Time-series biological responses toward decellularized bovine tendon graft and autograft for 52 consecutive weeks after rat anterior cruciate ligament reconstruction

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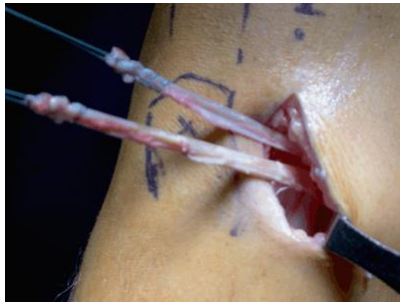
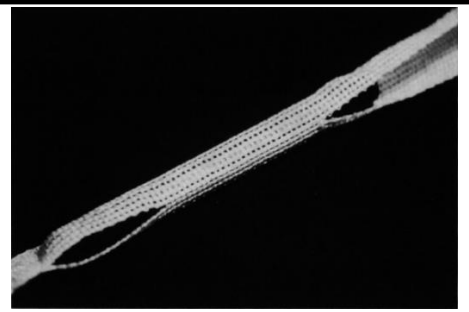
- Conflict of interests

  The authors declare no competing interests.

Background: Issues in Grafts for ACL Reconstruction

	Autograft: First choice	Artificial ligament: Almost withdrawn from the market[1]	Allograft： Mainly U.S.
	 2)	 3)	 4)
Invasiveness	Taken from healthy legs	No harvesting required	No harvesting required
Supply	✓ Limited supply ✓ Difficult to predict size[5]	Stable supply available	Poor availability
Outcomes	Rupture rate: 5-10%[4]	Rupture rate: 47% (Leeds-Keio@5yrs.)[6]	Rupture rate: 29.0%[7]

The ideal graft

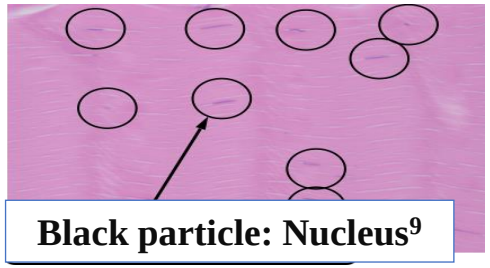
1. **No volume limits**
2. **No sacrifice of autologous tissue**
3. **Biocompatibility**
4. **Initial strength and durability**

Tissue structures are difficult to create, so **biological tissues** are preferable

Utilization of xenograft via decellularization

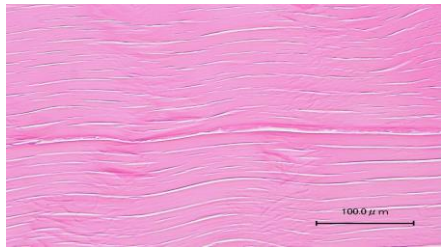
Tissue "decellularization" technology

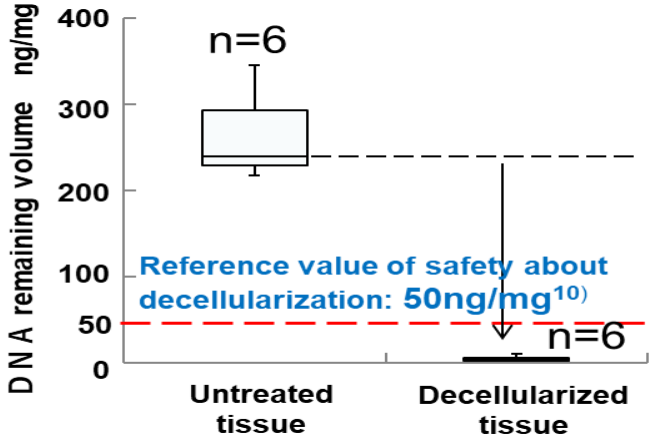
Untreated



Black particle: Nucleus[9]

Decellularization





1st Step

1. Microwave Irradiation
2. Circulate deoxycholic acid under pulsatile flow and pulsatile pressure condition

2nd Step Endonuclease

Rupture stress (Mpa)	
Human Quadr-Fold Ham (-20℃ storage)[11]	30.8
Decellularized Bovine Tendon	73.5

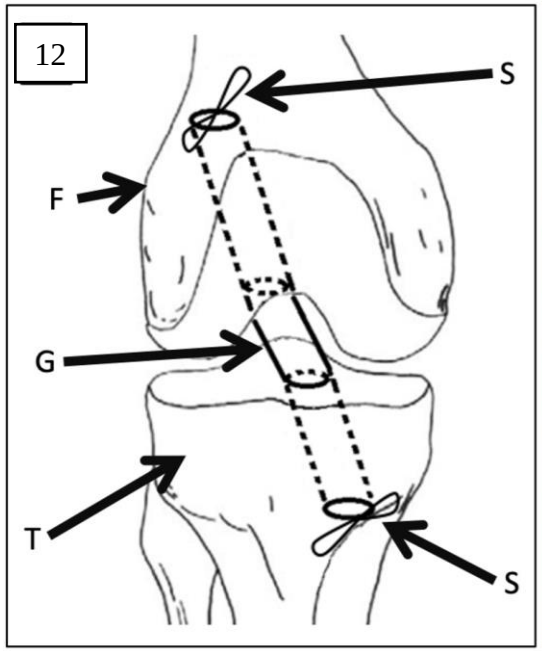
Decellularization maintaining the strength and flexibility of thick tissue

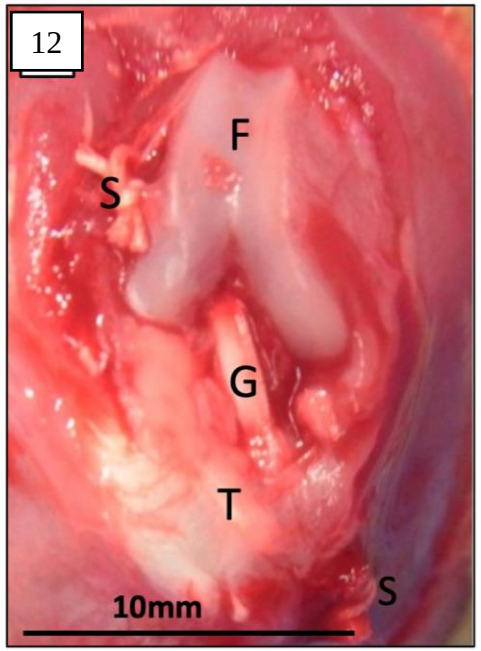
Aim

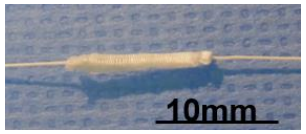
Evaluation of the time-series biological responses of bovine decellularized tendons in the rat ACL-R model

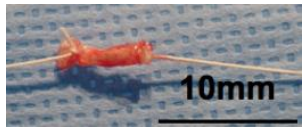
Methods

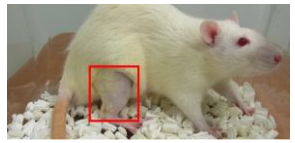
Rat ACL-R (N = **90**)











Group-D (N = **42**; **Bovine decellularized** tendon xenograft)

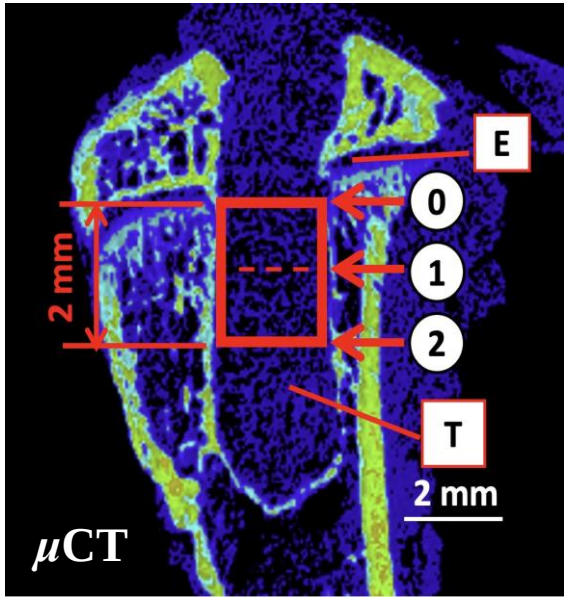
Group-A (N = **42**; Digitorum longus tendon **autograft**)

Group-N (N = **6**; **Control**. Native ACL)

F femur, T tibia, G graft, S 3-0 silk thread

Assessment BMD @graft–tibial tunnel interface

*** Inhibited BMD reduction → Enhanced bone-tendon healing[13]**



Setting tunnel diameter at 3 sites within 2 mm of growth plate @0 day

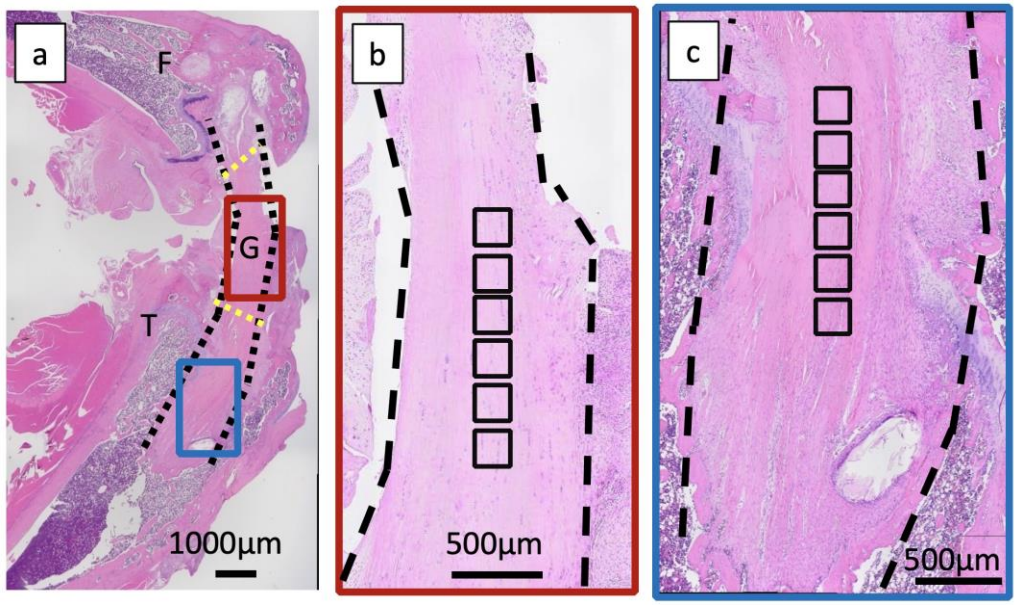
CT computed tomogreiphy, E epiphysis, T tunnel

Tibial-tunnel diameter @0-day(N=6)

Location	Major axis mm	Minor axis mm
①	**1.83**	**1.81**
②	**1.80**	**1.80**
③	**1.82**	**1.80**

Interpolate three locations to determine the measurement area

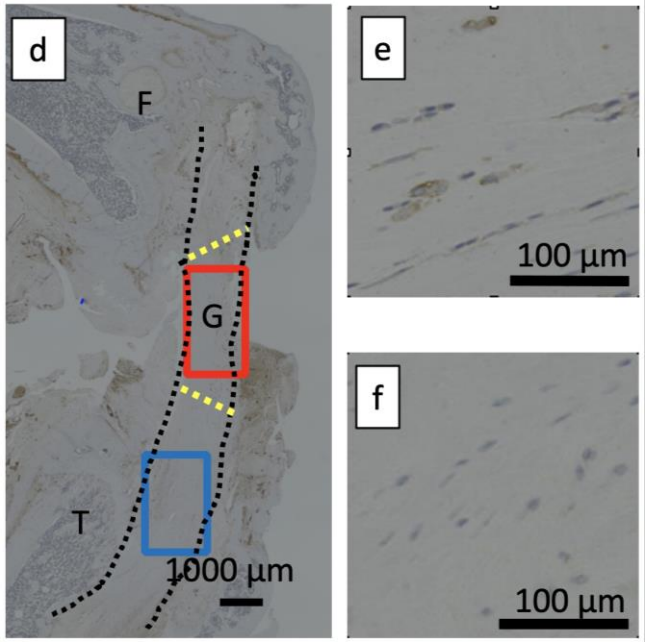
Methods



Assessment the cellularity of the grafts (HE stain)

- ✓ Cellularity was assessed in the intra-articular graft and the graft in the tibial tunnel.
- ✓ Cell counting was performed in six 200-μm2 square regions. Then, the average number of cells in the six squares was used.

a: Overall view of the femur–graft–tibia complex
b: Magnified view of the intra-articular graft
c: Magnified view of the graft in the tibial tunnel



Immunohistological staining of M1&M2 macrophages.

- ✓ **M1: Proinflammatory** activities[14]
- ✓ **M2: Tissue repair** properties[15]
- ✓ Counted in the same manner as above

d: Overall view of the femur–graft–tibia complex
e: M1 macrophages
f: M2 macrophages

F femur, G graft, T tibia

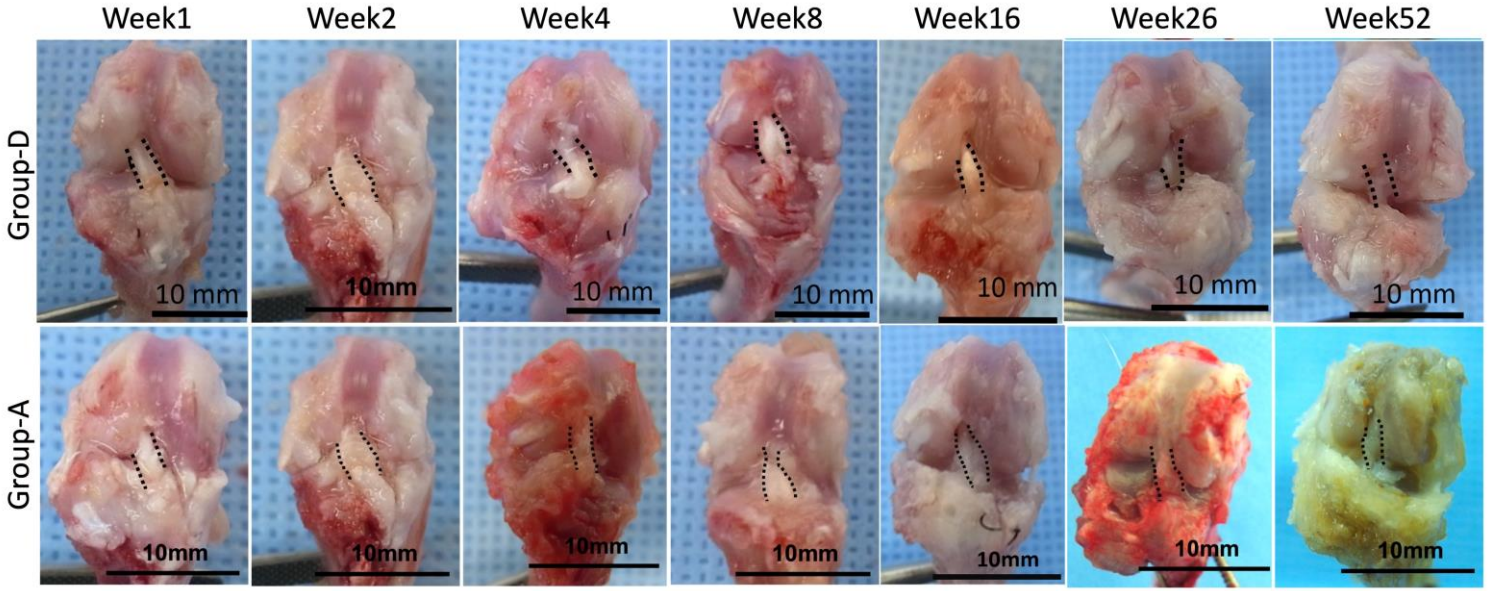
Evaluation timepoint

- ✓ Group-D&A; Evaluated @**1, 2, 4, 8, 16, 26, 52w** (7-timepoints; each n = 6)
- ✓ Group-N; Evaluated @0d

Statistics

- ✓ Student's t-test or Mann-Whitney U test
- ✓ Kruskal–Wallis test or one-way analysis of variance
- ✓ $P < 0.05$: Significant

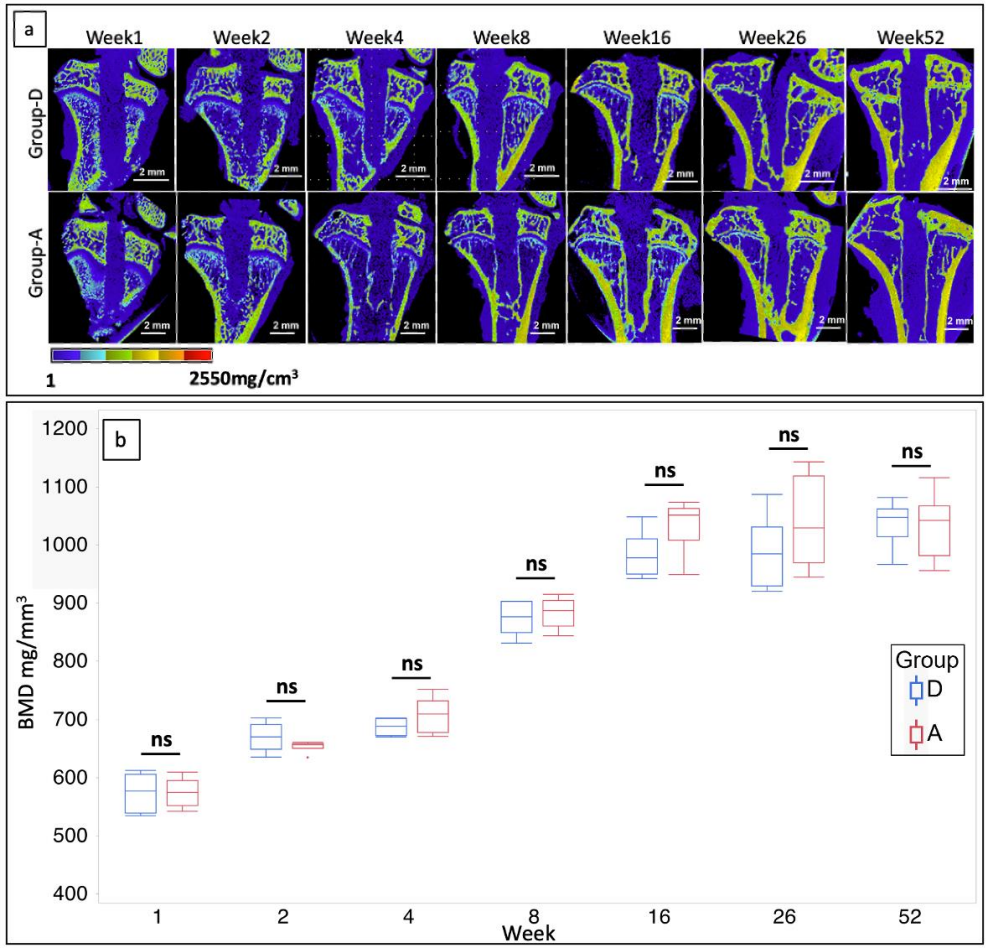
Results



The gross appearance of the right knee at each time point in representative cases

- ✓ The decellularized xenograft tissues were retained for 52 weeks in the knee joint in a manner similar to that of the autografts.
- ✓ The black dashed line shows the outline of the graft.

Group D, decellularized bovine tendon graft; group A, autologous tendon graft.



BMD at the interface between graft and peritibial tunnel

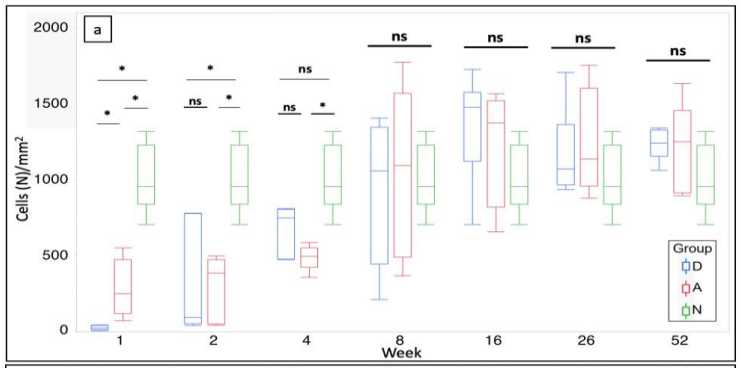
- ✓ No significant enlargement of the tibial tunnel and no difference of BMD between the groups over time.

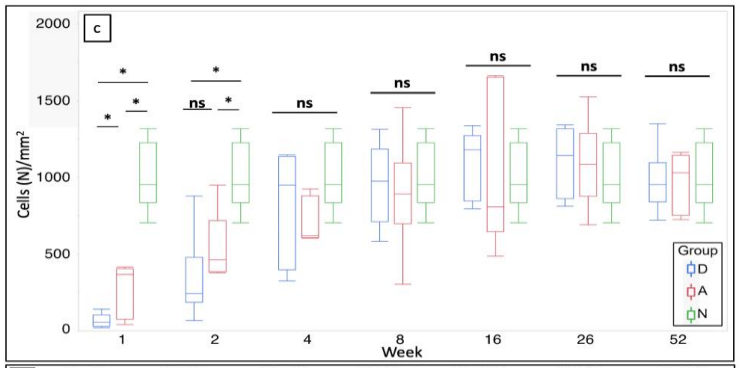
a: Micro-CT images of slices parallel to the tibial tunnel at each time point in each group.

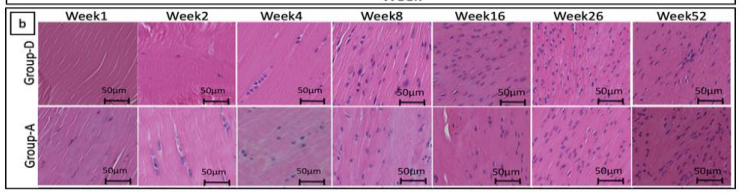
b: Box plots showing the BMD of the peritibial tunnel for each group at each time point. (each, n = 6)

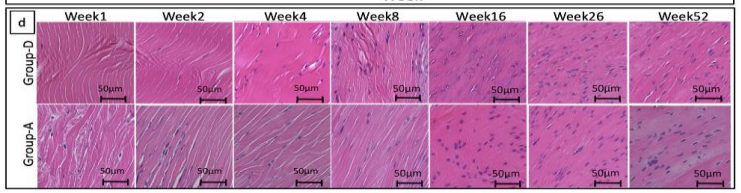
Results

*: $p < 0.05$





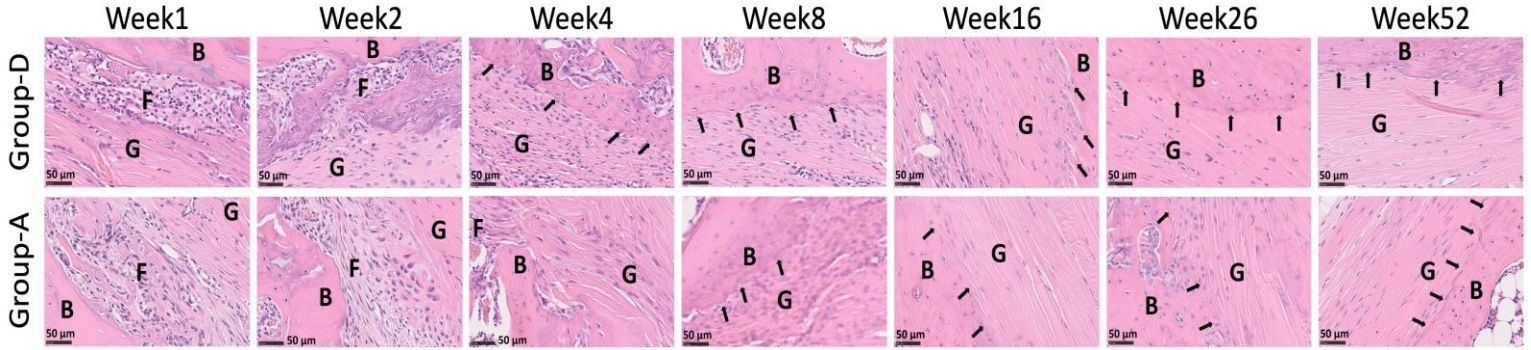




Cellularity of the decellularized bovine tendon and autologous tendon

- ✓ **Intra-articular** decellularized grafts were cellularized comparable to or faster than autologous tendons.
- ✓ **Intratibial tunnel** decellularized grafts were cellularized at a rate comparable to autografts.

a: Cell counts in the intra-articular grafts of both groups at each period.
b: Representative HE-staining showing cell infiltration in the intra-articular graft at each period.
c: Cell counts in the intratibial tunnel grafts of both groups at each period.
d: Representative HE staining showing cell infiltration in the intratibial tunnel graft at each period.



1w
F (fibrous tissue) width : D<A

2w
Decrease in F width : D>A

4w
Sharpey-like fibers appeared in group D

8w
Sharpey-like fibers appeared in group A

16w~
The graft-tibial tunnel interface was well integrated and maintained in both groups

The graft–tibial tunnel interface healing in both groups at each period

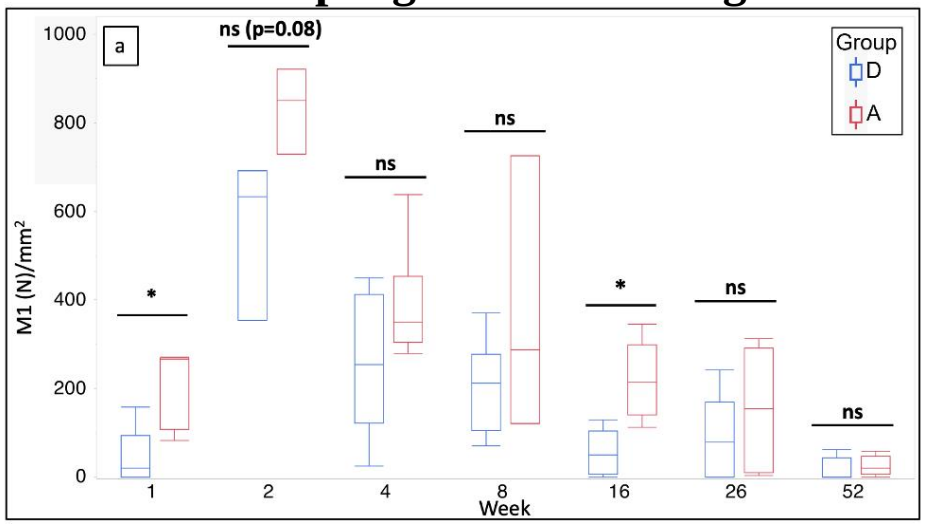
- ✓ **Sharpey-like fibers** appeared earlier in group D (4w) than in group A (8w).
- ✓ Decellularized grafts suggested faster "**tendon-bone healing**" than autografts.

The black arrow indicates a Sharpey-like fiber. B bone, T tendon, F fibrous tissue.

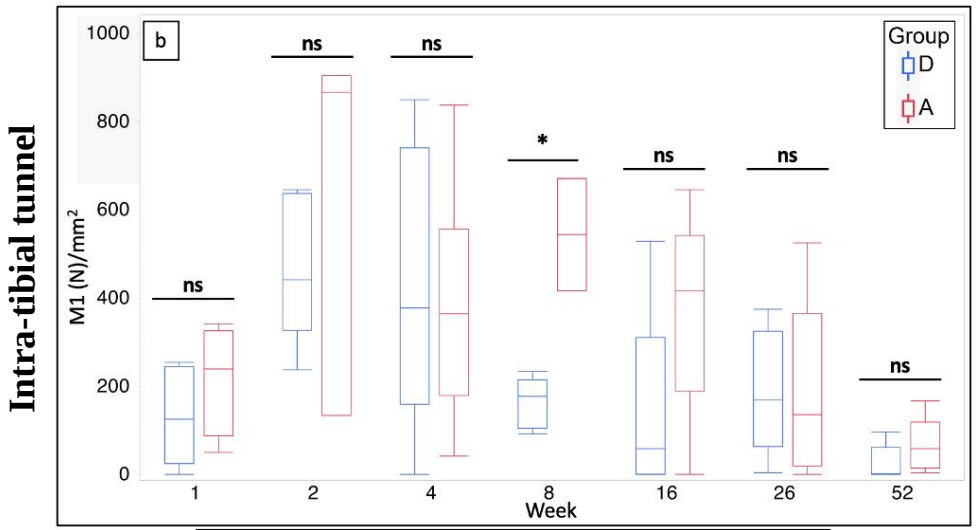
Comparison of the count of M1 and M2 macrophages that appeared in the decellularized and autologous tendons

- ✓ **M1:Proinflammatory** activities[14]
- ✓ **M2: Tissue repair** properties[15]
- ✓ *: $p < 0.05$

M1 macrophage counts in the grafts

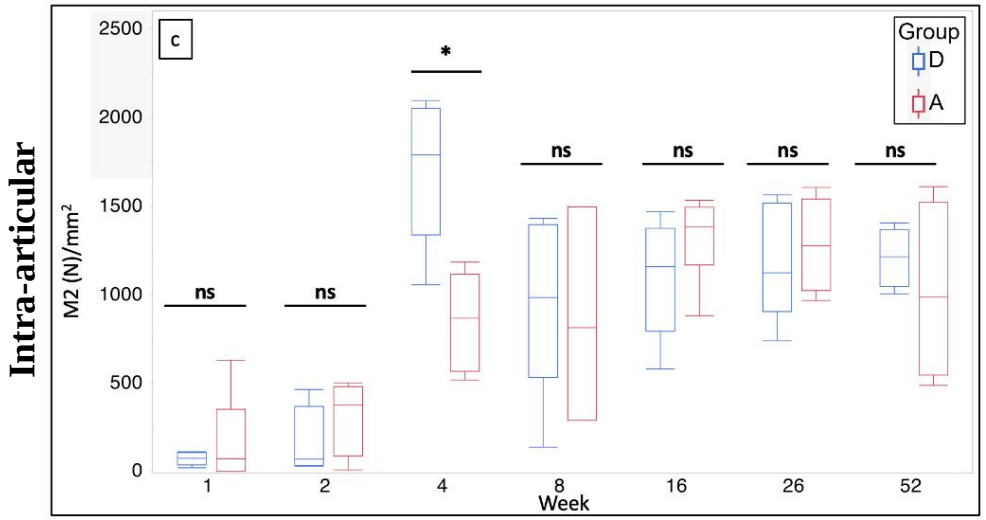


M1 counts of intra-articular graft
- ✓ D<A @1w & 16w
- ✓ Otherwise, comparable

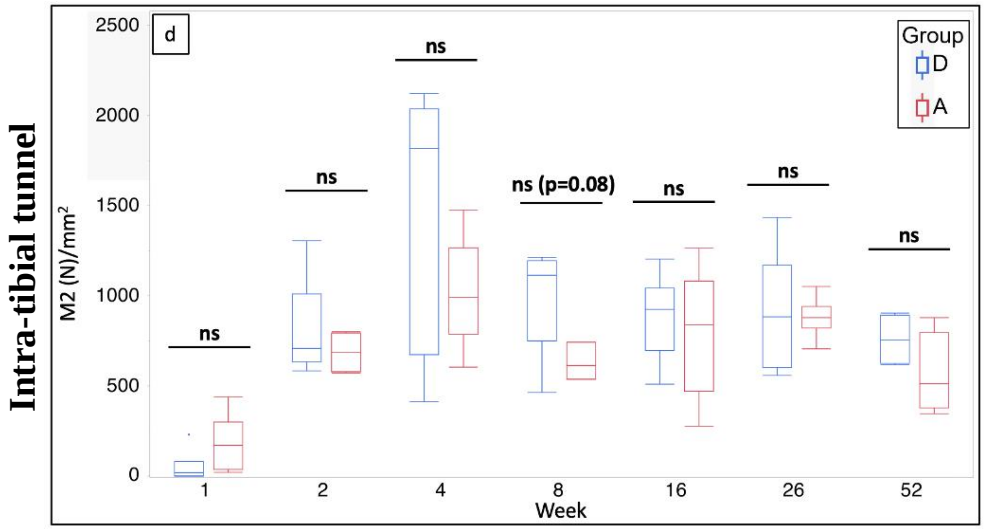


M1 counts of intra-tibial tunnel graft
- ✓ D<A @8w
- ✓ Otherwise, comparable

M2 macrophage counts in the grafts



M2 counts of intra-articular graft
- ✓ D>A @4w
- ✓ Otherwise, comparable



M2 counts of intra-tibial tunnel graft
- ✓ D>A @8w (Numerically)
- ✓ Otherwise, comparable

Discussion

The most important findings

- ✓ **Decellularized xenografts** were **repopulated** with recipient rat cells as were autografts.
- ✓ The **decellularized xenografts** induced an **M2**-dominant host response and earlier cell infiltration compared with the autografts.
- ✓ In contrast, the **autografts** resulted in **M1**-type inflammation and cell infiltration was slower than decellularized xenografts.

- ✓ **Cell infiltration speed into intra-articular grafts; D≧A**
- ✓ **Cell infiltration speed into intra-tibial tunnel grafts; D=A**

Number of cells in the grafts

Group	Intra-articular	Intra-tunnel
D	No significant difference with native @4w	Plateau @4w
A	Plateau @8w	Plateau @4w

Reasons for faster cell infiltration into intra-tunnel grafts

↓

Bone marrow cells infiltrate directly into the intra-bone tunnel graft

Ovine ACLR model (autograft)[16]

Decreased number of cells in autografts @2w

→Cell necrosis within autograft in early stage

→ Subsequently, cell infiltration

Period*16			
0 Weeks	2 Weeks	6 Weeks	12 Weeks
1187 (212)	25 (18)^{a}	557 (220)^{a}	762 (263)^{a,b}

The current study

The reason the number of cells @1w in group A was lower than that of the native autologous cells was thought to be in the process of cell necrosis or after necrosis.

(Rats have faster timelines than sheep)[17]

M1 (Inflammation): Tendency for D<A
M2 (Tissue repair): Tendency for D>A

Number of M1 and M2 macrophages in the grafts		
	M1(Inflammation)	M2(Tissue repair)
Intra-articular	D<A @1, 16W	D>A @4w
Intra-tibial tunnel	D<A @8w	D≒A @entire period

Consistent with Brown's "Tissues containing cellular components, even autologous, exhibit an M1-dominant inflammatory response."

It was suggested that decellularized tendons were as biocompatible or more biocompatible than autologous tendons.

Limitations

1. The **biomechanical properties** of explanted decellularized and autologous tendons were **not tested**.
2. Whether the observation period of 52 weeks in rats is sufficient to evaluate in vivo remodeling processes thoroughly is unclear.

Conclusions

1. Bovine tendon-derived decellularized grafts have excellent time-series recellularization and tendon–bone integration abilities compared with autografts in the rat ACLR model for up to 52 weeks.
2. Sharpey-like fibers appeared earlier in group D than in group A.
3. The number of M2 macrophages responsible for tissue repair tended to be higher in the decellularized xenograft tendon at 4–8 weeks after ACLR than in the autografts.

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