12], with
91 absorbance measured at 405 nm against alkaline phosphatase standards. Total protein
92 content was determined by Pierce BCA assay (ThermoFisher, UK) and Alkaline phosphatase
93 activity expressed as units of activity per mg of total protein.

94

95 **2.5 Statistical Analysis**

96 Data was analysed by 1-way ANOVA with Dunnett's post-hoc tests. Data within figures

97 represents mean ± SEM, with significance accepted as $p<0.05$.

98

3. Results

3.1 The effect of LIA treatments on human hip OA osteoblast morphology and proliferation.

We first examined the effect of the LIA reagents and their combinations (Table 1) on osteoblast morphology. Osteoblasts exposed for 30 min to the PBS control maintained a spindle like morphology and aligned to the surface of the culture plates over the course of 7 days of culture. Conversely, cells which were exposed for 30 min to 0.5% Bupivacaine showed a clear reduction in confluency and cell homogeneity after 7 days of culture. Furthermore, the combination of Bupivacaine with either 0.0005% Adrenaline or 0.5% Ketorolac, and the combination of 0.5% Ropivacaine with Ketorolac also reduced cell confluence after 7 days post-exposure (Figure 1). The effects of cell morphology and confluence were particularly pronounced in the combination of LIA reagents. Osteoblasts exposed to 1% Lidocaine either with or without Adrenaline appeared to maintain a similar morphology to the control PBS treated cells (Figure 1).

We next examined the effect of the LIA regimens on osteoblast proliferation as determined by MTA assay. At 24 h post-exposure to LIA reagents, there was no significant reduction in cell proliferation in osteoblasts exposed to any of the LIA monotherapies at clinically relevant concentrations, namely 0.5% Bupivacaine, 1% Lidocaine, 0.5% Ropivacaine or 0.5% Ketorolac (Figure 2A). However, osteoblasts that were exposed to the combination treatments of either Bupivacaine/Adrenaline or Bupivacaine/Ketorolac showed a significant reduction in proliferation after 24 h compared to PBS treated cells ($p<0.05$; Figure 2A).

At 7 days post-exposure, osteoblasts exposed to either Bupivacaine alone ($p<0.001$), or the combination treatments of either Bupivacaine/Adrenaline ($p<0.001$), Bupivacaine/Ketorolac ($p<0.001$) or Ropivacaine/Ketorolac ($p<0.01$) showed a significant reduction in proliferation compared to control cells (Figure 2B).

3.2 The effect of LIA treatments on human hip OA osteoblast function

We next investigated whether the LIA treatments affected osteoblast function by first examining their effect on the secretion of OPG and RANKL, which are known mediators of bone remodelling. Osteoblasts treated with either 0.5% Bupivacaine or 0.5% Ketorolac exhibited a significant reduction in OPG production after only 24 h post exposure ($p<0.0001$). Furthermore, the combination treatments of either Bupivacaine or Ropivacaine with either Adrenaline or Ketorolac were also found to significantly reduce OPG production at 24 h post exposure (Figure 3A). The reduction in OPG production was sustained at 7 days post-exposure in osteoblasts treated with either Ropivacaine alone or the combination treatments of Bupivacaine/Adrenaline, Ropivacaine/Adrenaline, Bupivacaine/Ketorolac and Ropivacaine/Ketorolac (Figure 3B). Production of RANKL was not significantly altered by any of the LIA treatments (Figure 3B), and therefore LIA treatments that significantly reduced OPG production also increased the RANKL:OPG ratio (Figure 3C).

Given the differential effects of Bupivacaine and Lidocaine on both osteoblast proliferation and OPG production we then further examined the effect of these reagents on osteoblast function by determining alkaline phosphatase activity. At 24 h post-exposure, 0.5% Bupivacaine, but not 1% Lidocaine, significantly reduced alkaline phosphatase activity, compared to control cells ($p<0.01$). However, both the combination of either Bupivacaine/Adrenaline ($p<0.01$) or Lidocaine/Adrenaline ($p<0.001$) also significantly reduced alkaline phosphatase activity, compared to control cells (Figure 4A). At 7 days post-exposure, osteoblast alkaline phosphatase activity in cells treated with either Lidocaine alone, or the combination of Lidocaine/Adrenaline was no different to control cells (Figure 4B). By comparison, osteoblasts exposed to either Bupivacaine alone, or the combination of

148 Bupivacaine/Adrenaline exhibited a sustained reduction in alkaline phosphatase activity at 7
149 days, comparted to control cells (Figure 4B).
150
151
152
153

154 **4. Discussion**

155 This is the first study to determine the effect of LIA reagents at clinically relevant
156 concentrations on primary human OA osteoblasts isolated form the joints of patients with hip
157 OA undergoing joint replacement surgery. Our data shows that the LIA reagents, most
158 notably Bupivacaine and its use in combination regimens, have a negative effect on many
159 aspects of osteoblast health at concentrations advocated for clinical use.

160 Of all the LIA reagents examined in our study 0.5% Bupivacaine had the most pronounced
161 effect on osteoblast morphology and viability, either when used alone or in combination with
162 Adrenaline or Ketorolac. After just 30 min exposure to 0.5% Bupivacaine osteoblasts
163 exhibited a 28% reduction in proliferation at 7 days post exposure. Even greater cytotoxicity
164 was observed in osteoblasts exposed to the combination of Bupivacaine with Adrenaline,
165 with a 46% reduction in proliferation at 24 h post exposure which was sustained up to 7 days
166 post-exposure. Of note, the added toxicity of Adrenaline in combination with Bupivacaine
167 appears to be analgesic dependent as we did not observe added toxicity in cells treated with
168 the combination of Adrenaline with either Lidocaine or Ropivacaine. Considering that the
169 combination of Bupivacaine and Adrenaline is typically used in orthopaedics [13], the
170 detrimental and sustained cytotoxic effect on osteoblasts we report here is concerning.

171 Further evidence of the adverse effects of combining LIA reagents was observed with
172 Ropivacaine and Ketorolac, which had no significant effect on osteoblast proliferation when
173 used individually, but when combined significantly reduced proliferation by 27% at 7 days
174 post exposure. Previously, greater cytotoxicity of chondrocytes has been reported using either
175 Bupivacaine or Ropivacaine in combination with the glucocorticoids Prednisolone or
176 Betamethasone [14]. Our findings add significant weight to the paradigm that the combination
177 of such reagents has the potential to increase their cytotoxicity.

178 This study also showed that LIA reagents negatively impact on osteoblast function by
179 reducing both OPG production and alkaline phosphatase activity. Again, the exposure of
180 osteoblasts to the combination of LIA reagents had a more marked effect on osteoblast
181 function. Both Bupivacaine and Ketorolac significantly reduced osteoblast OPG production
182 at 24 h post exposure, but recovered by 7 days post-exposure. However, the combination
183 treatment of either Bupivacaine/Adrenaline or Bupivacaine/Ketorolac elicited both a larger
184 and a sustained reduction in osteoblast OPG production. Furthermore, although Ropivacaine
185 alone had little effect on OPG production, osteoblasts exposed to the combination of
186 Ropivacaine/Ketorolac exhibited a 75% and a 90% reduction in OPG production at 24 h and
187 7 days post exposure respectively. Importantly, OPG acts as a decoy receptor to prevent
188 binding of RANKL to the RANK receptor on osteoclasts and therefore inhibits bone
189 resorption . Given that osteoblast RANKL production was not significantly affected by
190 exposure to LIA reagents, the LIA-mediated decline in OPG production resulted in marked
191 increases in the ratio of RANKL:OPG which would be expected to promote greater bone
192 resorption.

193 Interestingly, osteoblasts exposed to Lidocaine exhibited the fewest adverse effects, with no
194 significant reduction in proliferation or OPG production, even when combined with
195 Adrenaline. However, similarly to Bupivacaine, osteoblasts exposed to Lidocaine in
196 combination with Adrenaline did exhibit a significant reduction in alkaline phosphatase
197 activity at 24 h post exposure. Alkaline phosphatase is a membrane associated protein found
198 on the surface of active osteoblasts functioning to reform bone matrix. It functions to supply
199 high quantities of phosphate to assist in bone mineral deposition . Therefore, our data
200 suggests that this combination of either Lidocaine or Bupivacaine with Adrenaline might be
201 detrimental to post-surgical bone remodelling and thus patient outcomes.

202 It is important to note that this study is limited by its conduction in an *in vitro* environment,
203 which may not accurately represent the reality of LIA regimens *in vivo*. For example,
204 cultured osteoblasts likely exhibit a different phenotype to osteoblasts within the *in vivo*
205 multicellular bone microenvironment [15]. However, to mitigate potential phenotypic changes
206 our study has utilised primary osteoblast cultures. Furthermore, the cells were cultured from
207 human OA subchondral bone tissue that was collected on the day of surgery.

208

209 **4.1 Conclusion**

210 In summary, this study shows that LIA reagents at concentrations advocated for clinical use,
211 particularly when used in combination, have detrimental effects on osteoblast viability and
212 function *in vitro*. Clearly, further studies, both *in vitro* and *in vivo* are warranted in order to
213 provide definitive recommendations. While the ability to progress a patient through a
214 recovery pathway at an accelerated rate is undoubtedly attractive, it must not come at the cost
215 of long-term wellbeing. For this reason, extensive study is required into the potential long-
216 term side effects of LIA.

217

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223 screening.

224

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266

267

268 **Figure Legends**

269 **Figure 1. Human osteoblast morphology following acute exposure to LIA reagents.**
270 Primary human osteoblasts were seeded in into 24-well plates at 50,000 cells per well and
271 exposed to either PBS or LIA reagents (n=3) for 30 min. Cells were viewed by confocal
272 microscopy 7 days after exposure to reagents.

273

274 **Figure 2. The effect of acute LIA exposure on human hip OA osteoblast proliferation.**
275 Primary osteoblasts cultured in 96-well plates were exposed to either PBS or LIA reagents for
276 30 min. Cellular proliferation was determined by MTS assay at (**A**) 24 h and (**B**) 7 days post
277 exposure to reagents. Bars represent mean ± SEM (n=4). *=p<0.05, **=p<0.0,
278 ***=p<0.001, significantly different from PBS control cells, as determined by 1-way
279 ANOVA using Dunnett's post-hoc tests.

280

281 **Figure 3. The effect of acute LIA exposure on human hip OA osteoblast OPG and**
282 **RANKL secretion.** Primary osteoblasts cultured in 96-well plates were exposed to either
283 PBS or LIA reagents for 30 min. (**A**) OPG and (**B**) RANKL production was determined by
284 ELISA at 24 h post-exposure and 7 days post-exposure. (C) Represents the RANKL/OPG
285 ratio. Bars represent mean ± SEM (n=3). *=p<0.05, **=p<0.0, ***=p<0.001, significantly
286 different from PBS control cells, as determined by 1-way ANOVA using Dunnett's post-hoc
287 tests.

288

289 **Figure 4. The effect of acute anaesthetic exposure on human osteoblast alkaline**
290 **phosphatase activity.** Primary osteoblasts cultured in 96-well plates were exposed to either
291 PBS or LIA reagents for 30 min. Alkaline phosphatase activity in cell lysates was
292 determined at (**A**) 24 h post-exposure and (**B**) 7 days post-exposure. Bars represent mean ±

293 SEM (n=4). **=p<0.0, ***=p<0.001, significantly different from PBS control cells, as

294 determined by 1-way ANOVA using Dunnett's post-hoc tests.

295