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Review article

Biological modulations to facilitate graft healing in anterior cruciate ligament reconstruction (ACLR), when and where to apply? A systematic review

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A R T I C L E I N F O

Keywords:
Anterior cruciate ligament reconstruction (ACLR)
Graft healing
Tendon-bone interface
Biological modulation

A B S T R A C T

Background: When and where to apply the biological modulations is effective to promote healing in the anterior cruciate ligament (ACL) reconstruction remains unclear.
Purpose: To perform a systematic review of preclinical animal studies on biological modulation in anterior cruciate ligament reconstruction (ACLR) concerning the time and site of delivery.
Study design: Systematic review of controlled laboratory studies.
Methods: PubMed, Ovid, and Scopus were searched until December 2020 using a combination of keywords and their synonym to retrieve all animal studies about biological modulation in ACLR. Studies that assessed mechanical strength after ACLR and compared with negative control were included. The methodological quality of animal studies was evaluated.
Results: 33 studies were included in this review and the majority reported mechanical strength improvement. 79 % of studies applied the biological modulations intra-operatively with different delivery systems used. For 21 % of post-operative delivery studies, intermittent delivery was tried. 21 of the included studies directly applied the biological modulations in the bone tunnels, 5 studies applied intra-articularly while 7 studies applied both in the bone tunnels and intra-articular part. Biological modulations applied intra-operatively and those applied in both parts showed better mechanical strength increase. A shift of the failure mode of pull-out from the bone tunnel in the early healing phase, to mid-substance rupture in the later phase was observed in most studies.
Conclusion: The improvement of the mechanical strength depends on how the biological modulations (delivery phase, delivery site, delivery form) are applied. The intra-operative delivery showed an overall higher mechanical strength increase and bone tunnel only delivery or intra-articular and bone tunnel both delivery are preferred than intra-articular only delivery. In addition, intra-articular and bone tunnel both delivery can have better mechanical strength increase for a long follow-up time. Thus, intra-operative application with a carrier to control release rate in both parts should be recommended. Further studies are needed to achieve a better healing outcome and more attention should be given to the intra-articular remodeling of the graft along with the tendon bone healing to increase the final mechanical strength.
The Translational potential of this article: Here, a systematic review of preclinical evidence of the time, site and the method the biological modulations being applied for ACLR to improve the graft healing would be performed. After reviewing the available studies, a choice of when and where to apply the biological modulations can achieve better mechanical strength after ACLR can be obtained. It provides evidence for both researchers and clinicians to decide when and where to apply the biological modulations can achieve their best effectiveness for ACLR before implementing. Promoting graft healing with targeted time and targeted site may reduce the risk of graft failure, safeguard return to sport.

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1. Introduction

Anterior cruciate ligament reconstruction (ACLR) is the most widely performed procedure in dealing with anterior cruciate ligament (ACL) injuries [1]. However, the outcomes of biological graft healing are not satisfactory with graft failure after ACLR, which was still around 1.5 %–5.7 % with a minimum of 2 year follow-up [2].

Graft healing of ACLR involves slow biological processes, which takes more than 12 months for the maturation of the graft [3,4]. In general, the healing process consists of three phases as described by many researchers: an "early healing phase", during which the graft should survive acute inflammation with necrosis, followed by a "proliferation phase", the time of most intensive revascularization and cellularity and finally, a "ligamentization phase" with characteristic maturation towards the properties of an intact ACL with tendon-bone integration [5,6].

The insufficient graft healing results in poor mechanical strength and studies using animal models have shown that the initial ACL strength has never been achieved. McFarland et al. [7] developed a dog ACL reconstruction model and by 16 weeks, the grafts remained only 40 % as strong as controls. Another study [8] examined the biomechanics of goats for as long as 3 years after surgery and the strength and stiffness of the grafts were only 44 and 49 % those of the control ligaments, respectively.

To improve the healing outcome, researchers have a great interest in the use of various biological modulations (growth factors, stem cells, drugs, biomaterial and biophysical interventions, etc. (Fig. 4) [9]) both in the intra-articular part and in the bone tunnels at different time points to completement surgical reconstruction. The healing environment in these two parts are quite different. The synovial fluid from ACLR knees contains a lot of pro- and anti-inflammatory cytokines and matrix metalloproteinases (MMPs) [10] which may interfere with the healing process of the intra-articular part. While in the bone tunnel, bone healing continues with graft incorporation. As a result, the biological modulations should be applied in different sites and healing phases, according to their individual properties and therapeutic effects.

Here, a systematic review of preclinical evidence of the time, site and also the method the biological modulations being applied for ACLR to improve the graft healing would be performed. After reviewing the available studies, a choice of when and where to apply the biological modulations can achieve better mechanical strength after ACLR can be obtained.

2. Methods

2.1. Article identification and selection

A systematic review of articles on the time and site of biological modulations in promoting tendon-bone interface healing after ACLR was performed using the PubMed, Scopus, and Ovid; the queries were performed on 17th December 2020. The keywords in combination with search operant were as follows: (ACLR OR ACL reconstruction OR anterior cruciate ligament reconstruction) AND (growth factor OR stem cell OR drug OR biomaterial OR biophysical intervention).

The inclusion criteria for studies in this systematic review consisted of the following:

- **Study types:** Controlled laboratory studies, concerning the usage of biological modulations. Studies included in searched reviews were also tracked.
- **Study group:** ACLR animal models using free tendon graft.
- **Intervention type:** Biological modulations (growth factor, stem cell, drug, biomaterial, biophysical intervention) that promote graft healing in ACLR.
- **Outcome assessment:** The main outcomes were to identify the time and site of delivery and to detect differences in mechanical strength increase.
- **Language:** English.

The exclusion criteria were: in vitro studies, ex vivo studies, or ACLR studies without the mechanical test.

2.2. Study selection

The search results from 3 different databases were merged, and duplicated studies were removed. Application of inclusion/exclusion criteria on the search results was started by screening the title, article type and then the abstracts. 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. The full text was then obtained for data extraction, such as first author, publication years, biological modulations, animal, delivery time, delivery site, delivery method, failure mode, and mechanical strength.

2.3. Assessment of study quality

The evidence level of animal studies can be stratified into 5 ranks based on outcome measures [9]:

A: Quantitative outcome measures analogous to clinical outcome measures (eg, 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/histological assessment
E: Qualitative imaging/histological assessment

The quality of animal studies was assessed according to the criteria adapted from the checklist of Hooijmans et al. [11], an arbitrary cutoff score ($\geq$5) was made to identify good-quality studies (Table 1).

3. Results

3.1. Search results

The literature search identified 1348 studies from the aforementioned databases. After duplicates were removed, 1198 articles were screened, and 33 articles met the inclusion criteria (Fig. 1). 22 of the animal studies used a rabbit model, 9 studies used a rat model, 1 study used a dog model and 1 another used a sheep model. Quality assessment of included studies was done by two assessors and good interobserver reliability was obtained (intraclass correlation coefficient average measures, 0.879), and consensus on scoring was reached by discussion (Table 2).

3.2. Delivery time

Most studies (26 out of 33) applied the biological modulations during the surgery (intra-operative). Different carriers and gene transfer technology were used as methods to control the release of the modulations. Only 7 studies delivered the biological modulations post-operatively and these interventions were all delivered for more than one time. Among them, multiple injection was the most used method to prolong the effective time [12–16]. The intra-operative delivery showed an overall higher ultimate failure load (UFL) increase (Fig. 2).

3.2.1. Intra-operative delivery without carriers

Alendronate [17], ACL-derived CD34+ cell [18], Brushite calcium phosphate cement [19], TCP [20], α2-macroglobulin [21], transforming growth factor-β1 (TGF-β1) [22], calcium phosphate cement (CPC) [23] have been applied simply by injection. And the mechanical strength improvement varies depending on the modulations used.

Biodegradable magnesium fixation screws [24–26] were used to enhance the healing of the tendon-bone interface by magnesium ions released while degrading, which may result in a continuous effect of magnesium ions. Wang et al. [27] reported the biodegradable

Table 1
Assessment criteria of methodological quality of animal studies of ACLR.

	Criteria	Score	Remarks
1	Unit of sample	Unilateral: 1 Bilateral: 0	Studies with bilateral operation may regard each limb as independent sample and assign to different treatment groups. Unless the sample unit was specified as number of animal 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 post-operative complications and follow-up	Yes: 1 No: 0	Records of post-operative complications such as broken sutures, wound infection, 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 test as primary outcome, reports of failure mode is important to reveal the quality and the implications of the mechanical tests.
5	Variation (SD/Mean)	<50 %: 1 >50 %: 0	For quantitative measure, large standard deviation may imply poor precision or large intra-group 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 un-adjusted 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	Semi-quantitative 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

magnesium screws did accelerate fibrous tissue mineralization at the tendon-bone insertion but the ultimate failure load (UFL) did not increase compared with the Ti group. These results were in accordance with that shown by Cheng et al. [24] using high-purity magnesium screws in 2016. Later, Wang et al. [26] developed Mg-6Zn-0.5Sr screws and showed that the bony ingrowth rapidly filled the cavity left by the complete degradation of the screws at 12 weeks with 68.4 % increased UFL at 12 weeks.

3.2.2. Intra-operative delivery with carriers

In order to control the releasing duration, Zhang et al. [28] used a gelatin sponge (GS) to deliver Platelet-Rich Plasma (PRP) and in vitro release kinetics test showed a sharp burst of 25 % release in the first 12 h and accounted for about 85 % of the loaded growth factor to day 7. However, although 16 % UFL was achieved compared with the non-treated control group, between the PRP and PRP-GS groups, similar failure loads were detected, and no significant difference was shown.

Furthermore, collagen was also applied as a carrier for slow release of the acidic fibroblast growth factor (aFGF) [29] and bone growth factor [30] to promote tendon-bone interface formation but characterization of the release rate is still warranted. 89.4 % and 75 % increased UFL has been achieved respectively at 8 weeks after ACLR but both studies lacked a no carrier control group.

Apart from gelatin and collagen, Weiler et al. [31] incorporated the platelet-derived growth factor (PGDF)-BB into 4 polyglactin sutures resulting in approximately 60 μg in the graft but they did not know how high the growth factor level was during the following healing period. The polyglactin has been shown to be effective in continuously delivering other growth factors over a period of 7 weeks, but significant UFL was revealed only at 6 weeks after ACLR but no difference was shown at other time points. According to the author, the desired peak release of the growth factor to optimize ACL graft remodeling might be around the third week; therefore, the delivery vehicle used in the study needs further improvement. Furthermore, Lui et al. used tendon-derived stem cell (TDSC) sheet [32] to wrap around the tendon graft while Mifune [33] et al. evaluated the effect of ACL-derived CD34+ cell sheet wrapping. The cell sheet showed significant mechanical strength improvement.

As an alternative, gene transfected cell has been developed as a technology to overcome the limited-release kinetics of the delivery vehicles. Dong et al. [34] embedded the tendon graft with BMP-2 transfected BMSCs 8 weeks after ACLR, a 107.8 % UFL increase was detected. While the BMP-2 combined with CPC [35] directly delivered in bone tunnels can only achieve 32.9 % increase in UFL.

3.2.3. Post-operative delivery

Sun et al. [36], Sauerschnig et al. [13], Fu et al. [14] intra-articular injected stem cell-conditioned medium (CM), COX-2 inhibitor, and GHK-Cu post-operatively. The GHK-Cu modulation showed no significant mechanical improvement and the CM treatment with 40.7 % increase. However, the mechanical strength of the COX-2 inhibitor treatment dropped by 37 % at the endpoint.

Lui et al. [37] reported that continued subcutaneous administration of alendronate could reduce peri-tunnel bone resorption and promoted graft-bone tunnel healing at the early stage after ACLR with 52.5 % UFL increase at 2 weeks post-operation, which is greater than single local administration into the bone tunnel [17]. However, systemic increase in bone mineral density (BMD) in the contralateral intact knee was observed. A similar method was tried by Bi et al. [15] to deliver the Parathyroid Hormone (PTH) with 101.8 % UFL increase.

In addition to those injectable interventions, biophysical interventions can be applied regularly after ACLR. Sun et al. [38] used the intermittent negative pressure 2 times a day for 5 days through a drainage tube and in the study of Song et al. [39], animals' knees were moved passively by the continuous passive motion (CPM) apparatus for 60min every other day for the two weeks after surgery.

3.3. Delivery site

More than half of the included studies directly applied the biological modulations in the bone tunnels while 5 studies injected the agents into the intra-articular part. Bi et al. [15] and Lui et al. [16] injected the drugs subcutaneously for several times post-operatively and positive biomechanical results had been achieved. Furthermore, the CPM study [39] moved the whole knee passively after surgery. The bone tunnel only delivery method showed higher UFL increase, while we can also see good results from the intra-articular and bone tunnel both methods. Among these categories, the intra-articular only method showed the lowest UFL increase (Fig. 3).

3.3.1. Intra-articular delivery

The α2-macroglobulin [21] injected intra-articularly had only 27 % mechanical strength improvement. Lower (7.1 % increase) mechanical

strength improvement was shown in the intermittent negative pressure study [38]. And tripeptide–copper complex glycyl-L-histidyl-L-lysine--Cu(II) (GHK-Cu) [14] delivered in the intra-articular part achieved no significant mechanical improvement although several injections have been applied. Even more, the COX-2 inhibitor [13] injected into the knee joint demonstrated negative results in the mechanical strength.

3.3.2. *Bone tunnel delivery*

For localization in the bone tunnels, BMP-2 combined with calcium phosphate cement (CPC) [35] directly delivered in bone tunnels can achieve 32.9 % increase in UFL while the BMP-2 delivered in the injectable CPC [40] can only increase 37.7 % the failure load at the 12-week post operation time period. And Pan et al. [40] combined the BMP with two biomaterials, the result showed that the localization ability of fibrin sealant (FS) covered only the first 7–14 days, which was relative shorter than CPC.

Besides, the fibrin glue applied in the graft-tunnel interface by Setiawati et al. [41] improved the graft press-fit to the walls of the femoral tunnel and reduced tunnel widening. Fibrin glue [42] was also useful to blend MSCs from bone to allograft to enhance the effect of MSCs and accelerated the osteointegration.

3.3.3. *Intra-articular + bone tunnel delivery*

Teng et al. [43] applied the PRP with BMSC both in the intra-articular and bone tunnel part with 85 % UFL increase. Similarly, Zhai et al. [44] used DPB in these two parts but only with 9 % mechanical improvement. Cell sheets, like TDSC [32] and ACL-derived CD34+ cell [18] sheets were wrapped around the graft so that they were both effective in the two different parts. Apart from cell sheets, Dong et al. [34] and Soon et al. [45] embedded the graft in the BMP-2 transfected BMSC and MSC,

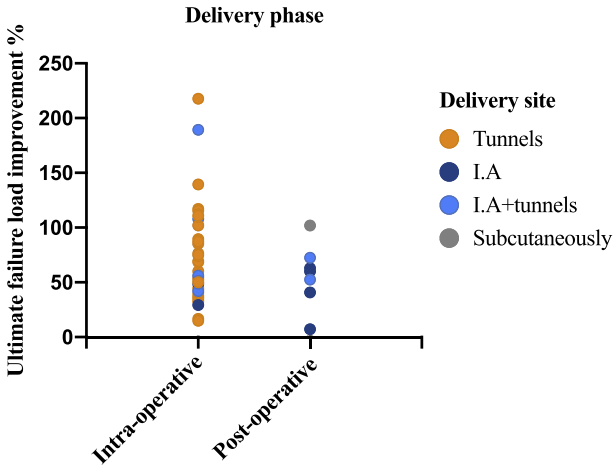

Fig. 2. The bubble diagram shows the relationship between the UFL and the delivery phase. The x-axis represents the delivery phase. The y-axis represents the UFL improvement percentage compared with the control group. The different colors of the bubble represent different sites the biological modulations were used respectively (For details see figure note). Abbreviation: UFL=Ultimate failure load.

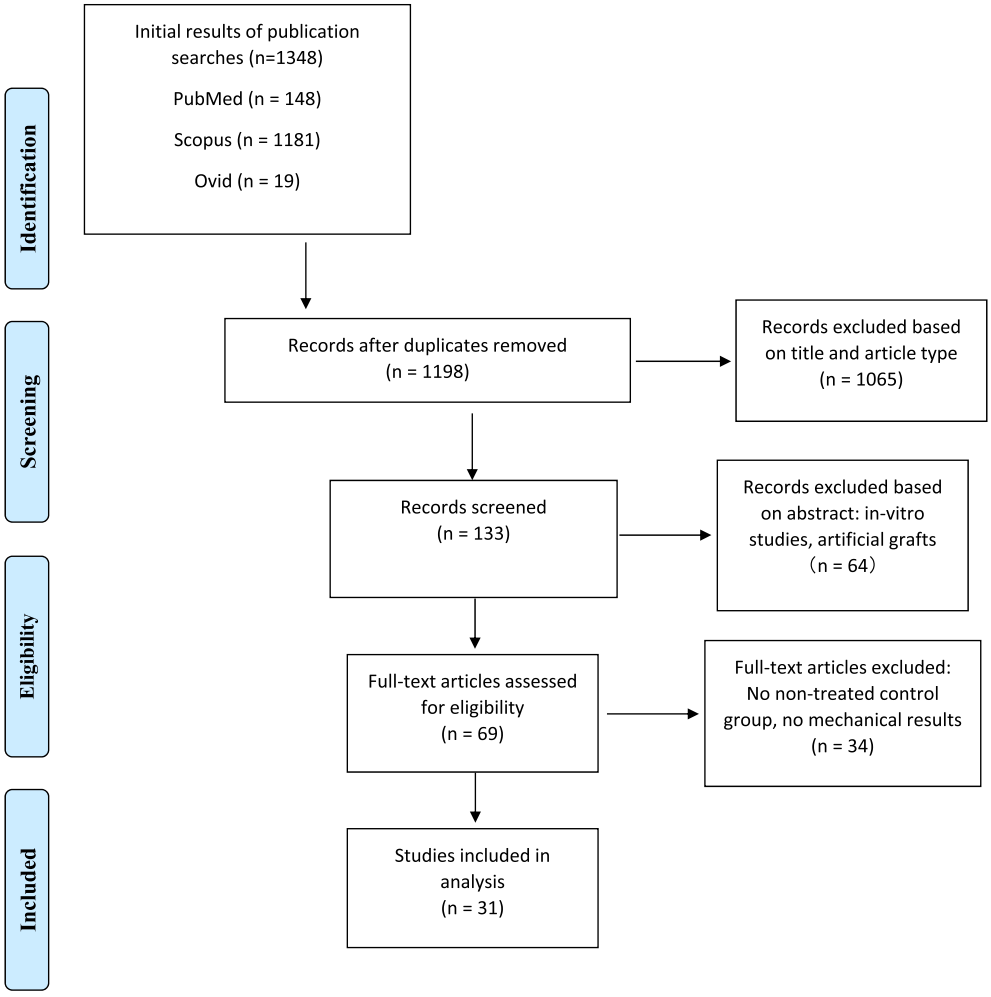

Fig. 1. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flowchart showing the selection criteria used to identify studies with the search strategy.

Fig. 3. The bubble diagram shows the relationship between the UFL and the delivery site. The x-axis represents the delivery site. The y-axis represents the UFL improvement percentage compared with the control group. The different colors of the bubble represent different phases the biological modulations were used respectively (For details see figure note). Abbreviation: UFL=Ultimate failure load.

respectively. 107.8 % and 56 % mechanical strength increase were achieved accordingly. Besides, Weiler et al. [31] incorporated the PDGF-BB in the Poly(D,L-lactide) for delivery but no significant mechanical increase was observed.

3.4. Failure mode

A shift of the failure mode of pull-out from the bone tunnel in the early healing phase, to mid-substance rupture in the later phase was observed in most studies (Fig. 5).

4. Discussion

The principal finding of this systematic review is that the improvement of the mechanical strength depends on how the biological modulations (delivery phase, delivery site, delivery form) are applied. In general, the intra-operative delivery showed an overall higher UFL increase and bone tunnel only delivery or intra-articular and bone tunnel both deliveries are preferred than intra-articular only delivery. In addition, intra-articular and bone tunnel both delivery can have better mechanical strength increase for a long follow-up time.

4.1. Delivery time

Most of the included studies delivered the modulation intra-operatively since it is a more direct and convenient way to do along with the surgery process. From Fig. 2, we can see that compared with the post-operative delivery, the intra-operative delivery achieved better UFL improvement compared with their control side. However, the quick clearance of the biological modulations will influence the general effectiveness. So, different delivery systems have been utilized in many studies, like gelatin [28], collagen [29,30], fibrin glue [42,43], etc. Improvement in UFL has been detected in all these studies, but further researches are needed to clarify the effect of different carriers. These studies also provide evidence that growth factor transfected BMSCs may

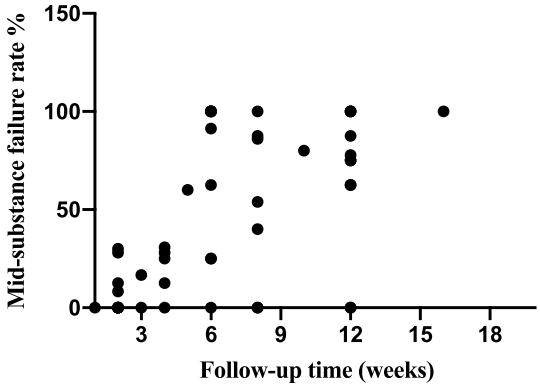

Fig. 5. The bubble diagram shows the relationship between the follow-up time and the mid-substance failure rate. The x-axis represents the follow-up time. The y-axis represents the mid-substance failure rate.

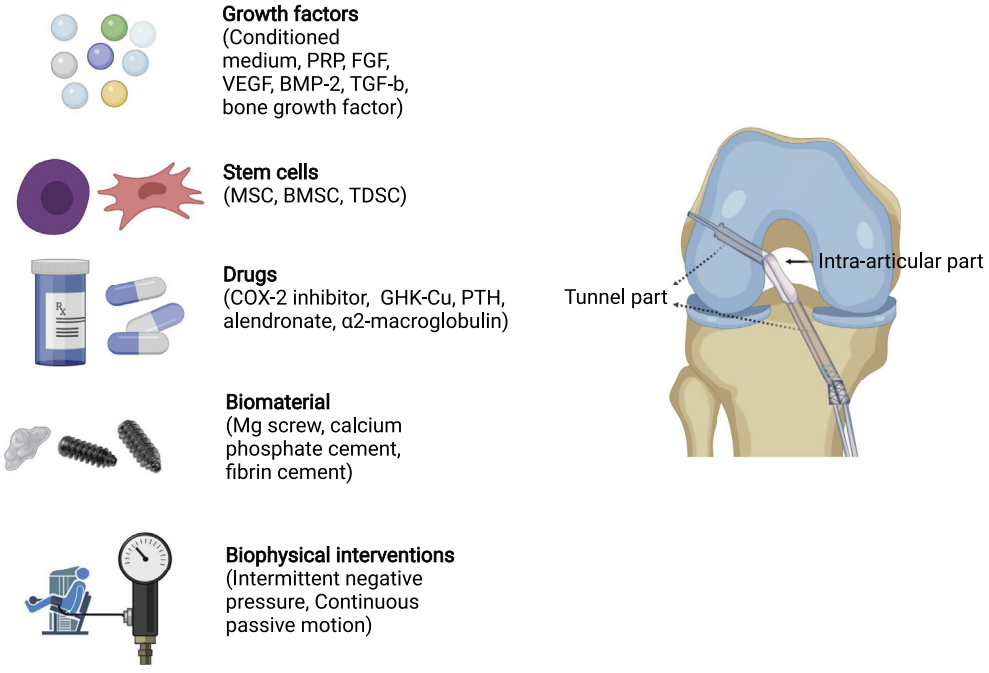

Fig. 4. Different biological modulations to facilitate anterior cruciate ligament reconstruction: growth factors, stem cells, drugs, biomaterial and biophysical interventions (Created with BioRender.com).

Table 2
Search results of studies investigating biological modulations in animal models in ACLR.

Year/Author	Modulation	Animal	Number/ Group	Tendon type	Delivery phase	Delivery site	Delivery form	% midsubstance graft failure	Ultimate failure load	Quality Score
2019/Zhang et al.	PRP	Rabbit	6	ST	Intra-op; Early healing	Tunnels	Gelatin sponge delivery	8wk: 0 %	8wk: 16.6 % increase	B, 7
2019/Sun et al.	CM	Rat	40	FLEX	Post-op; Early healing	Intra-articular	Injection	4wk: 25 %; 8wk: 100 %	4wk: 62.8 % increase; 8wk: 40.7 % increase	B, 6
2019/Wang et al.	Magnesium screw	Rabbit	6	EXT	Intra-op: Early healing	Tunnels	Fixation	Not reported	12wk: 68.4 % increase	B, 7
2019/ Sauerschnig et al.	COX-2 inhibitor	Rabbit	8	ST	Post-op: Early healing	Intra-articular	Injection	3wk: 16.7 %	3wk: 60 % increase	B, 6
2018/Lu et al.	aFGF	Rabbit	21	EXT	Intra-op; Early healing	Tunnels	Collagen delivery	Not reported	4wk: 44.4 % increase; 8wk: 60 % increase; 12wk: 89.4 % increase	B, 5
2017/Wang et al.	Magnesium Screw	Rabbit	56	EXT	Intra-op; Early healing	Tunnels	Fixation	6wk, 12wk, 16wk: 100 %	6wk, 12wk, 16wk: NS	B, 5
2017/Wang et al.	TGF-β transfected BMSC	Rabbit	18	FLEX	Intra-op; Early healing	Tunnels	PRP delivery	Not reported	6wk: 76.5 % increase; 12wk: 54.7 % increase	B, 4
2017/Sun et al.	Intermittent negative pressure	Rabbit	12	ST	Post-op; Early healing	Intra-articular	Drainage tube delivery	6wk: 91.3 %	6wk: 7.1 % increase	B, 6
2017/Song et al.	CPM	Rabbit	36	ST	Post-op; Early healing	Total knee	Continuous passive motion	Not reported	6wk: 52.4 % increase; 12wk: 72.4 % increase	B, 5
2017/ Setiawati et al.	BMSC + VEGF	Rabbit	6	ST	Intra-op; Early healing	Tunnels	Fibrin glue localization	Not reported	3wk: 36.4 % increase; 6wk: 14.8 % increase	B, 4
2016/Teng et al.	PRP + BMSC	Rabbit	15	ST	Intra-op; Early healing	Intra-articular + tunnels	Fibrin glue localization	Not reported	8wk: 85.2 % increase	B, 6
2016/Cheng et al.	Magnesium screw	Rabbit	30	ST	Intra-op; Early healing	Tunnels	Fixation	0wk: 0 %; 12wk: 0 %	0wk, 12wk: NS	B, 4
2015/Fu et al.	GHK-Cu	Rat	24	FLEX	Post-op; Early healing + Proliferation phase	Intra-articular	Injection	6wk, 12wk: 100 %	6wk, 12wk: NS	A, 8
2014/Lui et al.	TDSC sheet	Rat	52/45	FLEX	Intra-op; Early healing	Intra-articular + tunnels	Wrap	2wk: 0 %; 6wk: 62.5 %; 12wk: 75 %	2wk: 52.5 % increase	B, 7
2014/Bi et al.	PTH	Rat	10	FLEX	Post-op; Early healing	Subcutaneously	Injection	12wk: 0 %	12wk: 101.8 % increase	B, 6
2013/Zhai et al.	DPB	Rabbit	4	ST	Intra-op; Early healing	Intra-articular + tunnels	PRG delivery	2wk: 12.5 %; 4wk: 25 %; 8wk: 87.5 %; 12wk: 87.5 %	4wk: 101.8 % increase; 8wk: 49.6 % increase	B, 5
2013/Pan et al.	BMP-2	Rabbit	30	EXT	Intra-op; Early healing	Tunnels	CPC	Not reported	24wk: 32.9 % increase	B, 4
2013/Mifune et al.	ACL-derived CD34+ cell sheet	Rat	9	Flex	Intra-op; Early healing	Intra-articular + tunnels	Wrap	Not reported	8wk: 189.2 % increase	B, 4
2013/Lui et al.	Alendronate	Rat	6	Flex	Intra-op; Early healing	Tunnels	Injection	2wk: 0 %; 6wk: 100 %	2wk: 100 % increase	B, 6
2012/Mifune	ACL-derived CD34+ cell	Rat	20	Flex	Intra-op; Early healing	Tunnels	Injection	8wk: 0 %	8wk: 32.9 % increase	B, 5
2013/Lui et al.	Alendronate	Rat	16	Flex	Post-op; Early healing	Subcutaneously	Injection	2wk: 0 %; 6wk: 100 %	2wk: 139.3 % increase	B, 7
2012/Dong et al.	BMP-2 transfected BMSC	Rabbit	10	GAS	Intra-op; Early healing	Tunnels + Intra-articular	Embed	Not reported	4wk: 75 % increase; 8wk: 107.8 % increase	B, 3
2011/Zhang et al.	BMP/RBX	Rabbit	17	Flex	Intra-op; Early healing	Tunnels	BMP fibrin glue localization; RBX bone cylinders fixation	Not reported	Fibrin glue group 6wk: 42.9 % increase; RBX group 6wk: 52.4 % increase; 12wk: 50 % increase	B, 3
2011/Pan et al.	BMP + ICPC/ BMP + IFS	Rabbit	17	EXT	Intra-op; Early healing	Tunnels	CPC/Fibrin sealant localization	6wk: ICPC 25 %; IFS 0 %; 12wk: ICPC 62.5 %; IFS 75 %	6wk: ICPC group 85.3 % increase; IFS group 40.4 % increase; 12wk: ICPC group 37.7 % increase; IFS group 32.6 % increase	B, 4
2010/Wang et al.	BMP-transfected cells	Rabbit	18	EXT	Intra-op; Early healing	Tunnels	Calcium alginate gel localization	12wk: 77.8 %	12wk: 35.1 % increase	B, 5

(*continued on next page*)

Table 2 (*continued*)

Year/Author	Modulation	Animal	Number/ Group	Tendon type	Delivery phase	Delivery site	Delivery form	% midsubstance graft failure	Ultimate failure load	Quality Score
2009/Wen et al.	Brushite calcium phosphate cement	Rabbit	14	EXT	Intra-op; Early healing	Tunnels	Injection	6wk: 25 %; 12wk: 62.5 %	6wk: 117 % increase; 12wk: 55 % increase	B, 5
2007/Soon et al.	MSC	Rabbit	18	AT allograft	Intra-op; Early healing	Intra-articular + tunnels	Fibrin glue localization	2wk, 4wk: 28 %; 8wk: 86 %	8wk: 56 % increase	B, 5
2007/Huangfu et al.	TCP	Dog	24	Flex	Intra-op; Early healing	Tunnels	Injection	2,4wk: 0 %; 6wk: 40 %; 8wk: 60 %; 10wk: 80 %; 12wk: 100 %	2wk: 101.9 % increase; 4wk: 87.2 % increase	B, 5
2005/ Yamazaki et al.	TGF-β1	Dog	7	Flex	Intra-op; Early healing	Tunnels	Injection	3wk: 0 %	3wk: 115.3 % increase	B, 4
2005/Demirag et al.	α2-macroglobulin	Rabbit	14	ST	Intra-op; Early healing	Intra-articular	Injection	2wk: 30 %; 5wk: 60 %	2wk: 48.6 % increase; 5wk: 29.2 % increase	B, 7
2004/Weiler	PDGF–BB	Sheep	24	Flex	Intra-op; Early healing	Tunnels + Intra-articular	Poly(D, L-lactide) delivery	Not reported	6wk: 41.9 % increase	A, 6
2004/Tien et al.	CPC	Rabbit	11	ST	Intra-op; Early healing	Tunnels	Injection	1wk, 2wk: 0 %	1wk: 217.6 % increase; 2wk: 110.8 % increase	B, 6
2001/ Anderson et al.	Bone growth factor	Rabbit	35	ST	Intra-op; Early healing	Tunnels	Collagen sponge delivery	2wk: 8.3 %; 4wk: 30.8 %; 8wk: 53.9 %	2wk: 50 % increase; 4wk: 69.2 % increase; 8wk: 75 % increase	B, 5

AT, achilles tendon; αFGF, α fibroblast growth factor; BMP, bone morphogenetic protein; BMP-2, bone morphogenetic protein–2; BMSC, bone marrow–derived mesenchymal stem cell; CPM, continuous passive motion; CM, cell-conditioned medium; EXT, extensor; Flex, flexor tendon; GHK-Cu, tripeptide–copper complex glycyl-L-histidyl-L-lysine-Cu(II); HS, hamstring tendon; MSC, mesenchymal stem cell; PDGF-BB, platelet-derived growth factor–BB; PRP, platelet-rich plasma; PTH, Parathyroid Hormone; ST, semitendinosus; TCP, tricalcium phosphate; TDSC, tendon derived stem cell; TGF-β1, transforming growth factor–beta 1; VEGF, vascular endothelial growth factor; PRG, platelet-rich gel; DPB, deproteinized bone; RBX, recombined bone xenograft (RBX); NS, no significant difference

be effective in promoting tendon-bone graft healing [46,47,48], which is designed to overcome the limited-release kinetics of the delivery vehicles since the growth factors will be able to act with the cellular activities of these cells. Despite the positive results shown by growth factor transfected BMSCs, complications of stem cell therapy and immunologic problems are still needed to be tackled when used in human therapy. What is also needed to figure out is that whether sustained delivery throughout the healing process or during a targeted period will achieve mechanical strength improvement.

It seems that repeated intra-articular injection post-operatively can apply the modulations at an appropriate timing. However, the UFL increase result was not that good compared to the intra-operative delivery studies. And they all tried several time injections in the intra-articular part [12,21,49], which may be easily cleared by the synovial fluid. In addition, from Table 3, we may conclude that it lacks studies to test the effect of long-term post-operative delivery until the maturation phase. But what is more important is to understand the healing process and apply the modulations during the targeted period.

4.2. Delivery site

As for the delivery site, in the included studies, modulations delivered in the intra-articular part showed little [12,21,49] or no mechanical strength improvement [14]. It may be due to the intra-articular synovial environment and the acute inflammatory reaction after ACLR. It should be noted that these modulations were directly injected into the intra-articular part, which indicates that intra-articular biological modulations should be applied with some carriers to retain them in the synovial environment and take effect gradually. Several-time injection should also be considered for long-term delivery.

For the bone tunnel delivery, studies trying to localize the biological agent in the tunnel part using CPC [19,23,40] or fibrin glue [40,50] showed positive mechanical strength improvement, which implies that localization of the biological modulation in the targeted place may have a better healing result. In the result of Tien et al.'s study [23], the UFL even had a 217.6 % increase compared to the control group at week 1, but immediately dropped to 110.8 % at week 2. It may be because during the early healing phase, the tendon-bone interface hasn't formed strong connection while the localization agents can provide support for the tendon graft to avoid pull-out.

It was shown that the intra-articular and bone tunnel duo delivery can lead to significant UFL increase, especially during the late healing phase [33,34,42–44,53]. The ACL-derived CD34+ cell sheet resulted a 189.2 % UFL increase compared to the control group at week 8, which is the best result among the included studies for a relative long follow-up time. So, for the delivery site, intra-articular and bone tunnel duo delivery may

Table 3
Time and site each study targeted (post-op 1–4wk: early healing phase; 4–8wk: proliferation phase; 8–12wk: maturation phase).

Site	Time Intra-op	Post-op (0–4wk)	Post-op (4–8wk)	Post-op (0–4wk)
Intra-tunnel	[18–20,22, 23,24,26,27, 37]			
Intra-tunnel (carrier)	[28–30,40, 41,46,47,50]			
Intra-articular	[21]	[13,14,36,49]	[14]	
Intra-articular (carrier)				
Both parts	[32–34]	[39]		
Both parts (carrier)	[42–44,51]			
Others		[15,52] (subcutaneously)		

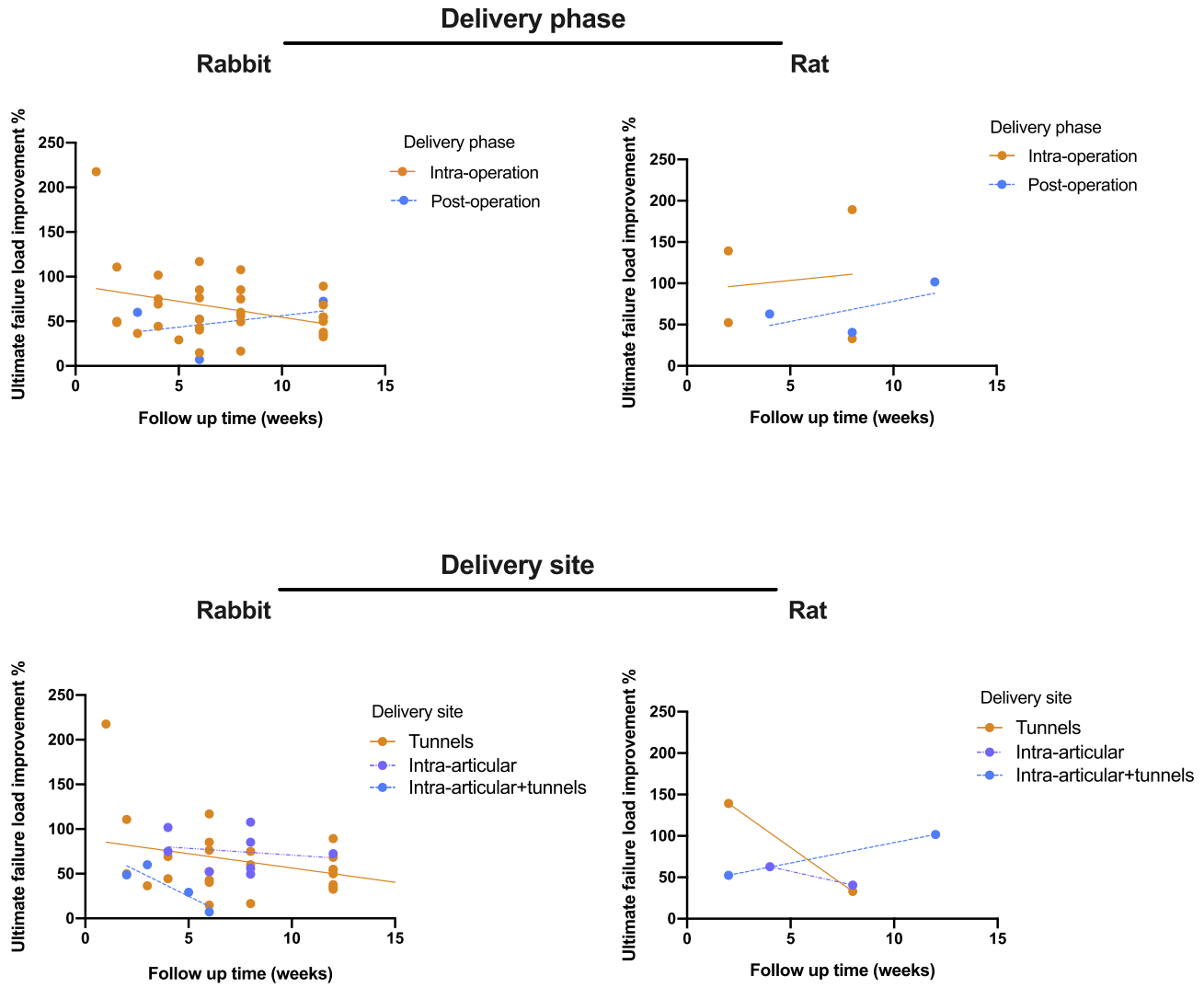

Fig. 6. The bubble diagram shows the relationship between the UFL and the follow-up time in rabbit and rat, respectively, showing different delivery phase, delivery site. The x-axis represents the follow up time. The y-axis represents the UFL improvement percentage compared with the control group. The different colors of the bubble represent different phases and different sites the biological modulations were used respectively (For details see figure note). Abbreviation: UFL=Ultimate failure load.

result in a better mechanical strength for the ACLR. What should be noticed is that the subcutaneous injection of parathyroid hormone [15] showed a 101.8 % increase as compared with the control group. But this site of application and the biological modulation used need more studies to support.

What's more, 22 studies reported the failure mode of the tendon graft after the ultimate load test and a shift of the failure mode of pull-out from bone tunnel in the early healing phase to mid-substance rupture by the end point was observed. During the early healing weeks, 72 % [42]-100 % [17] failures occurred inside the bone tunnels and most of them were pullout failures [22]. And during the later healing period, mid-substance rupture accounted for 53.85 % [30]-100 % [12]. This suggests that we need to shift the priority of different concerns for intra-articular and bone-tunnel healing at different time points.

4.2.1. *Other considerations for translating the conclusion into clinic*

As mentioned previously, it is difficult to effectively synthesize the available evidence due to the wide heterogeneity of animal models, type of free tendon graft used, observation time points, and intervention modalities and procedures. So, the increase in mechanical strength was calculated as a percentage relative to their own non-treated control group at each end point. To simplify the analysis, here, we focused on the evidence in two mainstream animal models, rabbits, and rats; while observing the fluctuation of UFL improvement with different delivery phases and delivery sites.

For the delivery phase, most of the studies focus on intraoperative delivery, which apparently has a high clinical operation. Whether using a rabbit or a rat model, the overall UFL improvement was superior to post-operation delivery. These data combined with the clinical practicability all make intraoperative delivery a more favored modality.

For the delivery sites, intra-tunnel delivery is the predominant delivery site. Not surprisingly, different delivery sites have large variability in the improvement of UFL. In the rabbit model, its overall distribution was slightly inferior to that of intra-articular delivery. In the rat model, it was instead superior to intra-articular delivery. It is worth noting that the delivery site is related to the material and biological function of the modulation itself, and such a simple trend analysis may not give us much additional information. But due to the shift of the graft rupture site to the intra-articular part, more attention should be given to the intra-articular remodeling of the graft along with the tendon bone healing to increase the final mechanical strength (see Fig. 6).

4.2.2. *Analysis of biases*

This systematic review may be subject to publication bias as most of

the included studies reported improvement (32 positive findings, 1 negative findings), many negative results may not have been reported. Besides, since the primary outcome we focus is mechanical strength, some good quality studies may be missed since they only compare the histology findings. Furthermore, Clinically, we use re-tear rate, return to sports rate, and functional scores to evaluate their short-term and long-term effects. Way more confounding compared with pre-clinical studies, but it is important to conduct more high-quality clinical studies to assess the true value of these supplements. This kind of clinical information may also be missed here.

4.2.3. Clinical implication

Before implementing any biological modulations, it is important to have evidence of when and where to apply to achieve their best effectiveness for ACLR. Further research is required to determine the time, site and method used to deliver these biological modulations.

Although this review showed great potential, biological modulations are still in the exploratory stage. More evidence from both preclinical and clinical studies is required for implementation in clinical practice.

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What is known about this subject

After ACLR, in order to improve the healing outcome, researchers have used various biological modulations (growth factors, stem cells, drugs, biomaterials, and biophysical interventions, etc.). However, the mechanical strength required for normal walking has not been achieved and there was a shift from tendon-bone junction rupture in the early healing phase to intra-articular part rupture in the late healing phase.

What this study adds to existing knowledge

Here, a systematic review of preclinical evidence of the time, site and the method the biological modulations being applied for ACLR to improve the graft healing would be performed. After reviewing the available studies, a choice of when and where to apply the biological modulations can achieve better mechanical strength after ACLR can be obtained. It provides evidence for researchers to decide when and where to apply the biological modulations can achieve their best effectiveness for ACLR before implementing.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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