Tripeptide–Copper Complex GHK-Cu (II) Transiently Improved Healing Outcome in a Rat Model of ACL Reconstruction

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ABSTRACT: After anterior cruciate ligament reconstruction (ACLR), the biological healing of the graft is a rate-limiting step which can contribute to graft failure. The tripeptide–copper complex glycyl-L-histidyl-L-lysine-Cu(II) (GHK-Cu) is a well-known activator of tissue remodeling. We investigated whether GHK-Cu can improve graft healing following ACLR. Seventy-two rats underwent unilateral ACLR were randomized to saline, 0.3 or 3 mg/ml GHK-Cu groups ($n = 24$). Post-operational intra-articular injections were given from week 2, once a week, for 4 weeks. Gait analysis was performed pre-injury and at harvesting time. At 6 or 12 weeks post-operation, knee specimens were harvested for knee laxity test, graft pull-out test, and histology. At 6 weeks post-ACLR, GHK-Cu groups resulted in a smaller side-to-side difference in knee laxity as compared to the saline group ($p = 0.009$), but there was no significant difference at 12 weeks post-operation. The graft complex in the 0.3 mg/ml GHK-Cu group had higher stiffness than saline group at 6 weeks post-operation ($p = 0.026$), but there was no significant difference in ultimate load, gait parameters, and histological scores among treatment groups. All grafts failed mid-substance during pull-out test. Intra-articular supplementation with a bioactive small molecule GHK-Cu improved graft healing following ACLR in rat, but the beneficial effects could not last as treatment discontinued. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 33:1024–1033, 2015.

Keywords: anterior cruciate ligament reconstruction; tripeptide copper complex; graft remodeling; biological modulation; rat

Anterior cruciate ligament reconstruction (ACLR) is a standard operation to restore knee function after ACL rupture. After surgical fixation, the tendon graft undergoes phases of necrosis, hypocellularity, cell recruitment, revascularization, and finally ligamentization.[1] Nevertheless, non-traumatic ACLR failure can occur due to poor graft healing,[1] which involves slow tissue remodeling, such as intra-tunnel graft incorporation and intra-articular ligamentization.[2] At the graft-tunnel interface, few connections composed of collagen fiber continuity resembling Sharpey's fibers are formed 1-year post-operation[3] and grafts that fail to incorporate may lead to excessive laxity during the early healing process, resulting in subsequent graft failure. On the other hand, in successful ACLR cases, the intra-articular ACL graft acquires extensive biological and mechanical properties of the native ACL, at least 1-year post-operation.[4,5] Various surgical methods for ACLR have been suggested for improving clinical outcomes, including graft choice,[6] graft preparation, and graft fixation.[7] In addition, the uses of stem cells,[8] growth factors,[9] and biomaterials[10] have also been proposed. Based on the systematic review performed by our group,[11] the proposed mechanisms for the biological modulation include bone-tendon healing at graft tunnel interface, angiogenesis, osteogenesis, cell supplementation for general healing capacity and reduction of local inflammation. Optimization of direct graft remodeling is likely to be the most efficient method to reduce recovery time and risks of surgical failure.

Glycyl-histidyl-lysine (GHK) tripeptide and its copper (II) chelated form (GHK-Cu) is well known for its uses in tissue remodeling processes.[12] For years, it has been widely used in the cosmetic industry for skin tissue remodeling, due to its high safety profile in humans.[13] It is involved in the activation of the synthesis of matrix compounds *in vivo*, including the synthesis of collagen, glycosaminoglycans,[14] metalloproteinases (MMPs),[15] and tissue inhibitors of MMPs.[16] Moreover, GHK-Cu may act as a biological source of copper for copper-dependent enzymes, such as lysyl oxidase which is involved in the formation of collagen crosslinks.[17] GHK-Cu can promote bone healing[18] and enhance the attachment of the implant to the bone.[19] It is also a matrikine for repair cell recruitment and angiogenesis.[20]

As GHK-Cu is well characterized for its involvement in tissue healing and remodeling, we sought to determine the effect of post-operative, intra-articular injections of GHK-Cu on the graft healing process by assessing: (1) anterior–posterior (A-P) knee laxity, (2) graft pull-out strength, (3) graft histology, and (4) gait analysis. We hypothesize that GHK-Cu can improve the healing outcome following ACLR.

METHODS

The animal experiments were approved by the Animal Experimentation Ethics Committee in The Chinese University of Hong Kong (Ref. no.: 11/054/GRF and 460611).

Experimental Design

The study design is shown in Figure 1. Seventy-two male Sprague–Dawley rats (12 weeks old, 418.2 ± 22.2 g) were used. ACLR was performed as previously described[21] on

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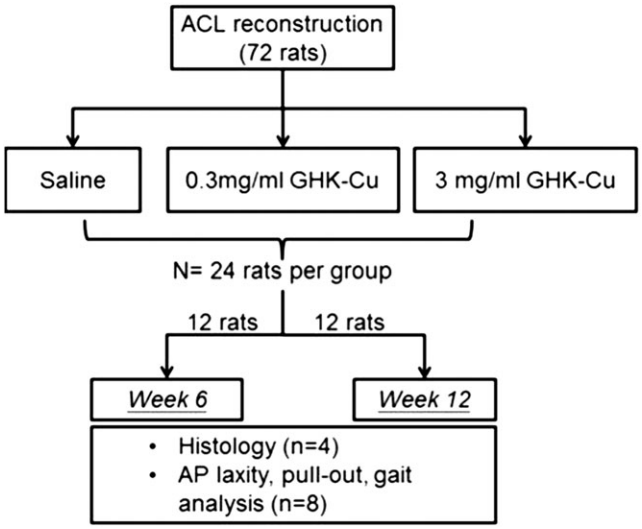

Figure 1. Experimental design to study the effect of post-operative intra-articular injection of GHK-Cu following ACLR.

the right knee using ipsilateral flexor digitorum longus tendon. The graft was inserted to femoral and tibial bone tunnels of 1.1 mm diameter and was fixed by suturing to periosteum under 4 N graft tensioning using a freely suspended weight. Intra-articular injection of saline or GHK-Cu solution was performed weekly from the second to the fifth week post-operatively. Gait analysis was performed pre-injury and at harvesting time ($n = 8$). At 6 and 12 weeks post-operation, knee specimens were harvested for the static knee laxity test, the graft pullout test ($n = 8$) and hematoxylin and eosin (H&E) staining for histological scoring ($n = 4$).

Post-Operative Intra-Articular Injection of GHK-Cu

GHK-Cu was prepared by dissolving GHK acetate (#G1887, Sigma-Aldrich, St. Louis, MO) and copper (II) chloride 2-hydrate (#27834, BDH Laboratory Supplies, Poole, England) in 0.9% saline in a 2:1 molar ratio. The resulting 10 mg/ml GHK-Cu stock solution was filtered through a 0.22 μm syringe filter, aliquoted and kept frozen until use. To avoid injection in a swollen joint, intra-articular injection was performed from the second to the fifth week, once a week, post-operatively. Saline or GHK-Cu solution at 0.3 or 3 mg/ml was loaded into a 50 μl syringe with a 26G removable needle (Hamilton Company, Reno, NV). The doses were determined based on the positive effects on wound healing.[14,16] Under general anesthesia, with the knee extended, the needle was inserted from the medial side of the knee to the bottom of the patella to access the joint space, with reference to clinical practice,[22] and 50 μl of treatment solution was injected slowly.

Anterior–Posterior (A-P) Knee Laxity Test

The test was performed according to our established protocol[21] with minor modifications as below. Both knees were freshly trimmed with tibia and femur shafts embedded in adhesive polymer (Ciba Specialty Chemicals, Cambridge, UK) in plastic tubes. The plastic tubes were aligned axially by tilting the tibia shaft inside the tube in order to preserve the natural varus/valgus angle of the knee. The samples were then mounted onto jigs on a mechanical testing machine (H25KM, Tinius Olsen Inc., Horsham, PA) with a 50 N load cell (H25KM, Tinius Olsen Inc.) (load measurement accuracy: ±0.5% of max. load). The jigs were positioned to keep the knee in a neutral A-P position and maintained at 70° flexed from full extension. Before the test, a posterior displacement of the tibia from the neutral A-P position was applied (0.5 mm). It was reset as force zero and an anterior displacement of 1 mm was applied and then returned to starting position for four cycles at a test speed of 40 mm/min and upper force limit of 20 N. The static A-P knee laxity was measured as total displacement caused by a fixed anterior and posterior loading (0.5 N) at the neutral position.

Load-to-Failure Test

After the laxity test, the specimens were kept at −20°C and thawed at room temperature for 2 h before the load-to-failure test.[21] The femur–graft–tibia complexes were positioned to align the femur and tibia tunnels with the graft vertically along the direction of applied force. The tensile test for failure load was carried out at a cross head speed of 40 mm/min with a 50 N load cell until an abrupt drop in loading was detected. Failure load was measured as the maximum force until graft failure, and the mode of failure was recorded. The stiffness of the femur–graft–tibia complex was measured as the slope at the linear region of the force–displacement curve.

Histological Analysis

At 6 and 12 weeks post-operation, the rats were euthanized ($n = 4$) and the harvested knee joints were fixed, decalcified, and paraffin-embedded. Five-micrometer-thick paraffin sections along the sagittal plane of the knee were collected in groups of 10–20 sections at 500 μm intervals. From each animal, 2–3 sections from each group of sections were chosen for histological examination of femoral tunnels, intra-articular graft mid-substance or tibial tunnels. Sections from the epiphyseal region were chosen for comparison of graft healing inside tunnels. H&E-stained sections were examined under bright field and polarized illumination (Leica Microsystems, Wetzlar, Germany). Histological evaluation was carried out by two independent examiners (S.C.F. and Y.C.C.) blinded to treatments according to a developed scoring system,[21,23] based on the extent of matrix degeneration of the tendon graft, and the healing responses in the graft–tunnel interface. Image analysis of cell density in graft mid-substance was performed using Image-Pro Premier 9.1 (Media Cybernetics, Bethesda, MD). In H&E images of the graft mid-substance region captured under 400× magnification, thresholding was performed to include cell nucleus only and the number of cells were counted.[24]

Gait Analysis

Individuals with ACLR are reported to have a higher risk of developing knee osteoarthritis (OA).[25] Evaluation of pain in a rat model of OA has been previously demonstrated with the Limb Idleness Index (LII).[26] To examine the effect of GHK-Cu treatment on functional recovery of ACLR, gait analysis[26] with Catwalk XT 9.0 (Noldus Information Technology, Wageningen, The Netherlands) was performed pre-injury and 6 and 12 weeks post-operation. Recorded runs with a steady walking speed (variation <30%) were accepted. Compliant runs for paw prints were auto-classified by the built-in software. Three to five runs were kept for calculation of gait parameters for every trial. A value of LII > 1.3 indicates pain-associated gait changes.[26]

Statistical Analysis

Statistical analysis was done using the Statistical Package for Social Science (SPSS) 20.0 (IBM Corp, Armonk, NY). All parametric tests were performed after checking for normal distribution by the Kolmogorov–Smirnov test. Repeated measures analysis of variance (ANOVA) was used to log-transform gait data with respect to temporal changes (within-subject factor) and experimental groups (between-subject factor). Data of knee laxity and pull-out force were also analyzed by repeated measures ANOVA with operated/contralateral sides as the within-subject factor and experimental groups as the between-subject factor. The likelihood ratio test was used to determine the association between the treatment and failure mode of the femur–graft–tibia complex, and the association between treatment and occurrence of highly asymmetric gait, where high LII may indicate gait adaptation to pain in rats. For histological scoring, non-parametric Kruskal–Wallis tests were used for multiple group comparison, while Wilcoxon signed rank test was used to determine the within-sample-difference with respect to regions. A two-way ANOVA was employed to calculate difference in cell density in graft mid-substance. Significant difference was determined at $p < 0.05$.

RESULTS

Animal Model

Among 72 operated rats, 3 rats died during anesthesia and were replaced. Surgical accidents were recorded, including fixation failure (one rat) and sub-optimal tunnel placement (four rats). Two rats (from the 0.3 and 3 mg/ml GHK-Cu groups, respectively) were found dead after the third session of the intra-articular injections, possibly due to incapability to wake up from anesthesia. Post-surgical complications were also recorded, comprised of re-sutures of the wound due to bites (four rats), patella dislocation (one rat), and infection (one rat). Rats with surgical accidents or post-surgical complications were excluded for statistical analyses of gait data and mechanical test data. The minimum sample sizes were seven per group for mechanical/gait analysis and three per group for histological scoring.

A-P Knee Laxity Test and Graft Pull-Out Test

At 6 weeks post-operation, rats in the 0.3 and 3 mg/ml GHK-Cu treatment groups had a significantly smaller side-to-side difference in A-P-knee laxity ($p = 0.012$, 0.049, Scheffe post hoc test) as compared to the saline group (ANOVA, $p = 0.009$), but no difference in A-P laxity was found at 12 weeks post-operation between all groups ($p = 0.531$) (Fig. 2A). In spite of the insignificant difference in failure load of the graft complex between GHK-Cu groups and saline group at 6 and 12 weeks post-operation ($p = 0.301$, 0.834) (Fig. 2B), the graft complex in the 0.3 mg/ml GHK-Cu group had significantly higher stiffness ($p = 0.026$, Scheffe post hoc test) than the saline group at 6 weeks post-operation ($p = 0.007$, ANOVA) (Fig. 2C). All grafts failed mid-substance during the pull-out test.

Histological Analysis

The results of histological scoring were summarized in Table 1. Intra-class correlation coefficient (ICC) for inter-observer reliability was 0.654, indicating a fair degree of agreement. At 6 weeks post-operation, better graft incorporation at tibial tunnel was noticed in the 0.3 mg/ml GHK-Cu group but the difference was not statistically significant (Kruskal–Wallis test, $p = 0.087$) (Fig. 4A–I). A complete bony layer surrounding the graft at the tibial tunnel was observed in the GHK-Cu groups (Fig. 4E and F) while a leaky tunnel interface was common in the saline group (Fig. 4D), suggesting a better bone healing but the bone healing score was not significantly different ($p = 0.060$). It was noticeable that these histological differences are less pronounced in femoral tunnel (Fig. 3A–I). At 12 weeks post-operation, no significant difference was found between groups (Figs. 3–5J–R). In both time points, graft degeneration, indicated by reduction of collagen birefringence, was less severe inside tibial tunnel as compared to femoral tunnel ($p = 0.01$, Wilcoxon signed rank test) and intra-articular mid-substance ($p = 0.01$). The 0.3 mg/ml GHK-Cu group exhibited reduced graft degeneration as compared with the control at 6 weeks post-operation (Figs. 3–5D–I), but the difference in histological score is not statistically significant ($p = 0.255$). Results from image analysis were shown in Table 1. GHK-Cu treated samples exhibited increased cellularity at the intra-articular mid-substance, but the difference was not statistically significant ($p = 0.053$, two-way ANOVA).

Gait Analysis

As compared to pre-injury levels, weekly intra-articular injections of GHK-Cu caused the injured limb to become significantly idled in rats with ACLR and ipsilateral flexor tendon donor site injury during walking at 6 and 12 weeks post-operation (Fig. 6). The target print ratio (TPR) ($p = 0.006$; Fig. 6B), swing duration ratio (SWR; $p < 0.001$) (Fig. 6C) and Limb Idleness Index (LII) ($p < 0.001$; Fig. 6D) were significantly altered with time, indicating the decreased use of the operated limb by decreased loading (as reflected by increased TRP) and increased paw elevation time (as reflected by increased SWR) on operated hindlimb. However, no significant change was observed in the anchor print ratio (APR) ($p = 0.702$; Fig. 6A). Differences among experimental groups in all gait parameters were also insignificant ($p = 0.576$, 0.726, 0.896, and 0.597 for APR, TPR, SWR, and LII, respectively). The increased idleness was observed regardless of treatments. At 6 weeks post-operation, 30% of rats in every group developed an LII > 1.3, indicating pain-associated gait changes. At 12 weeks post-injury, percentages of LII > 1.3 in saline, 0.3 and 3 mg/ml GHK-Cu groups were 30%, 30%, and 50%, respectively, but the difference was not statically significant (Likelihood ratio test, $p = 0.477$).

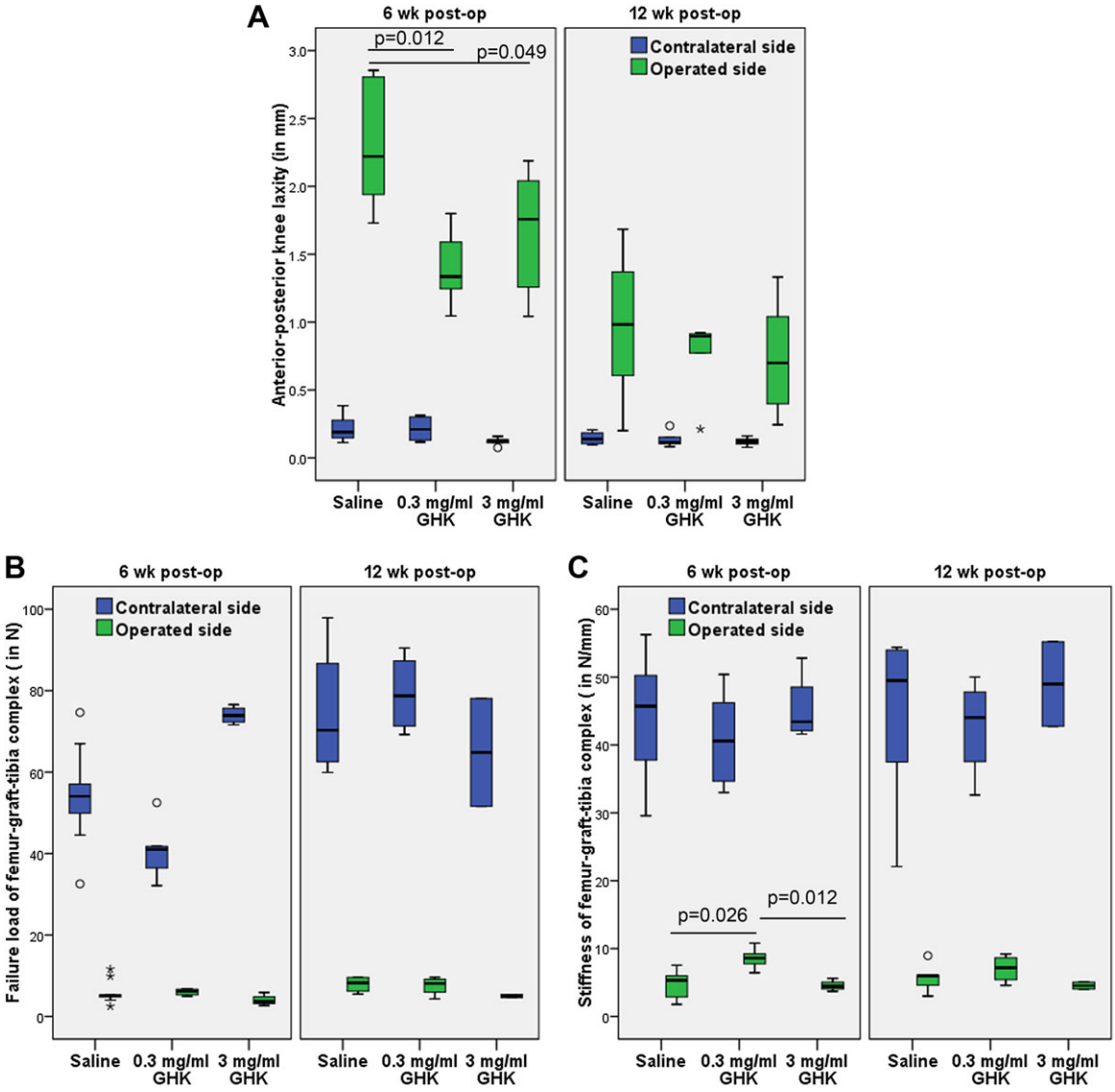

Figure 2. Effect of GHK-Cu on (A) A-P knee laxity, (B) pull-out strength of the femur-graft-tibia complex, and (C) graft stiffness of the femur-graft-tibia complex in a rat model of ACLR. The *p* values showing significant differences in (A) are comparisons of side-to-side differences in A-P knee laxity. The circle (O) represent an outlier which exceeds 1.5× interquartile range (IQR, the box width), whereas the asterisk (*) represents an extreme value which exceeds 3× IQR.

DISCUSSION

Graft healing and survival are critical factors affecting the ACLR clinical outcome. Tissue remodeling involves coordinated matrix degradation and synthesis, and is essential for graft incorporation inside bone tunnels and ligamentization. Direct modulation of tissue remodeling may serve to improve graft healing. In this study, we investigated if GHK-Cu, a renowned activator for skin tissue remodeling, could speed up graft remodeling in ACLR.

Our results demonstrated that intra-articular injections of GHK-Cu at 0.3 mg/ml could transiently promote graft healing in ACLR at 6 weeks post-operation. GHK-Cu has been known to be effective in promoting bone healing[18] and recruitment of repair cells,[20] therefore, it is not surprising to observe enhanced bone formation at the tunnel interface (Figs. 3 and 4) and increased cell repopulation in the graft mid-substance (Fig. 5) in GHK-Cu-treated groups. It was speculated that the positive effect of GHK-Cu on A-P laxity may be attributed to improved anchorage at the graft tunnel interface and enhanced tissue remodeling in the graft mid-substance, as indicated by increased stiffness of the femur-graft-tibia complex in the 0.3 mg/ml GHK-Cu group (Fig. 2C). A higher dose of GHK-Cu did not lead to further improvement. In the rat model of ACLR, 3 mg/ml GHK-Cu induced greater cell infiltration into the graft mid-substance as compared to 0.3 mg/ml GHK-Cu, resulting in a corresponding decrease of collagen birefringence (Fig. 5). Cell repopulation of the tendon graft is essential for graft survival and remodeling. During the early graft

Table 1. Histological Analysis of Effects of GHK-Cu on a Rat Model of ACLR[a]

Time post-operation	Saline	0.3 mg/ml GHK-Cu	3 mg/ml GHK-Cu
Graft degeneration[b]			
6 weeks	F: 1.5 (1–2)	F: 2 (1–4)	F: 2.5 (1–4)
	T: 1 (1–1)	T: 1 (1–1)	T: 1 (1–1)
	MS: 3 (2–4)	MS: 2 (1–3)	MS: 3 (2–3)
12 weeks	F: 2.5 (1–3)	F: 1.5 (1–4)	F: 2.5 (1–4)
	T: 1 (1–2)	T: 1 (1–1)	T: 1.5 (1–2)
	MS: 3 (2–4)	MS: 2.5 (2–4)	MS: 2 (2–3)
Graft incorporation			
6 weeks	F: 1.5 (1–3)	F: 2 (1–4)	F: 3 (1–4)
	T: 3 (1–4)	T: 1 (1–2)	T: 2 (1–2)
12 weeks	F: 2.5 (2–3)	F: 3 (1–4)	F: 1.5 (1–4)
	T: 2.5 (1–3)	T: 2.5 (1–3)	T: 2.5 (1–4)
Bone tunnel healing			
6 weeks	F: 1.5 (1–3)	F: 1 (1–3)	F: 1.5 (1–4)
	T: 2.5 (2–4)	T: 3 (1–4)	T: 1 (1–1)
12 weeks	F: 2 (2–4)	F: 2.5 (2–4)	F: 1.5 (1–2)
	T: 1 (1–4)	T: 2.5 (1–3)	T: 1.5 (1–4)
Cell density at mid-substance (cell number in ROI of 0.2 mm × 0.2 mm)[c]			
6 weeks	82 (80–122)	111 (98–156)	126 (98–155)
12 weeks	105 (85–111)	137 (106–142)	109 (77–122)

ACLR, anterior cruciate ligament reconstruction; GHK-Cu, glycyl-histidyl-lysine-copper (II); ROI, region of interest.[a]The results of the histological scoring are shown as the individual score at femoral tunnel (F), graft mid-substances (MS), and tibial tunnel (T). The median score with range for each experimental group is presented. Lower score indicates better healing. Kruskal–Wallis test shows no significance difference between treatment groups in all three scores ($p > 0.05$).[b]In all treatment groups, the graft was less degenerative in tibial tunnel than in femoral tunnel ($p = 0.01$, Wilcoxon signed rank test) and intra-articular mid-substance ($p = 0.01$).[c]Image analysis of cell density at the graft mid-substance was performed in H&E-stained images. Under 400× magnification, three squares of 0.2 mm × 0.2 mm were counted within the section showing mid-substance region from each rat, and the counts from three ROI were averaged. Median value with range from each experimental group is presented. There was increased cell density with increased dose of GHK-Cu at 6 weeks post-operation, but no statistical significance was found ($p = 0.053$, two-way ANOVA).

healing phase, it is supported by evidence that the graft undergoes necrosis and demonstrates hypocellularity, especially in the center of the graft.[2] Cytokines such as TNF-α and interleukin 1-β are released as a consequence of necrosis, which then trigger the expression of growth factors for cell migration, proliferation, extracellular matrix synthesis, and revascularization.[2] In the 0.3 mg/ml GHK-Cu group, there was increased expression of angiogenic factor (Fig. S1), bone mophogenetic protein (Fig. S2), and increased collagen synthesis (Fig. S3) at 6 weeks post-operation. The tissue remodeling phase starts with cell-mediated restructuring of the extracellular matrix as an adaptive response to mechanical loading on the tendon graft. However, rigorous re-cellularization induced by a high dose of GHK-Cu (3 mg/ml) may antagonize the protective effect of GHK-Cu on graft healing, as excess cell infiltration would inevitably weaken the graft integrity. Hence, instead of cell number, it was speculated that cell types and their activities to mediate matrix remodeling may be more crucial for the success of graft healing. The recruited cells need to distribute evenly inside the tendon graft to mediate pericellular matrix remodeling and hence graft remodeling as a whole; these cells need to synthesize new matrix components and degrade unfit matrix components. Interestingly, GHK-Cu has been reported to stimulate synthesis of MMPs[15] and tissue inhibitors of MMPs,[16] which further suggests GHK-Cu may promote graft remodeling by regulating cellular activities. On the contrary, our preliminary findings showed that there was decreased MMP1 expression in tendon graft in GHK-Cu treated groups at 6 weeks post-operation (Fig. S4). It is therefore important to further characterize temporal cellular responses to GHK-Cu and determine the underlying mechanisms during graft remodeling during future studies, so as to devise the time frame and dosage in order to modulate specific biological processes.

The failure load of the femur–graft–tibia complex did not improve and all grafts failed mid-substance in the present study. It was possible that the graft ligamentization process was not complete at 6 weeks post-operation and active tissue remodeling may result in mechanical weakness on the graft mid-substance. Moreover, to avoid trauma caused by repeated intra-articular injection, GHK-Cu treatment was completed 5 weeks post-operation in this study. The treatment effects were evident at 6 weeks post-operation immediately after the treatment regime at weeks 2–5. Due to fast intra-articular clearance of GHK-Cu, discontinuing GHK-Cu supply may lead to wash-out of treatment effects from 6 to 12 weeks post-operation, resulting in insignificant differences among experimental groups

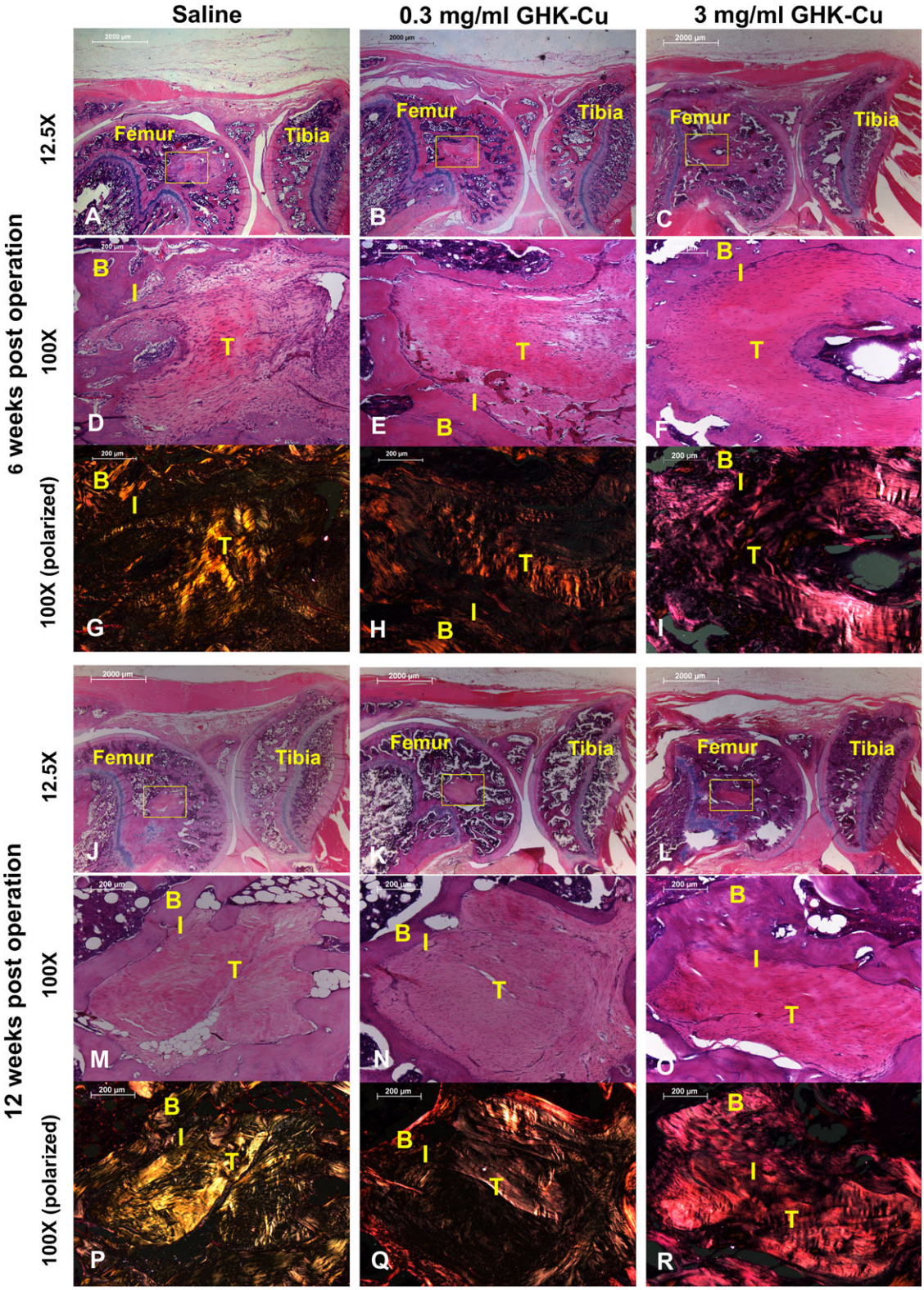

Figure 3. H&E-stained femoral tunnel under bright field and polarized illumination for the evaluation of the effect of GHK-Cu on ACLR rat knees at (A-I) 6 weeks and (J-R) 12 weeks post-operation. T: tendon graft; B: bone; I: bone-tendon interface. Squares marked in the 12.5× images are shown as high power images at 100× magnification in the same column.

after 12 weeks post-operation. Although graft healing was improved by 0.3 mg/ml GHK-Cu, experimental groups demonstrated no significant changes in LII. As ACL in situ force during walking/descending stairs approached 7.8%/20% of ACL ultimate load in humans,[27] an even lower loading force in ACL may be involved for quadrupedal rat. This may be the reason why the walking gait of the rats was not significantly

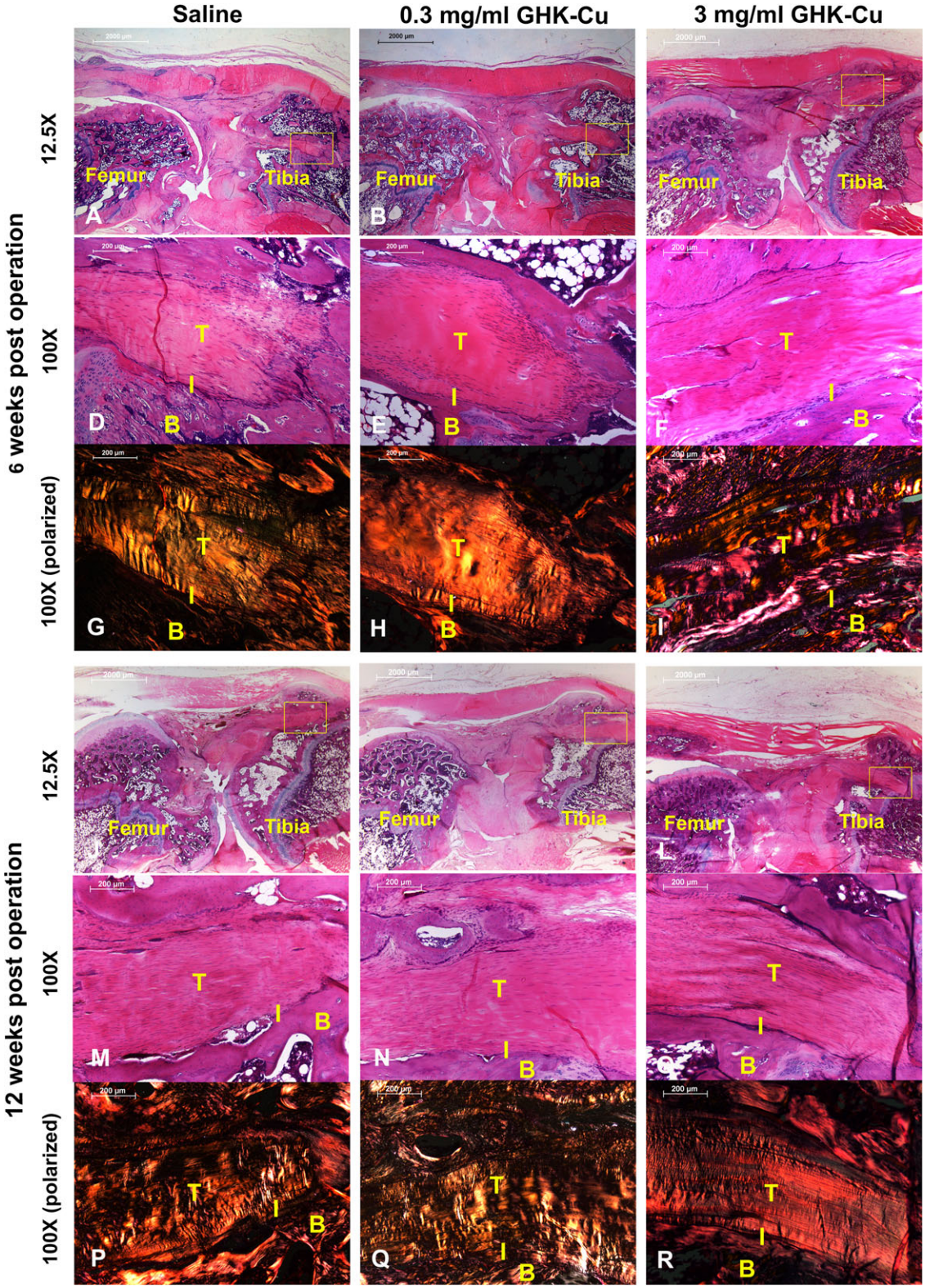

Figure 4. H&E-stained tibial tunnel under bright field and polarized illumination for the evaluation of the effect of GHK-Cu on ACLR rat knees at (A-I) 6 weeks and (J-R) 12 weeks post-operation. T: tendon graft; B: bone; I: bone-tendon interface. Squares marked in the 12.5× images are shown as high power images at 100× magnification in the same column. Graft integrity was best preserved in low dose GHK-Cu group (E and H). There was a bony layer surrounding the graft in GHK-Cu groups (E and F), whereas in control group there was a leaky tunnel interface (D).

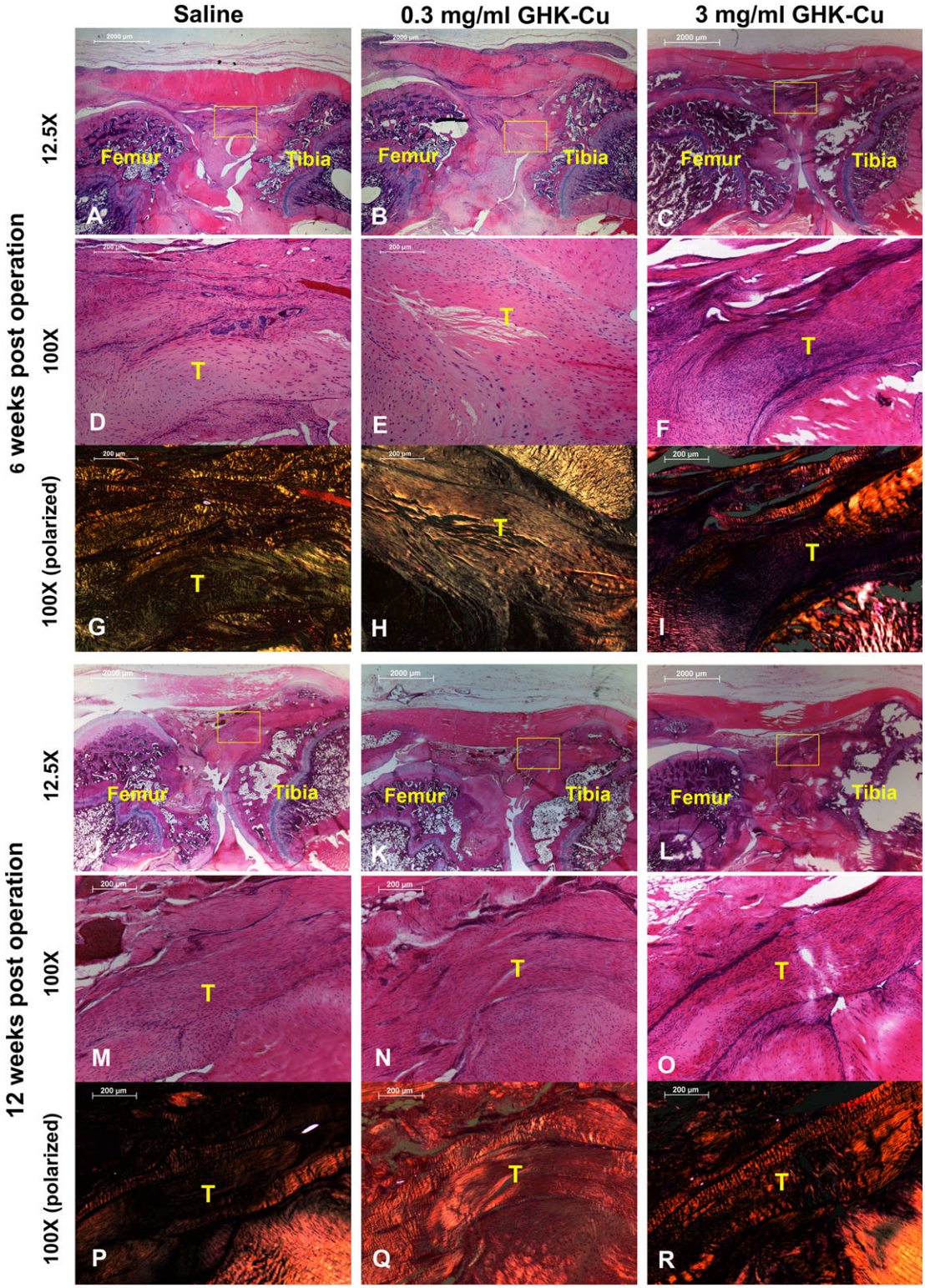

Figure 5. H&E-stained intra-articular graft mid-substance under bright field and polarized illumination for the evaluation of the effect of GHK-Cu on ACLR rat knees at (A-I) 6 weeks and (J-R) 12 weeks post-operation. T: tendon graft. Squares marked in the 12.5× images are shown as high power images at 100× magnification in the same column. Rats treated with 3 mg/ml GHK-Cu exhibited highest cell infiltration in the graft mid-substance (F) as compared to (D) saline control group and (E) 0.3 mg/ml GHK-Cu group.

affected with the weakened graft to replace the ACL in rats.

There are some limitations in the current study. Firstly, current delivery methods and the treatment regime require optimization. Trauma resulting from repeated intra-articular injections may lead to sustained neovascularization and inhibited graft survival. After the injection, the amount of GHK-Cu retained in

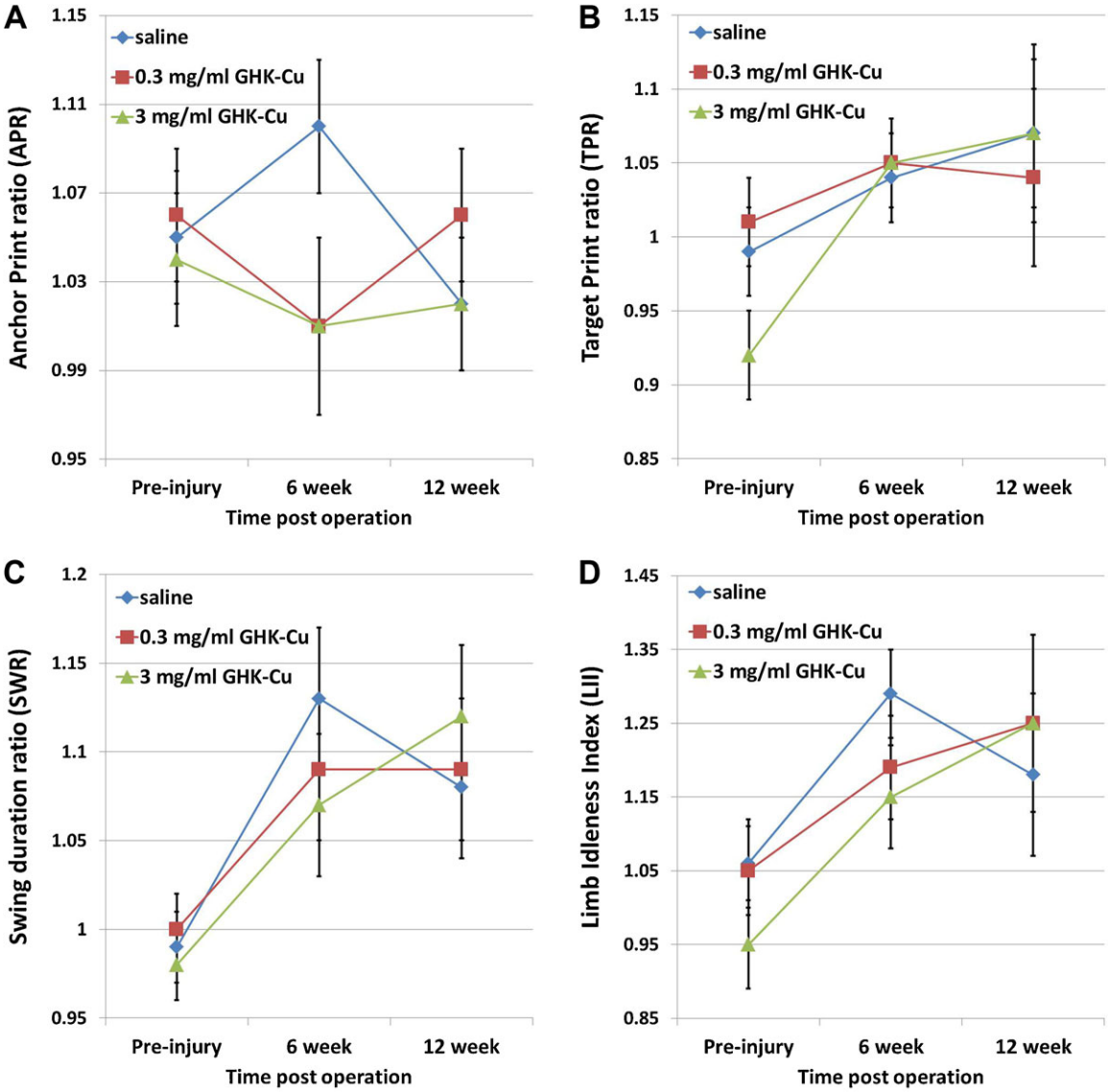

Figure 6. Effect of GHK-Cu on walking gait of ACLR rats. Except (A) APR, other gait parameters including (B) TPR, (C) SWR, and (D) LII were significantly altered ($p = 0.702$, 0.006, <0.001, <0.001, respectively, repeated measures ANOVA). There were no differences among treatment groups in all gait parameters ($p > 0.05$ for APR, TPR, SWR, and LII). Anchor print ratio (APR) is calculated as ratio of paw print intensity in forelimb of contralateral knee to operated knee. Target print ratio (TRP) is calculated as ratio of paw print intensity in contralateral hindlimb to operated hindlimb. Swing duration ratio (SWR) is calculated as the ratio of swing time in operated hindlimb to contralateral hindlimb. Limb Idleness Index (LII) is a product of these three ratios. Limb idleness caused by pain or other functional deficits may lead to increase in these gait parameters which reflect different aspects of gait adaptation for an idled limb.

the joint space and the amount reached the graft was not measured. Thus the actual amount of GHK-Cu available remained unknown. In addition, dosage in terms of drug concentration, volume, frequency, and duration of the treatments may also affect the final outcome. We tested two different GHK-Cu concentrations with fixed injection volume and weekly injections for 4 weeks. Positive treatment effects were observed at 6 weeks post-operation (after cessation of GHK-Cu treatment), while no significant improvement was observed at 12 weeks post-operation. The second limitation of the current study was the discrepancies between rat and human models which may restrict extrapolation of the findings from the experimental model to clinical application, which has been discussed in previous reports.[23] The third limitation was the use of LII when evaluating functional recovery after ACLR in rats, which has previously been discussed.[26] Donor site injury and the presence of bone tunnels resulting from ACLR, and repeated injections may also contribute to gait adaptation to these traumas which were irrelevant to the treatment effect of GHK-Cu. Lastly, the safety of GHK-Cu treatment on cartilage and the surrounding tissues in knee joint was not evaluated.

To conclude, post-operative intra-articular injection of 0.3 mg/ml GHK-Cu transiently enhanced some of the healing outcomes in a rat model of ACLR, but the beneficial effect could not last after the treatment

regime was discontinued. Previous approaches of biological modulation in ACLR mainly utilize protein growth factors and cell supplementation, the use of bioactive small molecules may present a more cost-effective option. Further investigation is necessary to develop drug delivery system for sustained release for these small bioactive molecules.

REFERENCES

1. Ekdahl M, Wang JH, Ronga M, et al. 2008. Graft healing in anterior cruciate ligament reconstruction. Knee Surg Sports Traumatol Arthrosc 16:935–947.
2. Scheffler SU, Unterhauser FN, Weiler A, 2008. Graft remodeling and ligamentization after cruciate ligament reconstruction. Knee Surg Sports Traumatol Arthrosc 16: 834–842.
3. Nebelung W, Becker R, Urbach D, et al. 2003. Histological findings of tendon-bone healing following anterior cruciate ligament reconstruction with hamstring grafts. Arch Orthop Trauma Surg 123:158–163.
4. Seitz H, Menth-Chiari WA, Lang S, et al. 2008. Histological evaluation of the healing potential of the anterior cruciate ligament by means of augmented and non-augmented repair: an in vivo animal study. Knee Surg Sports Traumatol Arthrosc 16:1087–1093.
5. Zaffagnini S, De Pasquale V, Marchesini Reggiani L, et al. 2007. Neoligamentization process of BTPB used for ACL graft: histological evaluation from 6 months to 10 years. Knee 14:87–93.
6. Baer GS, Harner CD, 2007. Clinical outcomes of allograft versus autograft in anterior cruciate ligament reconstruction. Clin Sports Med 26:661–681.
7. Marumo K, Saito M, Yamagishi T, et al. 2005. The "ligamentization" process in human anterior cruciate ligament reconstruction with autogenous patellar and hamstring tendons: a biochemical study. Am J Sports Med 33:1166–1173.
8. Lim JK, Hui J, Li L, et al. 2004. Enhancement of tendon graft osteointegration using mesenchymal stem cells in a rabbit model of anterior cruciate ligament reconstruction. Arthroscopy 20:899–910.
9. Rodeo SA, Suzuki K, Deng XH, et al. 1999. Use of recombinant human bone morphogenetic protein-2 to enhance tendon healing in a bone tunnel. Am J Sports Med 27: 476–488.
10. Murray MM, Spindler KP, Abreu E, et al. 2007. Collagen-platelet rich plasma hydrogel enhances primary repair of the porcine anterior cruciate ligament. J Orthop Res 25: 81–91.
11. Fu SC, Cheuk YC, Yung SH, et al. 2014. Systematic review of biological modulation of healing in anterior cruciate ligament reconstruction. Orthop J Sports Med 2: 2325967114526687.
12. Pickart L, 2008. The human tri-peptide GHK and tissue remodeling. J Biomater Sci Polym Ed 19:969–988.
13. Pickart L, Vasquez-Soltero JM, Margolina A, 2012. The human tripeptide GHK-Cu in prevention of oxidative stress and degenerative conditions of aging: implications for cognitive health. Oxid Med Cell Longev 2012:324832.
14. Simeon A, Wegrowski Y, Bontemps Y, et al. 2000. Expression of glycosaminoglycans and small proteoglycans in wounds: modulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu(2+). J Invest Dermatol 115:962–968.
15. Simeon A, Emonard H, Hornebeck W, et al. 2000. The tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu^{2+} stimulates matrix metalloproteinase-2 expression by fibroblast cultures. Life Sci 67:2257–2265.
16. Simeon A, Monier F, Emonard H, et al. 1999. Expression and activation of matrix metalloproteinases in wounds: modulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu^{2+}. J Invest Dermatol 112:957–964.
17. Siegel RC, Pinnell SR, Martin GR, 1970. Cross-linking of collagen and elastin. Properties of lysyl oxidase. Biochemistry 9:4486–4492.
18. Pohunkova H, Stehlik J, Vachal J, et al. 1996. Morphological features of bone healing under the effect of collagen-graft-glycosaminoglycan copolymer supplemented with the tripeptide Gly-His-Lys. Biomaterials 17:1567–1574.
19. Godet D, Marie PJ, 1995. Effects of the tripeptide glycyl-L-histidyl-L-lysine copper complex on osteoblastic cell spreading, attachment and phenotype. Cell Mol Biol (Noisy-le-grand) 41:1081–1091.
20. Raju KS, Alessandri G, Gullino PM, 1984. Characterization of a chemoattractant for endothelium induced by angiogenesis effectors. Cancer Res 44:1579–1584.
21. Fu SC, Cheng WH, Cheuk YC, et al. 2013. Effect of graft tensioning on mechanical restoration in a rat model of anterior cruciate ligament reconstruction using free tendon graft. Knee Surg Sports Traumatol Arthrosc 21:1226–1233.
22. Courtney P, Doherty M, 2013. Joint aspiration and injection and synovial fluid analysis. Best Pract Res Clin Rheumatol 27:137–169.
23. Fu SC, Cheng WH, Cheuk YC, et al. 2013. Development of vitamin C irrigation saline to promote graft healing in anterior cruciate ligament reconstruction. J Orthop Transl 1:67–77.
24. Chuen FS, Chuk CY, Ping WY, et al. 2004. Immunohistochemical characterization of cells in adult human patellar tendons. J Histochem Cytochem 52:1151–1157.
25. Hall M, Stevermer CA, Gillette JC, 2012. Gait analysis post anterior cruciate ligament reconstruction: knee osteoarthritis perspective. Gait Posture 36:56–60.
26. Fu SC, Cheuk YC, Hung LK, et al. 2012. Limb Idleness Index (LII): a novel measurement of pain in a rat model of osteoarthritis. Osteoarthritis Cartilage 20:1409–1416.
27. Morrison JB, 1970. The mechanics of the knee joint in relation to normal walking. J Biomech 3:51–61.

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