Experimental Cell Research 408 (2021) 112841

Contents lists available at ScienceDirect

Experimental Cell Research

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

Combining stretching and gallic acid to decrease inflammation indices and promote extracellular matrix production in osteoarthritic human articular chondrocytes

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ARTICLE INFO

Keywords:
Articular cartilage tissue engineering
Chondrocytes
Collagen
Cyclic tensile stress
Gallic acid
Glycosaminoglycan
And inflammation

ABSTRACT

Osteoarthritis (OA) patients undergo cartilage degradation and experience painful joint swelling. OA symptoms are caused by inflammatory molecules and the upregulation of catabolic genes leading to the breakdown of cartilage extracellular matrix (ECM). Here, we investigate the effects of gallic acid (GA) and mechanical stretching on the expression of anabolic and catabolic genes and restoring ECM production by osteoarthritic human articular chondrocytes (hAChs) cultured in monolayers. hAChs were seeded onto conventional plates or silicone chambers with or without 100 μM GA. A 5% cyclic tensile strain (CTS) was applied to the silicone chambers and the deposition of collagen and glycosaminoglycan, and gene expressions of collagen types II (COL2A1), XI (COL11A2), I (COL1A1), and X (COL10A1), and matrix metalloproteinases (MMP-1 and MMP-13) as inflammation markers, were quantified. CTS and GA acted synergistically to promote the deposition of collagen and glycosaminoglycan in the ECM by 14- and 7-fold, respectively. Furthermore, the synergistic stimuli selectively upregulated the expression of cartilage-specific proteins, COL11A2 by 7-fold, and COL2A1 by 47-fold, and, in contrast, downregulated the expression of MMP-1 by 2.5-fold and MMP-13 by 125-fold. GA supplementation with CTS is a promising approach for restoring osteoarthritic hAChs ECM production ability making them suitable for complex tissue engineering applications.

1. Introduction

Osteoarthritis (OA) is the most common musculoskeletal disorder in the world [1]. It has been recently considered a major public health concern as it is becoming more prevalent due to the population aging. OA affects more than 50% of the population above 75-years-old, and 25% of them cannot perform major life activities [1,2]. Until recently, the disease was traditionally defined solely as the degradation of articular cartilage (AC) and was not considered an inflammatory disease [3,4]. However, studies have revealed this to be incorrect. Inflammation of the synovial membrane and the presence of inflammatory markers including interleukins, nitric oxides, and matrix metalloproteinases, have been observed in a significant portion of OA patients [5,6]. Therefore, AC degradation is not merely mechanical, but matrix proteases, oxidative stress, and reactive oxygen species (ROS) are also key players in OA progression [7]. These findings are transforming how researchers define and develop treatments for OA. OA is known today as a whole joint disease rather than just a degenerative disease of AC because it also affects the subchondral bone, synovium, and periarticular muscles [8–10]. What is needed are studies that investigate chemical and mechanical cues well-known to suppress inflammatory

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https://doi.org/10.1016/j.yexcr.2021.112841
Received 2 March 2021; Received in revised form 21 August 2021; Accepted 22 September 2021
Available online 24 September 2021

Abbreviations

ACI	autologous chondrocyte implantation
ADAMTS4	a disintegrin and metalloproteinase with thrombospondin motifs-4
CM	Control Medium
COL	collagen
COL10A1	collagen type X chain alpha 1
COL11A2	collagen type XI chain alpha 2
COL1A	collagen type I chain alpha 1
COL2A1	collagen type II chain alpha 1
CTS	cyclic tensile strain
ECM	extracellular matrix
ELF-3	E74-like factor-3
ESE-1	epithelium-specific Ets-like factor-1
FRAP	ferric reduction antioxidative power
GA	gallic acid
GAG	glycosaminoglycan
hAChs	human articular chondrocytes
I- κB	inhibitor of NF- κB
ICRS	International Cartilage Regeneration Society
IL-1β	interleukin I beta
MAPK	mitogen-activated protein kinase
MMP-1	matrix metalloproteinase-1
MMP-13	matrix metalloproteinase-13
NADP+	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa betta
OA	osteoarthritis
ROS	reactive oxygen species
RT-qPCR	real-time quantitative polymerase chain reaction
TNF-α	tumor necrosis factor alpha

pathways and evaluate whether osteoarthritic chondrocytes may be used as suitable candidates for regenerating a healthy extracellular matrix (ECM) upon treatment with these cues while maintaining their phenotype.

Regulation of inflammatory markers, especially ROS, is key to mitigating OA [11]. ROS are oxygen-containing free radicals, e.g., the superoxide anion ($O_2^{\bullet-}$), naturally produced with Nicotinamide adenine dinucleotide phosphate ($NADP^+$) in chondrocytes by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes per the reaction $2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$ [12]. The free electrons in radicals lead to the oxidization of cellular components including DNA. This harmful phenomenon is termed "oxidative stress" and strongly associated with the upregulation of catabolic events in OA [13]. It is desired to minimize oxidative stress to prevent OA progression, and nutraceuticals are known to play a major role as antioxidative molecules. Logically, it is important to determine which antioxidant works best and if they can restore diseased chondrocyte ability to produce healthy cartilage.

Antioxidants, and more specifically, nutraceutical compounds, reduce inflammation through several mechanisms. Nutraceuticals including gallic acid, vitamins E and C, and other compounds containing aromatic rings react directly with ROS, accepting or donating and delocalizing unpaired electrons, and rendering the ROS molecule less reactive and far less harmful [14]. Other nutraceuticals, including curcumin, act as chelators, binding transition metals such as iron and copper and preventing their continuous participation in reactions with hydrogen peroxide that generate ROS [15]. Finally, nutraceuticals have been shown to interact with enzymes that both produce ROS and act as ROS scavengers [16,17]. Further, in chondrocytes, various nutraceuticals have been shown to specifically inhibit the downstream effects of the proinflammatory cytokine interleukin 1-beta (IL-1β), found commonly in osteoarthritic cartilage, which promotes the upregulation of inflammatory and catabolic pathways and molecules [18].

Chondrocytes, similar to other cells in the musculoskeletal system with a mechanical function, are mechanosensitive and respond to their mechanical environment starting from development, to maintenance and to regulation of cell activities [19]. A plethora of studies have reported enhanced ECM expression by chondrocytes upon exposure to mechanical loads like compression [20–22], shear [23,24], and hydrostatic pressure [25–28]. Furthermore, recent findings show that, in contrast to supraphysiological loads, proper magnitudes of mechanical strain reduce expression of proinflammatory cytokines in chondrocytes, even when chemically induced by proinflammatory cytokines like TNF-α and IL-1β [29]. An important research question then is whether strain and specific anti-inflammatory nutraceuticals work in concert and what underlying mechanisms for their effect on ECM production and gene expression may be at play.

In this study, we aim to determine the effects of mechanical strain and nutraceutical supplementation, applied separately and in concert to osteoarthritic human articular chondrocytes (hAChs) by examining impacts on ECM protein production and gene expression of chondrogenic, fibrogenic, and inflammatory markers. While monolayer culture alone can lead to dedifferentiation of chondrocytes [30] proper levels of mechanical strain in the two-dimensional space and corresponding fluid shear of overlaying medium leads to upregulation of chondrogenic indices [31]. Moreover, the ability to finely control magnitude, frequency, and duration of strain in the two-dimensional space [32] is attractive and results because chondrocytes and their surrounding matrix is directly attached to the mechanical surface. Furthermore, we can study these effects in combination with other chondrogenic promoters such as the nutraceutical in this study and results in two-dimensional studies provide the basis for more complex three-dimensional studies. To this end we first present results on the antioxidative power of six different nutraceuticals: gallic acid, catechin hydrate, ascorbic acid, curcumin, α-tocopherol, and carvacrol. Then, we assess collagen and glycosaminoglycan deposition, as the major cartilage ECM components, under supplementation with the most promising nutraceutical from our list and mechanical strain. Next, we quantify the gene expression of cartilaginous, type II and XI, and the fibrogenic and hypertrophic collagens, type I and X, as well as genes for matrix metalloproteases, MMP-1 and –13, the major catabolic factors in OA. Later, we discuss signaling pathways through which applied stimuli are likely acting for individual and simultaneous applications of nutraceutical supplementation and mechanical strain. Finally, we address implications about the potential of combined stretching and nutraceutical treatment in preparing tissues for implantation. The approach we suggest is potentially useful for rehabilitating osteoarthritic chondrocytes, by enhancing their ability to produce a healthy ECM, while maintaining their phenotype during the expansion phase, before manufacturing three-dimensional tissue structures *in vitro* and before using chondrocytes in tissue engineering based OA treatments.

2. Materials and methods

2.1. Ferric reduction antioxidative power (FRAP) assay

To identify the strongest and most potentially useful antioxidative nutraceutical, the ferric reducing/antioxidant power (FRAP) assay was performed [33] in the absence of chondrocytes. In the FRAP assay, aqueous ferric tripyridyltriazine (Fe^{3+}-TPTZ) is added in excess to be reduced by antioxidants. The redox reaction is coupled with a color change from pale yellow to blue that is maximally absorbed at 593 nm.

The higher the antioxidative power of a nutraceutical, the better its ability to reduce iron from its ferric to ferrous form, and the larger the measured absorbance. Here, the assay was conducted following the procedure described by Benzie and Strain [34]. Briefly, the FRAP reagent was prepared by mixing 10 mL of 0.3 M acetate buffer at a pH of 3.6, with 1 mL of 10 mM tripyridyl-*S*-triazine (TPTZ) in 40 mM hydrochloric acid (HCl), and 1 mL of 20 mM ferric chloride. An amount of 100 μL of each nutraceutical, i.e. gallic acid, catechin hydrate, ascorbic acid, curcumin, α-tocopherol, or carvacrol, sample was placed inside a well of a 96-well plate, and to that, 300 μL of FRAP reagent was added. Mixtures were allowed to react and equilibrate for 6 min at 37 °C. The absorbance was then read at 594 nm using a Cytation 5 multi-plate reader (BioTek Instruments, Inc). A standard curve was prepared using an aqueous solution of ferrous sulfate at 7 different dilutions, 50, 100, 250, 500, 170, and 1000 μM, and deionized water was used as a blank. FRAP values were reported as mM equivalents of ferrous (Fe^{2+}) species and were calculated using the following equation [35].

$$FRAP\ Value = \frac{Absorbance\ at\ 593\ nm\ of\ test\ sample}{Absorbance\ at\ 593\ nm\ of\ ferrous\ standard}\ x\ ferrous\ standard\ concentration\ in\ mM$$

2.2. Isolation of human chondrocytes (hAChs) from human knees

Human knee AC was aseptically collected post total knee arthroplasty from four consenting Caucasian study participants (56- and 58-year old males and two 59-year old females, average BMI of 35.25 ± 3.05) under the approval of the Institutional Review Board at Washington State University. All participants had International Cartilage Regeneration Society (ICRS) level 4 changes, but cartilage samples were typically harvested from locations with ICRS grade level 2 and 3 changes. The superficial cartilage layers were shaved off the bone specimen and cut using No. 15 surgical scalpel blades. The pieces were then digested in 100 mg/mL collagenase type I (Worthington Biochemical Corporation) in high glucose Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (HG-DMEM/F12) (Gibco®) for 16 h in a shaking incubator at 125 rpm and 37 °C. The digest was then filtered through a 40-μm cell strainer (BIOLOGIX) and washed twice with HG-DMEM/F12 with 10 min centrifugation at 1500 rpm between washes.

2.3. Cell culture and mechanical stimulation

Primary hAChs were suspended at a high density of 1.5×10^7 cell/mL in culture medium (CM) containing HG-DMEM/F12 (Gibco®), which has 17.25 μg/mL L-proline in its formulation to support collagen formation, supplemented with 10% fetal bovine serum (FBS, Gibco®), 1% penicillin-streptomycin (Sigma-Aldrich), and 1% amphotericin b (Gibco®). To facilitate cell adherence and assure CTS acts on cell membranes, even before ECM formation, 1X Corning™ Collagen I [Rat] solution was used to coat the bottoms of cytostretcher chambers, to which chondrocytes strongly adhere [36–39]. Tissues formed in monolayer cultures were studied with cells seeded into four groups, two in a 96-well plate for static culture, and two in a 24-well Cytostretcher chamber (Curi Bio Inc.), to be mechanically stimulated by cyclic tensile stretching (CTS), as shown in Fig. 1. A 4 μL droplet of hACh suspension was placed into each well of either well-plate. In each group, half of the wells were supplied with CM, and the other half with 100 μM nutraceutical supplemented CM, a typical concentration for nutraceutical treatment of chondrocytes [40,41] which showed no adverse effects on chondrocyte viability in our study (data not shown). The Cytostretcher groups were stretched by 5% at 1 Hz for 2 h/day, a total of 7200 cycles per day for 14 days. This loading protocol was chosen to match the loads to which knees are exposed *in vivo*. Chondrocytes in AC are compressed by 15% under normal body movement, which leads to 5% tensile strain [31] as the localized indention at one point in the cartilage stretches the surrounding surface inward to make the indention [31]. All experimental groups were maintained at standard conditions at 37 °C under a 5% CO_2 atmosphere, and the medium was changed every two days. In summary, the groups studied were as follows:

i. Static negative control (NCS): Static + CM
ii. Static nutraceutical: Static + CM + 100 μM nutraceutical
iii. Cyclic tensile strain with no GA (CTS): Stretched by 5% at 1 Hz for 2 h/day + CM
iv. Cyclic tensile strain with nutraceutical (GA + CTS): Stretched by 5% at 1 Hz for 2 h/day + CM + containing 100 μM nutraceutical

2.4. Quantitative biochemistry

For total collagen quantification, samples were first digested in 0.1 mg/mL pepsin (0.1% pepsin (Sigma-Aldrich) in 0.5 M acetic acid) at 60 °C for 1 h, followed by incubation at 37 °C for 15 h. Total collagen in the digest was then quantified using the Sircol assay kit (Biocolor Life Science Assays), following the manufacturer's directions. For sulfated glycosaminoglycans and DNA quantification, samples were digested in 125 μg/mL papain (Sigma-Aldrich) in 100 mM pH 6.5 sodium phosphate buffer containing 10 mM L-cysteine and 10 mM EDTA at 60 °C for 16 h. The Blyscan assay kit (Biocolor Life Science Assays) was used to quantify glycosaminoglycans and the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher) was used to quantify DNA, following the manufacturers' directions.

2.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

To quantify the gene expression of chondrogenic markers, COL2A1 and COL11A2, hypertrophic markers COL1A1 and COL10A1, and catabolic markers MMP-1 and -13, real-time RT-qPCR was used. Briefly, cells were lysed by adding 250 μL of TRIzol Reagent (Invitrogen) into each well and homogenized by repetitive pipetting. Then, lysates from 4

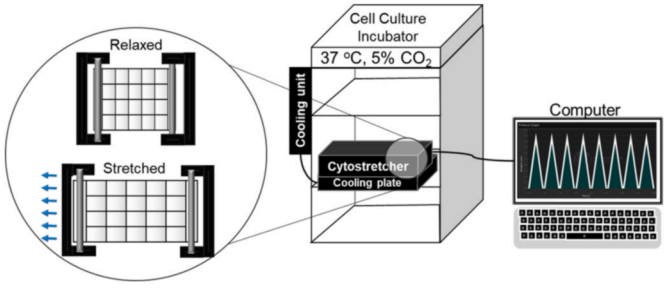

Fig. 1. Schematic view of the cell stretching apparatus. The loading protocol is designed on the NanoSurface Operational Mechanics Interface (NaOMI) and is transferred from a computer to the Cytostretcher, where the stretchable silicone cell culture plates are inserted. The Cytostretcher is placed on top of a cooling plate that prevents Cytostretcher from over-heating and maintains the unit at 37 °C. A top view of the relaxed (top) and stretched (bottom) 24-well Cytostretcher chambers are shown to the left.

Table 1
RT-qPCR primer sequences for markers probed.

Gene	Probe sequence	Forward primer	Reverser primer
Col2A1	CAGGCCTTCCTGGTGTCAAAGG	GGTGCTCGTGGTTTCCCA	GGCCTGGATAACCTCTGTGA
COL11A2	TGGTGCCCGTATTCTGGATGAAGA	GACACCCATGGAGTGATCATCT	GGCCAGCTCCTGGACATC
COL10A1	CAGGGAGTGCCATCATCGATCTCACA	CACCAAAGGCTACCTGGATCAG	GGCCATTTGACTCGGCATTG
MMP-1	TTGAAGCTGCTTACGAATTTGCCGA	TGGCCACAACTGCCAAATG	CCCTGAACAGCCCAGTACTTA
MMP-13	CAGCAAGCTCCATGACTGAGAGGC	GCGGGAATCCTGAAGGAGAATG	CACCTCTAAGCCGAAGAAAGACT
β-ACTIN	CAAGATCATTGCTCCTCCTGAGCGC	GGCACCCAGCACAATGAAG	GCCGATCCACACGGAGTA

identical wells were pooled into a 1.5 mL Eppendorf tube to ensure adequate amounts of mRNA (>2.5 μg) were collected for each RT-qPCR replicate. Chloroform was then used for phase separation, and total mRNA was reverse transcribed into cDNA using SuperScript® VILO™ Master Mix (Thermo Fisher Scientific). cDNA was amplified with the TaqMan® Gene Expression Master Mix (Applied Biosystems by Life Technologies) on an ABI 7900HT Sequence Detection System (Applied Biosystems) and probes for beta-actin (β-actin) as a housekeeping gene, COL2A1, COL11A2, COL1A1, COL10A1, MMP-1, and MMP-13 were used. For COL1A1, the Hs00164004_m1 probe from ThermoFisher was used. Primer sequences for the other probes are shown in Table 1. For each experimental group, triplicates were analyzed and compared to a day zero sample as a reference. The relative gene expression was calculated using the ΔΔCT method, and fold differences were determined using the $2^{-\Delta\Delta CT}$ expression [42].

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.4.3. Two-way analysis of variance (ANOVA) was used to compare group means. Groups were considered significantly different when the ANOVA F test had a p-value less than 0.05, in which case, a Fisher's test was conducted as a post hoc procedure to determine specific group differences. The mRNA values in all experiments are compared to the amount present on the final day of culture relative to a reference sample taken at day 0. Then the relative expression values on the final day are calculated using the ΔΔCT method and compared to those on the final day for an NCS control group, void of both nutraceutical and CTS. In addition to the statistical significance analysis, we determined effect sizes or Hedges' g values as an indication of practical differences between treatment groups, calculated as the size of the difference between mean values divided by the pooled standard deviation and multiplied by a correction factor for sample numbers less than 10 [43]. Effect sizes are often enlightening when p-statistics, due to small sample sizes, do not reveal significance and in our data where all or at least 2 of 3 data points are substantially larger or substantially smaller for a given index, such as for COL2A1 in this study, than the corresponding index for the control group.

$$Hedges' g \text{ or } Effect\ Size = \frac{Mean\ of\ experiemental\ group - Mean\ of\ control\ group}{Pooled\ standard\ deviation} \times \left(1 - \frac{3}{4(N-2)-1}\right)$$

where N is the sum of samples from the two groups being compared. The effect size can be designated as small, medium, large, or very large, and interpreted as a percentage of the control group (NCS) indices that would have values below or above the average sample value in the experimental group. Effect sizes in this paper are categorized as follows:

1. Small (ˆ), $g = 0.2 - 0.5$, when the percentage of NCS values above or below is between 62 and 69%

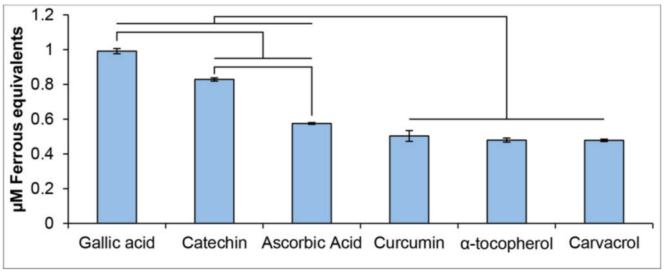

Fig. 2. FRAP value (antioxidative power) of nutraceuticals at 100 μM concentrations. Error bars represent the standard deviation, and connected columns represent significant differences (N = 3, $p < 0.01$).

2. Medium (ˆˆ), $g = 0.5 - 0.8$, when the percentage is between 69 and 79%
3. Large (ˆˆˆ), $g = 0.8 - 1.2$, when the percentage is between 79 and 88%
4. Very Large (ˆˆˆˆ), $g > 1.2$, when the percentage is between 88 and 99.9%

The effect size relative to the NCS is indicated as ˆ to ˆˆˆˆ on all figures, groups connected by lines are significantly different at a $p < 0.05$ level, and error bars are standard deviations.

3. Results

3.1. The FRAP value of nutraceuticals

Gallic acid (GA) is the strongest antioxidant among compared nutraceuticals, based on FRAP assay analyses. As illustrated in Fig. 2, the measured FRAP value of GA was 0.99 ± 0.03 (mean ± σ) which was statistically higher ($p < 0.0001$) than those of catechin at 0.82 ± 0.01, curcumin at 0.50 ± 0.02, ascorbic acid at 0.58 ± 0.01, α-tocopherol at 0.48 ± 0.01 and carvacrol at 0.48 ± 0.01. Based on its superior reduction capability, 100 μM GA was added to the growth medium for the cell culture experiment under mechanical strain.

3.2. Deposition of glycosaminoglycans and collagen by engineered AC tissues

Moving forward with our strongest antioxidant nutraceutical GA, when chondrogenic biochemical indices were quantified, we observed that simultaneous stretching of hACh and GA supplementations, compared as always throughout the manuscript to a control group without stretching or nutraceutical, worked in synergy, remarkably enhancing the production of glycosaminoglycans (GAG) and collagen

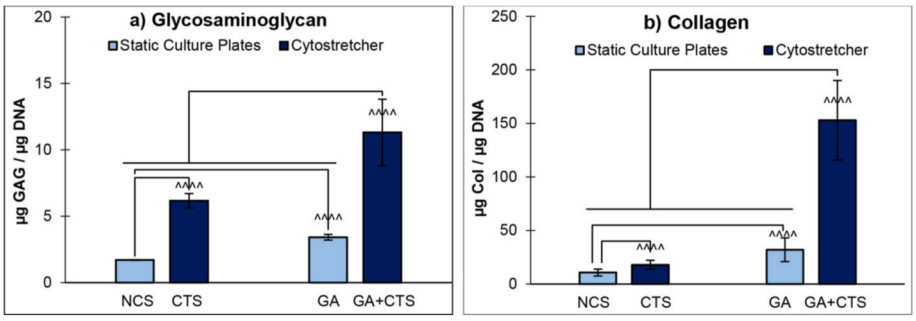

Fig. 3. Total GAG and collagen data. a) normalized sulfated glycosaminoglycan (µg GAG/µg DNA) and b) normalized collagen content (µg Col/µg DNA) for chondrocytes in static culture plates and under CTS with and without GA. Error bars represent the standard deviation and connected columns represent significant differences (N = 4, $p < 0.05$). Column labels as ^^^^ show very large effect sizes compared to NCS.

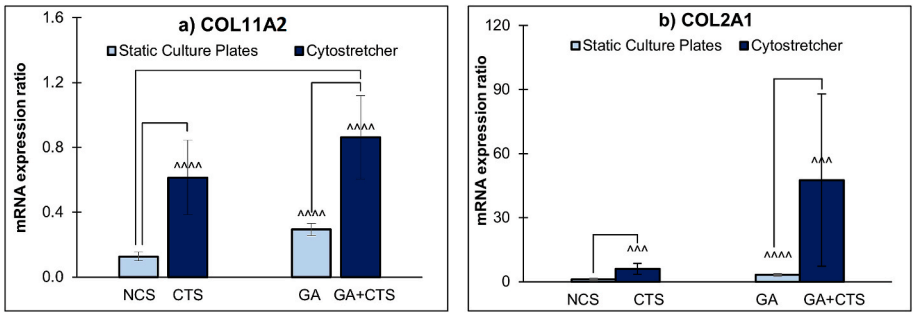

Fig. 4. Relative mRNA gene expression of cartilage-specific collagens. a) COL11A2 and b) COL2A1 for the CTS and GA groups each ratioed to the NCS group without GA on the last day of culture. Error bars represent the standard deviation, and connected columns represent significant differences (N = 3, $p < 0.05$). Column labels show effect sizes compared to NCS, ^^^ for large, and ^^^^ for very large effect sizes.

(Col) normalized to DNA content over the increases observed when each factor was applied individually and when compared to the negative control without any stimulation whatsoever. As shown in Fig. 3a, progressive improvements were observed over the control in GAG content in hAChs treated with GA, subjected to stretching without GA, and then hAChs treated with a combination of GA and stretching. Compared to the NCS group, GAG production was enhanced by 2.6-fold with GA treatment ($p = 0.0002$; $g = 1.4$), 3.6-fold with stretching ($p < 0.0001$; $g = 1.5$), and by 6.6-fold ($p = 0.021$; $g = 1.4$) when GA treatment and stretching were combined. Similarly, increases were observed in Col/DNA content when GA and CTS were applied as shown in Fig. 3b. Compared to the NCS, the collagen content increased by 1.7-fold with CTS and no GA ($p < 0.001$; $g = 1.2$) and by 3.0-fold with GA treatment ($p < 0.001$; $g = 1.3$), but without CTS. However, there is a large and significant increase in Col production over the NCS, by more than 14-fold ($p < 0.0001$; $g = 1.5$), when both GA and CTS were simultaneously applied. In all cases differences show very large effect sizes. The interaction effects were found significant through the two-way ANOVA analysis, suggesting a synergistic, i.e., more than the sum of two individual impacts, and not just additive, effect of GA and CTS on improving GAG and Col contents.

3.3. Gene expression of cartilaginous collagens

The respective expressions of the articular cartilage-specific collagen genes, COL11A2 and COL2A1, are shown in Fig. 4a&b. COL2A1 encodes for the alpha-1 chain of type II collagen, which makes up 80–90% of the collagen in AC, and COL11A2 encodes for the alpha-2 chain of type XI collagen, which constitutes 3–10% of the collagen in AC [44]. Data in Fig. 4a for COL11A2 show up-regulation that is both significant and with very large effect sizes with 4.9-fold increases on average with CTS ($p = 0.0007$; $g = 1.3$), GA has no significant effect on COL11A2 expression, but it increased with a very large effect size by 2.3-fold 4.9-fold ($p = 0.081$; $g = 1.3$), and by 6.8-fold ($p = 0.748$; $g = 1.4$) with simultaneous GA and CTS. We note how use of the effect size adds to our arguments, i.e. Hedges' g values indicate that 92, 90, and 92% of the NCS data are likely to fall below the mean values for the respective treatments.

As shown in Fig. 4b, COL2A1 expression was upregulated by 3.2-fold with very large effect sizes with GA treatment ($p = 0.406$ $g = 1.4$), significantly by 6.1-fold by CTS ($p = 0.011$; $g = 1.0$), and a synergistic 47.5-fold ($p = 0.354$; $g = 0.8$) increase with GA treatment under CTS. We note in all cases effect size calculations reveal that 92%, 84% and 79% of the NCS samples are likely to fall below the average values for the GA, CTS and GA + CTS treatment groups, respectively, though p-statistics, due to the small sample sizes (N = 3) and large relative standard deviations do not show p-values less than 0.05. This is corroborated by reviewing the raw data collectively where 8 of 9 COL2A1 values for the entire set of treatment groups are substantially above the 0.130 ± 0.029 COL2A1 level for the NCS group, i.e. 1.9 – 114-fold higher, and the remaining 1 of 9 falls in the upper bracket of the standard deviation for the NCS group. More specifically, for the GA only static experiments, COL2A1 values ranged from 1.93 to 2.4-fold higher than the NCS without GA, and for the stretching only group 0.8, 4.5 and 9.8-fold higher. For the GA + CTS experiment two of three replicates that constitute the average 46.5-fold average increase in COL2A1 show substantial increases over the NCS by 25.7- and 114-fold with just one value on par with the NCS yet with a 0.11-fold increase over the average and hence within the higher end of the standard deviation brackets for the NCS data.

3.4. Gene expression of fibrogenic and hypertrophic collagens

Fibrogenic COL1A1 and the hypertrophic COL10A1 collagens are typically expressed by dedifferentiated chondrocytes or in fibrillated and osteoarthritic cartilage [45] and both were nearly constant, decreased or showed only small increases with GA, CTS, or their

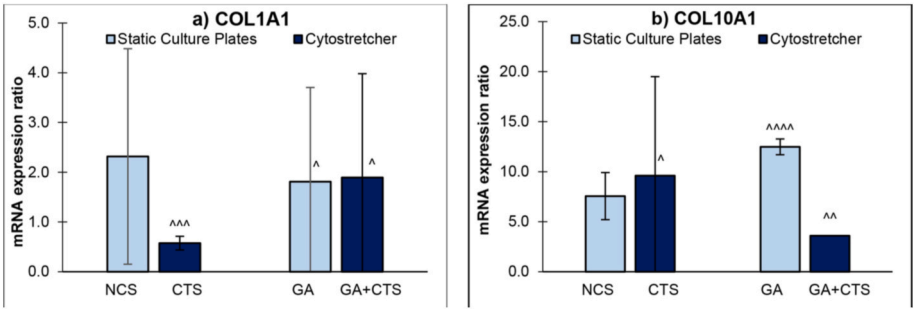

Fig. 5. Relative mRNA gene expressions of cartilage-specific collagens. a) COLA1 and b) COL10A1, for the CTS and GA groups normalized to the negative control static (NCS) group in the static plates without GA. Error bars represent the standard deviation (N = 3). Column labels show effect sizes compared to NCS, ^ for small, ^^ for medium, ^^^ for large, and ^^^^ for very large effect sizes.

combination, as shown in Fig. 5 a&b. COL1A1 mRNA data show a decrease or at least a non-significant change with GA and CTS with a 1.3-fold decrease and small effect size with GA ($p = 0.689$ $g = 0.3$) and 4-fold decrease with an insignificant but large effect size for CTS without GA ($p = 0.418$; $g = 1.0$) and 1.2-fold decrease with a small effect size for GA + CTS ($p = 0.358$; $g = 0.2$). Similar results are shown for COL10A1 changes with GA or CTS with a significant 1.7-fold increase and a very large effect size ($p = 0.882$, $g = 1.3$) with GA and a nonsignificant 1.3-fold increase with CTS ($p = 0.341$, $g = 0.2$), and a nonsignificant 1.4-fold decrease ($p = 0.145$; $g = 0.5$) is observed in the GA + CTS group with medium effect size. Interestingly, GA and CTS do not enhance the production of all types of collagen fibers, but genes encoding collagens of interest to AC, i.e. COL11A2 and COL2A1, are upregulated selectively while fibrinogenic and hypertrophic genes, COL1A1 and COL10A1 do not change with statistical significance and even when effect sizes are large or large or very large the relative magnitudes of change are on the order of 1.3 – 1.7-fold in contrast to the 1.93 – 114-fold increases in a favorable direction for chondrogenic indices along with large and often very large effect sizes. From the effect size analysis, we see that 62% and 76% of GA + CTS samples express less COL1A1 and COL10A1, respectively, than an average NCS sample.

3.5. Gene expression of matrix metalloproteases

MMP-1 and MMP-13 are critical players in cartilage destruction in OA patients [46–49]. In fact, MMP-13 is considered the principal catabolic factor in OA due to its destructive action of degrading collagen type II [50,51], which is the dominant collagen species in healthy articular cartilage. While we expect antioxidants to reduce ROS and MMPs, as illustrated in Fig. 6 a&b, we observed with GA alone, a non-significant yet 1.4-fold increases in MMP-1 ($p = 0.416$; $g = 0.8$) and in MMP-13 ($p = 0.525$; $g = 0.5$) expression compared to NCS. The two-way ANOVA results indicate that GA causes 0.087% of the variation only ($p = 0.884$). However, CTS is the source for 52% of variations ($p = 0.006$) resulting in nonsignificant decreases with large effect sizes observed when CTS is introduced. MMP-1 and MMP-13 expressions were downregulated by 1.4- ($p = 0.299$, $g = 0.7$) and 2-fold ($p = 0.646$, $g = 0.6$) in the CTS group. In the GA + CTS group, the interaction term caused significant 18% of the variations ($p = 0.044$) with further downregulations of very large and large effect sizes are observed, resulting in a statistically significant decrease ($p = 0.035$; $g = 1.2$) of 2.6-fold for MMP-1 and though non-significant a large 131-fold decrease for MMP-13 ($p = 0.067$, $g = 1.1$). We observe from the effect size analysis that 88% and 86% of NCS samples would express more MMP-1 and MMP-13, respectively, than an average GA + CTS sample.

3.6. Summative results

In Table 2 we summarize the above results indicating the fold changes, p-statistics, Hedges' g effect sizes and corresponding percent of experimental data points expected to be higher than the NCS control. We do this for the four chondrogenic indices, total GAG and Col, and COL11A2 and COL2A1 mRNA, and the four non-chondrogenic indices COL1A1, COL10A1, MMP-1 and MMP-13. Indices with positive increases over NCS are indicated by ▲ for small increases in the 1 to 5-fold range, ▲▲ for large increases above 5-fold, and ▲▲▲ for very large increases above 10-fold, and decreases indicated by ▼ for small decreases in the 1–5 range, and ▼▼▼ for very large decreases greater than 10-fold. There were no decreases in the 5 to 10-fold range. All chondrogenic parameters show increases with CTS and GA, being substantially larger for the combined influences of both parameters acting in concert. In three of eight instances for either CTS or GA non-chondrogenic indices are decreased, and for combined CTS and GA all non-chondrogenic indices are decreased. The collective information creates a compelling visual picture that will support conclusive statements described in the Discussion section.

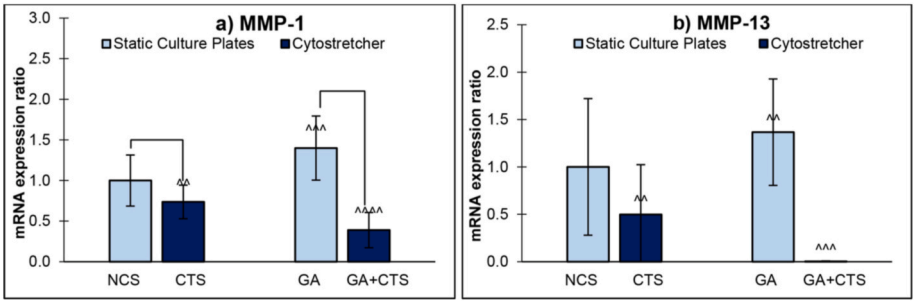

Fig. 6. Relative mRNA gene expression of matrix metalloproteases. a) MMP-1 and b) MMP-13, for the CTS and GA groups normalized to the negative control static (NCS) group in the static plates without GA. Error bars represent the standard deviation, and connected columns represent significant differences (N = 3, $p < 0.05$). Column labels show effect sizes compared to NCS, ^ for small, ^^ for medium, and ^^^ for large effect sizes.

Table 2
Statistical summary of results.

Corresponding figure	Expression	Parameter	GA	CTS	GA + CTS
Chondrogenic Indices					
3a	GAG	Fold change	▲2.6	▲3.6	▲▲6.6
		p-value	<0.0001	<0.0001	0.0003
		Hedges' g	1.4	1.5	1.4
		% higher than NCS	92	94	92
3b	Col	Fold change	▲ 3	▲1.7	▲▲▲14.3
		p-value	0.0104	0.0382	0.0003
		Hedges' g	1.3	1.2	1.5
		% higher than NCS	90	88	94
4a	COL11A2	Fold change	▲ 2.3	▲ 4.9	▲▲ 6.8
		p-value	0.0075	0.0220	0.0079
		Hedges' g	1.4	1.3	1.4
		% higher than NCS	92	90	92
4b	COL2A1	Fold change	▲ 3.2	▲▲ 6.1	▲▲▲ 47.5
		p-value	0.0013	0.1443	0.2480
		Hedges' g	1.4	1.0	0.8
		% higher than NCS	92	84	79
Non-Chondrogenic Indices					
5a	COL1A1	Fold change	▼▼▼1.3	▼▼▼4	▼▼▼1.2
		p-value	0.5151	0.2463	0.2475
		Hedges' g	1.3	1.0	0.6
		% higher than NCS	90	84	73
5b	COL10A1	Fold change	▲1.7	▲1.3	▼▼▼1.4
		p-value	0.0259	0.7432	0.1117
		Hedges' g	1.3	0.2	0.5
		% higher than NCS	90	58	69
6a	MMP-1	Fold change	▲1.4	▼▼▼1.4	▼▼▼2.6
		p-value	0.2532	0.2986	0.0340
		Hedges' g	0.8	0.7	1.2
		% higher than NCS	79	76	88
6b	MMP-13	Fold change	▲1.4	▲2	▼ 131.6
		p-value	0.5252	0.6956	0.0754
		Hedges' g	0.5	0.6	1.1
		% higher than NCS	69	73	86

4. Discussion

Our discussion begins with analyzing the FRAP assay results to justify the selection of the best nutraceutical candidate for restoring ECM production by osteoarthritic chondrocytes. We then move to the analysis of ECM matrix components changes in concert with up or down-regulation of chondrogenic, hypertrophic, and catabolic genes. Finally, we review cellular mechanisms known to be influenced by antioxidants and cellular stretching to provide support for the findings observed from a fundamental physiological standpoint. We conclude with a section stressing the implications of our findings for cartilage regenerative therapies.

Our FRAP assay results agree with the findings of Soobrattee et al., where GA had a significantly higher antioxidant activity when compared to catechin, Trolox, a water-soluble α-tocopherol analog, and ascorbic acid [52]. The high antioxidant activity of GA is hypothesized to be correlated to several features of its structure. GA has three hydroxyl groups organized in an ortho position with respect to each other, which allows stabilization after free radical scavenging *via* short intramolecular hydrogen bonds. Further, GA has a carboxylic acid group at the para position, which, due to its tendency for hydrogen donation, has been shown to positively contribute to its antioxidant properties [53]. At physiological pH, a computational study showed that GA reacts with OOH radicals primarily through sequential double proton loss electron transfer reactions at the carboxylic acid and phenolic OH groups, while reaction with OH radicals can proceed through several mechanisms [53]. Experimental work shows that phenoxyl-type radicals are formed upon reaction of GA with OH radicals at a near-physiological pH (37). These studies, along with our corroborating data, provide strong evidence that GA does indeed neutralize ROS and is an obvious choice for studies on synergistic influences when combined with stretching.

Moving to GA + CTS experiments, collectively Table 2 summary data unequivocally support our premise that each factor promotes chondrogenesis individually and, when combined, synergistically. While histological staining studies are the norm in many chondrogenic studies, this is not possible to accomplish within non-transparent cytostretcher wells. We therefore rely on total collagen and GAG content, which is more quantitative. And corroborate these findings with gene expression data. To confirm that gene presence leads to deposition of corresponding extracellular matrix proteins Western blots could be done. However, the enhanced production of total collagen along with upregulation of the requisite genes for COL2A1 and COL11A1 with corresponding reductions in COL1A1 and COL 10A1 are in strong support that enhanced total collagen is indeed related to an increased chondrogenic phenotype. The four chondrogenic indices, total GAG and Col, and COL11A2 and COL2A1 mRNA, have positive 1.7 – 6.1-fold increases over NCS, as indicated by ▲ for increases, ▲▲ for large increases above 5-fold, and ▲▲▲ for very large increases above 10-fold. All are statistically significant except for CTS effects on COL2A1, which is approaching significance ($p = 0.248$), and importantly all have large to very large effect sizes including that for COL2A1. Combined applications of both GA and CTS strongly suggest the individual effects are additive for total Col, and COL11A2 mRNA, and more importantly that there is a large second order multiplicative effect for GAG, being nearly 4 times the sum of the individual effects, and for mRNA coding for COL2A1, the more prominent proteins in the extracellular matrix, being 5 times the sum of the individual effects. When viewing the undesirable non-chondrogenic indices there are a mixed set of negative, 1.3 – 5-fold decreases for mRNAs coding for fibrogenic COL1A1 with both GA and CTS, and for catabolic MMP-1 with CTS, and positive 1.3 – 1.7 increases for mRNAs coding for hypertrophic COL10A1 and catabolic MMP-1, and MMP-13. Yet, when the two treatments are combined all non-chondrogenic indices in this study decline in the 1.35 – 2.5-fold range with mRNA coding for catabolic MMP-13 falling by 125-fold. While one p-statistic

approaches $p < 0.05$ significance, two are nearly so, and all have meaningful effect sizes – of importance is that mRNA coding for COL1A1 has a large effect size and that for MMP-1 and MMP-13 have very large effect sizes in the negative direction.

The findings for the individual indices are consistent with the literature, and yet the synergisms go beyond what has been reported.

We note that while GAG enhancement through GA treatment was not found significant in a previous study by Lu et al. [54] where GA supplementation in medium did not cause significant changes in glycosaminoglycan production by rat AChs, there was a significant 2-fold increase in our studies. Our results observed with stretching alone agree exactly with the work by Fukuda et al. [37], where exposing calf AChs to 5% strain led to an increase of proteoglycan synthesis by 1.7-fold as measured by assessing the incorporation of [^{35}S]-sulfate into cetylpyridinium chloride (CPC) precipitable material. However, in our study when GA was added to stretched cultures, it caused a significant non-linear 14.3-fold increase in GAG deposition with a large effect size. These results observed with stretching in combination with GA hAChs are unique and suggest that two mechanisms are involved with interplay between them and that GA and CTS use in concert is important for optimizing cartilage formation *in vitro*.

Regarding collagen, other studies show mixed results with GA, but that it does have a protective effect. GA did not significantly increase collagen expression in healthy chondrocytes as shown by Lu et al., where cytoplasmic collagen II levels were 1.2-fold lower in healthy rabbit chondrocytes treated with GA alone than in a negative control [54]. In contrast, GA is shown to protect cells from collagen degradation caused by inflammation as shown by Wen et al., where GA increases collagen II expression in a dose-dependent manner in inflamed rabbit chondrocytes, approaching the level expressed in healthy cells at a concentration of 80 μM [55]. From these studies, it can be inferred that while GA alone may not promote the synthesis of new collagen, it can play a protective role in preventing the degradation of existing collagen. Because our study shows the combination of GA and CTS leads to a significant increase in total collagen and COL11A2 mRNA and an increase with a large effect size for COL2A1 mRNA, the collective information, between our data and the literature suggests that the protective effect for GA when combined with a stimulator effect from the mechanical loading enhances chondrogenic indices beyond the sum of the two factors alone.

Not only does our study provide strong evidence that combining CTS and GA enhances production of cartilaginous collagens, but that the combination also downregulates the expression of matrix metalloproteases (MMPs) a crucial factor in preventing OA progression. The tendency for GA alone to reduce MMP levels in inflamed chondrocytes is not supported by our work as there is an apparent increase in MMP-1 and -13 with medium effect sizes, but there are large to very large effect sizes for a drop when CTS is applied in concert with GA. This is in agreement with several reports comparing the anti-inflammatory properties of GA and synthetic GA derivatives that show GA alone is ineffective in preventing the upregulation of MMP-1, -3, and -13 in chondrocytes inflamed through IL-1β stimulation [56]. Yet there is evidence that GA has a regulatory inhibitory impact on MMP production as supported in a study by Pang et al. showing GA effectively reduced MMP-1 mRNA by 2-fold and corresponding protein levels by 2.4-fold in human carcinoma cells [57], and a study by Yoon et al. showing that GA reduced MMP-9 mRNA levels in fibroblast-like synoviocytes [58]. This suggests that cell culture in the presence of GA conditions cells for when CTS is applied.

Gene expression data strongly reveal that chondrogenic indices are displayed in deposition studies confirming that CTS and GA supplementation allow the desired chondrogenic phenotype to be maintained and do not induce cartilage hypertrophy. When taking the data collectively, there is strong support that the 47.5-fold increase in COL2A1 mRNA and 6.8-fold increase in COL11A2 indeed are the major factors contributing to the statistically significant 14.3-fold increase in total collagen deposition. We base our argument on the fact that the 1.35-fold COL1A1 decrease nearing significance and bordering on a very large effect size and the decrease in COL10A1 with a medium effect size suggest an overall decrease in the corresponding fibrogenic and hypertrophic ECM proteins inferring that the COL1A1 and COL11A2 are solely responsible for the increase in total collagen in the ECM when combining GA and CTS. Since COL2A1 typically constitutes 80–85% of the total articular collagen, and COL11A2 a mere 3–10% of the statistically significant ~7-fold in COL11A2, which is associated with enhanced chondrogenesis, gives credence to the 47.5-fold increase and large effect size for COL2A1 though the p-value is not <0.05. Moreover, this corresponding deposition of COL2A1 along with COL11A2 has been shown by many studies [59–61]. These findings, when taken together with the reduction in MMP-1 and MMP-13 mRNA levels under combined stimuli, strongly suggest that GA and CTS together have a positive, synergistic effect on the production and maintenance of collagen proteins in the ECM of diseased chondrocytes.

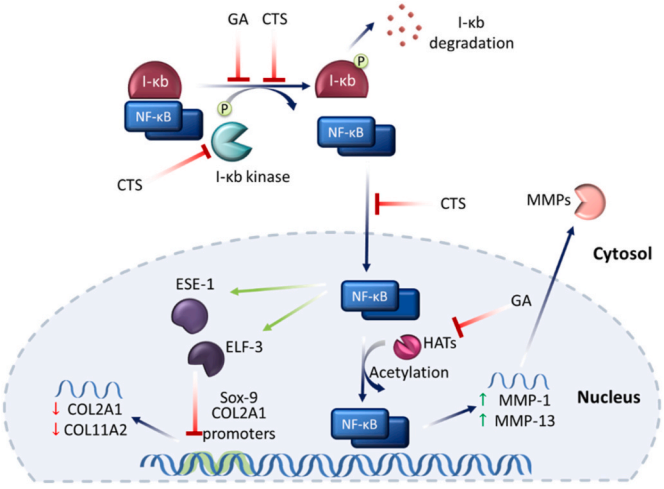

Fig. 7. The NF-κB signal transduction pathway during inflammation and the inhibitory roles of CTS and GA. CTS attenuates inflammation through the NF-κB signal transduction pathway by inhibiting I-κb kinase, I-κb degradation, and NF-κB translocation. GA works together with CTS to inhibit I-κb degradation and inhibits the activation of NF-κB by acetylation inside the nucleus.

There is also strong support in the literature for the increases in chondrogenic collagen and decreases in the MMP protein enzymes in response to GA. An illustration adapted from Dossumbekova et al. [62], simplified and modified by us to include the mechanisms of action of GA, is shown in Fig. 7. Huang et al. recently studied the mechanism of action of GA in nucleus pulposus cells, which, similar to chondrocytes, produce both collagen type II and proteoglycans [63]. They found that GA significantly reduces protein and mRNA levels of a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS4), a metalloproteinase that degrades proteoglycans, through inhibition of the nuclear factor-kappa betta (NF-κB) inflammatory pathway in cells exposed to tumor necrosis factor-alpha (TNF-α). Their study reveals that GA reduces the phosphorylation of p65, one of many NF-κB transcription factors which form dimers with other NF-κB transcription factors in the cytosol, and decreases the activity of several histone acetyltransferases (HATs) which acetylate p65 and limit the ability for the complex to be transported out of the nucleus. The role of p65 in the NF-κB pathway is complex. In healthy cells, NF-κB transcription factor dimers are maintained in an idle state by the inhibitor of NF-κB (I- κB). Upon disassociation from I-κB, which is phosphorylated and degraded during inflammation, these dimers are free to translocate to the nucleus, as shown in Fig. 7. Once in the nucleus, they induce the transcription of target inflammatory genes, including MMPs [56]. Given this information, it is surprising that GA was unable to decrease the production of MMP-1 and MMP-13 mRNA in the static study results, as these enzymes

are regulated through p65 activity [64]. However, it is also possible that MMP-1 and MMP-13 production were induced *via* other known inflammatory pathways, including mitogen-activated protein kinase (MAPK) and that GA had no inhibitory effect on these alternative pathways [65,66].

Regarding mechanical loads associated with chondrocyte cultures, they are shown to act in a magnitude-dependent fashion either as pro-inflammatory or anti-inflammatory signals. This is shown by studies in Agarwal [62] and Athanasiou labs [62,67,68]. Loads of physiological magnitudes are anti-inflammatory; they upregulate matrix synthesis and suppress the actions of cytokines including IL-1β and TNF-α [29,62,69]. In chondrocytes, mechanical signals are transduced through integrin-β1, a cell surface receptor that links the ECM to the cytoskeleton [70]. When integrins are stimulated, they activate several cytoskeleton proteins, including focal adhesion kinase, and act through the NF-κB pro-inflammatory signal transduction pathway [71]. Mechanical tension of appropriate magnitudes attenuates inflammation by acting on three sites in the NF-κB signal transduction pathway, as shown in Fig. 7. First, it inhibits the translocation of NF-κB from the cytosol to the nucleus, thereby hindering its mechanistic effect beyond the nucleus. Second, it protects I-κB from degradation by cytoplasmic phosphorylation, thereby rendering NF-κB inactive. Finally, it downregulates I-κB kinase activity, thereby inhibiting phosphorylation reactions. The results shown previously in Fig. 6 a&b and Table 2, show stretching, combined with GA, leads to greater reduction in MMP-1 and MMP-13 gene expression than either stimulus alone, presumably because of the combination of mechanisms explained above.

Moving to the effect of GA and CTS on COL2A1 and COL11A2 expressions, we observe that both treatments upregulate the expressions of these chondrogenic collagens. To our knowledge, the effect of GA on COL11A2 production by inflamed chondrocytes has not been previously reported. Interestingly, it has also been shown that COL2A1 expression is downregulated in response to inflammation. Specifically, members of the Erythroblast Transformation Specific (ETS) transcription factor family, including epithelium-specific Ets-like factor-1 (ESE-1) and E74-like factor-3 (ELF-3) which are activated during the NF- κB pathway, suppress the promoter of COL2A1, leading to reduced COL2A1 transcription [72]. Sex determining region Y- box 9 (SOX-9), a critical regulator of COL2A1, COL11A2, and aggrecan gene transcription, are sequestered and inhibited by excess ELF-3 [73]. This further explains why COL2A1 and COL11A2 mRNA expression in this study were highest in groups where NF- κB activity was likely lowest as indicated by a reduction in MMP mRNA expression.

Results presented in this paper are important for the clinical implications of articular cartilage tissue engineering. Current surgical treatments of OA including the state of the art, autologous chondrocyte implantation (ACI), can temporarily relieve the pain associated with OA. However, it does not fully restore the functionality of articular cartilage [74]. ACI induces the formation of a fibrocartilaginous tissue that is consisted of collagen type I instead of type II, which holds 50–100% less water, making cartilage less resistant to compression [75,76]. In ACI, chondrocytes are harvested from articular cartilage, expanded *in vitro*, and re-implanted in the cartilage defect; clinical shortcomings are due to chondrocyte de-differentiation, and loss of phenotype during expansion [77,78]. In this work, we suggest using combined CTS and GA to treat chondrocytes during the expansion phase to enhance the overall outcome for procedures like ACI. The rationale behind our suggestion is that the tendency for chondrocytes to lose their phenotype upon expansion and passaging in traditional static culture is a well-recognized challenge in cartilage tissue engineering [79]. Levels of collagen type II and aggrecan typically decrease in cells cultured *via* traditional static methods [80]. This is a critical issue, as both collagen type II and aggrecan play important roles in the structural properties of cartilage and its ability to absorb loads. Furthermore, chondrocytes isolated from osteoarthritic knees are more active catabolically and express larger amounts of inflammatory markers compared to normal chondrocytes [81]. The results of this study provide a promising alternative to the traditional expansion of diseased chondrocytes; our data suggest that use of CTS and GA will downregulate the expression of catabolic genes, upregulate expression of cartilage specific proteins, and maintain chondrocyte phenotype. We hypothesize that better ACI outcomes will occur if the chondrocytes used in the procedure are prepared with our GA + CTS approach instead of using the conventional 2D expansion protocols.

In conclusion, our data are in support of a strong synergistic impact of GA and CTS first from composition data for the resulting ECM and then from the mRNA profile, which regulates cell physiology. It is worth noting that this is the first study that reports enhanced ECM matrix for osteoarthritic human knee chondrocytes upon exposure to appropriate CTS and anti-inflammatory treatment making them more suitable for tissue engineering applications. At a concentration of 100 μM, GA is shown to be a more potent anti-oxidant based on its ferric reducing ability compared to curcumin, catechin hydrate, carvacrol, ascorbic acid, and α-tocopherol, all of which are naturally occurring and recommended for degenerative diseases. We also show that combining CTS and GA results in the greatest downregulation of catabolic, matrix-degrading enzymes present in osteoarthritic tissue, and critically, the greatest upregulation of COL2A1, COL11A2, and total collagen and glycosaminoglycan deposition in the ECM. Taken together, our results suggest that mechanical stimuli and nutraceuticals can be used synergistically as a pre-treatment to inhibit the downstream negative effects of the NF-kB signaling pathway, such as increased production of matrix-degrading enzymes, downregulation of cartilage specific-collagens, and destruction of ECM proteins, in inflamed cells. We recommend further *in vivo* studies to explore the properties of engineered cartilage produced using chondrocytes pre-treated in this manner compared to freshly harvested chondrocytes.

Credit author statement

Haneen A. Abusharkh: Conceptualization, Methodology, Validation, Investigation, Data Curation, Writing – Original, Writing – Review & Editing, Visualization; Olivia M. Reynolds: Conceptualization, Methodology, Writing – Review & Editing, Visualization; Juana Mendenhall: Conceptualization, Methodology, Resources, Writing – Review & Editing, Funding acquisition; Bulent A. Gozen: Writing – Review & Editing, Funding acquisition; Edwin M. Tingstad: Methodology, Resources, Writing – Review & Editing, Funding acquisition; Vincent Idone: Methodology, Resources, Funding acquisition; Nehal I. Abu-Lail: Conceptualization, Methodology, Writing – Review & Editing, Funding acquisition; Bernard J. Van Wie: Conceptualization, Methodology, Validation, Data Curation, Writing – Original, Writing – Review & Editing, Visualization, Supervision, Project Administration, Funding acquisition.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported financially by a National Science Foundation GOALI grant No. 1606226 and Haneen Abusharkh was partially supported by a National Institute of Health T32 Protein Biotechnology Training Program, through grant No. GM008336-31. The authors would like to thank: 1) Alia Mallah, Mahmoud Amr, and Samina Yasmeen for their contributions to discussions of principles tangentially related to this work; 2) Trey Reppe, an NSF REU research assistant, who received training and experience in isolating human chondrocytes from tissues to the extent he was able to provide the bulk of the chondrocytes used in this study; 3) the consenting study participants who donated cartilage post-TKA surgery for the research; 4) Curi Bio Inc. for an academic

discount on the lease for the Cytostretcher; and 5) Hyon Kim and the DNA Core at Regeneron Pharmaceuticals, Inc. for RT-qPCR processing.

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