Author's response to reviews

Title: Synoviocytes protect cartilage from the effects of injury in vitro

Authors:

Christina M Lee (Christina.Lee@colostate.edu)
John D Kisiday (John.Kisiday@colostate.edu)
Wayne McIlwraith (Wayne.McIlwraith@colostate.edu)
Alan J Grodzinsky (alg@MIT.edu)
Frisbie D David (David.Frisbie@colostate.edu)

Version: 2 Date: 14 December 2012

Author's response to reviews: see over
Dear review committee,

Please find our attached revised manuscript entitled “Synoviocytes protect cartilage from the effects of injury in vitro” we eagerly re-submit for publication in the journal *BMC musculoskeletal disorders*. We believe the recommendations made by the reviewers greatly strengthened the presentation of the manuscript. The authors had no issues relating to the editorial policies of this publication.

Specific changes made:
The image of passage 3 primary synoviocytes seen below was inserted as Figure 1 and referenced in the methods section. After 32 days in culture the cells show the same phenotype but are at 100% confluency. This image could be provided in the end of January 2013.

![Figure 1. Fibroblastic synoviocyte phenotype. Digital Image of synoviocytes harvested from a horse and expanded to passage 3. Image obtained at 10x magnification.](image)

The methods to mechanically injure the cartilage were expanded upon and the manuscript detailing the techniques was referenced.

The cartilage – synoviocyte co-culture section of the methods was described in more detail to more clearly describe the experimental conditions and the rationale for choosing the variations time points for each portion (the gene expression and the Histology) of the study.

The sample collection and sectioning was clarified to read “For histology and immunohistochemistry (IHC) cartilage samples at days 8, 16 and 32 were removed from culture and sectioned across the diameter perpendicular to the fissures when present (arbitrarily in the middle of the sample when not) and always along a plane perpendicular to the superficial surface.”

The protocol used to extract RNA from the pulverized cartilage samples was described in detail.

The table of primer and probe sequences generated at the ORC was provided.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1B</td>
<td>AGTCTTCAGTGCTCAGGTTTCTGA</td>
<td>TGCCGCTGCAGTAAGTCATCCAGCCATGGCAGCAGTACCCGA</td>
<td>CAGCCATGGGCAGCAGTACCCGA</td>
</tr>
<tr>
<td>IL-10</td>
<td>TTCAGCAGGGTGGAAGACTTT</td>
<td>CTTGGCAACCCAGGTACCC</td>
<td>TGTGAACGGGTCCCTGCTGGA</td>
</tr>
</tbody>
</table>
The section grading piece of the methods was described in more detail to detail the maximum score of 12.

The figures have been modified to include a table of the delta CT values. The modified figures are below.
A summary figure of representative images of histology and immunohistochemistry was inserted as figure 7.

![Representative images of cartilage samples in each culture condition after 32 days in culture. Images represent slides stained with SOFG and H&E or by immunohistochemistry to detect collagen type II and aggrecan. Box inserted into the images showing collagen type II and aggrecan show the negative control slides.]

Figure 7. Representative images of cartilage samples in each culture condition after 32 days in culture. Images represent slides stained with SOFG and H&E or by immunohistochemistry to detect collagen type II and aggrecan. Box inserted into the images showing collagen type II and aggrecan show the negative control slides.

All figure numbers have been modified to reflect the insertion of the new figures.

The discussion was shortened by the removal of information that was not directly related to the finding or explanation of the results. A paragraph was added as requested to provide explanation about the lack of IL-1 gene expression and MMP-13 gene expression not being affected by injury.

Sincerely,

Christina Lee, John Kisiday, Wayne McIlwraith, Alan Grodzinsky and David Frisbie
Corresponding Author:

David D. Frisbie
Orthopaedic Research Center
Colorado State University
300 W. Drake Road
Fort Collins, CO 80523
Phone: 970-297-4555
Fax: 970-297-4138

dfrisbie@colostate.edu
Reviewer 1 Anna Plaas

Major Compulsory Revisions:

1) Synoviocyte Cultures:

The authors point out in the Conclusion that the phenotypic characterization of the synoviocytes used in this study is unclear. It would be helpful to include a picture of the typical appearance of the cultured cells pre-and post-co-culture (i.e. up to 32 days).

The image of passage 3 primary synoviocytes seen below was inserted as Figure 1 and referenced in the methods section. After 32 days in culture the cells show the same phenotype but are at 100% confluency. This image could be provided in the end of January 2013.

What is the cell viability of synoviocytes at 32 days post-culture?

After 32 days all cells are presumed viable as they remain adhered to the plate.

In the Methods Section, p 6, the authors state that the synoviocytes were cultured in HAM’s F12 medium. The co-culture is performed in a mixture of DMEM/F12. In Figure 1 changes in synoviocyte gene expression during co-culture were expressed relative to synoviocytes only. Does the medium composition alone, (F12 vs. DMEM/F12) affect the gene expression? What effect does inclusion of HEPES during co-culture have on synoviocyte metabolism?

To clarify, the synoviocytes were expanded in culture to passage 3 in Ham’s F12 with Hepes, the manuscript has been modified. At the commencement of the experiment, all samples including the synoviocytes, the cartilage and synoviocyte co-cultures, and the cartilage cultures were cultured in media of half F12 and half DMEM. Changes in gene expression were relative to the synoviocytes cultured alone which were treated with the same media as all other experimental conditions; therefore there is little concern about the effect of the medium composition on the relative gene expression results. Hepes is commonly used in synoviocyte medium, including commercially produced complete media, and is not expected or considered to have ill effects on synoviocyte metabolism.

2) Quantitative PCR data:

• The authors should include information in the Methods (p 7 and 8) describing
the yields and purity of RNA preparations from the cartilage samples. How well was RNA extracted without pulverization and/or homogenization of the tissue?

Without pulverizing or homogenizing the samples RNA was not able to be extracted from cartilage.

The authors state that cells were rinsed with ice-cold PBS prior to the addition of lysis buffer. What effect would the cold temp. rinses have on ‘stability’ of RNA?

Rinsing the monolayer with cold PBS was recommended by the manufacturer of the RNeasy kit as this removed any trace amounts of nucleases that may have been present in the culture media and thus left behind on the monlayer while keeping the temperature cool which stabilizes RNA.

A table should be included with the delta CT values (including GAPDH). This would provide important information on the ‘major’/minor' genes active in the two systems as well as over time.

The tables below have been created to display the delta CT values represented in the graphs. GAPDH was not included in the tables to save space as the delta CT value of GAPDH is always 0.

<table>
<thead>
<tr>
<th></th>
<th>MMP1</th>
<th>MMP3</th>
<th>MMP13</th>
<th>ADAMTS4</th>
<th>ADAMTS5</th>
<th>TIMP-1</th>
<th>cox-2</th>
<th>TGFB</th>
<th>IL-6</th>
<th>FGF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.63</td>
<td>7.05</td>
<td>7.04</td>
<td>8.32</td>
<td>4.78</td>
<td>-0.48</td>
<td>8.62</td>
<td>8.49</td>
<td>4.31</td>
<td>4.63</td>
</tr>
<tr>
<td>Injured</td>
<td>6.39</td>
<td>8.29</td>
<td>7.11</td>
<td>8.84</td>
<td>5.84</td>
<td>0.26</td>
<td>6.55</td>
<td>9.61</td>
<td>5.56</td>
<td>6.29</td>
</tr>
<tr>
<td>Alone</td>
<td>7.44</td>
<td>8.60</td>
<td>8.39</td>
<td>8.22</td>
<td>4.80</td>
<td>-0.58</td>
<td>5.48</td>
<td>8.45</td>
<td>4.52</td>
<td>5.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1 Day</th>
<th>2 Day</th>
<th>3 Day</th>
<th>4 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.00</td>
<td>6.86</td>
<td>9.30</td>
<td>6.23</td>
</tr>
<tr>
<td>Injured</td>
<td>4.63</td>
<td>5.65</td>
<td>9.65</td>
<td>6.74</td>
</tr>
<tr>
<td>Alone</td>
<td>5.13</td>
<td>7.70</td>
<td>9.25</td>
<td>6.68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Col2</th>
<th>Agg</th>
<th>MMP1</th>
<th>MMP3</th>
<th>MMP13</th>
<th>ADAMTS4</th>
<th>ADAMTS5</th>
<th>TIMP-1</th>
<th>cox-2</th>
<th>TGFB</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injured</td>
<td>0.46</td>
<td>2.16</td>
<td>7.50</td>
<td>6.12</td>
<td>3.06</td>
<td>7.83</td>
<td>8.19</td>
<td>1.87</td>
<td>7.77</td>
<td>10.04</td>
<td>9.56</td>
</tr>
<tr>
<td>Injured co-culture</td>
<td>-0.13</td>
<td>2.37</td>
<td>5.53</td>
<td>6.82</td>
<td>1.89</td>
<td>5.77</td>
<td>5.78</td>
<td>1.64</td>
<td>5.36</td>
<td>7.57</td>
<td>6.59</td>
</tr>
<tr>
<td>Control</td>
<td>1.15</td>
<td>2.69</td>
<td>5.57</td>
<td>7.26</td>
<td>3.46</td>
<td>5.98</td>
<td>5.98</td>
<td>1.53</td>
<td>7.32</td>
<td>2.80</td>
<td>6.00</td>
</tr>
<tr>
<td>Control co-culture</td>
<td>1.59</td>
<td>2.41</td>
<td>6.15</td>
<td>6.86</td>
<td>3.10</td>
<td>6.33</td>
<td>6.32</td>
<td>2.54</td>
<td>7.04</td>
<td>8.20</td>
<td>7.31</td>
</tr>
<tr>
<td></td>
<td>1 Day</td>
<td>2 Day</td>
<td>3 Day</td>
<td>4 Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injured</td>
<td>8.83</td>
<td>8.40</td>
<td>11.67</td>
<td>11.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injured</td>
<td>6.68</td>
<td>8.61</td>
<td>6.59</td>
<td>11.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>co-culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.32</td>
<td>-3.52</td>
<td>7.935</td>
<td>11.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.29</td>
<td>6.16</td>
<td>8.51</td>
<td>10.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>co-culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

What is the rationale behind the choice of TIMP1? TIMP3 is the potent inhibitor for ADAMTS4 and 5, and should be included also.

In this study we found an increased expression of MMP-1 by synoviocytes cultured in the presence of injured cartilage cultured. TIMP-1 expression has repeatedly been shown to coincide with MMP-1 expression (Tang et al. 2009, Dahlberg et al. 1994), therefore we sought exploration into TIMP-1 expression. By no means were the genes examined expected to be an exhaustive evaluation of all genes affected by these conditions. Gene expression analysis was limited by the amount of sample available for gene expression analysis, and a limited number of real time PCR assays available. Indeed the reviewer brings up a good point, yet we do not have an assay for the screening of the equine TIMP-3 gene. This recommendation will certainly be considered for future studies.


A figure showing typical examples of cellularity, Safranin O staining and IHC for collagen II and aggrecan for each of the experimental conditions should be provided.

Figure 7 inserted
Figure 7. Representative images of cartilage samples in each culture condition after 32 days in culture. Images represent slides stained with SOFG and H&E or by immunohistochemistry to detect collagen type II and aggrecan. Box inserted into the images showing collagen type II and aggrecan show the negative control slides.

As per Method description, p9, one section was used for estimating cartilage integrity. How representative is that section? Was the scoring done blinded?

The authors believe a stained section of the cartilage from the middle of each sample that included the fissure sufficiently represents the histologic changes in the cartilage occurring from injury. Each sample of cartilage was harvested from the proximal region of the medial trochlear ridge. The cartilage in this region is known to be rather uniform in thickness as we observed with this study and our previous [1, 2]. The cartilage plugs were 5 mm in diameter and injured by a 10 mm in diameter impermeable platen thereby invoking compression uniformly across the cartilage sample. Examining a section in the middle of the cartilage eliminates the inclusion in the analysis of artifacts associated with harvesting the sample from the joint. All scoring was conducted without the evaluators being aware of treatment group (Dr Lee and Dr Frisbie).
• Figs 3 and 4. Cell death and cell proliferation should also be biochemically quantitated, i.e. by assaying for total DNA contents and/or performing specific evaluation for ‘apoptosis’ (QPCR or IHC for markers) and proliferation (e.g. IHC using Ki67).

The purpose of this study was to compare the effects of synoviocytes on the characteristic of osteoarthritis that develop in equine full thickness cartilage after injury as previously conducted [2]. In the previous study we found regional changes in chondrocyte cell death and focal cell loss that biochemical evaluation would not detect. The reviewers suggestions are indeed of interest to the authors and may be carried out in a later study that further explores the modality of cell death and proliferation.

• The authors should provide information where the changes in cell numbers occurred relative to the injury and/or the cut edges of the explant.

The cut edges of the sections were not evaluated for histologic changes. When grading the slides the outer 5% of the tissue around the left and right edges and below the deep zone were not graded. This statement was added to the manuscript.

• As above, a biochemical confirmation of cartilage matrix destruction should be provided. This could be done for example by western analyses for aggrecan or DMMB assay for S-GAG contents, OH-Proline assay or SDS-PAGE electrophoresis solubilized collagen.

As mentioned, the goal of this study was to compare the previously conducted study of cartilage injury to the effects of synoviocytes on injured cartilage. Much of the data of interest in the previous study demonstrated regional changes associated with early osteoarthritis as opposed to global changes throughout the tissue that would be detected by the suggested biochemical analysis. Indeed we did evaluate GAG content via DMMB analysis for the 4 of the experiments and did not detect any differences in GAG content.

4) Discussion:
• The discussion should be significantly shortened and aimed at describing the potential mechanism. From the data shown, the major effect of the synovial co-culture is the protection against overall cell loss in the cartilage. What mediators could be responsible for this? Have the authors considered other anabolic factors such as BMP7, EGF, and IGF1? Can they be assayed in this experimental system (e.g. QPCR, immunoassay, blocking antibodies, addition of exogenous factors)

The discussion has been shortened. Suggested mechanisms have been provided in the discussion as they are supported by the data.

Minor Essential Revisions
• *Figures 1A, 2A, 3A – what time point post-culture do those data represent?*

Figures 1A, 2A, 3A are graphical representations of the gene expression when all time points are combined for each condition as for these genes time in culture did not have a significant effect on gene expression. Figure legends have been modified to include “Data presented as mean relative expression across all time points for each culture condition (± SEM).”

• *Figure Legend 1A- Please correct – it should be for synoviocytes cultures in the presence of control and injured cartilage.*

Figure legend has been corrected.
Reviewer 2: Milena Fini

Major Compulsory Revisions:

Synoviocyte isolation
I understood that the synovial tissue was harvested from 6 different animals and not from the 12 animals used for the extraction of the osteochondral plugs. This methodology should be discussed in the paper.

This section of the methods has been modified to read “Cartilage-synoviocyte co-culture: Synoviocyte and cartilage were co-cultured using a transwell plate with a 0.4 µm microporous insert (Corning Inc., Corning, NY). Cartilage and synoviocytes were randomly paired for co-culture to form 2 sets of experiments. One set of experiments involved the culture of synoviocytes with cartilage for gene expression analysis at 1, 2, 4 and 8 days in co-culture. The other set of experiments involved the culture of synoviocytes with cartilage for histologic and immunologic evaluation at days 8, 16 or 32 in culture. The time points to evaluate gene expression was determined based on previous studies that have evaluated changes in gene expression in cartilage after injury [3, 4]. For histologic evaluation we extended the duration of culture because in previous studies (Lee 2013) we found injured cartilage required culture for at least 28 days to develop histologic changes. Each experiment was conducted in duplicate, and all experiments repeated a total of 6 times using cartilage from a new horse for each experiment and synoviocytes from 6 different horses total; synoviocytes from each horse were used for one gene expression and one histology experiment. Each experiment consisted of four conditions, Injured and control (uninjured) cartilage cultured alone or with synoviocytes. Experiments for gene expression analysis included the fifth condition of synoviocytes cultured alone. Forty-eight hours prior to commencement of experiment synoviocytes were recovered from liquid nitrogen and seeded into the bottom well of a 24-well transwell system at a seeding density of 5000 cells/cm² and cultured in growth media composed of half cartilage DMEM media and half synoviocyte F12 media. Immediately after injury, cartilage plugs were added to the top well of the transwell system, uninjured control cartilage samples were maintained in parallel. All samples remained in culture for 1, 2, 4, 8, 16 or 32 days, with media changes every 2-3 days. “

Mechanical injury
The protocol to induce injury in equine cartilage may be explained or referenced because it is of fundamental importance for the entire study. In particular, the similarity between laboratory induced lesions and the in vivo ones should be better documented.

This section has been modified to reference the manuscript where the mechanical injury model is thoroughly defined as follows;“Mechanical injury: Just prior to injury, the thickness of each cartilage plug (plane perpendicular to the articular surface; average thickness 1.33 mm) was measured using digital calipers. Cartilage plugs were injured as previously defined (Lee et al 2013). In brief, cartilage plugs were removed from
media and placed into a sterile polysulphone loading chamber consisting of a well aligned coaxially with an impermeable platen (10 mm diameter) in the absence of media. Using a H1KS benchtop universal testing machine (Tinius Olsen, Horcham PA) an initial compressive tare load of 4 N was applied. After creep equilibrium was attained for 5 seconds, an injurious compression at a rate of 100% strain/second was applied until 60% final cartilage strain was achieved. Compression was then released at the same rate and plugs were immediately placed into the appropriate culture condition.”

Sample collection

It is reported that when it was not possible to identify the specimen fessures, specimens were sectioned in an arbitrarily plan. I think that the section plane is important for the comparative evaluation of tissues and that the arbitrarily selected plan could not be idoneous.

All samples were sectioned in the same plane by sectioning the sample in half perpendicular to the superficial layer. In the event a fissure was present the sample was sectioned such that the fissure was bisected. In the absence of a fissure there was no discerning architecture or characteristic on the surface of the cartilage so consistently section. Our goal was to section each sample to one half could be formalin fixed for paraffin embedding and section with the mirrored face frozen embedded for immunohistochemistry. That way the plane examined in the paraffin samples was the mirror section of the plane examined for IHC. We wanted each plane to include all regions of the cartilage (the superficial, middle and deep zone) and since cellular architecture and ECM composition often differs in the regions around the fissure, we wanted to capture those areas in the plane when present.

Real time PCR

It is of importance to specify the primer sequences and the TAQMAN probes used in the study.

The primer and probe sequences used for this study have been included in the methods section for the assays we have access to. The authors do not have access to the sequences for the Gene assays purchased from the Lucy Whittier Molecular and Diagnostic core facility at University of California, Davis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1B</td>
<td>AGTCTTCAGTGCTCAGGTTTCTGA</td>
<td>TGCCGCTGCAGTAAGTCATC</td>
<td>CAGCCATGCGCAGTACCCGA</td>
</tr>
<tr>
<td>IL-10</td>
<td>TTCACGGAGGGTGAAGACTTCC</td>
<td>CTTGGCAACCCAGGTAACCCTTA</td>
<td>TGTTGAACGGGTCCTGCTGAG</td>
</tr>
<tr>
<td>TGF-B</td>
<td>GTTAAGCGTGAGACGACAT</td>
<td>AGTGACATCAAAGGACAGCC</td>
<td>CTGCTGACCCCCAGCGACTCG</td>
</tr>
<tr>
<td>ADAMTS4</td>
<td>TGTGATCGTGCTAATGGCTCC</td>
<td>TGGTTCAGCAGCTAAGACCATC</td>
<td>AGTTTGACAAGTGCGATGGTGGGT</td>
</tr>
<tr>
<td>ADAMTS5</td>
<td>AAGGTGACTGATGGGACCAGATGT</td>
<td>TTTGAGCCAAATGATGCGTCACAG</td>
<td>AGGCCATACAGTAATTCCGTCTG</td>
</tr>
</tbody>
</table>

Histology
The use of only one section for each sample could be a limitation for the qualitative and quantitative histologic evaluations. The execution of an histological score usually require 3 sections of the same sample. Usually to evaluate GAGs Toluidine Blue staining is used. Some sections stained with Safranin O and Fast Green should be added to see the presence of GAGs with these stainings.

While serial sections or multiple sections are certainly better than a single section the multiple sections of various staining and immunohistochemistry limited the sections that were prepared. It is important to realize that a difference was seen in the current study, it is noted in the manuscript that this was accomplished with a single section.

**Minor Essential Revisions**

References 1 and 7 are not directly related to the statements.

Reference 1 has been removed.

References 2, 8, 9, 10, 12, 46 are old.

The references mentioned are from the early times of research in this field but the authors feel are still relevant and valid references.

*In References 12, 28, 36, 39 the year of publication is not reported.*

This has been corrected.

**Isolation of cartilage plugs**

- Please specify the height of the osteochondral plugs and the surgical aseptic conditions.

To harvest the osteochondral plugs from the joints the a commercial harvester was used (Sontec, Centennial Colorado) was used as per the manufacturers recommendation as well as the authors clinical experience (DDF). The exact length of bone attached varied based on where the bone snapped which was anywhere from 3 mm to 10 mm below the calcified cartilage interface. The height of each osteochondral plug was not a critical or important parameter in this study. Osteochondral plugs were merely collected to ensure the harvest of full thickness cartilage.

This section was modified to read “Using aseptic technique (sterile gloves, instruments and media) osteochondral plugs 5 mm in diameter (varying heights) were harvested from the trochlear ridges of the left and right limbs using a customized cylindrical osteotome (Sontec Instruments, Centennial CO).

- The sentence “Due to the non-uniform swelling….after extraction from the joint” require more details.
Due to the non-uniform swelling of the cartilage that occurs during culture when the cartilage remains attached to the subchondral bone, the cartilage was removed from the subchondral bone at the calcified and non-calcified cartilage junction immediately after extraction from the joint.

- Was the culture medium changed at 24 hrs?

The samples in culture were checked after 24 hours of harvest to ensure there was no contamination, but the media was not changed until after 48 hours at the onset of the experiment.

**Cartilage-synoviocyte co-culture**

*What is meant with “Free swelling control cartilage samples”?*

The term “free swelling control” was referring to the culture condition of the sample, meaning that the cartilage plugs were maintained in culture without any load applied or opposing forces to keep samples in a defined shape or space. The authors realize that this terminology can be confusing and is not necessary given that all samples were maintained in culture in this same manner and have thus eliminated this phrase from the next to refer to controls and “uninjured controls”.

*The co-culture conditions are not exhaustively explained in the text*

The text has been modified to read “Cartilage-synoviocyte co-culture: Synoviocyte and cartilage were co-cultured using a transwell plate with a 0.4 µm microporous insert (Corning Inc., Corning, NY). Cartilage and synoviocytes were randomly paired for co-culture to form 2 sets of experiments. One set of experiments involved the culture of synoviocytes with cartilage for gene expression analysis at 1, 2, 4 and 8 days in co-culture. The other set of experiments involved the culture of synoviocytes with cartilage for histologic and immunologic evaluation at days 8, 16 or 32 in culture. The time points to evaluate gene expression was determined based on previous studies that have evaluated changes in gene expression in cartilage after injury [3, 4]. For histologic evaluation we extended the duration of culture because in previous studies [2] we found injured cartilage required culture for at least 28 days to develop histologic changes. Each experiment was conducted in duplicate, and all experiments repeated a total of 6 times using cartilage from a new horse for each experiment and synoviocytes from 6 different horses total; synoviocytes from each horse were used for one gene expression and one histology experiment. Each experiment consisted of four conditions, Injured and control (uninjured) cartilage cultured alone or with synoviocytes. Experiments for gene expression analysis included the fifth condition of synoviocytes cultured alone. Forty-eight hours prior to commencement of experiment synoviocytes were recovered from liquid nitrogen and seeded into the bottom well of a 24-well transwell system at a seeding density of 5000 cells/cm² and cultured in growth media composed of half cartilage DMEM media and half synoviocyte F12 media. Immediately after injury, cartilage plugs were added to the top well of the transwell system, uninjured
control cartilage samples were maintained in parallel. All samples remained in culture for 1, 2, 4, 8, 16 or 32 days, with media changes every 2-3 days.”

**RNA extraction**
- There are some spelling errors in the section.

This has been corrected.

- For the RNA extraction: was the integrity of the sample verified?

The quality but not integrity of the RNA was determined by way of 260/280 ratios. All samples had a 260/280 ratio between 1.8 and 2.0.

- Please explain the steps for the sample processing.

The section has been modified to read “The homogenized sample was then allowed to incubate at room temperature for 20 minutes in Trizol followed by centrifugation at 12,000 x g for 12 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube, mixed with 400 ul chloroform, incubated at room temperature for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new microcentrifuge tube, mixed with 200 ul chloroform, incubated at room temperature for 3 minutes then centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new microcentrifuge tube to which 500 ul isopropanol was added. The samples were incubated at room temperature for 10 minutes then centrifuged at 12,000 x g for 10 minutes at 4°C to pellet the RNA. The RNA fraction was cleaned with 70% ethanol and re-suspended with RNase free water followed digestion of genomic DNA using RNase free DNase (Qiagen, Valencia, CA).”

**Real time PCR**

It is reported on “Equal concentration of RNA…” but what is the concentration?

The sentence has been modified to read “Equal concentrations of RNA (5 ng) from each sample”

**Histology**

- Which is the percentage of formalin used?

10% neutral buffered formalin as indicated in the sample collection section.

- Please add information on the used microtome and image analysis system

This section has been modified to read “Histology: Formalin fixed samples were paraffin embedded, and sectioned (5 µm thick) using a Leica RM2255 microtome (Leica Biosystems, Buffalo Grove IL). One slide each from an injured and control sample was stained with Hematoxylin and Eosin (H&E) to evaluate cellular pathologic changes or
Safranin O Fast Green (SOFG) to detect changes in regional glycosaminoglycan (GAG) content. All images of slides were obtained using Q-capture (QImaging, Surrey BC).

- It is unclear why the maximum score is 12 and, Please explain it better in the text.

This section has been modified to read “For each OA characteristic, scores for each region (superficial, middle, deep and fissures) were evaluated separately then summed together for each slide, the maximum score for each of the 4 regions was 3 creating a maximum score for the regions combined of 12.”

Results
Gene expression
- The standard deviation of MMP-13, COX-2 TIMP-1 and Agg are very high. How it is explained?

These samples were harvested from different animals. The high standard deviation is likely attributable to variation in gene expression between animals.

- The same p-value (or p= or p<) should be used thorough the paper.

The authors have reported the p-value as accurately as possible. When an exact p-value was calculate then p= was used. When the p-value was calculated at less than 0.0001 an exact value was not able to be determined and thus must be presented as p<. The authors have made sure this format was consistent throughout the paper, this is also how the p-values are reported in SAS.

Discussion
In my opinion, Discussion paragraph should be shortened

The discussion has been shortened to include only information pertaining to the interpretation of the results.
Reviewer 3: Lia Pulsatelli

Discretionary Revisions
- **Experimental design:** The experiments were performed at different time-points. The Authors should clarify why they chose these time points. On the basis of preliminary time-dependent experiments?

The authors have inserted the following into the methods; “The time points to evaluate gene expression was determined based on previous studies that have evaluated changes in gene expression in cartilage after injury [3, 4]. For histologic evaluation we extended the duration of culture because in previous studies (Lee 2013) we found injured cartilage required culture for at least 28 days to develop histologic changes.”

- Results:
  a) **One of the main features of joint injury is the increased production of pro-inflammatory cytokines, such as IL-1 beta.** Furthermore, among matrix degrading enzymes, MMP-13 has been recognized as having an important role in inducing OA cartilage damage. Data presented in this study showed a total absence of IL-1 beta gene expression in injured cartilage samples and no significant difference in expression levels of MMP-13 between injured cartilage and control samples. The Authors should discuss these findings.

  The following section has been added to the discussion “Interleukin -1β and MMP-13 have both been extensively demonstrated to play a prominent role in the progression of OA yet IL-1β gene expression by chondrocytes was not detected in the present study and MMP-13 expression levels were not affected by injury or culture condition. Interestingly this finding is consistent with a recent study conducted by Ross et al 2012 involving an in vivo model of experimentally induced synovitis in the horse [5]. In the in vivo study the data strongly demonstrated the development of a model consistent with traumatic arthritis and OA, yet IL-1β gene expression levels were not consistently detectible and MMP-13 expression did not differ between normal and inflamed joints. These results collectively suggest IL-1β and MMP-13 gene expression may not be a critical component of traumatic joint injury in horses.”

  b) **Why the Authors reported the results concerning time-dependent gene expression only for MMP-1 (synoviocyte samples) and TGF beta (cartilage samples)? Concerning the results reported in Figure 1A e Figure 2A, may the Authors specify the corresponding time point?**

  The results for MMP-1 (synoviocytes) and TGF-B (cartilage) were reported as time-dependent changes in gene expression because the expression of those genes in those culture condition were significantly affected by both time in culture and culture condition. For all other genes expressed, time in culture did not have a significant affect expression levels, only the culture conditions had an effect. The data presented in figure 1A and 2A are the changes in gene expression averaged across all time points for each culture condition.


