The Effects of Freeze-Thaw Cycle and Application of Proteolysis Inhibitors and Cryopreservants on Biomechanical Properties of Articular Cartilage
+1Hirviniemi, M; 1Lammi, MJ; 1Jurvelin, JS; 1,2Töyräs, J
+1University of Eastern Finland, Kuopio, 2Kuopio University Hospital, Kuopio, Finland
Senior author juha.toyras@uef.fi

INTRODUCTION
For practical reasons, articular cartilage (AC) samples are often frozen for further analyses. This fact has raised questions, whether biological and biomechanical properties of AC change during a freeze-thaw cycle. The effects of freezing and thawing on articular cartilage have been studied by several research groups (e.g. [1, 2]). Many of these studies, however, have focused on detection of glycosaminoglycan (GAG) loss, which may happen due to possible collagen network damage during thawing, and enzymatic degradation of proteoglycan (PG) core protein via activation of proteases.

PGs have significant effect on cartilage permeability and equilibrium modulus. Thus, the loss of PGs has significant effect on cartilage functional properties. Zheng et al. [1] reported a significant GAG loss due to freezing without cryoprotective agents (CPAs).

The aim of this study was to investigate the effects of freezing and thawing on biomechanical properties of AC. The effectiveness of protecting cryopreserved cartilage was our main interest. For this purpose, a biomechanical stress-relaxation test was conducted before 21-24 h freezing, and again after 18 h storage at room temperature.

METHODS
Intact, mature bovine knee joints (age 18 to 24 months) were obtained from a local slaughterhouse (Atria Oyj, Kuopio, Finland). Osteochondral disk (d = 25.4 mm) was prepared from each patella (PAT) within 12 h post mortem. Four 6 mm diameter plugs were then detached from each disk (n = 9) using a biopsy punch (Figure 1).

The samples were divided into four groups: control group (1) and three experimental groups (2, 3 and 4). Each plug in the groups 1, 3 and 4 was immersed in 5 ml of Dulbecco’s phosphate buffered saline (PBS, pH = 7.4, Euroclone, Siziano, Italy) containing inhibitors of proteolytic enzymes (5 mM benzamide and 5 mM EDTA) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Each plug in the group 2 was immersed in 5 ml of PBS without enzyme inhibitors or antibiotics.

Subsequently, the thicknesses of the cartilage plugs were measured using a stereomicroscope (Nikon SMZ-10, Nikon Co., Tokyo, Japan) before biomechanical testing.

Biomechanical properties of the samples were measured using a custom made computer controlled material testing device [3]. A stepwise stress-relaxation indentation (three 5% compressions with 30 min relaxation time) was done with a plane-ended indenter (d = 1.01) at room temperature. At all times (also during biomechanical testing and freezing) the samples were kept in same solution bath in groups 1, 2 and 3. Before freezing, samples in group 4 were immersed into 30% dimethylsulfoxide (DMSO)/saline solution bath, containing same concentration of enzyme inhibitors and antibiotics as the PBS solution, for at least 1 h 30 min [4]. This was done to protect cartilage from possible damages caused by ice formation during freezing [1].

In the group 1 (control), the water content analysis of the samples was performed immediately after biomechanical analyses, while the samples in groups 2, 3 and 4 were frozen for 21-24 h at -21°C. Freezing was followed by thawing and storage at room temperature (22-33°C) for 18 h. After approximately 1 h of thawing, the samples in group 4 were moved back to PBS saline containing 5 mM benzamide, 5 mM EDTA and 100 U/ml penicillin and 100 mg/ml streptomycin. After 18 h storage at room temperature, a biomechanical stress-test was performed with the same protocol as before.

After the second biomechanical stress-relaxation test, the water content of the samples in groups 2, 3 and 4 was analyzed. The samples were cut vertically in half, and subchondral bone was removed with a razor blade. The wet weight of each sample (half of the articular cartilage plug) was measured. Subsequently, the samples were freeze-dried (Christ Alpha 1-2, B Braun Biotech Inc., Allentown, USA). After lyophilization (18 - 68 h), the dry weight of the samples was measured.

Young’s modulus determined in indentation geometry was calculated from equilibrium modulus. Dynamic modulus was determined from peak-to-peak values, of the second compressive step, using the stress/strain information. [5] Statistical analyses were conducted using the SPSS Statistics software (SPSS Inc. Chicago, USA). A Wilcoxon signed ranks test was performed to test differences between parameter values before and after the freeze-thaw cycle.

RESULTS
There were no significant differences in water content between the groups (mean values 77.7-78.1 %). A slight trend towards lower dynamic modulus was seen in all sample groups (Table 1), but it was statistically significant only in samples of group 3, which were kept in the presence of enzyme inhibitors and antibiotics. A larger amount of samples is needed to find out whether this unexpected finding will remain, since the equilibrium modulus in the same group was slightly higher after the freezing.

Table 1. Mean values ± SD of biomechanical parameters of the samples before and after the freeze-thaw cycle.

<table>
<thead>
<tr>
<th>Group</th>
<th>Equilibrium modulus (MPa)</th>
<th>Dynamic modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BF</td>
<td>AF</td>
</tr>
<tr>
<td>1</td>
<td>0.76 ± 0.16</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.90 ± 0.19</td>
<td>0.87 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>0.81 ± 0.34</td>
<td>0.84 ± 0.32</td>
</tr>
<tr>
<td>4</td>
<td>0.88 ± 0.20</td>
<td>0.89 ± 0.19</td>
</tr>
</tbody>
</table>

1 = Control group; 2 = Samples in PBS bath; 3 = Samples in PBS bath with enzyme inhibitors and antibiotics; 4 = Samples in PBS bath with enzyme inhibitors and antibiotics, freezing in 50 % DMSO; BF = Before freezing; AF = After freezing.

DISCUSSION
In this study we investigated the effects of freeze-thaw cycle on articular cartilage mechanical properties. Furthermore, the effectiveness of the use of CPAs and inhibitors of proteolytic enzymes was studied.

Based on our results, a freeze-thaw cycle does not appear to significantly affect the biomechanical properties of articular cartilage. Zheng et al. [1] reported a significant GAG loss caused by freezing and thawing on articular cartilage without the use of DMSO during the freezing. Such a loss can be expected to affect also the biomechanical properties of the AC remarkably, which was not observed in this study. This difference in the results could be due to the small number of samples (n = 9) used in our study. The only statistically significant difference was a slight decrease in the dynamic moduli in samples immersed in PBS containing proteolytic inhibitors and antibiotics. Logically, this group should have shown the smallest differences due to freeze-thaw cycle. Thus, collection of larger amount of samples and their further biochemical and quantitative histology analyses, which are used to evaluate the proteoglycan content and integrity of the collagen network, are needed to be able to evaluate the effects of freeze-thaw cycle on the articular cartilage structure.

REFERENCES