The Effect of Butyric Acid on Normal Tendons: A Potential Stimulus for Extracellular Matrix Expression

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Abstract

We propose comparing angiogenic effects of butyric acid (BA)–impregnated suture vs control suture on an aged tendon model.

Twenty-four 3-year-old rabbits underwent bilateral Achilles tendon exposure. BA-impregnated orthopedic suture was sutured into one side, and a control orthopedic suture into the contralateral side similarly. The rabbits were sacrificed at 7, 30, and 45 days and the tendons harvested for gross, histologic, and biochemical study.

Histologically, there was increased vascularity/cell migration at all time points in the BA-treated tendons; proteoglycan expression (ie, safranin O staining) increased at 30 and 45 days. DNA concentration was significantly (P = .05) higher in the BA-treated tendon group relative to the control group at 7 days but was unchanged at 30 and 45 days. Similarly, messenger RNA (mRNA) expression of vascular endothelial growth factor (VEGF) was significantly (P = .05) higher in the BA-treated tendon at 7 days. A trend (P = .12) for higher expression in the BA group also was found at 30 days.

BA-treated tendons showed significantly increased angiogenic activity 7 days after surgery, as well as cell proliferation. This was seen with increased mRNA expression of VEGF and with increased DNA concentration. This continued for up to 30 days, leveling off by 45 days.

Much of tendon repair and reconstruction in orthopedics is centered on repairing compromised tendons—tendons with tendinosis or significant degenerative problems before the tear or rupture occurred. By enhancing this repair with biological stimulation, as demonstrated in this BA suture study, we may effectively improve results of tendon repair, such as in the older rotator cuff population.

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Tendon conditions and injuries are common in orthopedics. The tendons most often affected are the lateral and medial epicondyles of the elbow, the rotator cuff, the Achilles tendon, and the patellar tendon.[1] Repetitive loading of a tendon above its physiologic threshold is thought to lead to inflammation and degeneration.[2,3] If a degenerated tendon is not actively repaired, it will continue to weaken and eventually will rupture.[4]

In addition to extrinsic factors, intrinsic tendon factors are thought to play a role in tendon degeneration. Ahmed and colleagues[5] postulated that tendon rupture often occurs in hypovascular areas—that inadequate vascular supply results in a decreased ability of cells to perform their reparative functions. This theory is supported by the work of Kraus-Hansen and colleagues,[6] who showed experimentally that intratendinous ligation of blood vessels in the superficial tendon of horses led to necrosis and fibrillation at the core of the tendon.

"Results of a meniscal healing study using BA-soaked sutures revealed increased neoangiogenesis at the repair site."

A tendon that has undergone degenerative tendinopathy is characterized by absence of inflammatory cells, poor healing response, scattered vascular ingrowth, and noninflammatory intratendinous collagen degeneration.[7] This clearly is not an ideal situation for healing when a tendon is reattached to bone, such as occurs in rotator cuff repair, or for intratendinous healing, such as in an Achilles tendon repair. It is thought that, if there were a substance that promoted tendon healing, it would

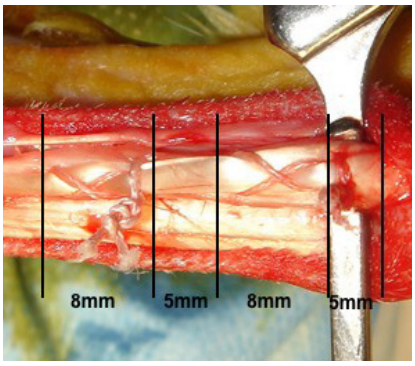

Figure 1. Figure-of-8 suture construct spanning 8 mm was used to increase contact between tendon and suture without strangulating the tendon. Sutures were spaced 5 mm apart.

have a useful role in tendon repair. One such substance, which has been shown to have proangiogenic properties, is butyric acid (BA). Results of a meniscal healing study using BA-soaked sutures revealed increased neoangiogenesis at the repair site.[8] In our study, we attempted to see if we could replicate these proangiogenic findings.

It has been shown that, when compared with mature rabbit Achilles tendons, aged tendons were characterized by hypocellularity, less even distribution of fibroblasts, more frequent gaps longitudinally, and a less regular crimping pattern.[9] In addition, the vascular markers α_v integrin and vascular endothelial growth factor (VEGF) both were expressed at lower levels in aged tendon as compared with mature tendon. These properties in aged rabbit tendons are similar to histologic and biochemical effects on tendons during tendinosis.[10,11]

The purpose of our study was to see if we could detect histologic and biochemical markers of angiogenesis in an aged rabbit Achilles tendon model using BA-impregnated sutures.

Materials and Methods

Twenty-four 3-year-old New Zealand rabbits with closed epiphyses were studied. The protocol used has been approved by the University of California San Diego Institutional Animal Care and Use Committee. With the rabbits under general anesthesia, both Achilles tendons were exposed in a traditional fashion. The paratenon was incised, and the middle bundle of the Achilles tendon was isolated. A BA-impregnated size 0 ultra-high-molecular-weight polyethylene suture was placed at 2 sites 0.5 cm apart in the Achilles tendon. BA concentration was 6.2 µg/cm. A figure-of-8 suture construct, which tightly spanned 8 mm, was used to increase contact between tendon and suture without strangulating the tendon (Figure 1). On the contralateral side, an identical suture and construct without BA was used to serve as the control group. The paratenon and skin were closed as separate layers with 4-0 Vicryl. The animals were sacrificed for gross, histologic, and growth factor analysis at 7, 30, and 45 days.

Histologic and Biochemical Assessments

The extracted tendon was examined grossly, histologically, and for specific biochemical parameters. Grossly, the tendon was examined for size, inflammatory response, paratenon reaction, and suture condition. Histologic evaluation was performed on paraffin sections using hematoxylin-eosin staining for cellular expression.

Biochemically, the tendon was examined for hydration, DNA content for cell proliferation, and messenger RNA (mRNA) gene expression for vascular expression. Tendon hydration was determined by measuring the wet and dry tissue weights. Results were expressed as percentage of tissue in water weight. DNA concentration as a relative measure of cell content in the tendon was assessed as follows: 5 to 10 mg (dry) of H_2O-washed and lyophilized tissue were solubilized by incubation for 2 hours in 1N NaOH at 67°C. Quantitation of the released deoxyribose was determined using a colorimetric assay.[12] Results were expressed as micrograms of DNA per milligram of dry tissue.

The mRNA expression levels of the vascular genes VEGF and α_v integrin were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), as previously reported.[13-15] Tendons were pulverized in liquid nitrogen, and total RNA was isolated using the acid-guanidinium-thiocyanate-phenol extraction procedure. First-strand cDNA is synthesized using oligo $(dt)_{15}$ primers. Based on published sequences, PCR primer sets specific to selected coding regions of VEGF, α_v integrin, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene were constructed (Table I). Cycle studies are undertaken for all genes to determine the linear range of PCR amplification for each gene. The appropriate cycle and temperature then were used for each primer pair. National Institutes of Health image analysis software (version 1.61; NIH, Bethesda, Maryland) is used to quantitatively scan RT-PCR profiles after agarose gel electrophoresis. Expression of VEGF and α_v integrin are expressed as ratios relative to GAPDH±SD.

Table I. Primer Sequence for Polymerase Chain Reaction

Vascular endothelial growth factor	5′-ACTCTCACTGCGGATGCTTT-3′ 5′-AATTCCTGGGAGTGCAACTG-3′
α_v Integrin	5′-AACGGATCCCACTCTCACTG-3′ 5′-AATTCCTGGGAGTGCAACTG-3′
Glyceraldehyde 3-phosphate dehydrogenase	5′-TCACCATCTTCCAGGAGCGA-3′ 5′-CACAATGCCGAAGTGGTCGT-3′

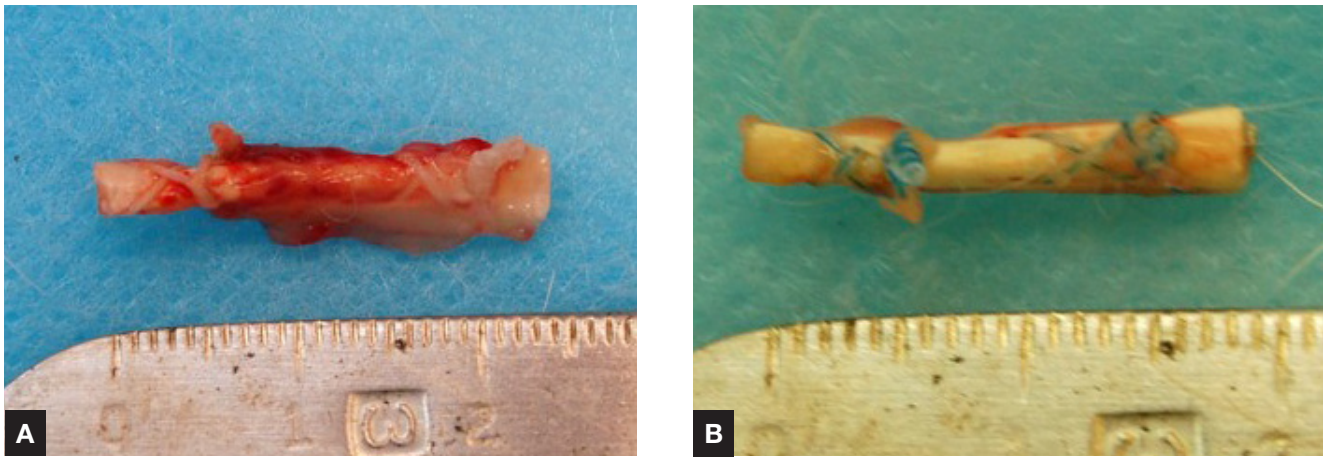

Figure 2. (A) A mild inflammatory response of paratenon is evident in this butyric acid–treated tendon at 7 days. (B) Minimal inflammatory response in paratenon of this control tendon at 7 days.

Statistical Analysis

Results were statistically analyzed with Statview 5.0 (Statsoft, Tokorozawa, Japan) and are presented as means and SDs in each group. Study groups on days 7, 30, and 45 were compared using a nonpaired Student *t* test with significance defined as $P<.05$.

Materials and Methods

Gross Assessment

The study was completed with 22 of the 24 rabbits. Two rabbits, both from the 7-day group, were euthanized early because of failure to thrive. The final groups were 7 days (n = 6), 30 days (n = 8), and 45 days (n = 8). Blinded gross examination of the tendons by 2 orthopedic surgeons did not reveal any statistically significant differences between the 2 tendon groups at any of the time points.

At 7 days, inflammation of the paratenon and tendon was observed in 50% (3/6) of the BA group and 33.3% (2/6) of the control group. Paratenon inflammation, which did not extend to the tendon proper, was identified by redness that may represent vasodilatation (Figures 2A, 2B).

At 30 days, hypertrophy of the paratenon was observed in 62.5% (5/8) of both groups (BA, control). Hypertrophy is described as a width increase of approximately 15%. Paratenon inflammation was decreased compared with the 7-day group. Each suture was found to be loosely wrapped around the tendon and adhering to the surrounding tissue.

At 45 days, tendon hypertrophy tended to continue in both groups:

Table II. Histologic Differences Between Butyric Acid and Control Specimens 7, 30, and 45 Days After Treatment

	Butyric Acid Specimens			Control Specimens		
Follow-up Period	Cellular Distribution[a]	Vascular Expression[b]	Proteoglycan Expression[c]	Cellular Distribution[a]	Vascular Expression[b]	Proteoglycan Expression[c]
7 days						
8343	++	++	++	+	+	+
33	++	++	+	+	+	+
8344	+	++	+	+	+	+
30 days						
24	+	+	++	+	+	+
26	++	++	+	+	+	+
28	++	++	++	+	+	+
45 days						
8347	++	++	+	+	+	+
8348	++	++	++	+	+	+
8349	++	++	+	+	+	+

+ = Baseline of assessed histologic parameters; ++ = Higher expression when compared with baseline.
[a]Hematoxylin-eosin stain.
[b]Verhoeff elastic tissur stain.
[c]Safranin O stain.

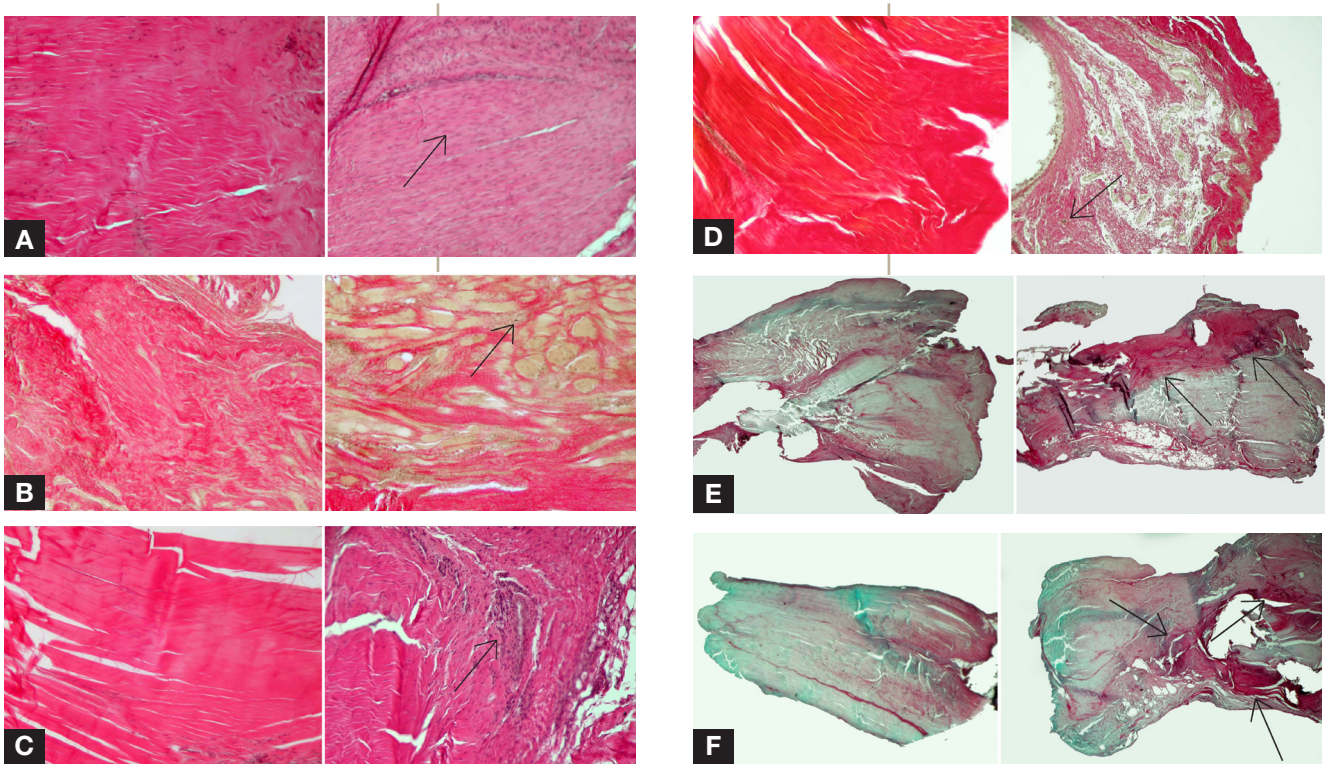

Figure 3. (A) Butyric acid (BA)–treated tendon on right shows increased cellular distribution (arrow) compared with control tendon at 30 days (hematoxylin-eosin staining, original magnification ×50). (B) BA-treated tendon on right shows increased vascular expression (arrow) compared with control tendon at 30 days (Verhoeff elastic tissue staining, original magnification ×50). (C) BA-treated tendon on right shows increased cellular distribution (arrow) compared with control tendon at 45 days (hematoxylin-eosin staining, original magnification ×50). (D) BA-treated tendon on right shows increased vascular expression (arrow) compared with control tendon at 45 days (Verhoeff elastic tissue staining, original magnification ×50). (E) BA-treated tendon on right shows increased expression of proteoglycan at 30 days (safranin O staining, original magnification ×50). (F) BA-treated tendon on right shows increased expression of proteoglycan at 45 days (safranin O staining, original magnification ×50).

BA (62.5%, 5/8) and control (50%, 4/8). Paratenon inflammation was absent, and the suture remained unchanged from the 30-day group.

In summary, a mild inflammatory reaction occurred in some of the specimens in the early stages of control (ie, normal) suture and BA-treated suture and then returned to normal. No differences were observed between the 2 groups. This was a gross observation based on a slight increase in redness of the paratenon and the tendon itself. Hypertrophy, judged on a width increase of approximately 10% to 15%, was noted in some specimens and was considered to be relatively mild.

Histologic Assessment

Histologically, there were increased vascularity and increased cellular distribution in all BA-treated tendons at 30 and 45 days (Figures 3A–3D). Proteoglycan concentration was also increased in the BA-treated tendon at 30 and 45 days (Figures 3E, 3F). Table II summarizes the histologic results.

Biochemical Assessment

DNA Concentration. DNA concentration as a relative measure of cell content was statistically higher in the BA group relative to the control group at 7 days ($P<.05$) but was unchanged at 30 and 40 days (Ps = .9 and .7, respectively) (Figure 4).

Hydration. There were no statistical differences ($P>.05$) in mean (SD) water content between the 2 tendon groups at any time point during the study: 7 days (BA, 69.6 [3.8]; control, 71.2 [2.4]), 30 days (BA, 65.6 [3.1]; control, 66.5 [4.4]), 45 days (BA, 64.7 [2.9]; control, 65.9 [3.8]) (Figure 5).

Gene Expression. The mRNA expression of VEGF was significantly ($P = .05$) higher in the BA group at 7 days (Figure 6A). A strong trend for higher expression in the BA group also was observed at 30 days ($P = .12$). At 45 days, the BA and control groups were similar. α_v Integrin also showed a trend ($P = .15$) for higher expression at 7 and 30 days in the BA group; there was no difference at 45 days ($P = .75$) .

A representative illustration of RT-PCR results after agarose gel electrophoresis and ethidium bromide visualization demonstrates up-regulation of VEGF and α_v integrin in the BA-treated sutures at 7 and 30 days (Figure 6B).

Discussion

Tendon vascular supply has 3 sources: the musculotendinous junction, the osseotendinous junction, and the extrinsic system through the synovium or paratenon.[16] In general, tendon blood flow has been found to be decreased at sites of

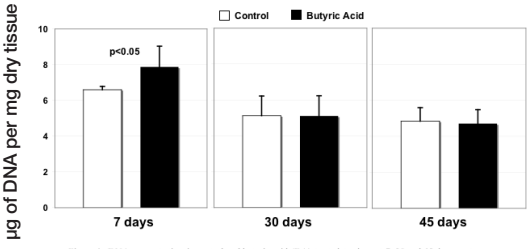

Figure 4. DNA concentration in butyric acid–treated and control tendons 7, 30, and 45 days after treatment.

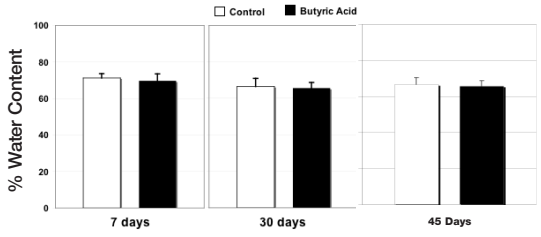

Figure 5. Water content for butyric acid–treated and control tendons 7, 30, and 45 days after treatment.

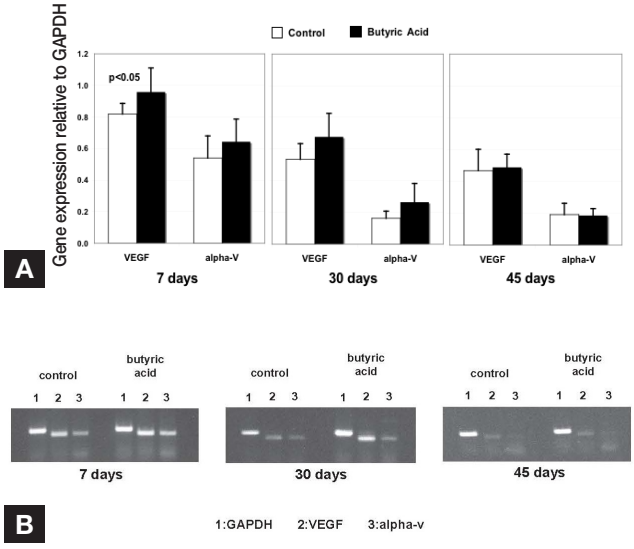

Figure 6. (A) mRNA expression of vascular endothelial growth factor and α_v integrin in butyric acid–treated and control tendons 7, 30, and 45 days after treatment. (B) Representative illustration of reverse transcriptase polymerase chain reaction results after agarose gel electrophoresis and ethidium bromide visualization.

increased mechanical loading and with increasing age.[17-19] In addition, several tendons, including the supraspinatus, the Achilles tendon, and the patellar tendon, appear to have regions of reduced vascularity.[16] It has been hypothesized that this decreased vascularity plays a crucial role in the development of tendinopathy and tendon tears and ruptures.

Normal tendons are characterized by a well-organized collagenous fibrillar network sparsely interspersed with fibroblastic cells and vascular structures.[1] Tendons that have undergone tendinosis are characterized by noninflammatory intratendinous collagen degeneration, scattered aberrant vascular ingrowth, and poor healing response.[7] These areas of tendinosis are thus more susceptible to rupture. Jozsa and Kannus[19] found that 97% of 891 spontaneously ruptured tendons exhibited degenerative changes, including hypoxic degenerative tendinopathy and mucoid degeneration. Similarly, Hashimoto and colleagues[20] showed that the degenerative changes of rotator cuff tendons were present before rupture. They performed histopathologic, histochemical, and morphometric studies on 80 medial stumps of torn rotator cuff tendons to clarify the cause of tears and found a high incidence and diffuse distribution of degenerative changes, including thinning and disorientation of collagen fibers and myxoid degeneration. No inflammatory reaction was found.

The first phase of tendon healing has been described as inflammation. This involves complex mechanisms, including release of vasoactive and chemotactic factors that initiate angiogenesis and stimulate tenocyte proliferation.[21] The degenerated tendon edge, with its lack of inflammatory reaction and myxoid degeneration, does not present the best picture of healing potential. In addition, Longo and colleagues[22] histologically examined 88 supraspinatus tendons at time of arthroscopic repair for rupture and found that degenerative changes were not only localized to the rupture site but also appeared in the macroscopically intact supraspinatus tendon. The implication is that, even if the edge of the ruptured tendon is débrided back, the remainder of the surrounding tendon does not present an optimal healing situation.

In their study on supraspinatus tendon healing, Boileau and colleagues[23] showed that arthroscopic repair produced a completely healed cuff only 71% of the time. This percentage fell to 43% when the data were stratified for patients older than age 65. It is likely that this older population had a supraspinatus more characterized by degenerative tendinopathy. Harryman and colleagues[24] reported similar results in their ultrasound study of rotator cuffs repaired after a mean of 5 years. They found a healed cuff in 80% of isolated supraspinatus repairs and in less than 50% of multitendon cuff repairs. Here again, older patients, with a presumably worse picture of tendinopathy, had a significantly lower rate of healing.

Several techniques for promoting better healing of tendons are being studied—including cell therapy, growth factors, gene therapy, electromagnetic field stimulation, and radiofrequency coblation.[7,25] BA is another option being investigated, because of its proangiogenic properties. BA is a naturally occurring compound formed in large amounts in the gut. It is known to have antiangiogenic effects at high doses but also has been shown to be proangiogenic at low doses.[14,26] The specifics of this

paradox have not been fully elucidated, but it is hypothesized that BA, at certain concentrations and depending on oxygen levels, is capable of exerting either effect.

Acton and colleagues,[8] recognizing the potential clinical application of BA, incorporated it into a Ticron suture used to repair sheep meniscal tears. They found that treating suture with BA did not decrease the mechanical properties of the suture. In addition, treatment time of the suture with BA predictably affected the release profile. When the menisci were examined histologically after animal sacrifice, neoangiogenesis at the repair site was seen, and new blood vessels were found within the BA suture itself. Mechanical testing performed on the menisci revealed that meniscal tears were significantly stronger in the BA group than in the control group.

In our study, we used an aged rabbit tendon model that is physiologically and histologically similar to a tendon that has undergone tendinosis. The histologic results demonstrated that BA-treated tendons had an increased vascular response and increased cell migration. Biochemically, we used VEGF and α_v integrin as markers of angiogenesis. Previous studies have shown that the angiogenic VEGF is expressed by cells involved in the early stages of tendon healing.[27] The statistically significant increase of VEGF at 7 days and the positive trends shown by VEGF and α_v integrin at 30 days further suggest that BA provides a stimulus to angiogenesis.

Although tendon length and width are relatively small in this study, there are enough histologic and angiogenic changes occurring in this small area to reflect increased healing potential. We know that tendon–bone and tendon–tendon interface healing occurs through a very limited area. In rotator cuff repair, multiple sutures are usually used, and they supply an adequate amount of BA. A larger animal model simulating this current experiment used a sheep meniscus.[8]

Augmentation of tendon healing is being approached in several ways. Many techniques require specialized instrumentation and involve some difficulty. Use of BA-impregnated sutures potentially provides a simple solution for augmenting tendon healing. BA-impregnated sutures could come loaded as part of a standard suture anchor construct and be applied with existing techniques. There would be no learning curve required, and no additional time would be added to the case.

Our data indicate that angiogenesis at the repair site would be improved, which could lead to better healing at the tendon–bone and tendon–tendon attachment sites.

Conclusions

Angiogenic activity was significantly increased in BA-treated tendons 7 days after surgery. This was seen both with increased mRNA expression of VEGF and with increased DNA concentration. This trend continued for up to 30 days and leveled off by 45 days. These biochemical findings are further supported by the histologic findings of increased cellularity and vascularity of the BA-treated tendons. These findings could indicate a simple and economic supplement to attempts to repair compromised tissue.

Authors' Disclosure Statement and Acknowlegement

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