Systematic Review

Current Evidence of Adult Stem Cells to Enhance Anterior Cruciate Ligament Treatment: A Systematic Review of Animal Trials

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Purpose: To systematically review the available preclinical evidence of adult stem cells as a biological augmentation in the treatment of animal anterior cruciate ligament (ACL) injury. **Study Design:** Systematic review. **Methods:** PubMed (MEDLINE) and Embase were searched for the eligible studies. The inclusion criteria were controlled animal trials of adult stem cells used in ACL treatment (repair or reconstruction). Studies of natural ACL healing without intervention, in vitro studies, ex vivo studies, and studies without controls were excluded. Evidence level, methodologic quality, and risk of bias of each included study were identified using previously established tools. **Results:** Thirteen animal studies were included. Six of 7 studies using bone marrow–derived mesenchymal stem (stromal) cells (BMSCs) reported a positive enhancement in histology, biomechanics, and biochemistry within 12 weeks postoperatively. Four studies using ACL-derived vascular stem cells showed a promoting effect in histology, biomechanics, and imaging within 8 weeks postoperatively. Two studies focusing on animal tendon-derived stem cells (TDSCs) and human umbilical cord blood–derived mesenchymal stem cells (hUCB-MSCs) reported promotable effects for the early healing in a small animal ACL model. **Conclusions:** BMSCs, ACL-derived vascular stem cells, TDSCs, and hUCB-MSCs were shown to enhance the healing of ACL injury during the early phase in small animal models. **Clinical Relevance:** Results of clinical trials using adult stem cells in ACL treatment are conflicting, and a systematic review of the current best preclinical evidence is crucial to guide further application.

See commentaries on pages 341 and 343

Anterior cruciate ligament (ACL) injury is among the most common orthopaedic trauma, especially in professional and amateur athletics. Surgical treatment is the most widely applied procedure for young and active patients. The incidence of ACL reconstruction in United States increased from 32.9 per 100,000 person-years in 1994 to 43.5 per 100,000 person-years in 2006.[1] Despite the general positive outcomes of ACL reconstruction, graft failure remains a major clinical problem.[2-5] To enhance the graft healing process, biological augmentation using growth factors, stem cells, and scaffolds has been investigated for more than a decade.[6]

With additional application of adult stem cells in ACL treatment, overall positive outcomes in various in vitro and animal studies indicate the potential possibility of clinical translation.[7] In contrast, several uncontrolled clinical case series reported improvements in knee imaging and function after ACL surgery enhanced by autologous bone marrow stem cells compared to preoperative levels,[8-12] whereas other controlled clinical studies presented no superior outcomes of stem cell–augmented ACL healing compared with stem cell–free controls.[13,14]

Systematic review of animal trials contributes heavily to the decision making, safety, and efficacy of the further clinical translation.[15,16] Whether there is any discrepancy between different characteristics of grafts, stem cells, and application techniques remains to be elucidated. The purpose of this review was to

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systematically summarize the best available evidence in animal studies of adult stem cells as a biological augmentation in ACL treatment. We hypothesize that additional application of several types of stem cells effectively promote ligament healing in animal models.

Methods

Eligibility Criteria

The inclusion criteria for studies consisted of the following:

- Study type: Controlled animal trials, concerning the usage of adult stem cells. Studies included in searched reviews were also tracked.
- Study group: Animals with ACL injuries (native ACL dissection and partial or complete ACL transection).
- Intervention type: ACL surgery with application of adult stem cells. Stem cells were not tested with other biological agents or materials (cell factors, synthetic scaffolds, or artificial ligaments). Same interventions without stem cells were taken as positive controls.
- Outcome assessment: The main outcomes were to detect differences in graft-bone integration, graft maturation, and/or knee function between interventions with/without adult stem cells.
- Language: English.

The exclusion criteria were embryonic stem cell, in vitro, ex vivo, clinical studies, and studies without controls.

Literature Search

A comprehensive search was conducted in the electronic databases PubMed (MEDLINE) and Embase, using the Preferred Reporting Items for Systemic Reviews and Meta-Analyses (PRISMA) checklist and flow diagram.[17] The search strategy was composed of three elements: adult stem cells, ACL and animals (see Appendix Table 1, available at www.arthroscopyjournal.org). To broadly capture all studies, the term *stem cell* was used in the literature search phase, then "pluripotent and embryonic stem cell" studies were excluded in the screening phase. Besides, previously established animal filters for both PubMed and Embase were used to identify all animal studies.[18,19] The final search was performed on May 1, 2017. Reference lists of included papers and top hits from Google Scholar were screened for potentially missed papers.

Study Selection

Studies were initially screened on the abstracts and titles. Full texts were then obtained for all studies matching the inclusion criteria and reviewed to reconfirm the eligibility. The study selection was performed independently by 2 authors (R.G. and L.G.), and disagreement was resolved by discussion among all authors.

Methodologic Quality Assessment and Risk of Basis

Scientific level of effectiveness in animal studies is commonly low, and they were further stratified into 5 ranks based on outcome measures according to the previously published review of biological modulation in ACL surgeries.[20]

A: Quantitative outcome measures analogous to clinical outcome measures (e.g., knee laxity, activity level, and gait)

B: Mechanical test of graft complex strength (ultimate load, linear stiffness) as quantitative outcome measures

C: Biochemical measurement as quantitative outcome measures

D: Semiquantitative imaging/histologic assessment

E: Qualitative imaging/histologic assessment

The quality (methodologic score) of animal studies was assessed according to the criteria of the checklist from Fu et al.[20] (see Appendix Table 2, available at www.arthroscopyjournal.org). Studies with $\geq$5 points were recorded as "good methodologic quality" and studies $<$5 points were graded as "poor methodologic quality." The SYstematic Review Centre for Laboratory animal Experimentation's risk of bias tool (SYRCLE's RoB tool), based on the Cochrane risk of bias tool, was used to assess the internal validity of animal studies.[21] Ten signaling questions were used for judging 6 types of bias (selection, performance, detection, attrition, reporting, and other bias). Because of the methodologic issues inherent in animal studies, as well as the significant risks for selection, performance and detection biases, 2 more questions regarding randomization and blinding were added (see Appendix Table 3, available at www.arthroscopyjournal.org). The assessments were performed by 2 authors (R.G. and L.G.) independently. Any discrepancy was discussed with the senior author (B.X.) for the final decision.

Data Synthesis

The following data were extracted from the included studies, including animal species, number in treated and control groups, methods of allocation to treatment group, types of intervention, duration of follow-up, methods to assess efficacy (blinded assessment), and results of treatment. The source of cells, number of applied cells, and application methods were recorded. For each study, we defined whether a positive (beneficial effect) or negative (no difference or deleterious effect) result was reported. Compared with stem cell free controls, significant improvement in histology, biomechanics, imaging, or biochemistry in the stem cell–treated group is defined as "positive effect." In contrast, no significant difference or deterioration is defined as "negative effect."

Results

Study Selection

A total of 206 studies were obtained from the databases after the removal of the duplicates. After screening of the titles and abstracts, 177 articles were filtered out because of irrelevance with in vivo ACL or adult stem cells. Finally, 13 studies with full text were included in this review after applying inclusion and exclusion criteria (Fig 1).

Methodologic Quality Assessment and Risk of Bias

In the 13 included studies, the average methodologic score was 4.4 ± 1.8 (range 1-8) (Fig 2 and Appendix Table 4, available at www.arthroscopyjournal.org). Nine studies were ranked B with mechanical test. Their methodologic scores ranged from 3 to 7, with 5 studies scoring ≥5 points. One study was ranked C with quantitative biochemical outcome, and the study quality was low (score 3).[22] Two studies were ranked D with semiquantitative outcome measures, and their methodologic score were 3 and 7, respectively. Only 1 study ranked E with the lowest score 1, indicating a low quality.[23] In total, only 6 studies were scored 5 or higher in methodologic quality assessment and were appraised with a satisfactory study quality.

Risk of bias analysis reported 3.4 ± 1.1 of 12 entries in the included studies (range 1-5) (Fig 3). Several items were scored as unclear, indicating the overall high risk of bias and poor reliability of the included studies. Ten of the 13 studies stated randomization at any level, but none or not enough randomization information could be found regarding selection, performance, and detection bias. Nine of the 13 studies reported blinding at any level, which referred to blinded result assessment.

Study Characteristics

The general characteristics of the included studies are summarized in Table 1.

Injury Models. Among the included studies, varieties of animal models have been investigated. Twelve studies utilized small animals (6 in rabbits and 6 in rats) to create the ACL injury model, and large animal (dog) was used in only 1 study.[24] Moreover, 2 types of ACL injury were employed: ACL resection (rabbit 6/6, rat 4/6, dog 1/1) and ACL partial transection (rat 2/6). Both male and female animals were applied.

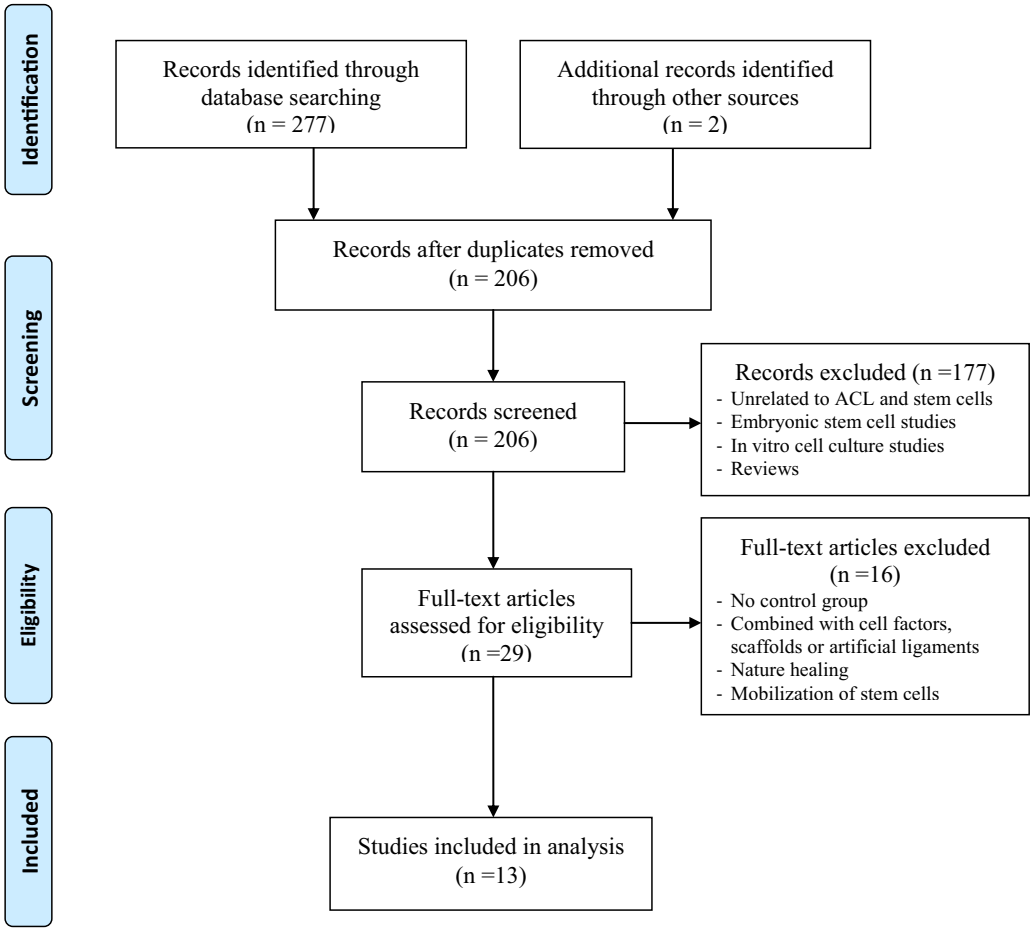

Fig 1. Search strategy according to PRISMA guidelines. Thirteen studies were identified for inclusion.

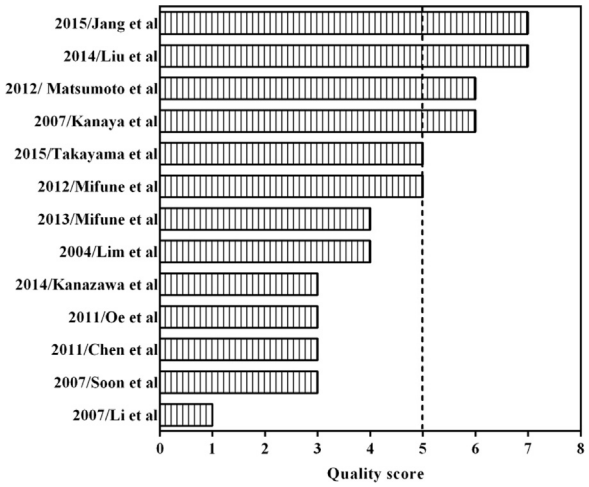

Fig 2. Quality score of the included studies. The dotted line represents the cut-off score of quality: ≥5 points is regarded as good methodologic quality and <5 points is regarded as poor methodologic quality.

ACL Treatments. Both uni- and bilateral interventions were executed. Graft reconstruction after resection was carried out on 11 studies and stem cell therapy after partial transection was performed in 2 studies. Concerning the graft source, allograft was used in 2/11 and autograft in 9/11. In addition, the grafts were harvested from different sources, including semitendinosus (3/11), Achilles tendon (2/11), long digital extensor tendon (1/11), and flexor digitorum longus tendon (5/11).

Cell Parameters. Five types of adult stem cells were isolated from different sources, including bone marrow—derived mesenchymal stem (stromal) cell (BMSC), ACL-derived $CD34^+$ cell, tendon-derived stem cells (TDSCs), and human umbilical cord blood—derived mesenchymal stem cells (hUCB-MSCs). BMSC was the commonest type in the included articles (7/13). All BMSCs were isolated from autologous bone marrow of the ilium or femur, and the applied cell number ranged from 7.5×10^5 to 1×10^7. ACL-derived $CD34^+$ cell, a type of vascular stem cell, was applied in 4 studies. Matsumoto et al.[24] directly tested autologous ACL rupture tissue to avoid isolation, culture, and expansion of $CD34^+$ cells. The remaining 3 studies in rats isolated $CD34^+$ cells from xenogeneic (human) ACL-ruptured tissue.[25-27] The applied cell number was 5×10^5 per rat. Besides, hUCB-MSCs and TDSCs were used in only 1 study separately and cells were isolated from the human umbilical cord blood and the allogeneic patellar tendon.[28,29] Various methods concerning cell isolation, culture, and counting were applied in included studies. Regarding the application method, whole grafts were coated with cells or cell sheet in 5 studies. Cells were coated to ends of graft and/or injected into the bone tunnel in 5 studies, and injection into articular cavity was adopted in 4 studies. Concerning efficacy among different cell application methods, only 1 study compared the outcome of autograft reconstruction between cell sheet technique and direct cell injection with ACL-derived $CD34^+$ cells.[26]

Outcomes

Twelve studies reported positive outcomes of adult stem cells in tendon maturation or tendon-bone healing, and only 1 study described negative results for BMSC-enhanced ACL reconstruction with autografts in rabbits. Of note, the follow-up period was less than 8 weeks in most animal trials.

BMSCs and BMCs. In rabbits, Lim et al.[30] presented that BMSC-enhanced ACL reconstruction with hamstring tendon autografts yielded large areas of chondrocytes at the tendon-bone junction at 2 weeks and a mature zone of cartilage by 8 weeks, whereas

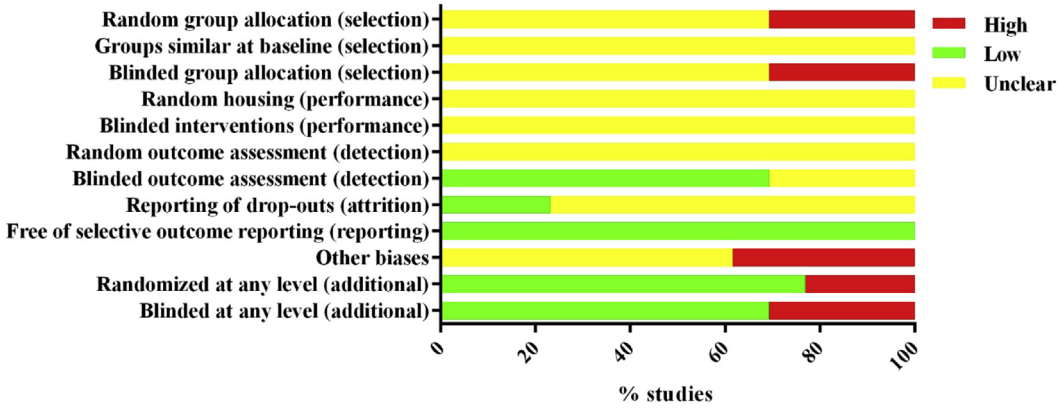

Fig 3. Risk of bias of the included studies.

Table 1. Characteristics of the Included Studies

Study	Study Characteristics						Stem Cells Characteristics				
Year/Author	Species	N	Protocol	Surgery/Graft	Follow-up, Min-Max	Outcome Measures	Source	Number	Application Method	Finding	Evidence Level
2004/Lim et al.[30]	Rabbit	48	Au + BMSCs vs Au	ACLR/ST	2 wk-4 wk-8 wk	Histology, mech	Autologous marrow from ilium	3-4 × 10^6	Coated to whole graft	Positive	B
2007/Li et al.[23]	Rabbit	36	All + BMSCs vs All	ACLR/AT	3 wk-6 wk-12 wk	Histology	Autologous marrow from ilium	7.5 × 10^5	Coated to whole graft	Positive	E
2007/Soon et al.[31]	Rabbit	36	All + BMSCs vs All	ACLR/AT	2 wk-4 wk-8 wk	Histology, mech	Autologous marrow from ilium	4 × 10^6	Coated to both ends of graft	Positive	B
2011/Chen et al.[22]	Rabbit	18	Au + BMSCs vs Au	ACLR/EXT	1 wk-4 wk	Histology, biochem	Autologous marrow from ilium	1 × 10^6	Injection into femoral tunnel	Positive	C
2014/Kanazawa et al.[35]	Rabbit	14	Au + BMSCs vs Au	ACLR/ST	4 wk-8 wk	Histology	Autologous marrow from ilium	1 × 10^7	Transplanted between tibial bone pit and tendon	Negative	D
2007/Kanaya et al.[33]	Rat	98	BMSCs vs PBS	ACLT partial	1 wk-2 wk-3 wk-4 wk	Histology, mech	Allogeneic marrow from tibiae	1 × 10^6	Injected into knee after surgery	Positive	B
2011/Oe et al.[32]	Rat	—	BMCs vs BMSCs vs Saline	ACLT partial	0 wk-2 wk-4 wk	Histology, mech, biochem	Marrow from femur	1 × 10^6	Injected into knee 1 wk after surgery	Positive	B
2012/Matsumoto et al.[24]	Dog	20	Au + ACL ruptured tissue vs Au	ACLR/Flex	2 wk-4 wk	Histology, mech, imaging	Autologous ACL ruptured tissue	—	Sutured to tibial end of the graft	Positive	B
2012/Mifune et al.[25]	Rat	40	Au + $CD34^+$ cell vs Au + NS cell vs Au+ $CD34^-$ cell vs Au + PBS	ACLR/Flex	0 wk-2 wk-4 wk-8 wk	Histology, mech, imaging	Xenogeneic (human) ACL ruptured tissue	5 × 10^5	Injected into knee 3 days after surgery	Positive	B
2013/Mifune et al.[26]	Rat	27	Au + $CD34^+$ cell sheet vs Au + $CD34^+$ cell injection vs Au	ACLR/Flex	2 wk-8 wk	Histology, mech	Xenogeneic (human) ACL ruptured tissue	5 × 10^5	1. Cell sheet wrapped around whole graft; 2. Injected into knee after surgery	Positive	B
2015/Takayama et al.[27]	Rat	60	Au + $CD34^+$ cell vs Au	ACLR/Flex	2 wk-4 wk-8 wk	Histology, mech	Xenogeneic (human) ACL ruptured tissue	5 × 10^5	Cell sheet wrapped around whole graft	Positive	B
2014/Lui et al.[29]	Rat	97	Au + TDSCs vs Au	ACLR/Flex	4 wk-8 wk	Histology, mech, imaging	Allogeneic Patellar tendon	—	Cell sheet wrapped and sutured around whole graft	Positive	B
2015/Jang et al.[28]	Rabbit	30	Au + hUCB-MSCs vs Au	ACLR/ST	4 wk-8 wk-12 wk	Histology, imaging	Xenogeneic (human) umbilical cord blood	4 × 10^6	Injected into femoral and tibial tunnel	Positive	D

ACLR, anterior cruciate ligament reconstruction; ACLT, anterior cruciate ligament transection; All, allograft; AT, Achilles tendon; Au, autograft; Biochem, biochemical assay; BMCs, bone marrow cells; BMSCs, bone marrow–derived mesenchymal stem (stromal) cells; CD, cluster of differentiation; CST, conservative treatment; DB, double-bundle; EXT, extensor; Flex, flexor tendon; FS, functional score; HRT, healing response technique; HS, hamstring tendon; hUCB-MSCs, human umbilical cord blood–derived mesenchymal stem cells; Mech, mechanical test; mo, month; NC, noncultivated; NS, nonsorted; PBS, phosphate-buffered saline; PE, physical examination; ST, semitendinosus; TDSCs, tendon-derived stem cells; wk, week; y, year.

the cell-free treatment showed only the mature scar tissue. Similar histologic findings were also found in ACL reconstruction with BMSC-coated Achilles tendon allografts in rabbits.[31] Similarly, Li et al.[23] found that BMSCs accelerated the cellular infiltration into ACL and enhanced collagen deposition by 12 weeks. Moreover, autologous BMSCs were also proved to promote the expression of collagen type II and aggrecan, and to reduce the expression of Runx2 and osteocalcin in the bone tunnel at 4 weeks, indicating an improved tendon-bone healing.[22] Besides, in ACL repair study in rats, Kanaya et al. reported a significantly higher histologic score in the BMSC(+) group than the BMSC(−) group,[25] and Oe et al. found that both BMC and BMSC groups were significantly higher in the tenocyte score, collagen score, and collagen type I than the saline control group at 4 weeks.[32,33] Furthermore, the number of nuclei of fibroblasts, secreting collagen type I, increased significantly in the BMC group compared with that in the BMSC group.[34] However, Kanazawa et al.[35] revealed no significant histologic differences at the tendon-bone interface between the BMSC and the control group, using a novel ACL reconstruction technique without a tibial tunnel.

All biomechanical results in the included studies supported the positive effect of BMSC-enhanced ACL treatment. Lim et al.[30] presented that ACL reconstruction with BMSCs in rabbits significantly improved the failure load and stiffness within 8 weeks. Similarly, BMSC-enhanced graft was proved to generate significantly higher load-to-failure rates and lower Young's modulus than controls after 4 and 8 weeks in rabbits.[31] In rats, the ultimate failure load of the femur-ACL−tibia complex in the BMSC(+) group was significantly higher than that in the BMSC(−) group at 4 weeks after surgery.[33] Similarly, the tensile strength of the BMC and BMSC groups were also significantly higher than in the saline group after 2 and 4 weeks.[32] Besides, no significant differences were observed between the BMC and BMSC groups after 2 and 4 weeks.

Two studies discovered the positive effect of BMSCs using a biochemical analysis of gene expression and cell factor level. Chen et al.[22] found the upregulated expression of chondrogenic genes and downregulated expression of osteogenic genes, representing the formation of fibrocartilage and the enhanced tendon-bone integration.[31] Oe et al.[32] found that both the BMC and BMSC groups were higher in transforming growth factor (TGF)-beta1 levels and expression of TGF-beta1 mRNA than the saline group after 4 weeks, indicating the acceleration of both intrinsic healing in the ACLs and extrinsic healing from the articular cavity.[36]

ACL-Derived CD34$^+$ Cells. Matsumoto et al.[24] tested the effect of ACL ruptured tissue, containing abundant vascular stem cells, for the maturation of bone-tendon integration in a dog model of ACL reconstruction with autografts. An induced endochondral ossification-like integration with enhanced angiogenesis and osteogenesis was seen in the grafts of tissue-treated group at week 2. Two rat studies using ACL-derived CD34$^+$ cells detected a significant improved expression of collagen type II and enhanced angiogenesis and osteogeneisis in the ACL-derived CD34$^+$ cell−treated group at week 2.[25,26] Similarly, Takayama et al.[27] observed that the ACL-derived CD34$^+$ cell−treated group yielded a significant improvement in intrinsic vascularization, healing, and maturation of the grafted tendon than controls. Biomechanical results in all studies indicated the enhanced tendon-bone healing by ACL-derived CD34$^+$ cells, as shown by the significant improved ultimate failure load at 4 and 8 weeks.[24-27] Imaging assessment using micro-CT showed a significantly smaller tibial bone tunnel and higher bone volume/tissue volume in ACL-derived CD34$^+$ cell−treated group, indicating a better peri-graft bone mass increase.[25]

TDSCs and hUCB-MSCs. Lui et al.[29] performed an allogeneic TDSC-enhanced ACL reconstruction in rats. Histologic assessment showed better bone-tendon integration and higher intra-articular graft integrity with lower cellularity and vascularity, better cell alignment, and higher collagen birefringence in the TDSC group. The ultimate load at week 2 and stiffness at week 6 were significantly higher in the TDSC group. Furthermore, significantly higher tunnel bone mineral density and bone volume/total volume at tibial and femoral tunnels were also found in the TDSC group.

hUCB-MSC-enhanced ACL surgery by Jang et al.[28] did not detect any evidence of immune rejection. Histologic scores of bone-tendon healing were significantly higher in the MSC-treated group at 4, 8, and 12 weeks, showing a superior fibrocartilaginous healing with mature chondrocytes. Micro-CT assessment at 12 weeks showed significantly smaller tibial and femoral tunnels in the MSC-treated group compared with the cell-free control.

Discussion

The principal finding of this systematic review is that the adult stem cells effectively promote the early healing of ACL in animal models. In the enrolled studies, 6 out of 7 BMSC studies reported positive enhancing effect up to 12 weeks follow-up, and all 4 ACL-derived vascular stem cells studies showed superior promoting effect within 8 weeks postoperatively. Two studies using TDSCs and hUCB-MSCs also presented the

promising evidence in graft healing at 8 and 12 weeks after surgery, respectively.

Stem cells include both pluripotent (embryonic and induced pluripotent cells) and adult stem cells, possessing the potential of differentiation and self-renewal. Embryonic stem cells have the pluripotent capacity to generate into any mature cell of the three germ lines; however, their research is impeded by restrictions of the ethical concerns and uncontrollable malignant differentiation.[37] Adult stem cells, including MSCs, are multipotent stem cells with the gene activation potential to differentiate into discrete cell types and are commonly chosen as augmentations for cell therapy, regenerative medicine, and tissue engineering.[38,39]

Three kinds of MSCs were investigated in this review including BMSCs, TDSCs, and hUCB-MSCs. Six studies reported the improved histologic and biomechanical performance of transected ligament or transplanted graft in both bone tunnel and articular cavity, and showed increased expression of chondrogenic genes and TGF-beta1, indicating the possible potential of BMSCs in stimulating human ACL healing.[22,23,30-33] Both autologous and allogeneic grafts have been tested and displayed similar positive results. Interestingly, only one study concerning the use of BMSCs in a novel ACL reconstruction technique revealed a negative result.[35] However, the failure is likely attributed to such nonconventional technique without a tibial tunnel, which results in small contacting area and stress. Besides, BMCs, consisting of BMSCs, hematopoietic stem cells, other stem cells, and differentiated cells and soluble factors, showed comparable (even better) outcomes than BMSC-enhanced ACL repair in rats. Of note, imaging evidence of the effectiveness of stem cell–augmented ACL repair is lacking in all BMSC studies, and no studies have ever been performed in large animal models. Importantly, only outcomes of early healing phase (within 4 weeks) and proliferation phase (4-12 weeks) were followed up, and data are still lacking regarding the performance of adult stem cells in the later ligamentization phase (after 12 weeks).[40]

Another promising cell type, ACL-derived vascular stem cells, was experienced in 4 animal trials, highlighting the biological efficacy to enhance both graft maturation and tendon-bone integration.[24-27] Three of these 4 studies are consistent in animal model (rat), transplanted graft (autologous flexor digitorum longus tendon), and applied cell number (5×10^5), making the results more convincing and comparable.[25-27] Moreover, Mifune et al.[26] compared different cell application methods and figured out that ACL-derived $CD34^+$ cell sheet wrapped grafts could provide a promising strategy to revitalize tendon autografts after the ACL reconstruction.

Two novel multipotent cell populations, TDMCs and hUCB-MSCs, have recently displayed great capability in tissue engineering and regenerative medicine. In the present review, these 2 cell types showed a stimulation effect in ACL reconstruction with autografts. Meaningfully, the methodologic quality of these studies were scored as high as 7 of 8, depicting the prospects of TDMCs and hUCB-MSCs as a new biological modulation in ACL surgery.

Autograft and Allograft

"Autograft or allograft" is a topic with various controversies in clinical practice for ACL reconstruction. A previous animal study has revealed that allografts yielded a similar but slower healing than autografts after implantation.[41] A recent meta-analysis including 56 clinical articles reported a significantly improved knee stability with autografts compared with allografts.[42] Additionally, another cohort study reported a significantly higher retear rate in young patients treated with allograft.[43] In the present review of animal studies, both graft substitutes were investigated: autograft (9/11) and allograft (2/11). To compare the outcome of autografts and allografts for BMSC-enhanced ACL reconstruction, 2 series studies by E. H. Lee's group did not detect significant difference histologically and biomechanically in rabbits.[30,31] Only stiffness was significantly higher in BMSC-enhanced ACL treatment with autografts. Thus, more in-depth preclinical and clinical studies are warranted to elucidate the influence of graft sources for ACL treatment with additional stem cells.

Cell Parameter and Administration Techniques

The optimal cell parameter and administration method are unclear for the stem cell–enhanced ACL treatment. A horse study comparing different application routes of MSCs showed intralesional injection more effective than intravenous injection for local homing of MSCs.[44] Furthermore, without using additional scaffolds, the advanced contractile cell sheet technique harvests intact sheets from temperature-responsive culture dishes by simple temperature changes, and presents significant advantages to minimize the intra-articular off-target deposition of the additional applied stem cells.[45] ACL-derived $CD34^+$ cell sheet was also shown to be more efficient than direct stem cell injection for perigraft cell deposition, favoring the early tendon-bone healing and graft maturation.[26] Regarding the ideal cell concentration and number, the number of applied ACL-derived $CD34^+$ cells was consistent among included studies (5×10^5 per rat), but methods of cell isolation and culture were particularly varied, yielding diverse numbers of BMSCs ranging from 7.5×10^5 to 1×10^7.[25-27] Previous studies reported that free bodies of scar tissues occurred in 1×10^7 BMSC-injected knees, whereas 1×10^6 BMSC-injected group remained normal.[46,47] Further studies

are needed to determine the appropriate cell parameters and administration modalities in the stem cells—enhanced ACL treatment.

From Bench to Bedside

Based on the in vitro and animal studies, preliminary clinical trials of adult stem cells—enhanced ACL treatment have been explored. Several case series presented improved outcomes in imaging and knee function.[8-12] However, these studies with a generally low evidence level are insufficient to validate the potency of these adjunct treatments without proper controls. So far, only 2 controlled clinical trials are available in the literature.[13,14] One study from Silva et al. recruited 43 adult patients with ACL injury treated with autologous ACL reconstruction. Three milliliters of autologous noncultured BMSCs were injected into the femoral end of the graft and femoral tunnel. No radiographic difference was found between the noncultivated BMSC-treated group and control group treated without BMSCs at 3 months postoperatively. The other study tested a novel Healing Response Treatment (HRT), similar to the marrow stimulation for cartilage repair, to recruit the marrow stem cells from the drilled marrow cavity. Compared with conservative treatment, one third of HRT-treated patients necessitated definitive ACL reconstruction, and the rest did not show any improvement in knee function, physical examination, and MRI after 4 years' follow-up. Such negative results might attribute to the low cell concentration in uncultured BMSC study and high proportion of complete ACL ruptures and high average age in HRT study. Remarkably, cautious interpretation of these clinical results is demanded because of the limited sample size, undefined cell number, various patient selection criteria, and unstandardized surgical techniques.

Limitations

The major limitation of this systematic review is the quality of the included studies. Only 6 of the 13 included studies are of high methodologic quality, and majority items for risk of bias are judged as unclear. Another limitation is that publication bias compensation was not performed considering the low methodologic quality of included studies. Moreover, the considerable heterogeneity of the included studies, for example, injury model, graft type, surgical technique, cell preparation and administration, and outcome assessment,[48,49] makes the interstudy comparison difficult and hinders drawing additional convincing conclusions.

Conclusions

BMSCs, ACL-derived vascular stem cells, TDSCs, and hUCB-MSCs were shown to enhance the healing of ACL injury during the early phase in small animal models.

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Appendix Table 1. Search Strategy

PubMed	
Component 1: Stem cell	Stem Cell[MeSH Terms] OR Stromal Cell[MeSH Terms] OR Bone Marrow [MeSH Terms] OR Stem cell[tiab] OR Progenitor cell[tiab] OR Stromal cell[tiab] OR Marrow[tiab] OR Marrow[tiab] OR Bone marrow[tiab] OR Red marrow[tiab] OR Yellow Marrow[tiab]
Component 2: Anterior cruciate ligament	Anterior Cruciate Ligaments[MeSH Terms] OR Cranial Cruciate Ligament [tiab] OR Anterior Cruciate Ligament[tiab] OR Anterior Cranial Cruciate Ligament[tiab] OR ACL[tiab]
Component 3: Animal	Search filter for animal studies [Hooijmans 2010]
Embase	
Component 1: Stem cell	exp stem cell/ or exp progenitor cell/ or exp stromal cell/ or exp bone marrow/ or exp marrow/ or (Stem cell or stem cells or Progenitor cell or progenitor cells or mother cell or precursor cell or Stromal cell or stromal cells or Bone marrow or Marrow or red marrow).ti,ab.
Component 2: Anterior cruciate ligament	exp anterior cruciate ligament/ or (ACL or anterior cranial cruciate ligament).ti,ab.
Component 3: Animal	Search filter for animal studies [de Vries 2014]

NOTE. Source: PubMed (US National Library of Medicine, National Institutes of Health) from 1946 to April 30, 2017; Embase 1974 to 2017 April 30 (under OvidSP).

Appendix Table 2. Assessment Criteria of Methodologic Quality of the Included Studies

Criteria	Score	Remarks
1. Unit of sample	Unilateral: 1 Bilateral: 0	Studies with bilateral operation may regard each limb as an independent sample and assign them to different treatment groups. Unless the sample unit was specified as number of animals instead of number of limbs, animal studies with unilateral operation with animal as sample unit will be better
2. Standardization of surgical procedure	Yes: 1 No: 0	Standardization of surgical procedure includes the descriptions about graft harvest, approaching intra-articular region, drilling tunnels, graft tensioning, and fixation method. Studies with these descriptions would be regarded as standardized procedures as major surgical variables are controlled.
3. Description of postoperative complications and follow-up	Yes: 1 No: 0	Records of postoperative complications such as broken sutures, wound infection, and early death are regarded to have better study quality.
4. Report of failure mode in mechanical test	Yes: 1 No: 0	Since most ACLR animal studies used mechanical testing as the primary outcome, report of failure mode is important to reveal the quality and the implications of the mechanical tests.
5. Variation (ratio of SD to mean)	$<50\%$: 1 $>50\%$: 0	For quantitative measure, large standard deviation may imply poor precision or large intragroup variations, which is regarded to have lower study quality.
6. Statistical method	Appropriate: 1 Questionable: 0	Questionable statistical analyses include the use of unpaired test for paired samples, parametric test for ordinal data with a few ranks, the use of unadjusted multiple comparisons instead of ANOVA or Kruskal-Wallis test.
7. Description of selection region of interest	Yes: 1 No: 0	For histology/imaging outcome measure, description of systematic/ random sampling of region of interest is considered to provide better study quality.
8. Semiquantitative scoring/image analysis	Yes: 1 No: 0	For histology/imaging outcome measure, implementation of scoring systems or image analysis protocol is considered to provide better study quality

ACLR, anterior cruciate ligament repair; ANOVA, analysis of variance; SD, standard deviation.

Appendix Table 3. Risk of Bias in the Included Studies

	Risk of Bias		Lim et al., 2004[30]	Kanaya et al., 2007[33]	Li et al., 2007[23]	Soon et al., 2007[31]	Chen et al., 2011[22]	Oe et al., 2011[32]	Matsumoto et al., 2012[24]	Mifune et al., 2012[25]	Mifune et al., 2013[26]	Kanazawa et al., 2014[35]	Lui et al., 2014[29]	Jang et al., 2015[28]	Takayama et al., 2015[27]
1	Selection bias	Random group allocation	?	H	H	?	H	?	H	?	?	?	?	?	?
2	Selection bias	Groups similar at baseline	?	?	?	?	?	?	?	?	?	?	?	?	?
3	Selection bias	Blinded group allocation	?	H	H	?	H	?	H	?	?	?	?	?	?
4	Performance bias	Random housing	?	?	?	?	?	?	?	?	?	?	?	?	?
5	Performance bias	Random interventions	?	?	?	?	?	?	?	?	?	?	?	?	?
6	Detection bias	Random outcome assessment	?	?	?	?	?	?	?	?	?	?	?	?	?
7	Detection bias	Blinded outcome assessment	L	L	?	L	?	L	L	L	L	?	L	L	?
8	Attrition bias	Reporting of drop-outs	L	?	L	?	?	?	?	?	?	?	?	L	?
9	Reporting bias	Free of selective outcome reporting	L	L	L	L	L	L	L	L	L	L	L	L	L
10	Other	Other biases	?	?	H	H	H	H	?	?	?	H	?	?	?
11	Additional	Randomized at any level	L	L	H	L	L	H	L	L	L	H	L	L	L
12	Additional	Blinded at any level	L	L	H	L	H	L	L	L	L	H	L	L	H

NOTE. L indicates a low risk of bias, H indicates high risk of bias, and "?" indicates an unclear risk of bias.

Appendix Table 4. Quality Score of the Included Studies

Criteria	Lim et al, 2004[30]	Kanaya et al., 2007[33]	Li et al., 2007[23]	Soon et al., 2007[31]	Chen et al., 2011[22]	Oe et al., 2011[32]	Matsumoto et al., 2012[24]	Mifune et al., 2012[25]	Mifune et al., 2013[26]	Kanazawa et al., 2014[35]	Lui et al., 2014[29]	Jang et al., 2015[28]	Takayama et al.2015[27]
1. Unit of sample	0	1	0	0	0	0	0	0	0	0	1	1	0
2. Standardization of surgical procedure	1	1	1	1	1	1	1	1	1	1	1	1	1
3. Description of postoperative complications and follow-up	1	0	0	1	0	0	0	0	0	0	1	1	0
4. Report of failure mode in mechanical test	1	1	0	1	0	0	1	1	0	0	1	0	0
5. Variation (ratio of SD to mean)	0	0	0	0	1	0	1	0	1	0	0	1	1
6. Statistical method	1	1	0	0	0	0	1	1	1	0	1	1	1
7. Description of selection region of interest	0	1	0	0	1	1	1	1	0	1	1	1	1
8. Semiquantitative scoring/image analysis	0	1	0	0	0	1	1	1	1	1	1	1	1
Sum	4	6	1	3	3	3	6	5	4	3	7	7	5

SD, standard deviation.