Introduction
Medial Collateral Ligament (MCL) injuries represent a significant cost to society in lost work and recreational time. The ability to better understand the factors involved in MCL healing would allow us to potentially improve or speed the healing process. Smoking is a common co-morbidity in people who sustain ligament injury. The effect of smoking, while studied in other disease conditions, has never been evaluated in ligament healing. We hypothesize that smoking is deleterious to the multifactorial cascade of MCL injury healing. The purpose of this study is to examine this hypothesis in vivo.

Methods
One hundred and twenty male SV129 white mice, 24-28 weeks old were used for the study. The experiments were performed under the approval of animal committee of Washington University. The smoking group of mice were exposed to cigarette smoke in a smoking chamber for one month prior to MCL surgery. The mice were exposed to the equivalent of two unfiltered cigarettes per day. MCL surgery was performed on the anatomical left knee. With 2.5 loupe magnification, the MCL underwent a complete transverse laceration with a 30G needle 2 mm distal to the joint line. The anatomical right knee on the same smoking mouse served as a sham control, with an identical skin and fascia incision but with no disturbance to the MCL. The animals were sacrificed at 7 and 28 days following surgery using a carbon dioxide chamber. For the histological studies the distal femur just above the knee joint and the proximal tibia just below the knee joint, were completely cut. The specimens were fixed in 3% paraformaldehyde in Phosphate Buffered Saline (PBS) for 24 hours. The specimens were then placed in Ethylenediamine Tetraacetic Acid (EDTA) at a pH of 7.6 for bone decalcification before being embedded in paraffin blocks. Approximately, twenty, 5 micron sections were selected from each specimen block. For the biomechanical study, the mice were kept frozen in a – 70 degree Celsius freezer till the day of testing.

In situ hybridization for type I collagen: Prehybridization treatment of the fixed tissue sections was carried out under Rnase free conditions. A hybridization solution containing type I procollagen (HF 667) antisense cRNA probe labelled with [35S]-uridine triphosphate, were used to detect procollagen synthesis. Hybridization was allowed to proceed overnight. Next day the slides were washed and dipped in nuclear track emulsion for autoradiographic analysis.

Biomechanical testing: Each lower limb was disarticulated at the hip joint. The femur and tibia were potted in 6. Saline (PBS) for 24 hours. The specimens were then placed in Ethylenediamine Tetraacetic Acid (EDTA) at a pH of 7.6 for bone decalcification before being embedded in paraffin blocks. Approximately, twenty, 5 micron sections were selected from each specimen block. For the biomechanical study, the mice were kept frozen in a – 70 degree Celsius freezer till the day of testing.

In situ hybridization for type I collagen:
A grading scale (0-5) was used to evaluate type I procollagen gene expression for in situ hybridization activity: 0 equals no gene expression above normal activity; 1 equals light signal throughout the ligament; 2 equals strong signal at the laceration site involving several cells with light signal in the remaining ligament; 3 equals strong signal in the laceration site and light signal in the remaining ligament; 4 equals strong signal at the laceration site and some strong signal in the remaining ligament; 5 equals strong signal throughout the ligament with no difference between the laceration site and the remaining ligament. The data showed that at day 7 post laceration the laceration site could not be identified in either the smoking and non-smoking mice. The smoking mice showed a trend in an increased level of type I procollagen mRNA (grade 4 to 5) indicating a retarded and less mature ligament healing when compared to the non smoking mice which showed a decreased level of type I procollagen mRNA (grade 3 to 4). When reviewing the ligament healing at day 28 post laceration both groups showed similar expression of type I procollagen mRNA.

Smoking effect on functional MCL healing:
Our data showed a trend for the smoking mice to be weaker when compared to the non smoking mice as regards the average ultimate force at which the ligament fails and also for the rigidity.

<table>
<thead>
<tr>
<th>Count</th>
<th>Mean</th>
<th>S.D.</th>
<th>Standard error</th>
</tr>
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<tbody>
<tr>
<td>Non smoker, day 7</td>
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<td>0.980</td>
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Discussion:
Smoking has been shown to have a probable adverse relationship with several orthopaedic conditions including osteoporosis, hip fracture rate and bone and wound healing (1). Collagen synthesis and fibroblast proliferation are closely related to oxygen availability. Oxygen is required for hydroxylation of proline and lysine, a step that is essential for the synthesis of collagen by fibroblasts (2). Both our collagen synthesis and biomechanical data support the finding of retardation of healing in the smoking animals. However, levels of cigarette smoke inhaled by the mice in this study are equivalent to light smoking in humans. In further studies, the dose and duration of smoking will be increased to model moderate to heavy smoking.

References:
(2) Ishii, Miyanaga; J Ortho Research.20 (2002) 353-356