Hyperosmolar Potassium (K^+) Treatment Suppresses Osteoarthritic Chondrocyte Catabolic and Inflammatory Protein Production in a 3-Dimensional *In Vitro* Model

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Abstract

Objective. The main goal of this study was to provide a proof-of-concept demonstrating that hyperosmolar K^+ solutions can limit production of catabolic and inflammatory mediators in human osteoarthritic chondrocytes (OACs). **Methods.** A 3-dimensional *in vitro* model with poly(ethylene glycol) diacrylate (PEGDA) hydrogels was used. Catabolic and pro-inflammatory protein production from encapsulated OACs was assessed following culture for 1 or 7 days in the presence or absence of 80 mM K^+ gluconate, 80 mM sodium (Na^+) gluconate, or 160 mM sucrose, each added to culture media (final osmolarity ~490 mOsm). **Results.** Relative to untreated controls, OACs treated with hyperosmolar (80 mM Na^+ gluconate or 160 mM sucrose) solutions produced lower levels of catabolic and inflammatory mediators in a marker- and time-dependent manner (i.e., MMP-9 after 1 day; MCP-1 after 7 days ($P \leq 0.015$)). In contrast, OAC treatment with 80 mM K^+ gluconate reduced catabolic and inflammatory mediators to a greater extent (both the number of markers and degree of suppression) relative to untreated, Na^+ gluconate, or sucrose controls (i.e., MMP-3, -9, -13, TIMP-1, MCP-1, and IL-8 after 1 day; MMP-1, -3, -9, -13, TIMP-1, MCP-1, and IL-8 after 7 days ($P \leq 0.029$)). **Conclusions.** Hyperosmolar K^+ solutions are capable of attenuating protein production of catabolic and inflammatory OA markers, providing the proof-of-concept needed for further development of a K^+ -based intra-articular injection for OA treatment. Moreover, K^+ performed significantly better than Na^+ - or sucrose-based solutions, supporting the application of K^+ toward improving irrigation solutions for joint surgery.

Keywords

chondrocyte activation, osteoarthritis, potassium, catabolism, inflammation

Introduction

In osteoarthritis (OA), chondrocytes become activated (in part by inflammation) and lose the balance between anabolic and catabolic activity, favoring matrix degradation (catabolism) and hypertrophic differentiation.^{1–3} The long-term goal of this work is to develop a potassium (K^+)-based intra-articular injection treatment for OA. Our working hypothesis is that K^+ can be used to limit 2 processes associated with cartilage matrix degeneration: (1) chondrocyte hypertrophic differentiation/catabolism and (2) production of pro-inflammatory mediators. This hypothesis was derived from 2 fields. First, studies from the bioelectricity field posit that cell state is related to a cell's transmembrane potential (V_{mem}), with depolarized and hyperpolarized V_{mem} values being associated with stem/proliferative/cancer cells or terminally differentiated cells, respectively.^{4–7} Adding extracellular hyperosmolar K^+ to culture media depolarizes V_{mem} and has been demonstrated to prevent adipocytic and osteoblastic differentiation of human mesenchymal stem

cells, even in the presence of the most potent chemical factors known to drive differentiation down these lineages.⁸ Second, the powerful capacity of K^+ to modulate cell phenotype was recently reinforced, albeit in a different, immunosuppressive context. Here, hyperosmolar extracellular K^+ was demonstrated to suppress T-cell effector function, independent of its influence on V_{mem} and separate from other immunosuppressive chemical mediators.⁹ Thus, hyperosmolar K^+ may exert positive influences on major OA disease processes coupled to cartilage matrix degradation—chondrocyte hypertrophic differentiation/catabolism and inflammation.

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Separate from our intentions for the development of an intra-articular OA therapy, using K^+ to improve hyperosmolar saline joint irrigation solutions for surgery represents another goal of this work. Hypertonic saline solutions (~600 mOsm) are currently being developed and validated for limiting cartilage damage (and posttraumatic OA) during surgical procedures, conferring chondroprotective effects in a number of model systems.¹⁰⁻¹⁴ Although the underlying mechanisms have not been investigated, the hyperosmolarity of the solution, rather than the ionic content, is considered to be most important.¹⁰⁻¹⁵ Interestingly, the sodium ion (Na^+) in saline has recently been demonstrated to be pro-inflammatory¹⁶⁻¹⁹ and therefore may exacerbate joint inflammation. Thus, a hyperosmolar K^+ solution may retain beneficial effects of hyperosmolarity and provide independent immunosuppression while circumventing potential inflammation induced by Na^+ .

In the present study, we first show that treatment of human osteoarthritic chondrocytes (OACs) with hyperosmolar K^+ promotes an anabolic/healthy gene signature with a concomitant decrease in catabolic/OA gene levels. The superior capacity of hyperosmolar K^+ solutions (over Na^+ or sucrose-based solutions) to attenuate production of catabolic, inflammatory, and OA markers in OACs was then examined in depth at the protein level.

Materials and Methods

Polymer Synthesis and Functionalization with Cell Adhesive Peptides

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized from PEG-diol (6 kDa, Sigma Aldrich) at ~99% acrylation as reported previously.²⁰ NH_2 -Arg-Gly-Asp-Ser-COOH (RGDS; American Peptide Company) was included to facilitate cell attachment within the PEGDA network. The product (ACRL-PEG-RGDS) was purified by dialysis, lyophilized, and stored at $-80^\circ C$ until further use.

Osteoarthritic Chondrocyte (OAC) Culture and Encapsulation

Primary human OACs (Cell Applications Inc.) were thawed and expanded in chondrogenic growth medium (Lonza) within a $37^\circ C$ -5% CO_2 jacketed incubator. One passage before experiments, the cells were transitioned to regular growth medium: high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Fisher Scientific).

Prior to cell encapsulation, a 10% (w/v) PEGDA hydrogel precursor solution was prepared in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) with 3.9 mg/mL ACRL-PEG-RGDS. OACs (passage 5) were harvested and the resulting cell pellet was resuspended in the

hydrogel precursor solution at 5×10^6 cells per mL. Aliquots (100 μL ; 0.5×10^6 cells per construct) of the cell suspension were dispensed into the wells of a 96-well plate (Corning) and cured by exposure to longwave ultraviolet light (~ 10 mW/cm²) for 6 minutes. Importantly, human OACs have been demonstrated to form hyaline cartilage in high-density culture, even after extended 2-dimensional (2D) passaging (9 population doublings) prior to seeding as high-density cultures²¹ and have previously been used to model OA inflammation *in vitro*.²²

Postfabrication, all hydrogel discs were washed with DPBS for 5 minutes and immersed in cell culture medium. After 24 hours of equilibration in regular growth media, the OAC-laden hydrogels cultured with or without 80 mM K^+ gluconate, 80 mM Na^+ gluconate, or 160 mM sucrose (all from Sigma) for 1 or 7 days. At culture endpoints, the hydrogels were washed in DPBS for 5 to 10 minutes, harvested by flash-freezing in liquid nitrogen, and stored at $-80^\circ C$ until further analysis.

Reverse Transcriptase Quantitative Polymerase Chain Reaction (qPCR)

mRNA and protein extraction from hydrogels were performed as previously described.²² qPCR was performed to compare mRNA levels across the various experimental groups using a StepOne Real-Time PCR system and the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies) according to the manufacturer's instructions. Validated qPCR primers were purchased from Qiagen or OriGene. Available primer sequences are provided in **Table 1**. Gene expression was normalized to 3 reference genes (GAPDH, L32, and β -actin) and expressed relative to day 1 control OACs. Melting temperature analysis was performed for each reaction to verify the appropriate amplification product.

MAGPIX Immunoassay Multiplexing

The protein levels of matrix metalloproteinase (MMP)-1, -3, -9, and -13, tissue inhibitor of metalloproteinase-1 (TIMP-1), monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) were measured from cell lysates using a human premixed magnetic bead analyte kit (R&D Systems) and a MAGPIX detection system (Luminex). Briefly, samples were loaded into a 96-well plate, after which magnetic bead suspensions, detection antibodies, and streptavidin-phycoerythrin were added to sample wells. Sample concentrations of proteins were obtained on the basis of a median fluorescence intensity (MFI) relative to a standard curve. The resulting measures were then normalized by sample DNA content, assessed with the PicoGreen assay per the manufacturer's instructions (Life Technologies). Data were

Table 1. Primer Sequences Used for qRT-PCR Analysis of Genes Associated with Anabolic/Healthy and Catabolic/OA Chondrocyte Phenotypes.

Function	Gene Marker	Primer Sequence, Forward (F), Reverse (R)
Anabolic/Healthy markers	SOX9	F: AGGAAGCTCGCGGACCACTAC R: GGTGGTCCTTCTTGCTGCAC
	Aggrecan (ACAN) Col 2A1	Qiagen Qiagen
Catabolic/OA markers	RUNX2	F: CCCAGTATGAGAGTAGGTGTCC R: GGGTAAGACTGGTCATAGGACC
	MMP-13	F: CCTTGATGCCATTACCACTCTCC R: AAACAGCTCCGCATCAACCTGC
	Col 1A1	Qiagen
Reference/Housekeeping	β -actin	F: CACCATTGGCAATGAGCGGTTTC R: AGGTCTTTGCGGATGTCCACGT
	GAPDH	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA
	L32	F: ACAAAGCACATGCTGCCCACTG R: TTCCACGATGGCTTTGCGGTTTC

OA = osteoarthritis; qRT-PCR = quantitative reverse transcriptase–polymerase chain reaction.

then further normalized to the control, untreated OACs for each experimental time point.

Justification of Experimental Parameters

Three-Dimensional Culture with PEGDA Hydrogels. PEGDA hydrogels were selected as the material for the 3D *in vitro* model due to their widespread use in cartilage research^{23–27} and for the ability of these materials to restrict cell proliferation and protein adhesion, even in serum-containing culture environments.²⁸ The latter property enables tight control over the study of the treatment in question on cell phenotype, without confounding influences from the selection of specific phenotypes over time or due to presence of adhered proteins. In the present studies, we have tethered the peptide RGD to the PEGDA network to enable consistent initial cell-matrix adhesion levels across experimental groups.

Concentration of Osmolytes. The concentrations of added osmolytes (i.e., 80 mM for ionic and 160 mM for nonionic) were selected based off literature from the bioelectricity field^{8,29–32} and for achieving osmolarities similar to those being investigated for joint irrigation solutions.^{10–15} The base osmolarity of high glucose DMEM is ~330 mOsm/L, which is raised to ~490 mOsm/L with 160 mOsm of additional osmolytes. These values are comparable, although not as hyperosmolar, to those used in hypertonic saline studies, where 300 and 600 mOsm/L solutions represent control and hyperosmolar formulations, respectively.^{10–15}

Endpoint Marker Assessment. For gene expression analyses, all proteins selected play major roles in cartilage

biology and/or OA. Sex determining region Y Box 9 (SOX9) and runt-related transcription factor 2 (RUNX2) were analyzed because they are major transcription factors involved in maintaining a healthy/anabolic and hypertrophic/catabolic chondrocyte phenotype, respectively.^{33,34} Aggrecan (ACAN) and collagen type II, alpha-1 chain (Col 2A1) comprise most of the protein/extracellular matrix (ECM) content found in hyaline cartilage³⁵ and are associated with an anabolic phenotype.^{2,3,33} Finally, MMP-13 and collagen type I, alpha-1 chain (Col 1A1) are proteins associated with the catabolic, hypertrophic differentiation of chondrocytes in addition to being upregulated in all stages of OA.^{33,36}

For protein analyses, the selected markers all have been demonstrated to stimulate OA disease progression (for reviews, see references^{1–3,37–39}). Generally, in cartilage and OA, MMP-1, -3, -9, and -13 are widely studied.^{37,40–42} In particular, MMP-13, which degrades collagen type II, the major collagen protein found in articular cartilage, has received considerable attention.^{33,43} Although TIMP-1 limits matrix degradation, TIMP-1 was analyzed because this protein has been shown to be increased in OA,^{44,45} aging-related processes,^{46,47} angiogenesis,⁴⁸ and fibrosis^{49,50} — all of which are considered undesirable in OA. The inflammatory proteins IL-8,^{51,52} VEGF,^{53,54} and MCP-1^{55,56} were selected because they have been demonstrated to exacerbate OA progression/cartilage degradation. To help segment the study and aid in presentation of the results, the MMPs and TIMP-1 have been grouped together under “catabolic/OA markers” while the inflammatory proteins have been grouped together under “inflammation/OA markers.”

Statistical Analyses

All data are reported as the mean $\pm$ standard deviation and are representative of 2 independent experiments with the same OA donor. In each experiment, results presented for each treatment group were based on measures from 4 independent hydrogel samples. Means were compared using either a 2-way or 1-way analysis of variance (ANOVA; $n = 4$ samples per group), as appropriate. In the first experiment, a 2-way ANOVA was used to determine significance resulting from main effects of 80 mM K^+ gluconate treatment or culture time. Homogeneity of variance was confirmed using Levene's test. For the second experiment, comparison of experimental group means was performed using Tukey's post hoc test or a Games-Howell post hoc test (in cases where Levene's test returned a significant result). For all tests, $P < 0.05$ was considered significant and SPSS software was used.

Results

Initial Characterization of Hyperosmolar K^+ -Treated OACs

Before moving into more complex experiments, we first asked what happens at the gene level when OACs are treated with solutions of hyperosmolar K^+ . Briefly, OACs were encapsulated in 3D PEGDA hydrogels and treated with 80 mM K^+ gluconate for 1 and 7 days (**Fig. 1A**). Relative to control OACs, 80 mM K^+ gluconate treated OACs exhibited significantly increased mRNA levels of all anabolic/healthy markers investigated, including SOX9, ACAN, and Col 2A1 ($P < 0.015$; **Fig. 1B**). In addition, the mRNA levels of critical catabolic/OA markers MMP-13 and Col 1A1 were significantly suppressed ($P < 0.0005$) whereas RUNX2 remained unchanged in OACs treated with 80 mM K^+ gluconate (**Fig. 1C**). Cumulatively, these data suggest that hyperosmolar K^+ gluconate treatment promotes an anabolic/healthy chondrocyte phenotype and suppresses a catabolic/osteoarthritic phenotype.

Isolating Effects of K^+ , Gluconate, Ionic Strength, and/or Osmolarity

Based off our initial results, we conducted an additional experiment to determine if the effects noted previously were from the K^+ ions, gluconate ions, ionic strength and/or osmolarity of the solution (**Fig. 2**). An 80 mM Na^+ gluconate group was included to control for the effects of the gluconate ion and the ionic strength of the solution whereas a 160 mM sucrose group was included as an osmolarity/non-ionic osmolyte control. In addition to these controls, we also analyzed a more complete set of phenotypic markers at the protein level using MAGPIX multiplexing technology

Figure 1. (A) Experimental design for the pilot study to investigate influence of hyperosmolar K^+ on gene expression of osteoarthritic chondrocytes (OACs.) (B) Relative mRNA expression of anabolic/healthy and (C) catabolic/OA markers in OACs cultured for 1 or 7 days in the presence of 80 mM K^+ gluconate. A combination of L32, GAPDH, and α -actin were employed as house-keeping/reference genes. *Denotes a significant main effect of 80 mM K^+ gluconate. #Denotes a significant main effect of culture time.

(**Fig. 2**). Emphasis was placed on catabolic and inflammatory markers known to be elevated in OA because of their important role in OA pathology and cartilage degradation in general. For these experiments, we were particularly interested in effects noticed after 1 day of stimulation, as this time point is more comparable to joint irrigation during surgery than the 7-day time point. A longer culture time was included to yield further information about how these

Figure 2. Experimental design for determining the influence of hyperosmolar solutions of differing osmolyte composition on the production of catabolic and inflammatory proteins from osteoarthritic chondrocytes (OACs) in 3-dimensional poly(ethylene glycol) diacrylate (PEGDA) hydrogels. Similar to the pilot study, culture was performed for 1 and 7 days before sample analyses. Na^+ gluconate (80 mM) was included to control for effects of ionic strength and the gluconate ion. Sucrose (160 mM) was included as a nonionic osmolyte control.

Figure 3. Relative protein production of several catabolic/OA markers in OACs cultured for (A) 1 day or (B) 7 days in the presence or absence of hyperosmolar solutions of differing compositions. *Denotes a significant difference relative to control (Cntl) osteoarthritic chondrocytes (OACs). #Denotes a significant difference relative to 80 mM K^+ gluconate.

solutions influence OACs and for experimental design consistency with our pilot study.

Catabolic/Osteoarthritis Markers. Figure 3 displays the relative protein production of several catabolic, matrix-degrading enzymes (MMP-1, MMP-3, MMP-9, and MMP-13) as

well as TIMP-1 in OACs treated with various hyperosmolar solutions for 1 or 7 days. Data are presented relative to control/untreated OACs for each time point and normalized to DNA content shown in **Supplementary Figure 1**. Absolute values of protein levels (expressed as pg protein/ng DNA) are shown in **Supplementary Figure 2**.

After 1 day of treatment, all 3 hyperosmolar solutions significantly reduced the production of MMP-9 ($\leq 80\%$, $P \leq 0.012$; **Fig. 3A**). Aside from this single difference, there were several instances where K^+ gluconate further limited the production of matrix-degrading/OA markers. Specifically, OACs treated with K^+ gluconate produced significantly less MMP-3 (40%, $P = 0.002$) and MMP-13 (65%, $P = 0.002$) relative to control OACs (**Fig. 3A**). In contrast, treatment with either 80 mM Na^+ gluconate or 160 mM sucrose did not significantly alter levels of MMP-3 or MMP-13 relative to control OACs (**Fig. 3A**). Moreover, OACs treated with K^+ gluconate exhibited significantly reduced MMP-13 (70%, $P = 0.011$) levels relative to Na^+ gluconate treated OACs and TIMP-1 levels relative to Na^+ gluconate (71%, $P = 0.011$) and sucrose (76%, $P = 0.045$) treated OACs (**Fig. 3A**). These day 1 data suggest that K^+ gluconate is superior at suppressing catabolic/OA related markers compared with Na^+ gluconate or sucrose.

Similar to day 1, the influence of osmolarity was limited after 7 days, although the markers that were significantly altered changed with time (**Fig. 3A vs B**). After 7 days, relative to control OACs, all 3 hyperosmolar solutions significantly reduced the production of TIMP-1 ($\leq 75\%$, $P < 0.0005$), and solutions with greater ionic strength (Na^+ and K^+ gluconate) significantly reduced MMP-13 ($\leq 71\%$, $P \leq 0.025$; **Fig. 3B**). Several additional differences were also observed in the K^+ gluconate group at 7 days. Here, OACs treated with K^+ gluconate produced significantly less MMP-1 (74%, $P = 0.029$) relative to control OACs (**Fig. 3B**). In contrast, treatment with either Na^+ gluconate or sucrose did not elicit any change in MMP-1 levels (**Fig. 3B**). Furthermore, relative to Na^+ gluconate, OACs treated with K^+ gluconate produced significantly reduced levels of all catabolic/OA markers investigated ($\leq 66\%$, $P \leq 0.023$; **Fig. 3B**). Similarly, relative to sucrose, K^+ gluconate treatment resulted in significantly lower production of MMP-3, MMP-13, and TIMP-1 ($\leq 57\%$, $P \leq 0.018$; **Fig. 3B**).

Cumulatively, these data suggest that (1) hyperosmolar solutions suppress several important catabolic/OA markers in a limited, marker-specific, and time-dependent manner; (2) treatment of OACs with 80 mM K^+ gluconate further attenuates the number and production of these markers; and (3) K^+ is likely responsible for the additional beneficial response.

Inflammation/Osteoarthritis Markers. **Figure 4** displays the relative protein production of several inflammatory (MCP-1, VEGF, IL-8) markers in OACs treated with various hyperosmolar solutions for 1 or 7 days. Data are presented relative to control/untreated OACs for each time point and normalized to DNA content shown in **Supplementary Figure 1**. Absolute values of protein levels (expressed as pg protein/ng DNA) are shown in **Supplementary Figure 3**.

Figure 4. Relative protein production of several inflammation/osteoarthritis (OA) markers in osteoarthritic chondrocytes (OACs) cultured for (A) 1 day or (B) 7 days in the presence or absence of hyperosmolar solutions of differing compositions. *Denotes a significant difference relative to control (Cntl) OACs. #Denotes a significant difference relative to 80 mM K^+ gluconate. +Denotes a significant difference relative to 80 mM Na^+ gluconate.

After 1 day of treatment, all 3 hyperosmolar solutions significantly reduced the production of IL-8 ($\leq 59\%$, $P \leq 0.002$) while none of the treatments influenced VEGF (**Fig. 4A**). However, OACs treated with 80 mM K^+ gluconate produced significantly less MCP-1 ($\leq 80\%$, $P < 0.048$) relative to all other experimental groups (**Fig. 4A**). In contrast, treatment with either 80 mM Na^+ gluconate or 160 mM

sucrose did not significantly alter levels of MCP-1 relative to control OACs (**Fig. 4A**). These day 1 data suggest that K^+ gluconate is superior at suppressing inflammation/OA related markers compared to Na^+ gluconate or sucrose.

After 7 days of culture, relative to control OACs, all three hyperosmolar solutions significantly reduced production of IL-8 ($\leq 28\%$, $P < 0.0005$) and MCP-1 ($\leq 83\%$, $P < 0.015$; **Fig. 4B**). However, similar to day 1, OACs in the K^+ gluconate group produced significantly less MCP-1 relative to Na^+ gluconate and sucrose ($\leq 78\%$, $P < 0.019$; **Fig. 4B**).

Cumulatively, these data suggest that (1) hyperosmolar solutions suppress several important inflammation/OA markers in a limited, marker-specific, and time-dependent manner; (2) treatment of OACs with 80 mM K^+ gluconate further reduces production of select markers; and (3) K^+ is likely mediating the additional beneficial effects.

Discussion

The long-term goal of this work is to develop a K^+ -based intra-articular injection for the treatment of OA and to improve irrigation solutions currently used for joint surgery. However, the capacity of K^+ to suppress catabolic, inflammatory, and OA disease markers in chondrocytes has never been explicitly investigated. Thus, the objective of the current study was to provide an initial proof-of-concept and to highlight the therapeutic potential of K^+ for OA research. In all studies, 3D cell culture in PEGDA hydrogels was used as an *in vitro* model. The concentrations of osmolytes (i.e., 80 mM for ionic and 160 mM for nonionic) were selected based off literature from the bioelectricity field^{8,29-32} and for achieving osmolarities similar to those being investigated for joint irrigation solutions.¹⁰⁻¹⁵ Markers selected for end-point analyses were chosen based off of their important roles in chondrocyte biology and for their prevalence in OA.

In our first experiment, OACs were treated with 80 mM K^+ gluconate for 1 or 7 days (**Fig. 1A**). At the gene level, qPCR analyses revealed that OACs treated with 80 mM K^+ gluconate exhibited significantly increased amounts of SOX9, ACAN, and Col 2A1 mRNA levels with a concurrent significant decrease in MMP-13 and Col 1A1 mRNA levels (**Fig. 1B and C**). These data indicated that hyperosmolar K^+ gluconate treatment promotes an anabolic/healthy and suppresses a catabolic/osteoarthritic chondrocyte gene signature.

Based off our initial results, we conducted an additional experiment to gain deeper insight into what might be eliciting the beneficial changes noted (**Fig. 2**). In a treatment of 80 mM K^+ gluconate, the cell response could be a result of any 1 of 3 factors: (1) the individual K^+ or gluconate ions, (2) the ionic strength of the solution, and/or (3) osmolarity of the solution. Decoupling these variables is necessary for demonstrating that K^+ may have therapeutic potential for

limiting cartilage catabolism and inflammation in OA and for determining the optimal composition of solutions used for the irrigation of joints during surgery.

With regard to catabolic and inflammatory proteins, hyperosmolarity exerted a suppressive influence on OACs in a limited, marker-dependent, and time-dependent manner. For instance, only MMP-9 was significantly reduced by all three hyperosmolar groups after day 1, and this significance was lost by day 7 (**Fig. 3**). Conversely, increased osmolarity did not significantly alter MCP-1 levels after 1 day of stimulation, but did significantly decrease this inflammatory chemokine by day 7 (**Fig. 4**). In contrast to these selective effects of hyperosmolarity, both the number of markers and/or their degree of suppression were significantly increased by K^+ gluconate (**Figs. 3 and 4**). Treatment with K^+ gluconate favorably diminished almost every protein investigated (MMP-1, MMP-3, MMP-9, MMP-13, TIMP-1, MCP-1, IL-8), with many of the changes occurring even after 1 day of culture (**Figs. 3 and 4**). Importantly, relative to 80 mM Na^+ gluconate and 160 mM sucrose, there was not a single instance where 80 mM K^+ gluconate stimulation resulted in significantly greater production of catabolic, inflammatory, and other proteins involved in OA progression. Cumulatively, these data provide the proof-of-concept that hyperosmolar K^+ gluconate suppresses production of catabolic and inflammatory proteins, and that the K^+ ion is the driving force behind many of these changes. Because of the significant capacity of K^+ to modulate key proteins involved in OA progression/cartilage degradation, future development of K^+ -based intra-articular injections is warranted. As OA is a disease of the entire joint, ongoing work is investigating K^+ influences on other tissues and cells, such as the macrophages in the synovium. Furthermore, the enhanced suppressive effect of K^+ gluconate over the other hyperosmolar solutions supports the notion that hypertonic solutions comprised of K^+ , rather than currently utilized Na^+ , should be considered for the development of joint irrigation formulations to promote cartilage repair/prevent degradation.

How could K^+ influence OACs, and what other molecular pathways are mechanistically involved? Whether V_{mem} alterations from 80 mM K^+ gluconate and/or a pure chemical influence of K^+ was responsible for the changes noted remains unknown and deserves further investigation. Interestingly, the changes in gene expression elicited by K^+ gluconate occurred independently of RUNX2, an essential transcription factor for hypertrophic chondrocyte differentiation whose expression often couples with MMP-13 expression.^{33,57} Similar to our gene results, enhanced hypoxia inducible factor-1 (HIF-1) stability has been shown to promote SOX9, ACAN, and Col 2A1 while concurrently suppressing Col 1A1 mRNA expression in chondrocytes.⁵⁸⁻⁶⁰ Finally, K^+ has recently been demonstrated to increase phosphorylated Smad 2/3 levels in T-cells.⁶¹ Smad

2 and 3 are important for maintaining an anabolic chondrocyte phenotype.^{3,33,62,63} Thus, future work will focus on the HIF and Smad signaling proteins in response to hyperosmolar K^+ stimulation.

A few limitations to the present study merit comment. First, extrapolating these findings to cartilage present in a diseased/injured environment may be difficult because of the experimental system used (which lacked applied inflammatory stimuli, involved extended preculture of cells from a single donor, and incorporated limited chondrogenic stimuli aside from a rounded cell morphology induced by PEGDA hydrogels). Inclusion of an inflammatory cytokine in culture media would help mimic inflammation present in OA and may further highlight the therapeutic potential of K^+ . In addition, expanding chondrocytes in 2D is known to induce dedifferentiation which may be responsible for some changes noted. That said, 3D culture has been shown to be the most effective way for inducing redifferentiation of chondrocytes expanded in 2D,⁶⁴ and 3D culture is commonly used to study chondrocyte biology.

In terms of applying K^+ -based solutions toward improving joint irrigation solutions, the exact formulation implemented in this study may not fully recapitulate those currently used clinically (i.e., 490 mOsm culture media with 10% FBS vs 600 mOsm saline). The precise formulation of osmolytes will need to be elucidated and validated with previous *in situ* models^{10,11} in subsequent work. Next, our study did not examine any potential (negative or positive) effects of K^+ on nondiseased cells that would be present in cartilage during early OA or undergoing surgery. Finally, the time of K^+ exposure (1 day) used is longer than would be feasible for intra-articular injections of small molecules (up to a 13-hour half-life),^{65,66} or for irrigation during surgery (up to 2.5 hours).^{10,11,13,14} However, K^+ has influenced key cell signaling pathways within 5 minutes of exposure.⁹ Future work will address normal and diseased cell responses following short-term stimulation utilizing several donors at lower passage numbers. Assessing these parameters will yield a deeper understanding of K^+ effects on 3D cultured chondrocytes.

In summary, this study demonstrated that hyperosmolar K^+ solutions are capable of attenuating protein production of catabolic and inflammatory OA markers, providing the proof-of-concept needed for further development of a K^+ -based intra-articular injection for OA treatment. Moreover, K^+ performed significantly better than Na^+ - or sucrose-based solutions, supporting the application of K^+ toward improving irrigation solutions for joint surgery. Future and ongoing studies will aim to characterize (1) underlying signaling mechanisms involved, (2) cell response to shorter stimulations, and (3) K^+ influence on other cell types involved in OA progression.

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Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: JEM and MSH have a provisional patent (U.S. Provisional Patent, Application Number 62/474,891) for this work.

Ethical Approval

Ethical approval was not sought for the present study because the cells were obtained from patients through a third party vendor (Cell Applications).

Informed Consent

Informed consent was not sought for the present study because it was previously obtained through the third party vendor in which the cells were purchased from (Cell Applications).

Trial Registration

Not applicable.

Supplementary Material

Supplementary material for this article is available online.

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