# EFFECTS OF SHOCK WAVES ON OSTEOGENETIC POTENTIAL OF MESENCHYMAL STEM CELLS DERIVED FROM FIBROUS HAMARTOMA IN CONGENITAL PSEUDARTHROSIS OF TIBIA

A dissertation submitted in partial fulfilment of the rules and regulations for MS Orthopaedics examination of the Tamil Nadu Dr. M. G. R Medical University, Chennai, to be held in May 2019.

# CERTIFICATE

This is to certify that the dissertation titled, "**Effects of shock waves on osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia**" is a bonafide work of Dr. John Premnath, in the Department of Orthopaedic Surgery, Christian Medical College and Hospital, Vellore in partial fulfillment of the rules and regulations of the Tamil Nadu Dr. M.G.R Medical University for the award of M.S Degree Branch II (Orthopaedic Surgery), under the supervision and guidance of Dr. Vrisha Madhuri during the period of his post-graduate study from April 2016 to May 2019.

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# DECLARATION

I hereby declare that this dissertation titled "**Effects of shock waves on osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia**" was prepared by me in partial fulfillment of the regulations for the award of the M.S Degree (Final) Branch II (Orthopaedic Surgery) of the Tamil Nadu Dr. M.G.R Medical University, Chennai towards examination to be Held in May 2019. This has not formed the basis for the reward of any degree to me before and I have not Submitted this to any other university previously.

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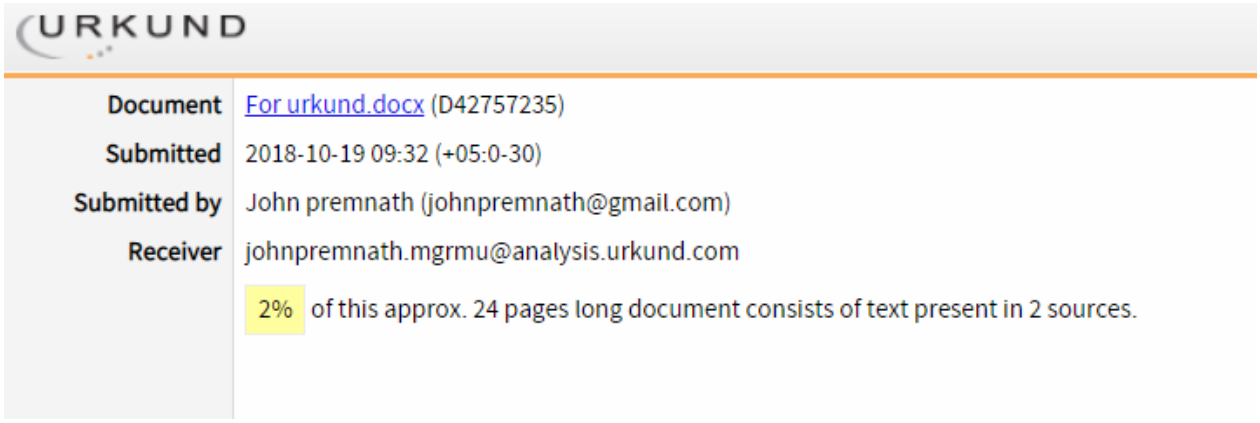
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# AIM & OBJECTIVES

## AIM

To describe effects of shock waves on the Osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia.

## OBJECTIVES

1) To assess the effects of focal shock wave treatment on proliferation of mesenchymal stem cell derived from fibrous hamartoma in congenital pseudarthrosis of tibia.

2) To assess the effects of focal shock wave treatment on Osteogenic differentiation of mesenchymal stem cell derived from fibrous hamartoma in congenital pseudarthrosis of tibia.

# REVIEW OF LITERATURE

## Introduction:

Congenital Pseudarthrosis of tibia is a rare childhood disease , the incidence of CPT reported is 1:53,000 in Norwegian population(1) and 1:28000 in Finland (2). It is a disease of the tibial diaphysis which presents with Pseudarthrosis at birth, a pathological fracture or deformities like bowing, narrowing of the medullary canal or as a cyst, which fractures spontaneously or after minor trauma and progresses to pseudarthrosis if treated conservatively.(3)

The treatment of congenital pseudarthrosis of the tibia (CPT) is challenging due to difficulty in achieving and maintaining fracture union while providing a functional extremity. There are multiple studies which evaluated the effectiveness of different surgical methods, local administration of Bone morphogenetic proteins, systemic administration of bisphosphonates and pulsed electromagnetic field therapy in the management of congenital pseudarthrosis, but still there is no single procedure which has the desired outcome of high union rates and low rates of re-fracture or recurrence of pseudarthrosis.

Congenital pseudarthrosis of tibia is often associated with Neurofibromatosis type I (NF I). 6% of NF type 1 patients had deformity of the tibia, while 55% of patients with anterolateral bowing and Pseudarthrosis were found to be associated with NF1.(4)

**Pathophysiology:**

Congenital Pseudarthrosis of Tibia is associated with autosomal dominant Neurofibromatosis type I (NF 1). NF1 is caused by a mutation in NF1 gene in chromosome 17q11.2. The NF1 gene encodes an mRNA, which produces neurofibromin protein.(5) A 360 amino acid domain of the neurofibromin protein is homologous with the GTPase activating protein (GAPs), this domain functions by converting active GTP bound Ras to inactive GDP bound Ras. Thus functions as a tumor suppressor gene by negative regulation of the Ras gene, resulting in inactive Ras proteins. (6). $p21^{ras}$ is an oncogene, which is involved in cell differentiation and proliferation. Mutation associated with NF1 is a loss of function mutation in which the GTPase activating protein (GAPs) domain is truncated, resulting in a non-functional neurofibromin protein and over expression of the active form of Ras.

Overexpression of the Ras pathway results in an increase in osteoclast activity and their precursors, explaining bone resorption in CPT and the high incidence of recurrent fractures. The loss of function mutation of neurofibromin causes a Ras-MAPK (Mitogen activated protein kinase) pathway anomaly, which results in higher cell proliferation, premature apoptosis and defective osteoblastic differentiation. The alkaline phosphatase and mRNA expressions of osteogenic markers (osteonectin and osteocalcin) were reduced in MSCs derived of NF1 mice.(7)

In the canonical Wnt pathway, Wnt (Wingless/Integrated) ligand upon attachment to the Frizzled (Fz) receptor results in accumulation of the β catenin, which enters the nucleus resulting in gene transcription responsible for cell

proliferation and differentiation. A deficiency of Wnt ligands contributes to the disturbed osteoblastic differentiation. (8)

**Pathology:**

There is a cuff of fibrous tissue in the Pseudarthrosis site called fibrous hamartoma, found to be in continuity with the abnormal periosteal thickening. (9)The affected tibia has poor mechanical strength and poor osteogenic capability. (10)A highly cellular fibro vascular tissue encroaches the bony cortex promoting in coordinated osteoclastic activity hampering the normal bone remodeling. Reactive changes cause medullary sclerosis or cystic changes. The blood circulation is impaired due to the constricting fibrous hamartoma. There is generalized osteoporosis in congenital pseudarthrosis of tibia associated with Neurofibromatosis 1(11)

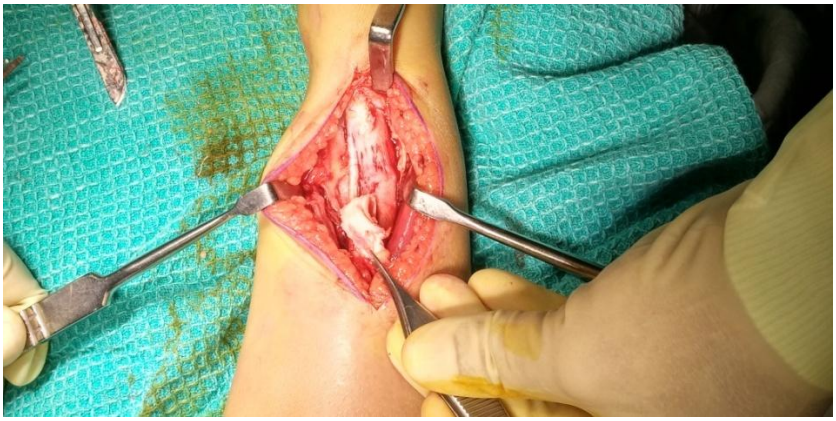


Fig 1: Congenital pseudarthrosis of tibia with a cuff of fibrous tissue(12)

Ultra structural studies showed a vast majority of cells in the fibrous hamartoma were fibroblasts which lacks a basement membrane and were found to be in stationary phase. These cells are undifferentiated and can differentiate into different mesenchymal cell lineages. (7,13)

At the bone Pseudarthrosis junction, there was resorption lacuna with tartarate resistant acid phosphatase (TRAP) positive giant Osteoclasts. The Pseudarthrosis junction was irregular with areas of sclerosis, cysts and multiple small lytic areas(14). This results in hourglass deformity, atrophy and sclerosis of bone ends.

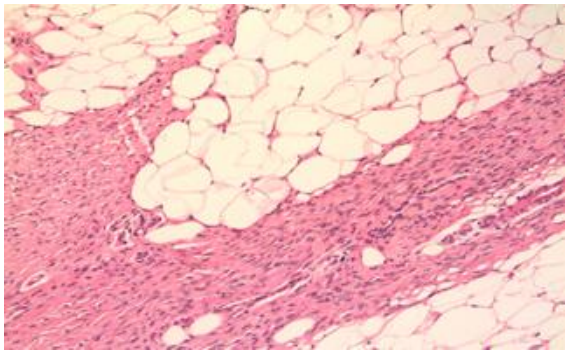


Fig 2: Tissue obtained from the site of pseudarthrosis showing mature lamellar bone with spindle cell proliferation and lipid cells suggestive of fibrous hamartoma.

**Regulators of osteogenesis:**

Osteoblastogenesis is a process of mesenchymal cell differentiation into osteo-progenitor cells, pre-osteoblast and osteoblast. Wnt, TGFβ and BMPs are signaling proteins that regulate osteoblastogenesis. RUNX2 and Osterix are transcription factors which aid in differentiation of MSC into Osteoblast. Osteocalcin, Alkaline phosphatase and COL1A1 are mediators of osteoblastic differentiation. The relative gene expression of these proteins, factors and enzymes are quantified to assess osteogenic differentiation.

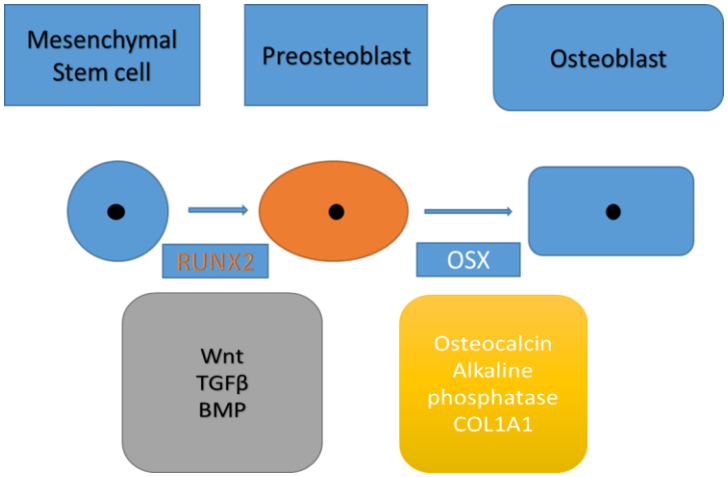



Fig 3: Osteoblastogenesis in normal MSC

**Characteristics of CPT MSC:**

The immuno-phenotyping of Mesenchymal stem cells of fibrous hamartoma of CPT shares similar characteristics to that of healthy MSC (CD 73,90 and CD 105).(7)

**Poor Osteoblastic differentiation of CPT MSC:**

The cells from the fibrous hamartoma are known to have poor osteogenic differentiation. Wnt signaling is one of the important mediators of bone formation. Studies have shown that the mRNA levels of Wnt ligands expressed from the disease periosteum MSC are lower than the normal healthy periosteal MSC. However, this change was not reflected at the receptor levels suggesting that the issue is the ligands in the diseased condition.(8) Thus the relative deficiency of Wnt ligand contributes to the poor osteoblastic differentiation of the CPT MSC.(15)

Gene expression studies revealed that the levels of COL1A1 and alkaline phosphatase are expressed by the CPT MSC without any osteogenic stimulus; while the levels of osteocalcin are not found to be uniformly expressed by all fibrous hamartoma cells. This differential response of CPT MSC is probably due to accumulation of gene expression when they are in different stages of differentiation.

There have been ways to improve the osteogenic potential of CPT MSC. Interestingly, the diseased cells upon treatment with hBMP, the mRNA levels of COL1A1, osteocalcin and alkaline phosphatase are found to be downregulated when compared to normal tibial periosteal cells suggesting poor osteogenic potential. (7,8) On the other hand, treatment with pamidronate, there was increase in cell proliferation but the ability to differentiate into osteoblast as assessed by gene expression of RUNX2 and ALPL is poor. (16) Considering that both strategies have been used in the clinics for patients with CPT for improving bone quality, the data from *in vitro* studies may provide crucial information on its exact action on the diseased cells devoid of systemic influence.

**Strong osteoclastogenicity of CPT MSC:**

RANKL (Receptor activator of Nuclear factor κ Ligand), a cytokine, is a promoter of osteoclastogenesis when it binds to RANK receptor on osteoclasts. OPG(Osteoprotegrin) is a competitive inhibitor of RANKL. As expected, the relative expression of RANKL in diseased periosteum was higher than the normal tibial periosteal cells; whereas the levels of OPG was found to be lower than the normal tibial periosteal cells.

$RAW_{264.7,}$ a macrophage cell line, is a promoter of osteoclast differentiation. When the fibrous hamartoma cells were co-cultured with $RAW_{264.7}$ there was a significantly higher osteoclastic activity compared to normal periosteal cells.

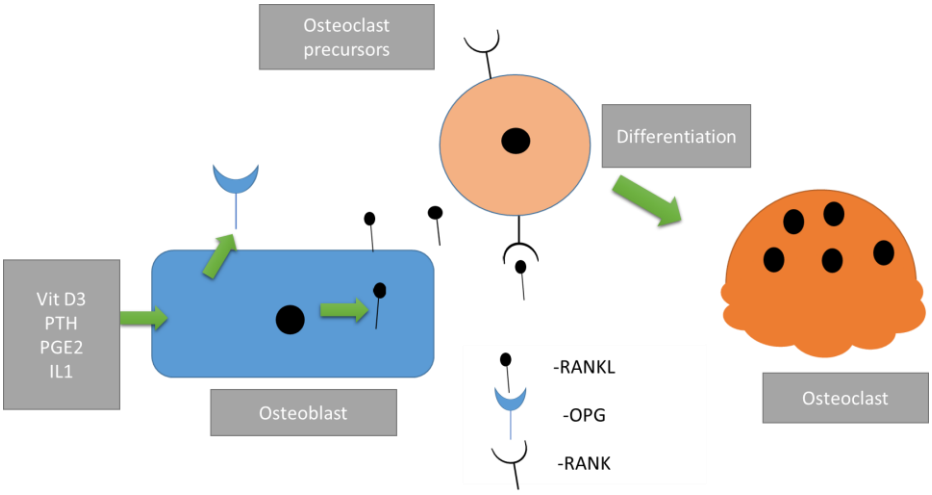



Figure 4: Osteoclastogensis in CPT MSC

The low osteogenic and high osteoclastogenic potential in CPT is one of the major contributor for the pathogenesis and progression of fibrous hamartoma. This hamartoma is the key pathology in CPT(14) and its complete removal is needed to support the bone grafting procedure during the treatment of CPT.(17)

**Clinical manifestation:**

Congenital Pseudarthrosis of Tibia presents with

- Pain,
- Deformity or
- Limp of the affected limb.

CPT is characterized by anterolateral angulation of the tibia.(5,18) The deformity has an apical prominence in the leg laterally, with the ankle in valgus. This bowing and overall decrease in distal tibia growth will result in shortening of the limb. Limp is due to shortening or impending fracture.

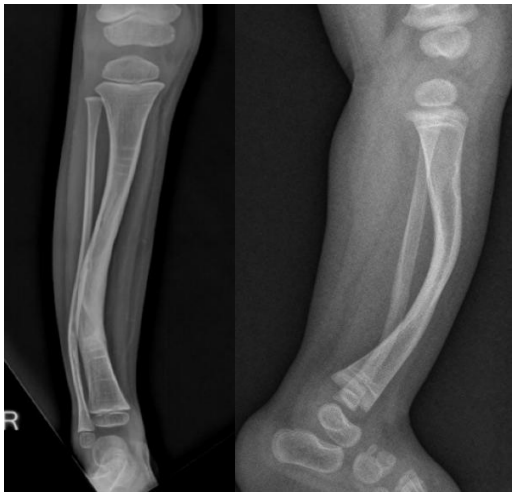


Fig 5: Radiograph showing anterolateral bowing in Congenital pseudarthrosis of Tibia

CPT patients associated with Neurofibromatosis Type I will also present with signs and symptoms of NF1 which will aid in diagnosis. In general, the diagnostic criteria for diagnosis of Neurofibromatosis 1 is NIH Diagnostic criteria for NF1.(19)The signs and symptoms of NF1 vary in presentation among patients. A Retrospective analysis of appearance of symptoms and the effectiveness of the NIH guidelines suggested that 94% of children under the age of 6 were accurately diagnosed with the NIH Diagnostic criteria for NF1.(19–21)

The appearance of café au lait spots and first degree relative with NF1 are the criteria to be identified at birth. The predictive value of café au lait macules (CALMs) in the diagnosis of Neurofibromatosis has been studied extensively.(22,23) Dennis Whitehouse proposed 5 or more café au lait spots in children under the age of 5 and CALMS >5 should be considered as a criteria for diagnosis of NF1(24,25). It was advised that children with CALMs<5 or atypical CALMs are followed up till the age 6 if they don't fulfill other criteria for the diagnosis of NF1.(19,24) Individuals who have isolated CALMS without any other manifestations of NF1 may also have a milder mutation of NF1.

If any long bone dysplasia's are present like congenital pseudarthrosis of tibia or radius, they are identified at birth or within the first year of birth. Axillary and inguinal freckling presents at 6 years of age. Lisch's nodule presents at 20 years of age. Plexiform neurofibroma and optic glioma appears within the age of 6. Because of the varied presentation of the signs and symptoms, individuals at higher risk for NF1 should be followed up at regular intervals.

The **Criteria for diagnosis of NF1** was proposed in the National institutes of health consensus conference in 1981. The NIH Diagnostic criteria for diagnosis NF1is(21)

1. Six or more café-au-lait spots or hyper pigmented macules at least 5 mm in diameter in pre pubertal children and 15 mm post pubertal
2. Axillary or inguinal freckles (>2 freckles)
3. One plexiform neurofibroma or Two or more typical neurofibromas
4. Optic nerve glioma
5. Lisch nodules -Abnormal clumps of pigment on the colored portion of the Iris
6. Typical long-bone abnormalities such as Pseudarthrosis or Sphenoid dysplasia
7. First-degree relative (mother, father, sister, brother) with NF1

The presence of 2 of 7 criteria confirms the diagnosis of NF1.

**Classification of congenital pseudarthrosis of tibia:**

Congenital pseudarthrosis of tibia is a challenge to the treating pediatric orthopaedician. Multiple classification systems had been proposed to classify congenital pseudarthrosis based on the radiological characteristics or the presence of

fracture a birth and associated deformities. Unfortunately, none of the classifications can guide in management and predict the successful outcome of the management, because of the difficulty in achieving and maintaining union. The successful union is usually transient and requires repeat procedures until the patient is skeletally mature. In general, Congenital pseudarthrosis is classified based on the radiological appearance whether they are cystic, atrophic, sclerotic and clubfoot type. The disadvantage of these classifications are that the children may progress from one stage to another as they grow and their failure to prognosticate the outcome. Few commonly used classification. (10,26)

- Anderson classification – 1973
- Boyd's classification -1982
- Crawford classification -1986
- El rosasy Paley herzenberg classifcation-2007

- **<u>Anderson classification (1973)</u>**:(27)
- Based on morphology of the pseudarthrosis
    - Dysplastic
    - Cystic
    - Sclerotic
    - Clubfoot type

❖ **Boyd classification (1982):**(9)

Type I: Congenital anterior bowing

- Radiograph - Deformity present in tibia

Type II: Congenital anterior bowing with hourglass constriction of tibia

- Radiograph - Medullary cavity non continuous, Fracture ends are Tapered, rounded & sclerotic
- Spontaneous fracture secondary to minor trauma, Age < 2 years
- Most common with worst prognosis

Type III: Fracture develops at site of bone cyst

Type IV: Sclerotic bony segment

- Radiograph -Complete or partial obliteration of medullary canal
- Fracture develops like stress fracture

Type V**:** Congenitally dysplastic tibia

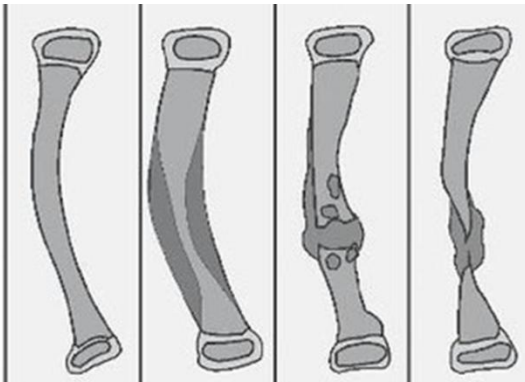
- Radiograph -Mild bowing ± pseudarthrosis

Type VI: Intraosseous NF or schwannoma

- Radiograph -± pseudarthrosis

❖ **Crawford classification (1986):**(28)

- Type 1: Anterolateral bow with a dense medullary canal.
- Type 2: Anterolateral bow with a widened medullary canal and a tubulation defect.
- Type 3: Anterolateral bow and a cystic lesion.
- Type 4: Anterolateral bow with evident fracture, cysts, or pseudarthrosis.



| | | | | | Fibula Pseud-arthrosis | Intra osseous Neuro-Fibroma | Club foot |
|---|---|---|---|---|---|---|---|
| Crawford | I | II | III | IV | | | |
| Andersen | | Sclerotic | Cystic | Dysplastic | | | Club foot |
| Boyd | I | IV | III | II | V | VI | |

Fig 6: Classification of CPT

❖ **El-rosasy's &Paley Classification (2007):**

This classification is based on

1. Time of presentation in relation to previous treatment,
2. Radiological - atrophic or hypertrophic
3. Mobility of pseudarthrosis site - stiff or mobile.

Based on this, 3 types are classified.

- Type I - Atrophic bone ends, mobile pseudarthrosis without previous surgical intervention.
- Type II - Atrophic bone ends, mobile pseudarthrosis, previous surgical intervention with or without retained hardware,
- Type III-Hypertrophic bone ends, stiff pseudarthrosis and with or without previous surgical intervention.

These classification systems are rarely relevant because most of the children present with fracture and the treatment for different morphological types usually requires excision of pseudarthrosis or hamartoma. Following resection, the treatment is achieving union for tibia with a bony gap.

Johnston suggested that two criteria to be considered to initially classify anterolateral bowing of the tibia, which carries a prognostic value are

- The presence or absence of fracture
- The age at which fracture occurs
    - Early onset < 4 years' old
    - Late onset > 4 years' old
    -

**Prognostic factors:**

General -

- CPT associated with NF 1 gene is a negative predictor for bone union.
- Fracture under the age of 1 carries poor prognosis, disease progression and fracture occurring at a later age has good prognosis.(3)

Local –

- Pseudarthrosis at the distal tibial metaphysis, it is difficult to achieve stability of the distal fragment, hence fixation includes ankle and foot.
- Fibular pseudarthrosis have a poorer prognosis and a severe valgus deformity of ankle.(29)

- Bone atrophy, sclerosis results in poor fracture healing and associated with a poor prognosis.
- Localized osteoporosis (11)
- Shortening of the limb has a poor prognosis (30)

Surgical –

- The total number of surgeries and remaining bone capital. If multiple procedures were not able to achieve successful union resulting in residual deformity and limb length discrepancy, it is associated with a poor prognosis.
- The resorption of the graft has a poor prognosis. If more than 3 bone graft procedures have failed to achieve union, it is associated with a poor prognosis.(31–33)
- Surgery under the age of 4 is associated with poor prognosis.

**Management:**(3,4,10,34,35)

**CONSERVATIVE – Prophylaxis:**

Congenital pseudarthrosis if untreated, disease progresses to worsening deformity, fracture and shortening. In sclerotic type of pseudarthrosis, there is worsening of deformity and cystic type usually fractures. Once the fracture occurs, the fracture rarely heals spontaneously. The application of a protective brace before the child starts walking can delay fractures. A clamshell type orthosis provides circumferential support is usually recommended. The protection of the unfractured bowed tibia should be continued till skeletal maturity.

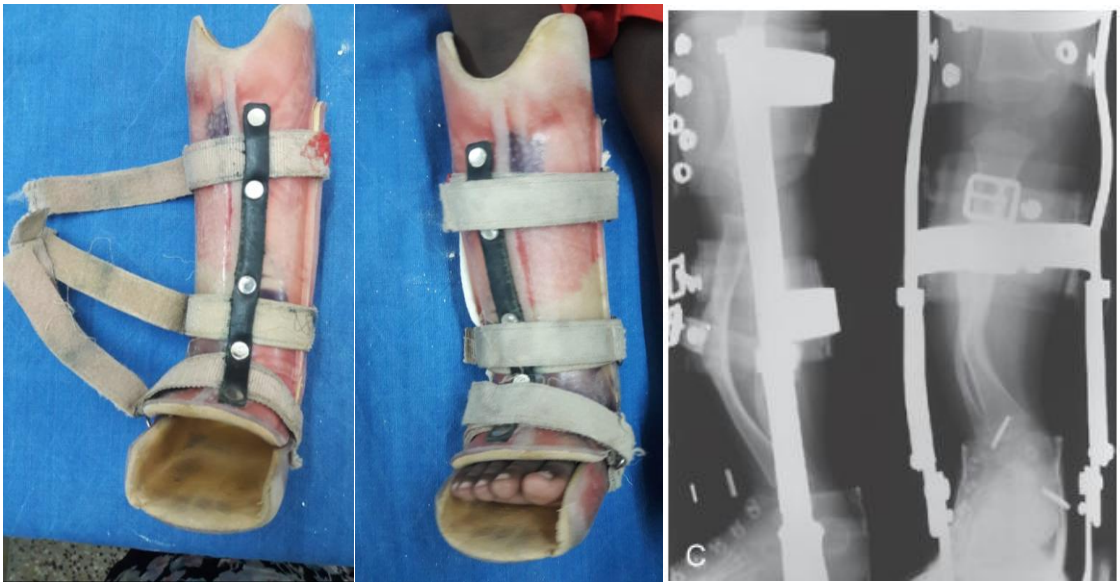


Fig 7: Knee Ankle Foot orthosis

**SURGICAL –**

Multiple surgical procedures have been tried in the past to prevent fractures and to achieve union in fractures, but no single procedure has so far been proven to be superior to the other. The surgical management of congenital pseudarthrosis can be

- Prophylactic surgery
- Surgery for fracture fixation and deformity correction:

**Prophylactic surgery:**

McFarland procedure:

McFarland proposed bypass grafting with contralateral tibia or de vascularized fibula grafting in congenital pseudarthrosis of the tibia.(36) It based on the principle that the anterolateral bowing s responsible for pseudarthrosis and failure of fracture union. He provided mechanical support by placing the strut graft posteromedial. Out of his 11 patient's, union was achieved in 9 patients. The contralateral tibia was

harvested and it was placed in the concavity of the deformity bypassing the pseudarthrosis. The leg is protected with a brace once union is achieved till skeletal maturity. Multiple modifications to this bypass grafting were suggested, Tachdjian proposed delayed McFarland grafting in which the graft is raised from the normal tibia and is grafted after an interval of 4-6 weeks.(37) Strong and Wong-Chung used ribs and fibular allografts as a modification for the Mac Farland procedure.(38)

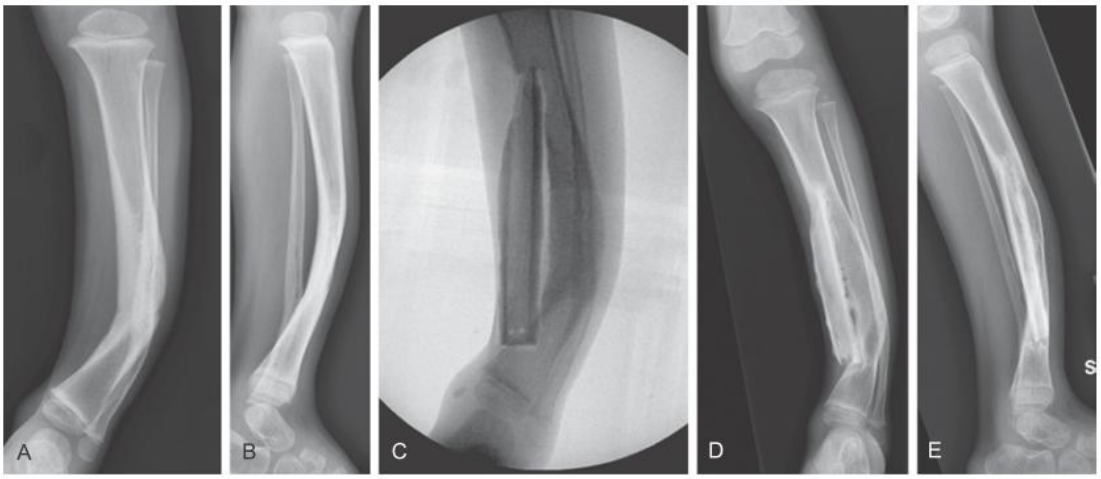



Fig 8: Radiograph of Mcfarland procedure

Adapted from the journal Pseudarthrosis of the tibia in childhood. McFarland B. J Bone Joint Surg Br. 1951 Feb;33-B (1):36–46.

The disadvantages of this surgery was that the normal tibia is sacrificed for the graft and the deformity was not corrected during the surgery. Hence the only preferred prophylactic treatment accepted in bracing of the deformed tibia.

**Surgery for fracture fixation and deformity correction:**

In treating fractures in patients with congenital pseudarthrosis of tibia, Regardless of the treatment preferred, the general concern is about the quality and longevity of the union achieved. No single treatment method has proven superiority over the other or had shown consistent results.

The primary aim of surgery is

1. Achieving fracture union,
2. Restoring satisfactory alignment of the affected leg to prevent recurrence of fractures,
3. Limiting limb length discrepancies and
4. Preserving articular functions of the affected limb.

Once the fracture occurred, the deformity should be corrected and alignment has to be maintained with an intramedullary fixation both during growth and at maturity to prevent any late fractures or reoccurrence of the deformity.

The surgical options are

- Internal fixation
    - Intramedullary nailing with bone grafting.
        - On lay tibia grafting
        - Vascularized fibular grafting
    - Plate osteosynthesis
- External fixation
- Amputation.

❖ **Intramedullary nailing:**

The procedure of choice for attaining union for fractures in congenital pseudarthrosis of tibia using Intramedullary stabilization with a nail was introduced by Charnley. (39)Multiple nail models have been used in the treatment of CPT but none has shown superiority over the other.

1. Fassier-DuvalTelescopic nails in which the ends were fixed to the epiphysis were used. As the child grows, the nail will telescope within each other and lengthen. (40)
2. Williams devised a 2-part nail with a threaded male and female component. The nail is inserted ante grade through the pseudarthrosis site and is brought out through the heel, followed by retrograde insertion of the nail into the proximal tibial segment. Immobilization of the ankle is necessary as the distal segment is usually small and to prevent valgus deformity of the ankle. As the kid grows the nail which is transfixing the ankle will migrate to the distal tibial segment leaving the ankle to regain its movements.
3. Flexible intramedullary titanium Rush rodding of the tibia is used in a child with a narrow medullary canal. (10,39–41)

All these intramedullary devices are used after resection of the pseudarthrosis with either a tibia on lay graft, fibular graft or free vascularized fibula graft, iliac crest bone graft.

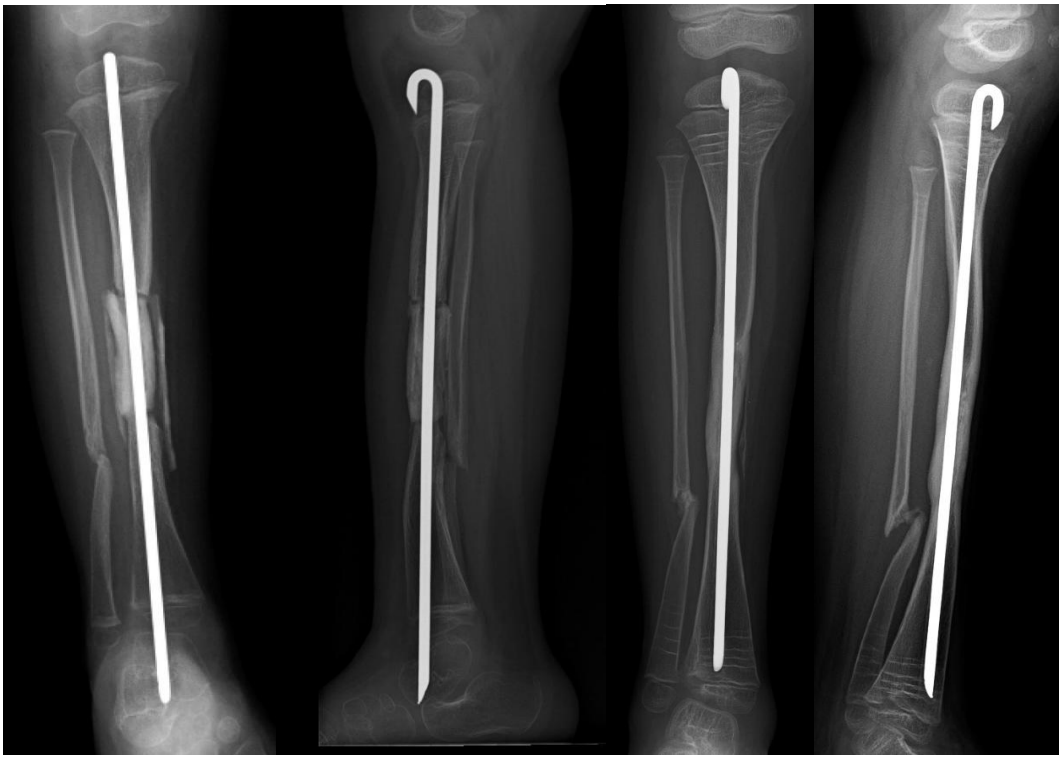


Fig 9: Radiographs of CPT treated with intramedullary nail

❖ **Plate Osteosynthesis:**

Locked plates are used in conjunction with the intramedullary nails as an internal external fixator to provide rotational stability. It can also be used internal fixator for patients undergoing surgical correction of congenital pseudarthrosis of tibia. (34,41)

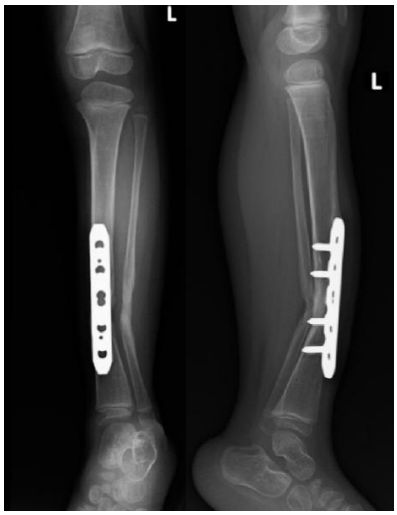



Fig 10: Radiographs with CPT treated with a locking plate

❖ **Free vascularized fibular grafting:**

The non-vascularized fibular grafting has been associated with failure in maintaining long lasting solid union. Hence since the advent of Free vascularized fibular grafting, it is used to achieve definite and lasting union during pseudarthrosis resection in congenital pseudarthrosis of tibia. (41,42)The fibula is resected to the desired length with a vascular pedicle and is anastomosed to either the anterior or posterior tibial artery and a vein.(43) The graft is stabilized with an external fixator or a minimal internal fixation(44). The morbidity associated with the surgery is valgus deformity and the loss of dorsiflexion of the toes in the donor leg. To prevent the ankle valgus deformity, the distal fibula is synostosed with the distal tibia (Langenskiöld procedure).

The Free vascularized fibular graft is anastomosed with the anterior tibia artery and the peroneal vein is anastomosed with the long saphenous vein. The ankle is transfixed with a kirschner wire and the Distal tibio-fibular joint is transfixed with a kirschner wire above the level of the physis. The graft is then stabilized with an intramedullary rod or an external fixator.

❖ **External fixator:**

Treatment of congenital pseudarthrosis of tibia is associated with limb length discrepancy. The Ilizarov circular external fixator was introduced in thelate 1980s.(4,45) Since the advent of Circular ring fixators, they are used to correct deformities, maintain alignment , rotation in CPT and it also aids in lengthening(46). The number of rings depends on the length of the defect to be transported and the

length of the bone. When he pseudarthrosis is distal, the foot is also included in the construct. One ring just distal to the proximal tibial physis and one ring just proximal to the distal tibia physis is applied. The pseudarthrosis is excised and primarily docked with or without bone graft. The corticotomy is based on the location of the pseudarthrosis. The major disadvantage of ring external fixator is the acceptability by young children. Few reported complications are pin tract infection, joint stiffness, muscle atrophy and rarely neurovascular compromise while lengthening and refracture. (26,47).

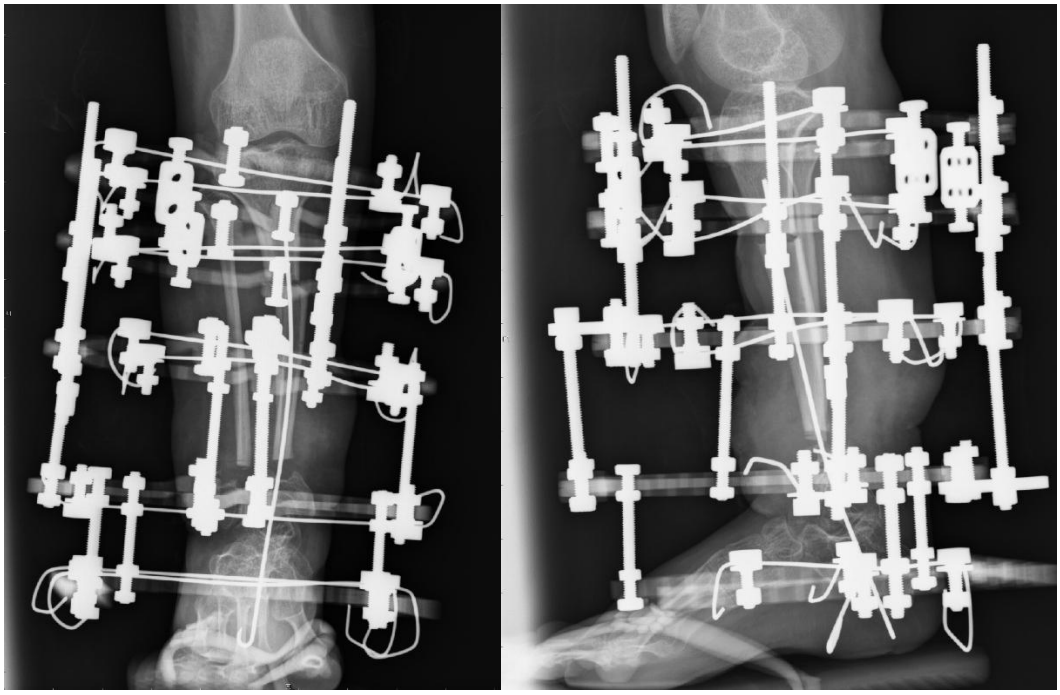


Fig 11: Radiograph of CPT treated with pseudarthrosis excision and Ilizarov fixator application.

❖ **Paley's X union technique:**

Paley had proposed a surgical protocol to achieving lasting union based on the observation that most of his patient who had cross union did not have a re-

fracture.(34)He proposed that the large cross sectional area of the union of the tibia and fibula prevented refracture. He suggested the small cross sectional area of the tibia in young children may be reason for increased incidence of fracture in the young than the adolescent patient. Based on his experience he proposed the Paley's X-Union Technique where after excision of the pseudarthrosis, the tibia is fixed with a FD nail and he used periosteal grafts, bone graft and BMP2 to achieve cross union.

❖ **Amputation:**

Amputation is considered for children with congenital pseudarthrosis of tibia, who have chronic treatment failure and left with a deformed nonfunctional extremity.(48) After considering multiple factors, McCarthy proposed a few indications for amputation in children with congenital pseudarthrosis of tibia.

(1) Failure to satisfactorily achieve solid bony union after a minimum of three surgical attempts.

(2) Significant limb length discrepancy (i.e., >5 cm), which needs a cosmetically unacceptable orthotic shoe to achieve equal limb lengths.

(3) Permanently deformed extremity with poor function.

(4) Functional loss of the extremity from prolonged medical care and hospitalization.

Amputation at the level of the pseudarthrosis is avoided due to poor bone quality and thin narrowed bone ends, and the scarred soft tissue as a result of the previous surgeries provide a poor end bearing stump. Hence amputation through the ankle with the heel pad as the stump is more acceptable.

Currently Amputations are rarely used in treatment of Congenital pseudarthrosis of tibia due to advances in the management of congenital pseudarthrosis of tibia. (41,49)

- **Refracture:**

Surgical management of congenital pseudarthrosis of tibia is challenging due to the occurrence of refracture.(47,49) Refracture is the most common indication for repeat surgery in children operated for congenital pseudarthrosis of tibia. A multicenter study to identify factors influencing refracture in children with congenital pseudarthrosis of tibia undergoing surgery suggested ilizarov technique was more effective in preventing refracture and hBMP had a poorer outcome. They also suggested leaving the fibula and the use of cortical bone graft alone yielded better outcome.

- **Bonemorphogeneticproteins(BMPs):**

Since the discovery of osteogenic potential of Human Bone morphogenetic proteins, it has been tested in treatment of nonunion and long bone diaphyseal defects.(50,51) Bone morphogenetic proteins belongs to the transforming growth factor-β (TGF β) family. They induce and regulate differentiation of mesenchymal stem cells into bone and cartilage. Exogenous BMPs are proteins which upon binding to specific receptors which in turn transduce the signals via the Smad proteins to genes responsible for differentiation. They turn on specific markers of

osteoblast differentiation like osteocalcin. The activity of BMPs are regulated by inhibitory proteins outside the cell and inhibitory Smad proteins inside the cells.(50)

The osteogenic potential of the human bone morphogenetic protein is explored in the treatment of nonunion, spinal fusions and diaphyseal defects along with cancellous bone grafts and was found to be beneficial. They achieved union after local administration of hBMPs along with skeletal stabilization(17,51)

In CPT, where there is increased osteoclastic activity, BMPs are tried to increase the anabolic stimulus at the pseudarthrosis site after excision of the fibrous hamartoma and intramedullary nailing. It was not found to be beneficial in the treatment of CPT alone or with cancellous bone graft in achieving lasting union.(17,49,52).

- **Bisphosphonates:**

Bisphosphonates, an anti resorptive agent, a pyrophosphate analogue used in the treatment of diseases with increased bone resorption like osteoporosis, tumor-associated osteolysis and Paget's disease. They P-C-P structure is similar to native pyrophosphate P-O-P. They attach to hydroxyapatite at sites of active resorption and impairs the ability of osteoclasts to adhere to the bone and form ruffled borders. They also decrease osteoclast progenitor cell development.(53)Bisphophonates causes apoptosis of osteoclasts by negative regulation of Ras activity.(54)

CPT is characterized by increased osteoclastogenesis. The conventional treatment for CPT is hamartoma excision and bone grafting. Following bone grafting, there was increased graft resorption and recurrence of the pseudarthrosis. (40,41,55)

Bisphosphonates, an anti resorptive agent was tried as an anti-catabolic agent in the treatment for CPT.

In a previous study when mesenchymal stem cells derived from fibrous hamartoma was treated with pamidronate, it was found that pamidronate has no role in increasing osteoblast differentiation.(16) Birke et al study in which they used BMP as an anabolic agent and Bisphosphonates as an anti-catabolic agent found no significant improvement in achieving union rates .(17)

- **Pulsed electromagnetic field therapy:**

Pulsed electromagnetic field therapy was used in the treatment of fractures and nonunion. The oscillating external electric field exerts an oscillating force on the free ions inside and outside the cell membrane causing a forced vibration of the free ions(56). In turn, these ions signal through the electrically sensitive gated channels to release growth factors. In animal models, when the fractured bone was treated with pulsed electromagnetic field therapy, it was found to increase TGF-β expression, a growth factor released during fracture healing.(57) It was found to increase healing of full thickness articular defects.(58). The advantage of PEMF therapy is that, it is noninvasive and relatively cheap compared to the other treatment modalities.

PEMF is also used in the treatment of CPT, but the frequency of union is poor and requires additional surgery to achieve union. Further studies are needed to provide further long term outcomes.(59)

**Extra corporeal shock wave therapy:**

**Introduction:**

In 1980, extracorporeal shockwaves(SW) were tried to break Kidney stones in humans for the first time in medicine. Since then, researches on possible effects on tissue as the wave travels through tissue were done and it has been found to have both destructive and regenerative effects were found in bone. High energy shock waves lead to destructive effects on tissue and lower energy shockwave leads to more regenerative effects on the treated tissue(60)

Shock waves are longitudinal acoustic wave, intense, short energy wave which travel faster than speed of sound. They transmit the energy from the point of generation to the area of interest. An ideal shock wave machine should be able to

1) Generate effective shock waves
2) Generate waves of range of energies,
3) Target the shock waves from the point of generation to a specific site
4) Minimize or cause no adverse effects.

**Mechanism of action:**

1. Mechano-transduction. Mechanical pressure generated increases cell membrane permeability.(61)
2. Acoustic waves cause micro capillaries in tissue to rupture, which increases growth factors to the area
    - SW produces "cavitation effect", by which microbubble are formed when the wave pass through water-tissue(62)

- These bubbles expand within a few microseconds following the shock wave, and usually collapses within 100 microseconds, generating a second spherical shock wave(63).

PHYSICAL SHOCK WAVE ENERGY

↓

BIOLOGICAL RESPONSES

↓

INCREASED BMP RELEASE

↓

BONE REPAIR

↓

TISSUE REGENERATION

3. Micro fractures, Shockwaves are found to cause microfractures in rabbit trabecular bone and periosteal elevation, inturn stimulating new bone formation.(64,65)
4. Shock waves increases release of angiogenesis-related markers including eNOS, VEGF and PCNA(66)
5. Shock waves stimulates osteoblasts – increases new bone production by enhancing osteoblast metabolic activity, particularly the proliferation of mg63 osteoblast-like cells(67)

The methods of shock wave generation are (68)

1. Piezoelectric
2. Electrohydraulic
3. Electromagnetic
4. Radial or electro-pneumatic

❖ **Piezo electric shock wave therapy:**

**Principle:**

The piezo crystals are mounted geometrically in a spherical manner which will focus the wave towards the center. These piezo crystals receive a rapid electrical charge. This charge causes deformation (expansion and contraction) of the crystals resulting in a pressure pulse. This is the piezoelectric effect. (68)

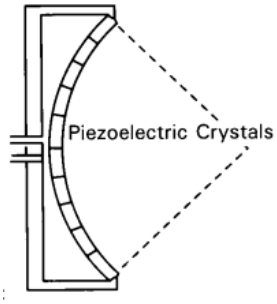



Fig 12: Arrangement of piezo crystals and the focal point

The amount of energy transmitted per unit area of surrounding tissue is measured as Energy flux density (EFD in $mJ/mm^2$). It is found that lower energy flux density shock waves are found to be useful in soft tissue conditions and high energy flux density shock waves are found to be effective in bone regeneration. (69) . High energy shock waves (0.40 and 0.70 $mJ/ mm^2$) had higher rates of fracture union in long bone nonunion without significant adverse effects. (67) We will be using SW with least cytotoxicity and high proliferation on CPT MSCs .

| | Level | EFD range ($mJ/mm^2$) |
|---|---|---|
| Mainz | Low | 0.08-0.27 |
| | Medium | 0.28 |
| | High | >0.60 |
| Kassel | Low | <0.12 |
| | High | >0.12 |

Classification of shock wave therapy based on Energy flux density(EFD)

❖ **Conditions where shock wave therapy is found beneficial**

- Extracorporeal shock wave therapy was found to be beneficial in insertional tendinopathies like Plantar fasciitis, Achilles tendinopathy, Epicondylitis, Calcific tendinopathy of the shoulder and Patellar tendinopathy.(70,71)
- It was also found to be effective in treating Post-traumatic myositis ossificans, Trigger points, Frozen shoulder, Dupuytren's contracture and Dequervain tenosynovitis.
- Past research has shown good union in the treatment of Fracture nonunion and pseudarthrosis.(61,67,69,69,72)

❖ **Contraindications:**

ESWT is relatively safe, the occurrence of severe adverse events reported due to its use. However, it should be used in caution or avoided in the following conditions.

- Bleeding conditions
- Pacemakers
- Near open growth plates (children)
- Tumors

❖ **Adverse effects**:

There are a few adverse events documented in literature. Most of them are localized adverse events with no systemic complications. These adverse effects usually come and go within 3 to 5 days

- Redness
- Swelling
- Pain
- Hematoma
- Petechiae

**Piezo wave 2:**

Piezowave2 is a compact, powerful desktop linear focal shock wave generator produced by Richard wolf and Elevation medical which is used for this study. It can modulate the energy produced by the shock wave generator.


Fig 13: Piezo wave 2

| | |
|---|---|
| Penetration depth | 5-40mm |
| Energy density | 0.03-0.4mJ/mm$^2$ |
| Pressure range | 11-80MPa |

**Osteogenic differentiation**:

Osteogenic differentiation in cultured cells is assessed using gene expression of the enzymes and markers found during osteoblastic differentiation. The few frequently used markers to study the osteogenic differentiation are RUNX2, Osterix, Osteocalcin and Bone alkaline alkaline phosphatase. Alizarin red staining is used to assess the calcium deposition during osteoblastic differentiation.

- ***RUNX2:***(73)

RUNX is the term given to genes coding for RUNT related proteins by Nomenclature Committee of the Human Genome Organization (HUGO). RUNX2 plays an important role in differentiation of osteoblasts, cartilage hypertrophy and vascular invasion of bone. It was located in located in 6p21 in the chromosome.

RUNX2 is the precursor for pre-osteoblast differentiation from mesenchymal stem cell. Mutations in RUNX 2 mutation results in cleidocranial dysplasia's. RUNX2 overexpression causes increase in osteoblast but matrix production and matrix mineralization are impaired.

- ***OSTERIX (SP7):***(73)

Osterix is a zinc containing transcription factor responsible for conversion of pre-osteoblasts in to functional osteoblast. In animal model, Osx null mice, type I collagen, bone sialoproteins, osteonectin and osteopontin were not expressed. Hence affecting osteoblastic differentiation despite normal expression of RUNX2.

Pre-osteoblasts have a potential to differentiate into osteoblasts and chondroblasts, but due to osterix, negative regulator of the chondrocyte differentiation

promotes osteoblastic differentiation. Osterix is a late marker of osteogenic differentiation.

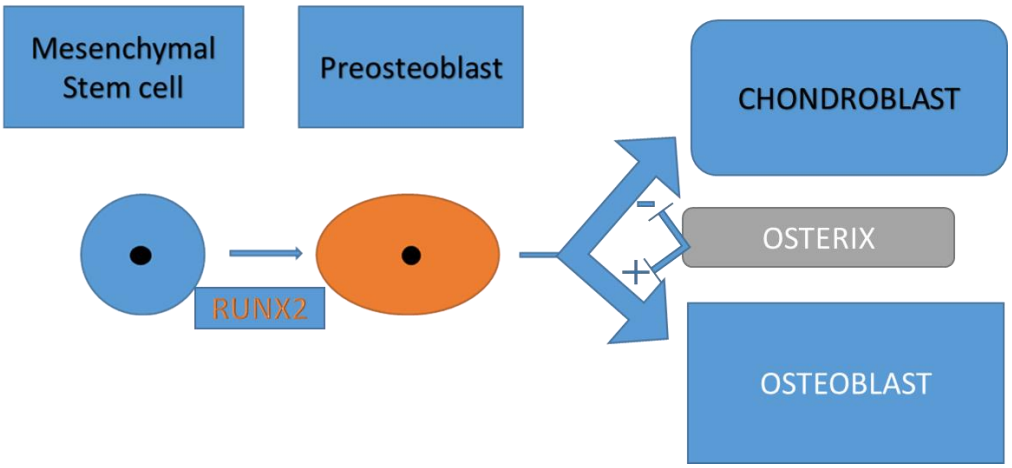



Fig 14: Action of Osterix is osteoblast differentiation

❖ ***Osteocalcin(BGLAP):***(74)

Osteocalcin is a bone sialoprotein, no collagenous bone matrix protein. It is a small γ-carboxyglutamate protein expressed by osteoblasts and binds to calcium ions. It is a marker of bone formation. It is more sensitive than alkaline phosphatase. The γ-carboxylation of osteocalcin increases its affinity to calcium ions. It is found to increase bone formation. It enhances chemotaxis and activity of osteoblasts. It influences osteoblast maturation in the late stages of osteoblast differentiation. (75)

❖ ***Alkaline phosphatase:***

Bone alkaline phosphatase is a glycoprotein, a phosphatase enzyme, which is expressed in the early stages of differentiation of osteoblasts. There are four different types of alkaline phosphatases in the alkaline phosphatase family,

- Intestinal,
- Placental,
- Placental-like, and
- Liver/bone/kidney (Tissue non-specific).

The First 3 genes were found in chromosome and the Tissue nonspecific alkaline phosphatase is found in chromosome 1. Osteocalcin is found to be more sensitive than bone alkaline phosphatase in monitoring bone formation, its use is limited in vivo testing due to its diurnal variation. Hence Bone alkaline phosphatase is cheaper and effective marker in monitoring bone formation in in vivo testing. (76)

**Osteogenic potential of Extracorporeal shock wave therapy:**

The effects of shock wave therapy on the osteogenesis of mesenchymal stem cells have been studied in the past and governed by multiple mechanisms. Upon treatment, shock waves result in membrane hyperpolarization and activation of ras and growth factor induction, resulting osteogenesis. (77,78) This is accompanied by significant increase in the expression of alkaline phosphatase activity and osteocalcin at days 6 and 12 respectively.

Shock waves induce local release of TGF β and endogenous NO synthesis (eNOS). (79) *In vivo* studies have shown there is increased neovascularization in the shock wave treated tissue.(66) There is increased expression of eNOS, VEGF and PCNA. The site of nonunion and pseudarthrosis is found to have hypo vascular fibrous tissue. This increase in vascularity in the shock wave treated site allows

stimulation of osteo-progenitor cells by influx of cytokines and osteo-inductive proteins, thereby promoting osteogenesis. (79,80)

The effect of SWT on osteoblast are due to signaling cascade activation, which promotes phosphorylation of extracellular signal–regulated kinases (ERK1/2), which results in gene expression that induces osteoblast proliferation and differentiation(81).

Shock waves induced ATP release from hMSCs and activation of p2x7 receptors enhances osteogenic proliferation and mineralization. (15)

ESWT causes "nonsurgical" damage and enhances the release of growth factors from the bone matrix, thereby promoting osteoblast proliferation and differentiation(82).

Shock wave treatment results in release of superoxides, which induces TGF β resulting in a cascde of signaling resulting in increased osteoblasti differentiation. (83,84).

All these studies suggest increased osteogenic potential of mesenchymal stem cells when treated with shock waves.

**Justification for the use of shock wave treatment in fibrous hamartoma:**

Surgery remains the gold standard treatment for patients with CPT, with high failure rates and high re-fractures rates.(10,49) As a non-invasive modality, shock wave therapy has been used in the clinics of various musculoskeletal disorders. It is found to be useful in the treatment of fracture nonunion(<5mm) and is being used as a first line of therapy for the same in some of the countries(61). The results of shock

wave therapy in the treatment of fracture nonunion have advantages of non-invasiveness and lower complications rate. During a procedure as early as 1992, one of the patients with congenital pseudarthrosis of tibia had callus formation following shock wave therapy(85)

It is a well-established fact that the fibrous hamartoma cells in congenital pseudarthrosis of tibia is found to have lower osteogenic potential and increased osteoclastogenesis.(7,8,16,86) So far literature provides evidence that focal shock wave treatment triggers osteogenesis of healthy MSC. We propose to elucidate the effects of focal shock wave treatment on the diseased periosteal MSC that lacks inherent potential to commit to osteoblastic lineage.(15,81–84)

The cells in the fibrous hamartoma are heterogeneous in nature and arearrested at different stages of osteogenesis. This is one of the major challenges in the treatment of CPT.None of the studies so far have addressed how shock wave therapy could potentially stimulate osteogenesis in CPT MSC. Our results may be of clinical significance in terms of future treatment and management of CPT.

In 1997, Haupt wrote ‘In patients in whom conservative treatment has failed, surgery is the only choice, but if its success rate barely exceeds that of shock wave therapy and surgery can still be done if shock wave therapy fails’.

# MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB no-10630). Fibrous hamartoma tissue was obtained from three children with CPT after informed consent from the parents. (Annexure 3).

**Setting**: The study was carried out in Laboratory IV - Centre for Stem Cell Research (a unit of in stem, Bengaluru), Christian medical college, Bagayam

**No. of patients**: 3

| | Age | Sex | Tibia | Histopathology | NIH criteria |
|---|---|---|---|---|---|
| Sample 1 | 2 | F | Left | Lipofibromatosis | 2/7 |
| Sample 2 | 4 | M | Right | Lipofibromatosis | 2/7 |
| Sample 3 | 2 | F | Left | Lipofibromatosis | 3/7 |

**Research period**: March 2017 to September 2018

## Methods:

### 1. Excision of the Fibrous hamartoma:

The fibrous hamartoma tissue was harvested surgically from three patients (2 females, one male with a mean age 2.66 years) with CPT, with clinical features of NF1 who underwent excision of the hamartoma and corrective surgery. The excised hamartoma was sent for histopathology and for isolation of mesenchymal stem cells(MSCs). The histopathology showed lipofibromatosis consistent with fibrous hamartoma associated with CPT.

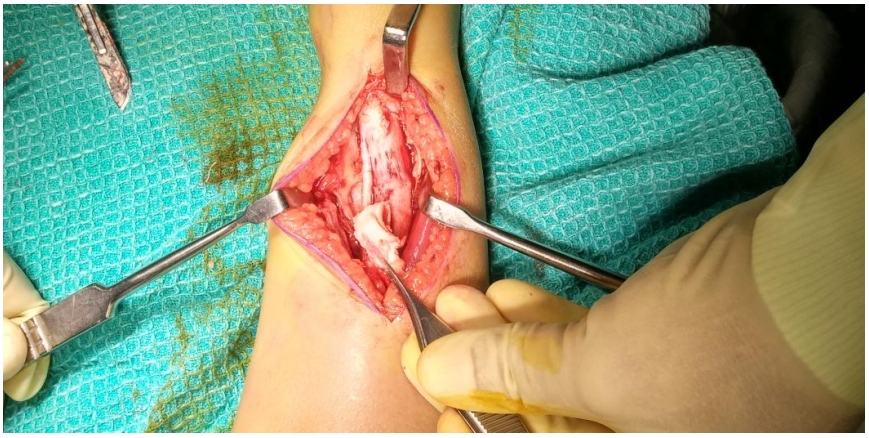


Fig 15: Intraoperative picture of Fibrous hamartoma excision

### 2. Isolation of MSCs from fibrous hamartoma tissue:

The excised hamartoma was transported to the lab in a culture medium. The mesenchymal stem cells were isolated from fibrous hamartoma excised and were cryopreserved with 70% DMEM, 20% FBS and 10%DMSO.

## 3. Morphology and Characterization of MSCs:

Cryopreserved third passage cells of Mesenchymal Stem Cells (MSCs) derived from human lipofibromatosis tissue isolated from CPT were thawed at 37ºc from liquid nitrogen and centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in medium and cultured in DMEM/F12 culture medium. Cell number and viability was performed by tryphan blue dye exclusion assay and live dead analysis and immunophenotypic characterization using flow cytometry for cluster of differentiation (CD) markers of MSCs.

## 4. Application of focal shock wave treatment to CPT MSCs:

The focal shock waves are applied to the culture medium using a customized chamber to prevent any loss of energy and uniform application of shock waves.

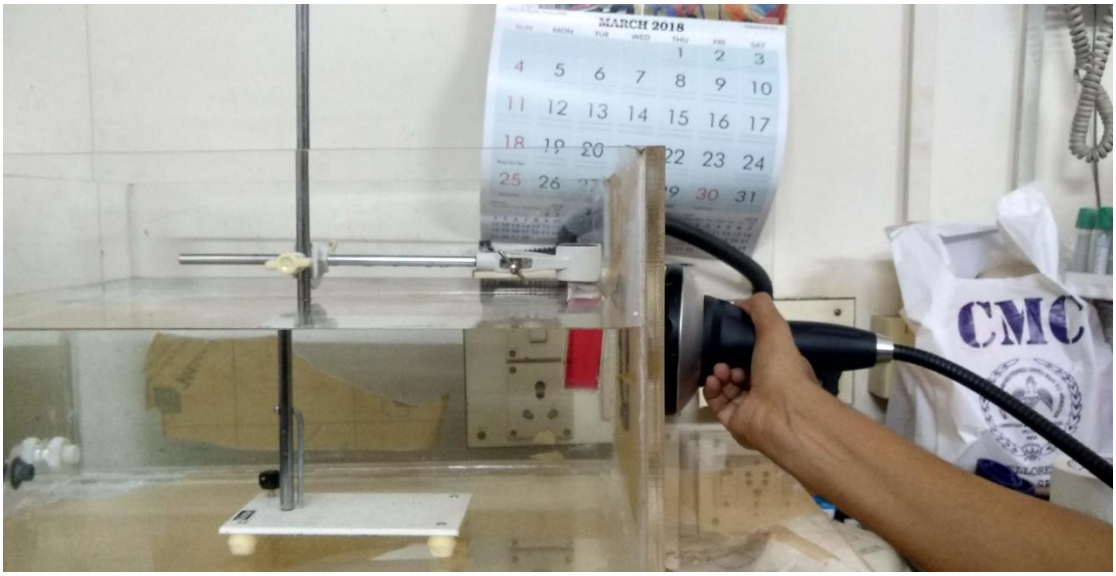



Fig 16: Standardization of shock wave application

## 5. Cytotoxicity test:

The cytotoxic effect of focal shock waves on CPT MSCs was evaluated using the MTT assay. It is a calorimetric assay based on NAD(P)H-dependent cellular oxidoreductase enzymes, present in metabolically active cells reduces tetrazolium dye in to insoluble formazan crystals, which is evident by the formation of the purple colour.

CPT MSCs(N=1) was treated with Shock waves of increasing frequency and varying energy flux density (Low/ Medium/High energy flux density shock waves). Cell proliferation and cytotoxicity was assessed after assessed by flow cytometry (immediately) and MTT assay after 24 hours. The dose with minimum cell death was chosen for subsequent experiments.

The focal shock wave treatment using piezo wave 2 with high energy shock waves at 4000 impulses which did not affect the metabolic activity of CPT MSCs was chosen for our study.

## 6. Osteogenic differentiation:

The cultured CPT MSCs that were treated with shock waves and the untreated control CPT MSCs were kept for differentiation in the osteogenic differentiation medium. The experiment was performed in triplicates (n=3); each treated sample had a respective control group (untreated).

The cells were cultured in DMEM f12 and 10% FBS medium for 24 hours prior to addition of osteogenic differentiation medium The osteoblastic differentiation will be induced under the influence of osteogenic differentiation

medium (dexamethasone, β-glycerol phosphate and ascorbic acid) and in the presence of 10% v/v fetal bovine serum (FBS).

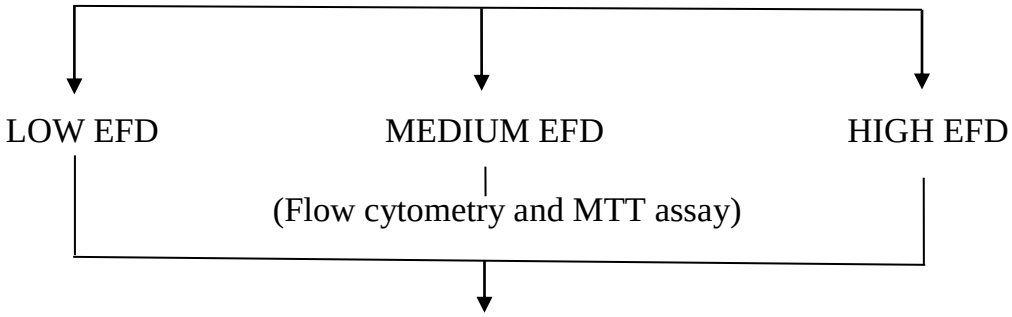
The quality of differentiation was assessed on day 21 by Alizarin Red S staining and gene expression of eNOS, RUNX2, Alkaline phosphatase, Osteocalcin and Osterix(SP7) were quantified using real time PCR (RT PCR)) for the osteogenic differentiation on days 7, 14 and 21. Alizarin red S staining is used to assess the calcium deposition during osteogenic differentiation. Calcium forms an alizarin red S-calcium complex during chelation process. This staining was assessed using Image J software.

The steps of the experiment are mentioned in detail in the annexure 1.

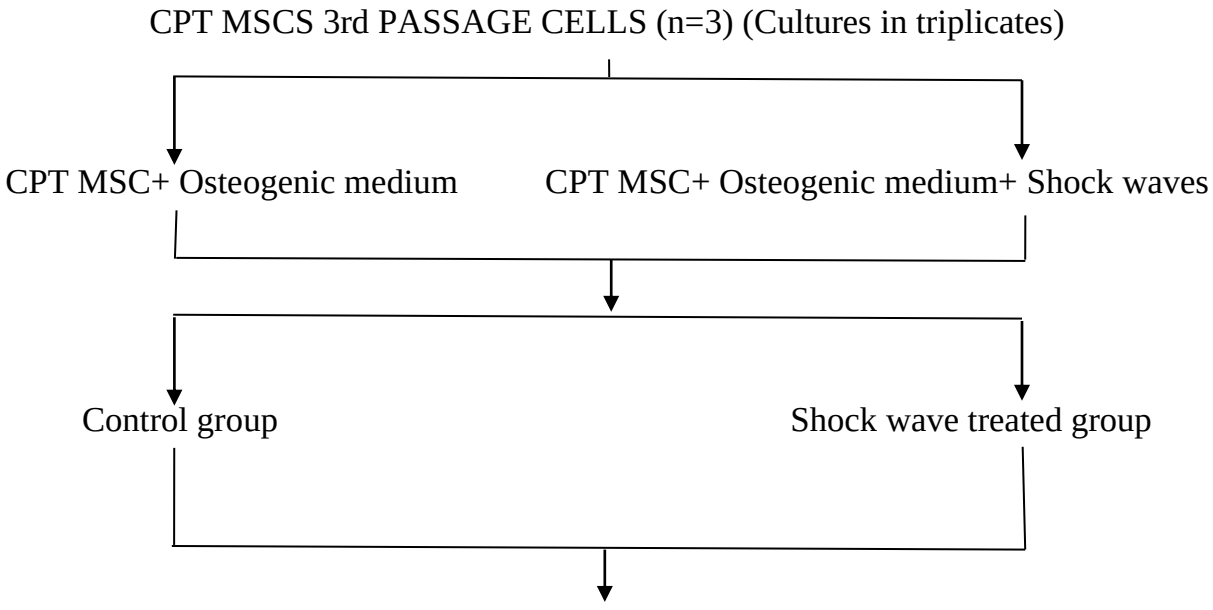
## Detailed algorithm of the study

Cryopreserved CPT MSCS (2nd passage cells)

The cells will be thawed at 37ºC from liquid nitrogen and centrifuged at 2000 rpm for 10 minutes. The pellet will be resuspended in medium and cultured.




High energy shock waves with Energy flux density of 0.831mJ/mm$^2$ at 4000 impulses which did not affect the metabolic activity of CPT MSCs was chosen for our study.



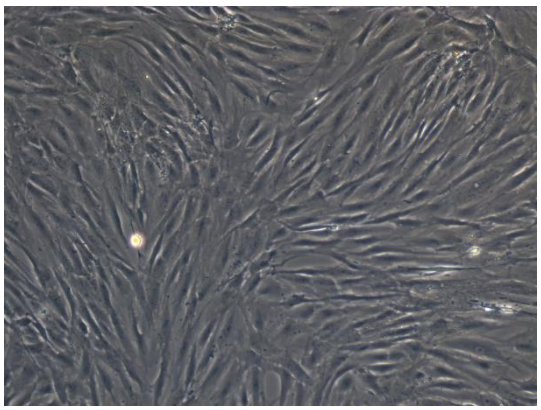

Differentiation will be evaluated at 3 weeks will be measured quantitatively by gene expression of osteogenic markers (eNOS, Alkaline phosphatase, osteocalcin, Osterix-SP7 and RUNX) using RT-PCR and

Calcium deposition using alizarin red s staining.

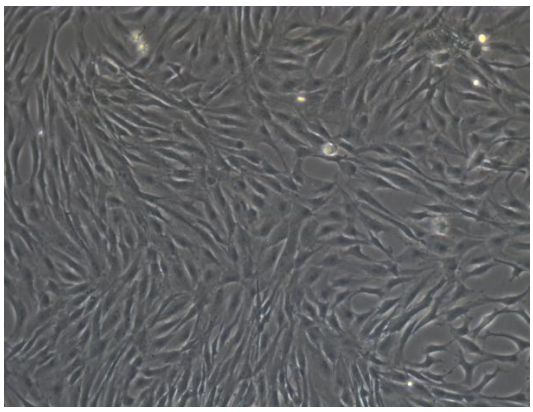
# RESULTS &ANALYSIS

## 1. Morphology:

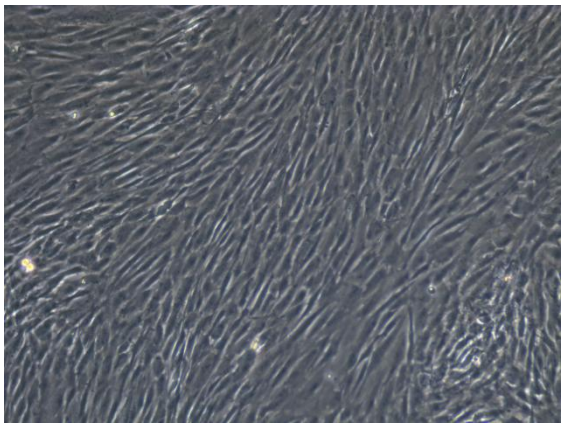
Cryopreserved human mesenchymal stem cells (MSC) harvested from fibrous hamartoma were revived and cultured. Cells on day 2 were plastic adherent; showed spindle shaped elongated morphology under phase contrast microscopy as shown in the Figs.



Patient sample 1



Patient sample 2

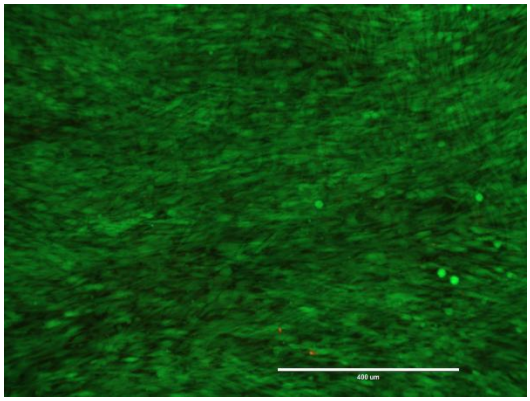


Patient sample 3

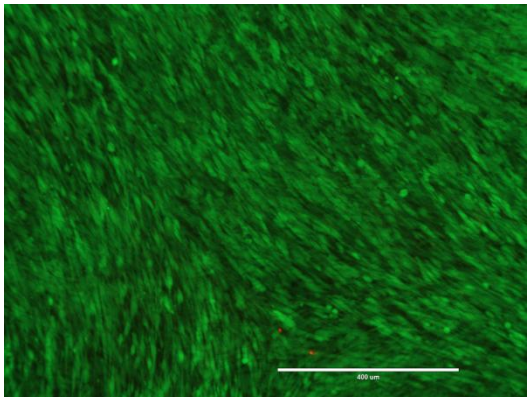
Fig17: **Phase contrast images of CPT MSC showing spindle shaped elongated MSC cells on day 2 of culture, x10**

## 2. Live dead assay:

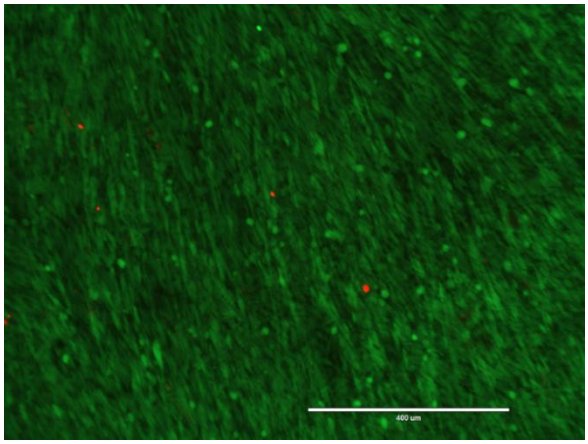
Cell viability was performed by live dead assay where calcein and ethidium homodimer was used. Fluorescence microscopy showed more than **98%** cells to be viable after focal shock wave treatment as indicated in green; red dots indicate dead cells.



Patient sample 1



Patient sample 2



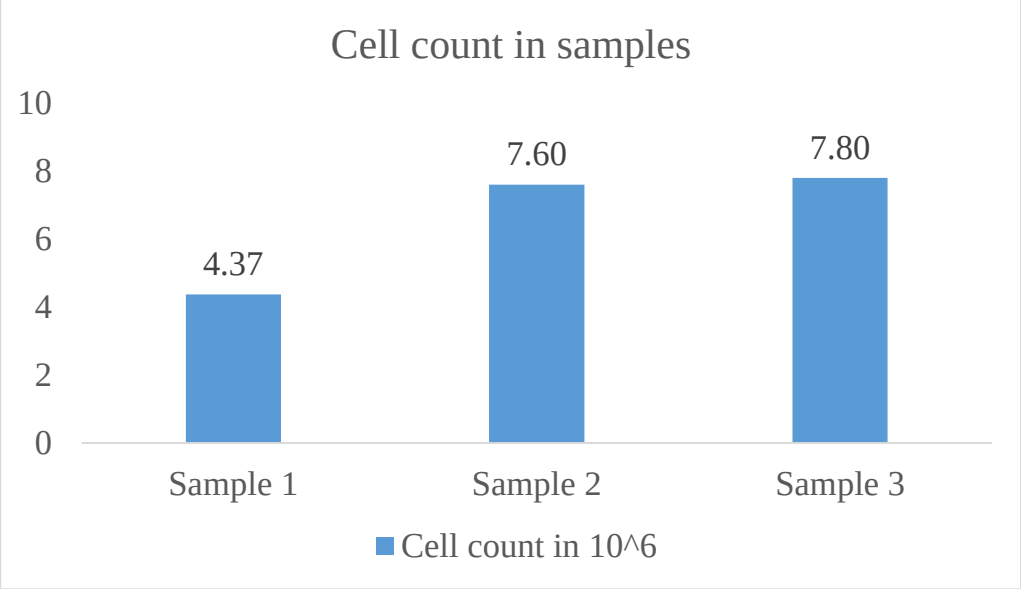
Patient sample 3

Fig 18: **Fluorescence microscopy image of Live dead assay showing 98% viable cells, x10**

### 3. Cell proliferation:

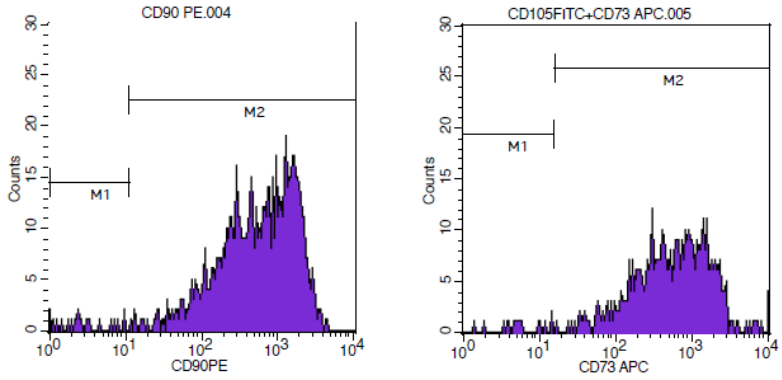
Cells on day 3 were trypsinized and counted using tryphan blue dye exclusion assay. The cell count obtained from each patient sample is tabulated. The average cell yield and viability was 6.59 million cells and 98% respectively. A good mesenchymal stem cell yield is necessary for subsequent downstream analysis.

| | Cell count in 10^6 |
|---|---|
| Sample 1 | 4.37 |
| Sample 2 | 7.6 |
| Sample 3 | 7.8 |



## 4. Characterisation of MSCs:

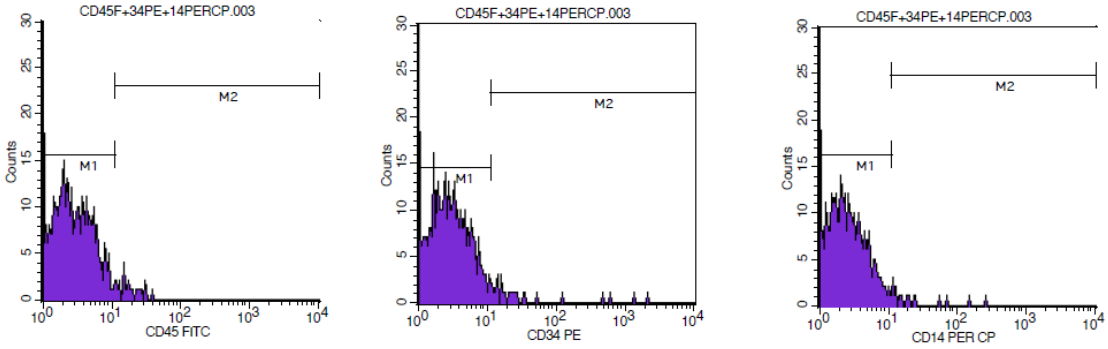
The harvested Human CPT MSC was characterized for cell surface markers by immunophenotyoping. Cells were positive for CD73 (98.5%), CD90 (99.19%), CD 105 (99.56 %) and negative for CD 34(3.22%), CD 45 (1.93%) and CD 14 (1.89%).




| Marker | Events | % Gated | % Total |
|---|---|---|---|
| All | 2801 | 100.00 | 29.45 |
| M1 | 27 | 0.96 | 0.28 |
| M2 | 2774 | 99.04 | 29.17 |

| Marker | Events | % Gated | % Total |
|---|---|---|---|
| All | 1578 | 100.00 | 31.40 |
| M1 | 18 | 1.14 | 0.36 |
| M2 | 1560 | 98.86 | 31.04 |

Fig19: **Fluorescence activated cell sorting analysis for Positive markers of MSC**




| Marker | Events | % Gated | % Total |
|---|---|---|---|
| All | 1682 | 100.00 | 33.37 |
| M1 | 1642 | 97.62 | 32.58 |
| M2 | 42 | 2.50 | 0.83 |

| Marker | Events | % Gated | % Total |
|---|---|---|---|
| All | 1682 | 100.00 | 33.37 |
| M1 | 1635 | 97.21 | 32.44 |
| M2 | 48 | 2.85 | 0.95 |

| Marker | Events | % Gated | % Total |
|---|---|---|---|
| All | 1682 | 100.00 | 33.37 |
| M1 | 1653 | 98.28 | 32.80 |
| M2 | 30 | 1.78 | 0.60 |

Fig 20: **Fluorescence activated cell sorting analysis for Negative markers of MSC**

## 5. Standardization of shock wave administration:

The water bath for the in vitro shock wave treatment was made according to the manual published by Dr. Johannes Holfeld (87). He proposed, the acoustic impedance between the cell culture medium and air reflects 99% of shock waves and the best way to reduce the impedance is to conduct in vitro shock wave treatment in a water bath.

Based on the description in his manual, we made our own customized water bath for in vitro shock wave treatment of CPT MSCs. For coupling between the Focal shock wave applicator and the membrane, ultrasound transmission jelly was used. Water is preheated and added to the water bath. We kept the working distance between the membrane and sample as 5 cm. Briefly the cells at 70% confluence were filled with completed medium and used for focal shock wave treatment as shown in the Fig below.

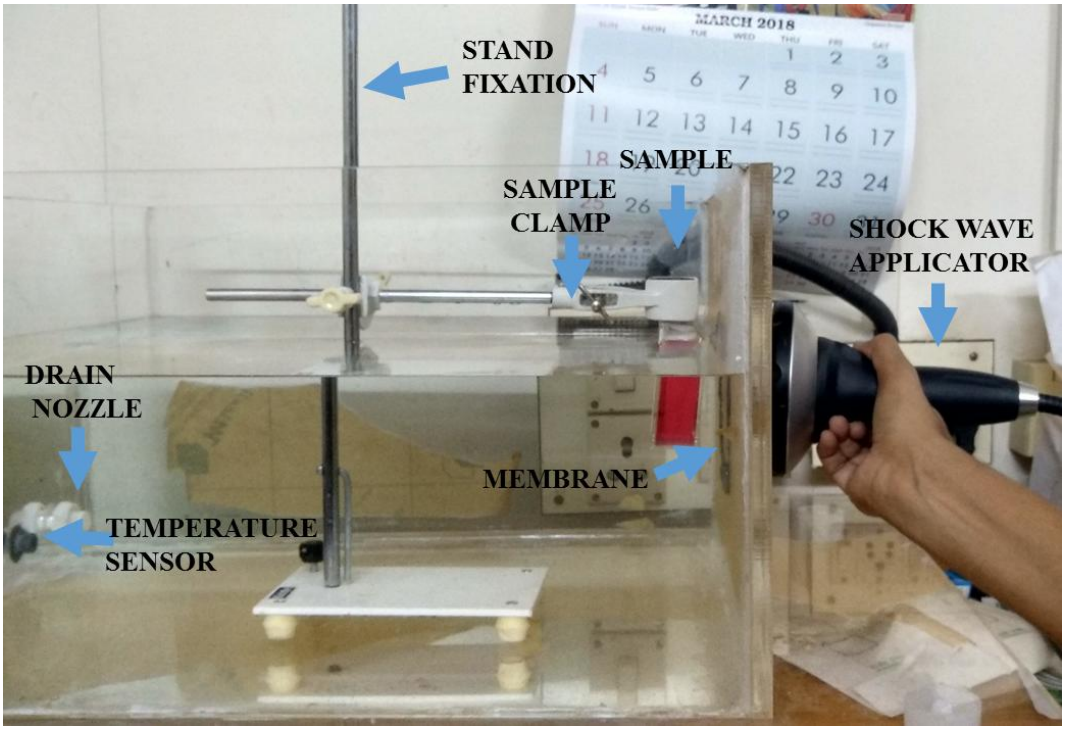


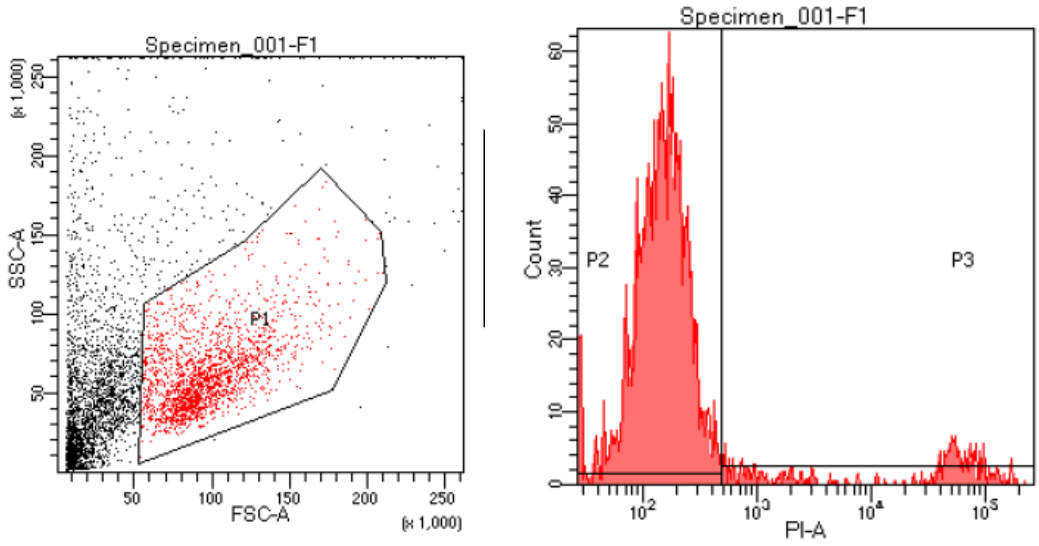

Fig 21: Customized water bath

## 6. Viability of CPT MSC after focal shock wave treatment:

To identify the optimal dose of focal shock wave treatment with minimal cell death, samples were treated at low, mid and high energy levels and viability was assessed using propidium iodide. The percentage of cell viability was 87%,89% ,86.4 in low, mid and high dose respectively**.**

- **Low energy:**

  The mesenchymal stem cells were treated with low energy shock waves with Energy flux density of 0.088mJ/mm$^2$and a positive peak pressure of 11.5MPa .A total of 2000 shock waves were given. We observed 87%viable cells after1 hour of shock wave treatment in low energy .

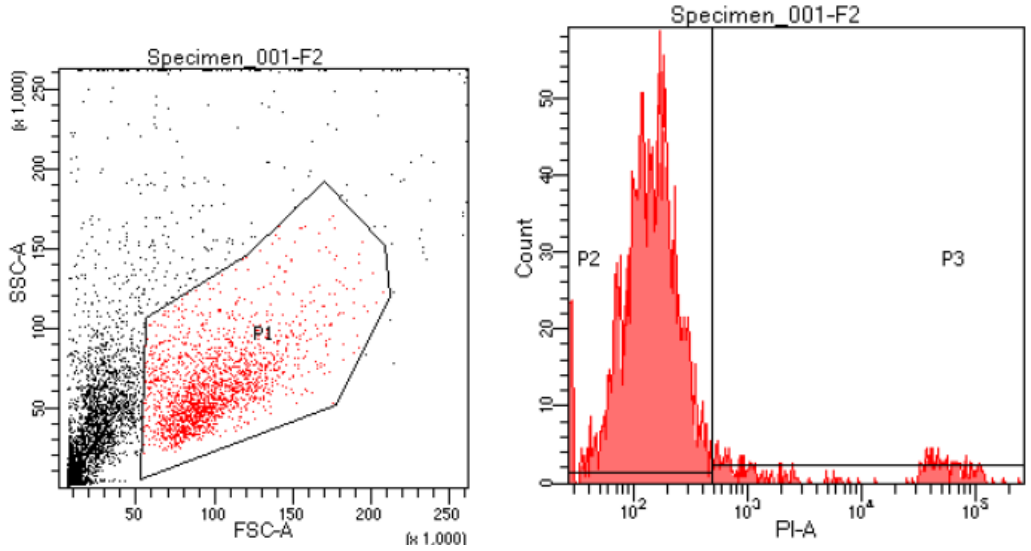



| Tube: F1 | | | |
|---|---|---|---|
| Population | #Events | %Parent | %Total |
| All Events | 5,138 | #### | 100.0 |
| P1 | 2,003 | 39.0 | 39.0 |
| P2 | 1,761 | 87.9 | 34.3 |
| P3 | 242 | 12.1 | 4.7 |

Fig 22: **Flow cytometry results showing 87%viable cells after1 hour of Low energy shock wave treatment**

- **Medium energy:**

The mesenchymal stem cells were treated with Medium energy shock waves with Energy flux density of 0.328mJ/mm$^2$and a positive peak pressure of 30.5 MPa .A total of 2000 shock waves were given. We observed 89%viable cells after1 hour of shock wave treatment in low energy group.



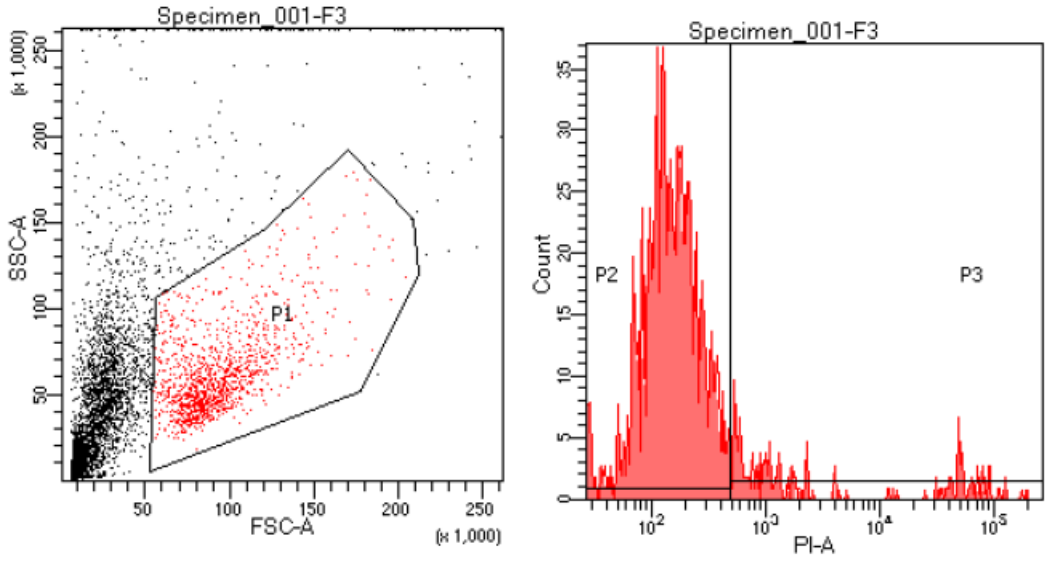

Tube: F2

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 5,009 | #### | 100.0 |
| P1 | 1,873 | 37.4 | 37.4 |
| P2 | 1,668 | 89.1 | 33.3 |
| P3 | 205 | 10.9 | 4.1 |

Fig 23: **Flow cytometry results showing 89%viable cells after1 hour of Medium energy shock wave treatment**

- **High energy:**

The mesenchymal stem cells were treated with high energy shock waves with Energy flux density of 0.831mJ/mm$^2$ and a positive peak pressure of 82.2MPa .A total of 2000 shock waves were given. We observed 86.4%viable cells after1 hour of shock wave treatment in low energy group.




Tube: F3

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 5,035 | #### | 100.0 |
| P1 | 1,217 | 24.2 | 24.2 |
| P2 | 1,052 | 86.4 | 20.9 |
| P3 | 165 | 13.6 | 3.3 |

Fig 24: **Flow cytometry results showing 86.4%viable cells after1 hour of High energy shock wave treatment**

| Energy level | Energy flux density ($mJ/mm^2$) | Positive Peak pressure (MPa) | Cell viability at 1 hour. |
|---|---|---|---|
| Low | 0.088 | 11.5 | **87%** |
| Mid | 0.328 | 30.5 | **89%** |
| High | 0.831 | 82.2 | **86.4%** |

We did not observe any cellular toxicity when CPT MSC were treated with high energy focal shock waves. For our subsequent experiement we used 2000 high energy shock waves with Energy flux density of $0.831mJ/mm^2$ and a positive peak pressure of 82.2MPa.

7. **<u>Cytotoxicity -MTT assay:</u>**

We further confirmed the metabolic activity of CPT MSC after shock wave treatment at 24h, 48h by MTT assay. The formation of the purple colored formazan crystals indicated metabolically active cells.

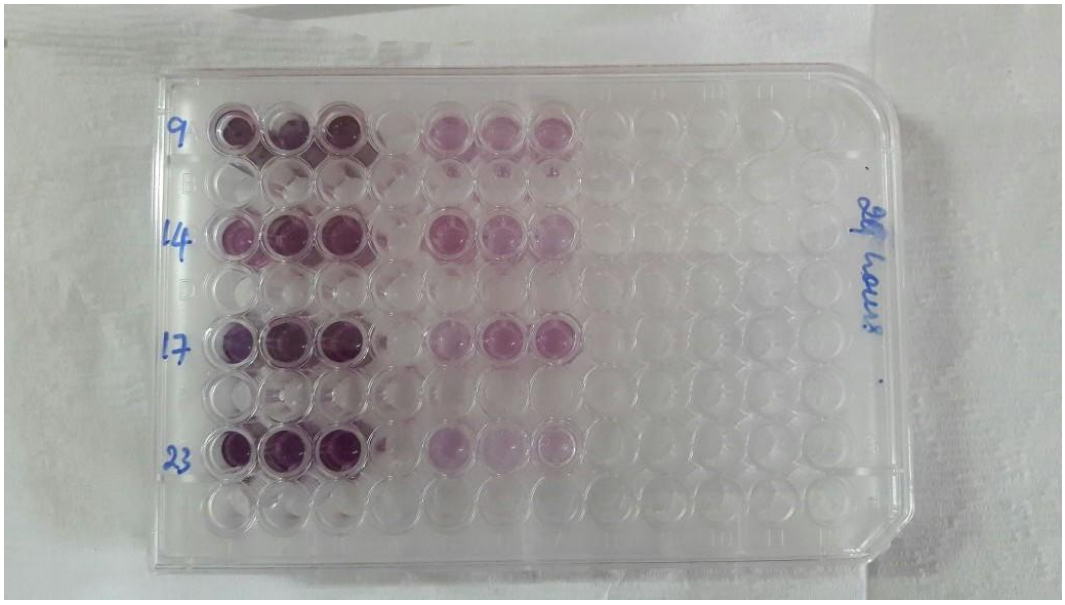



Fig 25: MTT assay at 24 hours showing formation of purple formazan crystal after focal shock wave treatment in high energy doses

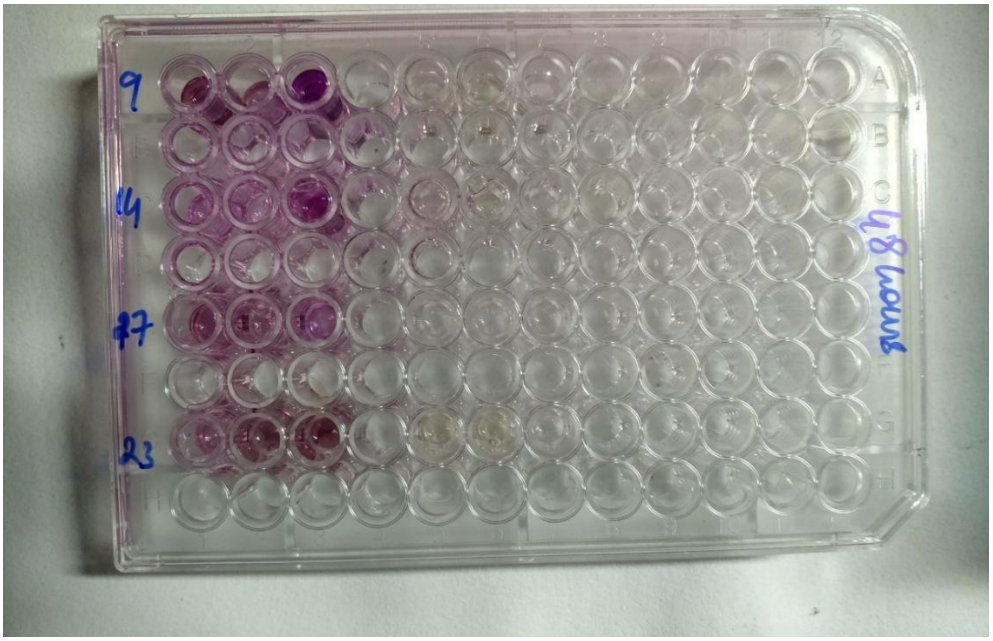



Fig 26: MTT assay at 48 hours showing formation of purple formazan crystal after focal shock wave treatment in high energy doses

From the Figs, it is evident that there is there is active cell proliferation indicated by purple formazan crystal at 24 and 48 hours after focal shock wave treatment in high energy doses. Thus focal shock wave treatment with high energy shock waves with Energy flux density of 0.831mJ/mm$^2$ and a positive peak pressure of 82.2MPa does not affect the metabolic activity of CPT MSC.

## 8. RNA concentration:

The purity and yield of RNA is essential for subsequent downstream analysis. The A260/280 scores were within the acceptable range for all the samples.

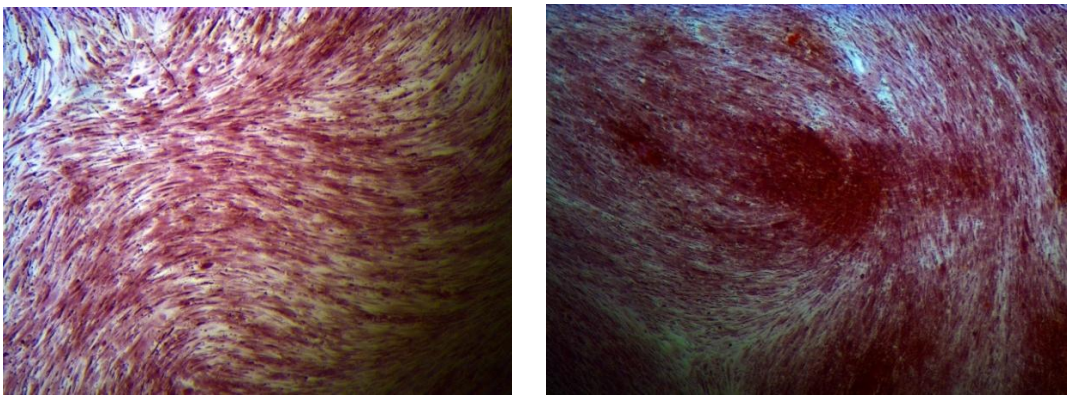
9. **Osteogenic differentiation:**

The Osteogenic differentiation is assessed qualitatively using Alizarin red staining and quantitatively using Gene expression of RUNX 2, ALPL, BGLAP and SP7.

❖ **Alizarin red Staining:**

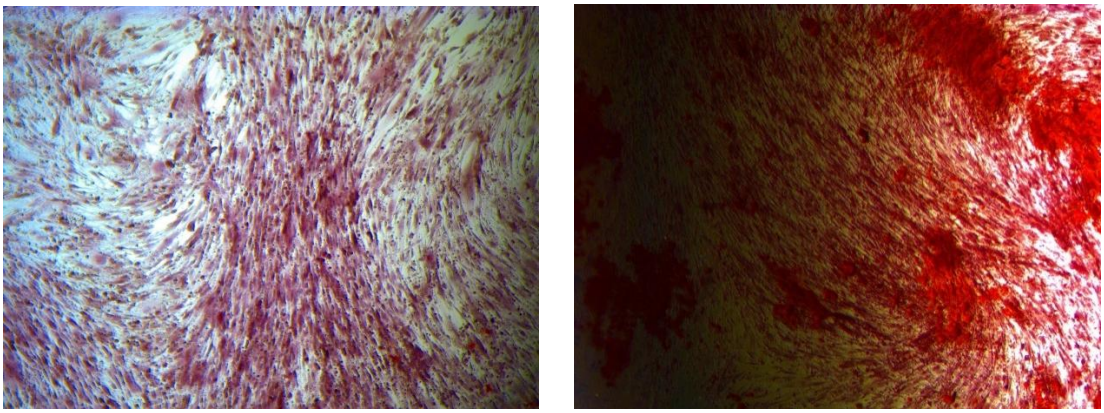
The average area of staining in control group is 55.33 and the average staining in treated group is 69.33. It is found that there is an average increase of 14% was found when samples are treated with focal shock waves, as indicated by calcium deposits in the Figs in calcium deposits.

The Alizarin red staining was quantified by Image J using automatic threshold.



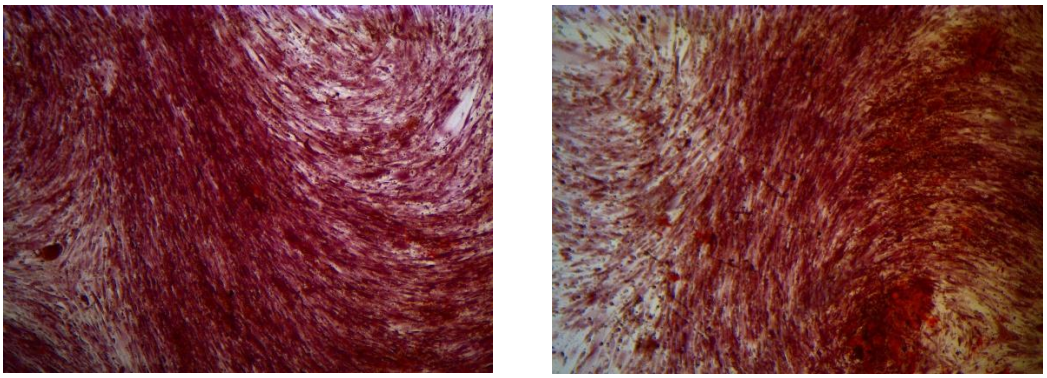
Control Treated

Fig 27: Phase contrast image Alizarin red stained Sample 1 MSCs showing **47.90%** staining in control and **64 %** staining in the treated, x10



Control Treated

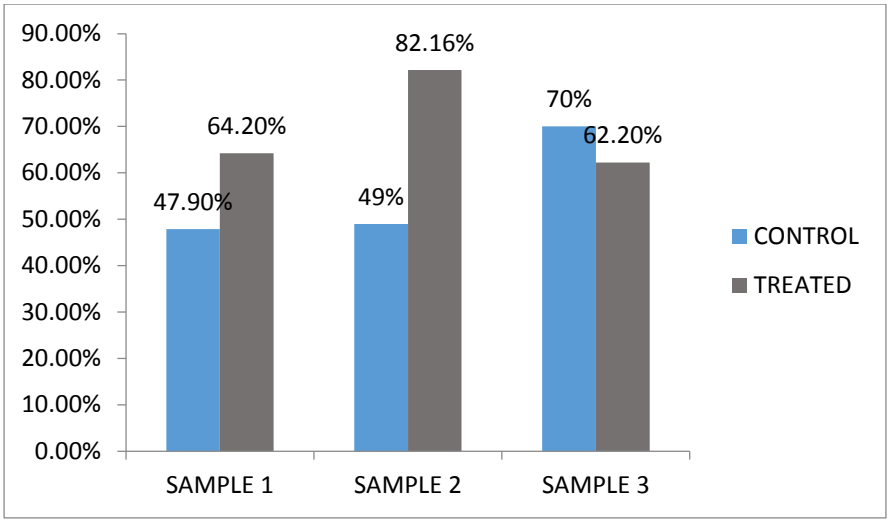
Fig 28: Phase contrast image Alizarin red stained Sample 2MSCs showing **49%** staining in control and **82.16%** staining in the treated, x10



Control Treated

Fig 29: Phase contrast image Alizarin red stained Sample 3 MSCs showing **70%** staining in control and **62.20%** staining in the treated, x10

| | CONTROL | TREATED |
|---|---|---|
| SAMPLE 1 | 47.90% | 64.20% |
| SAMPLE 2 | 49% | 82.16% |
| SAMPLE 3 | 70% | 62.20% |
| AVERAGE | **55.33%** | **69.33%** |




❖ **Change in Gene expression of osteogenic markers by shock wave treatment:**

The mRNA level of osteoblast markers eNOS, RUNX 2, ALPL, BGLAP and SP7 were analyzed in all treated and untreated hamartoma samples (n=3, in triplicates). The shock wave treated samples showed upregulation of mRNA expression of eNOS, RUNX2, SP7, ALPL and BGLAP on days 7, 14 and 21.

The tables and graphs below give the gene expression across all 3 samples at day 7, 14 and 21. Fold change is expressed as 2^-DDCT and normalized to Day 0 of untreated control.

- **eNOS:**

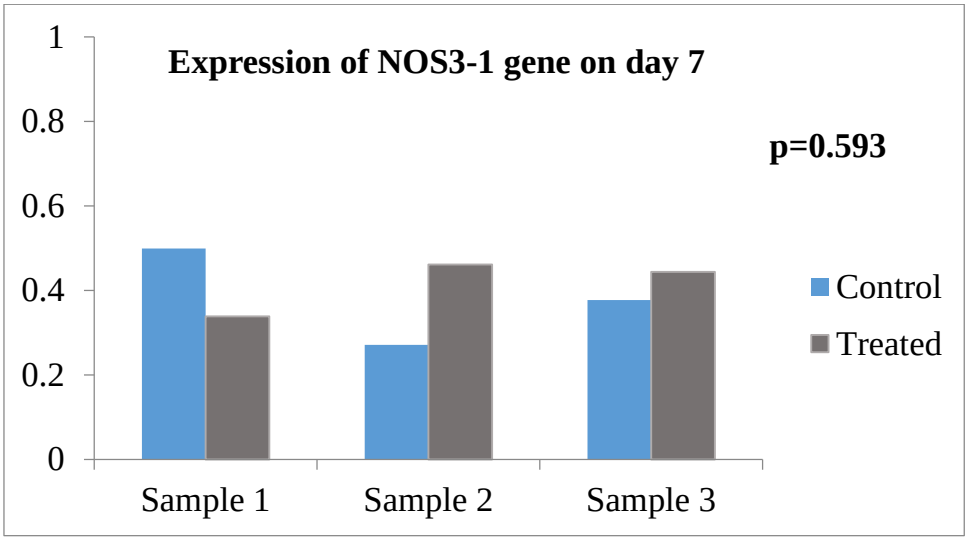
eNOS a product of Nitric oxide synthase pathway was found to play an important role in osteoblast differentiation and bone healing and deletion of the NOS gene resulted in poor osteoblastic differentiation(88) . Previous in vivo studies in rabbit have shown early release of endothelial nitric oxide synthase. (66)

To confirm the early release of angiogenic growth factor after focal shock wave treatment, we assessed the gene expression of eNOS by real time PCR on day 7 of osteogenic differentiation.

We found that with shock wave treatment eNOS was upregulated by 0.086 fold in CPT MSC than the untreated control. In Sample 1, The expression of eNOS was downregulated by 0.33 and in sample 2 and 3 the fold increase was 0.70, 0.17 respectively, which was not statistically significant.

| eNOS expression | Control | Treated |
|---|---|---|
| Sample 1 | 0.498961 | 0.338681 |
| Sample 2 | 0.270556 | 0.461 |
| Sample 3 | 0.376834 | 0.44498 |

.

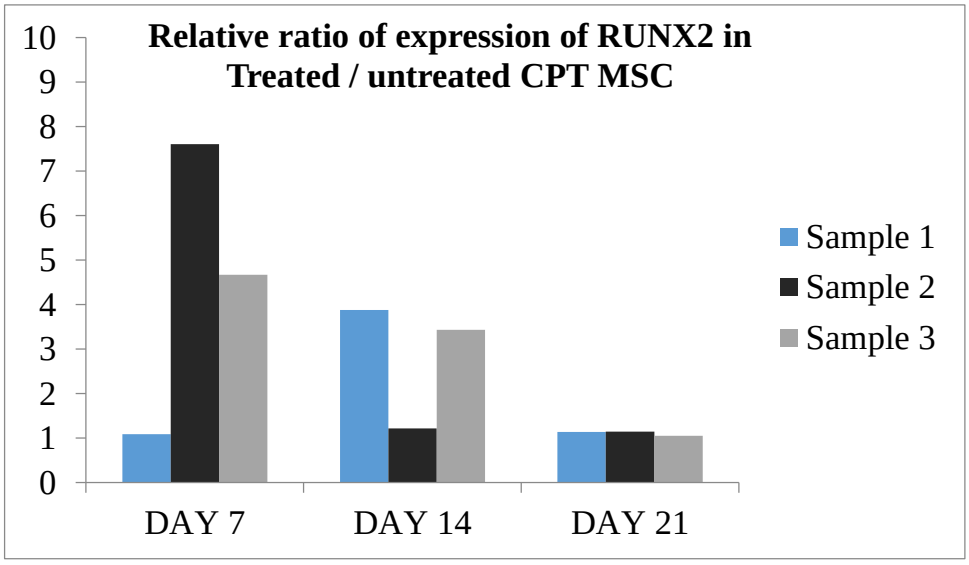


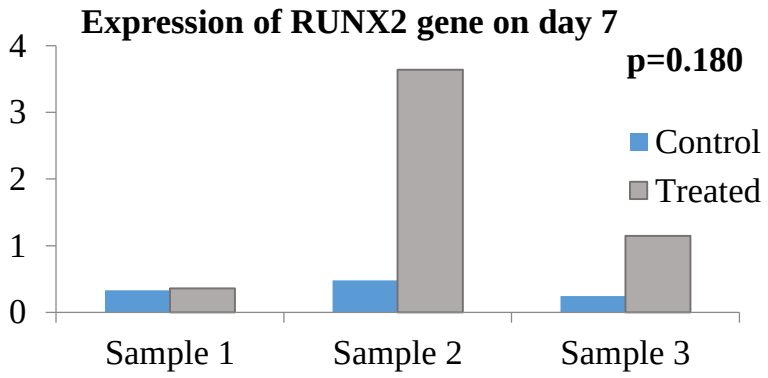
- **RUNX2:**

The expression of RUNX2 is crucial in the early phase of osteogenic differentiation from mesenchymal stem cells.(73).RUNX2 is determines the osteoblast lineage and directs to bone lineage.

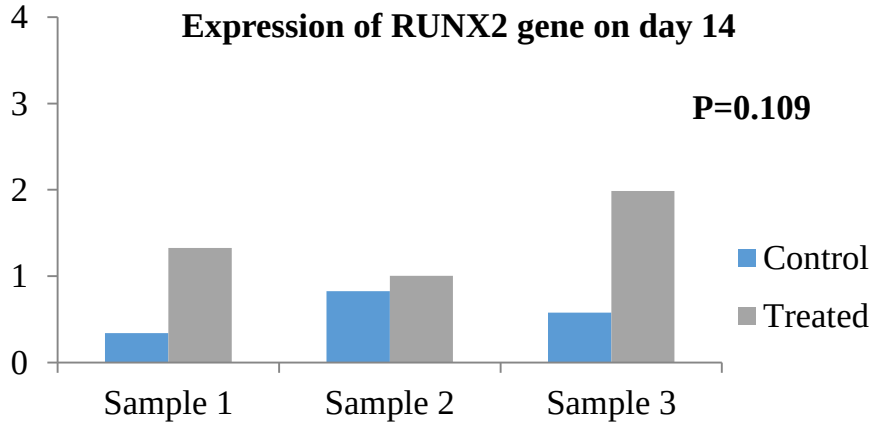
The relative ratio of gene expression of RUNX2 between the shock wave treated CPT MSC and untreated control CPT MSC on day7,14,21 was 4.45,2.84,1.1. There was a steady gene expression of RUNX2 throughout the osteogenic differentiation with a peak increase in day 7-14. This was not found to be statistically significant.

| RUNX | Control 1 | Treated 1 | Control 2 | Treated 2 | Control 3 | Treated 3 |
|---|---|---|---|---|---|---|
| DAY 7 | 0.3285 | 0.3577 | 0.4786 | 3.64 | 0.2457 | 1.1479 |
| DAY 14 | 0.3415 | 1.3250 | 0.8258 | 1.0034 | 0.5783 | 1.9848 |
| DAY 21 | 0.5403 | 0.6151 | 1.3286 | 1.5209 | 1.2397 | 1.3022 |

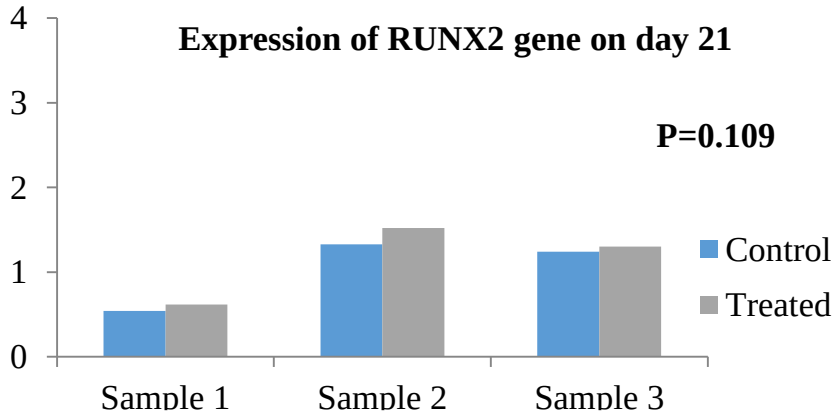





On day 7, In sample 1, 2 and 3, the expression of RUNX-2 was 0.08, 6.6,3.67-fold increase compared to the control respectively.




On day 14, In sample 1, 2 and 3, the expression of RUNX-2 was 2.87,0.21,2.43-fold increase compared to the control respectively.

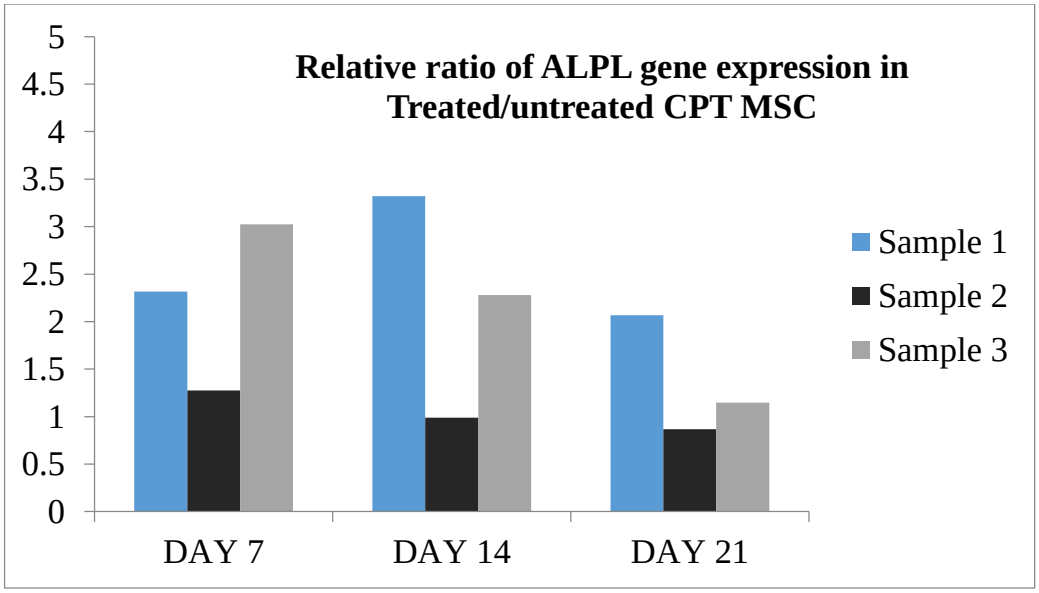


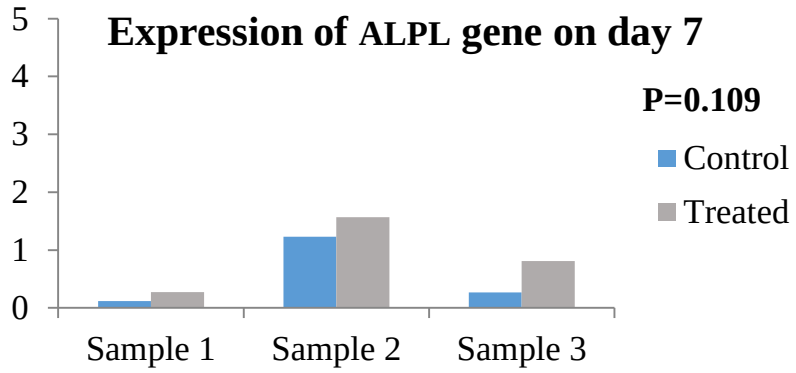

On day 21, In sample 1, 2 and 3, the expression of RUNX-2 was 0.13,0.144,0.050-fold increase compared to the control respectively.

❖ **ALPL:**

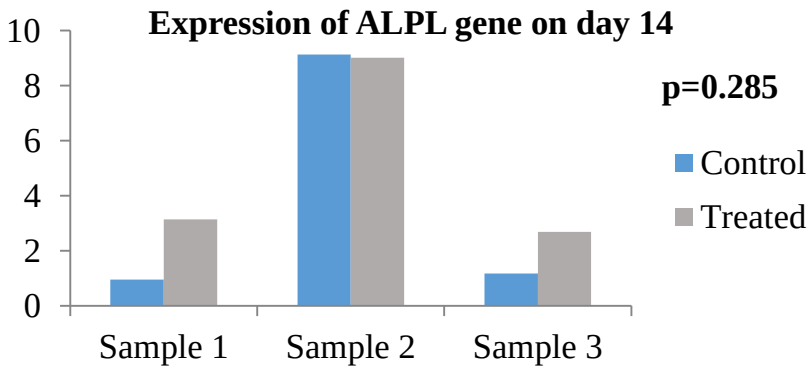
Alkaline phosphatase increases calcium deposition. It is expressed in the early stages of differentiation of osteoblasts. The relative ratio of gene expression of ALPL between the shock wave treated CPT MSC and untreated control CPT MSC on day7,14,21 was 2.205,2.195,1.360 respectively. There was a steady gene expression of ALPL throughout the osteogenic differentiation with a peak increase in day7-14. There is heterogeneity in the gene expression of ALPL between samples.

| | Control 1 | Treated 1 | Control 2 | Treated 2 | Control 3 | Treated 3 |
|---|---|---|---|---|---|---|
| DAY 7 | 0.1181 | 0.2736 | 1.2294 | 1.568 | 0.2683 | 0.8111 |
| DAY 14 | 0.9460 | 3.142 | 9.126 | 9.0129 | 1.1760 | 2.6795 |
| DAY 21 | 0.8139 | 1.6817 | 3.9449 | 3.4271 | 1.1941 | 1.3707 |

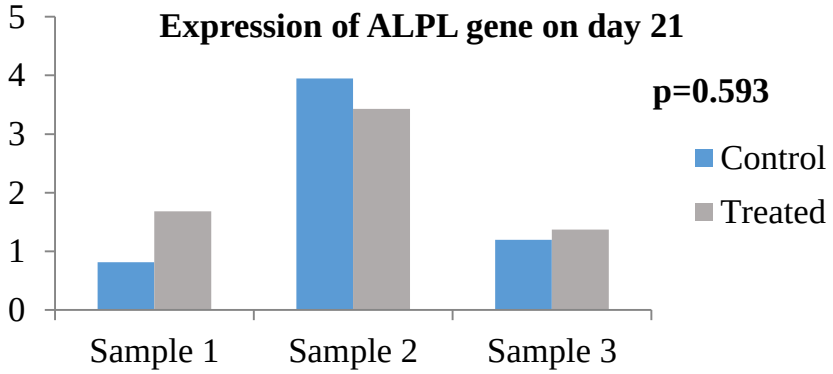





On day 7, In sample 1, 2 and 3, the expression of ALPL was 1.31,0.27,2.02-fold increase compared to the control respectively.




On day 14, In sample 1, 2 and 3the expression of ALPL was 1.27, -0.013,2.32-fold increase compared to the control respectively.



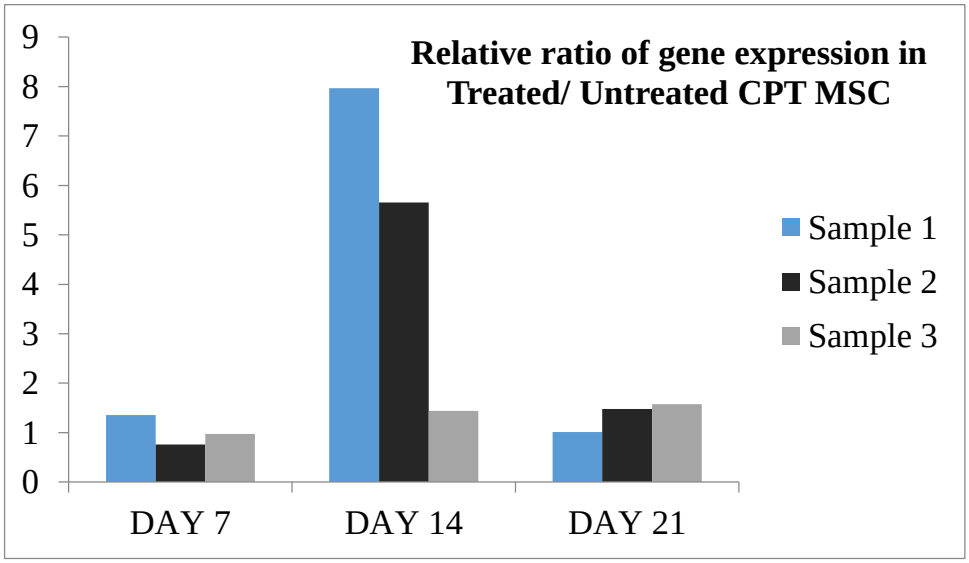

On day 21, In sample 1, 2 and 3, the expression of ALPL was 1.06, -0.142,0.147-fold increase compared to the control respectively.

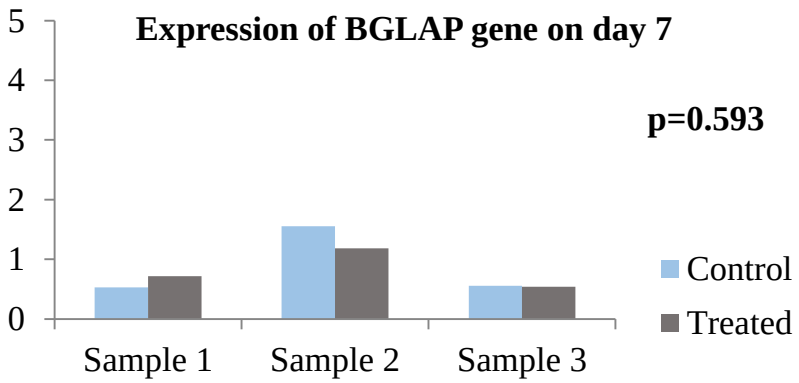
❖ **BGLAP:**

Osteocalcin is one of the late markers of osteogenic differentiation.

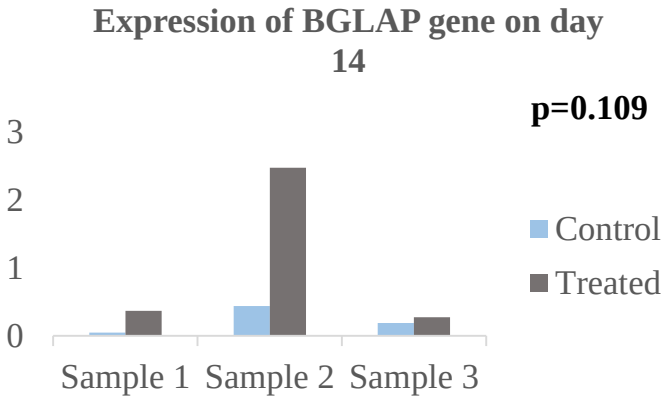
The relative ratio of gene expression of BGLAP between the shock wave treated CPT MSC and untreated control CPT MSC on day7,14,21 was 1.03,5.020,1.355 respectively. There was a decreased gene expression of BGLAP in day 7 and with a peak increase in day 14. There is heterogeneity in the gene expression of BGLAP between samples.

| | Control 1 | Treated 1 | Control 2 | Treated 2 | Control 3 | Treated 3 |
|---|---|---|---|---|---|---|
| DAY 7 | 0.5289 | 0.7185 | 1.1554 | 1.181 | 0.5548 | 0.5404 |
| DAY 14 | 0.40465 | 0.3708 | 0.4379 | 2.4776 | 0.1913 | 0.2752 |
| DAY 21 | 0.1751 | 0.1778 | 6.5070 | 9.6130 | 0.2928 | 0.4610 |

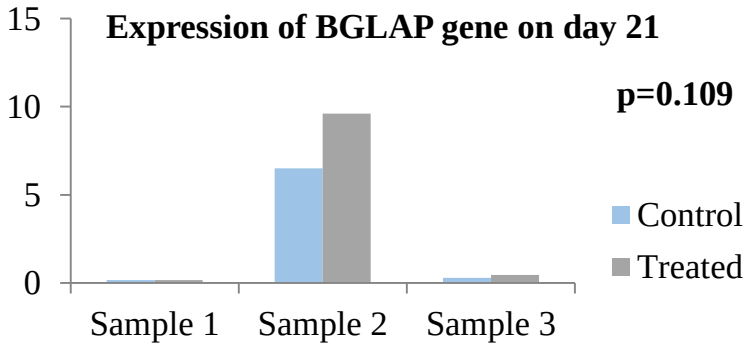





On day 7, In sample 1, 2 and 3, the expression of BGLAP was 0.358, -241, -0.036-fold increase compared to the control respectively.




On day 14, In sample 1, 2 and 3, the expression of BGLAP was 6.96,4.656,0.438-fold increase compared to the control respectively.

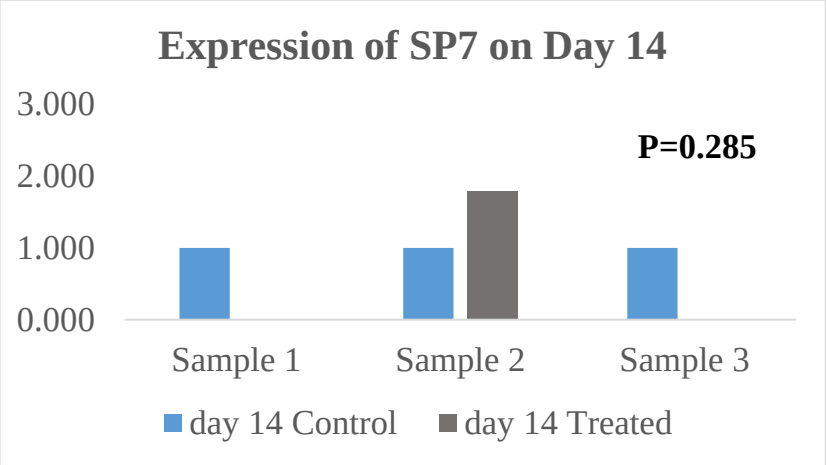



On day 21, In sample 1, 2 and 3, the expression of BGLAP was 0.015,0.477,0.574-fold increase compared to the control respectively.

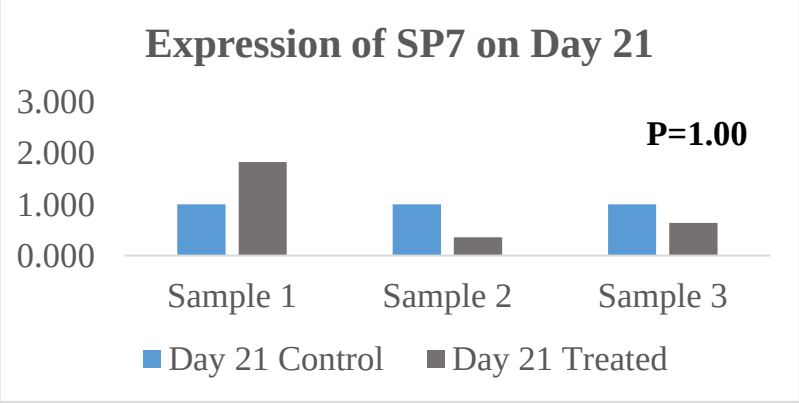
❖ **Osterix SP7:**

SP7 determines the fate of preosteoblasts derived from mesenchymal cells to osteoblasts, blocking their differentiation into chondrocytes.(73)

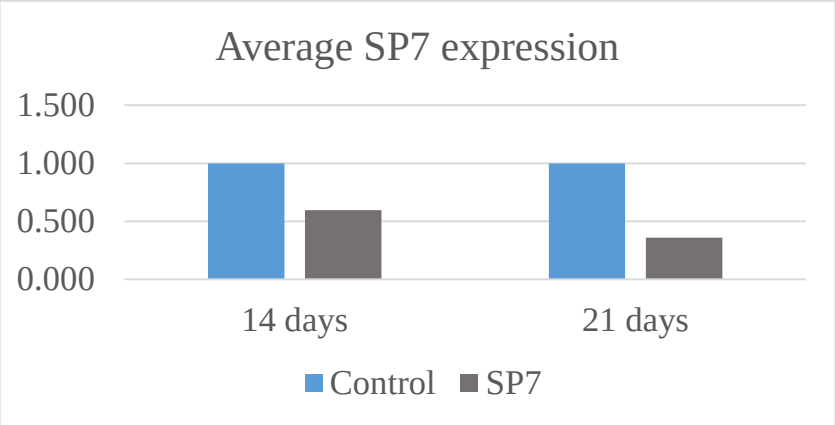
| SP7 | Control | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|---|
| DAY 14 | 1.000 | Undetermined | 1.790 | Undetermined |
| DAY 21 | 1.000 | 1.825 | 0.359 | 0.636 |




On day 14, expression of SP7with shock wave treatment was downregulated by 0.404 fold.




On day 21, expression of SP7with shock wave treatment was downregulated by 0.641 fold.




- The expression of SP7 was downregulated by an average of 0.5225 fold in CPT MSC than the untreated control.

# DISCUSSION

The treatment of congenital pseudarthrosis still remains a challenge to the paediatric orthopaedician due to the difficulty in achieving sound union and to maintain long lasting union.(10) The current standard of care for management of congenital pseudarthrosis of tibia is fibrous hamartoma excision + skeletal stabilisation with intramedullary rod or ilizarov fixator and cortical bone grafting.(41) The rate of union following surgery has been found to be around 86 %(49).The result of the surgery is still not uniform and the incidence of re-fracture are high.(49)

The major pathology has been found to be in the fibrous hamartoma which surrounds the affected bone. The fibrous harmartoma at the site of non-union occurs due to the abnormal proliferation of periosteal cells. The Mesenchymal stem cells derived from this hamartoma have good proliferative ability however has poor ability to differentiate. The cells express low osteogenic markers when differentiated in osteogenic media.(16) One of the strategies for dealing with the poor osteogenesis has been to improve the osteogenic potential with bio-molecules such as BMP however without significant success. (17,41,52)

Several *in vitro* studies have been performed to characterize the progenitor cells isolated from the abnormal periosteum(9). We isolated the cells from 3 fibrous hamartomas of CPT and found that the hamartomatous tissue shares similar phenotypic characteristics of mesenchymal stem cells (CD73+, CD90+, CD105+, CD34-, CD45- and CD14-). This is consistent with the previous studies and shows that we were successful in isolating the MSC from the hamartoma.(16,17).

Shock waves have been used in treatment of non-union and had similar results compared to surgical management of non-unions. (61,67,72) Shock wave therapy has a probable role in increasing osteogenic differentiation and osteoblast proliferation.(64)

Our study concentrated on improving the osteogenic behavior of the MSCs of this hamartoma using high energy focal shock waves which are already shown to improve fracture healing and nonunion. We postulated that focal shock wave treatment could enhance the osteogenic differentiation of the Mesenchymal stem cells derived from the fibrous hamartoma of congenital pseudarthrosis of tibia. Characteristically, the fibrous hamartoma at the site of pseudarthrosis decreases the bone formation and bone remodeling. (17)

Our first goal was to establish the safe dose of shock waves. The MSCs of fibrous hamartoma were treated with shock waves of different energy levels (Low, Mid and high). None of these were found to be toxic to the cells in both healthy control and MScs derived from CPT based on MTT assay and cell proliferation, since higher energies are associated with fracture healing this group was taken forward. Shock waves of high energy was used finally as it would enhance osteoblastic differentiation of CPT MSCs to replicate the desired effect in vivo.

Since there is no study comparing the effect of focal shock waves on CPT mesenchymal stem cells. We compared our data with results from our previous study with pamidronate, another non-invasive treatment method for treating congenital pseudarthrosis of tibia.

The cells treated with 4000 High energy shockwaves were primed in to osteogenic lineage. When assessed for calcium deposits with Alizarin red staining, wefound increased deposits in the treated sample. This was in contrast to pamidronate treatment where they found no difference.(17)

We further quantified the osteogenic differentiation by measuring gene expression of various markers of differentiation using Real time PCR.

**1. <u>eNOS2:</u>**

eNOS a product of Nitric oxide synthase pathway was found to play an important role in osteoblast differentiation and bone healing and deletion of the NOS gene resulted in poor osteoblastic differentiation(88) . Previous in vivo studies in rabbit have shown early release of endothelial nitric oxide synthase. (66)In our study we found eNOS2 to be upregulated after shock wave treatment.

**2. <u>RUNX2:</u>**

RUNX2 is determines the osteoblast lineage. The expression of RUNX2 is crucial in the early phase of osteogenic differentiation from mesenchymal stem cells.(73) Our study shows upregulation of RUNX2 while studies with pamidronate alone showed fold down regulation upon treatment.

**3**. **Osterix- SP7:**

SP7 determines the fate of pre-osteoblasts derived from mesenchymal cells to osteoblasts, blocking their differentiation into chondrocytes.(73)We found that there is a downregulation of the osterix and this is due to the inherent nature of CPT MSC being in different stages of aberrant osteogenic differentiation.

**4. BGLAP- Osteocalcin:**

Osteocalcin is a late marker of osteogenic differentiation. We found upregulation when treated with shock waves as early as day 14 as opposed to downregulation of osteocalcin when treated with pamidronate in the other study.

**5. ALPL:**

Alkaline phosphatase increases calcium deposition. It was found that shock waves increased calcium deposition when stained with alizarin red as well as upregulation in the expression of ALPL when treated with shock waves.

# CONCLUSION

The impaired osteogenic potential of fibrous hamartoma cells has been investigated previously. In this study we attempted to use shock wave therapy, a non-invasive modality to improve the osteogenesis of CPT MSC and found upregulation of all associated osteogenic markers at different time points, though this increase in trend did not reach statistical significance because of wide variations known to occur in human primary cells, they might be of clinically relevance. Furthermore, we found that comparing our data with existing studies on pamidronate (*in vitro*) we found that shock wave treatment may provide a better osteogenic stimulus than the bisphosphonate or rhBMP treatment. Further studies with increased sample size supported by in vivo preclinical animal studies are warranted to establish its clinical significance.

## LIMITATIONS

- Sample size
- The variability in the results is mainly due to the subset of mesenchymal stem cells within the hamartoma which are at different stages of aberrant osteogenesis depending on the case and severity of the disease, in turn affecting the outcome.

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# ANNEXURE

## Annexure 1 – Steps of the experiment

### 1) Excision of the Fibrous hamartoma:

1) The fibrous hamartoma tissue was harvested surgically from three patients (2 females, one male with a mean age 2.66 years) with CPT who underwent excision of the hamartoma and corrective surgery.
2) All of these patients had café au lait spots of more than six in numbers and their surgical specimen had histological fibrous hamartoma consistent with the diagnosis of CPT.

### 2) Isolation of MSCs from fibrous hamartoma tissue:

1. Tissue was collected in a 50 ml centrifuge tube containing α-MEM (Sigma Aldrich, St Louis, USA) supplemented with gentamycin (50 μg/ml).
2. The time taken from harvest and to transport the sample to the lab was 1 hour.
3. The tissue was then washed twice with phosphate buffered saline (PBS) and minced, tissue was kept for digestion overnight digestion in 1 mg/ml collagenase type II (Worthington Bio-chemicals, New Jersey, U.S.A.) in a $CO_2$ incubator.
4. Following incubation, the cell suspension was filtered through 100 μm cell strainer (BD Falcon, Bedford, USA) and centrifuged at 2000 rpm for 10 min at room temperature. This step was repeated twice to remove the undigested debris.

5. Then the cell pellet was suspended and cultured in medium supplemented with 10% FBS and gentamycin.
6. Cells were cryopreserved with 70% DMEM, 20% FBS and 10%DMSO
7. Cells were centrifuged at 2000rpm and supernatant was removed.
8. Cells were transferred to -80˚C following which they were stored in liquid nitrogen.
9. These cryopreserved cells were revived and used for our study.

**3) <u>Morphology and Characterization of MSCs:</u>**

1. The cell morphology and confluence were documented using phase contrast microscopy.
2. Cells at passage 3/4 were characterized for human MSCs cluster of differentiation (CD) markers by flow cytometry.
3. The positive markers are CD73, CD 90, CD105 and negative markers are CD34, CD45, CD14 (flurochrome conjugated antibodies)
4. Briefly, cells were incubated for 15 minutes at room temperature individually with 2 µl of CD73 PE, CD90, CD105 PE and CD 34 PE, CD 45 FITC, CD 14 PerCp; IgG FITC, IgG PE and IgG PerCp served as negative controls to ensure the gating of accurate positive signals without non-specificity. Unstained cells served as controls.
5. The cells were diluted with PBS and centrifuged at 500 g; cell pellet was resuspended in PBS and acquired using BD FACS Celesta (10, 000 events).

6. Data was analyzed using BD FACS Diva software. The signal falling beyond the control was considered as positive and expressed as percentage of the total population.

**4) <u>Application of focal shock wave treatment to CPT MSCs:</u>**

1. For accurate administration and preventing the loss of dissipation of focal shock waves, a chamber was customized as per a previously published protocol.(88)
2. Briefly, the chamber was filled with warm water (approx. 37°C); culture flask with cells (filled with complete medium) at 80% confluence was suspended on a tube holder. The flasks were kept at 5 cm from the applicator.
3. The focal shock wave treatment using piezo wave 2with high energy shock waves at 4000 impulses with Energy flux density of 0.831mJ/mm$^2$ and a positive peak pressure of 82.2MPa which did not affect the metabolic activity of CPT MSCs was chosen for our study.
4. Untreated cells served as controls.

5) **<u>Cytotoxicity test:</u>**

1. Cells were cultured in 96 well plates (seeding density 25,000 cells/cm$^2$) with complete media (αMEM with 10% FBS) and kept in a $CO_2$ incubator.
2. After 48 h of incubation, culture media was removed and replaced.

3. MTT assay was performed after further incubation of 24, 48 hours in triplicates.
4. MTT reagent was added to the culture and the cells were incubated in dark condition.
5. The formation of insoluble formazan crystals is qualitatively assessed by the formation of the purple colour.

**6) <u>Osteogenic differentiation:</u>**

1. MSCs were cultured in 24 well plates at a seeding density of approximately 8000 cells per well.
2. The cells treated with and without shock waves (high energy, 4000 Shocks) were cultured for 48 hours in complete medium
3. After 48 hours, osteogenic differentiation medium (Hyclone) was added to the cells.
4. The medium was changed regularly at when the colour of the medium changes.
5. Differentiation was carried out for 21 days.
6. The differentiation was qualitatively assessed by Alizarin red staining and gene expression.

- **<u>Alizarin red S staining:</u>**

1. The medium was removed and the culture plate was washed with Phosphate buffer solution.
2. 70% Ethanol was added to the culture plate and was incubated in the 4C.
3. The culture plate was removed and was washed with water.

4. 2% Alizarin Red S stain was mixed with water (1:3) and was added to the culture plate. The culture plate was kept in a shaker for equal distribution of the stain.
5. Image J software was used to compute the percentage of area stained by a blinded observer.
6. The analysis was repeated minimum three times and expressed as percentage area stained ± SD.

- **Gene expression of markers of osteogenic differentiation:**

1. The differentiation medium on days 7, 14 and 21 was removed and the culture plate was washed with phosphate buffer solution.
2. Total RNA was isolated using TRIZOL Reagent (Sigma Aldrich, St Louis, USA) as per the manufacturer's protocol
3. The collected RNA sample with TRI reagent was centrifuged and stored on days 7,14 and 21.
4. The quantity and quality of RNA was obtained using NanoDrop (Thermo)
5. Isolated total RNA was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (PrimeScript TAKARA).
6. Template DNA was used in gene-specific RT-PCR. The relative gene expression of osteogenic markers (RUNX2, ALPL, OSTERIX, OSTEOCALCIN) was quantified using a real time PCR.

## Annexure 2- Informed consent

## Paediatric orthopaedics unit, CMC Vellore

## Isolation and in-vitro characterization of the mesenchymal stem cells from the lipofibromatous tissue around human congenital pseudarthrosis tibia and in vivo induction of pseudarthrosis in mice tibia.

**1. What is congenital pseudarthrosis of tibia (CPT)?**

Congenital pseudarthrosis of tibia is the disease where the fracture at the leg bone takes long time to unite despite of many surgeries. This disease occurs because of some tumour like tissue grows around the leg bone which prevents it from uniting.

2. **What is the work plan in this study?**

Under this study, after your consent, part of the excised sample after your child's surgery limb will be taken to laboratory and stem cells will be isolated from that tissue and their possible source, quantity will be assessed. Those cells will be injected in the mice bone to check whether these cells create the same disease in mice bone. This information will be helpful to treat the future patients as the source of the CPT will be targeted.

**3. What your child will have to do if you participate in this study?**

You don't need to do anything. The tissue will be collected only from the excised tissue which is usually discarded. So it won't harm your child and need not to

worry about any consequences and in fact the removal of that extra tissue is necessary step for the treatment of CPT. Treatment of your child will not be changed even slightly for this study.

**4. Is there any benefit by accepting for this study?**

There is no direct benefit by accepting to participate in this study. But the knowledge generated by this study will be of immense help to the scientific and research community. To put in simple words the participation will help for the well being of the future society.

**5. Any compensation will be provided for this study related injury?**

There will not be any side effect related to this study hence we will not be compensating.

**6. Can you withdraw from this study?**

This is a onetime event so the question of withdrawal does not arise. You can however refuse consent.

For study related queries you can feel free to contact

Dr Vrisha Madhuri

Paediatric orthopaedic unit,

Christian Medical College,

Vellore- 632004.

Phone number: 0416 2282172.

**கிருத்துவ மருத்துவக் கல்லூரி, வேலூர்**

**குழந்தை முடநீக்கியல் துறை**

குழந்தை கால் நீண்ட எலும்பில் ஏற்படும் பிறவி செயற்கை மூட்டு நோயின் திசுவியிருந்து மூல உயிரணுவை பிரித்தெடுத்து ஆராய்தல் மற்றும் இந்த மூல உயிரணுவைக் கொண்டு எலியில் குழந்தையின் நோய் மாதிரியை உருவாக்குதல்

1. பிறவி செயற்கை மூட்டு நோய் என்றால் என்ன?

இந்த நோய் குழந்தையின் நீண்ட எலும்புகளில் ஏற்படுகிறது இந்த நோயினால் நீண்ட எலும்பு பலவீனம் அடைந்து விரிசல் ஏற்படுகிறது. இந்த விரிசல் தானாகவே கூடாமல் சில நாட்களில் செயற்கையான மூட்டாக மாறுகிறது இந்த நோயில் ஒரு வகை திசு நீண்ட எலும்பை சுற்றி உருவாகி, விரிசல் அடைய செய்து பின் அதனை கூடாமல் தடுக்கிறது.

2. இந்த ஆராய்ச்சியில் என்ன செய்யப்படும் ?

இந்த ஆராய்ச்சியில், தங்களுடைய ஒப்புதலுக்குப் பின், நோய் உண்டாக்கும் திசுவை நீண்ட எலும்பில் இருந்து அறுவை சிகிச்சை மூலம் பிரித்தெடுக்கபடும். பிரித்தெடுத்த திசுக்களை ஆராய்ச்சி நிலையத்திற்கு எடுத்துச் சென்று, அதில் இருந்து மூல உயிரணுக்களைப் பிரித்து எடுக்கபடும். இந்த மூல உயிரணுக்களை எலியின் காலில் செலுத்தி நோய் ஏற்படுகிறதா என்று ஆராயப்படும். இந்த எலிகளை பின் ஆராய்ச்சிக்கு உட்படுத்தி, பிற குழந்தைகளுக்கு ஒரு சிறந்த சிகிச்சை முறை கண்டுப்பிடிக்கப்பட்டு அளிக்கபடும்.

3. இந்த ஆராய்ச்சியில் பங்கேற்க உங்கள் குழந்தை என்ன செய்யவேண்டும் ?

தங்கள் குழந்தைகள் எதுவும் செய்ய தேவையில்லை குழந்தைக்கு நோய் உண்டாக்கும் திசுக்களை அறுவைசிகிச்சை மூலம் அகற்றபடும் இத்திசுகக்கள் தான் ஆராய்ச்சிக்கு பயன்படுத்தப்படும். இதனால் உங்கள் குழந்தைக்கு எந்த பாதிப்பும் இல்லை.

4. இந்த ஆராய்ச்சியில் தங்களது குழந்தை பங்கு பெறுவதால் எவ்வாறு பயனடைவார் ?

இந்த ஆராய்ச்சியில் தங்கள் குழந்தை பங்குபெறுவதால் எந்த ஒரு நேர்முக பலனும் பெற முடியாது. ஆனால், இந்த ஆய்வின் இறுதியில் பெறப்படும் அறிவிகைக் கொண்டு வருங்காலத்தில் குழந்தையின் இவ்வகை நோய்க்கு சிறந்த சிகிச்சை அளிக்க முடியும்.

5. இந்த ஆய்வின் விளைவாக ஏற்படும் விளைவுகளுக்கு நஷ்டயீடு கிடைக்குமா?

தங்கள் குழந்தைக்கு நோய் உண்டாக்கும் திசுக்களை சிகிச்சைக்காக அகற்றப்படும் திசுவில் இருந்து சிறு திசுவை ஆராய்ச்சிக்கு பயன்படுத்தபடும் இதனால் குழந்தைக்கு எந்த பாதிப்பும் ஏற்படாது. அதனால் நஷ்டயீடு எதுவும் தரப்படாது.

6. நீங்கள் இந்த ஆய்வில் இருந்து விலகமுடியுமா?

இந்த ஆய்விலிருந்து நீங்கள் எந்த நேரமும் விலகிக் கொள்ளலாம். ஆனால் தாங்கள் இந்த ஆய்வில் பங்கு பெறுவது உங்கள் சொந்த விருப்பமே. அதனால் விருப்பம் இல்லையெனில் நீங்கள் ஒப்புதல் தர மறுக்கலாம்.

7. உங்களுடைய குழந்தையின் திசுக்கள் மற்றும் தகவல்களின் நீண்ட காலம் திட்டம் என்ன?

உங்களுடைய குழந்தையின் திசு மாதிரிகள் காலவரையற்ற காலம் ஆய்வகத்தில் சேமிக்கப்படும். அது எதிர்கால ஆய்வுகளில் மற்ற ஆராய்ச்சியாளர்களால் பயன்படுத்தபடும் இது மருத்துவ ஆராய்ச்சியாளர்களுக்கு ஒரு நிலையான மூலத்தைக் கொடுக்கிறது.

தங்களுக்கு மேலும் சந்தேகம் இருந்தால் தொடர்பு கொள்ள வேண்டிய முகவரி:

மரு. விருஷா மாதுரி.

குழந்தை முடநீக்கியல் பிரிவு

கிருத்துவ மருத்துவக் கல்லூரி

வேலூர் தொலைபேசி எண் (0416) 2282172

## जानकारी पत्र

## पिडीयाट्रीक ऑर्थो युनिट, सी एस सी वेलोर

शिर्षक : वुंजेनायल सुजर्शोसीस हिबीया मतलब पैर की हडडी का जन्मजात बिमारी से ना जुडना'' इस बिमारी से मूलकोशिकाओं को निकालना और उनका अभ्यास करना और इन मुलकोशिकाओं से चुहों में इसी बिमारी से उत्पन्न करना।

1. कंजेनायटन सुजर्थोसीस टिबीया क्या है? (Congenital Pseudarthrosis)

यह एक बिमारी है जिसमें पैर की हडडी के उपर मांसपेशीया जमा हो जाती है और धीरे–धीरे पैर की हडडी को खत्म करना शुरू करती है।

2. इस अभ्यास में क्या होगा?

इस संशोधन के अंतर्गत इसी बिमारी के मांसपेशीयों में से मुलकोशिकायें अलग की जाएगी और उनका अभ्यास किया जायेगा। इन्हीं कोशिकाओं को चुहों के हडडी में डाला जायेगा। और इसी बिमारी को चुहों में उत्पन्न किया जाएगा।

3. आपको इसमें सदभाग के लिए क्या करना है?

इस संशोधन में वही मांसपेशीयां ली जाएगी जो प्रायः फेकी जाती है। इसलिए आपको इसमें सहभागी होने के लिए कुछ भी नहीं करना पडेगा। यह मांसपेशीया निकालने से ही आपकी/आपके बच्चे की बिमारी ठीक होगी और हडडी जुड जाएगी।

4. आपको इस अभ्यास में सहभागी होने के क्या लाभ है?

कुछ भी नही। आपको सिधे से इसका कोई लाभ नहीं है परन्तु इसमें से जो जानकारी मिलेगी उससे आपके जैसे और रीजों के ंलाज में फायदा हो सकता है।

5. क्या मुझे कुछ भुगतान मिलेगा?

नहीं। इस संशोधन से आपको कोई भी नुकसान नहीं है। इसलिए भुगतान का सवाल नहीं उठता है।

6. क्या मैं इस अभ्यास से नाम वापस ले सकता हूँ?

नहीं। यह अभ्यास सिर्फ एक बार आपके मांसपेशीयों को प्रयोगशाला में ले जायेगा। आपको उसके बाद कुछ भी नहीं करना है। इसलिए नाम वापस लेने का प्रयोजन नहीं आता। आपका नाम अपने आप वापस हो जाता है।

और जानकारी के लिए :

डॉ वृषा माधूरी
प्रोफेसर और मुख्य
पीडीयाट्रीक ऑर्थो युनीट
वेल्लोर
0416 2282172

## ANNEXURE 3-INFORMED CONSENTS

### Informed Consent form to participate in a clinical trial

Study Title: **Isolation and in-vitro characterization of the mesenchymal stem cells from the lipofibromatous tissue around human congenital pseudarthrosis tibia and in vivo induction of pseudarthrosis in mice tibia.**

Study Number: _____

Subject's Initials: _____

Subject's Name: _____

Date of Birth / Age: _____

Please initial box (Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any future research that may be conducted in relation to it, even if I

withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/ parents: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date:____/_____/_____

Name of the Witness: _____

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**ஆய்வில் பங்கேற்பதற்கான ஒப்புதல் படிவம்**

**ஆராய்ச்சியின் தலைப்பு:**

குழந்தையின் நீண்ட கால் எலும்பில் ஏற்படும் பிறவி செயற்கை மூட்டு நோயின் திசுவில் இருந்து மூல உயிரணுவை பிரித்தெடுத்தல் மற்றும் இந்த மூல உயிரணுவை கொண்டு எலியில் குழந்தையின் நோய் மாதிரியை உருவாக்குதல்

ஆய்வு வரிசை எண்: ஆய்வு செய்பவரின் பெயர் :

தொடக்கப் பெயர் :

பிறந்த தேதி:

வயது:

1) நான் முன் காணக்கூடிய ஆய்வு பற்றிய தகவல் படிவத்தை படித்து சந்தேகங்களை கேட்டு அறிந்து கொண்டேன்.

2) இந்த ஆராய்ச்சியில் எனது குழந்தைக்கு எந்த பாதிப்பும் வராது என்பதை நான் அறிவேன். அதனால் எனக்கு எந்த நஷ்டயிடும் கிடைக்காது என்பதை அறிவேன்.

3) இந்த ஆய்வில் சம்மந்தப்பட்ட அனைவரும் எனது குழந்தையின் மருத்துவக் குறிப்பை ஆய்வு காரணமாக பயன்படுத்துவார்கள் என்பதை நான் அறிவேன். எனது குழந்தையின் அடையாளம் மற்றவர்களுக்கு வெளிபடுத்தபட மாட்டாது.

4) என்னுடைய குழந்தையின் திசுக்கள் காலவரையற்ற காலம் ஆய்வகத்தில் சேமிக்கப்படும் மற்றும் பிற மருத்துவ ஆய்வுகளில் பயன்படுத்தப்படும் என்பதை நான் அறிவேன்.

5) ந ான் இந்த ஆய்வில் என் குழந்தை பங்கேற்க ஒப்புக்கொள்கிறேன்.

- குழந்தையின் பெற்றோர்/ பிரதிநிதியின் பெயர்

  கையொப்பம் :

  நாள் :

- ஆய்வாளரின் கையொப்பம் :

  நாள் :

  பெயர் :

- சாட்சிக் கையெழுத்து :

  நாள் :

  பெயர் :

## संमली पत्र

## प्रयोगाभ्यास और संशोधन में सहभाग की संमली

शिर्षक : कंजेनायटल सुडार्शोसीस टिकीया मतलब पैर की हडडी का जन्मजात बिमारी से ना जुडना इस बिमारी से मुलकोशिकाओं को अलग निकाल कर उनका अभ्यास करना और इन्हीं कोशिकाओं से चुहों में इसी बिमारी को उत्पन्न करना।

**नाम :**

**संख्या :**

**जन्मतारिख :**

मैं यह घोषित करता हूँ कि जानकारी पत्रिका मैंने ..... तारिख को पढी और समझी है और मुझे उसमें सवाल पुछने का मौका भी दिया गया।

मैं यह घोषित करता हूँ कि मेरा सहभाग इस अभ्यास में पूर्णतः ऐच्छित है और मैं कभी भी मेरी सहमती वापस ले सकता हूँ।

मैं यह जानता हूँ कि इस अभ्यास के संशोधक, प्रायोजक नियंत्रण अधिकारी कभी भी मेरी जानकारी जो अभ्यास में है, ले सकते है। वही जानकारी बाद में होने वाले अभ्यास में उपयोग कर सकते है। यह मेरा नाम वापिस लेने के बाद में भी कर सकते है।

मैं इस अभ्यास में दी गई जानकारी को सिर्फ वैज्ञानिक उपयोग के लिए नियंत्रित करता हूँ।

मैं इस अभ्यास में सहभागी होने की संमली देता हूँ।

**हस्ताक्षर :**

**नाम :**

**दिनांक :**

**गवाह के हस्ताक्षर :**

**नाम :**

**दिनांक :**

## ANNEURE 4 – CENTRE FOR STEM CELL RESEARCH APPROVAL

PAEDIATRIC ORTHOPAEDICS UNIT
CHRSITIAN MEDICAL COLLEGE, VELLORE

March 08, 2017

Dr. Alok Srivastava,
Head, Centre for stem cell research,
CMC.

Subject: Permission to use laboratory IV for our research on "Effects of shock waves on osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia"

Dear Dr. Alok,

John premnath, 1st year PG registrar from the Department of Orthopaedics is going to do his thesis under my guidance on "Effects of shock waves on Osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia". I kindly request you to grant the permission to use our laboratory for the study.

Thank you,

Most welcome!

With regards,

Dr. Vrisha Madhuri,
Prof. & Head

CENTRE FOR STEM CELL RESEARCH
(A Unit of inStem, Bengaluru)
8 MAR 2017
Christian Medical College Campus,
Bagayam, Vellore - 632 002, Tamil Nadu India.

# Annexure 5 – IRB Approval

**OFFICE OF RESEARCH**
**INSTITUTIONAL REVIEW BOARD (IRB)**
**CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA**

**Dr. B.J. Prashantham,** M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

**Dr. Anna Benjamin Pulimood,** M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

**Dr. Biju George,** M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

June 15, 2017

Dr. John Premnath,
PG Registrar,
Department of Orthopaedics,
Christian Medical College,
Vellore – 632 002.

Sub: **Fluid Research Grant NEW PROPOSAL:**
Effects of shock waves on Osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia.
Dr. John Premnath, 1st Year Postgraduate Registrar, Employment Number: 29537 Department of Orthopedics. Dr. Vrisha Madhuri Professor and Head, Employment No.: 08689, Pediatric Orthopedic unit, Mr. Karthikeyan.R Senior Research Fellow, Employment No.: 32134, Ms. Sowmya Ramesh, EMP no: 33340, Senior Research Fellow, Pediatric Orthopedics Unit.

Ref: IRB Min. No. 10630 [OBSERVE] dated 03.04.2017

Dear Dr. John Premnath,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
M.B.B.S., MD., DM.
SECRETARY (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Vrisha Madhuri, Dept. of Paediatric Orthopaedics, CMC, Vellore



Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

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Ref: IRB Min. No. 10630 [OBSERVE] dated 03.04.2017

Dear Dr. John Premnath,

The Institutional Review Board **(Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Effects of shock waves on Osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia" on April 03rd 2017.

The Committee reviewed the following documents:

1. IRB Application format
2. Consent Form (English, Tamil and Hindi)
3. Cvs of Drs. Vrisha, Karthikeyan and Sowmya.
4. No. of documents 1- 3..

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on April 03rd 2017 in the CK Job Hall, Christian Medical College, Bagayam, Vellore 632002.



Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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Director, Christian Counseling Center,
Chairperson, Ethics Committee.

**Dr. Anna Benjamin Pulimood,** M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

**Dr. Biju George,** M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

| Name | Qualification | Designation | Affiliation |
|---|---|---|---|
| Dr. Biju George | MBBS, MD, DM | Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore | Internal, Clinician |
| Dr. B. J. Prashantham | MA(Counseling Psychology), MA (Theology), Dr. Min (Clinical Counselling) | Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore | External, Social Scientist |
| Dr. Ratna Prabha | MBBS, MD (Pharma) | Associate Professor, Clinical Pharmacology, CMC, Vellore | Internal, Pharmacologist |
| Dr. Rekha Pai | BSc, MSc, PhD | Associate Professor, Pathology, CMC, Vellore | Internal, Basic Medical Scientist |
| Dr. Jayaprakash Muliyil | BSC, MBBS, MD, MPH, Dr PH (Epid), DMHC | Retired Professor, CMC, Vellore | External, Scientist & Epidemiologist |
| Mr. C. Sampath | BSc, BL | Advocate, Vellore | External, Legal Expert |
| Ms. Grace Rebekha | M.Sc., (Biostatistics) | Lecturer, Biostatistics, CMC, Vellore | Internal, Statistician |
| Dr. Sowmya Sathyendra | MBBS, MD (Gen. Medicine) | Professor, Medicine III, CMC, Vellore | Internal, Clinician |
| Dr. Santhanam Sridhar | MBBS, DCH, DNB | Professor, Neonatology, CMC, Vellore | Internal, Clinician |
| Dr. Thomas V Paul | MBBS, MD, DNB, PhD | Professor,Endocrinology, CMC, Vellore | Internal, Clinician |
| Dr Sneha Varkki | MBBS, DCH, DNB | Professor, Paediatrics, CMC, Vellore | Internal, Clinician |
| Dr. Sathish Kumar | MBBS, MD, DCH | Professor, Child Health, CMC, Vellore | Internal, Clinician |



Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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**Dr. B.J. Prashantham,** M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

**Dr. Anna Benjamin Pulimood,** M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

**Dr. Biju George,** M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

| | | | |
|---|---|---|---|
| Dr. Anuradha Rose | MBBS, MD, MHSC (Bioethics) | Associate Professor, Community Health, CMC, Vellore | Internal, Clinician |
| Dr. Ajith Sivadasan | MD, DM | Professor, Neurological Sciences, CMC, Vellore | Internal, Clinician |
| Dr. Visalakshi. J | MPH, PhD | Lecturer, Biostatistics, CMC, Vellore | Internal, Statistician |
| Mrs. Pattabiraman | BSc, DSSA | Social Worker, Vellore | External, Lay Person |
| Dr. Mathew Joseph | MBBS, MCH | Professor, Neurosurgery, CMC, Vellore | Internal, Clinician |
| Dr. Shyam Kumar NK | MBBS, DMRD, DNB, FRCR, FRANZCR | Professor, Radiology, CMC, Vellore | Internal, Clinician |

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Effects of shock waves on Osteogenetic potential of mesenchymal stem cells derived from fibrous hamartoma in congenital pseudarthrosis of tibia" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

*Fluid Grant Allocation:*

*A sum of 98,868/- INR (Rupees Ninety eight thousand eight hundred and sixty eight Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 48,868/- INR (Rupees Forty eight Thousand eight hundred and sixty eight only) will be released at the end of the first year as 2 nd Installment*

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min. No. 10630 [OBSERVE] dated 03.04.2017 4 of 4

# Annexure 6- Gene expression Data

| Day 7 | | CPT 14 | | | | | CPT 17 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NOS3-1 | RUNX2 | BGLAP | ALPL | | NOS3-1 | RUNX2 | BGLAP | ALPL | CPT 23 | NOS3-1 | RUNX2 | BGLAP | ALPL |
| C | 0.498961 | 0.328508 | 0.528875 | 0.118093 | C | 0.2705562 | 0.478636 | 1.554015 | 1.229439 | C | 0.376834 | 0.245705 | 0.554785 | 0.268315 |
| T | 0.338681 | 0.357744 | 0.71847 | 0.273573 | T | 0.461 | 3.64 | 1.181 | 1.568 | T | 0.443498 | 1.147902 | 0.540363 | 0.811127 |
| Relative ra | 0.678772 | 1.088997 | 1.358486 | 2.316586 | | 1.7038976 | 7.604948 | 0.759967 | 1.275379 | | 1.176907 | 4.67187 | 0.974004 | 3.02304 |
| | | | | | | | | | | | | | | |
| Day 14 | | | | | | | | | | | | | | |
| CPT 14 | RUNX2 | ALPL | BGLAP | | CPT 17 | RUNX2 | ALPL | BGLAP | | CPT 23 | RUNX2 | ALPL | BGLAP | |
| C | 0.34151 | 0.946058 | 0.046552 | | C | 0.8258777 | 9.12611 | 0.437999 | | C | 0.578344 | 1.176091 | 0.191312 | |
| T | 1.325007 | 3.14269 | 0.370874 | | T | 1.0034717 | 9.012954 | 2.477697 | | T | 1.984809 | 2.679567 | 0.275285 | |
| Relative ra | 3.879848 | 3.32188 | 7.966798 | | | 1.2150368 | 0.987601 | 5.656854 | | | 3.431882 | 2.278367 | 1.438934 | |
| | | | | | | | | | | | | | | |
| Day 21 | | | | | | | | | | | | | | |
| CPT 14 | RUNX2 | ALPL | BGLAP | | CPT 17 | RUNX2 | ALPL | BGLAP | | CPT 23 | RUNX2 | ALPL | BGLAP | |
| C | 0.540363 | 0.813943 | 0.175191 | | C | 1.3286858 | 3.944931 | 6.507034 | | C | **1.239708** | 1.194163 | 0.292803 | |
| T | 0.615146 | 1.681793 | 0.177883 | | T | 1.5209788 | 3.427128 | 9.613088 | | T | 1.302244 | 1.370783 | 0.461052 | |
| Relative ra | 1.138394 | 2.066229 | 1.015366 | | | 1.1447242 | 0.868742 | 1.477338 | | | 1.050445 | 1.147902 | 1.574616 | |
| | (Ctrl) | | | | | | | | | | | | | |