Incomplete Eradication of Persistent Infection May Impede Union: Our Experience in Treating Fracture Non-Unions with Allogenic MSC

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Abstract

Introduction : Non-union remains a major clinical challenge for orthopaedic surgeons, as the treatments are associated with risks for complications, and sometimes multiple surgeries are required. Mesenchymal stem cells (MSCs) have been found to aid in osteogenesis and fracture healing; however, the number of studies on MSC application for treating non-unions is still sparse. We present a translational study of 8 subjects treated with MSC implantation, along with those considered as standard treatments in treating non-unions. To our knowledge, this is the most extensive clinical study on the use of MSCs to treat fracture non-unions.

Methods: We performed 20x10 6 units of MSC implantations derived from adipose tissue, bone marrow, and umbilical cord on subjects diagnosed with fracture non-union of the long bone, along with internal fixation and hydroxyapatite-calcium sulphate (HA-CaSO 4) pellets. We excluded pathological fractures, subjects with immunological deficiencies (type II diabetes mellitus, and HIV/AIDS), and subjects with a history of immunosuppressive therapies. All subjects were assessed using the Disabilities of the Arm, Shoulder, and Hand (DASH) or Lower Extremities Functional Scale (LEFS), and visual analog score (VAS). Serial radiological images were also assessed using Tiedeman and Lane-Sandhu scoring to determine union. Follow up assessments were performed every three months for at least 12 months or until clinical and radiological union was achieved.

Results: Four (50%) out of eight subjects developed union in a median of five (3-12) months. There was a reduction of VAS, from a median of 1 (0-6) to 0 (0-4), and an increase in mean LEFS/DASH of 56.25 ± 10.71 to 65 ± 22.72. However, the infection was identified in 3 (37.5%) subjects. Methicillin-resistant Staphylococcus aureus (MRSA) was identified in two (25%) subjects, while one was infected with Escherichia coli . No other adverse events occurred during the follow-up period.

Conclusion: Allogenic MSC implantation can be used as a potential and safe therapy for fracture non-union. However, the presence of infection may interfere with bone healing; thus, thorough eradication of infection must be ensured to achieve fracture union. Further clinical studies are required to investigate the safety and efficacy of allogeneic MSC implantation.

Introduction

Treating non-unions remains an arduous task in orthopaedics and traumatology surgery. Previous studies discovered the overall relative risk of non-union in long bone fracture to range from 1.9–10%, with observable peaks in young adults.[1,2] Inadequate care of several factors may contribute to the diminution of bone healing, which includes mechanical, biological, and infection.[3,4] Among these, an infection may be present in up to 40% of non-union cases.[4] Failure to address and treat these problems may lead to devastating outcomes for patients, such as permanent disability.

While there is no universal consensus on standardized diagnostic criteria and therapy to treat fracture non-unions,[5–7] standard procedures include reconstructive surgery using bone grafts or synthetic granules to achieve union. However, outcomes from this procedure vary widely and can lead to a series of revision surgeries.[2] Different bone grafts are used in cases where defects are present; these can range from autograft, allograft, or synthetics grafts. After ten years, graft failure is still observed in approximately 60% of cases, leading to recurring non-union.[8]

Fracture healing consists of several complicated processes from hematoma formation until bone remodeling, which includes mesenchymal stem cell (MSC) recruitment in the acute inflammatory phase. The recruited MSCs, which were hypothesized to be derived from the surrounding tissues, will aid in the bone synthesis and regulate bone remodeling and angiogenesis.[8,9] Previous studies have found that bone marrow mesenchymal stem cell (BM-MSC) implantation has improved bone healing in patients with non-union and critical-sized bone defects. However, the obtainment of BM-MSC may expose donors to potential morbidities, such as sciatic nerve injury, hemorrhage, pain, and infection.[10–13] Stem cells derived from umbilical cord and adipose tissue have attracted researchers as the accretion is not as invasive; therefore, may avert donors from the previously mentioned drawbacks.[14,15] Adipose-derived stem cells (ADSCs) and umbilical cord mesenchymal stem cells (UC-MSCs) were shown to aid in osteogenesis and fracture healing as demonstrated in the previous studies.[14–17]

This study is aimed to investigate the efficacy of allogeneic MSC implantation, regardless of the origin, in treating non-union of the long bones.

Methods

Subject Selection

This case series included subjects diagnosed with fracture non-union of the long bone, defined as a disturbance of bone growth after nine months or absence of the bridging callus in the first three months consecutively, in Cipto Mangunkusumo General Hospital (Jakarta, Indonesia) between 2014 and 2018. Subjects with long bone fracture non-union aged 0 to 55 years old were enrolled in this study. We excluded cases of non-union due to pathological fracture (e.g., primary or secondary bone malignancy), subjects with immunologic deficiencies (e.g., HIV/AIDS, type II diabetes mellitus, hepatitis), and subjects undergoing immunosuppressive therapy (e.g. chemotherapy, corticosteroid regimens).

MSC Acquisition

MSC used for implantation were derived from adipose tissue, bone marrow, and umbilical cord. All donors were screened for HIV, Hepatitis B, and Hepatitis C. BM-MSC was extracted from donors aged 19 to 30 without comorbidities (e.g., type II diabetes mellitus, cardiovascular diseases, autoimmune diseases). All donors are positioned on an operating table under local anesthesia. Aspiration needle is inserted 45° into the iliac crest, and then the needle hub is removed and connected to a 20 ml syringe filled with 1 – 2 ml of 1,000 IU/ml heparin. The bone marrow is aspirated by pulling the syringe plunger backward rapidly. Several syringe rotations are made to retrieve aspirate in different sites. Next, the aspirate is transferred into a 50 ml sterile polypropylene tube. Lastly, the aspiration needle is removed, and pressure is applied on the skin, followed by dressing of the wound. UC-MSC was obtained from elective cesarean sections from mothers with uncomplicated full term (37 – 42 weeks) pregnancy, and ADSC was obtained from adipose tissue residues from liposuction procedures. The MSCs attained were collected and stored in sterile containers filled with 0.9% NaCl at 4°C. Cells were processed within 8 hours following the corresponding procedure.

Culture, Characterization, Cryopreservation, and Activation of MSC

The obtained bone marrow, umbilical cord, or adipose tissue was processed in a Good Manufacturing Practices (GMP)-standardized culture laboratory at the Stem Cell Medical Technology Integrated Medical Service Unit of Cipto Mangunkusumo General Hospital at the Faculty of Medicine at Universitas Indonesia (Jakarta, Indonesia). Processing was performed using the multiple harvest-explant methods as detailed by Pawitan et al.[18]. Cell culture was performed using the appropriate medium and subcultured until confluence was achieved, and then the cells can be harvested (approximately in 21 – 28 days). Characterization is conducted using flow cytometry and cells are declared as MSC with CD105, CD90, and CD73 expression ≥95 % with CD34 and CD45 expression ≤2%. Cells from passages 3 to 6 were then implanted onto recipients with non-union. Part of the characterized MSC was then stored inside -180°C nitrogen tanks for cryopreservation. Cryopreserved MSCs were activated and analyzed for its viability every three months.

Intervention

Each subject was given $20x10^6$ units MSC and hydroxyapatite-calcium sulphate (HA-$CaSO_4$) pellets (Bongros-HA, Bioalpha, Seongnam, Korea) per defect site. MSC was diluted in 10cc per cm^3 defect conditioning medium, then transferred into a container filled with HA-$CaSO_4$. Each container was incubated for 5 minutes before implantation. MSC and HA-$CaSO_4$ components were then implanted into the non-union / defect site while installing bone fixation. After the soft tissue was sutured, the remaining serum was then injected into the defect surroundings using a 5cc syringe. For cases with infected non-unions, subjects were treated with gentamicin-loaded cement spacer on the fracture site before definitive fixation surgery.

Outcome Measurement and Follow Up

The clinical and radiological evaluation was performed every three months postoperatively until clinical and radiological union was achieved, or up to 12 months post-op. Clinical evaluation was performed using the Disabilities of the Arm, Shoulder, and Hand (DASH)[19] questionnaire for non-union in the upper extremities, and the Lower Extremities Functional Scale (LEFS)[20] questionnaire for lower extremities. DASH scores ranges from 0 (most functional) to 80 (least functional); LEFS scores ranging from 0 (least functional) to 10 (most functional). All DASH and LEFS scores were translated into percentages for comparative purposes. Each subject's visual analog score (VAS) was assessed in every follow-up meeting as well.

Radiological results were assessed using Tiedeman[21] (Table 1) and Lane-Sandhu[22] (Table 2) scoring. The Tiedeman score is calculated by totaling the score in each aspect. A radiological union is considered achieved when Lane-Sandhu score is ≥2 and Tiedeman score ≥6.

Results

Of the eight subjects included in this study, four subjects achieved union, with the time of union ranging from two to 12 months. One subject requiring reimplantation during follow up: Subject 3 underwent three separate MSC implantations due to recurrent infections resulting in non-unions. (Fig. 1–8) Despite four reported cases of radiological non-unions, three subjects showed a slight improvement in functional activity and pain, as assessed using the the LEFS/DASH questionnaires and VAS score.

Discussion

Non-union remains a challenging problem for orthopaedic surgeons, as it delays recovery and often requires multiple follow-up procedures. Numerous methods of treatment, such as autologous bone graft and allograft bone chips, are available for this condition; however, they are often associated with drawbacks and high costs.[23] In recent years, mesenchymal stem cell implantation has garnered interest worldwide due to its regenerative capacity.

Moreover, both preclinical and clinical studies have shown that MSC can aid in bone regeneration.[15,24–27] The most common source of MSC is bone marrow; however, its isolation requires an invasive procedure, often causing pain and discomfort to the donors. Other accessible sources include adipose and umbilical cord tissue, which both obviate the need for invasive isolation as they are byproducts of liposuction and childbirth. In this study, we investigate the safety and efficacy of ADSCs and UC-MSCs for treating nonunion.

In this study, 5 (62.5%) subjects developed union in 2–12 months. To our knowledge, this is the most extensive series of allogeneic MSCs in subjects with nonunion. Previously, we administered allogeneic UC-MSCs for infected non-union femoral shaft fracture with a 12 cm bone defect.[15] There was a reduction of VAS, from a median of 1 (0–6) to 0 (0–4), and increase in mean LEFS/DASH of 56.25 ± 10.71 to 65 ± 22.72 in one year of follow-up. In an animal model, Qu et al. found that the administration of UC-MSCs resulted in disappearance of fracture line at eight weeks.[28] Most subjects still have significant LLD following the procedure that may interfere with daily activities. However, the primary outcome of this study is to achieve union, while this issue can be managed with additional procedures following bone union, such as bone lengthening using the Ilizarov apparatus or lengthening and intramedullary nailing. Regardless the rarity of the occurrence, it should be noted that this procedure might expose the subjects to risks of complications, such as pain, infection, and failure of treatment.[29–31]

The mechanisms by which exogenous MSC implantation enhances fracture healing have been extensively studied. It was previously postulated that fracture healing occurs due to differentiation of transplanted cells; however, in non-unions without critical bone loss, the implanted MSCs largely act as cellular modulators, instead of directly differentiating, as the differentiation of transplanted MSC at the site was less efficient.[15,32–34] Contrary to previously held beliefs, it is the release by MSCs of their secretome, an assemblage of paracrine factors (e.g. cytokines, growth factors, etc.), into the extracellular environment that plays the crucial role in fracture healing.[34,35] Several cytokines contained in secretome include insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and hepatocyte growth factor (HGF). These factors enhance migration and recruitment of osteoprogenitor cells (osteoblasts) to the implantation site and assists in

upregulation of cell proliferation and differentiation.[15,35] Besides, these factors also aid in angiogenesis, in order to fulfill oxygen and nutritional requirements during fracture healing.[35]

In comparison from other types of stem cells, MSC poses a shallow risk when implanted. Implantation of exogenous MSC, whether differentiated or undifferentiated, does not cause alloreactive lymphocyte proliferation and does not elicit further immune responses. This suggests that MSC is a safe and potential alternative therapy for treating nonunion.

Contact between fracture fragments should be established to assist bridging between the two fragments. In several cases in our study, we utilized double-plating to ensure contact, alignment, and mechanical stability of the fracture site. In a report by Steinberg et al., double-plating may help in aiding mechanical stability in femoral distal or supracondylar fractures by reducing lever arm and load on the fracture size, which improves fracture stabilization and prevents implant loosening.[36,37] We also performed dynamization on subject Bub (case no. 4) by removing the distal screw in the tibia after intramedullary (IM) nailing. Dynamization may promote fracture healing and can be proposed as the primary treatment in non-union cases.[6,38,39] However, it should be taken into consideration that the use of intramedullary reaming and nailing, in particular, affects the microvasculature of the bone. As demonstrated in an animal study, bone reaming may destroy the endosteum microvasculature, which is usually followed with vasculature hypertrophy of the periosteum.[40]

Status of infection may affect bone healing. Changes in the biological environment caused by open fractures may alter the healing process and lead to further septic complications.[41] Infection, in coexistence with metal fixtures, may cause osteolysis, loosening, and mechanical failure, eventually making union harder to achieve.[42,43] Therefore, eradication of infection must be assured to establish bone union. Radical resection should be performed as needed, debriding all infected and potentially infected non-viable tissue.[44] One approach to aid eradication of infection is by performing the Masquelet procedure, which includes local antibiotic delivery as its first step. Subject compliance is another external aspect to be taken into consideration, as antibiotic therapy may be prolonged in this condition.

We observed several complications during our study, in which three experienced infections during follow up. Tissue culture examination revealed two methicillin-resistant Staphylococcus aureus infection. Both subjects were referred to the tropical diseases and infection specialists in our hospital and treated with appropriate antibiotics according to the sensitivity test. Subject 7 (Tas) was administered oral rifampicin and trimethoprim/sulfamethoxazole and underwent sequestrectomy, debridement, and antibiotic-loaded bone cement application. Subject MYu (case no. 2) was given oral trimethoprim/sulfamethoxazole. Multiple MSC implantations with Masquelet procedure stage II were performed on Subject 3 (Suy); however, radiological union has been achieved. The subject complained of mild to moderate pain during follow up (VAS: 3–5) and wound dehiscence. Culture examination revealed Escherichia coli infection, and the subject was treated with oral trimethoprim/sulfamethoxazole according to the sensitivity test. The subject was planned for another debridement and another round of the Masquelet procedure.

Currently, the application of bone morphogenetic protein (BMP) as an osteoinductive agent in treatment is being investigated. It is hypothesized that the addition of recombinant human bone morphogenetic protein 2 (rhBMP2) may assist proliferation and differentiation of MSC, while recombinant human bone morphogenetic protein 7 (rhBMP7) may support osteoblast differentiation.[45] Unfortunately, results of multiple studies regarding the use of BMP remain inconclusive.[46,47] We are further exploring the possibility of incorporating BMP and MSC treatment.

Conclusion

Allogeneic mesenchymal stem cells could be used as a potential therapy for fracture nonunions. However, the presence of infection may interfere with bone healing; thus, thorough eradication of infection must be ensured to achieve fracture union. However, further more extensive clinical studies are required to investigate their safety and efficacy.

Abbreviations

AIDS
acquired immunodeficiency syndrome

ADSC
adipose-derived stem cells
BM-MSC
bone marrow mesenchymal stem cells
BMP
bone morphogenetic protein
DASH
the Disabilities of the Arm, Shoulder, and Hand
GMP
Good Manufacturing Practices
HA-$CaSO_4$
hydroxyapatite-calcium sulphate
HGF
hepatocyte growth factor
HIV
human immunodeficiency virus
IGF-1
insulin-like growth factor-1
IM
intramedullary
LEFS
Lower Extremities Functional Scale
LLD
leg length discrepancy
MSC
mesenchymal stem cells
MRSA
methicillin-resistant Staphylococcus aureus
rhBMP2
recombinant human bone morphogenetic protein 2
rhBMP7
recombinant human bone morphogenetic protein 7
TGF-β
transforming growth factor-β
UC-MSC
umbilical cord mesenchymal stem cells
VAS
visual analogue score
VEGF
vascular endothelial growth factor

Declarations

Ethical Approval and Consent to Participate

This study has undergone ethical review and received approval from the Health Research Ethics Committee at Cipto Mangunkusumo General Hospital – Faculty of Medicine, Universitas Indonesia, reference no. 165/H2.F1/ETIK/2014. The protocol has been registered in ClinicalTrials.gov Protocols Registration and Results System, registration no. NCT02307435.

All patients have signed written consents to participate in this study.

Consent for Publication

All patients have signed written consents for their clinical information to be published in this study.

Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

All authors declared no conflict of interests.

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Authors' Contributions

IHD constructed the concepts of this research, performed the operation, and performed the data analysis.

ALH collected the data, performed the data analysis, and wrote the article.

JAP led the manufacturing of MSC used in this study, performed the data analysis, and reviewed the article.

IKL led the manufacturing of MSC used in this study, performed the data analysis, and reviewed the article.

NDY analysed and interpreted the radiological results.

All authors have read the final version of this article and approved for publication.

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Tables

Table 1. Lane-Sandhu[22] radiographic scoring

Score	Description
0	No callus
1	Minimal callus formation
2	Callus evident and beginning of the osseous formation
3	Callus evident and fracture line almost obliterated
4	Complete union with complete remodeling

Table 2. Tiedeman[21] radiographic scoring

Score	Description
Bone formation	
0	No bone formation
1	25% of defect
2	50% of defect
3	75% of defect
4	100% of defect
Union	
0	Intact fracture line
2	Partial fracture line
4	Absence of fracture line
Remodeling	
0	No remodeling
1	Remodeling on 1 side of the cortex
4	Remodeling on both side of cortices

Table 3. Results

No	Patient	Sex	Age	Diagnosis	Procedure	MSC Source	Time to Union	VAS		LLD		LEFS / DASH		Follow Up Period
								Initial	Follow Up	Initial	Follow Up	Initial	Follow Up	
1	SMa	F	54	NU, right humerus (Previous ORIF P/S)	ORIF reconstruction with double plate and bone reconstruction	UC	6 months	6	0	n/a	n/a	68.7 %	12.96 %	16 months
2	MYu	M	51	Infected NU, right proximal tibia (Previous ORIF P/S)	ORIF P/S with double plate	AD	-	0	3	2 cm	2 cm	61.25 %	48.75 %	12 months
3	Suy* (3)	M	49	Infected NU, right supracondylar femur (Previous 7x ORIF P/S)	Masquelet procedure, ORIF P/S	1. AD 2. BM 3. UC	-	3	3	2 cm	2 cm	41.25 %	50 %	36 months
4	ESu	M	61	NU, left femoral shaft (Previous ORIF P/S and IM nail)	ORIF P/S, bone grafting	UC	-	3	0	1 cm	1 cm	36.25 %	73.75 %	12 months
5	Bub	M	37	NU, left tibia (Previous IM nail)	IM nail, dynamization / distal screw removal	UC	3 months	2	0	2 cm	2 cm	55 %	80 %	23 months
6	Sut	M	20	Atrophic NU, right proximal femur (Previous 2x ORIF P/S)	ORIF P/S	UC	12 months	0	0	12 cm	8 cm	51.25 %	82.5 %	15 months
7	Tas	M	63	Infected NU, left femoral shaft (Previous ORIF P/S)	IM nailing, bone cement	UC	-	0	4	9 cm	7 cm	60 %	61.25 %	14 months
8	JNe	M	23	NU, left tibia (Previous IM nail, ORIF P/S, and bone grafting)	ORIF P/S, double plate	UC	4 months	0	0	6 cm	6 cm	57.5 %	68.75 %	12 months

*DASH: disabilities of the arm, shoulder, and hand score; IM: intramedullary; LEFS: lower extremities functional scale; LLD: leg length discrepancy; NU: non-union; ORIF: open reduction internal fixation; P/S: plate and screw; VAS: visual analog scale; * indicates patient received MSC implantation more than once*

Table 4. Complications observed during our study

Complications	n
Number of subjects	8
Radiological non-union	4
Infection	3
Methicillin resistant *Staphylococcus aureus*	2
Escherichia coli	1
Follow up surgery	
MSC reimplantation	2

Figures

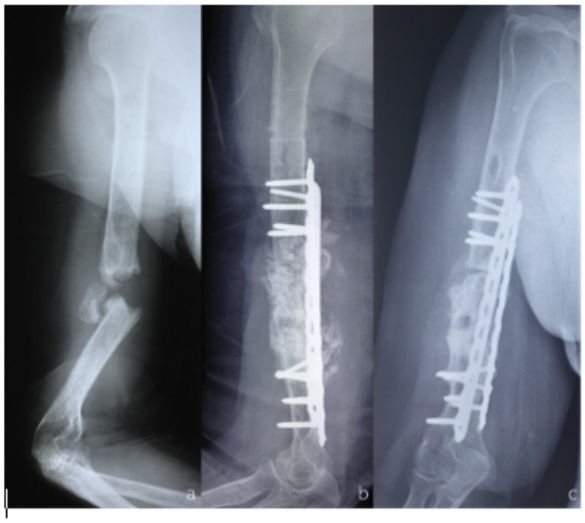

Figure 1

Radiograph of patient SMa (a) Pre-implantation; (b) Post implantation; (c) 12 months post implantation.

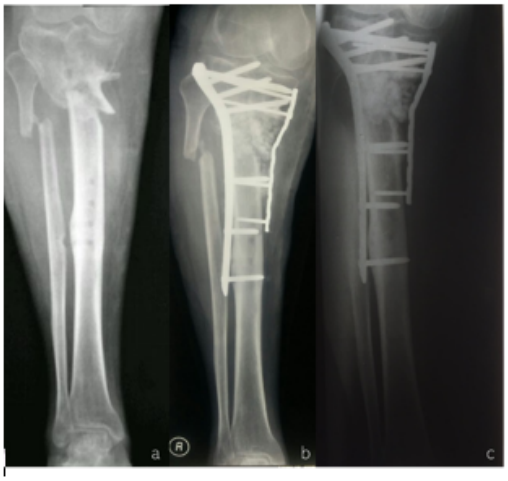

Figure 2

Radiograph of patient MYu (a) Pre-implantation; (b) Post implantation; (c) 6 months post implantation.

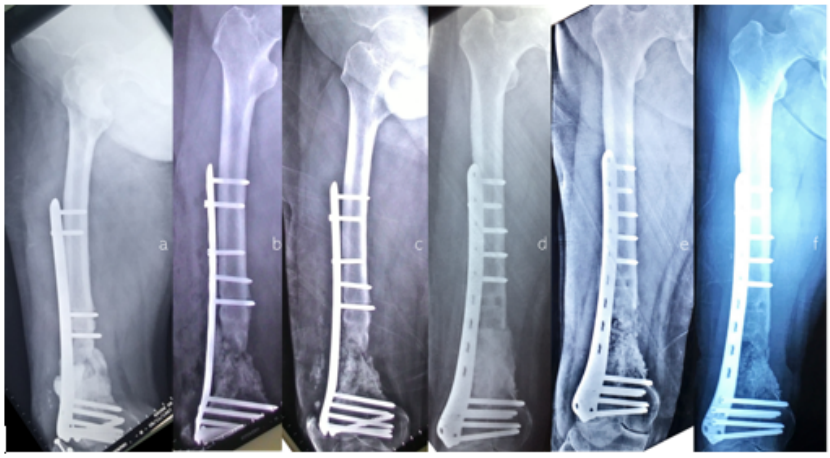

Figure 3

Radiograph of patient Suy (a) Pre ADSC implantation; (b) 10 months post ADSC implantation; (c) Immediately post BM-MSC implantation; (d) 8 months post BM-MSC implantation; (e) Immediately post UC-MSC implantation; (f) 12 months post UC-MSC implantation.

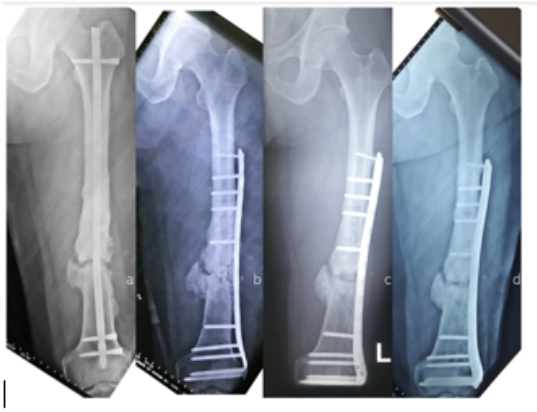

Figure 4

Radiograph of patient ESu (a) Pre-implantation; (b) Post implantation; (c) 7 months post implantation; (d) 12 months post implantation.

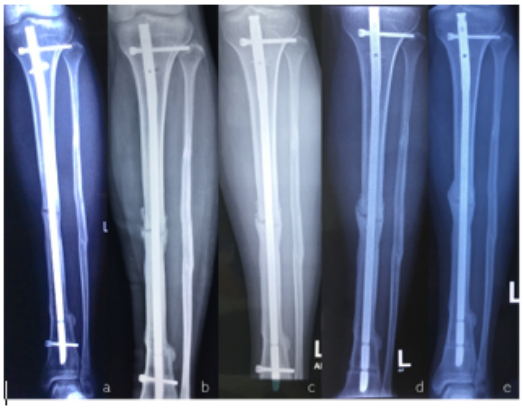

Figure 5

Radiograph of patient Bub (a) Pre-implantation; (b) Post implantation; (c) 6 months post implantation; (d) 14 months post implantation and distal screw removal; (e) 23 months post implantation

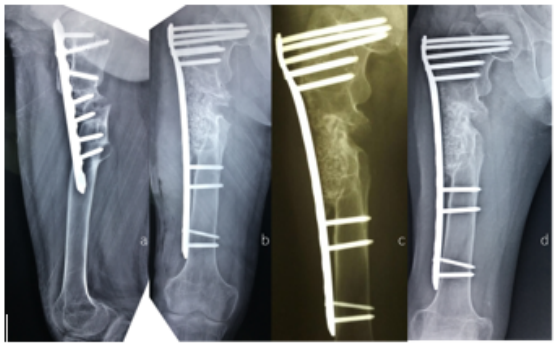

Figure 6

Radiograph of patient Sut (a) Pre-implantation; (b) Post implantation; (c) 6 months post implantation; (d) 12 months post implantation.

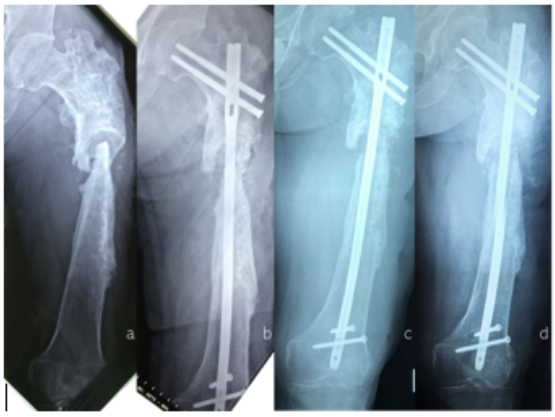

Figure 7

Radiograph of patient Tas (a) Pre-implantation; (b) Post implantation; (c) 6 months post implantation; (d) 12 months post implantation.

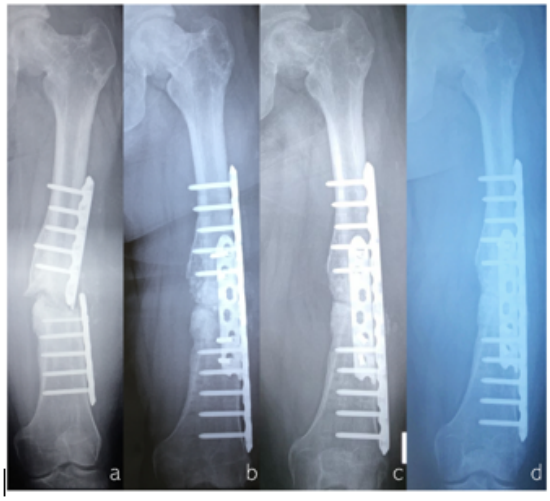

Figure 8

Radiograph of patient JNe (a) Pre-implantation; (b) Post implantation; (c) 6 months post implantation; (d) 12 months post implantation