Surface Treatment with 5-Fluorouracil After Flexor Tendon Repair in a Canine in Vivo Model

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Background: Topical 5-fluorouracil has been reported to reduce adhesions in animal models of tenolysis. The purpose of this study was to investigate the effects of topical 5-fluorouracil on adhesion formation after tendon repairs were subjected to immediate postoperative rehabilitation in a canine model in vivo.

Methods: Sixty dogs were randomly assigned to either a 5-fluorouracil treatment (thirty dogs) or a control group (thirty dogs). Each treatment group was then divided into three survival time points: ten days, twenty-one days, and forty-two days. The second and fifth flexor digitorum profundus tendons from each dog were fully lacerated at the zone-II area and then were repaired. Passive motion therapy started at day 5 postoperatively and continued until the dogs were killed. The repaired tendons were evaluated for normalized work of flexion, gliding resistance, repair strength, gene expression for type-I and type-III collagen and transforming growth factor-β1, and histological appearance.

Results: The normalized work of flexion of the repaired tendons treated with 5-fluorouracil was significantly lower than that of the repaired tendons without 5-fluorouracil treatment at ten days. However, there was no significant difference between treated and untreated tendons at twenty-one and forty-two days. There was also no significant difference in gliding resistance, repair failure strength, or stiffness between treated and untreated tendons at any time point, or in the gross or histological appearance of adhesions at the time of killing. The expression of types-I and III collagen and transforming growth factor-β1 of the repaired tendon with 5-fluorouracil treatment was significantly lower than that of the tendons without treatment at ten days postoperatively, but not at twenty-one or forty-two days.

Conclusions: Although 5-fluorouracil treatment can reduce adhesions in in vivo models of tenolysis, this treatment had only a transient effect in an in vivo model of tendon repair that included passive motion.

Clinical Relevance: Topical 5-fluorouracil, at the dosing regimen tested in this study, does not appear to offer a major benefit over early postoperative motion protocols alone in the treatment of flexor tendon lacerations.
esized that exposing the repaired tendon to topical 5-fluorouracil for five minutes would decrease tendon adhesions but not adversely affect intrinsic tendon healing, thus allowing for an early return to postoperative motion therapy. We also hypothesized that 5-fluorouracil would not result in an increase in tendon rupture or gaping compared with untreated tendons. Finally, we wished to determine whether 5-fluorouracil therapy offered a benefit over those already seen with postoperative motion therapy alone.

Materials and Methods

Experimental Design

The project was reviewed and approved by our Institutional Animal Care and Use Committee, and all National Institutes of Health animal care guidelines were followed. Sixty mongrel dogs weighing 20 to 25 kg were used. Each dog was randomly assigned to either the 5-fluorouracil treatment group (thirty dogs) or the control group (thirty dogs). Each group was then divided into three survival time points, ten days (ten dogs), twenty-one days (ten dogs), and forty-two days (ten dogs). The second and fifth flexor digitorum profundus tendons from each dog were fully lacerated at the zone-II D area and then repaired. The therapy started at day 5 postoperatively and continued until the dogs were killed. Following the killing of the dogs, one of the two repaired tendons in each paw was randomly assigned for biomechanical testing. Biomechanical testing consisted of evaluation of the work of flexion (ten dogs at each time point), gliding resistance, and repair strength. The other repaired digit was evaluated for gene expression for types-I and III collagen, transforming growth factor (TGF)-β1, and fibronectin (eight dogs at each time point) by reverse transcriptase-polymerase chain reaction and histological analysis (two dogs at each time point). The details of each test are described below.

Surgical Procedure and Postoperative Therapy

The dogs were anesthetized with intravenous ketamine (13 mg/kg) and diazepam (6 mg/kg) and maintained under anesthesia with 1.5% isoflurane. The selected forepaw was shaved, scrubbed with povidone-iodine, and steriley draped. An elastic bandage was then used to exsanguinate the forelimb and act as a tourniquet for the procedure. In the second and fifth digits, the flexor digitorum profundus tendons were carefully exposed at the proximal, middle, and distal phalanges, respectively. The preparation of the operatively treated forelimb was first performed for ten repetitions of flexion-extension to prevent any joint contracture due to immobilization. Then, therapy was performed with proximal interphalangeal and distal interphalangeal joint flexion and metacarpophalangeal and wrist extension, and proximal interphalangeal and distal interphalangeal joint extension with metacarpophalangeal and wrist flexion for ten times in each operatively treated digit. The dogs were killed with an overdose of pentobarbital at postoperative day 10, 21, or 42.

Measurement of Digit Work of Flexion

To quantitatively evaluate the resistance generated by adhesions, the work of flexion was measured in the digit. Briefly, the second and fifth digits in both operatively treated and non-operatively treated paws were harvested. The flexor digitorum profundus tendons were carefully exposed at the proximal metacarpal level, transected, and sutured to a cable that connected to a load transducer. The repaired tendon within zone II was not exposed. A Kirschner wire was inserted longitudinaly through the metacarpal bone to fix the metacarpophalangeal joint in extension. A T-shaped piece of hardware with a pair of reflective markers (2 mm in diameter) was pinned to the proximal, middle, and distal phalanges, respectively. The prepared digit was then mounted on the testing device by fixing the proximal Kirschner wire to a custom jig. The testing device consisted of a testing frame, actuator, linear potentiometer, and one load transducer. A 0.5-N weight was attached to the extensor tendon to ensure full extension of the digit as a starting position, and to apply an initial tension of 0.1 N to the flexor digitorum profundus tendon (Fig. 1).

The actuator pulled the tendon proximally at a rate of 2 mm/sec, causing flexion of the digit. The tendon excursion was determined by the collateral normal digit tendon excursion during full range of motion of the proximal interphalangeal
and distal interphalangeal joint. Data from the linear potentiometer and the proximal load transducer were recorded at 60 Hz. During testing, motion of the digit was recorded simultaneously (from extension to flexion) by Motion Analysis System software (Motion Analysis, Santa Rosa, California). Work-of-flexion data were calculated from the tendon displacement versus loading curve during flexion of the digit, and then were normalized (divided) by total proximal interphalangeal and distal interphalangeal joint motion angle at the point when the distal interphalangeal joint reached 40°. If the distal interphalangeal joint could not reach a 40° arc of motion, maximum proximal interphalangeal and distal interphalangeal joint motions were used to normalize the work of flexion.

**Tendon Gliding Resistance Measurement**

Following the work-of-flexion measurement, the tendons were further dissected, keeping the proximal pulley intact. The gliding resistance between the tendon graft and the proximal pulley was then measured with use of a custom tendon-pulley friction testing device, as previously described. Briefly, the dissected proximal phalanx with proximal pulley was mounted in a custom jig with the palmar side upward. Two tensile load transducers were attached to the proximal and distal ends of the flexor digitorum profundus tendon with the repair site between them. A mechanical actuator with a linear potentiometer was connected to the proximal flexor digitorum profundus tendon, and a 500-g weight was attached to the distal end of the flexor digitorum profundus tendon. The tendon was pulled proximally by the actuator against the weight at a rate of 2.0 mm/sec. The actuator movement was then reversed, causing the tendon to be pulled distally by the weight. The gliding resistance of the repaired tendon against the pulley was calculated as the difference between two load transducers during tendon gliding.

**Measurement of Repair Strength**

To measure breaking strength, the tendons, following frictional testing, were secured in a servohydraulic testing machine and distracted to failure at a rate of 20 mm/min. A differential variable reluctance transducer (DVRT; MicroStrain, Williston, Vermont) was attached to the tendon spanning the repair site to measure gap formation during testing. Tensile force, grip-
to-grip displacement, and gap displacement measured by the DVRT transducer were collected at a rate of 20 Hz. Throughout testing, the tendons were kept moist by spraying with physiologic saline solution. Maximum breaking force was recorded. In addition, a regression line was fit to the linear region of the force versus gap formation (as measured by the DVRT transducer) to measure the resistance to gap formation.

Reverse Transcriptase-Polymerase Chain Reaction Quantification of Metabolic and Functional Markers

After gross evaluation, the tendons were processed for real-time reverse transcriptase-polymerase chain reaction to measure the gene expression for type-I collagen, type-III collagen, fibronectin, and TGF-β1. Briefly, each specimen was homogenized in TRIzol reagent (Invitrogen, Carlsbad, California) with a Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany). Following the addition of chloroform (Sigma-Aldrich, St. Louis, Missouri), the homogenized tissue samples were centrifuged for ten minutes at 12,000 g, and the upper aqueous phase was transferred to a clean tube. Total RNA from the aqueous phase was precipitated by mixing with isopropl alcohol (Sigma-Aldrich) and collected by centrifugation at 12,000 g for fifteen minutes. The above extracted RNA was purified with an RNeasy Mini spin column (QIAGEN, Valencia, California). Any potential remaining DNA was further removed by DNase treatment. The RNA concentration was determined with use of a RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, Oregon). RNA was reverse-transcribed into single-stranded cDNA with a random primer with use of a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). The reverse transcriptase was inactivated by heating to 85°C for five minutes. The expression of type-I col-

| Table I The Sequences of Polymerase Chain Reaction Primers of Canine Genes and the Length of Amplicons |
| --- | --- | --- |
| Gene | Accession No. | Primers (5’ to 3’) | Size (bp) |
| Type-I collagen | AF153062 | TGGTTCTCCTGGCAAGAT ATCACGGGGTTCACTTTA | 232 |
| Type-III collagen | XM_535997 | ACAGCAGCAAGCTATTTGA GGACAGCTAATCTTGTCTGT | 156 |
| Fibronectin | CFU52106 | GATGACTGTGGCTGAC CTTCTGGAATCTTGTACCTT | 183 |
| TGF-β1 | NM_001003309 | ACCATCATGGCATGAAACC CAGATCTTGGCGAGTC | 174 |

Fig. 2

The work of flexion normalized by proximal interphalangeal and distal interphalangeal joint angle (nWOF) in repaired tendons with 5-fluorouracil (5-FU) treatment or repaired tendons without such treatment (control) and a normal digit as well at three time points: ten, twenty-one, and forty-two days. Bars with similar letters at the top (i.e., a and a) have no significant difference; however, bars with different letter designations (i.e., a and b) indicate there is a significant difference (p < 0.05) between them. The bars and l-bars indicate the mean and the standard deviation.
lagen, type-III collagen, fibronectin, and TGF-β1 was quantified with LightCycler FastStart DNA Master SYBR Green I kit (Roche) in a LightCycler instrument (Roche). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the housekeeping gene. The polymerase chain reaction primers designed from canine-specific cDNA sequences are listed in Table I.

**Histological Analysis**

After gross evaluation, two repaired flexor digitorum profundus tendons from each group were carefully dissected from the flexor sheath, fixed in 10% formalin, embedded in paraffin, sectioned longitudinally, and stained with hematoxylin and eosin. Because only two tendons were used in each group,
Fig. 5
Resistance to gap formation of the repaired tendons with 5-fluorouracil (5-FU) treatment or without such treatment (control) and a normal digit as well at three time points: ten, twenty-one, and forty-two days. Bars with similar letters at the top (i.e., a and a) have no significant difference; however, bars with different letter designations (i.e., a and b) indicate there is a significant difference (p < 0.05) between them. The bars and I-bars indicate the mean and the standard deviation.

Fig. 6
Reverse transcriptase-polymerase chain reaction analysis of the expression of type-I and III collagen and TGF-β1 of the repaired tendons with 5-fluorouracil (5-FU) treatment or without such treatment (control) and a normal digit as well at three time points: ten, twenty-one, and forty-two days. A: Type-I collagen expression. B: Type-III collagen expression. C: Fibronectin expression. D: TGF-β1 expression. Bars with similar letters at the top (i.e., a and a) have no significant difference; however, bars with different letter designations (i.e., a and b) indicate there is a significant difference (p < 0.05) between them. The bars and I-bars indicate the mean and the standard deviation.
we did not attempt to quantify any of the histological observations.

**Statistical Analysis**

Animal numbers were determined on the basis of a power analysis and previously published studies with use of this canine model\(^5,12\). With a sample size of ten, we had 80% power at a significance level of \(p < 0.05\) to detect a 30% change in work of flexion and ultimate strength and 35% change in gliding resistance, which would be considered large changes. The thirty dogs in the control group also served as a control group for other experimental studies not reported in this manuscript. The data obtained from work of flexion and frictional force were analyzed with use of two-factor (time and treatment) analysis of variance, followed by the Tukey Studentized range (HSD; honestly significant difference) post hoc test to compare control, 5-fluorouracil-treated, and normal digits at three time points (day 10, day 21, and day 42). The Fisher exact test was used for the tendon rupture and gap comparisons. In all cases, a level of \(p < 0.05\) was considered to be significant.

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**Results**

The rates of tendon rupture (two [3%] in the control group and three [5%] in the 5-fluorouracil group) and tendon gaps of >3 mm (ten [17%] in controls and twelve [20%] in the
5-fluorouracil group) were found to be similar (p > 0.05). The 5-fluorouracil group had a higher number of tendons with a small (1-to-2-mm) gap (seven; 12%) compared with the controls (three; 5%), although this difference did not reach significance (p > 0.05).

Figure 2 illustrates the results of normalized work of flexion (nWOF) at the three examined time points. The normalized work of flexion of the 5-fluorouracil-treated tendons was significantly lower than the repaired tendons without 5-fluorouracil treatment at the day-10 time point (p < 0.05), but not at day 21 or day 42. The normalized work of flexion of 5-fluorouracil-treated tendons significantly increased with time, so that the normalized work of flexion in the day-10 group was significantly lower than that in the day-21 group, which in turn was significantly lower than the day-42 group (p < 0.05). In the repaired tendons without 5-fluorouracil, although the normalized work of flexion of the day-42 group was significantly increased compared with the day-10 and day-21 groups (p < 0.05), there was no significant difference between the day-10 and day-21 groups.

The gliding resistance of all of the repaired tendons was significantly higher than the normal flexor digitorum profundus tendons (p < 0.05). However, there was no significant difference in gliding resistance among any repaired tendon groups, regardless of surface treatment or time point (Fig. 3). The maximum force to break the repaired tendons at forty-two days was significantly higher than that at ten days and twenty-one days in both treated and untreated repaired tendons (p < 0.01). There was no significant difference (p > 0.05) between 5-fluorouracil treatment and untreated repaired tendons and no significant interaction between the two variables (p > 0.05) (Fig. 4). Similarly, the day-42 tendons had a significantly higher resistance to gap formation than did the day-10 and day-21 tendons (p < 0.01) (Fig. 5). Again, no significant difference was noted between 5-fluorouracil-treated and control tendons.

The expression of types-I and III collagen and TGF-β1 of the repaired tendons with 5-fluorouracil treatment was significantly lower than that of the tendons without treatment at ten days postoperatively (p < 0.05). Type-I collagen expression of the 5-fluorouracil group was significantly higher than that of the untreated group at forty-two days (p < 0.05). In a comparison of the different time points, the expression of types-I and III collagen after 5-fluorouracil treatment was significantly increased in the day-42 group compared with the day-10 and day-21 groups (p < 0.05) (Fig. 6).

Histologically, the healing of tendons in the control and 5-fluorouracil-treated tendons improved similarly with the increase in postoperative time. The epitenon cells increased at the repair site and migrated into the laceration over time. There appeared to be fewer cells on the surface of the day-10 5-fluorouracil-treated tendons than there were on the day-10 control tendons. On the basis of the two tendons evaluated in each treatment group, there were no dramatic differences in histological appearance between control and 5-fluorouracil tendons at twenty-one or forty-two days (Fig. 7).

**Discussion**

5-fluorouracil is one of the antimetabolites that interferes with DNA production and therefore cell proliferation. Besides its use in cancer treatment, 5-fluorouracil has also been shown to inhibit the formation of postoperative flexor tendon adhesions. This effect could be detrimental to intrinsic tendon healing, especially in the clinical setting where early motion protocols can subject tendons to strain at the repair site. In a study examining the antiadhesive effects of 5-fluorouracil in a chicken model, we found that higher doses of 5-fluorouracil resulted in an increase in inflammation and were less effective in preventing adhesions than were lower doses of 5-fluorouracil. Subsequent studies found no detrimental effect on gap formation or rupture strength, but they were limited to a rabbit model in which tendons were immobilized throughout the healing course. While these studies have helped to establish 5-fluorouracil as a potential antiadhesive agent with use of small-animal models with a postoperative immobilization protocol, one must still verify that repaired tendons exposed to 5-fluorouracil when they are subjected to early motion protocols show no signs of increased tendon rupture or gap formation. In addition, in order to translate this pharmacotherapy to the clinical setting, one must establish that 5-fluorouracil provides a benefit over early motion therapy alone.

In the current study, we found that the antiadhesive effect of 5-fluorouracil, which was indirectly measured by the work of flexion, was only beneficial at the early stages of tendon healing (day 10) and that these beneficial effects were not observed at day 21 or 42. Our results also showed that 5-fluorouracil inhibited the gene expression of types-I and III collagen and TGF-β1 compared with untreated tendons at the early time point only. With time, this antimitabolic effect gradually subsided and expression of type-I collagen as well as TGF-β1 rebounded. This might be the reason that we did not observe any difference in adhesion and healing strength between the two groups at later time intervals of healing.

5-fluorouracil can reduce adhesion formation by inhibiting ingrowth of connective tissue from the digital sheath. It may also postpone healing by affecting the proliferation and migration of cells from the epitenon. We found that tendon strength increased over the course of six weeks to an average value of 69 N for the control repaired tendons and 63 N for the 5-fluorouracil-treated repaired tendons. Structurally, the stiffness of the 5-fluorouracil-treated tendons was not significantly different from that of the control tendons. In addition, the resistance to gap formation was not significantly different between the two groups.

Histologically, there appeared to be fewer cells on the surface of tendons in the day-10 5-fluorouracil group than were present on the surface of tendons in the day-10 control group. Although this observation was associated with gene expression levels at ten days, as mentioned above, there was no significant difference in the tensile strength of the repaired tendons between the treated and untreated groups. This may be due to the suture holding power, which is the major fac-
tor determining tensile strength in the early stages of tendon repair, masking any differences in the intrinsic tendon healing strength. However, the single five-minute exposure of 5-fluorouracil at a concentration of 50 mg/mL also had little effect on tendon healing at twenty-one or forty-two days, when the tendon healing strength might be more likely to contribute to breaking strength.

The measurements of work of flexion and gliding resistance are commonly used to evaluate digit function in animal studies of flexor tendon repair.2–5 Gliding resistance of the repaired tendon directly reflects the smoothness and lubrication of the tendon surface. Together, these measures allow a more precise quantification of adhesion formation and, of course, directly measure tendon mechanics. Especially early in the tendon healing process, low gliding resistance and work of flexion are clinically extremely important, as they provide a lower limit of the force necessary to initiate tendon movement. The upper bound, the breaking strength of the repair construct, is quite limited in the early phases of tendon healing, so a low gliding resistance and low work of flexion increase the size of the so-called safe zone, in which the tendon can move without jeopardizing the mechanical integrity of the repair. Low-friction suture techniques have produced fewer adhesions following flexor tendon repair in a canine in vivo model. In the current study, the modified Pennington suture was chosen because of its low frictional force and high tensile strength characteristics. We did not observe any effect of the 5-fluorouracil on tendon surface lubrication.

One limitation of this study is that mechanical strength was measured following both the gliding resistance test and the work-of-flexion measurement. This sequential testing might have affected the results. It was not possible to randomize the testing procedures within the same digit because of the sequentially disruptive assessment. However, even as the last testing procedure, the tendon tensile strength was comparable with the previous published data, suggesting that any effect of the testing sequence was limited. A second weakness of the investigation is that we studied only a single concentration of 5-fluorouracil. The 5-fluorouracil concentration and treatment duration were based on published results with use of a small-animal model; the ideal dose and duration might be lower than optimal for a canine study. A final weakness of the investigation is that we did not study all cytokines involved in tendon healing; we chose instead to focus on what we considered some key measures: TGF-β1, collagen type-I, and collagen type-III.

In conclusion, 5-fluorouracil at the dosing regimen tested in this canine study does not appear to offer a major benefit over early postoperative motion protocols alone in the treatment of flexor tendon injuries.

References


