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Poly-N-Acetyl Glucosamine (sNAG) Enhances Rotator Cuff Tendon Healing in a Rat Model

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Abstract

Rotator cuff injuries frequently require surgical repairs which have a high failure rate. Biological augmentation has been utilized in an attempt to improve tendon repair. Poly-N-acetyl glucosamine (sNAG) polymer containing nanofibers has been shown to increase the rate for healing of venous leg ulcers. The purpose of this study was to investigate the healing and analgesic properties of sNAG in a rat rotator cuff injury and repair model. 144 adult male Sprague Dawley rats underwent a transection and repair of their left supraspinatus tendons. Half of the animals received a sNAG membrane on the tendon-to-bone insertion site. Animals were further subdivided, receiving 1 or 3 days of analgesics. Animals were sacrificed 2, 4, or 8 weeks post-injury. Animals sacrificed at 4 and 8 weeks underwent longitudinal in vivo ambulatory assessment. Histological properties were assessed at 2, 4, and 8 weeks, and mechanical properties at 4 and 8 weeks. In the presence of analgesics, tendons receiving the sNAG polymer had significantly increased max load and max stress. Ambulatory improvements were observed at 14 days in stride length and speed. Therefore, sNAG improves tendon-to-bone healing in a rat rotator cuff detachment and repair model.

Key Terms

supraspinatus repair; animal model; analgesia; mechanics; orthopaedics

INTRODUCTION

Rotator cuff injuries are a common musculoskeletal problem frequently requiring surgical intervention to restore shoulder function and decrease pain. Although advancements in surgical techniques and rehabilitation protocols have been made, this procedure continues to have a high failure rate.⁶ The tendon-to-bone repair site consists primarily of fibrotic scar

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CONFLICTS OF INTEREST

The author (LJS) has received funding during the conduct of the study related directly to the subject of this manuscript from Marine Polymer Technologies Inc, and has also received funding from commercial entities related indirectly to the subject of this manuscript. The author (JV) is employed by Marine Polymer Technologies, owns stock in the company, and supplied the materials used in this study.

tissue, which is mechanically inferior to native tissue. Biological augmentation can take several forms including the use of patient-derived growth factor enriched platelet-rich plasma (PRP) and autologous cell delivery such as mesenchymal stem cells (MSCs). However, these therapies are not well-established. Both approaches have shown mixed results in tendon repair, and there is little clinical evidence that patient-derived materials can reliably improve healing over a diverse patient population.^{4,9,22}

Due to the variability in cells and plasma obtained from the patient population, there is a need for additional biological materials with which to augment tendon healing. Poly-N-acetyl glucosamine (sNAG) polymer containing nanofibers has been shown to interact with tissues and cells and facilitate downstream responses. Unlike previously discussed materials, this polymer is produced by oceanic microalgae (Talymed®, Marine Polymer Technologies Inc, Burlington, MA, USA). sNAG has been shown to mediate red blood cell aggregation due to its ionic interactions with cell-surface proteins¹⁸ which in turn can activate platelets.¹⁹ Adherence of biomaterials to human tissues is an important aspect of augmentation success, and is dependent on a net positive charge of the material. The positive surface charge of sNAG is similar to that of human skin¹⁴, promoting strong adhesion. In a mouse cutaneous injury model, sNAG was shown to decrease scarring and increase failure load by immune activation via Akt1 signaling.¹³ Clinically, sNAG membranes have been shown to accelerate the rate of healing in venous leg ulcers, with an 86% success rate.¹⁰ Animal studies utilizing this material for diabetic wound healing indicate enhanced cell migration, cell metabolic rates, vascularity within wound beds, and inflammatory cell recruitment.¹⁷ These findings suggest that this material might also be helpful in tendon healing.

Additionally, N-acetyl glucosamine is part of the glycosaminoglycan family that has been demonstrated to provide pain relief from osteoarthritic knee pain and stiffness.^{15,21} There is anecdotal evidence that this material may possess analgesic effects when applied directly to a tissue (*personal communication with Vournakis and Finkelstein, 2016*). As non-steroidal anti-inflammatory drugs have been implicated in impairment of rotator cuff healing², and opioid dependency can develop during surgical pain management¹¹, there is a need for alternative analgesic approaches. Therefore, the purpose of this study was to investigate the potential healing and analgesic properties of sNAG membranes in a rat rotator cuff tendon injury and repair model. We hypothesized that sNAG would improve tendon-to-bone healing and reduce pain in animals after supraspinatus detachment and repair.

MATERIALS AND METHODS

Study Design

144 adult male Sprague-Dawley rats (400–450g) were used in this University of Pennsylvania Institutional Animal Care and Use Committee approved study. Animals were housed in a conventional facility in 12-hour light/dark cycles and were fed standard rat chow ad libitum. All animals underwent a full thickness transection and repair of the left supraspinatus tendon^{1,2}. Prior to surgery, animals were randomized into treatment groups. Half of the animals received a standard repair, while the other half the animals received a thin membrane of sNAG (4 mm diameter) placed on the “foot print” of the supraspinatus tendon to bone insertion. Therefore, animals were further subdivided, receiving only 1 day

of analgesics (buprenorphine) on the day of surgery or receiving the standard 3 days of analgesics. Therefore, animals were randomized into one of four groups receiving: 1) only 1 day of buprenorphine and no sNAG (1DB.NS), 2) the standard 3 days of buprenorphine and no sNAG (3DB.NS), 3) 1 day of buprenorphine with sNAG (1DB.S), and 4) 3 days of buprenorphine with sNAG (3DB.S). All groups were allowed normal cage activity after surgery. Animals were sacrificed at either 2 (n=4 per group), 4 (n=16 per group), or 8 weeks (n=16 per group) post-injury and repair. Those sacrificed at 2 weeks underwent histological analysis only, while animals sacrificed at 4 and 8 weeks underwent either quasi static mechanical testing (n=12 per group), or histological analysis (n=4 per group) and longitudinal in vivo ambulatory assessments with measurements 1 day pre-injury and 3, 7, 14, 28, 42, and 56 days post-injury and repair.⁷

Detachment and Repair Surgery

All animals underwent a full thickness transection and repair of the left supraspinatus tendon as described previously.¹ Buprenorphine (0.05 mg/kg) was administered subcutaneously 30 minutes prior to surgery and 6–8 hours post operatively in the groups receiving one day of analgesics. The standard analgesics group received the same doses on day one, followed by a dose every 12 hours for the next 48 hours. Briefly, with the left forearm held in external rotation and adduction, the deltoid muscle was split transversely to expose the supraspinatus tendon. The tendon was grasped using double-armed 5-0 polypropylene suture (Surgipro II, Covidien, Mansfield, MA) and was sharply transected from its insertion on the humeral head. For repair, a 5 mm diameter high speed bur (Multipro 395, Dremel, Mt. Prospect, IL) was used to remove remaining fibrocartilage from the footprint of the tendon insertion site. A 0.5 mm bone tunnel was drilled anterior to posterior through the greater tuberosity of the humerus. The suture was passed through the bone tunnel and tied down, affixing the tendon to the greater tuberosity using a modified Mason-Allen technique. Before tightening the suture for repair in the groups receiving sNAG (1DB.S and 3DB.S), a 4 mm diameter circle of Tallymed® was placed over the insertion site, apposed between the tendon and bone interface (Fig 1). The size of the construct was consistently achieved by removing circles with a 4 mm biopsy punch at the time of surgery. The surgical site was flushed with saline, and the deltoid and skin was sutured closed with 4-0 Vicryl suture (Ethicon, Bridgewater, NJ).

Quantitative Ambulatory Assessment

Ground reaction forces (medial/lateral, vertical, braking, propulsion) and temporal spatial parameters (step length and width) were measured using an instrumented walkway.¹⁶ Measurements were taken longitudinally, and included baseline measures taken 1 day pre-injury and then at 3, 7, 14, 28, 42, and 56 days post-injury and repair. Parameters were averaged across walks on a given day. Ground reaction force measurements were normalized to body weight.

Tendon Mechanical Testing

Animals sacrificed at 4 and 8 weeks (n=12 per group) were frozen at –20°C and later thawed for dissection at the time of quasi static mechanical testing.^{3,16} Left supraspinatus and humerus tendon-bone units were dissected from each rat and cleaned of excess soft

tissue under a stereomicroscope. Stain lines were placed along the length of the tendon using Verhoeff's stain for optical strain measurement. Cross sectional area was measured using a custom laser device as previously described.⁵ The humerus was embedded in polymethylmethacrylate (PMMA) and held in a custom fixture. The end of the tendon was fixed between fine grit sand paper using cyanoacrylate and then fixed in a custom grip. Samples were immersed in a 37°C PBS bath and subjected to a mechanical testing protocol consisting of a preload to 0.08 N, ten cycles of preconditioning (0.1–0.5 N at 1% strain/s), a stress relaxation to 5% strain (5%/s) followed by a 600s hold, and finally a ramp to failure at 0.3%/s. Maximum stress was calculated as force divided by cross sectional area and 2D Lagrangian optical strain was determined from stain line displacements measured from images taken throughout the test using custom tracking software.³

Histological Analysis

Histological analysis was performed on animals sacrificed at 2, 4, and 8 weeks (n=4 per group) to assess cell shape, cellularity, and collagen fiber organization at the injury site and midsubstance of the repaired supraspinatus tendon. At the time of sacrifice, supraspinatus–humerus units were immediately dissected, fixed in formalin, and processed using standard paraffin techniques. 7µm sections were stained with hematoxylin and eosin (H&E) to assess cell shape, cellularity, and collagen fiber orientation at both the tendon insertion (injury site) and midsubstance. Cell shape and cellularity were evaluated by three blinded graders using a semi-quantitative method. Images were graded on a scale between 1 and 3 for both measures (where 1= fewer cells/more rounded shape and 3= greater number of cells/more spindle shaped). Grading standards were created within the set of images at each time point, and at least 2 images per region were assessed per specimen. Circular standard deviation of collagen fibers was determined by images taken with a polarizing microscope and analysis with custom software as described previously.^{7,8,20}

Statistical Analysis

Sample sizes were determined using a priori power analyses. Statistical comparisons were made between 1DB.NS and 1DB.S (to test the effect of sNAG on tendon to bone healing with abbreviated analgesics), 3DB.NS and 3DB.S (to test the effect of sNAG on tendon to bone healing with standard analgesics), and 1DB.S and 3DB.S (to test the effect of sNAG in the presence of abbreviated versus standard analgesics) at each time point. Mechanical testing and collagen fiber organization comparisons were made using two-tailed t-tests at each time point with significance set at $p = 0.05$. Semi-quantitative histological comparisons were made using non-parametric Scheirer-Ray-Hare tests with significance set at $p = 0.05$. Ambulatory assessment comparisons were made using a 2-way ANOVA with repeated measures on time with follow-up t-tests between groups at each time point. Multiple imputations were performed to replace missing data points (~10% per time point) necessary for repeated measures analysis. Significance was set at $p = 0.05$.

RESULTS

Tendon mechanical properties

Differences in some mechanical properties were observed between groups with and without sNAG that received 3 days of analgesics (3DB.NS and 3DB.S) (Fig 2). Specifically, in the presence of analgesics, tendons receiving the sNAG polymer had significantly increased max load (Fig 2A) and max stress (Fig 2B) at 4 weeks compared to tendons without sNAG. At 8 weeks, max load (Fig 2A) was significantly decreased and max stress (Fig 2B) trended toward decreased with the addition of sNAG. No differences in tendon modulus or stiffness were detected (Fig 2C,D). Additionally, no changes in tendon cross-sectional area or percent stress relaxation were identified across treatment groups (data not shown). Failure data excluded samples that failed at the grip or growth plate. These data are represented in Figure 3, with full data sets shown on the right (Fig 3E, F, G, H, excluded values indicated by red markers) and analyzed data shown on the left. These forms of non-physiological mechanical failure do not indicate the true maximum load values of the healing tendon, and were therefore excluded from analyses. At 4 weeks, only a small number of data points were excluded, and they were distributed similarly for each group (Fig 3E,F). At 8 weeks, a large percentage of samples were affected (up to 58% per group) and importantly, the distribution of excluded data are not uniform (Fig 3G,H), skewing the max load and max stress data for 3DB.S towards lower overall average values.

Similar to the use of sNAG in the presence of analgesics, maximum stress trended toward an increase at 4 weeks when comparing groups with and without sNAG when only 1 day of analgesics was provided (Fig 2B, 1DB.NS vs. 1DB.S).

Some differences were also identified between analgesic groups in the presence of sNAG. At 4 weeks, max load was also significantly greater for animals receiving both 3 days of analgesics and sNAG when compared to 1 day of analgesics and sNAG (Fig 2A, 3DB.S vs 1DB.S). Max stress also trended toward an increase for this comparison (Fig 2B, 3DB.S vs 1DB.S).

Overall, the use of analgesics alone did not have an effect on mechanical properties (1DB.NS vs 3DB.NS). No differences were seen between any groups in other mechanical parameters (cross-sectional area, stiffness, modulus, or % relaxation; data not shown).

Histological analysis

No significant differences were detected between treatment groups at any time point based on the power available in this study. Significant differences were seen across time in insertion and midsubstance cellularity (Fig 4A,B). Similarly, cell shape trended toward more spindle shaped across time at the insertion (Fig 4C). Collagen organization was increased (decreased CSD) at 8 weeks in the insertion of the 3DB.S group compared to the 1DB.S group (Fig 5A). No differences were seen in collagen organization at other time points (data not shown).

Quantitative ambulatory assessment

There were no significant differences between any groups for measurements of medial lateral force, propulsion force, braking force, and vertical force. Stride width and stride length of the uninjured limb were also not different between groups. Speed of the injured left limb demonstrated that at 3 days, rats that were still receiving analgesics walked faster regardless of the presence of sNAG (Fig 6A, 3DB.S vs 1DB.S). This group continued to show increased speed at 28 and 56 days. Additionally, animals who received 3 days of analgesics walked faster in the presence of sNAG than those without (Fig 6A, 3DB.S vs. 3DB.NS) at both 7 and 28 days.

Similarly, stride length of the injured left front limb demonstrated that at 3 days, rats still receiving analgesics have longer strides than those with only one day of analgesics, regardless of sNAG (Fig 6B, 3DB.NS vs 1DB.NS and 3DB.S vs 1DB.S). Lastly, at 7 days, rats with sNAG load their injured limb faster regardless of analgesics (Fig 6C, 3DB.S vs. 3DB.NS). This parameter was also increased at later time points for animals receiving sNAG without extended analgesics (Fig 6C, 1DB.S vs 1DB.NS). Modest differences were also identified in functional assessments pre-operatively.

DISCUSSION

This study determined the effect of sNAG on tendon-to-bone healing and the potential analgesic properties of sNAG containing membranes in a rat rotator cuff tendon injury and repair model. Overall, results suggest that sNAG has positive effects on tendon strength during early healing in a rat rotator cuff model. Animals treated with sNAG showed an increase in max load and max stress at 4 weeks, demonstrating that the presence of this polymer improves the mechanical strength of the repaired tendon-to-bone healing construct in relatively early healing. Given that the primary complication of rotator cuff repair surgery is early retear, these findings are particularly important. Although decreases in these mechanical properties were identified in sNAG treated groups at 8 weeks, we do not believe that the data support the idea that sNAG is detrimental at later stages of healing. This is due to a cluster of samples in the 3DB.S group that failed at higher loads requiring exclusion from the data analysis after non-physiological failure. In fact, these tendons may have failed physiologically at much higher loads than were measured. Therefore, we believe there are no important differences in tendon mechanics with or without sNAG at 8 weeks.

Overall, mechanical differences were observed between groups treated with and without sNAG along with 3 days of analgesics (3DB.NS vs 3DB.S), and between groups receiving sNAG but receiving different time courses of analgesics (1DB.S vs 3DB.S). Modest ambulatory differences were also identified between these groups. Therefore, the combined observations in both mechanical and ambulatory metrics could be a result of differences in both pain and structural deficit. Taken together, these results do not support an analgesic effect in this model. A significant increase in collagen organization (decreased CSD) at 8 weeks in the insertion of the 3DB.S group compared to the 1DB.S group also suggests that sNAG was likely not able to replace the analgesic effects of buprenorphine. We suggest that without buprenorphine, changes in cage activity during early healing processes may have impacted long-term matrix remodeling. Although many different biological agents have

been utilized in attempts to decrease retear rates after rotator cuff repair, there is little clinical evidence that patient-derived materials can reliably improve healing over a diverse patient population. For example, Dolkart et al. utilized the rat rotator cuff repair model and injected PRP into the intraarticular space. Three weeks post-operatively, load failures, stiffness, and histological parameters were enhanced with the addition of PRP.³ On the other hand, Weber et al. reported no significant differences in pain, range of motion, or structural integrity after administering platelet-rich fibrin matrices in all size rotator cuff tears in a patient cohort.²² Delivery of MSCs has shown promising results, but cell quality can be variable based on numerous patient characteristics. A long-term study reported that 100% of patients who received bone marrow derived MSCs (BM-MSCs) had intact rotator cuffs at 6 months post-op, compared with 67% of those who did not receive BM-MSCs. These results were consistent over 10 years post-op, with intact cuffs maintained in 86% of BM-MSC recipients compared to 44% in the control group.⁹ This study identified a new treatment application for sNAG polymer, a product isolated from microalgae. Previous pre-clinical and clinical studies have demonstrated improved wound healing with sNAG treatment, and Talymed® is currently indicated for management of wounds including diabetic ulcers, burns, abrasions and lacerations, and pressure wounds.^{10,17} Our study, the first that investigates the effects of internal use of sNAG polymer after a musculoskeletal injury, suggests that this material also improves early tendon-to-bone healing in the rat rotator cuff. Unlike other forms of rotator cuff biological augmentation, no secondary procedures are needed to isolate cells or blood from the patient at the time of surgery. sNAG polymer sheets require no preparation, are stable at room temperature, and are non-immunogenic. Additionally, the material is thin and shows strong adherence to wound beds, unlike other materials that prevent healing due to thickness or unknown growth factor temporal availability.¹²

This study has some limitations that should be acknowledged. First, though the rat rotator cuff repair model is well established and used extensively for such studies, quadruped models do not exactly imitate clinical scenarios. Second, this study used an acute supraspinatus detachment and repair although clinically, rotator cuff tears are commonly chronic subsequent to frequent overhead motions and overuse. However, we are using a well-established rat surgical and repair model as well as other well-established assays. Our methods are repeatable and the inclusion of longitudinal functional assays allow for improved clinical relevance. Third, because the method of administration for sNAG is the placement of a quickly dissolvable membrane, we are unsure of the exact distribution of sNAG after delivery. Utilizing a tagged polymer that can be tracked over time would provide the confirmation of material maintenance at the site of healing. Additionally, polymer uptake and clearance may diminish the effects of this treatment. However, given all the differences quantified in this study, we are confident that the sNAG was present for important phases of tendon healing.

This study evaluated the mechanical, histological, and functional properties of injured tendons with and without sNAG polymer delivery as well as in the context of analgesics. Overall, sNAG was shown to have positive effects on tendon strength during early healing, but no effects during later healing. Further, there was no confirmed analgesic effect as previously speculated in this model. Future studies should incorporate additional molecular characterization, which may elucidate the mechanism of action for the changes identified.

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Figure 1.

A thin membrane of sNAG (4 mm diameter) is placed on the “foot print” of the supraspinatus tendon to bone insertion site before the tendon is reattached.

Figure 2.

Mechanical properties of treatment groups at 4 and 8 weeks. In the presence of analgesics, tendons receiving the sNAG polymer had significantly increased (A) max load and (B) max stress at 4 weeks compared to tendons without sNAG. At 8 weeks, max load was significantly decreased and max stress trended toward decreased with the addition of sNAG. No differences were seen in (C) tendon stiffness or (D) tendon modulus across any treatment group.

Figure 3.

Included data points for max load and max stress (A–D) and all collected data points (excluded data indicated by red symbols, E–H). At 4 weeks, only a small number of data points were excluded, and they were distributed similarly for each group (A,E). At 8 weeks, a large percentage of samples were affected (up to 58% per group). Here, distribution of excluded data are not uniform (C,G), in turn skewing the max load and max stress data for 3DB.S towards lower overall average values.

Figure 4.

Semi-quantitative grading results for tendon histology. No differences were seen between groups at any time point. (A) Cellularity significantly decreased over time at the insertion ($p=0.01$) and (B) midsubstance ($p<0.001$). (C) There was a trend toward a more spindle like cell shape over time at the insertion ($p=0.1$). (D) No differences were seen in cell shape at the midsubstance.

Figure 5.
Collagen organization was increased (decreased CSD) at 8 weeks in the insertion of the 3DB.S group compared to the 1DB.S group.

Figure 6.

Ambulatory parameters are altered by the presence of sNAG. (A) Speed of the injured left limb demonstrated that at 3 days, rats that were still receiving analgesics walked faster regardless of the presence of sNAG (3DB.S vs 1DB.S). This group continued to show increased speed at 28 and 56 days. Additionally, animals who received 3 days of analgesics walked faster in the presence of sNAG than those without (3DB.S vs. 3DB.NS) at both 7 and 28 days.

(B) Stride length of the injured left front limb demonstrated that at 3 days, rats still receiving analgesics have longer strides than those with only one day of analgesics, regardless of sNAG (3DB.NS vs 1DB.NS and 3DB.S vs 1DB.S).

(C) At 7 days, rats with sNAG load their injured limb faster regardless of analgesics (3DB.S vs. 3DB.NS). This metric was also increased at later time points for animals receiving sNAG without extended analgesics (1DB.S vs 1DB.NS).