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Nicotine Delays Tendon-to-Bone Healing in a Rat Shoulder Model

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Investigation performed at the Department of Orthopaedic Surgery, Washington University School of Medicine at Barnes-Jewish Hospital, St. Louis, Missouri

Background: Many studies have shown that nicotine negatively impacts fracture healing and bone fusion processes. However, very little is known about its effect on tendon and ligament healing. The goal of the present study was to evaluate the effect of nicotine on tendon-to-bone healing.

Methods: Supraspinatus tendons in both shoulders of seventy-two rats were transected and repaired to the humeral head. Osmotic pumps were implanted subcutaneously, and nicotine or saline solution was delivered for ten, twenty-eight, or fifty-six days. Cell morphology was evaluated with use of histologic sections. Cells were counted, and proliferating cell nuclear antigen (PCNA) immunohistochemistry was performed to assess cellular proliferation. In situ hybridization was performed to measure type-I collagen mRNA expression. Biomechanical and geometric properties were assessed.

Results: Inflammation persisted longer in the nicotine group than in the saline solution group. Cellular proliferation was higher in the saline solution group than in the nicotine group at the early time-points. Type-I collagen expression was higher in the saline solution group at twenty-eight days. Mechanical properties increased over time in both groups. Maximum stress was significantly lower in the nicotine group than in the saline solution group at ten days. Maximum force was significantly lower in the nicotine group than in the saline solution group at twenty-eight days. Maximum force was significantly higher in the nicotine group than in the saline solution group at fifty-six days. Stiffness was not different between the groups at any time-point.

Conclusions: Nicotine caused a delay in tendon-to-bone healing in a rat rotator cuff animal model. Mechanical properties increased over time in both groups, but the properties in the nicotine group lagged behind those in the saline solution group. Chronic inflammation and decreased cell proliferation may partly explain the inferior biomechanical properties in the nicotine group as compared with the saline solution group.

Clinical Relevance: Failure of rotator cuff repair is a major clinical problem. The adverse effect of nicotine on rotator cuff healing noted in this clinically appropriate animal model may be an important clinical consideration.
Two specimens were obtained from each group for histological properties would be reflected in histological changes. We hypothesized that nicotine would delay tendon-to-bone healing, as demonstrated by a decrease in ultimate force and stiffness at early time-points, and that decreased mechanical properties be reflected in histological changes.

**Materials and Methods**

**Animal Injury Model**

All procedures were approved by the Institutional Animal Studies Committee. Supraspinatus tendons in both shoulders of seventy-two adult male Sprague-Dawley rats (weight, 350 to 400 g) were transected and repaired to the humeral head as described previously. Briefly, a skin incision was made over the craniolateral aspect of the scapulohumeral joint. The deltoid muscle was detached sharply from the posterior, lateral, and anterior aspects of the acromion and was split distally. The supraspinatus was detached sharply at its insertion on the greater tuberosity. A 0.5-mm drill-hole was made transversely in an anteroposterior orientation through the proximal part of the humerus. Any fibrocartilage at the insertion was removed by scraping with a scalpel blade. The tendon was grasped with a double-armed 5-0 proline suture with use of a technique similar to the Mason-Allen method. The suture was passed through the drill-hole, and the tendon was reapposed to its anatomic position. It is important to note that this is an injury model that approximates an acute rotator cuff tear.

**Histology-Based Assays**

Two specimens were obtained from each group for histological analysis. The tendon with its attachment to bone was processed with use of standard techniques. Specimens were fixed overnight in 4% paraformaldehyde and were decalcified in 14% EDTA. Precautions were taken to maintain mRNA integrity (e.g., all solutions were treated with 0.1% diethylpyrocarbonate). Specimens were embedded in paraffin, sectioned at 5 to 7 µm, and dried for one hour at 60°C. Sections were stained with toluidine blue to examine fibrocartilage formation and with hematoxylin and eosin to examine cell morphology. Tissue sections were evaluated blindly by a pathologist (N.H.) for inflammation, fibrocartilage formation, vascular proliferation, and fibroblast proliferation.

**In Situ Hybridization**

A rat cDNA probe for type-I collagen was used to measure mRNA expression. A 1.5-kb EcoRI/EcoRI fragment from clone HF677 coding for pro-α(I) collagen was subcloned into the pBluescript II KS(+) vector (Stratagene, La Jolla, California). To synthesize an antisense riboprobe for pro-α(I) collagen, the resulting vector was linearized with Hind III and transcribed with use of T3 RNA polymerase. The sense riboprobe was synthesized with use of T7 RNA polymerase, while linearizing with Bam HI. Specimens for collagen probes were hybridized to gene-specific 32P-labeled sense and antisense riboprobes (2.5 × 106 cpm/µL) according to the methods of Lyons et al. and as described previously.

**Immunohistochemistry for PCNA**

Proliferating cell nuclear antigen (PCNA) immunohistochemistry was done in accordance with the manufacturer’s protocol (Zymed Laboratories, San Francisco, California) as a measure of proliferation. Antibody was visualized by incubation with diaminobenzidine (DAB) for four minutes. Tissue was counterstained with Mayer’s hematoxylin.

**Semiquantitative Evaluation of Protein Levels and mRNA Expression**

Levels of protein and mRNA at the healing insertion site were graded by three investigators (L.M.G., S.T., and S.Y.R.) who were blinded with regard to specimen group. Grading was performed with use of printed standards representing high (4), intermediate (3 and 2), low (1), and undetectable (0) levels.

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**TABLE I Serum Nicotine and Cotinine Levels Over Time**

<table>
<thead>
<tr>
<th></th>
<th>Nicotine (nm/mL)</th>
<th>Cotinine (nm/mL)</th>
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<tbody>
<tr>
<td>2 Weeks</td>
<td>65.8 ± 28.5</td>
<td>385.3 ± 60.9</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>50.9 ± 25.7</td>
<td>272.3 ± 106.3</td>
</tr>
<tr>
<td>6 Weeks</td>
<td>48.9 ± 18.2</td>
<td>278.5 ± 74.7</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>56.5 ± 15.9</td>
<td>297.0 ± 105.4</td>
</tr>
</tbody>
</table>

*The values are given as the average and the standard deviation.
Cell Density
To measure cellularity, sections were stained with propidium iodine (Invitrogen/Molecular Probes, Carlsbad, California) to label cell nuclei and were viewed under fluorescent light. Images of the healing tissue were captured and thresholded, and cell nuclei were counted with use of Scion Image (Scion Corporation, Frederick, Maryland). Cell density was then calculated by dividing the total number of cells by the measured tissue area.

Geometry and Biomechanics
Ten specimens from each group were used for biomechanical testing. The tendon-humerus unit of each specimen was dis-
sected in a standard fashion. The overlying deltoid and acromion were removed. The supraspinatus muscle was removed subperiosteally from the supraspinatus fossa, leaving the bone-tendon-muscle intact. Specimens were stored in a freezer at 20°C until testing. Specimens were thawed, and the humerus was embedded in an aluminum tube with use of polymethylmethacrylate. Testing was performed with the shoulder at 90° of abduction in a materials testing machine (model 8841; Instron, Norwood, Massachusetts). The humerus was clamped with its long axis in the horizontal plane. The proximal end, at

Fig. 2
Photomicrographs demonstrating increased type-I collagen expression (evident as black grains) in the saline solution group as compared with the nicotine group at twenty-eight days (magnification, ×20). Arrows indicate cells expressing type-I collagen. Type-I collagen expression decreased over time in both groups. A, Nicotine group. B, Saline solution group.
the origin of the supraspinatus tendon, was glued between two pieces of sandpaper. The sandpaper-tendon was clamped vertically in a soft-tissue clamp. Stress was calculated as the tensile force divided by the cross-sectional area. Specimens were subjected to a preload of 0.2 N and were preconditioned for five cycles to 0.38 mm of displacement (approximately 5% of gauge length at a rate of 0.1 mm/s). A stress relaxation test was then performed for 300 seconds at 0.38 mm of displacement followed by 300 seconds of recovery. Specimens were then tested to failure in tension at a rate of 0.1 mm/s. Ultimate stress, ultimate force, and stiffness were determined for each specimen. For geometry calculations, cross-sectional area was calculated at the distal aspect of the tendon (i.e., at the insertion site). Tendon thickness was quantified with use of a laser displacement sensor (LK-081; Keyence, Woodcliff Lake, New Jersey). Tendon width was measured with use of optical methods. The cross-sectional area was then calculated by assuming an elliptical geometry. These methods provided a fully noncontact procedure for the measurement of cross-sectional area.

Statistical Methods
Biomechanical and geometric results were compared with use of a two-factor analysis of variance for group (saline solution or nicotine) and time (ten, twenty-eight, or fifty-six days) followed by a post hoc pairwise comparison with use of the Fischer least-squares-differences test. The level of significance was set at p < 0.05. Histology-based results (e.g., cell morphology, mRNA expression, cellular proliferation, and cell density) were semiquantitative in nature, with only two specimens per group, and were not statistically compared.

Results
Histology
At ten days, the interface between tendon and bone displayed focal damage, with scattered acute inflammatory cells mixed with a predominant mononuclear infiltrate in both groups. In both groups, vascular and fibroblast proliferation was at its highest level at this stage as compared with the other time-points. At twenty-eight days, the interface displayed focal damage in both groups. The inflammatory infiltrate was slightly less in the saline solution group than in the nicotine group. Vascular and fibroblast proliferation was higher in the saline solution group than in the nicotine group. At fifty-six days, the interface displayed normal to slight focal damage in both groups. Acute inflammation was absent in the saline solution group but persisted in the nicotine group. Vascular and fibroblast proliferation was similar in both groups.

Cell Density and Cellular Proliferation
Cell density was decreased by 13%, 8%, and 1% in the nicotine group as compared with the saline solution group at ten, twenty-eight, and fifty-six days, respectively. Cellular proliferation (i.e., PCNA grade) was higher in the saline solution group than in the nicotine group at ten and twenty-eight days (Fig. 1). Proliferation was similar in both groups at fifty-six days. Cell density and cell proliferation decreased over time in both groups.

mRNA Expression for Type-I Collagen
Type-I collagen expression was higher in the saline solution group than in the nicotine group at twenty-eight days (Fig. 2). Type-I collagen expression was highest at the earliest time-point and decreased over time.

Geometry and Biomechanics
Geometric and biomechanical results are summarized in Figure 3 and Table II. Maximum stress was significantly lower in the nicotine group than in the saline solution group at ten days (p < 0.05) (Fig. 3, Table II). Maximum stress significantly increased over time in both groups. Maximum force was significantly lower in the nicotine group than in the saline solution group at twenty-eight days (p < 0.05) (Fig. 3, Table II). Maximum force was significantly higher in the nicotine group than in the saline solution group at fifty-six days (p < 0.05). There was a significant increase in maximum force over time. Stiffness was not significantly different in the nicotine group as compared with the saline solution group at any time-point (Fig. 3, Table II). There was a significant increase in stiffness over time (p < 0.05).

<table>
<thead>
<tr>
<th>TABLE II Geometric and Biomechanical Properties*</th>
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<tr>
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<td>-----------------------------------------------</td>
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<tr>
<td><strong>Nicotine group (n = 10)</strong></td>
</tr>
<tr>
<td>10 days</td>
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<tr>
<td>28 days</td>
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<tr>
<td>56 days</td>
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<tr>
<td><strong>Saline solution group (n = 10)</strong></td>
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<tr>
<td>10 days</td>
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<tr>
<td>28 days</td>
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<tr>
<td>56 days</td>
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*The values are given as the average and the standard deviation. †The difference between the nicotine group and the saline solution group was significant (p < 0.05) ‡The difference was significant over time (p < 0.05).
Discussion

To our knowledge, this is the first study to evaluate the effects of nicotine on rotator cuff repair. Smoking has a known detrimental effect on the healing of bone\textsuperscript{12-14} and skin\textsuperscript{7-11}. It also has been associated with intervertebral disc disease and low-back pain\textsuperscript{28-36}. Its effect on tendon healing remains unknown. The decreases in maximum stress and maximum force that we observed suggest inferior healing and decreased remodeling due to nicotine. This inhibition of the natural healing process has potential clinical implications as tendon injuries are among the most common orthopaedic injuries, both during recreational activities and in the workplace\textsuperscript{37}. The present study is most relevant to rotator cuff repair, a frequently performed procedure\textsuperscript{37}.

The delay in tendon-to-bone healing associated with the administration of nicotine in this rat rotator cuff model is consistent with what has been demonstrated in studies of fracture healing\textsuperscript{8}. Raikin et al. demonstrated a lag in the formation of cortical continuity in a rabbit tibial osteotomy model when the animals were exposed to nicotine\textsuperscript{38}. There also was a 13% higher rate of nonunion in the nicotine group. Hollinger et al. demonstrated a negative impact on the healing of parietal bone defects in rats that had been exposed to nicotine\textsuperscript{39}. Other studies have shown that nicotine delays and may prevent spinal fusion\textsuperscript{12-14}. Silcox et al. reported that fusion did not occur in the lumbar spines of rabbits that had received a continuous infusion of nicotine\textsuperscript{12}. Brown et al. reported a significantly higher rate of pseudarthrosis after lumbar fusions in humans with a heavy smoking history\textsuperscript{16}. Delayed healing due to smoking also has been demonstrated after soft-tissue injury\textsuperscript{7-11}. In the study by Jorgensen et al., a quantitative analysis of collagen production demonstrated that the production of subcutaneous collagen in smokers was impeded\textsuperscript{7}.

In the present study, maximum stress, maximum load, and stiffness increased over time in both groups, but the mechanical properties in the nicotine group lagged behind those in the saline solution group at ten and twenty-eight days. The maximum stress (a material property) in the nicotine group was significantly inferior to that in the saline solution group at the early time-points. The inferior material properties are an indication of less mature scar and less scar remodeling in the
nicotine group. Thus, nicotine may have a negative effect on extracellular matrix degradation. Additionally, it may have no effect on extracellular matrix production or it may promote the production of a lower-quality matrix (e.g., type-III collagen rather than type-I collagen).

Cell density and cellular proliferation were higher in the saline solution group than in the nicotine group at ten and twenty-eight days, and they were similar in both groups at fifty-six days. Differences in type-I collagen expression were evident only at twenty-eight days. At ten days, maximum stress was significantly higher in the saline solution group whereas maximum load was not significantly different between the groups. At twenty-eight days, maximum load was significantly higher in the saline solution group whereas maximum stress was not significantly different between the groups. This finding indicates that the structural properties that were tested were dominated by the suture repair early on. As time progressed and the properties of the healing tissue began to contribute to strength at the repair site, the delay in matrix remodeling in the nicotine group became evident.

Unexpectedly, the maximum load was significantly higher in the nicotine group than in the saline solution group at fifty-six days. This difference could be explained by increases in collagen crosslinking caused by advanced glycation end products. These end products are formed by the reaction of sugars with lysine and arginine residues in proteins. Their accumulation leads to the formation of irreversible crosslinks, resulting in a more brittle collagen network. Accumulation of proteins by non-enzymatic glycation is one of the underlying factors associated with diabetes complications, age-related arthritis, cardiomyopathy, and other protein-deposition disorders. Smoking also has been associated with increased advanced glycation end product formation in gingival fibroblasts, plasma proteins, and myocardium. These changes are thought to cause increased susceptibility to injury in affected tissues. This may explain the difference in maximum force that was seen in our study, especially given the fact that this difference was seen only at the later time-point (i.e., only after these end products had accumulated in the matrix). This change is likely detrimental, as it may lead to increased susceptibility to injury or failure as the result of a more brittle matrix and decreased viscoelasticity.

Our histology-based assays could not entirely explain the geometric and biomechanical changes that were observed. Chronic inflammation and decreased cell proliferation may partly explain the inferior biomechanical properties in the nicotine group. The delayed healing also was evident in the decreased expression of type-I collagen. However, these historical differences between the nicotine and saline solution groups were small and could only partly explain the large differences in terms of geometric and biomechanical properties.

A strength of our study is the use of an established rodent model for rotator cuff repair. The use of this model makes our findings particularly relevant to rotator cuff injury and repair. Our use of an osmotic delivery pump is also well established in the literature for studying the effects of nicotine and cotinine levels in order to ensure adequate delivery throughout the time-period studied. A limitation of our study is that the animal model used is that of an acute injury and repair. The more common clinical injury to the rotator cuff involves chronic degeneration followed by tendon tears. However, an acute repair would likely heal better than a chronic tear, so the effects of nicotine demonstrated in the present study may be less than those seen in association with the repair of a chronic tear. Finally, rats may metabolize nicotine faster than humans do. In the present study, we were careful to maintain levels at those consistent with an adult smoking twenty to thirty cigarettes per day.

By fifty-six days, there were few differences between the nicotine and saline solution groups. This finding suggests that there may be a vulnerable time-period in tendon-healing during which early failure secondary to the effects of nicotine may be more of a problem. This is important clinically because often it is during this time-period that repairs are exposed to early rehabilitation protocols.

Whether or not the effects of smoking are reversible is unknown. We are not aware of any conclusive studies that have generated definitive guidelines about perioperative cessation of smoking. Therefore, it is still not known whether requiring patients to stop smoking prior to surgery will improve or change the biologic outcome.

Failure of rotator cuff repair is a major clinical problem. Treatment may require additional procedures, and there are often no reliable or predictable surgical options. On the basis of the findings of the present study, we conclude that nicotine causes a delay in tendon-to-bone healing and also causes a delay in scar degradation and remodeling. Therefore, it is advisable that patients cease smoking or using tobacco products before undergoing a rotator cuff repair. Our results also may affect the advisability of using nicotine patches during the perioperative period. Additional studies are necessary in order to elucidate the changes in geometry and biomechanics reported here and to provide potential targets for therapeutic intervention.
References


