Introduction: Previous epidemiologic studies revealed that the injuries of anterior cruciate ligament (ACL) were more frequently seen in female athletes than in male athletes [1]. However, the principal pathogenetic factor for this sex difference still remains unclear. Scanning acoustic microscopy (SAM) has been used for measuring the tissue acoustic properties including sound speed and attenuation that closely correlate to the mechanical properties at a microscopic level. Especially, it is known that tissue sound speed is directory proportional to the square root of its Young’s modulus. In the current study, we hoped to assess hormonal effects on the tissue sound speed of ACL at a microscopic level.

Materials and Methods: Animal model
Forty age-matched female Japanese White rabbits (32 weeks old, the average body weight was 4.3 kg) were used for the current study. The animals were divided into 4 groups (L, M, H and C) after ovariectomy under general anesthesia. Intramuscular injection of 1β-estradiol was performed 1, 2, 3 and 4 weeks after surgery. The doses of 17β-estradiol in Groups L, M, and H were 50, 100, and 500 μg/kg, respectively. For Group C, which served as control, neither estradiol nor any types of vehicles were administered.

Preparation of the specimens
All rabbits were euthanized at 5 weeks after the ovariectomy. The right hind limb was disarticulated at the hip, and the knees were dissected to expose the ACL. The lateral portions of ACL (lACL) were fixed in 10% neutralized formalin and embedded in paraffin. Then, they were cut perpendicularly to the ligament fibers in the thickness of 10 μm. Serial sections were made both for the SAM measurements and routine histologic staining including haematoxylin-eosin.

Measurement of the tissue sound speed of ACL
A SAM system specially developed in Tohoku University, operating in the frequency range of 50-150 MHz, was used for this study [2]. The sections for SAM measurements were mounted on glass slides but not covered by cover slips. The paraffin was removed from the sections by the graded alcohol method prior to the ultrasonic measurement. Distilled water was used as the coupling medium, which maintained the specimen at 20°C during the measurement procedure. A single ultrasound pulse of 5 ns width was emitted and received by the same transducer above the specimen. The reflections from the tissue surface and those from the interface between the tissue and glass were introduced into a digital oscilloscope. Four values of the time taken for a pulse response at the same point were averaged in the oscilloscope in order to reduce the noise in the measurement. The transducer was mounted on an X-Y stage with a microcomputer board that was driven by the computer installed in the digital oscilloscope. The X-scan was driven by a linear servo motor, and the Y-scan was driven by a stepping motor. The area of measurement was 2.4 × 2.4 mm (300 × 300 pixels). A two-dimensional distribution of sound speed in a specimen was displayed and saved as image files using both a color-coded scale and a gray scale. To exclude the artifacts caused during the cutting process, the area where ligament tissue was properly transected was determined histologically as the region of interest (ROI). In each specimen, a gray-scale image was imported to the software, Adobe Photoshop (version 7.0). With this image, the average value of density (between 0 and 255) of ROI was measured 5 times using the analysis option, “histogram”. Then, the mean value of these 5 measurements (mean density) in each specimen was converted again to the sound speed (mean sound speed). The mean sound speed was compared among 4 groups (L, M, H and C).

Statistical analyses
Dunnett test (Statview 5.0, SAS Institute Inc.) was used to determine the differences in the tissue sound speed among 4 animal groups.

Results: One rabbit in Group M died due to an anesthetic problem during surgery, thus this animal was excluded from analysis. In the other 39 rabbits, no postoperative infections were seen. A two-dimensional distribution of the tissue sound speed was successfully measured with SAM (Figure 1, 2). The mean sound speed and the standard deviation of the Group C, Group L, Group M, and Group H were 1727±32, 1683±53, 1665±63, and 1690±56 m/sec, respectively. All 3 estrogen-administered animal groups (Groups L, M and H) indicated lower sound speed than that of Controls (Group C). Especially, Group M indicated the lowest value of the tissue sound speed among 4 groups, which could be interpreted as the lowest Young’s modulus. A statistically significant difference was found in the tissue sound speed between Groups C and M (p=0.028).

The tissue sound speed of anterior cruciate ligament in 4 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean sound speed (m/sec)</th>
<th>Standard Deviation</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>1727</td>
<td>32</td>
</tr>
<tr>
<td>L</td>
<td>1683</td>
<td>53</td>
</tr>
<tr>
<td>M</td>
<td>1665</td>
<td>63</td>
</tr>
<tr>
<td>H</td>
<td>1690</td>
<td>76</td>
</tr>
</tbody>
</table>

Discussion: The pathogenetic roles of estrogen in ACL ruptures have not been fully clarified yet. Controversies still continue whether estrogen alters the strength of the ACL tissue or not. In the current study, the authors developed rabbit models with 4 different doses of estradiol. Since the estrogen receptors were localized in the ligament proper (i.e., nuclei of synoviocytes, fibroblasts and cells in the blood vessel walls) [3], we measured their sound speed that closely correlates to the tissue elasticity. Our results clearly demonstrated that all three estradiol-administered groups showed lower sound speed than that of group C. Moreover, a significant difference in the tissue sound speed was seen between Groups M and C. In other words, the ligament proper of Group M indicated the lowest material property among 4 animal groups. Based on these results, we assumed that estrogen might constitute one of the pathogenetic factors of the ACL ruptures in the female athletes.