

Studies on Augmentation of Fracture Healing using Stem Cell Seeded on β -Tricalcium Phosphate in Goats

Dharmendra Kumar^{1*}, Mahesh Kumar Bhargava², Apra Shahi², Randhir Singh², Neelam Tandia¹, Priya Singh¹

ABSTRACT

Twelve clinical cases of goats having long bone fracture, presented at College in Jabalpur (India), were included in the study. These animals were randomly divided into two groups. Fracture segment of group I were immobilized by dynamic compression plate (DCP). In group II fracture immobilization was done as in group I and the gap between the fracture segments was filled by bone marrow mesenchymal stem cells (BMMSCs) seeded on β -tricalcium phosphates scaffold. Fracture healing was evaluated on the basis of inflammation, exudation, pain, weight bearing and radiographic interpretation. Goat mesenchymal stem cells were established from bone marrow aspirates under aseptic conditions. Fibroblast like appearance of MSCs was observed after 3-4 days of seeding. Early cessation of inflammation, exudation and pain was observed in group II as compared to group I. Significant increase in weight bearing was observed from 7th day onwards in group II, while from 15th day onwards in group I. Complete weight bearing by all the goats was observed on 45th day in group II and 90th day in group I. Radiographic interpretation on 7th post-operative day revealed periosteal reaction on the fractured segment adjoining the fracture line in group II. On 30th post-operative day high density radiopaque area was visible at the fracture site in group II. Radiograph of 45th day in group II revealed more organized periosteal as well as intercortical callus, which reduced on 60th day, indicative of initiation of remodeling. On 90th day, homogenous bone with patent bone marrow was observed, indicative of completion of healing process in group II.

Key words: β -tricalcium phosphate, Dynamic compression plate, Fracture, Radiograph, Stem cells, Weight bearing.

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INTRODUCTION

Fracture is a break in the continuity of hard tissue. Healing of injured tissue is innate quality of an individual. Wound and fracture heals on its own, if proper congenial environment is provided to it. Fracture repair demands, immobilization and filling of the gap between fractured fragments with bone or its substitutes. Immobilization of fractured fragments can be done either by external or internal immobilization and fixation techniques. External immobilization can be achieved using bandages and splint, external fixation by various fixation devices and internal fixation using different types of pins and plates. Internal fixation provides rigidity to fractured segment until bone heals. Rigid fixation is responsible for early ambulation of the patient along with body weight bearing. Optimal movement of the limb keeps the muscles and joints healthy by providing nutrition to muscles, bone and cartilage (Roe, 2003).

Successful management of bone defects is a challenge in orthopedic practice. Several surgical procedures using biological or synthetic bone substitutes have been developed, for the treatment of bone defects and to promote closure with new bone tissue with morphological and functional characteristics, similar to those of the original tissue (Ruhaimi, 2000). The ideal graft material should be biocompatible, non-antigenic, non-carcinogenic, osteoinductive, osteoconductive, cheaper and provide

¹Department of Surgery and Radiology, College of Veterinary Science and Animal Husbandry, Rewa-486001, NDVSU, Madhya Pradesh, India

²Department of Surgery and Radiology, College of Veterinary Science and Animal Husbandry, Jabalpur-482001 NDVSU, Madhya Pradesh, India

Corresponding Author: Dharmendra Kumar, Department of Surgery and Radiology, College of Veterinary Science and Animal Husbandry, Rewa- 486001, NDVSU, Madhya Pradesh, India. e-mail: dharmendrabrooke@gmail.com

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gradual substitution of the bone tissue (Frame, 1980). Autologous bone graft is still considered as "gold standard" but its limited availability and difficult collection procedure makes it a hard nut to crack (Jensen *et al.*, 1996). Tissue engineering strategies for the replacement of load bearing tissues require a combination of a scaffold and a source of bone forming cells and growth factors. Bone marrow stromal cells (BMSCs) seeded in ceramic scaffold can hasten the fracture healing and can be used in areas with bone loss

(Langer and Vacanti, 1993). This study was aimed to evaluate the augmentation of fracture healing using stem cell seeded on β -tricalcium phosphate (TCP) in goats.

MATERIALS AND METHODS

Twelve clinical cases of goats of either sex, aged between one to six years, having long bone fracture presented at Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Jabalpur (India), were included in the present study. The animals were randomly divided into two groups. Fracture segments in group I were brought into apposition and immobilized by dynamic compression plate (DCP), while in group II fracture immobilization was done as in group I and the gap between the fracture segments was filled by bone marrow mesenchymal stem cells (BMMSCs) seeded on β -tricalcium phosphates scaffold.

Isolation and Culture of Bone Marrow Mesenchymal Stem Cells (BMMSCS)

Isolation: After collection, bone marrow aspirate was mixed with equal volume of phosphate buffer saline. 5 mL of the bone marrow suspension was carefully layered on equal volume of histopaque solution in a 15 mL test tube. The test tube was then centrifuged at 447 g for 30 min at 28-30°C. After centrifugation, the intermediate buffy coat was carefully transferred into another 15 mL centrifuge tube having 4-5 mL of washing medium, *i.e.* Dulbecco's phosphate buffer saline (DPBS). The tube was re-centrifuged at 447 g for 20 min at 28-30°C to get cell pellet. The pellet was washed twice by DPBS. Finally the cell pellet was re-suspended in 5 mL of growth media, *i.e.*, Dulbecos modified eagles medium (DMEM) with 10% fetal bovine serum (FBS).

Primary Culture: Cells were plated @ 5×10^6 cells in 25 cm² culture flasks and were incubated under humidified atmosphere of incubator at 38.5°C and 5% carbon dioxide (CO₂). The growth media was replaced at three days interval with new growth media and observed regularly for attachment of mesenchymal stem cells (MSCs) under inverted microscope. Once 70-80% confluency was achieved, the primary monolayer of MSCs was trypsinized for subculture.

Secondary Culture: Spent growth media was discarded and the cell monolayer was given two washing with phosphate buffer saline. The cells were trypsinized by brief treatment of 0.25% trypsin EDTA. After observing partial cell detachment from the surface under inverted microscope, pipetting was done to completely detach the cells from the surface of tissue culture flask. The cells were then centrifuged, washed and reseeded @ 5×10^6 cells in 25 cm² culture flasks. The MSCs of 3rd-6th passages were used for transplantation in bone defects. The MSCs were characterized at Animal Biotechnology Centre, Nanaji Deshmukh Veterinary Science University, Jabalpur (MP).

Graft Preparation (Gan *et al.*, 2008): 0.5 gm of β -tricalcium phosphate whitish granules of 2-3 mm in diameter were

loaded into 10 mL syringe. About 1.5 mL of cell suspension containing approximately $4-5 \times 10^6$ cells was pooled into the syringe containing β -TCP granules so as to soak the granules completely in cell suspension. After gentle mixing of the suspension with β -tricalcium phosphate granules, a negative pressure of 0.25-0.5 atmosphere was applied to the mixture. During this vacuum exhausted air from the granule pores facilitated the cells to enter inside the β -tricalcium phosphate granules. Finally the composite was incubated under humidified atmosphere of incubator at 38.5°C and 5% CO₂ for 2 h.

Transplantation of MSCs in Segmental Defects:

Approximately $4-5 \times 10^6$ cells of MSCs seeded on β -TCP were transplanted at fracture site with the help of sterilized forceps after fixing the fractured segment with dynamic compression plate.

Evaluation of Results

The results were evaluated on the basis of inflammation such as exudation and pain was assessed on 2nd, 4th, 7th, 10th and 15th post-operative day. Exudation of the of the wound was recorded on 2nd, 4th, 7th, 10th and 15th post-operative day on the basis of visual assessment and arbitrary score, as per modification done in the method of Bhowmick (2014). Grading and score were given as per following table.

Exudation	Grade	Score
Nil	-	0
Mild	+	1
Moderate	++	2
Marked	+++	3

Pain at the fracture site was evaluated on 2nd, 4th, 7th, 10th and 15th post-operative day on the basis of palpation and pressure and arbitrary score was given as per modification done in the method of Hellyer *et al.* (2007). Grading and score were given as mentioned for exudation. Assessment of weight bearing was done on 2nd, 7th, 10th, 15th, 30th, 45th, 60th and 90th post-operative day as per modification done in the method of Aithal (1996).

Standing	Score
Carrying the limb away from ground	0
Touching the toe on the ground	1
Touching the sole on the ground	2

Radiographs were taken prior to surgery and subsequently on 7th, 15th, 30th, 45th, 60th and 90th post-operative day, and the evaluation was done as per modification done in the method of Hammer *et al.* (1985)(Table 1).

RESULTS AND DISCUSSION

Development of Primary and Secondary BMMSCs

The goat mesenchymal stem cells were established from bone marrow aspirates under aseptic conditions. The fibroblast like appearance of MSCs was observed after



Table 1: Radiographic evaluation of fracture healing (Hammer *et al.*, 1985)

Callus formation	Fracture line	Stage of union	Grade/Score
No callus	Distinct	Not achieved	0
Trace: No bridging of fracture line	Distinct	Not achieved	1
Apparent : Bridging of fracture line	Discernible	Uncertain	2
Massive: Bone trabeculae crossing fracture line	Barely discernible	Achieved	3
Homogenous bone structure	Obliterated	Achieved	4

3-4 days of seeding. The confluency of 50-60% was achieved in 8-10 days and 70-80% in 10-12 days post-seeding.

After attainment of 70-80 % confluency of BMMSCs, the cells were trypsinized to get secondary cell lines. After second passage, only fibroblasts like cells were present in the culture plates. These fibroblasts like cells formed multiple colonies like structure, which were observed under microscope. The cells of 3rd- 6th passages were used for implantation at fracture site.

Exudation and Pain

Examination for exudation revealed significant decrease in exudation from 7th day to 15th day intervals. Slight exudation was present even on 15th day in group I. In group II non-significant decrease in exudation was observed on 4th day, while from 7th day onward no exudation was observed till the end of observation period. In the present investigation, exudation was absent on 7th post-operative day in group II, which might be due to invasion of blood vessels at the fracture site in response to hypoxic state of MSCs. These findings were in agreement with the reports of Singh (2015) and Kumar *et al.* (2021). Initial inflammation at implantation site of MSCs in turn spontaneously increases vascularization at the area leading to decrease in inflammation (Wang *et al.*, 2010).

A gradual decrease in pain was observed from 2nd to 15th post-operative day in control and treatment groups. However, it was significantly low from 4th day onwards in group II goats. These findings were in corroboration with the observations of De'Souza (2012), Singh (2015) and Kumar *et al.* (2021), who observed decrease in inflammatory sign from 7th day onwards and attributed this to cessation of inflammation at fracture site. In the present study, lower pain in group II in which stem cells was implanted on β -tricalcium phosphate, might be due to tissue growth factor β 1 (TGF- β 1), which is responsible for analgesic effect in injured tissue. Stem cells increases production of TGF- β 1, and hence prolong the analgesic effect at the injured tissue (Chen *et al.*, 2015).

Weight Bearing

Significant increase in weight bearing was observed from 7th day onwards in group II, while from 15th day onwards in group I. Complete weight bearing while walking by all the goats was observed on 45th day in group II and 90th day in group I. Gradual increase in weight bearing while walking was also observed by Singh (2015) in canine, and

Kumar *et al.* (2021) in goat. Early weight bearing in group II could be attributed to enhanced fracture healing, as compared to group I. These findings corroborated with the report of Liu *et al.* (2008), who studied the efficacy of synthetic β -tricalcium phosphate, as scaffolds to repair weight-bearing bone defects in large animals and reported that the mechanical properties of the bone marrow mesenchymal cells and β -tricalcium phosphate composites gets improved via tissue engineering approach in goats.

Radiographic Examination

Radiographic interpretation (Fig. 1) on 7th post-operative day revealed periosteal reaction on the fractured segment adjoining the fracture line. Callus at the interface between the host bone and BMMSCs seeded on β -tricalcium phosphate was visible on 15th day. On 30th post-operative day high density radiopaque area was visible at the fracture site. Both endosteal and periosteal callus were well differentiated at this stage. Radiograph of 45th day revealed more organized periosteal as well as intercortical callus, which reduced on 60th day, indicative of initiation of remodeling at this interval, but it was not prominent. β -tricalcium phosphate at fracture site was not visible on 60th post-operative day indicative of its complete resorption. On 90th day, homogenous bone with patent bone marrow was observed, indicative of completion of healing process. These findings were in accordance with the observations of Kon *et al.* (2000) in sheep, Nair *et al.* (2008) in goats, Seebach *et al.* (2010) and Kondo *et al.* (2016) in rats, Zhou *et al.* (2010) and Ismail *et al.* (2014) in rabbits, and Yaokai *et al.* (2010) in humans, who found an enhanced fracture healing using BMMSCs seeded β -tricalcium phosphate.

Arinze *et al.* (2003) reported that allogeneic mesenchymal stem cells loaded on hydroxyapatite-tricalcium phosphate implants, enhanced the repair of fracture in the canine femur without use of immunosuppressive therapy and concluded that mesenchymal stem cells has the potency to differentiate into osteogenic cells. Liu *et al.* (2008) studied the efficacy of synthetic β - tricalcium phosphate (β -TCP) as scaffolds to repair weight-bearing bone defects in goat. They implanted β -tricalcium phosphate scaffold seeded with bone marrow stromal cells (BMSCs) and found that it was completely replaced by bony tissues at 32 weeks interval. Nair *et al.* (2008) compared the regeneration and repair of segmental femoral defects, subsequently to implantation of either triphasic ceramic bone graft substitute or stem cells coated triphasic

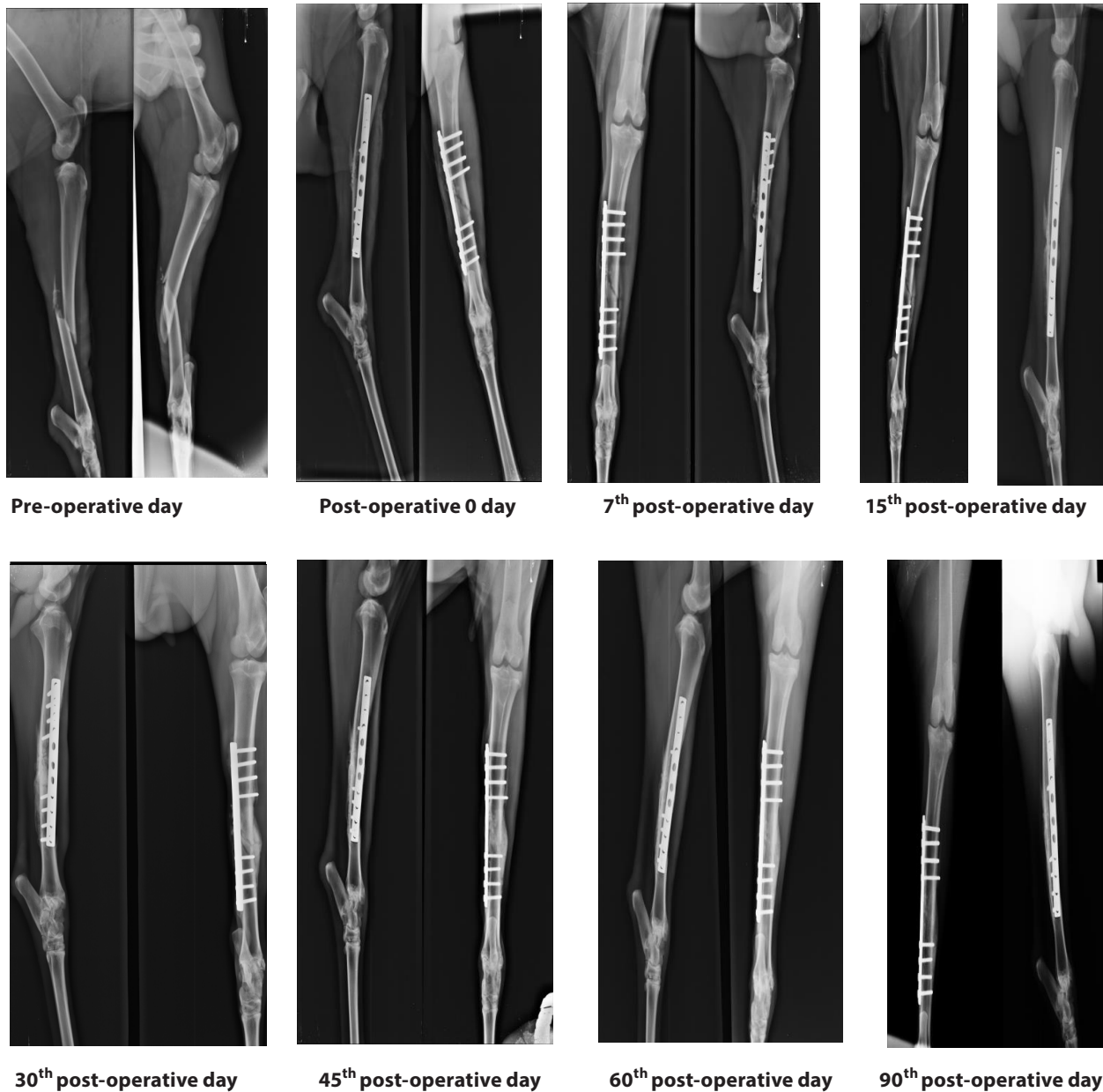


Fig. 1: Radiograph at different time intervals in group II (DCP + β -TCP + Stem cells)

ceramic in gaps. They observed continuous external bridging in both groups at 6 week interval, whereas at 4th month, the radiolucent zone reduced, to which they attributed to union or integration of implant with host bone in both groups. Early initiation of periosteal callus, followed by an early primary and secondary callus, succeeded by early remodeling phase can be attributed to osteo-conductive and osteogenic nature of BMMSCs seeded on β -tricalcium phosphate, which provides this group an edge on other group.

CONCLUSION

On the basis of above findings, it was concluded that combination of bone marrow mesenchymal stem cells and

β -tricalcium phosphate is good bone substitute, to accelerate fracture healing.

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