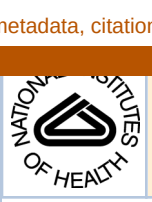

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Changes in chondrocyte gene expression following *in vitro* impaction of porcine articular cartilage in an impact injury model

Melissa S. Ashwell[1], **Michael G. Gonda**[1,3], **Kent Gray**[1,4], **Christian Maltecca**[1], **Audrey T. O'Nan**[1], **Joseph P. Cassady**[1], and **Peter L. Mente**[2]

[1]Animal Science Department, North Carolina State University, Raleigh, NC, USA

[2]Joint Department of Biomedical Engineering, North Carolina State University, Raleigh, NC, USA and University of North Carolina, Chapel Hill, NC, USA

Abstract

Our objective was to monitor chondrocyte gene expression at 0, 3, 7, and 14 days following *in vitro* impaction to the articular surface of porcine patellae. Patellar facets were either axially impacted with a cylindrical impactor (25 mm/sec loading rate) to a load level of 2000 N or not impacted to serve as controls. After being placed in organ culture for 0, 3, 7, or 14 days, total RNA was isolated from full thickness cartilage slices and gene expression measured for 17 genes by quantitative real-time RT-PCR. Targeted genes included those encoding proteins involved with biological stress, inflammation, or anabolism and catabolism of cartilage extracellular matrix. Some gene expression changes were detected on the day of impaction, but most significant changes occurred at 14 days in culture. At 14 days in culture, 10 of the 17 genes were differentially expressed with *col1a1* most significantly up-regulated in the impacted samples, suggesting impacted chondrocytes may have reverted to a fibroblast-like phenotype.

Keywords

Articular cartilage; Chondrocyte; Gene expression; Joint injury; Osteoarthritis

Introduction

OA is an extremely debilitating condition that affects upwards of 20 million people in the US alone (1). The causes of degenerative joint diseases such as OA are multifactorial and in many cases there may be no known initiating factor. Because many cases are idiopathic in nature, it is difficult to reproducibly initiate the disease process in a research model. Joint injury and instability are known predisposing factors, making injury models useful in the study of joint degeneration (2-5). Ligament cutting (6-11) is a common injury model used to create joint instability; however, the complexity and ongoing nature of the unstable loading makes it difficult to quantify the injury and establish the timing of degenerative changes. Impact injuries, another common model used to initiate cartilage degeneration, have included *in vivo* single impaction (12-17) and multiple impactions (18, 19) as well as *in vitro* impactions to cartilage explants (20-29). Impact injuries have the advantage of initiating the degeneration using a known event that can be more easily followed. The cascade of events following an impact injury is very complex, influenced by factors within

reprint requests: Melissa S. Ashwell, North Carolina State University, Campus Box 7621, Raleigh, NC 27695 USA, Phone: 001-919-513-7488, Fax: 001-919-515-6884, msashwel@ncsu.edu.

[3]Current address: Animal and Range Sciences Department, South Dakota State University, Brookings, SD, USA

[4]Current address: Smithfield Premium Genetics, Rose Hill, NC, USAProofs

the cartilage tissue, underlying bone, and surrounding synovial tissue, however, the progressive degeneration that occurs in the articular cartilage is the most significant symptom.

We developed an *in vitro* model for joint injury in which the articular surface of intact porcine patellae are impacted and maintained, intact, in culture (30). In previous work with this model we observed cell necrosis increasing with time, peaking at 7 days post-impaction (15) while apoptosis peaked at 14 days, the longest time point examined. The timing of cell death and apoptosis indicates chondrocyte behavior is changing over the first few weeks following an injury. Understanding what happens to chondrocytes at the molecular level early in the disease process is critical to understanding the degenerative process and determining potential therapeutic interventions.

Previous studies have examined gene expression changes at various times following mechanical damage to cartilage. Burton-Wurster *et al.* (31) identified 172 significantly differentially expressed transcripts 24 hours following mechanical damage to canine cartilage explants. Chan *et al.* (32) used bovine cartilage explants and found 14 up-regulated genes and 5 down-regulated genes 3 hours after mechanical damage to the explants. The up-regulated genes included cytokine and chemokine receptors and signal transduction molecules, while the down-regulated genes included adhesion molecules and genes involved in apoptosis. Previously we used SAGE analysis to measure gene expression at 14 days post-impaction in *in vitro* impacted patellae (30). We found 30 differentially expressed genes following mechanical damage to the cartilage where the affected genes were involved in pathways affecting matrix remodeling, iron transport, protein synthesis, skeletal development, cell proliferation, lipid metabolism and biological stress. The above mentioned studies assayed differences in gene expression either within 24 hours or at 2 weeks after mechanical damage to the tissue. Our objective here was to use our model to measure expression of 17 genes at earlier time points (0, 3, and 7 days), in addition to 14 days, to ascertain the timing of gene expression changes and to help clarify how the chondrocytes' response is changing following an injury.

Methods

Tissue collection, impaction, and culture

Twenty-three porcine knee joints from sows (180 kg) were obtained from a local slaughterhouse. Patellae with visually healthy cartilage were harvested using a sterile technique as previously described (30) and randomly assigned to the impacted or non-impacted control group and culture times of 0 (no culture), 3, 7, or 14 days. Each patella was impacted on both the medial and lateral facets using a hydraulic load frame (MTS Minibionix 858, MTS, Minneapolis, MN) at a loading rate of 25 mm/sec to a load level of 2000 N. Contact was made using a 10 mm long, 10 mm diameter stainless steel cylindrical impactor oriented with the cylinder axis perpendicular to the loading direction. Following impaction, intact patellae were immersed in culture media [Delbecco's MEM/Ham's F12 with 10% fetal calf serum, ascorbic acid (25 μg/ml), and antibiotics (100 units/ml penn., 100 μg/ml strep, and 25 μg/ml amphotericin B) (Gibco, Grand Island, NY)] that was changed daily and kept at 37°C in a humidified incubator with 5% CO_2. After the proscribed culture time, full thickness cartilage samples from directly beneath the impaction measuring 5 mm wide by 10 mm long, were harvested. Tissue from the non-impacted patellae was treated and harvested in the same manner. Tissue from 0 day patellae was collected within two hours following completion of the impactions.

Quantitative real-time PCR (qPCR)

RNA was isolated using TRI Reagent as previously described (30). Seventeen genes were examined: *aggrecan* (*agc*), *calpain small subunit 1* (*capns1*), *chitinase-3 like-1* (*chi3l1*), *clusterin* (*clu*), *collagen type I alpha 1* (*col1a1*), *collagen type II alpha 1* (*col2a1*), *cathepsin B* (*ctsb*), *chemokine (C-X-C motif) ligand 16* (*cxcl16*), *decorin* (*dcn*), *epidermal growth factor receptor feedback inhibitor 1* (*errfi1*), *ferritin heavy polypeptide-1* (*fth1*), *ferritin light polypeptide* (*ftl*), *matrix metalloprotease* (*mmp) 1*, *mmp3*, *S100 calcium binding protein A11* (*s100a11*), *tissue inhibitor of metallopeptidase* (*timp)1*, and *timp3*. *Capns1, chi3l1, clu, col1a1, col2a1, ctsb, cxcl16, fth1, ftl, mmp3,* and *s100a11* were chosen because they were differentially expressed between impacted and non-impacted samples at 14 days in culture in our previous study using this *in vitro* model (30). *Agc* was selected because many studies have identified it as being differentially expressed in arthritic cartilage (33). *Dcn* was chosen because of its high abundance in normal and osteoarthritic human cartilage (34). *Errfi1* was selected because it was the most differentially expressed in a study comparing mechanically injured and control canine cartilage explants (31). *Mmp1* was selected because studies found that it was differentially expressed in cartilage tissue following mechanical pressure treatment (35). *Timp1* and *timp3* were selected because of their ability to down-regulate metallopeptidase activity (31, 33, 36-38) . PCR primers (Table S1) were designed as previously described (30). *Col1a1, col2a1* and *dcn* primer sequences were obtained from Chou *et al*. (39). Agc primer sequences were obtained from Bevill (40). cDNA was synthesized and amplified as previously described (30). Reactions were heated for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Statistical analysis

A mixed model theory, as developed by Steibel *et al*. (41), was used to analyze the expression of target and housekeeping genes:

$$y_{ijkl}=A_{i(j)}+TG_{kl}+e_{ijkl}$$

where i references the animal number, j the date the patella was collected from the slaughterhouse (accounting for potential differences due to timing of experiments), k the treatment (impacted or control in culture for 0, 3, 7, or 14 days), and l the gene of interest. The value y_{ijkl} is the cycle threshold (CT) obtained from the thermocycler for the random i animal within j day, corresponding to the k treatment for l gene (housekeeping or target gene). Expression was normalized to *beta actin* because we found it to be one of the most stably expressed genes in porcine cartilage (42). The factor TG_{kl} modeled the interaction of a combination of two effects; time in culture (four levels: 0, 3, 7 and 14 days) and impaction (two types: impacted and control) with each gene. Animal within date $A_{i(j)}$ was included as a random effect, to account for variability associated with each subject. A random effect accounting for technical replicates in qPCR was first fitted and subsequently dropped from the final model due to lack of statistical significance. Finally, heterogeneous residual variances were allowed between the housekeeping gene and each of the target genes. In all cases variances followed assumptions of normality. Patellae were collected from a total of 15 different animals. Of these, 8 animals had both left and right patellae collected while 7 animals had only one patella collected. When both left and right patellae were collected from the same animal, one patella was impacted and the contralateral was used as a non-impacted control. The use of paired and unpaired patellae from impacted and control samples created a partial confounding effect between treatment and animal which was accounted for in our statistical model. The model was fit using the mixed procedure of SAS (SAS Institute, Cary, NC). Differential expression among groups was tested for the

interaction of time by impaction and pair-wise treatment differences. False discovery rate (43) was used to adjust for multiple testing procedures (q value). Estimates of fold changes were approximated from linear contrasts by back transformation. Q values less than 0.05 were considered significant and q values between 0.1 and 0.05 were considered to be trending towards significance.

Results

Twenty-three patellae (46 facets) were collected—22 impacted facets and 24 control facets (Table 1). Fewer facets were used for 7 genes because we did not recover enough total RNA to examine all genes. Therefore, 20 impacted facets and 19 non-impacted control facets were used for *agc*, *capsn1*, *col1a1*, *col2a1*, *dcn*, *ftl*, and *s100a11*. Expression levels were compared between impacted and non-impacted cartilage samples at each time point (0, 3, 7, and 14 days) (Table 2). On Day 0, *col1a1* and *mmp3* had a tendency to be up-regulated in impacted samples. At Day 3, only *col2a1* showed any difference with a tendency to be up-regulated in impacted samples. At Day 7, *col2a1* was significantly down-regulated and *col1a1* had a tendency to be up-regulated in the impacted samples. At Day 14, *col1a1* was significantly up-regulated at 847-fold, *agc* was significantly down-regulated, and *col2a1* showed a tendency to be up-regulated. *Mmp1* was significantly up-regulated, *mmp3* down-regulated, and a *timp1* had a tendency towards up-regulation. Of the biological stress and inflammatory factor transcripts assayed, *s100a11* was significantly down-regulated and there was a tendency for *clu*, *cxcl16* and *ftl* to be up-regulated.

Expression levels were also compared within impacted and non-impacted samples across time, using Day 0 samples as the reference time point (Table 3). At Day 3, seven genes in the impacted samples were significantly differentially expressed and one had a tendency to be differentially expressed, with two having higher expression and six having lower expression at Day 3 than at Day 0. When Day 7 and 0 samples were compared, 10 genes in the impacted samples were significantly differentially expressed, with 7 having lower expression at Day 7 when compared to Day 0. Only 3 genes (*col1a1*, *mmp1* and *chi3l1*) had higher expression in the impacted Day 7 samples when compared to Day 0 impacted samples. Nine genes were significantly differentially expressed when Day 14 was compared to Day 0 in impacted samples. Six of these had lower expression at Day 14 and three (*col1a1*, *mmp1* and *timp3*) showed severe up-regulation of gene expression at Day 14.

Examination of non-impacted control samples across time showed similar trends to the impacted specimens when Days 3 and 0 were compared. Expression changes were in the same direction for all genes with similar magnitudes. When Day 7 non-impacted samples were compared to Day 0 non-impacted controls, all but one gene (*s100a11*) changed in the same direction as the impacted samples and magnitudes of change were similar for all but *col2a1*. In impacted samples where Day 7 was compared to Day 0, *col2a1* was down-regulated 33-fold while it was only down-regulated 8-fold in the non-impacted sample comparison. When Day 14 non-impacted controls were compared to Day 0 non-impacted controls, three genes (*mmp3*, *clu*, and *ftl*) had a direction of change opposite from what was observed in their Day 14 impacted counterparts. While the changes in expression were not significant for *ftl*, they were significant for *mmp3* and *clu*. Magnitudes of changes were similar for 10 of the 17 genes examined in these samples.

Discussion

The rationale for this study was to characterize how genes important for the cartilage matrix or known to alter their expression significantly following an injury, change their expression patterns in the first two weeks following an *in vitro* impact injury. In this model an intact

patella was used, preserving normal bone and cartilage boundaries during impaction and throughout the culture period. Gene expression changes were measured on full thickness cartilage samples taken from directly beneath the area of impaction. Our results represent an average chondrocyte response throughout the thickness, combining the response of surface, mid and deep zone chondrocytes. Overall we believe this system provides a relatively inexpensive, controlled test bed to examine early tissue changes following an impact injury. The current culture system only allows for a relatively short term examination of tissue changes compared to the months or years to develop full blown osteoarthritis. However, if an impact injury acts as the catalyst for degeneration, it is important to understand the very early tissue changes following this initiating event because the best place to prevent or mitigate the disease process is before significant tissue degeneration takes place.

We targeted three gene categories—those whose gene products are matrix components (*agc*, *col1a1*, *col2a1* and *dcn*), degradative enzymes and their inhibitors (*capns1*, *ctsb*, *mmp1*, *mmp3*, *timp1* and *timp3*) and biological stress and/or inflammatory factors (*chi3l1*, *clu*, *cxcl16*, *errfi1*, *fth-1*, *ftl* and *s100a11*). *Agc* consistently showed lower expression in impacted samples when compared to controls, with significant down-regulation at Day 14, similar to that found by others (38, 44). *Col1a1* consistently showed higher expression levels in impacted samples relative to control, with significant up-regulation at Day 14 and tendencies toward up-regulation at Days 0 and 7. Relative to control, *col2a1* exhibited higher expression levels in impacted samples after 3 days in culture, significantly lower expression levels at Day 7 and then higher expression again 14 days post-impaction. There were no significant differences between control and impacted tissue in the *dcn* levels throughout the study. Collagen type I is normally synthesized by fibroblasts, so our results, in conjunction with reduced levels of *agc*, suggest that chondrocytes found in the impacted cartilage were reverting to a more fibroblast-like phenotype. This result is supported by other studies that found an increase in *col1a1* and decrease in *agc* expression one and two weeks post-impaction (38). These changes in collagen and aggrecan production may lead to changes in material properties that might contribute to OA development and progression.

The only difference observed in degradative enzyme gene expression levels at Day 0 in impacted samples when compared to non-impacted was the *mmp3* level, exhibiting a tendency to be increased. Previous studies found that *mmp3* was up-regulated 3 and 24 hours after impaction (32, 33), similar to our findings on Day 0, where full thickness cartilage samples are collected approximately two hours after impactions. No other significant changes were observed in this group of genes until Day 14, where *mmp1* was significantly up-regulated and *mmp3* was significantly down-regulated. The inhibitors did not appear to be different when we compared impacted and non-impacted controls until 14 days in culture, when *timp1* showed a tendency toward up-regulation. Some of these results are similar to those reported by Natoli and colleagues (38), who examined temporal differences in expression of genes including *agc*, *col1*, *col2*, *mmp1* and *timp1* in bovine elbow cartilage explants undergoing high and low energy impactions. In their high energy impaction samples, they reported significant decreases in *collagen 2* transcript levels at one week in culture. For *col1a1*, they reported a 25-fold up-regulation after one week in culture, which agrees with our findings. They also found significant increases in *agc* in their high impact model but no significant differences in their low impact model.

No significant differences were found when we compared impacted to non-impacted controls within time point for the genes associated with biological stress and inflammation until Day 14. Biological stress and molecules associated with inflammation were selected because we previously found them to be differentially expressed in our earlier study and because they are more important in OA than once thought. In studies completed by others, *clu* was up-regulated in response to oxidative, mechanical and thermal stress (45). Ferritin

(formed from its subunits fth1 and ftl) is a regulator of iron availability and excess iron has been implicated in OA progression, due to its role in the production of reactive oxygen species (46). Previously ferritin was up-regulated in response to rheumatoid arthritis (47) and inflammatory stimuli in general (48, 49). It recently was examined in macrophages of patients with OA and both the heavy and light subunits had higher expression in OA patients than in controls (46) and we found *ftl* to be marginally up-regulated in impacted samples. *Chi3l1* expression levels were correlated with the degree of joint inflammation in rheumatoid arthritis patients (50) and have been linked to the biological stress response of articular cartilage (51). CXCL16 is a scavenger receptor implicated in control of the oxidative stress response in atherosclerosis (52) and age-related macular degeneration (53). In addition, many inflammatory and biological stress molecules, such as S100A11, have been associated with changes to the chondrocyte phenotype (54). After 14 days in culture, our impacted samples showed significant down-regulation of *s100a11* and tendencies for up-regulation of *clu*, *cxcl16* and *ftl*.

When results within treatment were examined over time, significant differences were observed, similar to those reported by Natoli *et al.* (38). In impacted samples, *col1a1* was drastically higher at 7 and 14 days when compared to expression levels on the day of impaction. *Agc, col2a1* and *dcn* were down-regulated over time. This is most likely an indication that impacted chondrocytes are not reproducing a normal cartilage matrix.

The degradative enzyme *mmp1* was significantly up-regulated in impacted samples over time when compared to Day 0 counterparts. Similar results were reported by Leong *et al.* (55), who found excessive loading induced *mmp1* transcription, while moderate daily loading suppressed it. *Mmp3*, *capns1* and *ctsb* were significantly down-regulated or unchanged in the impacted samples over time. *Timp1* tended to be lower while *timp3* was significantly up-regulated at Day 14 compared to Day 0, similar to that reported by Wei *et al.* (56). In their study, they found that *timp3* was up-regulated and *timp1* levels were unchanged 3 days and 21 days after a meniscus tear was surgically induced in rats.

Molecules associated with the inflammatory response, such as chemokines (i.e., CXCL16), have previously been identified in cartilage and are thought to send repair signals to surrounding tissue (57). When examining the change over time in impacted samples for genes involved in biological stress and inflammation, we found that *chi3l1* was the only gene that showed any significant up-regulation while the remaining six genes were mostly unchanged, or down-regulated if significantly affected in our model. Similar results for *chi3l1* were reported by Wei *et al.* (56) who found this gene up-regulated 3, 7 and 21 days post medial meniscus tear surgery. Dissimilar results were reported by Burton-Wurster *et al.* (31) for *errfi1*, in which we found significant down-regulation of this gene across time. They used canine articular cartilage shoulder explants to examine changes in gene expression for this gene and found it to be up-regulated when a load was applied. However, the difference may be explained by the fact that they applied cyclic loading while we used a single impaction in our model. Zhang *et al.* (58) produced *errfi1* knockout mice and found an early onset of degenerative joint disease in young mice, providing additional evidence that our model represents an impact injury model of OA with the down-regulation of *errfi1* in the impacted samples.

Examination of changes to gene expression in the non-impacted samples showed *col1a1* was up-regulated (significant at Day 7 compared to Day 0 and trending towards significance at Day 14) while the other matrix components were significantly down-regulated over time. *Mmp1* was significantly up-regulated but not to the same extent it was in the impacted samples, and *mmp3* had a tendency to be higher while it was down-regulated in impacted samples. Significant down regulation of three biological stress and inflammatory factors was

observed in the non-impacted samples over time. Similar results have been observed in recent studies examining gene expression changes during joint immobilization. The loss of joint loading that occurs when patellae are placed into *in vitro* culture and the reduced loading that occurs with immobilization may affect cartilage similarly. Recent studies have examined the effects of joint immobilization on gene expression and found results similar to our findings from the non-impacted control samples; significant up-regulation of *mmp1* (55) and *mmp3* (59) was reported with some significant effects observed after only 6 hours of immobilization.

Although our model has many unique properties that allow us to study early changes in chondrocyte gene expression following an injury, there are also several limitations. First, we used an *in vitro* culture model that ignored effects other tissues may have on chondrocyte gene expression as well as effects from differences in oxygen and load levels. Second, by examining only changes in gene expression, we were ignoring differential expression of proteins and production of important lipids and carbohydrates. Although gene expression is often correlated with protein expression, the correlation is not perfect, but preliminary results from ongoing proteomics studies indicate that many of the mRNA molecules we have identified as being differentially expressed are differentially expressed at the protein level (MSA, unpublished results).

This experiment was designed to assess how an impact injury affects gene expression at 0, 3, 7, and 14 days in culture post-impaction. Following an injury there may be an initial short term response, but if the response dissipates quickly it may not be sufficient to account for degenerative changes that may take a year or more to manifest clinically. We wanted to look for early changes that persist for weeks which may represent a more chronic threat to the tissue. For the selected genes we examined in this model most changes occurred between 7 and 14 days post-impaction. The changes observed suggest that the chondrocytes in the impacted cartilage were reverting to a more fibroblast-like phenotype and that the impaction abrogated or delayed a stress response due to the introduction of the tissue into culture and reduced loading of the cartilage tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Number of patellae facets used at each culture time point.

	Days in Culture				
Treatment	**0**	**3**	**7**	**14**	**Total**
Impacted	5 (4) *	5 (4) *	6	6	22 (20) *
Non-impacted	5 (4) *	6 (4) *	6	7 (5) *	24 (19) *
Total	10 (8) *	11 (8) *	12	13 (11) *	46 (39) *

* A smaller number of facets were used for *agc*, *capns1*, *col1a1*, *col2a1*, *dcn*, *ftl*, and *s100a11* due to insufficient RNA amounts from some facets to perform qPCR on all genes. Number of facets used for these genes are in parentheses.

Table 2

Differential expression between impacted and non-impacted cartilage samples at 0, 3, 7, and 14 days after culture.

Gene	Fold Change Day 0	Fold Change Day 3	Fold Change Day 7	Fold Change Day 14
Matrix Components				
agc	0.89	0.94	0.80	0.15 *
col1a1	3.39 †	1.68	3.07 †	847.34 ****
col2a1	1.13	3.65 †	0.265 *	3.15 †
dcn	0.76	1.86	0.85	1.94
Degradative Enzymes and Their Inhibitors				
capns1	1.57	2.10	0.506	2.22
ctsb	1.21	0.96	0.71	1.94
mmp1	0.40	2.17	1.53	7.44 *
mmp3	5.10 †	2.66	2.46	0.150 *
timp1	1.54	1.17	1.98	2.46 †
timp3	0.90	0.79	1.57	1.74
Biological Stress and Inflammatory Factors				
chi3l1	0.79	1.40	1.52	0.56
clu	0.521	0.976	0.645	2.21 †
cxcl16	0.85	0.64	1.39	2.28 †
errfi1	0.89	1.02	0.61	2.19
fth1	1.10	1.17	1.21	0.93
ftl	1.63	1.26	0.83	2.65 †
s100a11	0.73	0.64	1.39	0.303 **

Genes up-regulated in impacted tissue have a fold change > 1; down-regulated—fold change < 1.

† = q < 0.1;

* = q < 0.05;

** = q < 0.01;

*** = q < 0.001;

**** = q < 0.0001

Table 3

Differential expression between time points within impacted and non-impacted cartilage.

Days in Culture Comparison						
Gene Name	**Impacted**			**Non-impacted**		
	Day 3 vs. 0	**Day 7 vs. 0**	**Day 14 vs. 0**	**Day 3 vs. 0**	**Day 7 vs. 0**	**Day 14 vs. 0**
Matrix Components						
agc	0.49	0.30	0.06**	0.46	0.34	0.34
col1a1	0.35	4.35*	769.23****	0.70	4.83*	3.03†
col2a1	0.13**	0.03****	0.24*	0.04****	0.12***	0.09****
dcn	0.17**	0.12****	0.21**	0.07****	0.10****	0.08****
Degradative Enzymes and Their Inhibitors						
capns1	0.30*	0.17**	0.57	0.22**	0.54	0.41†
ctsb	0.50	1.08	1.75	0.63	1.85	1.10
mmp1	200****	666.66****	1923.08****	37.04***	169.49#x0002A;***	104.17****
mmp3	0.86	2.56	0.12*	1.64	5.35†	4.17†
timp1	0.53	0.28*	0.93	0.69	0.22**	0.59
timp3	1.11	2.13	5.10***	1.92	1.85	4.02**
Biological Stress and Inflammatory Factors						
chi3l1	3.57*	3.23**	2.08	2.00	1.69	2.94*
clu	0.40†	0.88	1.54	0.21**	0.71	0.36*
cxcl16	0.56	0.30*	0.50	0.75	0.18***	0.19***
errfi1	0.15***	0.14****	0.26*	0.13****	0.20***	0.11****
fth1	0.70	1.47	1.49	0.66	1.35	1.75
ftl	0.18**	0.27*	1.25	0.23*	0.53	0.76
s100a11	1.82	1.54	0.28*	2.04	0.80	0.68

Fold changes > 1 were more highly expressed at the day time point than at Day 0. Fold changes < 1 were more highly expressed at Day 0 than at the other time point.

† = q < 0.1;

* = q < 0.05;
** = q < 0.01;
*** = q < 0.001;
**** = q < 0.0001