

Oral Supplementation of Chondroitin Sulfate to Facilitate Fracture Healing: A Pilot Study

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Oral Supplementation of Chondroitin Sulfate to Facilitate Fracture Healing: A Pilot Study

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Introduction:

Despite decades of experience in fracture management, not until recently have the biochemical markers of fracture healing been discovered. Fibroblasts produce dermatin sulfate in the early fracture callus (1,2,3). During the second week after a fracture, chondroitin sulfate is expressed in large amounts by the chondrocytes (2,4,5). However, by the third week, the amount of proteoglycans and their aggregates decrease, and mineralization of the fracture callus begins. Approximately 9 days after a fracture, there is an abundant expression of type II collagen, the major structural protein of cartilage. Chondrocytes produce chondroitin sulfate during these first

9 days of callus formation. By the end of the second week the events involved in the production of cartilage switch off (1,2,3,6).

Pharmacological facilitation of the steps in callus formation and subsequent fracture healing remains unknown. During endochondral bone formation, proteoglycans are expressed in the extracellular matrix of the callus (1,2,3,6).

Recent literature has increased our understanding of the biochemical contributors in fracture healing. After an initial hematoma phase, local proliferation and differentiation of inflammatory cells initially occurs. This influx facilitates proteoglycan deposition and the formation of a cartilaginous callus which matures through discrete stages and is ultimately remodeled into bone (1,2,3,7,8,9,10).

Callus formation occurs in a dynamic process allowing the healing of bone. This process involves many changes in biochemical framework as the fracture callus evolves. The initial hematoma provides a scaffold for the deposition of collagens types I, II, and III, glycosaminoglycans, as well as several proteoglycans (1,2,3,11). These proteoglycans are expressed in the extra-cellular matrix of the callus and comprise the main ground substance of this connective tissue (2,3).

Heparan sulfate, dermatan sulfate and chondroitin sulfate are three of the proteoglycans that are vital components of callus formation in the first to second week of fracture healing (2,3,4). As healing progresses, there is abundant expression of type II collagen, and by day nine, it becomes the major structural component (2,3). By the third week of callus formation the amount of proteoglycans decreases and mineralization continues (2,3).

Chondroitin sulfate consists of linear repeating units of D-galactosamine and D-glucuronic acid with variable sulfation patterns as seen in Figure 1(12).

Jackson et al in 2006 examined fracture healing using the proteoglycan heparan sulfate (4). They found that local application of 5µg heparan sulfate to rat femoral fractures resulted in a significant increase in callus size, as well as increased expression of several growth factors. They concluded that heparan sulfate had

anabolic potential and may be a potential candidate therapy for enhancing bone repair.

Several studies have used chondroitin sulfate orally to examine its effect on cartilage (12,13,14,15,16). Oral supplementation reduces the degradation of cartilage matrix components, specifically collagen II, glycosaminoglycan and other proteoglycans. These studies have also suggested that the oral administration of chondroitin sulfate is safe (12,13,14,15,16,17,18,19).

Rammelt et. al. has shown that chondroitin sulfate can successfully facilitate bone healing when implants are coated with chondroitin sulfate (11). In this study, the hypothesis was tested that early administration of oral chondroitin sulfate will have similar effects on bone fracture healing and callus formation as the local direct application of chondroitin sulfate.

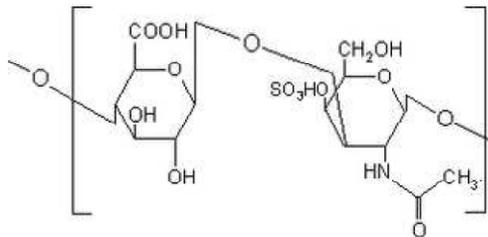


Figure 1: Chondroitin Sulfate Molecule

Material and Methods :

Experimental Design and Surgical Procedures:

Eighteen male Sprague-Dawley rats (mean weight 300g) rats were randomized into six groups of three animals and anesthetized using an intraperitoneal injection of 60mg/kg Ketamine and 10mg/kg Xylazine. For each rat, the fur of the left knee was removed and the skin was sterilized using an iodine preparation. A 5mm midline longitudinal incision was made (Figure 2). Using a standard medial parapatellar approach, the anterior intercondylar notch was appreciated. A 1.6mm Kirschner-wire was inserted manually into the femoral medullary canal until the canal was filled; the distal end of the wire was cut to sit flush with the knee (Figure 3). After insertion, the soft tissues were re-approximated and the incision was closed with a simple interrupted 4-0

Monocryl suture.



Figure 2: Longitudinal incision for dissection over knee.



Figure 3: Intra-Medullary insertion of 1.6 mm K-Wire.

While still anesthetized, a blunt guillotine-like blade device was used to generate a transverse mid-femoral closed fracture. Radiographs were immediately taken to confirm the fracture and confirm the intramedullary placement of the Kirschner-wire (Figure 4).



Figure 4: Post guillotine fracture pattern.

The rats were numbered 1-18 and individually housed. Daily buprenorphine 0.1ml was injected

subcutaneously for pain control until no signs of pain were appreciated; normal activity was resumed within a few days.

The eighteen rats were initially separated into experimental (#1-9) and control (#10-18) groups. These were further subdivided into three groups of three rats each to be euthanized at 1 week (Group A), 4 weeks (Group B), and 5 weeks (Group C) time points. All experimental rats were dosed once daily with a solution of 7mg Chondroitin Sulfate in 1mL deionized water delivered via oral gavage tubes. The animals were dosed for nine days following the procedure (except for Group A animals which were sacrificed at 1 week.).

Animals were euthanized at their respective time points by CO₂ asphyxiation and the left legs were disarticulated at the hip joint. After careful dissection of the surrounding soft tissue, the femurs were removed (Figure 5). The Kirschner-wires were removed and the femurs were placed in a 70% formalin solution at 4°C.

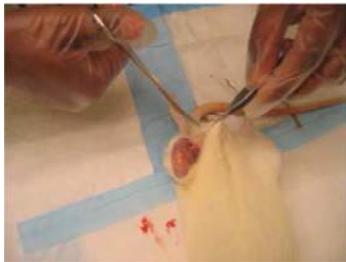


Figure 5: The soft tissue was removed prior to amputation of the limb.

MicroCT Scanning

To accurately describe the morphology of the healing fracture site, the diaphysis of each femur was scanned via micro-computed tomography (MicroCT40, Scanco Medical, SUI) at a resolution of 15 microns (70KV, 114uA). Gaussian filtering removed noise from the images and global thresholding segmented mineralized tissue from soft tissues and bone marrow. Within 216 slices of the mid-diaphysis, the following morphometric parameters were determined via software provided by the manufacturer: cross-sectional bone area, area of the periosteal envelope, area of the endocortical envelope, moments of inertia, transcortical thickness, cortical

porosity, and the volumetric density of the mineralized tissue (Figure 6).

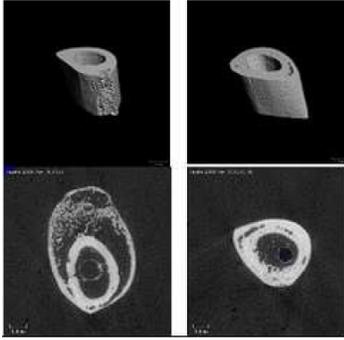


Figure 6: Micro-CT images.

Post Radiographs

Following the CT scans, the femurs were arranged on an 11x17 large radiographic cassette and anterior-posterior radiographs were taken at a distance of 100cm (Figure 7). Radiographs were evaluated by a senior orthopaedic surgical resident to describe callus formation. Using picture archiving and communication systems (PACS) computer imaging software we were able to quantify the callus formation. The widest transverse width and the longest longitudinal length were measured. A quantitative measure of the callus was calculated.



Figure 7: Radiograph of rat femurs after amputation and removal of the K-Wire.

Histology

The rat femurs were decalcified in 5% formic acid and saturated with ammonium oxalate and agitated for 24 hours. After decalcification, the tissues were prepared for paraffin embedding.

The free water was removed from the tissue with alcohol as a dehydrating agent. Xylene was used to remove the alcohol, which is not directly miscible with paraffin. The tissue was then submerged into melted paraffin on a tissue processing machine.

The specimens were kept in the VIP Surgical Processor program Overnight cycle. The 14 different stations contained the solutions of 10% formalin, 60% ethanol, 95% ethanol, 100% ethanol, xylene, and paraffin. Paraffin blocks were made next; this involved enclosing the tissue in the infiltration medium for processing allowing the medium to solidify. The final slides were made by the removing sections of uniform thickness using the microtome knife.

A drop of synthetic resin was used to remove the excess xylene. This formed a mounting medium allowing the slides to be stained. The slides were initially stained with hematoxylin and eosin. Lastly, the slides were stained with Sfog to view any cartilage formation.

A board certified blinded Pathologist reviewed all of the slides and gave mathematical percentages to granulation tissue, cartilaginous callus, and bony callus (Figure 8).



Figure 8: Experimental rat from group A showing premature healing.

Statistics:

Statistical evaluation was performed using Microsoft Excel for Windows. The significance level was set to 0.05 and all T-tests were two-sided. *P*-values were not adjusted for multiple testing.

Results :

The body weights of the rats between the two groups did not vary significantly. Of the 18 rats, three of the rats, all in the control group, died prematurely and were excluded from the study; (two had penetration of intra-medullary rod through the perineum, one was overdosed on anesthesia). Two of the rats from control group C were immediately replaced by control group A rats to maintain a similar quantity in each five week time frame.

During tissue processing, one of the femurs in the experimental group broke at the callus and was excluded from the analysis. Consequently we were able to fully analyze 14 (78%) of the initial 18 rats.

We analyzed eight experimental rats, three that were euthanized at 1 week (Group A; rats number 1,2,4), two were euthanized at 4 weeks (Group B; rats 5,6), and three that were euthanized at 5 weeks (Group C; rats 7,8,9). The control group was left with six rats, one was euthanized at 1 week (Group A; rats number 10), two were euthanized at 4 weeks (Group B; rats 11,13), and three that were euthanized at 5 weeks (Group C; rats 14,15,18).

MicroCT Results

| WK | Rat# | Periosteal Area [mm ²] | Endosteal Area [mm ²] | Bone Area [mm ²] | Cortical Porosity [%] | Cortical Thickness [mm] | Bone Density [mg/ccm HA] |
|--------------|------|------------------------------------|-----------------------------------|------------------------------|-----------------------|-------------------------|--------------------------|
| EXPERIMENTAL | | | | | | | |
| 1 | 1 | 16.2 | 3.3 | 10.5 | 18% | 1.3 | 803 |
| | 4 | 33.1 | 4.6 | 14.1 | 51% | 2.01 | 792 |
| | 5 | 11.5 | 3.6 | 7.3 | 7% | 0.91 | 909 |
| 4 | 6 | 29.7 | 3.6 | 18.9 | 27% | 2.08 | 714 |
| | 9 | 34 | 4.3 | 10.4 | 65% | 2.05 | 753 |
| 5 | 7 | 23.8 | 3.7 | 11.1 | 45% | 1.76 | 816 |
| | 8 | 11 | 4.7 | 6.2 | 2% | 0.65 | 928 |
| CONTROL | | | | | | | |
| 1 | 10 | 9.8 | 3.8 | 5.9 | 1% | 0.67 | 895 |
| 4 | 11 | 10.6 | 2.8 | 7.5 | 3% | 0.91 | 926 |
| | 13 | 12.9 | 3.6 | 8.8 | 6% | 1.01 | 885 |
| 5 | 15 | 16.2 | 4.7 | 10.4 | 10% | 1.07 | 880 |
| | 18 | 25.3 | 5.2 | 11.7 | 42% | 1.63 | 800 |

Table 1

MicroCT analyses showed that, one week after fracture, the amount of mineralized bone present in the mid-diaphysis was two-fold greater in the experimental than in the control bones (Table 1). This difference was entirely accounted for by the difference in periosteal area (152% greater)

while there was no difference in the endosteal area. Because of the much greater callus in experimental bones, cortical thickness was also 2.5 fold greater and the relative amount of cortical porosity had increased by an order of magnitude. Tissue mineral density, an indicator of the tissue mineralization was similar between the groups. However, with the number of rats in this study, statistical significance was not obtained with the MicroCt results.

When bones from the 4wk and 5wk time periods were pooled, the periosteal area of experimental rats was still 36% greater than that of control rats. Similar to the one week time point, there was no difference in the endosteal area, indicating that the treatment effect was confined to the fracture callus. The amount of bone present in the diaphysis was 12% greater, accompanied by a two-fold greater cortical porosity and a 30% greater transcortical thickness. The similarity in the density of the mineralized matrix suggested that the treatment had no effect on tissue mineralization. Again, P values were not significant using the student T-test.

Radiographs Results

| Rat # | Group | Status | Time Point Sacrificed | Gross Dimensions of Callus (cm) |
|--------------|-------|--------------|------------------------|---------------------------------|
| EXPERIMENTAL | | | | |
| 1 | A | Experimental | One Week | 1.45 x 0.18 |
| 2 | A | Experimental | One Week | 1.30 x 0.30 |
| 3 | | Experimental | Broken Callus | - |
| 4 | A | Experimental | One Week | 2.21 x 0.23 |
| 5 | B | Experimental | Four Weeks | 0.76 x 0.16 |
| 6 | B | Experimental | Four Weeks | Not Measurable |
| 7 | C | Experimental | Five Weeks | 1.15 x 0.28 |
| 8 | C | Experimental | Five Weeks | Not Measurable |
| 9 | C | Experimental | Five Weeks | 1.5 x 0.22 |
| CONTROL | | | | |
| 10 | A | Control | One Week | 0.94 x 0.24 |
| 11 | B | Control | Four Weeks | Not Measurable |
| 12 | | Control | Premature Death | - |
| 13 | B | Control | Four Weeks | Not Measurable |
| 14 | C | Control | Five Weeks | Not Measurable |
| 15 | C | Control | Five Weeks | 0.89 x 0.16 |
| 16 | | Control | Premature Death | - |
| 17 | | Control | Premature Death | - |
| 18 | C | Control | Five Weeks | 1.06 x 0.26 |

Table 2

Radiographs were evaluated by a senior orthopaedic surgical resident to evaluate callus formation. Using picture archiving and communication systems (PACS) computer imaging software, we were able to quantify the callus formation. The widest transverse width and the longest longitudinal length were measured (Table 2).

Although statistical significance was also not obtained with radiographic analysis, there was an

obvious trend towards more robust callus in the experimental group. Acknowledging the small sample size, no callus was appreciated in 2 (25%) of the experimental group, and 3 (50%) of the control group.

Histology Results

| Rat # | Group | Granulation Tissue % | Cartilagenous Callus % | Bony Callus % |
|--------------|-------|----------------------|------------------------|---------------|
| EXPERIMENTAL | | | | |
| 1 | A | 40 | 50 | 10 |
| 2 | A | 40 | 50 | 10 |
| 3 | | Broken Callus | | |
| 4 | A | 0 | 5 | 95 |
| 5 | B | 20 | 50 | 30 |
| 6 | B | 10 | 0 | 90 |
| 7 | C | 20 | 40 | 40 |
| 8 | C | 5 | 10 | 85 |
| 9 | C | 0 | 0 | 100 |
| CONTROL | | | | |
| 10 | A | 80 | 10 | 10 |
| 11 | B | 40 | 30 | 30 |
| 12 | | Premature Death | | |
| 13 | B | 0 | 20 | 80 |
| 14 | C | 50 | 50 | |
| 15 | C | 15 | 15 | 70 |
| 16 | | Premature Death | | |
| 17 | | Premature Death | | |
| 18 | C | 35 | 35 | 30 |

Table 3

A board certified Pathologist evaluated the histological slides. He was blinded to the experimental and control groups. The hematoxylin and eosin stained sections combined with the Sfog stain facilitated in the elucidation of granulation tissue, fibrocartilagenous and new bone formation (Table 3).

Rat bone number 2 in the experimental group A had significant amounts of necrotic bone and was the most premature of all the slides (Figure 8). On the other hand, experimental rats from groups B and C (rat number 4,6,9) showed significant advancement in fracture healing with rat number 4 in experimental group C showing complete healing and signs of marrow regeneration (Figure 9). These patterns of fracture healing were also evident in the MicroCT results with the most pronounced periosteal bone formation. The plain radiographs, although one dimensional, also had a significant increase in callus formation compared to the other specimens. At the same time points, there was a trend towards advanced histological progression of callus in the

experimental group (Figures 10, 11).



Figure 9: Experimental rat from group C showing almost complete healing.

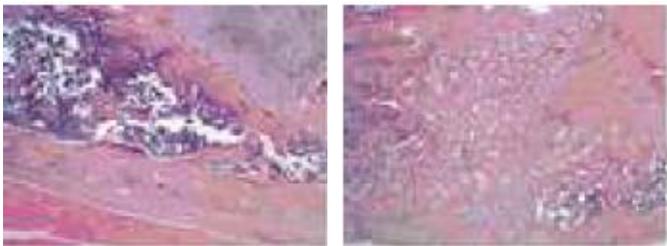


Figure 10, 11: Trend towards histological progression of callus in experimental group.

Discussion:

This pilot study demonstrated that supplementation of oral proteoglycans to acute fractures may facilitate bone healing. Chondroitin sulfate is a proteoglycan that has been found to be a key biochemical contributor in the early phases of bone healing. The initial fracture hematoma is organized by the infiltration of local inflammatory cells and subsequent proteoglycan deposition. Further remodeling yields a cartilaginous callus which matures through discrete stages and is ultimately remodeled into bone. (2,6). In this study, chondroitin sulfate given during the initial hematoma phase showed differences in the periosteal area of experimental rats indicating that the treatment effect was confined to the fracture callus. Additionally, the histology demonstrated advances in marrow formation and bony callus compared to the control group. However, P value of <0.5 could not be achieved with the small sample size of this pilot study.

This study showed that a single oral daily dose of chondroitin sulfate increased the callus size by 152% within the first week and over a period of 5 weeks the mean callus size was over 36%

larger than the control group. The chondroitin sulfate did not increase the rate of endochondral healing as evidenced by the same proportions of endosteal area in the experimental as well as the control group (Table 1). The increase in bone content suggests that more bone tissue formed through intramembranous ossification rather than endochondral ossification.

Heparan sulfate, dermatan sulfate and chondroitin sulfate are three of the proteoglycans that are vital components of callus formation in the first to second week of fracture healing. Song et. al. showed that heparin sulfate and chondroitin sulfate expression was generally found to increase in the days immediately following injury, reaching peak expression two weeks post-surgery (3). These authors suggested the possibility of using exogenous proteoglycans as an adjunct to fracture healing (3).

In 1962, Burger et. al. documented accelerated new bone formation using chondroitin sulfate in rat cranial bone defects (17). In 1962 they published a follow up study comparing their study arm of demineralized bone and chondroitin sulfate compared to a control group without chondroitin sulfate in Wistar albino rat's cranium. Thirty three percent less time (six weeks versus nine weeks) was required in the experimental group to achieve maximal healing (17).

Jackson et. al. in 2006 examined the augmentation of fracture healing using an exogenous proteoglycan (4). They found that local application of 5 μ g heparan sulfate to rat femoral fractures resulted in a significant increase in callus size, as well as increased expression of several growth factors. They concluded that heparan sulfate had anabolic potential and may be a potential candidate therapy for enhancing bone repair. Although effective, heparan sulfate is not routinely used, nor readily available for oral administration in humans. It would be difficult to perform this in vivo to humans with acute fractures. Oral chondroitin sulfate has been proven to be safe and may be effective in augmenting fracture healing if given during the hematoma phase of bone injury.

A pilot study has inherent limitations. Due to the small sample size in each group, statistical significance could not be obtained for each criteria measured. Radiographs demonstrated to be the least helpful tool in defining callus formation, and have been confirmed in the literature to be the least sensitive tool in addressing fracture healing in rats (20). Three dimensional imaging

and histology have been promoted in the literature as more sensitive tools and was found to be useful in this pilot study (20). There was a trend towards increased callus formation in all of the experimental arms, most notably with the micro CAT scan. The pathology results also demonstrated a strong correlation between chondroitin sulfate and increased callus formation.

The pharmacologic response to chondroitin sulfate in patients with knee osteoarthritis was analyzed (21,22). It was discovered that chondroitin sulfate is a slow acting drug. This study showed that pharmacologic response increases as a function of time until it reaches the maximal effect, even after cessation of treatment (21,22). We believe that by taking advantage of the long acting effects of chondroitin sulfate, it could be used as a safe and effective tool to facilitate bone remodeling, specifically in bones notorious for non-union (23,24,25,26,27).

To our knowledge, the mechanism of bone repair by chondroitin sulfate is yet unclear. Based on the results of our study we would suggest that chondroitin sulfate has the potential to enhance callus formation. This study provides a framework for future studies to investigate oral supplementation for the facilitation of fracture healing.

In addition, chondroitin sulfate chains often contain multiple protein binding sites, the particular sulfation pattern of bone-specific chondroitin sulfate, and its resultant growth factor-binding capabilities need to be investigated with further research to determine the best combination for enhancing bone repair.

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