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# Influence of chondroitin sulfate on the biochemical, mechanical and frictional properties of cartilage explants in long-term culture

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ABSTRACT

A recent study [Basalo et al., 2007. Chondroitin sulfate reduces the friction coefficient of articular cartilage. J. Biomech. 40(8), 1847–1854] has shown that the friction coefficient of bovine articular cartilage is reduced significantly by the supplementation of chondroitin sulfate (CS) at a concentration of 100 mg/ml. This result suggests that intra-articular injection of CS may be used as a prophylactic treatment against the progression of osteoarthritis. The objective of this study was to test the hypothesis that long-term culture of cartilage explants in CS produces no adverse mechanical, biochemical, or cytotoxic effects, while reducing the friction coefficient relative to the control group. Long-term cultures of live bovine articular cartilage explants were performed with incubation in media containing CS of three different concentrations (0, 10 and 100 mg/ml). Frictional tests (cartilage-on-glass) were performed under constant stress (0.5 MPa) for 3600 s and the time-dependent friction coefficient was measured. Samples incubated in a 100 mg/ml of CS solution exhibited a significantly lower equilibrium friction coefficient than the control ($0.05\pm0.01$ vs. $0.18\pm0.02$ on Day 0, $0.04\pm0.01$ vs. $0.14\pm0.04$ on Day 7 and $0.04\pm0.01$ vs. $0.15\pm0.06$ on Day 14). Samples incubated in 10 mg/ml of CS did not exhibit any significant decrease in the friction coefficient. Cell viability and DNA content were maintained in all groups. However, after 28 days of culture, the Young's modulus and glycosaminoglycan content of explants incubated in 100 mg/ml of CS decreased to 5% and 40% of their initial levels, respectively. Based on this adverse outcome the hypothesis of this study is rejected, dampening our enthusiasm for the use of intra-articular CS injections as a prophylactic treatment in osteoarthritis.



## 1. Introduction

In a series of recent studies on cartilage friction, we demonstrated that chondroitin sulfate (CS) serves as an effective boundary lubricant for articular cartilage. First, it was shown that cartilage that was enzymatically degraded with chondroitinase ABC exhibits a significantly higher friction coefficient than normal tissue (Basalo et al., 2005, 2006), complementing the findings of an earlier study (Kumar et al., 2001); then it was shown that normal cartilage that had been incubated or tested in a saline bath with 100 mg/ml CS, exhibited much lower friction than tissue tested in saline with 10 mg/ml CS, or saline alone (Basalo et al., 2007).

These *in vitro* findings motivate the consideration of CS, which is a natural constituent of articular cartilage, for intra-articular therapeutic injections for the treatment of painful joint degenerative diseases, and as a potential prophylactic against the progression of cartilage degeneration (Ghosh, 1988; Pinals, 1992; Kelly, 1998), in analogy to hyaluronic acid (Balazs and Denlinger, 1993). Prior to proceeding with animal studies, the objective of this study is to first investigate the influence of high concentrations of exogenous CS on live cartilage explants in long-term culture. The hypothesis is that long-term culture of cartilage explants in high-concentration CS produces no adverse mechanical, biochemical, or cytotoxic effects, while reducing the friction coefficient relative to the control group.

To test this hypothesis, we investigate the effect of CS on mechanical and frictional properties, biochemical composition and chondrocyte viability, during long-term culture of live bovine articular cartilage explants. Explants of articular cartilage are commonly used experimentally to study cartilage function and mechanoregulation (Sah et al., 1989; Asanbaeva et al., 2007). We adopt the protocol of our recent study (Bian et al., 2008) where it was demonstrated that functional properties of cartilage explants, such as mechanical stiffness and biochemical composition, can be sustained at normal levels in a serum-free medium (Byers et al., 2006) for up to 6 weeks.

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## 2. Materials and methods

### 2.1. Sample preparation and culture

Bovine cartilage plugs were harvested from the femoral condyles of three 2–6-month-old calves. Explant disks of ( $\varnothing$ 6 mm × 5 mm) including superficial, middle and depth zone were obtained using a corneal punch. Explants were then cultured in a chemically defined serum-free medium (DMEM, 1% ITS+ Premix, 50 μg/ml L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate) (Byers et al., 2006) and supplemented with ascorbate 2-phosphate (50 μg/ml) (37 °C, 5% $CO_2$). The serum-free medium is also referred to as a chondrogenic medium (CM), for its original use in inducing chondrogenesis of stem cells. The three experimental groups were: explants cultured in CM with no CS (Control), with 10 mg/ml (CS10) and 100 mg/ml of CS (CS100) (Sigma Chemical). The CS used in this study consists of 89.6% chondroitin-6-sulfate (CS-6) and 10.3% chondroitin-4-sulfate (CS-4) from shark cartilage, a proportion similar to that in human articular cartilage (93.3% CS-6). Media were changed three times a week.

### 2.2. Mechanical testing

The mechanical properties of explant disks were evaluated at days 0, 7, 14 and 28 of culture, using a custom tabletop testing device (Mauck et al., 2000). The samples were placed in a testing chamber filled with phosphate buffered saline (PBS) with the articular surface facing up. A loading platen positioned above the sample was moved down by a stepper motor to apply compression. A creep tare load of 0.1 N was applied first, to ensure full contact between the loading platen and the sample top surface. The sample was subsequently compressed to 10% tare strain at a strain rate of 0.05%/s, before the onset of the stress-relaxation test. The equilibrium Young's modulus ($E_Y$) was determined under unconfined compression at 10% strain at the end of the stress relaxation which lasted for 2000 s. The unconfined dynamic modulus was also measured, after reaching stress-relaxation equilibrium to 10% strain, by superimposing 1% strain amplitude at 0.1, 0.5 and 1 Hz. Tests of static and dynamic compressive properties were selected since the normal physiological loading mode of cartilage is compressive.

### 2.3. Frictional testing

Friction measurements were performed at room temperature, in a PBS bath, in a previously described custom-designed testing apparatus (Krishnan et al., 2004). The friction apparatus consists of a sliding stage (Nutec, Deer Park, NY) with a motion control module (ACS Tech 80, Maple Grove, MN) to provide reciprocal translational motion; a stepper micrometer to provide normal load application (Oriel Instruments, Stratford, CT), connected to a linear variable differential transformer to measure specimen deformation (HR100, Shaevitz Sensors, Fairfield, NJ). Normal and frictional loads were measured with a multiaxial load cell (JR3 Inc., Woodland, CA.). Cartilage specimens were placed within a small recess at the center of the test chamber, with the articular side facing up. Friction measurements (cartilage against a 1 mm thick glass slide) were performed in unconfined compression stress relaxation, at room temperature, with the specimen and glass surface immersed in PBS during the entire duration of the experiment. The friction measurements (cartilage-on-glass) were performed in unconfined compression creep, under reciprocal sliding motion consisting of 100 cycles over a range of ±4.5 mm, at 1 mm/s. Under this testing configuration, the friction coefficient rises monotonically over time, from a minimum value denoted by $\mu_{min}$ to a steady-state equilibrium value denoted by $\mu_{eq}$, as shown in previous studies (Krishnan et al., 2004; Basalo et al., 2006). The pausing interval between two consecutive sliding cycles was gradually increased as the friction coefficient increased. This resulted in fewer sliding cycles during the later stage of the test, when the friction was high, helping to reduce tissue wear. A creep load of 6.3 N (corresponding to a normal stress of 0.5 MPa) was ramped up in 5 s and held constant for the duration of the friction test (3600 s). The friction force and normal force were averaged over the back and forth portions of each cycle of reciprocal motion. This averaging procedure eliminates the contribution of the tangential force that may arise when the top and bottom faces of the cylindrical sample are not perfectly parallel. The time-dependent friction coefficient was determined from the ratio of the average friction force and normal force within each cycle; $\mu_{min}$ and $\mu_{eq}$ were used in the statistical analyses.

### 2.4. Biochemical analysis

One-half of each explant disk was weighed wet, lyophilized, reweighed dry, and digested in 0.5 mg/ml Proteinase-K (Fisher Scientific; in 50 mM Tris buffered saline containing 1 mM EDTA, 1 mM iodoacetamide and 10 mg/ml pepstatin A) at 56 °C for 16 h. The PicoGreen assay (Invitrogen, Molecular Probes) was used to quantify the DNA content of the explant disks with Lambda phage DNA (0–1 mg/ml) as a standard (McGowan et al., 2002). The GAG content was measured using dimethylmethylene blue (DMMB, Sigma Chemicals) dye-binding assay with shark CS (0–50 mg/ml) as a standard (Farndale et al., 1986). The overall collagen content was assessed by measuring the orthohydroxyproline (OHP) content via dimethylaminobenzaldehyde and chloramine T assay. Collagen content was calculated by assuming a 1:7.5 OHP-to-collagen mass ratio (Hollander et al., 1994). The collagen and GAG contents were normalized to the disk wet weight. Cell viability was assessed using the LIVE/DEAD Assay Kit (Molecular Probes) where live cells are stained green with Calcein-AM and dead cells stained red with propidium homodimer.

### 2.5. Statistical analysis

Statistica (Statsoft, Tulsa, OK) was used to perform statistical analyses using two-way ANOVA and the Tukey HSD Post Hoc test ($n = 4$–6 per group) for two independent factors: culture duration (Day 0, Day 7, Day 14 and Day 28) and treatment (Control, CS10 and CS100). Interactions between these two factors were also analyzed. Shapiro–Wilk test was performed to verify the normality of the data.

## 3. Results

The water content of explants remained unchanged over time in culture ($p = 0.51$), as a function of treatment ($p = 0.11$), or interactions thereof ($p = 0.28$), averaging 77.9 ± 2.6% by weight. After 4 weeks in culture, the equilibrium Young's modulus and dynamic modulus of the explant disks cultured with media containing no CS (Control) and 10 mg/ml of CS (CS10) remained stable near initial Day 0 values (0.95 ± 0.26 MPa and 27.8 ± 7.3 MPa, respectively) (Fig. 1A and B). However, the equilibrium modulus of the explant disks cultured with 100 mg/ml of CS in the media (CS100) decreased significantly on Day 14 and dropped to about 5% of the Day 0 value on Day 28 (Fig. 1A, $p < 0.005$). There was also a significant reduction in the dynamic modulus of the CS100 group on Day 28 (Fig. 1B, $p < 0.005$).

Frictional testing in a PBS bath indicated that the cartilage explants cultured with 100 mg/ml of CS in the culture medium exhibited significantly lower equilibrium frictional coefficient as compared to the control group ($p < 0.05$), whereas 10 mg/ml of CS did not significantly reduce the equilibrium friction coefficient ($p = 0.24$) (Fig. 1C). No significant difference in minimum friction coefficient was observed among any of the groups ($p > 0.05$).

On Day 28, the GAG content of the CS100 group dropped to 2.2 ± 0.2% of the disks wet weight, significantly lower than the other two groups ($p < 0.005$), whereas the GAG content of the control and CS10 groups stayed nearly constant at the Day 0 level (Fig. 1D). In contrast, the collagen and DNA content of all three groups did not change significantly throughout the 4 week culture and remained at the Day 0 level of 10.4 ± 0.4% and 0.09 ± 0.04% of the sample wet weight, respectively (Fig. 1E and F).

Cell viability staining on Day 28 showed that there was negligible cell death, with the great preponderance of cells in all groups remaining viable (Fig. 2).

## 4. Discussion

The objective of this study was to test the hypothesis that long-term culture of cartilage explants in high-concentration CS produces no adverse mechanical, biochemical, or cytotoxic effects, while reducing the friction coefficient relative to the control group. The long-term aim is to use intra-articular CS injections as a prophylactic treatment against the progression of joint degeneration in osteoarthritis. A strong rationale in favor of this treatment modality is the recent observation that CS, which has the benefit of being a molecule native to articular cartilage, is an effective boundary lubricant for cartilage, at a concentration of 100 mg/ml (Basalo et al., 2007).

However, the results of this study demonstrate a significant loss of native (endogenous) GAG in long-term culture of explants