Adenosine, lidocaine, and magnesium therapy augments joint tissue healing following experimental anterior cruciate ligament rupture and reconstruction

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Aims

Adenosine, lidocaine, and Mg^{2+} (ALM) therapy exerts differential immuno-inflammatory responses in males and females early after anterior cruciate ligament (ACL) reconstruction (ACLR). Our aim was to investigate sex-specific effects of ALM therapy on joint tissue repair and recovery 28 days after surgery.

Methods

Male (n = 21) and female (n = 21) adult Sprague-Dawley rats were randomly divided into ALM or Saline control treatment groups. Three days after ACL rupture, animals underwent ACLR. An ALM or saline intravenous infusion was commenced prior to skin incision, and continued for one hour. An intra-articular bolus of ALM or saline was also administered prior to skin closure. Animals were monitored to 28 days, and joint function, pain, inflammatory markers, histopathology, and tissue repair markers were assessed.

Results

Despite comparable knee function, ALM-treated males had reduced systemic inflammation, synovial fluid angiogenic and pro-inflammatory mediators, synovitis, and fat pad fibrotic changes, compared to controls. Within the ACL graft, ALM-treated males had increased expression of tissue repair markers, decreased inflammation, increased collagen organization, and improved graft-bone healing. In contrast to males, females had no evidence of persistent systemic inflammation. Compared to controls, ALM-treated females had improved knee extension, gait biomechanics, and elevated synovial macrophage inflammatory protein-1 alpha (MIP-1α). Within the ACL graft, ALM-treated females had decreased inflammation, increased collagen organization, and improved graft-bone healing. In articular cartilage of ALM-treated animals, matrix metalloproteinase (MMP)-13 expression was blunted in males, while in females repair markers were increased.

Conclusion

At 28 days, ALM therapy reduces inflammation, augments tissue repair patterns, and improves joint function in a sex-specific manner. The study supports transition to human safety trials.

Article focus

- New strategies are required to minimize surgery-induced inflammation in a sex-specific manner and improve outcomes in males and females after anterior cruciate ligament reconstruction (ACLR).
- In this study, we investigated sex-specific effects of adenosine, lidocaine, and magnesium (ALM) therapy on joint tissue repair and functional recovery in a rat model of ACL rupture and repair.

Bone & Joint Research

Key messages

- Males and females have contrasting inflammatory and tissue reparative processes after ACLR surgery.
- Perioperative ALM therapy accelerates tissue healing within the ACL graft and adjacent tissues of males and females after ACLR surgery.

Strengths and limitations

- ALM therapy is a promising strategy to regulate surgery-induced inflammation and improve patient outcomes in both sexes undergoing orthopaedic procedures.
- Follow-up beyond 28 days, and biomechanical strength testing of ACL grafts is required to confirm accelerated recovery of joint function in ALM-treated animals.

Introduction

Anterior cruciate ligament reconstruction (ACLR) is one of the most common orthopaedic surgeries performed. Despite advances in surgical techniques, rehabilitation, and prevention practices, secondary complications such as pain and limitations in knee function remain a concern.[1-6] Early dysregulation of inflammatory and immune responses initiated by ACL rupture, and compounded by surgical intervention, impede normal tissue repair processes and drive the pathogenesis of fibrotic and degenerative postoperative complications.[2,4,7-10] Current therapies are limited, and tend to address the symptoms as they occur, not the underlying 'upfront' causes of inflammation which begin immediately at the first incision,[11] and drive the altered tissue healing responses that delay functional recovery and impact quality of life after ACLR.

An emerging area of clinical significance is the role of sex in ACL injury and tissue repair responses following ACLR.[4,12] A number of preclinical studies have shown that males and females differ in the timing, and molecular and cellular composition, of the inflammatory and tissue repair responses to injury and surgery.[13-19] In a rat model of ACL rupture with no surgical repair, Morris et al[15] reported that tissue repair processes in the ACL remnant appeared slower in males than females. Similarly, males and females exhibit differential inflammatory responses to ACLR surgery in the first postoperative week.[14] Although the magnitude of systemic inflammatory cell activation and plasma inflammatory cytokine levels is greater, and occurs earlier (< 24 hrs) in females than males after experimental ACLR surgery, inflammation resolves earlier in females.[14] Interestingly, perioperative adenosine, lidocaine, and Mg^{2+} (ALM) therapy was shown to dampen early systemic and local inflammation in both sexes following ACL rupture and surgical repair.[14] ALM therapy was chosen because of its ability to blunt inflammation and reduce immune dysfunction in experimental models of total knee arthroplasty,[20,21] hemorrhagic shock, traumatic brain injury, and sepsis.[22] The aim of the present study was to investigate the effects of perioperative ALM therapy on knee function, inflammation, and joint tissue repair in males and females at 28 days postoperatively. We hypothesized that by dampening early inflammation, perioperative ALM therapy would improve joint tissue healing and functional recovery following ACLR surgery, and that tissue repair profiles may be more advanced in females than males.

Methods

Animals and study design

Conventional 16-week-old male (n = 21; 431 g (standard deviation (SD) 37)) and female (n = 21; 246 g (SD 12)) Sprague-Dawley rats were randomly divided into ALM (male, n = 11; female, n = 11) or Saline control (male, n = 10; female, n = 10) treatment groups. Animals were acclimated for at least seven days prior to experimentation and housed in individually ventilated cages (Tecniplast Australia, Australia) in a 14- to ten-hour dark-light cycle under controlled temperature (21°C to 22°C) and humidity (65% to 75%) conditions, with access to standard rodent pellets (Speciality Feeds, Australia) and water ad libitum. An ARRIVE checklist is included in the Supplementary Material to show that the ARRIVE guidelines were adhered to in this study.

Treatment

The ALM treatment group received a 0.5 ml/kg/h intravenous (IV) infusion for one hour via the left femoral vein, commencing immediately prior to skin incision (adenosine 18.7 mM, lidocaine 34.6 mM, $MgSO_4$ 41.5 mM in 0.9% NaCl).[14] Immediately following capsule closure, and prior to skin closure, animals also received an intra-articular (IA) bolus of ALM (0.1 ml; 1 mM adenosine, 3 mM lidocaine, and 2.5 mM $MgSO_4$ in 0.9% NaCl).[14] Saline control animals received a 0.9% NaCl IV drip, with an IA bolus of 0.9% NaCl.

Non-invasive ACL rupture and ACL reconstruction

Non-invasive ACL rupture of the right hind limb was performed on anaesthetized animals, as described previously.[15] Three days after ACL rupture, animals underwent remnant-sparing ACLR surgery on the right knee using a tail tendon autograft, with postoperative assessment over 28 days (Figure 1).[14,15] All surgeries were performed by a single surgeon (PCM) on eight to ten animals per surgery day, with equal numbers of animals per treatment group, blinding of the surgical team and data analyst (JLM) to treatment assignment, and randomization of the order of animals undergoing surgery. Intraoperative blood loss from vascular and ACLR surgery was measured as previously described.[20] Immediately after catheter removal, and prior to recovery from anaesthesia, animals received Carprieve (5 mg/kg, subcutaneous) with analgesic administered 24-hourly thereafter, according to pain indicators for individual animals.

Joint swelling

Joint swelling was assessed one day prior to surgery (day -1, 2 days after ACL rupture), and 1, 3, 5, 8, 14, and 28 days after ACLR surgery using methods described previously.[14] Data show the difference in size between the operated (right) and non-operated (left) knee.

Knee extension angle

Knee extension angles were measured at day -1, and 28 post-surgery (Saline, n = 10 per sex; ALM, n = 11 per sex), as described previously.[21] Data show the difference in extension angle between the operated and non-operated knees.

Pain

For pain assessment, the paw withdrawal threshold (PWT) was evaluated in the operated and non-operated knee (Saline, n

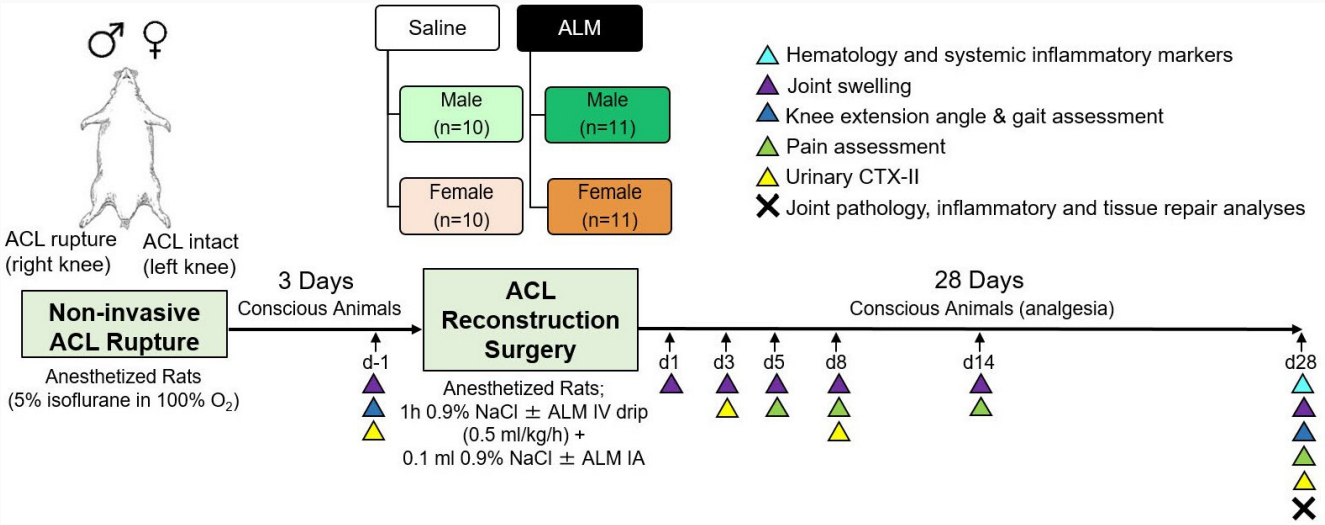

Fig. 1
Study protocol schematic. ACL, anterior cruciate ligament; ALM, adenosine, lidocaine, and magnesium therapy; CTX-II, C-terminal cross-linked telopeptide of type II collagen; d, days; IA, intra-articular; IV, intravenous.

= 10 per sex; ALM, n = 11 per sex) at day 5, 8, 14, and 28 postoperatively, using an up-down protocol for von Frey filaments, as previously described.[15] Data show the PWT for the operated and non-operated limb after ACLR surgery.

Gait analysis

To assess recovery of normal gait function after ACLR, temporal-spatial gait variables were assessed at day -1 and day 28 postoperatively. Gait was assessed using a custom catwalk system (Supplementary Figure a) within a darkened room, with each animal permitted to walk freely down the catwalk, and a minimum of two separate walks video-recorded (1,080 p, 60 fps) for each animal, at each timepoint. Borderline strides (first and last steps within the catwalk) were excluded to avoid acceleration and deceleration bias, and only walking trials that included a minimum of six core strides, without hesitation, were used for analysis. Gait parameters were analyzed using MTrackJ/ImageJ (Erasmus University Medical Centre, Netherlands)[23] and included: stride length (distance between two paw strikes on the operated limb), stride length variability (coefficient of variation (CV) of stride length), stride time variability (CV of stride time), stance width, step length (distance between two paw strikes of operated (right) and non-operated (left) hind limbs), paw angle (outward deviation of the paw from the midline), and track speed (time taken to traverse the catwalk with exclusion of first and last stride) (Supplementary Figure a).

Urinary CTX-II

C-terminal cross-linked telopeptide of type II collagen (CTX-II), a marker of cartilage degradation, was measured in urine collected at day -1, 3, 8, and 28 following surgery (Rat CTX-II ELISA kit, E-EL-R2554; Elabscience, USA), and normalized to urinary creatinine (Creatinine Urinary Detection Kit, EIACUN; Thermo Fisher Scientific, USA), according to manufacturer's instructions.

Systemic and local inflammation

At day 28 postoperatively, complete blood cell counts were performed (VetScan HM5 hematology analyzer; Abaxis, USA), and inflammatory cytokines and chemokines were measured in plasma and synovial wash (interleukin (IL)-1β, IL-6, interferon-inducible protein (IP)-10, lipopolysaccharide-inducible CXC chemokine (LIX), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein-1 α (MIP-1α), MIP-2, regulated upon activation, normal T cell expressed and presumably secreted (RANTES), tumour necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF)) using custom Milliplex Rat Cytokine/Chemokine Magnetic Bead Panels (Abacus ALS, Australia), as described previously.[15] Baseline ranges for haematology parameters (n = 10 per sex), and plasma and synovial wash inflammatory mediators (n = 8 per sex), were determined in healthy male and female animals.

Joint tissue assessments

Animals were euthanized at day 28 to assess joint pathology and tissue repair responses. Macroscopic scoring of the intact joint, the articular surfaces, and capsular and synovial tissue was performed using a modified grading system described previously.[20] Synovial lavage was performed on operated and non-operated knees.[15] Quadriceps atrophy was assessed by comparing wet weights of quadriceps muscles from operated and non-operated hind limbs. Samples of the ACL graft and articular cartilage of the medial femoral condyle (MFC) were collected for gene expression studies (Saline, n = 6 per sex; ALM, n = 7 per sex). Remaining joints were fixed in 4% paraformaldehyde (PFA) for 48 hours, decalcified with 14% ethylenediaminetetraacetic acid (EDTA), processed, paraffin-embedded, and sectioned (4 μm) in the coronal plane.

Semi-quantitative evaluation of synovitis, joint fibrosis, ACL graft remodelling and bone-tendon healing, and articular cartilage integrity was performed on two to four sections per knee, approximately 80 μm apart, by a blinded investigator (HLL). Haematoxylin and eosin (H&E) staining was used to assess synovitis, fibrotic changes, and cell infiltration within

the infrapatellar fat pad (IFP), using previously described scoring criteria.[20] The IFP was selected as the region of interest since it shows the largest fibrotic response after knee injury/surgery.[20] A five parameter, 0-to-3 scale scoring system was used to assess ACL graft inflammation,[24] remodelling, and degenerative changes within the graft midsubstance in H&E and Masson-Goldner trichrome (MGT)-stained sections. Assessment of bone-tendon healing within the femoral and tibial bone tunnels was performed on safranin O/fast green (SafO)-stained sections using a modified five parameter, 0-to-3 scale scoring system, where a score of 15 reflects the highest degree of graft osseointegration.[24] Since cartilage degenerative changes occur more frequently in the medial compartment after ACLR, cartilage degradation within the MFC, and medial tibial plateau (MTP), was assessed in SafO-stained sections according to the Osteoarthritis Research Society International (OARSI) scoring scale.[21,25] Average MFC and MTP scores were combined to provide a summed score joint value, with a maximum possible score of 10. Immunohistochemical localization of the macrophage marker, CD68 (ED1, 1:125 dilution), and α-SMA (1A4, 1:1,000 dilution) was performed using methods described previously.[14,15,20]

RNA isolation and quantitative RT-PCR

Total RNA was isolated from ACL graft and MFC cartilage, and complementary DNA (cDNA) was prepared by reverse transcription.[20] Real-time polymerase chain reaction (PCR) with custom-designed primers was used to assess gene expression of key markers of inflammation (nuclear factor kappa B subunit 1 (Nfkb1)), macrophage polarization (M1: nitric oxide synthase 2 (Nos2), M2: arginase 1 (Arg1)), extracellular matrix (ECM) constituents (collagen type 1 α 1 chain (Col1a1), collagen type II α 1 chain (Col2a1), collagen type III α 1 chain (Col3a1), fibronectin 1 (Fn1), elastin (Eln), aggrecan (Acan), cellular communication network factor 2 (Ccn2)), ECM remodelling enzymes (matrix metallopeptidase 9 (MMP9) and 13 (MMP13), tissue inhibitor of matrix metalloproteinase 1 (Timp1)), and stem cell/fibroblast activation and differentiation (transforming growth factor β 1 (Tgfb1), and actin α 2 (Acta2)) (Supplementary Table i). Expression of each gene was normalized using the average expression of the housekeeping hypoxanthine-guanine phosphoribosyl transferase (Hprt1) gene for each sample.[15] Data show relative expression in operated compared to non-operated knees of each animal.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 10.0.3; GraphPad, USA). Normality assumptions and equality of variances were assessed using Shapiro–Wilk and Levene's tests, respectively. Mixed analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test was used for between- and within-groups comparison, or mixed ANOVA with Tukey's HSD test for assessment of longitudinal data. Between-group differences for non-parametric data were assessed using Kruskal–Wallis with Dunn's test. Inflammatory mediator concentrations were analyzed using MILLIPLEX Analyst 5.1 software (Luminex Corporation, USA). Results are expressed as mean and standard error (SEM) unless otherwise stated, with significance set at $p < 0.05$.

Results

Operative metrics and post-surgery recovery

No animals showed signs of infection or lameness following non-invasive ACL rupture, or ACLR surgery, with all animals partial weightbearing immediately following recovery from anaesthesia. ACL rupture was confirmed at the time of surgery, with comparable injury profiles between treatment groups and sexes (Supplementary Table ii). Total surgery times and blood loss were comparable regardless of sex or treatment group (Supplementary Table ii). Weight loss and time taken to recover preoperative weight were greater for males than females after ACLR surgery (ALM-treated males, 22 days (SEM 5) vs ALM-treated females, 15 days (SEM 6); $p = 0.016$, Kruskal-Wallis test; Supplementary Figure b), however there were no statistically significant treatment differences.

Joint pain and swelling assessment

Pain (mechanical hyperalgesia) in the operated limb after surgery was determined as a reduction in PWT compared to the non-operated limb, and was evident in male and female operated knees to day 14, returning to normal ranges thereafter (Figures 2a and 2b). At day 5, mechanical hyperalgesia was 1.5-fold greater in male ALM-treated than Saline controls ($p = 0.017$, mixed ANOVA, Tukey's test), with no further treatment differences for the remainder of the experimental period (Figure 2a). After ACLR, joint swelling peaked at day 1 for both sexes, and returned to preoperative levels by day 14 (Figure 2c). Compared to male Saline controls, peak swelling was greater and more sustained over the first postoperative week for male ALM-treated animals ($p = 0.014$, mixed ANOVA, Tukey's test).

Recovery of joint function

To assess recovery of joint function after ACLR surgery, time taken to full weightbearing, knee extension angle, and temporal-spatial gait parameters were compared between male and female ALM-treated and Saline control animals. Median time taken to return to full weightbearing was comparable between treatment groups for females (ALM-treated, 12 days (SEM 5) vs Saline control, 12 days (SEM 4)) and males (ALM-treated, 11 days (SEM 13) vs Saline control, 9 days (SEM 7)). Compared to non-operated control knees, knee extension was reduced in operated knees of males and females (Figure 2d). In females, knee extension angles were 1.5-fold greater in ALM-treated animals, compared to controls ($p = 0.231$, one-way ANOVA; Figure 2d).

Preoperative and 28-day postoperative walking gait mechanics were compared to assess recovery of operated hind limb function (Supplementary Table iii). Consistent with recovery of pre-injury body weights, step length increased for males ($p = 0.019$, mixed ANOVA, Tukey's test) and females after surgery ($p = 0.007$, mixed ANOVA), and for females this was significantly higher in ALM-treated than Saline controls ($p = 0.005$, mixed ANOVA, Tukey's test; Supplementary Table iii). Compared to males, females traversed the catwalk more quickly after surgery ($p = 0.004$, mixed ANOVA, Tukey's test; Supplementary Table iii), with track speeds 1.2-fold faster for ALM-treated than Saline control females ($p = 0.091$, mixed ANOVA, Tukey's test). Increased track speed in ALM-treated females corresponded to a 13% increase in stride length ($p = 0.009$, mixed ANOVA, Tukey's test) and 10% decrease in stride

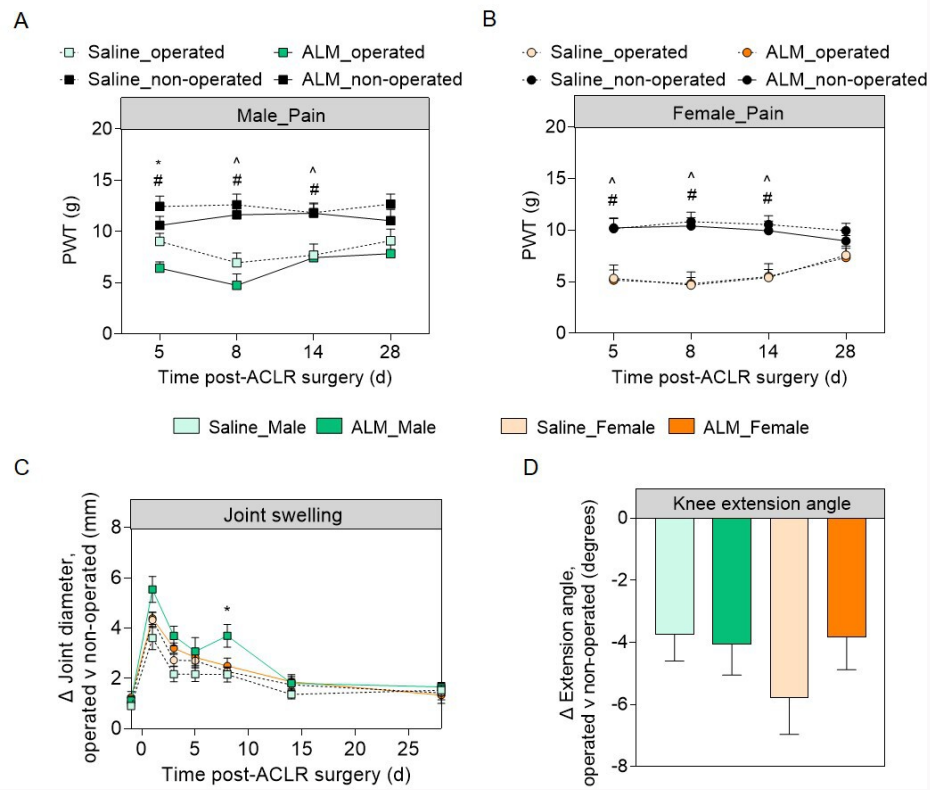

Fig. 2

A reduction in paw withdrawal threshold (PWT), as an indicator of pain sensitivity, was assessed using an up-down protocol for von Frey filaments in adenosine, lidocaine, and magnesium (ALM)-treated and Saline control a) males and b) females at days 5, 8, 14, and 28 postoperatively. Pain sensitivity was 1.5-fold higher in male ALM-treated than Saline controls at day 5, with no further treatment differences thereafter. c) Joint swelling was measured in male and female ALM-treated and Saline control animals postoperatively using digital calipers, with data expressed as the joint size difference between operated and contralateral, non-operated knees. Peak joint swelling was greater, and more sustained over the first postoperative week, for male ALM-treated, compared to male Saline controls. d) Differences in knee extension angles between operated (right) and non-operated (left) knees of ALM-treated and Saline control animals at day 28 postoperative were determined using an angulometer with 20 *g* force. Compared to Saline controls, a 1.5-fold improvement in extension angle was shown for ALM-treated females. Data show mean and standard error (SEM) (Mixed analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) test). *$p < 0.05$, ALM compared to Saline; #$p < 0.05$ ALM operated (right) compared to ALM non-operated (left) knee; ^$p < 0.05$ Saline operated (right) compared to Saline non-operated (left) knee.

variability ($p = 0.071$, mixed ANOVA, Tukey's test), compared to female Saline controls (Supplementary Table iii). Paw angles in ALM-treated females were comparable to preoperative values, however they remained elevated in female Saline controls at day 28 ($p = 0.017$, mixed ANOVA, Tukey's test; Supplementary Table iii). In males, while pre- and postoperative stride times were comparable for ALM-treated animals, stride times tended to be slower for Saline controls ($p = 0.068$, mixed ANOVA, Tukey's test; Supplementary Table iii).

Systemic inflammation

To determine the resolution of postoperative systemic inflammation, we assessed leucocyte subset proportions, and plasma levels of key inflammatory cytokines and chemokines involved in mobilization of neutrophils (LIX), monocytes (MCP-1), and lymphocytes (RANTES, IP-10) 28 days after ACLR surgery (Table I). Circulating neutrophils remained significantly elevated above baseline levels in male Saline controls ($p = 0.013$, mixed ANOVA, Tukey's test). In addition, plasma IP-10 remained elevated (1.4-fold; $p = 0.036$, mixed ANOVA), and was significantly higher than levels in ALM-treated males (1.5-fold; $p = 0.003$, mixed ANOVA) and Saline control females (1.9-fold; $p < 0.001$, mixed ANOVA, Tukey's test) (Table I).

Joint pathology and inflammatory profile

Quadriceps atrophy, a common response following ACLR surgery,[26,27] was evident in operated knees at day 28 post-operative, however muscle loss was comparable between sexes and treatment groups (males: ALM, 0.35 g (SEM 0.05) vs Saline control, 0.38 g (SEM 0.06); females: ALM, 0.38 g (SEM 0.06) vs Saline control, 0.35 g (SEM 0.05)). Mild-to-moderate effusion, joint capsule hypertrophy, and remodelling of articular surfaces was evident upon dissection of all operated knees (Figure 3a). Macroscopic pathology scores were 1.4-fold lower for female than male Saline controls ($p = 0.032$, Kruskal-Wallis test; Figure 3b).

Persistence of postoperative joint inflammation was assessed by levels of inflammatory mediators in synovial fluid, and histological evidence of synovitis. At day 28 postoperatively, synovial levels of pro-inflammatory (IL-1β, $p = 0.039$; MCP-1, $p = 0.015$, Kruskal–Wallis test) and angiogenic (VEGF, $p = 0.015$, Kruskal–Wallis test) mediators were significantly higher in males than females, with ALM dampening this response in males to levels comparable to those in females (Figure 3). In females, ALM treatment was associated with a 4.7-fold increase in MIP-1α, a pleiotropic mediator with chemotactic and pro-healing properties ($p = 0.041$, Kruskal–Wallis test).

Table I. Systemic leucocyte subsets and inflammatory cytokine and chemokine concentrations in adenosine, lidocaine, and magnesium-treated and Saline control male and female animals at day 28 following anterior cruciate ligament reconstruction surgery. Data are shown as mean and standard error of the mean.

Indices	Baseline*	Post-ACL reconstruction surgery	
		Saline	ALM
Leucocytes (× 10^6/ml)			
Total			
Male	11.2 (0.6)	11.9 (1.1)	12.5 (1.5)
Female	10.7 (1.1)	10.1 (0.7)	10.9 (0.9)
Neutrophils			
Male	1.6 (0.2)	3.4 (0.5)‡	2.7 (0.4)
Female	1.7 (0.3)	2.5 (0.3)	2.7 (0.4)
Monocytes			
Male	0.7 (0.2)	0.4 (0.2)	0.8 (0.2)
Female	0.7 (0.1)	0.4 (0.2)	0.8 (0.2)
Lymphocytes			
Male	9.1 (0.5)	8.0 (0.6)	9.0 (1.0)
Female	8.3 (0.8)	7.2 (0.5)	7.4 (0.6)
Inflammatory mediator (pg/ml)			
IL-6			
Male	177.6 (46.8)	92.6 (18.6)	82.0 (7.4)
Female	229.3 (80.9)	111.3 (29.2)	96.4 (23.3)
TNF-α			
Male	ND	ND	ND
Female	ND	ND	ND
IL-1β			
Male	5.7 (1.6)	4.3 (0.8)	2.6 (0.3)
Female	3.9 (1.5)	2.6 (0.3)	5.1 (2.1)
LIX			
Male	2,395.9 (426.2)	2,362.6 (572.1)	2,489.2 (462.7)
Female	2,097.8 (265.1)	2,734.0 (639.0)	1,856.5 (317.0)
MCP-1			
Male	543.8 (62.0)	585.8 (71.9)	555.8 (43.9)
Female	458.1 (26.9)	458.1 (74.6)	418.7 (56.7)
IP-10			
Male	500.3 (28.6)	684.5 (61.1)‡§	464.2 (21.5)†
Female	499.5 (109.0)	359.6 (23.0)	341.8 (25.9)
RANTES			
Male	2,071.1 (471.2)	1,476.1 (279.4)	1,186.7 (245.8)
Female	1,850.1 (293.5)	1,401.4 (285.0)	971.4 (130.2)

Inflammatory mediator assay limit of detection (pg/ml): IL-1β, 2.4; IL-6, 73.2; IP-10, 2.4; LIX, 24.4; MCP-1, 29.3; RANTES, 4.9; TNF-α, 2.4. Mixed analysis of variance, Tukey's post-hoc test.

*Baseline healthy male and female Sprague-Dawley rats: haematology, n = 10 per sex; inflammatory mediator, n = 8 per sex.

†$p < 0.05$ ALM compared to Saline.

‡$p < 0.05$ compared to Baseline.

§ $p < 0.05$, Saline male compared to Saline female.

ACL, anterior cruciate ligament; ALM, adenosine, lidocaine, and magnesium therapy; IL, interleukin IP-10, interferon gamma-induced protein 10; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein 1; ND, not detected; RANTES, regulated on activation, normal T cell expressed and presumably secreted; TNF-α, tumour necrosis factor α.

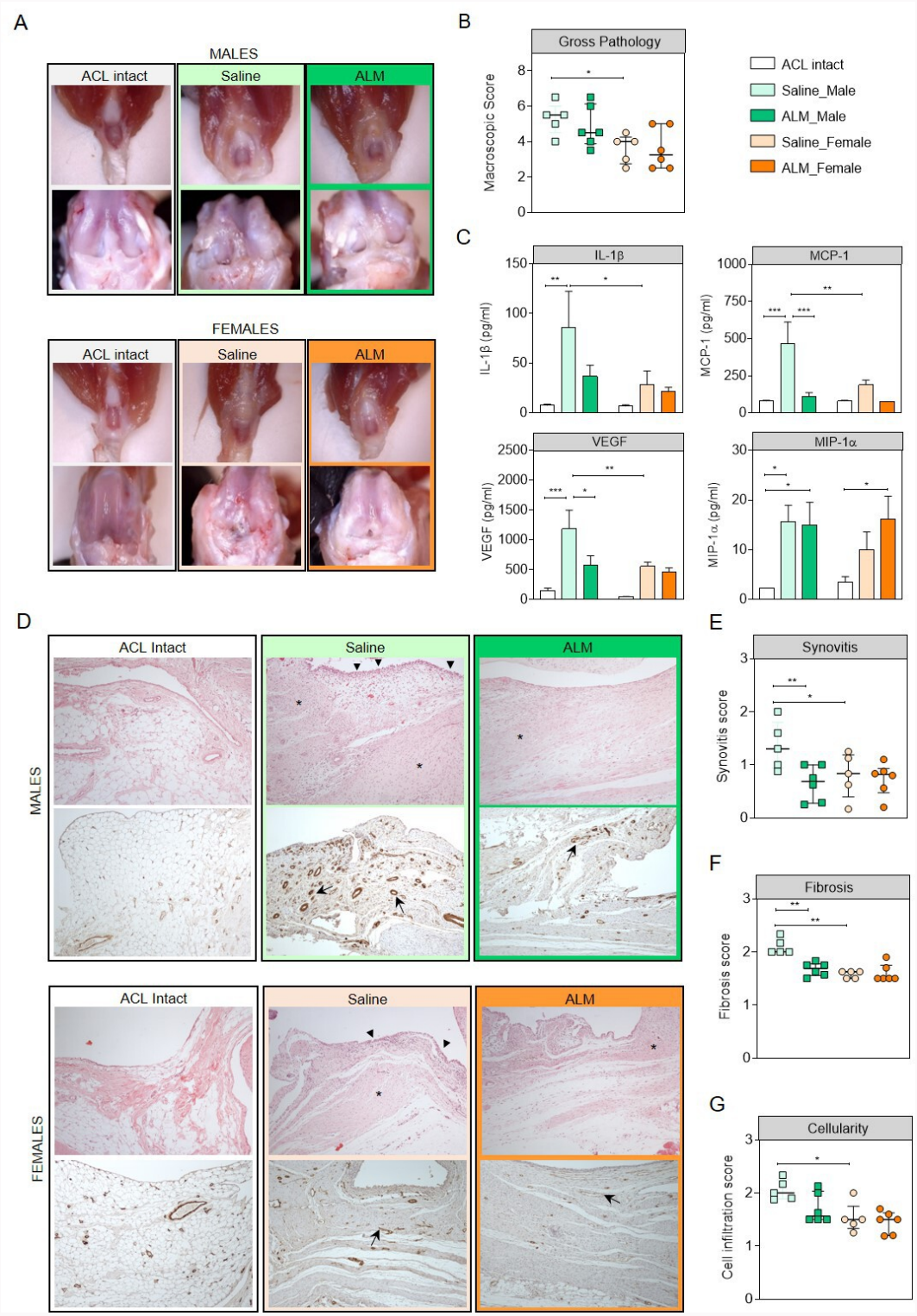

Fig. 3

Joint pathology and inflammation in male and female adenosine, lidocaine, and magnesium (ALM)-treated and Saline control animals, 28 days after anterior cruciate ligament reconstruction (ACLR) surgery. a) Representative images of dissected native (ACL intact) and operated knees. Mild-to-moderate effusion, joint capsule hypertrophy, and remodelling of articular surfaces was evident in all operated knees. b) Macroscopic pathology scores of operated knees were 1.4-fold lower for female than male Saline controls. c) Synovial fluid concentrations of inflammatory mediators in operated and native (ACL intact) knees of male and female ALM-treated and Saline control animals. Pro-inflammatory (interleukin (IL)-1β, monocyte chemoattractant protein (MCP)-1) and angiogenic (vascular endothelial growth factor (VEGF)) mediators were higher in males than females, with ALM dampening this response in males. In females, ALM treatment was associated with elevated levels of macrophage inflammatory protein (MIP)-1α. d) Representative haematoxylin and eosin (H&E)-stained (upper panels) and alpha-smooth muscle actin (α-SMA)-stained (lower panels) sections of infrapatellar fat pads (IFP) of operated knees showing synovitis (arrowheads), fibrosis (asterisk), and angiogenesis (arrows) in sub-synovial tissue (magnification, ×100). H&E-stained sections of IFP were scored for the presence of e) synovitis, f) fibrosis, and g) cellularity. Synovitis and fibrosis were lower in IFP from ALM-treated males than Saline controls. Data show mean and standard error (inflammatory mediators; Kruskal–Wallis test) or median and interquartile range (histology scores; Kruskal–Wallis test). *p < 0.05, **p < 0.01, ***p < 0.001.

Contrasting inflammatory mediator profiles of male and female synovial fluid were supported histologically. Synovial hyperplasia and sub-synovial fibrous metaplasia were evident in operated knees, with greater synovitis ($p = 0.048$, mixed ANOVA, Tukey's test), fibrotic changes ($p = 0.008$, mixed ANOVA), and cellularity ($p = 0.048$) in males than females (Figures 3d and 3e). IFP from operated knees of male ALM-treated animals exhibited less extensive histopathological changes than Saline control animals, with reduced synovitis ($p = 0.030$, Kruskal–Wallis test) and fibrosis at day 28 after surgery ($p = 0.004$, Kruskal–Wallis test). Consistent with contrasting synovial VEGF levels, immunohistochemical staining of α-SMA demonstrated reduced IFP angiogenesis in females than males, and in ALM-treated animals, compared to controls (Figure 3d). Cellular infiltration of sub-synovial tissue was predominantly composed of fibroblasts, mononuclear leucocytes, macrophages, and mast cells (Figure 3f).

ACL graft healing and maturation

To evaluate the effect of ALM therapy on ACL graft tissue healing, gene expression of key markers of inflammation (Nfkb, Nos2, Arg1), core constituents of the extracellular matrix (Col1a1, Col3a1, Ccn2, Fn1, Eln), and mediators of tissue remodelling (Tgfb1, Timp1) were measured in graft tissue, and histopathology of the graft mid-substance and bone-tendon healing within the femoral and tibial bone tunnels was assessed. Expression levels of tissue repair markers tended to be higher in females than males (Figure 4). However, Nfkb ($p = 0.005$, Kruskal–Wallis test) and Tgfb1 ($p = 0.041$, Kruskal–Wallis test) expression was significantly increased in ALM-treated males, to levels similar to females. Similarly, expression of Timp1, an inhibitor of ECM degradative enzymes, was 2.8-fold higher in females than males ($p = 0.013$, Kruskal–Wallis test), with ALM increasing levels 1.4-fold in males ($p = 0.317$, Kruskal–Wallis test). In ALM-treated females, expression of ECM synthesis markers was reduced (Nfkb, 1.9-fold; Tgfb1, 1.8-fold; Col3a1, 2.8-fold; Fn1, 2.3-fold; Ccn2, 1.7-fold; Tgfb1, 3-fold) in ACL grafts, compared to Saline controls (Figure 4). Ratios of Nos2:Arg1 expression (indicative of M1:M2 macrophage ratios) tended to be lower in females than males.

Sex-related transcriptional differences were supported histologically, with reduced cellularity and increased organization, and parallel orientation of collagen fibres in female ACL grafts, compared to males at day 28 postoperatively (Figure 5; Table II). Mild-to-moderate degenerative changes were evident in the intra-articular mid-substance of male and female graft tissue, with histological scores 1.5-fold lower in female ALM-treated than Saline controls (Figures 5a to 5h; Table II). Specifically, organization and continuity of collagen fibres was greater, and mucoid degeneration reduced in grafts of ALM-treated females. In ALM-treated males, collagen density and organization, and hypercellularity within the graft mid-substance, were more prominent than Saline controls (Figures 5e and 5f). In contrast to widespread foci of inflammatory cell infiltration in Saline control animals, grafts from ALM-treated males exhibited decreased inflammation, and an increased presence of fibroblasts and stromal cells with ovoid and spindle-shaped nuclei. Fewer $CD68^+$ macrophages were present within the graft mid-substance of ALM-treated animals, particularly females (Figures 5i to 5l). Within the femoral and tibial bone tunnels, ingrowth of a fibrovascular interface was apparent between the tunnel wall and ACL graft (Figures 5m to 5p; Table II). Compared to Saline control animals, the interface was narrower and more organized in ALM-treated males and females, with evidence of direct junction between graft and bone within the femur and tibia. The presence of chondrocyte-like cells and fibrocartilage staining within the healing femoral and tibial enthesis was also more prominent in females than males, and in ALM-treated animals compared to Saline controls (Figures 5q to 5x).

Articular cartilage degeneration

Since ACL rupture and surgical repair is associated with increased risk of cartilage degenerative changes, urinary CTX-II was monitored as a systemic biomarker of cartilage breakdown following ACLR surgery (Figure 6a). Compared to males, CTX-II levels were higher in females at days 3 ($p = 0.002$, mixed ANOVA, Tukey's test) and 8 ($p = 0.002$, mixed ANOVA, Tukey's test), with ALM reducing CTX-II levels 1.5-fold in females ($p = 0.209$, mixed ANOVA, Tukey's test). Histologically, cartilage damage and proteoglycan loss were minimal-to-mild, with occasional focal surface fibrillation in the MFC and MTP, and comparable OARSI scores between sexes and treatment groups (Figures 6b and 6c). No significant sex or treatment differences were observed in expression levels of Col2a1, the major constituent of articular cartilage. Expression of Acan, the most abundant articular cartilage proteoglycan, was similar between sexes, however levels were 2.1-fold higher in ALM-treated females, than Saline controls ($p = 0.065$, Kruskal–Wallis test; Figure 6d). MMP13 expression, a key catabolic enzyme, was significantly higher in males than females ($p = 0.022$, Kruskal–Wallis test), with ALM blunting this response ($p = 0.022$, Kruskal–Wallis; Figure 6d). Timp1, an inhibitor of MMP-13 activity, was comparable between sexes and treatment groups. Compared to Saline controls, expression of cartilage repair markers, Tgfb1 (9-fold; $p = 0.065$, Kruskal–Wallis test) and Acta2 (4-fold; $p = 0.093$, Kruskal–Wallis test) tended to be higher in ALM-treated females (Figure 6d) and were supported histologically, with hypercellularity and increased presence of α-SMA-expressing cells in the superficial and transitional zones of MFC cartilage in ALM-treated animals, particularly females (Figure 6e).

Discussion

Using a rat model of ACL rupture and ACLR surgery, we show that perioperative ALM therapy exerts differential effects on joint tissue healing profiles of males and females at 28 days postoperatively. In males, ALM therapy appears to dampen local and systemic inflammation, reduce fibrotic changes within the IFP, augment graft healing, and blunt expression of MMP13, a key catabolic enzyme, in articular cartilage. In females, ALM therapy appears to improve knee function, elevate synovial levels of tissue remodelling mediators, accelerate graft maturation, and promote cartilage regenerative changes. We will now discuss these findings.

ALM dampens persistent postoperative systemic and joint inflammation in males

Knee swelling, an indirect measure of postoperative joint inflammation, peaked at day 1, and receded by day 14 in both males and females. By day 28 postoperatively, pain and joint swelling had subsided in males and females, regardless

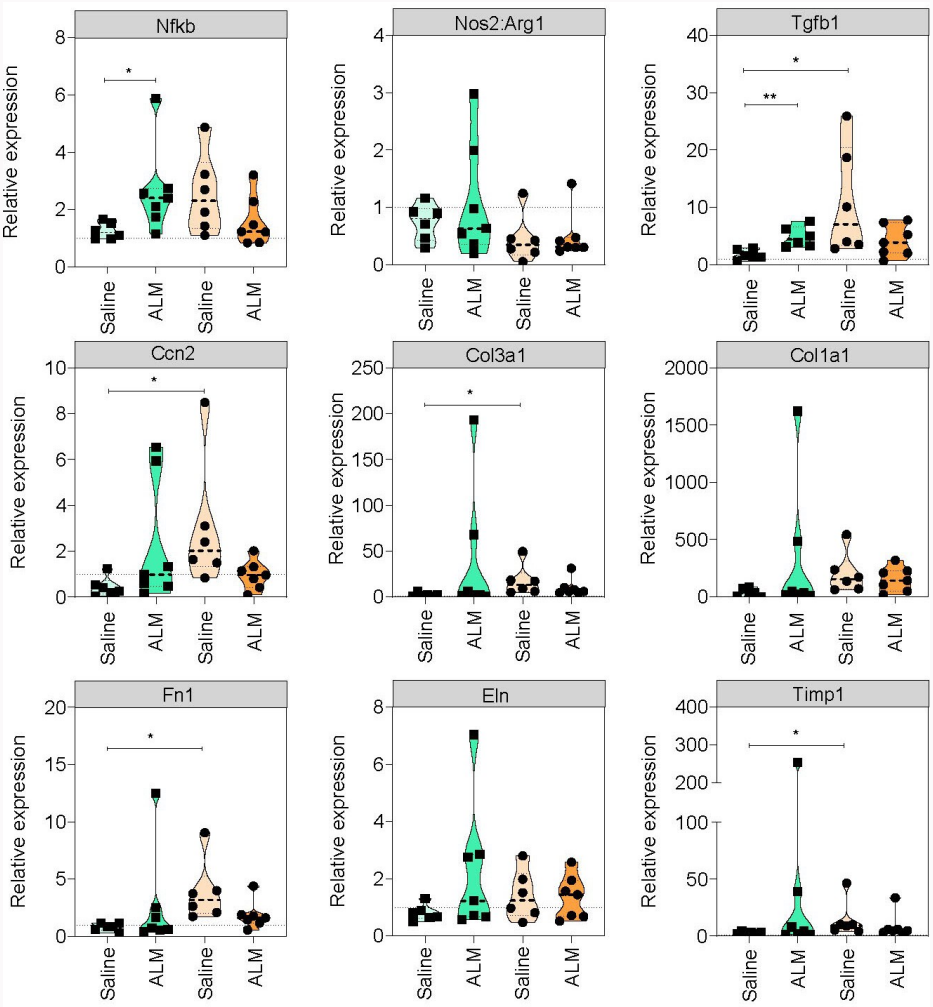

Fig. 4

Relative expression of markers of inflammation (nuclear factor kappa B, Nfkb; inducible nitric oxide synthase 2, Nos2; arginase 1, Arg1); extracellular matrix (ECM) components (collagen type 1, Col1a1; collagen type 3, Col3a1; fibronectin, Fn1; elastin, Eln); ECM remodelling enzymes (matrix metalloproteinase-9, MMP9; tissue inhibitor of MMP1, Timp1); and fibroblast/stem cell activation (transforming growth factor β 1, Tgfb1; α smooth muscle actin, Acta2) in the anterior cruciate ligament (ACL) graft of male and female adenosine, lidocaine and magnesium (ALM)-treated and Saline controls, at day 28 postoperatively. Expression of tissue repair markers tended to be higher in females than males, with ALM treatment increasing marker levels in males. Data show medians and interquartile ranges. *p < 0.05, **p < 0.01, Kruskal–Wallis test.

of treatment. In contrast to males, females had no evidence of persistent systemic inflammation. In males, circulating neutrophil numbers, and plasma levels of IP-10, a chemokine involved in recruitment of T cells, NK cells, and monocytes, remained elevated at day 28 postoperatively, with ALM blunting this effect.

Similarly, synovial levels of IL-1β, MCP-1, and VEGF at day 28 were significantly elevated within operated knees of males, but not females, with ALM dampening levels in males. In humans, elevated synovial IL-1β and MCP-1 is associated with knee pain, increased fibrosis severity, impaired graft healing, progressive cartilage degeneration, and poor functional outcomes after ACLR.[2,5,28,29] In our study, contrasting synovial inflammatory mediators between male ALM-treated and control animals corresponded to reduced synovitis, fibrotic changes, and angiogenesis within joint tissues. Within female operated knees, ALM treatment was associated with a 4.7-fold increase in synovial levels of MIP-1α, a peptide with pleiotropic effects that vary throughout the inflammatory, proliferative and remodelling phases of healing.[30] In the later stages of healing, MIP-1α promotes recruitment of tissue macrophages, inhibition of stem cell proliferation, and enhancement of tissue repair processes, including bone remodelling.[31-33] These features are consistent with our molecular and histological findings of enhanced tissue repair within ACL graft tissue, and the graft-bone interface within operated knees of ALM-treated females, compared to controls (discussed below). Consistent with the findings of Morris et al,[14] we conclude that systemic and local inflammation is resolved sooner in females than males after ACLR surgery, and that ALM alters the synovial milieu in a sex-specific manner resulting in decreased inflammation in males, and increased tissue remodelling mediators in females.

ALM improves joint function in females

There is a strong association between joint inflammation and joint dysfunction after ACLR surgery.[2,4,34,35] In this study, time to return to full weightbearing in the operated hind limb was comparable between sexes and treatment groups after surgery (~12 days), which corresponded to reduced

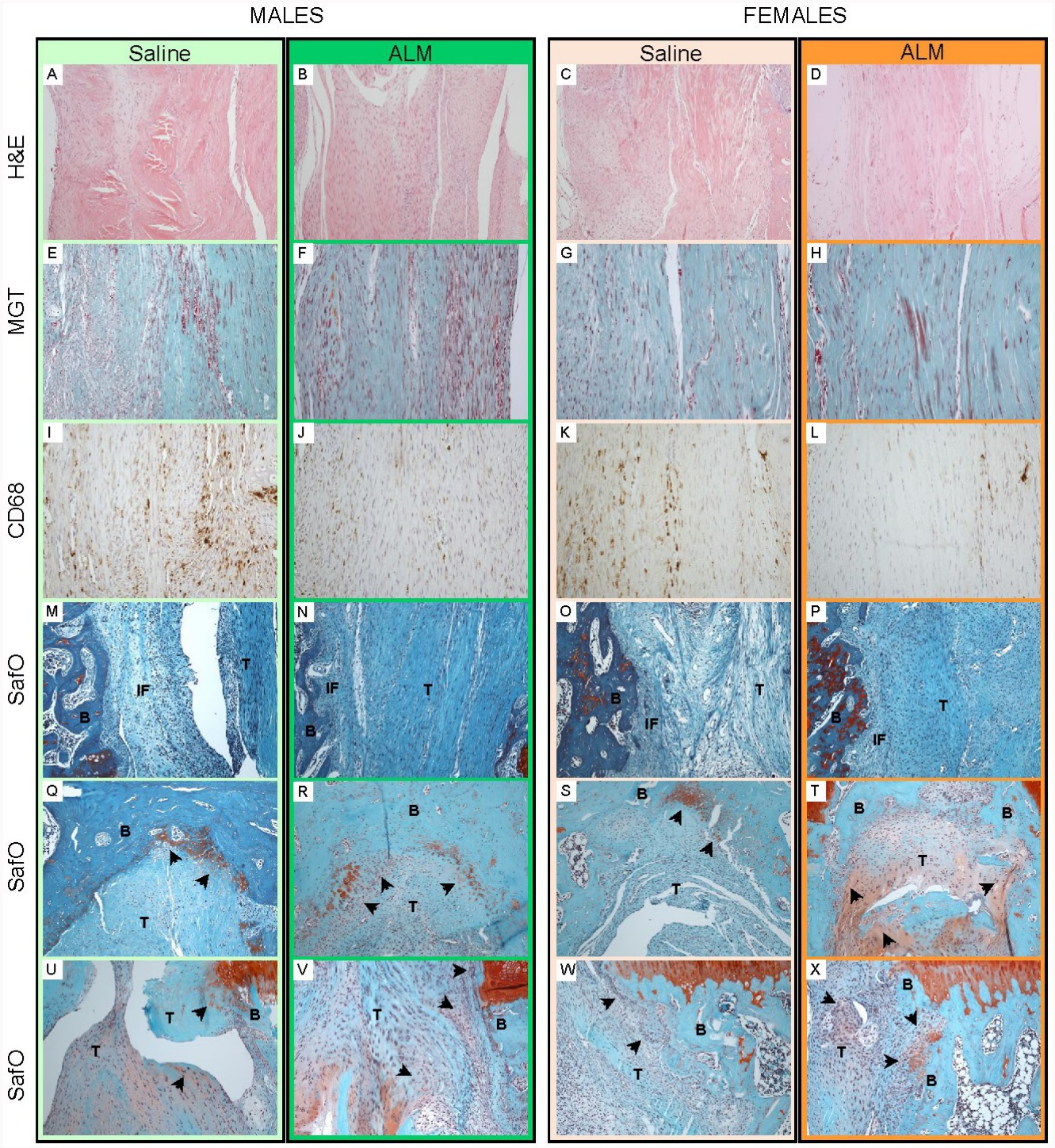

Fig. 5

Histological changes associated with anterior cruciate ligament (ACL) graft healing in male and female adenosine, lidocaine, and magnesium therapy (ALM)-treated and Saline control animals, 28 days after ACL reconstruction (ACLR). Representative images of a) to d) haematoxylin and eosin (H&E, magnification ×100), e) to h) Masson-Goldner trichrome (MGT, magnification ×100), i) to l) $CD68^{+}$ macrophages (magnification ×100), and m) to x) safranin O/fast green (SafO, magnification ×200) stained sections of the a) to l) ACL graft mid-substance, m) to p) femoral bone tunnel, and o) to t) graft insertion at the femoral and u) to x) tibial enthesis. Male and female ALM-treated animals exhibited increased collagen organization and reduction in macrophages within the graft mid-substance, a narrowing of the graft-tunnel interface, and increased fibrocartilage formation (arrows) at the graft enthesis, compared to respective controls. B, bone; IF, interface; T, tendon.

knee swelling and pain. Consistent with clinical outcomes,[26,27] a reduction in knee extension angles (~5°) and quadriceps atrophy (~0.4 g) following ACLR was evident in operated knees at 28 days postoperatively in this experimental model. No significant differences were observed in males, however minor improvements in knee extension (1.5-fold), and walking gait mechanics (increased step and stride length, decreased stride variability, increased track speed) were observed for ALM-treated females, compared to controls. Although joint functional improvements were minor, they are consistent with contrasting tissue repair profiles within the operated knees of treated and untreated females. Further comprehensive gait

Table II. Anterior cruciate ligament graft healing scores for adenosine, lidocaine, and magnesium-treated and Saline control male and female animals at day 28 after anterior cruciate ligament reconstruction surgery. Values are expressed as medians and interquartile ranges.

	Male			Female		
Variable	**Saline**	**ALM**	**p-value***	**Saline**	**ALM**	**p-value***
ACL graft degeneration score	6.75 (5.9 to 7.5)	6.1 (4.8 to 6.8)	0.242	6.5 (5.4 to 7.4)	4.3 (3.9 to 6)	0.113
Inflammation	1.8 (1.3 to 2.1)	1.3 (1 to 1.5)	0.110	2 (1.3 to 2)	1 (1 to 1.4)	0.134
Mucoid degeneration	1.5 (1 to 1.9)	1.6 (0.9 to 1.9)	0.946	1.5 (1.3 to 1.8)	0.8 (0.5 to 1.2)	0.045†
Chondroid metaplasia	1.8 (1.5 to 2)	1.3 (1 to 2)	0.314	1.5 (0.9 to 2)	1.3 (1 to 1.8)	0.794
Cystic changes	0.3 (0 to 0.4)	0.4 (0 to 0.8)	0.455	0.3 (0 to 0.5)	0.4 (0 to 0.7)	0.645
Collagen fibres orientiation	1.8 (1.1 to 2)	1.3 (1.2 to 1.8)	0.392	1.5 (1.3 to 1.8)	1 (1 to 1.1)	0.013†
Femur, graft-to-bone score	8.5 (7.3 to 10.5)	10.9 (9.4 to 12.2)	0.117	9.8 (9.4 to 10.4)	12.1 (10.8 to 12.3)	0.071
Fibrous interface width	1.5 (1.4 to 2)	2.4 (1.9, to 2.6)	0.048†	1.8 (1.5 to 1.9)	2.8 (2.3 to 3)	0.049†
Bony ingrowth	1.5 (1.3 to 2.1)	2.1 (1.9 to 2.5)	0.162	2 (2 to 2.3)	2.5 (2.4 to 2.5)	0.037†
Cellularity	1 (0.8 to 1.5)	1.6 (0.9 to 2)	0.251	1.5 (1.3 to 1.6)	1.8 (1.3 to 2)	0.437
Giant cells	2 (0.8 to 2)	2 (1.5 to 2.5)	0.448	1.5 (1.4 to 2.1)	2.3 (1.8 to 2.5)	0.245
Vascularization	3 (2.8 to 3)	3 (2.9 to 3)	> 0.999	3 (2.8 to 3)	3 (2.4 to 3)	0.848
Tibia, graft-to-bone score	8.3 (7.3 to 10.5)	9.5 (9.2 to 11.5)	0.225	10.8 (9.3 to 11)	11.5 (10.8 to 12.1)	0.049†
Fibrous interface width	1.5 (1.5 to 2)	2 (1.9 to 2.1)	0.190	2 (1.8 to 2.1)	2.4 (2 to 3)	0.091
Bony ingrowth	1.8 (1.4 to 2.3)	2 (1.8 to 2.1)	0.680	2.3 (2.1 to 2.3)	2.4 (2.3 to 2.5)	0.121
Cellularity	1 (1 to 1.6)	1.1 (0.9 to 2)	0.870	1.5 (1.3 to 1.9)	1.5 (1.4 to 2)	0.903
Giant cells	1.5 (0.8 to 2)	1.9 (1.5 to 2.5)	0.327	2 (1.6 to 2.5)	2.5 (1.7 to 2.5)	0.621
Vascularization	2.5 (2.4 to 2.9)	3 (2.8 to 3)	0.080	2.5 (2 to 3)	3 (2.4 to 3)	0.513

Animals per treatment group, per sex: Saline, n = 5; ALM, n = 6.
*Kruskal–Wallis test.
†$p < 0.05$ ALM compared to Saline.
ACL, anterior cruciate ligament; ALM, adenosine, lidocaine, and magnesium therapy.

and knee kinematic analyses are warranted to better elucidate sex differences in knee functional recovery after ACLR, and the potential benefits afforded by perioperative ALM therapy beyond 28 days postoperatively.

ALM augments ACL graft healing and maturation in both sexes

ALM therapy appears to alter the timing and nature of ACL graft tissue reparative processes, with molecular and histological evidence of distinct healing patterns within the ACL graft of males and females at day 28 postoperatively. Graft mid-substance remodelling and graft-to-bone tunnel healing are two key healing processes underpinning successful outcome after ACLR surgery.[36,37] However, they remain poorly characterized due to the difficulty in clinical monitoring of temporal changes using non-invasive methods, and the absence of a defined endpoint, given that graft tissue never reaches the appearance of the native ACL.[38] In this study, we show increased expression of key ECM synthesis markers associated with the proliferative phase of graft healing (NFkb, Tgfb1, Ccn2, Col3a1, Col1a1, Fn1, Eln, Timp1) in females, compared to males at day 28 after ACLR, with ALM treatment augmenting expression of these markers in males. Histologically, increased infiltration of non-inflammatory cells, and increased collagen and proteoglycan content, was evident within the graft mid-substance of ALM-treated males compared to controls. Together these changes suggest that ALM accelerates the proliferative phase of healing in males, to a phenotype more akin to females.

Following the proliferative phase, graft maturation into well-organized functional tissue (ligamentization) with good biomechanical properties is characterized by a decrease in cellularity, a shift to spindle-shaped, rather than ovoid nuclei within cells, gradual replacement of collagen type 3 with type 1, and formation of fibrocartilage within the bone tunnels followed by complete osseointegration.[6,38,39] In our study, both male and female ALM-treated animals exhibited increased collagen organization and reduction in macrophages within the graft mid-substance, a narrowing of the fibrovascular interface, and increased fibrocartilage formation within the bone tunnels, compared to their respective controls. In contrast to ALM-treated males, however, mid-substance cellularity was decreased in grafts from ALM-treated females, with a predominance of spindle-shaped nuclei. These histological features parallel decreased gene expression of ECM synthesis markers (Tgfb1, Ccn2, Col3a1, Fn1) in ALM-treated versus Saline control females, suggesting that ALM also augments graft maturation in females. Together, our findings demonstrate a more advanced graft healing phenotype in females than males after experimental ACLR, which was

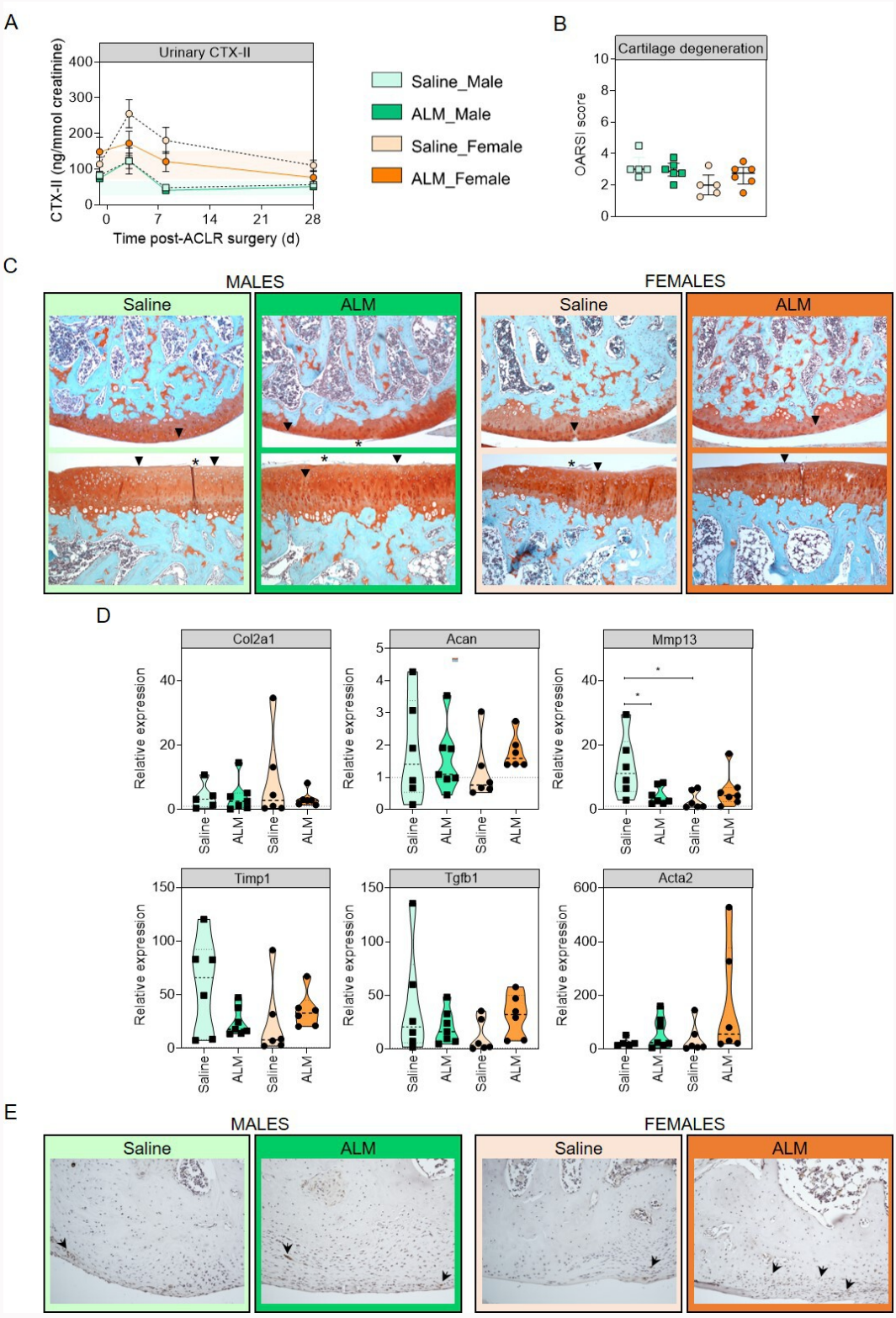

Fig. 6

Cartilage degenerative changes in male and female adenosine, lidocaine, and magnesium (ALM)-treated and Saline control animals, 28 days after anterior cruciate ligament reconstruction (ACLR) surgery. a) Postoperative urinary CTX-II levels peaked in the first postoperative week, and were higher in females than males. b) Osteoarthritis Research Society International (OARSI) scoring of medial articular cartilage surfaces, and c) representative safranin O/fast green-stained sections (magnification ×100) of medial femoral condyles (MFC, upper panels) and medial tibial plateau (MTP, lower panels). Minor proteoglycan loss (asterisk), and surface fibrillation (arrows) was evident, with no sex- or treatment-specific differences. d) Relative expression of cartilage extracellular matrix (ECM) components (collagen type 2, Col2a1; aggrecan, Acan), ECM remodelling enzymes (matrix metalloproteinase-13, MMP13; tissue inhibitor of matrix metalloproteinase 1, Timp1); and mediators of cell proliferation and activation (transforming growth factor β 1, Tgfb1; α smooth muscle actin, Acta2) in MFC cartilage. MMP13 levels were higher in males than females, with ALM dampening this response. In females, expression of Acan, Tgfb1, and Acta2 was increased in ALM-treated, than Saline control animals. e) Representative α-SMA-stained sections of MFC indicating the presence of positive-staining cells in the superficial and transitional zones (arrowheads) of ALM-treated animals. Data show medians and interquartile ranges. *p < 0.05, Kruskal–Wallis test.

indicated by contrasting sex-specific inflammatory profiles in the first postoperative week.[14] Molecular and histological data suggest that by modulating early inflammatory responses in a sex-specific manner,[14] perioperative ALM therapy augments healing at the graft-bone interface in both sexes. Further studies are required to determine the biomechanical implications of these changes, and to characterize the progression of ligamentization and osseointegration beyond 28 days.

ALM exerts chondroprotective changes in articular cartilage in both sexes

Consistent with adjacent joint tissues, males and females differed in the molecular signatures of the cartilage response to ACLR surgery, with ALM exerting sex-specific chondroprotective changes at day 28 postoperatively. In our study, urinary CTX-II, a biomarker of inflammatory-driven collagen type II breakdown,[2,8] peaked in the first postoperative week, returning to baseline levels thereafter in both sexes, and corresponded to postoperative joint swelling profiles. However, urinary CTX-II levels were notably higher in females than males at baseline and after ACLR surgery, reflecting increasing awareness of sex differences in cartilage degenerative and regenerative processes.[40] Despite similar histological appearance, articular cartilage in male and female knees exhibited contrasting transcriptional signatures at 28 days postoperatively, with ALM dampening markers of cartilage catabolism in males, and boosting anabolic markers in females. Cartilage degeneration involves progressive remodelling of the cartilaginous ECM, and is driven by an imbalance between matrix-degrading matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) released by chondrocytes under inflammatory conditions.[41] Strikingly, expression of MMP13, a catabolic enzyme and a marker for osteoarthritis severity,[42] was higher in cartilage of males than females at day 28 postoperatively, with ALM blunting this response.

In females, ALM was associated with increased expression of markers associated with anabolic and regenerative processes, including aggrecan, a major proteoglycan within cartilage, Tgfb1, and Acta2. Tgfb signalling pathways are essential for maintaining a differentiated chondrocyte phenotype, and preventing chondrocyte hypertrophy in healthy articular cartilage. However, within the context of joint inflammation, these pathways have the potential to switch on pro-catabolic and destructive processes that contribute to osteoarthritis progression.[43,44] α-SMA-positive chondrocytes, or myochondrocytes, are present in healthy, injured, and regenerated articular cartilage,[45-47] and are thought to possess heightened regenerative capacity, and play a key role in maintaining cartilage integrity.[47] Consistent with increased Acta2 expression, the presence of α-SMA positive cells was increased in articular cartilage of ALM-treated animals, particularly females, after ACLR in the present study. Together, these data suggest that ALM exerts sex-specific chondroprotective changes within the knee after ACLR surgery. Longer-term studies are required to determine whether these changes provide ongoing protection against osteoarthritis progression.

Clinical relevance

ACL injury and its surgical reconstruction is known to increase inflammatory cytokines in the synovial fluid and cartilage breakdown biomarkers which, if not resolved in a timely manner, can lead to poor outcomes.[1,2,8,9,48,49] Despite sex-specific phenotypic and temporal differences in joint tissue healing profiles, we have shown that perioperative ALM therapy facilitates timely resolution of postoperative inflammation, leading to augmented joint tissue healing profiles in both males and females, 28 days after ACLR surgery. Local infiltration analgesia, commonly comprising lidocaine, is a safe and effective technique widely used by orthopaedic surgeons during ACLR surgery to minimize postoperative pain.[50] However, debate persists concerning the potential for chondrotoxicity following intra-articular administration of 1% to 2% lidocaine.[51,52] Notably, no adverse inflammatory or chondrotoxic effects were shown following exposure to clinically relevant concentrations of ALM, which contains 0.08% (3mM) lidocaine.[53] The growing body of preclinical evidence of ALM's protection in experimental models of musculoskeletal injury and surgery provide support for human safety trials and clinical translation of ALM therapy.[20,21,54] The possibility exists that ALM therapy may be useful for other orthopaedic procedures.

We acknowledge several limitations to the study. First, the use of a rat model of ACLR necessitates open knee surgery, rather than the preferred arthroscopic approach used clinically. Second, our assessment of postoperative joint tissue healing and functional recovery was limited to a 28-day experimental period. Third, recovery of joint function was assessed by simple walking gait mechanics. Given the sex- and treatment-specific differences in early healing profiles within the synovial fluid, IFP, graft, and articular cartilage at 28 days, extension of the experimental period for several months is warranted to enable assessment of long-term functional outcomes including dynamic and kinematic gait analysis, microCT analysis of graft osseointegration, subchondral bone remodelling and cartilage integrity, and graft biomechanical strength. Despite these limitations, the model reproduces early molecular and histopathology changes in multiple joint tissues that are consistent with those described clinically.

We conclude that perioperative ALM therapy dampens inflammation and enhances inherent sex-specific tissue repair responses in the knee following experimental ACL rupture and ACLR surgery. Joint tissues in female operated knees show a more advanced healing phenotype than males, 28 days after ACLR. Contrasting inflammatory, immune, and tissue remodelling patterns between males and females following ACL rupture and repair require further investigation.

Supplementary material

Tables and figures describing the primer sequences, surgery details, gait variables, gait testing apparatus, and postoperative body weight recovery. An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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ICMJE COI statement

G. P. Dobson is the inventor of the ALM concept for trauma and surgery. J. L. Morris, P. C. McEwen, and H. L. Letson have no conflicts to declare.

Data sharing

All data generated or analyzed during this study are included in the published article and/or in the supplementary material.

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Ethical review statement

Study protocols were reviewed and approved by the James Cook University Animal Ethics Committee (A2684), and the US Animal Care and Review Use Office (ACURO) and are reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.