

Osteoarthritis and Cartilage



Meniscus is more susceptible than cartilage to catabolic and anti-anabolic effects of adipokines



J.F. Nishimuta †, M.E. Levenston †‡*

† Department of Mechanical Engineering, Stanford University, Stanford, CA 94305, USA

‡ Department of Bioengineering, Stanford University, Stanford, CA 94305, USA

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SUMMARY

Objective: This study compared the effects on cartilage and meniscus matrix catabolism and biosynthesis of several adipokines implicated in osteoarthritis (OA).

Design: Bovine cartilage and meniscus explants were cultured for 1 or 9 days in serum-free medium alone or with 0.02, 0.2, or 2 µg/ml of leptin, visfatin, adiponectin, or resistin. Media were supplemented with ³H-proline or ³⁵S-sodium sulfate to evaluate protein and sulfated glycosaminoglycan (sGAG) accumulation on the last day of culture. Explants were assayed for radiolabel, sGAG, and DNA contents. Cultured media were assayed for sGAG, nitrite and lactate dehydrogenase.

Results: Cartilage tissue was minimally affected by adipokines, with only the highest resistin dose increasing sGAG release and nitrite production compared to controls. In sharp contrast, meniscus tissue was responsive to several adipokines, with elevated sGAG and nitrite release following treatment with resistin, leptin, or visfatin. Cartilage sGAG content was unaltered by adipokine treatment whereas meniscal sGAG content significantly decreased with resistin dosage. Protein (³H) incorporation was unaffected by adipokine treatment in both tissues. sGAG (³⁵S) incorporation did not significantly vary with adipokine treatment in cartilage but was inhibited by treatment with leptin, visfatin, and resistin in meniscus.

Conclusion: Our results indicate that meniscal tissue is more susceptible to adipokine-stimulated catabolism than is cartilage. Resistin had the strongest effect of the adipokines tested, inducing sGAG release in both tissues and depleting sGAG content in meniscus. These results suggest that increased adipokine levels due to obesity or joint injury may alter the mechanical integrity of the knee joint through biological pathways.

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Introduction

Obesity in developed nations is at epidemic levels, with prevalence among US adults estimated at 36% in 2009–10¹ and predicted to reach about 50% by 2030². Obesity is a major risk factor for hip and knee osteoarthritis (OA), with every 5 unit increase in body mass index (BMI) doubling the risk of OA³. The association between obesity and OA has been viewed as predominantly biomechanical⁴, but overloading may not be the only link. Knee OA incidence and progression were more strongly associated with fat mass than with

BMI or total mass⁵, and obesity has been identified as a strong risk factor for hand OA^{6,7}, suggesting that systemic, biologic links between obesity and OA may exist in addition to direct mechanical effects.

Adipose tissue is now recognized as a metabolically active organ⁸ that secretes biologically active factors collectively described as adipokines. Altered adipokine levels have been implicated in diabetes, cardiovascular disease, metabolic syndrome, and inflammatory disorders including rheumatoid arthritis⁹. With observations of adipokines including leptin¹⁰, visfatin¹¹, adiponectin and resistin¹² in the synovial fluid of osteoarthritic joints, roles for adipokines in the onset and progression of OA have been hypothesized¹³. In the knee, the synovium and infrapatellar fat pad have been identified as major adipokine producers^{14,15}. Elevated leptin^{10,16}, resistin^{17,18}, and visfatin^{19,20} levels in both serum and synovial fluid have been associated with increased OA progression or

* Address correspondence and reprint requests to: M.E. Levenston, Department of Mechanical Engineering, Stanford University, Stanford, CA 94305-4038, USA. Tel: 1-650-723-9464; Fax: 1-650-725-1587.

E-mail addresses: j.nishimuta@gmail.com (J.F. Nishimuta), levenston@stanford.edu (M.E. Levenston).

severity, but the association of adiponectin with OA remains unclear^{21,22}.

In OA joints, synovial fluid concentrations range around 10–20 ng/mL for leptin^{10,14,16}, 10–40 ng/mL for visfatin^{19,20}, 0.3–2.3 µg/ml for adiponectin^{12,14,23}, and 3–50 ng/mL for resistin^{12,14,17}. Increased BMI correlates with elevated synovial fluid concentrations of leptin¹⁰ but lower synovial fluid concentrations of adiponectin^{21,24}. While studies have reported no correlation between synovial fluid concentrations of visfatin²⁰ or resistin²⁴ and BMI, both correlated with OA severity.

Various adipokines have induced cell-mediated cartilage catabolism *in vitro*. Leptin induced production of proinflammatory factors and enhanced matrix metalloproteinase (MMP) expression in OA cartilage^{25,26}. Visfatin stimulated chondrocyte aggrecanase expression and MMP production¹¹, while adiponectin induced production and expression of several MMPs and proinflammatory factors^{22,27}. Resistin induced production of proinflammatory factors and inhibited matrix synthesis in mouse cartilage¹⁷ and increased aggrecanase and MMP expression by human chondrocytes²⁸. Particularly relevant to the potential involvement of adipokines in OA development, serum and synovial fluid level of resistin were elevated following traumatic joint injury (median resistin level approximately 3 ng/ml 1 week following injury)¹⁷.

However, little is known about the effects of adipokines on joint tissues other than articular cartilage. A recent investigation demonstrated that 100 ng/ml visfatin increased nitric oxide production and MMP activity or matrix degradation in adult porcine cartilage and meniscus²⁹. Particularly given growing evidence that meniscal degeneration is an early event in knee OA that accompanies or precedes cartilage degeneration^{30,31}, a more detailed understanding of the effects of catabolic mediators on meniscal metabolism is desirable. The goals of this study were to evaluate dose-dependent catabolic and anti-anabolic effects of leptin, visfatin, adiponectin and resistin on cartilage and meniscus tissue explants, and to examine the relative susceptibility of these tissues to each adipokine.

Method

Materials

Immature bovine stifles were from Research 87 (Marlborough, MA). High glucose Dulbecco's modified Eagle's medium (DMEM) was from HyClone (Logan, UT). Non-essential amino acids (NEAA) and proteinase K were from Life Technologies (Carlsbad, CA). N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES), gentamicin and phosphate buffered saline (PBS) were from Mediatech (Manassas, VA). Insulin-transferrin-selenous acid premix (ITS+) was from BD Biosciences (Franklin Lakes, NJ). L-ascorbic acid 2-phosphate, proline, ammonium acetate, 1,9-dimethyl-methylene blue (DMMB), shark chondroitin sulfate, sodium nitrite, bisbenzimidazole (Hoechst 33258), and calf thymus DNA were from Sigma (St. Louis, MO). Sulfanilamide reagent and naphthylethylenediamine dihydrochloride solution were from Ricca Chemical (Arlington, TX). The CytoTox-ONE™ Homogeneous Membrane Integrity assay kit was from Promega (Madison, WI). ³H-proline and ³⁵S-sodium sulfate were from PerkinElmer (Waltham, MA). Recombinant human leptin was from Shenandoah Biotechnology (Warwick, PA), recombinant human visfatin was from Novus Biologicals (Littleton, CO), and recombinant human adiponectin and resistin were from BioVendor (Candler, NC).

Experimental design

Independent experiments evaluated the effects of initial (1 day) and sustained (9 day) exposure to adipokines. Both experiments

involved two substudies, each consisting of an untreated control group and three doses (0.02, 0.2 and 2 µg/ml) of two adipokines: leptin and visfatin in one substudy, adiponectin and resistin in the other. Tissue explants for each substudy were isolated from four immature bovine stifles (one each from four animals, total of sixteen donors), and samples from different donors and relative positions (anterior, central, posterior) were distributed across experimental groups ($n = 6–8$ explants/tissue/condition).

Tissue culture

Using a 4 mm biopsy punch, full thickness explants were isolated from the lateral femoral condylar cartilage and the caudal surface of the radially middle region of lateral menisci from bovine stifles. Explants were cultured at 37°C, 5% CO₂, and 95% relative humidity in basal, serum-free medium consisting of high glucose DMEM with 0.1 mM NEAA, 10 mM HEPES buffer, 50 µg/ml L-ascorbic acid-2-PO₄, 50 µg/ml gentamicin, 0.4 mM proline, and 1% ITS+ premix. After overnight culture, explants were trimmed to 2 mm thickness retaining intact articular surfaces.

Radiolabeled cultured media were prepared with 20 µCi/ml of ³H-proline and 10 µCi/ml of ³⁵S-sodium sulfate to label biosynthesis of proteins and sulfated glycosaminoglycans (sGAGs), respectively. In the first experiment, explants were cultured for 22 h in radiolabeled basal medium alone (control) or supplemented with 0.02, 0.2, or 2 µg/ml of adipokines, as described above. In the second experiment, explants were cultured for 8 days in radiolabel-free media supplemented with adipokines, with media exchanges every 48 h. Explants were then cultured for an additional 22 h in media supplemented with radiolabels to assess chronic effects of adipokines on biosynthesis. Conditioned media were collected and stored at –20°C for later analysis.

Biochemistry

Explants were washed four times for 30 min each at 4°C in PBS supplemented with 0.8 mM sodium sulfate and 1.0 mM L-proline to remove unincorporated radiolabels. Explants were weighed, lyophilized overnight and re-weighed dry, then digested overnight at 60°C in 1 ml proteinase K (1 mg/40 mg cartilage wet mass, 1 mg/20 mg meniscus wet mass) buffered with 100 mM ammonium acetate. Digest radiolabel contents were measured using a liquid scintillation counter. Explant DNA contents were measured using the Hoechst 33258 assay³² with calf thymus DNA standards. Sulfated glycosaminoglycan (sGAG) contents of digested explants and conditioned media were assayed using the dimethylmethylene blue (DMMB) assay³³ with chondroitin sulfate standards ranging from 0 to 100 µg/ml. To estimate production of nitric oxide (NO), a cell signaling mediator in inflammation³⁴, nitrite contents of conditioned media were measured using the Griess assay³⁵ with sodium nitrite standards ranging from 0 to 100 µM. Lactate dehydrogenase (LDH), a surrogate indicator of cell lysis, was measured in conditioned media from the 9-day culture studies using the CytoTox-ONE™ kit with purified LDH from fibrocytes as standards. As necessary, samples were diluted to remain in the linear region of each assay.

Data analysis

Radiolabel incorporation of each explant was normalized to its DNA content. All data were log transformed to improve normality. Statistical analyses were performed using Minitab 17 (Minitab, Inc., State College, PA). For each experiment, responses for each adipokine were analyzed using general linear models (GLMs) and Bonferroni's test for planned pairwise comparisons. Tissue and

adipokine dose were the main factors in each analysis, with donor animal as a random factor and position (anterior, central, posterior) nested within tissue to account for baseline regional differences. The Anderson-Darling test was performed to verify normality of residuals; in rare instances individual outcomes had non-normal residuals due to outliers, but elimination of outliers did not alter main findings. Significance was set at $P < 0.05$ and values are presented as means with 95% confidence intervals (lower limit, upper limit).

Results

sGAG release

Initial effects

In the adiponectin-resistin substudy, cartilage controls and adiponectin at all doses exhibited higher sGAG release than corresponding meniscus groups (Fig. 1; all $P < 0.001$). For cartilage, none of the adipokines significantly elevated sGAG release on day 1 compared to tissue-matched controls. In contrast, meniscus sGAG release on day 1 was significantly greater than tissue-matched controls for leptin at 2 $\mu\text{g/ml}$, visfatin at 2 $\mu\text{g/ml}$ and resistin at all doses (all $P < 0.001$). Leptin, visfatin, adiponectin and resistin induced release of 24.1% (2.6%, 45.5%), 22.8% (5.0%, 40.5%), 1.4% (1.2%, 1.6%) and 32.0% (17.8%, 46.3%) of total sGAG, respectively, as compared to 2.3% (1.6%, 2.9%) and 1.4% (0.8%, 1.9%) for controls in the two substudies. Additionally, meniscus treated with leptin (2 $\mu\text{g/ml}$, $P = 0.032$) visfatin (2 $\mu\text{g/ml}$, $P < 0.001$) or resistin (0.2 $\mu\text{g/ml}$, $P = 0.001$; 2 $\mu\text{g/ml}$, $P < 0.001$) exhibited higher sGAG release than cartilage with corresponding treatments, despite the substantially lower sGAG contents of meniscus explants.

Sustained treatment effects

sGAG release from controls was greater for cartilage than for meniscus throughout the 9-day culture duration in both substudies (Fig. 2). As a fraction of total measured sGAG, however, 8-day sGAG

release from controls was significantly greater for meniscus than for cartilage (24.6% (19.8%, 29.5%) vs 5.8% (5.3%, 6.2%)), consistent with previous observations³⁶. Cartilage sGAG release was not significantly affected by any adipokine treatment throughout the culture duration. In contrast, meniscus sGAG release on day 2 was elevated by treatment with 2 $\mu\text{g/ml}$ leptin, 2 $\mu\text{g/ml}$ visfatin and all concentrations of resistin (all $P < 0.001$ vs controls). Elevated sGAG release continued through 4 days for meniscus treated with 2 $\mu\text{g/ml}$ ($P < 0.001$) or 0.2 $\mu\text{g/ml}$ ($P = 0.023$) leptin, 2 $\mu\text{g/ml}$ visfatin ($P = 0.011$), and all concentrations of resistin (all $P < 0.001$). Later in the culture period, meniscus samples treated with resistin exhibited lower sGAG release than controls on day 6 at 2 $\mu\text{g/ml}$ ($P = 0.003$) and on day 8 at 0.2 $\mu\text{g/ml}$ ($P = 0.003$) and 2 $\mu\text{g/ml}$ ($P \leq 0.001$), likely reflecting substantial sGAG depletion. Over 8 days, leptin, visfatin, adiponectin and resistin induced cumulative release of 29.6% (32.9%, 35.4%), 38.4% (23.1%, 53.8%), 35.8% (24.9%, 46.8%) and 62.7% (51.6%, 73.8%) of measured sGAG, respectively, as compared to 20.0% (14.2%, 25.8%) and 29.3% (22.3%, 36.3%) for controls in the two substudies. Consistent with the results for immediate release, treatment with 0.2–2 $\mu\text{g/ml}$ resistin elevated meniscus sGAG release relative to cartilage with the same treatment on day 2 of culture (both $P < 0.001$), particularly notable given the substantial disparity in sGAG content between cartilage and meniscus tissues.

Nitrite release

Initial effects

Nitrite release patterns on day 1 (Fig. 3) generally paralleled sGAG release. In the adiponectin-resistin substudy, day 1 nitrite release was greater in cartilage for controls ($P = 0.035$) and adiponectin groups (all $P < 0.001$). In cartilage, nitrite release was not significantly elevated above controls for any adipokine. In contrast, leptin (2 $\mu\text{g/ml}$, $P < 0.001$), visfatin (2 $\mu\text{g/ml}$, $P < 0.001$) and all doses of resistin (0.02 $\mu\text{g/ml}$, $P = 0.004$; 0.2 and 2 $\mu\text{g/ml}$, $P < 0.001$) significantly elevated day 1 nitrite release in meniscus above

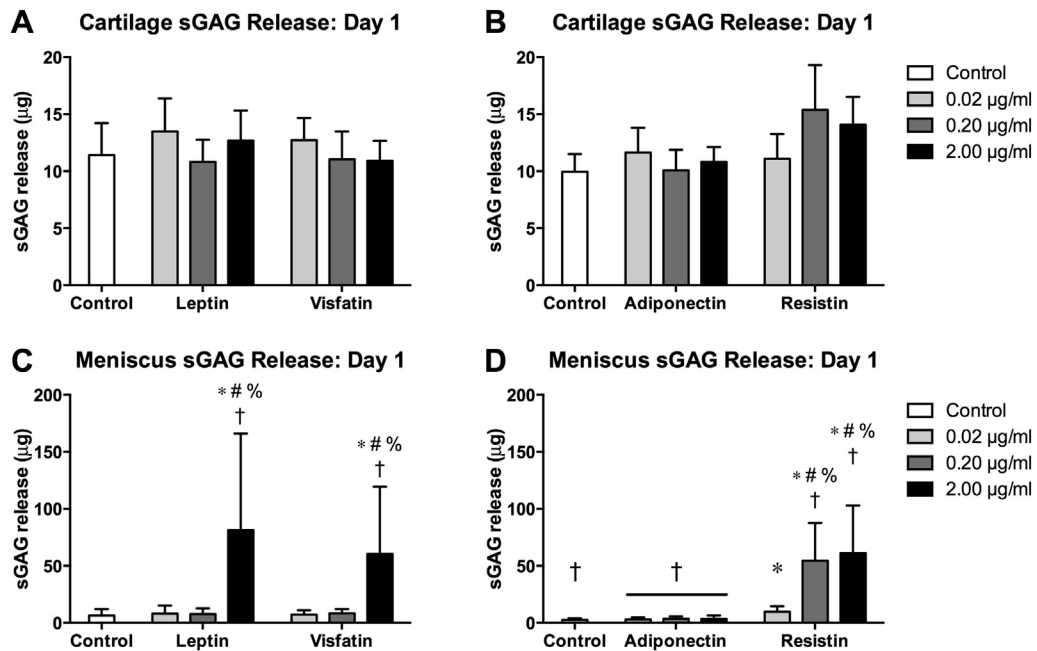


Fig. 1. Initial effects of adipokines on sGAG release. sGAG contents of conditioned media from cartilage (A–B) and meniscus (C–D) explants treated with adipokines for 22 h. Note the difference in scaling between cartilage and meniscus. * $P < 0.05$ relative to tissue-matched controls, # $P < 0.05$ relative to 0.02 $\mu\text{g/ml}$ treatment, % $P < 0.05$ relative to 0.20 $\mu\text{g/ml}$ treatment, † $P < 0.05$ relative to cartilage at the same treatment and dose.

sGAG Release: Sustained Treatment

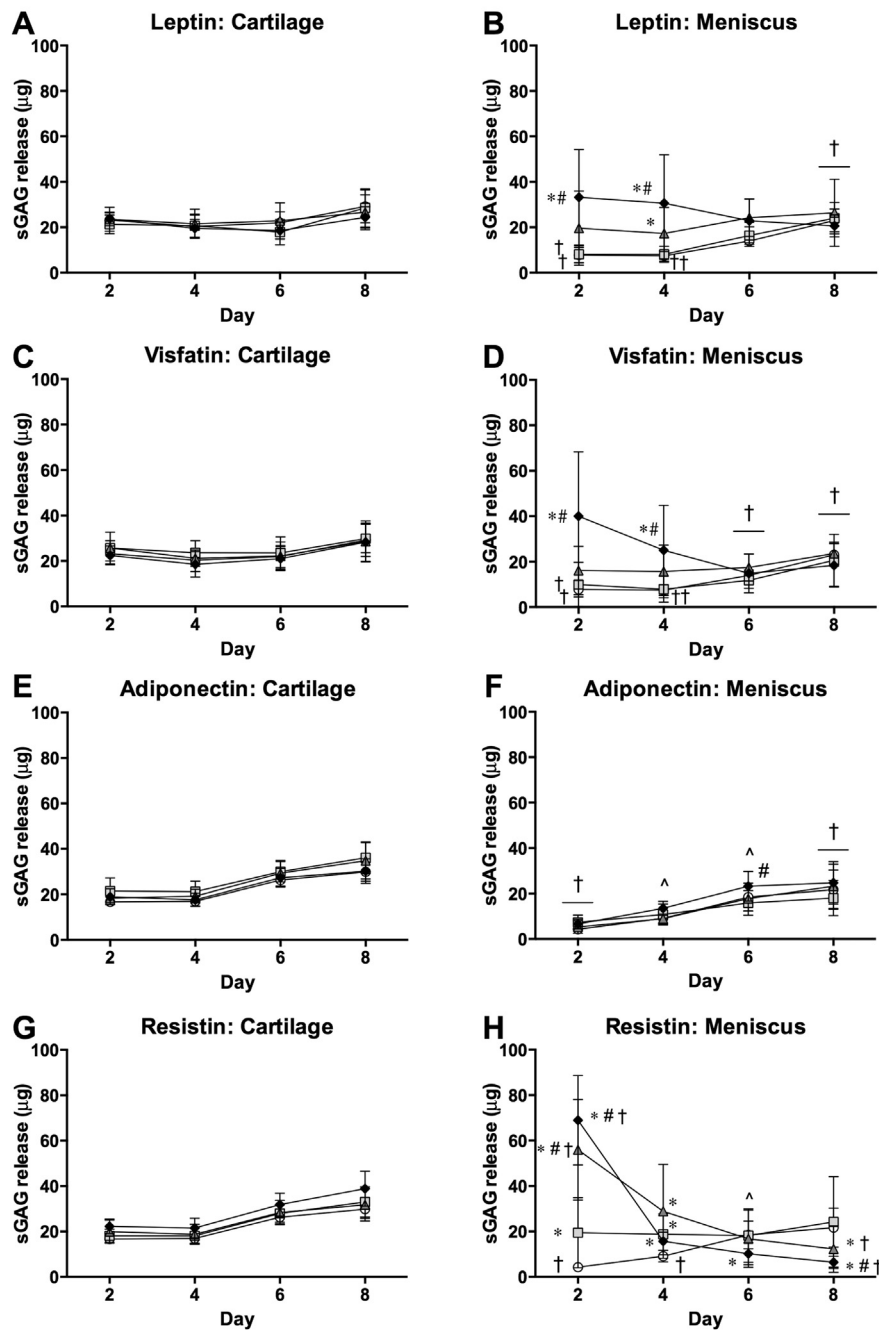


Fig. 2. Effects of sustained adipokine exposure on sGAG release. 48-hour sGAG release to media from cartilage (left column) and meniscus (right column) explants treated with adipokines for 8 days. * $P < 0.05$ relative to tissue-matched controls, # $P < 0.05$ relative to 0.02 µg/ml treatment, † $P < 0.05$ relative to cartilage at the same treatment and dose, ^ $P < 0.05$ relative to cartilage for some groups.

controls. Day 1 nitrite release was greater for meniscus than cartilage samples treated with visfatin (2 µg/ml, $P < 0.001$) or resistin (0.2 µg/ml, $P = 0.042$; 2 µg/ml, $P = 0.002$), with a trend towards greater release with leptin (2 µg/ml, $P = 0.059$).

Sustained treatment effects

Dose-dependent nitrite release patterns (Fig. 4) were similar to sGAG release patterns. On day 2 of both substudies, cartilage controls had greater nitrite release than meniscus controls (both $P < 0.001$). Cartilage nitrite release was significantly increased

relative to controls only by 2 µg/ml resistin on day 2 ($P = 0.001$) and day 4 ($P = 0.004$). Meniscus nitrite release on day 2 was elevated by treatment with all leptin doses (all $P < 0.001$), 2 µg/ml visfatin ($P < 0.001$) and all resistin doses (0.02 µg/ml, $P = 0.001$; 0.2 and 2 µg/ml, $P < 0.001$). Elevated nitrite release continued through day 4 of the culture duration for meniscus treated with 2 µg/ml resistin ($P = 0.004$). Additionally, 2 µg/ml visfatin ($P < 0.001$) and 0.2 and 2 µg/ml resistin (both $P < 0.001$) elevated meniscus nitrite release on day 2 relative to cartilage with the same treatment, despite the lower baseline (control) nitrite production for meniscus.

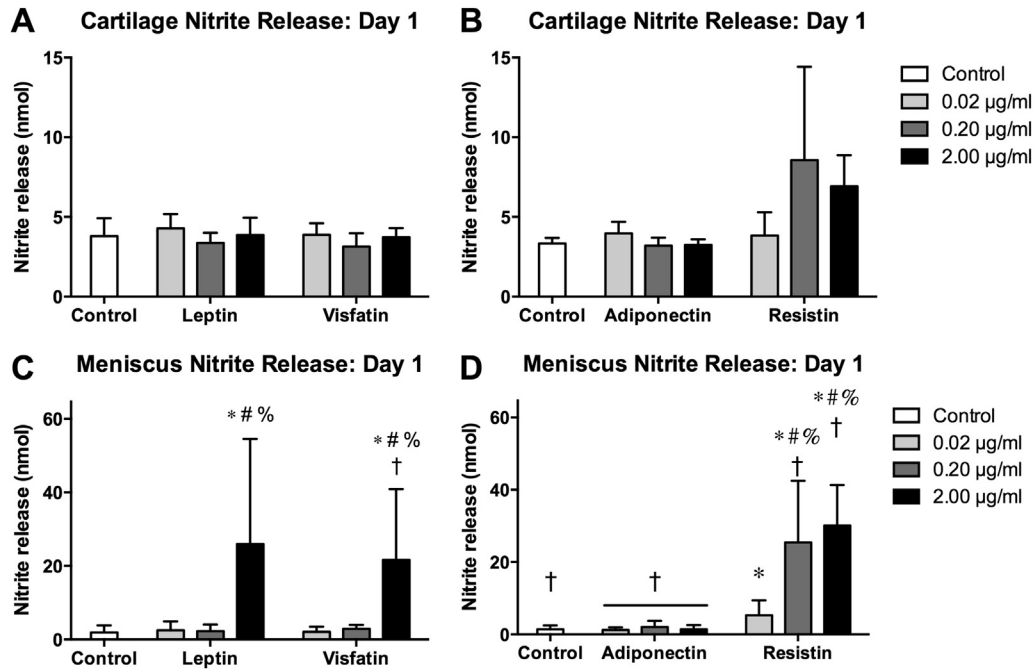


Fig. 3. Initial effects of adipokines on nitrite release. Nitrite content of conditioned media from cartilage (A–B) and meniscus (C–D) explants treated with adipokines for 22 h. Note the difference in scaling between cartilage and meniscus. * $P < 0.05$ relative to tissue-matched controls, # $P < 0.05$ relative to 0.02 $\mu\text{g/ml}$ treatment, % $P < 0.05$ relative to 0.20 $\mu\text{g/ml}$ treatment, † $P < 0.05$ relative to cartilage at the same treatment and dose.

Lactate dehydrogenase release

LDH release, a surrogate measure of cell lysis, was not significantly above controls for any adipokine for either tissue, suggesting that the doses examined were not cytotoxic. All leptin doses decreased LDH release relative to controls on days 2, 4 and 8 (all $P < 0.01$).

Explant composition

Initial effects

In both substudies, cartilage explants had greater water ($P < 0.001$) and DNA ($P < 0.001$) contents than meniscus explants, and water content was unaffected by any adipokine treatment. Cartilage DNA content was lower than controls for adiponectin at 0.02 $\mu\text{g/ml}$ ($P < 0.001$) but not for any other treatment, and meniscus DNA content did not vary with adipokine treatment. sGAG content (Fig. 5) was greater in cartilage than in meniscus ($P < 0.001$) in both substudies, indicative of differences in native tissue composition. Cartilage sGAG content did not vary with 1 day of adipokine treatment. Meniscus sGAG content after 1 day of culture was lower with 2 $\mu\text{g/ml}$ resistin than with 0.02 $\mu\text{g/ml}$ resistin ($P = 0.002$), but otherwise showed no significant variations.

Sustained treatment effects

Cartilage explants after the 9-day culture had greater water ($P < 0.001$), DNA ($P < 0.001$) and sGAG (Fig. 6, $P < 0.001$) contents than meniscus explants. Adipokine treatments did not significantly alter water or DNA contents for either tissue or sGAG content for cartilage. Meniscus sGAG content was significantly decreased relative to tissue-matched controls by resistin at 0.2 ($P = 0.001$) or 2 $\mu\text{g/ml}$ ($P < 0.001$), and was significantly lower at 2 $\mu\text{g/ml}$ than at 0.02 $\mu\text{g/ml}$ ($P < 0.001$), but was not significantly altered by other adipokines.

Biosynthesis

Initial effects

^3H -proline incorporation on day 1 of culture [Fig. 7(A–D)] was significantly greater for cartilage than for meniscus in the leptin-visfatin substudy ($P < 0.001$) but not in the adiponectin-resistin substudy. ^{35}S -sulfate incorporation on day 1 [Fig. 7(E–H)] was significantly (and substantially) greater in cartilage than in meniscus explants in both substudies ($P < 0.001$), consistent with the higher baseline sGAG synthesis rate of cartilage.

Treatment with adipokines did not significantly alter ^3H -proline incorporation on day 1 compared to tissue-matched controls. ^3H -proline incorporation was lower with visfatin at 2 $\mu\text{g/ml}$ than at 0.02 $\mu\text{g/ml}$ ($P = 0.037$) with no interaction with tissue, but there were no other significant effects of adipokine dose. In contrast, ^{35}S -sulfate incorporation on day 1 exhibited dose-dependent responses to several adipokines. ^{35}S -sulfate incorporation was lower for visfatin at 2 $\mu\text{g/ml}$ than at 0.02 $\mu\text{g/ml}$ ($P = 0.017$) or controls ($P = 0.045$), with no interaction with tissue. Leptin significantly affected meniscus ^{35}S -sulfate incorporation, with lower values at 2 $\mu\text{g/ml}$ than at 0.2 $\mu\text{g/ml}$ ($P = 0.004$), 0.02 $\mu\text{g/ml}$ ($P < 0.001$) or in controls ($P = 0.045$). Similarly, resistin significantly affected meniscus ^{35}S -sulfate incorporation, with lower values at 2 $\mu\text{g/ml}$ than at 0.02 $\mu\text{g/ml}$ ($P = 0.009$) or in controls ($P = 0.028$), and lower values at 0.2 $\mu\text{g/ml}$ than at 0.02 $\mu\text{g/ml}$ ($P = 0.043$).

Sustained treatment effects

Effects of adipokines on radiolabel incorporation rates were fairly minor by day 9 of culture. Both ^3H -proline incorporation and ^{35}S -sulfate incorporation (Fig. 8) were significantly greater in cartilage explants than in meniscus explants in both substudies (all $P < 0.001$). ^3H -proline incorporation was not significantly altered by any adipokine treatment in either tissue. For meniscus, ^{35}S -sulfate incorporation was significantly lower with 2 $\mu\text{g/ml}$ visfatin than

Nitrite Release: Sustained Treatment

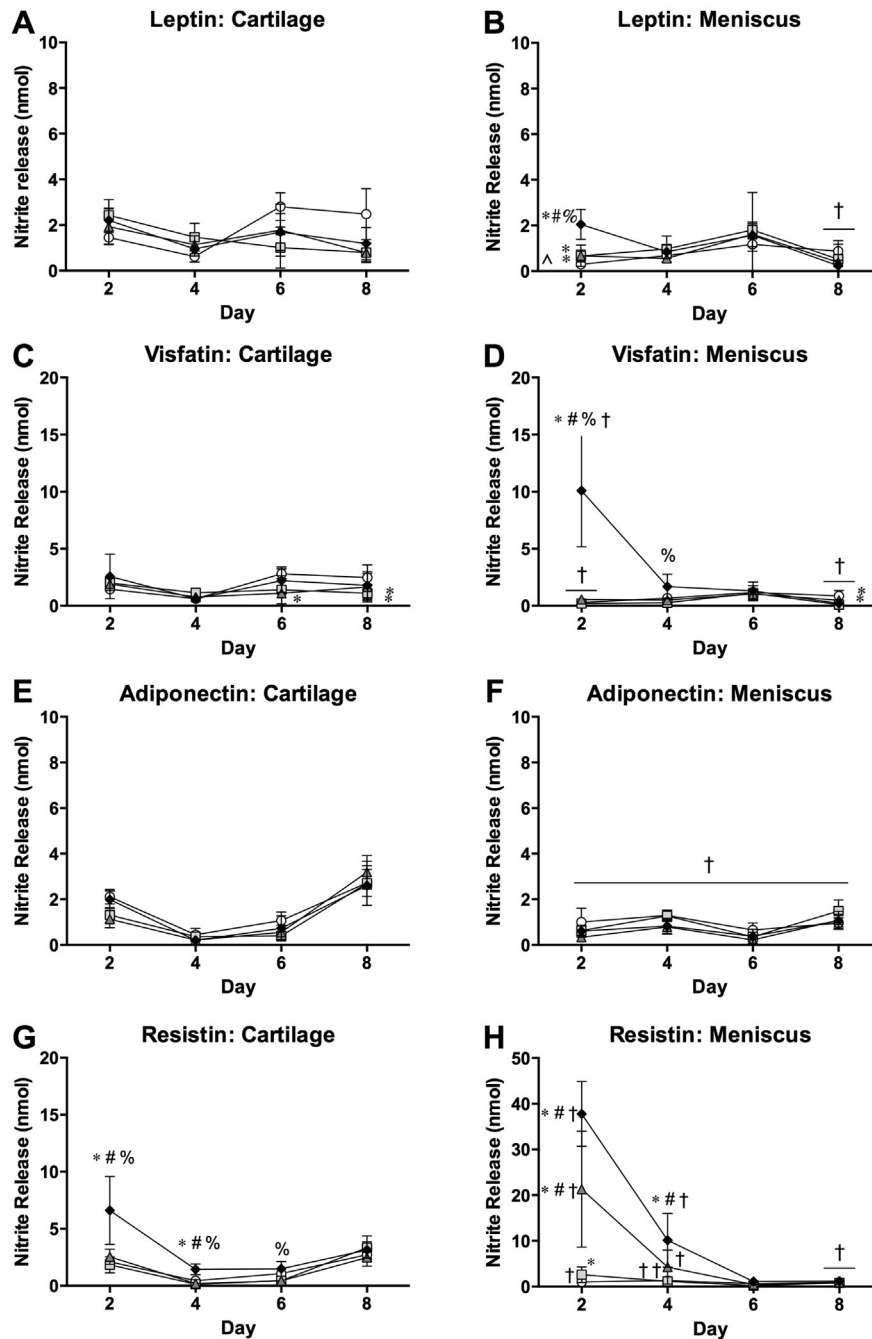


Fig. 4. Effects of sustained adipokine exposure on nitrite release. 48-hour nitrite levels in conditioned media from cartilage (left column) and meniscus (right column) explants treated with adipokines for 8 days. Note the differences in scaling. * $P < 0.05$ relative to tissue-matched controls, # $P < 0.05$ relative to 0.02 µg/ml treatment, % $P < 0.05$ relative to 0.20 µg/ml treatment, † $P < 0.05$ relative to cartilage at the same treatment and dose, ‡ $P < 0.05$ relative to cartilage for some groups.

with 0.02 µg/ml ($P = 0.023$) or in controls ($P = 0.045$), with no other significant effects of adipokines.

Discussion

While systemic and local effects of obesity-related biochemical signals have received increasing attention as non-mechanical links between excess fat and joint degeneration, few studies have compared the effects of these factors on different tissues involved

in early stage OA. The current studies evaluated catabolic and anti-anabolic effects on cartilage and meniscus explants of several adipokines present in synovial fluid and implicated in the development of OA. In meniscus explants, leptin, visfatin and resistin all stimulated sGAG release and nitrite production while inhibiting to varying degrees incorporation of newly synthesized sGAG. Effects on cartilage were relatively minor, with the only significant effects being initial reduction of matrix synthesis at the highest visfatin dose and elevated nitrite production with the highest resistin dose.

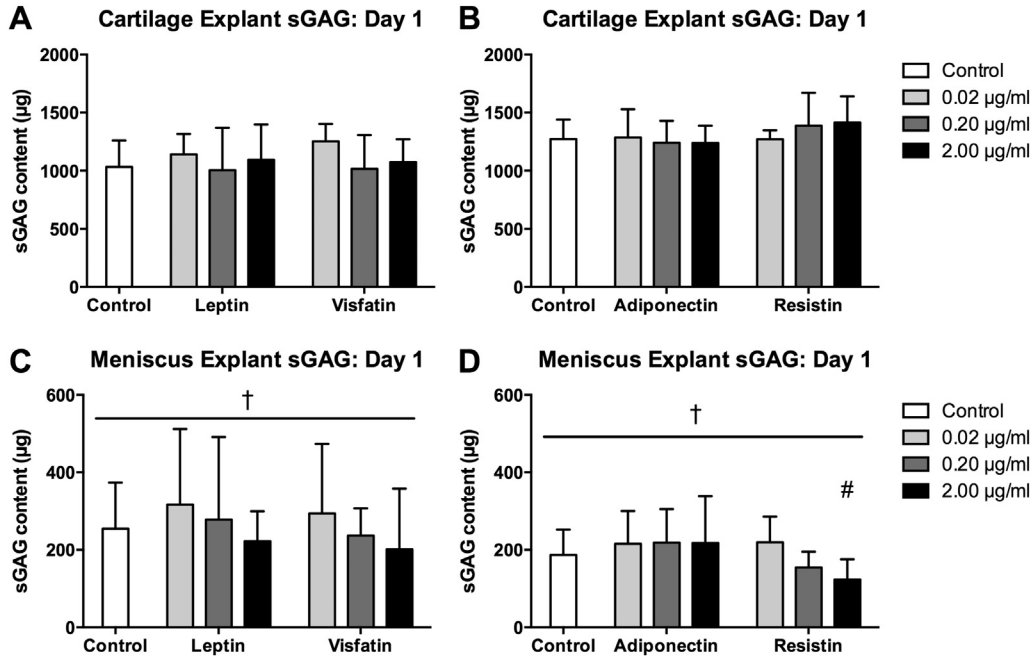


Fig. 5. Initial effects of adipokines on explant sGAG content. sGAG content of cartilage (A–B) and meniscus (C–D) explants treated with adipokines for 22 h. Note difference in vertical axes between cartilage and meniscus. #*P* < 0.05 relative to 0.02 µg/ml treatment, †*P* < 0.05 relative to cartilage.

Overall, our results indicate that meniscal tissue is more susceptible than cartilage tissue to metabolic alterations induced by these adipokines.

Interestingly, the greater relative effects of adipokines on meniscus are consistent with effects of interleukin-1 (IL-1) in similar culture conditions. Meniscus sGAG release was maximal immediately upon IL-1 α treatment and steadily decreased until sGAG depletion³⁷ while cartilage exhibited more gradual sGAG release that peaked at about 1 week of treatment³⁸. Although the

patterns of catabolism induced by adipokines (primarily resistin) and IL-1 are similar, further studies are required to determine the extent to which common pathways are involved in the response, an issue with implications for potential pharmacologic interventions to address early obesity- or injury-related degeneration. It should be noted that we used media including high glucose and insulin to ensure high metabolic activity³⁹ and for consistency with previous studies examining IL-1 effects on these tissues^{36,38}. Like cytokines such as IL-1 and TNF- α ⁴⁰, some adipokines may regulate glucose

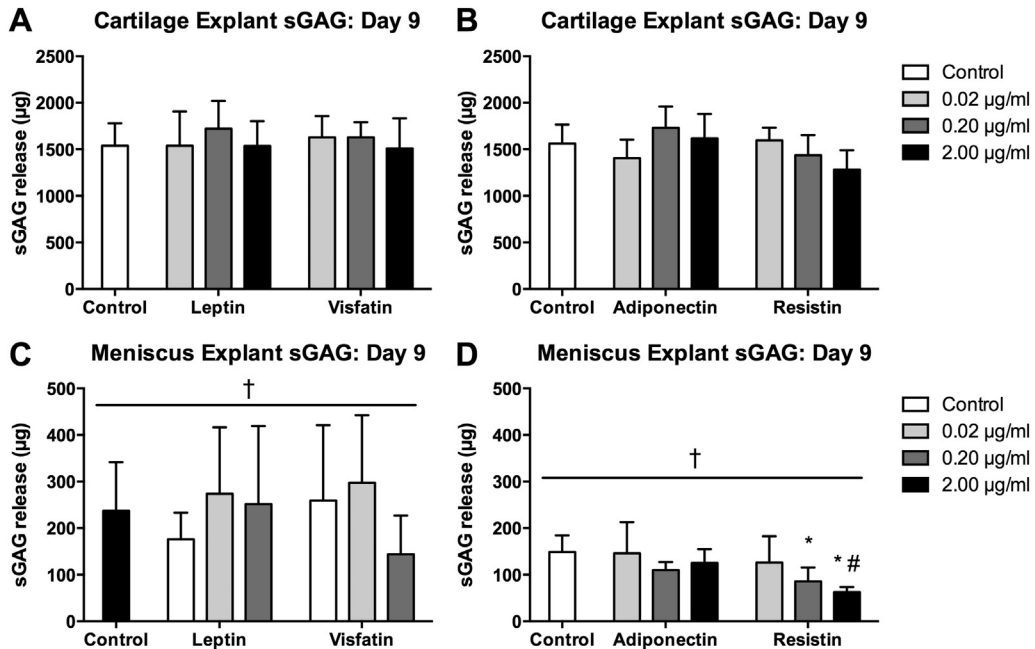


Fig. 6. Effect of sustained adipokine exposure on explant sGAG content. sGAG content of cartilage (A–B) and meniscus (C–D) explants treated with adipokines for 22 h. Note the difference in scaling between cartilage and meniscus. **P* < 0.05 relative to controls. #*P* < 0.05 relative to 0.02 µg/ml treatment, †*P* < 0.05 relative to cartilage.

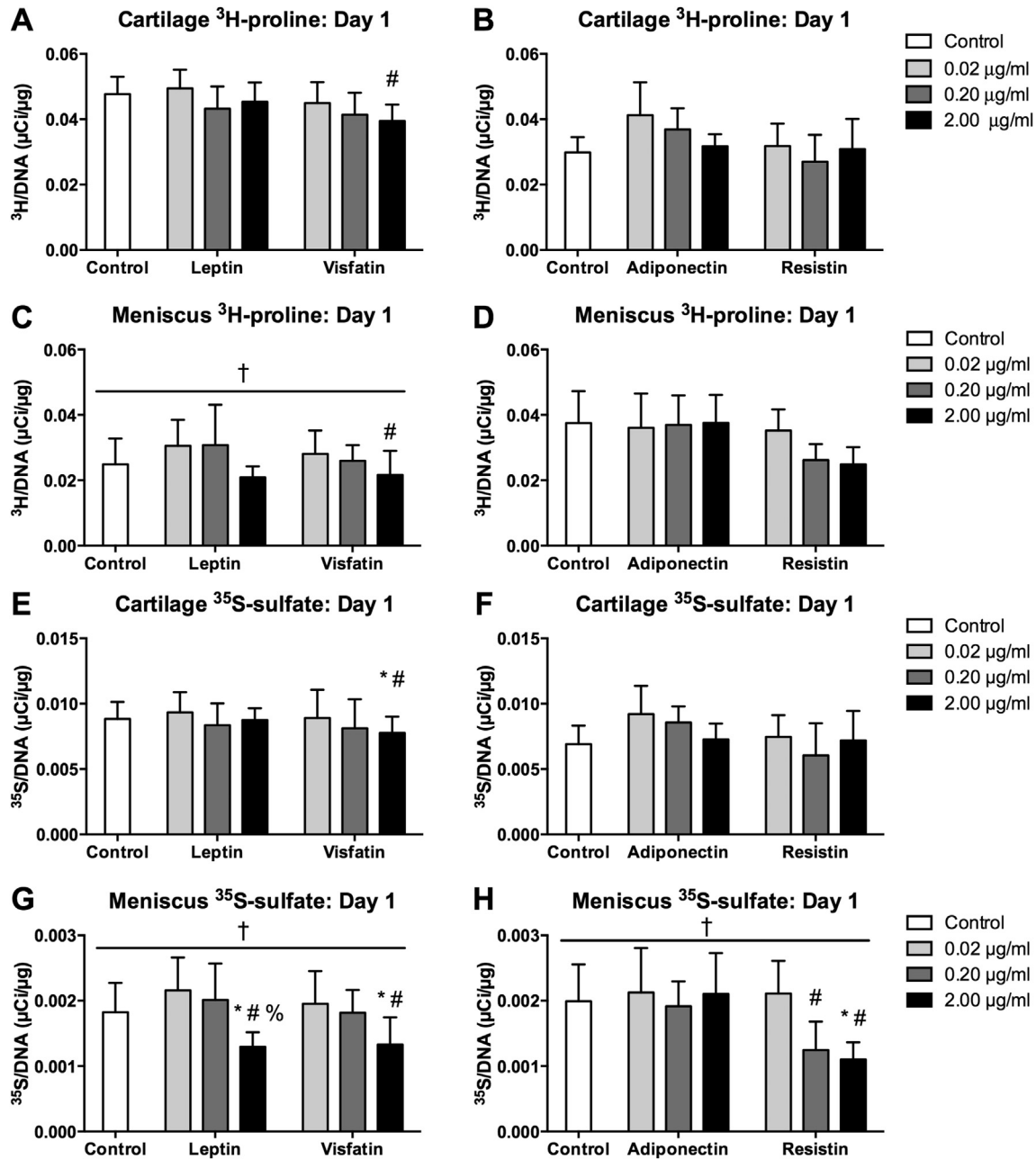


Fig. 7. Initial effects of adipokines on incorporation rates. Protein (^3H -proline, A–D) and sGAG (^{35}S -sulfate, E–H) incorporation rates of cartilage (A–B, E–F) and meniscus (C–D, G–H) explants over the first 24 h of culture with adipokines. Note the difference in scaling between cartilage and meniscus. * $P < 0.05$ relative to tissue-matched controls unless otherwise indicated, # $P < 0.05$ relative to 0.02 $\mu\text{g/ml}$ treatment, % $P < 0.05$ relative to 0.20 $\mu\text{g/ml}$ treatment, † $P < 0.05$ relative to cartilage tissue.

metabolism in cartilaginous tissues, and the effects of glucose levels (both *in vitro* and *in vivo*) on adipokine-stimulated tissue catabolism should be examined.

Several factors may contribute to the differential effects of adipokines on cartilage and meniscus tissues. Chondrocytes and meniscal fibrochondrocytes may respond differently to the adipokines, for example due to differences in receptor expression or differences in the cellular response to a given stimulus (e.g., different profiles of secreted proteases). The extracellular matrices (ECMs) may play a role via differential transport of adipokines into or through the tissues, differential transport of secreted proteases, or differential susceptibility of the ECMs to cell-mediated catabolism related to differences in ECM composition and structure. These intertwined factors could be separated to some extent by studying

the responsiveness of isolated cells to exogenous factors, detailed analysis of protease expression patterns, or selective use of protease inhibitors. The microscale arrangement of the ECM and resident cells are quite different between cartilage and meniscus, with more mesoscale heterogeneity of meniscal matrix constituents and localization of fibrochondrocytes in the proteoglycan-enriched matrix compartment surrounding the primary circumferential collagen bundles^{41,42}. This different microstructural arrangement may play a role in the tissue-specific responsiveness to exogenous factors, as a greater fraction of the tissue sGAG is near the cells in meniscal tissue. Examination of the spatial distribution of protease expression and activity through histological methods may provide additional insights into the mechanisms behind the differential susceptibility.

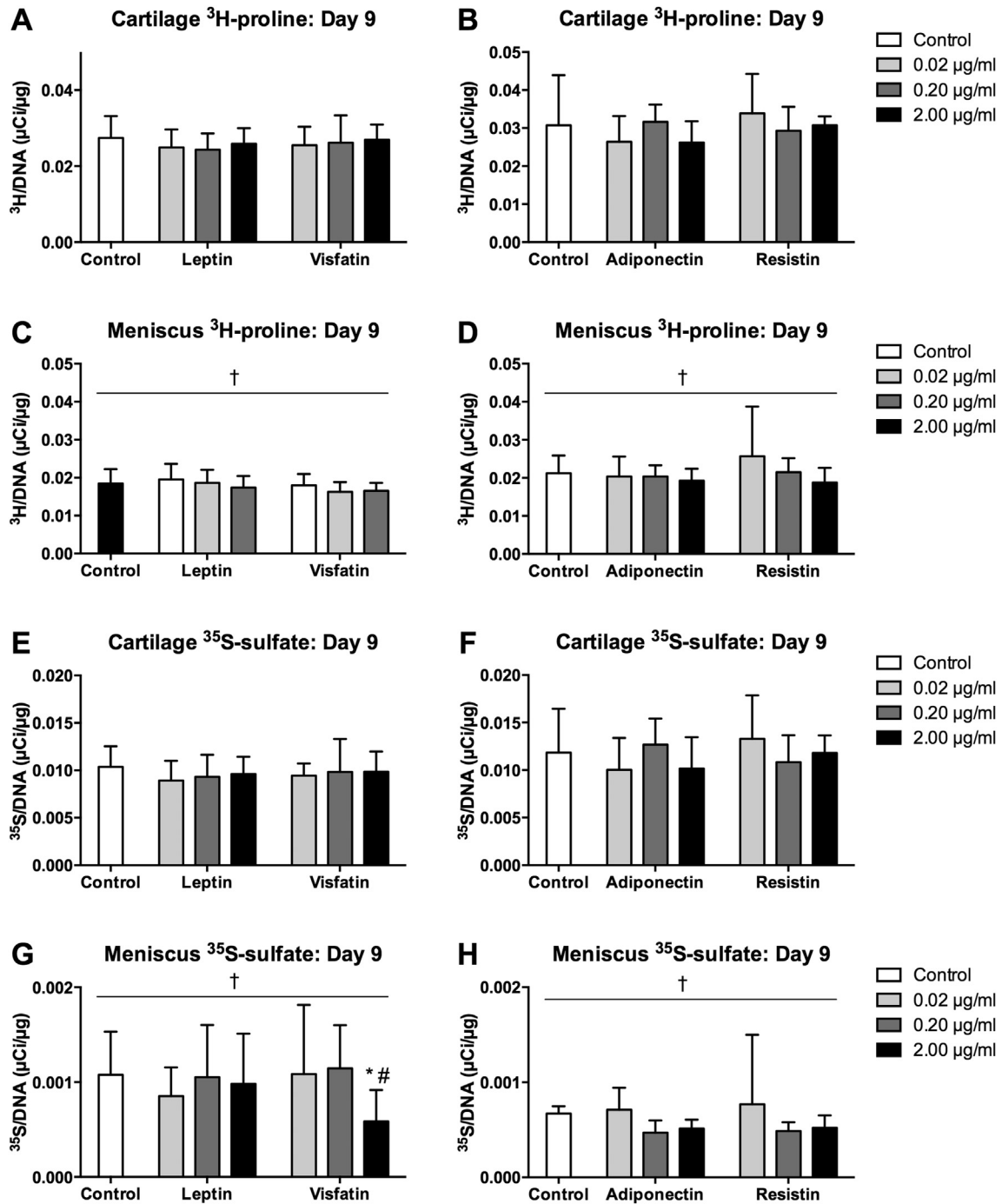


Fig. 8. Effects of sustained adipokine exposure on incorporation rates. Protein (³H-proline, A–D) and sGAG (³⁵S-sulfate, E–H) incorporation rates of cartilage (A–B, E–F) and meniscus (C–D, G–H) explants over the final 22 h of 9-day cultures with adipokines. Note the difference in scaling between cartilage and meniscus. **P* < 0.05 relative to tissue-matched controls, #*P* < 0.05 relative to 0.02 µg/ml treatment, †*P* < 0.05 relative to cartilage tissue.

Increased sGAG release was accompanied by increased nitrite release (indicative of NO production), suggesting the involvement of active inflammatory pathways. Visfatin (but not leptin) was previously reported to increase NO production in cartilage and meniscus explants and to increase sGAG release from cartilage explants²⁹. Differences with our studies include culture media (serum-supplemented vs serum-free), concentration ranges (up to 0.1 µg/ml vs 2 µg/ml) and tissue source (adult porcine vs juvenile bovine). Immature bovine tissue has often been used to study cartilage degradation *in vitro* as it provides a non-diseased blank

slate to study pathological mechanisms. However, maturity can affect cell metabolism⁴³ and responses to catabolic stimuli⁴⁴. While effects of recombinant human adipokines have been demonstrated on cartilaginous tissues from various species^{17,29,45}, caution should be exercised in directly extrapolating specific findings to human tissue without verification.

The dose ranges used in this study encompass pathological and hyper-pathological concentrations. For leptin^{10,14,16}, visfatin^{19,20}, and resistin^{12,14,17}, reported concentrations in the synovial joints of OA patients approach the lowest dose in this experiment (20 ng/

ml). It should be noted that *in vivo* adiponectin concentrations (up to 2 $\mu\text{g/ml}$ ^{12,14,23}) can be orders of magnitude higher than those of the other adipokines tested, so the adiponectin doses in this study did not reach the clearly hyper-pathological range.

In vivo animal studies with a single high dose injection of leptin into rat knees demonstrated a catabolic response in cartilage, with elevated MMP and aggrecanase expressions and proteoglycan depletion⁴⁶. While this response could have been mediated through other joint tissues such as the synovium, *in vitro* culture with 10 $\mu\text{g/ml}$ leptin upregulated MMP activity²⁵ and NO production²⁶ in human OA cartilage. In the current study, cartilage NO release was not modulated by leptin but NO production in meniscus was temporarily elevated, as observed previously for cartilage²⁶. Leptin also stimulated elevated sGAG release in meniscus through the first 4 days of the culture period and inhibited sGAG incorporation in meniscus on day 1. Although leptin decreased LDH release in both cartilage and meniscus in this study, we found no significant effects on LDH release in a preliminary study. Similarly inconsistent effects of leptin on porcine myoblasts have been reported^{47,48}.

Visfatin increased aggrecanase (ADAMTS-4 and -5) expression and MMP-3 and -13 synthesis by human OA chondrocytes in monolayer¹¹. In the current study, cartilage was minimally affected by visfatin at the concentrations tested, which may be attributable to differences in species and disease state but may also reflect differences between monolayer and 3D tissue culture. Meniscus sGAG release and NO production were elevated by exposure to visfatin while initial protein and sGAG incorporations were inhibited at the highest dose. Inhibition of meniscal sGAG incorporation persisted through day 9, suggesting that sustained exposure to visfatin might affect tissue homeostasis.

The role in cartilage catabolism of adiponectin, which is inversely correlated with BMI²¹ but associated with OA severity, remains controversial. Adiponectin has been reported to upregulate TIMP-2 and downregulate interleukin-1 β (IL-1 β)-induced MMP-13 production by OA human chondrocytes²¹, but has also been reported to increase NO and MMP production²². We observed no significant effects of adiponectin on cartilage or meniscus explants, again pointing to potential differences between monolayer and tissue culture.

Resistin downregulated collagen II and aggrecan expression and upregulated ADAMTS-4, TNF- α , and IL-1 β expression in cultured human chondrocytes²⁸, and induced dose-dependent depletion of sGAG from mouse cartilage and inhibition of sGAG synthesis in human cartilage¹⁷. In our studies, resistin was the only adipokine to substantially stimulate cartilage sGAG release, although cartilage sGAG content and incorporation rates were not significantly altered. Our results show for the first time that resistin dramatically increased sGAG release from meniscal tissue and substantially reduced meniscus sGAG content, even after just 22 h of exposure, and meniscal sGAG content was reduced to less than half that of controls after 9-day exposure to 2 $\mu\text{g/ml}$ resistin.

The strong effects of resistin are particularly interesting, as resistin was elevated in the knee joint for several weeks following traumatic injury¹⁷. Both meniscus and cartilage tissues undergo immediate cell death following relatively moderate overload^{49,50}, despite the greater resistance of meniscus to macroscopic failure. However, a greater susceptibility to inflammatory factors elevated as part of the injury response could contribute to earlier development of degeneration in meniscus than in articular cartilage. Meniscus has nearly an order of magnitude less proteoglycan content than cartilage, and small perturbations to the proteoglycan composition can induce larger relative loss of functional mechanical properties in meniscus than in cartilage^{37,38}. Following injury, the meniscus may be particularly susceptible to resistin-induced

sGAG depletion even in the absence of macroscopic damage directly related to the trauma itself.

In conclusion, these studies demonstrated a greater susceptibility to adipokine-induced catabolism by meniscus tissue than by cartilage. Resistin was the most active of the four adipokines tested, inducing substantial sGAG depletion from meniscal tissue explants. This finding is particularly relevant as traumatic injury, independent of obesity, can elevate synovial concentrations of resistin in the knee joint. Based on these results, even a short-term exposure to resistin can have catabolic effects in meniscal tissue. Degradation of the meniscus then may stimulate cartilage degradation, either by altering the local mechanical environment or biologically stimulating catabolism. As obesity continues to be a national epidemic, understanding the systemic consequences of increased adiposity is important in evaluating its impact on the lifestyles of those at risk. Investigations into the complex interactions between biologic and mechanical factors in knee joint health are not only valuable to understand the etiology of OA, but may also reveal novel targets for early diagnosis or pharmaceutical intervention.

Contributions

Both authors participated in the conception and design of the studies and interpretation of data. JFN performed the studies and drafted the manuscript. Both authors participated in revising the manuscript and approved the final version. Both James Nishimuta (j.nishimuta@gmail.com) and Marc Levenston (levenston@stanford.edu) take responsibility for the integrity of the work presented.

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The sponsors (NIH through R01AR052861, NSF through a graduate research fellowship) had no involvement in the study design and execution, data interpretation, writing of the manuscript or the decision to submit the manuscript.

Competing interests statement

Neither author has any competing interests to declare.

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