Knee Surg Sports Traumatol Arthrosc (2008) 16:935–947
DOI 10.1007/s00167-008-0584-0

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Graft healing in anterior cruciate ligament reconstruction

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Received: 22 February 2008 / Accepted: 30 June 2008 / Published online: 17 July 2008

Abstract Graft healing within the bone tunnel after anterior cruciate ligament (ACL) reconstruction is still a complex, poorly understood biological process that is influenced by multiple surgical and postoperative variables. However, remarkable advances in knowledge of this process have been made based primarly on animal models. According to the findings of this review, some surgical and postoperative variables are known to directly affect time-course and quality of graft-tunnel healing. The type of graft, graft motion, and fixation methods have shown to directly affect time-course and quality of graft-tunnel healing. Therefore, the application of early and aggressive rehabilitation protocols should be cautious when using soft-tissue graft, allografts, and direct or aperture type of fixation for ACL reconstruction. With regard to graft placement, several cadaveric models showed biomechanical advantages of a more anatomical graft location; however, there are no studies that explore the relationship between graft placement and healing process. The precise effect of graft tensioning, graft/tunnel diameter disparity, and graft length within the bone tunnel in the graft healing process remains unclear and requires more research. To enhance graft-tunnel healing, tissue-engineering approaches, including the use of growth factors, mesenchymal stem cells, and periosteum graft augmentation, have been tested on animal models. These have shown promising results in terms of enhancement of bone-graft healing rate.

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Keywords Anterior cruciate ligament · Graft · Healing · Tendon · Tunnel

Introduction

Although the treatment options for anterior cruciate ligament (ACL) tears include conservative management and extra-articular procedures, reconstruction with intra-articular grafts is the most broadly accepted procedure for the young and active population [33]. ACL reconstruction has been demonstrated to improve clinical instability of the knee joint, reduced knee laxity, and decrease risk of late meniscus tear and surgery [3, 33, 84, 98, 99, 115].

Nevertheless, the success rates of ACL reconstruction surgery have been reported to vary between 73 and 95% [33, 34, 128], and the return to pre-injury activity level varies from 37 to 75% [33, 128]. Despite the relatively high rate of positive outcome reported, graft failure continues to occur. Such graft failure may be attributed to traumatic injuries or non-traumatic causes. Non-traumatic causes include technical errors, fixation failure, and the failure of biological graft incorporation into the bone tunnel [30, 35, 54]. This intent to categorize failure in traumatic and non-traumatic failure is subjective and most cases probably fall in a mixed category of poorly healed graft and minor trauma.

After ACL reconstruction, stable graft-tunnel healing is desired, so that graft tissue can be incorporated into the bone tunnel. The graft-tunnel healing is a complex healing process, influenced by multiple surgical and postoperative variables, including the type of graft used (auto versus allograft, and soft tissue versus bone-plug grafts), method of graft fixation and tensioning, graft motion, and tunnel placement. To enhance graft-tunnel healing, tissue-engineering approaches, including the use of growth factors

[7, 63, 83, 109, 119, 123], mesenchymal stem cells [61] and periosteum graft augmentation [28, 59, 127], have been tested on animal models, showing promising results in terms of enhancement of bone-graft healing quality. Recently, in an attempt to improve graft incorporation within the bone tunnel, knee kinematics, and long-term outcomes, more anatomical reconstruction involving the replacement of both bundles of the ACL has also been proposed to better preserve the mechanical loading conditions of the grafts [16, 21, 24, 114].

This review begins with a brief description of the general healing process of graft tissue after implantation, followed by a discussion of the effect of different surgical and post-operative factors on the graft healing process, based on the findings of basic research studies. Moreover, current strategies to enhance the graft healing process and their possible clinical applications are evaluated. It should be noted that this review focuses on the biological healing process of the graft, not on its clinical aspect. The search of the studies was performed using keywords: anterior cruciate ligament, tendon, tunnel, graft, and healing. Finally, only published studies in English were included in this review.

Healing process of graft for ACL reconstruction

Shortly after graft implantation, an inflammatory response ensues. Neutrophils and recruited macrophages are present in the tendon–bone interface as early as 4 days after surgery, and after 10 days, resident macrophages are identified [55]. These cells progressively repopulate the tendon graft, and the cytokines produced by these infiltrating cells, including transforming growth factor-β (TGF-β), may contribute to the formation of a fibrous scar tissue interface between the graft and host bone [55]. After 6 weeks, the graft is completely covered by a vascular synovial envelope, and at 20 weeks, the intrinsic vasculature of the graft is complete [9]. Graft revascularization is originated mainly from the infrapatellar fat pad, posterior synovial tissue [9], and endosteal vessels within the femoral and tibial tunnels [5, 20].

Although graft necrosis is clearly demonstrated in the osseous part of the bone-plug grafts (e.g. the BTB autograft) according to some animal models [6, 9, 72], the intra-articular portion of the graft seems to survive and undergo an early revascularization process. Rougraff and Shelbourne performing biopsies in the central region of the BTB autograft in human subjects, demonstrated absence of necrosis and vascularity of the grafts as early as 3 weeks after reconstruction [87].

This phenomenon of partial graft necrosis does not occur clearly in a soft-tissue graft. Using dog and sheep models, Rodeo et al. [81] and Goradia et al. [38] did not find evidence of necrosis in the soft-tissue auto-graft such as the long digital extensor and hamstrings tendons, respectively. A rabbit model using flexor digitorum longus tendon autograft [55] demonstrated that there was no proliferation of intrinsic tendon cells. Despite no necrosis was reported, these cells probably does not contribute to the early healing processs.

The remodeling phase of the intra-articular portion of the graft tissue is characterized by replacement of large collagen fibrils with small fibrils [50, 100, 129]. Amiel et al. [6] proposed the term "ligamentization" to explain the histological changes of a tendon graft when implanted for replacement of the ACL. In a rabbit model, Amiel at al. [6] showed that the patellar tendon autograft gradually assumed the histological properties of the native ACL. For example, at 30 weeks, the percentage of type III collagen increased to 10% in the graft, a level comparable to that of normal ACL. The glycosaminoglycan content and collagen cross-linking were also similar to those found in native ACL. A more recent work showed that even in the environmental milieu of the native rat tendon, collagen type III expression was high in patellar tendon that underwent an in situ frozen-thawed process [104]. It is likely that both repopulation of cells in a devitalized graft and altered biomechanical environment are responsible for these observed histological changes.

During the healing process, the graft tissue is gradually weakened with decreased structural properties, including stiffness and ultimate failure load. Using a primate intra-articular model [17] the ultimate failure load and stiffness of the graft at 7 weeks were shown to drop to 16 and 24% of control ACL, respectively. At 1 year, these structural properties of the intra-articular portion of the graft improved to 39% of control failure and 57% of control stiffness, but did not fully return to those of control ACL. To the best of our knowledge, no study has shown that the graft returns to its original strength at the time of implantation [17, 51, 52, 69, 81, 95].

Factors of affecting graft healing

Type of graft

Autograft

Although the clinical outcomes of different types of autograft are comparable [3, 94, 98, 112], the rates and characteristics of the healing processes among autografts differ. Several animal models have shown a slower incorporation rate into the bone tunnel with soft-tissue grafts compared to bone-plug grafts such as bone-patellar tendon-bone (BTB) [73, 74, 81, 105].

The incorporation of a soft-tissue graft (long digital extensor tendon) into the bone tunnel is well described in a dog model [81]. As early as 2 weeks, progressive reestablishment of collagen fiber continuity between the bone and tendon through the presence of a fibrous interface starts. At 12 weeks, collagen fibers resembling Sharpey′s fibers which insert directly into the bone, are present. The presence of these fibers correlates with enhanced interface strength, as all the grafts failed by pullout testing only at the tunnel before 12 weeks but failed at the mid-substance or clamp sites after 12 weeks (Fig. 1).

Using an intra-articular dog model, Tomita et al. [105] investigated the histological and biomechanical differences in the healing outcome of two types of autograft. The same animals underwent ACL reconstruction with BTB graft in one knee and flexor digitorum superficialis (FT) in the opposite. At 12 weeks, the bone plug was completely incorporated into surrounding bone tissue. The ultimate failure load of the BTB at 3 weeks was significantly higher than the FT graft, but at 6 and 12 weeks, there were no significant differences between the BTB and FT grafts. The differences in the healing process between bone plug and soft-tissue graft was also evaluated in a goat model study [73]. The soft tissue portion of the patellar tendon autograft was inserted in the tibial tunnel while the bone plug was inserted in the femoral side. In the bone-to-bone tunnel, the bone plug showed complete incorporation at 6 weeks; however, in the tendon-bone tunnel—though there were some Sharpey′s like fibers—graft incorporation was incomplete. A rabbit model study found that the healing of bone-to-bone was mature at 8 weeks, whereas the healing of tendon-to-bone was mature at 12 weeks [74]. Although different animal models were used, the similarity of results in the aforementioned studies indicates that soft-tissue graft is slower to incorporate than bone-plug graft (Fig. 2)

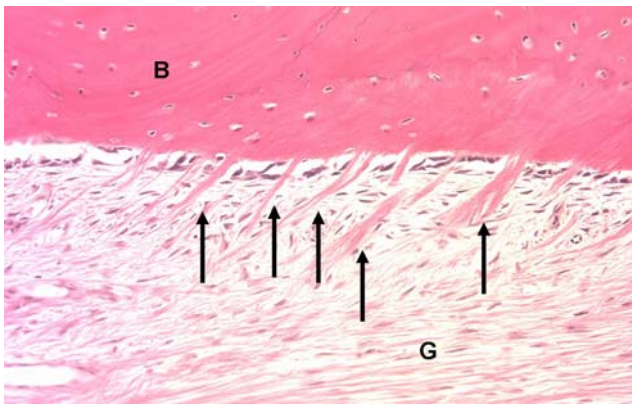

Fig. 1 Histological section of bone-graft interface. Present were the Sharpey′s-like fibers (*arrows*) anchoring the soft tissue graft (*G*) to the bone tunnel (*B*) after 12 weeks of healing in a goat model. The presence of these fibers correlates with the interface strength (H&E stain, 20×)

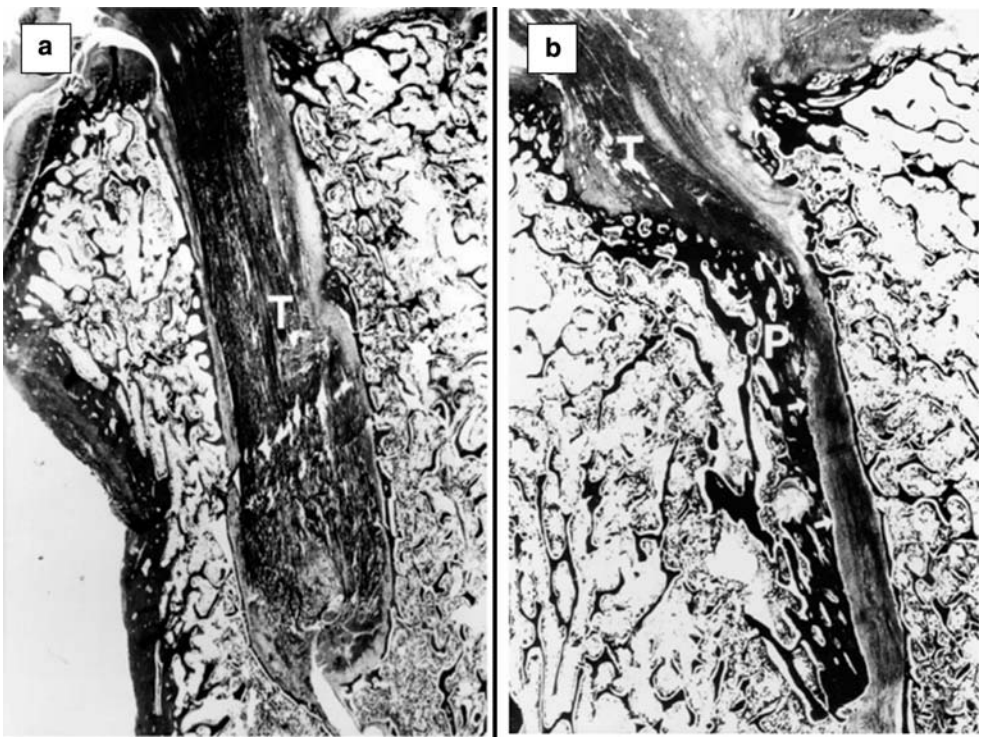

Fig. 2 Comparison between soft tissue graft and bone-plug graft healing in a dog model. **a** Histological section of a soft-tissue graft after 6 weeks of healing shows granulation tissue that filled the tendon-bone interface. **b** Histological section of a BTB graft after 6 weeks of healing shows graft incorporation on the trabeculae face of the bone plug (*T* tendon, *P* bone plug) (H&E staining, 100×. Adapted with permission from Tomita et al. [105])

[73, 74, 105]. Interestingly, although bone-plug graft shows complete histological incorporation between 6 and 8 weeks, the stiffness and ultimate failure load of the graft decrease to the lower values compared to control ACL at the same time points [17, 73].

The insertion site of patellar tendon consists of four zones (tendon, fibrocartilage, mineralized fibrocartilage, and bone) and is similar to the normal direct insertion site of ACL. Some authors think that this is an advantage of BTB graft over soft-tissue grafts [72, 73, 105, 126]. Using a BTB autograft in a dog model, Yoshiya et al. [126] showed that the structure of the insertion site of a patellar tendon at 12 weeks resembles the normal direct insertion pattern of native ACL. A rabbit model [72] showed that the tendon-bone junction underwent an early disappearance of the fibrocartilage when it was placed inside the bone tunnel and a new bone-graft junction was developed at 6 months. If the tendon-bone junction was placed at the articular exit, the fibrocartilaginous layer was present during the entire process of remodeling. The authors suggest that mechanical loading on the graft is responsible for the development of the new insertion site. This restoration of the insertion site with four zones offers the biomechanical advantages in terms of preventing stress concentration [8].

Allograft

In order to eliminate the donor site morbidity and decrease surgical time, allografts have become an option for ACL reconstruction [25, 42, 56]. Using a dog model, Shino et al. [95] showed no differences between deep frozen (−20°C) patellar tendon allograft and autograft in revascularization, infiltration of mesenquimal cells, and remodeling. At 30 weeks, the mean maximum tensile strength of the two grafts was also comparable. Nikolaou et al. [69] used a cryopreserved (−80°C) ACL allograft in a dog model and found no differences in revascularization rate and structural or biomechanical properties such as ultimate failure load and stiffness compared to those of ACL autograft.

Conversely, in other studies, these comparable properties of healing ACL autografts and allografts could not be reproduced. A comparative study between ACL reconstruction with BTB autograft and deep frozen (−85°C) allograft in a goat model [52], showed that at 6 months, the autograft group had better biomechanical properties such as higher ultimate failure load, less anterior tibial displacement, and increased density and number of small diameter collagen fibrils. Zhang et al. [133] investigated the differences in the insertion site between a fresh soft-tissue allograft and an autograft in a dog model. After 6 months, a clear four-layer insertion site was primarily formed in the autograft group. On the other hand, a less organized insertion site was found in the allograft group. In addition, a recent sheep model study demonstrated inferior mechanical properties at 52 weeks in fresh-frozen allograft when compared with autograft [89].

When using allograft, gamma radiation can be used to sterilize the graft to reduce the risk of disease transmission. It is known that the dose of radiation has a direct effect on the biomechanical properties of the graft such as ultimate failure load and stiffness [78, 88]. In a goat model study [88], reductions in maximum force and stiffness were found to depend on gamma radiation dose (4, 6, or 8 Mrad). Increased dose resulted in decreased structural properties of a patellar tendon graft at time point zero, including 46 and 18% reductions in maximum force and stiffness, respectively, compared to control. A more recent goat study showed that at 6 months, highly irradiated grafts (4 Mrad) had lower stiffness and lower ultimate failure load compared to controls [90]. To prevent these adverse effects, radiation doses between 1.5 and 2.5 Mrad are currently used in most tissue banks [64]. Although, this dose range is effective in reducing bacterial contamination, is not effective against viral agents.

Based on the aforementioned studies, the results of animal models comparing graft remodeling, graft structural properties, and incorporation processes of allograft versus autograft are inconsistent [52, 69, 89, 95, 133], probably due to the lack of uniformity in these studies, in terms of allograft processing and sterilization, different graft tissue used and different end-point evaluations, which make comparison between the studies difficult.

Graft placement

Although is known that tunnel misplacement is one of the main causes of allograft and autograft failure of ACL surgery [23, 37, 53, 54], correct tunnel placement is still a matter of debate and there is no consensus as to the correct location [19, 47].

An anatomical tunnel placement is necessary to achieve a physiological loading of the graft, avoid overstretching, promote bone-graft healing, and restore knee stability. The importance of a correctly placed graft is highlighted in a retrospective analysis of several different animal models [40]. The study found a statistically significant inverse correlation between the extent of anterior–posterior tibial translation and cross-sectional area of the graft. Grood et al. [40] suggested that a slack graft is stress shielded by other structures of the knee, which reduces the in vivo mechanical loads. This in turn decreases the cellular metabolism, resulting in a small cross-sectional area over time.

The mechanical stress has a role in modulating the healing process. In an extra-articular rabbit model [116], the extensor digitorum longus tendon was transplanted into

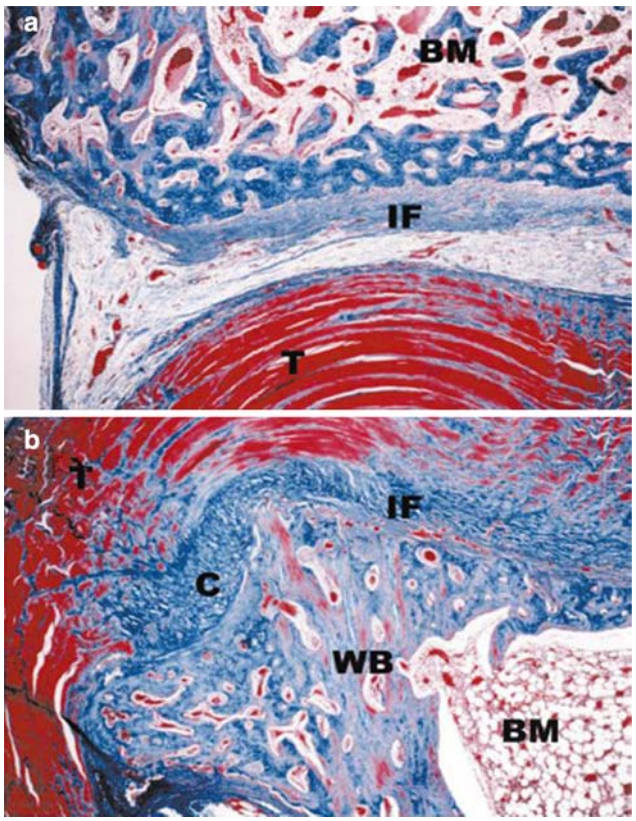

Fig. 3 Different type of healing in a soft tissue graft subjected to tensile forces (**a**) in the upper region of the tunnel and compressive forces (**b**) in the lower region of the tunnel after 4 weeks of healing (*BM* bone marrow, *IF* interface tissue, *T* tendon, *C* chondroid foci, *WB* woven bone). Extensive fibrovascular tissue in the upper region and foci of chondroid-like cells on the woven bone layer on the lower side were present (Masson trichrome staining, 40×. Adapted with permission from Yamakado et al. [116])

a tibial bone tunnel perpendicular to the long axis of the bone. As a result, the proximal aspect of the graft-tunnel exit was subjected to tensile stress and the distal aspect of the graft-tunnel exit to compressive forces. The tensile stress exit showed a different healing process with the presence of more abundant Sharpey′s-like fibers in the early phase and the development of a direct insertion type with four zones after 6 months, whereas the compressive stress exit showed chondroid formation and woven bone (Fig. 3). These tensile and compressive loads are certainly related to tunnel placement and tunnel direction [91, 92]. Therefore, when different graft/tunnel placement is used, the influence of the tensile and compressive loads on graft healing needs to be further clarified. An animal model that explore differences in the healing process when anatomical and non anatomical reconstructions are performed, could give us valuable information related to the effect of graft tunnel on the healing process.

The femoral tunnel placement has been shown to be critical, as small variations in graft placement have large effects on laxity and tension patterns during the complete range of motion [44, 66, 132]. Also, a more anatomical femoral graft placement offers biomechanical advantages in terms of better control of anterior tibial translation under anterior and rotatory tibial loads [62, 67, 132].

The knowledge that single bundle ACL reconstruction does not restore normal knee kinematics [36, 79, 101, 102] and the current trend of reconstructing the torn ACL by attatching it more to its anatomical insertion sites have led some surgeons to move the femoral tunnel lower in the notch (9–10 o'clock position) [18, 77] and to replace the two bundles of the ligament [2, 4, 16, 21, 24, 65, 114, 120]. Currently, little is known, however, about the healing process of the graft used in the double bundle (DB) ACL reconstruction. Cho et al. [29] performed a comparative study of double versus single bundle ACL reconstruction with hamstring autograft. They found that the diameter of collagen fibrils in the double bundle group was significantly larger than that in the single bundle group. Based on the histological finding, the authors speculate that the tensile strength may be greater in the double bundle group, but this remains to be verified by mechanical testing.

Although, there is a large amount of information about biomechanics regarding the different options for graft placement in cadaveric models, a large gap still exists between the ex vivo results and in vivo outcome relating load and biological healing. The importance of a more anatomical graft placement was emphasized in several biomechanical studies [62, 67, 132], which suggest that more physiological loading of the graft is achieved when graft placement is closer to the anatomical location. However, to our knowledge, there are no animal or humans studies that explore directly the relationship between tunnel placement and graft healing. Further research is needed to determine whether a more anatomical graft placement improves the graft incorporation in a bone tunnel.

Graft length within bone tunnel and graft-tunnel diameter disparity

It is commonly believed in the clinical setting that better healing of the graft can be achieved by having more graft tissue within the bone tunnel, as well as a tighter fit. However, support from basic research for this belief is rather weak. One study with an extra-articular dog model [39] showed that pull-out graft strength at 6 weeks was enhanced by increase in the length of tendon within the tunnel; however, a recent study that compared 5 and 15 mm tibial intra-osseous graft length [118] found no differences in histological and biomechanical properties of the healing graft at 6 weeks. The different animal models (extra-articular [39] and intra-articular [118]) could explain the different results.

A dog model was used to investigate the effect of graft-tunnel diameter on the intra-osseous healing of the flexor tendon graft [117]. The authors found no differences in ultimate failure load and histological properties of the healing graft with disparity of up to 2 mm. Surprisingly, there were more perpendicular fibers connecting the graft to the bone in the 2 mm disparity diameter group, where the space between the graft and bone was greater. It is unknown, however, whether graft-tunnel diameter disparity greater than 2 mm can affect graft-tunnel healing. Based on these studies, no sufficient evidence currently exists to make recommendations for either a specific graft length within the bone tunnel or maximum graft-tunnel diameter disparity.

Graft fixation

The demand for a rapid return of knee function requires a secure mechanical fixation in the early post-operative period, before "biological fixation" takes place through healing of the graft in the bone tunnel [93]. Biomechanical testing has shown that graft materials show higher initial strength than the ACL graft [22, 41, 70], and therefore, the competence of an ACL reconstruction immediately after surgery depends on the surgical fixation technique. Is important to note that early after surgery (6–7 weeks) the biomechanical properties of graft, such as stiffness and failure load decrease [17] and this is not related to the fixation device but to the intrinsic remodelling process of the graft.

The increasing interest in soft-tissue graft, such as hamstring autograft and allograft for ACL reconstruction has led to the development of many different fixation devices. Although there are a large number of publications exploring differences in biomechanical properties between different fixation devices at time 0 [10, 14, 43, 45, 57, 58, 122, 130], comparative studies exploring differences in biological incorporation of soft-tissue grafts between different fixation methods are lacking [96, 131].

The two different mechanisms of tendon-to-bone healing that occur in soft-tissue graft are intra-tunnel healing and surface healing. In an intra-articular sheep model study [110], the histological healing process of ACL reconstruction with autologous Achilles tendon graft fixed with biodegradable poly-(D,L-lactide) interference screw was evaluated. A fibrous inter-zone between the graft and the bone tunnel was only partially developed. At 24 weeks, some cases showed a bony separation between the intra-tunnel graft and its insertion site, and the intra-tunnel portion of the graft appeared unstructured and partially reabsorbed. At 52 weeks, a normal four zones direct insertion site was found (Fig. 4). The authors suggested that the use of a press-fit mechanism of fixation neutralizes the graft-tunnel motion and promotes the development of a direct type of ligament insertion. They also suggested that

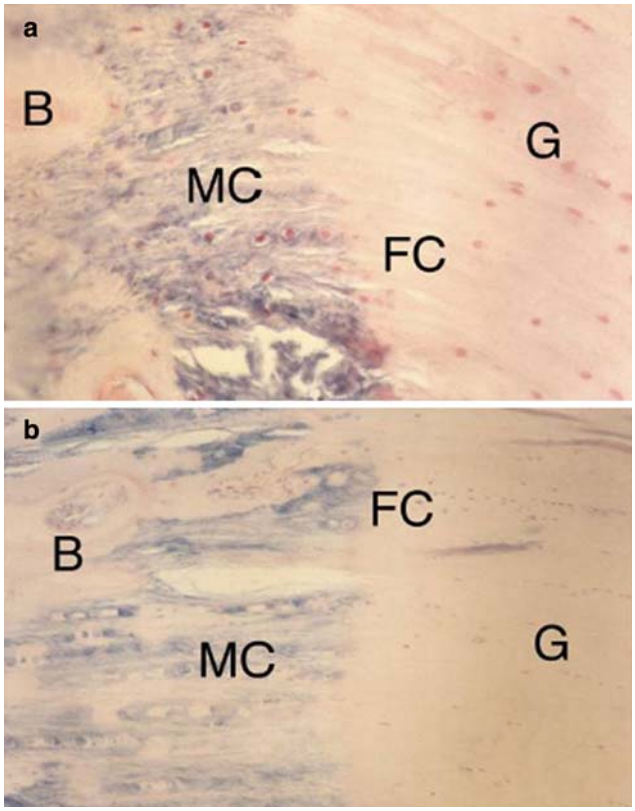

Fig. 4 **a** Tibial graft insertion after 52 weeks of healing in a sheep model showing the four zones of normal direct insertion site using bio-absorbable interference screw fixation. **b** Histological section of femoral insertion of extensor tibialis muscle for comparison (*B* bone, *MC* mineralized cartilage, *FC* fibrocartilage, *G* graft) (Alcian blue staining, 252×. Adapted with permission from Weiler et al. [110])

once the surface healing has been established, the intra-tunnel healing is stress shielded, and that portion of the graft is reabsorbed. These findings were confirmed in a 2-year study using the same animal model [48]. While the findings are important in understanding the healing process of soft-tissue graft using a direct type of fixation, no comparative group with different fixation device in these studies was used.

The presumed advantages of the direct type of fixation for soft-tissue graft are critically analysed during the early post-operative period by several animal models [96, 111, 131]. According to their findings, the use of interference screw has detrimental effects on the early healing process for soft-tissue graft. In a sheep extra-articular model, the long digital extensor tendon was transplanted into a bone tunnel drilled in the tibial metaphysis [96]. Two different fixation methods were compared: bio-absorbable interference screw (Bio-Interference Screw, Arthrex, Inc., Naples, Florida) and an extra-articular fixation device (WasherLoc, Arthrotek, Inc., Warsaw, Indiana). After 4 weeks of implantation, the strength and stiffness of the complex fixed with interference screws deteriorated 63 and 40%,

respectively. For the WasherLoc group, the strength at 4 weeks was similar to that at time point zero, whereas the stiffness improved 136% also compared to that at point zero. Also in a sheep intra-articular model [131], the biomechanical properties of soft-tissue graft fixed with bio-absorbable interference screw (Absolute Absorbable Interference Screw; Innovasive Devices, Marl-borough, MA, USA) was compared with a cross-pin fixation device (RigidFix, Ethicon, Mitek Division, Norderstedt, Germany) at 6 weeks of implantation. For knees fixed with interference screw, strength and stiffness deteriorated 81 and 67%, respectively. For knees with cross-pin fixation, the graft strength deteriorated by 48% and stiffness improved by 52%. Although the first study used an extra-articular model [96] and the second an intra-articular ACL reconstruction model [131], the results are similar: both showed a deterioration of the healing process with the use of bio-absorbable interference screws in the early post-operative period compared with an extra-articular fixation device and a cross-pin fixation device. Although a direct chondral insertion can be obtained with interference screws [110, 111], this fixation method might make the graft more vulnerable to failure during the early postoperative period. The findings of these animal models suggest that a less aggressive rehabilitation protocol might be recommended when bio-absorbable screws are used for soft-tissue graft fixation.

Graft tensioning

Clinically, the optimal graft tension to apply during fixation is still unknown [68, 108, 121, 125]. Few studies compare the effect of different graft tensioning on the healing process and the biomechanical behaviour of the graft [1, 60, 124], and there is disagreement on the effects of initial graft tension among them.

Higher initial graft tension (17.5 versus 1 N) resulted in improved pull-out force and stiffness at 32 weeks post-operatively in a rabbit model [60]. On the other hand, a dog model study compared 1 versus 39 N of load during graft fixation and found no differences after 3 months in laxity and mechanical measurements of graft strength [124]. However, histological examination showed evidence of poor vascularity and focal myxoid degeneration within the 39 N pre-tensioned graft. Similar findings were reported in a goat model study [1]. At time zero, high tension could better reproduce the biomechanical properties of the normal ACL, but after 6 weeks of healing, the anterior tibial translation, in situ forces, stiffness and ultimate failure load, were not significantly different between high (35 N) and low (5 N) tension group. Although the type of graft (BPTB) was the same in these three studies, the animal model, the tension values, the fixation devices, and the knee angle at fixation were different, making a definitive conclusion difficult. Probably not only excessively low tension, but also excessively high tension may reduce the biomechanical properties of the graft. Only adequate tension within a relatively narrow range may prevent the deterioration of the graft. However, at present, the extent of this range is unknown. To determine the optimal tension that should be applied to the graft, further ultra-structural, biochemical, and biomechanical studies should be conducted.

Graft-tunnel motion and rehabilitation

Delayed graft healing and tunnel enlargement can be explained by excessive graft-tunnel motion [45]. However, little is known about the relationship between graft-tunnel motion and graft healing. The amount of motion between graft and bone tunnel has been related to the type of fixation used and the postoperative rehabilitation program [12, 15, 110, 111]. In a recently published study [82], the authors first performed an ACL reconstruction in cadaveric rabbit knees with hamstring autograft fixed through sutures to the periosteum. Greater graft-tunnel motion was found in the femoral tunnel when compared to the tibial tunnel. Also, there was significantly greater graft-tunnel motion at the articular side than at both the mid-tunnel and the extra-articular side. An in vivo study was performed thereafter. Animals were subjected to ACL reconstruction with the same technique, and histomorphometry was used to compare tendon-to-bone healing. In the results, the healing rate was slowest at the articular side of the tunnel compared with the mid-tunnel and extra-articular side, and there was an inverse correlation between graft-tunnel motion and healing in the femoral tunnel.

There is a general consensus that an early aggressive rehabilitation protocol increases graft-tunnel motion. This may contribute to tunnel enlargement as the graft-bone interface is subjected to early stress before biological incorporation and the subsequent healing process could be affected [46, 113]. However, there is not enough evidence to explain how different exercise protocols influence the graft load and how this modulates the healing process. In vivo studies showed that closed kinetic chain exercises, which involve body weight loading and isometric contraction of the hamstring muscles, subject the normal ACL to lower strain values than open kinetic chain exercises, and are considered safe exercises during the early postoperative period to protect the graft after the reconstruction [11–13]. Early articular motion and early weight bearing are also considered to be beneficial [71, 85, 107]. No study, however, evaluated the strain limits or sufficient/detrimental load required for successful graft healing after ACL reconstruction.

Graft healing studies in human

Due to obvious technical difficulties, knowledge about graft healing process in human subjects is limited. Through core biopsy of tibial side during revision surgery, histology was performed in two patients and showed a complete integration of the graft when the failure was traumatic and graft degeneration to myxoid tissue in the non-traumatic case [31]. Two cases of traumatically failed hamstring ACL autograft showed, again through core biopsy, that a completely integrated graft with the surrounding bone occurred, with presence of Sharpey′s-like fibers as early as 12 weeks [76].

Analyzing the histology of a series of patients subjected to revision after BTB autograft fixed with interference screws, Ishibashi et al. [49] noted the presence of the original tendon-bone junction in the graft; however, in the cases with more than 1 year since the index surgery, this junction was not seen, and the tendon was linked to the host bone through Sharpey′s fibers. This insertion site healing was also studied performing biopsies in revision surgeries of BTB ($N = 8$) and failed hamstring grafts ($N = 6$) [75]. In the hamstring group, femoral fixation was performed through endobutton and tibial fixation through cortical cramp. The findings showed direct insertion of collagen fibers into the bone. In patellar tendon group, the insertion site in femur showed chondral pattern with the four zones of direct insertion type. However, the tibial side evidenced different insertion healing depending on type of tibial fixation. In all cases with screw interference fixation within the bone tunnel, the insertion site resembled direct chondral pattern, but when the graft was fixed outside of the tunnel (cramp), a fibrous insertion was present.

Rougraff et al. [86] perforrned biopsies at different time points in the central region of the graft in humans subjected to ACL reconstruction with BTB autograft. They demonstrated the viability of graft as early as 3 weeks postoperatively and an absence of the necrotic stage. According to their findings four stages of ligamentization were identified. The first stage is repopulation of cells during the first 2 months in which the graft undergoes a modest increase in fibroblast number and metabolic rate. The second stage is a rapid remodeling between 2 months and 1 year where the number of fibroblast increases dramatically. The third stage is one of maturation, happening between 1 and 3 years, where the graft becomes less cellular and less vascular. After 3 years, the graft becomes quiescent.

Despite the limited data in histological findings in humans, usually presented as case reports or small case series, the results support some findings of the animal model studies, which show that it is possible to achieve both direct and indirect type of healing, and that the fixation method plays a role in the healing process. Other findings of animal models such as graft necrosis were not reproduced in human studies [86, 87]. The relationship between the healing process of the graft and changes in its biomechanical properties is unknown in humans.

New strategies to enhance bone-graft healing

The slower healing rate of soft-tissue grafts compared to bone-plug graft and the interest in aggressive postoperative rehabilitation and early return to sports activities after ACL injuries have led to the development of several techniques to enhance bone-graft healing. These include the use of growth factors, periosteum augmentation, and mesenchymal stem cells.

The use of growth factors has been shown to enhance the graft healing process. For example, in a sheep model, platelet-derived growth factor increased load-to-failure at 6 weeks and vascular density and collagen fibril amount at 6 and 12 weeks, respectively [109]. The use of transforming growth factor-β1 (TGF-β1) in an intra-articular dog model improved the load-to-failure and showed higher density of perpendicular collagen fibers compared to controls [119]. The usually short half-life of these proteins limits their use; therefore, gene therapy is used to achieve a continuous release of a therapeutic protein [63]. The effect of bone morphogenetic protein-2 (BMP-2) has been studied in animal models [63, 83], and the treated side exhibited more extensive bone formation around the tendon and better biomechanical properties compared to paired control limbs.

A rabbit model study reported enhanced bone-graft healing using a sponge carrier vehicle containing a bovine mixture of bone-derived proteins, which included BMP-2, 3, 4, 5, 6, 7, TGF-β1, TGF-β2, TGF-β3, and fibroblastic growth factor-1 [7]. The treated knees showed extensive formation of new bone and cartilage in the tendon-bone interface and significantly higher load-to-failure rates. Using a sheep model [123], the introduction of vascular endothelial growth factor 12 weeks after ACL reconstruction reduced the stiffness of hamstring autograft. Despite the fact that the use of growth factors according to the aforementioned animal studies is promising, caution should be exercised in terms of direct extrapolation to human healing conditions. Certainly more research is required to determine the ideal growth factor, its dosage, release method, and safety.

The use of periosteum as a biological scaffold to enhance bone-graft has been tested in animal models [28, 59, 127] and also in some clinical trials [26, 80]. An extra-articular rabbit model study showed that the periosteal augmentation of a soft-tissue graft had higher ultimate failure load at 6 weeks compared to control group [127]. Comparable results are reported in previous animal models with similar design,

Table 1 Summary of evidence presented in basic research studies regarding the influence of different variables on graft-tunnel healing for ACL reconstruction

Surgical variables of affecting graft-tunnel healing	Effect on healing process	References
Type of graft: soft tissue versus bone-plug graft	Slower incorporation rate into the bone tunnel with soft tissue grafts compared to bone-plug grafts	[74, 75, 82, 106]
Type of graft: auto versus allograft	Controversial findings between studies regarding incorporation rate. Lack of uniformity between studies	[53, 70, 90, 96]
Graft placement	Large gap between ex vivo and in vivo studies exploring relationship of load and graft-bone healing	[41, 118]
Graft length within bone tunnel and graft-tunnel diameter disparity	Not enough evidence to make specific recommendation of precise graft length or maximum diameter disparity	[40, 119, 120]
Graft fixation	Direct chondroid insertion site achieved with interference screw fixation for soft-tissue graft, which might be biomechanically more vulnerable during the early phase of healing	[49, 97, 112, 113]
Graft tensioning	Controversial findings in literature. Lack of uniformity in studies. Best tension value currently unknown	[1, 61, 127]
Graft-tunnel motion	One study explores effect of graft motion in healing. Healing was inversely proportional to graft-tunnel motion	[83]

reporting also better biomechanical and histological healing when using periosteum-wrapped grafts [28, 59]. Some clinical experience exists with the use of periosteal augmentation in soft-tissue graft. A prospective case series study reported good clinical results in instrumented laxity measurement, Lysholm and IKDC scores, using hamstring autograft enveloped with autologous periosteum [27]. Significant reduction of enlargement at the articular side of the tunnel was reported in a prospective randomized study [80] using a periosteal-augmented graft in femoral tunnel widening after ACL reconstruction.

Although there is experience with the use of mesenchymal stem cells (MSCs) in different settings related to tissue healing [32, 103, 106], the work with its use in bone-graft healing is limited. In a rabbit model study, grafts were coated with fibrin glue containing MSCs [61]. Histologically, the treated knees showed healing through fibrocartilage junction resembling direct pattern of ligament insertion, whereas control knees healed by presence of Sharpey′s fibers. Biomechanically, the MSC-treated knees had significantly higher failure loads and stiffness when compared to contralateral controls at 8 weeks. The same group obtained similar results using allograft coated with MSCs in a rabbit model [97].

Summary

To improve bone-graft healing in ACL reconstruction, it is necessary to have a detailed knowledge of the effects of surgical variables on the graft-tunnel healing process. Despite the inherent limitations of animal models, including the differences in anatomy, biomechanics, and uncontrolled postoperative rehabilitation program, remarkable advances have been made in understanding the graft healing process. Due to these limitations and the differences between the study designs, i.e., extra-articular versus intra-articular models, direct application of these results to human cases must be considered with caution. However, it seems clear that surgical variables, which include type of graft, graft motion, and fixation methods, directly affect time-course and quality of graft-tunnel healing. Regarding graft/tunnel placement, several biomechanical studies have shown advantages with a more anatomical tunnel placement; however, no studies explore directly the effect of graft placement on biological graft-tunnel healing. A summary of the effect of different variables on graft-tunnel healing is provided in Table 1.

Although it is not possible to make recommendations for a specific rehabilitation protocol based on the studies reviewed here—given the differences in the healing quality at different time points reported with different types of graft and different types of fixation—early and aggressive rehabilitation protocols should be a concern when using soft-tissue graft, allografts, and direct or aperture type of fixation for ACL reconstruction.

Despite their ability to enhance graft healing, several techniques (the use of growth factors, periosteum augmentation, and mesenchymal stem cells) are still in development stages, and further studies are needed to investigate the potential clinical application of enhancing healing and decreasing recovery time after ACL reconstruction.

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