Histomorphometric and Biomechanical Strength Evaluation of New Bone Regeneration Effect of Low Intensity Pulsed Ultrasound on Mandibular Distraction Osteogenesis

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Abstract

The present study was to assess the effect of low intensity pulsed ultrasound on new bone formation during mandibular distraction osteogenesis in rabbit. Twenty four skeletally mature male New Zealand White rabbits underwent distraction osteogenesis at right side of mandible (3 days of latency period; 10 days of distraction period with rate of 0.5mm/12hr). Low intensity pulsed ultrasound (LIPUS) with 1.5 MHz, 30mw/cm^2 was conducted to 12 rabbits for a single 20 min treatment daily and started on the first day of distraction until rabbits were sacrificed on week 0, 2 and 4 after the distraction. Four rabbits were sacrificed at each time point, hence 3 ultrasound groups foumed according to the time intervals. Other 12 rabbits followed the same sacrifice Plain x-ray, microhardness test, micro-CT scan, histological examination were performed. The animals were well tolerated to the entire distraction and ultrasound treatments. No in infection and other complications occurred. All ultrasound groups showed more positive results, especially on week 0 and 2 after the distraction, significant more bone formation and higher surface microhardness were detected by plain x-ray, Micro-CT scan and microhardess test ($p<0.05$). The similar results revealed by histological evaluation. LIPUS accelerates new bone formation during mandibular distraction osteogenesis in rabbit, particularly during distraction and early weeks after the distraction. LIPUS may be an effective modality to shorten the treatment time of distraction osteogenesis.

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List of Abbreviations

DO	=	Disatraction Osteogenesis
LIPUS	=	Low Intensity Pulsed Ultrasound
MGL	=	Mean Gray Level
BVF	=	Bone Volume Fraction
PBA	=	Percentage of bone Area

1. Introduction

1.1 Introduction of distraction osteogenesis

Distraction osteogenesis is a technique to generate new bone for treatment of various skeletal deformities. This technique, also called callus distraction[1], callotasis[1], osteodistraction[1], osseous distraction, originated from orthopaedics in 1905[2]. Ilizarov[3] named the distraction osteogenesis to describe the induction of new bone formation between osseous surfaces that are gradually pulled apart. Because the uniqueness and value of distraction osteogenesis lie in its ability to simultaneously gain the new bone and expand the surrounding soft tissues without a donor site, it has achieved worldwide acceptance and great success in orthopedic surgery. Since 1992 this technique has been used in craniofacial region and rapidly extended in craniofacial surgery[4]. Distraction osteogenesis has opened up new perspectives for management of numerous congenital and acquired craniofacial skeletal anomalies.

1.1.1 History of distraction osteogenesis in orthopaedics

The history of distraction osteogenesis is primarily the history of lengthening operations used to treat war wounds, malunited fractures of the femur, and the sequelae of poliomyelitis. The history of leg lengthening can be traced back to the 19th century. Researchers such as von Langenbeck in 1869, Hopkins and Penrose in 1889, and von Eiselsberg in 1897 described various techniques, most of which appear to have been 1-stage lengthening

osteotomies[5]. Modern bone lengthening treatment began with the work of A. Codivilla in Bologna, who in 1905 described the use of an osteotomy of the cortex and immediate application of traction force to a calcaneal pin, with or without the use of narcotics[2] (Fig 1)

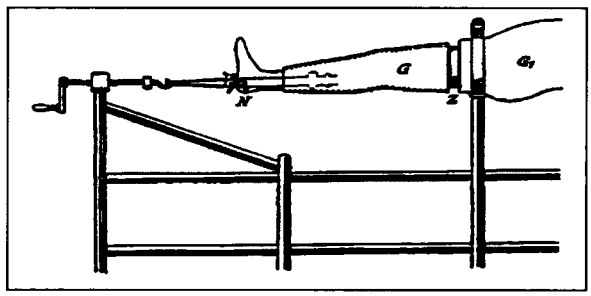


Fig 1. Codivilla's calcaneal pin traction plaster

First, he drove a pin measuring 5 to 6mm in diameter transversely through the heel, and then with a chisel he made an oblique osteotomy in the middle of the femoral shaft. Finally, with the patient on an extension table, he applied powerful traction (25-75kg) to the osteotomy by means of the pin in the heel. This traction was applied suddenly and was not repeated. The patient was then immobilized in a plaster jacket encircling the thorax, pelvis, and leg, the pin through the calcaneus being incorporated into the plaster by means of 2 metal strips. If the lengthening was insufficient, then the plaster was cut at the level of the osteotomy and vigorous traction was exerted (with or without anesthesia) as often as necessary. Therefore, his technique would be regarded as a 1-stage or multistage lengthening procedure. The calcaneal pin was removed after 30 days[5]. Codivilla's greatest contribution was the idea of exerting traction through a calcaneal pin, which allows for great force to be used without the need of plaster extension techniques (compression necrosis of bone at the ankle and wrist). Ilizarov stated that all subsequent publications represented nothing more than modifications of Codivilla's technique[6]. The idea behind all of the proposed techniques mirrored Codivilla's main concern, which was to overcome the resistance of the soft tissues without damaging their function and to focus on the response of normal muscle to gradual traction[5]. During the next few years, Codivilla's method became known and was adopted in Europe and America. The first experimental study was reported by Paul B. Magnuson in Chicago in 1908[7]. He achieved the single stage lengthening of 5 to 7.5cm without damaging soft tissue using the dog model. Ameliorations appeared in later years. In contrast with

the previous studies that applied the lengthening procedure by 1-stage or multistage, Ombredanne performed lengthening of femur at a rate of 5mm/day in 1913[7]; however, skin necrosis and infection occurred. In 1921, Putti[8] slowed the rate of distraction to two to three millimeters per day with a unilateral fixator and half-pins (Fig 2).

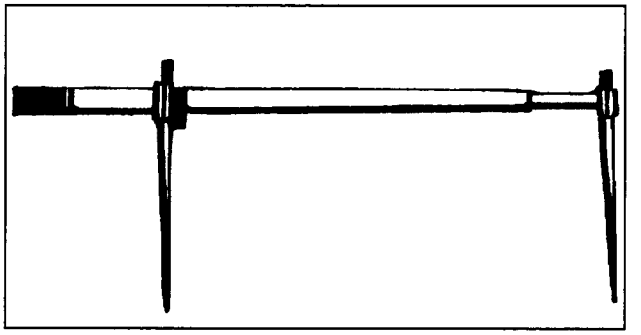


Fig 2. Putti's unilateral distraction frame

Later on Abbott[9] introduced the idea of a latency period to promote the formation of bone. He performed a step-cut osteotomy, with preservation of the periosteum, and then allowed a seven-to-ten-day latency period before applying distraction through a spring-loaded, force-controlled device in 1927 (Fig 3).

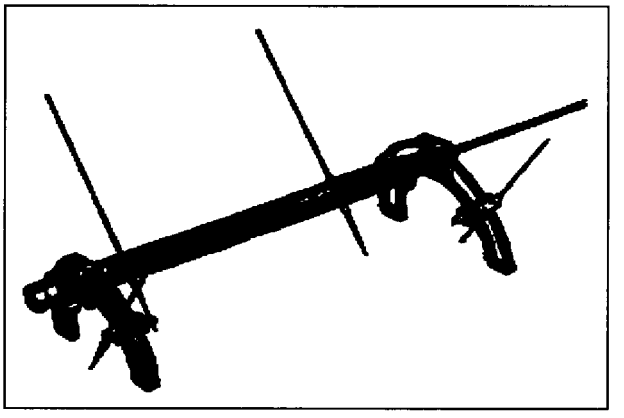


Fig 3. The Abbott's lengthening apparatus

In 1936, Anderson[10] reported several innovations for femoral lengthening, including the use of wires attached to the apparatus under tension and a technique for percutaneous osteotomy.

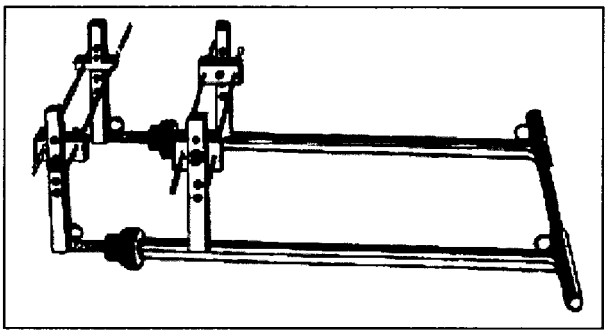


Fig 4. Anderson's modification of Abbott fixator

During the 1930s various modifications were introduced and the apparatus was simplified, but the limb lengthening technique fell into disrepute due to an increasing tendency to apply this technique to unsuitable cases.

Distraction osteogenesis had not been well developed until Gavril Abramovic Ilizarov, a Russian orthopedic surgeon, in Kurgan, established the basic biological principles of distraction osteogenesis grounded on pioneering experiments and greatly advanced the clinical utility of this technique from 1950s to 1970s[5, 7, 11]. He succeeded in learning the concepts that had already been recognized, and to them he added his new understandings. His technique was first used in 1951 for the treatment of a bone defect caused by tuberculosis. By developing his modular ring fixator in 1952 (Fig 5), Ilizarov allowed for precision in using the technique and rendered its results predictable, thus the surgeon could recommend the technique to a patient without the serious reservations attached to the older procedures.

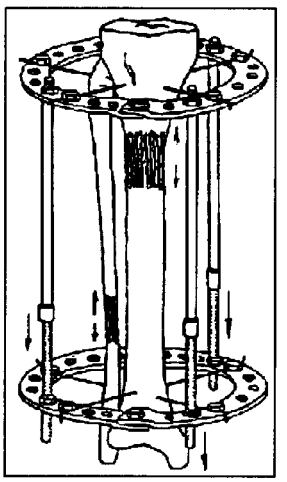


Fig 5. The Ilizarov ring fixator

Having operated on numerous patients and having performed countless scientific studies (more than 1000 papers from the Ilizarov Institute in Kurgan), Ilizarov strove to validate and refine the principles that he had established. His ideas influenced a generation of surgeons active in this field in Eastern Europe and by the mid 1970s influenced surgeons worldwide.

The principles of Ilizarov's method were as follows: 1. Superior biologic quality of the regenerated bone due to the performance of a percutaneous corticolomy (the osteotomy of the cortex only) that causes minimal trauma to the periosteum and bone marrow. 2. A postoperative waiting period. 3. Multistep, incremental distraction totaling 1mm/day. 3. Use of a compression and distraction procedure involving full weightbearing. 4. Use of a ring fixator in which the fragments are held by Kirschner wires under tension, which enables the surgeon to exert planned axial control in all planes and even to correct multidirectional deformities. 5. Development of segment transport for defects of the bone shaft. 6. Promotion of good tissue nutrition and joint mobility by means of a mobile device that allows full weightbearing and physiotherapy[7]. Based on the above practical principles, Ilizarov discovered two biologic principles of distraction osteogenesis known as the "Ilizarov effects": 1. the tension-stress effect on the genesis and growth of tissues; 2. the influence of blood supply and loading on the shape of bones and joints. The first Ilizarov principle postulates that gradual traction creates stress that can stimulate and maintain regeneration and active growth of living tissues. The second Ilizarov principle theorized that the shape and mass of bones and joints are dependent on an interaction between mechanical loading and blood supply. If blood supply is inadequate to support normal or increased mechanical loading, then the bone cannot respond favorably, leading to atrophic or degenerative changes. In contrast, if blood supply is adequate to support increased mechanical loading, the bone will demonstrate compensatory hypertrophic changes. Ilizarov's work remained unknown for some time outside Russia, and the technique mainly used in German speaking countries and later in the United States was the developed by Wagner[12, 13]. From 1970 to 1990, the Wagner method of lengthening became more popular than the Anderson technique among most pediatric orthopedists. The concept introduced by Wagner involves cutting of the periosteum, fascia, and other constraining tissues in order to minimize resistance; limiting the lengthening to a maximum of seven centimeters; relatively rapid distraction (as much as two millimeters per day) as tolerated by the patient, who is awake; and bone-grafting of the defect after the intended

amount of distraction is achieved. The mid-diaphyseal osteotomy is made with an oscillating saw, and a specially designed internal fixation plate replaces the external fixator after lengthening has been achieved. However, this method was technically simple and was in keeping with contemporary ideas but offered no major advantages regarding early mobilization of the patient. In addition, it disregarded the soft tissues and the biologic principles; it involved several major operations, and led to many complications[5]. By means of the great achievements in clinical, biological and non-biological understandings of distraction osteogenesis during 1950s to 1970s, this technique was adopted worldwide in orthopedics in the 1980s[5].

1.1.2 History of distraction osteogenesis in craniofacial surgery

Although distraction osteogenesis has been worldwide accepted and developed from orthopaedics to craniofacial surgery in recent decades, the application of tensile and compressive forces to bones of the craniofacial skeleton is not a new concept. As early as 1728, Fauchard[14, 15] described the use of the expansion arch. When ligating an ideally shaped metal plate to the teeth, the crowded dentition was broadened to a more normal form. This form of traction, however, was limited to tooth movement only and had little effect on the shape of the bone. Wescott first reported the placement of mechanical forces on the bones of the maxilla in 1859[16]. He used two double clasps separated by a telescopic bar to correct a crossbite in a 15-year-old girl. One double clasp was soldered to the bar; the other was soldered to a screw that fit into the tube, thereby allowing lengthening of the screw to widen the palate. By the author's own admission, however, the entire expansion procedure was slow and tedious and lasted several months. A year later, Angell[17] performed a similar procedure, using a differentially threaded jackscrew connected across the palate to both bicuspids on one side and the second bicuspid on the other (Fig 6).

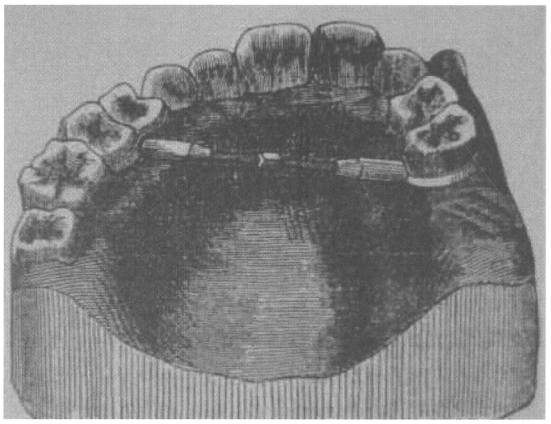


Fig 6. Angell's palatal expansion device on the maxillary arch

The patient was given a key to turn the screw and instructed to keep it uniformly firm. On her return 2 weeks later, she had developed a space between her central incisors, which Angell claimed "showed conclusively that the maxillary bones had separated". Goddard[18], in 1893, further standardized the palatal expansion protocol. He activated the device twice a day for 3 weeks, followed by a stabilization period to allow the deposition of "osseous material" in the created gap. In 1920, Mesnard[19] demonstrated radiographically that the midpalatal suture could be separated using fixed appliance and that the space would be filled with bone around 4-6 weeks.

Rosenthal is the first to perform a distraction osteogenesis on a human mandible, in a woman, to correct a dentofacial deformity secondary to a mandibular deficiency in 1927[20] (Fig 7).

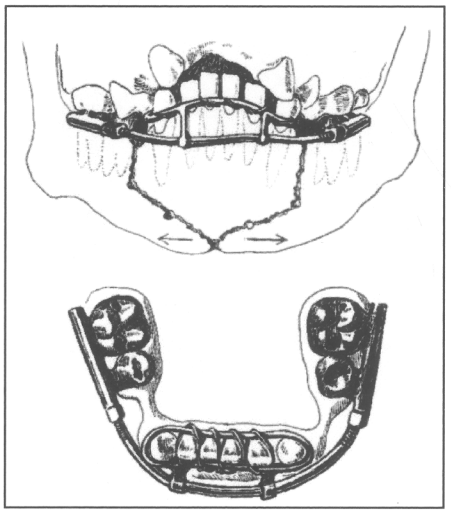


Fig 7. Rosenthal's tooth-borne mandibular distraction device

(Above) After removing the lower first premolar and applying a Kleeberg distraction device to the dental arch of the lower jaw, a subperiosteal oblique osteotomy was performed to the midline to produce three fragments. (Below) The frontal segment was slowly advanced by applying and activating daily the intraorally applied Kleeberg distraction device until the desired position of the frontal segment was achieved.

The first osteotomy of a Le Fort type I to correct a skeletal malocclusion was performed by the craniofacial surgeon Martin Wassmund on a patient with a frontal open bite in Berlin in 1926. The osteotomy included the anterolateral walls of the maxillary sinus bilaterally, but only the anterior part of the medial walls and the base of the nasal septum. Wassmund mentioned that after 1 week of "elastic traction" (distraction osteogenesis), normal occlusion was achieved (Fig. 8), but elastic intermaxillary fixation was continued 13 weeks until the upper jaw was firmly united[20].

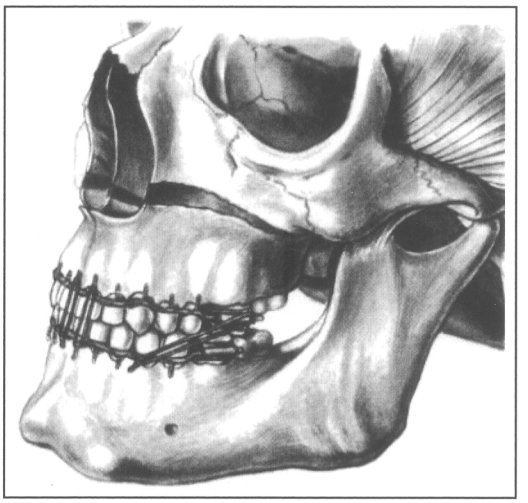


Fig 8. Wassmund's original drawing showing the correction of skeletal malocclusion of the upper jaw and a frontal open bite by intraoral distraction osteogenesis.

Even though the first distraction osteogenesis procedures applied gradual traction to the bone segments and surrounding soft tissues, this technique did not gain immediate acceptance. This was primarily due to the lack of control over bone segment manipulation, inadequacy of distraction devices, and the instability of osseous fixation. Instead, corrective osteotomies remained the principal treatment modality for the management of mandibular deformities, especially after the introduction of sagittal split osteotomies by Trauner and Obwegeser[21].

The first report demonstrating the application of Ilizarov's principles to the craniofacial skeleton appeared in 1973[22]. In order to simulate a mandibular deformity, Snyder and co-workers resected a unilateral 15mm bone segment from a canine mandible, thereby creating a crossbite. Ten weeks later, the shortened mandible was osteotomized and an external distraction device was placed. After a 7-day latency period, the device was activated at a rate of 1mm per day for 14 days, at which time the occlusion was restored. Reestablishment of the mandibular cortex and medullary canal across the distraction gap was noted following 6 weeks of consolidation. This work was repeated by Michieli and Miotii using the intraoral device in 1977[23].

In 1989, McCarthy and colleagues were the first to clinically apply distraction osteogenesis on four children with congenital craniofacial anomalies such as hemifacial microsomia and Nager's syndrome[4]. Bone division was initiated by placing a series of drill holes along the osteotomy line, which were then connected with a narrow osteotomy. After a latency of

7 days, lengthening began at a rate of 1mm per day performed in two increments of 0.5mm. Following 18 to 24 days of distraction, external fixation was maintained for an additional 8 to 10 weeks of consolidation (Fig 9, 10).

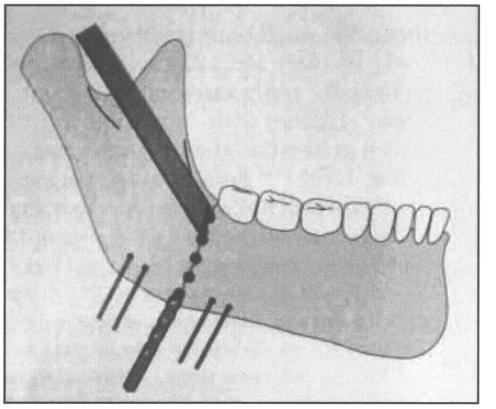


Fig 9. McCarthy's mandibular distraction technique. Predrilled osteotomy.

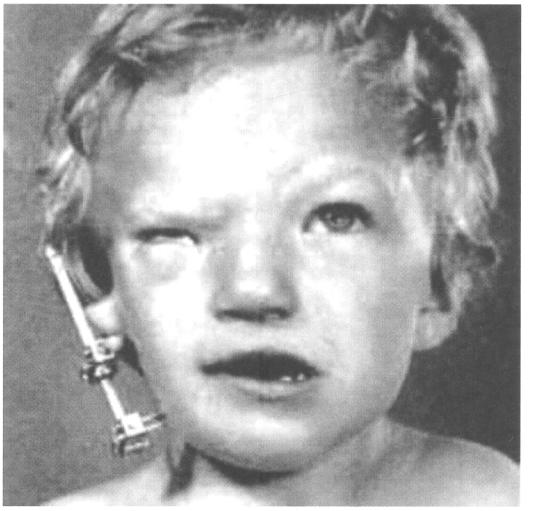


Fig 10. Position of the Hoffman mini lengthener during mandibular lengthening

Following the first report of McCarthy, which demonstrated successful lengthening of the human mandible by gradual distraction, the field of craniofacial distraction osteogenesis rapidly gained momentum. Many authors have reported successful results with corrective osteotomies followed by gradual mandibular distraction for deformity correction, lengthening, widening, bone transport, and alveolar ridge augmentation. Distraction osteogenesis has received the majority of clinical attention in craniofacial surgery.

1.1.3 Histology of distraction osteogenesis

Distraction osteogenesis reveals a unique biological phenomenon of new bone formation between the bony segments that are gradually pulled apart. As yet distraction osteogenesis remains one of the most mysterious phenomena of bone biology, which are not typical of those found elsewhere in the skeleton. The process of distraction osteogenesis involves osteotomy surgical phase, latency phase, distraction phase and consolidation phase. Different phases present different histological phenomena.

Osteotomy surgical phase is to create bone segments by surgical fracture and to place the distractor, which provides the separation as well as the stability between the bone segments. The loss of continuity and mechanical integrity of the bone triggers an evolutionary process of bone healing known as fracture healing.

Latency phase is the time between the osteotomy and activation of the distractor and is the period when very early stages of bone healing takes place at the osteotomized site. This is the phase to be no different than early stages of routine fracture healing, including inflammatory reaction-hematoma and soft callus formation stages. At the time of fracture the inflammatory reaction begins. Local macrophages and damaged cells release vasodilatory factors (e.g., TNF, IL-1) which cause the migration of polymorphonuclear leukocytes[24]. A few hours later, a hematoma forms between and around the bone segments, then it converts to a clot (Fig. 11-A). Within 1 to 3 days, the clot is replaced with granulation tissue consisting of inflammatory cells, fibroblasts, collagen, and capillaries[24, 25] (Fig. 11-B). Then the following is the soft callus formation stage. Granulation tissue is converted to fibrous tissue by fibroblasts. Cartilage also replaces the granulation tissue. This occurs more toward the periphery of the intersegmentary gap than in the central region[26] (Fig. 11-C).

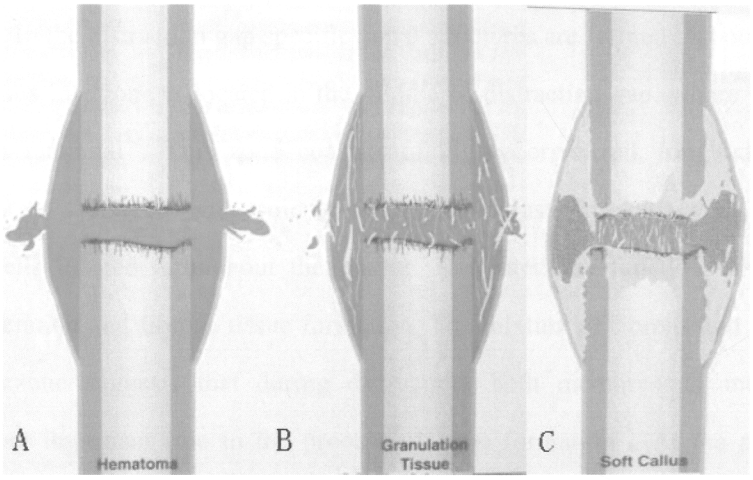


Fig 11. A: Hematoma. B: Granulation tissue. C: Soft callus.

Distraction phase is the time period of pulling apart the bony segments at a certain distraction rate. Distraction rate is the length or distance per day at which the bone segments are stretched. It means the amount of distraction per day. One millimeter one day is the most commonly used and primarily recommended by Ilizarov[27, 28]. Distraction rhythm is the frequency of distraction per day, in other words, the distraction rate is achieved by certain times of distraction in one day. It is believed that the higher frequency, the better result[29]. However, due to the practical limitation, distractions are performed 2-4 times a day mostly. Distraction phase is the most distinct phase in distraction osteogenesis. Unlike the fracture healing needing rigid immobilization, tension forces are applied gradually to the gap between bony segments by the distractor during distraction phase[30]. Hereon the normal process of fracture healing is no longer present. As distraction begins, the soft callus becomes elongated. Fibrous tissue orientating along the axis of distraction are found in distraction gap[31]. A few days later, capillaries grow into the fibrous tissues. The newly formed vessels have a spiral pathway and numerous circular folds suggesting growth rates much higher than the rate of distraction, and 10 times faster than vessel growth during fracture healing[32]. During the second week of distraction, primary trabeculae begin to form. The osteoblasts, located among the collagen fibers, lay down osteoid tissue on these collagen fibers and eventually become enveloped as bone spicules gradually enlarge by circumferential apposition of collagen and osteoid. Osteogenesis is initiated at the existing bone walls and progresses toward the center of the distraction gap. By the end of the second week, the osteoid begins to mineralize[31].

In the distraction gap specific zonal structures are formed. A poorly mineralized, radiolucent fibrous interzone is located in the middle of distraction gap, where the influence of tension force is maximal[33]. This zone consists of highly organized, longitudinally oriented, parallel bundles of collagen with spindle-shaped fibroblast-like cells and undifferentiated mesenchymal cells located throughout the matrix. The interzone functions as the center for fibroblast proliferation and fibrous tissue formation. The mixture of fibrous and cartilage tissues within the interzone suggests that during distraction, both membranous and endochondral processes play an important role in the process of bone formation[34]. At the periphery of the fibrous interzone, there are two zones with longitudinally oriented cylindrical primary trabeculae, which are covered by a layer of osteoblasts that grow toward each other[34] (Fig 12-A). At the end of distraction phase, two new zones of primary trabeculae remodeling may become evident at the junction of the mineralization zone and the host bone segments (Fig. 12-B).

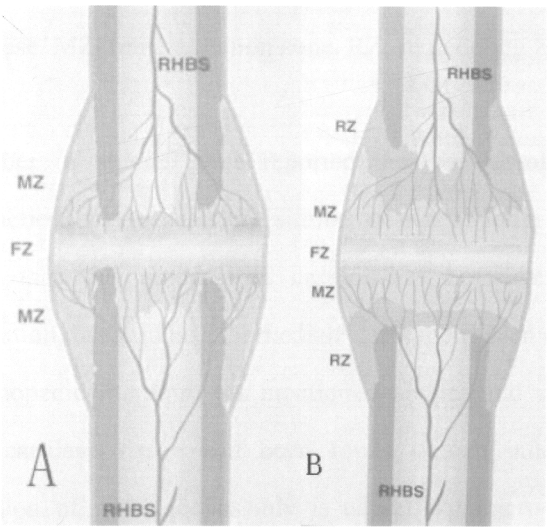


Fig 12. A: A three-zonal structure during distraction phase. FZ: fibrous zone. MZ: mineralization zone.

B: A five-zonal structure at the end of distraction phase. RZ: remodeling zone

Consolidation phase is the time period required for the bone regeneration. This phase is completed when the distractor is removed. This phase represents the time required for complete mineralization of the distraction gap. The fibrous interzone gradually ossifies and on distinct zone of fiber bone completely bridges the gap[34]. Although the distraction osteogenesis

forms predominantly via membranous ossification, isolated islands of cartilage may also be observed, suggesting endochondral bone formation[35]. As the end of the consolidation phase, the zone of primary trabeculae significantly decreases and later is resorbed completely (Fig. 13).

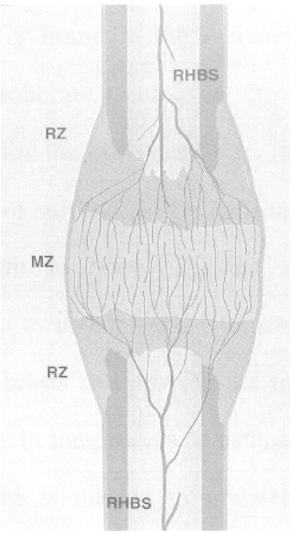


Fig 13. Consolidation phase. MZ: mineralization zone. RZ: remodeling zone.

A number of studies have reported that the histological processes during membranous bone distraction osteogenesis are similar to that of long bones[36]. The majority of research groups agree that the process of craniofacial bone regeneration is primarily intramembranous ossification, because no intermediate cartilage is seen at the fibrous interzone[36]. However, as in the orthopedic literature (as mentioned above) and some craniofacial animal studies, small foci of cartilage tissue had been found, which indicated the endochondral ossification. The formation of cartilage possibly is caused by micro-movements or result of decreased oxygen tension[36, 37].

1.1.4 Mechanism of distraction osteogenesis

So far the mechanism of distraction osteogenesis is poorly understood. Ilizarov's concept of distraction osteogenesis is based on the law of tension-stress: tissues subjected to slow steady traction become metabolically activated and maintain active growth and regeneration. Mechanical strain has been shown to regulate bone production during distraction[27, 28]. Stress across the distraction site may be an important part of the initiation of osteogenesis (cellular

recruitment and organization), the remodeling of the bone regenerate, and the completion of bone consolidation[38]. The study from Samuel T. Rhee[39] and co-workers substantiated the hypothesis that osteoblastic differentiation occurring in mesenchymal precursor cells during distraction osteogenesis is mediated by an ERK 1/2-dependent mechanotransduction pathway. In addition, they found that BMP 2/4 is initially found in the extracellular areas surrounding cells with upregulated ERK 1/2 expression corroborate previous in vitro findings[39]. Brian L. Schmidt et al.[40] assumed a possible functional role for the collagen fibers in their periosteal distraction animal study. The immature osteoblasts were surrounded by the longitudinally oriented collagen fibers that extended from the periosteum to bone. Tension on the periosteum might induce differentiation of mesenchymal cells to osteoblasts. Ultimately, these fibers could transmit the tensile forces to the immature osteoblasts and provide the mechanism to induce osteogenesis[40]. The importance of mechanical forces in the process as osteogenesis, and others needs the further study. Nevertheless more knowledge is needed to understand the mechanism of distraction osteogenesis in molecular level.

1.1.5 Clinical applications of distraction osteogenesis in craniofacial surgery

Even the exact mechanism of distraction osteogenesis is not clear, the clinical practices keep going. As previous mentioned, since 1992, when McCarthy et al. reported the first clinical application of distraction osteogenesis to the mandible in four patients, surgeons have applied this technique of gradual bone lengthening throughout the craniofacial skeleton[4]. In recent years, distraction osteogenesis has become increasingly popular and opened new therapeutic perspectives for the treatment of numerous congenital and acquired craniofacial skeletal anomalies.

1.1.5.1 Distraction osteogenesis in the mandible

Distraction osteogenesis has proved useful for severe bone deficiencies and deformities of the mandible[41-43]. The indications for mandibular distraction osteogenesis include severe bone deficiency, including those with associated malocclusion, masticatory dysfunction, temporomandibular ankylosis, failed costochondral grafts, obstructive apnea, apertognathia, and reflexive facial and maxillary canting with growth restriction. Syndromes and recognized anomalies with these problems can include Treacher-Collins syndrome, Goldenhar's syndrome,

hemifacial microsomia, Pierre Robin anomaly, Stickler syndrome, oralfacial-digital syndrome, and others[43]. Distraction osteogenesis of the mandible has become an important new adjunct to conventional orthognathic surgery. Now, distraction osteogenesis is being increasingly used to correct more moderate deformities because of its considerable advantages. These advantages include physiologic adaptation of associated soft tissues and joint structures, functional seating of the temporomandibular joint during correction, improved facial esthetics, and stability of result[43]. Distraction osteogenesis is emerging as the preferred method of treatment in the growing child[43]. A case was presented by McCarthy et al.[41], a 7-year-old girl with right-sided craniofacial microsomia characterized by underdevelopment of the right mandible was treated by mandibular distraction osteogenesis (Fig 14).

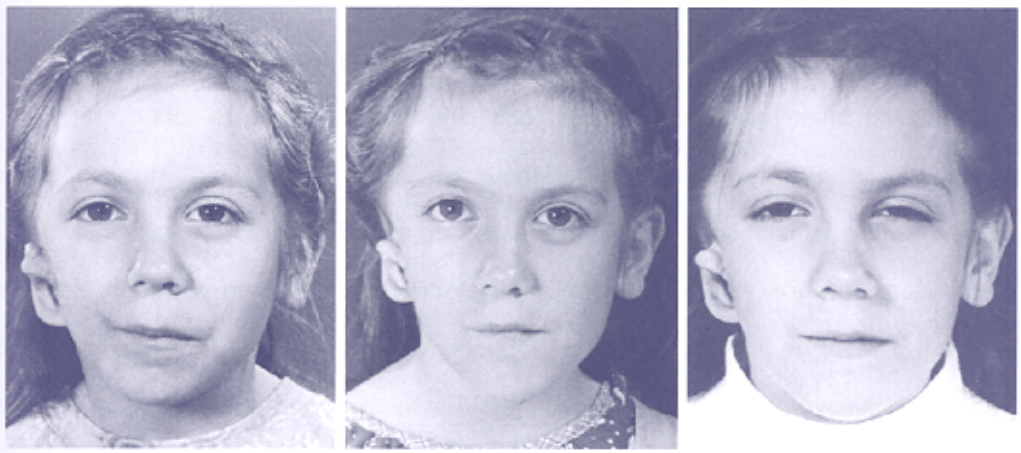


Fig 14. (Left) A 7-year-old girl with right-sided craniofacial microsomia characterized by underdevelopment of the right mandible, retrusion of the chin to the affected side, and elevation of the oral commissure. (Center) Appearance 1 year after right-sided mandibular distraction. Note the improvement in the position of the chin and the oral commissure. There is also more fullness in the affected lower third of the face. (Right) Appearance 2 years after unilateral mandibular distraction, with no evidence of relapse.

Another case also treated by mandibular distraction osteogenesis by McCarthy et al.[41] (Fig 15).

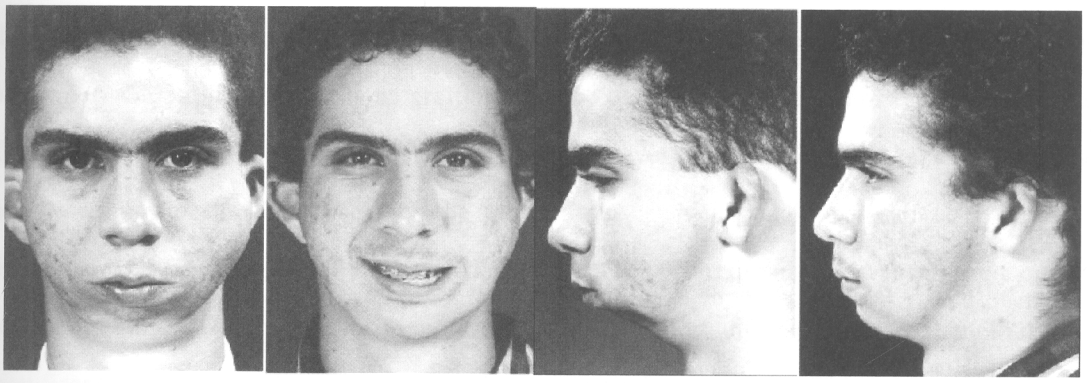


Fig 15. (From left to right, 4 pictures) (First picture) A 17-year-old boy with bilateral developmental micrognathia and ear anomalies. (Second picture) Appearance 2 years after bilateral mandibular distraction and genioplasty. (Third picture) Predistraction profile. Note the straightline relationship between the lower lip and the hyoid. (Fourth picture) Profile 2 years after mandibular distraction.

1.1.5.2 Distraction osteogenesis in the maxilla and the cranium

The indications for maxilla and cranium distraction could be postoncologic ablation; midface hypoplasias (maxillary deficiency, craniofacial synostosis); zygomatic deficiency (Treacher Collins syndrome); maldevelopment of the cranial vault (craniofacial synostosis)[42]. For the orofacial cleft patients, Le Fort I osteotomy was the most frequently performed procedure[44]. Maxillary distraction osteogenesis seems to be most efficacious for movements of more than 6 mm in cases of cleft palate surgery and for movements of more than 10 mm in cases of synostosis. Although a high degree of relapse is associated with advancement of the maxilla because of scarring in patients with cleft palate, Robinson et al.[43] found that advancement of the maxilla in cleft palate patients is more stable using distraction osteogenesis than with conventional surgery and bone grafting. Cheung et al.[45] had further confirmed this issue by a randomized controlled clinical trail. They concluded that the distraction provided better skeletal stability than conventional maxillary advancement which showed a significant amount of skeletal relapse in the first 12 weeks postoperatively.

At all three Le Fort osteotomy levels, distraction osteogenesis could be successfully performed. The cranium can be distracted at the various suture lines to correct variants of craniosynostosis. The orbits and frontal bone can also be distracted using the frontofacial monoblock advancement described by Tessier[46] and others. McCarthy et al.[42] stated

three advantages of midface distraction osteogenesis. First, it allowed the advancement to be performed without the need of bone grafting, thereby decreasing the length of the procedure and the donor-site morbidity. Second, it had significantly lower relapse rates than standard midfacial advancement, presumably because the soft tissue, which traditionally resists midface advancement, was also gradually distracted along with the bone. In this way, the entire functional matrix was altered, allowing for a more stable result. Third, the lack of initial mobilization significantly decreased the time needed for postoperative recovery. There was a significant decrease in blood loss and postoperative pain after this procedure, and patients were typically able to be discharged from the hospital in only 1 to 2 days. This also resulted in the ability to perform distraction in a younger patient population, allowing patients to have correction of their deformity before their teenage years, because the corticotomies were not injurious to the unerupted dentition as with traditional osteotomies.

1.1.5.3 Distraction osteogenesis in the alveolar

Augmentation of a deficient dentoalveolar segment secondary to congential deformity, trauma, lack of dentition, and so forth is becoming an increasingly popular application of distraction osteogenesis. Using this technique, the hypoplastic alveolar ridge of the edentulous mandible can be augmented to a bony volume capable of retaining osteointegrated implants. Chin & Toth[47] and Hidding et al.[48] were the first to report clinical use of distraction osteogenesis for alveolar ridge augmentation. The technique involves freeing a bone segment (the transport segment) from the basal bone, but retaining attachment via the lingual periosteum. The available distractors can be classified as juxtaosseous and intraosseous. Juxtaosseous distractors are placed on the buccal face of the maxillary bone[48]. Intraosseous distractors run through the transport segment in the direction of distraction[49]. Gaggl et al.[49] have described a simplified technique for alveolar ridge augmentation using "distraction implants", which do not require subsequent removal. Despite the relatively small number of patients studied to date and the relatively short periods for which implants have been monitored, distraction osteogenesis appears to be a reliable technique for alveolar ridge augmentation, reducing both postoperative morbidity and the length of the recovery period. Distraction osteogenesis appears to be at least as reliable as guided bone regeneration and bone grafting as a method for augmenting insufficient height of edentulous

ridges[50]. It enables bone formation between the transport segment and the basal segment in a relatively short period of time. Since there is no need to obtain bone from elsewhere, surgery time and postoperative morbidity are evidently reduced. Generally only local anaesthesia is required, again reducing postoperative morbidity. The regenerated bone appears to be highly resistant to resorption, is capable of supporting heavy functional loads, and enables the placement of implants with good aesthetics. Most complications arising during distraction osteogenesis can be considered minor, and are readily resolved[51]. In addition, small alveolar deficiencies such as alveolar cleft are also capable of being treated through both horizontal and vertical elongation of the deficient alveolus. If carefully planned in conjunction with an orthodontist, a single tooth or an entire segment containing teeth can be carefully advanced into a more anatomic position without risking devascularization of the dental roots[52].

1.1.6 Advantages and disadvantages of distraction osteogenesis

As above-described, distraction osteogenesis has been rapidly accepted and extended in craniofacial surgery through the world. Obviously, there are many advantages of distraction osteogenesis over traditional methods. Most advantages are above-mentioned in review of clinical applications of distraction osteogenesis. Generally and simply say the greatest advantages are the large amount of new bone formed accompanying by the expansion of surrounding soft tissues and the donor sites unnecessary[4, 53].

However, the disadvantages exist. Distraction osteogenesis is a technique and equipment sensitive surgery and mostly needed the second surgery to remove distraction devices. Certain complications were also reported[44, 54-56]. Long treatment periods and fibrous union or even non-union of bone are possible major impediments to its widespread clinical application[41, 57, 58]. Because the rate-limiting step of distraction osteogenesis is the consolidation phase. A comprehensive review by Swennen et al.[44] showed that the 6–8 week consolidation period was the most appropriate for all mandibular lengthening and expansion distraction osteogenesis procedures and for the reconstruction of segmental defects by bone transport or compression distraction osteogenesis, and even longer time for maxillary (2-3months), midfacial and/or cranial distraction osteogenesis (2-3,6 months). Thus at least 2/3 of the treatment time was dedicated to passive maturation, and most complications arose during consolidation time, such as infection,

fibrous union or even non-union, fracture of the residual bone, positional changes of the bony segments, and loosening or malposition device[42, 56, 59, 60]. Moreover, the distraction device gave significant impact to patient's psychological state, specially the external device[54]. If any method could accelerate the bone formation and maturation in distraction osteogenesis, the distraction device could be removed earlier, the treatment period could be shortened, complications would decrease and patients could return to daily activities more quickly. Hence, shorter consolidation period, more benefits to the patients.

1.1.7 Methods focused on the shortening of the consolidation phase

Therefore various efforts have been made in shortening the consolidation period by enhancement of bone quality or acceleration of bone formation. These included application of growth hormone[61], growth factors[59, 62-66], electrical stimulation[67, 68], stem cells[69, 70] and even the laser[71]. Recently, the low intensity pulsed ultrasound (LIPUS) has been applied to distraction osteogenesis for enhancement of bone formation during consolidation period and shown positive results[72-75]. The following content focused on introduction of LIPUS, review of its effect and utility in fracture and distraction osteogenesis.

1.2 Low intensity pulsed ultrasound (LIPUS)

1.2.1 Basic knowledge of low intensity pulsed ultrasound

Ultrasound is acoustic (sound) energy in the form of waves at a frequency above 20 KHz, which cannot be heard by humans. The intensity of ultrasound is defined as the energy transported by a wave per unit time across unit area perpendicular to the energy flow expressed as watt/meter^2 (W/m^2) or (W/cm^2)[76]. The limit between low and high-intensity is difficult to fix, but it can be approximately established for intensity values which depend on the medium, vary between 0.1W/cm^2 and 1W/cm^2. It means that if the intensity is lower than 0.1W/cm^2, it must be defined as the low intensity. There are two types of waveform that common ultrasound devices provided. One is continuous waveform and the other, pulses. The difference between the two is that the pulse waveform, unlike the continuous one having heat efficacy, produces more powerful

output suddenly in every circle. Currently, an ultrasound signal consisting of a burst width of 200μs containing frequency of 1.5 MHz sine waves with a repetition rate of 1 KHz and intensity of 30mW/cm^2 has been the most widely used for bone healing[77].

1.2.2 A brief history of the ultrasound for fracture healing

In 1927 Wood and Loomis first described the effects of high-frequency sound waves on living tissue, such as killing fish[78]. In 1938, in Germany, Pohlman expressed the opinion that ultrasound could be therapeutic; he was the first to construct a device for the treatment for patients with lower back pain, neuralgias and myalgias[79]. Then ultrasound became one of the most commonly used treatments in management of soft tissue injuries[80]. In 1950, Maintz published the first study in which the relationship between ultrasound and the bone healing was investigated in rabbits, but the results did not show an accelerated bone healing[81]. However, in 1952, Corradi and Cozzolino[82] first found that the ultrasound simulated the formation of callus in rabbit models. At the same period, a study from Murolo and Claudio[83] used the pulsed ultrasound to avoid the thermal effect and accelerate the fracture healing in pigs. In human studies, the earliest clinical application of ultrasound for treatment of osteomyelitis was reported by Strauβ in 1948[77]. In the 1950s, there were several studies concerning ultrasound treatment of bone disorders and presenting positive results[84-86]. However, less attention was paid toward the ultrasound stimulation of bone healing until the 1980s. Duarte and Xavier[77] could be the first doctors who used the low intensity pulsed ultrasound (30mW/cm^2) to treat 27 recalcitrant non-unions and show 70% of the cases healed in 1983. Duarte also reported 28% accelerated cortical bridging after fibular osteotomy in rabbits compared with that in controls[87]. Pilla et al.[88] found that treatment duration of 20 min/day of LIPUS (200 ms burst of 1.5 MHz sine waves, repeated at 1 kHz, delivery the intensity of 30mW/cm^2) accelerated the recovery of torsional strength and stiffness. Moreover, the ultrasound frequency of a 1.5-MHz burst was significantly greater than the 0.5-M burst in stiffness of the rat femoral fracture model[89]. The pulse width of 200 ms and a 1-kHz repetition rate was reflective of optimal ultrasound parameters for fracture repair[87, 88]. Thus, the parameters of therapeutic LIPUS were established as pulsed ultrasound of a 200-ms burst of a 1.5-MHz sine wave, repeated at 1 KHz, delivering the intensity of 30mW/cm^2 with the treatment duration of 20 min/day. In the States, the Food and Drug Administration

approved the use of low-intensity ultrasound for the accelerated healing of fresh fractures in October 1994 and for the treatment of established nonunions in February 2000. These first regulatory approval were based primarily upon two rigorous, double-blind, placebo controlled clinical trials, which showed 38% reduction of healing time for fresh fractures[90, 91]. Then the commercial products were available. LIPUS has been widely utilized in clinical practices for the bone healing in various conditions.

1.2.3 Biological effects of low intensity pulsed ultrasound on bone healing

There are many effects of LIPUS on living cells and tissues investigated. (Table 1)

Table 1. The Effects of Low Intensity Pulsed Ultrasound on Cells

Cell Type	Effect
Macrophage and–MMP9 expression	Increased phagocytic activity Increased MMP9 activity in macrophages[92]
Periosteal cells	Increased osteoblast differentiation (increased VEGF, osteocalcin, alkaline phosphatase) and increased mineralization[93]
Chondrocytes	Accelerated matrix formation, demonstrated by an increase in proteoglycan synthesis[94]
Osteoblasts	Accelerated maturation as shown by upregulation of bone markers (Cbfa1/Runx2, HIF-1, PGE2); Increased VEGFa levels; Increased osteocalcin mRNA[95-98]
Bone marrow stromal cells	Increased expression of osteocalcin, osteopontin and IGF-1[99]

Moreover, LIPUS elevated intracellular calcium in cultured chondrocytes and stimulate endochondral bone formation in vitro[100]. LIPUS influenced every phase of fracture-healing process. (Table 2)

Table 2. The Effects of Low Intensity pulsed Ultrasound on Fracture-healing Phases

Different Phases	Effects
Inflammatory phase	Shortening the inflammatory period and earlier commencement of the reparative phase[77, 101]
Reparative phase	Directly stimulates chondrogenesis and osteogenesis[94, 102]
Later period of reparative phase	Stimulates endochondral[100] and intramembranous ossification[91]

1.2.4 The mechanism of low intensity pulsed ultrasound on bone healing

The exact mechanism underlying the ultrasound effects on the living tissue remains unclear[103].

1) The characteristics of low intensity pulsed ultrasound

The ultrasound waves produced are transmitted by propagation through molecular collision and vibration, with a progressive loss of the intensity of the energy during passage through the tissue (attenuation), due to absorption, dispersion or scattering of the wave[104]. Low-frequency ultrasound waves have greater depth of penetration into the tissue such as 1-3MHz. Ultrasound at a frequency of 1 MHz is absorbed primarily by tissues at a depth of 3-5 cm[103]. Tissues can be characterized by their acoustic impedance, which is related to the density of the material and the speed of sound in the material. The greater the difference in impedance, the more ultrasound will be reflected rather than transmitted. Such as the waves transmit from the muscle to the bone, there will be a great intensity reflection.

2) The theories of low intensity pulsed ultrasound on the tissue

When ultrasound traverses through a tissue, vibrating forces are applied on every tissue component, such as intra- and extra- cellular fluids and cell membranes. Because of the vibrations of particles in tissue, ultrasound treatment is described in terms of 'internal tissue massage' or 'micromassage'. First, there is the principle that healing bone responds functionally and morphologically to mechanical stimuli. So that LIPUS produces micromotion, both directly

and through cavitation, also similarly may stimulate callus formation and regenerate bone stiffness and strength through secondary chemical mediators[105]. Second, cavitation occurs when gas- or vapor-filled bubbles expand and compress because of ultrasonically induced pressure changes in tissue fluids, with a resulting increase in flow in the surrounding fluid. Stable cavitation is considered to be beneficial to injured tissue, whereas unstable cavitation is considered to cause tissue damage[80]. However, the extent to which cavitation plays a role in vivo is not well understood and still needs to be determined[106]. Third, LIPUS is considered without thermal effect[107]. Unlike the physiotherapy which needs the thermal effect to increase blood flow, decrease the pain and increase extensibility of collagenous tissues, the thermal effects are not considered to play a role in the ultrasound treatment of bone[77]. Fourth, the LIPUS has ability to alter cell membrane permeability to ions and cell membrane electrophsiological properties[108]. The changes in the cell membrane may be the most important mechanism by which the ultrasound signal influences cellular changes and responses, such as above-mentioned the effects of LIPUS on cells[77]. However, the exact mechanism is not clear. Fifth, piezo-electric phenomenon is also supposed as the possible mechanism underlying the ultrasound applied on the bone[87, 109]. However, this effect remains to be determined[77].

In all, LIPUS stimulation of bone healing may be mediated through cavitation, piezo-electric phenomena, and effects on the cell membranes. This stimulation appears to be multilevel, involving different cell types in and during the healing process[77].

1.2.5 Low intensity pulsed ultrasound used in distraction osteogenesis

The application of LIPUS on distraction osteogenesis has been investigated in several animal and clinical studies. (Table 3, 4)

Table 3. Low Intensity Pulsed Ultrasound for Distraction Osteogenesis in Clinical Trail

Author, Year	No. of Patients	Indication, Type of Distraction	Intensity (mW/cm^2)	Conclusion
Sao et al. 1999[110]	1	Delayed Callotasis in Bilateral Leg Lengthening	30	US improved the poor callus formation.
Esenwein et al. 2004[111]	20	Delay Callotasis in Lengthening of legs and limbs	30	US could accelerate bone maturation and formation in DO.
Tsumaki et al. 2004[112]	21	Hemicallotasis at high bibial	30	US accelerated callus maturation
Dudda et al. 2005[113]	1	Humeral lengthening	30	US accelerated callus formation and the its early use should be considered.
El-Mowafi et al. 2005[73]	20	Tibial lengthening	30	US stimulated the bone maturation and reducing healing time.
Gebauer and Correll 2005[114]	13	Delayed union or nonunion from lengthening	30	30% reduction in healing time.
Schortinghuis et al. 2005[115]	8	Alveolar distraction in mandible	30	No effects of US were found.

Sato et al.[110] first reported the good result of using low intensity pulsed ultrasound in a 22-year-old woman who was treated for short stature by means of nine-centimeter bilateral leg lengthening. Most studies have shown positive results of the effects of LIPUS and suggested that LIPUS could be an adjunct treatment for patients having distraction osteogenesis. However, only one study showed no effect of using the ultrasound in alveolar distraction[115]. All the above-mentioned studies used the same property of low intensity pulsed ultrasound with

30mW/cm^2 and for applying 20 minutes per day, which is the same protocol as the application in treating the fracture and non-union[116].

Table 4. Low Intensity Pulsed Ultrasound for Distraction Osteogenesis in Animal Mode

Author, Year	Animal and No.	Part of Distraction	Intensity (mW/cm^2)	Conclusion
Shimazaki et al. 2000[117]	70; Rabbit	Tibia	30	US promoted bone maturation
Mayr et al. 2001[118]	18; Sheep	Metatarsus	30	US stimulated the bone healing
El-Bialy et al. 2002[119]	21; Rabbit	Mandible	30	US enhanced the bone formation
Machen et al. 2002[105]	20; Rabbit	Tibia	30	US stimulated cartilage formation in callus.
Tis et al. 2002[120]	26; Rabbit	Tibia	30	US increased the size of the callus.
Eberson et al. 2003[74]	34; Rat	Femora	30	US mature the newly formed bone.
Uglow et al. 2003[121]	34; Rat	Tibia	30	US showed no effects on DO.
Sakurakichi et al. 2004[122]	75; Rabbit	Tibia	30	US stimulated the bone formation and was most effective during the distraction phase.
Claes et al. 2004[123]	18; Sheep	Metatarsus	30	US stimulated the callus formation.
Chan et al. 2006[72]	17; Rabbit	Tibia	30	US was more effective in initial stage of consolidation.
Chan et al. 2006[75]	18; Rabbit	Tibia	30	US enhanced bone regeneration, and its effect was dose-dependent.
Taylor et al. 2007[124]	44; Rabbit	Tibia	30	US did not enhance distraction callus.

The LIPUS application was performed in most animal and human studies during the consolidation period[72-74, 105, 117-120], expect some investigations that the LIPUS was applied exclusively or additionally during the distraction period[75, 115, 121, 122, 124]. Shimazaki et al.[117] reported that LIPUS was administered during consolidation phase accelerated bone maturation in tibiae lengthening in rabbit, even when callotasis was poor, based on radiography, measurement of the BMD, mechanical testing and histology. Mayr et al.[118] demonstrated that application of LIPUS during consolidation phase stimulated bone healing in sheep bone transport model proved by plain and high resolution radiographs, and computed tomography scans. Many studies found a larger callus formation in the ultrasound treated animals compared with the control groups[74, 117, 120-123]. Shimazaki et al.[117] and Sakurakichi et al.[122] detected higher bone density and a corresponding higher biomechanical stiffness and strength in bones treated with LIPUS. However, Uglow et al.[121] could not find any significant influence of the LIPUS stimulation. They used a rabbit tibia distraction model (1 day latency, 1mm/day distraction rate, 10.5mm distraction, maturation period 2 and 4 weeks), and found a trend towards higher bending strength at 2 weeks maturation for the LIPUS treated group but this difference disappeared after 4 weeks of maturation. Taylor et al.[124] also found no positive effect of LIPUS to enhance distraction callus. For histological examinations, several studies showed that the percentage of fibrous tissue in the maturing callus was lower and the percentage of newly formed bone higher in the LIPUS treated groups than control group[120, 122, 123].

1.3 Statement of Problem

In all, to the best of our knowledge only one article evaluated the effect of the ultrasound on mandibular distraction model[119] and showed the positive evidences by assessment of bone photodenstiy, vibratory coherence, mechanical stiffness, and quantitative histological examination. But one human study showed negative results[115]. Therefore, there is a need for more investigations to assess the effect of LIPUS on mandibular distraction osteogenesis.

1.4 Objective

To evaluate the effect of low intensity pulsed ultrasound on new bone regeneration in distraction osteogenesis.

2. Material and Method

2.1 Material

2.1.1 Animal

Twenty four adult male New Zealand White rabbit are served as the experimental model, with the average weighing 3.5-4.0 kg were used in the present study. (Fig 16)

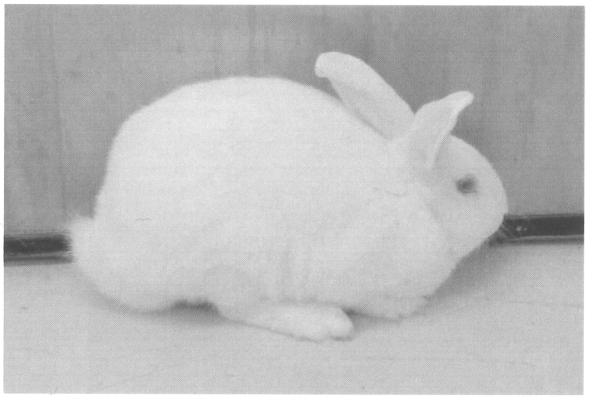


Fig 16. A New Zealand white rabbit

2.1.2 Distractor

The distractors were custom made by modification of orthodontic palatal expansion screw (Hyrax^{R}) (Fig 17) with maximal lengthening up to 10mm (Fig 19). A pair of metal legs was custom made and soldered to each site of the expansion screw, and there were two holes in each metal leg used for accommodation of microscrews (Fig 18).

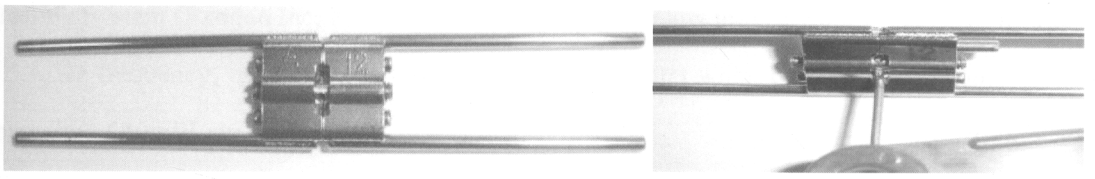


Fig 17. (Left) An orthodontic palatal expansion screw (Hyrax^{R}).

(Right) An activating pin inserted into the expansion screw for expanding

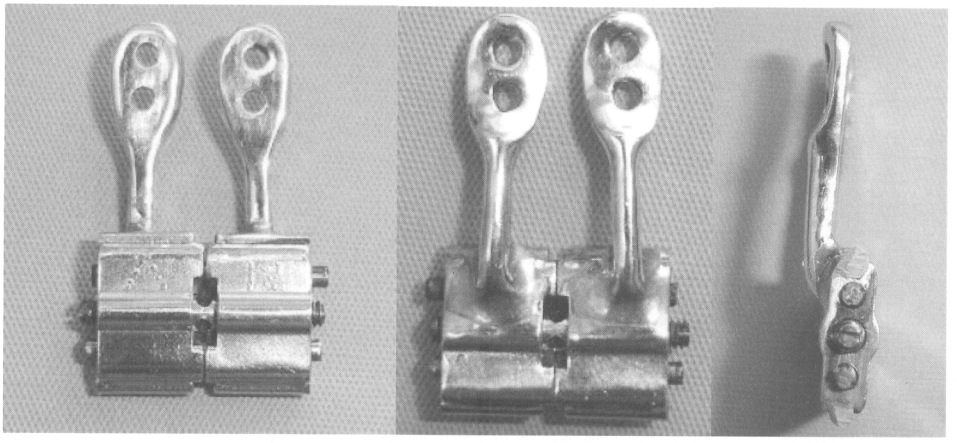


Fig 18. Custom made distractor

(Left) frontal view. (Middle) back view. (Right) lateral view

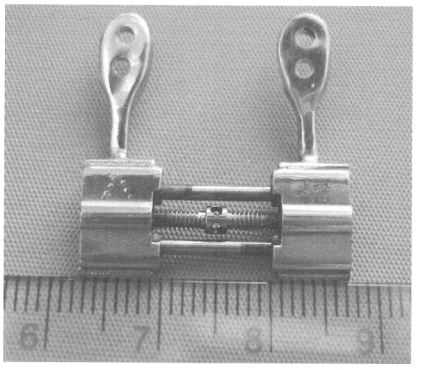


Fig 19. An activated distractor with 10mm length gained

2.1.3 Low intensity pulsed ultrasound device

The commercial device was used. The so-called Sonic Accelerated Fracture Healing System (Exogen Inc, Piscataway, NJ) provides the low intensity pulsed ultrasound with a 1.5 MHz frequency, modulated at 1 KHz with a signal burst width of 200 microseconds and an intensity of 30mW/cm^2 (Fig 20).

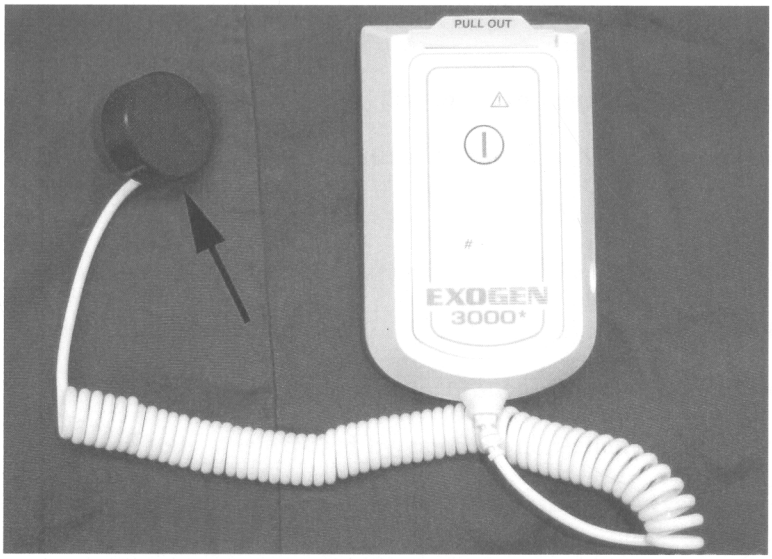


Fig 20. Low intensity pulsed ultrasound device.

(arrow): Transducer head

2.2 Method

2.2.1 Grouping

Total twenty four rabbits underwent distraction osteogenesis at right site of the mandible. They were randomized divided into two major groups (A group and B group). Group A received ultrasound treatment, group B served as control. Each of those two major groups was subdivided to three subgroups (termed as A1, A2, A3 and B1, B2, B3). Hence, there were four rabbits in each subgroup.

Division of the major groups was according to different consolidation periods. In group A1 and B1 animals were sacrificed immediate after completion of distraction phase without consolidation period. In group A2 and B2 animals had 2 weeks of consolidation period when sacrificed. Group A3 and B3 had longest consolidation period for 4 weeks.

2.2.2 Surgery

The surgery for mandibular distraction osteogenesis was performed under aseptic condition. All rabbits were anesthesized with an intramuscular injection of Ketamine Hydrochloride (35mg/kg) and Diazepam (5mg/kg). The Penicillin G Sodium (0.5 million units) was administered preoperatively 20 minutes intramuscularly. The right submandibular area was shaved and disinfected with iodine solution. 1.0 ml of 2% Lidocaine with 1:100,000 epinephrine solution was injected subcutaneously at surgical area. Submandibular incision was made with 3cm long. The mandibular body was exposed by carefully dissection the plastysma muscle and reflection of periosteum. The facial artery and mental nerves were preserved. The osteotomy line was made straight downward to the lower border of the mandible between the premolar and the mental foramen. The osteotomy of the mandible was achieved by two steps: the first step was to perform a corticotomy by fissure bur at buccal and lower border aspects of the mandible, thus a bony slot was made in order to locate the distractor, moreover if the osteotomy had been completed before placement of the distractor, it would have been more difficult to fix the distractor between two unstable bone segments rather than keeping continuity of the mandible by an incomplete osteotomy at beginning. Then the distractor was located according to the bony slot (osteotomy line) and fixed by four self-tapping titanium microscrews. In addition, the vector of distraction was parallel to the long axis of the mandible and perpendicular to the osteotomy line. Hence the second step of the osteotomy was finished by a complete osteotomy using fissure bur and chisel after the placement of the distractor. Intraoperatively the distractor was activated up to about 3mm of lengthening for testing the distractor and completion of the osteotomy. Then the distractor was drawn back without any lengthening. Every step in the operation was accompanied by thorough irrigation. Finally each layer of periosteum, plastysma muscle, subcutaneous tissue and skin was sutured. The rabbits returned to the normal diet immediately after surgery, which were fed by milled pellet. Postoperative care included intramuscular applications of Penicillin G Sodium (0.5 million units) and Acetaminophen 75mg per day for 3 days. (Fig 21)

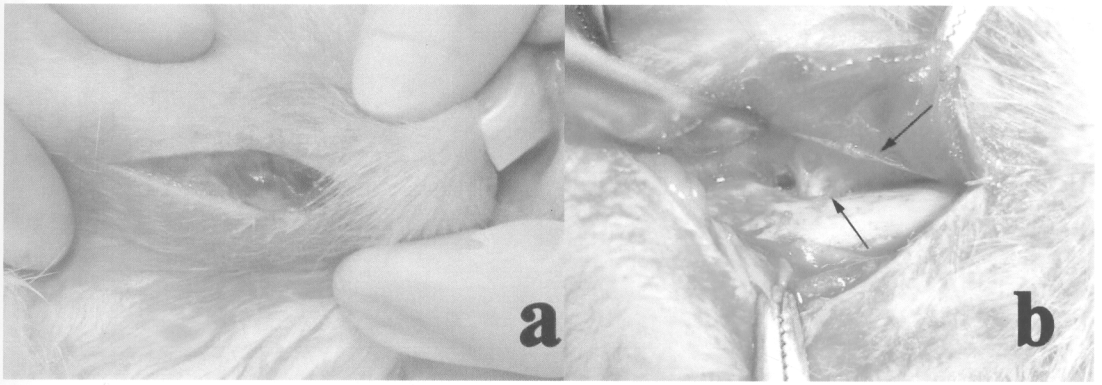


Fig 21. (a) A submandibular incision

(b) Exposure of buccal aspect of the mandible.

(upper arrow) shows the periosteum

(lower arrow) shows the mental nerve

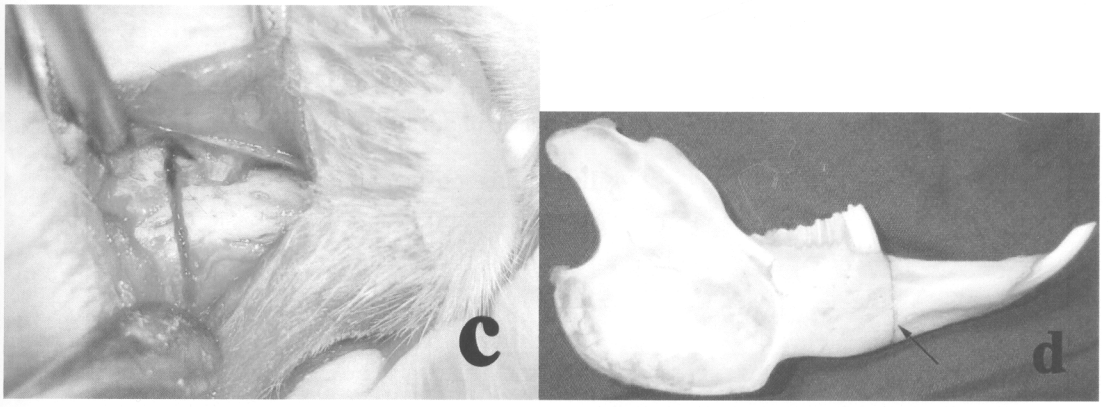


Fig 21. (c) A partial osteotomy (Corticotomy) was made just between the premolar and the mental foramen.

(d) A dry mandible showing the osteotomy line as the arrow indicated

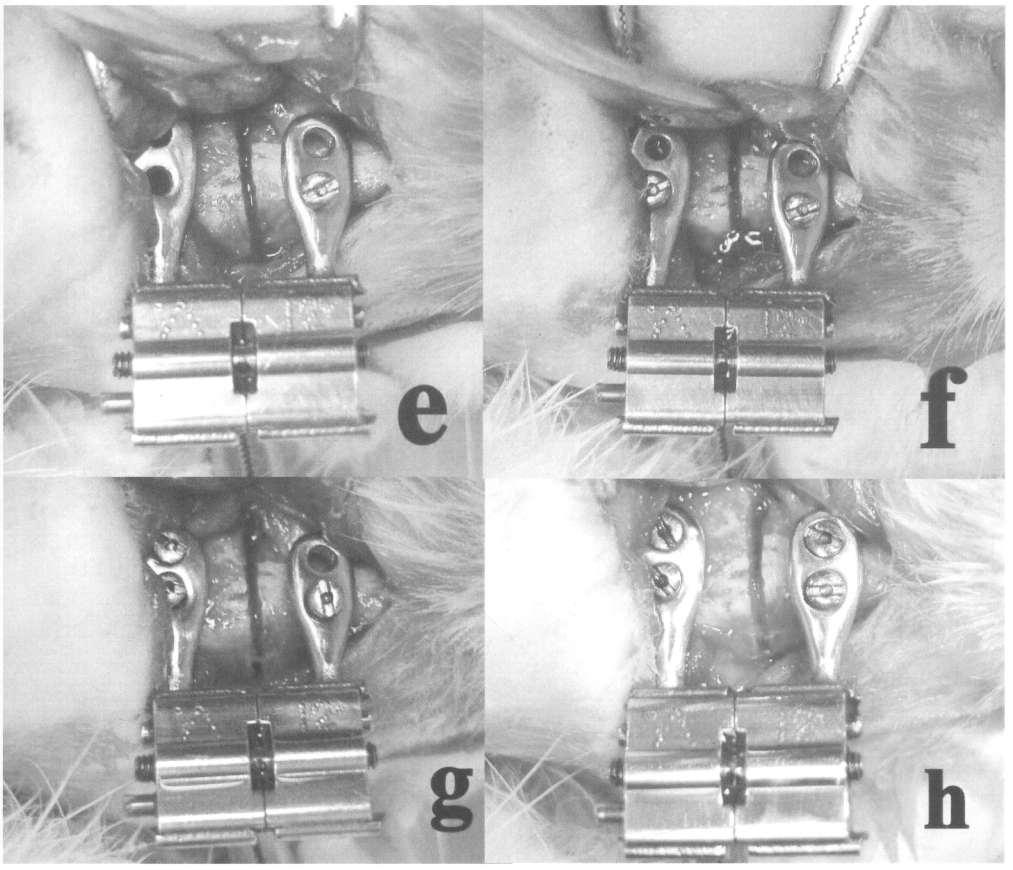


Fig 21. (e,f,g,h) The fixation steps of the distractor.

Note : (h) The osteotomy was not completed

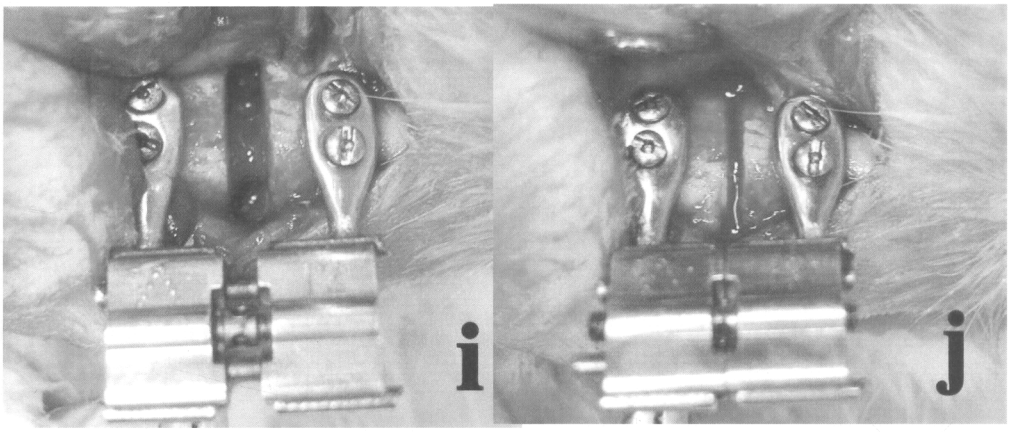


Fig 21. (i) The distractor was activated for test after complete osteotomy

(j) The distractor was drawn back to initial

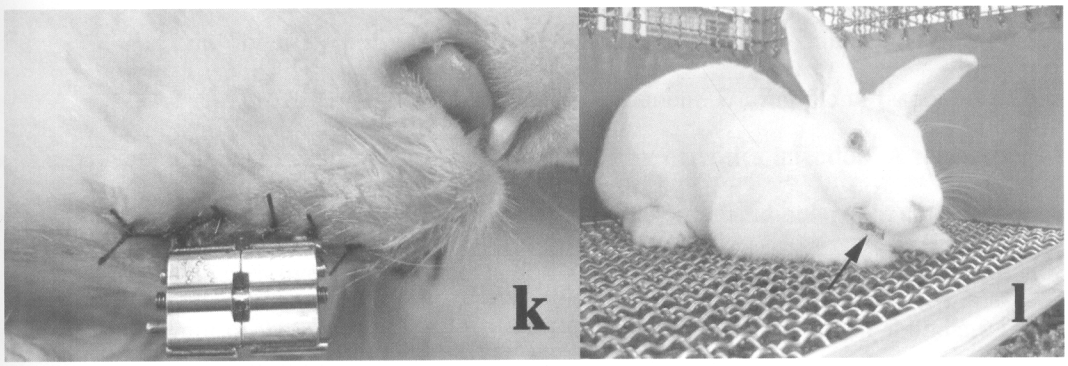


Fig 21. (k) Sutured

(l) Post-operative 12 days

(arrow) shows the distractor

2.2.3 Distraction protocol

Latency period: 3 days.

Distraction period: 10 days. Rate: 1mm/day.

Distraction rhythm: 2 times/day

Consolidation period: different in each group (0 day, 14days, 28days) (Fig 22).

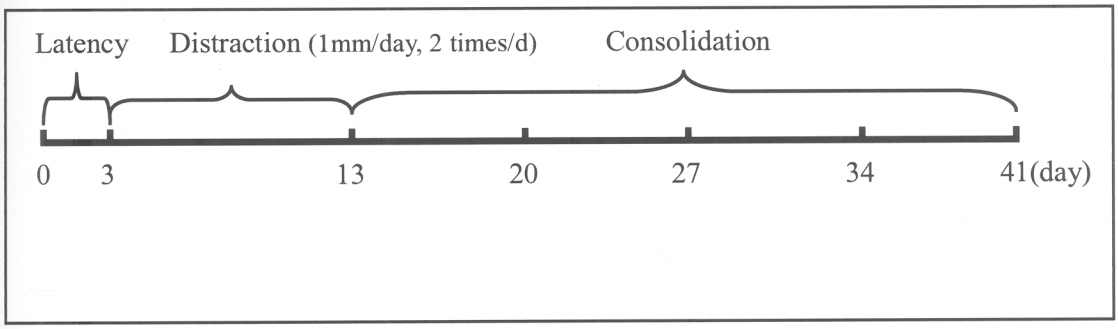


Fig 22. Distraction protocol

2.2.4 Application of low intensity pulsed ultrasound

Low intensity pulsed ultrasound was applied for 20 minutes per day only in group A1, A2, A3 and group B1, B2, B3 received no treatment. During the ultrasound treatment, the rabbits were trapped in a special wood box without any narcotics injection. A researcher held the head of the rabbit by right hand for exposure of the right site mandibular skin area. It was easy to determine the skin area overlying the distraction gap for receiving the ultrasound waves, where just above the border of the mandible and between the distractor legs. With an abundant working surface area of the transducer head (with diameter of 2.5cm), it was certain that the distraction gap had been fully covered by the ultrasound transducer head (Fig 23). Before pressing of the ultrasound head on the skin of the rabbit mandible, the ultrasound coupling gel was used for transmission ultrasound waves from transducer to the skin, while the distractor was covered by tape for preventing attachment of the ultrasound gel to the Jack screw of distractor causing mechanical problems such as the Jack screw adrift. Then the researcher had kept the ultrasound transducer head pressed on the shaved skin by left hand until the ultrasound treatment finished (Fig 24). All the ultrasound treatments had been smoothly conducted. The flounder of rabbits was not a problem, the rabbit head was immobilized by right hand easily. For most instances, the rabbits were calming during the ultrasound treatment.

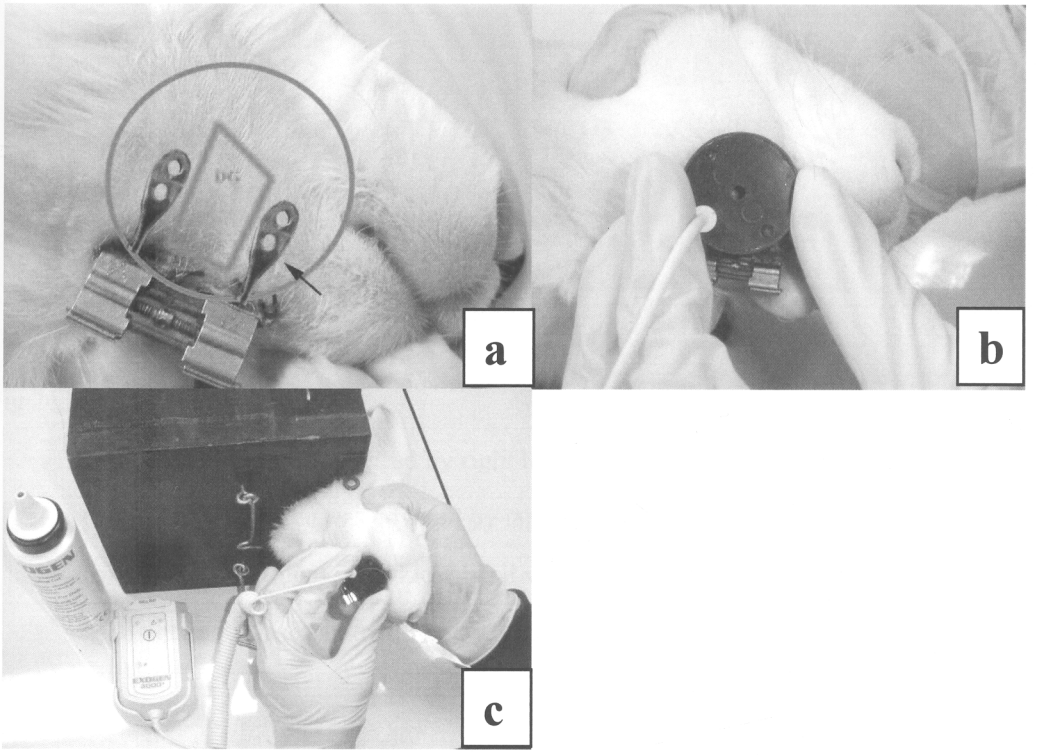


Fig 23. (a) Imitation of applying the ultrasound treatment, (arrow) shows the distractor leg underlying the skin; the blue circle shows the covering area (insider of the circle) of the ultrasound transducer head, the green trapezium and (DG) means the distraction gap.

(b) For showing the position of the ultrasound transducer head without starting ultrasound treatment

(c) For showing the control of rabbit for ultrasound treatment by hands (the ultrasound treatment was not started)

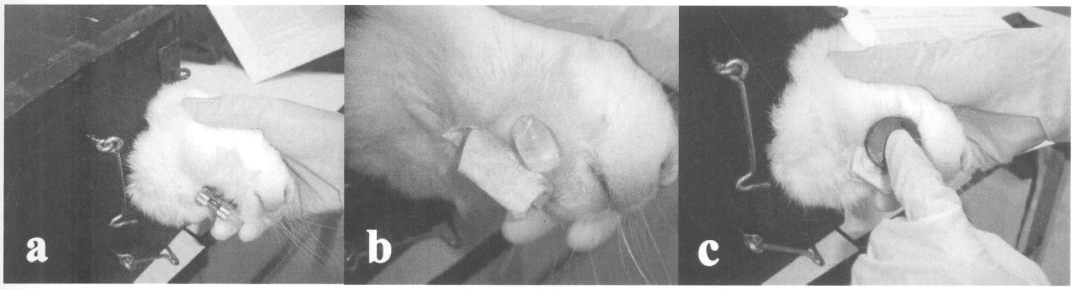


Fig 24. Ultrasound treatment

(a) Control the rabbit head by right hand

(b) The distractor was covered by tape, the ultrasound coupling gel was applied

(c) The ultrasound transducer head was pressed on the gel above the distractor by left hand, ultrasound treatment was started.

2.2.5 Protocol of low intensity pulsed ultrasound treatment

Ultrasound treatment was only for A1, A2 and A3 groups. In all groups, the ultrasound treatment started at the first day of distraction period and continued until the animal sacrificed. Group A1 had no consolidation period. Group A2 had 2 weeks of consolidation period. Group A3 had 4 weeks of consolidation period (Fig 25).

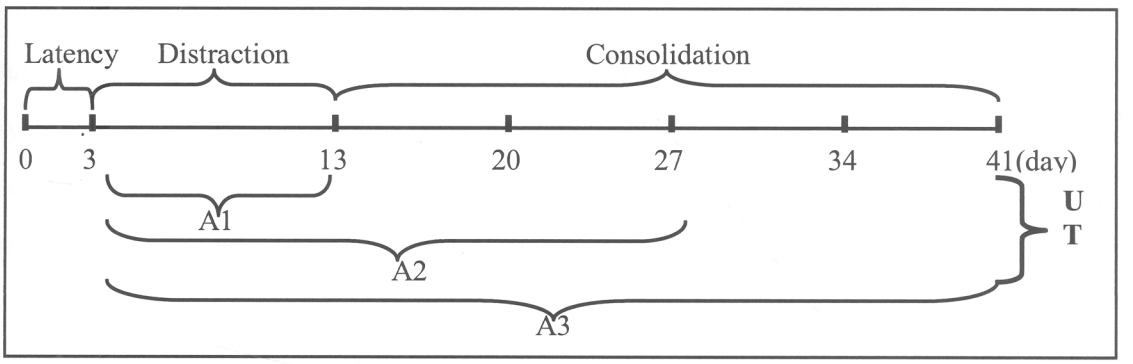


Fig 25. Scheme of ultrasound treatment protocol

UT : ultrasound treatment

2.2.6 Sacrifice scheme

The sacrifice was performed by an intravenous injection of sodium pentobarbitone (1ml/kg).

Group A1 and B1 were sacrificed at completion of distraction.

Group A2 and B2 were sacrificed at 2 weeks after completion of distraction

Group A3 and B3 were sacrificed at 4 weeks after completion of distraction (Fig 26).

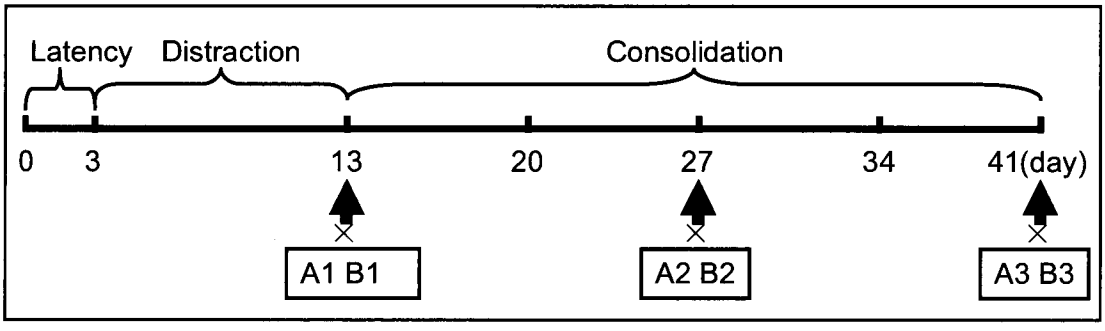


Fig 26. Scheme of sacrifice

2.2.7 Assessment method

2.2.7.1 Plain radiography

After the sacrifice, the whole mandible was carefully dissected from the surrounding soft tissue, and then the mandible was separated into two hemimandibles at the synthesis by scalpel. Both two hemimandibles were taken the plain radiograph. The hemimandible was placed on an occlusal film with the lingual side contacting to the film. The lateral film of hemimandible was taken (10 mA, 50 KVP, 0.26 sec, 12 inch FFD) with an aluminum step-wedge. All of the films were taken by the same machine (Gendex, Illinois, USA) (Fig 27) and processed by an automatic film processor (Dent X 9000, DentX/Logetronics GmbH, Kornberg, Germany) (Fig 28). Then the films were transformed into digital images by digital camera (JVC TK-C1380, Tokyo). The films were examined and recorded as descriptive study.

Further quantitative study was performed under the software (Image Pro Plus 5.0, Media Cybernetics Inc. Slier Spring, USA) to measure the mean gray level which was represented the projectional bone mineral density (BMD) in the distraction gap (Fig 29).

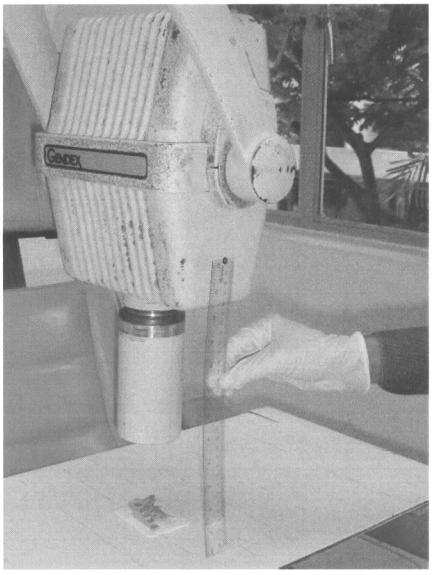


Fig 27. The lateral film of hemimandible, a ruler was used to keep the same distance between the X-ray tube and the film
An aluminum step-wedge used
The X-ray machine (Gendex, Illinois, USA).

Fig 28. The automatic film processor (Dent X 9000, DentX/Logetronics GmbH, Kornberg, Germany)

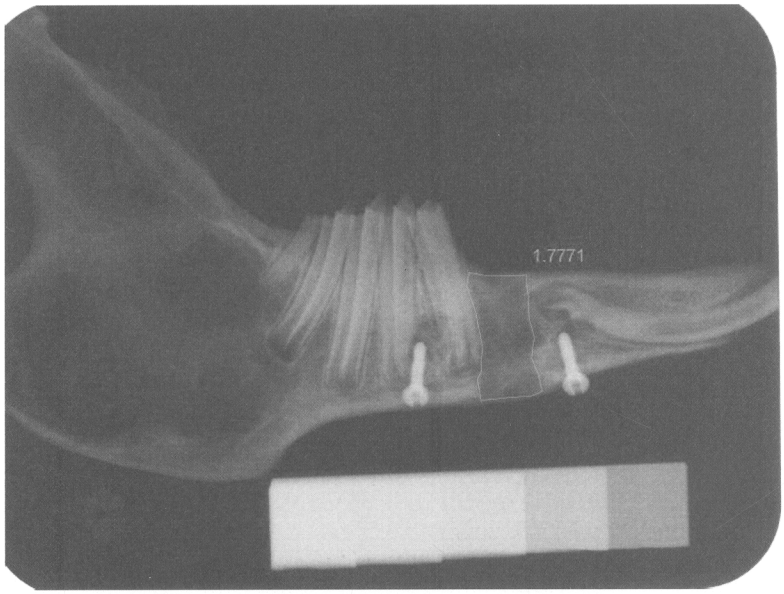


Fig 29. Analysis of mean gray level under the software (Image Pro Plus 5.0)

Note the yellow defined the interest of region, the green showed the mean gray level.

Cutting the specimen

After taken the plain radiograph, the cutting machine (Exakt-Cutting Grinding System, Norderstedt, England) (Fig 30) was use to separate the specimen from the hemimandible which was distracted. The specimen included the bony tissue in the distraction gap and parts of the original bone, anterior and posterior to the distraction gap. Another horizontal cutting further separated the specimen into upper and lower parts (Fig 31). All the specimens were saline-soaked and frozen at -80 for preservation before additional testing.

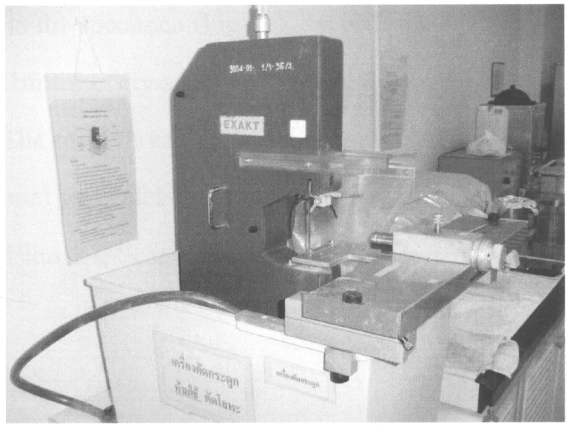


Fig 30. Exakt-Cutting Grinding System, Norderstedt, England.

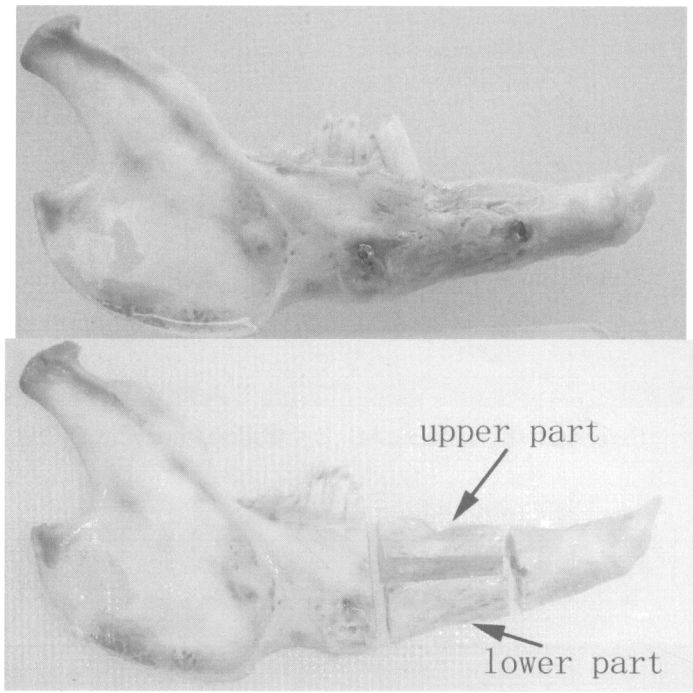


Fig 31. (upper) The distracted hemimandible with 2 screws, the distraction gap was between the screws

(lower) Two vertical cuttings and one horizontal cutting for harvesting the specimens

2.2.7.2 Micro-computerized tomography

The lower part was sent for Micro-CT scanning (μCT20, Scano Medical AG, Bassersdorf, Switzerland) (Fig 32). The lower part was placed into a 17mm diameter sample holder (cylinder shape) during the scanning (Fig 33). First, a gross 2-D image (similar to plain radiograph) of the whole specimen was conducted by a quickly scanning in order to choose the region of interest (ROI) in the specimen (Fig 34). Second, the ROI was transversely scanned with a slice thickness of 200μm for every specimen. Third, the image of every slice was obtained and then analyzed (Fig 35). The same threshold was set for all the analyses. Bone volume fraction (%, Bone volume/Total volume) was calculated. The analysis software was provided by the Micro-CT company (Software Revision 3.1, Scanco Medical AG).

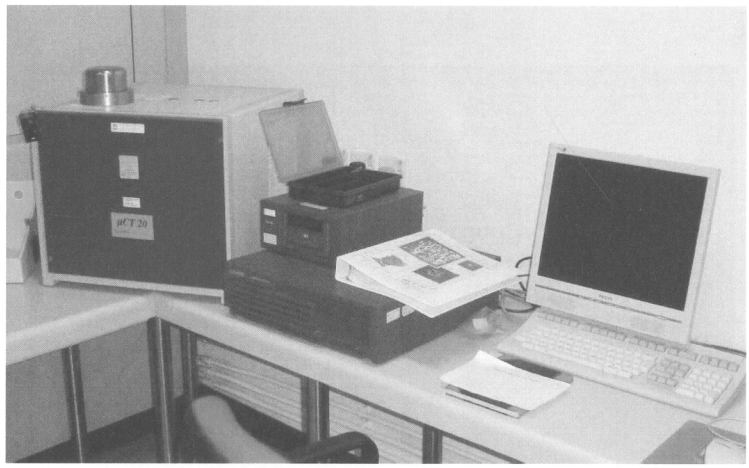


Fig 32. Micro-CT (μCT20, Scano Medical AG, BAssersdorf, Switzerland)

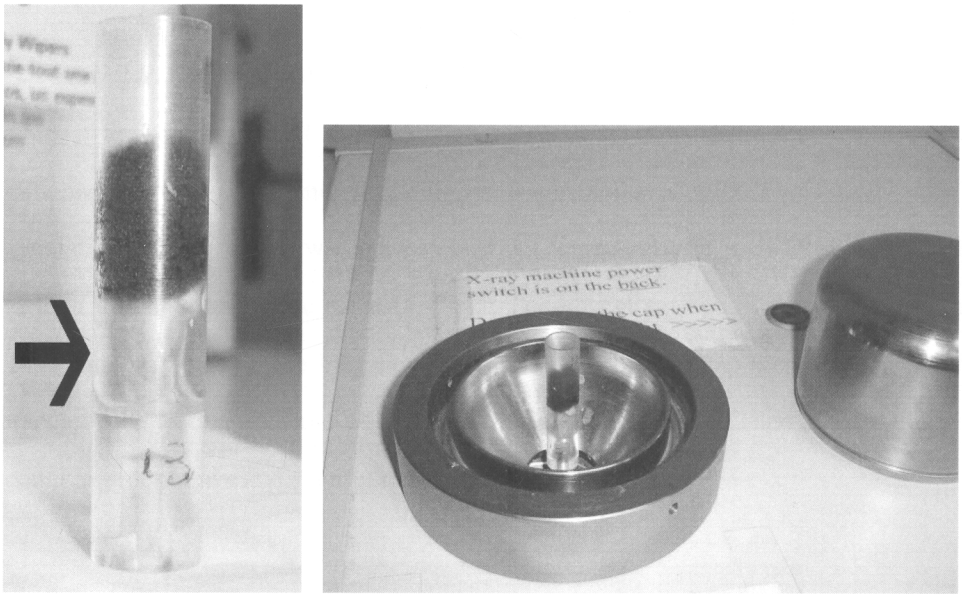


Fig 33. Placement of the specimen ("arrow" shows the specimen)

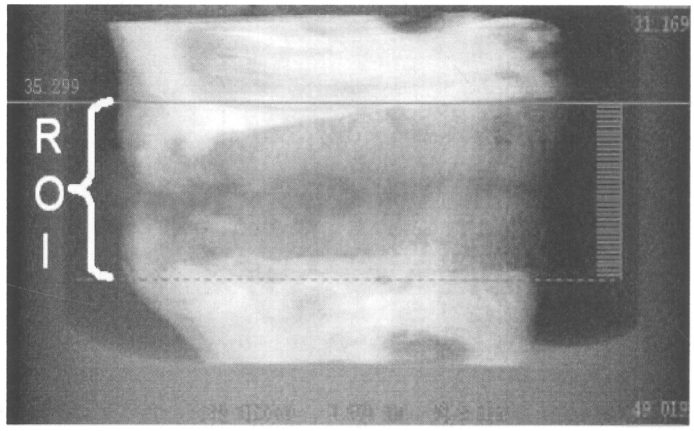


Fig 34. Choosing the region of interest (ROI)

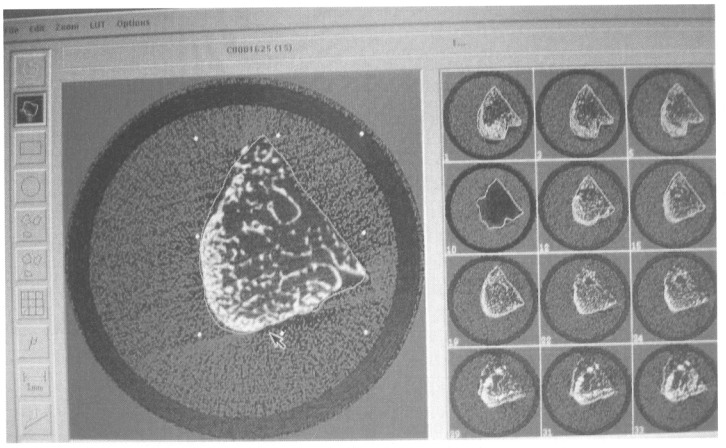


Fig 35. The green line defined the analysis area for each 2-D image

2.2.7.3 Microhardness test

The upper part of the specimen received microhardness test by Microhardness Tester (Buehler Mcromed, England) (Fig 36). The Vickers microhardness was used (more details in the appendix). The specimen was thawed to room temperature at least 20 minutes before the test, and then mounted to acrylic mold (Fig 37). The buccal surface of the distraction gap was tested. Five random areas on the buccal surface of the distraction gap were chosen for the test. The distance of the testing area should be apart from each other for at least 2 times the length of the diagonal in order to avoid detecting the same region for 5 times.

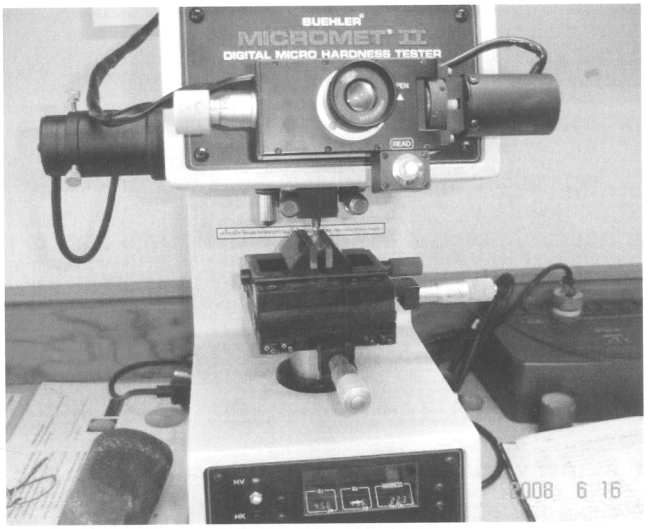


Fig 36. Microhardness Tester (Buehler Mcromed, England)

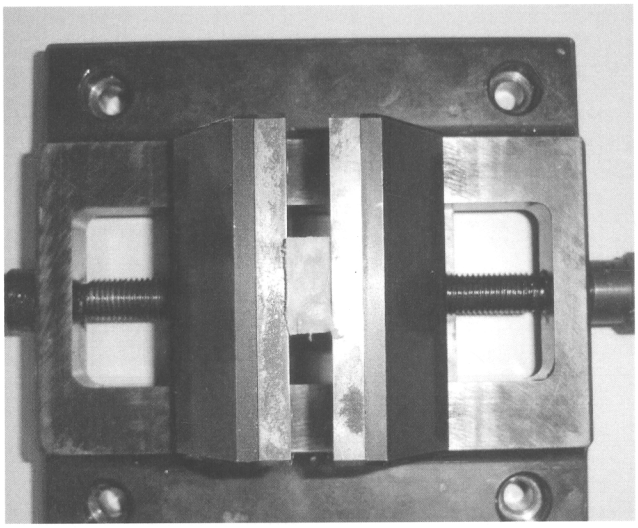


Fig 37. The specimen was mounted

2.2.7.4 Histological examination

After surface microhardness test, the upper part of the specimen was fixed in 10% formalin for 2 weeks and decalcified with 50% formic and 20% sodium citrate then dehydrated in increasing concentrations of alcohol until 100% is reached, finally embedded in paraffin. 10 μm thickness of sections were sectioned (horizontal direction, so that the whole distraction gap was present in every section) and stained with hematoxylin and esion for light microscopy (Carl Zeiss, Axioskop 40, Germany). In histomorphometric analysis, the distraction gap was analyzed as three major horizontal parts, upper, middle and lower parts. Then in each horizontal part, four regions of interest (ROI) were chosen that included two near to the original bone ends (not included), another two in the middle of the distraction gap. The regions of interest were not overlapping to each other. Hence, totally 12 regions of interest were selected for one specimen to represent the whole distraction gap. This sampling method was reported by Cope et al.[37]. Percentage of bone area (%, PBA) was conducted in each ROI under the software (Image Pro Plus 5.0, Media Cybernetics Inc. Slier Spring, USA). The final PBA of each specimen was the mean of total 12 ROI. PBA is the proportion of area of newly formed bone to the total area. The software allows the researcher to select a specific color of the new bone, so total surface area of this same color range is automatically calculated and divided by the total area (Fig 38).

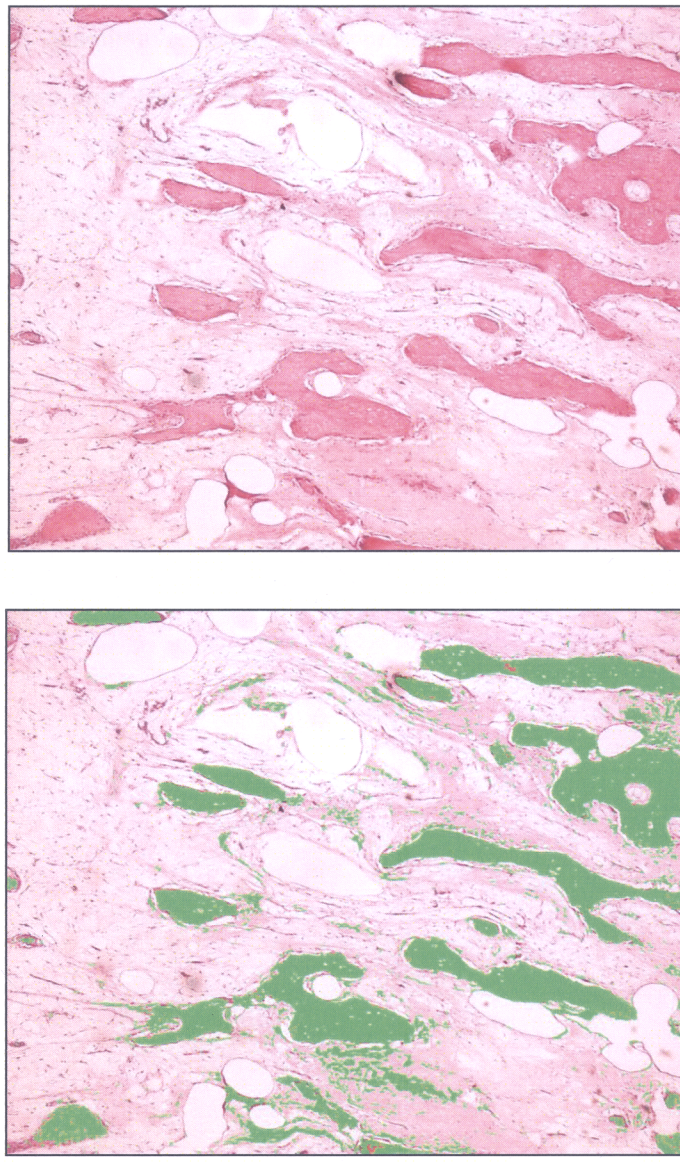


Fig 38. Analysis of percentage of bone area

(upper) before the color of new bone was chosen

(lower) new bone area was selected and drew green color

2.2.7.5 Statistics

The quantitative results were presented as mean with standard deviation (mean±SD) or standard error (mean±SE) for all values. Differences between the two groups in mean gray level, bone volume fraction, microhardness and percentage of bone area were compared by non parametric Mann-Whitney test. Statistical significance was set at $p<0.05$.

3. Result

3.1 Clinical evaluation of the animal

All the surgery was performed smoothly and consistently. Simple intramuscularly injection of Ketamine Hydrochloride (35mg/kg) and Diazepam (5mg/kg) provided good surgical anesthesia lasting about 2 hours. Animals were tolerated well on the operation table. There was no animal death during and after the surgery.

Our custom-made distractors worked well. The distractor showed excellent stability and strength. No breakage or dislodgement of the distractors occurred. All rabbits received the successful distraction. After the completion of distraction, central midline of the mandible was severely shifted to left side causing the cross-bite (Fig 39). In the 2 and 4 weeks of consolidation period groups the rabbits developed severe over-erupted incisors (Fig 40). Meanwhile, the rabbit tolerated wearing the distractor. All rabbits returned to normal diet immediately after operations. It seems the distractor had little impact to the activities of rabbit such as eating.

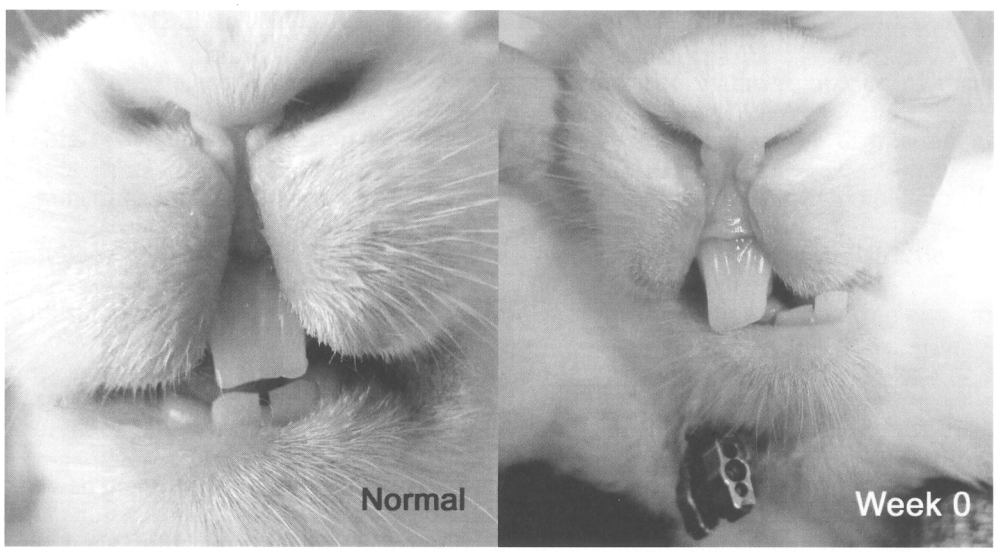


Fig 39. (Left) normal incisal occlusion

(Right) At the completion of distraction, it showed the left shifted mandible. The over-eruption of the incisors was not obvious.

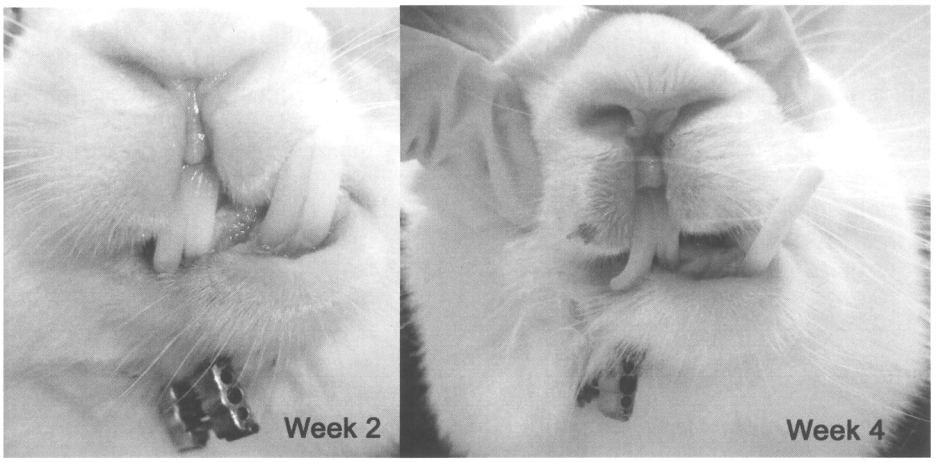


Fig 40. (Left) 2 weeks after the distraction

(Right) 4 weeks after the distraction, note the more severe over-erupting of the incisors comparing with 2 weeks after the distraction. One lower incisor was broken.

The ultrasound treatment was conducted to rabbits easily and smoothly without injection of narcotics. The rabbit was calming and well tolerated to the ultrasound treatment.

3.2 Gross morphological evaluation of the specimen

Distraction osteogenesis was accomplished at the right side of mandible in all rabbits. No sign of infection and no void space were found in the distraction gap. In both treatment and control groups, soft tissue mainly bridged the gap at the completion of distraction; thin bone with porous and rough surface was found at 2 weeks after the distraction; thicker bone with smoother surface was showed at 4 weeks after the distraction. Comparing two groups in each time point, the treatment groups revealed distinguishable differences on week 0 and 2 after distraction. On week 0 after the distraction (at the completion of distraction), the treatment group showed more thin bone coverage near the original bone segments. On week 2 after distraction, the thin bone fully covered the distraction gap in the treatment group, whereas incomplete bony coverage presented in the middle of the distraction gap in the control group. However, we can not find the morphological differences between the groups on week 4 after distraction.

At the completion of distraction, in both two groups, the distraction gap was easily identified from the original bone. The soft tissue with red color occupied the gap dominantly. Interestingly, the tiny soft tissue bundles displayed an orientation parallel to the direction of distraction. In the treatment group, significant more thin bone coverage was easily recognized by naked eyes comparing to the control group. There was significant more thin bone presenting in the gap at both sites near the original bone segments in the treatment group (Fig 41).

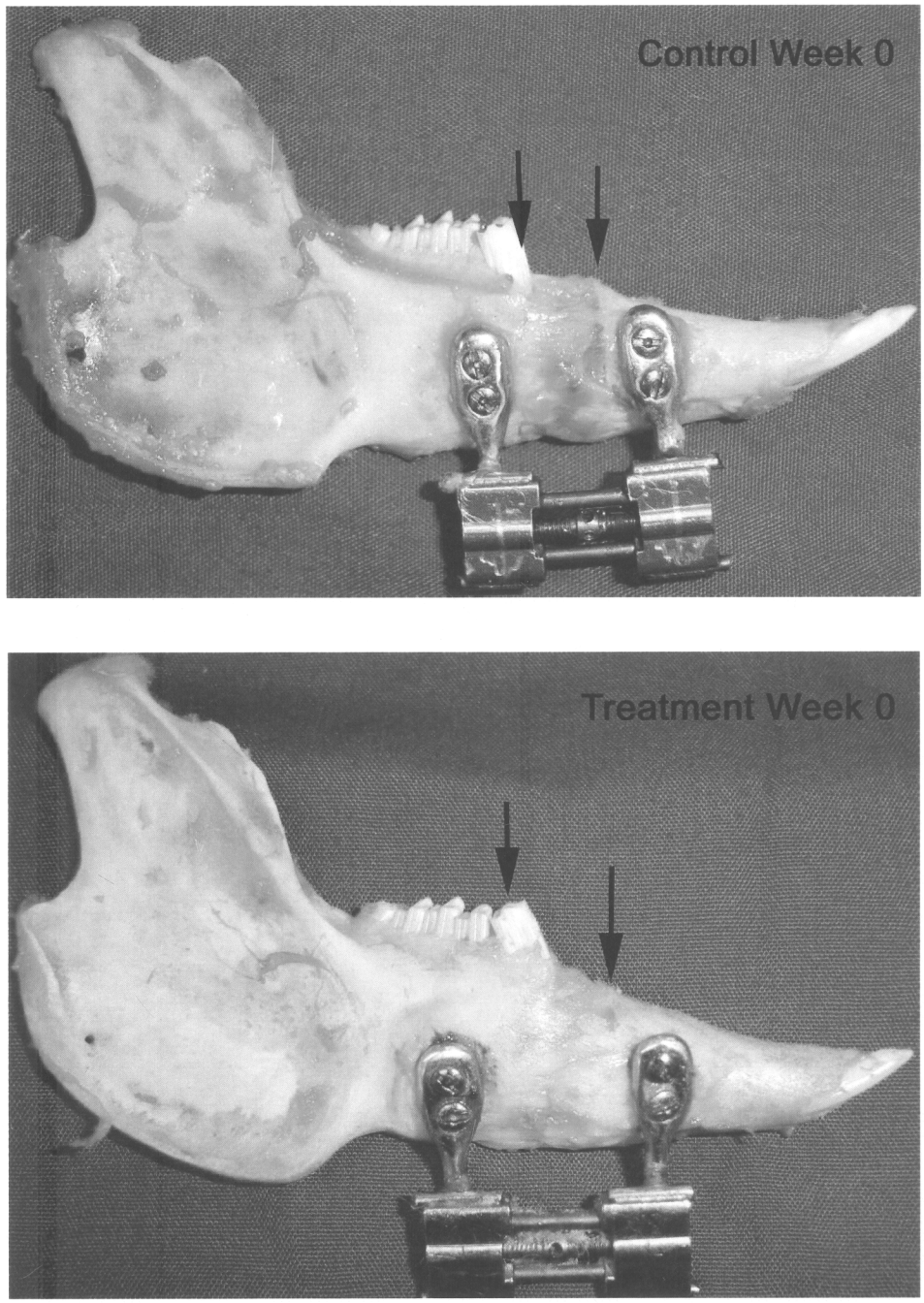


Fig 41. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group showed significant more bone coverage in the gap near the original bone.

At 2 weeks after the distraction, in both two groups, the border of the distraction gap was no longer distinct. The red soft tissue was significant decreased in the gap. Hence, it seems the distraction gap narrower. Although more bony tissue bridged in the gap comparing with the shorter period groups, the majority of the bony tissue formed in the gap presented dull red color which was very different from the original bone. The newly formed bone was thin with porous and rough surface. The differences were also found between the groups by eye observation. The specimens in the treatment group showed more bony coverage, especially in the middle of the gap. In contrast, the large area of soft tissue in the middle of the gap was found in control group (Fig 42).

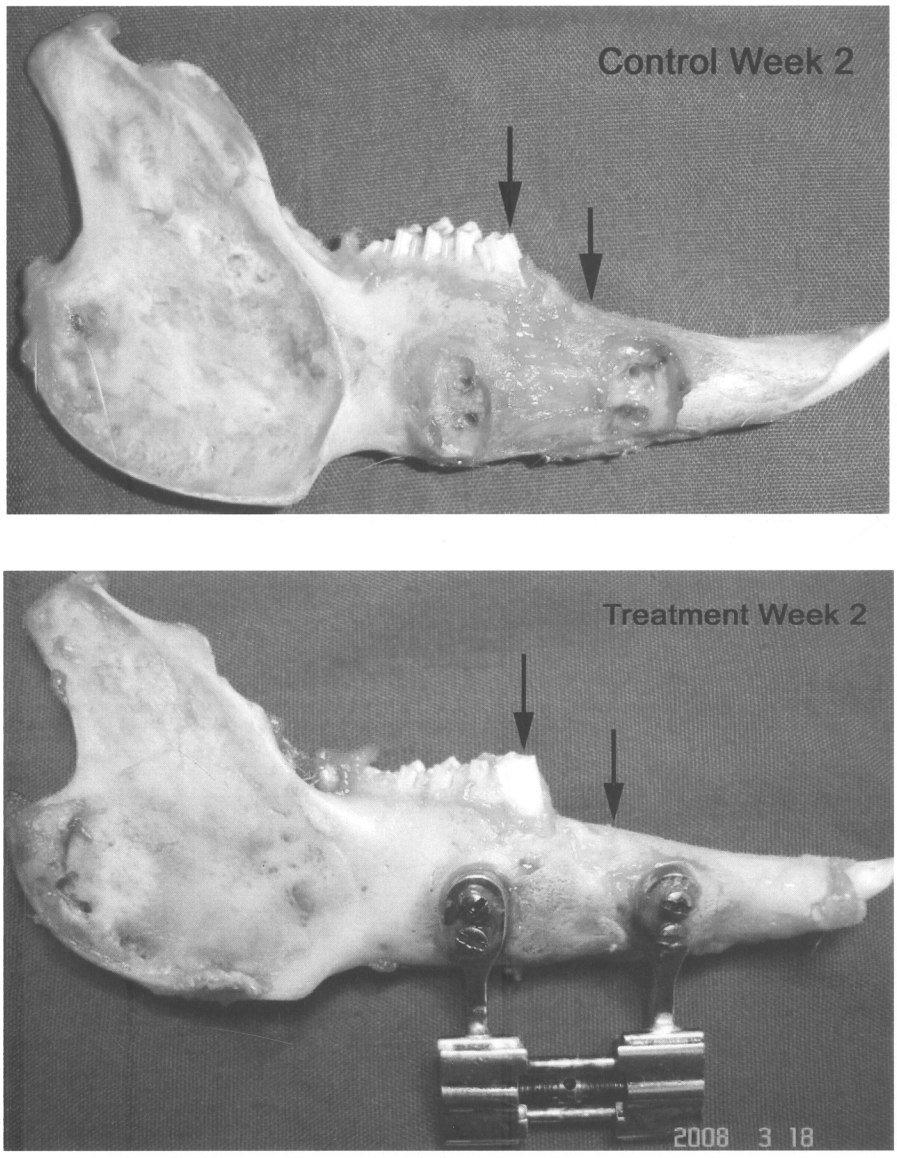


Fig 42. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group. Showed more bone in the middle of the gap.

At 4 weeks after the distraction, it was impossible to outline the border of the distraction gap from the original bone. Thick bone with the white color fully filled in the gap without soft tissue. However, topographically irregular bone surface revealed, comparing with the normal bone surface which was very flat and smooth. There was no difference could be distinguished by eyes between the groups (Fig 43).

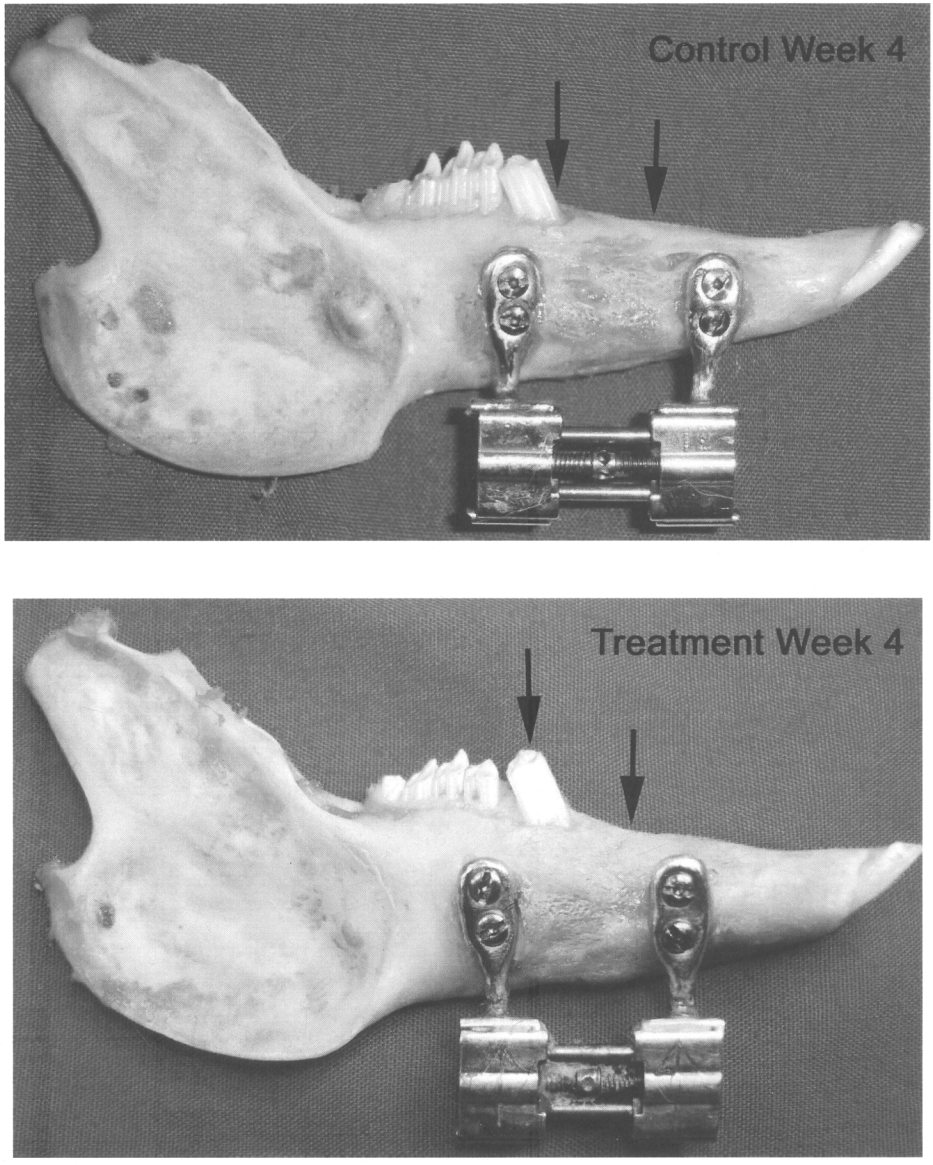


Fig 43. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group. No difference could be found between the groups.

3.3 Radiographic evaluation

All the distracted hemimandible was taken the lateral view film. In general, mineralization took place progressively from the ends of bone segments to the middle of distraction gap, decreased the radiolucent zone in the middle of distraction gap. Increasing radiopacity and gradually indistinct borders between the distraction gap and the original bone were observed.

At the completion of distraction, in both groups, a clear radiolucent gap with distinct border was present. However, there was a significant increase of radiopacity in the treatment group, which presented as more bony spicules toward to the middle of the radiolucent gap (Fig 44).

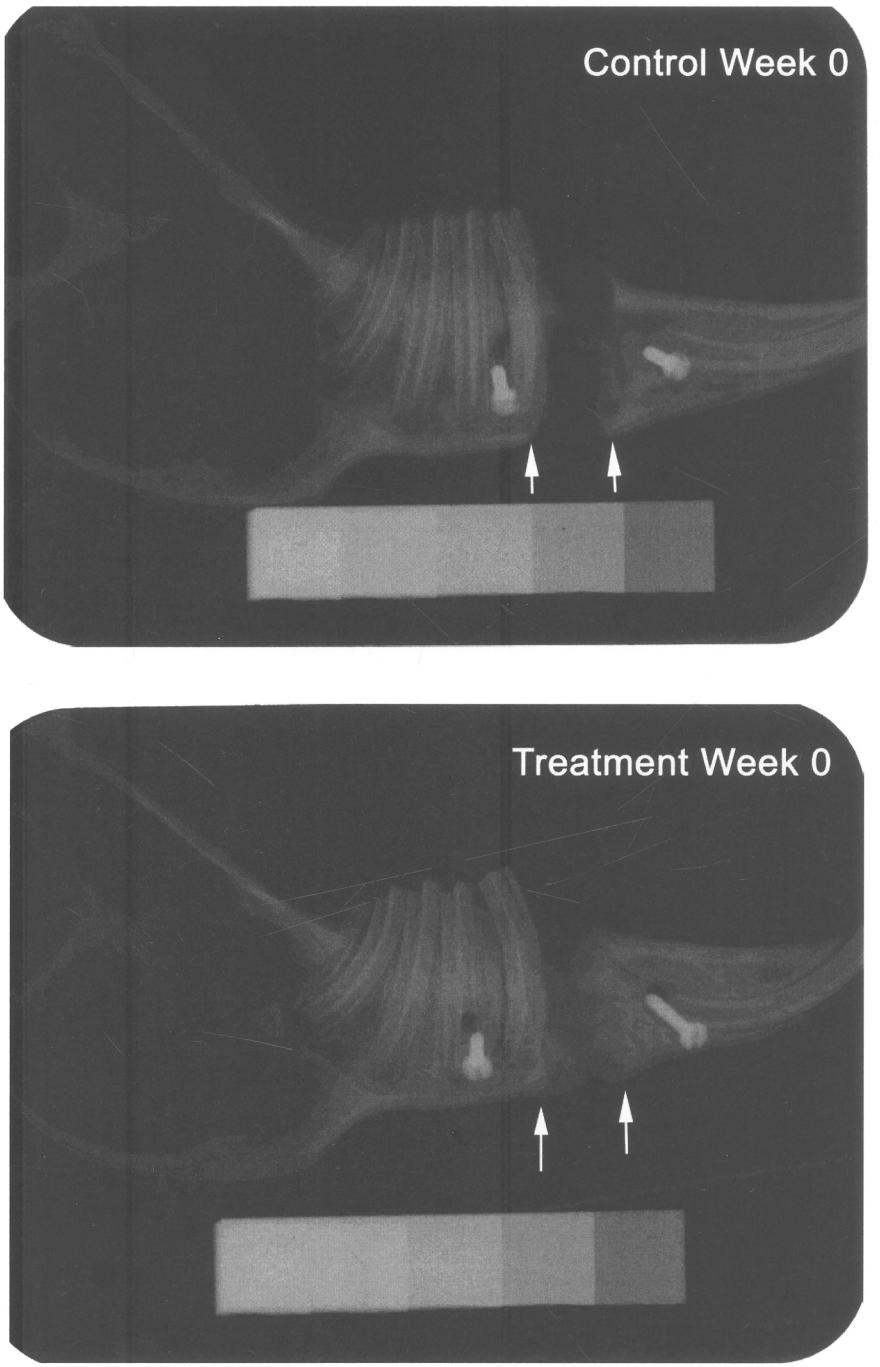


Fig 44. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group. More bony spicules (radiopacity) were present in the gap.

At 2 weeks after the distraction, in both groups, a dramatically increased radiopacity was showed in the distraction gap. Previous radiolucent gap became very narrow or even disappeared. The border of the gap was less defined. The cortical and medullary bone structures were not recognizable in most specimens expect one specimen (Fig 45). More radiopacity was also noted in the treatment group (Fig 46). However, in both groups, morphological structure of the newly formed bone in the gap was not well organized, which presented rather cloudy radiopaque image (Fig 45).

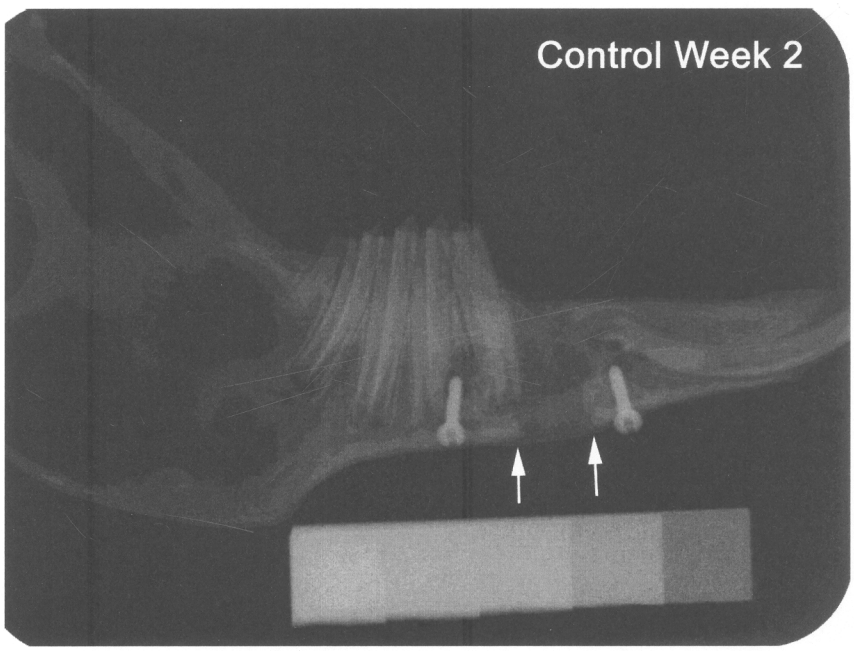


Fig 45. (arrow) indicates the distraction gap

A rare specimen presented the cortical and medullary like structure in the gap, in the control group.

Note, less radiopacity than the treatment group in (Fig 47)

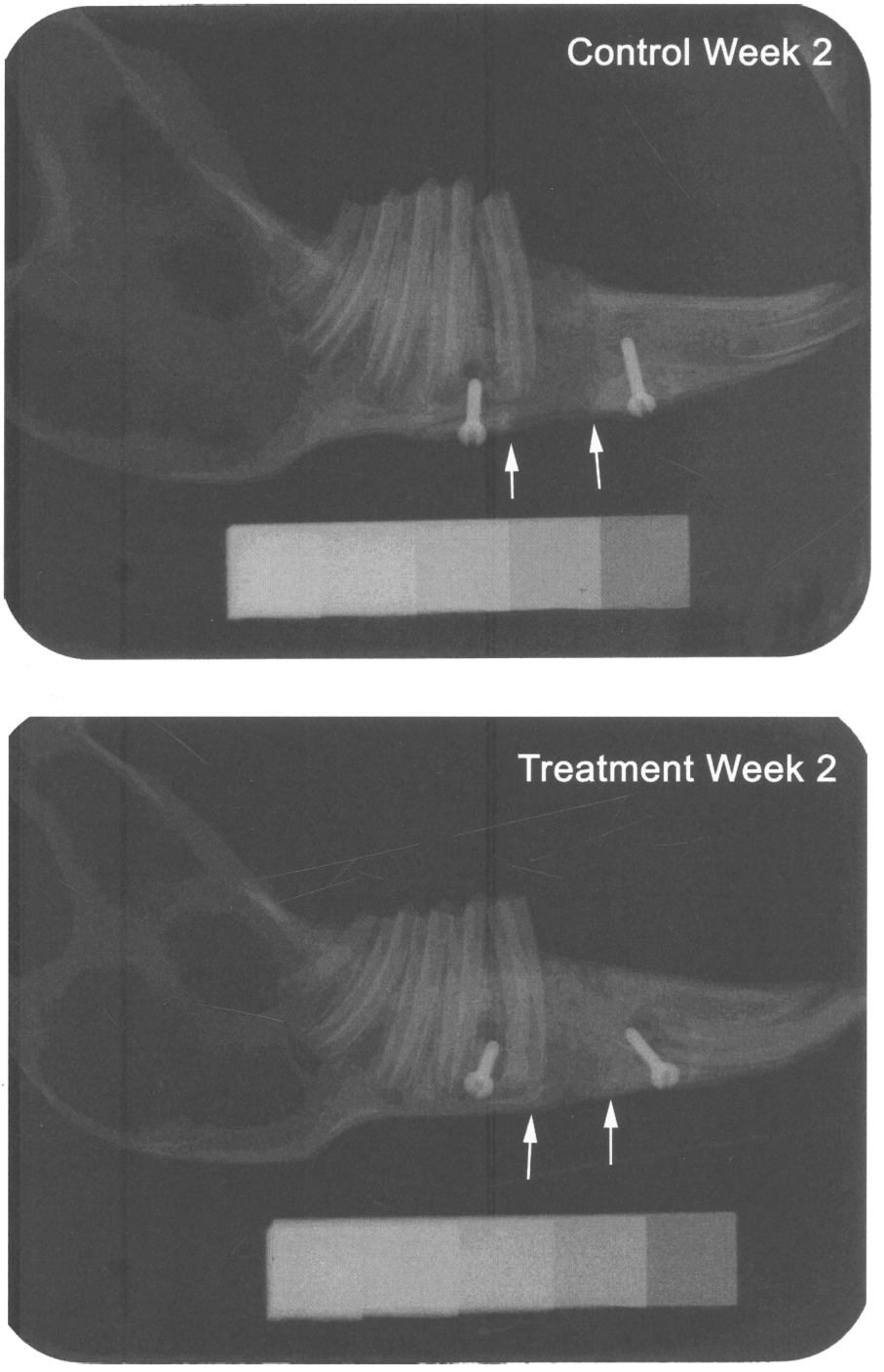


Fig 46. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group. Showed more radiopacity.

Note in both groups, the image of bone in the gap was cloudy.

At 4 weeks after the distraction, in both groups, the radiopacity in the distraction gap kept increasing. Due to the remodeling process of the new bone in the gap, the borders between new and original bone were hardly distinguished. Moreover, the structure of the bony tissue in the gap was clearer and more organized than earlier groups. However, the cortical and medullary bone structures were still not present in the distraction gap. No difference between the control group and treatment group was found by eye observation (Fig 47).

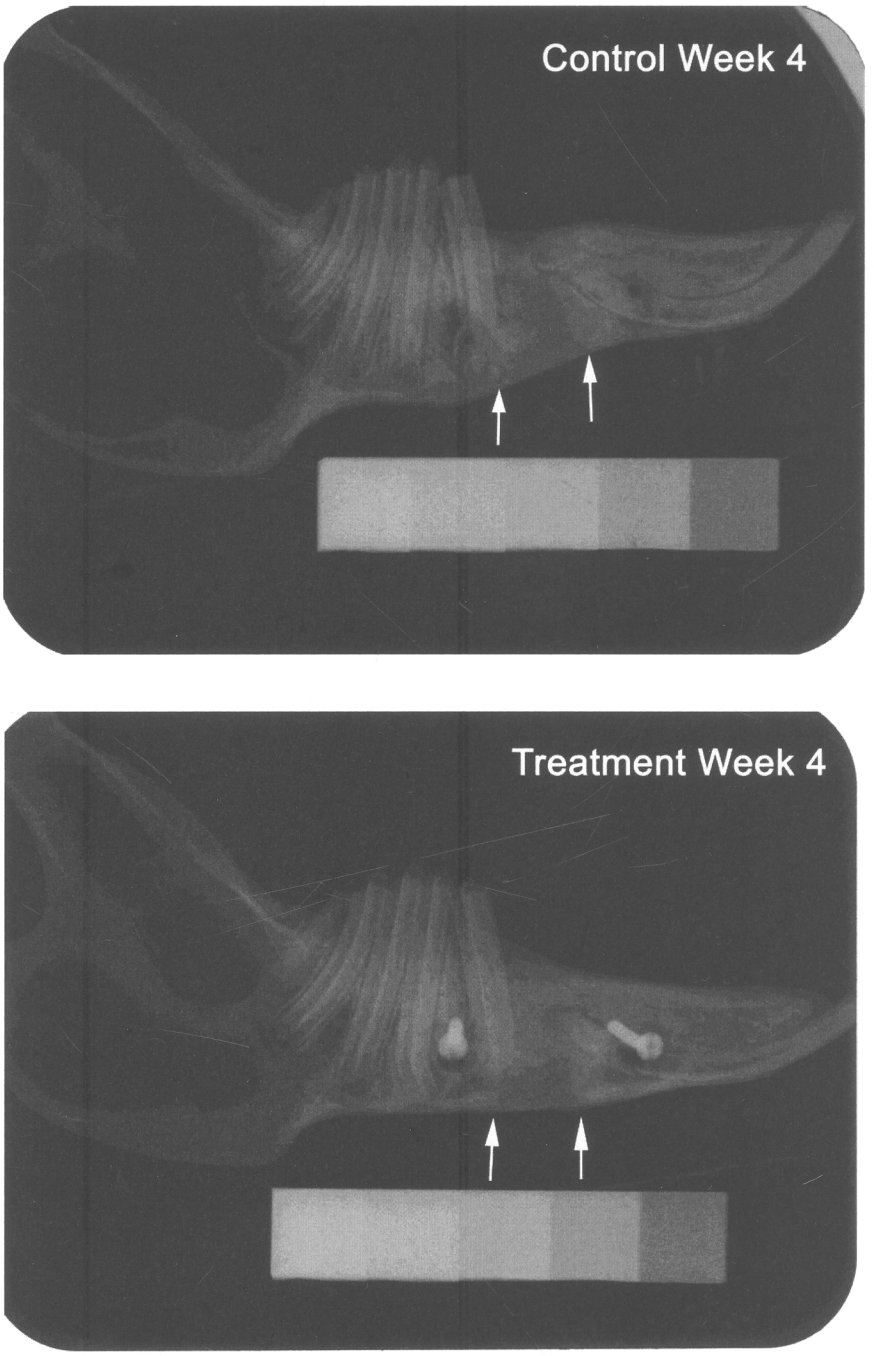


Fig 47. (arrow) indicates the distraction gap

(upper) Control group

(lower) Treatment group. No difference could be found between the groups.

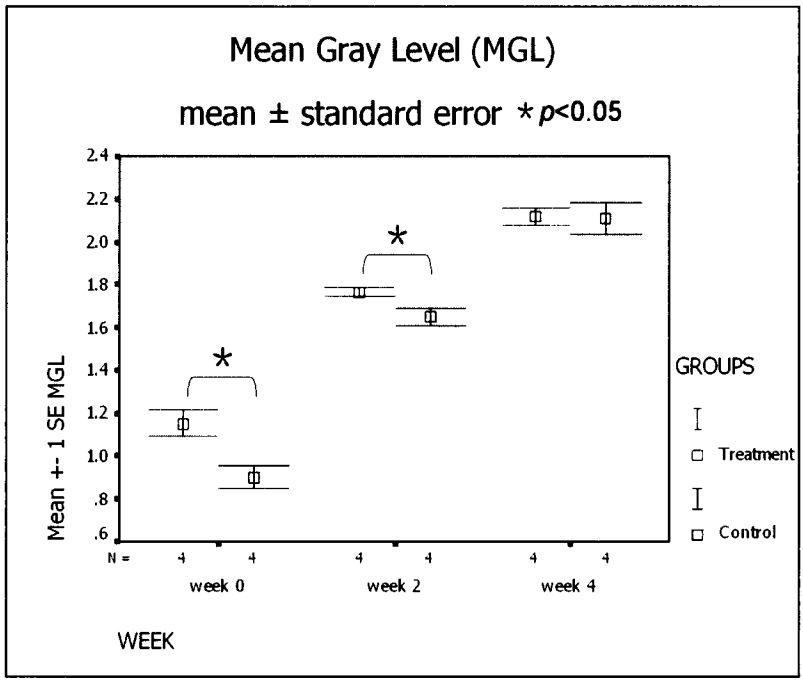
Mean gray level

This is the quantitative value to indirectly represent the bone mineral density in the distraction gap. In both groups, the mean gray level was significantly increasing over time (Table 5) as a result of compared the groups between the time points. Significant differences were also found on week 0 and 2 after the distraction between the control and treatment groups. On week 0 after the distraction (at the completion of the distraction), the control group had the mean gray level of 0.90±0.05 (mean ± standard error) compared with 1.15±0.06 of the treatment group (p=0.021). On week 2 after the distraction, the mean gray value of the treatment group was also significant higher than the control (p=0.043). There was no significant difference between the two groups at 4 weeks after the distraction (p=0.564) (Graph 1).

Table 5. Results of Mean Gray Level

Significances were on week 0 and 2 between the treatment and control groups (Mann-Whitney Test)

Mean Gray Level of Plain Radiography (mean ± standard error)			
	Week 0	Week 2	Week 4
Treatment group	1.1492 ±0.060	1.7637±0.021	2.1181±0.038
Control group	0.9007±0.054	1.6502±0.043	2.1091±0.073
p Value	0.021	0.043	0.564
The level of significance was set at $p<0.05$			



Graph 1. Mean Gray Level of Plain Radiography

Significances were on week 0 and 2 after the distraction between the treatment and control groups

(Mann-Whitney Test, the level of significance was set at $p<0.05$)

3.4 Micro-computerized tomography evaluation

Micro-CT is a nondestructive, fast, and very precise procedure that allows the measurement of cancellous and compact bone in unprocessed biopsies or small bones, as well as a fully automatic determination of three-dimensional morphometric indices[125]. The image of each transverse slice precisely showed the internal structure of bony tissue in the distraction gap. By eye observations, only one difference was visibly distinguished on week 0 after the distraction between the two groups. The treatment group showed more radiopacity in each slice contrast to more radiolucency observed in the control group (Fig 48). At other time points, no obvious difference was found by means of radiopacity or radiolucency (Fig 49, 50). Meanwhile attentions were paid to observe the bony wall of each slice which was covering on the distraction gap. Continuity of the buccal bony wall was noted both in control and treatment groups on week 2 and 4 after the distraction (Fig 49, 50).

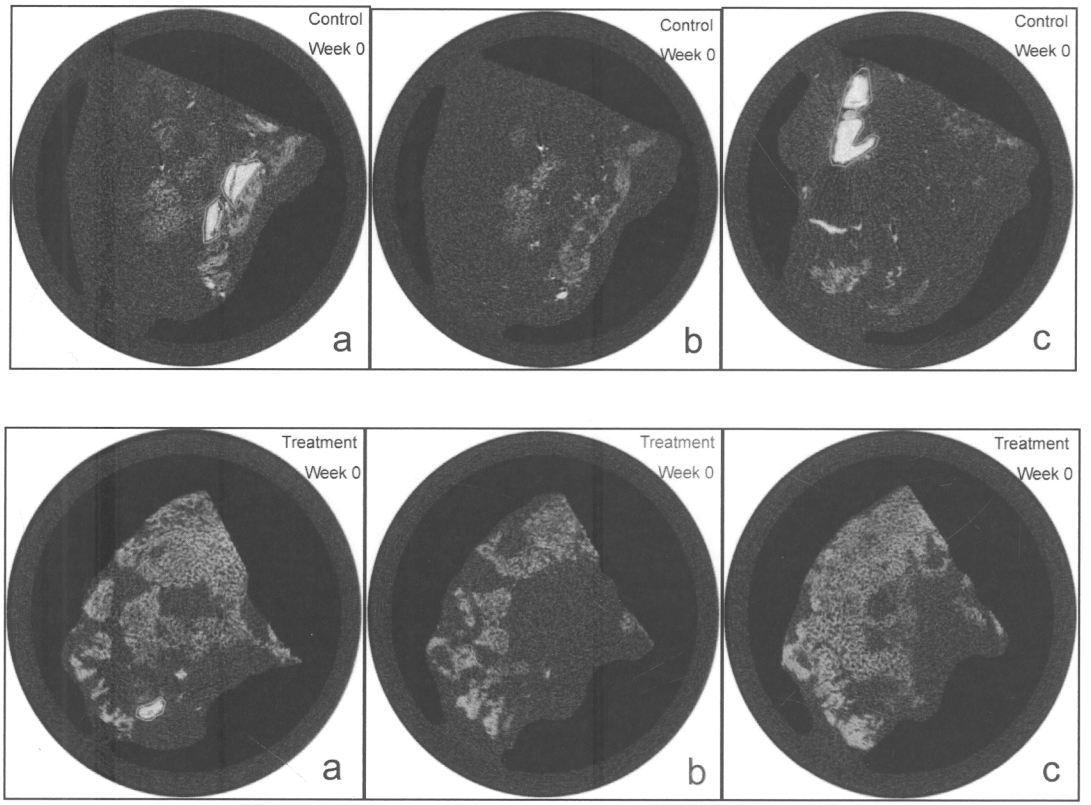


Fig 48. Micro-CT image on week 0 after the distraction

Upper images in control, lower images in treatment.

Three sections were showed, **(a)** and **(c)** images showed the section near the original bone, the **(b)** image showed the middle section of the distraction gap.

The green line showed the dental tissue

Note the treatment group showed more radiopacity in each section.

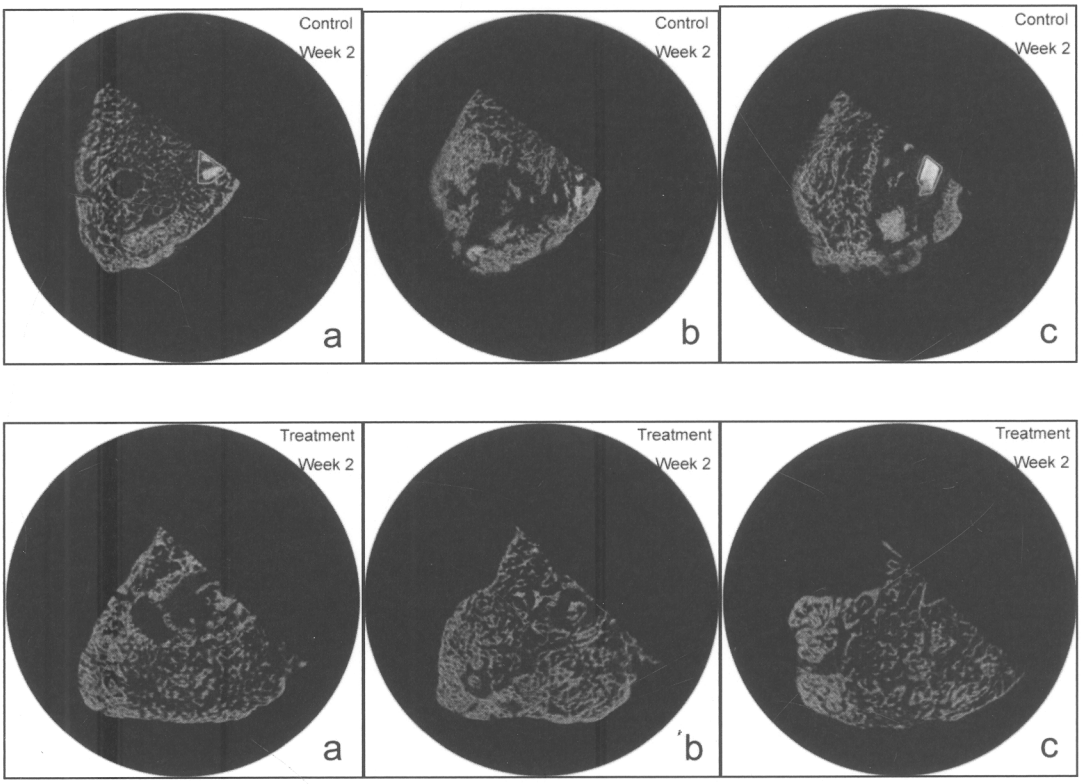


Fig 49. Micro-CT image on week 2 after the distraction

Upper images in control, lower images in treatment.

Three sections were showed, **(a)** and **(c)** images showed the section near the original bone, the **(b)** image showed the middle section of the distraction gap.

The green line showed the dental tissue

Note it was hardy to distinguish the difference in radiopacity or radiolucency between the two groups. However, treatment group showed slightly denser.

In both groups, bony walls continuously presented at both sites (regardless lingual or buccal) covering the internal bony tissue.

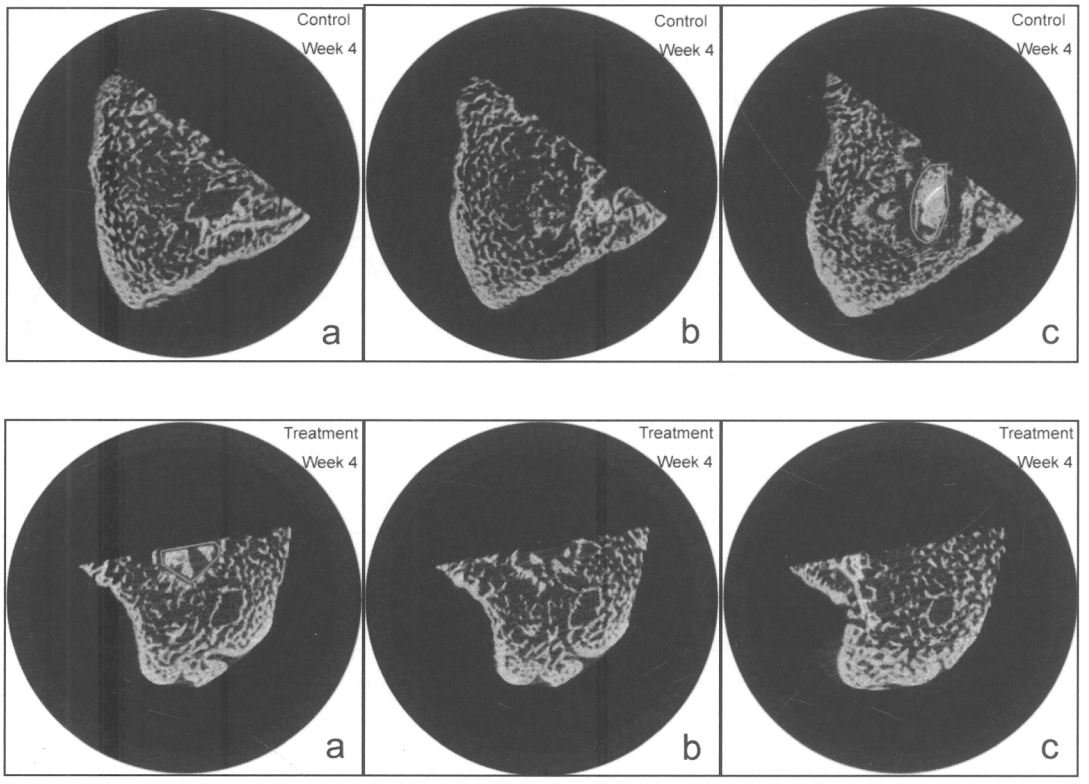


Fig 50. Micro-CT image on week 4 after the distraction

Upper images in control, lower images in treatment.

Three sections were showed, **(a)** and **(c)** images showed the section near the original bone, the **(b)** image showed the middle section of the distraction gap.

The green line showed the dental tissue

It was impossible to recognize the difference between the two groups

In both groups, bony walls continuously presented at both sites (regardless lingual or buccal) covering the internal bony tissue.

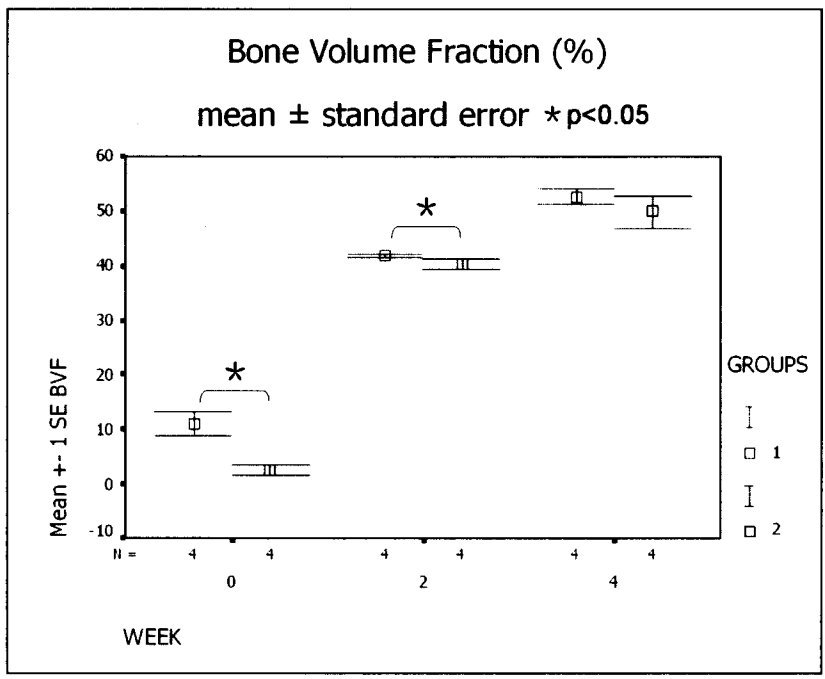
The bone volume fraction (%, bone volume/total volume, BVF) is the ratio of the bone volume to the specimen's volume. BVF of each slice was calculated. Because one specimen had dozens of slices, the final data of each specimen was the mean of all slices conducted automatically by the software (Software Revision 3.1, Scanco Medical AG). There were significant differences between the control and treatment groups on week 0 and 2 after the distraction with the *p* value of 0.021 and 0.043 respectively (Table 6). No difference had been found on week 4 after the distraction (Graph 2)

Table 6. Results of Bone Volume Fraction

Significances were on week 0 and 2 between the treatment and control groups

(Mann-Whitney Test)

Bone Volume Fraction (%, mean ± standard error)			
	Week 0	Week 2	Week 4
Treatment group	11.0903±2.12028	41.7985±0.21952	52.5470±1.45824
Control group	2.5090±0.91616	40.2365±1.02770	49.8905±2.99146
p Value	0.021	0.043	0.564
The level of significance was set at $p<0.05$			



Graph 2. Bone Volume Fraction of Micro-CT Radiography

Significances were on week 0 and 2 after the distraction between the treatment and control groups

(Mann-Whitney Test, the level of significance was set at $p<0.05$)

3.5 Microhardness evaluation

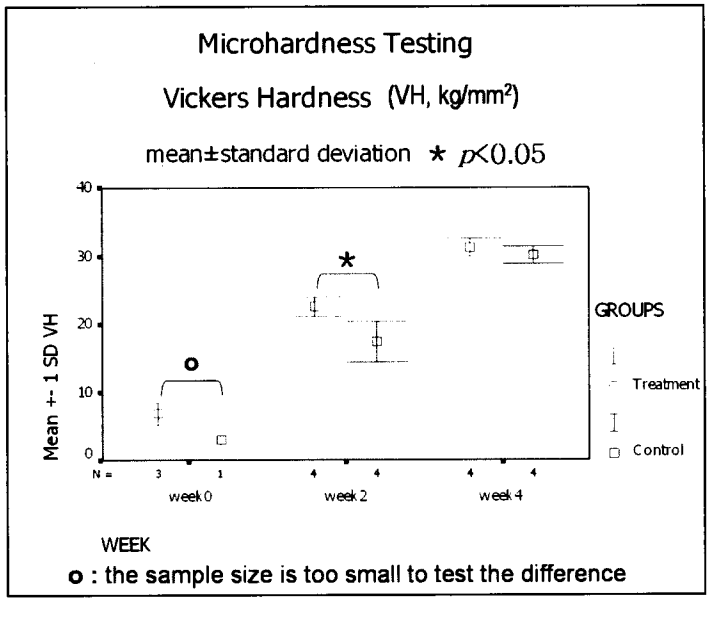
The data yielded from the microhardness test represented the surface hardness on the distraction gap. In both groups, the values were rising steadily over time. But on week 0 after the distraction (at the completion of distraction), three and one specimens were failed to be obtained the data in the control and treatment groups respectively. The reason is that they were too soft to be studied by the microhardness testing. Although the data gained on week 0 after the distraction are too few to be tested the significance, the treatment group still showed a remarkable result. Three specimens were hard enough to be tested compared only one specimen could be tested in control group. Moreover, those three specimens showed a dramatically higher hardness than that only one specimen showed in the control group (Table 7). In the later time points, there was a significant difference between the control and treatment groups on week 2 after the distraction. The Vickers hardness value was 17.455±3.587 kg/mm^2 (mean ± standard deviation) in control, while the value was 22.490±2.955 kg/mm^2 in treatment (p=0.021), on week 2 after the distraction. However, the difference between the control and treatment was not significant at 4 weeks after the distraction (Graph 3).

Table 7. Results of Microhardness Test

On week 0 after the distraction, the sample size was too small to test the significance

On week 2 after the distraction, the significance was detected between the control and treatment groups. (Mann-Whitney Test)

Microhardness Testing (Vickers hardness) (mean ± standard deviation)			
	Week 0	Week 2	Week 4
Treatment group	6.820±1.778(n=3)	22.490±2.955(n=4)	31.215±2.931(n=4)
Control group	3.020±0.750(n=1)	17.455±3.587(n=4)	30.165±2.878(n=4)
p Value		0.021	0.248
The level of significance was set at $p<0.05$			



Graph 3. Vickers Hardness of Microhardness Testing

The significance was on week 2 after the distraction between the treatment and control groups (Mann-Whitney Test, the level of significance was set at $p<0.05$)

3.6 Histological evaluation

At the completion of distraction, in both groups, fibrovascular tissue mainly filled in the distraction gap, and primary bony trabeculae were only present near to the original bone ends and beneath the periosteum (Fig 51, 53). Thus three different tissue zones were present in the distraction gap (Fig 52, 54). Both fibrous tissue and newly formed trabeculae oriented parallel to the direction of distraction. The mesenchymal cells with osteoblastic morphology (large basophilic cytoplasm, prominent Golgi region, and eccentrically place nucleus) were found in the region of the periosteum and around the trabeculae (Fig 51). Two specimen of each group showed the cartilage islands in the distraction gap (Fig 53). The treatment group showed more trabeculae near the original bone ends and near the middle of the distraction gap (Fig 54).

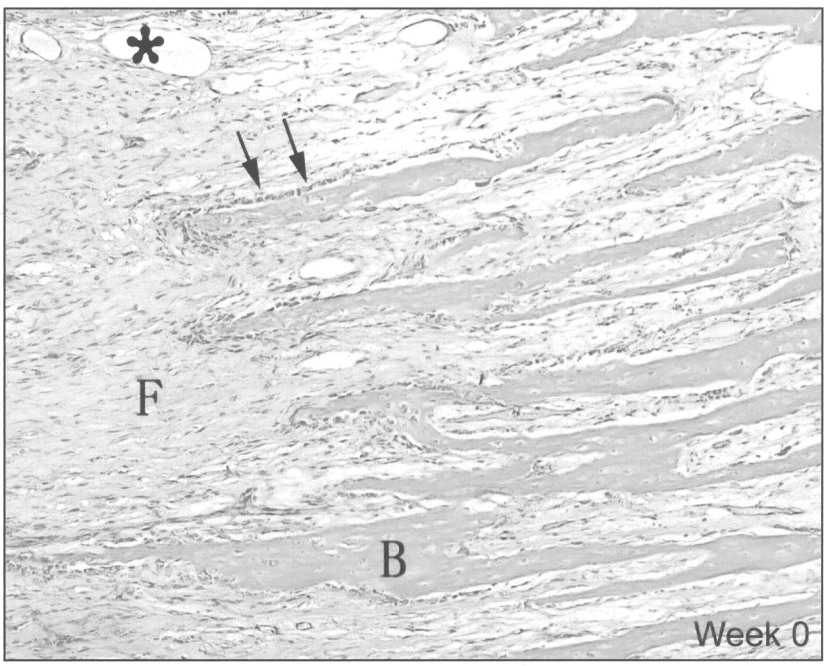


Fig 51. Fibrovascular tissue and newly formed trabeculae oriented parallel to the direction of distraction

*: small blood vessel. F: fibrous tissue (collagen boundless). B: newly formed bony trabeculae Arrow: Mesenchymal cells with osteoblastic morphology. (H&E stain, original magnification × 10)

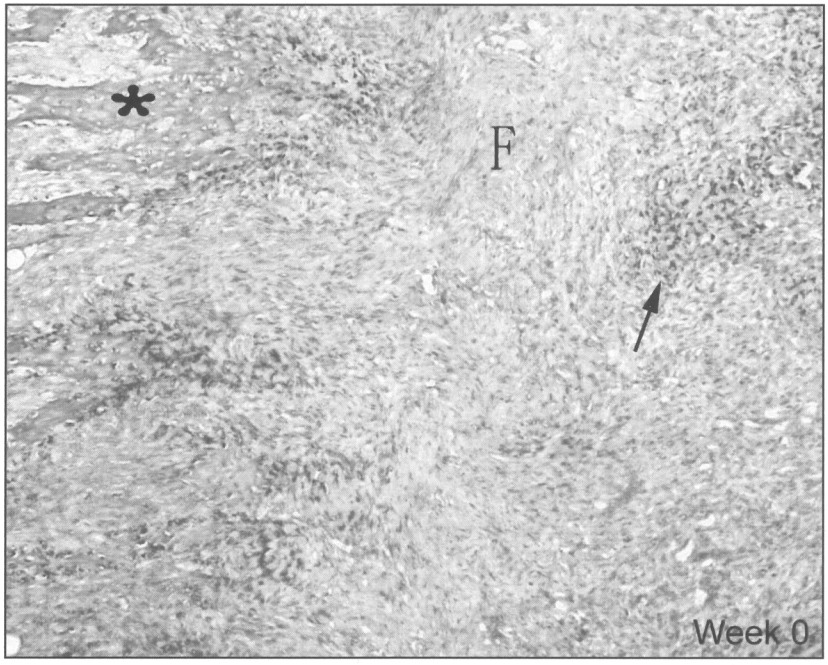


Fig 52. Fibrous tissue in the middle of distraction gap. Note the direction of the collagen came from two sites and met in the middle

*: newly formed bony trabeculae (woven bone). F: fibrous tissue (collagen boundless)

Arrow: Mesenchymal cells with osteoblastic morphology. (H&E stain, original magnification × 5)

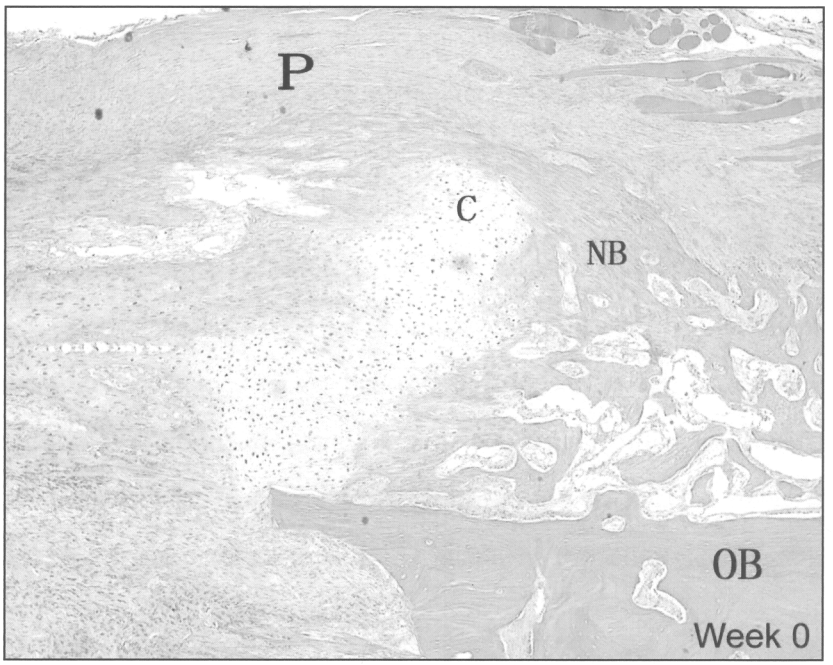


Fig 53. A cartilage island presented beneath the periosteum.

P: periosteum. C: cartilage island. NB: new bone. OB: original bone (H&E stain, original magnification × 5)

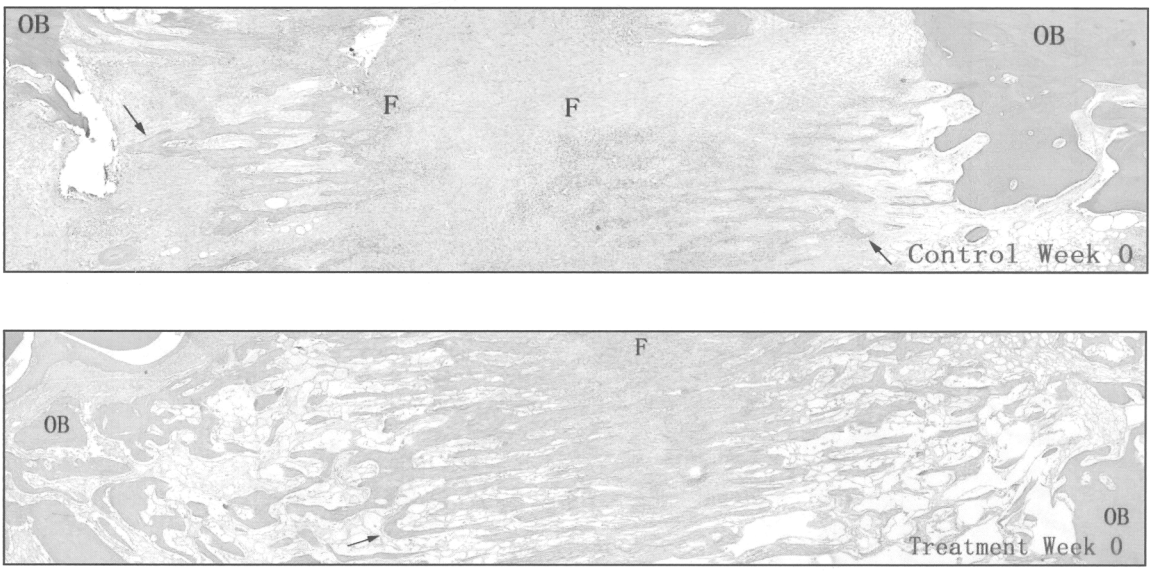


Fig 54. (upper picture, control group) shows wider fibrous tissue zone in the distraction gap, less bony trabeculae presented near the original bone

(lower picture, treatment group) shows trabeculae nearly bridged the whole distraction gap, more trabecular presented. F: fibrous tissue. OB: original bone. Arrow: bony trabeculae(H&E stain, original magnification × 5)

At 2 weeks after the distraction, in both groups, fibrous connective tissue dramatically reduced, but some remained in the middle of distraction gap (Fig 55, 56, 57, 59). We found 3 specimen and 1 specimen with incomplete cortical bone formation in the control and treatment groups (each group 4 rabbits) respectively (Fig 56, 57). Bone trabeculae significantly increased in terms of amount and location that became more calcified and extended to the middle of distraction gap. Numerous osteoblastic cells were embedded in the bony trabeculae. Meanwhile, the trabeculae started remodeling (Fig 55). In each group, 2 specimens showed the cartilage islands in the distraction gap (Fig 58). No visible difference could be found in comparison of bone trabeculae between the two groups by eye observation. However, the control group presented slightly more fibrous tissue in the middle of distraction gap (Fig 59).

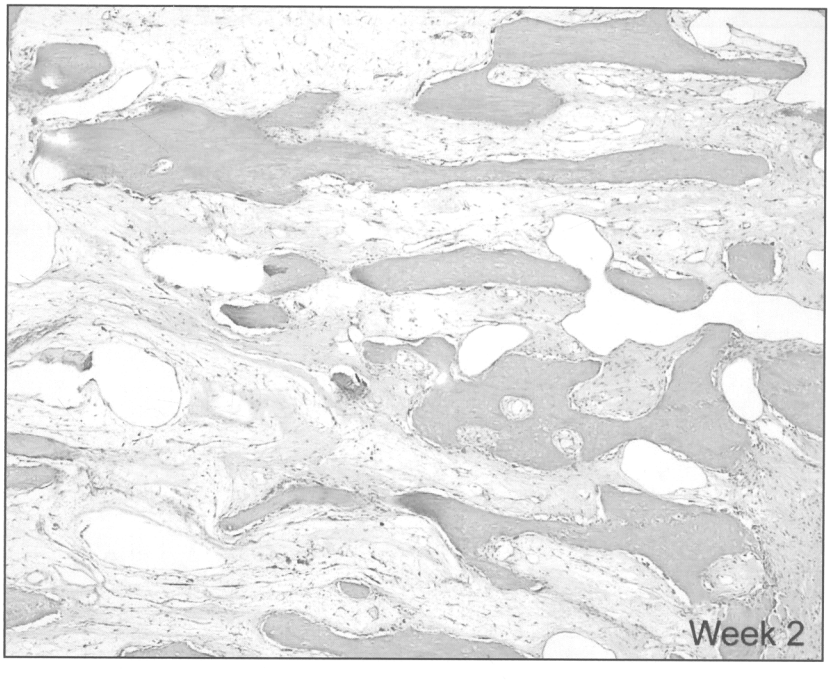


Fig 55. More calcified bone trabeculae. (H&E stain, original magnification × 5)

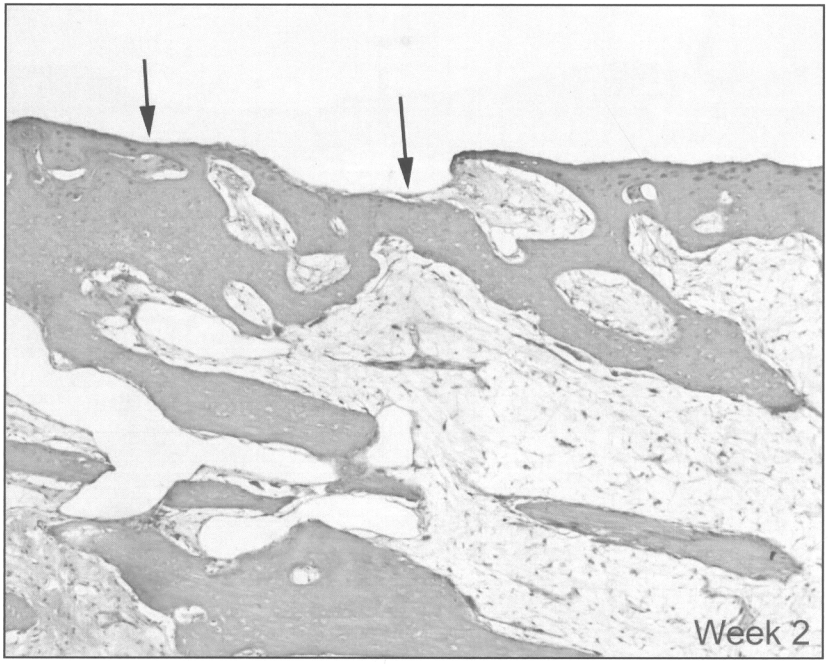


Fig 56. Complete coitical bone formation covering the distraction gap

Arrow: newly formed cortical bone (H&E stain, original magnification × 5)

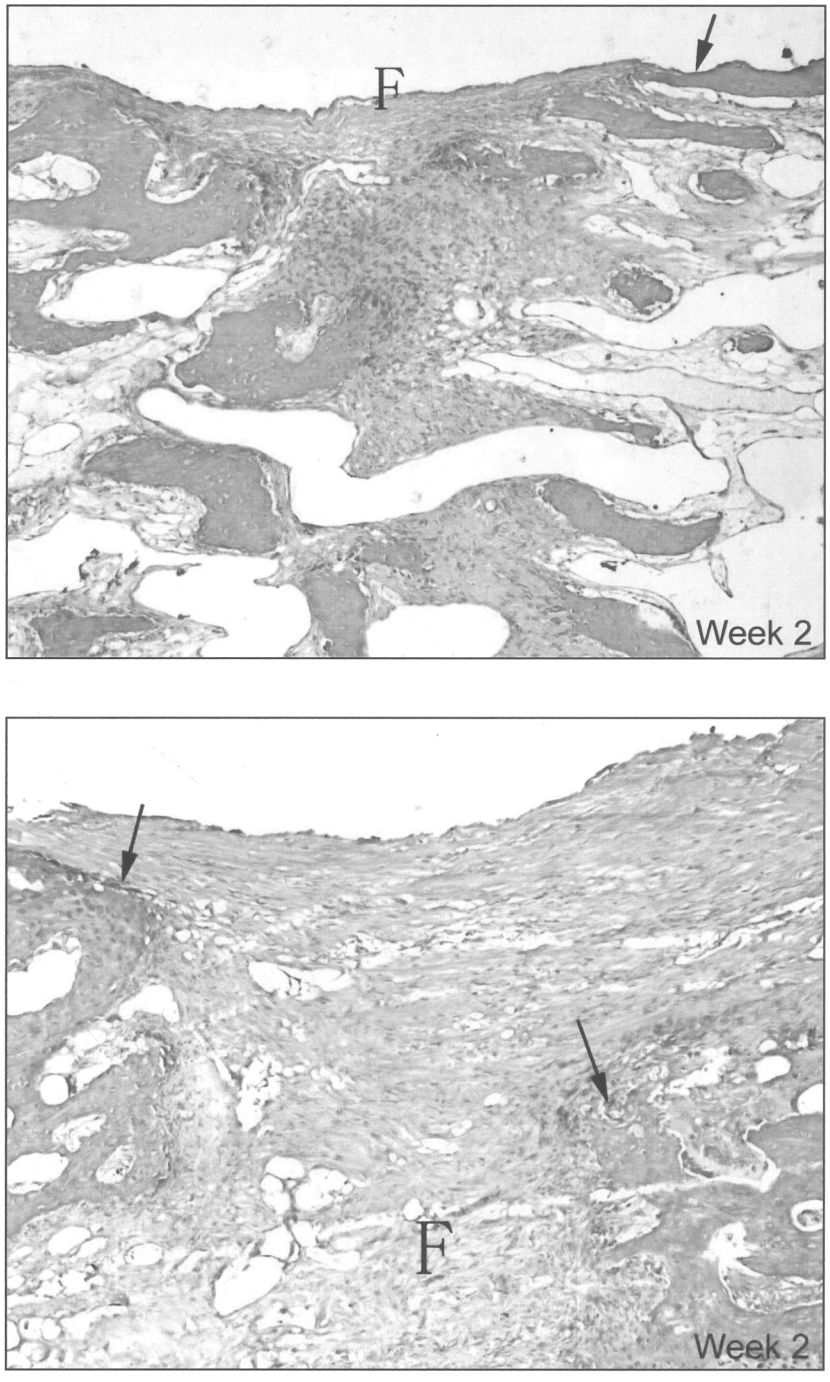


Fig 57. Two specimens showed incomplete coitical bone formation in the middle of distraction gap F: fribrous tissue. Arrow: newly formed cortical bone (H&E stain, original magnification × 5)

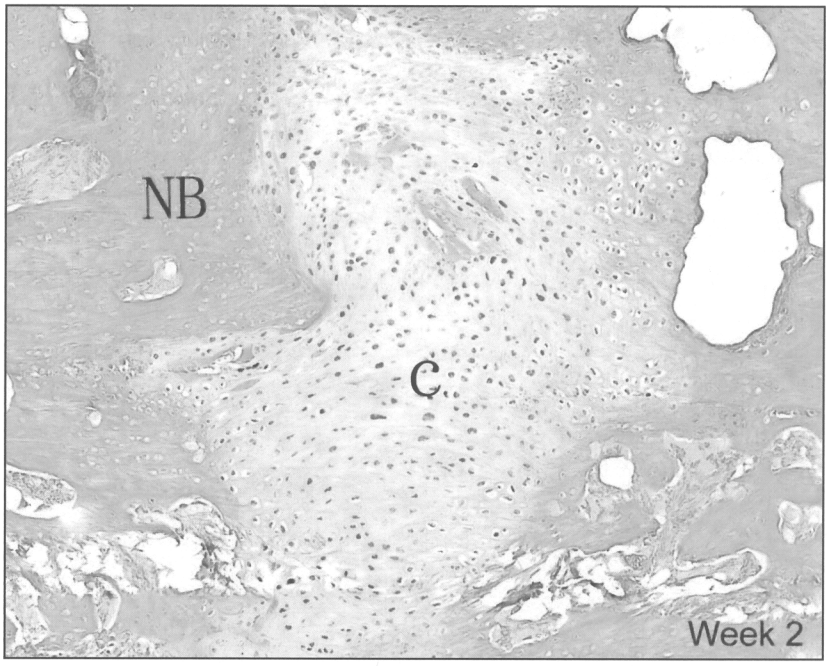


Fig 58. A cartilage island.

C: cartilage island. NB: new bone (H&E stain, original magnification × 10)

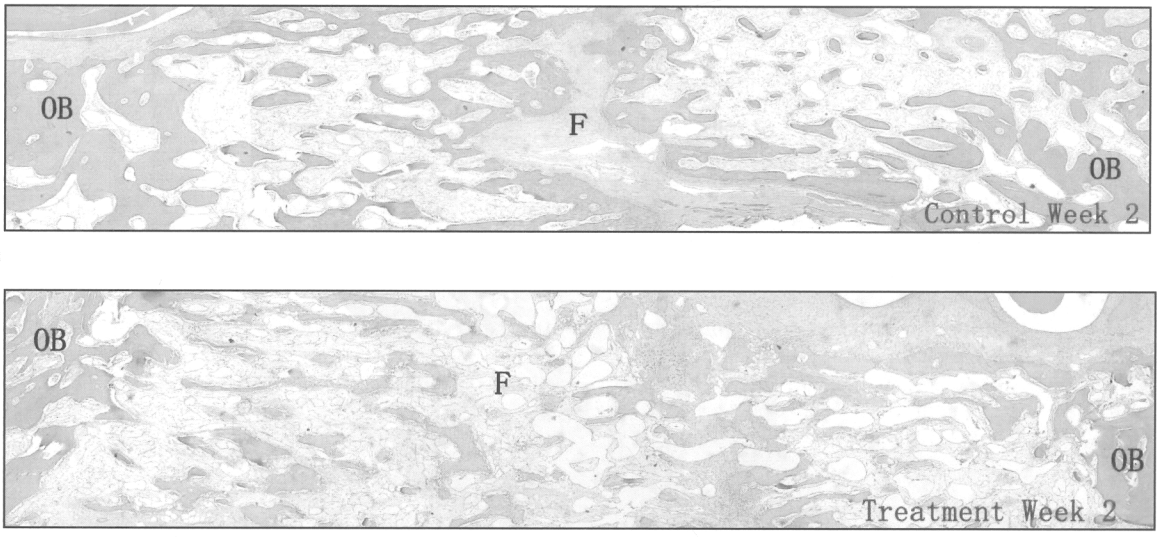


Fig 59. (upper picture, control group) shows slightly wider and more obvious fibrous tissue zone in the distraction gap.

(lower picture, treatment group) shows less fibrous tissue

However, similar amount of bone trabeculae between the two groups by eye observation

F: fibrous tissue. OB: original bone. Arrow: bony trabeculae (H&E stain, original magnification × 5)

At 4 weeks after the distraction, in both groups, there was no obvious fibrous tissue showed in the distraction gap. Thick and continuous cortical bony wall, clear bone marrow cavity and medullary tissue were present (Fig 60). More mature bone trabeculae and osteons were found in the whole distraction gap (Fig 61). No cartilage was found. No visible difference was found in terms of area of bone trabeculae (Fig 62).

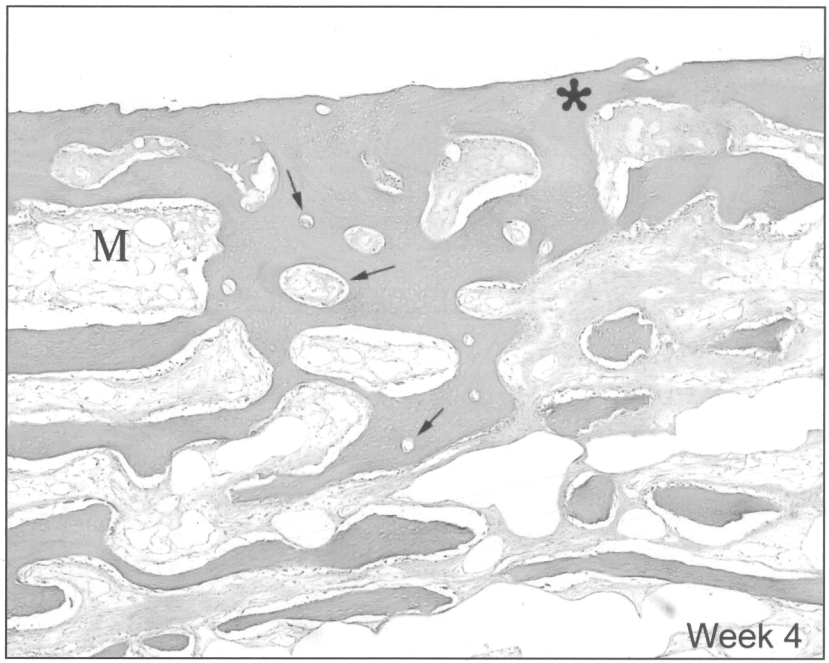


Fig 60. Thick cortical bone covering the distraction gap and more mature bone trabeculae. *: cortical bone. M: medullary cavity and tissue. Arrow: osteon (H&E stain, original magnification × 5)

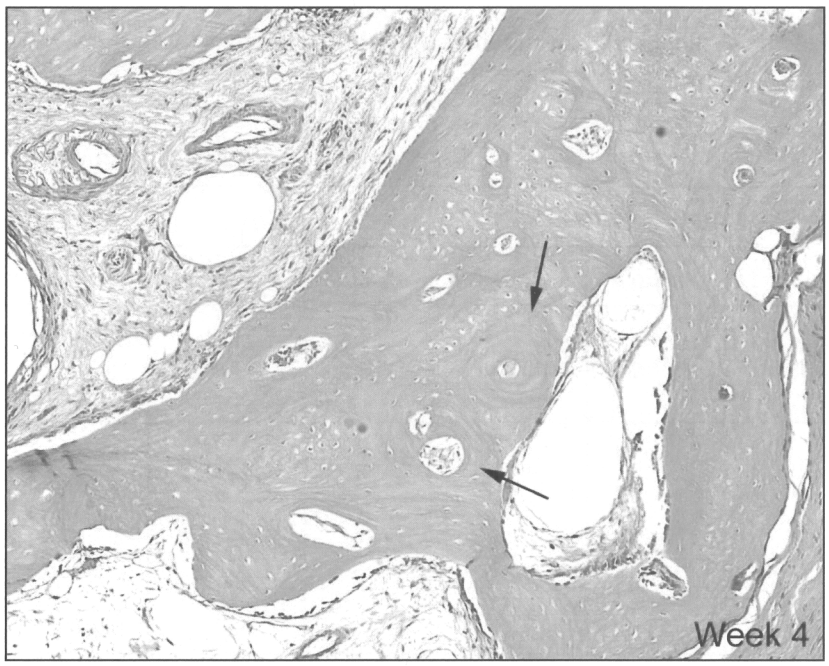


Fig 61. Highly remodeling of bone trabeculae

Arrow: osteon (H&E stain, original magnification × 10)

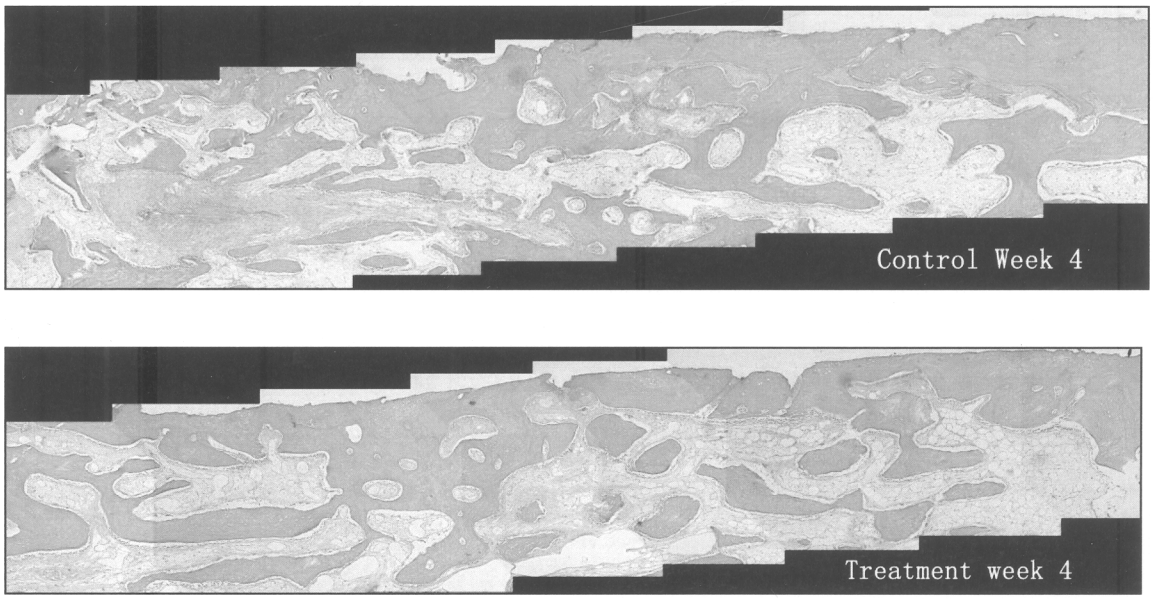


Fig. 62 (upper picture, control group) (lower picture, treatment group) shows the cortical region of the distraction gap, hard to distinguish the original bone and new bone.

In both groups, it was hard to distinguish the original bone and new bone.

Advanced calcification and remodeling were present. No obvious difference was found between the groups (H&E stain, original magnification × 5)

A brief summary of histological findings was showed in the table 8.

Table 8. A summary of Histological Findings

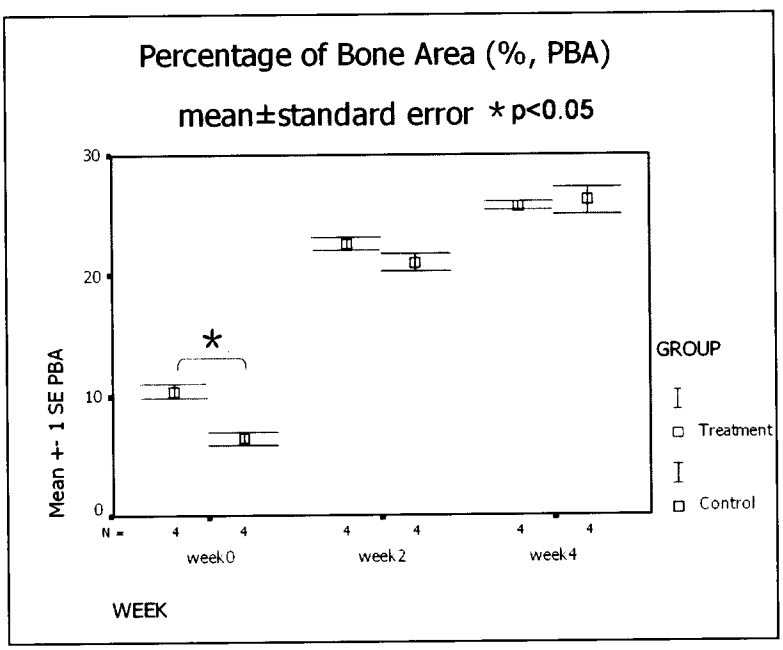
Time points		**Week 0**		**Week 2**		**Week 4**	
Each group 4 rabbits		Treatment	Control	Treatment	Control	Treatment	Control
Findings	Bone formation	More	Less	Similar	Similar	Similar	Similar
	Complete cortical bone formation	Non	Non	3	1	All	All
	Fibrous tissue	Less	More	1 presented in the middle	3 presented in the middle	Non	Non
	Cartilage	2	2	2	2	Non	Non

In histomorphometric analysis, on week 0 and 2 after the distraction, treatment groups showed more bone area occupied in the distraction gap compared with the control. However, on week 2 after the distraction, the difference did not reach to significant level (P=0.149) (Mann-Whitney Test, the level of significance was set at p<0.05). On week 4 after the distraction, the treatment group even showed slightly less percentage of bone area (Table 9 and Graph 4).

Table 9. Result of Percentage of Bone Area

Percentage of Bone Area (%, mean ± standard error)			
	Week 0	Week 2	Week 4
Treatment group	10.4242±0.6617	22.6453±0.5593	25.7422±0.3674
Control group	6.4532±0.5745	21.0259±0.7452	26.1878±1.1337
p Value	0.021	0.149	0.773
The level of significance was set at $p<0.05$			

(Mann-Whitney Test, the level of significance was set at $p<0.05$)



Graph 4. Percentage of Bone Area

The significance was on week 0 after the distraction between the treatment and control groups

(Mann-Whitney Test, the level of significance was set at $p<0.05$)

4. Discussion

Acceleration of bone healing has long been an interesting and challenging topic in medical field. As a special form of bone healing, distraction osteogenesis becomes more and more popular in recent decades. Obviously it is very meaningful to achieve acceleration of bone healing either in treatment of fracture or distraction osteogenesis.

In the present study, we had used the rabbit model to evaluate the effect of low intensity pulsed ultrasound on new bone formation during mandibular distraction osteogenesis. Determining the appropriate animal model is essential for the success of the study. The outmost concerns in choosing the appropriate animal model should depend on the level of hypothesis testing and the expected extrapolation of results to the human condition[126, 127]. Siegel and Mooney[126] suggested the three levels of appropriate animal choices. 1) Generic animal models such as the rat and rabbit are appropriate models to evaluate the biology of the distraction osteogenesis or histiogenesis and functional cranio-facial skeletal adaptations after cranio-facial distraction osteogenesis, because as mammals they share equal primitive bone and soft tissue responses as humans[36]. Hence, the rat[59] and rabbit[62, 64, 128, 129] are the most appropriate animal models to elucidate the molecular mechanisms that mediate cranio-facial distraction osteogenesis, especially in the mandible. Moreover, an interesting study from Norris S. A. et al.[130] claimed that rabbits showed patterns of bone accretion and peak bone mass profiles similar to humans and thus might serve as a viable model for human bone physiology. 2) Phylogenetically closer animal models, such as the cat, dog, minipig, sheep and non-human primates are supposed to possess an anatomic and developmental cranio-facial characteristic that is closer to humans compared to generic animal models. Theses larger models can help in developing new applications of distraction osteogenesis, new distraction devices and new surgical techniques[36]. 3) "Fitting" the appropriate animal model to specific regional craniofacial growth patterns or anatomic conditions of interest, such as chimpanzee[126].

In the present study, the level of hypothesis testing was focused on bone biology rather than testing the new distractor or new surgical techniques that required the human-like anatomic animal models such as phylogenetically closer or "fitting" animal models. Therefore, the generic animal model such as the rabbit fulfilled the hypothesis test of the present study, and

also showed the highly expected extrapolation of the results to the human condition by means of sharing equal primitive bone and soft tissue responses as humans even in molecular level as above-mentioned[36, 130]. In addition, comparing with phylogenetically closer and "fitting" animal models, generic animal models are less expensive because of cost, housing (space saving) and care (cost-effective to feed), and easy handling (no sedation required during the activating the distractor)[36], in the present study, even no sedation during applying the ultrasound treatment. The rabbit mandibular distraction model represents the best compromise between cost and size of the mandible[131]. In the literature, the rabbit mandibular distraction model has been studies and proved reliable by numerous researches[36, 62, 64, 128, 129, 132-134]. There were three very first studies set the rabbit mandibular distraction model in 1994[132-134]. Up to 2001, 12.9% of the total mandibular distraction models were performed on rabbits among other mandibular distraction animal models[36], this number has been increasing in recent years. Therefore based on the level of hypothesis testing and the expected extrapolation of the results to the human condition in the present study, while concerning the advantages of the rabbit model[36, 131] and hard data supported by previous researches[36, 62, 64, 128, 129, 132-134], we chose the rabbit mandibular distraction model for the present study.

The surgical techniques used in the present study to achieve rabbit mandibular distraction osteogenesis were first described by Komuro Y. et al.[132] and Califano L. et al.[134]. The osteotomy line was just between the mental foramen and the first premolar. Hence, it was easy to perform the consistent osteotomy lines in different rabbits owing to those stable anatomic landmarks.

Successful intervention studies require a stable and accurate characterization of the animal model. The latency period[135-137], distraction rate and rhythm[128, 138-140] had been demonstrated as important factors influenced the results. In Ilizarov's original works[27, 28], the latency period was considered as the healing time for soft tissue particularly periosteum. We also considered the latency period as the time for animals to adapt to the distraction device and regain masticatory function. According to Swennen et al.[36], among the rabbit mandibular distraction researches, the latency period of 3-5 days was reported in 87.1%. Base on those previous studies[36, 135, 136], 3-5 days for the latency period was recommended. Distraction rate and rhythm had been studied extensively[27, 28, 128, 138-140], most researchers[135, 138, 140] agreed that a rate faster than 1mm/day

resulted in unreliable bone formation, whereas a rate of less than 1mm/day cause premature ossification. Although a higher rhythm recommended by Ilizarov[28] and demonstrated by Zheng et al.[139], twice daily activation was performed in most of the animal studies and clinical practices. For setting a stable and accurate rabbit mandibular distraction model, we chose the most accepted parameters for rabbit mandibular distraction osteogenesis[36], 3 days for the latency period and 1mm/day, twice daily for the distraction rate and rhythm.

The distractor plays another important role in distraction osteogenesis. However, the distractors used in the studies are diverse. Most of the studies used custom-made distractors[128, 131, 134, 139], others used commercial distractors[71, 132, 141]. The custom-made distractors used in the studies are unduplicated for other research groups. For the commercial distractors, they are also variable from company to company, and they are designed for the human mandible rather than the rabbit mandible which is small, thin and fragile. Moreover they are too expensive to be widely used for a large number of rabbits. We designed our own external distractor remodeled from the orthodontic palatal expansion screw (Hyrax^{R}). The strength of this distractor was tested by a universal testing machine and direct measurement by digital caliper (Autograph AG-100 KNG, Kyoto, Japan). The result proved satisfactory strength of the device with minimal relapse of only 0.5mm on average. When fixed on the rabbit mandible by 4 micro-screws, the distractor provided excellent stability and strength to resistant the scratching or being knocked against the cage wall or floor by any movement from the rabbit. So it eliminated the need for the plastic facial shield which would disturb the rabbit. The small volume (14×10×3mm) of distractor emerged out of the skin may also play a favorable role in minimizing the comfortlessness to the rabbits. No breakage or dislodgement of the distractors occurred. All the rabbits tolerated with the distractors very well.

Easy handling is one of remarkable merit of the rabbit. However, in previous ultrasound-used studies, researchers had to sedate rabbits during applying LIPUS by injections of certain solutions such as Domitor[119], Ketamine/Xylazine[72, 122], Acepromazine/Ketamine[105, 120, 124] etc. Although there has not been a report correlating the narcotics with the bone formation, the narcotics do somewhat disturb the normal activities of rabbits from physiology to psychology. For instance, when Ketamine (35 mg/kg) was applied for sedation of the rabbit, the anaesthetic effects of Ketamine lasted at least 2 hours and much longer time for regaining full consciousness. When the Ketamine had to be administrated daily for facilitating the application of ultrasound to the

rabbits, we think it would disturb the normal activities of rabbits such as eating and resting which were considered important for recovery or bone healing of the rabbits. Further, such disturbing would impact to the results in the ultrasound treatment groups, if no sham treatments (with narcotic injections, but no ultrasound treatments) to the control groups. However, some studies did not treat the control groups by the sham treatments[72, 119, 122], including the present study. Thus we found the way to apply the ultrasound on the mandible of rabbits without narcotics. The rabbit was trapped in a special wood box, then the ultrasound transducer head and the rabbit head was held by each hand. During the ultrasound treatment, most rabbits were very calming, no floundering. Even occasionally floundering, it was just easy to handle. Especially, after about 3 times being treated by the ultrasound, most rabbits seemed acceptable to this treatment manner. So we considered that it was a proper way to apply the ultrasound without narcotics injections which disturbed the rabbits too much, and also it fulfilled the ethical demands.

There were three time points for sacrifice of animals in the present study. The reasons for choosing those time points were based on the previous rabbit mandibular distraction osteogenesis studies and LIPUS-related studies. Same to all kinds of tissue healing, the bone formation process in distraction osteogenesis especially in the consolidation period reveals highly heterogeneous in spatial and temporal dimensions[142]. According to this nature of distraction osteogenesis, there is a definite need to monitor the bone formation process of distraction osteogenesis in different time points rather than a single check at the final stage of distraction osteogenesis when the new bone was formed completely. Checking at different time points was even more important for the present study aiming to dynamically evaluate the effect of LIPUS on the new bone formation during distraction osteogenesis. Apparently, the results yielded at each time point would indicate the efficacy of LIPUS in the different time intervals. Hence we would know the most effective time to use the LIPUS by checking different time points. 1) Many studies applied LIPUS treatment after completion of the distraction[72-74, 105, 117-120], whereas the others started the LIPUS treatment at the first day of the distraction[75, 115, 121, 122, 124]. The positive effect by early application of LIPUS has also been advocated by some studies[72, 113, 116, 117, 120, 122]. Whether the LIPUS has the positive effect during the distraction phase of distraction osteogenesis has become an interesting issue. Therefore we started the LIPUS treatment at the first day of distraction and chose the first time point of sacrifice at the completion of distraction. The effect of LIPUS during

the distraction phase would be revealed. 2) Two weeks after distraction was an important landmark point presenting what was happening during the consolidation period. The new bone formation was approaching to the peak, different ossification zones presented in the distraction gap. It was also the golden time to evaluate the effect from LIPUS that had been applied for 10 days of the distraction period and 2 weeks of the consolidation period. Thus the second time point of sacrifice was chosen at 2 weeks after distraction. 3) Most studies chose 4 weeks of consolidation period as the longest time interval to evaluate the effect from the interventional methods comparing to the controls[36, 67, 72, 119, 122, 140]. It is believed that the end of new bone formation in the animal distraction models was proximately 4 weeks after distraction[37, 132, 143]. A study from Cope et al.[37] showed the mineralization in the distraction gap gradually increase from the end of distraction and peak between 2 to 4 weeks after distraction detected by mineral apposition rate. This implied the new bone was formed mainly during 4 weeks after distraction rather than any other longer period. Hence the longest time interval for 4 weeks of consolidation period with LIPUS treatment would be long enough to assess the effect of LIPUS on the new bone formation during distraction osteogenesis, which was the purpose of the present study.

In the current study, plain radiography, Micro-CT, microhardness and histological examinations served as assessment methods.

The plain radiography is easy to perform, cheap, quick and non-destructive. It has become a routine assessment for most hard tissue studies. The results obtained from the plain radiograph were coincided with results of Micro-CT, which again proved its usefulness. However, the shortcomings of plain radiograph are not only its 2-D overlapped image but also poor resolution and insensitivity[144]. It has long been suggested that the loss of bone density is not apparent on a plain X-ray film until approximately 40% of the bone is lost[144-146]. Therefore, Micro-CT had been used for further study of the specimens in the present study. Micro-CT radiograph has been proven useful in a wide variety of applications by providing high-resolution images and quantitative parameters of 3-D image[147]. Micro-CT is particularly well-suited for imaging small animal models[147]. In the present study, Micro-CT provided a precise image of internal micro-structure of regenerated bone in the distraction gap. We observed the continuity of bony wall covering the distraction gap on week 2 and 4 after the distraction in both groups. Furthermore, a quantitative data was also conducted by Micro-CT. Bone volume fraction (BVF)

is ordinarily measured by histomorphometry from bone biopsies, it is the space occupied by mineral tissue relative to the specimen's volume. Tissue density was taken as the ratio between the mass of specimen and the space occupied by mineral tissue. Bone volume fraction is also highly associated with the mechanical competence of trabecular bone[148]. Therefore, higher BVF presented in the LIPUS group on week 0 and 2 after the distraction indicated LIPUS accelerated bone formation significantly. These findings are consistent with the study performed by Eberson et al.[74] who also used the Micro-CT in evaluation of LIPUS in rat distraction osteogenesis. In addition, regarding no torsional or bending test had been taken in the present study, the results from plain radiography and Micro-CT strongly correlated to the mechanical properties of the specimens that has been well proved[148-151].

Hardness refers to various properties of matter in the solid phase that gives it high resistance to various kinds of shape change when force is applied. Microhardness testing of metals, ceramics, and composites is useful for a variety of applications for which 'macro' hardness measurements are unsuitable: testing small materials, measuring individual microstructures within a larger matrix, or measuring the hardness gradients of a part along the cross section. Microhardness testing gives an allowable range of loads for testing with a diamond indenter; the resulting indentation is measured and converted to a hardness value. The actual indenters used are Vickers (more common; a square base diamond pyramid with an apical angle of 136°). The result for Vickers microhardness is reported in kg/mm^2 and is proportional to the load divided by the square of the diagonal of the indentation measured from the test. To out best knowledge, none of the studies related to distraction osteogenesis with LIPUS has used the microhardness test. Eberson et al.[74], Tis et al.[120], Sakurakichi et al.[122], Shimazaki et al.[117], Taylor et al.[124] etc used torsion test generated the torsional stiffness (N-mm/degree). Claes et al.[123] had used the compression tests for the whole distracted bony tissue and the bony tissue inside of the distraction gap generated the compression stiffness (N/mm). However, the stiffness is the resistance of an elastic body to deflection or deformation by an applied force, which is different from the hardness. Simply saying, the microhardness represents the surface property of the material, whereas the stiffness represents the whole property of the material. In the present study, the microhardness test showed the hardness of the newly formed bone that covered the distraction gap. From this unique parameter, we knew the bone was increasingly covering the distraction gap and how hard it is.

More important is that the treatment group represented harder bone or may also be considered as earlier bone coverage than the control group on week 0 and 2 after the distraction. In addition, the valuable data from the microhardness test not only indicated a positive effect from LIPUS at early stage of the distraction osteogenesis but also provided a possible evidence to interpret why LIPUS works at early stage of the distraction osteogenesis. (discussed in the later part)

Histological examination is the study of microscopic anatomy of cells and tissues. It is extremely useful to provide additional information in evaluation of hard tissue coupling with radiological methods. In the present study, on week 0 after the distraction, LIPUS showed significant acceleration of bone formation in terms of percentage of bone area and eye. On week 2 after the distraction, 4 specimens (3 in treatment, 1 in control) showed that bony trabeculae (or cortical bone formation) completely covered the distraction gap at both lingual and buccal sites. More bone area was also found in treatment group, but it failed to reach the statistical significance. We think it may be due to the variability of histology of distraction osteogenesis. The heterogeneity of the healing processes of distraction osteogenesis was revealed in the histological examination. Different areas showed different histological morphologies of new bone formation. This was indicated by the data of percentage of bone area, which had a large variance. Moreover, the sampling error would also play a role in the final result of percentage of bone area. The histological healing processes of distraction osteogenesis were well studied by many researchers[36]. The majority of research groups agree that the process of craniofacial bone regeneration is primarily intramembranous ossification, because no cartilage is seen in the distraction gap. However, cartilage was found in many studies[36] as well as the present study. We found on week 0 and 2 after the distraction (2 treatment and 2 control groups), in each group (4 rabbits), two specimens presented the cartilage islands, and no cartilage was present on week 4 after the distraction in both groups. According to our findings, we agree the bone formation during craniofacial distraction osteogenesis predominantly via the intramembranous ossification. Cartilage formation is considered due to decreased oxygen tension[37]. Moreover, it is suggested that LIPUS stimulates cartilage formation[75, 94, 105, 122, 152]. However, the controversial remains whether LIPUS promotes intramembranous or endochondral ossification or both. At least, our results do not support the promotion of endochondral ossification from LIPUS because the cartilage was present equally in both groups. We may consider LIPUS promotes both

intramembranous and endochondral ossification rather than shift one form to another. The mechanical factor such as fixation methods (rigid or less-than-rigid), distractors, distraction protocol, early function and physiological factors such as blood supply may play more important roles in determination of types of bone formation. LIPUS may only play a role to stimulate bone formation rather than to determine or change the types of bone formation. However, this ideal needs for further study.

To our best knowledge, there was only one animal study conducted by El-Bialy et al.[119] to evaluate the effect of LIPUS on mandibular distraction osteogenesis. The positive effect of LIPUS on mandibular distraction osteogenesis was confirmed both in El-Bialy et al.'s study and our study. However, there are some differences between the two studies from the methods to the results (Table 10).

Table 10. The Differences between El-Bialy et al.'s Study and the Present Study

		El-Bialy et al.'s study[119]	The present study
Methods	No. of rabbits	21	24
	Distraction rate	3mm/day, for 5 days	1mm/day, for 10 days
	Location of the osteotomy	At anterior site of the mandible (Cutting through the incisor)	Between the premolar and the incisor (without cutting through the incisor)
	The side of mandible for distraction	Bilateral distraction	Unilateral distraction
	Time for ultrasound treatment	During consolidation period (4 weeks)	During distraction and consolidation period (10 days and 4 weeks)
	Sacrifice time	At week 4 after the distraction	At week 0, 2 and 4 after the distraction
Results	Effective time duration of LIPUS	During the 4 weeks of consolidation period	During the distraction period and 2 weeks of consolidation period.

Regardless the different methods, the major differences are the effective times of LIPUS between their study and our study. We did not find the significant differences between the control and treatment groups that LIPUS had been applied on the first day of the distraction until 4 weeks after the distraction. But we did find the remarkable differences on week 0 and 2 after the distraction between the two groups respectively. In animal studies of long bone distraction osteogenesis with LIPUS, our findings are consistent with Sakurakichi et al.[122], Chan et al.[72] and Shimazaki et al.[117] in conclusion of the effective timing of LIPUS, and similar to Machen et al.[105], Claes et al.[123], Mayr et al.[118], Eberson et al.[74] and Chan et al.[75] demonstrated the effect of LIPUS. However, opposite results exist. Schortinghuis and co-workers[115] did a double blind randomized clinical pilot trait to investigate the effect of LIPUS during alveolar distraction osteogenesis in human mandible. They did find the positive effect from LIPUS. Nevertheless, they explained their results: First, the physiological conditions of the elderly patients were very different from the health animal long bone distraction models. Second, the consolidation period might be too short and it was difficult to obtain the precise assessment of the distracted bony tissue serially. Third, medications of the patients could affect the results. In our opinion, the relatively small sample size (8 patients) and thick soft tissue coverage at chin area that attenuated the energy of LIPUS propagating into the distraction gap may also play roles in the final results of their study. Uglow et al.[121] and Taylor et al.[124] also did not detect the favorable effect of LIPUS in animal long bone distraction models. Tis et al.[120] reported a dilemmatic result. The LIPUS group had larger callus but similar density and mechanical properties compared with the control group. In all, based on searching the PubMed, there were 12 articles (Table 4) studied the effect of LIPUS on distraction osteogenesis in animals. Nine articles showed the positive effect of LIPUS on distraction osteogenesis, which were confirmed with our findings.

Again, our data showed the positive effect of LIPUS on week 0 and 2 after the distraction, no difference was found on week 4 after the distraction.

To date, with the advances made in molecular medicine and molecular biology, our knowledge of the cellular and molecular events taking place in the bone healing processes has vastly expanded[153]. However, it is still far from fully understanding the every consequence happened either in fracture healing or distraction osteogenesis at the cellular and molecular levels[154]. Unsurprisingly, we also do not know the exact mechanism of ultrasound effects on the

bone healing[103]. With the detective methods used in the current study which focused on the tissue level, we tried to provide the evidence of ultrasound effects in case of mandibular distraction rather than research the fundamental mechanism of ultrasound effects at the molecular level. Nevertheless, we still tried to interpret our results at the tissue level with a physical understanding of the nature of ultrasound and distraction osteogenesis. To our best knowledge, there was no research given an explanation for why LIPUS works effectively at the early stage and why no difference at 4 weeks after the distraction. We supposed that there were 2 reasons.

First reason is that LIPUS may not increase new bone formation at the later stage of distraction osteogenesis. To interpret this we should know a physical characteristic of the ultrasound first. Whatever the intensity is, LIPUS shares the same characteristics as other types of ultrasound. Refection is one of the characteristics of the ultrasound. Reflection of the ultrasound occurs when it hits a boundary between materials having different acoustic impedance. The reflection (echo) is the portion of a sound that is returned from the boundary (Fig 63).

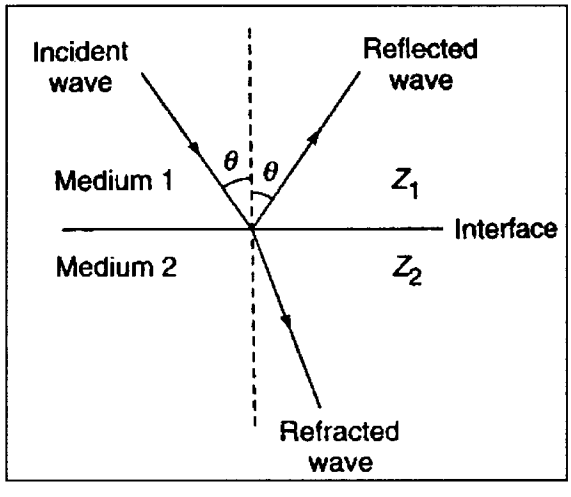


Fig 63. Reflection and refraction of ultrasound at an interface between two media having different acoustic impedances

Acoustic impedance is the property of tissue causing resistance to the propagation of ultrasound. Acoustic impedance is defined as Z = r c, where r is the tissue density and c is the propagation velocity of ultrasound in the tissue. Ultrasound propagation is dependent partly upon the particle mass (which determines the density of the tissue), partly upon the elastic forces binding the particles together (which determine the propagational speed of sound). A fraction of the

ultrasound is reflected whenever there is a change in acoustic impedance. The larger the change in acoustic impedance, the larger the fraction reflected. This means larger difference between the two media, more ultrasound energy reflected rather than through-penetration. For example, more ultrasound energy reflected at the interface between the muscle and the bone compared with the interface between the muscle and the skin. Second, we have learnt a great deal of using ultrasound as an assessment method to monitor the healing of mandibular distraction in animal[155] and human[156, 157], which would be very useful to interpret out results. Osteogenesis occurred in the distraction gap, with increasing mineralization, produces a change in the acoustic impedance of the tissue inside the distraction gap relative to that of the soft tissue surrounding the distraction gap[157] (Fig 64-66) , from Thurmuller et al. [155]).

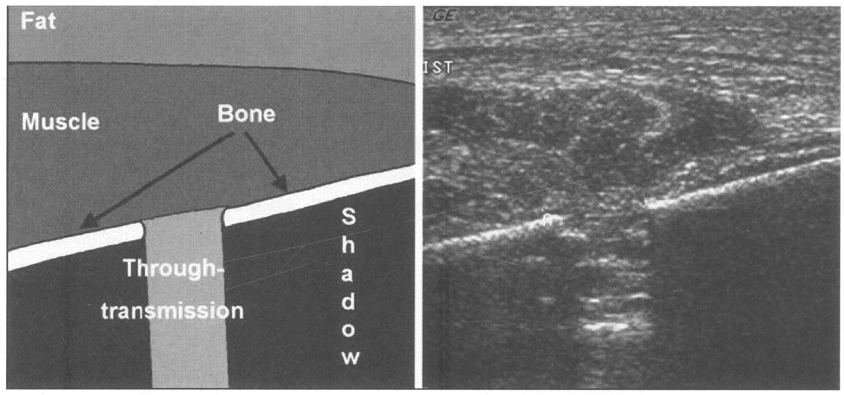


Fig 64. (Left) Diagram

(Right) An actual image of an animal showed complete through-transmission of the ultrasound waves, clear gap margins, and no echogenic material. (Picture from Thurmuller et al. [155])

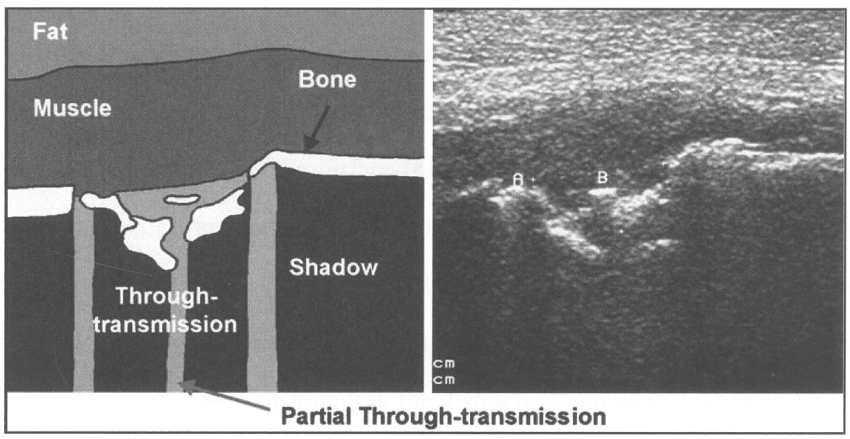


Fig 65. (Left) Diagram

(Right) An actual image of an animal showed partial through-transmission of the ultrasound waves, partially obscured gap margins, and more than 50% echogenic material. (Picture from Thurmuller et al.[155])

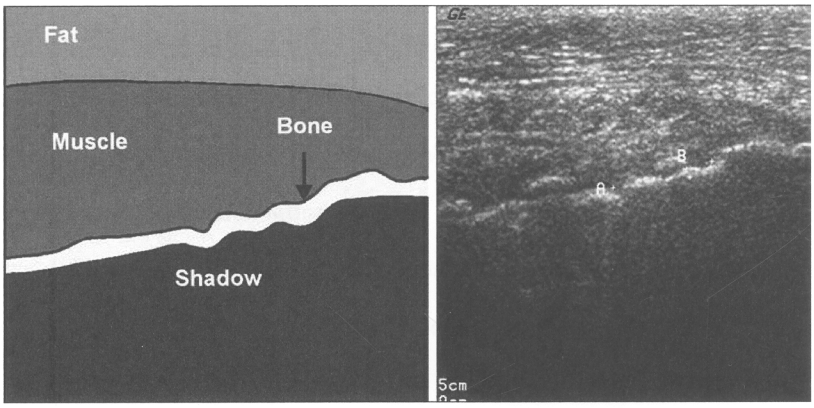


Fig 66. (Left) Diagram

(Right) An actual image of an animal showed no through-transmission of the ultrasound waves, invisible gap margins, and 100% echogenic material. (Picture from Thurmuller et al.[155])

As the pictures and results reported by Thurmuller et al.[155], the ultrasound reflection closely revealed the bone formation processes in the distraction gap. More bone formed, less ultrasound penetration. However, the diagnostic ultrasound with 10MHz frequency used in those studies are different from the LIPUS (1.5MHz) used in the present study. We believe that the LIPUS acts similar to the diagnostic ultrasound. Third, in the present study, our microhardness testing provided unique evidence that the bony tissue forming on the surface of the distraction gap was increasingly harder and harder. And the precise radiographies generated by the Micro-CT gave a vivid and inside examination of the bony morphology in the distraction gap, where the bony tissue was continuously present on the surface (both lingual and buccal sites) of distraction gap in almost every radiographic transverse slice (2-D image). Moreover, our histological findings also showed more specimens with complete cortical bone formation in LIPUS group on week 2 after the distraction. In other words, the acoustic impedance differences between the tissue in the distraction gap and the soft tissue surrounding the distraction gap were becoming larger and larger

overtime, as Thurmuller[155] mentioned. Therefore, more and more LIPUS waves would be reflected from the surface of the distraction gap coinciding with the bone formation in the gap, especially after 2 weeks of the distraction that the bony tissue mostly covered the distraction gap detected by the microhardness test and Micro-CT scan in the current study. The newly formed bone on the surface of distraction gap acted as a natural shield to block the penetration of LIPUS. At lest this would interpret why LIPUS works effectively at the early stage of distraction osteogenesis. In the early stage of distraction osteogenesis, soft tissue mainly filled in the distraction gap that had similar acoustic impedance to the surrounding soft tissue, thus LIPUS would easily transmit into the distraction gap and affect the bone cells. But in the later stage of distraction osteogenesis, most of the LIPUS waves would be blocked by newly formed bone on the surface of the distraction gap. So if the new bone formed earlier by the effect of LIPUS, the effectiveness of LIPUS was also reduced earlier. We may say earlier bone formed by applying LIPUS, earlier uselessness of applying LIPUS. Therefore, the early use of LIPUS is recommended, also early disuse of LIPUS might be concerned due to its reduced effectiveness at the later stage of distraction osteogenesis. However, LIPUS is less effective at the later stage of distraction osteogenesis still could not totally explain why similar bone formation on week 4 after the distraction between the two groups while more bone formation was found on week 2 after the distraction in the treatment group. Supposed if LIPUS had not been used between week 2 and 4 after the distraction, bone formation would still be significant on week 4 after the distraction owing to the effect of LIPUS on the distraction period and 2 weeks of consolidation period. But in the results, both the LIPUS and control groups had a similar level of bone formation on week 4 after the distraction. Even we considered LIPUS did not work between week 2 and 4 after the distraction, why the significance of bone formation on week 2 after the distraction did not maintain to the final week 4 after the distraction. We hold another explanation.

Second reason is that LIPUS may only accelerate bone formation rather than increase the total amount of bone formation. This point was supported by Yang et al.[94], Takayama et al.[158] Mayr et al.[118], Eberson et al.[74], Chan et al.[72], Shimazaki et al.[117] and Sakurakichi et al.[122]. Yang proposed that the LIPUS was more an effect of cell differentiation and an increased synthesis of extracellular matrix proteins, such as aggrecan and proteoglycan, rather than an effect of cell proliferation[94]. Mayr reported that the control group showed the same amount of new bone

formation with the LIPUS treated group[118]. No significant differences were found in bone mineral content or bone mineral density at 5 weeks after the distraction for rat femoral lengthening reported by Ebersion et al.[74]. Sakurakichi also demonstrated that at 4 weeks after the distraction all groups including the control group, end up with the same amount of new bone[122]. In the current study, it would be assumed that the bone formation in the LIPUS group from week 2 to 4 after the distraction would be slow down until reach the similar peak bone formation as in the control group. However, because of lack of the data on week 3 after the distraction, we do not know whether the peak bone formation was reached earlier than or just on week 4 after the distraction in the LIPUS group. Supposingly, the LIPUS may be capable of shortening the time needed to reach the peak bone formation. Therefore, more studies are needed to evaluate whether the bone formation peak comes earlier by applying LIPUS.

We consider that the above mentioned two reasons might be sufficient to explain our results. At the early stage of distraction osteogenesis, soft tissue contained high cellularity and less mineralization filled in the distraction gap, which facilitated LIPUS to transmit into the distraction gap. Thus the bone cells responded to LIPUS very well, which had been substantially proved in many studies[95-98]. Our results on week 0 and 2 after the distraction reconfirmed the positive effect from LIPUS. At the later stage of distraction osteogenesis, less and less LIPUS could penetrate the bony coverage into the distraction gap. It implied that LIPUS would be no effective at the later stage of distraction osteogenesis. Meanwhile, LIPUS may only accelerate bone formation rather than increase the total amount of bone formation. Therefore the longest LIPUS treatment group that received LIPUS at the first day of distraction until 4 weeks after the distraction showed the similar amount of bone formation of the control group. However, based on the data yielded on week 0 and 2 after the distraction, we suppose the LIPUS group earlier reached the peak bone formation than the control. The additional studies are needed.

Concerning other methods to enhance bone formation presented in previous articles, electrical stimulation[67, 68], growth hormone[61], growth factors[59, 62-66], stem cells[69, 70] and even the laser[71] have been studied, all showed a good result. Electrical stimulation was applied for 12-24 h every day and it would be inconvenient for patients to use[67, 68]. In their studies, external distractors were used for facilitating conduction of the electrical stimulation. However, patients and doctors prefer to use the internal distractor especially in maxillofacial region, it is also the

tendency of distractor designing. Moreover, availability of the electrical device was another concern. Effects of growth factors are promising[59, 61-66], but little is known about possible side effects (e.g., stimulation of other tissue growth or development of tumors) or the optimal dose and therapeutic regimen for human. The cost of growth factors is high. Transplantation of autologous stem cells has been tried[69, 70]. However the treatment was invasive, technically demanding and required more operations such as harvesting the transplant. The usefulness of the laser to enhance bone formation requires more supports. In contrast to these shortcomings, LIPUS therapy shows remarkable advantages. 1) Noninvasive (same to ultrasonography). 2) No complications and adverse effects have been reported (with similar intensity to diagnostic ultrasound). 3) Easy to use (20min/day) and patient friendly (use in home). 4) The ultrasound device is commercially available. 5) Inexpensive.

5. Conclusion

A meticulous judgment should be taken to every animal study. Based on the qualitative and quantitative results of the present study, we could conclude our study with 4 points.

1. LIPUS accelerates new bone formation during distraction period and 2 weeks after the distraction in rabbit mandibular distraction osteogenesis.

2. LIPUS did not show positive results when it was applied during distraction period and 4 weeks after the distraction. The possible explanations might be that less LIPUS could penetrate the bony coverage into the distraction gap after 2 weeks of consolidation period, and the effect of LIPUS may only accelerate bone formation rather than increase the total amount of bone formation when LIPUS works during distraction period and 2 weeks after the distraction.

3. More studies should be taken to determine whether LIPUS shortens the time needed to reach the peak bone formation or LIPUS heightens the peak bone formation (increase the total amount of bone formation).

4. LIPUS may be an effective modality to shorten the treatment time of craniofacial distraction osteogenesis. Further clinical study is needed.

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