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Studies on the efficacy of stem cells for fracture healing in goats

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Abstract
The study was conducted on twelve clinical cases of goats having long bone fracture randomly divided into two groups. In group I the fracture segment was immobilized by dynamic compression plate whereas, in group II after immobilization with dynamic compression plate mesenchymal stem cells were transplanted at fracture site. Exudates and pain at fracture site revealed a decreasing trend in both the groups from 7th to 15th post - operative day. A significant increase in weight bearing was observed from 7th post-operative day in both the groups of goat. Radiographic interpretation at different interval revealed early periosteal reaction, formation of soft and hard callus and initiation of remodeling in group II as compared to group I, The values of alkaline phosphatase increased non-significantly in all groups of goat up to 15th post-operative day, with maximum value at this interval. After reaching its maximum values, a decline in the values was observed in both the groups.

Keywords: fracture, dynamic compression plate, stem cells, weight bearing, radiograph

1. Introduction
Fracture of long bones is a common orthopedic condition encountered in goats and other small ruminants. Goats mostly become frightened while being captured and often injures their limb leading to fractures or dislocations (Smith and Sherman, 2009) [8]. Automobile accident is one of the major etiological factors for fracture in veterinary field. Fracture healing involves various physiological steps occurring throughout the body and it is controlled by a range of cytokines and signaling proteins (Giannoudis *et al.*, 2007) [7]. The process of fracture healing is chronological in nature and various steps intermingle with each other for the formation of new bone. New bone formed by this complex process is structurally and mechanically similar to the pre-fracture state (Gerstenfeld, 2003) [6].
Various biological or synthetic bone substitutes have been developed, with an aim to accelerate the process of fracture healing so that new bone formed may regain its original structure and function. (Ruhaimi, 2000) [17]. The ideal graft material should be biocompatible, non antigenic, non carcinogenic, osteoinductive, osteoconductive, cheaper and provide gradual substitution of the bone tissue (Frame, 1980) [5]. The autogenous bone is considered as gold standard for treatment of a fractured bone but its limited availability and complications associated with its procurement, has led the researcher to think for an alternate (Jensen *et al.*, 1996) [10]. Stem cells are the cells that have the ability to differentiate into any type of tissue under the appropriate environment and can additionally activate surrounding cells to aid in tissue healing and repair. When stem cells are transplanted near an injured area, they act in number of ways to repair and regenerate tissue. They have the ability to differentiate into the surrounding tissue types, like bone, cartilage, tendon, ligament, muscle, and nervous tissue. Moreover, it also activates surrounding resident stem cells and hastens the process of tissue repair. Keeping in view the above facts present research work was designed to evaluate the therapeutic efficacy of bone marrow mesenchymal stem cells (BMMSCs) with internal fixation for fracture healing in goats.

2. Material and Methods
The work was approved by institutional ethical committee. The study was conducted on twelve clinical cases of goats having long bone fracture, presented at Teaching Veterinary

Clinical Complex, College of Veterinary Science and Animal Husbandry, Jabalpur, during the study period. Goats of either sex, aged between one to six years having long bone fracture were selected for present study. The goats selected were randomly divided into two groups. In group I the fracture segment was immobilized by dynamic compression plate whereas, in group II after immobilization with dynamic compression plate mesenchymal stem cells were transplanted at fracture site. The surgical procedure was performed under general anaesthesia using Diazepam hydrochloride @ 0.5 mg/Kg body weight intravenous, followed by Ketamine hydrochloride @ 5 mg /Kg body weight intravenous till effect. Maintenance of anaesthesia was done by repeated intravenous administration of Ketamine hydrochloride as and when required. The animals were administered amoxicillin sulbactam@ 10 mg /kg intramuscular twice a day and continued up to 7th post-operative day. Similarly, Meloxicam @ 0.3 mg /Kg b. wt. intramuscular was administered for four postoperative days to take care of pain. Bone marrow aspirates were collected from the epiphyseal region of fractured long bone of goats undergoing internal immobilization. Stem cells were isolated, cultured and characterized at Animal Biotechnology centre, Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.). The MSCs of 3rd - 6th passages were used for transplantation in bone defects. Approximately 4-5 x 10^6 cells of MSCs suspended in 0.3 ml of Dulbecos phosphate buffer saline (DPBS) were transplanted at the fracture site using micropipette, after immobilization of the fracture ends by dynamic compression plate. Fracture healing was evaluated on the basis of inflammation, exudation, pain, weight bearing by the animals, radiographic evaluation and haemato-biochemical studies at different time intervals. Inflammation, exudation, and pain at the fracture site was observed on 2nd, 4th, 7th, 10th and 15th post-operative day, Assessment of weight bearing was done on 2nd, 7th, 10th, 15th, 30th, 45th, 60th and 90th postoperative day, as per modification done in the method of Aithal (1996) [1].

Weight bearing by the animal	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 postoperative day, and the evaluation was done as per modification done in the method of Hammer *et al*. (1985) [8].

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. Results and Discussion

The goat mesenchymal stem cells were established from bone marrow aspirate under aseptic condition. The fibroblast like appearance of mesenchymal stem cells was observed after 3 to 4 days of seeding. The confluence of 50-60% was achieved in 8-10 days and 70-80% in 10 - 12 days of seeding. Similar findings were observed by Liu *et al*. (2016) [14] who observed a confluence of 90% in about 12 days of seeding.

3.1 Exudates and Pain

Exudates at fracture site revealed a decreasing trend in both the groups from 7th to 15th post - operative day. Complete absence of exudates was noticed in group II on 7th post-operative day whereas, slight exudates were present in group I even on 15th post-operative day. Early cessation of exudation in group II can be explained by the findings of Wang *et al*., (2010) [22] who opined that implantation of mesenchymal stem cells at fracture site causes inflammation at initial stage, which in turn spontaneously increases vascularization at the area leading to decrease in exudates and its complete absence at later stage. Progressive decrease in degree of pain was observed in both the groups from 7th to 15th post - operative day. Throughout the observation period, a lower value of pain was observed in group II in which stem cells were implanted as compared to group I where only plating was done. During study, lower pain in group II might be due to stem cells that initiates secretion of Transforming growth factor–beta 1 (TGF-beta 1), which is liable for analgesic effect in injured tissue. Stem cells increases production of TGF-beta 1, and hence prolong the analgesic effect at the injured tissue (Chen *et al*., 2015) [4].

3.2 Weight bearing

A significant increase in weight bearing was observed from 7th post-operative day in both the groups of goat. Complete weight bearing by all the animals was observed on 30th day in group II however, complete weight bearing by all the animals of group I was observed on 60th post-operative day. These findings were similar to the findings of Kushwaha *et al*. (2011) [12], Avasthi *et al*. (2012) [3] and Vinit (2018) [21] who reported gradual increase in weight bearing from 7th day onwards. An early weight bearing by all the animals of group II can be explained by the fact that there was early alleviation of pain in group II as compared to group I.

3.3 Radiographic examination

Radiographs taken immediately after surgery depicted proper alignment of the fracture in all the animals of both the group. Radiographic evaluation on 7th post-operative day in group II exhibited proper fixation of implant without any incidence of implant failure. 15th day radiograph of the goats implanted with stem cells, revealed slight increase in the soft tissue density in group II. A periosteal callus in proximal, distal or both segments adjoining the fracture line was discernible in some of the cases. Fracture line was distinct and stage of union was not achieved. Callus formation at this interval was regarded as nil to trace with a score of 0.40 ± 0.21. Contrary to this radiographic score of group I at this stage was 0.17^a± 0.17. Radiographic interpretation on 30th post-operative day showed distinct callus and bridging of the fracture line in all the cases of group II. There was an evidence of both endoosteal as well as periostel callus at this time interval. Fracture line was discernible and the stage of union was

uncertain. The implant was found to be stable in all the cases, which rejected any possibility of non-union or mal-union. The radiographic score achieved in this group, at this interval was 2.00 ± 0.00 and the stage of callus formation could be graded as apparent whereas, in group I bridging of fracture line was incomplete.

45th day radiograph of group II revealed massive callus formation with complete radiopaque area in all the cases. Fracture line was not visible in any of the case of this group. Bone trabeculae were found to be crossing the fracture line. Stage of union was ascertained as apparent to massive and the radiographic score was 2.83 ± 0.17. In group I fracture line was still discernible and the callus formation was graded as apparent.

Radiographic examination of 60th post-operative day depicted complete union with radiodense callus between the two fractured segments of the bone. The newly formed periosteal, as well as intercortical callus became more organized and bridged across the fracture gap, with obliteration of fracture line. Initiation of remodeling of fractured area could well be appreciated at this time interval. The callus formation was ascertained as massive to homogenous. On the other hand complete union with radiodense callus between fractured segments was observed in group I. Interpretation of radiograph on 90th day, of group II animals exhibited almost homogenous bone. Fracture line was completely obliterated and stage of union was achieved. Radiograph of group I animal at this stage revealed union of fractured segment with an initiation of remodeling stage which was indicated by start of homogenesity of bone.

These findings were in accordance with the observations of Arnizeh *et al*. (2003) [1], who reported that mesenchymal stem cells has potency to differentiate into osteogenic cells and enhance large segmental bone defects in canine, Ismail *et al*. (2014) [19], reported that mesenchymal stem cells, when implanted at fracture site enhances callus formation and accelerates its thickness leading to early healing in rabbits, while Kim *et al*. (2014) [11], stated that mesenchymal stem cells have potential healing capabilities. They further reported that, these cells contain growth factor and signaling protein that can instigate the regeneration of damaged tissue.

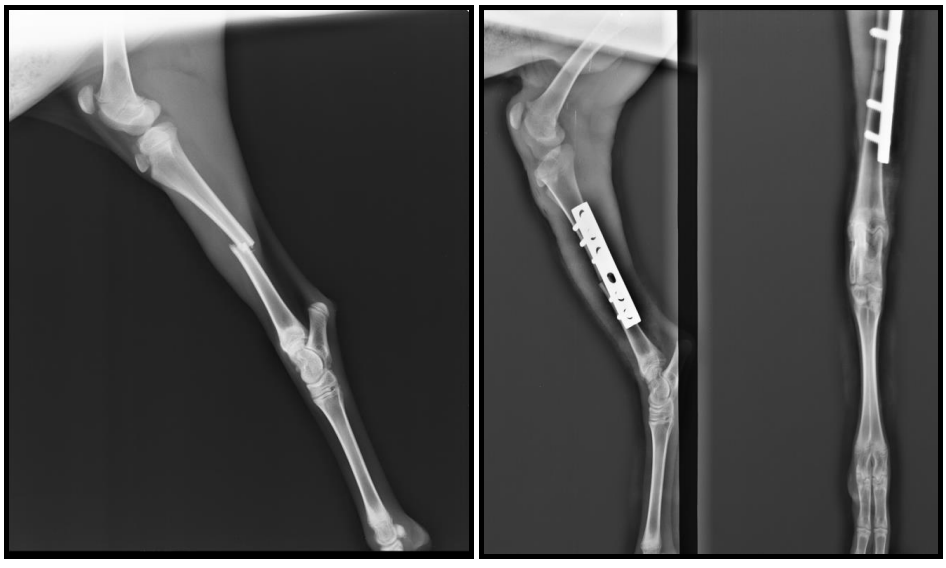
Early healing of fracture in case of BMMSCs implanted at the fracture site may be due to the capacity to stem cells to alleviate pain and its osteogenic activity.

3.4 Biochemical changes

The values of alkaline phosphatase increased non-significantly in all groups of goat up to 15th post-operative day, with maximum value at this interval. After reaching its maximum values, a decline in the values was observed in both the groups and minimum values were seen on 60th day in group I and 45th day in group II. Decrease in the value of alkaline phosphatase might be indicative of cessation of osteoblastic activity and receding of the values towards its base value due to ossification and consolidation of fractured bone. This higher activity of alkaline phosphatase on 15th day and its gradual decrease was too supported by the findings of radiographs. After 45th day interval, there was a gradual increase in the values of alkaline phosphatase till 90th day, in group II and from 60th day till 90th day in group I, which might be due to the fact that remodeling phase may have started after these time intervals, which correlates with the radiographic findings. The above observations are in agreement with the findings of Rani and Ganesh (2003) [16], Phaneedra *et al*. (2016) [15], Szponder (2018) [19, 20], Vinit (2018) [21] and Yadav *et al*. (2020) [23] who reported that alkaline phosphatase increases in initial days of fracture healing and then its value gradually decreases till 60th post-operative day.

Creatinine kinase showed significant decrease from 7th day to 90th day interval in both the groups of goats. The present findings corroborate with the findings of Laurence (2000), who observed increased activity of creatinine kinase after surgery, which receded back to its normal value after healing. Higher values of creatinine kinase at initial stage might be attributed it to the extent of muscle damage at this stage which decreased as the healing progressed.

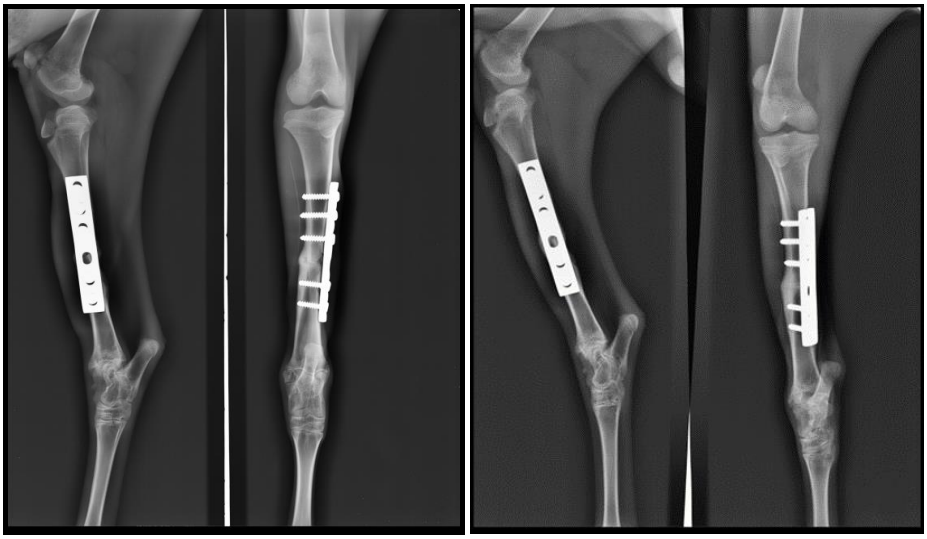
There was a gradual decrease in the values of serum calcium till 45th post-operative day after which there was a gradual increase in these values up to 90th post-operative day. The above findings are in accordance with the findings of Rani and Ganesh (2003) [16] and Vinit (2018) [21], who observed an increase in the value of serum calcium on first day of fracture repair, followed by marked reduction in goats. Higher value of serum calcium in initial intervals can be attributed to increased osteoclastic activity, leading to resorption of dead bone in initial stage.



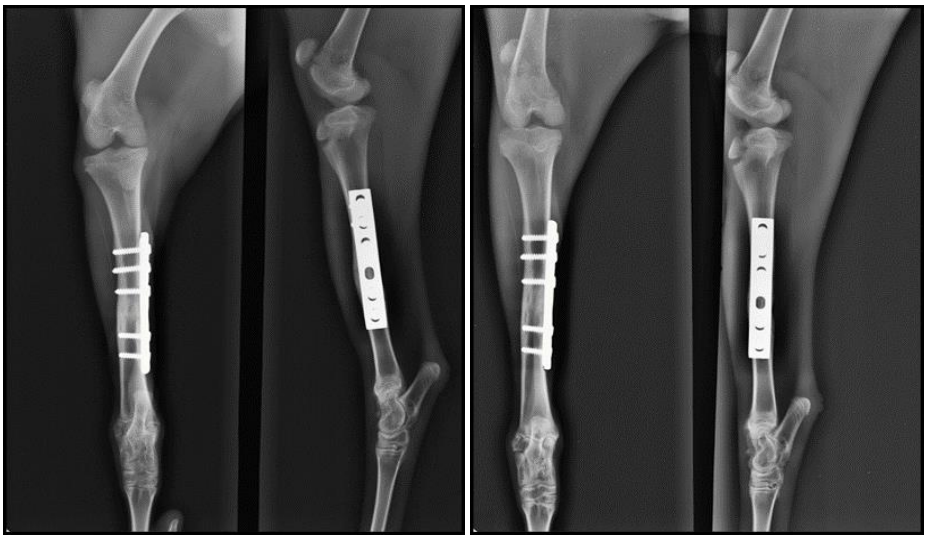
Pre-operative day Post- operative day

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

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



30th post-operative day 45th post-operative day



60th post-operative day 90th post-operative day

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

4. Conclusions

From the above discussion it can be concluded that β tricalcium phosphate can be used as a bone substitute to accelerate the process of fracture repair since it acts as a scaffold and help to alleviate pain, early weight bearing by the animal, initiates early periosteal reaction, callus formation and its remodeling. Radiograph and biochemical parameters can be used as an indicator of fracture healing. Use of mesenchymal stem cells for weight bearing long bones is still in its infancy and requires a lot of research work.

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