INTRODUCTION:
Female athletes are 2-8 times more likely to suffer a knee and ankle ligament injury than male athletes. Sex hormones are considered to be critical in this gender difference. Since the ligaments are always under mechanical loading, stress also plays an important role. Previous study showed that a 24-hours combined administration of cyclic mechanical loading and estrogen dramatically reduced collagen types I and III gene expressions in primary cultured porcine anterior cruciate ligaments (ACL) fibroblasts [1]. However, it remains undefined if this regulation is sensitive to time. The goal of this study is to determine the time course of the combined effect of cyclic mechanical loading and estrogen on the gene expressions of collagen types I & III and two important small leucine-rich proteoglycans—decorin and biglycan.

METHODS:
Fibroblasts were primarily cultured from freshly harvested ACLs from 3-month-old pigs as described previously [1]. After the cells reached confluence in the culture wells, they were subcultured to six-well tissue BioFlex culture plates with 3x10⁵ fibroblasts per well. Estrogen administration followed the standard protocol used previously. Once the fibroblasts reached confluence, the medium was changed to a hormone-free medium 24 hours prior to the experiments. This medium was phenol red-free DMEM containing 10% dextran-coated charcoal-stripped FBS. 10⁻⁹ M 17p-estradiol (Sigma Chemical Co.) was administered with the vehicle of 100% ethanol.

Mechanical loading was applied on fibroblasts via a Flexercell™Tension Plus system (Flexcell International Co.). The fibroblasts were subjected to equibiaxial cyclic tensile stress at a rate of 0.5 Hz with 5% strain for 4, 8, 12 and 24 hours. The gene expressions of collagen types I & III, decorin and biglycan were evaluated by examining their messenger RNA (mRNA) level using reverse transcription—polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from fibroblasts in TRIzol reagent (Gibco Technologies Inc.) according to the manufacturer’s instruction. One microgram of total RNA then served as a template for reverse transcription and PCR amplification using the Superscript One-Step RT-PCR System (Gibco Technologies Inc.) according to the manufacturer’s instruction. Primers specific for type I collagen (g1), type III collagen, decorin, biglycan and β-actin (as an internal control) were derived from GenBank sequences. PCR cycle numbers were carefully selected to be within the linear zone of each gene. Ten-microliter aliquots of the PCR products were electrophoresed in 4% agarose gels stained with ethidium bromide. Densitometric analysis was performed using image analysis software AlphaEase™ (Alpha Innotech Co.). The resulting data were expressed as a ratio to the internal control value.

RESULTS:
The gene expressions of collagen types I & III and biglycan showed dynamic decreases according to exposure time (Fig. 1). The relative amount of mRNA of collagen type I was 1.04 at 4 hours, 1.06 at 8 hours, 0.81 at 12 hours and 0.74 at 24 hours. That of collagen type III was 0.31 at 4 hours, 0.25 at 8 hours, 0.24 at 12 hours and 0.09 at 24 hours. For biglycan, the relative amount of mRNA was 0.83 at 4 hours, 0.77 at 8 hours, 0.35 at 12 hours and 0.29 at 24 hours. The decreases seemed not to be linear with a dramatic drop between 8—12 hours for collagen type I and biglycan and between 12—24 hours for collagen type III. Gene expression of decorin also altered dramatically with combined administration of estrogen and mechanical force, but in the opposite direction (Fig. 2). The relative amount of mRNA of decorin was 0.45 after 4 hours’ exposure to mechanical loading, 0.69 at 8 hours, 0.79 at 12 hours and finally 1.02 at 24 hours. The increase slowed after 8 hours exposure.

DISCUSSION:
Dynamic observations showed non-linear decreases of gene expressions in collagen I, III, biglycan and increase in decorin under cyclic mechanical loading and exposure to 10⁻⁹ M 17p-estradiol. The turning point for each gene occurred at around 8 hours exposure. These results suggest 8 hours is an important time point for the gene expression alterations and also implied more complicated mechanisms rather than simple accumulation might be involved.

It is very interesting that the gene expression of decorin was altered opposite to that of biglycan. These two SLRPs have similar structures and belong to the same class of SLRPs [2]. Different alterations of gene expression of decorin and biglycan implied these two SLRPs might play different roles in the ACL.

In summary, this study determined the time-dependence of the alterations of collagen I, III, decorin and biglycan gene expressions of ACL fibroblasts, which were exposed to a cyclic mechanical loading and estrogen. The relative amount of mRNA of collagen I, III and biglycan decreased with exposure time, while that of decorin increased with exposure.

REFERENCES:

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