In Vivo Heat-stimulus Triggered Osteogenesis

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Introduction:
The treatment for large bone defects following musculoskeletal tumor resection and fractures of delayed union remains difficult. Although a variety of bone substitutes such as tricalcium phosphate and hydroxyapatite have been developed, they have mostly osteoconductive characteristics and no or insufficient capacity of osteoinduction as a substitute for autologous bone graft. However, several issues remain to be addressed concerning autologous bone graft. The development of novel techniques is required to achieve more stimulatory effects of bone formation.

Several in vitro studies that analyzed the possible roles of heat stress in enhancing osteogenesis found that mild heat stress between 40ºC and 42.5ºC increased the rate of osteogenic differentiation of stem progenitor cells.¹-⁴ A previous study reported the beneficial effects of heat stimulus at 44ºC on osteogenesis in vivo with an external microwave modality.⁵ However, the most important issue associated with hyperthermia is the difficulty in heating only the targeted region without damaging the surrounding normal tissues.

To address the problems that heat stimulus should be forced on target tissues, magnetic nanoparticles have been applied. Magnetic nanoparticles generate heat in an alternating magnetic field (AMF) as a result of hysteresis and relaxational losses. Target tissue could be heated where magnetic nanoparticles are present in an AMF, and the temperature is easily modulated. Preliminary results revealed that hyperthermia induced ectopic bone formation in mouse. We hypothesized that targeted heating in bone, using magnetic nanoparticle in an AMF could induce new bone formation. The aims of this study were to investigate the effects of hyperthermia (43-46ºC) induced by magnetite cationic liposomes (MCLs) under AMF on new bone formation, and to determine appropriate temperatures for the effect in a rat tibial defect model.

Methods:
Forty-four male Sprague Dawley rats were subjected to the experiment in this study. A 3-mm circular defect was created in the proximal
tibia, and filled with alginate beads containing MCLs. Thirty-two rats in the hyperthermia group were subjected to an AMF (43-46°C) immediately after implantation of MCLs composites. Twelve rats in the control group were implanted with MCLs composite without AMF. The rats in the hyperthermia group were further subdivided into 4 groups (8 rats each) according to the temperature applied (43°C, 44°C, 45°C, 46°C). Radiographs were evaluated with lateral views of right (implanted) and left (non-implanted) tibias using soft X-rays at the time of implantation and every 2 weeks until 6 weeks after implantation. Radiographs were scanned, and the consolidation areas adjacent to the tibial defects (2 mm around the implantation area) were defined as regions of interest (ROI). Because MCLs are radiopaque substances, regions of implantation were excluded from ROI for analyses. Consolidation area of ROI was measured with Image J software. Consolidation areas of ROI were evaluated at the time of implantation and 2 weeks afterwards.

At 2 weeks after implantation with or without hyperthermia, 8 rats in the control group and 4 rats in each temperature group were subjected to the histological analyses. The most central and sagittal sectioned surface of each tibial defect was stained with hematoxylin and eosin, and subjected to histomorphometric analyses. The total area for analyses was delineated at 2 mm from the edge of the tibial defects, which was approximately identical to ROI in the radiographic evaluation. The newly formed bone was marked, and subjected to quantification with Image J software. Newly formed bone was assessed by adding areas of formed trabecular bone surrounding MCLs composites and callus on the cortex close to the defects within ROI. The amount of newly formed bone area was normalized by the area of posterior cortex.

To more precisely assess the effect of hyperthermia on bone formation histologically, the expression of alkaline phosphatase (ALP) was examined using rabbit anti-ALP polyclonal antibody. Tissue sections were also subjected to tartrate-resistant acid phosphatase (TRAP) staining. TRAP-positive multinucleated cells adjacent to the bone were estimated as osteoclasts.

**Results:** Radiographically, more accelerated ossification at 2 and 4 weeks after implantation in the hyperthermia group as compared with the control (Fig. 1A). The degree of consolidation around the tibial defects in the hyperthermia group was increased compared with that in the control group, particularly at 2 weeks after implantation. Radiological assessment at 2 weeks after the treatment showed that significantly stimulated osteogenesis was observed in the hyperthermia group as compared to the control group \((P = 0.003)\) (Fig. 1B). Dividing the hyperthermia group into sub-groups with different temperatures ranging from
43°C to 46°C, the groups treated at 45°C and 46°C showed a significant increase in consolidation ($P = 0.013$ and $P = 0.031$, respectively), while the group treated at 44°C showed such a trend compared with the control group ($P = 0.087$) (Fig. 1C). Although the consolidation increased in the 43°C-treated rats, the difference did not reach significance ($P = 0.104$). Sections of all hyperthermia groups showed increased newly formed bone around the tibial defects (Fig. 2b-d, arrows) compared with the control group (Fig. 2a, arrowheads). Histomorphometrical evaluation revealed that the ratio of newly formed bone area to posterior cortex area was significantly higher in the hyperthermia group (43°C-46°C) than control group at 2 weeks after implantation ($P < 0.001$) (Fig. 2f). Analyzing the hyperthermia group independently, this ratio was all significantly higher in each temperature group compared to that in the control group at 2 weeks after implantation (43°C; $P = 0.005$, 44°C; $P = 0.019$, 45°C; $P = 0.003$, and 46°C; $P = 0.003$, respectively) (Fig. 2g). ALP was overexpressed in the cells on the border of newly formed bone adjacent to MCLs composites in the hyperthermia group (Fig. 2i, arrows), whereas less ALP expression was observed in the control group (Fig. 2h). TRAP-positive multinucleated cells were observed in areas of osteogenesis close to MCL composites in the control group (Fig. 2j, arrowheads). Few TPAP-positive multinucleated cells were observed at the boundary in the hyperthermia group compared with those in the control group, but were observed at the center of osteogenesis (Fig. 2k, arrowheads).

**Discussion:** Our study demonstrated that intramedullary application of hyperthermia using MCLs under AMF effectively enhanced bone formation in a rat tibial defect model. Hyperthermia has been applied to treat human malignant tumors in Japan. However, targeted hyperthermia has not been in clinical use. Moreover, hyperthermia has never been utilized to stimulate osteogenesis. Compared to a gene therapeutic approach or the systemic application of pharmacological compounds, hyperthermia with MCLs seemed to achieve local effects with greater efficacy. The results of our study suggest that targeted hyperthermia is a promising modality to stimulate osteogenesis, and provide the novel insight that heat stimulus to the intramedullary region may be tolerable as well as beneficial in some settings.

**Significance:** Our results demonstrate for the first time that heat stimulus accelerates osteogenesis in vivo, and may thus be of interest as a novel and promising tool to induce osteogenesis clinically as well.
Fig. 1. Radiographic evaluation for targeted hyperthermia
(A) Temporal change in radiographs of the right tibia in the control and hyperthermia groups.
(B) Changes in the radio-density of right tibia from baseline to 2 weeks after implantation (**P<0.003).
(C) Changes in radio-density in each hyperthermia group from baseline to 2 weeks after implantation (*P<0.05).
Fig. 2. Representative sections at 2 weeks after implantation with hematoxylin and eosin staining in the control group and all types of the hyperthermia group. Bars indicate 2 mm. 
(a) Control. 
(b) Hyperthermia at 43°C. 
(c) Hyperthermia at 44°C. 
(d) Hyperthermia at 45°C. 
(e) Hyperthermia at 46°C. 
(original magnification, ×20).
(f) Ratios of newly formed bone area.
(g) Ratios of newly formed bone at each temperature (43°C–46°C) and control. Immunostaining for evaluation of bone turnover (h–k). ALP staining (h, i), and TRAP staining (j, k), (original magnification, × 200). Bars indicate 200μm.

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