Fibrous membrane tissue formed around liquid nitrogen-treated bone promotes osteogenesis and revitalization of frozen bone: A rat study

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INTRODUCTION:
Reconstruction of large bone defects resulting from the wide resection of malignant bone tumors in the extremities is sometimes challenging. We have developed and clinically applied a reconstruction technique using tumor-bearing autografts treated with liquid nitrogen. The union rate of frozen bones may be higher than that of the bones treated with other methods, and revitalization of frozen bones has been shown in histopathological examination. This study hypothesized that the fibrous membrane tissue formed around frozen bones treated with liquid nitrogen has a structure and osteogenesis-promoting capacity similar to the induced membrane in the Masquelet technique and aimed to reveal this hypothesis in animal experiments. Furthermore, the study aimed to clarify the involvement of this membrane tissue in the osteogenesis and revitalization of frozen bones.
METHODS:
Fifty male Sprague–Dawley rats (weight: 300 g) were randomized into two experimental groups. In Experiment 1, after a 6-mm segmental defect was created in the right femoral diaphysis and fixed with an external fixator, the rats were divided into two groups in which (i) liquid nitrogen-treated excised bone was inserted into the defect (FB group; n=20) and (ii) polymethylmethacrylate (PMMA) was inserted using the Masquelet technique (PMMA group; n=20) (Figure 1). The liquid nitrogen treatment method was followed a previously described protocol: the resected bone was soaked in liquid nitrogen (−196 °C) for 20 minutes, thawed at room temperature (20 °C) for 15 minutes, and subsequently thawed in distilled water (30 °C) for 15 minutes. Five animals from each group were euthanized at 2, 4, 6, and 8 weeks postoperatively, and the membrane tissue around each spacer was collected for immunohistochemical analysis and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) for bone morphogenetic protein 2, transforming growth factor-β 1 , and vascular endothelial growth factor. In Experiment 2, frozen bone and PMMA were removed from both groups (n=5 each) in week 2 and autologous cancellous bone (ACB) harvested from the left ilium was grafted into the defect, preserving the membrane tissue around the spacers as in the Masquelet technique. Radiological analysis was performed every 2 weeks to evaluate the osteogenic process.
RESULTS:
In week 2, two-layered membrane structures were observed in the FB and PMMA groups, with the inner part consisting of neutrophils, monocytes, and lymphocytes, suggesting an inflammatory response, and the outer part consisting of fibroblasts and loose fibrous tissue. The membrane in both groups matured into fibrous tissue and thickened over time (Figure 2). Angiogenesis was active in both groups until week 4, with a significant increase in vessel number observed in the FB group compared to the PMMA group ($p<0.01$), and then decreased. In the FB group, chondrocytes invaded the osteotomy site from the membrane in week 4, and bone union was observed in week 8, suggesting that endochondral ossification may be involved in osteogenesis (Figure 3). Additionally, fibroblasts and capillaries in the membrane invaded the frozen bone's surface in week 2, and osteocytes were observed around the invasion area in weeks 6 and 8 (Figure 4). This invasion may be involved in initiating the revitalization of the frozen bone. Immunohistochemically, stained-positive areas for all factors were larger in the FB group at all evaluation points. qRT-PCR showed higher expression of all factors at each evaluation point in the FB group, and the expression dynamics of these factors were the same as those of the stained-positive area dynamics in immunohistochemical analysis. In the ACB graft model, the mean period to bone union and new bone volume were significantly shorter (6.0 weeks vs. 8.0 weeks, $p=0.01$) and greater (week 2, $p=0.01$; week 4, $p<0.01$; week 6, $p<0.01$) in the FB group.
DISCUSSION AND CONCLUSION:
This study showed that fibrous membrane tissue, rich in blood vessels and growth factors associated with osteogenic potential, was formed around liquid nitrogen-treated bones. Fibroblasts within this membrane tissue differentiate into chondrocytes and may promote endochondral ossification during osteogenesis in the treated bone. Furthermore, fibroblasts and capillaries invaded the treated bone and promoted maturation into osteocytes, suggesting their relationship with the initiation of frozen bone revitalization.

Figure 1. Surgical procedure for rats in Experiment 1. (A) A 6-mm segmental defect was created at the right femoral diaphysis and fixed with an external fixator. (B) Resected bone was treated with liquid nitrogen (FB group). (C) Polymethylmethacrylate (PMMA) was created to match the defect as a control (PMMA group). (D, E) Liquid nitrogen-treated bone or PMMA, respectively, were inserted into the defect. (F, G) Fibrous membrane tissue that formed around each spacer was collected for analyses at 2, 4, 6, and 8 weeks postoperatively.

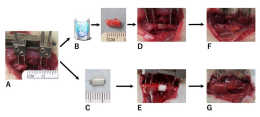

Figure 2. Histological analysis by hematoxylin and eosin staining of membrane tissue forming in the frozen bone (FB) and polymethylmethacrylate (PMMA) groups. (★: FB, ●: PMMA)

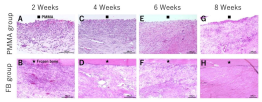

Figure 3. (A) In the frozen bone group, chondrocytes were observed at the osteotomy site in weak 4. In addition, periosteal reaction, including osteocytes and bone matrix, was observed from the host bone side to the osteotomy site. (B) In weak 8, the bone union of the osteotomy site was achieved, and thickening of cortical bone was observed covering the union site. (★: Frozen bone, ●: host bone, ※: screw site)

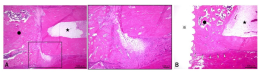

Figure 4. In the frozen bone group, fibroblasts and capillaries invaded the liquid nitrogen-treated bone from the membrane tissue in weak 2 (A). Osteocytes were observed around the invasion area in weeks 6 (B) and 8 (C), respectively.