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Intracellular Na^+ and Ca^{2+} Modulation Increases the Tensile Properties of Developing Engineered Articular Cartilage

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Objective. **Significant collagen content and tensile properties are difficult to achieve in tissue-engineered articular cartilage. The aim of this study was to investigate whether treating developing tissue-engineered cartilage constructs with modulators of intracellular Na^+ or Ca^{2+} could increase collagen concentration and construct tensile properties.**

Methods. **Inhibitors of Na^+ ion transporters and stimulators of intracellular Ca^{2+} were investigated for their ability to affect articular cartilage development in a scaffoldless, 3-dimensional chondrocyte culture. Using a systematic approach, we applied ouabain (Na^+/K^+-ATPase inhibitor), bumetanide ($Na^+/K^+/2Cl^-$ tritransporter inhibitor), histamine (cAMP activator), and ionomycin (a Ca^{2+} ionophore) to tissue-engineered constructs for 1 hour daily on days 10–14 of culture and examined the constructs at 2 weeks or 4 weeks. The gross morphology, biochemical content, and compressive and tensile mechanical properties of the constructs were assayed.**

Results. **The results of these experiments showed that 20 μM ouabain, 0.3 μM ionomycin, or their combination increased the tensile modulus by 40–95% compared with untreated controls and resulted in an increased amount of collagen normalized to construct wet weight. In constructs exposed to ouabain, the increased percentage of collagen per construct wet weight was secondary to decreased glycosaminoglycan production on a per-cell basis. Treatment with 20 μM ouabain also increased the ultimate tensile strength of neo-tissue by 56–86% at 4 weeks. Other construct properties, such as construct growth and type I collagen production, were affected differently by Na^+ modulation with ouabain versus Ca^{2+} modulation with ionomycin.**

Conclusion. **These data are the first to show that treatments known to alter intracellular ion concentrations are a viable method for increasing the mechanical properties of engineered articular cartilage and identifying potentially important relationships to hydrostatic pressure mechanotransduction. Ouabain and ionomycin may be useful pharmacologic agents for increasing tensile integrity and directing construct maturation.**

Because many cartilage injuries are irreversible, tissue replacement techniques are desirable (1,2). Loading via hydrostatic pressure (HP) affects articular cartilage matrix synthesis (3,4), and hydrostatic loading of chondrocytes alters intracellular ion concentrations by either stimulating or inhibiting the action of numerous ion transporters (3). Therefore, changing intracellular ion concentrations using agents known to affect specific ion transporters or signaling pathways could lead to new strategies to alter the biochemical and mechanical properties of tissue-engineered articular cartilage.

Two major controllers of Na^+ transport in chondrocytes are the Na^+/K^+ pump and the $Na^+/K^+/2Cl^-$ cotransporter. The application of HP to articular chondrocytes inhibits the action of these transporters (5). The Na^+/K^+ pump is an ATPase that pumps ions against a concentration gradient to keep the level of intracellular K^+ higher than that of Na^+; therefore, inhibition of this pump leads to increased levels of intracellular Na^+. The

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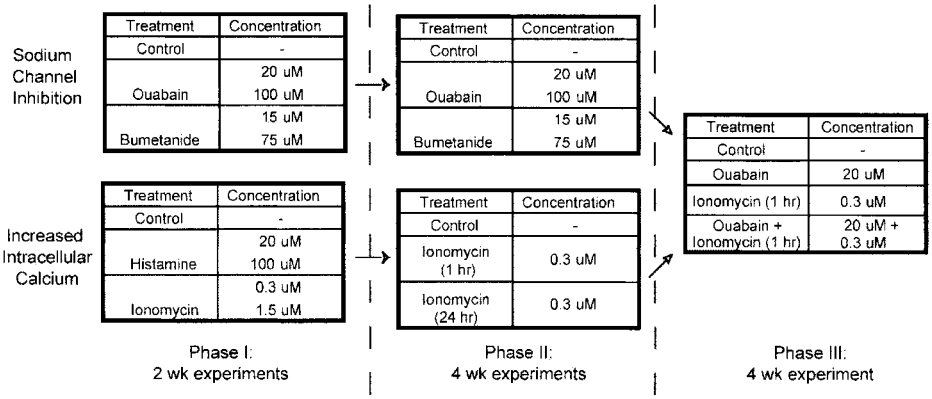

Figure 1. Experimental designs for each phase in the study. In phase I, a low concentration and a high concentration of several intracellular Na^+ and Ca^{2+} modulators (ouabain, bumetanide, ionomycin, and histamine) were examined at 14 days. In phase II, the effects of the Na^+ modulators were examined at 4 weeks, and the effects of continuous treatment with 0.3 μM ionomycin from day 10 to day 14 were compared with the effects of treating for only 1 hour per day on days 10–14. Phase III experiments were also 4 weeks in duration. Based on the phase II results, combined treatment with 0.3 μM ionomycin and 20 μM ouabain for 1 hour per day on days 10–14 of culture was compared with individual treatment with these agents.

$Na^+/K^+/2Cl^-$ cotransporter is a homeostatic regulator of cell size that allows influx of these ions to prevent cell shrinkage in hyperosmotic conditions (3). Inhibition of this cotransporter diminishes the ability of the cell to regulate its volume and decreases the level of intracellular Na^+.

The application of HP also causes the release of intracellular Ca^{2+} in chondrocytes (6), suggesting that Ca^{2+} is involved in the signal transduction pathways initiated by HP. Similarly, histamine stimulates Ca^{2+} release from intracellular stores within chondrocytes (7). For example, Taylor et al (8) demonstrated dose-dependent increases of cAMP concentrations in chondrocytes treated with histamine, and cAMP mobilizes intracellular Ca^{2+} (9). Additionally, Tomita et al (10) showed that ionomycin induces chondrogenesis. Ionomycin is a calcium ionophore that increases intracellular calcium in a dose-dependent manner (11).

In a recent experiment performed at our laboratory, 10 MPa of HP was applied to developing articular cartilage constructs for 1 hour daily on days 10–14 of culture, with a resulting increase in the amounts of sulfated glycosaminoglycan (sGAG) and collagen (12). Furthermore, treated constructs had improved mechanical properties. In the present study, we investigated whether chemical modulation of intracellular sodium and calcium, which is similar to the effects of HP on ion transporters, would also change the biochemical composition and mechanical properties of articular cartilage constructs. Based on the observations described above, ouabain and bumetanide were used to inhibit the Na^+/K^+ and $Na^+/K^+/2Cl^-$ transporters, respectively, while histamine and ionomycin were chosen to increase the level of intracellular Ca^{2+}. It was hypothesized that these treatments would lead to increased collagen concentrations and tensile properties, which are two aspects of articular cartilage tissue engineering that are notably difficult to achieve (13–15). This hypothesis was tested through a series of experiments conducted in sequential phases. First, intracellular Na^+ and Ca^{2+} were altered individually. Then, based on the results from the individual experiments, the constructs were treated with ouabain and ionomycin simultaneously.

MATERIALS AND METHODS

Chondrocyte isolation and construct self-assembly, culture, and processing. Bovine chondrocytes were isolated and self-assembled, as previously described (1,12,16). Separate cell harvests were used for each experiment. The constructs were formed by seeding 5.5×10^6 live cells in 150 μl of medium into 5-mm–diameter nonadherent, cylindrical wells made of 2% agarose, with an additional 350 μl of medium added 4 hours later. The 500 μl of medium was changed 24 hours after seeding and once daily throughout the experiments. The chemically defined medium that was used consisted of Dulbecco's modified Eagle's medium with 4.5 mg/ml glucose and L-glutamine (BioWhittaker, Walkersville, MD), 100 nM dexamethasone (Sigma, St. Louis, MO), 1% fungizone, 1% penicillin/streptomycin, 1% ITS+ (insulin–transferrin–

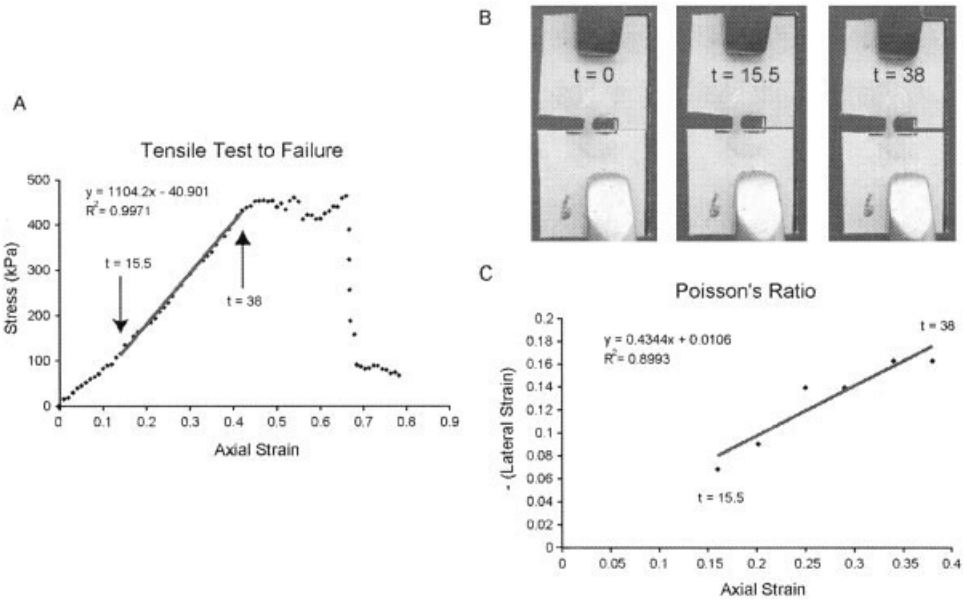

Figure 2. Representative data from construct tensile testing. **A,** Stress versus axial strain. **B,** Digital images obtained during the test for determination of the Poisson ratio. **C,** Linear regression of axial strain versus the negative of lateral strain. In **B,** note the subtle decrease in the lateral dimension of the construct. "Time-stamps" are provided in each figure to demonstrate the way in which lateral strain was matched in time to axial strain.

selenium; BD Biosciences, Franklin Lakes, NJ), 50 μg/ml ascorbate-2-phosphate, 40 μg/ml L-proline, and 100 μg/ml sodium pyruvate (Fisher Scientific, Pittsburgh, PA) buffered at pH 7.3 by 10 m*M* HEPES. This medium formulation has 151 m*M* Na^+, 5.2 m*M* K^+, and 1.7 m*M* Ca^{2+}, which are near-physiologic serum concentrations (17). The medium osmolarity was ~346 mOsmoles. All cultures took place at 37°C in an atmosphere of 10% CO_2, 19% O_2, and 71% N_2.

Figure 1 shows the different phases of the experiments performed in this study. For all experiments, the constructs were randomly assigned across treatment groups. In any given experiment, the constructs were unconfined (18) on day 10 and transferred to tissue culture plates for which only the well bottoms were coated with agarose. On days 10–14, the constructs were treated with their respective agents. During treatment, the constructs (including controls) were cultured in petri dishes for 1 hour with ~4 ml of chemically defined medium containing the appropriate concentration of agent. Treatment was followed by a 30-minute wash step in medium without agent before the constructs were returned to their wells. Treatment occurred at the same time every day over the course of 5 days. In phase I experiments, the effects of a high concentration and a low concentration of ouabain, bumetanide, ionomycin, and histamine (all from Sigma) were examined at 14 days to ascertain the immediate response to treatment. The concentrations were chosen based on previously published reports (5,8,11). The phase II experiments were 4 weeks in duration. In one experiment, the Na^+ modulators were examined. In the other, the effects of continuous treatment with 0.3 μ*M* ionomycin from day 10 to day 14 were compared with the effects of treatment for only 1 hour daily on days 10–14. The phase III experiment was also 4 weeks in duration. In this experiment, combined treatment with 0.3 μ*M* ionomycin and 20 μ*M* ouabain for 1 hour daily on days 10–14 of culture was compared with individual treatment with these agents. These two treatment regimens were chosen based on the results of the phase I and phase II experiments.

Depending on the phase, the constructs were processed at either 2 weeks or 4 weeks for quantitative biochemical analysis, mechanical testing, and immunohistochemical analysis. From culture, the constructs were photographed, weighed while wet, and portioned for analysis. A 3-mm–diameter punch biopsy specimen was obtained from the center of the construct for testing of creep indentation. The remaining outer ring was portioned at ~60% for biochemical analysis and ~40% for tensile testing. Samples for immunohistochemical analysis were prepared from separate constructs.

Gross morphology and immunohistochemistry. The construct diameter was measured using ImageJ software (National Institutes of Health, Bethesda, MD). For immunohistochemical analysis, the constructs were cryoembedded and sectioned at 14 μm. Samples were fixed with chilled acetone, rinsed with immunohistochemistry buffer, quenched of peroxidase activity with hydrogen peroxide/methanol, and blocked with horse serum (Vectastain ABC kit; Vector, Burlingame, CA). Sections were then incubated with either mouse anti–type I collagen diluted 1:1,000 (Accurate, Westbury, NY) or rabbit anti–type II collagen diluted 1:300 (Cedarlane, Burlington, NC). The secondary antibody (anti-mouse or anti-rabbit IgG; Vectastain ABC kit) was then applied, and color was developed using the Vectastain ABC reagent and diaminobenzidine. Additionally, bovine articular cartilage was used as a

Table 1. Construct growth metrics and biochemical content in phase I and phase II experiments*

Group	Diameter, mm	Thickness, mm	WW, mg	Collagen, % WW	GAG, % WW	Collagen, $\mu g/10^6$ cells	GAG, $\mu g/10^6$ cells
Phase I							
Control	5.1 ± 0.1	0.26 ± 0.03	5.4 ± 0.3	15 ± 3.5	1.9 ± 0.2	136 ± 35	17.7 ± 3.6
20 μM ouabain	5.1 ± 0.1	0.24 ± 0.01	4.9 ± 0.7	20.6 ± 5.4	2.4 ± 0.3	122 ± 13	14.3 ± 1.6
100 μM ouabain	4.9 ± 0.1	0.24 ± 0.02	5.0 ± 0.1	17.8 ± 5.4	2.4 ± 0.5	112 ± 31	15.1 ± 3.5
15 μM bumetanide	5.0 ± 0.1	0.25 ± 0.04	5.3 ± 0.4	18.8 ± 5.7	2.1 ± 0.4	120 ± 18	14.1 ± 3.5
75 μM bumetanide	5.1 ± 0.1	0.24 ± 0.01	5.3 ± 0.3	17.4 ± 2.8	1.5 ± 0.1	119 ± 21	10.4 ± 1.3†
Control	5.3 ± 0.1	0.26 ± 0.03	7.1 ± 0.1	13.7 ± 2.1	2.2 ± 0.4	242 ± 63	39.3 ± 10.0
20 μM histamine	5.3 ± 0.1	0.28 ± 0.02	7.6 ± 0.5	15.3 ± 2.8	2.9 ± 0.3	239 ± 21	46.3 ± 9.3
100 μM histamine	5.3 ± 0.1	0.30 ± 0.03	7.5 ± 0.5	12.6 ± 2.0	2.8 ± 0.6	209 ± 35	46.5 ± 13.9
0.3 μM ionomycin	5.3 ± 0.1	0.29 ± 0.02	7.4 ± 0.3	17.8 ± 2.5†	2.7 ± 0.7	274 ± 35	41.3 ± 8.0
1.5 μM ionomycin	5.5 ± 0.2	0.31 ± 0.02	7.3 ± 0.4	15.3 ± 2.2	2.2 ± 0.3	258 ± 42	36.9 ± 4.6
Phase II							
Control	7.4 ± 0.3	0.76 ± 0.04	39.9 ± 3.0	6.3 ± 1.0	6.3 ± 0.3	403 ± 62	409 ± 83
20 μM ouabain	6.5 ± 0.2†	0.69 ± 0.09	24.9 ± 2.5†	9.0 ± 2.0†	6.8 ± 1.0	345 ± 44	267 ± 60†
100 μM ouabain	6.3 ± 0.2†	0.56 ± 0.05†	19.6 ± 1.2†	12 ± 1.6†	6.8 ± 0.6	426 ± 77	236 ± 38†
15 μM bumetanide	7.1 ± 0.2	0.73 ± 0.05	34.2 ± 2.3	8.2 ± 2.5	6.7 ± 0.3	412 ± 167	335 ± 86
75 μM bumetanide	7.1 ± 0.4	0.73 ± 0.10	36.0 ± 3.7	7.7 ± 0.7	6.8 ± 0.4	484 ± 120	426 ± 88
Control	5.9 ± 0.2	0.54 ± 0.04	15.6 ± 1.3	8.3 ± 1.0	9.2 ± 0.5	319 ± 25	354 ± 23
0.3 μM ionomycin (1 hour)	6.0 ± 0.2	0.52 ± 0.04	15.3 ± 1.7	8.0 ± 0.4	8.8 ± 0.6	311 ± 32	345 ± 52
0.3 μM ionomycin (24 hour)	5.9 ± 0.1	0.47 ± 0.05	13.1 ± 1.7	8.9 ± 1.6	9.3 ± 0.4	297 ± 35	313 ± 36

* Values are the mean ± SD. In phase I experiments, the effects of a high concentration and a low concentration of ouabain, bumetanide, histamine, and ionomycin were examined at 14 days to ascertain the immediate response to treatment. Phase II experiments were 4 weeks in duration. See Materials and Methods and Figure 1 for details. WW = wet weight; GAG = glycosaminoglycan.
† Significantly different versus control, by post hoc test.

positive control for type II collagen and as a negative control for type I collagen, while bovine tendon was used as a positive control for type I collagen and as a negative control for type II collagen. Additional controls consisted of tissue samples stained as described, but without application of the primary antibody. The slides were examined with a light microscope.

Biochemical analysis. Samples were digested in pepsin–elastase as previously described (16). Cell numbers were determined with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA), assuming a value of 7.8 pg DNA/cell (19). The sGAG content was determined using the Blyscan sGAG assay kit (Accurate). Following hydrolyzation of the digest by 2*N* NaOH for 20 minutes at 110°C, samples were assayed for collagen content using a chloramine T hydroxyproline assay (20). The Sircol collagen assay standard (Accurate) was used, such that the standard curve was reflective of the collagen amount.

Compressive testing. As previously described (14), the compressive mechanical properties were determined by creep indentation testing, assuming a linear biphasic model (21). A creep indentation apparatus was used to determine the compressive creep and recovery behavior of constructs (22), using a 0.2*g* tare load and a 0.7*g* test load applied through an 0.8-mm–diameter, flat-ended, porous, rigid tip. Specimen thickness was measured using a micrometer. To calculate the material properties of the specimens (aggregate modulus, Poisson ratio, and permeability), a semianalytical, seminumeric, linear biphasic model was used, followed by finite element optimization (22,23).

Tensile testing. Samples were cut into a dog-bone shape and affixed to paper tabs for gripping (24). ImageJ software was used to determine the sample gauge length and width in the photographs. The gauge length was defined as the distance between paper tabs. Thickness was measured with a micrometer. Tensile tests were performed until failure at a strain rate of 1% of gauge length/second, using an electromechanical materials testing system (Instron model 5565; Instron, Canton, MA). The apparent Young's modulus was determined by least squares fitting of the linear region of the stress–strain curve. The ultimate tensile strength (UTS) was determined as the maximum stress reached during a test.

The tensile Poisson ratio was determined in phase II and phase III experiments. The Poisson ratio is the negative of the ratio of lateral-to-axial strain. Axial strain data were acquired via the Instron system. Lateral strain was determined via digital image analysis, as follows. First, CHDK, a free firmware add-on for Canon (http://chdk.wikia.com/wiki/CHDK) was installed onto a Canon SD600 6-megapixel digital camera to enable time-lapse videography. The software script was set so the camera would take a photograph at 2 seconds and every 4.5 seconds thereafter. Settings were validated by running the script while photographing a digital timer. Just before a test, a photograph of the specimen loaded into the Instron was obtained. This image defined time 0. The camera script and tensile test were then begun simultaneously, such that acquired images corresponded temporally to the Instron data. During data analysis, the photographs were matched in time to the linear portion of the stress–strain curve. As shown in Figure 2A, the linear portion begins at 15.5 seconds and ends at 38 seconds. Lateral strain was determined using ImageJ software. The width of the specimen (in pixels) from the time 0 photograph determined the "lateral gauge length."

The width in pixels was measured in subsequent photographs (Figure 2B) corresponding to the linear portion of the stress–strain curve, allowing the lateral strain to be calculated. Figure 2C shows a plot of axial strain versus lateral strain. Linear regression of this plot gives the Poisson ratio.

Statistical analysis. For each experimental group in this study, 5–7 samples were used. For all assays, a one-way analysis of variance was performed (StatView; Abacus Concepts, Berkeley, CA). *P* values less than 0.05 were considered significant. If significance was identified, a post hoc test was performed. For phase I and phase II Na^+ experiments, Dunnett's post hoc test was used. The Dunnett's test compares each experimental group with control but does not compare experimental groups with each other. For the phase II ionomycin experiment and phase III, a Student-Newman-Keuls post hoc test was performed. This test allows comparisons among all groups to be made. All data are presented as the mean ± SD.

RESULTS

Phase I. In phase I, the results of treating tissue-engineered articular cartilage constructs with low and high concentrations of Na^+ transport inhibitors (ouabain and bumetanide) and intracellular Ca^{2+} modulators (histamine and ionomycin) were examined at 2 weeks (Figure 1). This time point was chosen to immediately follow up treatment on days 10–14.

Tables 1 and 2 show the growth metrics (diameter, thickness, and wet weight), biochemical content, and mechanical properties for phases I and II. The only significant differences observed for the Na^+ transport inhibitors experiment were an increase in the number of cells per construct for all treatment groups and a decrease in GAG production per cell for the 75 μM bumetanide treatment group. Control constructs had a mean ± SD of 6.0 ± 0.7 million cells, and treatment with Na^+ transport inhibitors caused a 32–35% increase in the cell number. For the intracellular Ca^{2+} modulators experiment, the only significant difference observed was that treatment with 0.3 μM ionomycin resulted in a 30% increase in the percentage of collagen per construct wet weight compared with control constructs. This increase was similar to what was observed at 2 weeks following application of 10 MPa of static HP to self-assembled cartilage constructs for 1 hour daily on days 10–14 (12).

Phase II. Based on the results from the phase I experiments, phase II experiments of 4 weeks duration were designed (Figure 1). Because none of the Na^+ transport inhibitors showed a distinct advantage over the others at 2 weeks, each inhibitor was studied again. It was hypothesized that the increased cell number seen at 2 weeks would translate to beneficial increases in biochemical and mechanical properties over the subsequent 2 weeks. Table 1 shows that treatment with ouabain yielded an increased percentage of collagen per construct wet weight compared with control. There were 43% and 90% increases in the percentage of collagen per construct wet weight following treatment with 20 μM and 100 μM ouabain, respectively, suggesting dose dependence. There was also an ~50% increase in Young's modulus for both the 20 μM and 100 μM ouabain treatment groups compared with control (Table 2), while the tensile Poisson ratio increased only with 20 μM ouabain treatment. Treatment with 20 μM ouabain yielded a 56% increase in UTS, although this increase was not statistically significant. Again, these changes are tantamount to those observed at 4 weeks following 10 MPa of HP applied to self-assembled cartilage constructs. Additionally, treatment with either 20 μM or 100 μM ouabain significantly decreased construct diameter, wet weight, and GAG production per cell. Treatment with 100 μM ouabain also decreased construct thickness compared with control, but 20 μM ouabain did not yield constructs significantly thinner than control. There were no significant differences following ouabain treatment in the number of cells per construct, the percentage of GAG per construct wet weight, or compressive mechanical properties. Furthermore, bumetanide treatment caused no significant differences in any of the parameters assessed.

With respect to intracellular Ca^{2+} modulators, results from the phase I experiments showed that 0.3 μM ionomycin deserved further study. It was hypothesized that the significant increase in the percentage of collagen per construct wet weight would lead to a delayed increase in tensile properties. Additionally, the effects of treatment for 1 hour daily on days 10–14 were compared with the effects of continuous treatment on days 10–14. Table 2 shows that both of these treatment regimens resulted in at least a 40% increase in Young's modulus compared with control, but there was no significant difference when comparing the treatment regimens with each other. There were no significant differences in any of the other parameters assessed following these 2 treatment regimens.

Phase III. Based on results from the phase I and phase II experiments, the phase III experiment compared individual treatment with 20 μM ouabain or 0.3 μM ionomycin for 1 hour with combined treatment with these two agents (Figure 1). As before, treatment was for 1 hour daily on days 10–14 of culture. Biochemical assays, mechanical testing, and immunohistochemistry staining for type I and type II collagen were performed at 4 weeks.

Table 2. Construct compressive and tensile properties in phase I and phase II experiments*

Group	Tension			Compression		
	Young's modulus, kPa	UTS, kPa	Poisson ratio	Aggregate modulus, kPa	Permeability, 10^{-15} $m^4/N \cdot$ second	Poisson ratio
Phase I						
Control	592 ± 199	260 ± 81	–	66 ± 10	3.8 ± 1.0	0.01 ± 0.01
20 μM ouabain	628 ± 398	372 ± 159	–	64 ± 17	9.0 ± 8.6	0.01 ± 0.01
100 μM ouabain	543 ± 222	271 ± 82	–	76 ± 25	6.6 ± 5.1	0.04 ± 0.08
15 μM bumetanide	644 ± 328	270 ± 100	–	71 ± 22	8.0 ± 3.6	0.01 ± 0.01
75 μM bumetanide	481 ± 258	234 ± 41	–	69 ± 27	5.9 ± 3.2	0.04 ± 0.07
Control	421 ± 145	337 ± 93	–	65 ± 20	5.4 ± 3.3	0.09 ± 0.11
20 μM histamine	425 ± 110	313 ± 97	–	66 ± 17	4.6 ± 2.4	0.07 ± 0.07
100 μM histamine	477 ± 269	265 ± 92	–	82 ± 31	5.3 ± 2.5	0.09 ± 0.06
0.3 μM ionomycin	423 ± 179	288 ± 90	–	63 ± 13	10.9 ± 14.9	0.15 ± 0.12
1.5 μM ionomycin	354 ± 184	287 ± 89	–	61 ± 19	2.6 ± 0.6	0.03 ± 0.03
Phase II						
Control	485 ± 150	228 ± 132	0.23 ± 0.06	129 ± 65	19 ± 14	0.11 ± 0.08
20 μM ouabain	733 ± 140†	356 ± 101	0.38 ± 0.14†	191 ± 53	16 ± 11	0.12 ± 0.09
100 μM ouabain	738 ± 127†	364 ± 66	0.34 ± 0.09	157 ± 73	14 ± 14	0.06 ± 0.09
15 μM bumetanide	527 ± 117	216 ± 65	0.29 ± 0.08	136 ± 72	43 ± 47	0.09 ± 0.11
75 μM bumetanide	574 ± 253	225 ± 125	0.33 ± 0.11	165 ± 51	42 ± 36	0.12 ± 0.10
Control	977 ± 266	273 ± 87	0.54 ± 0.19	286 ± 160	64 ± 60	0.22 ± 0.13
0.3 μM ionomycin (1 hour)	1372 ± 304†	402 ± 106	0.51 ± 0.15	399 ± 95	24 ± 22	0.26 ± 0.07
0.3 μM ionomycin (24 hours)	1409 ± 262†	425 ± 108	0.45 ± 0.12	357 ± 30	40 ± 24	0.22 ± 0.13

* Values are the mean ± SD. In phase I experiments, the effects of a high concentration and a low concentration of ouabain, bumetanide, histamine, and ionomycin were examined at 14 days to ascertain the immediate response to treatment. Phase II experiments were 4 weeks in duration. See Materials and Methods and Figure 1 for details. UTS = ultimate tensile strength.
† Significantly different versus control, by post hoc test.

Figure 3 shows images of the gross morphology and immunohistochemical staining for type I and type II collagen in samples for the 4 groups studied. Note the grossly appreciable decrease in diameter of the ouabain-treated samples compared with control. Type II collagen was present in all groups. Type I collagen was absent from the control and ouabain groups but stained weakly in the ionomycin and combined treatment groups. Congruent with the gross morphology observations, the diameters of the constructs treated with ouabain or the combination of ouabain and ionomycin were significantly less than the diameters of controls or ionomycin-treated constructs. The mean ± SD diameters measured were 6.77 ± 0.27, 6.18 ± 0.14, 6.71 ± 0.28, and 6.40 ± 0.18 mm for control, ouabain, ionomycin, and combined treatment, respectively. Similarly, for construct wet weights, treatment with ouabain or the combination of ouabain and ionomycin resulted in constructs with significantly less wet weight than controls or ionomycin-treated constructs. The mean ± SD construct wet weights were 28.7 ± 6.0, 17.8 ± 1.1, 30.0 ± 2.7, and 19.7 ± 1.8 mg for control, ouabain, ionomycin, and combined treatment, respectively. For construct thickness, ouabain-treated and combined treatment constructs were significantly thinner than ionomycin-treated constructs but were not significantly different from control construct thickness. The mean ± SD construct thicknesses measured were 0.74 ± 0.08, 0.62 ± 0.04, 0.84 ± 0.14, and 0.62 ± 0.13 mm for control, ouabain, ionomycin, and combined treatment, respectively. These growth metric findings recapitulate phase II results.

In terms of the biochemical composition of the constructs, there were no significant differences in the number of cells per construct or the percentage of GAG normalized to construct wet weight. The mean ± SD percentages of GAG per construct wet weight were 8.0 ± 0.7, 8.0 ± 0.5, 7.3 ± 0.6, and 7.2 ± 0.3% for control, ouabain, ionomycin, and combined treatment, respectively. Figure 4B shows the results for total collagen normalized to construct wet weight. Treatment with ouabain or the combination of ouabain and ionomycin resulted in constructs with significantly increased collagen concentrations compared with controls or ionomycin-treated constructs. There was an ~30% increase over control in the percentage of collagen normalized to wet weight for both the ouabain and the combination treatment groups. When normalized to construct cellular content, treatment with ouabain or the

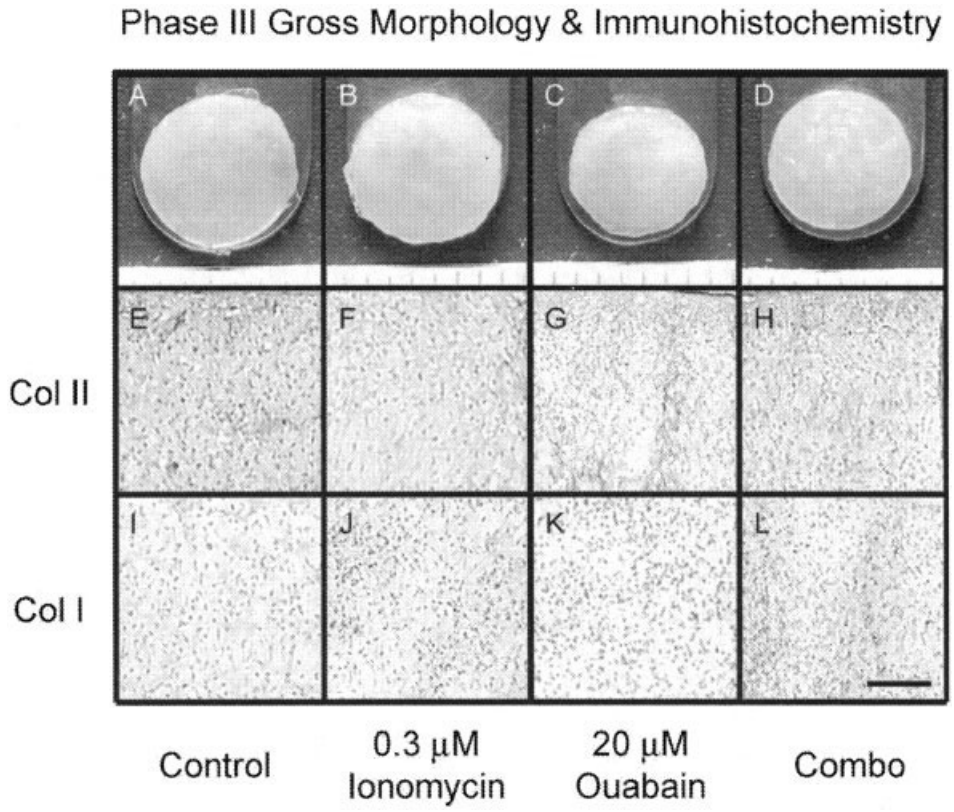

Figure 3. Images of gross morphology (**A–D**) and immunohistochemical staining for type II collagen (Col II) (**E–H**) and type I collagen (**I–L**) from the phase III experiment. Note the smaller size of the ouabain-treated constructs. All of the groups showed staining for type II collagen, while the group treated with ionomycin also showed trace staining for type I collagen. Combo = combination. Bar = 100 μm.

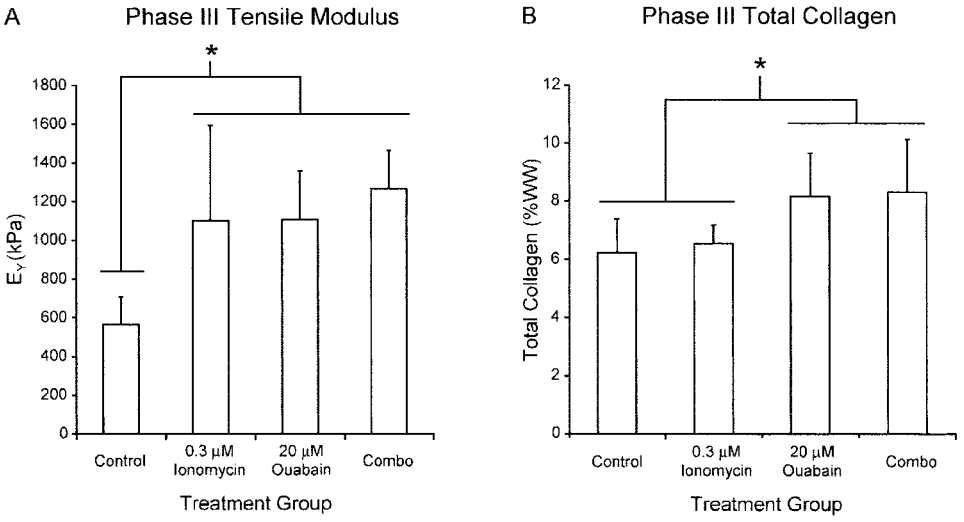

Figure 4. Phase III tensile stiffness (**A**) and total collagen normalized to construct wet weight (WW) (**B**). All 3 treatments resulted in an ~95% increase in tensile stiffness compared with control, while groups treated with ouabain contained significantly more total collagen per wet weight than controls. Bars show the mean and SD. $* = P < 0.05$.

combination of ouabain and ionomycin resulted in significantly less GAG production per cell compared with control or ionomycin-treated constructs. The mean ± SD amounts of GAG produced per 10^6 cells were 429 ± 39, 324 ± 19, 418 ± 23, and 305 ± 18 μg for control, ouabain, ionomycin, and combined treatment, respectively. As measured by hydroxyproline, there were no differences in the amount of collagen production per cell. The mean ± SD amounts of collagen produced per 10^6 cells were 346 ± 41, 328 ± 51, 373 ± 23, and 343 ± 57 μg for control, ouabain, ionomycin, and combined treatment, respectively.

In terms of construct mechanical properties, as seen in phase II, there were no significant differences in compressive properties. The mean ± SD construct aggregate moduli measured were 174 ± 120, 132 ± 71, 130 ± 54, and 186 ± 105 kPa for control, ouabain, ionomycin, and combined treatment, respectively. The permeabilities were 42 ± 20, 49 ± 27, 51 ± 35, and 43 ± 48 $\times 10^{-15}$ $m^4/N \cdot$ second, while the compressive Poisson ratios were 0.16 ± 0.14, 0.18 ± 0.10, 0.22 ± 0.07, and 0.21 ± 0.09 for control, ouabain, ionomycin, and combined treatment, respectively. Figure 4A shows the construct tensile stiffness. All treatment groups had significantly increased Young's moduli compared with control. There was at least a 95% increase in tensile stiffness compared with control for all treatments. In terms of the UTS, only the ouabain-treated constructs had significantly greater strength than control (an 86% increase) (mean ± SD 258 ± 78, 479 ± 74, 340 ± 144, and 407 ± 192 kPa for control, ouabain, ionomycin, and combined treatment, respectively). In terms of tensile Poisson ratios, there were no significant differences between groups, although there was a trend toward an increased tensile Poisson ratio compared with control ($P = 0.12$) in ouabain-treated constructs (mean ± SD 0.38 ± 0.14, 0.55 ± 0.16, 0.43 ± 0.10, and 0.45 ± 0.19 for control, ouabain, ionomycin, and combined treatment, respectively).

DISCUSSION

In this study, a series of experiments identified 20 μM ouabain, an inhibitor of the Na^+/K^+ pump, and 0.3 μM ionomycin, a calcium ionophore, as modulators of intracellular ion concentrations that led to increased tensile properties in developing articular cartilage constructs. Specifically, 20 μM ouabain, 0.3 μM ionomycin, or the combination increased the tensile modulus 40–95% compared with untreated controls. Moreover, treatment with 20 μM ouabain increased construct UTS and trended toward an increased tensile Poisson ratio. The results of this study suggest that chemical modulation of intracellular ion concentrations may be useful for functional tissue engineering of articular cartilage.

Although modulation of both intracellular Na^+ and Ca^{2+} yielded similar functional improvement at 4 weeks, intracellular Na^+ and Ca^{2+} modulation showed differential behavior over the course of these experiments. First, the growth metrics of developing constructs were affected by intracellular Na^+ modulation with ouabain but not by intracellular Ca^{2+} modulation with ionomycin. Ouabain treatment resulted in decreased diameter and wet weight, with 100 μM causing a greater decrease in both metrics compared with 20 μM. Ionomycin treatment, for either 1 hour daily for 5 days or for 5 days continuously, did not significantly alter construct wet weight or thickness. These changes in growth metrics reflect the fact that ouabain treatment significantly reduced GAG production on a per-cell basis, whereas ionomycin treatment did not. Lower GAG levels are associated with decreased wet weight and size.

Second, the tensile property increase after treatment with ouabain was related to an increase in the percentage of collagen per wet weight at the same time point, while the increase in the percentage of collagen per wet weight after treatment with ionomycin was observed at 2 weeks, with increased tensile stiffness at 4 weeks. A delay in tensile stiffness compared with increased collagen has been previously observed following HP application (12), with the delay likely attributable to the relatively slow rate of collagen remodeling within the matrix.

Finally, ionomycin caused trace type I collagen staining, while ouabain treatment did not. The effects of Ca^{2+} have previously been related to the chondrocyte phenotype (9), and it was suggested that increased levels of intracellular Ca^{2+} may induce the production of type I collagen. These findings demonstrate that Na^+ and Ca^{2+} modulation have different effects on developing articular cartilage constructs. Further understanding of these differences will contribute to improved control over articular cartilage tissue-engineering processes.

It has been suggested that continued matrix deposition with relative maintenance of the construct or tissue explant size represents maturational growth (16,25). Thus, treatment with ouabain may be a pharmacologic method for directing the maturation of engineered cartilage. Articular cartilage tissue-engineering efforts are hampered by poor collagen content (15,26–28), whereas attaining GAG levels that are in the range of the values for native tissue is readily accomplished

(16,27,29–32). This imbalance results in engineered constructs with reasonable compressive properties but poor tensile integrity. Although GAG production on a per-cell basis was decreased following ouabain treatment, there was net positive GAG deposition between 2 weeks and 4 weeks (compare the percent wet weight of GAG multiplied by the construct wet weight at these time points). However, the net deposition was less than that for controls. The decrease in GAG deposition in ouabain-treated constructs compared with control constructs likely allows for a more appropriate balance between GAG and collagen, resulting in increased tensile integrity.

The engineering of articular cartilage with increased tensile stiffness is a recognized hurdle (26–28). Results from this study show that treatment with 20 μM ouabain or 0.3 μM ionomycin is effective toward achieving this goal. Further experiments should investigate whether these treatments can be combined with other known methods of increasing tensile properties of tissue-engineered articular cartilage (13,14,16,33). Moreover, although measurement of tensile stiffness is becoming more common in articular cartilage tissue engineering (12–14,16,31,34–38), to our knowledge, the data presented in this study are the first measurements of the tensile Poisson ratio in engineered articular cartilage; furthermore, this material property may change in response to altered intracellular Na^+. These data may be useful in growth mixture models of articular cartilage and the collagen network (39), which are aided by experimentally measured biomechanical properties.

Literature on the effects of intracellular ion regulators in cartilage tissue engineering has been sparse. For example, gadolinium, an inhibitor of stretch-activated Ca^{2+} channels, was shown to abrogate proteoglycan synthesis that occurred in response to dynamic compression of chondrocyte-embedded agarose constructs (40). Mouw et al (41) have also examined the relationship of ion channels and compression-induced mechanotransduction. Chondrocyte-seeded agarose gels treated with different ion channel modulators were subjected to dynamic compression. In the presence of nifedipine (a Ca^{2+} channel blocker) or thapsigargin (a releaser of intracellular Ca^{2+}), compression significantly increased collagen synthesis, while the presence of gadolinium led to an increase in both collagen and sGAG synthesis. The present study is the first to demonstrate that directed manipulation of intracellular ion concentrations can result in changes in the functional properties of developing neo-tissue.

The downstream mechanisms of how the alteration of ion concentrations results in changes in matrix synthesis and mechanical properties should be deciphered in future studies, now that a direct effect has been shown. Other stimuli known or suspected to function via changes in intracellular ion concentrations, such as dynamic compression (42), fluid-induced shear loading (11,43–45), and HP (3,5,6), are notable here. Results from this study may be related to mechanotransduction occurring during static HP application. Supporting this idea, the intracellular Na^+ and Ca^{2+} modulators chosen for systematic study were based on the known effects of HP application to chondrocytes (3,5,6). These chemical agents were also applied following the same regimen used for HP treatment of developing engineered articular cartilage, a regimen that is known to alter the biochemical composition and mechanical properties of constructs formed in the same system used in the present study (12,31). Several of the findings by Elder and Athanasiou (12) are paralleled in the present study, such as an increased percentage of collagen per wet weight at 2 weeks (with 0.3 μM ionomycin) and 4 weeks (with 20 μM ouabain or the combination) and increased tensile properties at 4 weeks (20 μM ouabain, 0.3 μM ionomycin, or the combination).

The fact that only some of the findings of HP application were recapitulated by treatment with ouabain, ionomycin, or their combination is not surprising given that HP is known to do more than inhibit the Na^+/K^+ pump and alter intracellular Ca^{2+}. For example, the application of HP has known effects on the Na^+/H^+ exchanger (46), the glucose carrier (47), and stretch-activated channels (48). The treatments used in this study are not known to directly affect these proteins. Additionally, the effects of bumetanide may have been mitigated in the present experiment due to the culture medium used. Because the medium osmolarity is not isotonic with chondrocytes in situ, the $Na^+/K^+/2Cl^-$ cotransporter is relatively inactive in the experimental environment (3). Bumetanide could not be expected to inhibit an already-quiescent transporter. Furthermore, the present study did not assess whether changes in intracellular ion concentrations following loading with 10 MPa of static HP and treatment with 20 μM ouabain or 0.3 μM ionomycin are similar. It could be that the doses of ouabain or ionomycin studied do not produce changes similar to what occurs during HP in our 3-dimensional chondrocyte culture. These are potential reasons for why no changes were observed in the percentage of GAG per wet weight or compressive properties with the treatments investigated in this study, and

they suggest a plethora of future experiments to be performed.

How oubain or ionomycin treatment leads to increased tensile properties, the minimal duration of treatment necessary to effect these changes, or what the optimal culture conditions are for the use of these treatments are unanswered questions. In this study, treatment was applied for 1 hour daily. It is possible that the observed effects may have been dominated by processes initiated early during treatment, because prolonged alteration of ionic concentrations can lead to changes in membrane transport proteins. For example, the ionic and osmotic environment of chondrocytes is known to alter Na^+/K^+ pump abundance (49,50). Furthermore, because ion transporter activity is influenced by extracellular ionic and osmotic conditions, experiments using these treatments in different medium formulations could lead to enhanced effects. Despite these unanswered questions, treatment with 20 μM ouabain and 0.3 μM ionomycin for 1 hour daily in the present medium formulation improved construct tensile integrity.

In summary, this study investigated whether chemical modulation of intracellular sodium and calcium would alter articular cartilage construct development. Through a series of experiments, both 20 μM ouabain and 0.3 μM ionomycin were demonstrated to increase the percentage of collagen per wet weight and the tensile properties of tissue-engineered constructs. The data presented provide insight into potentially important aspects of mechanotransduction during HP application and demonstrate that it may be possible to find chemical equivalents to some forms of mechanical stimulation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Natoli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Natoli, Skaalure, Bijlani, Chen, Hu, Athanasiou.

Acquisition of data. Natoli, Skaalure, Bijlani, Chen, Hu.

Analysis and interpretation of data. Natoli, Skaalure, Bijlani, Chen, Hu, Athanasiou.

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