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

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Abstract

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

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

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

2. Methods

2.1 Cell culture

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

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

2.2 Osteoblast proliferation and morphology

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

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

2.3 Osteoprotegerin and RANKL ELISAs

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

2.4 Alkaline phosphatase assay

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

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 $p<0.05$.
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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).
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4. Discussion

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

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

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

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

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

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

4.1 Conclusion

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

5. Acknowledgments

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6. References

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Figure Legends

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

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

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

Figure 4. The effect of acute anaesthetic exposure on human osteoblast alkaline phosphatase activity. Primary osteoblasts cultured in 96-well plates were exposed to either PBS or LIA reagents for 30 min. Alkaline phosphatase activity in cell lysates was 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.
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