The bone regeneration using bone marrow derived mesenchymal stem cell with platelet-rich plasma in femoral segmental defect of rats.

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INTRODUCTION
Bone marrow derived mesenchymal stem cells (MSCs) have been shown to be progenitor that have the potential to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes, fibroblasts and neuronal cell. In recent studies, MSCs are used to treat bony defects. However, adequate bone regeneration was not obtained from transplantation of MSCs alone. Bone morphogenetic proteins (BMP) are known to drive differentiation of MSCs along the osteogenic lineage. It has been well established that BMP-2 can induce orthotopic and ectopic bone formation in vivo.

Our previous studies showed that MSCs transplantation with recombinant human BMP-2 can induce bone formation. Furthermore, MSCs which introduced BMP-2 gene by a recombinant adenovirus showed high quality bone formation in rat femoral segment defect model.

Platelet-rich plasma (PRP), a platelet concentrate made of autogenous blood, was capable of increasing soft-tissue healing or bone regeneration in recent study. PRP includes growth factors such as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-b), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF). It is thought that these growth factors promote wound healing and bone regeneration. But there are controversies about the effect of PRP on newly bone formation in the literature regarding. PRP provides an assortment of growth factors, however it is still unknown influence of the platelet concentration in the PRP.

The aim of the present study was to determine the optimal concentration of PRP for a bone regeneration and evaluate the effect of PRP on MSCs in a rat model.

METHODS
This study was approved by the Animal Ethical Committee of Yamagata University. Platelet-rich plasma was isolated by traditional two-step centrifugation. Concentration of platelets in PRP was adjusted as follows, 500x10^3/ml, 250x10^3/ml, 125x10^3/ml, 62.5x10^3/ml, 31.25x10^3/ml, 15.625x10^3/ml and 0 (control). Activated PRP were added to rat bone marrow derived mesenchymal stem cell in different concentrations to assess cell proliferation and osteogenic differentiation in vitro. The proliferation of MSCs was measured on 1, 2, 3, 4 and 5 days using MTS assay. Osteogenic differentiation were confirmed by detecting alkaline phosphatase (ALP) activity. ALP activity was measured spectrophotometrically at 405nm on 2, 4, 6, 10, 12 and 14 days using p-nitrophenolphosphatase as a substrate.

A rat femoral segmental defect model was used in this study. Male Lewis rats were used as donor of MSCs. MSCs were harvested from femur with flush-out method, and cultured for 2 weeks. 36 female Lewis rats were used as recipients. A 6mm defect was made on femoral diaphysis of recipient rat and divided into four groups, Group 1 8x10^3 MSCs/PRP-collagen mix, Group 2 8x10^5 MSCs-collagen mix, Group 3 PRP-collagen mix, Group 4 Defect (control).

Serial radiographs of rat femur were examined at 2, 4, 6 and 8 weeks after implantation. Amount of new bone formation was evaluated by soft X-ray and quantify the bone fill percentage of newly formed bone using a digital image software package (ImageJ V.1.41, NIH, USA).

On day of 2, 4, 6 and 8 weeks after transplantation, the operated femur was harvested and decalcified. Microscopic evaluation was performed on paraffin embedded section stained with HE and Safranin-O.

To evaluate the survival of transplanted MSCs, Fluoroscence in situ hybridization analysis was performed on paraffin-embedded histological sections of the operated femur. The number of hybridization signals in 100 non-overlapping nuclei was counted under fluorescent microscopy.

Group means and standard deviations were calculated for each measured parameter. Data were analysed using Dr. SPSS. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA). A p value of <0.05 indicated statistical significance.

RESULTS:
On day 3, the number of MSCs adding PRP (500x10^3/ml, 250x10^3/ml, 125x10^3/ml, 62.5x10^3/ml, 31.25x10^3/ml, 15.625x10^3/ml and control) was 34.34x10^5/ml, 33.49x10^5/ml, 31.49x10^5/ml, 27.17x10^5/ml, 25.93x10^5/ml, 19.21x10^5/ml, 12.9x10^5/ml.

On day 5, the ALP absorbance of MSCs adding PRP (500x10^3/ml, 250x10^3/ml, 125x10^3/ml, 62.5x10^3/ml, 31.25x10^3/ml, 15.625x10^3/ml and control) were 0.323, 0.431, 0.412, 0.414, 0.437, 0.402, 0.357 (Fig.1). PRP (125x10^3/ml, 62.5x10^3/ml, 31.25x10^3/ml and 15.625x10^3/ml) significantly increased ALP activity compared to the control group (p<0.05).

The bone fill percentage of newly formed bone in the MSCs/PRP-collagen mix, MSCs-collagen mix, PRP-collagen mix and control groups were 24.0%, 8.8%, 7.0% and 7.5% at 2 weeks, and 35.0%, 10.5%, 11.8% and 14.1% at 6 weeks, and 46.6%, 10.9%, 12.5% and 18.5% at 8 weeks, respectively (Fig.2). At 8 weeks, the MSCs/PRP-collagen mix Group showed a significant increase in newly formed bone compared with other groups (p<0.05).

In MSCs/PRP-collagen mix Group, new bone formed on the proximal fragment extended the length of the defect to the distal fragment in histrogical section. The MSCs-collagen mix, group sparse new bone formation at the proximal or the distal end with little or no bone within the defect in histogical section.

DISCUSSION:
In this study, we showed that PRP enhanced the proliferation significantly compared to the control group on day 3 (p<0.05) and had a concentration-dependent effect on the cell proliferation. PRP induced differentiation of MSCs in low concentration. We consider that it is necessary for PRP to use low concentration as compared with high concentration to obtain proliferation and osteogenic differentiation of MSCs.

New bone formation was observed in the MSCs/PRP-collagen mix Group but few bone formation was seen in MSCs-collagen mix Group.

Our results demonstrate that PRP can be candidate for safe and useful activator in clinical application.

Fig.1 ALP activity was measured spectrophotometrically at 405nm.

Fig.2 The bone fill percentage of newly formed bone.

*P < 0.05, compared with other groups