Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i7.7040

COMPARATIVE ANALYSIS OF HISTOLOGICAL AND HEMATOLOGICAL OF CRITICAL-SIZED FEMUR FRACTURES TREATED WITH IM-PIN IN COMBINATION WITH PMMA AND CAP IN A CANINE MODEL

Abdul Salam Khoso[1], Ahmed Nawaz Tunio[1*], Muhammad Ghiasuddin Shah[2], Akeel Ahmed Memon[3], Atique Ahmed Behan[4], Mansoor Tariq[5], Muhammad Bilawal Arain[6]

[1*]Department of Surgery and Obstetrics, Sindh Agriculture University Tandojam
[2]Department of Veterinary Anatomy and Histology, Sindh Agriculture University Tandojam
[3]Department of Animal Breeding and Genetics, Sindh Agriculture University Tandojam
[4]Department of Livestock Management, Sindh Agriculture University Tandojam
[5]Department of Veterinary Pathology, Sindh Agriculture University Tandojam
[6]Department of Veterinary Pharmacology, Sindh Agriculture University Tandojam

[*]**Corresponding author:** Prof. Dr. Ahmed Nawaz Tunio (PhD)
*Department of Surgery and Obstetrics, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, 70060 Sindh, Pakistan, Email: antunio@sau.edu.pk

ABSTRACT:
This study aimed to assess critical-size bone defect reconstruction using polymethylmethacrylate (PMMA) and calcium phosphate (CaP) bone materials in comparison, focusing on histological, and hematological parameters. Thirty-six healthy dogs were divided into three groups, with each group receiving different treatments: intramedullary pin (IM-pin) in Group A, PMMA with IM-pin in Group B, and CaP-based bone graft material with IM-pin in Group C. Histological evaluations at various post-surgery time points revealed differences in healing responses, trabeculae formation, and osteon numbers among the groups. Group B exhibited significant growth and dense connective tissue formation, indicating a rapid healing process. IM-Pin PMMA has been recognized as the optimal implant material for femoral bone fractures in dogs, exhibiting histological advantages, including biocompatibility, corrosion resistance, and minimal bone resorption. Throughout all groups, serum calcium levels were maintained within the normal range. Hematological parameters, encompassing red blood cell (RBC) count, hemoglobin, packed cell volume (PCV), white blood cell (WBC) count, neutrophils, lymphocytes, and monocytes, were thoroughly assessed. Variations in RBC count and WBC count were observed post-surgery, with eventual normalization. Overall, this study provides valuable insights into the histological and hematological aspects of critical-size bone defect reconstruction, highlighting the effectiveness of IM-Pin PMMA and the need for long-term safety considerations in veterinary practices.

Keywords: critically sized; canine model; femur fracture; IM-Pin; Histological; hemato-biological

Introduction

Fractures, characterized by soft tissue damage and bone separation, present therapeutic challenges, especially in critical-sized defects like femoral fractures (PURNOMO et al., 2022). The femur is one of the most frequently and commonly affected bones (GADDAM et al., 2022) Furthermore, critical-

sized defects are represented as a major therapeutic challenge (RODDY et al., 2018). Treatment options include mechanical stabilization, bone grafting, and induced membrane techniques. However, fractures in elderly patients, particularly comminuted or unstable ones, may exhibit inconsistent healing (SAUNDERS et al., 2022). Moreover, to accurately diagnose those fractures, clinical examination including hematological and biochemical parameters provides excellent detail to understand the degree of fracture healing (KUMAR et al., 2020). Bone types of cement and implants aid in extensive damage treatment, with polymethylmethacrylate (PMMA) being a commonly used and successful option due to its properties such as nontoxicity and functionalization (VAISHYA et al., 2013; REDDY et al., 2020).

Biological osteosynthesis emphasizes proper anatomical reconstruction to enhance bone healing potential (AYRE et al., 2014; MERTA et al., 2017). Various techniques and materials, including autografts, allografts, and hydroxyapatite, are employed in fracture treatment (GOPINATHAN et al., 2021; KUMAR et al., 2020). Metallic implants may face failures, but PMMA can stabilize them effectively. Calcium phosphates (CaP) are commonly used as bone scaffolds due to their bioactivity and ability to bond directly with natural bone (SAHARAN and MATHEW, 2019).

Calcium phosphates (CaP) serve as bioactive bone scaffolds, forming chemical bonds with natural bone (GALOVICH et al., 2011; MAYFIELD et al., 2022). Histological assessment, crucial for evaluating bone repair, utilizes stains like Hematoxylin and Eosin (H&E), Safranin O-fast green, Masson's trichrome, and Toluidine blue (KAZEMI et al., 2017; WANCKET, 2015). Challenges include tissue variability during repair. Calcium phosphate-based ceramics are osteoconductive and suitable for bone repair. Calcium phosphate cements, alongside PMMA, offer solutions in orthopedic procedures (MORGAN et al., 2014; JACKSON et al., 2019).

Histological processing poses challenges, but standardized terms and staining protocols aid in evaluating bone healing responses. Calcium phosphate-based ceramics offer osteoconductive and bioactive properties, making them suitable for bone repair (JACKSON et al., 2019).

Our study aims to evaluate histological findings, serum calcium levels, and hemato-biological parameters in critically sized femur fractures treated with intramedullary pin alone and in combination with PMMA and CaP bone material.

Materials and methods

The study was conducted on dogs, which were captured two weeks before to start of the experiment. The dogs were examined thoroughly before being placed in the indoor patient ward under standard environmental conditions (18 to 21 °C, 30% to 70% humidity). Deworming of the dogs was done by using the tablet Caniverm 0.7g (Bioveta, Czech Republic) and vaccinated with Biocan-LR (Bioveta, Czech Republic) to remain protected against deadly diseases during research. The study was made on open fracture management of the femur in the dog model using two different bone graft materials with the combination of intramedullary pin (IM-pin), the bone graft materials are polymethylmethacrylate (PMMA), calcium phosphate (CaP) based bone graft material.

Experimental design: The study was conducted on a total of 36 healthy street dogs, aged between two to three years, comprising both male and female individuals. They were divided into three groups (A, B, and C), with 12 dogs allocated to each group. In group A, fractures of the dogs (n=12) were managed with the intramedullary pin (IM-pin), in group B, fractures of the dogs (n=12) were managed by using polymethylmethacrylate (PMMA) with the metallic combination of intramedullary pin (IM-pin) and in group C, fractures of the dogs (n=12) were managed using calcium phosphate (CP) based bone graft material with the combination of intramedullary pin (IM-pin). However, to evaluate the results of the operated dogs, after surgery, (n=3) animals were sacrificed of each group on different endpoints i.e., 14^{th}, 28^{th}, 45^{th} and 60^{th} day for analysis of the operative site by histological evaluation and hemato-biological parameters were taken after surgery on different days to compare IM-Pin, IM-Pin+PMMA, IM-Pin+CaP in critically sized femur defects in dogs.

Ethical Approval. The protocols applied in this study were approved Research Ethics Committee of the Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, Pakistan, Approval No DAS-525-2022.

Pre-operative preparation: Before anesthesia and surgery, each dog was properly examined. On the day of surgery, usual feed was withdrawn to avoid anesthetic complications. Experiments were conducted in the postgraduate research surgery room Department of Surgery and Obstetrics early in the morning. An intravenous catheter 20G×1 1/4 was placed into the cephalic vein and a T-connection cannula was also attached to it for the induction of intravenous anesthesia and fluid therapy. Clipping of hair was performed using an Oster hair clipper (Jarden Consumer Solution United States) blade no 40 at the site of the incision and the skin was aseptically prepared by using topical antiseptic povidone iodine.

Pre-medication and Anesthesia: All the dogs were off-feed 8 to 12 hours before surgery. The dogs were premedicated 10 minutes before induction with Butorphanol 0.2mg/kg body weight intramuscularly, Atropine Sulphate 0.02 mg/kg body weight with a combination of Xylazine 0.5mg/kg body weight.

Preparation of Bone Graft: Fresh and unclothed non-heparinized bone graft material was used in powder and granular form. They were mixed with bone graft substitutes just before application on the fracture site. Care was taken to complete hemostasis before the graft application as non-blood may occur at graft application.

Surgical procedure: A skin incision was made along the cranio-lateral side of the thigh, extending from greater trochanter to the patellar level. The superficial leaf of tensor fascia lata was incised along the cranial border of the biceps femoris muscle for the length of incision. The biceps femoris muscle and vastus lateralis muscle were separated by blunt dissection. The dissection was carried proximally along the mid-shaft of the femur. A 1-cm bone fracture gap was created manually with a bone cutter. Care was done to avoid nerve and blood vessel damage. At the site of the fracture the IM-pin was placed, and the gap of fractured bone was poured with polymethylmethacrylate (PMMA), calcium phosphate (CaP) based bone graft material with correct technique and with management of stabilization. Although insertion of the IM pins, care was taken to avoid longitudinal splitting of the diaphysis, and IM pin was inserted according to the size, weight and age of the dogs. The fracture stability was assessed and ensured that none of the hardware impinged on soft-tissue elements. After the process muscles and subcutaneous area were closed with vicryl 2-0 and skin was closed with silk 2-0.

Post-operative care: After closure and application of sterile dressing, the hind legs were hobbled proximal to the hock by using elastic veterinary wrap in a figure-eight pattern to prevent limb abduction and formation of pressure ulcer. After surgery, the analgesic drugs were administrated routinely to control pain buprenorphine (0.05 mg/kg twice daily) for 3 days and antibiotic intramuscular Amoxycillin (15mg/kg once daily) for 10 days. Dogs were provided with a warm soft place after surgery as they can feel stressless and painless. Sutures were checked daily to ensure wound healing, if in the case of chewing the E-collar was used for 3 to 5 days. After recovery from anesthesia maximum water was offered to dogs as they need much water post operatively, as a well-hydrated animal can recover quickly.

Tissue collection: The tissues of the operated dogs were taken under general anesthesia, and IM-Pins were removed from all the samples. The specimens from the fracture site were taken under sterilized conditions. After collecting specimens, they were washed with normal saline to clean from blood or any other debris. Then the specimens were kept in buffered neutral 10% formalin for preservation till the Laboratory analysis. Furthermore, the histological conformation for fracture wound healing after specimen collection, the animals were sacrificed (n=3) animals were sacrificed on every endpoint. However, after the sample collection, all the samples were taken to the Liaquat Medical Research and Diagnostic Lab Liaquat University of Medical and Health Sciences (LUMHS) Hyderabad branch for tissue processing.

Histology examination: For the histological evaluation, the bones were decalcified using Nitric Acid 5% for 5 days. After decalcification, each specimen of the femur was harvested in 5 mm slices from fractured sites and was submitted for paraffin embedding with SLEE MPS/P EMBEDDING CENTRE after 3 times thorough washing with 90% alcohol. The tissue was sectioned using a microtome at around 5-8 µm thickness to produce histological slides which were stained. The slides were then stained using Hematoxylin & Eosin (H&E) (SHEKHO et al., 2022). In addition, inflammatory reactions and healing responses were also assessed. The histological data were recorded accordingly.

Haemato-biochemical evaluation: Whole blood was collected from the cephalic vein and samples were added to EDTA for hematological assessment. The sample was analyzed for hemoglobin (Hb) (gm%), Packed cell volume PCV (%), Total leukocytosis count (TLC) (cell/uL) and differential leucocyte count (DLC) (%) using standard method on the sample collection day. The analysis was made before and after surgery on days 0,1, 3, 7, 14, 28, 45 and 60.

Serum calcium blood test: Blood for serum calcium test was taken from cephalic vein and collected in a yellow top tube to ensure the proper results. Samples were taken before and after surgery on days 0, 1, 3, 7, 14, 28, 45 and 60.

Statical Analysis: The collected data was analyzed using a two-way Analysis of variance (ANOVA) followed by Tukey's test with the interaction between days and groups also done concurrently, and the level of significance was determined at $p=<0.05$. Analysis and graphs were generated by using the computer software Origin (Pro), ("Version 2023"). Origin Lab Corporation, Northampton, MA, USA.

Results

Three dogs of each group were euthanized on days 14^{th}, 28^{th}, 45^{th} and 60^{th} following a bone fracture treatment to evaluate the proper histological results. The results of histology of all groups on different days were described. Day 14 histological photomicrograph showed undifferentiated cells in bone marrow. While on day 28 considerable differentiation of osteoprogenitor cells to osteoblasts and their secreted matrix indicated embedded osteocytes in trabiculae of spongy bones. Moreover, on day 45 number of osteocytes increased and dispersed trabiculae were observed besides large number of stem cells in bone marrow. In last 60 days photomicrograph revealed remarkable number of bone cells and an ossification center which leads to formation of compact bone.

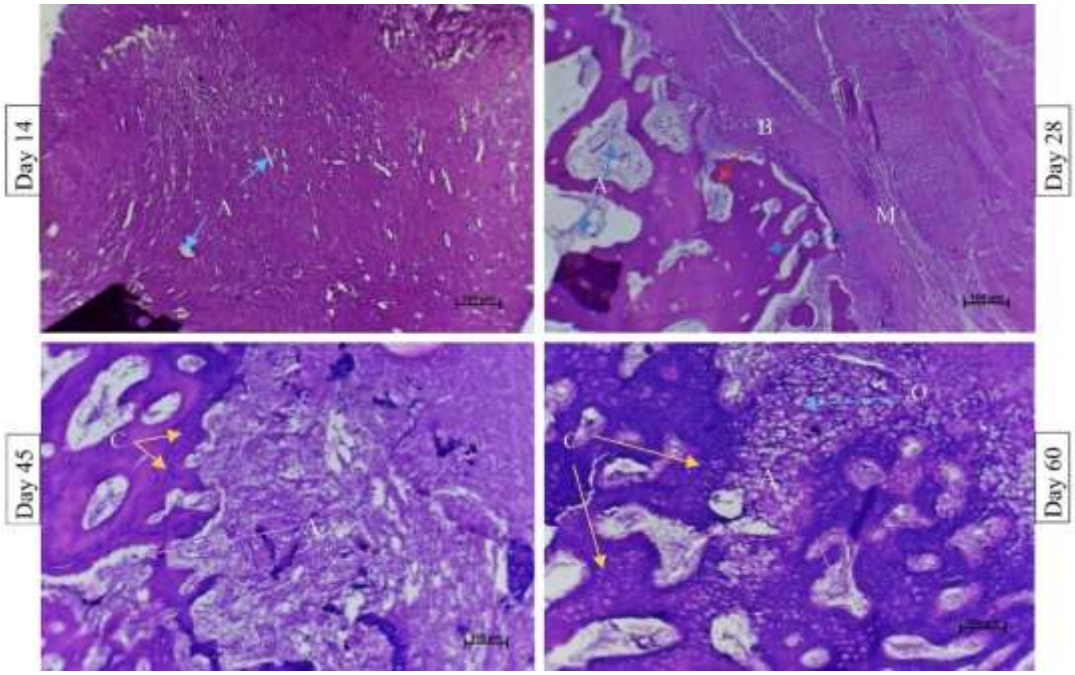

Fig. 1. Photomicrographs of bones of group A : (A= Bone marrow, B= Osteoblasts, C= osteocytes, O= ossification center, M= Bone matrix) at 10x

Magnification photomicrograph made at 14th, 28th, 45th, and 60th days after surgery demonstrating bone healing by using PMMA and IM pin. On day 14th photomicrograph indicated cell differentiation from bone marrow stem cells and trabeculae formation at initial stage. After four weeks (day 28th) a remarkable number of osteoblasts forming matrix and embedded mature osteocytes in trabiculae were noted along with lamellae at 10x. On day 45th bone ossification started deposition of minerals and matrix increased and differentiation of dense connective tissue from cancellous bones was also observed. Besides all other developmental stages of bone growth dense connective tissue formation was noted remarkably on day 60th of surgery at 10x magnification.

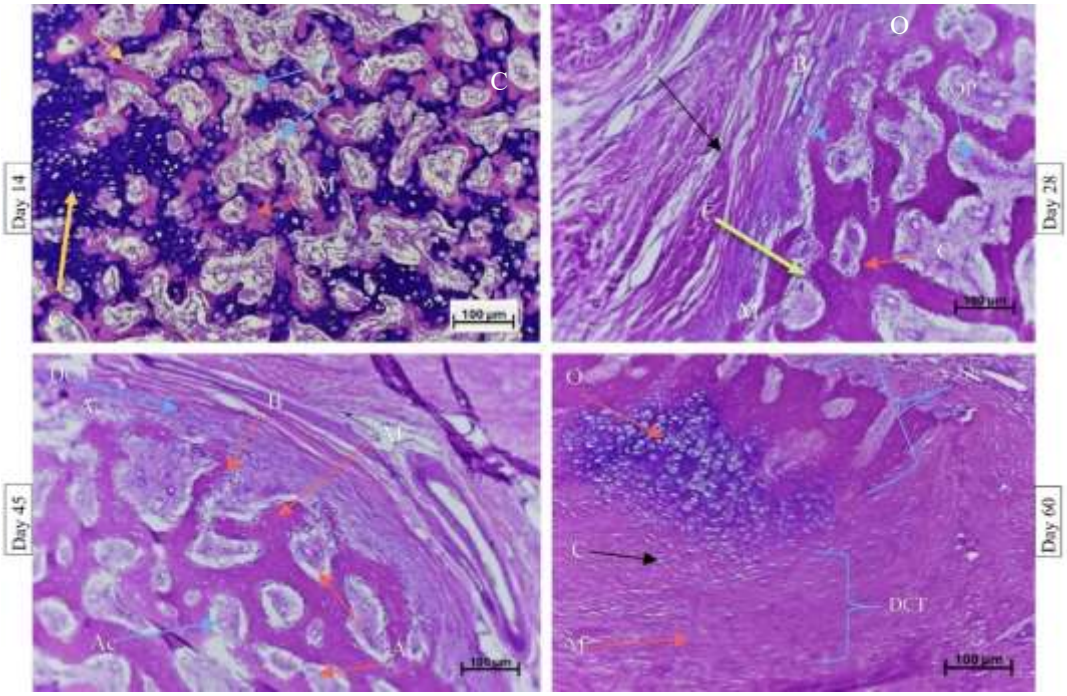

Fig. 2. Photomicrographs of bones of group B: (A= Bone marrow, B= Osteoblasts, C= osteocytes, O= ossification center, M= Bone matrix, OP= osteoprogenitor cells, SB= Sponge bone, CB= Compact bone, AC= Adipocytes, DCT= Dense connective tissue)

Histological examination of bone healing in CaP and IM pin was made at 10x on days 14th, 28th, 45th and 60th after surgery. Magnified results at 10x revealed that on day 14th small spongy bone formation started in the form of osteoblasts and trabicular formation from osteocytes. On day 28th remarkable dispersion of spongy bones and deposits of CaP were recorded. Deposition of bone matrix and compact bone formation had just begun. But after 60th days histology demonstrated the bone ossification and dispersed trabeculae highly embedded with osteocytes and osteoblasts differentiation from stem cells was also prominent.

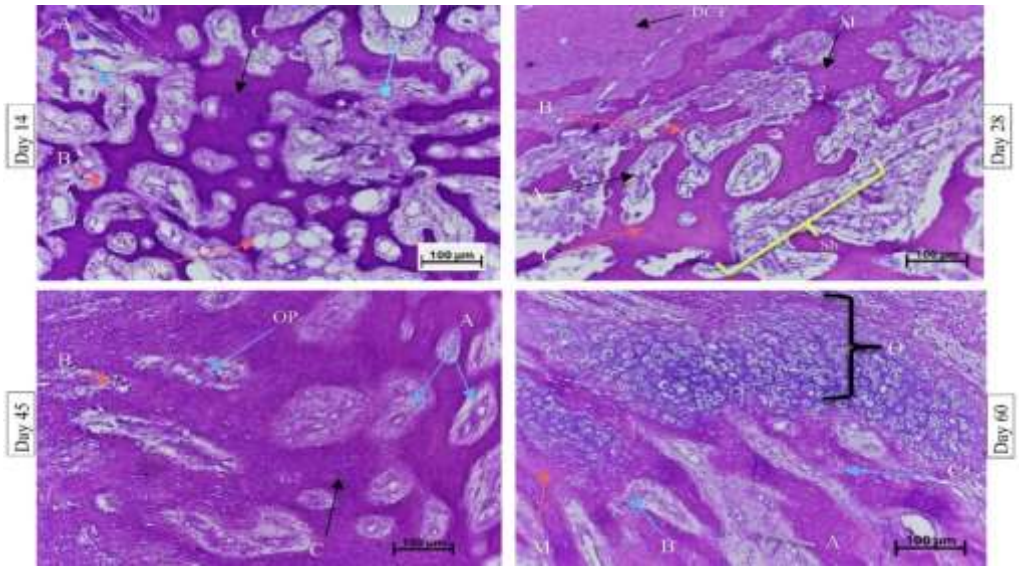

Fig. 3. Photomicrographs of bones of group C: (A= Bone marrow, B= Osteoblasts, C= osteocytes, O= ossification center, M= Bone matrix, OP= osteoprogenitor cells, SB= Sponge bone, CB= Compact bone, AC= Adipocytes, DCT= Dense connective tissue)

Comprehensive Histological Evaluation of Fracture Healing in Dogs

Bone healing response to each protocol demonstrated significant differences at different intervals. Histological photomicrographs after 14th day in group A showed no callus formation at all. An insignificant difference between animals of groups B and C was noted as an early rapid healing response was recorded in both groups on day 14th. The only difference of both later groups was that animals of group B showed diversified areas of spongy bones as compared to group C on the same day 14 (i.e., bone healing response to PMMA+ IM pin was greater than CaP + IM pin on day 14). On the second sampling on day 28th, histological evaluations indicated significant growth in cancellous bone formation and bone matrix in all groups. The only difference to all groups was that trabeculae formation and the number of osteocytes in group A was lesser than the other two (i.e., B and C group). In group C connective tissue was also observed which was not recorded in groups A and B. However, the overall healing in Group B was better than A and C groups in terms of cellular growth and matrix formation.

After 45 days histological evaluations showed significant differences in all groups. In groups A and C dense connective tissue formation was not indicated while in group B significant growth along with the formation of dense connective tissue indicated a rapid healing process. However, the remarkable number of osteocytes embedded in the bone matrix in Group C was recorded as higher than in Groups A and B. The last sampling for histological examination was made on day 60. In histological evaluation, ossification centers were observed in all groups with the highest number of osteoblasts in Groups A, B and C, respectively. Ossification centers surrounded by dense connective tissue were significantly higher in group B with dense connective tissue and osteocytes.

Blood Hematology

Serum Calcium: The Mean values of serum calcium levels were recorded as normal within all groups. Similarly results of all groups at level $p<0.05$, mean values of days and groups were not significantly **different (Table 1).**

Table 1. Serum Calcium level for different treatment groups following use of IM pin in combination with PMMA and CaP

	Serum Calcium Level							
Groups	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	9.4±0.7^{a}	9.6±1.1 ^{a}	9.7±0.75 ^{a}	10±1.2 ^{a}	9.9±1.1 ^{a}	9.2±0.97 ^{a}	9.6±0.99 ^{a}	9.1±0.75 ^{a}

B	9.4±0.7 [a]	9.8±0.81 [a]	9.9±1.6 [a]	9.9±1.5 [a]	9.9±1.3 [a]	9.8±1.1 [a]	9.7±0.87 [a]	9.6±0.94 [a]
C	9.9±1.2 [a]	9.9±1.3 [a]	9.8±0.97 [a]	9.8±1.3 [a]	9.6±0.87 [a]	9.8±0.98 [a]	9.6±0.88 [a]	9.8±0.85 [a]

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Red Blood cells (RBCs): The RBCs count was observed in every experimental animal till the last day of the experiment. The RBCs count revealed that on day 0 the values were observed normal in all groups. However, from day 1, the mean values of RBCs count were recorded low in range till day 30th in all groups. After day 30th increased RBCs were recorded in mean values of groups A and B, but little decreased variations in mean values of group C. However, on day 60th all mean values of RBCs were mostly recorded within normal range, which is 5.5 to 8.5 million/uL (Table 2).

Table 2. RBC level for different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**RBC Days**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	6.9 ±0.72^{a}	5.2 ±0.94^{bc}	5 ±0.83^{bc}	5 ±0.78^{bc}	4.92 ±0.75^{bc}	4.9 ±0.67^{bc}	5.2 ±0.66^{bc}	5.7 ±0.78^{b}
B	6.96 ±0.8^{a}	5.4 ±0.84^{bc}	4.9 ±0.68^{bc}	4.7 ±0.67^{c}	4.6 ±0.53^{c}	4.8 ±0.50^{bc}	5 ±0.34^{bc}	5.3 ±0.28^{bc}
C	7.1 ±0.68^{a}	5.3 ±0.81^{bc}	4.9 ±0.63^{bc}	4.7 ±0.58^{bc}	4.8 ±0.51^{bc}	5.12 ±0.39^{bc}	5.2 ±0.35^{bc}	5.4 ±0.36^{bc}

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Hemoglobin: The hemoglobin analysis revealed that on day 0 the mean values were normally observed in all groups. However, on day 1 post-surgery the mean values of hemoglobin were recorded low in two groups A and B but with little increase in C groups compared to the control day. There was not a significant difference in groups, but on the contrary, days showed some difference. To conclude all the mean values of Hemoglobin were mostly recorded within the normal range (Table 3).

Table 3. Hemoglobin level for different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**Hemoglobin**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	12±2.2 [ab]	11±2.5 [abc]	12±0.72 [ab]	12±0.87 [ab]	12±0.59 [ab]	11±0.87 [abc]	11±1.1 [abc]	11±1.1^{c} [abc]
B	12±2.1 [ab]	12.00±1.84^{ab}	12±0.67^{ab}	13±1.2^{a}	12±0.66^{ab}	11±1.2 [abc]	10±1.2^{bcd}	9.9±0.79^{cd}
C	11±1.9 [abc]	11±2 [abc]	12±0.72 [ab]	12±1.1 [ab]	12±0.59 [ab]	11±0.93^{abc}	11±1.1 [abc]	10±1 [bcd]

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day Means with different superscripts differ at $p < 0.05$

Packed Cell Volume (PCV): The Mean values of PCV were recorded as normal in all groups which is 37 to 55%. Only variations were recorded in the days, but all the variations were recorded within the normal range. However, days and groups were Significantly not different (Table 4).

Table 4. PCV level for different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**PCV**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	49±5^{a}	47±6.4 [a]	47±5 [a]	46±4.8 [a]	46±4.3 [a]	47±7.2 [a]	47±7.2 [a]	48±6.2 [a]
B	50±4.1 [a]	46±6.1 [a]	48±6.5 [a]	47±6.5 [a]	48±6.7 [a]	48±6.6 [a]	48±6.3 [a]	49±3.4 [a]
C	49±2.6 [a]	45±5.3 [a]	45±5.3 [a]	47±6.4 [a]	47±6.6 [a]	48±6.2 [a]	48±5.2 [a]	48±2.4 [a]

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day

Means with different superscripts differ at $p < 0.05$

White Blood Cells (WBC) count: The analysis of WBC count revealed that on day 0, the mean values were normal in all groups. However, from day 3 the mean values of WBC count were recorded high in groups till day 7th in all groups. As antibiotics were continuously used through the first week of post-surgery WBCs recorded in normal ranges on most days. The mean values of WBC count within groups were within the normal range (Table 5).

Table 5. WBC level of different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**WBC**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	9.3±1.4 de	10±3.1cd	13±0.8 a	13±0.94a	13±0.9 a	12±0.79 ab	12±0.78 ab	11±1 bc
B	8.9±1.2de	9.2±1.3de	10±1.4 cd	11±1.3 bc	11±1.3bc	9.8±0.94 cde	9.5±0.67 cde	8.4±0.93e
C	9.2±1.5de	9.5±1.2 cde	9.8±0.94 cde	11±1.1 bc	10±1.7 cd	10±1.8 cd	9.6±1.2cde	9.2±1.3de

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Neutrophils: The mean values of neutrophils were recorded as normal in all groups on all days, slowly decreased values were recorded in post-surgical days, but all results were found in normal ranges. There was no significant difference in groups (Table 6)

Table 6. Neutrophil levels of different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**Neutrophils**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	70±4.8a	70±4.6 a	69±4.7 a	66±4.4 bc	64±4.3 cde	63±4 def	63±4 def	61±3.5 f
B	70±4.8 a	70±4.8 a	69±4.7 a	66±4.4 bc	64±4.3 cde	62±3.5 ef	63±4.1 def	61±2.9 f
C	70±4.6 a	68±5.0 ab	68±5.0 ab	66±4.2 bc	66±3.5 bc	65±3.3 cd	64±2.7 cde	63±2.6 def

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Lymphocytes: The mean values of lymphocytes were recorded normal in all groups during the study period. There was no significant difference in groups and days (Table 7).

Table 7. Lymphocytes level for different treatment groups following use of IM pin in combination with PMMA and CaP

Groups		**Lymphocytes**						
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60
A	21±4.4ef	22±3.8 ef	22±3.3 ef	23±2.9 def	27±2.9 abcd	27±3.4 abcd	29±2.3 ab	30±1.5a
B	24±4.9 cdef	24±4.7 cdef	25±4.1 bcde	25±4.1 bcde	27±2.9 abcd	27±3.9 abcd	27±3.9 abcd	28±3.8 abc
C	20±5.1f	21±4.8ef	21±4.3ef	22±3.7 ef	24±5.1 cdef	23±4.8 def	23±4.8 def	23±4.8 def

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Monocytes. The mean values of monocytes in all groups recorded significantly different but that difference was observed within the normal ranges which is 3-10% (Table 8).

Table 8. Monocytes level for different treatment groups following use of IM pin in combination with PMMA and CaP

Groups	**Monocytes**							
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 30	Day 45	Day 60

A	5.8±1.9 [a]	3.03±0.54 [b]	3.13±0.42 1	3.26±0.432 [b]	2.73±0.34 9 [b]	2.83±0.31 4 [b]	2.98±0.043 [b]	3.03±0.191 [b]
B	5.6±1.6 [a]	2.97±0.328 [b]	3.03±0.39 4 [b]	3.17±0.389 [b]	3.05±0.32 3 [b]	3.13±0.43 3 [b]	3±0 [b]	2.93±0.176 [b]
C	5.9±1.7 [a]	3.21±0.396 [b]	3.38±0.48 3[b]	3.27±0.431 [b]	3.48±0.5 [b]	3.37±0.50 [b]	3.28±0.369 [b]	2.92±0.156 [b]

Group-A: IM-Pin Group-B: IM-Pin+PMMA Group-C: IM-Pin+CAP Day=0 control Day
Means with different superscripts differ at $p < 0.05$

Discussion

In the present research, all groups were compared on different days, revealing significant differences in the bone healing response to each protocol. Histological photomicrographs after 14 days in group A (treated with IM-Pin only) showed no healing cells found in all animals, aligning with SAKAMOTO et al. (2019) suggestion that four weeks may be needed for bone turnover in normal healing. Conversely, an early rapid healing response was noted in both groups B and C on day 14, supported by SHEKHO et al. (2022) report of cartilage tissue formation initiating the bridge between fractured bone parts. Photomicrographs of group C using Calcium Phosphate (CaP) bone cement indicated minimal cement absorption, consistent with FRAKENBURG et al. (1998), along with limited cellular activity.

The primary distinction between groups A and B lies in the diversified spongy bone areas observed in group B (PMMA) on day 14, indicating superior healing response to PMMA+ IM pin compared to CaP + IM pin. This finding is corroborated by MA et al. (2018), who reported cellular ossification and endochondral ossification after two weeks with PMMA during bone healing. By day 28, histological evaluations showed significant growth in cancellous bone formation and bone matrix in all groups. Notably, group B exhibited a remarkable number of osteoblasts forming matrix and embedded mature osteocytes in trabeculae, indicating robust cellular growth and matrix formation. Similarly, microphotographs during the sixth week showcased fibrous tissues, vascular sections, and endochondral ossification, demonstrating good bone healing reactions with PMMA, consistent with previous research.

After 45 days, histological evaluations revealed significant differences in all groups, with group B exhibiting rapid healing characterized by dense connective tissue formation. Group C showed a higher number of osteocytes embedded in the bone matrix compared to groups A and B. DUNKLEY et al. (2018) also indicated healing with CaP, even after defects. On day 60, ossification centers were observed in all groups, with group B showing significantly higher levels of osteoblasts surrounded by dense connective tissue and osteocytes. These findings are in line with OOMS et al. (2003), who reported remodeling of lacunae with mature bone cells after PMMA implementation. Additionally, histological findings of PMMA showed promising healing indications compared to other groups, supporting PAHLEVANZADEH et al. (2019) assertion of PMMA's potential in clinical applications. This study demonstrated positive responses across all treatment groups, showcasing diverse clinical outcomes regarding fracture healing with different bone materials. Throughout the hematological analysis, most observations fell within normal ranges. However, to ensure proper comparison between groups, uniform treatment protocols were administered to all animals. While statistically significant differences ($P < 0.05$) were noted in various hematological parameters across different days among the groups, these variances remained within normal physiological ranges. AYYAPPAN et al. (2011) similarly reported normal hematological parameters in dogs undergoing orthopedic surgery.

Regarding serum calcium analysis, results did not significantly differ between groups, with variations within normal ranges. Interestingly, findings from days 0 to 30 diverged from those of SINGH et al. (2017), likely due to differences in study populations. SINGH et al. focused on road accident cases in dogs, whereas our study controlled for serum levels after orthopedic surgery. However, our findings aligned with SINGH et al. (2017) on days 45 and 60.

Hemoglobin levels remained within normal ranges across all groups, with no significant differences observed before surgery and on various postoperative days. Similar findings were reported by

YADAV et al. (2021). Throughout the study, packed cell volume (PCV) percentages remained consistent with normal ranges, suggesting minimal impact from orthopedic surgery, in line with YADAV et al. (2021). Efforts to control infection through antibiotic administration yielded consistently normal results across all groups. While SINGH et al. (2017) observed differing results, likely due to their inclusion of traumatic stray dogs, SINGH et al. (1998) highlighted that increased white blood cell counts may indicate a heightened trauma response.

Increased lymphocyte counts across all groups indicated favorable fracture healing, contrasting with TOBEN et al. (2011), who suggested decreased lymphocytes might signify poor healing. Significant differences in red blood cell (RBC) levels were observed among groups in our study, with temporary decreases post-surgery followed by increases from days 45 to 60. These findings are in line with ALIMI et al. (2022).

Monocyte levels remained within normal ranges across all groups, consistent with findings by ALIMI et al. (2022) and WASHINGTON et al. (2012), suggesting stability even in the presence of chronic inflammation.

Conclusion

In conclusion, our investigation into critical-size bone defect reconstruction using polymethylmethacrylate (PMMA) and calcium phosphate (CaP) bone materials has yielded valuable insights into the histological, and hematological parameters associated with these treatments. The histological assessments demonstrated distinct healing responses, trabeculae formation, and osteon numbers among the groups, with Group B showing significant growth and dense connective tissue formation, indicative of a rapid healing process. IM-Pin PMMA emerged as the optimal implant material for femur bone fractures in dogs, exhibiting favorable histological characteristics such as biocompatibility, corrosion resistance, and minimal bone resorption. The maintenance of normal serum calcium levels across all groups underscores the biocompatibility of the materials used. Hematological parameters, including red blood cell (RBC) count, hemoglobin, packed cell volume (PCV), white blood cell (WBC) count, neutrophils, lymphocytes, and monocytes, were systematically analyzed. While variations in RBC count and WBC count were observed post-surgery, these parameters eventually normalized, emphasizing the overall safety and adaptability of the interventions.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

1. AYRE, W.N., S.P. DENYER, S.L. EVANS (2014). Ageing and moisture uptake in polymethyl methacrylate (PMMA) bone cements. J. Mech. Behav. Biomed. Mater. 32, 76-88. https://doi.org/10.1016/j.jmbbm.2013.12.010
2. AYYAPPAN, S., M.S. SIMON, B. DAS, A.A. PRASAD, R. KUMAR (2011). Management of diaphyseal humeral fracture using plate rod technique in a dog. Tamilandu J. Vet. Ani. Sci. 7, 35-38.
3. DUNKLEY, I.R., S.M. VICKERS, J. BADURA, J. TOTH (2018). A Histological Assessment of the Mechanism of Early-Stage Healing of a Biphasic Calcium Phosphate in an In Vivo Rabbit Model. Paper presented at the Key Engineering Materials. https://doi.org/10.4028/www.scientific.net/KEM.782.275
4. FRAKENBURG, E.P., S.A GOLDSTEIN, T.W. BAUER, S.A. HARRIS, R. POSER (1998). Biomechanical and histological evaluation of a calcium phosphate cement. J. Bone Jt. Surg. 80, 1112-1124.
5. GADDAM, V., V. PODARALA, S.K. RAYADURAM VENKATA, S.L. MUKKU, R. DEVALAM, B. KUNDU (2022). Multi-ion-doped nano-hydroxyapatite-coated titanium

intramedullary pins for long bone fracture repair in dogs—Clinical evaluation. J. Biomed. Mater. Res. Part B: Appl. Biomater. 110, 806-816. https://doi: 10.1002/jbm.b.34960

6. GALOVICH, L.A., A. PEREZ-HIGUERAS, J.R. ALTONAGA, J.M. GONZALO ORDEN, M.L. MARIÑOSO BARBA, M.T. CARRASCAL MORILLO (2011). Biomechanical, histological and histomorphometric analyses of calcium phosphate cement compared to PMMA for vertebral augmentation in a validated animal model. Eur Spine J.l. 20, 376-382. https://doi.org/10.1007/s00586-011-1905-4
7. GOPINATHAN, A., K. SINGH, A. KHAN, S.D. SIDDARAJU (2021). Open and closed intramedullary pinning of tibial fractures in dogs.
8. JACKSON, N., M. ASSAD, D. VOLLMER, J. STANLEY, M. CHAGNON (2019). Histopathological evaluation of orthopedic medical devices: the state-of-the-art in animal models, imaging, and histomorphometry techniques. Toxicol. Pathol. 47, 280-296. https://doi.org/10.1177/01926233188210
9. KANCHANA, P., C. SEKAR (2012). Effect of magnesium on the mechanical and bioactive properties of biphasic calcium phosphate. J. Mine. Mater. Charact. Eng. 11, 982.
10. KAZEMI, D., K.S. ASENJAN, N. DEHDILANI, H. PARSA (2017). Canine articular cartilage regeneration using mesenchymal stem cells seeded on platelet rich fibrin: Macroscopic and histological assessments. Bone Joint Res. 6, 98-107. https://doi.org/10.1302/2046-3758.62.BJR-2016-0188.R1
11. KUMAR, A., B. QURESHI, V. SANGWAN (2020). Biological osteosynthesis in veterinary practice: A review. Int. J. Livest. Res. 10, 24-31. doi: http://dx.doi.org/10.5455/ijlr.20200718062546
12. KUMAR, K.M., V.D. PRASAD, N.D. LAKSHMI, N. RAJU (2018). Evaluation of biochemical parameters for assessment of fracture healing in dogs. Phar. Innova. J. 7, 577-580.
13. MA, Y.F, N. JIANG, X. ZHANG, C.H. QIN, L. WANG, Y.J. HU, B.W. WANG (2018). Calcium sulfate induced versus PMMA-induced membrane in a critical-sized femoral defect in a rat model. Sci. Rep. 8, 1-12. https://doi.org/10.1038/s41598-017-17430-x
14. MAYFIELD, C.K., M. AYAD, E. LECHTHOLZ-ZEY, Y. CHEN, J.R. LIEBERMAN (2022). 3D-Printing for Critical Sized Bone Defects: Current Concepts and Future Directions. Bioeng. 9,680.. https://doi.org/10.3390/ bioengineering9110680
15. MERTA, I., L. BERGER, G. HEIDFOGEL, K.D. KUHN, G. LEWIS, E.K. TSCHEGG (2017). Size and boundary effects on notch tensile strength and fracture properties of PMMA bone cement. Polymer Testing, 59, 441-448. https://doi.org/10.1016/j.polymertesting.2017.02.018
16. MORGAN, E.F., A. DE GIACOMO, L.C. GERSTENFELD (2014). Overview of skeletal repair (fracture healing and its assessment). Skeletal Development and Repair: Methods and Protocols, 13-31.
17. OOMS, E., J. WOLKE, M. VAN DE HEUVEL, B. JESCHKE, J. JANSEN (2003). Histological evaluation of the bone response to calcium phosphate cement implanted in cortical bone. Biomaterials, 24: 989-1000. https://doi.org/10.1016/S0142-9612(02)00438-6
18. PAHLEVANZADEH, F., M. EBRAHIMIAN-HOSSEINABADI (2019). Poly (methyl methacrylate)/biphasic calcium phosphate/nano graphene bone cement for orthopedic application. J. Med. Signals Sens, 9, 33. https://doi.org/10.4103/jmss.JMSS_34_18
19. PURNOMO, A., S. BUDHI, D. ADJI, D. ANGGRAENI, D. ANGGORO, S. WIDYARINI, M.T.E. PURNAMA (2022). Radiographic and Histological Evaluation in Canine Femur after Implantation of 304 Stainless-steel-based Plate. Pharmacogn. J. 14. https://doi: 10.5530/pj.2022.14.112
20. REDDY, G., V.G. KUMAR, K. RAGHAVENDER, D.P. KUMAR (2020). Evaluation of haemato-biochemical parameters for assessment of fracture healing in dogs. Pharma. Innovation. J. 9, 123-125. https://doi.org/10.22271/tpi.2020.v9.i9Sc.5187
21. SAHARAN, S., R.V. MATHEW (2019). Current and future perspectives of polymethylmethacrylate bone cement in veterinary orthopaedics: A review.

22. SAKAMOTO, A., P. QI, S. OHBA, S. OHTA, Y. HARA, T. OGAWA, M. MOCHIZUKI (2019). Bone regeneration by calcium phosphate-loaded carboxymethyl cellulose nonwoven sheets in canine femoral condyle defects. J. Biomed. Mater. Res. Part B: Appl. Biomater. 107, 1516-1521. https://doi.org/10.1002/jbm.b.34243
23. SAUNDERS, W., L. DEJARDIN, E. SOLTYS-NIEMANN, C. KAULFUS, B. EICHELBERGER, L. DOBSON, C. GREGORY (2022). Angle-stable interlocking nailing in a canine critical-sized femoral defect model for bone regeneration studies: In pursuit of the principle of the 3R's. Front. Bioeng. Biotechnol. 10, 1432. https://doi.org/10.3389/fbioe.2022.921486
24. SHEKHO, H.A., S.A. WADI, E.R. SARHAT (2022). Histopathological evaluation of Tilduronate on healing of femoral bone in dogs. Tikrit J. Vet. Sci. 1.
25. VAISHYA, R., M. CHAUHAN, A. VAISH (2013). Bone cement. J. Clin. Orthop. Trauma. 4, 157-163. https://doi.org/10.1016/j.jcot.2013.11.005
26. WANCKET, L. (2015). Animal models for evaluation of bone implants and devices: comparative bone structure and common model uses. Vet. Pathnol. 52, 842-850. https://doi.org/10.1177/0300985815593124