The Analysis Of The Natural Healing Process Of Partial Defects Of Rats' Rotator Cuff Tendons

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Disclosures:

Introduction: Tendon degeneration and rupture is a common disorder that affects both athletes and workers. Ultrasound studies have reported incidence rates for full-thickness rotator cuff tears of 16% in 40-60 year olds and 46% in a 70 year old or higher age group 1). The capacity of the tendon to heal varies depending on its magnitude, duration, and location. However, most of repaired tissue appear scar-like and never completely regain the biomechanical properties it had prior to injury, even after surgical procedures. And underlying mechanism of tendon healing has not fully understood in the first place. We previously reported that small deficit made at rats' patellar tendons led to macular lesions of chondrometaplasia and ectopic ossifications beyond the original deficit at the annual meeting of ORS 2011 and 2012. We considered this phenomenon as one of the potential cause of failed tendon healings. Understanding how different tendons heal is an important consideration for treatments and rehabilitations of the rotator cuff tear. We therefore made the partial defects of the rat supraspinatus tendons, and examined the change of factors involving the tendon healing.

Methods: Animal model
88 Sprague-Dawley male rats (10-11 weeks, body weight of 310-350 g) were used in this study. All rats were anesthetized by intraperitoneal injection of sodium pentobarbital. Through a 2-cm skin incision on the left shoulder, the supraspinatus (SSP) tendon was fully exposed, and a 1.5mm × 1.5mm full-thickness defect was created on the SSP tendon with a scalpel, 1.5 mm proximal to the humeral insertion. Subsequently, the skin and subcutaneous tissue were closed with 5-0 nylon. The same procedures were performed in the right shoulder except that the tendon defect was not created, and this was used as a control.

At surgery, all the rats were allowed to move freely inside a cage. At 3 days, 1, 2, 4, 6, 9, 12 and 20 weeks following the operation, post-surgical animals were sacrificed and bilateral SSP tendons were dissected for histological (n = 3 each time point) and quantitative PCR (qPCR) (n = 8 each time point) analysis.

Histological analysis
Specimens were immediately fixed in 10% neutral buffered formalin, dehydrated through an alcohol gradient, cleared, and embedded in paraffin blocks. Histological sections (6 mm) were prepared using a microtome and subsequently stained with Hematoxylin and Eosin (HE) staining and Alcian Blue staining.

RNA isolation and quantitative PCR
Extramuscular potion of the harvested tendons was frozen in liquid nitrogen, and powdered using the bead beater. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions, and RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of the genes described below was quantified by qPCR using a LightCycler 480 SYBR Green I Master kit (Roche) in a LightCycler 480 instrument (Roche); Scleraxis (Scka), Tenomodulin (Tnmd), Mohawk (Mkx), Type1 Collagen (Col1a1), Elastin (Eln), Proline/arginine-rich end leucine-rich repeat protein (Prelp), SRY box 9 (Sox9), Type2 Collagen (Col2a1), Aggrecan (Acan), Hypoxia-inducible factor 1 (Hif1a), Vascular endothelial growth factor (Vegf), Notch homolog 1 (Notch1).

Statistical analysis
Comparison of the results of qPCR was done using Mann-Whitney U-test. All the data analysis was done using SPSS software (SPSS, Inc.). p > 0.050 was regarded as statistically significant.

Results: Histological analysis
At Day 3, the defect site was infiltrated by many cells (Fig.1 A). Thereafter, the cellularity of the injured tendons remained higher even at Week 20 postoperatively (Fig.1 B-E) than that of control (Fig.1 F). While the collagen fibrils around the original defect sites as well as in the original defects were randomly oriented until Week 6 (Fig.1 C), they became gradually aligned after that (Fig.1 D, E).

Alcian Blue stain revealed the widespread proteoglycan deposits beyond the original defect sites from Week 2 to Week 20 (Fig1 G-J).
qPCR analysis
The qPCR showed that expression of SCX was upregulated from Day 3 to Week 4, that TNMD was from Week 1 to Week 12, and that MKX was from Week 1 to Week 9 (Fig. 2). Expression of each 3 gene increased until Week 2, and then gradually decreased. Expression of extracellular matrix genes such as Col1a1 and Dcn also increased until Week 2, remained high till Week 6, and then decreased quickly (Fig. 2). Eln was finally downregulated significantly than control at Week 20. Expression of Sox9 increased gradually until Week 6, and then quickly returned as low as that of controls. While Acan was upregulated significantly all over the observation period, expression of Col2a1 was never significantly high. Expression of Hif1a and Notch1 was significantly higher from early period to Week 12. Vegf was upregulated at Week 4, 6, and
Discussion: Alcian Blue staining showed the proteoglycan deposits not only in the regenerated tissues, but also in the surrounding tissues, even at Week 20. That might be considered as a sort of the chondrometaplasia. While Scx, Mxk, Co1a1, and so on, were expressed as much as controls at the latter period, expression of Acan was significantly higher throughout the study period. HIf1a and Notch1 were up-regulated until Week 12. Some histological studies reported that a higher incidence of chondrometaplasia was detected in the articular side of rotator cuff tendon tears, in areas that have few fibroblasts. Lui et al. reported that chondrometaplasia and ossification were observed in both the collagenase-induced injury model and the window injury model of rats' patellar tendons. However, in this study, it was not observed that such a severe metaplasia with ectopic ossifications in the rotator cuff. It is speculated that it could come from the difference of the nature between patellar tendons and rotator cuff (e.g. vascularity). The exact mechanism of chondrometaplasia is still unknown, but some researchers considered it as an adaptation to hypoxia.
due to compressive loading or overuse\textsuperscript{5}. Hif1a is reported to directly regulate Sox9 expression in rat MSCs\textsuperscript{6}, and mouse limb bud mesenchymal cells\textsuperscript{7}, and so on. Moreover, some reports showed that Notch1 induces Sox9 expression in embryonic stem cells\textsuperscript{8}. In this study, expression of Hif1a and Notch1 was significantly higher from early period to Week 12. This upregulation might affect the induction of chondrometaplasia through the upregulation of SOX9.

In conclusion, we confirmed that small defects of the rotator cuff tendons of rats led the chondrometaplasia, and that it extended beyond the original defects. Early increase of expression of tendon-specific, chondrogenic and angiogenic genes was found. These results suggested that the expansion of chondrometaplasia might adversely affect the tendon healing, and that hypoxia may be a potential inducer of that. Further studies should be needed to investigate the exact mechanism of the induction of chondrometaplasia after tendon injuries.

**Significance:** The exact mechanism of tendon healing is not fully understood. This study provides some insights that chondrometaplasia and hypoxia might affect the process adversely.

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**References:**

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