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Surgical angiogenesis with short-term immunosuppression maintains bone viability in rabbit allogenic knee joint transplantation

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Abstract

Background—Vascularized Composite Allotransplantation (VCA) has potential for reconstruction of joint defects, but requires life-long immunosuppression (IS), with substantial risks. This study evaluates an alternative, using surgical angiogenesis from implanted autogenous vessels to maintain viability without long-term immunotherapy.

Methods—Vascularized knee joints were transplanted from Dutch Belted donors to New Zealand White rabbit recipients. Once positioned and revascularized microurgically, a recipient-derived superficial inferior epigastric fascial (SIEF) flap and a saphenous AV bundle were placed within the transplanted femur and tibia, respectively, to develop a neoangiogenic, autogenous circulation. Ten transplants comprised Group 1. Group 2 (n=9) were no-angiogenesis controls with ligated flaps and AV bundles. Group 3 rabbits (n=10) were autotransplants with patent implants. Tacrolimus was used for 3 weeks to maintain nutrient flow during angiogenesis. At 16 weeks, we assessed bone healing, joint function, bone and cartilage mechanical properties and histology.

Results—Group 1 allotransplants had more robust angiogenesis, better healing, improved mechanical properties and better osteocyte viability than ligated controls (group 2). All 3 groups developed knee joint contractures and arthritic changes. Cartilage thickness and quality were poorer in allograft groups than autotransplant controls.

Conclusions—Surgical angiogenesis from implanted autogenous tissue improves bone viability, healing and material properties in rabbit allogenic knee transplants. However, joint contractures and degenerative changes occurred in all transplants, regardless of antigenicity or blood supply. Experimental studies in a larger animal model with improved methods to maintain joint mobility are needed before the merit of living joint allotransplantation can be judged.

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Introduction

Large osseous or osteoarticular defects can result from trauma, oncologic resection and infection. There is a substantial incidence of complications with most available reconstructive methods, such as *custom prosthetic replacement* or *bone transport*, including infection, nonunion, prosthetic failure, or periprosthetic fracture. Functional loss from large joint *arthrodesis*, and stress fracture or nonunion resulting from poor revascularization of *cryopreserved allografts* are common problems. *Frozen joint allografts* have provided generally unsatisfactory results. The use of *vascularized bone and joint autografts* improves healing and lessens fracture and infection problems. Unfortunately, there are few expendable autogenous sources, often poorly matched in geometry and function to the defect. *Vascularized allotransplants* of bone and joint may provide the same advantages as vascularized autografts, without donor morbidity and be closely matched to any bone or joint defect. Their use currently requires life-long immune modulation, with significant risks and side-effects and disappointing results in reports to date.

We have previously successfully transplanted living allogenic femora in rats and rabbits without long-term IS (1, 2) using a unique method termed surgical angiogenesis. In this method, vascularized host-derived tissue is placed within the microsurgically transplanted bone. Over a two-week period of IS, neoangiogenesis arising from this tissue builds a new autogenous blood supply within the allograft. After cessation of drug treatment and subsequent nutrient artery thrombosis, the new autogenous vascular bed takes over perfusion and bone viability persists thereafter without need for any immune modulation. Isolation of the rabbit knee joint has been described (3), including allogenic knee joint transplantation with postoperative IS (3–5).

Here, we describe the results of the first vascularized composite allotransplantation study evaluating rabbit vascularized knee joint transfer with surgical angiogenesis instead of long-term immune modulation to maintain transplant viability and function.

Methods

Experimental design

This study was approved by the Institutional Animal Care and Use Committee (IACUC). Three experimental groups were devised. In groups one and two, vascularized whole knee grafts were microsurgically transplanted from male Dutch Belted rabbits to New Zealand White (NZW-) recipients using our previously described method (6). Additionally, a pedicled fascial flap (superficial inferior epigastric artery fascia [SIEF] flap (7)) from the recipient abdomen was introduced in the femur and an arteriovenous saphenous bundle including the surrounding fascia was implanted in the tibia to induce surgical angiogenesis (figure 1). These tissues were then ligated distally and pulled through the bone using a pull through suture. Care was taken to avoid kinking or twisting. These vascularized tissues were left patent (Group 1, n=10) or were ligated before entering the bone (Group 2, n=9). Group 3 (n=10) animals were autotransplant controls. In Group 3, the knee was isolated on a vascular pedicle in NZW-rabbits and orthotopically replanted. A SIEF-flap and saphenous AV-bundle were similarly introduced in femur and tibia, respectively. All animals received three weeks of IS (Tacrolimus: 0.1 mg/kg/day IM for 3 days for induction, and every other day thereafter for 21 days to maintain therapeutic blood levels (3–12 ng/ml whole blood) and were sacrificed after 16 weeks).

Radiographic evaluation

Plain radiographs were taken postoperatively and after 1, 2, 3, 4, 8, 12 and 16 weeks using a high-resolution mammography X-ray film (Min-R 2000 Film, Kodak, Rochester, NY) in two different oblique views (General Electric, Fluoroscope Model 46, Milwaukee, WI; 54 kV, 6 mA). Osseous healing was evaluated as described before (8). Additionally, extent of arthrosis was evaluated using an arthrosis score (1 [no arthrosis] to 6 [joint destruction]). Radiographic evaluation was blindly performed by one observer (T.K.)

Microangiography

The density of the vasculature was evaluated using a colored polymer (Microfil®, FlowTech Inc., Carver, MA), that was injected intra-arterially(6). After sacrifice the joints were fixed in 10% formalin and decalcified in 14% EDTA in a laboratory microwave (Pelco Biowave 3450, Ted Pella, Redding, CA) with a previously described technique (1, 9) and subsequently cleared with the Spalteholz method 6, 27. Two standardized perpendicular digital images were taken and the vessel area-to-transplant area ratio determined with scientific imaging software (Image-Pro Plus 7.0, Media Cybernetics Inc. Bethesda, MD, USA). The two measurements of each diaphyseal bone were averaged for later comparison.

Biomechanical testing – passive range of motion

Directly before sacrifice at 16 weeks x-rays were performed in maximum extension and the contracture angle was measured in vivo. In addition, the extension lag of the joint transplants was evaluated after knee explantation and soft tissue removal using fluoroscopy (OEC Mini 6600, OEC Medical Systems Inc., Salt Lake City, UT). A force gauge (AccuT-4, Yamoto Corp., Colorado Springs, CO) was attached to the tibia 2.8 cm from the joint space and a force of 2 kg was applied perpendicular to the tibia axis.

Histology - Bone viability

5 µm sections of femurs and tibias were evaluated after decalcification and Hematoxylin/Eosin (H/E) staining using a specialized image analysis software (Image-Pro Plus 7.0, Media Cybernetics Inc. Bethesda, MD, USA) at 20 × magnification. Bone viability was estimated with osteocyte counts in six random fields. The number of filled lacunae (defined as lacunae containing an osteocyte with nucleus and normal cytoplasm) was expressed as a percentage of the total number of empty + filled lacunae.

Histology – Cartilage

The tibia plateau cartilage was likewise decalcified and stained (H/E) and 5 µm sections were analyzed at 10× magnification and the cartilage quality was assessed using a previously described histology score (0; normal, 10; complete destruction) ^{S8}.

Biomechanical testing – cantilever bending

A micro-computerized tomography (CT) scan (Inveon mikroCT, Siemens Medical Solutions, Inc., Knoxville, TN) of the knee transplant was performed for dimensional analysis required for biomechanical calculation purposes (6). Thereafter, the knees were disarticulated and stripped of soft tissue. A bending moment was applied to each bone using a hydraulic material testing machine (MTS, Minneapolis, MN) and the ultimate stress at the fracture site was calculated using established methods (6).

Biomechanical testing – Elastic modulus of cartilage

The elasticity of the tibia plateau cartilage was evaluated using a hydraulic material testing machine (EnduraTEC Elf 3220, Minnetonka, MN). Force (N) and Displacement (mm) were

measured using a 4.46 kg load cell (MOB-10 Load Cell 10LBS, Transducer Techniques, Temecula, CA) and the Young's elastic modulus of the cartilage was calculated as described before (6)

Statistics

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). Results of bone healing scores, arthrosis scores and histologic quality assessment of tibia plateau cartilage were analyzed using a Chi-Square test. The results are expressed as medians. Capillary density, contracture angles, bone viability, cartilage thickness, and biomechanical results were analyzed for normal distribution using a Kolmogorov-Smirnov test. Differences between groups were analyzed using a One way analysis of variance followed by Bonferroni's adjustment procedure. Differences were considered significant at $P < 0.05$. Results in the manuscript are expressed as means $\pm$ SEM.

Results

Radiographic evaluation

Union rate—Irrespective of the groups osseous union was achieved in all tibias. 1, 4 and 0 rabbits were observed with non-union of the femur in groups 1 to 3, respectively. These differences were significant ($p < .05$, figure 2)

Transplant fracture—Transplant fractures were only observed in femurs. 1, 4 and 0 femoral failures were found during the follow-up period in groups 1 to 3, respectively. These differences were significant ($p < .05$). Overall, the untoward outcomes nonunion or fracture occurred in 2 rabbits in group 1 (1 fracture, 1 nonunion) and 5 rabbits in group 2 (3 fractures as well as nonunion, 1 transplant fracture, 1 nonunion).

Bone healing score—Median bone healing scores were 22, 20 and 24 (12 weeks) and 22.5, 22 and 24 (16 weeks) in groups 1 to 3, respectively. The distribution of score results between groups was significantly different ($p < .05$). Median bone healing scores at 1 to 8 weeks showed no differences ($p > .05$, figure 3).

Arthrosis scores—All rabbits irrespective of group, developed arthritic changes. Median arthrosis scores at 8 weeks were 3 in group 2 and 2 in groups 1 and 3. These differences were significant ($p < .05$). At 16 weeks, allografts with ligated intramedullary bundles showed a trend for more severe arthrosis (median score 4) when compared to the other groups (median scores 3 [group 1] and 2 [group 3]). These differences were not significant ($p > .05$).

Microangiographic results

All saphenous AV-bundles (in the tibias) as well as the SIEF flaps (in the femurs) were confirmed patent in groups 1 and 3. The nutrient allogenic knee pedicles in groups 1 and 2 were all thrombosed, whereas the vascular hilus in group 3 stayed patent. The ratio of vessel area to bone area of the femurs was 0.15 ± 0.05 , 0.06 ± 0.04 and 0.19 ± 0.1 in groups 1 to 3, respectively. Paired comparison of allotransplants with patent intramedullary bundles (group 1) with allotransplants with ligated bundles (group 2, $p = 0.043$) as well as group 2 with the autotransplant control (group 3, $p < 0.001$) revealed significant differences. Capillary density difference between groups 1 and 3 was not significant ($p = 0.59$; figure 4). Analysis of capillary density in the tibia was essentially the same, results being significantly worse in allotransplants with ligated intramedullary bundles (group 2, 0.06 ± 0.02) when compared to allografts with patent intramedullary vessels (group 1, 0.21 ± 0.05 ; $p = 0.001$) or

autotransplants (group 3, 0.17 ± 0.10 , $p=0.005$). Differences between groups 1 and 3 were not significant ($p=0.59$).

Passive range of motion *in vivo*—Contracture angles (extension lag) *in vivo* before sacrifice were 132.6 ± 35.94 degrees (group 1), 140.22 ± 14.64 degrees (group 2) and 126.2 ± 26.32 degrees (group 3). Differences were not significant ($p>0.05$).

Passive range of motion *ex vivo*—Irrespective of groups all animals developed joint contractures. Contracture angles after knee explantation were 73.1 ± 51.3 degrees (group 1), 84.2 ± 33.9 degrees (group 2) and 58.8 ± 60.9 degrees (group 3). Differences were not significant ($p>0.05$).

Histology results - Bone viability—The osteocyte viability ratio in femurs was 44.1 ± 16.2 % (group 1), 16.3 ± 3.8 % (group 2) and 78.2 ± 19.1 % (group 3). Tibias showed ratios of 44.6 ± 8.7 % (group 1), 11.8 ± 3.1 % (group 2) and 72.2 ± 14.5 % (group 3). Differences between groups were significant (all comparisons $p<0.001$; figure 5) and demonstrated the value of angiogenesis from implanted autogenous vessels in group 1.

Histology results - Cartilage quality score—The worst result in histologic arthrosis scoring was seen in group 2 (median score 8); Better results were observed in allotransplants with patent bundles and autotransplant controls (group 1; median score 7, group 3; median score 2.5). Differences between groups were significant ($p=0.035$, figure 6).

Histology results - Cartilage thickness—Cartilage thickness similarly showed a trend towards preservation of cartilage in allografts with patent intramedullary bundles (mean thickness 411.8 ± 127.5 μm) when compared to allotransplants with ligated bundles (mean thickness 330.13 ± 97.43 μm). The highest measures were observed in autograft controls (group 3; 489.6 ± 183.3 μm). Group comparisons showed no significance ($p>0.05$, figure 6).

Cantilever bending—Cantilever bending results revealed the autotransplant control to have the strongest osseous union, as only one fracture occurred through the osteotomy. Strength of the union of allotransplants with patent bundles was intermediate, and those with ligated bundles the weakest (4 versus 6 fractures at graft/host junction in groups 1 and 2, respectively). If fractures did not occur at the graft/host junction, the bones fractured directly at the site of osseous fixation in the hydraulic testing machine – the site with the longest lever arm. Differences between groups were significant ($p=0.038$). Similarly, 2, 5 and 3 fractures occurred through the tibia osteotomy sites in groups 1 to 3, respectively. However, group comparisons showed no significant difference ($p>0.05$).

The ultimate force at the femur fracture site during cantilever bending was higher in group 3, when compared to group 1 (88.8 ± 42.7 MPa versus 59.4 ± 38.1 MPa; $p=0.24$), whereas group 2 was observed with the lowest forces (35.9 ± 26.7 MPa). Differences between group 2 and 3 were significant ($p=0.021$). The mean ultimate forces at the fracture site of the tibias were the highest in allotransplants with patent intramedullary bundles when compared to the other groups (150.2 ± 83.4 MPa, 100.63 ± 77.0 and 116.7 ± 86.6 MPa in groups 1 to 3, respectively). None of the groups comparisons showed significant differences ($p>0.05$).

Cartilage elasticity—A trend towards higher elastic modulus in the autotransplant control group (group 3; 49.3 ± 20.7 MPa) was observed. The average elastic modulus of group 1 (allotransplant with patent intramedullary bundles; 48.2 ± 22.1 MPa) was higher than the

group 2 results (allotransplants/ligated bundles; 45.2 $\pm$ 28.9 MPa). None of the groups comparisons showed significant differences ($p > .05$).

Discussion

Vascularized composite allotransplantation, including the much publicized recent hand and face transplantations presents an ethical dilemma (10). The need for lifelong IS requires careful consideration not only of expected benefits, but also known significant risks in the context of non-life-critical transplantation. Such considerations are even more important in bone or joint allotransplantation, as other reconstructive options do not carry known complications of drug immunotherapy. A handful of allogenic knee joint transplantations have been performed in the past, showing poor outcomes since the majority of cases had to be converted to above knee amputations or arthrodesis due to immunologic problems or infection (11). The risk-benefit ratio of allogenic knee joint transplantation would be considerably better if tissue viability could be maintained without lifelong IS.

In this paper, we have evaluated a novel method of accomplishing long-term tissue survival requiring only short-term drug therapy in rabbits. The use of a larger species was necessary to allow vascularized knee allotransplantation and functional as well as biomechanical evaluation. However, the degree of immunological mismatch (major histocompatibility mismatch) is uncertain using these animals since they are outbred. However, the present as well as previous studies showed pedicle occlusion attributable to rejection after VCA since pedicles stayed patent in autotransplants. Heart allotransplantation studies using the same species similarly showed acute rejection after withdrawal of IS (12). In this VCA model we used surgical angiogenesis as a means to replace allogenic blood vessels with a neoangiogenic source of nutrition. At the time of microsurgical whole knee joint transplantation, we have simultaneously implanted vascularized tissue from the recipient into the tibia and femur. The result is angiogenesis of autogenous vessels within the allotransplant. Short term IS maintains graft perfusion for 3 weeks during this process. Thereafter, the neoangiogenic vessels maintain transplant viability despite withdrawal of IS. We have previously demonstrated that this method maintains bone blood flow in a rat femoral transplantation model, with viable cortical osteocytes and active bone remodelling that was not the result of tolerance induction (9, 13, 14). Surgical angiogenesis was also applied to frozen segmental bone allografts in rats and was similarly shown to induce revascularization. However, the resulting capillary density measurements were significantly lower when compared to our technique with vascularized composite allotransplantation and no-IS groups were generally observed with lower capillary density measurements when compared to groups with IS (2, 13, 15). Here, we transferred the principle of surgical angiogenesis from bone allotransplantation to VCA for the first time and therefore chose vascularized knee transplantation as a model rather than using frozen avascular knee transplants. Giessler *et al.* proved that this approach leads to increased capillary density and allows incorporation and remodelling of bone allotransplants after withdrawal of IS in orthotopic rabbit femoral allotransplantation (1). All previous studies used short term immunosuppression with tacrolimus for two weeks only. This approach effectively increased capillary density and bone blood flow in rats (9) but did not significantly improve biomechanical properties in rabbits (1). Previous studies have confirmed that short-term IS is not only necessary to prevent acute rejection with nutrient pedicle thrombosis, but also desirable in promoting surgical angiogenesis (1, 2, 9). Consequently, we applied tacrolimus for three weeks to allow a more complete restoration of the bone blood supply by host derived neoangiogenic vessels. Using this approach in this orthotopic whole knee joint allotransplant study, we have confirmed neoangiogenesis within both tibia and femur to occur when autogenous AV bundles were implanted at the time of transplantation. Results were superior to control group animals without autogenous surgical angiogenesis.

Significantly, capillary density has been proven to correlate with bone blood flow (16, 17). In this study we have additionally found capillary density to correlate with osteocyte viability.

Previous experimental knee allotransplantation studies suggest that long term immune modulation is required to maintain viability (4, 5, 18–21). Muramatsu and colleagues further demonstrated transplant viability only while IS with Cyclosporine A was maintained (22). In the present model we confirmed the occlusion of all nutrient pedicles at sacrifice, attributable to cessation of tacrolimus after three weeks. Unlike any previous study, however, we confirm that it is possible to maintain long-term viability in this scenario, when one uses surgical angiogenesis to develop a neoangiogenic autogenous circulation. Bone tissue undergoes constant bone remodelling and osteoprogenitor cells originate from a mesenchymal stem-cell line and reside mainly in the bone periosteum and marrow (23). In a previous study, we have demonstrated most of the viable osteocytes within bone using surgical angiogenesis with short-term IS to be the result of transplant chimerism—that is, to be of recipient origin rather than surviving allogenic cells (24). This was similarly observed by others applying bone allotransplantation with long term immunosuppression in rats (25, 26). To date, to our knowledge chimerism studies were not performed in calcified tissues in rabbits. Future studies should evaluate intragraft and systemic chimerism after joint allotransplantation in different species, since life spans of osteoblasts and osteocytes as well as bone turnover rates differ.

Previous studies have demonstrated bone healing in femoral allotransplants to be enhanced using our novel transplant methodology (1). Union rates in knee joint transplantation are either not presented (5, 27) or the results are controversial (4, 5, 22, 27–31). The only study reporting radiographic results after knee joint allotransplantation in rabbits reports satisfactory union rates (7 unions / 1 non-union) in immunosuppressed animals, but with complete failure without IS. Interestingly, we were able to achieve similar union rates (9 unions / 1 non-union) using only short-term IS when combined with surgical angiogenesis. The observed trend towards better bone healing was paralleled by a similar trend to increased fracture resistance in cantilever bending not previously reported. Segmental bone autotransplants are significantly weaker than undisturbed controls (1). Consequently we compared material properties of the allo- to vascularized autotransplants. We found allotransplants with patent bundles to be comparable to autotransplants, and those with ligated bundles to be significantly weaker. These findings suggest viable allotransplanted bone material properties are similar to those of autotransplanted bone after a 16 week survival period. It is likely, however, that completion of the remodelling process in allotransplanted bone may take longer than 16 weeks. Analysis of this process will require future studies not only to measure bone material properties as a function of time post-transplant, but also quantify new bone formation and osteoclasts with histomorphometric analysis.

The knee joint itself did not fare as well as the bone. After 16 weeks all transplanted knees (both auto- and allotransplants) developed radiographic signs of advanced arthrosis. Histologic analysis of cartilage showed a trend towards better-preserved thickness and quality in allotransplants with surgical angiogenesis (group 1) when compared to allograft controls (group 2). Values for both groups were inferior to autotransplant controls, however. Previous studies of allogenic knee transplantation using long term IS report normal cartilage histology (4, 18, 22, 30), and minimal evidence of rejection (4, 22). We believe the less satisfying cartilage outcome of our study may be related to several potential problems. One of these could be synovial rejection after cessation of immunosuppression. However, immunological problems are unlikely to be the only reason for arthrosis after knee joint transplantation since all three groups, including autotransplant controls developed arthritic

changes. Additionally, our previous results using autotransplants showed comparable joint alterations that were not observed in the intraindividual contralateral control legs (6). Another potential explanation for arthrosis in the knees could be the loss of proprioception after joint isolation, since similar changes and fatigue fractures that were addressed to joint denervation were observed in humans after vascularized knee joint transplantation with long term immunosuppression (11, 32). Joint denervation may therefore lead to pathologic changes that are similar to the development of a Charcot joint. However, we neither assessed rejection nor evaluated innervation of knee joint soft tissues and therefore, these potential problems should be evaluated in future studies. Additionally, all rabbits developed joint contractures that most likely result from the extensive dissection of soft tissues and the normal knee resting posture in maximal flexion. These contractures may have contributed to arthrosis and to the poor functional results in the rabbit knee. No other rabbit knee allotransplantation study reports on postoperative range of motion; the reported results in the canine are controversial (18, 30, 31). Since the poor functional outcome in rabbits seems to be at least partially attributable to the size of the study animal in relation to the extensive soft tissue dissection and the resting posture we believe that our results merit future studies using larger animals that allow easier dissection, postoperative ambulation or physical therapy as well as a closer gait analysis. These experiments will need to explore the feasibility of both improving autogenous circulation to all tissues as well as maintaining a full arc of knee motion. Once this is achieved further studies are necessary comparing our new model to currently available techniques such as acellular and avascular allotransplantation as well as vascularized autotransplantation and prosthesis. Additionally, surgical angiogenesis should be applied to processed cadaveric grafts to completely avoid immunosuppression.

Life-long IS and/or tolerance induction is necessary at present to maintain viability of vascularized composite allotransplants. We have evaluated an alternative method, requiring only short-term IS that maintains bone viability by development of a neoangiogenic autogenous (host-derived) blood supply. We have demonstrated such bone to have improved healing and better material properties than identical transplants without surgical angiogenesis. These results are similar to previously published bone allotransplantation data from our laboratory (1, 2, 9)

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Figure 1. Whole knee allotransplants were microsurgically transplanted from Dutch belted donors to New Zealand White recipients. Anastomosis was performed end-to-side to the femoral artery and end-to-end to the femoral vein (C). A fascial flap (SIEF-flap) was introduced in the femur (A) and a saphenous av- bundle was implanted in the tibia (B) to induce surgical angiogenesis. (Courtesy of Mayo Clinic)

Figure 2. Three dimensional reconstructions of knee joints after micro-CT evaluation. Free microvascular knee joint transplants showed complete union at 16 weeks with surgical angiogenesis (B) and non-union of the femoral graft host junction when intramedullary vascular bundles were ligated (A).

Bone Healing Score

Figure 3.

Bone healing was assessed using a score from 1 to 25 that was based on conventional x-rays in to different views (*; significant differences between groups, group 1; allotransplant with patent intramedullary bundle , group 2; allotransplant with ligated intramedullary bundle , group 3; autotransplant control with patent intramedullary bundle [group 3] (8)).

Capillary Density

Figure 4.

Capillary density was evaluated using a colored compound (Microfil®) in cleared bone. The ratio of vessel area to bone area (capillary density) was calculated from 2 dimensional digital pictures in two perpendicular views (*; significant differences between groups).

Figure 5.
The ratio of filled lacunae to all lacunae as a measure of bone viability was evaluated using H&E slights (*; significant differences between groups)

Figure 6. H&E slights of tibia plateau cartilage and conventional x-rays showed development of arthrosis in all groups. However, arthritic changes were more severe in allografts with ligated intramedullary bundles when compared to autografts and allografts with surgical angiogenesis.