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STUDIES ON THE EFFICACY OF STEM CELL SEEDED ON B- TRICALCIUM PHOSPHATE FOR FRACTURE HEALING IN GOATS

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Abstract

Twelve clinical cases of goats having long bone fracture presented at College of Veterinary Science and Animal Husbandry, Jabalpur, were included in the present 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 mesenchymal stem cells(BMMSCs) seeded on β-tricalcium phosphate 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 remodelling. 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

INTRODUCTION

Fracture is the 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 orthopaedic 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 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 make 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).

MATERIAL METHODS

Twenlve clinical cases of goats having long bone fracture presented at Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Jabalpur, were included in the present study. Apparentlyhealthy goats of either sex, aged between one to six years were selected for present study. The animals included in the present study were randomly divided into two groups.Fracture segment group I was brought into apposition and 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 mesenchymal stem cells(BMMSCs) seeded on β-tricalcium phosphate scaffold.

II. A. ISOLATION AND CULTURE OF BONE MARROW MESENCHYMAL STEM CELLS (BMMSCS)

a. ISOLATION

i. After collection, bone marrow aspirate was mixed with equal volume of phosphate buffer saline.

ii. Five milliliter of the bone marrow suspension was carefully layered on equal volume of histopaque solutionin a 15 mltest tube.

iii. The test tube was then centrifuged at 2000 revolution per minute (rpm) for 30 minutes at 28-30^{0}C.

iv. 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).

v. The tube was re-centrifuged at 2000 rpm for 20 minutes at 28-30°C to get cell pellet. The pellet was washed twice by DPBS.

vi. Finally the cell pellet was resuspended in 5ml of growth media i.e. dulbecos modified eagles medium (DMEM) [4] with 10% fetal bovine serum (FBS).

b. PRIMARY CULTURE

i. Cells were plated 5×10^6 cells in 25-cm^2 culture flasksand were incubated under humidified atmosphere of incubator at 38.5^0C and 5% carbon dioxide (CO_2).

ii. 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.

iii. Once 70-80% confluency was achieved, the primary monolayer of MSCs was trypsinized for subculture.

c. SECONDARY CULTURE

i. Spent growth media was discarded and the cell monolayer was given two washing with phosphate buffer saline.

ii. 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.

iii. The cells were then centrifuged, washed and reseeded,5×10^6 cells in 25 cm^2 culture flasks.

iv. The MSCs of 3^{rd} -6^{th} passages were used for transplantation in bone defects (Plate-03).

v. The MSCs were characterized at Animal Biotechnology centre, Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.).

B. GRAFT PREPARATION (Gan *et al*., 2008)

i. 0.5 gm of β-tricalcium phosphate whitish granules of 2-3 mm in diameter were loaded into 10 ml syringe.

ii. About 1.5 ml of cell suspension containing approximately 4-5 x 10^6 cells was pooled into the syringe containing β-TCP granules so as to soak the granules completely in cell suspension.

iii. 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.

iv. Finally the composite were incubated under humidified atmosphere of incubator at 38.5^{0}C and 5% CO_2 for 2 hours.

C. TRANSPLANTATION OF MSCS IN SEGMENTAL DEFECTS

Approximately 4-5 x 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.

D. RESULTS WERE EVALUATED ON THE BASIS OF

a. INFLAMMATION

Inflammation such as exudation and pain was assessed on 2nd, 4th, 7th, 10th and 15th post-operative day.

i. EXUDATION

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

ii. PAIN ASSESSMENT

Pain at the fracture site was evaluated on2nd, 4th, 7th, 10th and 15th post- operative day on the basis of palpation and pressure and arbitrary score was given as permodification done in the method of Hellyer *et al.* (2007).Grading and score were given as mentioned for exudation.

b. WEIGHT BEARING

Assessment of weight bearing was done on 2nd, 7th, 10^{th,} 15th, 30th, 45th, 60th and 90thpostoperative 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

c. RADIOGRAPHIC EXAMINATION

Radiographs were taken prior to surgery and subsequently on 7th, 15th, 30th, 45^{th,} 60th and 90thpostoperative day, and the evaluation was done as per modification done in the method of 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

RESULTS AND DISCUSSION

a. DEVELOPMENT OF PRIMARY BMMSCs

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

SECONDARY CELL CULTURE

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.

b. EXUDATION

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 are in agreement with the findings of Singh (2015) and Kumar *et al*.(2021).

Inflammation at implantation site of MSCs causes inflammation at initial stage, which in turn spontaneously increases vascularization at the area leading to decrease in inflammation (Wang *et al*., 2010).

c. PAIN

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 are in corroboration with the finding 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 groups 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).

d. 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 too observed by Singh (2015) in canine, Kumar *et al*. (2021) in goat.
Early weight bearing in group II could be attributed to enhanced fracture healing in group II, as compared to group I. These findings, corroborated the findings 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.

e. Radiographic examination

Radiographic interpretation 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 findings of Kon *et al*. (2000) in sheep,Sempuku *et al*. (2005), Seebach *et al.* (2010) and Kondo *et al*. (2016) in rats and Nair *et al*. (2009) in goats, Yaokai *et al*. (2010) in humans and Zhou *et al*. (2010) in rabbits, who found an enhanced fracture healing using BMMSCs seeded8β – tricalcium phosphate.

Arinzeh *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.

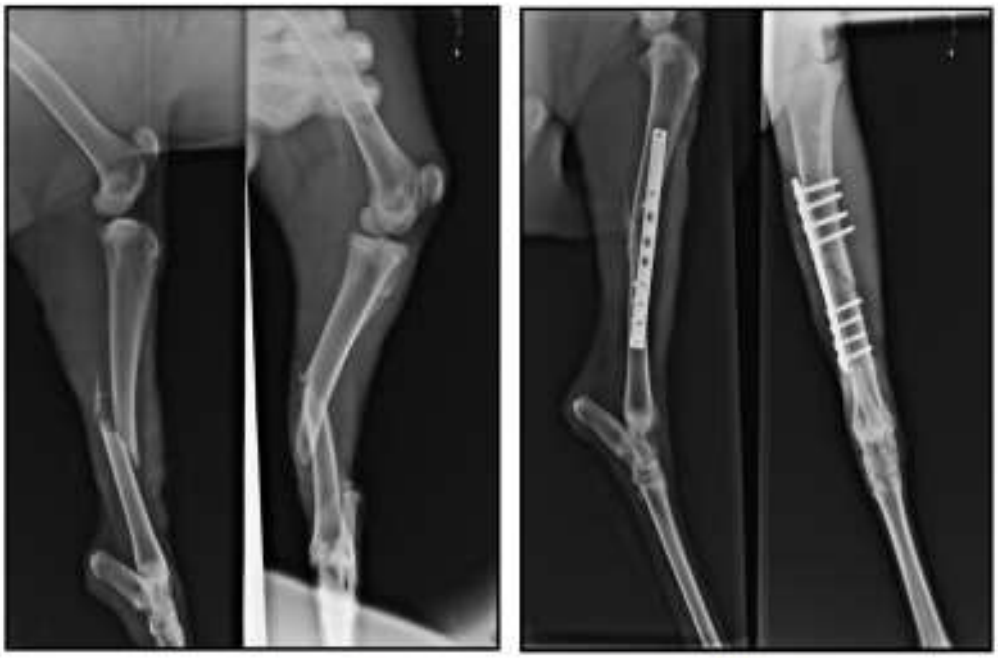
Liu *et al*. (2008)studied the efficacy of synthetic β- tricalcium phosphate (β-TCP) as scaffolds to repair weight-bearing bone defects in goat. They implanted β- tricalcium phosphate scaffold seeded with bone marrow stromal cells (BMSCs) and found that it was completely replaced by bony tissues at 32 weeks interval.

Nair *et a*l. (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 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.

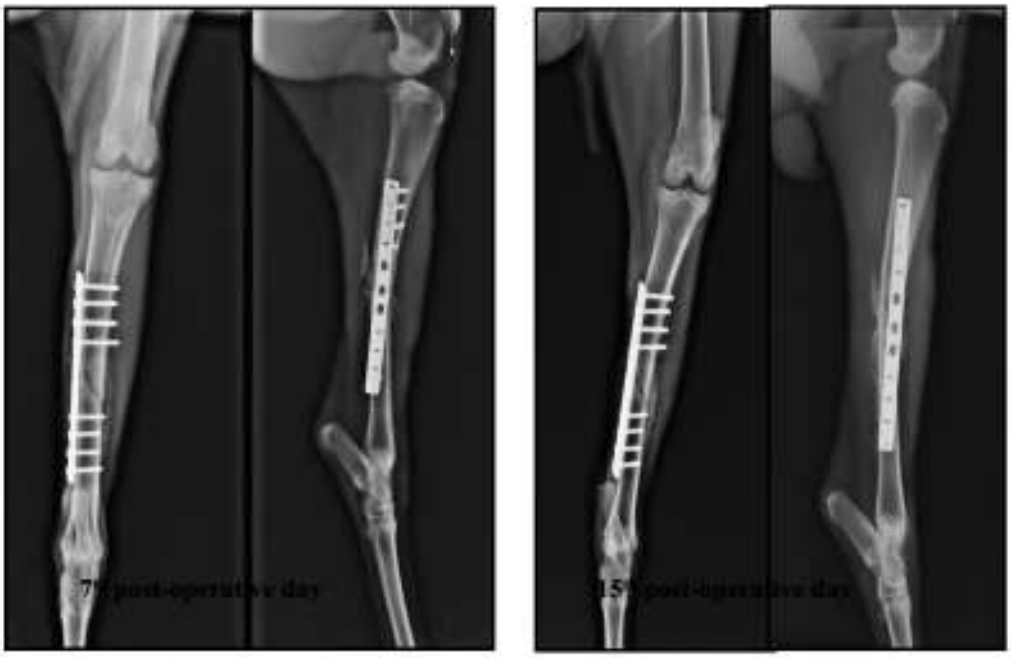
Ismail *et al*. (2014) evaluated the role of allogenic mesenchymal stem cells in the reconstruction of bone defects in rabbits on the basis of radiograph .They concluded that mesenchymal stem cells transplantation on bone defect results, significantly faster callus formation and tend to generate a thicker callus.

Early initiation of periosteal callus, followed by an early primary and secondary callus, succeeded by earlyremodeling 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 groups.

Thus 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.

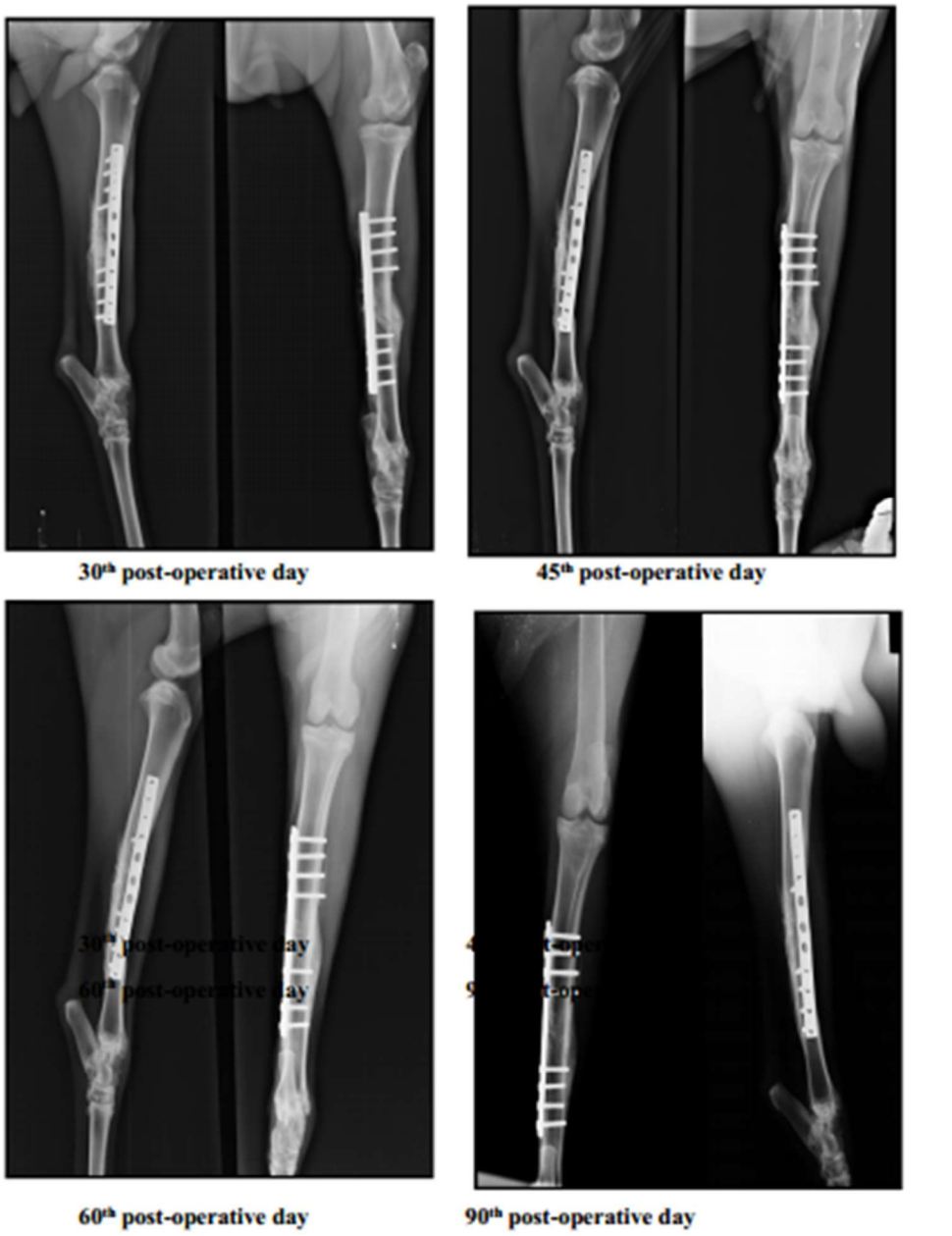


Pre-operative day Post-operative day



7th post-operative day 15th post-operative day

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



30th post-operative day

45th post-operative day

60th post-operative day

90th post-operative day

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

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