INTRODUCTION:
In degenerative lumbar spinal stenosis, neurogenic claudication is often caused by hypertrophied ligamentum flavum and/or osteoarthritic hypertrophy of a facet joint. Facet joint is synovial joint with hyaline cartilage on each side. Therefore osteoarthritic of a facet joint eventually occurs with aging and other degenerative conditions of the spine. In lumbar spinal degeneration, inflammatory mediators or cytokines are released from facet joint tissue\(^1\), which consequently affect adjacent ligamentum flavum (LF), since LF covers posterolateral aspect of spinal canal near facet joints. However, there were no studies on the relationship of degenerated facet joint fluid and LF in the lumbar spine. Accordingly, in this experimental study, we cultured human LF cells under the stimulation of synovial fluid from degenerated facet joint and investigated the effect of synovial supernatant on cell viability, collagen mRNA expressions and the expressions of osteogenic phenotype and various transcriptional factors on osteogenesis in LF.

MATERIALS AND METHODS:
Cell isolation: Ligamentum flavum surgical specimens were obtained from patients with lumbar spine stenosis. The chips were digested in serumless medium containing 250 U/ml type I A collagenase at 37 ℃ in 5% CO\(_2\). The supernatant, which contained collagenase-released cells, was removed after 90 min and the collagenase treated ligament chips were washed with serum containing medium to inhibit collagenase and placed in 35 mm Petri dishes in DMEM-10% FBS. Cultures were incubated at 37 ℃ in a humidified atmosphere, 5% CO\(_2\). The medium was changed at two day intervals. They were allowed to grow out of ligament explants maintained in DMEM-10% FBS until the third passage.

Preparation of the conditioned medium: Each of the synovium tissues, together with 25ml PBS, were weighted and recorded. Each tissue was cut into small pieces (about 3 mm) with a pair of scissors, placed in a Petri dish and subsequently washed 3 times with PBS in order to eliminate possible interference from serum and growth factors in the blood. The washed tissue pieces were then cultured for 96hr at 37 ℃, 5% CO\(_2\) in DMEM-F-12-0.1% FBS with a density of 200mg/ml medium. The supernatant medium was collected after 96hr and centrifuged at 12,000 rpm for 5min. The supernatant was next put into sterile tubes in aliquots and stored at -80 ℃ until use.

Cellular metabolic activity and viability assays: To measure quantitatively the proliferation of cells, the AlamarBlue assay was used\(^2\). Target cells were counted and resuspended to a final concentration of 1×10\(^5\) cells/ml. A 200ul of the target cell suspension was added to each of the test wells. Cultures were terminated after 3 days for cell proliferation test.

Reverse transcription-polymerase chain reaction for mRNA expression: Total cellular RNA was extracted from the cells and concentration was measured by a spectrophotometer, and cDNA was synthesized from 1ug of total RNA. Amplification reactions specific for the following cDNAs were performed: GAPDH, three osteogenic master transcription factors, which are Dlx5, Runx2, osterix, also collagen type I, II, XI and osteocalcin. The intensity of the PCR products was quantified by TINA 2.0.

Alkaline phosphatase activity assay: For ALP staining, cultured cells were rinsed twice with PBS, fixed in 100% methanol, rinsed with PBS, and then overlaid with 1.5ml of 0.15mg/ml 5-bromo-4 chloro-3 indolylphosphate plus 0.3mg/ml nitro blue tetrazolium chloride in 0.1M Tris-HCl, pH 9.5, 0.01N NaOH, 0.05M MgCl\(_2\), followed by incubation at room temperature for 2hr in the dark.

RESULTS:
Human LF cell culture with supernat from facet synovium showed upregulation of osteogenic master transcription factors, Dlx5, Runx2, and osterix after 1 hours to 12 hours (Figure 2). In RT-PCR of collagens mRNA, human LF cell cultures with supernat of facet synovium demonstrated increase in collagen type I mRNA expression in 72 hours (Figure 3-A), collagen type II and XI mRNA expressions in 48 hours (Figure 3-B, C). LF cell culture with supernat from facet synovium showed slight positive stain for alkaline phosphatase (Figure 3-D).

DISCUSSION:
Supernatant of facet joint from patients with degenerative spinal stenosis affected LF cells in terms of increase in cellular proliferation, upregulation of mRNA expression of collagen type I, II, XI and finally increase in the expression of osteogenic transcription factors i.e., Dlx5, Runx2, osterix and positive alkaline phosphatase stain. Hence degenerated synovial fluid from facet joint renders an important mechanism of LF hypertrophy and ossification.

REFERENCES:

ACKNOWLEDGMENT
This study was supported in part by Brain Korea 21 Project.