

Effect of Autologous Mesenchymal Stem Cells on Biological Healing of Allografts in Critical-sized Tibial Defects Simulated in Adult Rabbits

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Abstract

Introduction: This study evaluated the effect of autologous bone marrow derived adult Mesenchymal Stem Cells (MSCs) on the biological healing of weight bearing diaphyseal bone allograft in the tibia of adult rabbits. **Materials and Methods:** Forty Adult New Zealand White Rabbits divided into 3 groups (Autograft, Allograft or Allograft impregnated with MSCs) with 12 rabbits in each group were used for the study. A 1.5 cm of cortical bone segment was excised from the rabbit's right tibia. The segment was replaced by an Autograft, Allograft or Allograft loaded with MSCs, depending on which group the rabbit was assigned. Internal fixation was performed using a 9-hole Mini-compression Plate and Cerclage Wires. Rabbits were sacrificed at end of observation periods of 12, 16 and 24 weeks. Specimens procured were assessed clinically and radiologically and fixed in 10% buffered formalin. For each specimen, 5 µm undecalcified sections were cut and stained with Von Kossa and Toluidine Blue stains. Histomorphometry was then performed. **Results:** Our study showed that addition of autologous MSCs to diaphyseal allograft segments enhances and accelerates not just the union at host graft junctions and also the biological incorporation of the allograft segment as shown by Resorption Index, New-Bone Formation Index and Osteocyte Index. **Conclusions:** The addition of autologous MSCs to deep frozen cortical allograft segments improved the host – allograft union rate and biological incorporation of diaphyseal allografts as shown by resorption activity, new bone formation and osteocyte cell counts.

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Introduction

Bridging of large bone defects is a challenging problem in orthopedic and reconstructive surgery. Options include vascularised bone autografts,¹ non-vascularised bone autografts,² allografts,³ bioceramics⁴ and custom-made prostheses.⁵ With the advent of tissue engineering, a new option available is tissue engineered bone. Adult Mesenchymal Stem Cells (MSC) in scaffolds such as Hydroxyapatite and Polycaprolactone - tricalcium phosphate (TCP) could be used to bridge bone defects. However the scaffolds were not strong enough for functional weight bearing. Hydroxyapatite and Polycaprolactone-TCP composite have the strength of cancellous bone only (5 to 6 Megapascals).⁶ Scaffolds used should preferably have at least cortical bone strength of 150 to 160 Megapascals if

weight-bearing is to be allowed.⁶

Allografts have been used to bridge large bone defects.³ However, the disadvantage is the small risk of disease transmission and other specific problems like fracture, infection and nonunion.³

Allografts act like the inert grafts with little evidence of biological incorporation.⁷ With non-vascularised cortical autografts, healing occurred by “creeping substitution”. Experimental study in adult cats showed good biological incorporation of the graft with bone resorption reaching 13% of the graft at 12 weeks, cortical new bone formation reaching 4.3% at 16 weeks and callus encasement of the graft reaching 8% at 16 weeks.^{8,9} In contrast, the biological repair processes occurring in cortical bone allografts showed little reparative activity. Experimental studies in the same

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cat model showed resorption activity to be only 1.8% at 9 months and cortical new bone formation to be only 0.74% at 9 months.⁹ No callus encasement occurred as periosteum has been removed in all bone allografts before they were transplanted.⁹ Without active biological repair activity, allografts remained weak and did not reach normal bone strength levels. Despite the lack of repair activity, it is important to point out the intrinsic biomechanical strength of the cortical bone allograft scaffold used is high compared to the strength of other scaffolds such as Hydroxyapatite and Polycaprolactone-TCP. The torsional biomechanical strength of allograft was about 64% of normal bone – far stronger than Hydroxyapatite and Polycaprolactone-TCP.^{6,10}

Bone marrow has been a source of progenitor cells for mesenchymal tissues. MSC derived from bone marrow has the potential to differentiate into distinct lineage cells, including osteoblasts,¹¹⁻²⁰ chondrocytes,^{13-16,21-24} adipocytes,^{13,14,16,25,26} tenocytes,²⁷ and marrow stromal cells.²⁸ The multipotent nature of bone marrow derived stem cells is based on the ability to differentiate into 3 distinct mesenchymal cell types – osteoblasts, chondrocytes and adipocytes.^{16,29}

MSCs have been shown to improve bone regeneration in various bone models using hydroxyapatite ceramic.^{30,31} Lucarelli et al³² studied the effect of bone marrow derived adult autogenous stromal stem cells combined with platelet rich plasma using allograft as a scaffold in a 3 cm defect in midshaft of metatarsal bone in 10 adult sheep. They showed that MSCs and platelet rich plasma increased new bone formation in the fracture callus at both host-allograft osteotomy junctions. However, Lucarelli et al³² did not study the biological incorporation of the allograft itself (resorption index, new bone formation index and osteocyte index). The objective of this paper is to study the effect of bone marrow derived autogenous adult mesenchymal stem cells on the healing of diaphyseal allografts in a large weight-bearing bone – the tibia using adult rabbits. Parameters studied include union of host-allograft junctions, resorption index, new bone formation index and osteocyte index of the allograft itself. The latter has not been studied before.

Materials and Methods

Experiment Design

Forty adult, male, New Zealand White Rabbits (epiphyses closed on x-rays) weighing between 3 to 3.5 kg were used. General anesthesia was employed using Ketamine (35 mg/kg) + Xylazine (5 mg/kg) for induction, the rabbits being intubated and maintained with isoflurane and oxygen. Three different experiments were conducted using 12 rabbits in each group. In the first group, non-vascularised autograft was used, in the second group, deep-frozen allograft (–80°C) were transplanted and in the third group, allografts loaded

with bone marrow derived autologous mesenchymal stem cells were transplanted.

Operative Techniques

Group 1 (Non-Vascularised Autograft): Under general anesthesia, an incision was made on right shin of the rabbit's leg to expose the tibia. An osteotomy was made at the junction of the upper one-third and middle one-third of the tibia using an oscillating saw. Using a sterile metal ruler, 1.5 cm of the tibia segment was measured and a mark made distally. A distal osteotomy was then performed at this mark. A 1.5 cm segment of the tibial diaphysis was excised and removed. The graft was flushed with normal saline to remove all blood and debris from the bone (Fig. 1A). The excised segment was then replaced and internal fixation performed using a 9-hole mini-compression plate with 3 screws proximal to the segment and 3 screws distally. The segment was additionally secured with 2 cerclage wires (Fig. 1B). The soft tissues and skin were closed with absorbable vicryl sutures. A below knee plaster of paris cast was applied.

Group 2 (Allograft)

(a) Technique of Procurement and Processing of Allografts

Four rabbits were used for procurement of allografts. Under general anesthesia and using the same operative technique described for Group 1, 1.5 cm tibial allograft (8 specimens) was procured. The soft tissues and periosteum was excised from the bone segment and the marrow removed completely from the medullary canal. Flushing was done with normal saline. The tibial graft was then inserted into an inner and outer sterile jar ('sterile double jar technique'¹⁰) for storage at –80°C deep freezer in National University Hospital (NUH) Tissue bank.

(b) Transplantation of Allograft Segment

At the start of the operation, the deep-frozen allograft was removed from the sterile double jar and thawed in 50 mls of normal saline containing 250 mg of Ampicillin and 250 mg of Cloxacillin for about 30 minutes. Using similar operative technique, a 1.5 cm cortical segment from the mid shaft of right tibia was excised. The soft tissues and periosteum was excised and the marrow removed from the medullary canal of this segment. The segment was flushed with 50 mls normal saline and soaked in antibiotic solution containing 250 mg Cloxacillin+250 mg Ampicillin, for at least 30 minutes and processed for use as donor allograft for another recipient rabbit. The defect was reconstructed using the thawed deep-frozen 1.5 cm cortical segment allograft with a 9-hole mini-compression plate.

Group 3 (Allograft + MSCs): Under general anaesthesia

(intramuscular Ketamine 50 mg/kg and Xylazine 10 mg/kg), about 8 to 10 mls of bone marrow was aspirated using a bone marrow aspiration needle from the posterior iliac crest of the recipient rabbit under sterile conditions using a 20 ml syringe containing 0.5 ml of heparin (to prevent clotting). This was done about 2 to 3 weeks before transplantation to allow for in-vitro culture of the mesenchymal stem cells.

The sample of bone marrow was mixed with 10 to 15 mls of Phosphate Buffered Saline (PBS). The cells were concentrated by centrifugation at 1500 rpm for 10 minutes at 20°C. The marrow separated into an upper serous layer and a lower densely packed cellular layer. The supernatant was removed with a pipette and the residual pellet was re-suspended in 10 ml of PBS. This was again centrifuged at 1500 rpm for 10 minutes, following which the supernatant was discarded. The pellet of cells was reconstituted in 6 ml of complete medium and transferred to a T-75 culture flask,³³ with an initial cell density of about 1×10^4 cells/cm³.

The sample was incubated at 37°C in a humidified 5% CO₂ environment generated in a CO₂ incubator. On day 5 of culture, the non-adherent cells were discarded along with the culture medium. The adherent cells were mixed with 15 ml of complete medium. The culture medium was changed every 3 to 4 days to ensure adequate cellular nutrition and removal of metabolic waste products. The cells were also routinely inspected using an inverted microscope.³³

Once 80% to 90% confluence of cells was achieved, the cells were detached by trypsinization and reseeded onto two new T-75 flasks for passage 1.

Bone marrow aspirate contains hematopoietic stem cells and mesenchymal stem cells. The cells cultured in our primary culture showed the property of adhering to plastic surfaces, a property exhibited by only mesenchymal stem cells.³⁴ These cells are indeed mesenchymal stem cells. The haematopoietic stem cells and other blood cells present in the bone marrow aspirate do not show this property³⁴ and such cells are discarded, and therefore removed from the primary culture, leaving only mesenchymal stem cells to be present in the primary culture using our standardised protocol.

After the first passage, the MSCs cultured were characterised. These cells were shown to be multipotent, being able to differentiate into at least 2 different mesenchymal tissues, namely cartilage and bone.³⁵

Chondrogenic differentiation of MSCs was induced using a chondrogenic medium containing 1 mM pyruvate, ascorbate 2-phosphate (37.5 mg/mL), 10^{-7} M dexamethasone, and transforming growth factor β 1 in Dulbecco's Modified Eagle's Medium (DMEM).³⁵ Immunohistological staining using vimentin test kit (UltraVision detection system) was performed to visualise Collagen Type II.

For osteoblastic differentiation of MSCs, osteogenic medium used contained 100 nM dexamethasone, 50 μ M ascorbic acid 2-phosphate, and 100 mM β -glycerophosphate in DMEM.³⁵ Mineralisation was demonstrated by staining the cells with von Kossa stain.³⁵

In our study, the cells were transplanted after passage 2. The number of cells attained from the 2 passages ranged from 3 to 4 million cells in the cell pellet as verified by the hemocytometer.

Just before transplantation, the cells were detached by trypsinization and mixed with complete medium and centrifuged to form a residual cell pellet. Tisseel (Baxter, CA, USA) was used as a carrier for the cells. To form a gel with cells, 1ml of fibrinogen from the Tisseel kit was mixed with the cell pellet and 1 ml of thrombin was added to this mixture.³⁶

For transplantation, the same technique as for group 2 was used except that the donor allograft used to reconstruct the tibial defect was loaded with the autologous MSCs. The medullary cavity of the allograft was packed with the gel containing MSCs (Fig. 1C). The gel containing the MSCs was also applied to the cortical surface of the graft and at both host-graft junctions.

All animals were examined daily. Subcutaneous Buprenorphine (0.5 ml/kg) for analgesia and prophylactic antibiotic—Cephalexin (20 mg/kg) were administered once daily for 3 days post operatively to all rabbits. The plaster cast was removed after 6 weeks.

Plan of Experiment

The healing of Autograft, deep-frozen Allograft and deep-frozen Allograft loaded with autologous MSCs were studied at observation periods of 12, 16 and 24 weeks with 4 rabbits for each observation period. At the end of the period, the rabbits were sacrificed under general anesthesia with intra-venous pentobarbitone (>200 mg/kg). The right tibia was procured. Soft tissues were dissected to expose the tibia and photograph (Fig. 1D) and radiographs taken (Fig. 1E). The clinical union at both ends of the grafts was assessed by testing for mobility after removing the implant and the specimens fixed in 10% buffered formalin.

Histological Studies of the Specimens: The specimens were sectioned longitudinally, perpendicular to the plate. Undecalcified sectioning of the specimens were done to produce sections of 5 μ m thickness to be stained with Von Kossa and Toluidine Blue stains. Histological examination was then performed and histomorphometry was conducted in the middle 50% (i.e. middle 0.75 cm) of the graft.

Union of Host – Graft Junctions: Union was studied clinically, radiologically and histologically. Union was

defined as bridging of host-graft junctions with osteoid tissue.

For biological incorporation of the graft the following parameters were quantitated using Pro Image Plus 5.0 software:

(a) Resorption Index: defined as the total area of all resorption cavities present in both cortices expressed as a percentage of the total area of both cortices⁸ - measured in undecalcified sections stained with Von Kossa at a magnification of 40X.

(b) Cortical New Bone Formation Index: defined as the total area of new bone formed within the cortex of the diaphyseal segment expressed as a percentage of the total area of the cortex⁸ - measured in undecalcified sections stained with Toluidine Blue at a magnification of 200X using an average of 3 microscope fields.

(c) Osteocyte Index: defined as total number of lacunae occupied by osteocytes expressed as a percentage of the total number of lacunae present in each microscope field. The counts were done in undecalcified sections stained with Toluidine Blue at a magnification of 200X and the average of 3 microscope fields in each cortex was taken to represent the osteocyte index of that specimen.⁸

Results

(1) Union at Host Graft Junction

Table 1 showed that in Group 1 (Autograft) union at both

Table 1. Union at Host-Graft Junctions

	Observation Period		
	12-weeks	16-weeks	24-weeks
Group 1: Autograft	4/4	4/4	4/4
Group 2: Allograft	0/4	2/4	4/4
Group 3: Allograft+ MSCs	4/4	4/4	4/4

MSCs : Mesenchymal stem cells

Table 2. Biological Incorporation of Grafts

Histology Index	Resorption Index			New bone formation Index			Osteocyte Index		
Weeks	12	16	24	12	16	24	12	16	24
Group 1: Autograft	11.91 (2.46)	7.73 (2.43)	2.88 (2.09)	3.29 (1.35)	4.29 (1.32)	2.39 (1.16)	23.12 (4.49)	21.25 (7.61)	19.53 (6.00)
Group 2: Allograft	1.05 (0.45)	0.91 (0.39)	0.86 (0.18)	0.00 (0.00)	0.13 (0.26)	1.16 (0.00)	1.63 (4.49)	2.50 (5.00)	1.13 (2.25)
Group 3: Allograft +MSCs	11.48 (1.03)	14.85 (1.06)	6.01 (0.97)	4.99 (1.02)	5.90 (1.39)	6.14 (2.07)	55.40 (18.17)	66.25 (19.89)	59.05 (16.21)
Statistics P (1 vs 3)	0.897 NS	0.111 NS.	0.252 NS.	0.024 NS.	0.289 NS.	0.042 Sig.	0.046 Sig.	0.039 Sig.	0.017 Sig.
P (2 vs 3)	0.011 Sig.	0.023 Sig.	0.025 Sig.	0.002 Sig.	0.004 Sig.	0.010 Sig.	0.008 Sig.	0.005 Sig.	0.008 Sig.

MSCs : Mesenchymal stem cells.

Standard Deviation is denoted () brackets

P < 0.05 = Significant (Sig.)

P > 0.05 = Not Significant (NS)

host-graft junctions was seen in all specimens by 12 weeks (Fig. 2A). However in Group 2 (Allograft alone) union was delayed and seen in all specimens only at 24 weeks (Fig. 2B). At 16 weeks, only 50% of allograft specimens showed union and at 12 weeks none of the allograft specimens showed any signs of union (clinically, radiologically and histologically). However, in Group 3 (Allograft + MSCs) union was seen in all 4 specimens by 12 weeks (Fig. 2C) - comparable to state of union seen in Group 1 (Autograft).

(2) Biological Incorporation

(a) Resorption Index: Fig. 3A and Table 2 showed that there was a significant increase ($P < 0.05$) in the resorption index of allografts loaded with MSCs (Fig. 3B) at all three observation periods as compared to allograft without MSCs (Fig. 3C). There was no significant difference ($P > 0.05$) in the resorption index of specimens of Group 3 (Allograft + MSCs) when compared to Group 1 (Autograft).

(b) New Bone Formation index: The New Bone Formation Index for allografts with MSCs (Fig. 4A and Table 2) showed a significant increase ($P < 0.05$) at 12, 16 and 24 weeks (Fig. 4B) when compared to allograft alone (Fig. 4C). When compared to autografts there was no significant difference ($P > 0.05$) in the new bone formation index for allografts with MSCs as compared to autografts at 16 and 24 weeks.

(c) Osteocyte Index: Table 2 and Fig. 5A showed the Osteocyte Index in all 3 groups. The number of osteocytes found in allograft+MSCs specimens at 12, 16 and 24 weeks (Fig. 5B) were found to be significantly higher ($P < 0.05$) than both Allograft alone (Fig. 5C) and autograft specimens.

Discussion

Though there are many studies showing the effects of mesenchymal stem cells with synthetic biomaterials,^{11,30,31,37} there are only a few studies reported on the effects of MSCs with allografts.^{32,38,39} Luceralli et al³² showed increased callus formation with MSCs in metatarsal allograft in sheep. Korda et al³⁸ likewise showed that MSCs added to

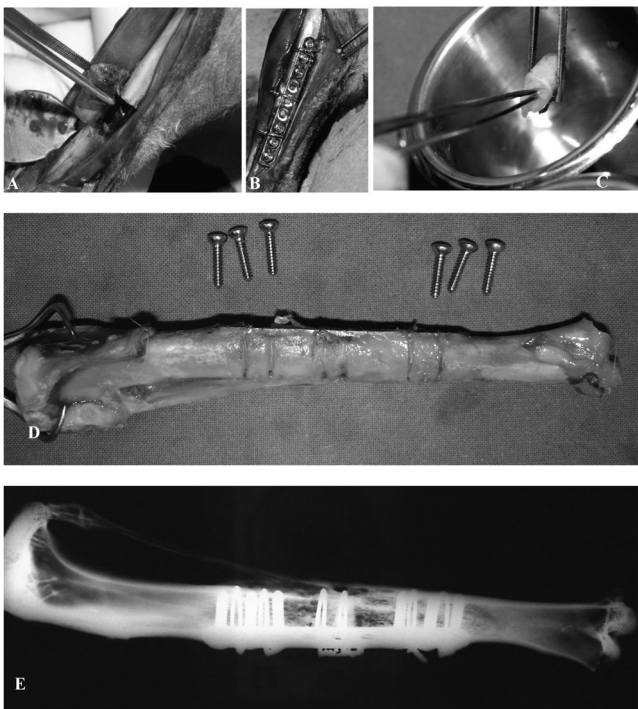


Fig. 1. Experimental model.

- (A) A 1.5-cm cortical defect (about 15% of the tibial length) simulated at the mid-shaft of the right tibia by a clean excision.
- (B) Fixation of the allograft with 9-hole plate and cerclage wire.
- (C) MSCs with Tisseel gel loaded into the medullary cavity of an Allograft segment at 16 weeks.
- (D) Photograph of allograft + MSCs (T3-7) showing good clinical union at both host-graft junctions.
- (E) Radiograph of allograft + MSCs (T3-7) showing good radiological union at both host-graft junctions.

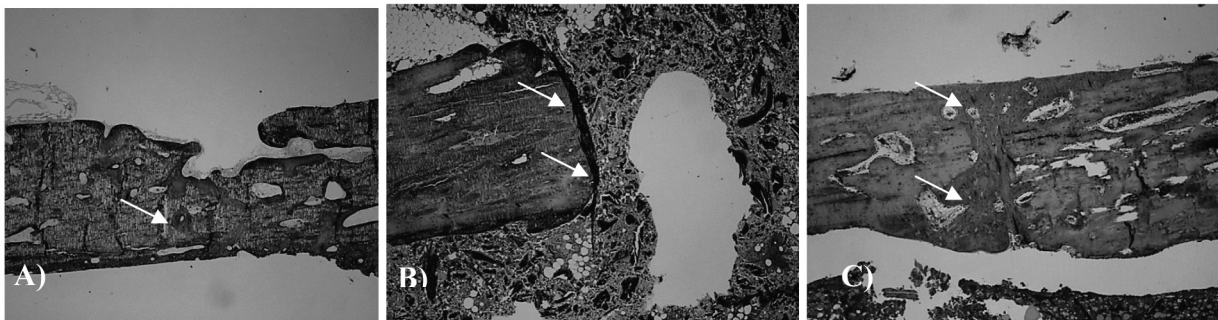


Fig. 2. Histological section stained with Toluidine Blue showing union at host graft junctions (arrows) at 12-weeks of

- (A) Autograft (T1-18) at 12 weeks,
- (B) Allograft (T2-4) at 24 weeks and
- (C) Allograft + MSCs (T3-2) at 12 weeks.

allografts significantly generated more new bone in implant/allograft interface region compared to allografts alone in a hemiarthroplasty model involving 10 adult sheep. This is the first study showing the effects of autologous adult Mesenchymal Stem Cells on a weight-bearing bone in

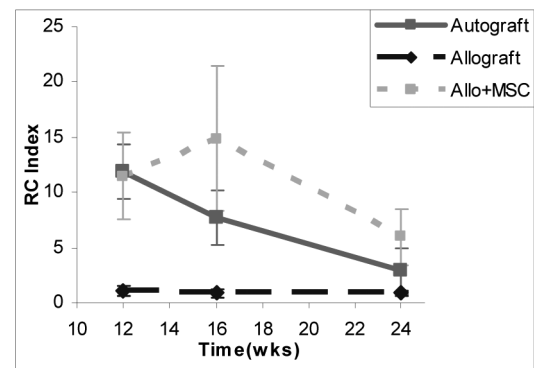


Fig. 3(A).

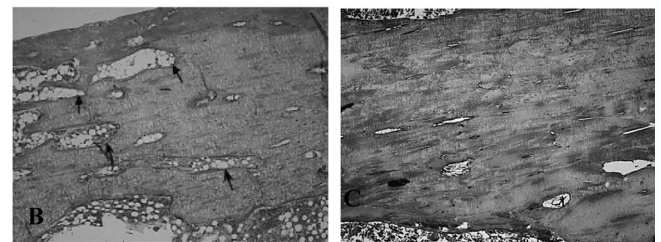


Fig. 3. Resorption Index Comparison (RC Index).

- (A) Graph showing Resorption Index (RC Index) of Autograft, Allograft and Allograft + MSCs.
- (B) Histological section of Allograft + MSCs (T3-10) at 24 weeks stained with Toluidine Blue showing large resorption cavities (arrows) at a magnification of 40X.
- (C) Histological section of Allograft (T2-4) at 24 weeks stained with Toluidine Blue showing minimal and small resorption cavities at a magnification of 40X.

a large bone- the tibia in adult rabbits. Zou et al³⁹ applied MSCs as a sheet of cells wrapped around the allografts using nude mice as the experimental models. They showed that MSCs could accelerate the repopulation of bone allograft.³⁹ In our study, MSC gel was applied onto the whole periosteal

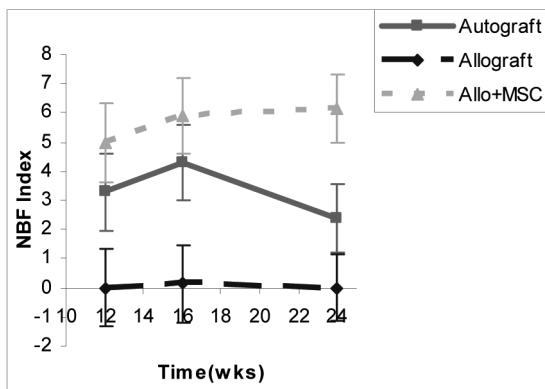


Fig. 4(A).

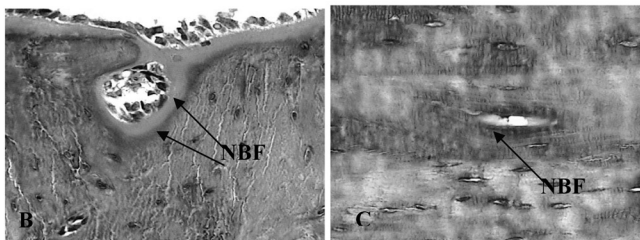


Fig. 4. New Bone Formation Index (NBF Index) comparison.

(A) Graph showing New Bone Formation Index (NBF Index) of Autograft, Allograft and Allograft + MSCs.

(B) Histological section of Allograft + MSCs (T3-2) at 12 weeks stained with Toluidine Blue showing abundant new bone formation (arrows) at a magnification of 200X.

(C) Histological Section of Allograft (T2-6) at 12 weeks stained with Toluidine Blue showing little new bone formation at a magnification of 200X.

surface of the allograft. In addition, MSC gel was loaded into the medullary canal of the allograft to fill the whole medullary cavity.

Allograft fracture and nonunion are some of the major problems with allografts besides infection.⁴⁰ Poor biological incorporation of the allograft and remodelling have been shown to be the major cause of failure in these structures.^{40,41} Introducing osteogenic cells at the host graft junctions and also in the medullary cavities of the allograft and on the periosteal surface of the allografts could improve the union of host-allograft junctions and also the biological incorporation of the allograft segment itself.

In our study, the parameters used for studying biological incorporation of the graft included Resorption Index, Cortical New Bone Formation Index, and Osteocyte Index.⁸ Resorption activity is an indication of revascularisation and cellular activity in a graft.^{8,41} In our study, we found a significant increase in this index when Mesenchymal Stem Cells were added to Allograft than when compared to Allograft alone which was relatively inert.

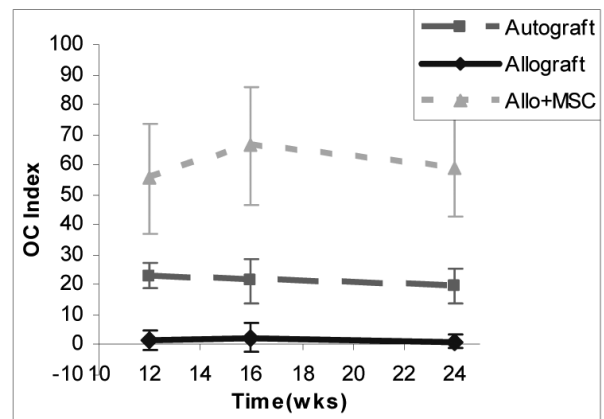


Fig. 5(A).

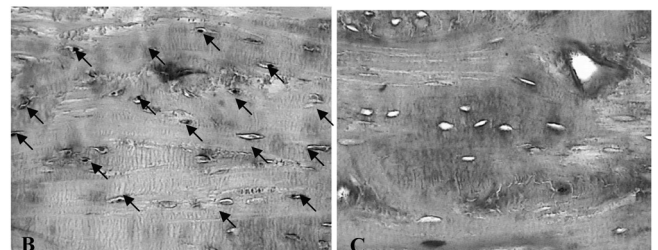


Fig. 5. Osteocytes Count Index (OC Index) comparison.

(A) Graph showing Osteocytes Count Index (OC Index) of Autograft, Allograft and Allograft + MSCs.

(B) Histological section of Allograft + MSCs (T3-3) at 16 weeks stained with Toluidine Blue showing increased number of osteocytes (arrows) at a magnification of 200X.

(C) Histological section of Allograft (T2-5) at 16 weeks stained with Toluidine Blue showing ghost cells seen at a magnification of 200X.

Cortical new bone formation in the allograft is a sensitive index to evaluate the amount of graft incorporation. Earlier studies have shown it to be not more than 20% and mainly at the peripheries of the allografts.⁴¹ In our study, there was a significant increase in the new bone formation in the Allograft segment when Mesenchymal Stem Cells were added to it as compared to Allograft alone.

Osteocyte Index is another good indicator of cellular activity in the transplanted allograft segment. Undifferentiated Mesenchymal Stem Cells have the potential to proliferate at sites of bone formation.¹¹ We found a significant increase in the Osteocyte index in Allograft with MSCs specimens as compared to Allograft without MSCs.

In our experimental studies, firstly, we found that the addition of MSCs improved the union of host-allograft junctions to 12 weeks compared to the union in Allograft where MSCs were not added.

Secondly, with regards to the biological incorporation of the Allograft itself, we found that the addition of MSCs into the medullary cavity of the allografts and on its periosteal

surface enhanced the biological incorporation of the allograft compared to the healing process that occurred in allografts where MSCs were not added as shown by Resorption Index, Cortical New Bone Formation Index and Osteocyte Index.

Conclusion

Our study showed that the addition of autologous bone marrow derived adult mesenchymal stem cells to deep frozen cortical allograft segments improved not only the host – allograft union rate but also the biological incorporation of diaphyseal allografts as shown by resorption activity, new bone formation and osteocyte cell counts.

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