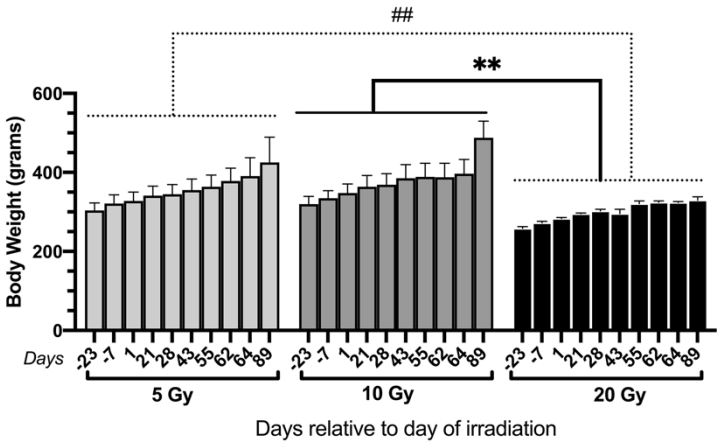
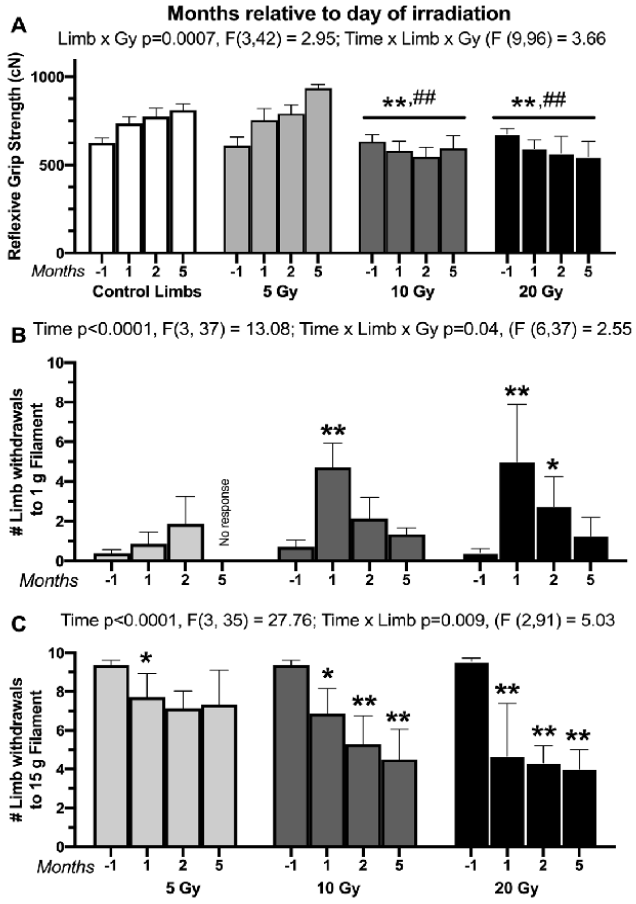
Supplemental File
Massage therapy for radiation-induced fibrosis: preliminary findings

S1: Appearances following 5 Gray (Gy), 10 Gy, and 20 Gy irradiation. Anesthetized rats were placed under the lead covers with only their arms extending into the exposure field as described in the main text. The forepaws of 20 Gy irradiated rats showed severe skin burns that largely recovered within 2 weeks, and hair loss in their forearms that recovered after 5 months (above, right, top row). Forepaws of 10 Gy irradiated rats (bottom row) showed transient forepaw skin redness for 2 days, and the health of these rats was otherwise unremarkable following irradiation. Irr = irradiated.

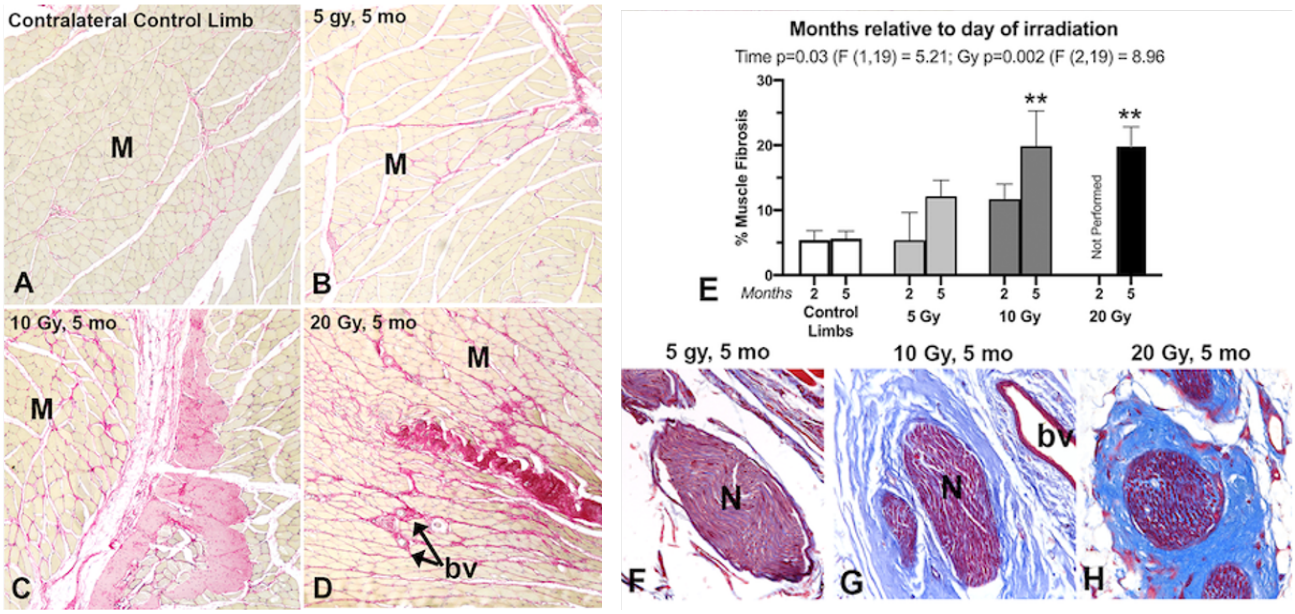
S2: Weight changes of irradiated rats. Rats irradiated with 5 or 10 Gy gained body weight normally. Rats irradiated with 20 Gy failed to gain weight across the time shown compared to the other groups (## and ** = p<0.01), suggestive of systemic sickness.



S3: Motor and sensory behaviors of irradiated rats. Major complaints in patients who have undergone radiation therapy include distressing paresthesia with progressive losses of touch and thermal perception, pain in some patients, and motor weakness.[1-3] Longitudinal assays showed that rats irradiated with 10 or 20 Gy doses did not show expected growth related increases in grip strength in their irradiated limb (S3-A), compared to control limbs and 5 Gy rats. Forepaw mechanical sensitivity was tested by probing forepaws with small and large sized monofilaments, with sensitivity indicated by withdrawal of the probed limb. Hypersensitivity of the irradiated forepaws to a 1-gram monofilament was seen at 1-month post-irradiation in 10 Gy rats, and at 1 and 2 months in 20 Gy rats, compared to baseline (S3-B). This is interpreted as allodynia. In contrast, a lowered sensitivity to a 15-gram monofilament was seen in both 10 and 20 Gy rats, compared to baseline levels (S3-C), suggestive of a loss of axons mediating cutaneous sensation. This is consistent with the nerve degeneration observed by histology (see below). * and **: p<0.05 and p<0.01 compared to non-irradiated limbs; ##p<0.05 compared to 5 Gy and control limbs combined.

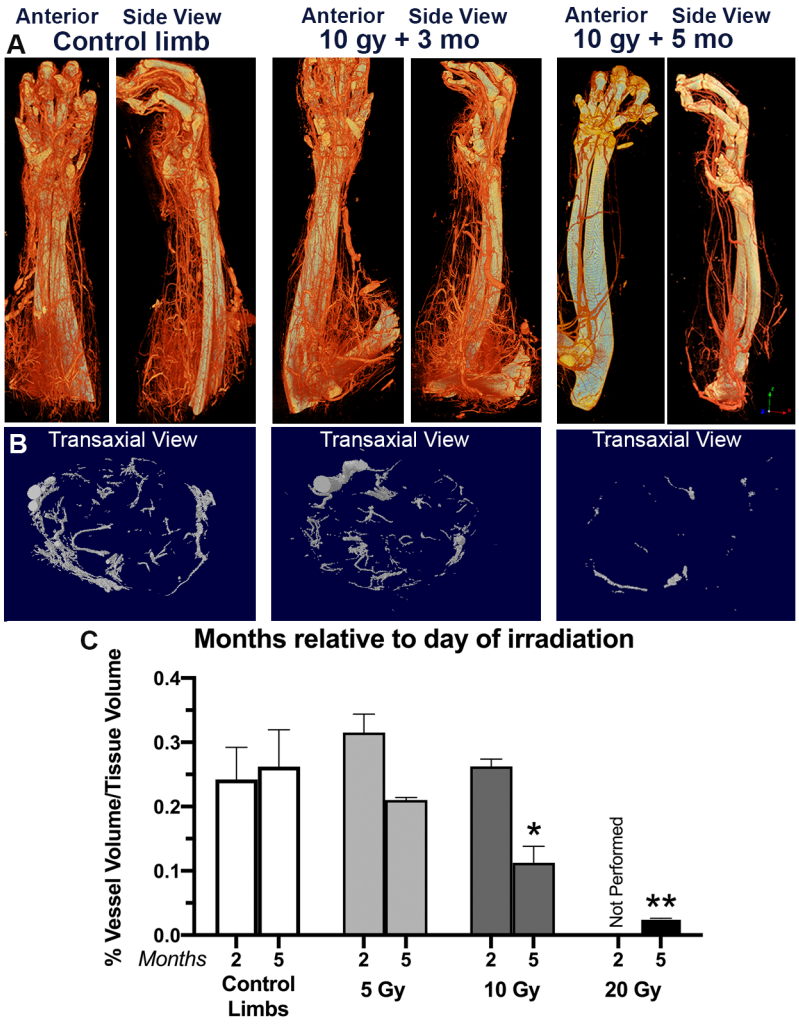


S4: Muscle and nerve fibrosis after a single exposure of 5, 10, or 20 Gy. Since radiation therapy of regions that includes muscles induces similar progressive fibrotic changes in muscle tissues,[4,5] we examined neuromuscular tissues collected from 5, 10, and 20 Gy exposed animals for evidence of RIF. Post-fixation, tissues were paraffin embedded and sectioned into 5 μm sections (proximal muscle regions) stained with picrosirius red (collagen is stained red), or with Masson's Trichrome (collagen is stained blue). Figures S4-A-E show clear increases in intramuscular fibrosis in 10 and 20 Gy flexor muscles at 5 months post-irradiation, compared to contralateral non-irradiated control limbs. Sections through the median nerve also showed similar dose-dependent fibrosis (now extraneural and intraneural) in 10 and 20 Gy irradiated rat limbs, as well as increased deposition of collagenous connective tissues around blood vessels (S4-D), similar to prior reports of radiation-induced vascular fibrosis.[6,7] N=nerve, bv = blood vessel.

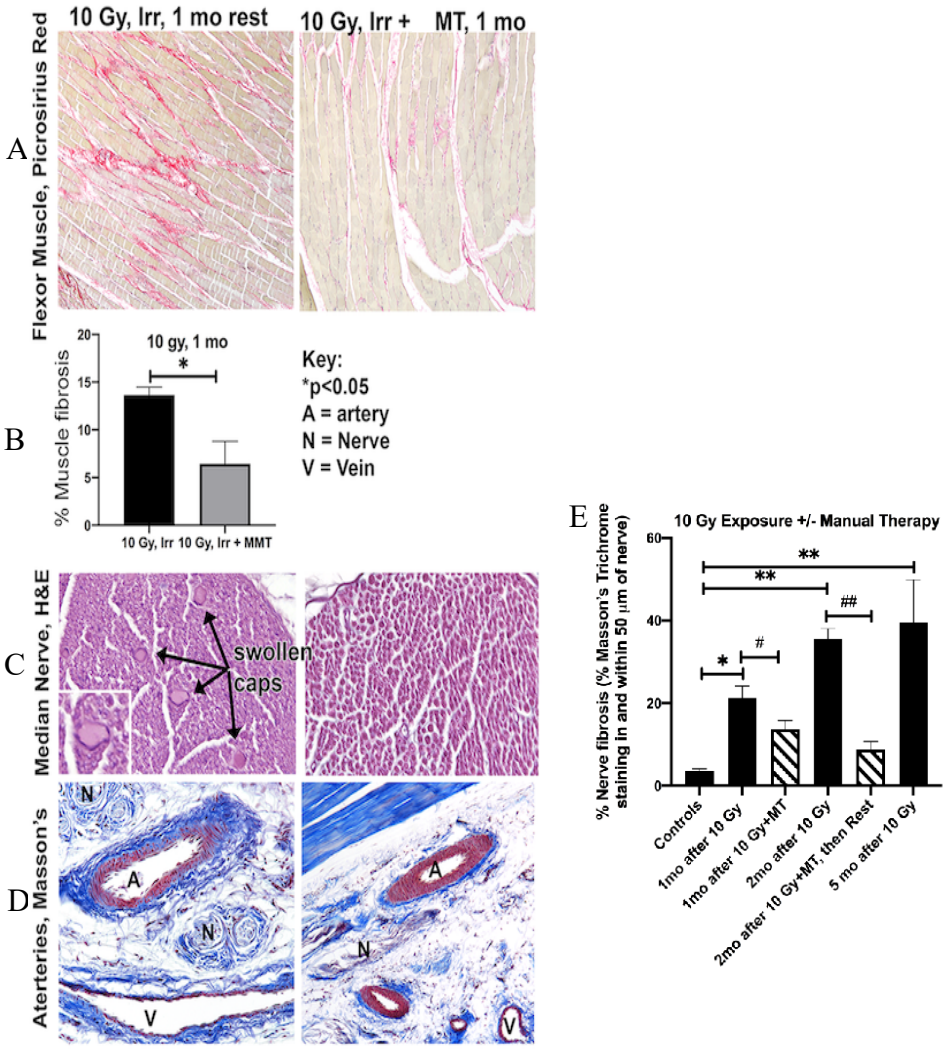


S5: Electrophysiological signs of neuropathy. Radiation therapy is associated with direct and indirect nerve damage.[1,8-12] Thus, we examined the effects of irradiation on neuronal function using electrophysiological methods developed by Dr. Bove.[13] A control experiment performed in the laboratory for this project led to recordings from 9 neurons with C-fibers (mean CV = 0.92m/s), of which one had ongoing activity at a rate of 0.15 Hz, and recordings from numerous neurons with faster fibers. There was no ongoing activity or pathological discharge from faster conducting neurons (other than Type II muscle spindles, which normally exhibit irregular ongoing activity). This result was like previous control experiments.[14] An experiment performed on a rat 2 months after a forelimb was irradiated with 10 Gy yielded recordings from 6 neurons with C-fibers (mean CV = 1.46 m/s), five of which (83%) had slow and irregular ongoing activity (mean 0.44 Hz). Although 2 of 9 faster neurons showed some ongoing activity, it was not deemed neuropathological (i.e., they did not have high rate and or bursting discharge.[14] This finding is consistent with previous findings during neuritis[15] and during the active inflammatory phase in rats with overuse injury.[13] An experiment performed on a rat 5 months after irradiation yielded recordings from 6 neurons with C-fibers (mean CV = 0.85 m/s) and 17 neurons with faster conducting fibers. None had ongoing activity, consistent with control experiments. While not enough data to make a conclusion, it is possible that the nerve changes observed at 2 months healed by 5 months post-irradiation.

S6: Vascular studies. Since vascular injury and capillary network failure have been reported to occur after radiation therapy,[1,12] additional rats were irradiated as described and used to examine vascular volume. These rats were anesthetized and then perfused intracardially with a radio-opaque polymerizing contrast agent, Microfil (Flow Tech). After polymerization, forearms were scanned in a Micro-CT system, and images compiled into whole forearm 3D models and 2D transaxial 50 "slice" reconstructions of Microfil casted vasculature. Percent vessel volume was calculated using previously described methods.[16] By 5 months post-irradiation, losses in % vessel volume was observed in 10 and 20 Gy irradiated limbs, compared to contralateral non-irradiated limbs. Although we did not find significant losses in vessels until 5 months after irradiation, as detectable using microCT at the lowest resolution of 3 μm, the significant loss of vessels by 5 months strongly supports the need to investigate earlier indices of endothelial cells damage in irradiation exposed tissues (muscle, tendon, and nerve). * and **: $p<0.05$ and $p<0.01$ compared to non-irradiated limbs



S7: Preliminary findings using manual therapy on the irradiated rats. We performed manual therapy on rats that had undergone a 10 Gy irradiation of one forearm and compared the results to irradiated rats that did not receive the manual therapy treatment. A. Manual therapy prevented intramuscular collagen formation (red; quantified in B). C. Manual therapy prevented vaculitis as indicated by swollen capillaries (caps). D. Arteriole intimal disruption seen in irradiated rats was not seen in rats that received MMT. E. Fibrosis in the nerve appeared by 1 month of treatment, and this effect lasted without further treatment for 2 months. We found that the manual therapy attenuated several key pathologies, including nerve, muscle, and arterial fibrosis. We also observed what appeared to be nerve vasculitis (swollen capillaries in the nerve) in nerves of untreated 10 Gy, 1-month forearms; a finding not seen in nerves of manual therapy treated rats.



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