INTRODUCTION:
Autologous chondrocyte transplantation (ACT) is a promising method for the treatment of chondral or osteochondral defect in articular cartilage. In this method, the patient’s chondrocytes are harvested, cultured in vitro for three weeks, and re-implanted to the joint. During the culture period, chondrocytes typically have a multi-fold increase, and become dedifferentiated. In this study, we proposed a modified method for the culture by applying additional low hydrostatic pressure at 250 Pa. The hypothesis was that low hydrostatic pressure would significantly promote the chondrocyte proliferation while maintaining its phenotype.

METHODS:
Chondrocytes from the patellofemoral joints of 3-month-old pigs were harvested and cultured in a medium containing Dulbecco’s Modified Eagle medium (DMEM) (Gibco) with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 mg/ml ascorbic acid (Sigma). Initial cell seeding was 5x10^5/cm². Chondrocytes were divided into two groups: 1) the control, cultured in a standard 24 well culture plate (1.9 cm²) with a medium height of 0.5 cm (equivalent to a hydrostatic pressure of 50 Pa), and 2) the loading group, cultured in a modified 24 well culture plate with a medium height of 2.5 cm (equivalent to a hydrostatic pressure of 250 Pa). The cultured cells were maintained at 37°C in a humidity incubator at 5% CO₂ and 95% air.

After a three-week culture, the cartilage-like membrane was removed from the culture medium. The membrane was weighed, the cell number was counted, and the syntheses of type II collagen and aggrecan were measured at the mRNA level using reverse transcription and polymerase chain reaction (RT-PCR). Chondrocytes were isolated from the extracellular matrix with 0.05% trypsin and 0.2% collagenase II in 5 ml DMEM. Under microscopy, non-viable cells were stained and counted using a hemocytometer. Total cellular RNA was extracted from cells after dissolving in TRIzol reagent (Gibco) according to the manufacturer’s instruction. One microgram of total RNA was used as a template for reverse transcription and PCR amplification using the Superscript One-Step RT-PCR System (Gibco) according to instructions. Amplification was performed within a thermocycler (PTC-200, MJ Research). The cytic parameters for PCR were 48°C for 30 min to reversely transcribe RNA to cDNA, 95°C for 2 min to activate the Taq DNA polymerase, followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at 30°C for annealing, and 60 s at 72°C for extension, then a final cycle of 7 min at 72°C for extension. The primers were derived from GenBank sequences. Twenty-microliter aliquots of the polymerase chain reaction products were electrophoresed in 4% agarose gels stained with ethidium bromide. The signals were quantified using image analysis software AlphaEase™ (Alpha Innotech) and were normalized against the expression of a housekeeping gene, β-actin. The results were analyzed by analysis of variance and post-hoc Tukey test with a sample size of 6.

RESULTS:
After the three-week culture, the net weight of the cartilage-like membrane in the loading group (53.7±11.03mg) was 4.0 times more than the control (13.5±1.51mg) (p<0.01) (Fig.1). Cell numbers were significantly increased by 7.4 times in the control and 23.1 times in the low pressure group. In other words, the cells under 250 Pa loading increased 3.1 times more than that in the control (p<0.01).

The mRNA expression of the type II collagen in the control was 0.51±0.20 (normalized ratio to β-actin) (Fig.2). This number was significantly lower than that from the fresh cartilage tissue (0.87±0.09) (p<0.01). Under the 250 Pa loading, however, type II collagen synthesis (0.85±0.10) was significantly higher than the control (p<0.01). More importantly, this value had no statistical difference from the fresh tissue sample. The type II collagen synthesis was largely preserved.

Aggrecan synthesis was decreased after the 3 week culture period from 0.69±0.07 to 0.30±0.04 in the control and 0.56±0.06 under 250 Pa loading (p<0.01) (Fig.2). However, the decrease in the control group was 57% while the decrease in the 250 Pa loading group was only 19%. The aggrecan synthesis under loading was 1.87 times higher than the control (p<0.01).

DISCUSSION:
This study introduced an improved method for ACT cell cultures. This method largely preserved the cell phenotypes and reduced the dedifferentiation of chondrocytes with a normal production of type II collagen and only a slight decrease in aggrecan.

Numerous studies have reported that chondrocytes are known to respond to compressive loading. However, most of the previous studies have either applied high pressure (at about the physiological loading level of ~10⁵ Pa) or dynamic compressive loading, or used three-dimensional polymer scaffolds. In this study, we applied a hydrostatic pressure at the relatively low magnitude of 250 Pa. The result showed an excellent preservation of chondrocytes. Although the mechanism is currently under investigation, this method provides a simple but efficient approach for ACT cell cultures.

Because medium volume in the 250 Pa culture was 5 times larger than the control, it was possible that the extra volume might have affected the results. We examined this factor by using a larger Petri dish in which the medium height was 0.5 cm but the volume was the same as that in the 250 Pa culture. The results showed no difference from the 50 Pa culture, proving that the medium volume did not contribute to chondrocyte preservation.

In summary, this study proposed a modified cell culture method for ACT technique with application of 250 Pa hydrostatic pressure. This method significantly increased the cell number and reduced the dedifferentiation of cultured chondrocytes.