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A Comprehensive Study on Bioengineering Strategies of Osseous Reconstruction by Using Autogenous Bone Graft with Osteogenic Biologics for Radial Diaphyseal Defect Repair

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Key words
Fracture healing, Autogenous bone graft, Bone marrow aspirate, Platelet rich plasma, Fish scale

ABSTRACT

Complex fracture reconstruction, malunion, delayed and nonunion are challenging problems often require special attention. Autogenous bone graft for osseous healing has been considered gold standard for decades. It contains viable osteoprogenitor cells, cytokines and growth factors with appropriate tissue matrix resulting in osteogenesis, osteoinduction and osteoconduction. Orthobiologics are biological materials used to augment bone and soft tissue cure, with multifactorial effects. In our study, three orthobiologics i.e., Bone Marrow Aspirate (BMA), Platelet Rich Plasma (PRP) and Decellularized Fish Scale (DFS) were used in conjunction to autogenous bone graft. Objective was to transplant as many cells as possible with osteogenic potential along with the graft. These three regimens were evaluated in terms of clinical vital parameters, hematology, serum analysis, bone biomarkers, mechanical assessment, radiographic evaluation and histomorphometry. We concluded that Bone-BMA combination showed best results in fracture healing and bone remodeling while Bone-PRP combination was below par than Bone-BMA but was superior to Bone-DFS combination. More clinical research may be required before widespread use of these regimens. Furthermore, it is paramount that veterinary orthopedic surgeons must have contemporary knowledge regarding autogenous grafts and orthobiologics, so they can utilize them correctly.

INTRODUCTION

Each year millions of bone graft surgeries are performed worldwide thus makes it second most transplanted tissue after blood (Campana *et al*., 2014). Bone is a dynamic tissue with marked ability to regenerate after fractures, making this unique restorative capacity comparable with liver and allowing complete bone healing to pre-injured forms (Armiento *et al*., 2020).

Critical sized fractures, malunions, delayed and

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nonunions multiple times need grafting, of which autogenous bone graft is gold standard (Cypher and Grossman, 1996). It shows lowest immunological rejection risk and has strong osteogenic, osteo inductive and osteoconductive properties. Bone autografts may be cortical, cancellous or corticocancellous. As they are harvested from same patient, so may entail some amount of risks like site morbidity, hematomas and prolonged wound healing times (Nandi *et al*., 2010).

Impaired bone healing complications may be overcome by bone autografts and various orthobiologic materials including cell-based therapies, cells and growth factors, biomaterials etc. for bone regeneration, tissue-engineering and to demonstrate potential of regenerative therapies (McDaniel *et al*., 2016).

Among autologous cell therapies, bone marrow aspirate (BMA) and platelet rich plasma (PRP) are quite significant. BMA contains bone marrow mesenchymal stem cells (MSCs) that are quite helpful in treatment of musculoskeletal issues. MSCs have ability to differentiate into osteoprogenitor cells, which in turn convert to osteoblasts with appropriate cytokines and growth factors. BMA by paracrine action, helps in angiogenesis induction and fractured site blood restoration (Schottel and Warner, 2017).

PRP is an autologous platelets suspension extracted by centrifugation. PRP has osteoconductive properties as they are rich in important growth factors in alpha granules i.e., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factors (TGF-β_1) etc. It has widespread use in musculoskeletal injuries especially in sports. Despite PRP extensive use, its effects on fracture healing remains unclear (Bray *et al*., 2017).

Natural biomaterials reveal better biocompatible properties in fracture fixations. Hydroxyapatite (HA), collagen, calcium and phosphorous are major components of bone tissue and thus are materials of choice as biomedical applications. Fish scale (FS) has abundance of natural hydroxyapatite and collagen type-I like bone extracellular matrix. It has unique biocompatibility with bone tissue along with low immunogenicity, good stretch strength and ability to stimulate cell differentiation and growth i.e., MSCs and osteoblasts (Granito *et al*., 2018; Qin *et al*., 2022). These factors may render fish scale as potential orthobiologic material to be used with autogenous grafts (Wickramasinghe *et al*., 2022).

Though bone autografts remain a powerful bone healing tool, but its use in conjunction with various orthobiologics (BMA, PRP and DFS) for fracture healing remains unclear. The intent of current study is to explore usage of autogenous bone grafts with various osteogenic biologics for osseus defect treatment in vivo studies to evaluate their respective efficacy. Given a little knowledge available for clinical trials in this area, the evidence will allow-in identification of most promising combination for fracture repair approaches and will be helpful for further clinical studies on various strategies and specific products.

MATERIALS AND METHODS

Animal selection

A total of 24 adult rabbits of either sex and breed, with age range of 1-3 years and average body weight of 2-4 kg were selected for study. Ten days prior to trials, all rabbits were acclimatized at experimental station, Department of Veterinary Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan for thorough clinical examination and to accustom them with the environs.

Experimental design

All animals were divided into four groups i.e. A, B, C and D with each group comprising 6 rabbits. Autogenous bone graft was obtained from individual's iliac crest for each animal. In group A, bone graft alone was used at fracture site. For group B, graft along with BMA was harvested at site. In group C, bone graft with autologous PRP was administered and for group D, bone graft and decellularized fish scale (DFS) was implanted at fractured site.

Parameters of techniques evaluation

The study was conducted for 4 months and all parameters were evaluated at day 0, 1, 7, 15, 30, 45, 60, 75, 90, 105 and 120 post-surgeries with a slight variation for serology.

Pre-operative clinical examination

All rabbits were undergone thorough physical probe, complete blood count, liver and renal function tests prior to trials to exclude any pre-operative issues. Rabbits were also examined for ectoparasites and bathed with fipronil (Frontline spray®, Merial). For endoparasitic infestations, they were given Mebendazole (Vermox®, Johnson^n Johnson Pharma) @ 20mg/kg orally. Three days prior to surgery, antibiotic enrofloxacin (Enroxsel®, Selmore Pharma) was given@ 10mg/ kg intramuscularly BID (Hedley, 2018) as prophylaxis to minimize chances of infection. During entire study period, rabbits were boarded in research facilities at Department of Veterinary Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan with all-time access to feed and water.

Patient preparation and surgical asepsis

Electric trimmer was used to clip proposed surgical

site prior to procedures, later the area was shaved with disposable razor to avert hair contamination. Rabbits were not fasted as they don't vomit (Mapara *et al*., 2012). Povidone-iodine surgical scrub (Pyodine® 7.5%, Brookes Pharma) was used to disinfect surgical site. Onwards, the site was scrubbed with methylated spirit (Micko Chemicals) and covered with disposable, sterile surgical drapes.

Premedication and anesthesia

All rabbits were sedated with combination of Xylazine hydrochloride (Xylaz®, Farvet) @ 3mg/kg and ketamine (Ketasol®, Indus Pharma) @ 20mg/kg, IM. Induction was done by propofol bolus (Pofol®, Dongkook Pharma) @ 5mg/kg IV. Maintenance of anesthesia was achieved by Isoflurane (Forane®, Abbott) @ 3% (Utexas.edu). Intravenous fluids, Dextrose 5% Solution (Otsuka Co.) and injection Tranexamic acid (Transamine®, Daiichi Sankyo Co.) @ 10-20mg/kg as intravenous homeostatic agent was dispensed during surgery.

Surgical procedure for fractures

Standard aseptic procedures regarding instruments, surgeon and patient preparation were followed as described by (Fossum, 2019). For radial bone exposure and fracture induction, after aforementioned anesthesia, 2-3 cm incision was given on cranial aspect of mid-shaft radius. Skin, sub-cut tissue and musculature was retracted and bone was exposed. An osteoperiosteal defect was induced in radius mid-shaft, which was filled with autogenous bone graft and osteogenic biologics in assigned research groups. Later incision site was closed as per standard procedure (Slatter, 2003).

Bone graft and osteogenic biologics collection

Autogenous bone graft was collected from iliac crest. For this purpose, incision was given parallel to iliac crest, incising skin, sub-cut tissue and then iliacus was elevated for deep exposure of iliac crest. Autogenous bone graft was harvested by trephine curettage technique. An incision was given over iliac tuberosity and a hole was made in bone cortex. Then a medium sized curette was used at 45º arc in each direction to collect cancellous bone graft. Area excavated for graft collection was filled with gelatin sponge to avoid post-operative complications (Ebraheim *et al*., 2001).

BMA was collected from iliac crest with help of 18 gauge bone marrow biopsy needle. A little force was used to insert needle through skin and muscle till it contacted bone, then needle was rotated slowly to penetrate bone cortex. Biopsy needle stylet was removed and 5 ml of BMA was collected in hypodermic syringe containing anticoagulant. Later this BMA was used at fractured site (Maiti *et al*., 2013).

For PRP preparation, whole blood was collected in tubes with anticoagulant. A double centrifugation method was used to get platelet rich plasma (PRP). After first spin @ 300g for 5 min, whole blood was separated into three layers i.e., upper layer (of WBCs and platelets), middle buffy coat (with WBCs) and a bottom layer (of RBCs). To produce pure PRP, upper layer and superficial part of buffy coat, were transferred to a vacant sterile tube. Then 2nd spin was executed @ 240g for 8 min to form soft pellets at tube bottom (mainly platelets). Upper layer of platelet poor plasma was discarded. In lower 1/3 portion, pellets were homogenized to prepare PRP (Amable *et al*., 2013; Dhurat and Sukesh, 2014).

Fresh grass carp fish (*Ctenopharyngodon idella*) were purchased from commercial market Lahore, Pakistan. It was instantly transported to laboratory and stored at 4Cº. Fish scales were harvested and thoroughly cleaned by distilled water. Cellular components of fish scale were removed by decellularization solution [0.05M Tris-buffer and 0.1MTriton X-100 (Sigma-Aldrich®)] at 4Cº for 3 days. Afterwards, these decellularized fish scales were rinsed with 70% ethyl alcohol and stored in sterilized phosphate-buffer saline (PBS) at 4Cº for future use (Chou *et al*., 2014).

Post-operative care and management

After surgery rabbits were shifted to recovery room to observe post-operative complications. Antibiotic enrofloxacin (Enroxsel®, Selmore Pharma) was given@ 15mg/ kg IM for 5 days to prevent infections. For pain relief, Flunixin meglumine (Loxin®, Selmore Pharma) was given IM @ 2.2mg/kg (Elmas *et al*., 2006). Rabbits were provided commercial feed and water ad-lib.

Parameters evaluated

Following parameters were evaluated at day 0, 1, 7, 15, 30, 45, 60, 75, 90, 105 and 120 with a minor variation for serum calcium and phosphates:

Clinical vital parameters

Clinical vital parameters i.e., temperature, pulse, respiration was monitored in each rabbit at stipulated days. Temperature was recorded from rectum, pulse from femoral artery and respiration from lungs (Di Girolamo *et al*., 2016; Reddy and Sivajothi, 2017).

Hematological parameters

1 ml jugular vein blood was added in blood vacutainer having anticoagulant (K2 EDTA). Complete blood count (CBC) was carried out by using hematological

analyzer (Abbott Cell-Dyn 4000) to rule out possible blood abnormalities e.g. anemia, blood loss, dehydration, infection and neoplastic growth etc.

Serum analysis

For calcium and phosphorous analysis, at day 0, 1, 3, 7, 15, 30, 45, 60, 75, 90, 105, and 120, 5ml blood was collected aseptically from Jugular vein in a vacutainer serum separator tube (SST). Vacutainer was stored at room temperature for 30 min and clot was allowed to be formed. Blood was centrifuged at 3000g for 20 min (Yang *et al*., 2019) to separate serum from blood cells. Then, serum was stored in plastic freezing vials at -20 °C. Later it was transported to University Diagnostic Laboratory (UDL) (ISO certified/17025, Lab 033), UVAS, Lahore for Ca and PO analysis.

Bone turnover markers

Serum collected as per 11.3 was also used to measure alkaline phosphatase (ALP) and osteocalcin (OC). For ALP, serum sample was dispatched to UDL (ISO certified/17025, Lab 033), UVAS, Lahore and for OC analysis, procedures were followed according to manufacturer's instructions (BT Lab, Bioassay Technology Laboratory, Birmingham, United Kingdom).

Mechanical assessment

Visual analogue scale (VAS) was employed to assess pain scale and limb deformity as per (Reed and Van, 2014). For pain scales, Colorado State University Institutional Animal Care and Use Committee (CSU- IACUC) recommendations to evaluate pain for animals during biomedical research was used. For rabbit orthopedic surgeries, pain score was measured from 0-3 (0=none, 1=mild, 2=moderate, 3=Severe) (Stasiak *et al*., 2003). Deformity severity scale were assigned grades from 1-3 (1=minor, 2=serious, 3=severe) as per (Dahl *et al*., 1994). For grade 2 or 3 deformity, appropriate external support was provided for their reduction.

Radiographic evaluation

Radiography was performed at Pet Center, University of Veterinary and Animal Sciences, Lahore by utilizing digital x-ray machine (collimator type R-20J-2015; Shimadzu Corporation) at day 30, 60, 90 and 120.

For radius union score (modified RUSS Scale) as per (Patel *et al*., 2014), radiographs were distributed to review panel of 2 surgeons and 3 radiologists with varying training and experience backgrounds, who were blinded to all clinical information. RUSS scoring assigns a score for antero-posterior (AP) and lateral radiographs based on union assessment and callus formation visible at both aforementioned views. Each cadre was assigned scoring from 0-2 and later these were summed up to assign a total score i.e., from 0-8.

Histomorphometry

Histomorphometry was done to evaluate cellular changes and healing efficiency at reconstruction site after completion of project. Bone sample was collected, immediately preserved in 10% buffered formalin (Vaught and Henderson, 2011) and dispatched to the histopathology Lab, Department of Pathology, UVAS, Lahore. For histomorphometric scoring method, amount of newly formed bone, cartilage, fibrous tissue and remnant defect size was measured. Histomorphometric score was graded from 1-10 for bone and cartilage while for fibrous tissue and remnant defect it was 10-1 as per (Han *et al*., 2018).

Statistical analysis

Graphical analysis was done by GraphPad Prism 6 software (La Jolla, CA 92037, USA). The data was analyzed by using two-factorial ANOVA with repeated measures. Significance among groups was evaluated by using Post Hoc test (Tuckey's test). Visual analog scale was statistically analyzed by non-parametric Friedman test analysis. Significance among groups was evaluated by using Dunn's multiple comparison test. Significant values were presented as *P≤0.05, **P≤0.01 and non-significance was denoted as (P≥0.05).

RESULTS AND DISCUSSION

Clinical vital parameters

Clinical vital signs of temperature, pulse and respiration were evaluated to identify animal health status throughout experiment. Temperature was observed above normal physiological range (101.5-104.2F°) at day 1. At day 7 and 15, it was noted within normal upper limits for each group. From day 30 and onwards, it slightly decreased and was seen at median levels till of end of trial period. There was no statistical difference between groups (P≥0.05) for whole study period. Pulse rate was observed from day 0 to 120 in all groups. At day 1, it was seen above normal physiological limits (180-350) in each group. From day 7 to end of experiment, pulse was noted within normal range for all rabbits. No significant significance (P≥0.05) was noted between groups during complete trial period. Respiratory rate was observed at upper normal physiological range (30-60) at day 1 for each group. Onwards it slightly decreased and was seen within middle normal limits throughout trial period. Only significant difference (P ≤ 0.05) was noted between groups A-D and A-C at day 7 and 15, respectively (Fig. 1). This

initial increase in TPR (temperature, pulse, respiration) immediate post-surgery may be associated with surgical manipulations, post-surgical stress, inflammation and pain. After first week, TPR values were noted within normal physiological limits with slight fluctuations for remaining trial period. These vital parameters related findings coincided with (Athanassious *et al*., 2011; Ashley *et al*., 2017; Sigmund *et al*., 2017; Tran and Nguyen, 2022).

Hematological parameters

White blood cells (WBCs) count was observed within normal ranges (5.2-12.5×10^{13}µl) in each group throughout trial period except at day1, where it was noted slightly above normal physiological levels in all groups. No statistical difference (P≥0.05) was seen between groups during experiment. This initial leukocytosis may be associated with post-operative systemic inflammatory response, invasiveness of surgeries, use of anesthesia and linked cell proliferations as a counter measure. These findings corroborated with (Czaplicki *et al*., 2011; Kraft *et al*., 2011).

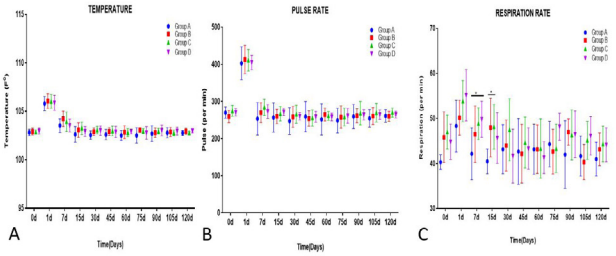

Fig. 1. Changes in temperature (A), pulse (B) and respiration (C) in each group with time points. For temperature and pulse, difference of values of groups A, B, C and D was non-significant (P≥0.05). For respiration, significant difference (P≤0.05) was noted between groups A-C at stipulated days.

Platelets levels, for all groups, were observed within normal physiological limits (250-650×10^{3}µl) throughout study period. At day 1, a slight decrease within normal range was noted. For rest of period, their values improved till end of trial. Furthermore, no statistical significance (P≥0.05) was noted between groups on stipulated days. The initial thrombocytopenia after orthopedic manipulations may be associated with site injury and hematoma formation resulting in perioperative platelet consumption and hem-plug formation at injured site, thus decreasing platelets numbers in general blood stream. These findings collaborated with (Wei *et al*., 1995; Wang *et al*., 2022).

A slight decrease below normal baseline values, for red blood cells (RBCs) and hemoglobin (Hb) were seen at day1(RBCs, 5-8×10^{6}µl) (Hb, 10-17g/dl) in all groups. From day 7 and onwards, an increase and stability in values was seen for rest of trial. No statistical significance (P≥0.05) for RBCs and Hb was observed between groups during study period (Fig. 2). This initial slight vascular decrease of RBCs and Hb may be linked to operative site tissue and vascular damage, intraoperative blood loss, increased surgical time, circulatory overload etc. These findings are in concurrence with (Jagow *et al*., 2015; Howell *et al*., 2019).

Serum analysis

Serum calcium levels showed an increase at day 1, with values near upper range of physiological limits (11-14mg/dl) for each group, that may be associated with body hemostatic response. At day 3, a drop below normal limits were seen in all groups, indicating bone spicules absorption post-surgery. At day 7, an abrupt sharp rise in Ca was observed in each group, showing its supersaturation in body fluids that favors bone salt deposition at fractured site. During Ca peak time, new callus formation was also noted at highest levels. From day 15 till end of study, Ca values were observed within upper normal physiological range, indicating that new bone trabeculae were replacing cartilage tissue, until each fragment's trabeculae met and joined. Statistical analysis revealed significant difference (P ≤ 0.01) between groups A and B at day 3, while at day 7 statistical difference (P≤0.01) was noted between groups A-B, A-C, A-D and B-C. For rest of trial period, no significant difference (P≥0.05) was noted between groups. Our findings of calcium studies correlated with (Meller *et al*., 1984; Kumar *et al*., 2018).

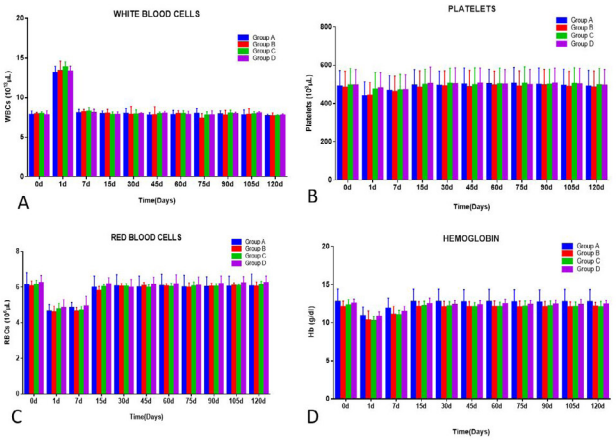

Fig. 2. Changes in WBCs (A), platelets (B), RBCs (C) and Hb (D) in each group with time points. For WBCs, platelets, RBCs and Hb, difference of values of groups A, B, C and D was non-significant(P≥0.05).

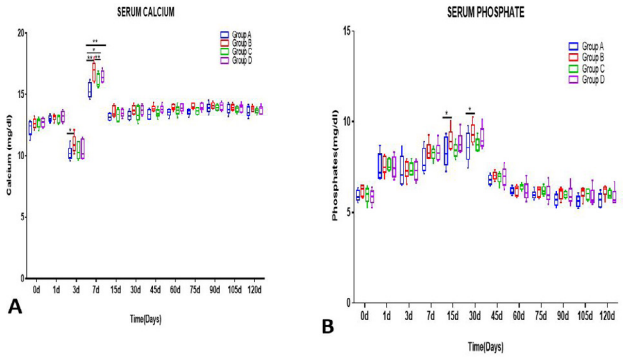

Fig. 3. Changes in serum calcium (A), phosphates (B) in each group with time points. For calcium and phosphates, significant difference of values i.e., $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$ of groups A, B, C and D was noted at marked days.

Serum phosphate showed an increase at day 1 above normal physiological range (4-6.5mg/dl), then a slight decrease at day 3 was noted but values were still above normal limits. At day 7, a sharp increase above normal range was observed that continued till day 30. From day 45 to end of study period, a gradual decreasing trend within normal physiological range was noted. Statistical analysis revealed, only significant difference ($P \leq 0.05$) between groups A and B at day 15 and day 30 (Fig. 3). These findings indicated necrotic disintegration of cells at fractured site during PO peak levels, that may be a stimulating factor for start of Ca deposition and overall fractured bone remodeling. These findings corroborated with (Nilsson and Westlin, 1972; Komnenou *et al*., 2005).

Bone bio-markers

For bone, alkaline phosphatase (ALP) and osteocalcin (OC) are special biochemical markers to assess osteoblasts activity. It has been noted that osteoblast activity considerably increases during fracture healing. Osteoblasts help in new tissue formation (bone matrix) and its mineralization, thus secrete large amounts of ALP and OC.

Alkaline phosphatase (ALP) values increased sufficiently above normal physiological range (12-96 U/L) at day 1. Maximum rise in ALP values was noted at day 7 in each group. A gradual decrease started from day 15, but values remained above normal limits till day 90. At day 105 and 120, ALP values were observed within normal range in all groups. Statistical analysis revealed, significant difference ($P \leq 0.01$) between all groups at day 7 and 15 but significant difference in groups A-D was noted at ($P \leq 0.05$). At day 30 and 45, there was statistical difference ($P \leq 0.01$) between all groups except group A-D. At day 60, significant difference was observed between groups A-B, A-C, B-C and B-D ($P \leq 0.01$). At day 75, significant difference was only present between groups A-B ($P \leq 0.01$) and B-D ($P \leq 0.05$). For rest of experiment period, no statistical difference ($P \geq 0.05$) was noted between groups. Alkaline phosphatase (ALP) helps in deposition of hydroxyapatite (HA) crystals in bone organic matrix. For this purpose, ALP is considered to increase concentration of local inorganic phosphates or neutralizes inorganic pyrophosphate (an inhibiter of HA crystal formation). These findings indicated extensive osteoblast activity and HA deposition in fractured bone matrix throughout study period. ALP values correlated with (Komnenou *et al*., 2005; Morsi *et al*., 2021).

Osteocalcin (OC) or bone gla protein is most abundant in bone and is produced exclusively by mature osteoblasts during their peak activity post bone trauma. It is significantly associated with serum alkaline phosphatase and is considered latest expression marker for bone turnover. Osteocalcin (OC) values demonstrated an increase at day 1 with maximum peak at day 7 for all rabbits. Onwards, a gradual and consistent decrease in OC values were observed till end of experiment. Statistical analysis revealed, significant difference ($P \leq 0.01$) between all groups except groups C-D at days 7, 15, 30 and 45. At day 60 and 75, significant difference ($P \leq 0.01$) was observed between all groups, except groups B-C and C-D. At day 90, statistical difference was noted between groups A-B, A-C ($P \leq 0.01$) and B-D ($P \leq 0.05$). At day 105, significant difference was observed between groups A-B ($P \leq 0.01$) and A-C ($P \leq 0.05$) while at day 120, statistical difference was seen between groups A-B ($P \leq 0.01$) and B-D ($P \leq 0.05$) (Fig. 4). Our observations for osteocalcin parameters coincided with (Olmedo *et al*., 1999; Nicholson *et al*., 2002).

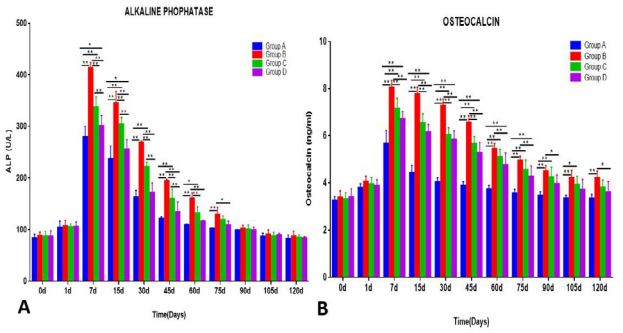

Fig. 4. Changes in alkaline phosphatase (A), osteocalcin (B) in each group with time points. For alkaline phosphatase and osteocalcin, significant difference of values i.e., $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$ of groups A, B, C and D was noted at marked days.

Mechanical assessment

Fracture related important issues are pain and limb deformity, due to significant changes in bone nociception and unpredictable bone healing. Visual analogue scale (VAS) seems a better way to detect subtle changes associated with them. Pain is considered an aversive sensory experience that results in learned avoidance and modifies social behavior. VAS scale was used to identify and follow-up the impact of bone-graft therapy on orthopedic pains, limb deformity and improvement of symptoms in stipulated timeframe. Pain score evaluation revealed that its intensity was maximum at day1 post-surgery, which gradually decreased with time to end of study for each group. Pain was noted at highest levels in group A, while a decreasing level (high to low) was seen in order of groups D, C and B respectively. Statistical analysis revealed significant difference ($P \leq 0.05$) between groups A-B and B-D. Our findings corelated with (Stasiak *et al*., 2003).

Limb deformity is leg angulation or length discrepancy that may be associated with unpredictable fracture healing or musculoskeletal pains. Limb deformity was observed in one rabbit of group A and D each. Grade-I deformity was observed in rabbit A4 at day 30, which persisted till end of experiment. Grade-II deformity was seen in rabbit D5 at day 30 that gradually improved with time and at day 90, it was ranked as grade-I that was seen till end of experiment. Statistical analysis revealed, significant difference ($P \leq 0.05$) between groups B-D and C-D (Fig. 5). Our findings were concurrent with (Dahl *et al*., 1994).

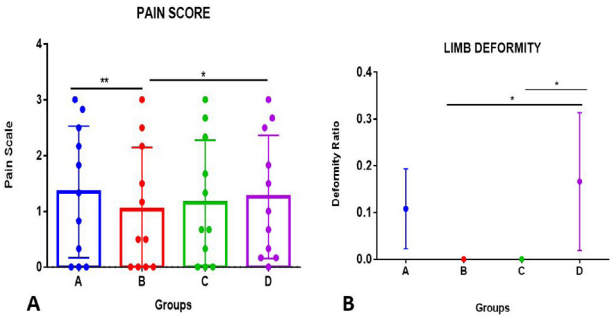

Fig. 5. Changes in pain score (A), limb deformity (B) in each group with time points. For pain score and limb deformity, significant difference of values i.e., $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$ of groups A, B, C and D was noted at marked days.

Radiographic evaluation

Radiographs were used to measure radius union score at stipulated days. RUSS score reliably addressed surgically induced radius fracture unions, callus formation and demonstrated substantial intra- and interobserver reliability. The RUSS scale was significant predictor of fracture healings i.e. RUSS score equal or greater to 6 showed better unions. In our studies, best radius union was noted in group B, then a decreasing trend was seen in groups C, D and A respectively (Fig. 7). Significant difference ($P \leq 0.01$) was noted only between groups A and B. Our findings coincided with (Patel *et al*., 2014) (Fig. 6)

Histomorphometry

Bone tissue defect healing was evaluated by histological scoring method. Osteogenic activity was not only measured by new bone formation but cartilage and fibrous tissue was also evaluated to validate the data, as these additional measurements allowed the variation in tissue type occupying the defect to be investigated, though fractured site seems to be healed appropriately.

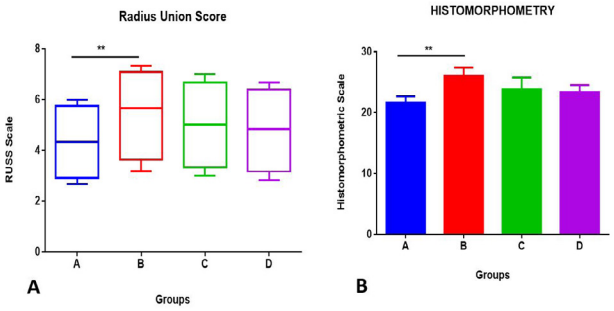

Fig. 6. Changes in radius union score (A), histomorphometry (B) in each group with time points. For radius union score and histomorphometry, significant difference of values i.e., $^{**}(P \leq 0.01)$ of groups A and B was noted at marked days.

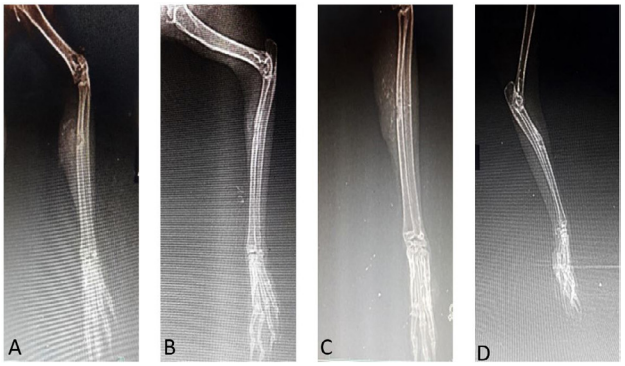

Fig. 7. Examples of Bone healing/callus formation in group A (A), group B (B), group C (C) and group D (D) at end of trial. Best to poor healing noted in group B, C, D and A, respectively.

Histomorphometric analysis revealed that group B showed maximum bone tissue formation with least

cartilage tissue remnants, furthermore fibrous tissue and defect size was minimum in this group. These trends were noted to tapered side in group C, D and A, respectively (Fig. 8). Statistical analysis revealed significant difference ($P \leq 0.01$) between group A and B only (Fig. 6). Our findings agreed with (Han et al., 2018).

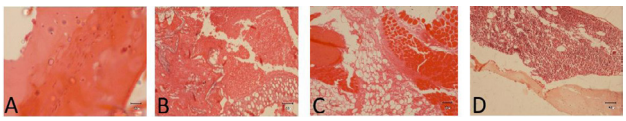

Fig. 8. Histomorphometric analysis in group A (A), group B (B), group C (C) and group D (D) at end of trial. Best to poor healing noted in group B, C, D and A, respectively.

CONCLUSION

Despite intensive research on potential bone graft alternatives, autogenous bone graft remained gold standard. In our studies, autologous fresh bone graft exhibited good mechanical and biological properties to address complex fracture healing and was cost effective. Bone graft biological activity in solo was complemented by addition of orthobiologics to facilitate and expedite autograft seeding, bone incorporation and remodeling. Our studies suggested that osseous defect healing by addition of autologous Bone-BMA graft showed excellent healing in terms of recovery speed and callus strength due to combination of enormous numbers of osteoblasts, MSCs, multiple pluripotent cell lines, various growth factors, cytokine and chemokines etc. Autogenous Bone-PRP graft also exhibited great fracture healing potential due to expansive bone graft cells facilitated by concentrated growth factors of platelets alpha-granules, but bone healing was noted below par than bone-BMA graft combination. bone-DFS graft also revealed reasonable bone healing capacity and callus strength due to presence of bone graft cells, HA and collagen-I but bone healing capability was found less than both aforementioned graft regimens. Our studies further suggested that all three treatment regimens are helpful in complex fracture healing, malunions, delayed and nonunions in terms of healing speed, callus strength etc. to descending order of bone-BMA, bone-PRP and bone-DFS but further randomized controlled trials (RCTs) can be performed to establish their efficacy.

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IRB approval

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Ethical statement

The current research study was conducted as per directives approved by Ethical Review Committee (ERCULA), University of Veterinary and Animal Sciences, Lahore, Pakistan with NO: DR-456, Dated: 17-05-2019.

Statement of conflict of interest

The authors declare no conflict of interest

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