A preliminary study of the effect of low intensity pulsed ultrasound on new bone formation during mandibular distraction osteogenesis in rabbits

Abstract. This study assesses the effect of low intensity pulsed ultrasound (LIPUS) on new bone formation during mandibular distraction osteogenesis (DO) in rabbits. Twenty-four rabbits underwent DO on the right side of the mandible. Twelve rabbits received a daily 20-min LIPUS (1.5 MHz, 30 mW/cm²) treatment on the first day of the distraction until they were killed at week 0 (immediately after the distraction), week 2 and week 4 after the distraction. Four rabbits were killed at each time point. The other 12 rabbits followed the same protocol without the ultrasound treatment. A plain radiography, a micro-CT scan, a microhardness test and a histological examination were used to evaluate new bone formation in the distraction gap. At week 0 and week 2 after the distraction, the treatment groups showed higher radiopacity and microhardness ($p < 0.05$), and more bone formation was detected by the histological examination. At week 4 after the distraction, there was no statistical difference between the two groups. In this study, LIPUS accelerated new bone formation during the distraction period and 2 weeks after the distraction, which implies that the effective time for using LIPUS is in the early stage of DO.

Distraction osteogenesis (DO) has the unique ability to gain new bone and simultaneously expand the surrounding soft tissues without a donor site. It has achieved worldwide acceptance and great success in the treatment of numerous congenital and acquired craniofacial skeletal anomalies. DO is a long term treatment, and it commonly takes 2–3 months for craniofacial DO. The rate-limiting step is the long waiting period for new bone formation, which takes at least two-thirds
of the whole treatment time. Great interest has been focused on reducing the treatment time by accelerating new bone formation during DO.

Low intensity pulsed ultrasound (LIPUS) is a form of mechanical energy that is transmitted through living tissues as acoustic pressure waves. Many studies have found LIPUS to be effective in the acceleration of fracture healing\(^6\). Two prospective, randomized, double-blind, placebo-controlled clinical trials have shown the same 38% reduction of healing time for tibia\(^9\) and radial\(^\text{11}\) fractures. The US Food and Drug Administration approved the use of LIPUS for the treatment of fresh fractures in 1994 and for the treatment of nonunion in 2000.

Several authors have reported the positive effects of LIPUS on long bone DO\(^2,7,17,19\). Few studies have evaluated the effect of LIPUS on membranous bone DO. EL-BIALY et al.\(^8\) found that LIPUS accelerated new bone formation in a rabbit mandibular DO model, but SCHORTINGHUIS et al.\(^18\) showed that LIPUS did not appear to stimulate bone formation in the severely resorbed vertical distracted human mandible. More investigations are required to clarify the effect of LIPUS on membranous bone DO. The aim of the present study is to assess the effect of LIPUS on new bone formation during mandibular DO in rabbits.

Materials and methods

24 skeletally mature, male, New Zealand white rabbits weighing 3.5–4.0 kg underwent DO on the right side of the mandible. In brief, anaesthesia was induced by intramuscular injections of ketamine (35 mg/kg) and diazepam (5 mg/kg). The skin on the right side of the mandible was shaved and disinfected with iodine solution, and the submandibular incision was 3 cm long. Once the mandibular body was exposed, the complete osteotomy was made just anterior to the first premolar straight down to the border of the mandible using a fissure bur. The custom-made distractor, a modification from an orthodontic palatal expansion screw (Hyrax, Germany) (Fig. 1), was fixed to the mandible with 4 self-tapping titanium microscrews, and the distracting direction of the distractor was made parallel to the long axis of the mandibular body and perpendicular to the osteotomy line. The wounds were closed in layers. Postoperative care included wound care, and intramuscular injections of penicillin G sodium (0.5 million units) and acetaminophen (75 mg) each day for 3 days. After a 3-day latency period, the distraction was started at a rate of 0.5 mm/12 h for 10 days. 24 rabbits were randomly divided into two main groups: the ultrasound treatment group and the control (without ultrasound treatment) group (n = 12). 4 rabbits from each group were killed at weeks 0, 2 and 4 after the distraction (week 0 means immediately after the distraction without a consolidation period).

Ultrasound treatment

A commercial ultrasound device was used. The Sonic Accelerated Fracture Healing System (Exogen Inc., Piscataway, NJ, USA) provided LIPUS with a 1.5 MHz frequency, modulated at 1 KHz with a signal burst width of 200 ms and an intensity of 30 mW/cm\(^2\). A single 20-min treatment to 12 rabbits per day started on the first day of the distraction and continued until they were killed at weeks 0, 2 and 4 after the distraction (Fig. 1).

Plain radiography

After the animal was euthanized with an intravenous injection of sodium pentobarbitone (1 ml/kg), its mandible was removed and separated at the synthesis, and a lateral film of the hemimandible (Kodak, Osceul film, Ultra-speed, USA) was taken (10 mA, 50 KVP, 0.26 s, 12 in. FFD) with an aluminium step-wedge using an X-ray machine (Gen-dex, IL, USA) and processed by an automatic film processor (Dent X 9000, DentX/Logetronics GmbH, Germany). The films were transformed into digital images by a digital camera (JVC TK-C1380, Tokyo), and quantitative analysis was carried out with the software (Image Pro Plus 5.0, Media Cybernetics Inc., USA) to measure the mean grey level of the distraction gap that indirectly represents the projectional bone mineral density.

When the plain radiography was done, the specimen was cut from the distracted hemimandible including the distracted regeneration tissue and parts of the original bone, which was anterior and posterior to the distraction gap. Another horizontal cut along the long axis of the mandible body further bisected the specimen into upper and lower parts, and all the specimens were saline-soaked and frozen at −80°C for preservation before additional testing.

Micro-CT

The lower part of the specimen was scanned transversely with a section of 200 µm thickness (µCT20, Scano Medical AG, Switzerland). The bone volume fraction (% bone volume/total volume) was calculated with software (Revision 3.1, Scanno Medical AG, Switzerland).

Microhardness test

The upper part of the specimen underwent a microhardness using a Microhardness Tester (Buehler Micromet, England), and the Vickers microhardness (kg/
mm\(^2\)), which represents the surface property of the distracted regeneration tissue, was used. The specimen was thawed to room temperature for at least 20 min before the test and the buccal surface of the distracted regeneration tissue was tested.

**Histology**

After the microhardness test, the upper part of the specimen was fixed in 10% formalin for 2 weeks, decalcified with 50% formic and 20% sodium citrate, then dehydrated in alcohol with increasing concentrations until it reached 100%, and finally embedded in paraffin. Sections of 5 µm thickness were cut longitudinally in the same direction as the distraction and stained with haematoxylin and eosin (H–E) for light microscopy examination (Carl Zeiss, Axioskop 40, Germany). In histomorphometric analysis, the distraction gap was longitudinally (the same as the direction of the distraction) divided into three horizontal rows (upper, middle and lower). In each horizontal row, four regions of interest (ROI) were chosen, two of which were near the original bone ends and the other two beside the middle of the distraction gap. 12 ROIs from one specimen were selected to represent the whole distraction gap. This sampling method was similar to that reported by Cope and Samchekov.\(^7\)

The percentage of bone area (%, PBA) of each ROI was calculated with the software (Image Pro Plus 5.0, Media Cybernetics Inc., Slier Spring, USA). The PBA is the proportion of newly formed bone area to the total area. The software allows the researcher to select a specific colour to represent the new bone, so the total surface area of this same colour range is automatically calculated and the percentage determined. The PBA of each specimen was the mean total PBA of 12 ROIs.

**Statistics**

All data were presented as the mean plus or minus the standard error of the mean (mean ± SEM). Differences between the treatment and control groups at each time point in mean grey level, bone volume fraction, microhardness and percentage of bone area were compared by the non-parametric Mann–Whitney test with SPSS (version 11, SPSS Inc., Chicago, IL, USA). The statistical significance was set at \( p < 0.05 \).

**Results**

The animals tolerated the DO and ultrasound treatments well, since no infection or other complications occurred. The custom-made distractor showed excellent stability and strength, and no breakage or dislodgement of the distractors occurred.

**Plain radiography**

In general, mineralization took place progressively from the ends of bone segments to the middle of the distraction gap decreasing the radiolucent zone in the middle of the distraction gap (Fig. 2). At week 0 after the distraction, more bony spicules appeared in the distraction gap in the treatment group, and it had a significantly higher mean grey level (1.1492 ± 0.060) than the control group (0.9007 ± 0.054) \( (p = 0.021) \).

At week 2 after the distraction, more radiopacity was noted in the treatment group. The mean grey level in the treatment group was 1.7637 ± 0.021, which was significantly higher than the 1.6502 ± 0.043 in the control group \( (p = 0.043) \).

At week 4 after the distraction, the treatment group presented slightly more radiopacity than the control groups, but no significant difference was found \( (p = 0.564) \). The mean grey level was 2.1181 ± 0.038 in the treatment group and 2.1091 ± 0.073 in the control group (Fig. 3).

**Micro-CT**

The image of each transverse section precisely showed the internal structure of the distracted regeneration tissue in the distraction gap. The difference in mineralization was visibly noticed between the two groups at week 0 after the distraction (Fig. 4). The significant difference in bone volume fraction was found \( (p = 0.021) \) to be 11 ± 2% in the treatment group and 3 ± 1% in the control group.

At week 2 after the distraction, dramatically increased mineralization and good continuity of the bony wall covering the distraction gap were seen, and slightly more radiopacity was noticed in the treatment group than in the control group. The difference in bone volume fraction between the two groups was significant \( (p = 0.043) \) with 42 ± 0.22% in the treatment group and 40 ± 1% in the control group.

At week 4 after the distraction, the two groups had similar radiological images. The bone volume fraction showed no significant difference between them \( (p = 0.564) \), but the treatment group had a higher value (53 ± 2%) than the control group (50 ± 3%).

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**Fig. 2.** Lateral films of the hemimandible. Time points at weeks 0, 2 and 4 after the distraction. The arrow shows the border of the distraction gap.
Fig. 3. The mean grey level is shown with standard error of the mean in the distraction gap at weeks 0, 2 and 4 after the distraction.

Microhardness test
At week 0 after the distraction, data were gained from three specimens in the treatment group and one specimen in the control group, the other specimens were too soft to be tested due to little bone formation on the surface of the distraction gap. Even though the data gained were too few to be of significance, three specimens in the treatment group had significantly higher microhardness than only one specimen in the control group (Table 1).

At week 2 after the distraction, a significantly higher microhardness value was detected in the treatment group compared with the control group ($p < 0.05$), which revealed that the treatment group had harder bony tissue covering the distraction gap. At week 4, no significant difference was found ($p > 0.05$).

Histology
At week 0 after the distraction, the distraction gap was mainly filled with fibro-vascular tissue, and primary bony trabeculae could be found near the original bone ends and beneath the periosteum. Both fibrous tissue and newly formed trabeculae oriented parallel to the direction of distraction. The treatment group showed more trabeculae than the control group (Fig. 5). The percentage of bone area in the treatment group was $10 \pm 1\%$, which was significantly higher than $7 \pm 1\%$ in the control group ($p = 0.021$). Cartilage islands were found in two specimens from each group.

At week 2 after the distraction, fibrous connective tissue dramatically reduced, bony trabeculae significantly increased in amount and location, which became more calcified and extended near the middle of the distraction gap. Complete cortical bone formation in the middle area of the distraction gap was found in 3 specimens from the treatment group and 1 specimen from the control group ($n = 4$). Although the percentage of bone area in the treatment group ($23 \pm 1\%$) was higher than that in the control group ($21 \pm 1\%$), the difference did not reach significance ($p = 0.149$). Cartilage islands were found in two specimens in each group.

Fig. 4. Micro-CT images of the lower half of distracted regeneration tissue in the distraction gap at week 0 after the distraction. The sections near the original bone ends in the distraction gap (a) and (c); and the middle section of distraction gap (b). The green line outlines the dental tissue.
Fig. 5. Photomicrographs of the longitudinal section of the distracted regeneration tissue at week 0 after the distraction. (OB) original bone. (F) fibrous tissue. The arrow shows bony trabeculae (H–E stain, original magnification ×5).

Table 1. Results of microhardness test (Vickers hardness, kg/mm²).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Week 0</th>
<th>Week 2</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>6.820 ± 0.459 (n = 3)</td>
<td>22.490 ± 0.660 (n = 4)</td>
<td>31.215 ± 0.655 (n = 4)</td>
</tr>
<tr>
<td>p value</td>
<td>NA</td>
<td>0.021</td>
<td>0.248</td>
</tr>
</tbody>
</table>

NA: not applicable.
Mean ± SEM, Mann–Whitney test, the level of significance was set at p < 0.05.

At week 4 after the distraction, both groups had similar appearances. No obvious fibrous tissue was seen, continuous thick cortical bone, clear bone marrow cavity and medullary tissue appeared, and more mature bone trabeculae and osteons were found in all the distractions gaps. The treatment group had slightly less percentage of bone area (26%) than the control group (26 ± 1%) (p = 0.773), and no cartilage was found in either group.

Discussion

The treatment groups were found to have more bone formation at weeks 0 and 2 after the distraction. This indicates that LIPUS has a positive effect on new bone formation during the distraction period and 2 weeks after the distraction. El-Bialy et al. evaluated the effect of LIPUS on mandibular DO in rabbits and found a positive effect from LIPUS². Compared with animal studies of LIPUS on long bone DO, the present findings are consistent with Shimazaki et al.¹⁹, Sakurakichi et al.¹⁷, Chan et al.² and Chan et al.³ in their conclusion of the effective timing of LIPUS, and similar to Mayr et al.¹⁴, Machen et al.¹³, Ebersen et al.⁷ and Claes et al.⁵, which demonstrated the positive effect of LIPUS.

During the early stage of DO, the distraction gap was mainly filled with soft tissue containing high cellularity and less mineralization which facilitated the transmission of LIPUS. Cells such as periosteal cells¹², chondrocytes²⁴ and osteoblasts³ responded well to LIPUS as there was increased cell differentiation¹², aggrecan gene expression²⁴ and osteocalcin mRNA expression⁵. Theoretically, the fluid shear flow produced by LIPUS may give favourable stimulation to bone cells during bone healing.⁶ The exact mechanism by which LIPUS accelerates new bone formation is unknown.

No significant difference was found between the two groups at week 4 after the distraction even though the treatment group had received the longest LIPUS treatment during the distraction period and 4 weeks of the consolidation period. There might be two explanations for this phenomenon.

First, LIPUS may not increase new bone formation at the later stage of DO (after 2 weeks of the consolidation period) because of ultrasound reflection and absorption. Approximately 25–40% of ultrasound energy is reflected at the soft tissue–bone interface in the intact bone²². A good example is given by Thurmuller et al.²² who used diagnostic ultrasound to monitor the bone healing of mandibular distraction in the minipig. In their study, there was no through-transmission of the ultrasound into the distraction gap at day 24 after the distraction. They concluded that the ultrasound reflection closely revealed bone formation processes during DO. The more bone formed, the more ultrasound reflected. In the present study, the micro-CT showed increasing mineralization and good continuity of the bony wall covering the distraction gap at week 2 after the distraction in both groups. Meanwhile, the data from the microhardness test indicated that the newly formed bone was increasingly covering the distraction gap and becoming harder (Table 1). The newly formed bone on the surface of the distraction gap may act as a natural shield to block the penetration of LIPUS. Ultrasound is severely absorbed by the bone. It has been reported that the majority of ultrasound energy (>80%) was attenuated within the first millimetre of propagation when the ultrasound was penetrating the cortex²³. Hence LIPUS energy into the distraction gap may be dramatically reduced due to the reflection and absorption at the later stage of DO (after 2 weeks of consolidation period). Certain parameters are needed, such as the acoustic impedance and absorption coefficient of the newly formed bone and surrounding soft tissue in order to calculate how much LIPUS energy is transmitted into the distraction gap. The portion of LIPUS energy lost between the ultrasound transducer head and the skin should also be considered.

Second, LIPUS may not heighten the peak bone formation level of DO. At the
cellular level, Yang et al. proposed that 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. Takayama et al. showed no effect of LIPUS on proliferation but an effect on differentiation in ROS 17/2.8 cells. At the tissue level, Shihazaki et al., Eberson et al., Sakurakichi et al. and Chen et al. found similar peak bone formation levels between the LIPUS treatment and control groups in DO animal models, which are consistent with the present results. Based on the data gained at weeks 0 and 2 after the distraction, the authors assume that LIPUS may be capable of shortening the time needed to reach peak bone formation level; this needs further study.

Cartilage islands appeared equally in both groups at weeks 0 and 2 after the distraction. It is thought that cartilage formation is due to decreased oxygen tension. LIPUS stimulated cartilage formation has been reported, but whether LIPUS promotes intramembranous ossification or endochondral ossification or both, remains controversial. The present findings do not support that LIPUS promotes endochondral ossification. Mechanical factors, such as the fixation method (rigid or less-than-rigid), the distractor, the distraction protocol, early function, and physiological factors, such as regional blood supply, play important roles in the determination of types of bone formation during DO. Whether LIPUS determines or changes the types of bone formation needs further study. Practically, LIPUS treatment has remarkable advantages. It is noninvasive (the same as ultrasonography), easy to use (20 min/day) and patient-friendly (can be used at home). There are no complications and adverse effects reported and the ultrasound device is commercially available and inexpensive. The efficacy of using LIPUS on human membranous bone DO needs further study. As discussed above, diagnostic ultrasound has been used to monitor bone formation during DO, and diagnostic ultrasound has a similar low intensity (1–50 mW/cm²) to LIPUS (30 mW/cm²) but a higher frequency (2–10 MHz) than LIPUS (1.5 MHz). If an ultrasound machine could combine diagnostic and therapeutic functions to monitor and accelerate new bone formation, it is worth trying.

In conclusion, LIPUS accelerated new bone formation during the distraction period and 2 weeks after the distraction. It implies that the effective time for using LIPUS is in the early stage of DO.

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**Competing interests**

None declared.

**Ethical approval**

0521.11/420 Ref. 05/52.

This research has been approved by The Animal Ethic Committee, Prince of Songkla University, Songkhla, Thailand.

**References**


23. Warden SJ, Bennell KL, McMeeken JM, Wark JD. Can conventional thera-


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