Effect of methylprednisolone and mechanical loading on canine articular cartilage in explant culture

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Summary

The objective of this study was to assess the effect of mechanical load on articular cartilage after in vitro corticosteroid exposure. Canine humeral cartilage was equilibrated for 4 days in defined medium with a serum substitute, then exposed to methylprednisolone sodium succinate for 20 h at 0, 0.01 or 1.0 mg/ml. After a drug-free recovery period, the explants were subjected to 0, 1 or 10 mega pascals (MPa) for 1 out every 5 s for 20 min, then incubated with [35S]-sulfate and [3H]-leucine for 4 h to measure proteoglycan and protein synthesis, respectively. When the loading occurred 22 h after drug exposure, proteoglycan synthesis was inhibited and protein synthesis was unaffected by the drug. Both were stimulated by load, relative to controls. When the loading was delayed until 142 h after drug exposure, there was no biosynthetic response to load whether or not the explant had been exposed to the drug. Proteoglycan and protein synthesis 142 h after 0 or 0.01 mg/ml were unchanged or slightly higher than at 22 h, in explants which did not receive load. In contrast, biosynthesis were strongly inhibited 142 h after 1.0 mg/ml, and there was a 40% loss of proteoglycan content, relative to 22 h controls. If explants receiving 1.0 mg/ml also received heavy (10 h/MPa) loads 142 h later, there was a 17% reduction in total dry content suggesting severe matrix damage. These in vitro results suggest that articular load can help maintain normal cartilage metabolism after corticosteroid exposure, but also suggest that heavy loading after a sub-clinical dose can cause a marked loss of matrix solids.

Key words: Cartilage, Corticosteroids, Methylprednisolone, Articular load.

Introduction

Corticosteroids are used to treat a variety of arthritic conditions in human medicine. Intrarticular corticosteroid injection is also used commonly in horses to reduce synovitis due to distal joint trauma and/or an osteochondral chip fracture [1, 2]. Corticosteroids act directly on nuclear steroid receptors in the synovial cells to reduce inflammatory responses to joint injury [3, 4]. In addition to providing symptomatic relief of joint pain and swelling, modern corticosteroids seem to inhibit the synthesis of proteolytic enzymes like stromelysin and collagenase [5-7]. However, their side-effects on the articular cartilage cells may accelerate joint degradation [8-11]. Of particular concern, cartilage proteoglycan (PG) sulfation is interrupted by clinical doses [9], leading to a net loss of cartilage matrix solids [1, 12, 13] and possibly reducing the joint’s ability to support biomechanical loads [14].

It is not uncommon for human or equine patients to resume strenuous physical training within a few days after corticosteroid injection therapy. This practice is controversial because the drug's analgesic effect may mask insidious effects on bony and soft tissues. Strenuous exercise after corticosteroid injection has been reported to accelerate joint degeneration in laboratory rats [15]. Clinical studies of ponies and horses also suggest that unrestricted activity after injection therapy can cause premature joint degeneration [13, 14, 16], but some of these data are open to interpretation because of the presence of a pre-existing joint abnormality. When horses with normal joints received corticosteroid treatment followed by paddock or treadmill exercise, there was no clear detrimental effect of exercise [18, 19]. Running alone has been reported to have positive effects on healthy cartilage [20], so moderate exercise may even be beneficial after corticosteroid treatment. These ideas could be tested experimentally at the...
who~e animal level, but it might be difficult to justify a comprehensive study involving a large athletic species like the horse or the dog or man.

The objective of this study was to assess the combined effects of methylprednisolone exposure and later mechanical loading on PG and protein synthesis and retention in normal canine cartilage, under well-regulated in vitro conditions.

Materials and Methods

Overview

Canine cartilage explants in defined culture were exposed to methylprednisolone sodium succinate, and later subjected to a brief period of pulsatile loading at one of two time points [Fig. 1(a)].

Drug Treatment

The explants were then exposed to methylprednisolone sodium succinate (Solu-Medrol, The Upjohn Co, Kalamazoo, MI, USA) for 20 h beginning 100 h after harvest [Fig. 1(a)] (this type of corticosteroid preparation is extremely soluble in water to speed clearance from the joint space after intra-articular injection). The drug was prepared according to the instructions enclosed with the manufacturer’s 1 gm Act-O-Vial packaging. Appropriate quantities of the stock injectable solution were diluted in medium and the final drug concentration was 0, 0.01 or 1.0 mg/ml. At the end of the drug exposure period, each disk was transferred to a clean well containing fresh medium. Henceforth, we refer to 0.01 mg/ml as a low dose and 1.0 mg/ml as a high dose.

To put the dose levels in perspective, a recent survey of equine veterinarians [12] found that the typical dose injected into an equine carpal joint was at least 10 mg/ml (assuming 2 ml of 40 mg/ml suspension into 6 ml of synovial fluid). Thus, our high dose was about 10 times less concentrated than a clinical dose, and our low dose was about 1000 times less concentrated than a clinical dose.
However, the constant high dose might exert metabolic effects comparable to the clinical dose because of the rapid clearance seen in vivo (~10 h half-life [23]).

MECHANICAL LOADING

The explants were subjected to pulsatile loading either 22 or 142 h after drug exposure ended [Fig. 1(a)]. Prior to loading, each cartilage disk was secured in a pre-warmed test chamber [Fig. 1(b)] which was then mounted in a special load frame inside an incubator [24]. A 2 mm diameter solid indentor applied 0, 1, or 10 mega pascals (MPa) to the center of the disk for 1 out of every 5 s, for 20 min. Since the fixtures did not restrict transverse expansion of the disk, the central core region would have experienced large compressive strains (up to 40% @ 10 MPa, [24]) during the pulsatile loading. However, the net volumetric strains stayed small (<1% @ 10 MPa) because of the recovery period between each pulse. Henceforth, we refer to the 1MPa protocol as moderate load and the 10 MPa protocol as heavy load.

To put the load levels in perspective, the contact area which transmits a dog's weight through its shoulder is such that the 1 MPa load would be comparable to a gentle walk and the 10 MPa load to vigorous running. In more familiar units, a pressure of ~ 10 MPa would be realized if a person weighing 150 pounds suddenly applied 10 times their weight to a contact area of one square inch. The period between drug exposure and load was chosen to mimic a recovery period between drug therapy and exercise, and the pulsatile waveform was chosen to approximate the loading cycle the cartilage might see in vivo.

BIOSYNTHESIS

Each disk was transferred to a tissue culture well within 2 min after the loading session, and incubated in fresh medium containing Na$_2^{[35]S}$SO$_4$ @ 10 uCi/ml and [3H]-leucine @ 20 uCi/ml (both from Amersham, Arlington Heights, IL, USA) for the next 4 h [Fig. 1(a)]. The disk was then rinsed 4 x 15 min in 0.5 ml ice-cold saline to remove free isotope, weighed, then frozen at -20°C to await later analysis. After thawing, each disk was divided physically into a core (high stress) and ring (low stress) using a 2 mm diameter dermal punch. Both pieces were digested in 1 mg/ml papain (Sigma, St. Louis, MO, USA), and aliquots in a scintillation cocktail (Ecosint, National Diagnostics, Atlanta, GA, USA) were counted using a Beckman LS6800 scintillation counter. After correction for label spillover, [35S] and [3H] content were calculated as measures of PG and protein synthesis 0–4 h after load, respectively.

COMPOSITION

Each explant was weighed at several time points. The explant was removed from its medium, gently blotted with sterile gauze, weighed on a Metler AT261 microbalance, then returned to defined culture. Weights were obtained at 3, 6, 9, 100, and either 142 and 148, or 262 and 268 h. The dry weights of the core and ring pieces were determined by our acetone drying method, as previously described [25]. The glycosaminoglycan weight of core and ring, as a measure of PG content, was determined using the dimethylmethylene blue dye-binding assay [26].

STATISTICAL ANALYSIS

We used the cartilage of healthy dogs culled from a research colony because the joints of large athletic animals like the dog are more similar than those of rodents to the joints of man. Ethical concerns aside, the economic and scientific value of these pure bred dogs mandated an efficient and meaningful experimental design. In our statistical analysis, the cartilage explant (N=108) was considered to be the smallest experimental unit. Six explants were randomly allocated to each treatment group in a complete block involving all possible combinations of (three levels of drug)x(three levels of load) x (two levels of time), counting no drug and no load as levels. The statistical analysis of the biosynthetic and compositional data involved multiple analysis of variance (ANOVA) with donor, shoulder, dose, load, and recovery time as possible sources of variation. LSD values at P < 0.05 were used to determine where differences lay.

Results

EFFECT OF TIME

The mean weight at 6 h was 8.808 mg (s.d. = 1.398, N=108), and did not vary between 3, 6, and 9 h (P < 0.01). Using each explant's weight at 6 h as a reference, wet weight increased and then decreased over the next 11 days (Fig. 2). At 100 h, the mean weight was 2.5% above the 6 h level. At 142 h (22 h after drug exposure), the mean weight was 2.1% above the 6 h level and was as yet unaffected by the drug. At 262 h, the mean weight was back to the 6 h level for no or low dose, and reduced 3.6% below the 6 h level for high dose (P < 0.05). These small changes are significant because they reflect a paired comparison of weights taken at several time points.
EFFECT OF DRUG ALONE

Corticosteroid exposure at 100–120 h had no effect on total protein synthesis at 142–146 h, relative to 3910 cpm/mg-wet uptake of [3H] in untreated controls [Fig. 3(a)]. Protein synthesis was the same at 142–146 h vs 262–266 h for no or low dose explants, but the high dose had inhibited protein synthesis 56% by 262–266 h. Drug exposure also inhibited PG synthesis at 142–146 h, reaching significance at the high dose level (-42%) relative to 104 cpm/mg-wet uptake of [35S] in untreated controls [Fig. 3(b)]. PG synthesis at 262–266 h was increased 39% after no dose, increased 89% after low dose, and decreased 49% after high dose, each relative to the 142–146 h level for the same dose. Differences between core and ring synthesis after drug exposure were not significant.

EFFECT OF LOAD ALONE

When moderate or heavy load was applied at 142 h, it stimulated total protein synthesis in the core (but not in the ring), relative to controls [Fig. 4(a)]. This stimulation reached significance after heavy loading (+31%) but there was no effect if the loading was delayed until 262 h. PG synthesis was insensitive to load alone at both time points [Fig. 4(b)]; when all three load levels are pooled (that is 0, 1, 10 MPa), proteoglycan synthesis was slightly higher (34%) at 262–264 h than at 142–146 h. Most of the variability in PG synthesis was due to dose or time effects, rather than to load.

INTERACTION OF DRUG, LOAD AND TIME

The effects of load on biosynthesis diminished with time in culture, but biosynthesis remained at or above control levels as the time between low dose and load was increased from 22 h to

![Graph](image-url)
FIG. 4. (a) Protein synthesis or (b) proteoglycan synthesis in core of cartilage explant vs load intensity. Cartilage disks in defined culture were subjected to repeated impact loading for 20 min ending at 142 (□) or 262 h (□), as described in the Methods. The disks were then incubated with [3H]-leucine and [35S]-sulfate for 4 h. The [3H] and [35S] content per wet weight of the 2 mm core of the explant provided measures of protein and proteoglycan synthesis 0–4 h after loading, respectively. In (a) and (b) the values are mean ± S.D. (6 × \(N=6\)) normalized by the mean of 142 h controls. Differences in biosynthesis are indicated by *, † (\(P \leq 0.05\)).

142 h (radiopulsed at 142–146 h or 262–266 h after harvest). At the earlier time point, protein synthesis was stimulated by load for each dose level [Fig. 5(a)]; when all three dose levels are pooled (0, 0.01, 1.0 mg/ml), protein synthesis was stimulated 24% by moderate load and 37% by heavy load. Although PG synthesis was insensitive to load when the explant had not been exposed to the drug, heavy load stimulated core PG synthesis 42% or 24% in explants exposed to low or high dose, respectively [Fig. 5(b)]. The mean biosynthetic levels for \(N=6\) untreated controls from each shoulder were within 5%. The power of our statistical testing was too low to rule out small differences between shoulders, but this would not diminish the significance of the effects of drug, load, and time described above.

The GAG content of untreated controls, as a percentage of 6 h wet weight, did not vary with time, as measured at 148 h or 268 h [Fig. 6(a)]. The GAG content of explants which had received heavy load at 262 h was similar to controls, but the GAG content of explants which had received a high dose at 100–120 h was reduced 44%. As a percentage of 6 h wet weight, the total dry content of explants which received heavy load at 262 h was similar to that of untreated controls at
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FIG. 6. (a) Glycosaminoglycan (GAG) weight or (b) dry weight of cartilage explants as a percentage of wet weight at 6 h, for selected treatments. GAG weight was assayed by the DMMB method [8] and dry weight was assayed by the acetone drying method [3], as described in the Methods. Group I and group II were 148 h and 268 h controls, respectively. The remaining groups were assayed at 268 h: group III was subjected to heavy (10 MPa) loading at 262 h, group IV was exposed to a high (1.0 mg/ml) drug dose at 100-120 h, and group V was subjected to the combined effects of a high dose at 100-120 h and heavy loading at 262 h, as described in the Methods. In (a) and (b), the values for each group are mean ± S.D. (N= 6) expressed as a percentage of the 6 h wet weight determined on the harvest day. Differences in GAG or dry weight are indicated by *, † (P ≤ 0.05).

148 h or 268 h [Fig. 6(b)]. There was no detectable change in the 268 h dry content of explants which received a high dose at 100-120 h. In contrast, the dry content of explants that received a high dose and later received heavy load at 262 h was decreased 17% (P < 0.05), relative to controls. Since the GAG content of untreated explants was only 12–13% of the total dry content, some of the lost dry content after high dose/heavy load must have been due to the loss of other matrix solids.

Discussion

Articular loading due to physical activity is always a factor after intra-articular corticosteroid therapy, but the exact levels of loading cannot be determined with precision. In the present study, drug dose and load intensity could be manipulated at will, and the effects of specific levels of these factors were isolated under controlled in vitro conditions. Several new observations suggesting important interactions between drug and load were made. On the positive side, drug-induced inhibition of PG synthesis was counteracted by a short period of loading 1 day after methylprednisolone exposure. On the negative side, the drug’s anti-anabolic effects involved protein as well as PG synthesis after a week, and heavy load exacerbated the drug-induced loss of matrix solids.

To our knowledge, the effects of in vitro corticosteroid exposure followed by loading have not been studied previously. The responses elicited from explanted cartilage may not be directly comparable to those occurring in the joints of living animals, but Fig. 3(b) suggested that methylprednisolone exposure could cause a dose-dependent inhibition of PG sulfation within 1–2 days. This matched reports of reduced PG synthesis and content after corticosteroid injection into equine joints [1, 12]. At the high dose level, the drug’s inhibitory effects were still in force a week later, and we can only conjecture that the explant metabolism might have eventually recovered from the high dose, as has been observed in ponies at 3 weeks after intra-articular injection [13]. There was a mild stimulation of PG synthesis a week after the low dose which did not quite reach significance. If confirmed, this might reflect a metabolic response to an earlier drop in PG synthesis and content, supporting the idea that PG synthesis is controlled by its concentration, as proposed by Sandy [27]. Low doses of one corticosteroid (triamcinolone hexacetonide) have been shown to slow the onset of surgically induced canine osteoarthritis [6], and this was possibly due in part to a net stimulation of biosynthesis during recovery from low dose exposure.

The ideal anti-arthritic drug would relieve pain and reduce synovial inflammation but it would also stimulate cartilage matrix synthesis. Corticosteroids at clinical doses do not satisfy the last requirement, but they are useful because of their potent anti-inflammatory effects. Modern drugs
like methylprednisolone also have a substantial effect on water retention, as confirmed by our in vitro results. The observed increase then decrease in explant wet weight over the first 11 days after harvest (Fig. 2) suggested a time-dependent injury response that affected extracellular water content. The low dose had no discernible effect on this swelling response, whereas the high dose reduced swelling a week after drug exposure, relative to controls (Fig. 2). Recognizing the limitations of our analogy between in vitro and in vivo swelling, this may support the idea that lower corticosteroid doses (high dose = 1/10 clinical dose) can still reduce joint swelling.

Corticosteroid exposure had no effect on protein synthesis a day later, but the high dose had an anti-anabolic effect on protein synthesis a week later. There was also a severe loss of GAG a week after the high dose [group IV, Fig. 6(a)] and it is possible that this distorted some osmotic signal required to maintain normal protein synthesis. The net decrease in total protein synthesis did not exclude the possibility that synthesis of specific injury-response proteins was increased, yet masked by decreased synthesis of more plentiful proteins (for example, aggregan core).

Protein synthesis was stimulated by loading on the 6th day after harvest [Fig. 4(a)], but not on the 11th day. Likewise, heavy load on the 6th day boosted PG metabolism back towards normal levels after drug exposure [Fig. 5(b)]. Other laboratories have reported anabolic effects of dynamic load on cartilage explants [28-31]. The drop-off in sensitivity to load between 6 and 11 days suggested that time in culture affected the explant's ability to modulate biosynthesis. It is therefore possible that a signal transduction mechanism which regulates normal cartilage metabolism deteriorated with time in defined culture.

Heavy load alone did not cause a detectable GAG loss (contrast to [32]) but high dose exposure lead to a drop in GAG content after a week [Fig. 6(a)]. The drug may have reduced the size of the aggregan monomer or of the PG aggregate [33] and this would facilitate GAG loss. However, our results suggested that heavy loading of drug-treated explants also promoted the loss of other matrix solids [Fig. 6(b)]. It seems reasonable to conjecture that drug-induced GAG loss might make cartilage more susceptible to matrix damage during loading. If so, this drug-load interaction might explain the putatively destructive effects of heavy exercise after corticosteroid injection. We hesitate to recommend changes in current medical practice on the basis of these in vitro results alone, but further study of the interaction between cortico-

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