



The role of nitric oxide in tendon healing

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Nitric oxide (NO) is a small free radical that is generated by a family of enzymes called the nitric oxide synthases (NOS). There are 3 isoforms of NOS: endothelial NOS (eNOS), brain or neuronal NOS (bNOS), and inducible NOS (iNOS). In experiments performed during the last 20 years, we have shown that NO is induced by all 3 isoforms of NOS after tendon injury and that NOS activity is upregulated in tendinopathy. In normal uninjured tendons, there is very little NOS activity. In injured rat and human tendons, NOS activity was found in healing fibroblasts in a temporal fashion. In animal models, competitive inhibition of NOS resulted in reduced tendon healing, whereas the addition of NO resulted in enhanced tendon healing. In cultured human cells, the addition of NO via chemical means and adenovirus transfection resulted in enhanced collagen synthesis. We performed 3 randomized, double-blinded clinical trials that demonstrated a significant positive beneficial effect of NO treatment on clinical symptoms and function in patients with Achilles tendinopathy, tennis elbow, and supraspinatus tendonitis. NO was delivered via glyceryl trinitrate (GTN) patches. We also conducted a 3-year prospective follow-up that demonstrated significant long-term efficacy of GTN patches in treating noninsertional Achilles tendinopathy. In a 5-year prospective comparison treating lateral epicondylitis, we found no additional benefits of GTN vs placebo at 5 years. The use of a new GTN patch, OrthoDerm, demonstrated no evidence for efficacy in treating chronic lateral epicondylitis.

Level of evidence: Review Article.

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Nitric oxide

Nitric oxide (NO) is a small, free radical molecule that is synthesized by a family of enzymes, the nitric oxide synthases (NOS). Free radicals are molecules that have unpaired electrons, making them very unstable and very reactive. Like other free radicals, NO is toxic in large doses; however, in small physiological doses, NO is an

important messenger molecule and part of the immune response in addition to other cellular processes.¹⁹ NO is produced as a coproduct when NOS catalyzes the reaction of L-arginine to L-citrulline. The NOSs are regulated by cofactors such as calmodulin, tetrahydrobiopterin, heme, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD).¹⁸

There are three isoforms of NOS:

- Endothelial NOS (eNOS) was originally found in endothelial cells.¹¹
- Brain or neuronal NOS (bNOS), originally found in neuronal tissue,¹ are low-output, constitutive and important in vascular function and memory.

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- Inducible NOS (iNOS) is a high-output isoform that can be induced by proinflammatory cytokines (such as interleukin-1 and tumor necrosis factor), bacterial cell wall products and is important in the host immune response.^{19,25}

NOS after tendon injury

Rat Achilles tendon model

In a normal, uninjured rat tendon, there is little or no NOS activity. After surgical division of the Achilles tendon, there was a significant increase (approximately 5-fold) in the conversion of L-arginine to L-citrulline, indicating increased NOS activity in the healing tendon.¹⁰ Activity peaked at 7 days and returned to baseline at 14 days. Semiquantitative polymerase chain reaction and immunoblotting were used to determine which NOS isoforms were expressed in tendon healing. Four days after surgical division, there were increases in the steady-state levels of messenger RNA (mRNA) and protein for all 3 NOS isoforms; therefore, all 3 isoforms of NOS were expressed: iNOS peaked at days 4 and 7 (23-fold increase), eNOS peaked at day 7 (24-fold increase), and bNOS peaked at day 21 (7-fold increase).^{5,6}

Rat rotator cuff tendon model

A defect approximately 50% the width of the supraspinatus tendon was created with a 3-mm-diameter biopsy punch. To prevent retraction of the tendon, at least a 1-mm strip of tendon was left on both sides. With competitive reverse-transcriptase polymerase chain reaction, we determined that in this acute rotator cuff injury model, all 3 isoforms of NOS were expressed. The expression of NOS was slightly different from that in the Achilles tendon model, bNOS expression peaking at day 4, eNOS on day 7, and iNOS on day 7.²⁰

Rat rotator cuff overuse model

In this model, rats were subject to treadmill running (1 km/h on a 10° incline) for 1 hour per day, 5 days per week for 4 weeks.^{2,3} Rats randomized to normal cage activities were used as controls. In rats subjected to treadmill running, we found over-expression of iNOS, eNOS, and bNOS mRNAs in the supraspinatus tendons at 14 days.²⁴ Our results suggest NOS activity is induced in tendon injury and NOS expression is a part of supraspinatus tendinopathy.

Human rotator cuff injury

The edges of torn tendons that are normally excised and discarded during the surgical repair of rotator cuffs in humans were evaluated for NOS activity. NOS activity was

found in 7 of 10 human rotator cuff samples. iNOS and eNOS mRNA expression was detected in each sample, whereas bNOS was detectable in 3 of 8.²¹ Our results indicate in humans, like that in rats, upregulation of NOS occurs after tendon injury.

Is NOS expression important to tendon healing?

When rats were fed NOS inhibitor (NG-monomethyl-L-arginine), we found a significant reduction in the healing of their Achilles tendon compared with rats fed its inactive enantiomer (N-nitro-D-arginine methyl ester). We found a 50% reduction in the cross-sectional area of the Achilles tendon at day 7 and a 24% reduction in the failure load in Achilles tendon constructs.¹⁰ We also looked at which NOS gene is important for tendon healing by deleting the gene for iNOS in mice. Our results suggest NO generated from eNOS and/or bNOS is more important or placed in a more important location for tendon healing than NO generated by iNOS.²⁴

Sources of NO in tendon healing

Using in situ hybridization and immunohistochemistry in Achilles tendon healing in rats, we demonstrated that fibroblasts expressed all 3 isoforms of NOS in a temporal fashion, with iNOS expressed first (4-7 days), then eNOS (4-14 days), and bNOS (14-21 days). As expected, iNOS was also expressed by macrophages and eNOS by endothelial cells.⁶

Role of NO in tendon healing

Our studies involving NOS inhibitors demonstrate that NO has a crucial role in new tissue synthesis during tendon healing. Nitric oxide is likely to be important to a number of processes, including blood flow and host defence. [Figure 1](#) illustrates the role of NO in tendon healing.

Human models

Work in our laboratory suggests NO may be important in collagen synthesis. When cultured human rotator cuff tendon cells were exposed to exogenous NO (in the form of S-nitro-N-acetyl-penicillamine) and when transfected with the iNOS gene via an adenovirus vector, they incorporated more collagenase-sensitive H-proline in their matrix. This increase in collagen synthesis was able to be inhibited a NOS inhibitor (NG-monomethyl-L-arginine).²³

After transfection with iNOS in tenocytes isolated from injured human rotator cuff tendons, we used microarray analysis to illustrate global gene expression. It is clear NO effects the expression of a wide range of genes, many of which are known to have roles in healing. Increased transcription and translation of extracellular matrix genes

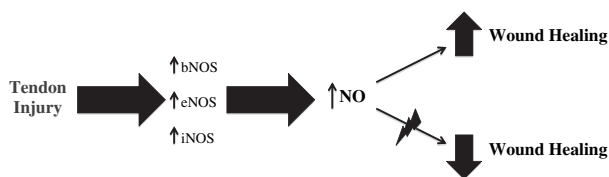


Figure 1 Schematic diagram of nitric oxide (NO) in tendon healing. Induction of NO by endothelial (*eNOS*), brain or neuronal (*bNOS*), and inducible (*iNOS*) nitric oxide synthases (*NOS*) leads to wound healing, whereas inhibition of NO leads to decreased wound healing.

important in the to the structure of connective tissues such as tendons, including collagen type I $\alpha 1$, collagen type III $\alpha 1$, collagen IV $\alpha 5$, biglycan, decorin, laminin, and matrix metalloproteinase 10, are of particular significance. These genes also responded to the stimulation by the NO donor S-nitroso-*N*-acetyl-penicillamine in a dose-dependent manner.⁸ Furthermore, varying levels of NO significantly affect cellular adhesion in tenocytes, a critical process during tendon repair.⁸

Rat models

We delivered NO via flurbiprofen (a nonspecific cyclooxygenase inhibitor) and via NO paracetamol. In both experiments, administration of NO had positive effects on collagen organization, tendon healing failure load, and stress (load/area).^{22,26} Enhanced collagen synthesis due to NO in the rat model are consistent with results achieved in cultured human tendon cells and in the results from clinical trials described in the next section.

Clinical trials

To assess whether NO might enhance tendon healing in humans, we conducted several double-blinded randomized clinical trials (RCTs) in humans. The patients and clinical examiner were both blinded to patient groups. Each RCT involved the use of commercially available glyceryl trinitrate (GTN) patch to deliver NO and identical placebo patches. The patches were applied to the area of maximal tenderness. Patients also received education and exercise; that is, GTN treatment was in addition to “best practice.” We also conducted 2 long-term comparison studies to assess the long-term efficacy of GTN patches in treating chronic lateral epicondylitis of the elbow and chronic noninsertional Achilles tendinopathy.

Tennis elbow

A double-blinded RCT involving 86 patients with chronic symptoms (>3 months) of tennis elbow randomized participants into 2 treatment groups: GTN (1.25 mg/24 h) or placebo patches. Both groups received tendon rehabilitation. The GTN group had reduced elbow pain at 2 weeks

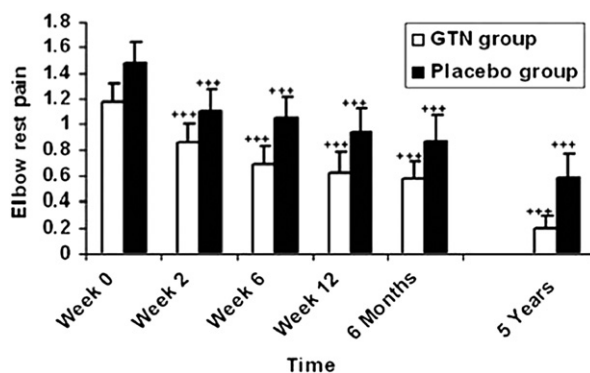


Figure 2 Effect of topical glyceryl trinitrate (GTN) on patient-rated pain at rest. Data are presented as mean with the standard error of the mean (error bars). $+++P < .001$ compared with week 0 using Wilcoxon signed-rank test. Reproduced from [McCallum S, Paoloni J, Murrell GAC. Five year prospective comparison study of topical glyceryl trinitrate treatment of chronic lateral epicondylitis at the elbow. *Br J Sports Med* 2011;45:416-20]⁷ with permission from BMJ Publishing Group Ltd.

($P = .01$), reduced elbow pain with activity at 2 weeks ($P = .01$), reduced epicondylar tenderness at 6 and 12 weeks ($P = .02$), and an increase in wrist extensor mean peak force and total work at 24 weeks ($P = .03$).¹² In activities of daily living, 81% of patients on GTN patches were asymptomatic compared with 60% of patients with tendon rehabilitation alone ($P = .05$).¹⁶

We conducted another double-blinded RCT involving 136 patients to evaluate whether a new GTN patch, OrthoDerm (Cure Therapeutics, Inc., New York, NY), was effective in treating chronic lateral epicondylitis. Patients were randomized into 4 groups; placebo, 0.72 mg/24 h, 1.44 mg/24h, or 3.6 mg/24 h OrthoDerm patches. Compared with placebo, we found a significant improvement in elbow pain with activity at 8 weeks with the 0.72 mg/24 h OrthoDerm patch ($P = .04$).¹⁷ There were no other significant results. The lack of demonstrated efficacy in this trial may be due to a different patch formula, lack of a rehabilitation exercise program (as in the previous tennis elbow trial), or too short a treatment period. Rashes and headaches were common side effects, with headaches appearing to be dose-related.¹⁷

We also conducted a 5-year prospective comparison study of the treatment of chronic lateral epicondylitis at the elbow with GTN. We monitored 58 patients from the original trial for 5 years after 6 months of GTN or placebo therapy. The GTN and placebo groups both demonstrated significant improvements (Figs. 2-6), but the GTN group did not show any additional benefits over the placebo group (rehabilitation therapy only) at 5 years.⁷

Achilles tendonitis

A double-blinded RCT was performed with 65 patients with chronic symptoms (>3 months) of Achilles tendonitis.

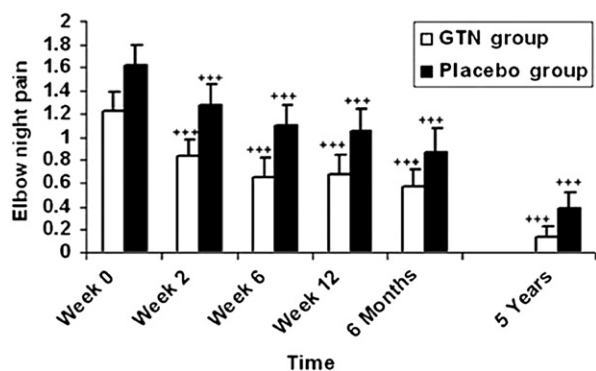


Figure 3 Effect of topical glyceryl trinitrate (GTN) on patient-rated pain at night. Data are presented as mean with the standard error of the mean (*error bars*), $n = 27$ for GTN group, $n = 31$ for placebo group. $+++P < .001$ compared with week 0 using Wilcoxon signed-rank test. Reproduced from [McCallum S, Paoloni J, Murrell GAC. Five year prospective comparison study of topical glyceryl trinitrate treatment of chronic lateral epicondylitis at the elbow. *Br J Sports Med* 2011;45:416-20]⁷ with permission from BMJ Publishing Group Ltd.

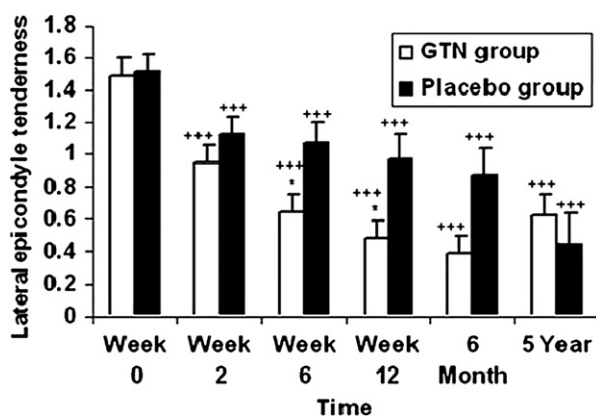


Figure 4 Effect of topical glyceryl trinitrate (GTN) versus placebo on lateral epicondyle tenderness. Data are presented as mean with the standard error of the mean (*error bars*). $+++P < .001$, compared with week 0 using Wilcoxon signed-rank test. Reproduced from [McCallum S, Paoloni J, Murrell GAC. Five year prospective comparison study of topical glyceryl trinitrate treatment of chronic lateral epicondylitis at the elbow. *Br J Sports Med* 2011;45:416-20]⁷ with permission from BMJ Publishing Group Ltd.

Patients were randomized into two treatment groups, GTN (1.25 mg/24 h) or placebo patches. The GTN group demonstrated reduced Achilles activity pain at 12 ($P = .02$) and 24 ($P = .03$) weeks, reduced night pain at 12 weeks ($P = .04$), reduced tenderness at 12 weeks ($P = .02$), decreased pain scores with the hop test at 24 weeks ($P = .005$), and an increase in ankle plantar flexor mean total work at 24 weeks ($P = .04$). Of patients on GTN patches, 78% were symptomatic with activities of daily living at 6 months compared with 49% of patients who received

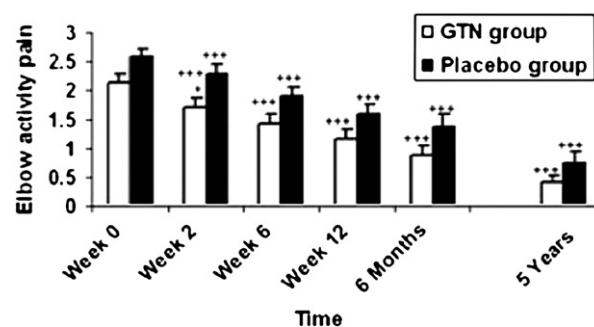


Figure 5 Effect of topical glyceryl trinitrate (GTN) on patient-rated pain with activity. Data are presented as mean with the standard error of the mean (*error bars*). $+++P < .001$, compared with week 0 using Wilcoxon signed-rank test. Reproduced from [McCallum S, Paoloni J, Murrell GAC. Five year prospective comparison study of topical glyceryl trinitrate treatment of chronic lateral epicondylitis at the elbow. *Br J Sports Med*. 2011;45:416-20]⁷ with permission from BMJ Publishing Group Ltd.

What we already know about this topic

- Nitric Oxide is important to tendon healing and NOS is upregulated in tendinopathy
- In animal models, when NO is inhibited, tendon healing is reduced while when additional NO is added, tendon healing is enhanced.
- Three clinical RCTs have demonstrated enhanced clinical recovery of tendinopathy in humans by delivering nitric oxide via GTN patches.

What this study adds

- Compared to rehabilitation alone, the use of GTN patches in chronic non-insertional Achilles tendinopathy has demonstrated efficacy and safety at 3 years.
- The use of GTN patches in chronic lateral epicondylitis at the elbow failed to demonstrate benefits over rehabilitation alone at 5 years.
- A new GTN patch, OrthoDerm, did not provide evidence for efficacy in treating chronic lateral epicondylitis. This was most possibly due to the absence of an exercise program.

Figure 6 Summarizing what we already know about this topic and what this study adds.

tendon rehabilitation alone ($P = .001$ with χ^2 analysis). Mean effect size for all outcome measures was 14%.¹³

We investigated the long-term efficacy of GTN patches by conducting a 3-year prospective comparison study involving the use of GTN patches to treat chronic non-insertional Achilles tendinopathy. Fifty-two patients previously treated with 6 months of GTN patches or placebo were assessed 3 years after the cessation of therapy. Patients treated previously with GTN patches had significantly less Achilles tendon tenderness ($P = .03$) and improved Victorian Institute of Sport Achilles tendon scale scores ($P = .04$). We found 88% of patients with GTN treatment were asymptomatic at 3 years compared with 67% of patients treated with tendon rehabilitation alone

Table I The effects of 1.25 mg/day glyceryl trinitrate transdermal patches and rehabilitation vs placebo and rehabilitation with supraspinatus tendinopathy*

Week	GTN (n = 35)	Placebo (n = 39)	P
Pain with activity			
2	2.2	2.1	.80
6	1.9	2.1	.34
12	1.6	1.9	.36
24	1.0	1.8	.006
Pain at rest			
2	1.7	1.9	.47
6	1.5	1.6	.68
12	1.1	1.4	.19
24	0.7	1.2	.09
Pain at night			
2	1.4	1.6	.61
6	1.4	1.5	.72
12	1.0	1.5	.09
24	0.8	1.4	.031
Tenderness			
2	0.1	0.4	.012
6	0.2	0.5	.017
12	0.2	0.4	.18
24	0.2	0.2	.60
Change from baseline in			
Abduction range of motion			
2	7.7	1.5	.15
6	7.8	2.0	.29
12	16.3	1.2	.020
24	26.6	8.7	.004
Adduction power			
2	5.6	1.3	.24
6	7.7	-0.6	.08
12	11.9	0.4	.023
24	9.9	1.6	.11
External rotation power			
2	4.7	0.5	.17
6	7.4	0.8	.11
12	11.5	0.5	.023
24	13.4	0.3	.013
External rotation range of motion			
2	1.9	6.7	.13
6	4.7	1.9	.45
12	7.7	3.3	.26
24	13.2	8.5	.17
Forward flexion			
2	7.8	0.8	.11
6	6.5	-2.3	.09
12	13.4	-2.2	.019
24	20.3	6.3	.027
Impingement external rotation (% with 0)			
2	40.7	32.1	.51
6	40.7	32.1	.57
12	70.4	57.1	.36
24	70.4	50.0	.15
Impingement internal rotation (% with 0)			
2	33.3	10.7	.059

(continued)

Table I (Continued)

Week	GTN (n = 35)	Placebo (n = 39)	P
6	48.2	17.9	.040
12	51.9	32.1	.15
24	74.1	39.3	.018
Change from baseline in			
Internal rotation power			
2	4.9	-0.9	.14
6	10.8	2.0	.055
12	11.1	-1.5	.029
24	13.9	1.1	.018
Internal rotation range of motion			
2	-2.3	0.5	.37
6	-4.3	-1.0	.22
12	-5.0	0.6	.15
24	-8.1	-0.3	.32
Subscapularis power			
2	9.0	0.9	.009
6	9.2	5.4	.24
12	14.0	5.8	.045
24	15.7	606	.018
Supraspinatus power			
2	3.6	-1.5	.10
6	11.0	0.7	.009
12	15.4	2.0	.010
24	16.2	2.9	.007

GTN, glyceryl trinitrate.

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($P = .03$). The mean estimated effect size for all outcomes was 0.21.¹⁴ This study demonstrates the benefits of GTN treatment in non-insertional Achilles tendinopathy continue at 3 years and suggests the mechanism of action of GTN therapy is more than an analgesic effect.

Supraspinatus tendinopathy

This trial produced the most significant results. A double-blinded RCT was performed with 53 patients with supraspinatus tendinopathy/impingement who were randomized to GTN (1.25 mg/24 h) or placebo patches. The NO group had significantly reduced shoulder pain with activity, at night, at rest, and internal rotation impingement, increased range of motion in abduction and internal rotation, and an increase in force with supraspinatus testing, external rotation, internal rotation, adduction, and subscapularis push-off (Table I).⁹ At 6 months, 46% of patients on GTN patches were asymptomatic with activities of daily living compared with 24% of patients with tendon rehabilitation alone ($P = .007$). Mean effect size of GTN treatment for all outcome measures was 26%.¹⁵

A Cochrane review of 3 RTCs on GTN use in rotator cuff disease found that although there is some evidence to

indicate GTN is more effective than placebo in treating rotator cuff diseases, further high-quality trials with larger patient samples are required to determine the overall efficacy and safety of GTN use for rotator cuff disease.⁴

Clinical considerations

After corticosteroid injection, GTN patches are our second line of treatment for the management of supraspinatus tendinopathy. The advantage of GTN patches is that it is a very benign intervention. The only significant side effect, headaches, can be dealt with by cutting the patches into smaller sizes. It is also good alternative for patients who do not want injections or operations and for patients who are likely to improve with time but want some form of benign treatment. We do not currently use GTN patches for tendon healing after rotator cuff repair and would not suggest so until there are RCTs on this topic with positive results. To further evaluate GTN's efficacy in tendon healing we would ideally like to see more RCTs from other groups.

Conclusions

NO is important to tendon healing. During tendon healing, all 3 isoforms of NOS are expressed by fibroblasts. Our animal studies, cell cultures, and clinical trials support our hypothesis that NO enhances extracellular matrix synthesis and results in injured tendons having better material and mechanical properties; that is, the healing tendons are stronger on a per unit area basis than those not exposed to additional NO. Clinical trials show that delivering NO via a patch enhances clinical recovery of Achilles tendonitis, chronic lateral epicondylitis, and supraspinatus tendinopathy, which is manifested by a reduction in pain, an increase in range of motion, and an increase in strength. Long-term efficacy of GTN patches was demonstrated in noninsertional Achilles tendinopathy but not in chronic lateral epicondylitis. In chronic lateral epicondylitis, OrthoDerm patches given in the absence of an exercise program failed to demonstrate evidence for efficacy, suggesting exercise rehabilitation may be important to GTN patch efficacy.

Disclaimer

George A. C. Murrell receives or has the potential to receive royalties from a patent on nitric oxide and tendon healing. The other author, his immediate family, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.jse.2011.11.001.

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