Mesenchymal Stem Cell Delivery via Topographically Tenoinductive Collagen Biotextile Enhances Regeneration of Segmental Tendon Defects

APPENDIX

Scaffold Fabrication

Scaffolds were fabricated as previously reported.[27] Collagen (type I, acid-soluble, 6 mg/mL, CS028, Collagen Solutions, Eden Prairie, MN) was diluted to 3 mg/mL with deionized water prior to dialysis against deionized water. The resulting solution was used to form ELAC threads between two stainless steel electrodes (30 V, 90 s) and 3-ply yarns were fabricated from single threads prior to crosslinking with genipin (2% weight/volume in 90% ethanol, catalog #078-03021, Wako Pure Chemical Industries, Richmond, VA) for 72 hours at 37°C. Crosslinked yarns were woven around a stainless-steel pin array (1.47 mm diameter, 2 mm center-to-center spacing) to a height of 5 mm to form a scaffold unit. Two scaffold units were stacked in parallel and consolidated using a weft fiber to produce a scaffold (dimensions: ~14 x 5 x 3 mm^3, Figure 2A). Woven scaffolds were then sterilized by treatment in peracetic acid (catalog #269336, Sigma-Aldrich) /ethanol solution (4hrs, 1%/22.5% weight/volume in deionized water).

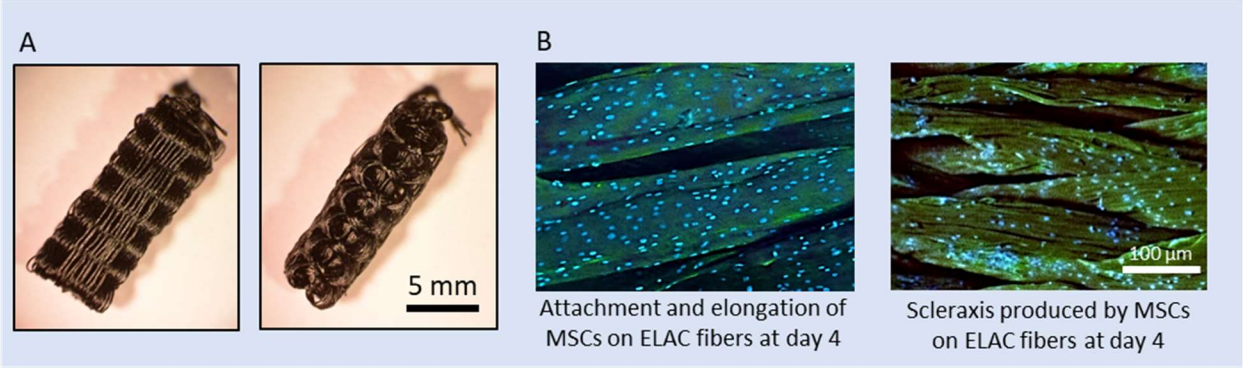


Figure A1. A) Bilayer scaffold composed of woven, genipin-crosslinked, electrochemically aligned collagen (ELAC). Left: top view of scaffold highlighting weaving pattern and right: side view of the same scaffold showing the two layers and the weft fiber pattern. B) Left: Confirmation of attachment of MSCs on ELAC at Day 4 following sequential cell-seeding (DAPI – nuclei – blue and phalloidin – actin cytoskeleton – green). Right: Presence of scleraxis (green) confirmed at Day 4 *in vitro* by immunofluorescence labeling indicates that aligned collagen fibers induce MSCs to commit to the tenogenic lineage.

Allogeneic Rabbit Mesenchymal Stem Cell (MSC) Isolation, Culture, Flow Sorting, and Seeding on ELAC Scaffolds

Bone marrow was harvested from the femurs of three, female New Zealand White (NZW) rabbits, aged 8-11 months. Adherent cells were cultured in standard monolayer conditions, provided fresh medium consisting of Dulbecco's modified eagle medium (catalog #11885, Gibco), 10% fetal bovine serum (catalog #12662029, Gibco), and 1% penicillin/streptomycin (10,000 U/mL, catalog #15140-122, Gibco) every 3 days, and passaged before reaching 70% confluence. Cells were expanded until passage 2, at which point they were detached using Accutase (Innovative Cell Technologies, Inc.) and flow sorted as detailed

previously to select for cells that were CD44+, CD45-, and CD90-.[27] Flow-sorted cells were cultured until passage 5. Scaffolds were then seeded once daily for 3 consecutive days ($5x10^5$ total cells day 1 and 2, $2.5x10^5$ total cells on day 3). The macroporous structure of the scaffold enabled facile cell seeding and suitable penetration of the MSCs. Nuclear and actin staining was performed on cells on one scaffold to confirm attachment and sufficient cell coverage and immunofluorescent staining for scleraxis was conducted on 2 additional scaffolds at the 4 day time point to assess early tenogenic differentiation of the seeded MSCs (Figure S1B).

Operative Procedures

All animal procedures were performed in accordance with established protocols pre-approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Thirty-four healthy adult female (aged, ~8-13 months; mean weight, 3.62 ± 0.43 kg) New Zealand white rabbits (Charles River Laboratories, Wilmington, MA) underwent acute surgical creation of a critically sized infraspinatus tendon defect in the right shoulder, measuring 6 mm, while the left shoulder served as an intact control. Rabbits were randomized to one of four groups: gap, direct repair, ELAC, and ELAC + MSC (Figure S2-A). In the gap group (n =6), the infraspinatus was detached sharply at its attachment on the proximal humerus and the resulting tendon-bone gap was left unrepaired. For direct repair (n = 8), the infraspinatus was detached, followed by direct reattachment via suturing the lateral aspect of the tendon using 3-0 Ethibond braided suture (Ethicon) in a Krackow pattern with three locking loops. The sutures were then passed through trans-osseous bone tunnels created from the enthesis to the bicipital groove laterally (Figure S2-B). The direct repair group served as an operative control and the operative standard of care, as it represents the current clinical standard of care for rotator cuff repair. For

the ELAC scaffold group (n = 10), a critically-sized infraspinatus tendon defect was created, followed by bridging of the defect using a woven ELAC scaffold. The scaffold was attached to the enthesis via trans-osseous bone tunnel fixation with 3-0 Ethibond suture in a Krackow pattern through the distal edge of the ELAC scaffold, ensuring three pairs of locking loops were incorporated at the tendon-scaffold interface. For ELAC + MSCs (n = 10), scaffolds were pre-seeded with P5 allogeneic marrow-derived CD44+/CD45-/CD90- MSCs and maintained in culture until surgery where scaffolds were fixed to the humerus in a similar fashion to the ELAC group. Prior to surgical closure, a small knot was placed at the distal end of the tendon (gap and direct repair) or the scaffold (ELAC and ELAC + MSCs) using 4-0 surgical stainless-steel suture (316L monofilament, Ethicon, Somerville, NJ, USA) to track tendon/scaffold retraction. Wounds were irrigated and closed using standard technique. Once awoken from anesthesia, rabbits were returned to their cages and allowed to ambulate without weight-bearing restrictions. Rabbits were euthanized 6 months following surgery. The time-point of 6 months was selected to provide adequate time for long-term resorption of the ELAC scaffold[24] and to allow a suitable comparison to the previous pilot study examining repair outcomes at 3 months.[27]

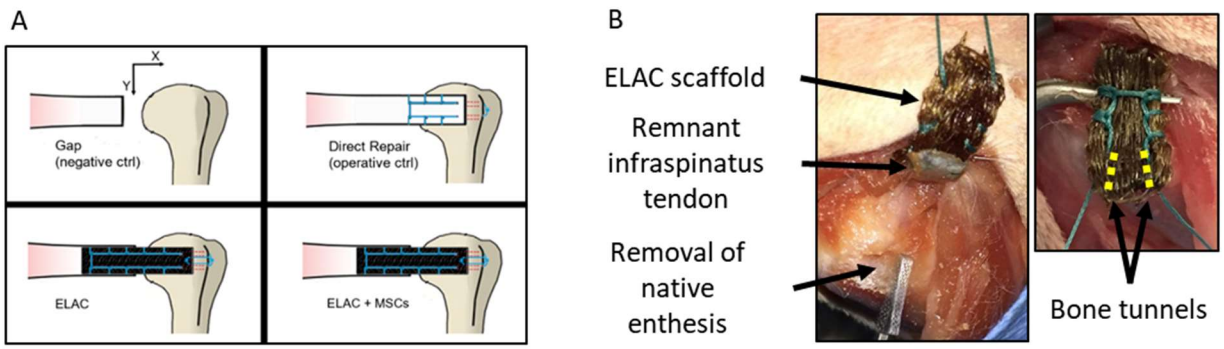


Figure A2. A) Schematic depiction of the four study groups: Gap (negative control), Direct Repair (operative control), ELAC, and ELAC + MSCs. B) Surgical implantation of the ELAC scaffold to bridge a 5 mm long rabbit infraspinatus tendon defect. Scaffold was sutured to the remnant tendon, the native enthesis was removed via deburring of the original footprint, and sutures were passed through transosseus bone tunnels and secured in the bicipital groove of the humerus.

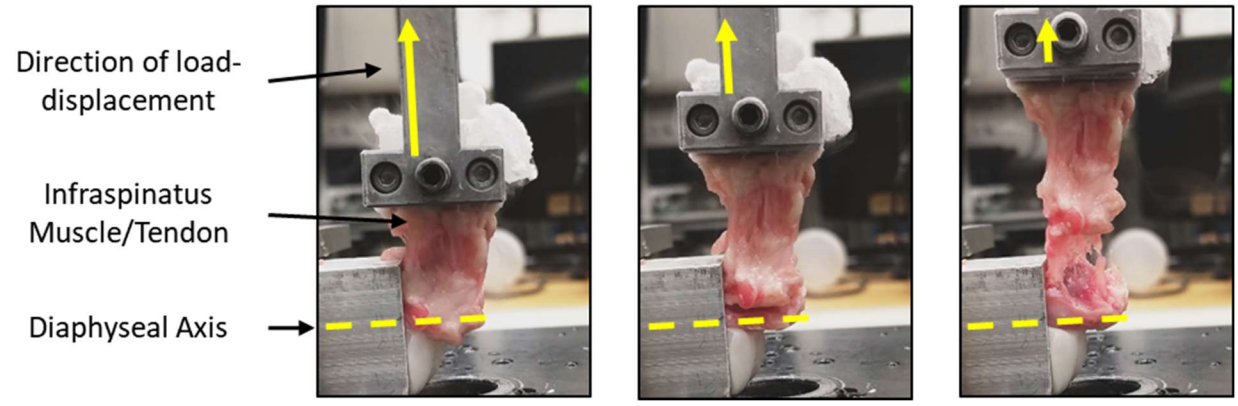


Figure A3. Mechanical testing setup and orientation for testing of the isolated infraspinatus tendon specimens. Infraspinatus tendon/muscle was clamped and the direction of the load-displacement in relation to the diaphyseal axis is depicted.

Histology

After fixation with neutral-buffered formalin (NBF), specimens for histological analyses were bisected on the long axis of the scaffold/tendon and sections primarily from the central regions of the repair site were collected for analysis. For histological scoring and point counting, a total of 6 slides from the central regions of the repair site were collected from each specimen (3 stained with hematoxylin and eosin and 3 stained with Masson's trichrome), giving a total of 12 sections scored in the gap and direct repair groups and 18 sections in the ELAC and ELAC+MSCs groups.

For assessments of collagen alignment, one section from all specimens in each group collected from the central regions of the repair was stained with picrosirius red. From each slide, a minimum of 6 high-power field images were collected under brightfield and cross-polarized lighting conditions to use for thresholding and calculation of the collagen orientation parameter in each case.

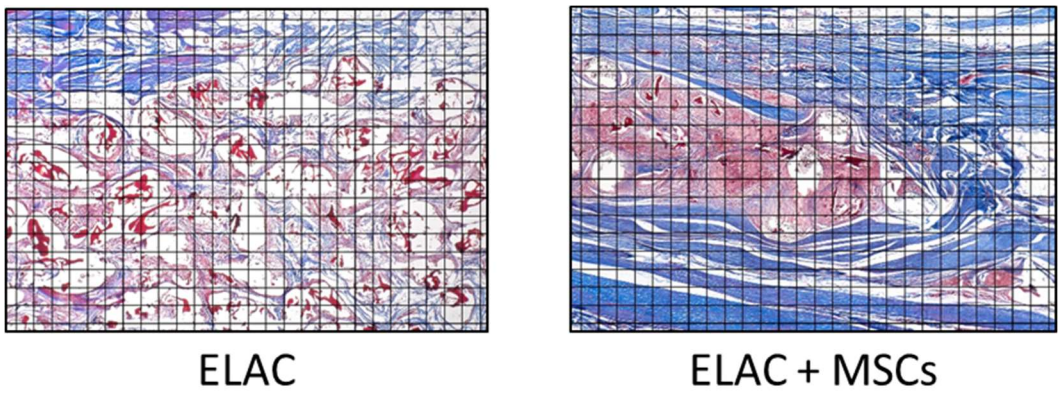


Figure A4. Examples of grids (200 x 200 µm) utilized for point counting assessment of ELAC and ELAC + MSC histological specimens.

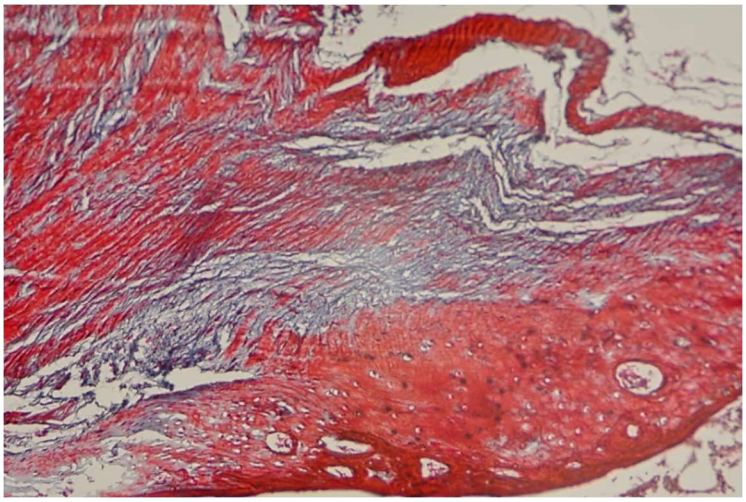


Figure A5. Representative histological section collected from the bone-tendon interface region of repaired shoulders. Lack of native enthesis structure (4 zones: bone-mineralized fibrocartilage-fibrocartilage-tendon) was noted in all histological specimens.

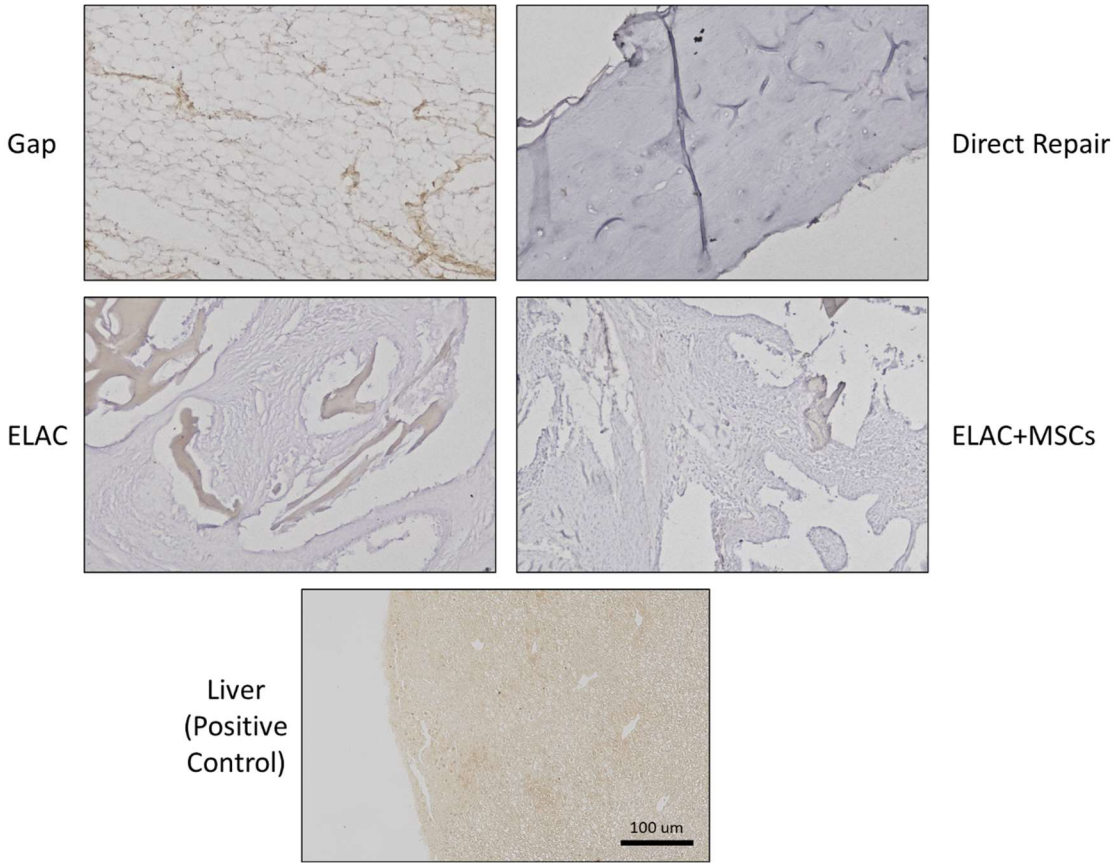
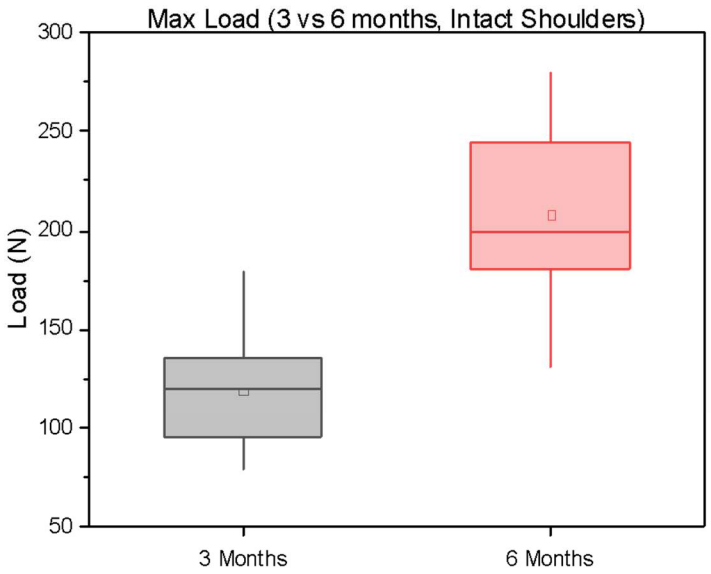


Figure A6. Representative images of immunohistochemical staining results for MMP-9 on Gap, Direct Repair, ELAC, and ELAC+MSCs. Minimal, if any, staining was noted in all groups except for Gap, which exhibited staining within the adipose tissue at the site of the defect. Positive control was liver tissue. Scale bar = 100 µm.

Table A1. P-values from Mann-Whitney comparisons of intact, contralateral shoulders at 3[27] and 6 months after surgery.

3 vs 6 months	Max Load	Mid 20% Stiffness
	0.000	0.000



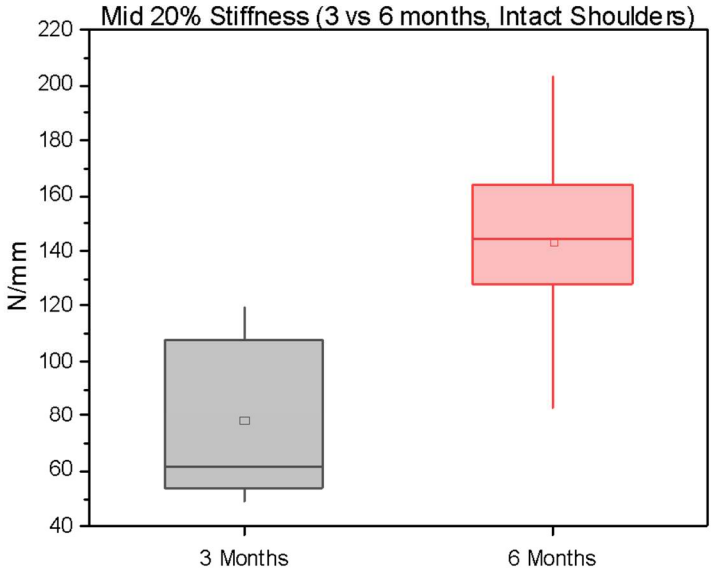


Table A2. P-values from Mann-Whitney comparisons of operative shoulders in each group at 3[27] and 6 months after surgery.

Max Load (op) (3 vs 6 months)	Direct Repair (3)	Direct Repair (6)	ELAC (3)	ELAC (6)	ELAC + MSCs (3)
Direct Repair (3)					
Direct Repair (6)	**0.014**				
ELAC (3)	0.312	0.337			
ELAC (6)	**0.018**	1.000	0.219		
ELAC + MSCs (3)	0.312	**0.014**	1.000	**0.073**	
ELAC + MSCs (6)	**0.030**	0.432	**0.073**	0.443	**0.018**

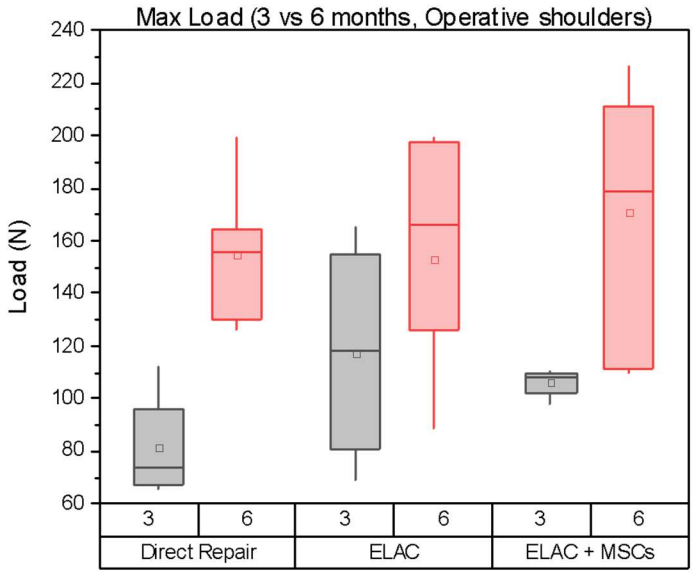


Table A3. P-values from Mann-Whitney comparisons of max load between operative and intact shoulders from each group at 6 months after surgery.

Max Load (Intact vs Operative)	Gap (in)	Gap (op)	Direct Repair (in)	Direct Repair (op)	ELAC (in)	ELAC (op)	ELAC + MSCs (in)
Gap (in)							
Gap (op)	**0.030**						
Direct Repair (in)	0.166	**0.014**					
Direct Repair (op)	**0.070**	**0.014**	**0.013**				
ELAC (in)	0.925	**0.011**	0.175	**0.038**			
ELAC (op)	0.156	**0.030**	**0.038**	1.000	**0.041**		
ELAC + MSCs (in)	0.637	**0.011**	0.175	0.074	0.307	0.125	
ELAC + MSCs (op)	0.299	**0.030**	0.054	0.432	0.097	0.443	0.523

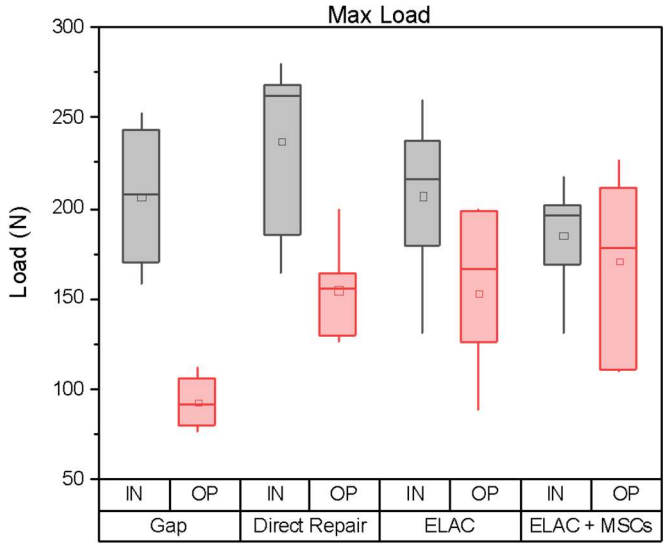


Table A4. P-values from Mann-Whitney comparisons of mid-20% stiffness of operative shoulders at 3[27] and 6 months after surgery.

Mid-20% stiffness (op) (3 vs 6 months)	Direct Repair (3)	Direct Repair (6)	ELAC (3)	ELAC (6)	ELAC + MSCs (3)
Direct Repair (3)					
Direct Repair (6)	**0.070**				
ELAC (3)	0.194	**0.014**			
ELAC (6)	0.219	0.617	**0.018**		
ELAC + MSCs (3)	0.665	**0.070**	0.194	0.108	
ELAC + MSCs (6)	**0.047**	0.432	**0.011**	0.160	**0.030**

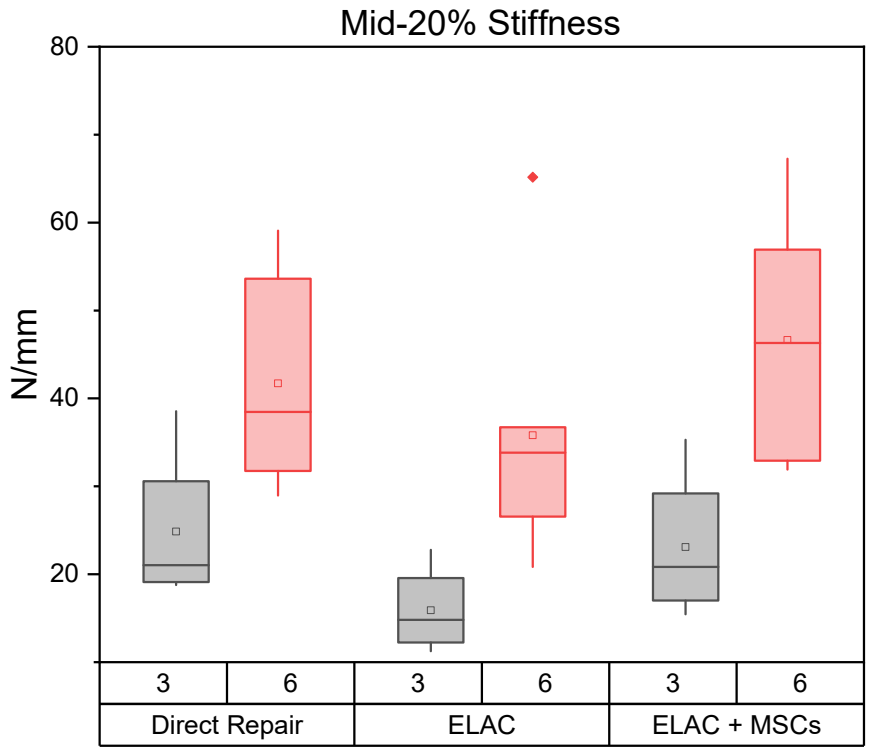


Table A5. P-values from Mann-Whitney comparisons of mid-20% stiffness between operative and intact shoulders from each group at 6 months after surgery.

Mid-20% stiffness (Intact vs Operative)	Gap (in)	Gap (op)	Direct Repair (in)	Direct Repair (op)	ELAC (in)	ELAC (op)	ELAC + MSCs (in)
Gap (in)							
Gap (op)	**0.030**						
Direct Repair (in)	0.166	**0.014**					
Direct Repair (op)	**0.014**	**0.070**	**0.005**				
ELAC (in)	0.156	**0.011**	0.432	**0.003**			
ELAC (op)	**0.011**	0.156	**0.003**	0.617	**0.002**		
ELAC + MSCs (in)	0.156	**0.011**	0.432	**0.003**	1.000	**0.002**	
ELAC + MSCs (op)	**0.011**	**0.030**	**0.003**	0.432	**0.002**	0.160	**0.002**

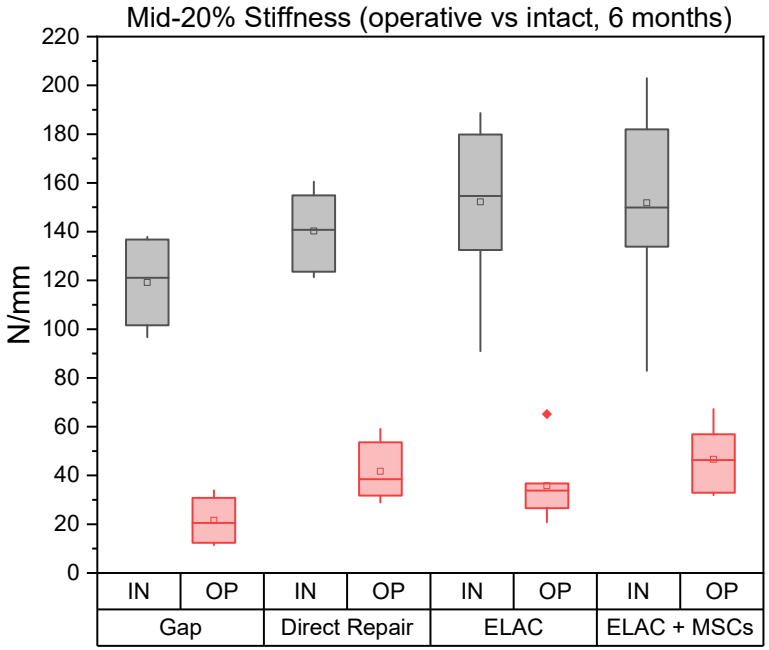


Table A6. P-values from Mann-Whitney comparisons of modulus between operative and intact shoulders from each group at 6 months after surgery.

Modulus	Gap (in)	Gap (op)	Direct Repair (in)	Direct Repair (op)	ELAC (in)	ELAC (op)	ELAC + MSCs (in)
Gap (in)							
Gap (op)	*						
Direct Repair (in)	*	*					
Direct Repair (op)	*	*	**0.030**				
ELAC (in)	*	*	0.665	**0.030**			
ELAC (op)	*	*	**0.030**	**0.030**	**0.030**		
ELAC + MSCs (in)	*	*	0.066	**0.020**	0.111	**0.020**	
ELAC + MSCs (op)	*	*	**0.020**	0.713	0.020	0.066	**0.012**

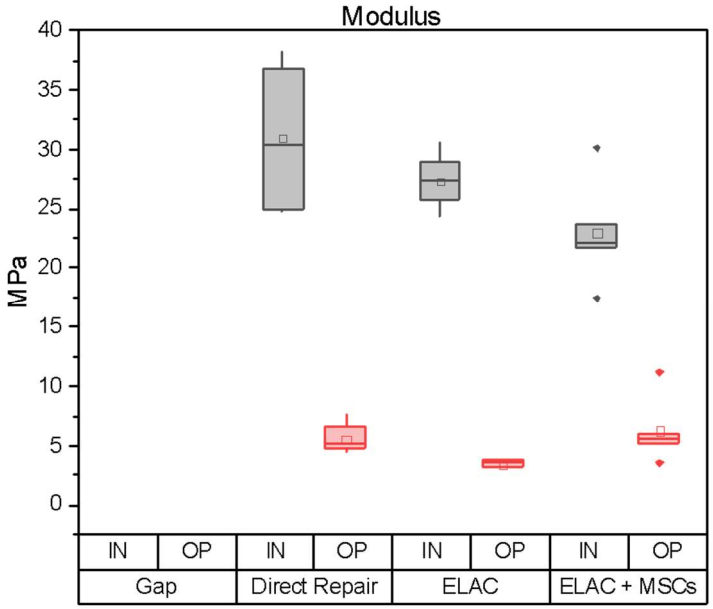


Table A7. P-values from Mann-Whitney comparisons of stress between operative and intact shoulders from each group at 6 months after surgery.

Stress	Gap (in)	Gap (op)	Direct Repair (in)	Direct Repair (op)	ELAC (in)	ELAC (op)	ELAC + MSCs (in)
Gap (in)							
Gap (op)	*						
Direct Repair (in)	*	*					
Direct Repair (op)	*	*	**0.030**				
ELAC (in)	*	*	0.194	**0.030**			
ELAC (op)	*	*	**0.030**	0.061	**0.030**		
ELAC + MSCs (in)	*	*	1.000	**0.020**	0.391	**0.020**	
ELAC + MSCs (op)	*	*	**0.020**	0.713	**0.020**	0.066	**0.012**