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Concentrated Autologous Bone Marrow-Derived Mononuclear Cells may have Positive Effect on Treatment of Long Bones Fracture Nonunion

Abstract

Background: Nonunion is a serious complication following long-bone fracture that is known as a therapeutic challenge for surgeons and is associated with significant morbidity. It has been shown that osteogenesis stimulating factors combined with optimization of the mechanical environment could facilitate and accelerate nonunion healing. In this study, we aimed to treat nonunion using autologous bone marrow-derived mononuclear cell (BMDMC) aspirate as a source of osteoprogenitor cells combined with internal fixation.

Methods: From November 2010 to May 2013, 19 cases of nonunion were treated with bone marrow-derived mononuclear cell (BMDMC) grafting, that included 15 males and 4 females with an average age of 37.8 years (range, 18-81 years). The time from injury to therapy was 7 to 28 months, with an average of 13.4 months. At first, decortications were performed around the nonunion site to prepare a suitable bed for bone marrow grafting. Then, 2 ml of bone marrow concentrated cells was applied to the nonunion site in a mixture with partially demineralized cortical cancellous allograft chips. The healing rate in each patient was clinically and radiologically evaluated every 4 weeks.

Results: Bone union was obtained in 18 of the 19 patients during 1.06 to 6 months with an average time of 3.5 months. No complications during anesthesia nor any infection, hematoma or chronic pain at the nonunion site were observed in any patient.

Conclusion: Transplantation of autologous BMDMC aspirate is a reasonable, effective and easy treatment option for tibial and femoral nonunion after internal fixation.

Trial registration: This study has been registrated in ClinicalTrials.gov (**https://clinicaltrials.gov).**
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Keywords: Fracture Ununited, Fracture Healing, Bone Marrow Cells, Bone Marrow Transplantation, Bony Callus

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Introduction

Distal Fracture healing is a complex physiological process involving sequential coordinated action of several different cell types, proteins and gene expression, working toward the restoration of its structural integrity without failure [(1)]. Complete bone regeneration requires a combination of biological and biomechanical therapeutic approaches [(2)]. Despite advanced and optimized surgical procedures, approximately 5 to 10% of fractures fail to achieve bony union, which is known as nonunion [(2)]. Nonunion is a problem in osteosynthesis due to several local and systemic factors; such as the severity of the soft tissue injury, patient-related factors and co-morbidity (heavy smoking, obesity, alcoholism, diabetes, peripheral vascular disease), iatrogenic factors or, simply, segmental bone loss [(3,4)].
An optimal approach for nonunion treatment involves a combination of adequate surgical intervention with some supportive actions, such as control of infection, improvement in nutrition and smoking cessation. Both inadequate stability of the fracture site and impairment of the biological.

response to fracture can be effective in nonunion's formation. Inadequate stability is most frequently addressed by the implantation of new fixation devices, either external or internal devices. Biological deficits are currently treated primarily by autogenous bone graft that provides growth factors and cellular mechanisms for osteoinduction [2,4].

The iliac crest or a bone site near the nonunion is the most commonly used donor site. Unfortunately, retrieval of the autograft may cause excessive morbidity in terms of blood loss, pain and risk of infection that are primarily related to the harvesting procedure. However, allografts are highly likely to trigger an immune response that causes graft rejection. Disease transmission is another problem in allografts (5). These challenges have led surgeons to consider less invasive treatment methods. Cell therapy has been suggested as another treatment approach for fracture nonunion [5]. Bone marrow-derived mononuclear cell grafting has been used in the treatment of nonunion. It is harvested by aspiration and delivered to the nonunion site percutaneously or via open routes. By this method, the rate of union formation and the volume of the callus are related to the number and concentration of progenitor cells in the aspirate [3,4,6].

Currently, different studies have shown that percutaneous autologous bone marrow injection provides a safe, easy and reliable treatment in union promotion. In those studies, bone marrow has been aspirated from the iliac crest and percutaneously injected into the fracture sites after the main operation even as an outpatient procedure. The percutaneous injection has been performed one to three times. Complete union has been observed in most of patients with an average healing time of 3 to 9 months [6,30]. It has been proposed that percutaneous autologous bone marrow grafting is an easy, effective and economic treatment for nonunion. However, the success of this treatment method is dependent on some factors, such as stable internal or external fixation, excessive bone defect and pre-existing angular deformity or shortening [6-14]. In another study, Healey et al. achieved good clinical outcomes under difficult clinical circumstances using bone marrow grafting as a reliable procedure for the nonunion treating. They attempted to treat delayed unions or nonunion by injection of aut ogeneic bone marrow into the site of failed healing in eight patients with primary sarcomas. Five of the patients received chemotherapy, with one patient also receiving radiation therapy. Bone formation was noted in seven (%87.5) patients after injection, and union was attained in five (%63) patients [15].

Herniguou et al. evaluated the effect of the concentration of progenitor cells to obtain bone repair in nonunion. They used the fibroblast colony-forming units method to estimate the number of the progenitor cells. Bone marrow was aspirated from iliac crest, and containing an average number of 612 ± 134 progenitors/cm^3. The aspirate was concentrated on a cell separator and had an average number of 2579 ± 1121 progenitors/cm^3. An average total number of 51×10^3 fibroblast colony-forming units in a relatively constant volume of 20 cm^3 were injected into each noninfected atrophic nonunion site of the tibia. Bone union was achieved in 53 (%98.1) patients who received an average total number of 54,962 ± 17,431 progenitors. These results showed that the concentration of progenitors injected into the nonunion sites of the seven patients in whom bone union was not obtained was significantly

Table 1: Patient demographics and medical history

	Gender	Age (yr.)	Time from initial injury to the present in our study (mo.)	Location of nonunion	Initial treatment	Treatment with bone marrow transfer	Co morbities
1	Male	48	9	Tibia	IMN[1]	IMN	Tobacco use
2	Male	30	18	Femur	IMN	IMN	Tobacco use
3	Male	53	16	Tibia	External fixator	Locking plate	Tobacco use
4	Male	81	13	Tibia	Compression plate	Locking plate	HTN[2]
5	Male	43	24	Tibia	External fixator	DCP[3] plate	NO
6	Male	18	6	Femur	IMN	IMN	NO
7	Female	24	15	Femur	IMN	IMN	NO
8	Male	22	21	Tibia	Compression plate	DCP	Tobacco use
9	Male	22	6	Tibia	Compression plate	Locking plate	Diabetes
10	Female	45	28	Femur	IMN	IMN	Tobacco use and Diabetes mellitus
11	Male	35	6	Femur	IMN	IMN	Tobacco use
12	Male	29	14	Tibia	IMN	IMN	Tobacco use
13	Male	41	8	Femur	Compression plate	Compression	Tobacco use
15	Male	19	13	Tibia	IMN	IMN	Tobacco use
16	Male	24	6	Femur	Compression plate	DCS[4]	NO
17	Female	36	8	Femur	IMN	IMN	NO
18	Female	44	6	Tibia	IMN	IMN	Diabetes
19	Male	33	21	Tibia	IMN	IMN	Tobacco use

[1]IMN: Intra Medullary Nailing
[2]HTN: Hypertension
[3]DCP: Dynamic Compression Plate
[4]DCS: Dynamic Compression Screw

lower than the 53 patients who obtained bone union. They concluded that the efficacy of percutaneous autologous bone-marrow grafting in the treatment of nonunion was related to the number of progenitors in the graft [6].

In this study, we aimed to treat nonunion using concentrated bone marrow-derived mononuclear cell (BMNCs) aspirate as a source of osteoprogenitor cells in an open route surgery. To combine biological and biomechanical therapeutic factors, BMNCs aspirate was applied to the nonunion site in a mixture with partially demineralized cortical cancellous allograft chips after internal fixation.

Methods

Patient Demographics:

Between November 2010 and May 2013, 19 patients were referred to our center and were included in the current study (Table 1). There were 4 females and 15 males with an average age of 37.8 years (range, 18-81 years) at the time of referral and an average of 13.4 months (range, 7–28 months) after their initial injuries. All nonunion were confirmed using the clinical examination like pain or motion in fracture site and inability to bear weight and radiological evaluation according to absence of callus formation and fracture line visibility in AP and lateral views. Eleven patients exhibited a tibial shaft and eight patients had femoral shaft nonunion. Seven primary tibial fractures were identified using the Gasstilo–Anderson classification as type IIIa, and the others were closed. Three patient had more than 2 surgeries for nonunion like exchange nailing or external fixator and others patients had no history of treatment for nonunion. All patients were treated surgically by open reduction and internal fixation (Table 1).

Prior to bone marrow grafting, a full blood count, ESR and CRP were performed for all patients to exclude infective cases. This study was approved by the institutional ethics committee, and all patients were fully informed with respect to the rationale of the study and the related risks and signed an informed consent form.

Marrow Aspiration

All 19 bone marrow samples were harvested from the posterior superior iliac spine while the patient was under general anesthesia and in the prone position. Under sterile conditions, a 2-mm skin incision was performed at the level of the posterior iliac crest. Then, a beveled Jamshidi needle (MEDax ®MODEL 13 G * 100 mm) for bone marrow aspiration was manually pushed approximately 1 cm into the spongy bone of the iliac crest. Next, the marrow was aspirated into a 50-ml plastic syringe. A total volume of 120 to 130 ml of bone marrow was harvested for each patient which was obtained from four different points of both iliac crests. Next, 40,000 IU heparin (HEPARODIC ® 25000 IU) was added to each syringes for 120 ml bone marrow to prevent coagulation.

Concentration and extraction of bone marrow-derived mononuclear cells

The bone marrow mononuclear cells were obtained using the manual Ficoll procedure. Fresh bone marrow aspirate was mixed in a volume ratio of 1:1 with normal saline and carefully layered upon 15 ml of Ficoll-Paque TM Plus. A concentrated buffy coat was obtained after a 30 min centrifugation at 400 g on the ficoll gradient. This buffy coat contained progenitor cells and other mononuclear cells in which some of these other cells are the sources of angiogenic or osteogenic cytokines with a clinical effect. The buffy coat was collected and washed in five volumes of normal saline (10 min at 400 g) for three times. At the end of the procedure, the bone marrow mononuclear cell (BMNC) fraction was resuspended in 2 ml of normal saline, quantified and placed into a syringe for injection (25,26). The bone marrow graft obtained after concentration contained an average of 33 to 560 million cells, with a mean of 195 ± 138.1 million of BMNCs.

SURGICAL PROCEDURE

After bone marrow aspiration and during bone marrow concentration and processing, the surgical interventions were performed by one expert surgeon for all patients. Five patients received a plate exchange, two of which underwent a nail exchange and 12 patients did not receive any additional treatment. In the next step, for preparing a suitable bed for the bone marrow grafting, decortications and freshening around the nonunion site were performed for all patients. Finally, the BMDMCs were applied to the nonunion site in a mixture with partially demineralized cortical cancellous allograft chips (Ceno bone company. Iran).

Postoperative Care

During the first two weeks after operation, the patients were followed for not having inflammation or infection at the site of surgery. When the callus was observed radiographically, partial weight bearing was allowed. The rate of healing in each patient was clinically and radiologically evaluated every four weeks. Radiographs were performed to evaluate the appearance of the callus and to monitor the progression of the callus until bone healing occurred. Successful treatment was determined when there was precise radiographical evidence of the fracture union and an achievement of the clinical criterion of healing within six months after bone marrow grafting. The clinical criterion of healing included the full weight-bearing and no tenderness at the fracture site on palpation [16].

Statistical Methods

All statistical analyses were performed using SPSS Statistics 16.0. Statistical significance was established at a probability value of <0.05. The outcome variables were the success of the treatment and the required time to complete the union after the administration of BMDMCs. The therapeutic factor that could affect the outcome variables was the total number of BMNCs injected at the nonunion site. The patients variables were age, sex, associated comorbidities, site of nonunion and time after their initial injuries. Descriptive statistics were performed for all variables, and a multivariate analysis was performed to evaluate the relationship between the outcome and variables.

Results

There were no complications during anesthesia; principally, no decrease in oxygen saturation or a change in pulse or blood pressure was observed during the procedure in any patient. Moreover, no occurrence of compartment syndrome was developed in any patient after bone marrow grafting. There were no infections, hematomas, or chronic pain at the nonunion site.

Patient and Bone Marrow Variables

An average volume of 120 ± 10 ml of marrow was aspirated from both iliac crests of each patient. The number of all mononuclear cells obtained from the individual patients ranged from 33 to 560 million cells, with a mean of 203.15 million cells. The number of mononuclear cells did not have a significant relationship with the patient's age. The mean number of mononuclear cells in males (172.3 ± 127.6 million) was lower compared to its number in females (295.7 ± 150.9 million), but this difference was not statically significant ($p = 0.111$). With the number of patients available, the comorbidities of smoking, diabetes, and use of pharmaceutical agents were not associated with a significant changes in the population of harvested cells.

Outcomes of Management of the Non-union

Bone union was obtained in 18 of the 19 patients (Nonunion outcome variables were defined for the success of the treatment as the starting time for the appearance of the callus and the complete healing time). The callus typically appeared on radiographs between 12 days to 10 weeks, with an average time of 4.9 weeks after bone marrow grafting (that was typically shown for patient No.5 in Fig 1). Radiographical evidence of the complete union was observed at an average of 3.5 months (ranged from 1.06 to 6 months). Statistical analysis showed that the sex, location of the nonunion, number of mononuclear cells and the time between the initial injury and administration of the BMDMC treatment did not significantly effect neither the starting time for callus appearance nor the healing time. Among the variables that were examined using t-test analysis, only age significantly affected the healing time ($p < 0.001$) without a significant effect on the starting time of union formation. Finally, there was a significant relationship between comorbidities and the time for union ($p = 0.004$): Twelve patients with one or more comorbidities showed a longer time to heal (average, 14 weeks) in comparison with the other patients (average, 10.5 weeks).

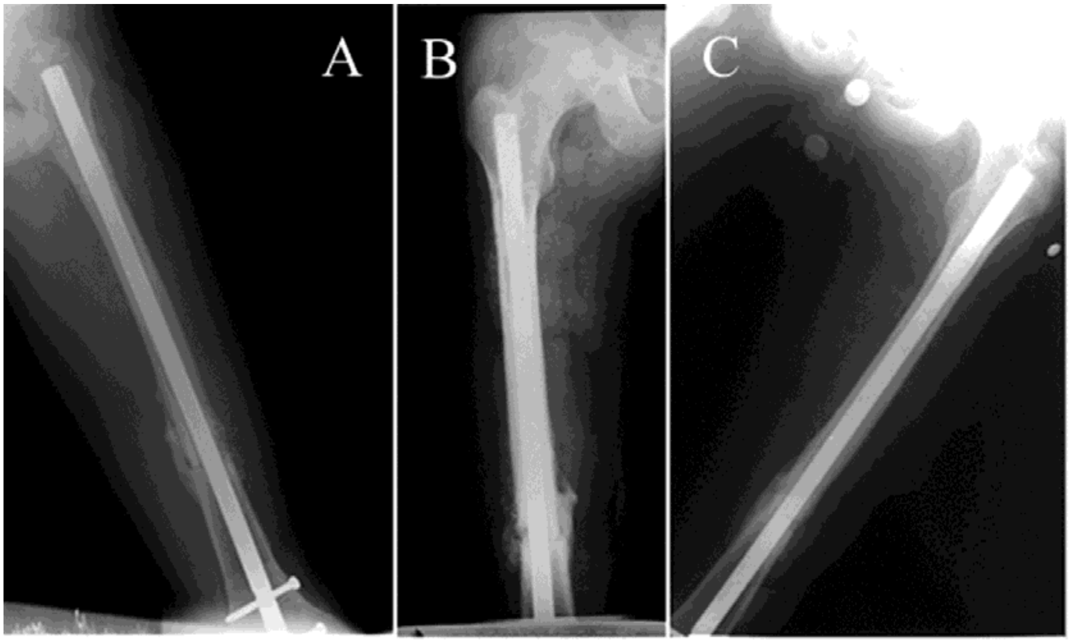

Fig. 1 Radiographs analysis of callus formation in patient No 5. A; one day, B; one month and C; five months after surgery.

Among the 19 patients, one patient did have neither union formation nor evidence of callous formation. In addition, she had pain at the nonunion site. This patient did not have any known risk factor for nonunion i.e., diabetes mellitus, smoking, etc., and her fracture site was in the subtrochantric region, which was fixed by the intramedullary nail.

Discussion

Even with using multiple common procedures, treatment of nonunion still remains a big challenge in orthopedics . Bone regeneration requires a combination of biological and biomechanical therapeutic approaches, so numerous treatment options have been developed to treat nonunion, including invasive interventions (such as internal fixation with or without the use of bone graft substitutes) and noninvasive procedures (such as using ultrasound or pulsed electromagnetic fields) [6,14]. Autogenous bone marrow graft is a biological resource that provides growth factors and cellular requirements for osteoinduction [2,4]. It is commonly harvested by aspiration from the iliac crest and can be delivered percutaneously or via open routes to the nonunion site [3,4,6]. Numerous studies have shown that percutaneous autologous bone marrow injection can be a safe, easy and reliable treatment for nonunion [27,28,29]. There are some limitations for this procedure such as there should not be any pre-existing angular deformity or shortening that requires direct access to the nonunion site. Also, the fracture fragment gap size should be less than 5 mm (6, 11, 14). Finally, the volume of callus and the healing rate are related to the number and concentration of osteoprogenitor cells in the aspirate (3, 4, 6).

In this study, we aimed to treat nonunion using autologous concentrated bone marrow aspirate in an open route surgery for avoiding the limitation of percutaneous bone marrow injection. Thus, a total volume of 120 to 130 ml of bone marrow was harvested from the iliac crest and concentrated using a manual Ficoll procedure (26). The concentrated buffy coat, which contained BMNCs, was resuspended in 2 ml normal saline and applied to the nonunion site in a mixture with partially demineralized cortical cancellous allograft chips. The concentrated iliac bone marrow grafts contained an average of 33 to 560 million mononuclear cells (most important factor is age). Seven patients had an implant exchange prior to receiving BMDMCs. The results indicated that 18 of the 19 nonunion patients achieved bony union between 1 to 6 months after this procedure. Infection or other complications were not observed in any patient. These results confirmed the usefulness of this method for the treatment of nonunion in long bones.

Historically, resection of the fibrous tissue at the nonunion site combined with mechanical stabilization has been described as an essential method for the treatment of an atrophic nonunion (6). In this study, we similarly used bone marrow grafting for bioactive cell stimulation in combination with internal fixation to treat nonunion. Based on this fact, there is no specific data regarding the number of cells that is necessary to obtain bone-healing, we used a similar volume of aspirate and similar volume of concentrated bone marrow aspirate for transplantation in all patients. The number of mononuclear cells obtained from the individual patients ranged from 33 to 560 million cells. Because the total number of progenitor cells represent the product of nucleated cells, more nucleated cells indicate more progenitor cells in the aspirate (6). Different studies have shown a direct relationship between the volume of the callus and the rate of nonunion healing with the number of progenitor cells in the graft (6, 17-19). Concentrating the aspirate on a cell separator and using a porous implantable material have been reported as alternative methods for the concentration and selection of connective tissue progenitors (6, 20-22). In this study, the bone marrow concentration was performed using a Ficoll procedure and the concentrated cells were transplanted to the nonunion site in a mixture with partially demineralized cortical cancellous allograft chips. However, the number of cells needed to start callus formation has not yet been determined. Hernigou et al. proposed that the importance of the number of cells may be related to their survival after transplantation, which is under the effects of limitative factors, such as the amount of available oxygen (6). Because the transplanted cells compete with other cells for oxygen, a higher number of transplanted cells can reduce the availability of oxygen that cause to decrease cell viability and thus no significant effect on the healing time.

Different studies have shown a decreased prevalence of connective tissue progenitors and bone marrow cellularity with increasing age, gender, and local or systemic disease [17, 23, 24]. However, this was not obvious in our small series of patients. Moreover, no significant effects of sex, location of the nonunion, number of mononuclear cells and the time between the initial injuries to bone marrow grafting was observed on the nonunion healing time. On the other hand, there was a significant relationship between age and nonunion healing time (p = 0.001). These dissimilarities could be observed in other studies due to the relatively small patient samples that are typically available for studies of nonunion. This limitation caused significant variation among selected patients with respect to diagnosis (i.e., nonunion or delayed union), long bones (i.e., tibia, femur and humerus), anatomical location (i.e., metaphysic or diaphysis), surgical fixation or other treatments in conjunction with BMDMCs. Each of these factors can affect the results, that provoke a challenge in the interpretation of the reported results across published studies [6,14,23]. The other potential weakness of these studies was the absence of a cohort with a placebo treatment, such as injection of saline solution [6]. Despite these limitations, the results of this study showed that about 94% of nonunion patients achieved bony union between 1 to 6 months after bone marrow grafting, which confirmed the usefulness of this method for the nonunion treatment. There are few limitation of the present study including: use of demineralized chips allograft, exchanging the fixation method with different implant and removal of fibrous tissue in non union site. Therefore the effect of aut ologous bone marrow aspiration on union can not be isolated as single factor also we can not give definite guideline on needed number of cells.

Conclusion

In conclusion, transplantation of autologous concentrated bone marrow aspirate is a reasonable, probably effective, minimally invasive and easy treatment option for tibial and femoral nonunion after internal fixation. This improves the activity of osteoblasts in fracture sites, which can accelerate fracture healing. On the other hand, bone marrow aspiration from the iliac crest did not limit treatment and did not cause a longer hospital stay. So, this procedure is proposed to be a suitable treatment for patients who have long-term nonunion with several previous unsuccessful and inconclusive surgeries.

List of abbreviations:

BMNCs: Bone Marrow Mononuclear Cells
IMN: Intra Medullary Nailing
HTN: Hypertension
DCP: Dynamic Compression Plate
DCS: Dynamic Compression Screw

Ethics approval and consent to participate:

This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Also, this study was approved by the institutional ethics committee of Mashhad University of Medical Sciences (code No:900939-93.5.21). Informed written consent was obtained from each participant.

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