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Research Article

Enhancement of Atrophic Non-Union Fracture Healing Using Autologous Progenitor Cell-Rich Bone Marrow

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Abstract

There is growing interest in the use of bone marrow derived stem cells for bone repair. Increasing evidence now indicates that physiological bone remodelling occurs in close proximity to blood vessels and that these vessels carry perivascular stem cells that differentiate into osteoblasts. Non-union of bones are serious complication, incidences of which can be as high as 5% to 20%, varies by fracture site and is influenced by a number of fracture characteristics and host factors. The healing of fractures is a complex physiological process which could be interrupted by various factors. Thus, development of a novel therapeutic method to treat non-union fractures' becomes a clinical necessity. The purpose of this study was to assess the safety and efficacy of the autologous bone marrow concentrate preconditioned with synthetic graft in the treatment of non-union bone fractures. Autologous Bone Marrow Concentrate (aBMC) was prepared from bone marrow aspirate utilizing rapid, intraoperative point-of-care system via a density gradient centrifugation. A total of 17 patients were treated with aBMC at the fracture site and evaluated for the length of bone fracture healing time during the study follow-up period. The mean Total Nucleated Cell Count (TNCC) and Mononuclear Cell Count (MNCC) in all the patients were 5.54 x 10^8 ± 1.99 and 1.64 x 10^8 ± 0.86, respectively. 82% of the patients showed considerable bone healing of the non-union and the mean time for bone union/healing was 6 months. Overall the study concludes aBMC administration to be safe and effective in treating patients with atrophic non-unions and may contribute as an alternative to autologous cancellous bone graft.

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Introduction

Bone is the tissue in which the ability to regenerate is more predictable than in any other tissue of the body. Most bone fractures heal without any complication, but at times due to a multitude of factors some may go into delayed union or non-union causing morbidity, prolonged hospitalization and increased expenses. The economic burden of non-union fractures is significant due to the cost and disability associated with the condition. Of the approximate 5.6 million bone fractures in the United States annually, up to 20% do not heal completely, and then they require subsequent treatment. The incidence of non-unions can be as high as 5% to 20% depending upon the fracture site and is influenced by a number of fracture characteristics and host factors [1]. Non-union leads to long lasting disability as the individual is not able to use the affected limb due to loss of movement and acute pain. The various predisposing factors leading to a non-union may be patient related such as age, physical activity, nutrition/catabolic states, anemia, smoking, alcohol intake, diabetes mellitus, immuno-compromised state, osteomalacia, intake of certain pharmacological agents such as steroids, cytotoxic drugs and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) [2,3]. It would also depend on the type of injury such as open fractures, significant soft tissue trauma, soft tissue interposition, infection, pathological fracture, anatomical location, excessive bone loss, displacement or intra-articular fracture. Treatment related factors may also lead to a non-union such as distraction of the fracture segments, inadequate stability with excessive movement or a surgical technique which has compromised the local vascular supply [4].

Non-union has been described as failure of the fracture to heal within six to nine months in a patient where progressive repair has not been observed radiographically, within six months following fracture. Non-unions can be classified as hypertrophic, atrophic and pseudoarthrosis. Hypertrophic non-unions are rich in callus and have a rich blood supply in the ends of the bone fragments, which can be managed by proper reduction and stabilization of the bone. However, they result from insecure fixation (inadequate stability) or premature weight bearing in a reduced fracture whose fragments are viable. While in atrophic or oligotrophic non-unions there is minimal or no callus formation with absent or poor blood supply to the ends of the fragments making the non-union inert and incapable of biologic reaction and these are the types of non-unions that may benefit most from cell based therapies. Pseudoarthrosis generally known as 'false joint' is the third type of non-union fracture that has no chances on mending without interventions. In pseudoarthrosis, cartilage cap or fibrous tissue form at the end of bone fragments which interrupt in healing the fracture, due to which the body perceives bone fragments as separate bones and does not attempt to unite them [1].

Most mal-union and non-unions require open surgery to realign the fractured fragments into their normal anatomical position (open reduction) and stabilize the fracture by use of metal plates, rods, screws, and/or wires (internal fixation). Bone graft is placed at the

surgical site to stimulate fracture healing. Treatment of non-union may be complemented with autograft (obtained from the same individual), synthetic bone graft or a graft from another individual (allograft, homogeneous graft). Some cases, whether treated surgically or with non-invasive techniques (closed reduction), benefit from the use of electrical, electromagnetic, or ultrasonic stimulation to promote fracture healing and bone growth. Infection requires surgical removal of any infected bone or tissue (debridement), followed by intensive antibiotic treatment. Newer approaches are using recombinant Bone Morphogenetic Protein (BMP) and bone marrow aspirates [5,6]. BMP use could lead to rising mutations in the body and it has been reported as not a very safe treatment method [7].

Fracture healing is a multidimensional process consisting of four well established remodeling stages; an initial inflammatory response, soft callus formation, initial bony union and bone remodeling. At the cellular level, inflammatory cells, vascular cells, osteochondral progenitors including stem cells and osteoclasts are fundamental in the repair process [8]. In the tissues surrounding non-unions, a decrease of progenitor cells is observed. This deficiency is often found in regions afflicted by infections, previous trauma, tissue defects and scars, as well as the compromised vascularity frequently associated with non-unions. This suggests that normal tissue repair may be limited by decreased population of progenitor cells locally. If the cells in the osteogenic layer of the periostium and endostium are too far apart and do not have sufficient osteogenic potential, bone consolidation can occur only by the osteoblasts present on the two fractured surfaces. In addition to necrosis, which can be a consequence of trauma at the fractured extremities, the osteoblasts are not numerous within the bone tissue [9].

Cell therapy with bone marrow cells has emerged as a promising new approach for bone regeneration. Several preclinical and clinical studies have shown that stem cells isolated from the bone marrow, can induce callus formation when injected in the non-union site of a fractured bone [10]. Bone marrow consists of progenitor cells that have the potential to promote angiogenesis, osteoinduction and osteogenesis thereby promoting bone healing/remodeling. We hypothesize that the presence of blood vessels, growth factors, and (proliferative) precursor cells are required to obtain successful fracture healing. Histologically it has been seen that in the tissues surrounding the non-union, there is a decrease in the number of progenitor cells. The concept of autologous cell therapy is based on the presence of beneficial growth factors and osteo-progenitors in the bone marrow and their property to differentiate into osteoblast cells [11].

The relationship between consolidation of fractures and activation of the bone marrow was apparently first observed by Ilizarov, who demonstrated that a 1% loss of blood volume induced an accelerated consolidation of osteotomy in rabbits [12]. This suggested a link between the haematopoietic activity of the iliac crest and osteogenesis localized around the focus of the osteotomy. This phenomenon has been confirmed by experiments in rats and mice by Bab [13] and in rabbits by Lippiello [14]. Animal studies done by Kadiyala et al (1997) on rat femora demonstrated that purified, culture-expanded syngeneic progenitor cells were capable of healing a clinically significant bone defect within 8 weeks [15]. The Kadiyala investigation further substantiates that compared to unprocessed marrow; concentrated mononuclear cells produce significantly more bone when placed in either an ectopic or an orthotopic site. Bruder et al., demonstrated bone formation at a segmental defect in adult athymic rats by implantation of human bone marrow-derived mesenchymal stem cells [16].

In 1990, Healey et al. [17] published good results in 7/8 cases of non-union after BM aspirate injection in humans. In 2005, Goel et al. presented results of Bone Marrow (BM) grafting in tibial non-unions [18]. Where 15/20 patients showed clinical and radio-graphical bone union after 14 weeks. Hernigou et al (2005) demonstrated significant results and union within 4-16 weeks (mean 12 weeks) in 53/60 patients who were treated using percutaneous injections of autologous bone marrow graft [8]. They also demonstrated a correlation between the volume of mineralized callus at four months and the number and concentration of fibroblast colony-forming units in the graft [8].

Surgical approach is still the most important tool in the management of non-union fractures. To date, autograft serves as the gold standard for bone grafts because they are histocompatible and non-immunogenic, and they offer all of the imperative properties required in a bone graft material. This is due to its high potential of osteogenesis (osteoprogenitor cells), osteoinduction and osteo-conduction [19-21]. However, the classical autograft strategy has certain limitations that may result in significant donor site injury and co-morbidity, deformity, scarring and are associated with surgical risks such as bleeding, inflammation, infection, and chronic pain [22-24].

Current practice of treatment of fractured non-unions employs methods to promote osteogenesis including the use of autologous bone marrow cells, autogenous /synthetic grafts and corrective fixation [25]. To optimize these methods we propose the preparation of autologous bone marrow concentrate using a rapid point-of-care technology (in the operation theatre). Several scaffolding strategies have been developed to administer concentrated progenitor cells in a lesion site. Earlier studies have shown that autologous bone marrow cells when applied together with a synthetic graft were a potentially safe and effective method for treating atrophic non-union in humans [8]. The proposed protocol would reduce patient morbidity, infection rates, and vascular disruption through administration of the bone marrow concentrate. As a result of the clinical benefits, the patient could benefit from a reduction in medium and long-term healthcare costs. This was a non-randomized, open label, feasibility study to evaluate the safety and efficacy of autologous Bone Marrow Cell concentrate (aBMC) in patients with atrophic non-union fractures. The aBMC was produced at the point-of-care in the operation theatre for all the treated patients using our proprietary device the Res-Q™ 60 BMC system. All patients were consented to the procedure before commencing the study using an informed consent process. Our study indicates that administration of autologous bone marrow cell concentrate along with synthetic graft can be safe and effective in long-term healing of bone non-unions resulting in successful clinical outcomes in all patients.

Materials and Methods

Patient selection

This was a non-randomized, open label, Phase Ib feasibility study designed to evaluate the safety and efficacy of autologous Bone Marrow Cell concentrate (aBMC) in patients with atrophic non-union fractures. A total of 17 patients in the age group of 18-75 years were enrolled in the study, who had a history of failed primary surgical intervention. All the enrolled patients had an Open Reduction Internal Fixation (ORIF) procedure that eventually persisted along

with the non-union of the bone (fractures of tibia (mid-shaft only), femur, wrist, and humerus) for more than 8 months on an average. The diagnosis of non-union of long bones was confirmed by X-ray or CT scan examination and clinically evaluated by the treating physician. Those patients who met the inclusion criteria were explained the entire study procedure, and only after obtaining voluntary written informed consent from the patients, were they enrolled in the study. The study was conducted in accordance to the Declaration of Helsinki and Good Clinical Practice (GCP) guidelines, and all care was taken to maintain patient safety and confidentiality. All the enrolled patients received single dose of autologous bone marrow cell concentrate injection on the day of procedure.

The inclusion criteria for the study was both male and female with atrophic non-union fracture diagnosed by X-ray or CT scan; a stable fracture with visible small gap (about 1-2 cm), and no signs of infection at the wound site or fracture site. Any patient with history of smoking but willing to quit was included in the study; corticosteroids were to be included only after cessation of smoking one month before cell therapy, (corticosteroids function by reducing the activity of immune system and decreasing inflammation). Also, patients must have a normal blood and marrow function as defined by: leukocytes ≥ 3000/µl, absolute neutrophil count ≥ 1500/ µl and platelets ≥ 100,000/ µl. Exclusion criteria for the study were patients with active systemic or local infection; any evidence of malignancy in the past five years; pregnancy or breastfeeding; patients who require corticosteroid or anti-inflammatory therapy after surgery; patients with genetic metabolic bone disease such as hypophosphatasia, or metabolic bone disorders such as primary or secondary hyperparathyroidism caused by chronic renal insufficiency or other disorders; patients that are positive by serology or PCR for HIV, hepatitis B or C infection; patients with uncontrolled Diabetes Mellitus (DM); patients under immunosuppressive therapy or taking anticoagulant agents; insufficient reduction of the fracture with displaced fragments; evidence of local sepsis by clinical signs; and multiple major fractures.

aBMC Preparation at point-of-care

The treatment procedure involved aspiration and processing of bone marrow and administration of autologous bone marrow cell concentrate mixed with synthetic graft material, that is, tri-calcium phosphate at the fracture site using our proprietary investigational device the Res-Q™ 60 BMC system (Point-of-Care Technology, License #MD-826, Central Drug Standard Control Organisation, India). This Point-of-Care integrated kit is inclusive of the devices required for aspiration, processing and delivery of aBMC, specifically aiding in the processing of cells (i.e. rapid purification/concentration) at the patients' bed side, with a single sitting in the operation theatre. Procedure was carried out under spinal or general anaesthesia and strict aseptic conditions. A total volume of 60 mL of bone marrow (with anticoagulant) was aspirated for all the treated patients from multiple sites from either the posterior superior iliac crest or anterior superior iliac crest in syringes containing anticoagulant (ACD-A) using an 11 gauge trocar needle. Small aliquot of aspirated bone marrow (1 ml) was sent to the laboratory for cell count analysis and sterility evaluation.

The harvested bone marrow was thoroughly mixed, and filter transferred to the device for processing and concentrating using our intraoperative rapid, closed, point-of-care device, the Res-Q™ 60 BMC technology (Thermogenesis Corp. USA). This point-of-care system is an automated cell processing medical device that concentrates the bone marrow by a density gradient centrifugation method. The processing was carried out in the operating room in less than 20 minutes and required minimum operator intervention. Following processing at the patient's bed side, 8 mL of aBMC was collected from the device that was rich in progenitor cells. Aliquot of 1 mL of aBMC (post-processed sample) was collected and later sent to the laboratory for analysis of cell counts and sterility evaluation, while the remaining 7 mL aBMC was preconditioned for 5-10 minutes with synthetic graft material i.e. tricalcium phosphate. During the 'preconditioning' of bone marrow concentrate, progenitor cells are bio-absorbed by the synthetic graft which makes a semi-solid matrix called 'putty'. This 'putty' is administered between the ends of non-union bones for rapid healing of the fracture. The pre- and post- processed bone marrow samples were quantified using Coulter cell counter and sterility was evaluated using BacT/ALERT.

aBMC Delivery procedure

For the aBMC delivery at the fracture site, a small incision was made and a needle identical to the one used for bone marrow aspiration was placed in the non-union fracture site, and positioned using a C-arm fluoroscope. The aBMC putty was slowly administered through the needle at the fracture site. After injection, the trocar was gradually withdrawn under fluoroscopic guidance, with small oscillating vibrations to fill in the path of the needle. The skin incision at the injection site was closed with circumferential sutures and properly plastered to avoid leakage of aBMC and prevent any sort of disruption of the healing and tissue regeneration process. Figure 1 gives a pictorial representation of the cell therapy procedure using bone marrow cell concentrate as described above. The treated patients were discharged on the first post-operative day and scheduled for regular follow-up at 1, 3, 6, 9 and 12 months post cell therapy, to ensure safe healing of the non-union fracture.

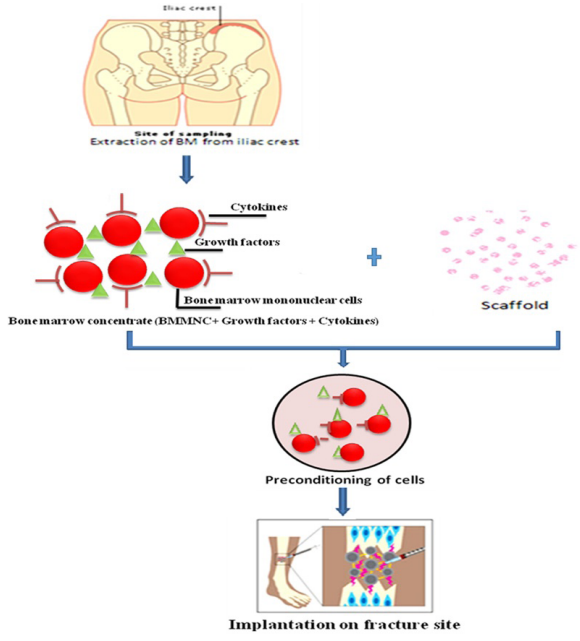

Figure 1: Flow chart to explain cell therapy.

Bone marrow is extracted from the posterior iliac crest of the pelvis. Bone marrow concentrate obtained (Bone marrow mononuclear cells along with paracrine factors) is mixed with a scaffold (synthetic graft) to be administered on fracture site. Preconditioning of cells is done for a 5-10 mins time period. After this, the solution 'putty' is administered at the fracture site.

Post-operative management and follow-up

All the patients were given standard-of-care medications and medical management as per the study protocol. Treated limb was immobilized and weight-bearing was not allowed during the first month after treatment procedure to avoid mechanical disruption of the tissue-regeneration and bone-healing process. After one month, if (and only if) callus was observed on radio-graphs, partial weight-bearing was allowed with the plaster cast in place. One-month transition period was given between the beginning of partial weight-bearing and that of full weight-bearing to each treated patient. At the end of that month, if the patient had no pain and there was cortical bridging or disappearance of the fracture lines on at least three of the four cortices viewed on the anteroposterior and lateral radiographs, the plaster cast was removed. Clinical end points that were evaluated during each follow-up visit of the patient were evaluation of the fracture site for abnormal mobility/normal function, radio-graphic evidence of fracture healing (Healing time, and change in displacement, shortening, or angulation during bone-healing viewed on the anterior-posterior and lateral radio-graphs) and any intervening procedures, hospitalizations, or major changes since the last visit.

The primary objective of this clinical study was to demonstrate the efficacy of autologous bone marrow cell concentrate (aBMC) in the treatment of atrophic non-union fractures by determining the formation of callus at the fracture site. While, the secondary end points were healing time and quantitative reduction in pain measured using the Visual Analogue Scale (VAS).

Statistical analysis

All statistical analysis for the data was performed using SPSS (Statistical Package for the Social Sciences) for Windows (Version 13.0). All endpoints were analyzed on an Intent-To-Treat (ITT) basis, that is, all patients who sign the study Informed Consent Form (ICF) and are enrolled in the study were included in the analysis. Descriptive statistical analysis of the data was carried out. Clinical event rates are presented in number and percentage (%) and continuous variables are presented as mean ± standard deviation. Intra-individual comparison of continuous variables at baseline with those at follow-up was performed with the paired T-test for normally distributed variables and Wilcoxon signed rank test for non-normally distributed continuous variables. All statistical tests were done using two-sided, $p<0.05$ level of significance.

Results

The baseline clinical characteristics of the enrolled patients are summarized in (Table 1). All the bone fracture patients screened had a persistent non-union fracture, that had been present for more than 8 months on an average following primary fixation of the bone and all the patients had undergone ORIF procedure that persisted with the non-union fracture. Out of the 24 patients screened, 17 met the eligibility criteria and were enrolled in the study. The mean age of the study patients was 45.64 ± 16.41 years. Of these 12 (70.58%) were male and 5 were (29.41%) female patients. Eight (47.1%) patients received treatment in the tibia, seven (41.2%) received treatment in the femur; one patient (5.9%) received treatment in the humerus and one (5.9%) in the wrist. Patients' co-morbidities in the study included smokers (23.5%), alcoholics (41.1%), diabetics (53%), and Cardio Vascular Diseases [CVD] (11.7%).

Bone marrow aspiration, processing and administration were accomplished in the operating room in a single sitting within 60 minutes using the Res-Q™ 60 BMC system. Patients tolerated the procedure well, and there was no bleeding, infection, or procedure related complication, including local injection site swelling in all the subjects on the day of treatment after aBMC administration. The mean (±Standard Deviation) Total Nucleated Cell Count (TNCC) and Mononuclear Cell Count (MNCC) of all the patients were 5.54 x 10^8 ± 1.99 and 1.64 x 10^8 ± 0.86

S. No.	Age (y)	Gender	Affected Bone	Diabetes (Y/N)	CVD (Y/N)	Infectious Disease (Y/N)	Smoking (Y/N)	Alcoholic	Time from Injury (months)	NU Status at 12 Months
1	19	Male	Tibia	N	N	N	N	N	9	United
2	59	Male	Tibia	Y	N	N	Y	Y	10	United
3	31	Male	Femur	N	N	N	N	Y	6	United
4	62	Male	Femur	Y	N	N	N	N	8	United
5	40	Male	Femur	N	N	N	N	Y	6	United
6	51	Male	Femur	Y	Y	N	Y	Y	10	Not United
7	72	Female	Femur	Y	Y	N	N	N	12	Not United
8	39	Male	Tibia	N	N	N	N	N	8	United
9	30	Male	Humerus	N	N	N	N	Y	8	United
10	31	Female	Tibia	N	N	N	N	N	6	United
11	59	Female	Tibia	Y	N	N	N	N	7	United
12	41	Male	Tibia	N	N	N	N	Y	9	United
13	71	Female	Femur	Y	N	N	N	N	8	United
14	42	Male	Wrist	Y	N	N	Y	Y	8	United
15	64	Female	Femur	Y	N	N	N	N	7	United
16	41	Male	Tibia	Y	N	N	Y	N	9	Not United
17	24	Male	Tibia	N	N	N	N	N	9	United

Table 1: Demographic data of the patients in the study.

y: years, Y : yes, N : no

respectively, in the bone marrow concentrate. The mean cell viability was found to be over 88% and the mean MNC dose administered to the patient was 1.34 x 10^8 cells.

Out of the 17 patients treated, signs of callus formation (confirmation of union of the bone) were observed in 14 patients with a union rate of 82%. The remaining 3 patients (18%) did not show any sign of bone union at the time of the last follow up (12 months). The average time for callus formation was observed to be 6 months following aBMC administration (Figure 2). Patient follow-up showed sign of clinical improvement at 3 month with respect to fracture healing, pain reduction and improved locomotive function of the limb. Radiographic images of the fractured bone, pre- and post- treatment are given in (Figure 2).

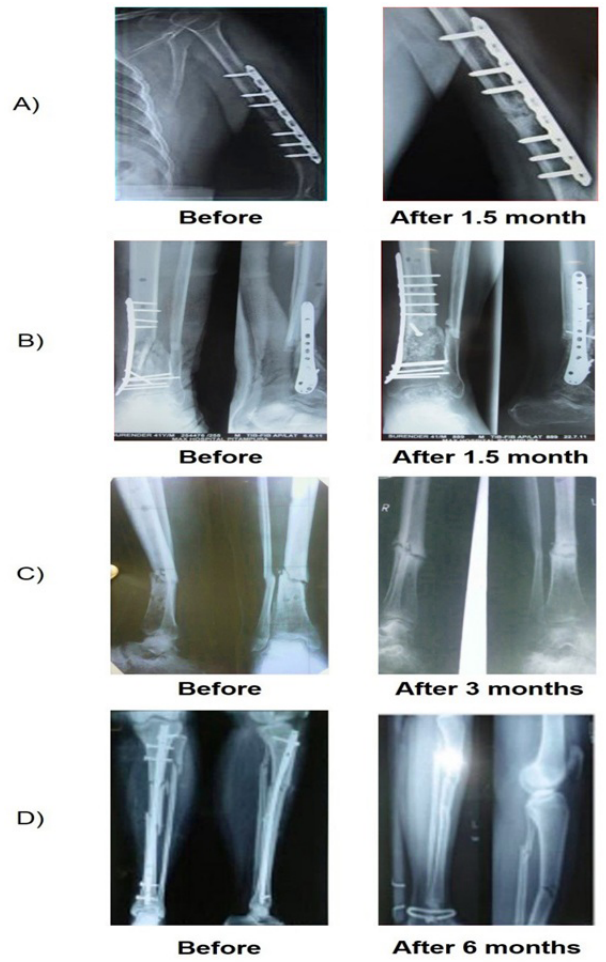

Figure 2: A) Figure showing the healing of a non-union fracture in the humerus bone
B) Bone healing is clearly evident in the tibia bone after 1.5 months
C) Figure showing callus formation in tibial bone after 3 months
D) Bone healing seen in non-union fracture in the tibia bone after 6 months

The bone marrow concentrate (post-processed) showed a significant increase in the Total Nucleated Cell Counts (TNCC) and Mono-Nuclear Cell Counts (MNCC) as compared to the bone marrow aspirate (pre-processed counts). The post-TNC counts showed a threefold increase from pre-counts and the post-MNC counts almost doubled compared to the pre-counts. The difference between the pre- and post- TNCC and MNCC data was found to be highly significant with a p value <0.001 as shown in (Figure 3A). The results showed a correlation between age and healing time. From the data it is evident that the 0-20 year age group has the fastest healing time in comparison to the 61-80 year age group (Figure 3B). The average healing time observed was 6 months. However, the comparison of healing time to age was not statistically significant between the different age groups with a p value > 0.05 as shown in (Figure 3b). The sample size was not sufficient to calculate significance via a paired t-test. In (Figure 3C) we have made a representation of the average MNC cell dose over the different age groups. Cell dose did not co-relate with age, co-morbidities or outcome.

Discussion

Bone healing after a fracture or small bone defect is a very unique process involving a complex, well-coordinated interaction between cells, cytokines, growth factors, osteo-conductive matrix and a mechanically stable environment with rich blood supply, according to the 'Diamond Concept' [26] to regenerate new bone instead of just fibrous scar tissue [6,8]. Skeletal healing is primarily a biological process, and depends upon a patients' cellular response. The most productive source of cells that influence osteogenesis is considered to be autologous bone marrow cells. Autologous bone marrow cell concentrate consists of progenitor cells, injection of which provides a graft that is osteogenic, osteoconductive and potentially osteoinductive through cytokines and growth factors secreted by the transplanted cells that enhance the paracrine effect. There are two major modes of bone formation, or osteogenesis, and both of them involve the transformation of a pre-existing mesenchymal tissue into bone tissue. The direct conversion of mesenchymal tissue into bone is called intramembranous ossification (desmal). While, the process by which a cartilage intermediate is formed and replaced by bone cells is called endochondral ossification.

Bone formation from autologous bone marrow combined with synthetic graft is believed to occur in two phases. During the first phase, which lasts approximately four weeks, the main contribution to bone formation is from the cells of the graft. However, during the second phase, cells from the host begin to contribute to the process. The endosteal lining cells and marrow stroma produce more than half of the new bone, whereas osteocytes make a small (10%) contribution [27]. This feasibility, non-randomized, open label, single arm study, was a scientifically rigorous study designed to evaluate the safety and efficacy of autologous bone marrow cell concentrate conjugated with synthetic graft (tri-calcium phosphate). The present study shows that the application of autologous bone marrow cell concentrate mixed with synthetic graft in 14 out of 17 patients (82%) resulted in bone union at 12 month follow up period. The callus formation in radiographic imaging is indicative of bone healing and union in these patients, confirming the effectiveness of autologous bone marrow graft in the treatment of atrophic non-union fracture.

When a fracture occurs, the repair or healing of the bone takes place in three steps; inflammatory stage, reparative stage and a remodeling stage. In a damaged bone, the soft tissue envelope, including the periosteum and surrounding muscles, is torn, and numerous blood vessels crossing the fracture line are ruptured. There is an accumulation of hematoma within the medullary canal, between the fracture ends, and beneath any elevated periosteum. This blood rapidly coagulates to form a clot. The effect of this vascular damage is of paramount importance. Osteocytes are deprived of their nutrition and die as far back as the junction of collateral channels. Thus, the

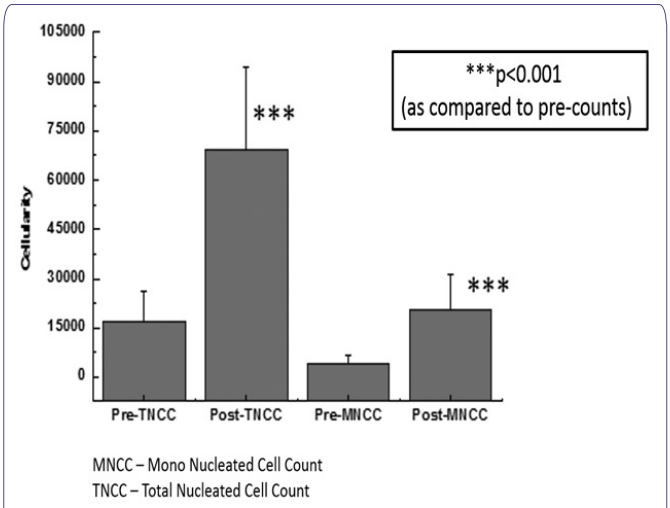

Figure 3A: Pre- and Post- TNC and MNC Counts.

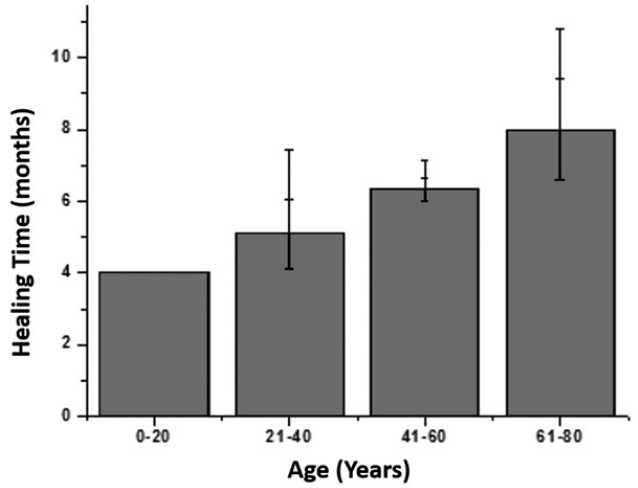

Figure 3B: Age vs Healing Time.

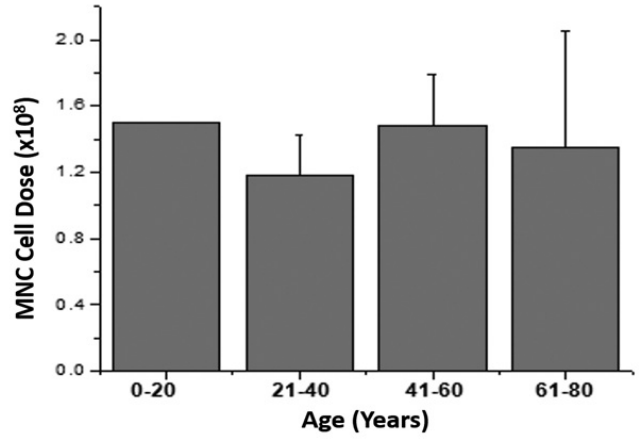

Figure 3C: Age vs MNC Counts.

A) Graph representing the pre and post counts of TNCC and MNCC of the patients. The TNCC shows an almost 3 times increase while the MNCC have doubled in the post-processed sample

B) Graph representing the age versus healing time of all the patients

C) Graph showing the correlation between age and MNC Counts

immediate ends of a fracture are dead; that is, they contain no living cells. The body begins to create cartilage around the fracture site to bridge the gap in the bone. This type of cartilage is called the soft callus, which is simply fibrous tissue. New blood vessels around the fracture site provide for nutrition. In the final stage of bone fracture healing, the body replaces old bone with new bone in a continual process called remodeling. Remodeling makes bones stronger and compact and blood circulation in the bone improves [28].

Historically, autologous bone grafts, usually the cancellous bone from the iliac crest is the gold standard and represents the most common therapeutic approach in orthopedic applications. The autologous graft is capable of providing all 3 elements of bone regeneration including osteogenic and osteoinductive as well as osteoconductive properties [21,25]. However, a bone autograft is limited in quantity and its harvesting represents an additional surgical intervention and donor site co-morbidity [6]. Therefore, investigations are needed to provide a safe and effective alternative approaches.

Bone marrow concentrate isolated contains progenitor cells such as Hematopoietic Stem Cells (HSCs), Mesenchymal Stem Cells (MSCs) and Endothelial Progenitor Cells (EPCs) along with abundant cytokines and growth factors. As bone marrow contains osteogenic progenitor cells, its implantation was proposed in order to potentially lead to efficient bone regeneration. In clinical practice, autologous bone marrow cells are harvested from the iliac crest and immediately transplanted into the site that is in need of skeletal repair. Low progenitor cell number is the major limiting factor of the direct bone marrow aspiration and injection into bone fracture site, and therefore concentration of bone marrow is important in order to provide abundant progenitor cells along with paracrine factors for bone healing.

This method of marrow-cell concentration and transfer or grafting is a relatively simple procedure that is inexpensive and can be performed intraoperatively at the bedside in a single sitting. Moreover, because it involves only the relocation of autologous tissue, it is not subjected to complex regulatory protocols. There have been some limitations observed with the traditional use of bone marrow derived cells for regenerative medicine. Earlier, after extraction of the bone marrow, the bone marrow sample had to be sent to a GMP facility for processing and isolation of progenitor cells. With the advent of technology, bedside processing of stem cells have become possible where the extraction, processing, as well as the injection of bone marrow cell concentrate can be done quickly. The advantage over traditional cell therapy processing is its rapid cell isolation at the bedside, single sitting procedure for the patient in comparison to the ex-vivo cultured autologous bone marrow cells, reduced cost, low to negligible infection rates and avoids the need for additional personnel. Another limitation of traditional cell therapy was the cost which has drastically come down owing to better technology for administering cell therapy. In our study, a rapid point-of-care device and technology, the Res-Q™ 60 BMC system, was used for processing the autologous bone marrow cell concentrate and has several advantages over the traditional method, as it is a closed, sterile, automated system that processes cells with minimal manipulation. Our proprietary device reduced time, cost and labour-intensive procedure for harvesting, processing and injecting bone marrow cell concentrate at the fracture site and the complete procedure was completed within 60 minutes in the operation theatre. The bone marrow concentrate was allowed to conjugate with synthetic graft. This process is called 'conditioning' of the bone marrow which makes 'putty' for administration between the ends of fractured bone.

Furthermore, the infused cell dose plays a pivotal role in cellular therapy. Hernigou et al. [8] demonstrated in their study that the number of progenitor cells injected play an important role in determining the volume of callus formed and thereby, determine the healing of atrophic non-union fractures. Their group measured the concentration of progenitor cells harvested from bone marrow using the 'Fibroblast- Colony Forming Unit' (CFU-F) assay, and found a direct relationship with the amount of healing and indirect relation with the time of healing versus the concentration of CFU-F. In the same study, they also showed that absolute CFU counts less than 634 ± 187/ mL led to the failure of the procedure [8]. Another study by Desai et al. [29] demonstrated a mean total nucleated cell counts of 66.52 ± 19.47/ mL after concentration and absolute CFU count was 1270 ± 1009/mL, which they concluded as higher than other commercially available concentrate systems. In our study, our point-of-care device was capable of achieving mean Total Nucleated Cell Counts (TNCC) of 5.54 x 10^8 ± 1.99 (69.31 ± 25/ mL), while the Mono-Nuclear Cell Counts (MNCC) were 1.64 x 10^8 ± 0.86 (20.5 ± 10.7/ mL) in the final aBMC product that was administered into the patients. These cell counts were higher than the previously reported studies, and therefore our device could effectively be used for processing at the point-of-care in treating patients with atrophic non-union fractures using concentrated bone marrow cells. The limitation with our study was the absence of CFU assay for better quantification of the injected cells for potency. The sterility testing of the post-processed bone marrow samples using BacT/ALERT 14 day culture showed no bacterial or fungal growth in any of the patient samples. Since, our device is a completely closed system; the chances of infection are minimized and provide a safe and effective treatment procedure.

Researchers are now beginning to understand the differences among the progenitor cells harvested from various individuals. These differences depend on many variables, such as age, gender, and local and systemic diseases [30-32], and the variability in the osteogenic potential from patient to patient represents a limitation of this autologous bone marrow grafting technique [8]. Previous studies have observed that bone marrow cellularity declines with age, and there is also a decrease in the prevalence of connective-tissue progenitors with increasing age [33,34]. However, this was not evident in our small sample size, which is in concurrence with previously published studies by Hernigou et al. [8] Furthermore, our data shows that, bone healing time and age are directly related. However, due to the small sample size statistical significance could not be demonstrated and further large sample size studies are required to prove the exact co-relation. Increased age is associated with decreased bone marrow cellularity and connective tissue progenitors, which may explain our findings of increased bone healing time with increase in age [29].

The exact reason for failure of bone union post cell therapy intervention has not been yet demonstrated in any of the previous studies. Several studies have shown that bone-fracture consolidation is often delayed in heavy smokers and drinkers. Experiments in animals confirmed the fact that nicotine had an adverse effect on bone consolidation [35]. Studies on humans did not find a significant relationship between the prevalence of osteogenic progenitors and smoking, but nicotine increased the activity of osteoclasts, which could be causally related to deficient bone regeneration. Nicotine decreases microperfusion and tissue oxygenation which can lead to platelet aggregation and result in micro clotting [35]. Conversely, a direct analysis of patients with a history of chronic alcohol intoxication has shown that they have abnormally low levels of mesenchymal progenitors in the iliac crests. Among other toxic agents, chemotherapy causes differential effects on mesenchymal progenitors, which are resistant to agents usually used for bone-marrow transplantation in onco-haematological patients but sensitive to a panel of commonly used cytotoxic agents. Therefore, problems of consolidation may be linked to an overall reduction in the number of progenitor cells in the bone marrow [36]. Our analysis identified another risk factor for failure to heal, that is, late treatment of non-union. However, the exact relationship between late treatment for non-union and poor prognosis is less clear. We do accept the possibility that patients treated earlier may not have a true non-union and may be able to heal intrinsically; biological changes may occur over time at the site of non-union, thereby perpetuating the inability to heal. Therefore, the three (3) patients who did not show union of bone in our study, 12 months post cell therapy intervention, could be due to the above mentioned inferences.

The procedure is safe and feasible with no major complications or side effects like hematomas, infections or chronic pain at the aspiration or injection site. In our study, 82% of patients attained bone healing and showed callus formation radiographically at an average of 6 months; however, signs of healing were visible as early as 1.5 months post cell therapy. These results are similar to previously published studies, where Desai et al. [29] reported healing in 79.6% of patients at an average of 4.7 months and Hernigou et al. [8] reported higher healing rate of 88% with faster healing time of 12 weeks on an average. In their study, all patients underwent external fixation or conservative therapy for the initial fracture repair and in our study the primary intervention for all the patients was open reduction internal fixation. The longer healing time in our study could be due to many factors related to the fracture gap size, the underlying co-morbidities and patient age (older age vs younger patients). The limitation of our study was a small sample size and improper quantification of the fracture gap size. Therefore, further studies with larger patient population and proper quantification of gap size are required to better understand the effect of the above mentioned factors on healing time and callus formation. Nevertheless, no major complications were observed, and we have successfully demonstrated the safety and effectiveness of our device and technique in the treatment of non-union of long bones as a reliable alternative to traditional techniques.

The use of autologous bone marrow in non-union fracture sites with plates that had been put previously was discussed in another study conducted on patients of non-union fractures where the site of fracture was the distal tibia [37]. 11 patients were recruited for the study and 9 had an average healing time of 6 months and showed considerable healing with the use of autologous bone marrow cells. A follow up was done for 4.5 years and all patients reported a decline in the pain intensity and interference. Another very small pilot study by Centeno and group [38], where culture expanded mesenchymal stem cells were used to treat non-union in 6 patients (4 females, 2 males), and the treatment intervention was at an average of 8.75 months post-fracture, all but one patient showed improvement. The patient who lacked healing had a chronic fracture that lasted for more than 40 years. Overall the treatment showed faster healing of the fractures and culture expanded MSCs could be used as an alternative cell source for treating non-union fractures. Mesenchymal stem cells have shown their therapeutic capacity in several in vitro and in vivo studies for the regeneration of bone defects and non-unions.

The supply of autologous mesenchymal stem cells is often limited. Nevertheless, their special immunological characteristics suggest that mesenchymal stem cells could be used in non-autologous applications. [39,40]. Also previous studies have shown that percutaneous injections of the autologous bone marrow has been effective in treating distal non-unions or delayed unions after internal fixation [8,41-43].

Some studies have also reported the use of PRP (platelet rich plasma) for the treatment of non–unions fractures. [44-49] Platelet Rich Plasma is the conglomeration of cytokines and growth factors isolated from whole blood using density gradient separation. PRP upon activation releases growth factors such as primarily Transforming Growth Factor-β (TGFβ) and Platelet Derived Growth Factor (PDGF), vascular endothelial growth factor (VEGF), Fibroblast Growth Factor (FGF) and insulin-like growth factor and proteins such as fibrin, fibronectin, vitronectin and thrombospondin, which play a vital role at several stages of tissue healing [50]. With the effect of growth factors, PRP stimulates and activates the local stem cells in the circulation and bone marrow, which plays a major role in fracture healing. In the paper by Simman et al. [44] an animal study was conducted on 48 male rats. The animals femurs' were fractured using a cat nail trimmer and bone fracture was analyzed in X-ray. PRP was injected on fracture sites and the healing was noted. In another study by Bielecki et al. [46], a single dose application of PRP was used for 32 cases of delayed and non-union. The results reported, showed union in the entire delayed union group and in 65% of the non-union group within 11 months after surgery. Therefore, they recommended the application of PRP in the treatment of delayed and non-union fractures. Similarly, in the study by Calori et al. [47], revision surgery together with PRP was compared with the application of bone morphogenetic protein-7 (BMP-7). In 120 cases of atrophic non- union, union was achieved in 86.7% of the BMP group and 68.3% of the PRP group and clinical and radiological healing was reported earlier in the BMP group. While, Griffin and group [48] reviewed the use of PRP in clinical studies and reported that PRP use was safe but no clinical evidence was shown of benefit in acute or delayed fracture union. Also, in the study by Say et al. [49], union was determined in 30% of patients after three doses of PRP application. Results with the application of PRP together with allograft or autograft in surgical treatment have been varied. Some researchers have maintained that PRP has positive effects, while others have claimed that there is no benefit of PRP [51,52].

Bone marrow derived cells are able to elicit angiogenesis by the presence of endothelial progenitors in the cell fraction [53]. The bone marrow concentrate supply abundant number of progenitor cells and angiogenic cytokines that actively engage in vasculogenesis in tissue devoid of vessels and in neoangiogenesis from the pre-existing capillaries [54]. The local ischaemia activates the Hypoxia Inducible Factor (HIF)-1a signalling and mobilisation of circulating progenitors through the Stromal-cell-Derived Factor (SDF1) dependent pathway stimulates the angiogenesis and neovascularization for bone regeneration [55]. These mechanisms may explain the long-lasting effect of cell therapy in bone healing.

Conclusion

In conclusion, our results are encouraging both in terms of patients' safety and bone repair efficacy. While the surgical approach still remains first choice for the management of non-unions. Biomechanical treatment options for non-union and bone healing defects are widely available, whereas biological resources appear to be limited. Cell therapy has also proven to be a highly effective treatment arm within the framework of regenerative medicine. Our data confirm that administration of aBMC may offer the patients, a new therapeutic option for treating non-union fractures where there is no response to the standard orthopedic procedures. Evidence indicates that a combination of factors stimulating neo-osteogenesis and neo-vascularization will restore hard and soft tissue deficits. Since it has not been known, whether the healing of non-unions is dependent on the graft that is being used while preparing bone marrow concentrate preconditioning mix or the presence of stem cells itself, a randomized controlled trial will have to be carried out on larger patient population to find the basis for healing of non-union. Synthetic grafts not only increase the efficacy of the treatment but also have much more controlled chemistry and quality. To clinically demonstrate the effectiveness of this proposed approach after bone injury, additional clinical studies are required, which should examine the roles of the biomaterial scaffold and various concentrations of orthobiologics and their impact on skeletal cell regeneration.

Author contribution

Venkatesh Ponemone: Conception and design, data analysis and interpretation; manuscript writing, final approval of manuscript.

Khushboo Choudhury, Saniya Gupta, Manish Suthar: Manuscript revisions, data collection, data analysis and interpretation

Kenneth Lee Harris, Dalip Sethi, Akshay Saxena, Manish Dalwani, Alok Sharma, Rakesh Mattoo, Nitiraj Oberoi: Data analysis and interpretation.

Harshavardhan Hegde: Conception and design, data analysis and interpretation; Manuscript revisions, final approval of manuscript.

Conflict of interest

None of the authors have a conflict to disclose

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