REVIEW ARTICLE

Non-vascularised Periosteal Graft Transplantation in Sheep: A practical Guide for Experimental Study

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ABSTRACT

The roles of experimental animals in orthopedic research is very crucial for the management of bone defects. Given the various drawbacks of the vascularised periosteal graft, researchers began to explore the effectiveness of non-vascularised periosteal graft in fracture healing management. Small laboratory animals especially rabbits have been widely used for non-vascularised periosteal transplantation research but with more challenges of obtaining a good graft size. Therefore, bigger animals, such as sheep—with more morphological and physiological similarities to human are used as animal model for surgical procedures. The article elaborates the features of periosteum, comparative anatomy of sheep's hind limb, pre-surgical preparation, and surgical procedure for the transplantation of non-vascularised periosteal graft, especially for researchers operating on sheep for the first time. A male sheep of 4-month-old was used for this procedure. Periosteal graft was harvested from tibia and transplanted to femur to mimic treatment of bone gap in orthopedic surgery.
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INTRODUCTION

Periosteal graft transplantation is a procedure used to enhance bone formation for over a century (1,2). Periosteal graft may be transplanted either with attached blood vessels (vascularised graft) or without blood vessels (non-vascularised graft) (3). Since vascularised periosteal graft demands advance microsurgical anastomose skills and prolong surgery, non-vascularised periosteal graft (NVPG) had been an option. It was observed that the disturbance of periosteum led to new bone formation, as periosteum has the osteogenic capabilities (4-6). The cambium layer of periosteum is an essential component for bone development as it was proven to result in bone formation when transplanted as a free graft (4). NVPG wrapping intercalary bone allograft can be used for the management of bone defects that resulted from infections, bone loss after trauma and tumour removal or specific illnesses including congenital pseudo arthrosis of the tibia (CPT) (7-9).

For periosteal transplantation, rabbit is among the most commonly used experimental animal (10-15). This might be due to the facts that rabbits can modify and rapidly replace their bones, and they also experience extensive intracortical and Haversian remodeling (16,17). Of all strains of rabbits, New Zealand White (NZW) rabbits is the most frequently used by researchers— this might be because the strains are less aggressive and have fewer health issues than other breeds (18). However, there are some drawback using rabbit as a models for experimental research. For instance, there are differences in joint loads and biomechanics between rabbits and humans, as well as a chance of synovitis and osteoarthritis following surgery (19).

Previous studies reported that NVPG aided fracture healing and contributed to new bone formation with less post-operative complications (14,20-22). Nevertheless, the use of NVPG for the management of bone defects is limited by the reduction in size of the graft, due to its contractile nature following harvest from donor bone (9,23). Apart from its potential healing effect in experimental animals, NVPG has been used in humans for the management of articular cartilage defects (13,24) and CPT (9).

A reliable way of expressing research ideas, which replicates the real human body system, either structurally or functionally is via animal model (25). The use of laboratory animal in biomedical research remains the best alternative to investigate and manage bone defects as well as fracture healing (25). Among the extensively utilised large animal model in biomedical research is sheep (Ovis aries sp). Although, sheep is less likely used in research when compared to mice, rats and rabbits, they have similar physiological characteristics with human, and is preferred to be used as a model for surgical procedures due to their large size and easy manipulation for surgical procedures (26).

Despite the morphological and physiological relevance of sheep as a model to study the effect of periosteal graft transplantation on fracture healing and bone formation, there is a scarcity of resources reporting on the surgical method and approach of the NVPG transplantation on sheep model. Since an important and commonly used donor site for harvest of periosteal graft is tibia, while a femur is used as a recipient site for experimental studies, understanding the anatomy of the sheep hind limb plays an important role to increase the success rate of NVPG transplantation.

This paper elaborates the features of periosteum, comparative anatomy of sheep's hind limb including its blood supply and pre-surgical animal preparation. The surgical procedure described harvesting of the NVPG from ipsilateral tibia, exposure to the recipient femur, fixation of bone allograft on the recipient femur followed transplantation of the NVPG on the allograft. The advantages and methodological challenges of this surgical method were also elaborated.

MORPHOLOGY OF PERIOSTEUM

The periosteum is made up of an inner cambium layer and an outer fibrous layer, which vary in the proportion of fibres, cells, and matrix. With the exception of articular surfaces, tendon insertions, and sesamoid bone surfaces, most bones possess a well-vascularised fibrous sheath covering them known as periosteum (27). The inner cambium layer, which is also known as osteogenic layer, is a proliferative layer that faces the bone and contains osteoblast and osteoprogenitor cells; while, the outer fibrous layer comprises of fibroblasts, collagen, reticular and elastin fibres (5,28). The outer fibrous layer is further sub-divided into superficial and deep layers. The superficial layer is strongly vascularised and innervated, has a loose collagenous matrix with a few scattered elastic fibres, and is typically inelastic and contains limited cells. The deep layer is less vascular and cellular than the superficial layer, but the majority of the fibroblasts in the periosteum reside in this layer (28).

Sharpey's fibres, which are collagen fibres that reach into bone, bind the periosteum and bones together (5). Tension forces determine the orientation of these collagen fibres. Tight connections of tendons and bones are the result of these fibres penetrating the whole cortex at the locations subjected to significant amounts of tension pressures (29). Varied areas of the bone have different periosteum thicknesses. For example, the periosteum is thicker and more easily detached from the underlying bone in the region of the diaphyses of long bones, but it is strongly bonded to the bones in the epiphyseal and metaphyseal region, where it is slender. Periosteum in children is strong, flexible and elastic, but it gets thinner and loses its strength and flexibility as a child grows (30). This explains why certain biomechanical characteristics of children's fractures are different from that of adults. For instance, periosteum is typically intact on the concave side of bone fractures in children which occurred without periosteum disruption (31). In contrary, the tensile pressure and shredding forces in a bone fracture of an adult can result in periosteal fracture (5).

GROSS ANATOMY OF SHEEP HIND LIMB

According to Allen et al (1998), there are some similarities between human lower limbs and sheep hind limbs (32). In terms of osteology, a human lower limb and a sheep hind limb are composed of a femur, patella, tibia, fibula, tarsal bones, metatarsal bones, and phalanges (33,34). In human, there are a pelvic bone—that is formed by the union of a pubis, iliac and ischium bones— a femur, a patella, a tibia, seven tarsals, five metatarsals, and 14 phalanges for each limb. While in sheep, the bones of the hind limbs are the os coxae (hip region), femur (thigh region), tibia and fibula (crus), tarsal bones (hock or tarsal region), fused third and fourth metatarsals (cannon or large metatarsal bone), and digits, whereby each weight-bearing digit is held together by proximal, middle, and distal phalanges (35). The bones of sheep hind limb are illustrated in Fig. 1a.

Sheep hind limb fascia is divided into superficial and deep fascia. Fats are stored mainly in superficial fascia (36). Deep fascia is arranged into fibrous sheet. Fascia lata is a deep fascia of the thigh that can be found in sheep hind limb. The tensor fascia lata is inserted into the patella and cranial tibia by the large connective tissue sheet that is modified from fascia lata (35).

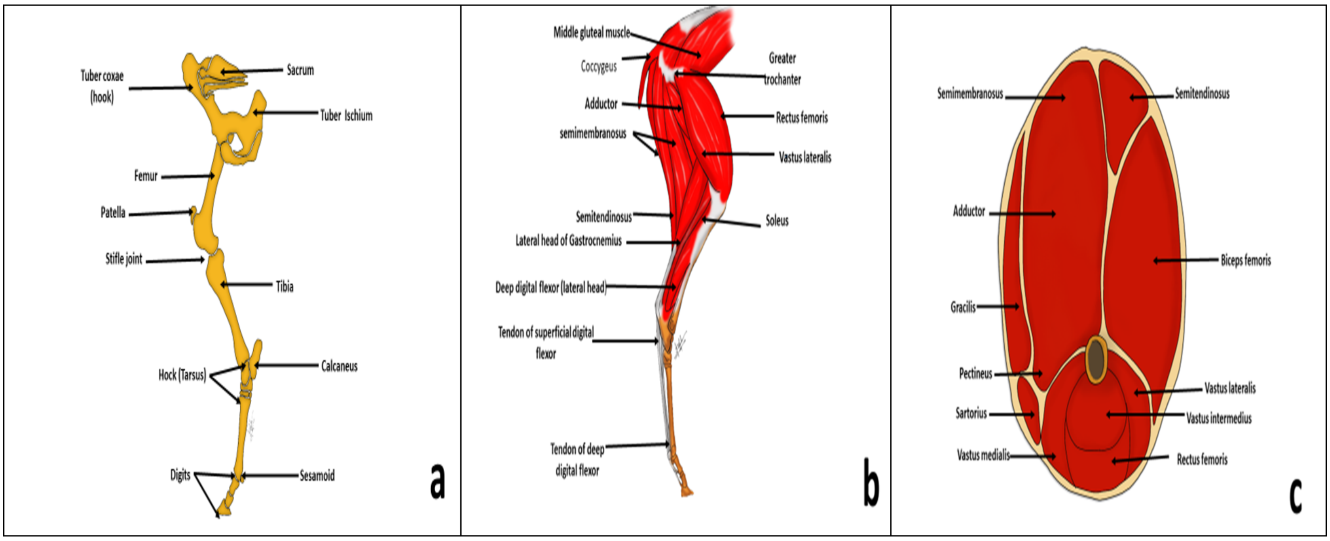

Figure 1: Anatomy of sheep hind limb. (a) Bones of sheep hind limb. (b) Lateral view of sheep hind limb muscles. (c) Cross sectional view of Sheep hind limb muscles.

Generally, the muscles of thigh and leg in sheep may be divided into three groups according to its action; muscles acting on the hip joint, stifle joint as well as hock and digits (35). The muscles acting of the hip joints include the hip flexors, extensors, and adductors (35). The hip flexors include iliopsoas, sartorius, tensor fasciae latae and rectus femoris.

The primary extensors of the hip joint are the gluteal and hamstring muscles, which are also referred to as the "rump muscles,"(35). In general, the gluteal muscle is composed of the superficial, medium, and deep gluteal muscles. On the other hand, the hamstring is formed by the biceps femoris, semitendinosus, and semimembranosus muscles. The superficial gluteal muscle and biceps femoris combine to form the gluteobiceps muscle, which is present only in ruminants (32,36). The semitendinosus and semimembranosus muscles are located on the opposite side of the medial aspect of the thigh, with semitendinosus located between the Gluteobiceps and semimembranosus muscles caudally (35).

As for the adductors of the hip joints, these muscles are the medial thigh muscles, which include pectineus, adductor femoris, gracilis and external obturator muscles (35,37,38). The pectineus is a small muscle that is located between the sartorius and gracilis and forms the caudal border of the femoral triangle (35,37). The Gracilis muscle is a broad and flat muscle that is closest to the skin and originates from the symphysial tendon, and it surrounds the semimembranosus on the medial thigh (35,38). The External obturator muscle in ruminants comprises intrapelvic and extrapelvic components. The ventral and dorsal surfaces of the obturator foramen are covered by it, which is located close to the Adductor muscle's origin (35,36).

For muscles acting on the stifle joint, they can be categorised into the extensors and flexors of the stifle joint. The main stifle extensor is the quadriceps femoris muscle, which is the largest muscle on the cranial surface of the femur is the quadriceps femoris. This muscle is composed of the rectus femoris, vastus lateralis, vastus medialis, and vastus intermedius (36).

The final group of hind limb muscles are the muscles acting on the hock and digits. This group of muscles can be roughly separated into two groups: caudomedial and craniolateral. The muscles in the craniolateral group can either flex the hock solely or stretch the digits. The cranial tibial, fibularis (peroneus) tertius, long and digital extensor, fibularis (peroneus) longus, and lateral digital extensor muscles make up the craniolateral muscle group. The soleus, gastrocnemius, superficial, and deep digital flexor, and popliteus muscles are included in the caudomedial group. They collectively stretch the digital joints and lengthen the hock (except from the popliteus) (35,38). The muscles of the sheep hind limb are illustrated in Fig. 1b and 1c.

BLOOD SUPPLY AND INNERVATION OF THE SHEEP HIND LIMB

The proximal hip region of sheep is supplied by the caudal gluteal artery—a terminal branch of internal iliac artery inside the pelvis—and the cranial gluteal artery, a branch of the internal iliac artery (35,38). The cranial gluteal artery exits the pelvic cavity through the greater ischiatic foramen, while the caudal gluteal artery exits pelvic cavity through the lesser ischiatic foramen (35,38). The femoral artery, which is the continuation of the external iliac artery is the main blood supply of the hind leg (32,35,38). In the distal thigh, the femoral artery divides into multiple branches, one of which being the saphenous artery. Along with the medial saphenous vein and saphenous nerve, the saphenous artery runs superficially between sartorius and gracilis. On the medial thigh, the saphenous artery obliquely traverses the distal end of gracilis muscle (32,35).

The popliteal artery runs through the popliteal notch between the medial and lateral heads of the gastrocnemius muscle before splitting into the cranial and caudal tibial arteries (32,35). Together with the deep peroneal nerve, the cranial tibial artery travels distally deep to the cranial tibial muscle on the dorsal surface of the crus. The cranial tibial artery continues as the dorsal pedal artery on the dorsal aspect of the hock, which eventually gives off the perforating tarsal branches. The dorsal pedal artery continues as the dorsal metatarsal artery III distal to this branch and extends distally to give rise to the tiny dorsal axial proper digital arteries III and IV at the distal portion of the big metatarsal bone (35,38). The veins of the hind limb are separated into superficial and deep satellite veins. With no satellite artery, the lateral saphenous vein travels along the lateral aspect of the hock, metatarsal, and digit bones. On the other hand, the medial saphenous vein, has a satellite saphenous artery (35,38). Deep in the medial thigh, the lateral saphenous vein empties into the medial circumflex femoral vein and flows proximally deep to the distal end of the gluteobiceps muscle. However, the medial saphenous vein enters the femoral vein in the middle of the thigh and runs alongside the saphenous nerve and a satellite artery (35,38).

The second sacral nerve of the lumbosacral plexus, which is connected to the last four spinal nerves, provides the pelvic limb with nerve supply (39,40). The femoral, obturator, cranial gluteal, sciatic and its two primary tributaries, the common fibular (peroneal) and tibial nerves, are the major nerves of the hind limb (35,36,38). Deep within the gluteobiceps femoris lies the big sciatic nerve, which gives off the caudal gluteal nerve that innervates the gluteobiceps muscle (35).

PREOPERATIVE PREPARATION

The key important feature for a successful experiment that involves non-vascularised periosteal graft transplantation surgery in sheep is the selection of sheep with appropriate age and sex, given that the osteogenic potential of periosteum is age- and sex-dependent (30,41). For the purpose of harvesting sufficient non-vascularised periosteal graft at the donor site, a sheep of four to six months old is suitable. It was reported that the progenitor cells which occupy the cambium layers as well as the thickness of the layer itself reduces as age advances (30). It was also proven that male develop more trabecular and cortical bone density than female. Periosteal growth is conspicuously seen during childhood, and males produce bones at a faster pace than females (42). Hence, male sheep within the age range of four to six months old is suitable for the experimental study involving the periosteal graft transplantation surgery.

While sheep is suitable for experimental study involving NVPG transplantation surgery due to its ability to be surgically manipulated for orthopedic research, there are diseases that may restrict its usage for this purposes. For example, sheep with lameness are not suitable for surgery as the condition would affect bone healing (43). Furthermore, lameness in sheep may be related to contagious foot diseases such as contagious ovine digital dermatitis (CODD) and footrot, which could subsequently infect other sheep kept in the same sheep pen. Hence, it is important to quarantine the selected sheep from other animals in the facility during the acclimatization phase—which is commonly conducted two weeks prior to the surgery—for health screening as to ensure it is clear of contagious diseases that might spread to other animals (44). Prior to surgery, the sheep is subjected to fasting for 24 hours to prevent rumen distension; however, water must be made available to prevent dehydration. Fasting is done to avoid nausea and prevent food or drink from entering the lungs.

ANESTHESIA PROTOCOLS AND METHODS

For the periosteal harvest and transplantation surgery, the sheep needs to be sedated and induced under general anesthesia. Although premedication is not essential prior to induction of general anaesthesia in small ruminant, introducing premedication such as opioid and sedative can provide muscle relaxation and analgesia, and decrease the dose of subsequent anaesthetic agents (45). Opioids like Butorphanol, 0.05–0.20 mg/kg—either be given via intramuscular or intravenous route—and buprenorphine, 0.01 mg/kg, which is given intramuscularly, are opioids that are excellent analgesia and muscle relaxation for small ruminants (46). Sedative agents such as Xylazine and medetomidine is sometimes used for premedication of sheep and goats but the degree of sedation is dose dependent (47). Given the high sensitivity of sheep to xylazine, small dose of xylazine (0.02-0.3 mg/kg) is required to reduce the risk of overdose. It was also argued that the use of xylazine in small ruminants is not always well-tolerated (48).

Besides that, the use of atropine as premedication in small ruminants is controversial, as it can result in undesirable side effects (i.e., tachycardia, ocular effects, and decrease intestinal motility). However, the use of atropine—which antagonises the effects of the acetylcholine—as a premedication drug, can reduce gastrointestinal secretion including salivation, respiratory tract secretion and dilation of bronchioles; and blockage of vagal impulse, and blockage of the effects of drugs that stimulate the parasympathetic nervous system, such as α2-adrenoceptor agonist (49). Hence, the use of atropine still has some value in the surgery of small ruminants if the advantages far outweigh the disadvantages. It is recommended that the dose for atropine, given as a prophylactic agent is 0.15-0.3 mg/kg via intramuscular route (49).

As for the anesthetic agents, its selection depends on various factors, namely the species, health condition of the sheep, surgical procedure that is to be performed, or whether the sheep is intended for food or not. Diazepam in combination with ketamine, given through intravenous route is a good induction for anesthesia. However, other anesthetic agents such as Tiletamine, zolazepam or Telazol can be administered if extended duration of anesthesia is required especially for major and complicated surgery. For a periosteal harvest and transplant surgery in sheep, intubation of the trachea should be performed but it is technically challenging, due to limited head and neck extension and narrowed jaw. To perform the tracheal intubation, the head and neck of the sheep need to be extended to create a straight line from mouth to trachea and a long-blade laryngoscope is used to visualise the larynx and depress the epiglottis (Fig. 2a). Stylet is placed into the proximal trachea, and then the endotracheal tube is advanced over the stylet and into the trachea (48). During the induction of sheep anesthesia, an orogastric tube should be inserted into the rumen, and the sheep should be placed in left lateral recumbency to reduce rumenal bloat (Fig. 2b). Although, anaesthetic management of sheep is usually uncomplicated, there is always a risk of getting regurgitation and pulmonary aspiration; hence, a proper monitoring through the surgery is crucial. It is recommended to switch sides throughout the surgery to reduce complications, and to record the name and dose of the administered drugs, types and rates of fluids give, and vital signs (Heart rate, respiratory rate, pupil size, protective blink reflexes, mucous membranes and body temperature) at specific intervals (49).

NON-VASCULARISED PERIOSTEAL GRAFT HARVEST AND TRANSPLANT PROCEDURE

Preparation of surgical tools and surgical site

The NVPG transplant procedure can mimic the bone gap that is sometimes treated with bone allograft. However, the surgical procedure could also be replicated without allograft. Surgical tools preparation and sterilization are important preliminary steps prior to the surgery. Among the commonly used tools are toothed and non-toothed dissecting forceps, scalpel blade, scalpel hand, scissors, needle holder, artery forceps, retractor, towel clip, periosteum elevator, surgical drapes, needle and syringe. The sterilised tools are placed in orderly manner for easy handling and maintaining sterility (Fig. 2c). To prepare the surgical site, the wool needs to be sheared using clippers or electric wool shearer, and the skin at the surgical site is cleaned with povidone-iodine (Fig. 2d), which is as an effective non-irritating bactericidal regimen (50). This will then be followed with draping as well as exposure of surgical site (Fig. 2e).

Surgical resection of periosteum in the tibia

The skin incision should be made on anterolateral part of proximal tibia, which curves anteriorly over the shin and curved posteriorly at distal level to end at the peroneus muscle (Fig. 3a). This incision should be followed by fascia incision to expose the peroneus muscle which is elevated off the periosteum. Retractors are placed on both sides to retract the peroneus muscle laterally as well as skin and fascia medially. This approach should be directed to the periosteum without meeting the Crania tibial and caudal tibial arteries that travel posterior to tibia and lateral to peroneal muscle. The periosteum is incised according to the required size and elevated from the bone starting from the edges using periosteum elevator and forceps (Fig. 3b). The harvested periosteum should then be placed on a sterilised small stainless bowl wet with buffered sodium chloride solution to prevent tissue shrinkage (Fig. 3c).

Surgical exposure to the femur

Surgical exposure through intermuscular plane is crucial to minimize the bleeding and muscular injury. skin incision was made through lateral plane in between the vastus lateralis, and biceps femoris. This plane would not encounter the femoral artery that lies in the medial compartment of the thigh. Surface anatomy in between the extensor and flexor muscles of the thigh should be used to identify skin and fascia incision. The

Figure 2: Anesthesia protocol and surgical sites preparation; (a) Tracheal intubation in sheep (b) Sheep in lateral recumbence. (c) Orderly arrangement of surgical tools. (d) Surgical site cleaning with povidone iodine solution. (d) Draping and exposure of surgical sites.

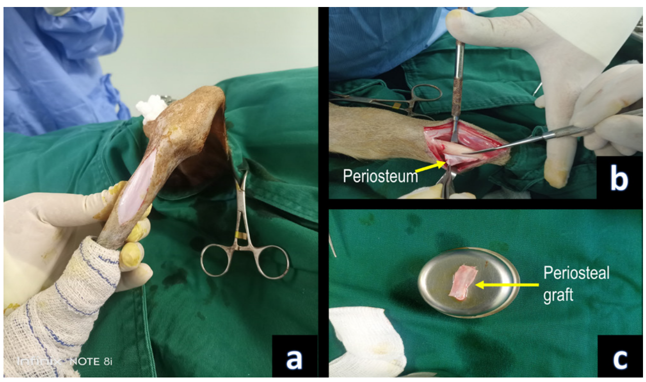

Figure 3: Periosteal graft harvest from donor site; (a) Skin incision on donor bone (Tibia); (b) Elevation of periosteum from donor bone (Tibia); (c) Harvested periosteum from donor bone wet with buffered sodium chloride solution

plane between the vastus lateralis and biceps should be identified and separated, and the incision should be done anterior to intermuscular septum to reach the bone. Perforator vessels is secured with artery forceps without requiring cauterization or suture ligation. The anterior compartment muscles were then separated from the periosteum and retracted anteriorly (Fig. 4a). The intermuscular septum will not be breached to prevent injury to sciatic artery. After the exposure of femur, the surface of the bone to receive allograft should be cleared from periosteum using a scalpel and a periosteum elevator. Allograft of required size will be transferred on femur shaft void of periosteum using two obliquely placed K-wire (Fig. 4b). Subsequently, the harvested periosteal tibia graft is transferred on the bone (Fig. 4c) and absorbable suture is used to anchor the periosteum to surrounding tissue periosteum. Before closure, the surgical area was rinsed with saline 5 times to dilute any possible bacterial contamination. Muscles were then allowed to fall back into placed. Fascia and skin were closed in layers with absorbable suture. Wound was coved with dry dressing. The whole surgery was between 2 to 3 hours.

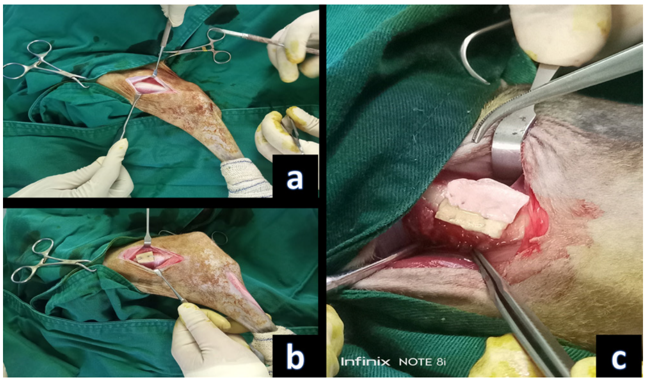

Figure 4: Periosteal graft transplantation on recipient bone; (a) Exposure of recipient bone (femur); (b) K-wires obliquely placed to hold allograft to recipient bone (femur); (c) Harvested periosteal graft from tibia transferred to femur with allograft.

CONCLUSION

Sheep with age of 4-6 months were feasible animal model for studies on periosteal autograft and bone allograft transplantation. The sheep could go through a 3-hour procedure safely. Anteromedial Surgical exposure to the donor tibia and lateral surgical exposure to the femur could be done safely without causing major bleeding. A manageable size of 3x2 cm tibia periosteum could be harvested, transferred and anchored to the recipient area with suture size 3.0. This model of fixing allograft with two Kirshner wire to the recipient femur eliminates the issue of stability of bone allograft that may interfere with bone formation.

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