Guided Tissue Regeneration with β-Tricalcium Phosphate and Platelet-rich Plasma for Fracture Repair in Dogs using Internal Fixation

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ABSTRACT

The study was conducted on 19 adult dogs referred for treatment of fracture in diaphyseal region of three long bones, viz, radius-ulna, femur and tibia-fibula. These dogs were divided randomly into three groups viz T1, T2 and T3. In group T1, the diaphyseal fractures of the long bones were immobilized by an internal fixation technique using reconstruction plate or locking compression plate. In group T2, β- tri calcium phosphate granules were incorporated at fracture site after fixation. In group T3, one milliliter of PRP was administered locally at fracture site just before the closing the first suture line. Clinical and radiographic examination were also conducted during the study. There was a significant reduction in mean inflammation score at 7th day in groups T2 and T3 and at 30th day than 0 day in all the groups. At 90th day, the mean radiographic score was highest for group T3 and it was followed by the score in group T2. At day 90th, a radiograph in both the views was not able to delineate the granules from osseous tissue. The mean weight bearing score was also higher in group T3 than in group T2 at 30th, 60th and 90th post operative day showing that restoration of the function was better and earlier in groups T2 and T3 than in group T1.

Keywords: Fracture, guided tissue regeneration, tri-calcium phosphate and platelet rich plasma

The fractures can be immobilised by external immobilisation/ fixation or internal fixation. Bone plates and screws are particularly useful when post-operative comfort and early limb use are desired (Piermattie *et al.*, 2006). The principle of guided tissue regeneration using a membrane technique has been used in periodontal surgery. By the membrane technique, cells originating from the surrounding bone are allowed to repopulate the wound without interference from other tissue types (Aaboe *et al.*, 1993). Guided tissue regeneration with polyglactin 910 mesh is used in combination with osseous grafting to obtain new bone formation over the surgical site (Balshi *et al.,* 1991). Autogenous bone grafting is considered the gold standard for filling bone defects even today, despite significant limitations. For this purpose, mostly, synthetic β-tricalcium phosphates (β-TCP) or calcium phosphates from corals are used (Kasten *et al.*, 2008).

Growth factors influence the chemotaxis, differentiation, proliferation and synthetic activity of bone cells, thereby regulating physiological remodeling and fracture healing. Numerous growth factors, such as bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), transforming growth factor-b (TGF- β), and insulin-like growth factors (IGF), have a stimulating effect on bone defect healing. Platelet-rich plasma (PRP) contains a number of these growth factors {PDGF, TGF- β1, TGF-β2, IGF}, epidermal growth factor (EGF) and epithelial cell growth factor (ECGF) in its natural composition. Considering the promising role of these new techniques in repair of fractures with bone defects, the present study was conducted.

MATERIALS AND METHODS

The study was conducted on 19 adult dogs referred for treatment of fracture in diaphyseal region of long bones. These dogs were divided randomly into three groups as below (Table 1).

Table 1: Treatment design

Group	Treatment
T1(n=7)	Internal fixation with mesh alone
T2 (n=6)	Internal fixation with mesh and β–tricalcium phosphate (β-TCP)
T3 (n=6)	Internal fixation with mesh, β-TCP and platelet-rich plasma (PRP)

Composite mesh

Composite mesh made up of polypropylene and polyglycolic acid was utilized for Guided tissue regeneration (GTR) of osseous tissue at fracture site. The mesh is available in size of 4"× 4" or 4"× 6" in a pre-sterlised pack.

β-tri-calcium phosphate

Gamma sterilized β-tri calcium phosphate (β-TCP) nanocrystalline granules having a particle size of 2000-3000μ were used at fracture site as an osteoconductive material to make up the bone loss.

Preparation of platelet-rich plasma

For the animals of group T3, platelet-rich plasma (PRP) was harvested by collecting 5 ml of peripheral venous blood aseptically collected from ten healthy dogs in heparinised test tubes and pooled together. The platelet count of the pooled whole blood was determined to be $183×10^3/$ μl. The blood was divided into 10 centrifuge tubes and centrifuged at 200 xg in a routine centrifuge for 10 minutes. After, the formation of three layers (a bottom layer composed of RBC; an upper layer composed of plasma, platelets and some WBC's and an intermediate layer or buffy coat composed mostly of WBC's), the upper layer was collected with pipette. This collection was performed carefully to avoid disturbing the bottom layer of RBC and the buffy coat layer. The collected volume

was approximately 3 ml per tube. The collected sample was then transferred to an empty siliconised glass tube to be homogenized. After the sample was adequately mixed, the collected sample, approximately 30 ml in volume, was again transferred to six centrifuge tubes and centrifuged at Relative Centrifuge Force (RCF) of 400xg for initial five minutes and subsequently at Relative Centrifuge Force (RCF) of 600xg for another five minutes. The upper half of the plasma volume, platelet poor plasma (PPP), was removed. The remaining volume of PRP was homogenized and analyzed for platelet count. The platelet count in the PRP was now determined to be $568×10^3/$μl. The PRP was stored in sterilized eppendorf tubes at -80ºC till use in a clinical case.

Radiographic examination

Radiographic examination in medio-lateral and cranio-caudal views of the affected limb was conducted on day 0 preoperatively to optimize the implant selection and post operatively to verify the position of the fractured segments. It was followed on 30th, 60th and 90th day to monitor implant stability and fracture healing. The radiograph was prepared on a conventional radiographic film or on Computerised Radiography (CR) System.

Surgical procedure

Pre-operative preparation

All the animals were fasted for 6 to 12 hours. The proposed site for the surgical procedure was aseptically prepared for internal fixation of fracture. Preoperative blood sample was collected and divided in two parts i.e. in EDTA coated vacutainer and in plain vacutainer.

Anaesthesia

The general anesthesia was obtained using inj. atropine sulphate @ 0.02 mg/kg I/M as pre-anaesthetic, followed by inj. diazepam hydrochloride @ 1 mg/kg I/V and induction of general anesthesia with inj. propofol 4 mg/kg I/V. After induction of the adequate level of anaesthesia in dog, endotracheal intubation was performed using a laryngoscope and a suitable sized cuffed endotracheal tube no. 6 to 7.5. Adequate anaesthetic depth was maintained

using inhalation anesthetic agent isoflurane at minimum alveolar concentration (MAC) of 1.0-2.5%. The flow rate of the oxygen was maintained approximately @ 10 ml/kg b.w per minute. The semi closed or closed breathing circuit was utilized during surgical procedure as per the requirement.

Instrumentation and implants

A standard set of orthopaedic instruments was used for internal fixation with a reconstruction or locking compression plate.

Table 2: Implants used for internal fixation

Sl. No.	Name
1.	Straight and T- locking compression plates – 7 holes to 10 holes
2.	Locking compression plates (T- plates)
3.	Self tapping cortical locking head screws 3.5 mm
4.	Reconstruction plates 2.7 mm
5.	Dynamic compression plates 2.7 mm
6.	Self tapping cortical screws 2.7 mm

The long bones radius-ulna, tibia and femur were exposed for internal fixation of fracture as described by Piermattie *et al.* (2006).

Technique for internal fixation

In group T1, the diaphyseal fractures of the long bones were immobilized by an internal fixation technique using reconstruction plate or locking compression plate. The reconstruction plate was used for internal fixation of the fracture in animals having body weight up to 12 kg. The LCP was used for internal fixation of the fracture in animals having a higher body weight up to 30 kg (Fig. 1a).

The fracture ends were retrieved from incision site and were reduced manually to their anatomical position. After reduction, LCP of suitable length was placed over bone surface and held with surface using bone holding forceps. The position of the plate and its alignment was checked and optimized. The LCP sleeve was fixed in hole to be drilled. Then, the holes were drilled in bone from the plate holes using universal drill. The depth of the hole was measured with orthopaedic depth gauze. This depth was

used to decide the length of screws. An appropriate screw was tightened in the hole using an orthopaedic screw driver. The same procedure was followed for each hole in proximal and distal fragment of the bone. The orthopeadic screws had purchase in minimum of six cortices of bone in one segment for rigid fixation. After fixation of the fracture, using a reconstruction plate or locking compression plate, a composite mesh was used in periphery of the bone at fracture site. Both the ends of the mesh were sutured with simple continuous sutures using polyglactin 910 No. 0 or 2-0, so as to fit the mesh snugly around the periphery of bone at fracture site (Fig 1a and 1b).

In group T2, the diaphyseal fractures were immobilized using an internal fixation technique using a suitable plate. B-TCP granules were incorporated at fracture site in a composite mesh cylinder after achieving haemostasis. One or more pack of 0.5 gm β-TCP was used to make up the bone loss at fracture site.

In group T3, the stable internal fixation of fracture fragments was achieved and β-TCP was filled in a cylinder of composite mesh. One milliliter of PRP was administered locally at fracture site just before the closing the first suture line.

Fig. 1: Internal fixation of fracture using **(a)** Locking compression plate **(b)** scaffold of composite mesh with β- tri calcium phosphate (β-TCP) and **(c)** application of Platelet rich plasma (PRP)

Rectal temperature

The rectal temperature of the dogs ranged from 101 to 103ºF at time of presentation of animals with fracture. The mean rectal temperature was non-significantly (p>0.05) higher at 0 day in three groups. Desouza (2012) has also reported a higher rectal temperature in dogs at day 0 pre-operatively. The increase in rectal temperature may be due to release of mediators of inflammation viz, prostaglandins, bradykinins and interleukins following trauma and fracture.

Recovery

A smooth recovery from general anaesthesia was observed after 10 to 30 minutes of turning off the isoflurane vaporizer. Raghunath *et al.* (2012) also maintained adequate general anaesthesia using isoflurane @ 1.0 to 2.0 MAC. In present study MAC for isoflurane ranged from 1.0 to 2.5. This could be attributed to different anaesthetic protocols used for induction of general anaesthesia.

Appearance of β-tricalcium phosphate

At day 90th, a radiograph in both the views was not able to delineate the granules from osseous tissue. The resemblance in radio-opacity of β-TCP and osseous tissue may be due to its resorption and osteoconductive properties. Jang *et al.* (2008) has also observed that an ideal bone graft should resorb fully and also provide three dimensional matrix to support bone ingrowth and on growth during resorption. On the same lines, Yamasaki *et al.* (2009) observed that the radio-opacity of the TCP decreased in a time dependent manner and was available on X-ray films at 12 weeks after implantation, but the margins of the implants were not clear indicating the resorption of β-TCP.

Radiographic score

At 90th day, the mean radiographic score was highest (4.50 ± 0.22) for group T3 and it was followed by the score of 4.33 ± 0.88 in group T2, showing osseous callus in evolution with presence of periosteal proliferation or exuberant osseous callus in evolution. The mean radiographic score was minimum (2.86 ± 0.88) for group T1 at 90th day showing organized periosteal proliferation.

The increase in mean radiographic score may be attributed to the progress in fracture healing at different intervals. In group T2, β-TCP implanted at fracture site probably acted as an osteoconductive material and leaded to increase in bone formation, hence, this bone substitute contributed to increase in radiographic score. The observation is well corroborated with the findings of Neamat *et al.* (2009) who stated that after resorption of Cerasorb M (β-TCP), the bone was regenerated completely.

In group T3, the mean radiographic score was noticed highest at 90th day expressing that fracture healing was better than rest of the two groups. This could be attributed to the role of PRP as an osteoinductive material in fracture repair as Fortier *et al.* (2011) has described the PRP as a milieu of growth factors, cytokines, perhaps some RBC's and all the proteins. A PRP clot serves as a fibrin matrix which acts as a scaffold for tissue repair and reservoir for retention and slow release of growth factors.
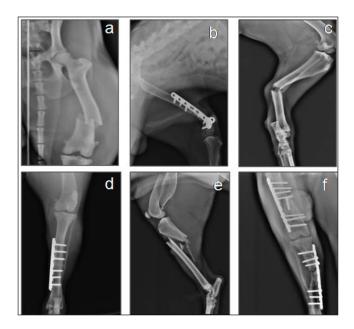

Fig. 2: Radiographic appearance of fracture **(a)**, **(c)** and **(e)** at day 0 **(b)** at day 90 in group T1 **(d)** at day 90 in group T2 **(e)** at day 90 in group T3

Table 3: Mean radiographic score at different intervals in three groups of animals

Groups	Days	Mean radiographic score
T1	0	0.00 ± 0.00[c]
	30	1.14 ± 0.26[bc]
	60	1.86 ± 0.59[ab]
	90	2.86 ± 0.88[a]
T2	0	0.00 ± 0.00[c]
	30	1.83 ± 0.31[b]
	60	2.83 ± 0.60[a]
	90	4.33 ± 0.88[a]
T3	0	0.00 ± 0.00[c]
	30	1.50 ± 0.22[b]
	60	3.17 ± 0.17[a]
	90	4.50 ± 0.22[a]

Note: [ab]values within groups with different superscripts differ significantly ($p < 0.05$).

Inflammation

The mean inflammation score at fracture site was recorded to be significantly (p<0.05) higher at 0 day in all the three groups. There was a significant reduction in mean inflammation score at 7th day in groups T2 and T3 and at 30th day than 0 day in all the groups. This decline was indicative of decrease in inflammation at 30th day in affected limb. Finally, at 90th day, the mean inflammation score reduced to almost normal values, suggesting cessation of inflammatory signs. Desouza (2012) observed clinical signs of inflammation at fracture site in affected limb at time of presentation, however; no quantification of these signs was carried out and the inflammation subsided in most of the cases after 30th day.

Weight bearing score

Weight bearing score (while standing)

The increment in this parameter was observed post operatively at 0 day itself, showing that animals were touching the toe (score 1) or touching the paw (score 2) in all the groups. The weight bearing (while standing) improved early because most of the animals started to touch the toe or paw just after some hours of the internal fixation. Similarly, Rajhans (2013) reported that weight bearing on affected limb after internal fixation using a reconstruction plate was observed at 0 day in few cases; however others did not show weight bearing on affected limb.

The mean weight bearing score (while standing) increased gradually from 0 to 30th day indicating that all animals were touching the toe or paw of the affected limb. It was observed better in group T3 followed by group T2 and group T1 at 30th day. This improvement in score may be due to the fact that most of the animals of group T3 showed early fracture healing as also supported by the radiographic scores. Kushwaha *et al.* (2012) reported full weight bearing at 18th day on affected limb after internal fixation of a radius-ulna in a non-descript dog.

In group T3, the mean weight bearing score was 2.00 ± 0.00 at 90th day as all the animals were showing full weight bearing on affected limb. This improvement in weight bearing could be attributed to better fracture healing probably attributed to local application of PRP as also suggested by the inflammation scores and radiographic scores in the present study.

Table 4: Clinical parameters at different intervals in three groups of animals

Groups	Days	Mean inflammation score	Mean weight bearing score (while standing)	Mean weight bearing score (while walking)	Mean weight bearing score (while running)
T1	0	2.43 ± 0.20^a	0.29 ± 0.18^b	0.57 ± 0.20^b	0.00 ± 0.00^d
	7	2.00 ± 0.00^a	0.86 ± 0.14^{ab}	1.00 ± 0.22^b	0.00 ± 0.00^d
	14	1.14 ± 0.14^b	0.86 ± 0.14^{ab}	1.00 ± 0.22^{b}	0.29 ± 0.18^{cd}
	30	1.00 ± 0.22^b	1.14 ± 0.26^a	1.71 ± 0.52^{ab}	1.00 ± 0.31^{bc}
	60	0.71 ± 0.18^{bc}	1.43 ± 0.30^a	1.71 ± 0.57^{ab}	1.43 ± 0.43^{ab}
	90	0.29 ± 0.18^c	1.43 ± 0.30^a	2.43 ± 0.69^a	2.14 ± 0.63^a
T2	0	2.17 ± 0.17^a	0.33 ± 0.21^b	0.67 ± 0.33^c	0.00 ± 0.00^c
	7	1.33 ± 0.21^b	0.67 ± 0.21^b	0.67 ± 0.33^c	0.00 ± 0.00^c
	14	0.83 ± 0.31^{bc}	0.67 ± 0.21^b	1.33 ± 0.49^{bc}	0.67 ± 0.33^{bc}
	30	0.67 ± 0.33^{bc}	1.50 ± 0.22^a	1.83 ± 0.70^{bc}	1.17 ± 0.54^b
	60	0.50 ± 0.22^c	1.67 ± 0.33^a	2.67 ± 0.61^{ab}	2.33 ± 0.61^a
	90	0.17 ± 0.17^{c}	1.67 ± 0.33^a	3.33 ± 0.67^a	3.17 ± 0.65^a
T3	0	2.50 ± 0.22^{a}	0.50 ± 0.22^b	0.83 ± 0.17^c	0.00 ± 0.00^c
	7	1.50 ± 0.22^b	0.83 ± 0.17^b	1.17 ± 0.31^c	0.00 ± 0.00^c
	14	0.83 ± 0.17^c	0.83 ± 0.17^b	1.67 ± 0.21^{bc}	0.83 ± 0.71^{bc}
	30	0.50 ± 0.22^{cd}	1.67 ± 0.21^a	2.67 ± 0.42^{ab}	1.33 ± 0.21^b
	60	0.17 ± 0.17^{cd}	1.83 ± 0.17^a	2.83 ± 0.48^{ab}	2.67 ± 0.42^a
	90	0.00 ± 0.00^d	2.00 ± 0.00^a	3.33 ± 0.42^a	3.33 ± 0.33^a

Note: [ab]values within groups with different superscripts differ significantly (p<0.05).

Weight bearing score (while walking)

The mean weight bearing score was found to be higher in groups T2 and T3 than group T1 at 14th, 30th, 60th and 90th day. The mean weight bearing score was recorded to be highest in group T3 at 30th, 60th and 90th day post operatively.

A response having fear was markedly witnessed while assessing the animals for weight bearing (while walking). The improvement in this score must have been depended upon the weight bearing score (while standing) at different time intervals as the animal showing an early weight bearing (while standing) can be predicted to express an early weight bearing (while walking) at further intervals.

Mean weight bearing score (while running)

The mean weight bearing score was also higher in group T3 than in group T2 at 30th, 60th and 90th post operative day showing that restoration of the function was better and earlier in groups T2 and T3 than in group T1. This finding is also based on the fact that animal showing an early weight bearing (while standing) will witness an early return of limb function with full range of motion. The same has been endorsed by Cruess and Dumont (1985) who stated that bone healing can be promoted by some factors like growth hormone, thyroid hormone, calcitonin, insulin, vitamin A and D, anabolic steroids, chondroitin sulphate, hyaluronidase, anticoagulants, electric currents, oxygen and physical exercise. Early weight bearing (while standing and while walking) in group T3 in comparison to remaining two groups, might have contributed in form of physical exercise to better weight bearing score (while running) from 60th day onwards. Further, the assessment of the weight bearing (while running) was found difficult in a clinical environment.

Fig. 3: Weight bearing status; **(a)** complete loss after fracture, **(b)** after 7th day, **(c)** at 90th day

Complications

The complications observed during the study were mostly related with implant failure. The failure of a locking compression plate might be due to the reason that the dog was allowed to run freely within a month. The implant failure observed in radius-ulna of a Pomeranian (10 kg) might be due to sub-optimal operative procedure. The high complication rate in radius-ulna fractures may be attributed to the fact that healing of radius-ulna fractures is potentially slowed by a limited blood supply, limited soft tissue around the bone (Boudrieau, 1993). Further, the bending of the plate may be attributed to the biomechanical forces applied during post operative period which vary according to temperament of animal and compliance from owner.

CONCLUSION

The composite mesh was found feasible to act as a tool for guided tissue regeneration. β-tricalcium phosphate was clinically suitable to act as osteoconductive material to improve the repair of diaphyseal fractures with bone loss. Platelet-rich plasma (PRP) was helpful as osteoinductive material to promote fracture healing with scaffolds like β-tricalcium phosphate in dogs as observed on basis of clinical and radiographic findings. Platelet-rich plasma (PRP) when used with osteoconductive material (tri-calcium phosphate) was found better than osteoconductive material alone.

Acknowledgements

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