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Original

Effects of Low-Intensity Pulsed Ultrasound on Callus Formation: A Comparative Morphological Study

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Ultrasound has a variety of applications as a noninvasive treatment. In particular, it is widely used for its accelerating effect on synostosis, through Low Intensity Pulsed Ultra-Sound (LIPUS). In this study, morphological evaluation was carried out of the effect of LIPUS on bone tissue, aimed at identifying the effective initiation timing for the treatment and the bone callus formation period.

A bone defect, 2 mm in both diameter and depth, was formed in the femur of 7-week-old male Sprague-Dawlay rats. 30 rats were assigned randomly into 5 groups: a group with no LIPUS stimulation (0 L); a group with LIPUS stimulation on day 1-3 (1-3 L); a group with LIPUS stimulation on day 1-14 (1-14 L); a group with LIPUS stimulation on day 4-14 (4-14 L); and a group with LIPUS stimulation for day 1-14 without a bone defect (Control). The inflammation period was considered to be 3 days. In order to evaluate the healing acceleration effect three-dimensionally, comparison took place of hard tissue volume, as the number of high CT value voxels, and bone density as the average CT value. LIPUS stimulation during the inflammatory period was found to be of significant importance: the 1-14 L group's bone volume was significantly higher at day 7 and day 10 compared to the non-LIPUS stimulated groups in the inflammation period. This study suggests that it is desirable for LIPUS stimulation to be continued from the time of bone injury to the osteogenesis stage, and that LIPUS promotes callus formation, especially by stimulation in the inflammatory phase.

Key Words: LIPUS, ultrasound, cullus formation, CT, bone

Introduction

With the spread of minimally invasive treatments, focus has grown on the use of non-invasive ultrasound, which now has applications in a variety of treatments. Among these, there has been particular attention paid to the application of low-power ultrasonic pulses (Low Intensity Pulsed Ultra-Sound: LIPUS). Reports based on animal experiments have suggested acceleration of both soft tissue[1)2)] and hard tissue healing[3)～5)], with further ap-

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plication reported for use on the nervous system[6]. LIPUS treatment has even been reported for use within the osteoporosis model[7)8)]. Analysis at a genetic level has also identified physical stimulation through LIPUS as promoting cell differentiation of osteoblasts and chondrocytes[9)~11)]. Furthermore, there have also been reports of increased mechanical strength[12)] and bone mineral content[13)] resulting from LIPUS stimulation. The results of LIPUS stimulation on bone union are clear and the application of LIPUS to aid post-fracture recovery has now become widespread. In addition to its utilization for post-fracture treatment, LIPUS application on implants has been reported to shorten the healing period[14)~18)]. Although there is research regarding the optimal working conditions for LIPUS frequency[19)], currently the only safe and effective conditions for fracture healing are a frequency of 1.5 MHz, a repetition frequency of 1.0 kHz, an ultrasound intensity of 30 mW/cm^2, a pulse width of 200 μs, and stimulation time of 20 minutes/day[20)].

LIPUS has been approved as a medical tool since the 1990s and is used in a variety of applications, such as bone fracture treatment. U.S Clinical trials have acknowledged the effects of LIPUS stimulation in the acceleration of bone union[21)22)]. While there exist studies looking at the optimal period for LIPUS stimulation[23)], review of these studies related to bone union following LIPUS stimulation shows that bone evaluation was predominantly done through assessment of mechanical strength[24)], using two-dimensional imaging[25)]. Within these studies there was also a lack of three-dimensional (3D) morphological evaluation of bone tissue. The fracture healing phase is made up of the inflammatory phase, the cell growth phase, the callus formation phase and the remodeling phase, with the callus formation phase reached between week 1 and week 4[26)27)]. However, the difference between the acceleration effect of LIPUS stimulation at each of these phases is unclear and there are also no reports on the duration or period of LIPUS stimulation necessary for effective bone union. There is, therefore, a possibility that more effective post-bone fracture healing acceleration may be achieved through LIPUS stimulation treatment if it is applied over an optimal period and duration. In this study, 3D morphological assessment is used on LIPUS stimulation on rats with the bone fracture model. The objective was to clearly show the optimal stimulation period and duration for post-fracture LIPUS treatment.

Materials and Methods

The experiment was done using 7-week-old, male Sprague-Dawlay rats (SD rats, Charles River). The entire bone fracture healing phase is made up of the inflammatory phase, the cell growth phase, the callus formation phase and the remodeling phase[26)]. However, in the pilot experiment the period necessary for the femur of the SD rats to recover enough strength was approximately two weeks. Therefore, based on this, the experiment duration was set to run over 15 days to reach the callus formation phase.

Preparation of the fracture model rats aimed for stable reproduction of the fracture site and was conducted using a drill (GC Corp., Depth Drill 6B341) to create a cavity diameter of 2 mm and a depth of 2 mm at the injury site of the femur. The drill was cooled between procedures in order to prevent burning and was thoroughly flushed with saline to ensure no fragments of bone remained.

In order to investigate the relationship between LIPUS stimulation period and post-bone damage bone union, with reference to Nakajima et al[28)], the inflammatory phase was set at three days and the experiment conducted with the subjects split between the five groups below.

1. Bone damage procedure applied, LIPUS stimulation not carried out (0 L group)
2. Bone damage procedure applied, LIPUS stimulation carried out from day 1-3 (1-3 L group)
3. Bone damage procedure applied, LIPUS stimulation carried out from day 1-14 (1-14 L group)
4. Bone damage procedure applied, LIPUS stimulation carried out from day 4-14 (4-14 L group)
5. Control group. Bone damage procedure not applied, LIPUS stimulation carried out from day 1-14 (Control group)

Each of the five groups contained six rats, making a total of 30 rats. (The day of the bone damage

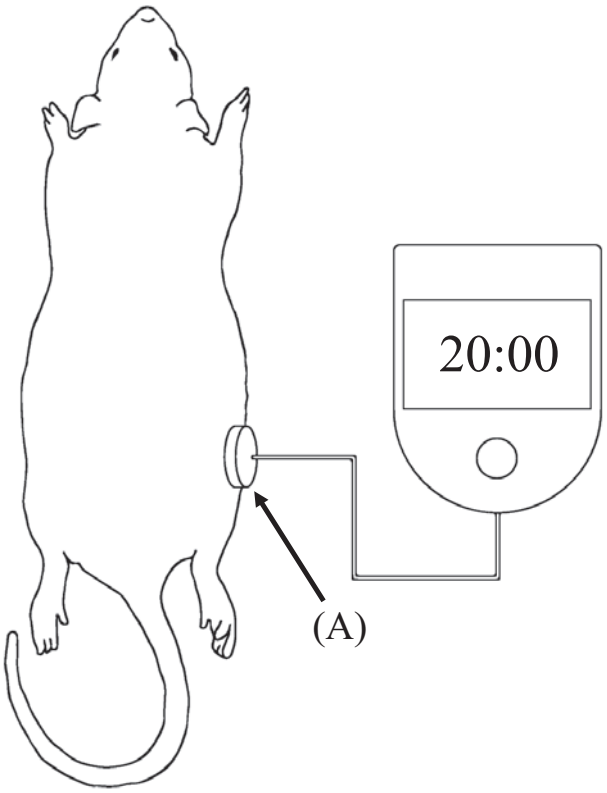

Fig. 1 LIPUS stimulation conditions
(A) is the LIPUS stimulation head unit.

procedure was counted as day 0).

LIPUS stimulation of the rats was carried out from day 1or day 4 and was conducted under anesthesia. During treatment the SAFHS LIPUS stimulation equipment was used (Teijin Pharma Ltd., SAFHS 4,000 J). Stimulation was carried out with a frequency of 1.5 MHz, a repetition frequency of 1.0 kHz, an ultrasound intensity of 30 mW/cm^2, a pulse width of 200 μs and a stimulation time of 20 minutes per day[9]. As shown in Fig. 1, during treatment the ultrasonic wave stimulation unit was affixed to the femur of the anesthetized rat using surgical tape and stimulation was then carried out. Shaving of the target site was conducted before the bone damage procedure. Shaving was repeated regularly to enable accurate repeat affixing of the stimulation head. X-ray micro-CT imaging of the rat femurs was performed for all groups on days 1, 3, 7, 10 and 15. The CT imaging was performed using the 3D micro-X-ray system R_mCT2 (manufactured by the Rigaku Corporation). The resolution of the CT device was 59-μm and the scan was carried out with an FOV diameter of 30 mm, a height of 30 mm, and an exposure time of two minutes.

The experiment received the permission of the animal experimentation ethics committee of Tokyo Women's Medical University (15-23) and was carried out in compliance with its standards.

Evaluation was carried out based on the CT values. A CT value of 200HU was set as the threshold for soft tissue; values below this threshold were excluded from the evaluation. Bone volume was calculated as the number of voxels. In addition, bone density was calculated as the sum of the CT values divided by the number of voxels, that is, the average CT value. It should be noted that due to the properties of ultrasound, LIPUS stimulation can be reflected and refracted depending on the tissue boundary surface. This is particularly the case for hard tissue boundaries, meaning the actual site stimulated by ultrasound is limited to the part facing the stimulation unit[29)30)]. In consideration of this, in the analysis of this study, the femur was divided into halves on the LIPUS stimulation surface and non-stimulation surface, and the volume of each bone and the density of bone were measured. Specifically, as shown in A in Fig. 2, the central axis of the hole was set as the x axis, the central axis of the femur was set as the y axis, and the z axis formed the right hand coordinate system defined by the x axis and the y axis. At this time, it was cut out into a cylindrical shape with a diameter of 9.4 mm and a height of 2.4 mm with the y axis as the central axis and the xz plane as the object plane, and it was divided into two on the yz plane, LIPUS stimulation side (Fig. 2B) and non-stimulation side (Fig. 2C).

Analysis of variance (ANOVA) and multiple comparison tests (Tukey-Kramer method) were applied on the measurement results from each group: results from the LIPUS stimulation side and non-stimulation side together and results solely from the LIPUS stimulation side (10 groups in total).

Results

Fig. 3(a) shows the bone volume results for the LIPUS stimulation side.

Over the LIPUS stimulation period no clear change in bone volume of the control group was ob-

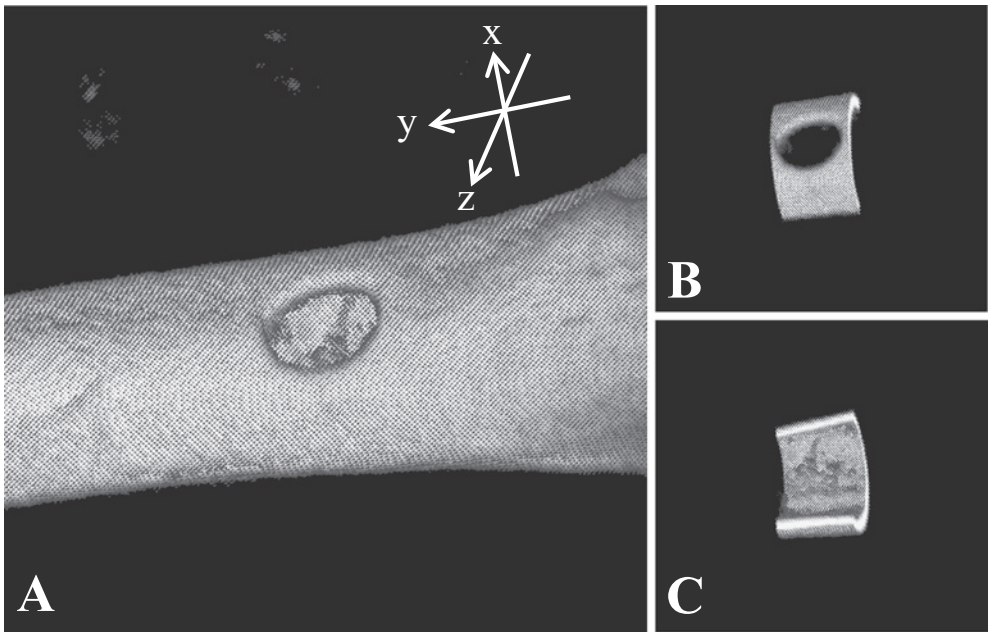

Fig. 2 An image obtained by Micro CT
A is a 3D reconstructed bone image. B shows the analyzed area including the bone defect. C is the other side of B.

served. Within the other groups, an increase of bone volume for bone union was confirmed regardless of the presence or absence of LIPUS stimulation or the period of the stimulation.

Comparing the results at day 1, the bone volume of the control group was significantly higher than that of the bone-damaged groups ($p < 0.05$), but there was no clear difference among the bone-damaged groups. The results for day 3 were the same as for day 1; however the differences between the bone-damaged groups and the control group had become smaller. By day 7, the bone volume of the bone-damaged groups had become higher than that of the control group. A trend was seen in the bone-damaged groups that underwent LIPUS stimulation during the inflammatory period (1-3 L group, 1-14 L group), with higher bone volume compared to the other bone-damaged groups (0 L group, 4-14 L group). In particular, the bone volume of the 1-14 L group (44,933 ± 2,958 voxels) was significantly higher ($p < 0.05$) in comparison to the 0 L group (36,288 ± 4,490 voxels) and 4-14 L group (37,035 ± 3,962 voxels). The same trend seen at day 7 was also apparent at day 10: the bone volume of the 1-14 L group (48,613 ± 3,738 voxels) was significantly higher ($p < 0.05$) than the 0 L group (40,461 ± 5,157 voxels) and the 4-14 L group (41,599 ± 2,354 voxels). The differences between the bone-damaged groups and the control group had also increased. By day 15, the difference in bone volume among the bone-damaged groups and the control group was significantly smaller ($p < 0.05$); however there was no significant difference between any combination of the bone-damaged groups.

Fig. 3(b) shows the transition result of the bone density for the LIPUS stimulation side.

At day 1 no differences in bone density were observed between each pair of groups. By day 3 differences in bone density between the control group and the bone-damaged groups had begun to appear, the bone-damaged groups having lower values than the control group and being relatively soft. At day 7, the average bone density of the control group was 505 ± 8, significantly different ($p < 0.05$) to those of the bone-damaged groups: the 0 L group (403 ± 29 HU), the 1-3 L group (397 ± 46 HU), the 1-14 L group (423 ± 20 HU), and the 4-14 L group (421 ± 18 HU). The results at day 10 were the same as for day 7 and a significant difference was again observed ($p < 0.05$): the average bone density of the

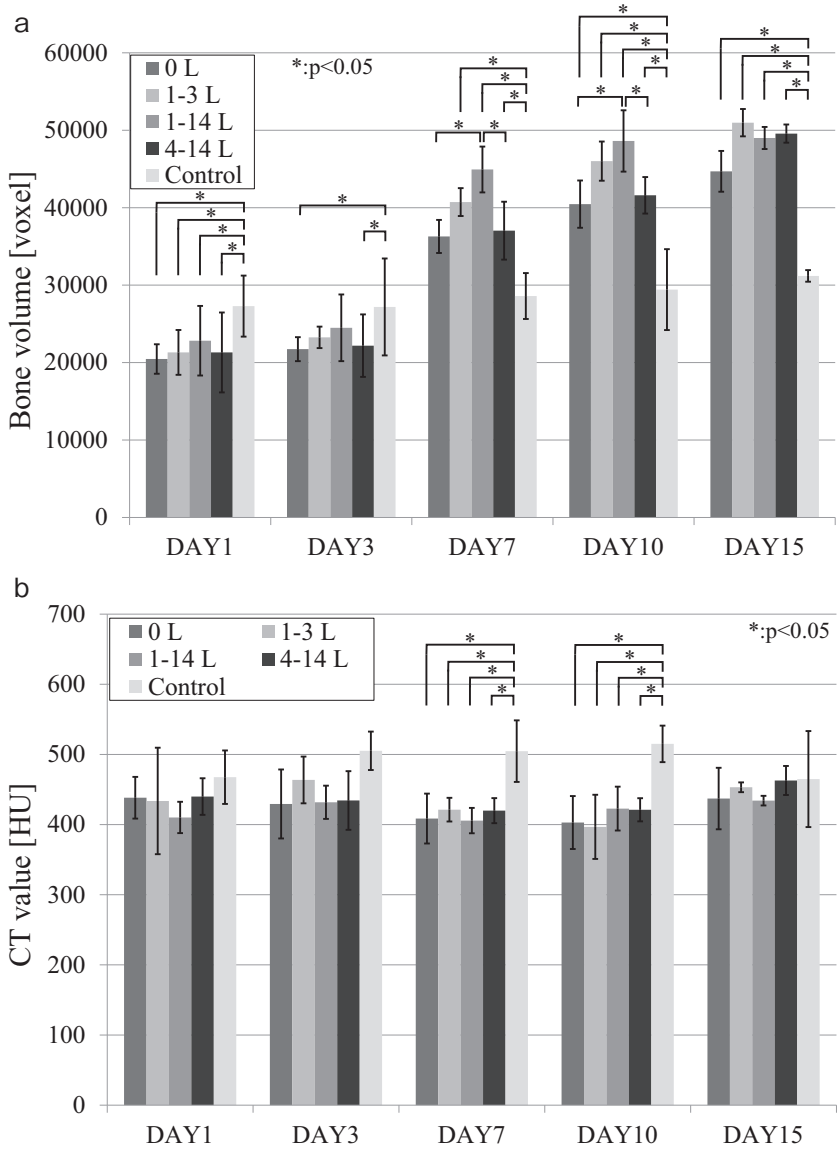

Fig. 3 Time series graph showing bone volume (a) and bone CT value (b) (LIPUS stimulated side).

control group was 515 ± 22, while the average density of the remaining bone-damaged groups showed no substantial change. By day 15, difference in average bone density among the groups was almost no longer apparent.

It should be noted that no clear change was observed in bone volume or bone density of the non-LIPUS stimulated side.

Fig. 4 shows a typical example of the micro-CT images from the 0 L and 1-14 L groups. A and B show the micro-CT images of the 0 L group on day 1 and day 7 respectively. D and E are the CT images of the 1-14 L group on day 1 and day 7. Images C and F are 3D diagrams of the CT images from the 0 L group and the 1-14 L group at day 7. Comparing B and E, it can be seen that by day 7 both cancellous bone impermeability and hard bone volume of the 1-14 L group had increased. It is also apparent from the 3D images that compared to C (0 L group) bone formation can be clearly confirmed at the bone injury site in F (1-14 L group).

Discussion

The experimental results show that at day 1 and day 3, although bone volume for the non-bone-damaged control group was significantly higher, no difference was observed among the bone-damaged groups. This difference in comparison to the control group is attributed to being due to the volume of

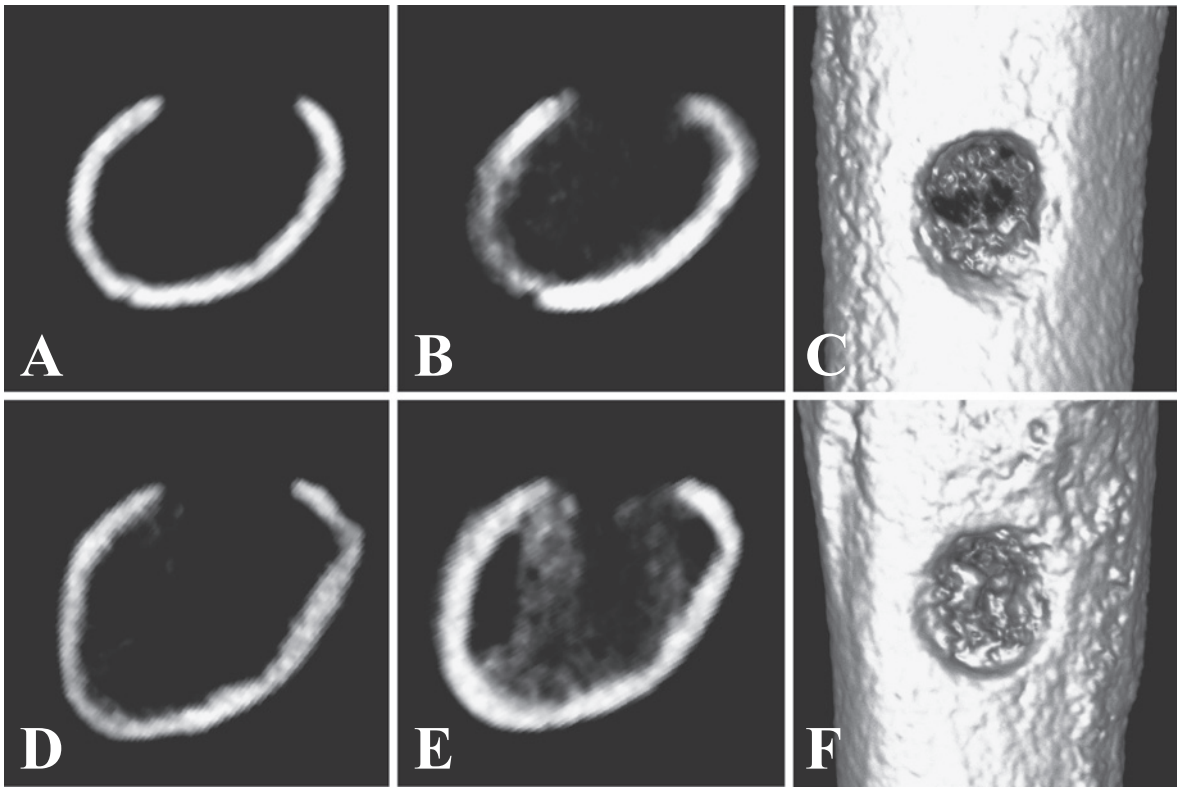

Fig. 4 Comparison between an LIPUS non-stimulated rat and a stimulated rat A and B are CT images from an LIPUS non-stimulated rat (0 L). A is a cross-sectional image on day 1. B is a cross-sectional image on day 7. C is a 3D reconstructed image of B. D-F show the LIPUS stimulated rat data (1-14 L).

bone removed as part of the bone damage treatment. By day 7 and day 10 the bone volume of the 1-14 L group, which underwent continued LIPUS stimulation, had greatly increased. In particular, a significant difference in bone volume was seen when compared to groups that had not undergone LIPUS stimulation during the inflammatory phase (0 L group, 4-14 L group). Based on this difference, LIPUS stimulation during the inflammatory phase is thought to be an important factor in accelerating bone recovery. The 1-3 L group, which had LIPUS stimulation only during the inflammatory phase, showed a tendency towards higher bone volume compared to the 0 L group; however, a significant difference was not seen. In regards to bone volume, even if LIPUS stimulation is only applied during the inflammatory phase, there may be a small healing acceleration effect. At day 15, significant differences in bone volume was observed between the bone-damaged groups and the control group: however no difference was found among the bone-damaged groups themselves. From these results it can be considered that application of LIPUS immediately after bone damage accelerates bone volume increase during the early period; however, after callus formation bone volume becomes roughly equal regardless of the presence, duration or period of LIPUS stimulation.

On the other hand, the bone density results show that at day 7 and day 10, the bone-damaged groups had a lower average bone density compared to the control group, suggesting the formation of immature bone as part of the bone healing process. However, clear differences among the bone-damaged groups themselves were not observed. At day 15 there was no longer a difference in average bone density among the groups, suggesting LIPUS stimulation does not affect bone density after healing.

The results above suggest that after bone injury, LIPUS stimulation in the inflammatory phase and the cell growth phase accelerate the formation of

immature bone, speeding up bone recovery in the early period. Furthermore, LIPUS stimulation does not affect bone volume or bone density at the injury site after healing and has little effect on normal bone. Therefore LIPUS should be used only during the healing process. Initiating LIPUS stimulation in the early period following bone injury, during the inflammatory phase and cell growth phase before the callus formation phase, is expected to accelerate bone union effectively. Also, while Azuma et al[23] reported an increase in mechanical strength resulting from LIPUS, the results from this experiment suggest an increase in mechanical strength could occur due to an increase in bone volume, without a change in bone density.

It also has been reported by Jingusi et al[15] and Iwabuchi et al[16] that as LIPUS stimulation is dampened by soft tissue the position of the stimulation head directly affects the therapeutic effect. In this experiment using rats, the comparison results from the LIPUS stimulation side and non-stimulation side suggest that healing acceleration is limited to the LIPUS stimulation side. Sasaki et al[19] reported that the LIPUS stimulation accelerates capillary vessel formation in the LIPUS stimulation side. Acceleration of capillary vessel formation is limited to the LIPUS stimulation side, suggesting that there is a relationship with our study. In terms of human application, for example in the case of a complete fracture, there is a possibility that further healing acceleration effects may be obtained through LIPUS stimulation of the entire fracture site.

LIPUS treatment has been reported as affecting osteoblasts, osteoclasts, cartilage cells and mesenchymal cells[31], but the specific mechanisms, stimulation times and duration involved remain unclear. This experiment suggests the optimal stimulation time as being the period before callus formation.

In the future, similar verification of reports that LIPUS stimulation is different on mature rats[25] is necessary to seek a more effective method of treatment. In addition to this, 3D morphological evaluation of bone tissue and implant treatment after tooth extraction is required to investigate if a similar bone regeneration effect can also be expected.

Conclusion

This study evaluated the callus formation acceleration effect of LIPUS stimulation, from the point of bone damage to callus formation, using CT imaging for 3D morphological evaluation, in order to verify the effective LIPUS stimulation period and duration. The group that underwent continuous stimulation directly following bone damage showed an increased bone volume in the early period compared to the other groups. This suggests that continuous LIPUS stimulation after bone injury occurs is effective in the acceleration of callus formation.

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低出力超音波パルス照射における仮骨形成への影響の形態的比較に関する研究

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超音波はその非侵襲性に注目が集まり，様々な治療に用いられるようになってきた．中でも低出力超音波パルス（Low Intensity Pulsed Ultra-Sound：LIPUS）による骨癒合促進効果が広く利用されている．しかしながら LIPUS 照射期間と時期の仮骨形成に関する研究では，骨の機械的強度や X 線画像による評価が主であり，骨組織の 3 次元的な形態評価は少ない．本研究は LIPUS 照射による仮骨形成を 3 次元的に形態評価し，骨癒合に最適な照射時期および期間を明らかにする．7 週齢の雄 SD ラットを使用し実験を行った．大腿骨にドリルを用いて直径 2 mm，深さ 2 mm の骨損傷を形成した．炎症期が 3 日であることを考慮し，5 群に分け実験を行った．骨損傷処置を行い LIPUS 照射を行わなかった群（0 L 群），骨損傷処置を行い Day 1〜3 に LIPUS 照射した群（1-3 L 群），骨損傷処置を行い Day 1〜14 に LIPUS 照射した群（1-14 L 群），骨損傷処置を行い Day 4〜14 に LIPUS 照射した群（4-14 L 群），コントロールとして骨損傷処置を行わずに Day 1〜14 に LIPUS 照射した群（Cont 群），各群 6 匹の計 30 匹で，実験日数は 15 日とした．LIPUS 照射は周波数 1.5 MHz，繰り返し周波数 1.0 kHz，超音波強度 30 mW/cm^2，パルス幅 200 μs，照射時間は 1 日あたり 20 分間とし，全群に対して Day 1, 3, 7, 10, 15 にラット大腿骨の X 線マイクロ CT 撮影を行った．骨の癒合促進効果を 3 次元的に評価するために，CT 値を元に軟組織を除く硬組織のボクセルデータの数を体積とみなした骨体積と平均 CT 値による骨の密度を比較した．Day 7, Day 10 では LIPUS を当て続けた 1-14 L 群の骨体積が大きく，特に炎症期における LIPUS 照射のない群との比較では有意に骨体積が大きいことから，炎症期の LIPUS 照射が特に重要であることがわかった．本研究より LIPUS 照射は，骨損傷直後から仮骨形成期までの間に照射し続けることが望ましく，LIPUS は特に炎症期に照射することで仮骨形成を促進させることが示唆された．