Contents lists available at ScienceDirect

## Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actbio

# Ion modulatory treatments toward functional self-assembled neocartilage

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#### ARTICLE INFO

Article history: Received 24 April 2022 Revised 6 September 2022 Accepted 8 September 2022 Available online 14 September 2022

Keywords: Cartilage tissue engineering Self-assembly Biomechanics Osteoarthritis

#### ABSTRACT

Signals that recapitulate in vitro the conditions found in vivo, such as hypoxia or mechanical forces, contribute to the generation of tissue-engineered hyaline-like tissues. The cell regulatory processes behind hypoxic and mechanical stimuli rely on ion concentration; iron is required to degrade the hypoxia inducible factor 1a (HIF1 $\alpha$ ) under normoxia, whereas the initiation of mechanotransduction requires the cytoplasmic increase of calcium concentration. In this work, we propose that ion modulation can be used to improve the biomechanical properties of self-assembled neocartilage constructs derived from rejuvenated expanded minipig rib chondrocytes. The objectives of this work were 1) to determine the effects of iron sequestration on self-assembled neocartilage constructs using two doses of the iron chelator deferoxamine (DFO), and 2) to evaluate the performance of the combined treatment of DFO and ionomycin, a calcium ionophore that triggers cytoplasmic calcium accumulation. This study employed a two-phase approach. In Phase I, constructs treated with a high dose of DFO (100  $\mu$ M) exhibited an 87% increase in pyridinoline crosslinks, a 57% increase in the Young's modulus, and a 112% increase in the ultimate tensile strength (UTS) of the neotissue. In Phase II, the combined use of both ion modulators resulted in 150% and 176% significant increases in the Young's modulus and UTS of neocartilage constructs, respectively; for the first time, neocartilage constructs achieved a Young's modulus of 11.76±3.29 MPa and UTS of 4.20±1.24 MPa. The results of this work provide evidence that ion modulation can be employed to improve the biomechanical properties in engineered neotissues.

#### Statement of significance

The translation of tissue-engineered products requires the development of strategies capable of producing biomimetic neotissues in a replicable, controllable, and cost-effective manner. Among other functions,  $Fe^{2+}$  and  $Ca^{2+}$  are involved in the control of the hypoxic response and mechanotransduction, respectively. Both stimuli, hypoxia and mechanical forces, are known to favor chondrogenesis. This study utilized ion modulators to improve the mechanical properties self-assembled neocartilage constructs derived from expanded and rejuvenated costal chondrocytes via  $Fe^{2+}$  sequestration and  $Ca^{2+}$  influx, alone or in combination. The results indicate that ion modulation induced tissue maturation and a significant improvement of the mechanical properties, and holds potential as a tool to mitigate the need for bioreactors and engineer hyaline-like tissues.

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#### 1. Introduction

Articular cartilage is a highly acellular and avascular tissue with little to no turnover that lacks the capacity to heal in response to degeneration or injury [1]. Current surgical therapies

such as microfracture, osteochondral grafts, and autologous chondrocyte implantation fail to provide long-term restoration, and tissue engineering strategies provide a promising strategy to produce biomimetic neotissues [2–5]. The self-assembling process, for example, allows for the development of hyaline-like neotissues by inducing chondrocytes into generating a cartilaginous extracellular matrix (ECM) without the need of a scaffold. Because clinical treatment of chondral diseases would require neotissues capable of resisting the harsh joint environment, methods to modulate neocar-



Full length article





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tilage stiffness have included investigations into the exogenous application of growth factors, proteins, hypoxia and mechanical stimuli, as well as the modulation of ions involved in translating such stimuli to ECM production and neocartilage maturation [6–10]. The development of clinically appropriate neocartilage constructs relies on efficient strategies to generate tissues with proper biomechanical properties.

As an avascular tissue, cartilage is recognized to exist under hypoxia, which is a well-characterized chondrogenic stimulus [11]. Under hypoxia, the presence of reactive oxygen species and lack of oxygen inhibits prolyl hydroxylase (PHD)-mediated hydroxylation of hypoxia inducible factor  $1\alpha$  (HIF1 $\alpha$ ), which allows HIF1 $\alpha$ to accumulate and interact with HIF1 $\beta$ . In the nucleus, the active HIF1 $\alpha$ /HIF1 $\beta$  complex binds to hypoxia response elements (HREs) and triggers the transcriptional activation of genes that control ECM remodeling, angiogenesis, glycolysis, mitophagy, and cell survival, among others [12]. Given the hypoxic nature of cartilage, HIF1 $\alpha$  activity plays a pivotal role in maintaining chondrocyte homeostasis; in cartilage, HIF1 $\alpha$  inhibits MMP13-mediated breakdown of the ECM, plays a role in controlling catabolism by inhibiting Wnt signaling, and promotes protective mitophagy [13,14]. In vitro hypoxic conditions are imparted by bioreactors to achieve low oxygen tension, typically  $\sim$ 2-4% (vol/vol) O<sub>2</sub> and improve neocartilage tissue properties. For example, neocartilage constructs cultured at 4% O<sub>2</sub> showed significant increases in their Young's modulus, and pyridinoline (PYR) crosslink content, the collagen crosslink typically found in mature articular cartilage [8]. Interestingly, the catalytic center of PHD requires  $\alpha$ -ketoglutarate and ferrous iron to hydroxylate and downregulate HIF1 $\alpha$  activity.

Mechanical stimuli have also been shown to be beneficial for the development of cartilage neotissues. The simulation of mechanical signals found in the joint enables the activation of signaling cascades with pleiotropic effects in the neotissue [15]. Example stimuli that have been used to improve the biomechanical properties of engineered neocartilage include compression, tension, fluid shear, and hydrostatic pressure [16-19]. The activation of mechanotransduction via mechanical stimulus has been shown to favor a chondrogenic ECM in terms of composition (e.g., collagen types and content), collagen alignment (anisotropy), and macromolecular bonds (e.g., PYR crosslinks) [20,21]. In self-assembled neocartilage constructs, particularly, mechanical stimuli result in multiple improvements. Tension stimulation induced a 1.6 fold increase in tensile properties of bovine neocartilage constructs, compared to untreated controls [17]. Microarray analysis revealed that the TRPV4 ion channel was involved in the matrix remodeling initiated by the tensile stimulus. Likewise, fluid-induced shear resulted in a 2.7-fold increase in the Young's modulus, a 1.9-fold increase in the aggregate modulus, and a 1.4-fold increase in the collagen content, compared to controls in human neocartilage [9]. The putative mechanisms for mechanical stimuli rely on Ca<sup>2+</sup> functioning as a second messenger toward gene transcriptional regulation [9,22].

Inasmuch ions such as  $Fe^{2+}$  and  $Ca^{2+}$  play key roles within signaling cascades of both hypoxia and mechanotransduction, it is instructive to consider their modulation for tissue engineering purposes. For hypoxia, the transcriptional activation of chondrogenic genes is mediated by HIF1 $\alpha$  [23]. Under normoxia, PHD hydroxylates HIF1 $\alpha$  in a reaction that requires Fe<sup>2+</sup>, marking it for ubiquitination via Von Hippel Lindau (VHL) and proteosomal degradation. At physiological pH, ferrous iron (Fe<sup>2+</sup>) is oxidized to ferric iron (Fe<sup>3+</sup>). Chemical sequestration of Fe<sup>3+</sup> by the iron chelator deferoxamine (DFO) inhibits hydroxylation of HIF1 $\alpha$  and promotes a hypoxic response via accumulation of the transcription factor [24,25]. For mechanical stimuli, mechanosensing proteins such as the mechanoelectrical transduction (MET) channels, found in the stereocilia of hair cells or the depolarizing non-selective cationic channel Piezo1, trigger an influx of Ca<sup>2+</sup> following membrane deformations resulting from mechanical displacement and flow-induced shear stress, respectively [26,27]. Likewise, in chondrocytes, the Ca<sup>2+</sup>-permeable transient receptor potential vanilloid 4 (TRPV4) mediates the response to osmotic changes and mechanical stimuli through its interactions with integrins and other elements of the cytoskeleton, using Ca<sup>2+</sup> as a secondary messenger [28]. Modulation of intracellular Ca<sup>2+</sup> concentration using ionomycin, a Ca<sup>2+</sup> ionophore, has the potential of replicating the effects elicited by mechanical stimuli, such as an increase in PYR content and improvement of the compressive and tensile properties [29]. Thus, it is expected that the discrete modulation of ions responsible for hypoxia and mechanotransduction may be associated with an increase in the biomechanical properties of tissue-engineered neocartilage.

In this work, we aimed to improve the mechanical properties of neocartilage constructs, derived from expanded and rejuvenated minipig costal chondrocytes, using two distinct strategies based on ion modulation: 1) sequestration of  $Fe^{3+}$  using DFO to induce a hypoxic response, and 2) the combined treatment of  $Fe^{3+}$ and Ca<sup>2+</sup> modulation using DFO and ionomycin. This work employed a two-phase approach. In Phase I, two concentration of DFO (10  $\mu$ M and 100  $\mu$ M) were tested and compared to an untreated group (control) and a group treated with glucose oxidase-catalase (GOX/CAT), an established system for depleting solubilized  $O_2$  and inducing hypoxia [30]. It was hypothesized that the induction of a hypoxic response using DFO, prompted by the sequestration of Fe<sup>3+</sup>, would outperform the untreated controls and mirror the hypoxic response induced by GOX/CAT treatment, in terms of neocartilage biochemical and mechanical properties. In Phase II, the DFO concentration yielding neocartilage properties most similar to those of native tissue was used in combination with ionomycin, a Ca<sup>2+</sup> modulator that mimics mechanotransduction, and compared to either treatment alone and to the untreated controls. It was hypothesized that modulating both Fe<sup>3+</sup> and Ca<sup>2+</sup> would result in neocartilage constructs with improved mechanical properties and an overall superior tensile properties compared to prior literature.

#### 2. Methods

#### 2.1. Isolation and expansion of costal chondrocytes

Juvenile porcine costal chondrocytes (CC) were isolated from the unmineralized portion of floating ribs of three juvenile Yucatan minipigs obtained from Premier BioSource (California, USA) no later than 48 hours after slaughter. Cartilage from ribs cleaned of all non-cartilaginous tissue was cut into 1 mm<sup>3</sup> pieces and washed three times with GlutaMAX Dulbecco's Modified Eagle Medium containing 4.5 g/L glucose (DMEM; Gibco) and 1% (v/v) penicillin/streptomycin/fungizone (PSF; Lonza, Basel, Switzerland). The cartilage was then digested with 0.4% pronase (Sigma) in DMEM for 1 hour at 37°C, and then in 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ) in DMEM supplemented with 3% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) for 18 hours at 37°C. Cells were then strained through a 70 µm strainer, washed with red blood cell lysis buffer [31] for 4 min, counted, and frozen in freezing medium containing 90% (v/v) FBS + 10% (v/v) DMSO (Sigma). Primary (P0) CC were thawed and seeded in T225 flasks at a density of  $\sim$ 2.5 million cells per flask in chondrogenic medium (CHG) consisting of GlutaMAX DMEM, 1% (v/v) PSF, 1% (v/v) insulin-transferrin-selenium (BD Biosciences, San Jose, CA), 1% (v/v) non-essential amino acids (Thermo Fisher Scientific), 100 mg/mL sodium pyruvate (Thermo Fischer Scientific), 50 mg/mL ascorbate-2-phosphate (Sigma, St. Louis, MO), 40 mg/mL L-proline (Sigma), and 100 nM dexamethasone (Sigma). Throughout expansion, medium was supplemented with 2% (v/v) FBS, 1 ng/mL TGF- $\beta$ 1, 5 ng/mL bFGF, and 10 ng/mL PDGF (PeproTech). Cells were cultured at  $37^{\circ}$ C in 10% CO<sub>2</sub>, and passaged to P3 at confluence using 0.05% trypsin-EDTA (Invitrogen) and 0.2% collagenase type II solutions.

## 2.2. Chondrogenic differentiation in aggregate rejuvenation and neocartilage construct seeding

In this study we used the process of "aggregate rejuvenation" as defined by Kwon et al., 2021 [32] to denote the methodology of recovering the chondrogenic phenotype of expanded chondrocytes to an original state. Specifically, passaged CC were seeded on 1% (w/v) agarose-coated plates at a density of 750,000 cells/mL (30 mL total) per plate with CHG supplemented with 10 ng/ml TGF- $\beta$ 1, 100 ng/ml GDF-5, and 100 ng/ml BMP-2 (PeproTech) [32]. Plates were placed on an orbital shaker at 50 rpm for 24 hours at 37°C in 10% CO<sub>2</sub>, and then kept static, changing medium every 4 days. At 14 days of aggregate rejuvenation, the aggregates were digested using 0.05% trypsin-EDTA and 0.2% collagenase type II solutions. Cells were strained through a 70 µm cell strainer, washed twice, resuspended in CHG, and seeded. For the self-assembling process of neocartilage constructs, 2 million cells were seeded in 5 mm diameter non-adherent 2% (w/v) agarose wells at a density of 20 million cells/mL. At 4 hours post self-assembly, additional 400 µL of CHG or CHG supplemented with 10 ng/ml TGF- $\beta$ 1 were added. This medium was replaced daily until unconfinement (at day 3 post self-assembly), after which, constructs were kept in 2 mL of medium, replaced every 2 days. The CHG control group was maintained in CHG medium. For all other groups, TGF- $\beta$ 1, chondroitinase ABC (c-ABC), and LOXL2 treatment (termed TCL) was used as follows: TGF- $\beta$ 1 was applied at 10 ng/ml after seeding and until day 28, c-ABC (Sigma) was applied at 2 Units per mL for 4 hours in day 7, and LOXL2 (Signal Chem) was applied at 0.15 µg/mL between days 7 and 21 post self-assembly, together with 0.146 µg/mL hydroxylysine (Sigma) and 1.6 µg/mL copper sulfate (Sigma).

#### 2.3. Ion modulation

For all experiments, the constructs were randomly assigned across treatment groups. Modulation of ions in the constructs was performed between days 12 and 16 post self-assembly [33,34]. For every group in Phase I, medium was replaced with 400  $\mu$ L of CHG containing 10  $\mu$ M DFO (Sigma), or 100  $\mu$ M DFO, or blank CHG for the untreated control group, based on the two most commonly used concentration of DFO employed in the scientific literature in cell culture [35–37]. For the enzymatic system GOX/CAT, 3 Un/mL glucose oxidase (GOX; Sigma) and 30 Un/mL catalase (CAT; Sigma) were used. Incubation was performed for 1 hour, after which constructs were washed twice using DMEM 1% (v/v) PSF before resuming culture in CHG medium. The same incubation procedure was followed for Phase II, using 400  $\mu$ L of 100  $\mu$ M DFO, 0.3  $\mu$ M ionomycin (Io; Sigma), and 100  $\mu$ M DFO + 0.3  $\mu$ M Io.

#### 2.4. Mechanical testing

Tensile properties were determined using uniaxial tension in an Instron model 5565 (Instron, Canton, MA). Dog bone-shaped samples were obtained from every engineered cartilage construct with a gauge length of 1.55 mm. The dog bones were photographed to measure thickness and width in ImageJ. The ends of the dog-bone were fixed to paper with cyanoacrylate to increase the gripping area for testing. A strain rate of 1% of the gauge length per second was used until failure. The Young's modulus was obtained from the linear region of the stress-strain curve, and the ultimate tensile strength (UTS) was defined as the maximum stress obtained.

Compressive properties of the neocartilage constructs were determined by creep indentation testing. Briefly, 2.5 mm punches obtained from every construct were submerged in PBS until equilibrium and indented with a flat porous 0.5 mm diameter tip perpendicular to the surface of the sample to ~10% strain. The aggregate modulus ( $H_A$ ) and shear modulus ( $\mu_s$ ) were obtained using a semi-analytical, semi-numerical, biphasic model and finite-element optimization [38,39].

#### 2.5. Biochemical testing

Cartilage constructs (~2-3 mg) were weighed to obtain wet weight, lyophilized for 3 days, and weighed again to obtain dry weight. Lyophilized samples were digested in 125 µg/mL papain (Sigma) +5 mM N-acetyl-L-cysteine +5 mM EDTA in phosphate buffer pH 6.5. for 18 hours at 60°C. GAG content was quantified using a Blyscan glycosaminoglycan assay kit (Biocolor, Newtown-abbey, Northern Ireland). Total collagen content was quantified using a modified chloramine-T hydroxyproline assay [40]. DNA content was quantification of PYR crosslinks, separate tissue samples (~0.2-1 mg) were weighed, lyophilized, and acid-digested for 12 hours in 6N HCl. After evaporation, the dried hydrolysate was resuspended in a 75%/25% (v/v) solution of 0.1% formic acid and acetonitrile. Samples were measured through mass spectrometry using a cogent diamond hydride column and a PYR standard [41].

#### 2.6. Histology

Samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at a thickness of 6  $\mu$ m for histological evaluation. Sections were subsequently processed and stained with hematoxylin and eosin (H&E), safranin-O, and picrosirius red using standard protocols.

#### 2.7. Real-time qPCR

To determine the gene expression of chondrogenic, hypoxic, and mechanotransduction markers, real-time quantitative PCR (RTqPCR) was performed. Chondrocytes at passage 3 were seeded in monolayer in T75 flasks until reaching 90% confluence. Cells were then treated during 1 hour with a final concentration of 0.3 µM ionomycin, 100 µM DFO, 0.3 µM ionomycin and 100 µM DFO, or CHG (control group). After treatment, the cells were washed with CHG three times and left in chondrogenic medium for 2 hours, after which RNA was extracted using Direct-zol RNA MiniPrep (ZYMO Research). RNA concentration and purity were measured on a Nanodrop spectrophotometer (Nanodrop Technologies), RT-qPCR reactions were performed using 100 ng of RNA per reaction and a Luna® Universal One-Step RT-qPCR Kit (New England BioLabs Inc.) in a Thermofisher Quantstudio<sup>™</sup> 7 flex, and Ct values were normalized to 18S ribosomal RNA housekeeping gene. The target genes corresponded to collagen type 2 (COL2A1), SRY-Box Transcription Factor 9 (SOX9), aggrecan (ACAN), lysyl oxidase (LOX), Vascular Endothelial Growth Factor (VEGF), tissue inhibitor of metalloproteinase 2 (TIMP2), and Jun proto-Oncogene (C-JUN). Relative gene expression was quantified using the  $2^{-\Delta\Delta Ct}$  method.

#### 2.8. Statistical analysis

All quantitative biochemical and biomechanical tests were performed using n = 6-8. All data are presented as means  $\pm$  standard deviations. A single factor one-way ANOVA was employed in each phase of the study to assess differences among experimental groups. Multiple comparisons were performed using a Dunnett's *post hoc* test. The Dunnett's test compares the means from every experimental group against a control group mean to determine significant differences. All statistical analyses were performed



Fig. 1. Gross morphology and histological staining of self-assembled constructs treated with GOX/CAT or DFO. (A) All constructs appear homogeneous, relatively flat with a minor upward curvature, and with no visible abnormalities. (B) The histological analysis of neocartilage constructs showed that overall cellularity and neotissue composition appears comparable among all groups as observed in hematoxylin and eosin staining. Similarly, none of the treatments elicit any noticeable variation in the Picrosirius red staining or the Safranin-O staining.

using GraphPad Prism version 8.4.1 for Windows (GraphPad Software, San Diego, California USA). Groups marked with \* indicate that there are statistically significant differences compared to the control group (p< 0.05).

#### 3. Results

## 3.1. Effect of iron chelation on the mechanical properties of neocartilage constructs

Self-assembled neocartilage constructs, formed using expanded and rejuvenated costal chondrocytes, were treated with a low dose of deferoxamine (DFO 10  $\mu$ M), or a high dose of deferoxamine (DFO 100  $\mu$ M). A treatment with a combination of glucose oxidase and catalase (GOX/CAT), an established method for inducing a hypoxic condition by depleting O<sub>2</sub> in the medium, was also examined, and all treated groups were compared to untreated construct. As depicted in the gross morphology images of representative samples (Fig. 1A), none of the treatments affected the morphological features of the constructs. No statistical differences were observed in the diameter, thickness, wet weight, and water content among untreated and various treatment groups (Table 1).

Histological evaluation was performed to assess any variations in the ECM structure of the neocartilage constructs (H&E staining) or in the GAG and collagen distribution within the constructs (safranin-O and picrosirius red, respectively). With H&E, all groups showed a homogeneous cell distribution (Fig. 1B). Similarly, safranin-O stain was found to be evenly distributed throughout constructs in all groups, albeit absent in the outermost acellular layer of all constructs. Likewise, no differences were observed for collagen staining among groups. For all constructs, the GAG-free layer showed increased staining for picrosirius red, indicating a collagenous composition in this layer. Biochemical quantification of GAGs and total collagens further supported these findings, showing no variation among groups (Table 1). Likewise, DNA content was unaffected.

The compressive and tensile biomechanical properties, along with the quantification of the PYR crosslinks, were assessed to determine the effects of the treatments. The compressive properties appeared unaffected by either treatment, and although a

#### Table 1

Gross morphology and biochemical data of neocartilage constructs. Data are presented as means  $\pm$  standard deviation.

	Diameter	Thickness	Wet weight	Hydration	DNA	GAG/DW	COL/DW
	(mm)	(mm)	(mg)	(%)	(µg/construct)	(%DW)	(%DW)
Control GOX/CAT DFO 10µM DFO 100µM	5.54±0.12 5.45±0.06 5.57±0.06 5.57±0.07	$0.45 {\pm} 0.02$ $0.44 {\pm} 0.06$ $0.44 {\pm} 0.06$ $0.47 {\pm} 0.05$	$\begin{array}{c} 13.17{\pm}0.87\\ 13.29{\pm}1.36\\ 13.73{\pm}1.77\\ 14.83{\pm}1.24 \end{array}$	$77.79 \pm 1.98$ $78.91 \pm 0.77$ $77.53 \pm 4.97$ $77.47 \pm 2.24$	$19.54{\pm}1.9\\18.94{\pm}1.87\\19.24{\pm}4.41\\19.29{\pm}2.06$	$\begin{array}{c} 44.58{\pm}1.48\\ 41.43{\pm}3.54\\ 46.23{\pm}2.50\\ 45.01{\pm}2.94 \end{array}$	$\begin{array}{c} 15.66{\pm}0.78\\ 15.77{\pm}1.00\\ 15.75{\pm}1.98\\ 15.30{\pm}0.84 \end{array}$

Table 2

Gross morphology and biochemical data of neocartilage constructs treated with ionomycin (Io), DFO, and ionomycin + DFO. Data are presented as means  $\pm$  standard deviation.

	Diameter	Thickness	Wet weight	Hydration	DNA	GAG/DW	COL/DW
	(mm)	(mm)	(mg)	(%)	(µg/construct)	(%DW)	(%DW)
Control Io DFO Io+DFO	$5.70 \pm 0.07$ $5.65 \pm 0.06$ $5.71 \pm 0.05$ $5.70 \pm 0.03$	$0.49 \pm 0.05$ $0.49 \pm 0.05$ $0.49 \pm 0.05$ $0.50 \pm 0.03$	$\begin{array}{c} 14.14{\pm}0.91\\ 14.22{\pm}0.49\\ 14.84{\pm}0.84\\ 14.72{\pm}0.79\end{array}$	$79.69 \pm 1.86$ $79.76 \pm 1.56$ $80.19 \pm 0.90$ $79.40 \pm 0.93$	$\begin{array}{c} 16.61{\pm}2.93\\ 15.80{\pm}1.82\\ 14.77{\pm}1.30\\ 15.38{\pm}1.14 \end{array}$	$\begin{array}{c} 40.21{\pm}0.46\\ 40.06{\pm}1.57\\ 41.78{\pm}1.91\\ 37.99{\pm}1.52\end{array}$	$15.14 \pm 1.12$ $15.16 \pm 0.92$ $14.16 \pm 0.67$ $14.09 \pm 1.12$

 $\sim$ 15% increase was observed in the aggregate modulus of DFOtreated constructs, this increase was not significant. On the other hand, the Young's modulus and the UTS values were higher in constructs exposed to the iron chelator compared to the untreated group (Fig. 2A-D). Constructs treated with 100 µM DFO showed a 57% significant increase in the Young's modulus over the untreated constructs. Treatment with GOX/CAT and 10 µM DFO also reported higher Young's modulus, with a 7% and a 40% increase, respectively, albeit not significant. Constructs treated with 10 µM or 100 µM DFO showed 72% and 112% significant increases in the UTS, respectively, compared to the untreated constructs. Similar effects were observed in the quantification of the crosslink content (Fig. 2E). All treated groups showed a significant increase of PYR crosslinks, with 58%, 64%, and 87% increases induced by GOX/CAT, 10 µM DFO, and 100 µM DFO, respectively, over the untreated group.

#### 3.2. Effect of combined ion treatment in neocartilage constructs

To study possible interactions between the two ion modulators, DFO and ionomycin were applied in combination to sequester Fe<sup>2+</sup> and increase intracellular Ca<sup>2+</sup> concentration, respectively. The effects of the combined treatment were interrogated in Phase II and compared to constructs treated with either ion modulator alone and untreated constructs. In agreement with Phase I data, neither ionomycin nor DFO induced changes in the gross morphology of the constructs. The same results were reported for the combined treatment (Fig. 3A). As in Phase I, morphological features were quantified, showing no differences among groups in terms of diameter, thickness, wet weight, and water content (Table 2). Likewise, and with the exception of the groups treated with DFO which showed a decrease in the total collagen content, the overall histological properties and biochemical content were unaffected by the treatments compared to the untreated group (Table 2 and Fig. 3B). The use of ion modulators did not affect gross tissue formation.

Ionomycin and DFO affect the mechanical properties of selfassembled neocartilage constructs alone and in combination. All treatment groups showed an increase in the compressive or tensile properties (Fig. 4A-D). While ionomycin-treated constructs showed a 37% significant increase in the aggregate modulus, the groups including DFO displayed the same non-significant ~20% increase compared to the untreated group observed in Phase I (Fig. 4A). A similar trend was observed for the shear modulus (Fig. 4B). The tensile properties, shown to increase after treatment with DFO, were further improved after combining the two ion modulators. Treatment with DFO alone produced a 109% significant increase in the Young's modulus over the untreated control. The simultaneous application of both ion modulators, however, resulted in a 150% significant increase in the measurement, resulting in constructs with a Young's modulus of 11.76  $\pm$  3.29 MPa (Fig. 4C). The same result was observed for the UTS of treated constructs, in which DFO induced 130% significant increase when administered alone, compared to a 176% increase when administered together with ionomycin. Thus, the combined treatment resulted in constructs with a UTS of 4.20  $\pm$  1.24 MPa (Fig. 4D). Lastly, all treatments increased the PYR crosslink content. Ionomycin, DFO, and the combination of both produced 51%, 45%, and 38% increases over the untreated group, respectively (Fig. 4E).

The expression of various chondrogenic, hypoxic, and mechanotransduction marker genes was quantified. Treatment of cells using ionomycin or DFO, alone and in combination, did not affect the expression of COL2A1, SOX9, or ACAN (Supplementary Figure 1). Both ion modulators, however, induced a significant increase in the expression of markers of hypoxia and mechanotransduction signaling pathways (Fig. 5); treatment of cells with ionomycin increased the expression of TIMP2 and C-JUN, whereas DFO increased the expression of LOX and VEGF. Lastly, the combined treatment of cells with ionomycin and DFO resulted in a significant increase of the expression of TIMP2, C-JUN, LOX, and VEGF target genes.

#### 4. Discussion

The development of biomimetic tissue-engineered neocartilage implants is emerging as a new solution for the treatment of articular cartilage lesions. Using ion modulation, this work's objective was to improve the biomechanical properties of self-assembled neocartilage constructs. Overall, it was hypothesized that the sequestration of  $Fe^{3+}$  (by DFO, Phase I), and the combination of  $Fe^{3+}$ sequestration with an induced cytoplasmic accumulation of Ca<sup>2+</sup> (by DFO and ionomycin, Phase II), would result in neocartilage constructs with improved mechanical properties. The rationale for these hypotheses comes from the capability of the iron chelator DFO to avoid HIF1 $\alpha$  degradation and induce a putative hypoxic response, and the capability of the Ca<sup>2+</sup> ionophore ionomycin to increase the cytoplasmic concentration of Ca<sup>2+</sup> and induce mechanotransduction-like conditions. Indeed, the functional properties were improved in both phases: in Phase I, of the dosages examined, treatment of constructs with 100 µM DFO improved the PYR crosslink content by 87%, Young's modulus by 57%, and UTS by 112% in self-assembled neocartilage constructs, engineered from minipig expanded and rejuvenated costal chondrocytes. In Phase II, the combined treatment of DFO and ionomycin improved the PYR



**Fig. 2.** Biomechanical and crosslink properties of neocartilage constructs treated with GOX/CAT and DFO. (A) Aggregate modulus, (B) shear modulus, (C) tensile Young's modulus, (D) UTS, and (E) PYR crosslinks. A high dose of DFO showed a 57% increase in the Young's modulus and a 112% increase in the UTS, so as an 87% increase in the PYR crosslink content. The asterisks denote significant differences compared to the control group based of Dunnetts *post hoc* test (\* denotes p<0.05; \*\* denotes p<0.01, \*\*\* denotes p<0.005).

crosslink content by 38%, Young's modulus by 150%, and UTS by 176% in the same neotissue model. This work's findings are significant, as this is the first study to show improved mechanical properties of neocartilage constructs via the use of soluble small molecules, 1)  $Fe^{2+}$  chelator and 2)  $Fe^{2+} + Ca^{2+}$  modulators, to achieve neocartilage with the best tensile properties thus far.

Toward achieving native tissue-level mechanical properties, the proper DFO concentration was identified. Because homeostasis of ions is required to maintain proper cell function, an appropriate concentration of DFO needed to be identified so as to improve the construct mechanical properties while avoiding any damaging effects on the chondrocytes and neocartilage constructs. In Phase I of this work, two concentrations of DFO were examined, 10  $\mu$ M and 100  $\mu$ M. These concentrations were determined based on the use of DFO in studies using osteoarthritic chondrocytes and cancer cell lines [42–44]. Treatment with 10  $\mu$ M DFO increased the Young's modulus and UTS by 40% and 72% over untreated controls, respectively, whereas the 100  $\mu$ M treatment increased the same tensile properties by 57% and 112%, respectively. These results suggest the existence of a dose-dependent response to DFO between the concentrations tested, and are comparable to previous studies that use hypoxic stimulation. For example, neocartilage constructs derived from primary bovine articular chondrocytes exposed to 4% O<sub>2</sub> had a 2-fold increase in tensile modulus, resulting in a Young's modu-



**Fig. 3.** Gross morphology and histological staining of self-assembled constructs treated with ionomycin (Io) and/or DFO. (A) All constructs appear homogeneous, relatively flat, with no visible abnormalities, and similar coloration and opacity. (B) The histological analysis of neocartilage constructs showed that  $Fe^{2+}$  and  $Ca^{2+}$  modulation did not affect the neotissue histological appearance, alone or in combination. Overall cellularity and neotissue composition appears comparable among all groups. Similarly, none of the treatments elicit any noticeable variation in the Picrosirius red staining or the Safranin-O staining.

lus of 0.82  $\pm$ 0 .53 MPa [8]; to place this in context, in the present study, under 10 µM and 100 µM DFO treatments, the construct Young's moduli were 9.47  $\pm$  2.69 and 10.57  $\pm$  1.95 MPa, respectively. Another study using tissue constructs formed using cell encapsulation exposed to 5% O<sub>2</sub> during 7 days not only showed an increase in equilibrium Young's modulus by 22% (510.01  $\pm$  28.15 kPa) compared to the group exposed to normoxic conditions (417.60  $\pm$  68.46 kPa), but also increased expression of various chondrogenic genes such as COL2A1, SOX9, and ACAN [45]. The increase in tensile properties of DFO-treated constructs, presumably due to HIF1 $\alpha$ -induced gene expression, demonstrates the feasibility of using the iron chelator as a tool in tissue engineering development efforts.

The modulation of ion concentration results in changes on the collagen crosslinks of the neotissue, which translate to an improvement of construct stiffness. Enzymatic collagen crosslinks, such as the mature trivalent PYR crosslink, play important roles in the mechanical properties of collagenous tissues. Strategies that increase crosslink content, such as hypoxia or *in vivo* maturation, improve

the mechanical properties of engineered neotissues, particularly the tensile properties [29,46,47]. In this work, self-assembled constructs exposed to DFO showed 64% and 87% significant increases in the PYR crosslink content in a dose-dependent manner, following treatment with 10  $\mu$ M and 100  $\mu$ M DFO, respectively. This finding is significant as it validates the hypothesized function of iron chelation as a medium of inducing HIF1 $\alpha$  activation, which prompts the expression of lysyl oxidase-like 2 (LOXL2), the protein that catalyzes the PYR crosslinks [8,24]. Neocartilage constructs derived from primary bovine articular chondrocytes exposed to 4% O<sub>2</sub> for 2 weeks not only showed a 2-fold increase in the Young's modulus, but also a 20-fold increase in the gene expression of LOXL2 [8]. While the changes in PYR crosslink content are not sufficient to explain the mechanical data and future experiments should aim at identifying the mechanisms behind the effects shown here, the literature is consistent in demonstrating that an increase in the crosslink content is associated with an increase in the tensile properties. This finding is also significant because it provides an explanation for the increase in the tensile properties, as the increase of



**Fig. 4.** Biomechanical properties and crosslink of neocartilage constructs treated with ionomycin (Io) and DFO. (A) Aggregate modulus, (B) shear modulus, (C) tensile Young's modulus, (D) UTS, and (E) PYR crosslinks. Constructs treated with a combination of ionomycin and DFO showed a 150% and a 176% increase in the Young's modulus and UTS, respectively, over the untreated control, and with better performance compared to both treatments alone. This effect cannot be attributed to an increase in of PYR crosslinks, where the combination treatment also increased the PYR content a 38% compared to the control, on par or below to the individual treatments. The asterisks denote significant differences compared to the control group based of Dunnetts *post hoc* test (\* denotes p < 0.05; \*\* denotes p < 0.01, \*\*\* denotes p < 0.005).

PYR crosslinks is directly associated with an increase in Young's modulus [47].

Separate cellular components are affected by Fe<sup>2+</sup> depletion and cytoplasmic Ca<sup>2+</sup> uptake, suggesting that the activation of distinct signaling cascades by different ion modulators may be used in combination. Ionomycin increases cytoplasmic Ca<sup>2+</sup> concentration, an early signal that follows a mechanical stimulus, presumably via calmoduline (CaM)-dependent factors [48–51]. The results shown here were expected based on prior work showing ionomycin's use for the improvement of tensile stiffness of constructs derived from primary bovine articular chondrocytes as well as constructs derived from expanded and rejuvenated minipig costal chondrocytes [10,29]. As hypothesized, the combination of DFO with the Ca<sup>2+</sup> ionophore resulted in a larger increase of the tensile properties compared to the untreated constructs or constructs treated with one ion modulator, with an average Young's modulus of 11.76  $\pm$ 3.29 MPa. This value corresponds to a 150% significant increase over the control group, and a 48% or 19% increase compared to the ionomycin or DFO treatments alone. Similarly, the UTS of the constructs exposed to the combined treatment was 176% higher compared to the control group, and 55% or 20% higher compared to the UTS of constructs treated with ionomycin or DFO alone, respectively. The findings shown in this work are significant, as they are, to the best of our knowledge, the first evidence that two different ion modulation strategies, one aiming at inducing a hypoxic effect and one aiming at inducing mechanotransduction, can be used together to attain a larger effect. Inasmuch as previous studies on self-assembled cartilage have examined the use of Na<sup>+</sup> and Ca<sup>2+</sup> modulation as a stimulation factor, this study showed that Fe<sup>2+</sup> and Ca<sup>2+</sup> modulation yield better results compared to Fe<sup>2+</sup> alone, Ca<sup>2+</sup> alone, and Na<sup>+</sup> and Ca<sup>2+</sup> combined, in terms of producing a mechanically robust neocartilage suitable for translation (Fig. 6).

The expression of COL2A1, SOX9, and ACAN genes, determined by RT-qPCR, was not affected by the treatment of chondrocytes



**Fig. 5.** Gene expression of chondrocytes treated with ionomycin (Io) and DFO. The dashed line marks the value 1 and corresponds to no change between groups. Asterisks mark significance of effects based on a Dunnett's *post hoc* test (\* denotes p < 0.05) using the corresponding control value for each gene assessed. Markers of hypoxia, LOX and VEGF, are increased in chondrocytes treated with DFO, whereas markers of mechanotransduction, TIMP2 and C-JUN, are increased in chondrocytes treated with ionomycin. All markers are significantly increased with the combined treatment of DFO and ionomycin.



Fig. 6. Modulation of ions via deferoxamine and ionomycin hypoxia and mechanotransduction signaling pathways which result in improved structure-function relationships of neocartilage constructs.

with DFO, ionomycin, or the combination of both (Supplementary Figure 1). In contrast, the use of the ion modulators triggered an increase in the expression of pathway-specific markers shown to be overexpressed in chondrocytes under mechanotransduction and hypoxia, respectively. Chondrocytes exposed to 0.3 µM ionomycin showed a significant increase in the expression of the TIMP2 gene [52], as well as an increase in the expression of the C-JUN [53] gene (Fig. 5). Similarly, the treatment with DFO resulted in a significant increase in the expression of LOX [8] and VEGF [54] genes (Fig. 5). The increase in these genes may directly explain the improvement of the mechanical properties observed in this work. For example, LOX isoforms, including Loxl2, contribute to increasing collagen crosslinking, which are directly associated with the tensile properties. Alternatively, TIMP2 halts ECM catabolism and protects collagen crosslinks, possibly affecting tensile and compressive properties [55]. Interestingly, the expression of LOX, TIMP, and C-JUN genes was further increased when ionomycin and DFO were applied in combination, indicating the possible existence of crosstalk between the hypoxia and mechanotransduction pathways, which merits further investigation.

Considering that Fe<sup>2+</sup> and Ca<sup>2+</sup> ions are involved in multiple processes within the cell, this work examined the potential toxic effects of ion modulation. Aside from its role in PHD function, iron is involved in various cell processes, including erythropoiesis, mitochondrial function, immune response, and DNA synthesis and repair [56]. In view of the possible adverse effects of iron depletion, a short-duration 1-hour daily treatment over 5 days was used. The rationale behind the dosage selection comes from the following findings: transcription factors of the same highly conserved family as HIF1 $\alpha$  elicit a 24-hour long sustained transcriptional activation in response to a 2-hour long stimulation [57]. A similar effect was found in fibrochondrocytes. Engineered meniscus exposed to 3% O<sub>2</sub> for 24 hours followed by 5 minutes of dynamic compression displayed over 40 genes supporting a chondrogenic phenotype, as revealed by RNA sequencing performed 30 min after the end of stimuli [58]. These data suggest that short Fe<sup>2+</sup> and Ca<sup>2+</sup> modulation stimuli are enough to elicit changes in the biomechanical

properties, but not long enough to induce adverse effects. Our results found no alterations in the appearance and structure of the neocartilage tissues, as evidence by their gross morphology and histology. Moreover, no differences were found in the DNA content, a measure of construct cellularity. These results are significant, as they demonstrate no toxicity induced by the ion modulation treatments.

The use of ion modulation via DFO and ionomycin allows for treatment modalities that cannot be replicated with the use of bioreactors. Hypoxia and mechanical stimuli have been used in the past to induce neotissue maturation. These strategies, however, need dedicated equipment, require significant manipulation of the tissues, and may be difficult to control temporally. As a result, neotissues may display higher variability and become prone to contamination and user errors, compared to non-bioreactor cultures [59]. For example, short-duration treatments are not feasible using hypoxic chambers, which rely on passive mixture of gases and their solubilization into the culture medium, and therefore, show a slow onset of hypoxia as opposed to the treatments used in this study [60]. Moreover, combination of stimuli results in added complexity that makes these strategies less feasible for translational purposes. A recent work combined hypoxia and dynamic compression by culturing tissue-engineered meniscus at 3% O<sub>2</sub> and exposing it to 5 min of dynamic compression, 4 times a day, at a 30-45% strain. The combined use of hypoxic and mechanical stimuli, termed mechano-hypoxia conditioning, resulted in upregulation of chondrogenic genes (e.g., SOX5/6) and down regulation of hypertrophic genes (e.g., COL10A1) on meniscus fibrochondrocytes [61]. The authors, however, reported several limitations in the study, including a high variability within the results and possible confounding effects due to the media not being changed during the whole 3-weeks culture to maintain hypoxia [61]. DFO and ionomycin are safe, long-lasting, low-cost exogenous agents with high reproducibility and efficacy without added complexity and costs of bioreactors.

Previously identified analog methods to induce hypoxia, for example via the use of GOX/CAT, do not necessarily result in functional improvements of neocartilage constructs. In Phase I, in addition to DFO, the enzymatic system GOX/CAT was also examined as a means of eliciting HIF1 $\alpha$  accumulation due to hypoxia [30]. This method uses two enzymes to induce hypoxia within the culture medium: glucose oxidase is used to consume oxygen by generating H<sub>2</sub>O<sub>2</sub>, and catalase to degrade H<sub>2</sub>O<sub>2</sub>. This treatment induced a 58% significant increase of the PYR content, suggesting that a hypoxic response may have been induced. However, the increase in crosslinks did not translate into improvements on the compressive or tensile properties. It is possible that the system, while depleting oxygen from the medium by consuming glucose and inducing a hypoxic response on the cells, also confers other effects that may not be conducive toward the improvement of functional properties. Such effects can include producing oxidative stress through the accumulation of  $H_2O_2$  in spite of the catalase reaction, or the consumption of glucose, also required by the cells [62,63]. Chondrocytes are particularly sensitive to H<sub>2</sub>O<sub>2</sub> accumulation, and the availability of glucose in the medium has been directly associated with ECM deposition [63,64]. The GOX/CAT system requires further characterization toward its application in cartilage tissue engineering platforms.

This work represents significant progress toward generating a tissue-engineered neocartilage solution for addressing articular cartilage lesions. Molecular assays were not employed in this work since the mechanisms of action of DFO and ionomycin have been examined by others [24,29,42,65]. However, future studies with  $Fe^{2+}$  and  $Ca^{2+}$  modulators should aim to identify the mechanisms that take place in self-assembled neocartilage to explain the mechanical data obtained in this work. The findings shown provide

a valuable new approach for the tissue-engineering field. The use of hypoxic and mechanical stimuli, often used to improve the biomechanical properties of engineered tissues, such as neocartilage, carry added limitations, technical complications, and costs. The use of ion modulation poses an analog method to elicit biomechanical improvements, while avoiding the potential complications of using bioreactors. As shown in this work, ion modulation is highly controllable, replicable, cost-effective, and allows the combination of treatments without added methodological difficulty. Particularly, this work used 1) costal chondrocytes, a novel cell source that eliminates donor site morbidity using an allogeneic approach; 2) cell expansion to eliminate cell scarcity limitations; and 3) rejuvenation of passaged chondrocytes to recapitulate a proper chondrogenic phenotype and ensure assembly of chondrocytes into hyaline cartilage. After a combined treatment of DFO and ionomycin, the neocartilage constructs achieved a Young's modulus of 11.76  $\pm$ 3.29 MPa and UTS of 4.20  $\pm$  1.24 MPa, the highest recorded tensile properties for self-assembled neocartilage constructs.

#### 5. Conclusion

This study examined the use of two ion modulation strategies to improve the mechanical properties of engineered neocartilage constructs derived from expanded and rejuvenated minipig costal chondrocytes: 1) sequestration of Fe<sup>3+</sup> using DFO, and 2) the combined treatment of Fe<sup>2+</sup> and Ca<sup>2+</sup> modulation using DFO and ionomycin. The results shown in this work demonstrate that DFO, an iron chelator, induces tissue maturation, as reflected in the PYR crosslink content, and an improvement in the tensile properties. In addition, this work shows the feasibility of combining different ion modulation strategies by using ionomycin, a calcium ionophore, together with DFO. The combined treatment of these ion modulators resulted in the highest tensile values ever obtained for selfassembled neocartilage constructs and provides evidence of the utility of ion modulation within tissue engineering efforts.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

The authors acknowledge support from the National Institutes of Health (NIH) R01 AR067821 and Fulbright Chile scholarship.

#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2022.09.022.

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