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A controlled release bupivacaine-alginate construct: Effect on chondrocyte hypertrophy conversion

Mollie S. Davis[a], **Ileana Marrero – Berrios**[a], **Xiomara I. Perez**[a], **Palangat Radhakrishnan**[b], **Devasena Manchikalapati**[b], **Khaja Ahmed**[b], **Hattiyangangadi Kamath**[b], **Rene S. Schloss**[a,*], **Joel Yarmush**[b]

[a]Rutgers University, Department of Biomedical Engineering, Piscataway, NJ, USA

[b]Department of Anesthesiology, NYP Brooklyn Methodist Hospital, Brooklyn, NY, USA

Abstract

Objective: Osteoarthritis is a degenerative disease of the joint, affecting over 30 million people in the US[1]. A key characteristic of OA is chondrocyte hypertrophy, characterized by chondrocyte changes to a more rounded and osteoblastic phenotype, characterized by increased IL-6 and IL-8 secretion[2]. While there are no cures for OA, treatments focus on mitigating pain and inflammation, the two main symptoms of OA. However, the analgesics, NSAIDS and corticosteroids commonly used, do not target regeneration and have negative side effects. Local anesthetics (LA) can be used as a pain management alternative but are usually short lasting and therefore, not suited for chronic conditions such as OA. Our engineered sustained release local anesthetic construct successfully delivers bupivacaine for an extended period of time[3–5]. This study is designed to evaluate the effect of the LA system on chondrocytes in an inflammatory OA-like environment.

Design: Chondrocytes were cultured with bolus, liposomal, or construct LA and either untreated or treated with TNF-α and IL-1α for 24 hrs, 48 hrs, or 96 hrs. Chondrocyte viability, interleukin-8 (IL-8), interleukin-6 (IL-6), collagenase activity and proteoglycan deposition were assessed.

Results: In the presence of the engineered construct, the chondrocytes retained viability and regenerative function. Moreover, the construct allowed for higher initial doses to be used, which promoted more regeneration and decreased inflammation without compromising cellular viability.

*Corresponding author. Department of Biomedical Engineering Rutgers, The State University of New Jersey 599 Taylor Road, Piscataway, NJ, 08805, USA. schloss@soe.rutgers.edu (R.S. Schloss).

Author Contributions

Mollie S. Davis, MSE ME, Research Fellow.
Ileana Marrero-Berrios, ME, Research Fellow.
Xiomara I. Perez, ME, Research Fellow.
Palangat Radhakrishnan, MD, Anesthesiologist, NYP Brooklyn Methodist Hospital.
Devasena Manchikalapati, MD, Anesthesiologist, NYP Brooklyn Methodist Hospital.
Khaja Ahmed, DO, Anesthesiologist, NYP Brooklyn Methodist Hospital.
Hattiyangangadi Kamath, MD, Anesthesiologist, NYP Brooklyn Methodist Hospital.
Rene S. Schloss, PhD, Assistant Research Associate, Rutgers University.
Joel Yarmush, MD, Anesthesiologist, NYP Brooklyn Methodist Hospital.

Declaration of competing interest

The authors report no competing interest in this work.

Conclusions: The construct promotes a less hypertrophic chondrocyte environment while promoting a more anti-inflammatory environment. These two factors are consistent with a less OA progressive environment when using the engineered construct, compared to bolus LA.

Keywords

Chondrocyte inflammation; Local anesthetics; Sustained release; Chondrocyte hypertrophy

1. Introduction

Osteoarthritis (OA) affects over 30 million people in the US [1], with an estimated economic burden of 80 billion dollars due to lost productivity [1]. OA, a disease of the entire joint, which affects cartilage, bone, and the surrounding synovia, is characterized by loss of articular cartilage and typically presents with pain, inflammation and stiffness or tenderness [1]. While risk factors have been identified, including age, obesity and gender, the cause of OA is currently unknown [1]. A key characteristic of OA is chondrocyte hypertrophy, a phenomenon where chondrocytes, in the late stage of chondrocyte differentiation, become specialized and involved in the chondrogenic-osteoblastic transition, which leads to osteophyte formation in OA [2]. Specifically, interleukins, such as interleukin 8 (IL-8) [3] and interleukin 6 (IL-6) [3], and matrix metalloproteinase and collagenases are upregulated while proteoglycan deposition decreases [4].

With no available OA cure, non-surgical treatment focuses largely on pain control via non-steroidal anti-inflammatory drug (NSAIDs), corticosteroids, opioids and hyaluronic injections [5–8]. However, the negative long term side effects of these treatments make them harmful for continuous use, especially for elderly patients [5,8]. A potential alternative is to use local anesthetics (LA) such as lidocaine, bupivacaine, and ropivacaine, which provide local numbness and/or analgesia, and have a history of safety and efficacy. These drugs act by reducing sensation within the localized area of administration by reversibly blocking the sodium gated nerve channels [9,10]. However, despite their benefits, LA have also been shown to have detrimental cellular effects. For example, common amide LA, such as bupivacaine, have cytotoxic effects in potency,- concentration,- and time-dependent manners on neurons [11] and non-target cells such as chondrocytes [12–16], fibroblasts [17] and macrophages [18], which indicate that LA could significantly affect healing and regeneration processes. In fact, several investigators have concluded that LA should not be used to treat chronic conditions such as OA [16]. In addition, while LA provide rapid onset of action, the effective duration is relatively short [19–22], a characteristic which has made LA suboptimal for treating chronic conditions, such as OA [16].

One approach to improve LA release profiles is via sustained release systems, which are designed to release drugs at pre-determined times and doses to control and prolong their availability for specific clinical applications. Previous formulations developed to provide controlled LA release include hydrophobic-based polymers [23], poly (lactic-*co*-glycolic acid) microspheres [24], injectable paste and solid polymers [25] and liposomes [26], among others [27]. Of these, only Exparel® (liposomal bupivacaine) has been approved for clinical use by the Food and Drug Administration (FDA) for provision of post-operative pain relief

for a limited number of surgical procedures [26]. However, while Exparel® consistently provides 24-hr pain relief it has failed to provide longer-term relief needed for chronic pain conditions.

Therefore, while liposomal formulations can slow the release of drugs such as bupivacaine [28,29], additional modifications are needed to further extend its release for chronic conditions such as OA, and to do so without compromising cell viability or promoting hypertrophy. Previously, we engineered an encapsulated bupivacaine-loaded liposome-alginate hydrogel construct, which reduced the release rate of 1 mM bupivacaine 4x relative to liposomes alone and therefore, provided a potential alternative chronic pain management formulation [30–32]. The goal of the present study was to investigate the effect of our bupivacaine construct on chondrocytes, including viability, function, and hypertrophic conversion, which, while alleviating local pain, could potentially also promote OA disease progression. These studies determined that our LA construct could be administered at clinically relevant levels without compromising chondrocyte viability or function.

2. Methods

2.1. Chemicals and reagents

Bupivacaine and other chemicals were purchased from Sigma Aldrich (Oakville, Ontario, Canada), unless otherwise stated. All cell culture reagents and growth factors were purchased from Thermo Fisher Scientific (Waltham, MA), unless otherwise stated.

2.2. Liposomal formulation containing bupivacaine

The liposomes were formed using a protocol described in Maguire et al. [30]. Briefly, hydrogenated soy phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) and cholesterol, in a 7:3 ratio, were dissolved in chloroform and dried on a rotovap under vacuum. The lipid film was then re-suspended in ultrapure water and incubated under constant rotation for 2 hr at 55 °C. The solution was then snap-frozen and lyophilized overnight. The lyophilized lipid was then re-suspended in 70 mg/mL bupivacaine-HCl. The lipid-bupivacaine mixture was then extruded through polycarbonate membranes (200 nm pore size) 21 times to create small unilamellar vesicles. The liposomes were then eluted through a Sephadex G-50 size exclusion column equilibrated with 0.9% saline. A UV/Vis spectrophotometer (DU730 Life Science UV/Vis Spectrophotometer, Beckman Coulter, Brea, CA) at a wavelength of 264 nm was used to determine bupivacaine concentration.

2.3. Alginate encapsulation of liposomes

Alginate encapsulation of liposomes was formed as previously described in Maguire et al. [30]. Briefly, 2.2 wt% ultrapure alginic acid (MW: 100,000–200,000 g/mol, G Content: 65%–70%, FMC Biopolymer AS, Sandvika Norway) was prepared using ultrapure water and stirring the solution at 65 °C until fully dissolved and then filtered using a 0.22 μm pore syringe filter (FisherBrand). Bupivacaine loaded liposomes were mixed, in various volumes based on the target drug concentration of 1 mM, 7 mM, or 9 mM, with 2.2 wt% alginate and deposited in Transwell inserts (Corning). The inserts were then submerged in a bath containing 100 mM $CaCl_2$, 145 mM NaCl, and 10 mM MOPS for 10 min. The hydrogels

were then washed with phosphate buffer solution (PBS) for 10 min before being introduced to the cells. Alginate constructs without liposomes and with empty liposomes were created and tested as controls, however no differences in cell viability or function were detected (unpublished work). Characterization of the engineered construct can be found in previously published work [30–32].

2.4. Chondrocyte cell culture

Multiple types of chondrocytes, both human and nonhuman, cell lines and primary culture, were analyzed for inflammatory cytokine production. Ultimately, C28/I2 cell line (obtained from Dr. Mary Goldring, Hospital for Special Surgery, NY, NY) was chosen for these initial studies because this cell line was shown to duplicate chondrocyte function and inflammatory responses [33] and a cell line reduces variability often found among primary cell sources. They were cultured as a monolayer at 50,000 cells/well in a 24 well plate in a humidified 37 °C, 5% CO_2 incubator. Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (same lot to reduce variability) (Atlanta Biologicals, Flowery Branch, GA) and 2 mM L-glutamine, was added to the cells. The cells attached overnight. Fresh media, with or without stimulating cytokines (10 ng/mL of Tumor Necrosis Factor Alpha (TNF-α) and Interleukin 1 alpha (IL-1α) (R&D Systems, Minneapolis, MN, USA)), replaced the previous media. Transwell inserts (Corning) containing the different treatment conditions (media, bolus bupivacaine, bupivacaine-loaded liposomes, alginate-liposome construct, empty liposomes, empty alginate-liposome construct, or alginate hydrogel) were added to the wells containing the chondrocytes and incubated for 24, 48, or 96 hr in the same conditions as described above (37 °C, 5% CO_2 incubator). The drug free control conditions contained secretions and viability comparative to media controls and were not published.

2.5. Assessment of cell viability/proliferation

At the end of each time point, the supernatants were removed and saved for cytokine analysis (see Cytokine Measurement below). The cell culture supernatants were replaced with medium containing CellTiter-Blue Cell Viability Assay reagent (Promega) according to the manufacturer's instructions. Viability can be assessed as the ability of viable cells to convert a dye to a fluorescent end product [34]. The cells were then returned to the incubator for continued culture. The fluorescence of the wells was read at 4 hr using a microplate reader (DTX880 Multimode Detector, Beckman Coulter), returning the cells to the incubator between readings.

2.6. Cytokine Measurement

Cell culture supernatants were thawed and tested for IL-6, IL-8, and collagenase, using enzyme linked immunosorbent assays (ELISA, Bio-legend, San Diego, CA and Cayman Chemical, Ann Arbor, MI, USA). Assays were run according to manufacturer's instructions and absorbance was read using a microplate reader (DTX880 Multimode Detector, Beckman Coulter, Brea, CA).

2.7. Assessment of proteoglycan formation

Following viability assay, the cells were fixed with 4% paraformaldehyde (PFA) for 20 min and then diluted down to 1% PFA using PBS for long term storage. Proteoglycan formation was analyzed using an alcian blue staining kit (ScienCell, Carlsbad, CA). The wells were washed with 0.1 N HCl and then covered with alcian blue overnight at room temperature. The wells were then washed with ultrapure water 3 times prior to dye extraction using 6 M guanidine HCl overnight at room temperature. The extracted dye was measured using a microplate reader (DTX880 Multimode Detector, Beckman Coulter) at 630 nm. A standard curve of alcian blue values from 635 to 9.76 μg/mL was used for quantification.

2.8. Statistics

Statistical differences among data sets were determined using two-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test. Significance level was $\alpha = 0.05$ in Prism 8 (GraphPad Software, San Diego, CA, USA). Data points represent the mean ± the standard error of the mean (SEM) for the indicated number of independent observations (n).

3. Results

3.1. Chondrocyte viability and function in the presence of bolus LA

In order to establish a comparative baseline and to evaluate the effect of LA exposure on chondrocyte viability, monolayer C28/I2 cells were first dosed with bolus bupivacaine in varying concentrations for 24–96 h rs. In addition, to more closely mimic the likely inflammatory OA environment, cells were cultured in the presence or absence of activating cytokines. As seen in Fig. 1, for both non-stimulated and stimulated cells, there was a dose dependent decrease in viability during the 96hr exposure period, especially when LA concentrations exceeded 150 μM. Both stimulated and non-stimulated chondrocytes could withstand a dose of 150 μM for 48 hrs with >90% viability. Therefore, for future chondrocyte studies, a 150 μM bupivacaine concentration was identified as the cutoff value.

Next, interleukin 6 (IL-6) and interleukin 8 (IL-8) secretory function was evaluated. IL-6 is a multi-functional chemokine secreted by chondrocytes, which for example, increases the production of cartilage degrading matrix metalloproteases (MMPs) [35]. IL-8 is a chemokine secreted by inflammatory cells as well as activated chondrocytes, its expression is associated with later stages of OA [33] and it is responsible for promoting immune cell infiltration. Therefore, reducing IL-6 and IL-8 may be consistent with a less degenerative chondrocyte environment. Interestingly, increasing the concentration of bolus bupivacaine led to a dose dependent reduction of IL-6 and IL-8, in both control and in a stimulated inflammatory environment (Fig. 2). While the overall secretion amount was higher in stimulated chondrocytes, the overall effect of LA on IL-6 reduction was more pronounced in unstimulated cells.

3.2. Effect of LA modalities on chondrocyte function

Having established the LA viability and secretory thresholds, we applied equal doses of LA using differing LA delivery modalities. The engineered construct was designed with a

concentration of 1 mM due to its sustained release of less than 10% at any given time point [31]. This enables an effective dose of 100 μM, which is below our cutoff of 150 μM. Table 1 indicates the highest effective LA concentration with each delivery method, as determined in Davis et al. [31]. As seen in Fig. 3, relative to bolus presentation, IL-6 and IL-8 secretion levels were similar in the presence of the construct in unstimulated conditions. However, after 96 hrs chondrocytes stimulated with inflammatory cytokines secreted significantly less IL-6 and IL-8 in the presence of the construct, compared to bolus or control media conditions.

ECM degradation by collagenase is typically increased during OA progression. In fact, *in vivo* injections of collagenase has been shown to induce OA [4]. Therefore, we evaluated the effect of LA on chondrocyte collagenase secretion. As indicated in Fig. 4A and B, when LA is added to chondrocytes, collagenase activity was unchanged, regardless of LA modality, in both unstimulated and stimulated environments. In all cases, collagenase activity was similar to media controls.

To further investigate the potential affects to the ECM that may be induced by LA, proteoglycan formation was analyzed via alcian blue quantification. As seen in Fig. 4C, the presence of LA, regardless of modality, decreased proteoglycan formation at 96 hrs in stimulated inflammatory conditions, though not significantly. Compared to bolus LA exposure, the construct did not reduce proteoglycan formation significantly.

3.3. Effect of higher LA construct doses on chondrocytes

The alginate construct allows for LA to be administered via sustained release. Our previous work demonstrated that our construct enables a steady release of LA four times longer than a bolus release [30], while maintaining a lower drug concentration at any given time point [31]. Therefore, the construct should allow for increased LA concentrations without compromising cellular viability, even though the same bolus doses were cytotoxic. To test their effects on chondrocyte viability and function, we developed higher dose constructs. 7 mM and 9 mM concentrations were chosen because they represent more clinically relevant LA doses [12,18] and our previous studies demonstrated that viability of mesenchymal stromal cells (MSC) [31,32,36] was not compromised following exposure to these bupivacaine doses.

Following incubation with higher dose LA constructs, chondrocyte viability was analyzed (Fig. 5). Overall, viability was maintained, with slight decreases compared to media controls, which while minimal, were significant. At 96 hrs, viability of both 7 mM and 9 mM in stimulated conditions decreased compared to media controls by 10% and 18% respectively. However, it is important to note that these concentrations administered, as a bolus dose, resulted in almost complete cell death, as indicated (Fig. 1) by viability decreases at higher LA bolus doses.

Next, IL-8 and IL-6 levels were analyzed to determine the effect of increased bupivacaine concentrations on the inflammatory properties of chondrocytes. As seen in Fig. 6A and B, over time non-stimulated chondrocytes demonstrate a trend toward increased IL-8 secretion relative to control cells. In contrast, in stimulated inflammatory conditions, there is a

decrease in IL-8 secretion at 96 hrs in the presence of the construct, regardless of LA concentration. IL-6 secretion from chondrocytes in the presence of the construct follows a similar trend. At 96 hrs, in stimulated inflammatory conditions, the 9 mM construct reduces IL-6 secretion. However, the 7 mM construct, while lower than media control, is not significantly different (Fig. 6C and D).

While collagenase activity did not increase in the presence of LA regardless of modality (Fig. 4A and B), we also wanted to determine if higher bupivacaine doses would have an effect on matrix degradation. As seen in Fig. 7A and B, collagenase activity is similar regardless of LA concentration, in both non-stimulated and stimulated conditions. While collagenase mediated matrix degradation remained constant even in the presence of higher LA concentrations, the effect of increased bupivacaine on matrix remodeling was still unknown. Therefore, proteoglycan formation was analyzed via alcian blue quantification. While our initial studies indicated that despite mode of application, proteoglycan formation was reduced relative to media controls (Fig. 4C), as seen in Fig. 7C, increasing LA construct concentrations in stimulated inflammatory conditions slightly increased proteoglycan formation significantly compared to lower LA doses.

To further analyze the effect of higher LA concentration constructs on chondrocyte hypertrophy, Collagen X and Alkaline Phosphatase (ALP) activity were measured from chondrocytes. Collagen X and ALP have been associated with hypertrophic chondrocytes [37–39] As seen in Fig. 8, in stimulated conditions, higher LA constructs do not increase collagen X and trend toward lower ALP activity. Collectively our results indicate that higher bupivacaine doses can be delivered via our liposome-alginate construct without compromising viability or function and without promoting hypertrophic conversion.

4. Discussion

Chondrocytes are significantly affected by OA and changes in their quiescent activity induces physical and chemical changes in the afflicted joint. The hypertrophic environment can induce other changes, including cartilage degeneration and osteophyte formation, which can induce pain in the area. Aside from arthroplasty, which is a surgical treatment for OA, and the current gold standard [40], mitigating this pain without significant side effects is currently the only non-surgical treatment option for OA patients. Given the growing opioid epidemic, finding alternative pain mitigating options that may be administered for chronic conditions, without harmful tissue side-effects, is clearly needed. While LA are not addictive, they have been shown to negatively impact chondrocyte cellular viability and function in a dose dependent fashion [12,13,16].

Drug delivery systems for LA may reduce these chondrotoxic side effects and may improve upon current pain management for chronic conditions such as OA. Similar to Exparel®, Posimir® (bupivacaine contained in a substituted sugar matrix) has been studied extensively and delivers bupivacaine over 72 hrs. However, Posimir®, which is not FDA approved, may only be used for minor surgical procedures because the alcohol based vehicle used for storage and delivery causes local tissue toxicity, limiting injection volumes to approximately 5 mL [30]. HTX-011 (Heron Therapeutics), a combination of bupivacaine

and meloxicam, an NSAID, is being investigated to reduce post-operative pain for 72 hrs [41] and is currently addressing FDA comments [42]. Bupivacaine loaded biomaterials have been developed for use in post-surgical applications and have been shown to release clinically relevant levels of LA for several days [43]. However, while these formulations have improved delivery profiles, all have been designed for post-surgical applications, with side effects and components that make them unsuitable for chronic conditions.

Our engineered construct helps combat some of these issues by extending the release of bupivacaine without toxic delivery vehicles or additional pharmaceutical components [30–32]. In theory, for chronic conditions, such as OA, longer time frames should be analyzed. However, the limitations of this *in vitro* system dictate a shorter time frame as longer culture times result in cell confluency and ultimately cell death. Regardless, our construct has been shown to improve cellular function and viability in mesenchymal stromal cells [31,32]. Using the same principle, the effect of LA loaded constructs on chondrocyte viability and function was assessed and we demonstrated improved viability and functionality compared to bolus bupivacaine doses. Our results indicate that the chondrocyte environment is less hypertrophic in the presence of the LA construct over time. IL-8, which has been associated with increased chondrocyte hypertrophy and differentiation, and IL-6, which is associated with increased MMP-13 production which promotes cartilage degradation, decrease in the presence of the construct. Higher dose constructs exhibit similar secretory profiles with increased proteoglycan formation and similar collagen X secretion and decreased ALP activity relative to the 1 mM construct. Future studies include primary cell analysis with FBS-free medium which may allow longer term survival in culture, especially if ECM proteins are included [44].

Aside from being associated with hypertrophy, IL-8 and IL-6 are associated with beneficial cellular function such as differentiation [45] and cell-cell signaling [3], as well as not always beneficial chondrocyte inflammation [3]. The reduction of both, may indicate a less inflammatory environment for the chondrocytes, which would be beneficial for OA. At a minimum, this drug delivery construct does not increase OA inflammation. LAs are known to increase the calcium concentration in the microenvironment, which should increase inflammation, however the opposite is seen. This is not a new phenomenon. Previously, researchers have shown that LA can inhibit the release of inflammatory factors, such as IL-1β, IL-8, IL-6, and TNFα, which are involved in OA inflammation and stimulate interleukin 1 receptor antagonist (IL1-RA) secretion, which has been shown to reduce inflammation [46–49]. The mechanism of this inflammatory depression is unknown. However researchers hypothesize that a transcription regulation may be responsible [46,48,49]. Regardless, the LA construct has been shown to have two applications, helping reduce pain (unpublished work) and not increasing inflammation, especially at higher LA doses.

In summary, the controlled release LA system may help improve the hypertrophic chondrocyte environment associated with OA. In addition, the LA dose can be increased without modifying chondrocyte viability or function, allowing for clinically relevant doses that may further depress OA inflammation. In addition, in the future our positionally

controlled construct may be co-delivered with other encapsulated drugs to further reduce OA mediated cartilage degradation and promote regeneration.

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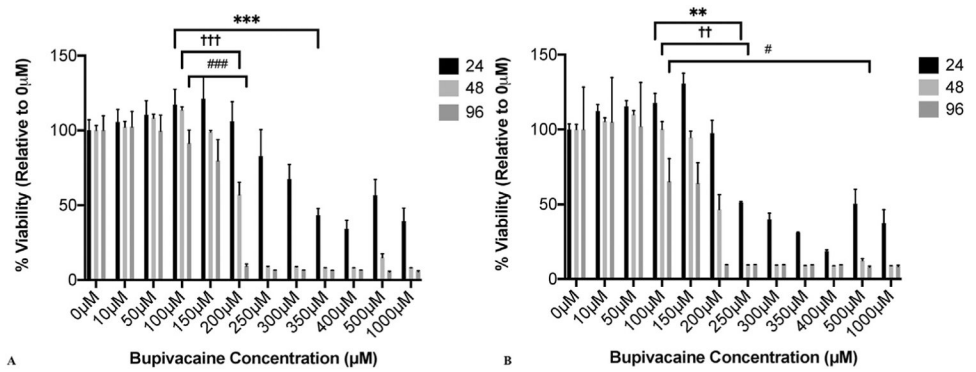

Fig. 1. C28/I2 viability dose response to bolus bupivacaine.

The bar heights represent the fluorescence intensities (FI) of reduced CellTiter-Blue reagent normalized by relative media control (0 mM). A) Non-stimulated media and B) Media stimulated with 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 4–18 independent observations. * or # or † $p \leq 0.05$, ** or ## or †† $p \leq 0.01$ and *** or ### or ††† $p \leq 0.001$.

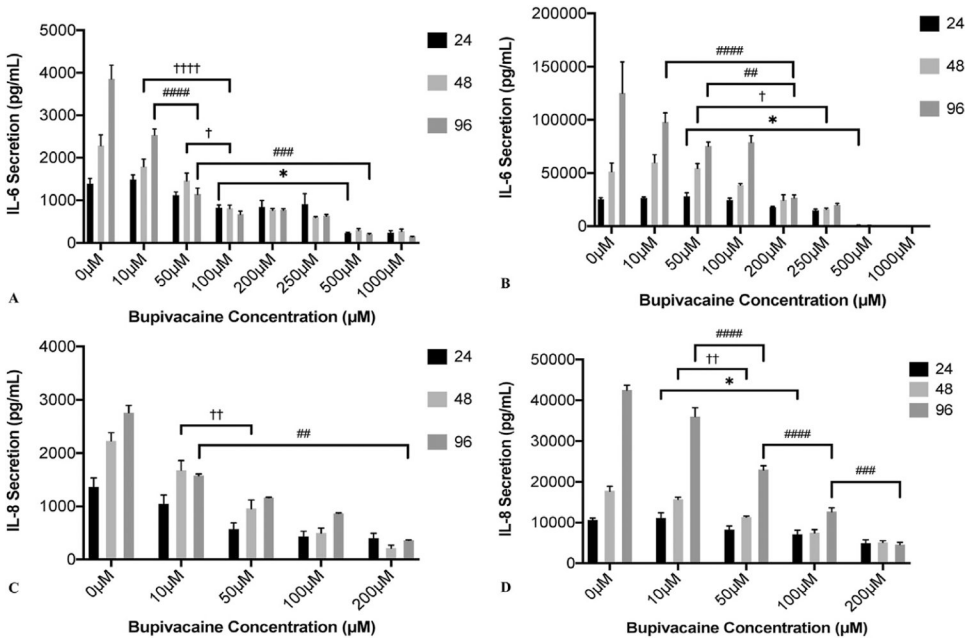

Fig. 2. C28/I2 IL-6 and IL-8 secretion dose response to bolus bupivacaine.

The bar heights represent the IL-6 or IL-8 concentrations (pg/ml) quantified from cell supernatants of chondrocytes dosed with bolus bupivacaine. A and C) Non-stimulated media and B and D) Media stimulated with 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 3–9 independent observations. * or # or †$p \leq 0.05$, ** or ## or ††$p < 0.01$ and *** or ### or ††† $p \leq 0.001$.

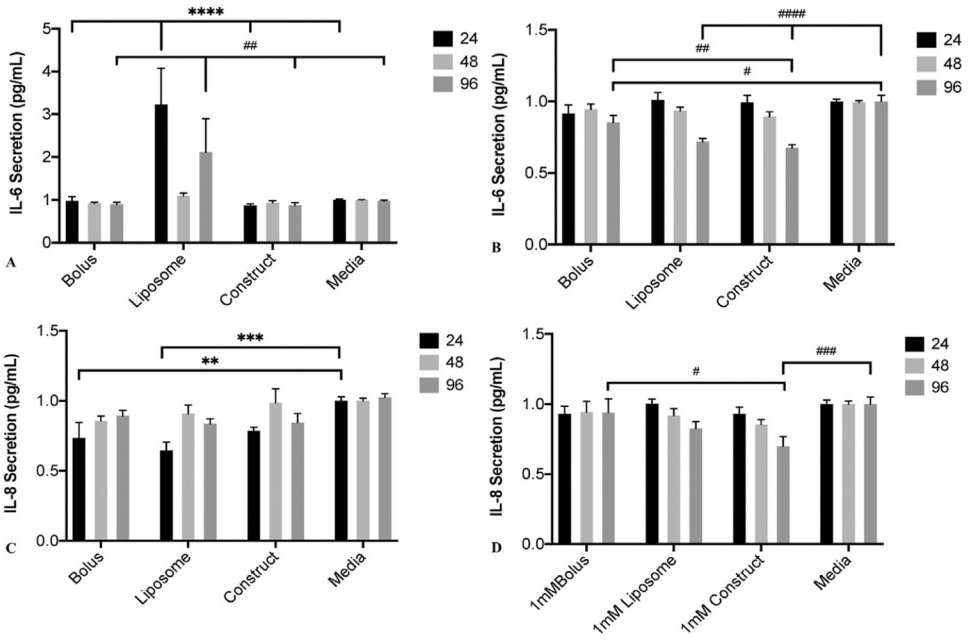

Fig. 3. Construct does not increase IL-8 secretion from stimulated C28/I2.

The bar heights represent the IL-8 concentration (pg/ml) quantified from cell supernatants treated with 1 mM bolus, liposomal, or alginate-construct bupivacaine or 0 mM media control. A) and C) Non-stimulated media and B) and D) Media stimulated with 10 ng/mL of TNF-α and IL-1α. Media Controls have secretion levels of 0 mM secretion levels of Fig. 2. The data is the mean ± SEM of n = 3–18 independent observations. * or # or † $p \leq 0.05$, ** or ## or †† $p \leq 0.01$ and *** or ### or ††† $p \leq 0.001$.

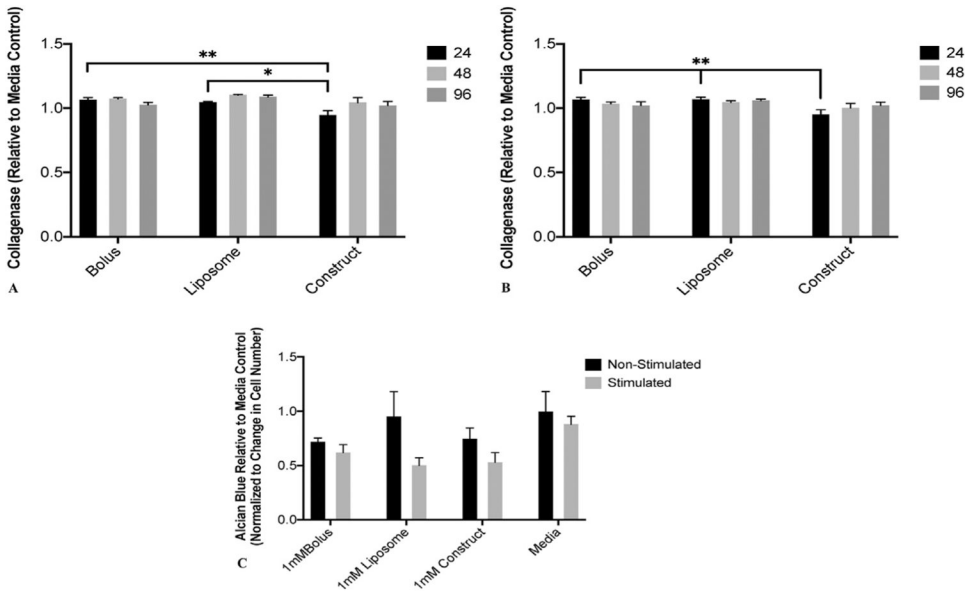

Fig. 4. C28/I2 degradation and regeneration does not change regardless of LA modality.

The bar heights represent A) and B) the collagenase activity quantified from cell supernatants or C) the alcian blue quantified from ECM deposits treated with 1 mM bolus, liposomal, or alginate-construct bupivacaine or 0 mM media control and normalized to 0 mM control. A) Non-stimulated media and B) Media stimulated with 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 4–18 independent observations. * or # or †p ≤ 0.05, ** or ## or ††p ≤ 0.01 and *** or ### or ††† p ≤ 0.001.

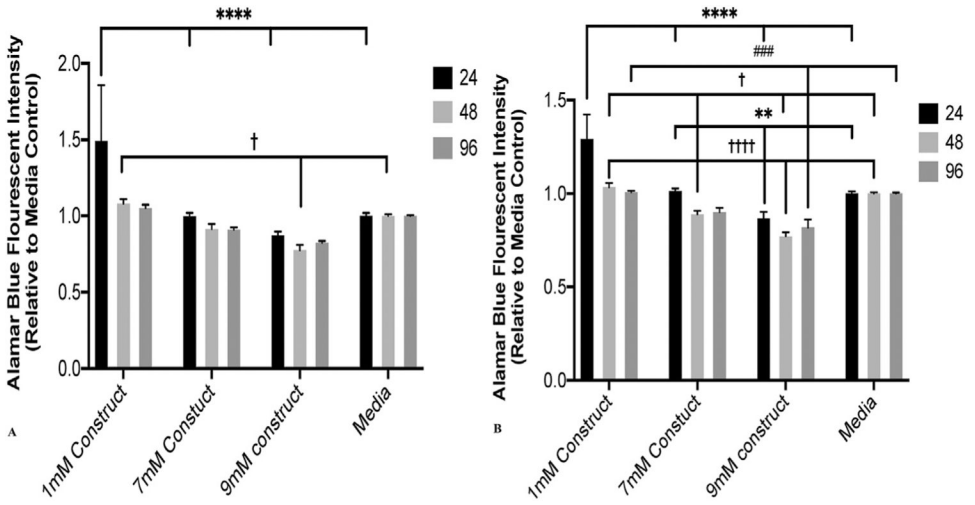

Fig. 5. Viability in C28/I2 remained consistent at higher LA construct doses.

The bar heights represent the alamar blue activity quantified from cell supernatants treated with 1 mM, 7 mM, or 9 mM alginate-construct bupivacaine or 0 mM media control and normalized to 0 mM media control. A) Non-stimulated media and B) Media stimulated with 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 9–24 independent observations. * or # or †p ≤ 0.05, ** or ## or †† p ≤ 0.01 and *** or ### or ††† p ≤ 0.001.

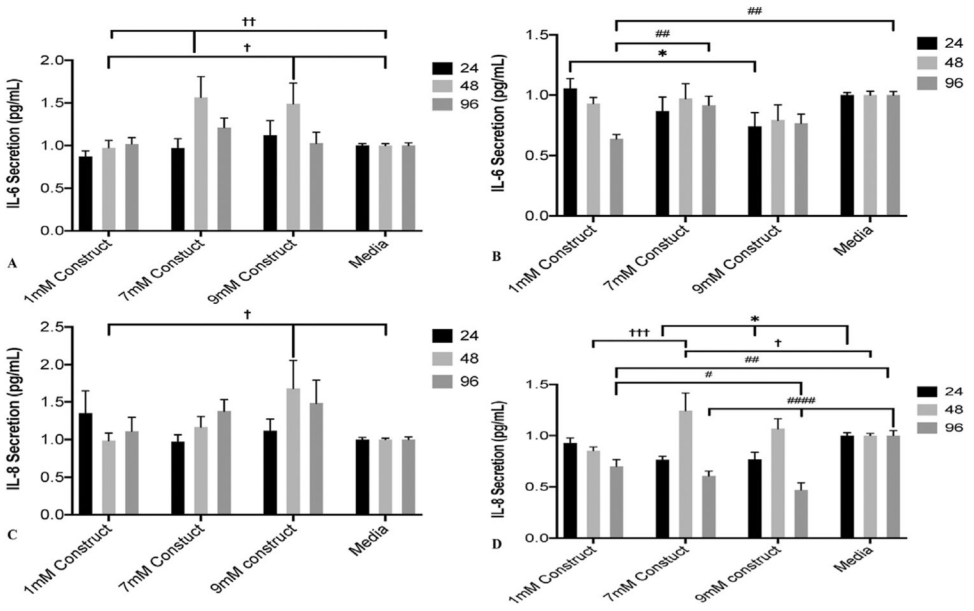

Fig. 6. Il-6 and IL-8 in C28/I2 decreases using a LA construct.

The bar heights represent the IL-6 or IL-8 secretion concentration quantified from cell supernatants treated with 1 mM, 7 mM, or 9 mM alginate-construct bupivacaine or 0 mM media control and normalized to 0 mM media control. A) Non-stimulated media and B) Media stimulated with 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 8–24 independent observations.. * or # or † $p \leq 0.05$, ** or ## or †† $p \leq 0.01$ and *** or ### or †††$p \leq 0.001$.

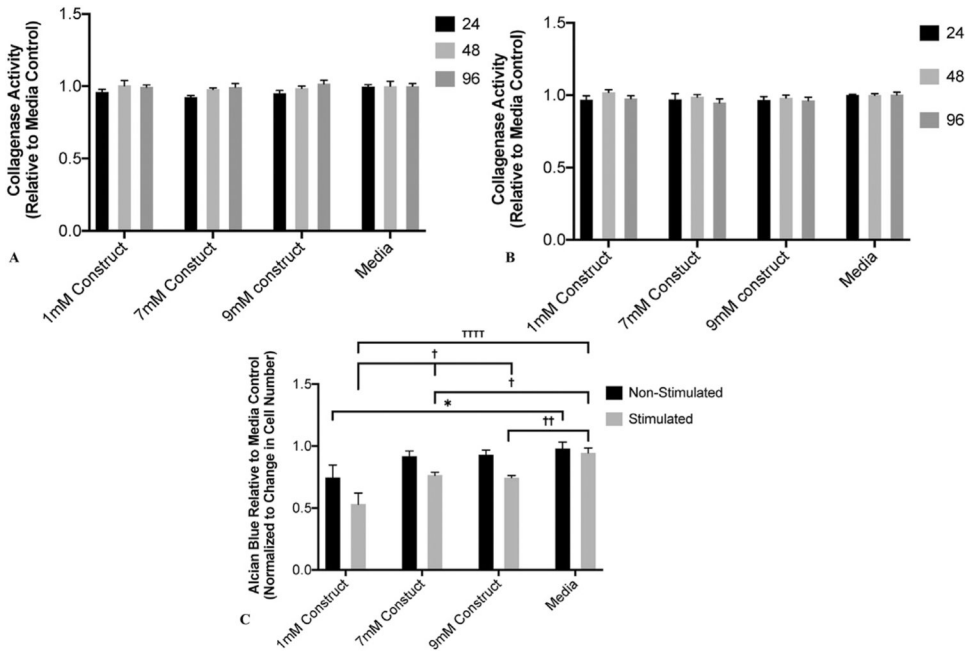

Fig. 7. C28/I2 degradation and regeneration in the presence of higher LA construct doses.
A) and B) Collagenase activity in chondrocytes does not change regardless of LA construct concentration.

The bar heights represent the collagenase activity quantified from cell supernatants treated with 1 mM, 7 mM, or 9 mM alginate-construct bupivacaine or 0 mM media control and normalized to 0 mM control. A) Non-stimulated media and B) Media stimulated with 10 ng/mL of TNF-α and IL-1α. **C) Chondrocyte proteoglycan formation increases in the presence of higher LA construct concentrations at 96 h**. The bar heights represent the concentration of alcian blue quantified from ECM deposits treated with 1 mM, 7 mM, or 9 mM alginate-construct bupivacaine or 0 mM media control and normalized to 0 mM control. Stimulated media contains10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 6–15 independent observations.. * or # or † $p \leq 0.05$, ** or ## or †† $p \leq 0.01$ and *** or ### or ††† $p \leq 0.001$.

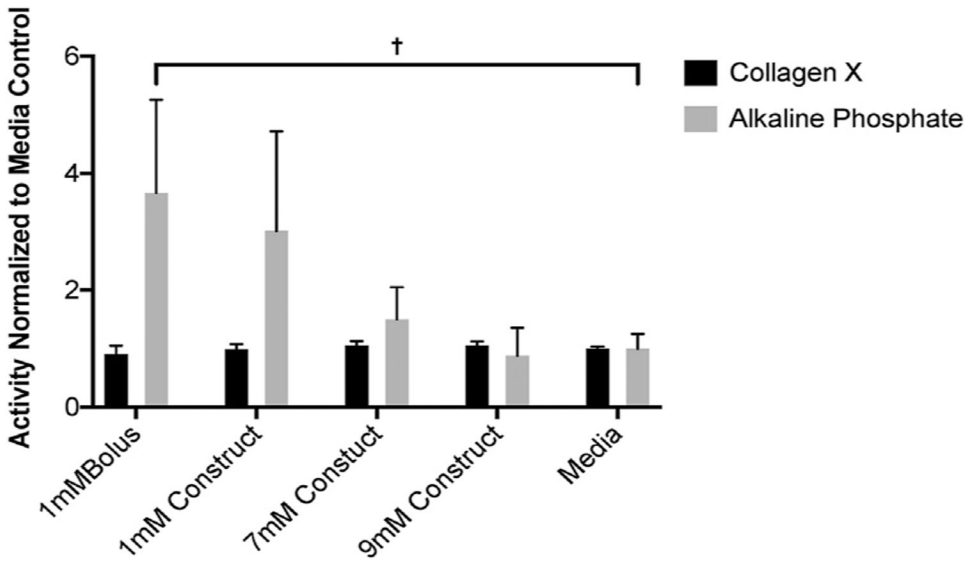

Fig. 8. C28/I2 hypertrophic markers are not increased in the presence of higher LA constructs.

Collagen X remains consistent regardless of LA modality or concentration. Alkaline Phosphatase activity decreases, though not significantly, at higher LA construct concentrations. Stimulated media contains 10 ng/mL of TNF-α and IL-1α. The data is the mean ± SEM of n = 3–10 independent observations. * or # or † $p \leq 0.05$, ** or ## or †† $p \leq 0.01$ and *** or ### or p ††† $p \leq 0.001$.

Table 1

Effective LA doses with various LA delivery modalities.

Summarized from Davis et al. [32].

Condition	Starting Concentration	Highest Effective Dose
Bolus	1 mM	1 mM
Liposome	1 mM	327 μM
Engineered Construct	1 mM	100 μM