Bone Reports 9 (2018) 19–26

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr

# Expression of pro-apoptotic markers is increased along the osteochondral junction in naturally occurring osteochondrosis☆

Stacy A. Semevolos*, Katja F. Duesterdieck-Zellmer, Maureen Larson, Marc A. Kinsley[1]

*Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA*

ARTICLE INFO

*Keywords:*
Osteochondrosis
Osteochondritis dissecans
Cartilage development
Apoptosis
Laser capture microdissection
Cartilage biology
Endochondral ossification

ABSTRACT

Osteochondrosis (OC) is a naturally occurring disease of the articular-epiphyseal cartilage and subchondral bone layers, leading to pain and decreased mobility. The objective of this study was to characterize gene and protein expression of apoptotic markers in chondrocytes surrounding cartilage canals and along the osteochondral junction of osteochondrosis (OC)-affected and normal cartilage, using naturally occurring disease in horses. Paraffin-embedded osteochondral samples (6 OC, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 6 normal controls) were obtained from the lateral trochlear ridge of femoropatellar joints in 14 immature horses (1–6 months of age). Equine-specific caspase-3, caspase-8, caspase-10, Fas, Bcl-2, BAG-1, TNFα, cytochrome *C*, thymosin-β10, and 18S mRNA expression levels were evaluated by two-step real-time quantitative PCR. Percentage of cell death was determined using the TUNEL method. Protein expression of caspase-10, Fas, cytochrome *C*, and thymosin-β10 was determined following immunohistochemistry. Statistical analysis was performed using the Wilcoxon rank sum test or two-sample *t*-test ($p < 0.05$). In OC samples, there was significantly increased gene expression of caspase-10, Fas, cytochrome *C*, and thymosin-β10 in chondrocytes along the osteochondral junction and increased Fas gene expression in chondrocytes adjacent to cartilage canals, compared to controls. In OC samples, higher matrix Fas and cytochrome *C* protein expression, lower mitochondrial cytochrome *C* protein expression, and a trend for higher cytoplasmic caspase-10 protein expression were found. Collectively, these results suggest that both extrinsic and intrinsic apoptotic pathways are activated in OC cartilage. Increased apoptosis of osteochondral junction chondrocytes may play a role in OC, based on increased gene expression of several pro-apoptotic markers in this location.

## 1. Introduction

Apoptosis is a form of programmed cell death that is typically initiated via the extrinsic or the intrinsic apoptotic pathway (Atasoy et al., 2003). In the extrinsic pathway, a ligand of the tumor necrosis factor (TNF) superfamily binds the extracellular domain of a death receptor, such as Fas or other receptors of the TNF receptor family (Atasoy et al., 2003). This binding results in intracellular activation of one of the apical caspases (caspase-8 and -10), which in turn activate downstream effector caspases (caspase-3, -6, and -7), leading to cell death (Wachmann et al., 2010). In the intrinsic pathway, mitochondrial release of cytochrome *C* into the cytosol stimulates the formation of apoptosomes, which activate caspase-9 (Kawamoto et al., 2016), followed by activation of downstream effector caspases (caspase-3, -6, and -7), leading to cell death (Wachmann et al., 2010). A multitude of anti-apoptotic proteins aid in regulation of apoptosis, among them B-cell lymphoma 2 (Bcl-2) (Opferman and Kothari, 2018) and Bcl-2 associated athanogene-1 (BAG-1) (Takayama et al., 1995), both of which inhibit the intrinsic pathway (Kinkel et al., 2004). In addition, some proteins play a role in regulation of apoptosis such as thymosin-β 10, a member of a group of actin monomer-sequestering proteins that inhibit actin polymerization (Shiotsuka et al., 2013; Viglietto et al., 1999).

In normal articular-epiphyseal cartilage, chondrocytes differentiate from proliferative to hypertrophic phenotypes, followed by ossification of the cartilage. Data is inconsistent on whether or not chondrocytes in this last step towards bone formation undergo apoptosis (Ahmed et al.,

*Abbreviations:* OC, Osteochondrosis; TNF, tumor necrosis factor; Bcl-2, B-cell lymphoma 2; BAG-1, Bcl-2 associated athanogene-1; QH, Quarter Horse; TB, Thoroughbred; POA, Pony of the Americas; LCM, laser capture microdissection; OCT compound, optimal cutting temperature compound

☆ Declaration of interest: none.

* Corresponding author.
*E-mail address:* stacy.semevolos@oregonstate.edu (S.A. Semevolos).
[1] Present address: Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI, 48824.

https://doi.org/10.1016/j.bonr.2018.06.003
Received 11 April 2018; Received in revised form 29 May 2018; Accepted 15 June 2018
Available online 21 June 2018

2007; Roach and Erenpreisa, 1996; Yang et al., 2014). In postnatal physeal cartilage, it has been suggested that hypertrophic chondrocytes undergo transformation into osteoblasts (Yang et al., 2014). However, another study (Roach and Erenpreisa, 1996) describes asymmetric cell division of hypertrophic chondrocytes leading to apoptosis of one cell and differentiation of the other cell into an osteogenic cell. Yet another study (Ahmed et al., 2007) identifies the presence of dark and light hypertrophic chondrocytes, each dying by different non-apoptotic means.

However, expression patterns of proteins involved in regulation of apoptosis in physeal cartilage suggest that this form of controlled cell death may well play a role in endochondral ossification. For example, late proliferative and prehypertrophic chondrocytes in growth cartilage appear to resist apoptotic cell death, as anti-apoptotic proteins such as Bcl-2 and BAG-1 are expressed prominently in these cells (Kinkel et al., 2004). Furthermore, expression of both proteins decreases with age in articular cartilage (Kinkel et al., 2004).

Osteochondrosis (OC) is a naturally occurring disease of the articular-epiphyseal cartilage and subchondral bone layers, with the hallmark feature of delayed ossification of epiphyseal cartilage, leading to pain and decreased mobility. One proposed etiology includes failure of cartilage canals due to crushing or shearing, resulting in cartilage necrosis and subsequent OC lesions (Carlson et al., 1995; Olstad et al., 2008; Olstad et al., 2011; Olstad et al., 2013). Other studies support a delay in endochondral ossification due to abnormal differentiation of chondrocytes into bone without apparent crushing or shearing injury (Kinsley et al., 2015; Laverty and Girard, 2013; Mirams et al., 2009; Riddick et al., 2012). Whether by necrosis or abnormal differentiation, OC may involve altered chondrocyte death, including altered apoptosis, along the osteochondral junction. Support for this premise includes the finding that thymosin-β4 is increased in experimentally-induced osteochondrosis (Mirams et al., 2016) and the fact that thymosins play a significant role in regulating apoptosis (Hall, 1995; Shiotsuka et al., 2013).

The objective of this study was to characterize gene and protein expression of apoptotic markers in chondrocytes surrounding cartilage canals and along the osteochondral junction of osteochondrosis (OC)-affected and normal cartilage, using naturally occurring disease in horses. Our hypothesis was that OC is associated with abnormal apoptotic cell death along the osteochondral junction during development and that expression of pro-apoptotic proteins increase in OC cartilage compared to normal controls.

## 2. Materials and methods

### 2.1. Samples

Archived paraffin-embedded osteochondral samples (6 OC, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 6 normal controls) were previously obtained from the lateral trochlear ridges of femoropatellar joints of 14 immature horses (Kinsley et al., 2015; Riddick et al., 2012). Institutional animal care and use committee approval was obtained for the previous study (Riddick et al., 2012). Foals were 1–6 months of age, including 4 intact males and 10 females (see Table 1).

At the time of harvest, osteochondral samples ($n = 2$ per trochlear ridge, 3–4 mm thick) were sharply dissected from mid-lateral trochlear ridges of both distal femurs. Cartilage was sharply cut with a scalpel down to bone and then a sharp thin osteotome was used to section the bone underneath. Osteochondral samples were either frozen in OCT medium (Tissue Tek OCT compound, VWR International, Radnor, PA, USA) and stored at −80 °C for laser capture microdissection, or fixed in 4% paraformaldehyde for 48 h and transferred to 10% EDTA solution for decalcification (2–4 weeks). Decalcified samples were embedded in paraffin and sectioned for immunohistochemistry and H&E staining (Histopathology Shared Resource Laboratory, Oregon Health & Science University, Portland, OR, USA).

**Table 1**
Age, sex, and breed characteristics of horses in OC and normal cartilage groups.

| | OC-affected | Normal |
|---|---|---|
| Age | Median (range): 4.5 months (1–5) | Median (range): 4 months (3–6) |
| Sex | 3 M, 3F | 1 M, 7F |
| Breed | 4 QH cross, 1 TB, 1 POA | 7 QH cross, 1 POA |

### 2.2. Sample evaluation and classification

All osteochondral samples were evaluated grossly at the time of harvest and histologically following H&E staining in order to classify them as normal or OC-affected, as previously described (Kinsley et al., 2015; Riddick et al., 2012). Normal cartilage was defined as having no gross or histologic abnormalities. OC was defined as samples having altered endochondral ossification (locally thickened cartilage only, loss of normal columnar arrangement of chondrocytes, chondrones) or separation (fissures, necrosis) along the osteochondral junction without concurrent superficial cartilage lesions (Weeren and Barneveld, 1999). Briefly, 6 foals were determined to have OC, and 8 were classified as normal. In OC samples, 5 foals had separation along the osteochondral junction and 3 foals had locally thickened cartilage (2 with concurrent osteochondral separation), all without concurrent superficial lesions.

### 2.3. Laser-capture microdissection (LCM)

Frozen osteochondral samples (4 OC, 6 normal) were sectioned using a cryomicrotome, mounted on slides using a tape transfer system (CryoJane, Instrumedics, Leica Biosystems, Inc., Buffalo Grove, IL, USA) and stored at −80 °C. Immediately prior to LCM, each slide was dehydrated in graded alcohol and xylene. LCM was performed using PIXCELL II Laser Capture Microdissection System (Arcturus Bioscience, Mountainview, CA, USA) and CapSure Macro LCM caps (Applied Biosystems, Foster City, CA, USA). Chondrocytes were captured immediately surrounding the cartilage canals, representing small rounded chondrocytes, and osteochondral junction, representing hypertrophic chondrocytes of each animal. Up to 8 caps from sequential sections were combined for each site (approximately 400–800 cells per site).

### 2.4. RNA isolation

PicoPure RNA Isolation Kit (Arcturus Bioscience, Mountainview, CA, USA) was used for RNA isolation of LCM samples with slight modifications. Briefly, cell lysate from up to 8 caps was loaded onto a pre-conditioned RNA-purification column. The column was then washed and treated with RNase-free DNase prior to RNA elution from the column. RNA quality control was performed using an Agilent 2100 Bioanalyzer and the RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA, USA).

### 2.5. Real-time quantitative RT-PCR

Two-step quantitative real time RT-PCR was performed as described in the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) to evaluate expression of equine-specific caspase-3 (NM_001163961), caspase-8 (XM_001496753), caspase-10 (XM_001498075), Fas (GQ429290), Bcl-2 (XM_001490436), BAG-1 (XM_001917727), TNFα (EU438779), cytochrome *C* (NM_001164014), and thymosin-β10 (AF506973) mRNA expression levels, using a real-time PCR system (ABI Step One Plus instrument and software, Applied Biosystems, Foster City, CA, USA) (Kinsley et al., 2015; Riddick et al., 2012). First strand cDNA synthesis was accomplished with reverse transcription, using random hexamers as primers. Logarithmic pre-

amplification (14 cycles) (Li et al., 2004) was performed on cDNA of LCM samples, using pooled equine specific primer pairs (Caspase-3, Caspase-8, Caspase-10, Bcl-2, BAG-1, Fas, TNFα, Cytochrome *C*, Thymosin-β10) and the Taqman PreAmp Master Mix kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR of diluted (1:20) pre-amplified LCM samples was then performed. Probes were labeled with FAM (6-carboxy-fluoroscein) (reporter dye) and TAMRA (6-carboxy-tetramethylrhodamine) (quencher dye). For each experimental sample, the amount of target cDNA was determined by comparing to a relative standard curve, constructed with six 10-fold serial dilutions of a calibrator sample of cartilage cDNA (starting concentration: 100 ng). The same calibrator sample was used for all experiments and 18S rRNA was used as the housekeeping gene for normalizing sample gene expression (Peng et al., 2012). PCR was performed in duplicate using a 20 μl final reaction mixture of 2× TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 250 nM probe, 900 nM forward and reverse primers, and 7.5 μl pre-amplified LCM sample cDNA. After 2 min incubation at 50 °C to activate uracil-DNA glycosylase (UDG) and 10 min incubation at 95 °C to deactivate UDG and activate AmpliTaq Gold DNA polymerase, 40 PCR cycles of 15 s of 95 °C followed by 1 min of 60 °C were run.

### 2.6. Percentage cell death

Percentage of cell death was determined using the TUNEL method, according to manufacturer's guidelines (In Situ Cell Death Detection Kit, AP, Roche Applied Science, Manheim, Germany). Briefly, osteochondral sections were permeabilized in 0.1 M Citrate buffer (pH 6) under microwave irradiation for 1 min on high power. Sections were then blocked for 30 min at room temperature with 3% BSA and 20% bovine serum in 0.1 M Tris-HCl, rinsed twice with PBS, and incubated in the dark with the TUNEL reaction mixture for 60 min at 37 °C. After rinsing, fluorescent signals were converted to permanent staining using an alkaline phosphatase enzyme and NBT/BCIP substrate (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate). Positive and negative controls were included in the staining protocol, according to manufacturer's guidelines. Slides were dehydrated and permanently mounted. Under light microscopy, live cells (no staining) and dead cells (blue staining) were counted to calculate percentage of cell death for 2 separate cell populations: 1) chondrocytes adjacent to cartilage canals, and 2) chondrocytes along the osteochondral junction. All chondrocytes were counted within approximately 100–150 μm of cartilage canals (32–158 total # cells) and within 200 μm of the osteochondral junction (80–179 total # cells).

### 2.7. Immunohistochemistry

Immunohistochemistry was performed on 6 μm osteochondral sections using 1:20 dilution of rabbit α-human polyclonal (Fas, caspase-10, thymosin-β10) (Abcam, Cambridge, MA, USA) or mouse α-human monoclonal (cytochrome *C*) primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and the Supersensitive Link-label Multilink Immunohistochemistry System (Biogenex, San Ramon, CA, USA), as described previously (Kinsley et al., 2015; Riddick et al., 2012). Negative procedural controls were confirmed by using non-immune serum in place of primary antibody. Positive tissue controls were confirmed for each antibody, including lung tissue for thymosin-β10 and cytochrome *C*, liver for caspase-10, and hemangioma for Fas. Following deparaffinization, osteochondral sections (6 OC, 8 normal) were either incubated at 37 °C for 60 min under a solution of testicular hyaluronidase (cartilage samples) or for 5 min under pepsin (connective tissue controls) to expose the antigen. Endogenous peroxidases were quenched with hydrogen peroxide and methanol. Non-immune goat serum was applied for 30 min (polyclonal primary antibodies only), and the primary antibody was applied for 60–90 min at room temperature. Secondary biotinylated multilink antibodies were applied, followed by labeling with streptavidin conjugated peroxidase, and then applying diaminobenzidine tetrachloride (DAB) as a chromogen for production of color product (brown). The sections were counterstained with Harris hematoxylin and mounted for microscopy.

#### 2.7.1. Immunohistochemistry scoring

Immunohistochemistry samples were evaluated and scored by two investigators (SAS and KDZ), using the HSCORE (Brun et al., 2012; Schatz et al., 2012). The HSCORE was determined for each specific cell population or cartilage layer by calculating the sum of the percentage of positive staining cells or matrix (Pi) at each intensity multiplied by its respective intensity score (i). HSCORE = $\Sigma i\, i * Pi$. The staining intensity was scored from 0 to 3, with 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = strong staining. Specific cell populations were scored first; namely, chondrocytes surrounding the cartilage canals and chondrocytes adjacent to the osteochondral junction (Duesterdieck-Zellmer et al., 2015). Scores of both investigators were then averaged at each location prior to HSCORE determination. Additionally, three cartilage layers (superficial, middle, deep) (Duesterdieck-Zellmer et al., 2015) were scored and then averaged between the two investigators prior to HSCORE calculation for each cartilage layer. For Fas samples, the middle cartilage layer was further divided into upper and lower halves for scoring, in an attempt to characterize perceived gradients of Fas expression in this layer. When scoring cytochrome *C* immunohistochemistry samples, a punctate pattern of discrete spots within the cytoplasm of chondrocytes was considered to be a mitochondrial pattern, while diffuse cellular immunostaining without distinct spots was considered a cytoplasmic pattern (Crowley et al., 2016).

### 2.8. Statistical analysis

Quantitative comparisons from real-time PCR assays were compared for each site (cartilage canal, osteochondral junction) between OC and normal horses using a Wilcoxon rank sum test. *P*-values for all gene expression comparisons for each site were then ordered from smallest to largest and the significance level was corrected (q*) to control the false discovery rate, according to Benjamin and Hochberg (Benjamini and Hochberg, 1995; JH, 2009; Presser et al., 2011). Using a false discovery rate (FDR) $< 0.1$, the corrected significance level for gene expression comparisons at the cartilage canal site was $q^* = 0.011$ and the osteochondral junction site was $q^* = 0.044$. Gene comparisons were considered to be significant if *P*-values $\leq q^*$ for the respective site. Percentage cell death and immunohistochemistry scores were compared between OC and normal horses using a two-sample *t*-test ($P < 0.05$). When data was not normally distributed, scores were compared using a Wilcoxon rank sum test ($P < 0.05$).

## 3. Results

### 3.1. Real-time quantitative RT-PCR

Chondrocytes along the osteochondral junction (Fig. 1) had significantly increased gene expression of caspase-10 ($p = 0.04$), Fas ($p = 0.02$), cytochrome *C* ($p = 0.01$), and thymosin-β10 ($p = 0.04$) in OC samples compared to normal controls. No significant difference was found in gene expression between OC-affected samples and normal controls for caspase-3, caspase-8, Bcl-2, BAG-1, or TNFα. In OC samples, chondrocytes adjacent to cartilage canals (Fig. 1) had significantly increased Fas gene expression ($p = 0.002$) compared to normal controls. No significant difference was found in expression of the remaining genes between OC and normal controls.

### 3.2. Percentage cell death

There was no significant difference in percentage of cell death of

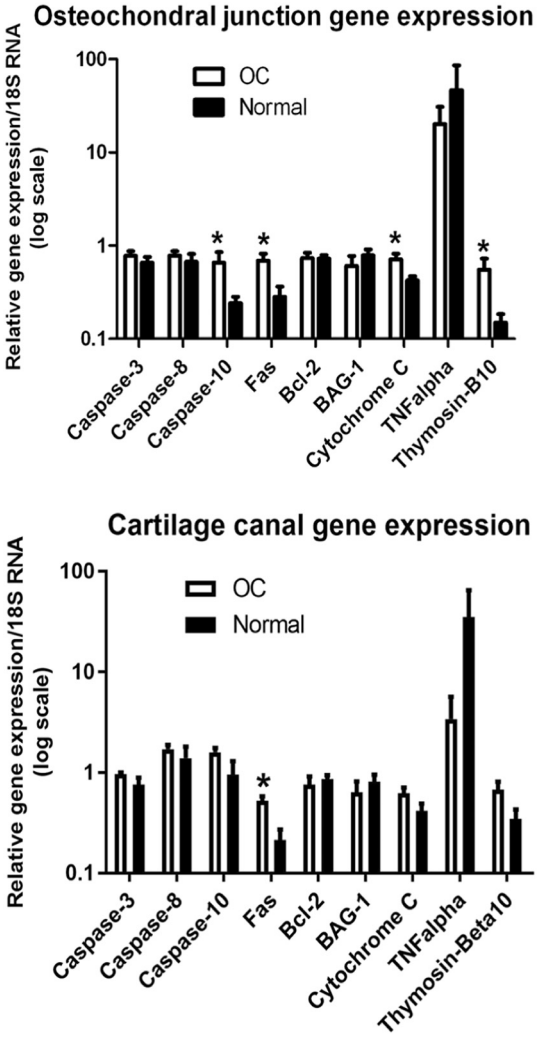


**Fig. 1.** Gene expression of laser-captured osteochondral junction chondrocytes and cartilage canal chondrocytes using real-time PCR. Relative gene expression (log scale) was determined using a relative standard curve and dividing the target gene by 18S RNA expression. * represents significant differences when comparing OC foals to normal controls.

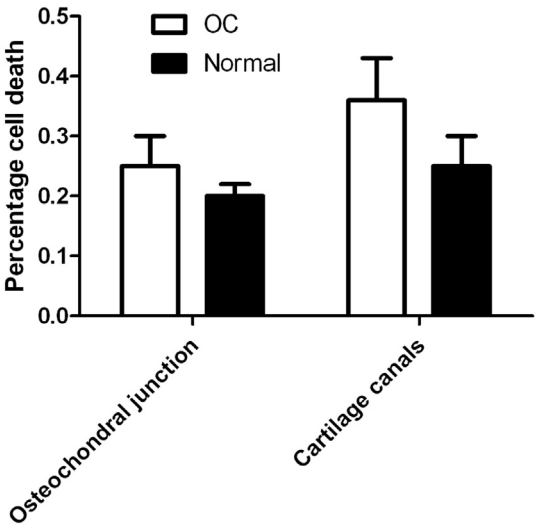


**Fig. 2.** Percentage cell death determined by TUNEL analysis of OC vs. normal cartilage surrounding cartilage canals and along the osteochondral junction.

**Table 2**
Caspase-10 mean ± SD immunohistochemistry HSCORES.

| Cartilage layer | | OC | Normal | *P*-value |
|---|---|---|---|---|
| Superficial | Cytoplasmic | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.0 |
| Middle | Cytoplasmic | 0.11 ± 0.11 | 0.025 ± 0.044 | 0.094 |
| Deep | Cytoplasmic | 0.11 ± 0.12 | 0.041 ± 0.053 | 0.35 |
| Cartilage canals | Cytoplasmic | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.0 |
| Osteochondral junction | Cytoplasmic | 0.029 ± 0.06 | 0.0063 ± 0.018 | 0.43 |

chondrocytes surrounding cartilage canals ($p = 0.20$) or along the osteochondral junction ($p = 0.43$) between OC and normal controls (Fig. 2).

### 3.3. Immunohistochemistry

#### 3.3.1. Caspase-10 immunohistochemistry

Overall, no or minimal caspase-10 immunostaining was apparent throughout most of the cartilage layers evaluated (Table 2, Fig. 3), and there were no significant differences were found between OC and normal control samples. No protein expression was apparent in the superficial layer. There was a trend for higher caspase-10 protein expression in the middle cartilage layer of OC samples ($p < 0.1$). Mild caspase-10 protein expression was observed in pre-hypertrophic chondrocytes near the junction of the middle and deep cartilage layers of approximately half of OC and normal samples (Fig. 3). When present, this expression was observed in 10–50% of cells in that location. No protein expression was apparent surrounding cartilage canals. Most samples had no expression along the osteochondral junction, although a few samples had mild expression of < 10% of chondrocytes at this location.

#### 3.3.2. Fas immunohistochemistry

Overall, Fas protein expression ranged from mild to moderate, with differential expression depending on the cartilage layer and cell type (Table 3; Fig. 4). In the deep layer, Fas immunostaining was mainly cytoplasmic with a thin rim of staining around lacunae. There was significantly greater Fas protein expression in the matrix of OC samples in the lower middle and deep cartilage layers than in normal samples. Conversely, Fas cytoplasmic staining was significantly lower in OC samples in the deep cartilage layer and along the osteochondral junction. Fas protein expression ranged from mild to moderate in most samples along the osteochondral junction, with variable expression in small chondrocytes surrounding cartilage canals (Table 3; Fig. 4).

#### 3.3.3. Cytochrome C immunohistochemisty

Cytochrome *C* immunostaining was mild to moderate in the superficial matrix, while mitochondrial staining was mild to moderate in the deep cartilage layer and along the osteochondral junction (Table 4; Fig. 5). There was significantly lower cytochrome *C* mitochondrial immunostaining in OC samples in the middle and deep cartilage layers compared to normal controls. A trend ($P < 0.1$) was found for higher matrix immunostaining surrounding cartilage canals and the osteochondral junction of OC samples compared to normal controls.

#### 3.3.4. Thymosin-β10 immunohistochemistry

Thymosin-β10 protein expression was apparent throughout all layers of cartilage, with moderate to strong expression in the superficial matrix and the deeper chondrocytes (Fig. 6; Table 5). Thymosin-β10 protein expression in chondrocytes along the osteochondral junction ranged from minimal to strong. Chondrocytes surrounding cartilage canals displayed either strong or mild immunostaining, suggesting two different cell populations. No significant differences were found between OC and normal samples.

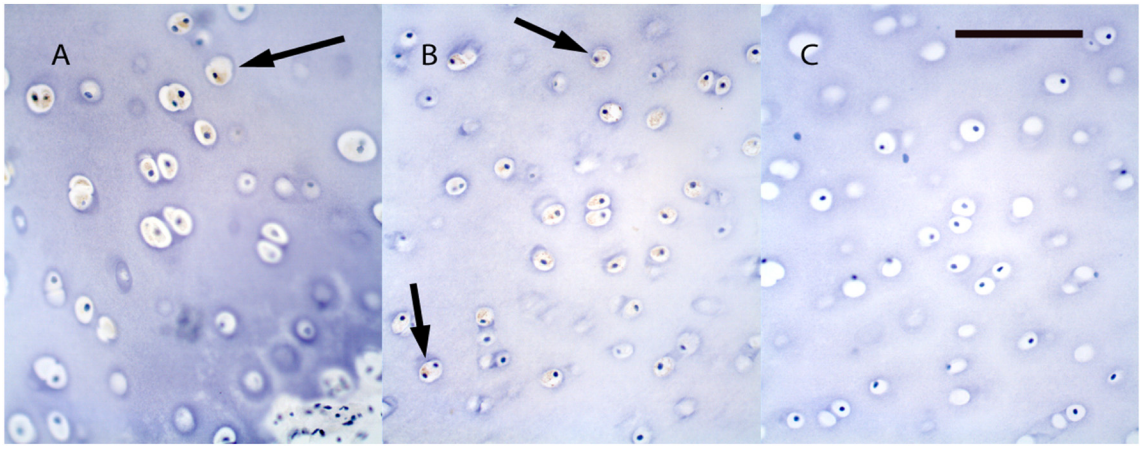


**Fig. 3.** Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against Caspase-10. A) Mild Caspase-10 expression in a few pre-hypertrophic chondrocytes (arrow) in the middle to deep zone of cartilage from a 5 month old foal having OC. B) Mild cytoplasmic immunostaining of chondrocytes (arrows) in the middle to deep cartilage layer of a normal 4 month old filly. C) Negative control for (B). Bar = 100 μm, hematoxylin stained, DAB chromogen (3′3′diaminobenzidine).

**Table 3**
Fas mean ± SD immunohistochemistry HSCORES.

| Cartilage layer | | OC | Normal | *P*-value |
|---|---|---|---|---|
| Superficial | Matrix | 2.2 ± 0.48 | 1.8 ± 0.54 | 0.14 |
| | Cytoplasmic | 1.5 ± 0.35 | 1.3 ± 0.35 | 0.24 |
| Upper middle | Matrix | 1.2 ± 0.43 | 0.78 ± 0.63 | 0.096 |
| | Cytoplasmic | 0.84 ± 0.55 | 1.1 ± 0.25 | 0.32 |
| Lower middle | Matrix | 0.92 ± 0.51 | 0.30 ± 0.29 | 0.0087 |
| | Cytoplasmic | 1.0 ± 0.44 | 1.4 ± 0.39 | 0.13 |
| Deep | Matrix | 0.57 ± 0.53 | 0.13 ± 0.12 | 0.012 |
| | Cytoplasmic | 1.4 ± 0.41 | 1.8 ± 0.39 | 0.048 |
| Cartilage canals | Matrix | 0.62 ± 0.68 | 0.30 ± 0.38 | 0.22 |
| | Cytoplasmic | 0.99 ± 0.35 | 0.82 ± 0.35 | 0.40 |
| Osteochondral junction | Matrix | 0.66 ± 0.82 | 0.14 ± 0.13 | 0.13 |
| | Cytoplasmic | 1.5 ± 0.22 | 1.9 ± 0.23 | 0.022 |

**Table 4**
Cytochrome *C* mean ± SD immunohistochemistry HSCORES.

| Cartilage layer | | OC | Normal | *P*-value |
|---|---|---|---|---|
| Superficial | Matrix | 1.9 ± 0.63 | 1.6 ± 0.40 | 0.63 |
| | Cytoplasmic | 0.25 ± 0.13 | 0.16 ± 0.15 | 0.34 |
| | Mitochondrial | 0.55 ± 0.29 | 0.66 ± 0.33 | 0.48 |
| Middle | Matrix | 0.80 ± 0.41 | 0.53 ± 0.34 | 0.15 |
| | Cytoplasmic | 0.069 ± 0.092 | 0.028 ± 0.025 | 0.51 |
| | Mitochondrial | 0.37 ± 0.25 | 0.74 ± 0.30 | 0.02 |
| Deep | Matrix | 0.53 ± 0.33 | 0.34 ± 0.26 | 0.27 |
| | Cytoplasmic | 0.23 ± 0.26 | 0.35 ± 0.36 | 0.29 |
| | Mitochondrial | 1.1 ± 0.37 | 1.5 ± 0.31 | 0.02 |
| Cartilage canals | Matrix | 0.44 ± 0.35 | 0.16 ± 0.19 | 0.089 |
| | Cytoplasmic | 0.22 ± 0.16 | 0.25 ± 0.18 | 0.80 |
| | Mitochondrial | 0.64 ± 0.31 | 0.72 ± 0.26 | 0.64 |
| Osteochondral junction | Matrix | 0.35 ± 0.31 | 0.12 ± 0.27 | 0.082 |
| | Cytoplasmic | 0.25 ± 0.19 | 0.30 ± 0.21 | 0.68 |
| | Mitochondrial | 1.0 ± 0.29 | 1.3 ± 0.46 | 0.19 |

## 4. Discussion

In this study, we hypothesized that OC chondrocytes along the osteochondral junction and cartilage canals express higher levels of apoptotic markers than normal chondrocytes, reflecting aberrant cell death. Our results partially support this hypothesis through increased gene expression of caspase-10, Fas, cytochrome *C*, and thymosin-β10 in OC chondrocytes along the osteochondral junction and increased Fas gene expression adjacent to cartilage canals. Further support for our hypothesis was provided by immunohistochemistry results, with higher protein expression of Fas and cytochrome *C* in the matrix, lower mitochondrial protein expression of cytochrome *C*, and a trend for higher caspase-10 protein expression found in OC samples. Contrary to our hypothesis, OC cartilage expressed lower cytoplasmic Fas protein than normal cartilage and had no difference in percentage of cell death along the osteochondral junction or cartilage canals. Collectively, these results suggest both extrinsic and intrinsic apoptotic pathways are activated in OC cartilage, possibly due to an earlier ischemic insult or

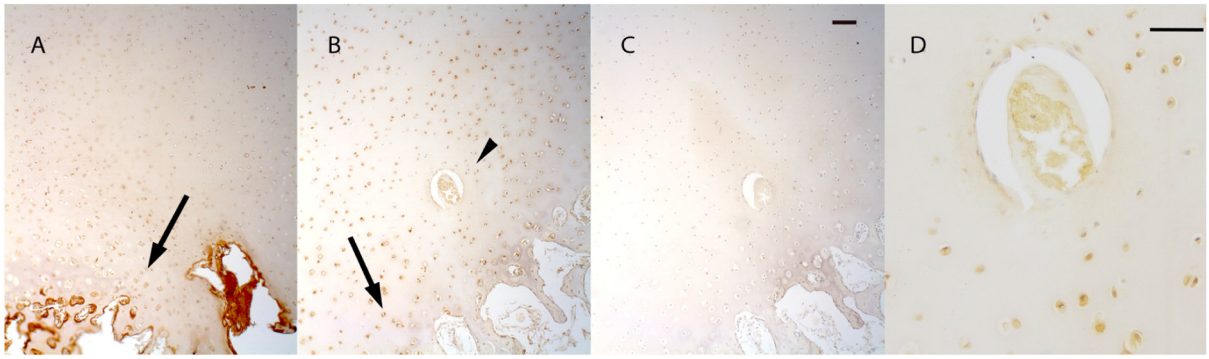


**Fig. 4.** Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against Fas. A) Mild cytoplasmic immunostaining of chondrocytes near the osteochondral junction (arrow) in a 5 month old colt with OC. B) Moderate Fas protein expression in chondrocytes along the osteochondral junction (arrow) and variable immunoexpression surrounding the cartilage canal (arrowhead) in a normal 4 month old filly. C) Negative control for (B). Bar = 100 μm for A, B, and C. D) Enlargement of cartilage canal shown in (B), Bar = 50 μm. Hematoxylin stained, DAB chromogen.

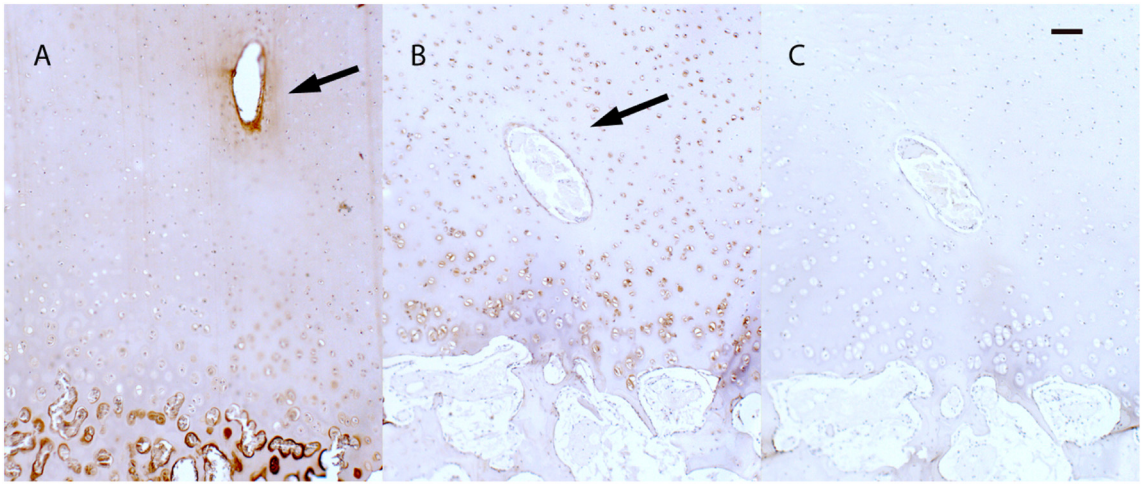


**Fig. 5.** Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against cytochrome *C*. A) Mild to moderate matrix immunostaining (arrow) surrounding a cartilage canal in a 5 month old foal having OC. B) No matrix immunostaining surrounding a cartilage canal of a normal 4 month old foal. C) Negative control for (B). Bar = 100 μm, hematoxylin stained, DAB chromogen.

**Table 5**
Thymosin β-10 mean ± SD immunohistochemistry HSCORES.

| Cartilage layer | | OC | Normal | *P*-value |
|---|---|---|---|---|
| Superficial | Matrix | 2.4 ± 0.35 | 2.3 ± 0.86 | 0.83 |
| | Cytoplasmic | 1.7 ± 0.30 | 1.6 ± 0.60 | 0.56 |
| Middle | Matrix | 0.94 ± 0.61 | 0.89 ± 0.86 | 0.64 |
| | Cytoplasmic | 1.8 ± 0.39 | 1.7 ± 0.49 | 0.66 |
| Deep | Matrix | 0.60 ± 0.74 | 0.26 ± 0.22 | 0.56 |
| | Cytoplasmic | 2.2 ± 0.33 | 2.1 ± 0.52 | 0.70 |
| Cartilage canals | Matrix | 0.70 ± 0.23 | 0.39 ± 0.32 | 0.13 |
| | Cytoplasmic | 1.6 ± 0.69 | 1.3 ± 0.57 | 0.36 |
| Osteochondral junction | Matrix | 0.56 ± 0.83 | 0.21 ± 0.33 | 0.49 |
| | Cytoplasmic | 1.8 ± 0.71 | 1.8 ± 0.59 | 0.89 |

response to failure of normal cell death along the osteochondral junction.

Caspase-8 and -10 are apical caspases that play a vital role in the extrinsic pathway of apoptosis via activation of downstream effector caspases (caspase-3, -6, and -7) (Schultz and Harrington, 2003; Wachmann et al., 2010). These caspases are highly homologous and may have overlapping functions (Wachmann et al., 2010). Based on our results, caspase-10 appears to be involved in OC pathogenesis, while caspase-8 does not. Increased caspase-10 gene expression along the osteochondral junction in OC samples supports possible involvement of the extrinsic apoptotic pathway, although no corresponding increase in protein expression in that location was found. The trend for higher caspase-10 protein expression in the middle cartilage layer of OC samples corresponded to increased expression in pre-hypertrophic chondrocytes. It would be interesting to determine if caspase-10 gene expression was also increased in pre-hypertrophic chondrocytes. However, because this study used laser captured samples from previously obtained areas of interest (not including pre-hypertrophic chondrocytes), this determination was not possible.

In addition to caspase-10 upregulation, increased Fas expression in OC further supports involvement of the extrinsic apoptotic pathway. Increased Fas gene expression in chondrocytes surrounding cartilage canals and the osteochondral junction and increased Fas protein expression in the matrix of middle and deep cartilage layers of OC samples suggest that Fas death receptor may play a significant role in OC. Despite these findings, cytoplasmic protein expression of Fas was significantly lower in OC chondrocytes in the deep layer and along the osteochondral junction. The reason for this difference in matrix and

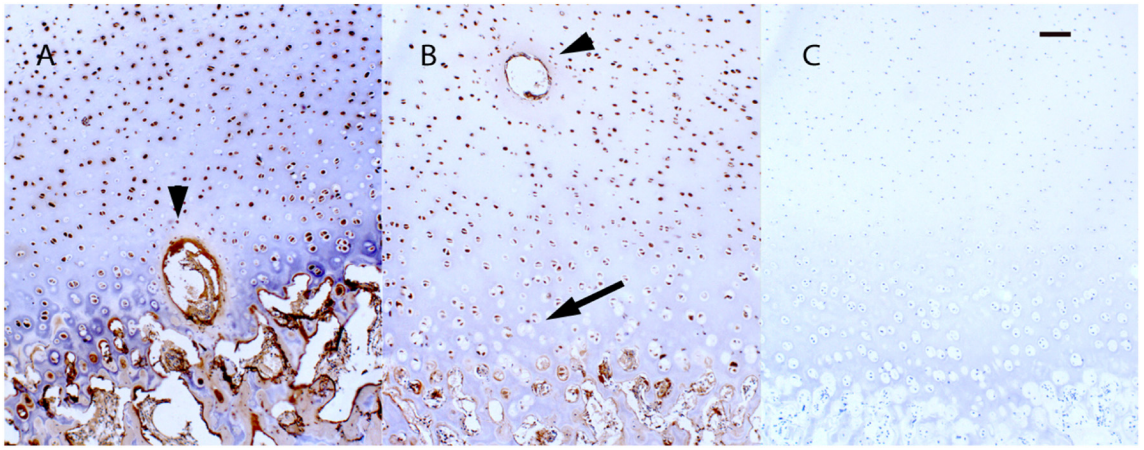


**Fig. 6.** Photomicrographs of osteochondral sections following immunohistochemical localization using antibodies directed against thymosin-β10. A) Strong cytoplasmic immunostaining in the middle and deep cartilage layers, with mild to moderate thymosin-β10 expression in chondrocytes surrounding a cartilage canal (arrowhead) in a 4 month old colt with OC. B) Moderate to strong cytoplasmic immunostaining in the middle cartilage layer of a normal 3 month old filly. Expression is variable along the osteochondral junction (arrow) and surrounding a cartilage canal (arrowhead). C) Negative control for (B). Bar = 100 μm, hematoxylin stained, DAB chromogen.

cytoplasmic expression of Fas in OC samples is not known. Fas is a membrane-bound receptor and typically is expressed in the cellular membrane. Matrix expression of Fas may be due to extruded cell membrane resulting from cell death of hypertrophic chondrocytes (Ahmed et al., 2007). However, increased matrix expression in some samples extended into the middle zone where there are no hypertrophic chondrocytes. Alternatively, matrix staining may be due to non-specific immunostaining, although this seems unlikely as the matrix was not positive in our negative controls.

Increased cytochrome *C* gene expression in OC cartilage suggests that the intrinsic pathway is also activated. Cytochrome *C* is a highly conserved mobile protein involved in electron transport and energy conversion when located in the inner mitochondrial membrane (Chandra et al., 2002). Upon translocation into the cytosol, however, cytochrome *C* becomes an activator of the intrinsic apoptotic pathway and ultimately may be released from the cell into the extracellular medium (Renz et al., 2001). Concurrently decreased mitochondrial protein expression and a trend for increased matrix protein expression found in our study may indicate translocation and extracellular release of cytochrome *C* in OC cartilage. Desjardin et al. (Desjardin et al., 2014) has previously reported abnormally large, empty mitochondria in the deep zone of OC cartilage, suggesting mitochondrial dysfunction as a part of this disease.

Thymosin-β10 is overexpressed in many neoplastic tissues (Santelli et al., 1999) and can induce apoptosis in ovarian cancer cell lines (Kim et al., 2012). In the current study, increased thymosin-β10 gene expression in OC cartilage indicates a possible role in aberrant regulation of apoptosis along the osteochondral junction. Although thymosin-β10 is a homolog to thymosin-β4, they appear to have different functions. Thymosin-β4 prevents apoptotic cell death and is increased in osteochondrosis (Mirams et al., 2016) and osteoarthritis (Wei et al., 2013), while thymosin-β10 expression has not been previously reported in these conditions.

Surprisingly, overall cell death percentages were not significantly different between OC and normal cartilage despite our finding increased expression of several apoptotic markers in OC chondrocytes. The TUNEL method detects DNA fragmentation associated with apoptosis, but also detects non-apoptotic cell death, including normal cell death in chondrocytes. In the current study, the lack of specificity of this assay may have made it difficult to detect differences in cell death between OC and normal cartilage. Additionally, false negatives can occur due to extracellular matrix steric hindrance; false positives can occur with certain forms of necrosis or in tissues with high metabolic activity.

Although there are no previous reports in OC, increased apoptosis is described in osteoarthritis (OA) (Thomas et al., 2011), affecting mainly the superficial and middle cartilage zones. Correspondingly, caspase-3 and -8 are increased in OA (Goggs et al., 2003; Toddallen et al., 2004), and Fas-mediated apoptosis occurs in OA chondrocytes in vivo and in vitro (Hashimoto et al., 1997). Fas positive cells are mainly located in the superficial and middle zones of OA cartilage (Goggs et al., 2003), and with cartilage fibrillation, can be found in the deep zone as well. TNF-α may also function as a mediator of apoptosis in OA cartilage (Fernandes et al., 2002; Yoshimura et al., 2006). Based on our study, however, TNF-α does not appear to be a factor in OC pathogenesis.

Based on our previous research (Kinsley et al., 2015), increased gene expression levels of β-catenin but not Wnt proteins occur in chondrocytes along the osteochondral junction in OC cartilage. In contrast, multiple genes associated with Wnt/β-catenin signaling have altered expression surrounding cartilage canals in OC cartilage. In the current study, we found the opposite was true, with increased apoptotic marker expression confined mainly to the osteochondral junction. Interestingly, β-catenin signaling of chondrocytes is associated with apoptosis in an age-dependent manner (Ning et al., 2012), and LiCl-activated β-catenin signaling of mature chondrocytes results in increased cell apoptotic events. However, other studies show conflicting effects of β-catenin and Wnt signaling on apoptosis, with most reports suggesting an anti-apoptotic effect (Hwang et al., 2004; Weng et al., 2010).

Overall, we found that in OC, compared to normal controls, more pro-apoptotic markers (a total of 4 markers) showed increased expression in chondrocytes along the osteochondral junction than surrounding cartilage canals (a total of 1 marker). This is not surprising given the stage of chondrocytic differentiation in each of these locations. Chondrocytes along the osteochondral junction are hypertrophic in phenotype compared to small rounded chondrocytes surrounding cartilage canals (Duesterdieck-Zellmer et al., 2015). Thus, osteochondral junction chondrocytes are expected to be entering terminal differentiation and apoptosis, while chondrocytes surrounding cartilage canals are in an earlier stage of differentiation.

## 5. Conclusion

In conclusion, these results indicate that both extrinsic and intrinsic apoptotic pathways are likely activated in OC cartilage. Increased apoptosis of osteochondral junction chondrocytes may play a role in OC, based on increased gene expression of several pro-apoptotic markers in this location. Alternatively, other mechanisms of cell death may be failing in OC, resulting in induction of apoptosis machinery in an attempt to bring about cell death and ossification.

Given these findings, it is tempting to speculate that in OC, apoptosis via the extrinsic (Fas/caspase-10 stimulation) and intrinsic (cytochrome *C* translocation) pathways are increased. However, no difference was found in TUNEL positive cells in this location, so overall cell death may not be affected in OC. In addition, protein expression of apoptotic markers did not always follow increased gene expression.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

## Acknowledgements

The authors would like to thank Dr. Chris Corless for use of laser capture microdissection unit at Oregon Health & Science University. The technical assistance of Carolyn Gendron with histologic processing at Oregon Health & Science University is much appreciated.

## Funding

This study was supported by Oregon State University College of Veterinary Medicine (VCS330-VG05). Archived samples were obtained from a previous study supported by the American Quarter Horse Foundation. The study sponsor did not play a role in the study design, collection or analysis of data, or writing of the manuscript.

## References

Ahmed, Y.A., Tatarczuch, L., Pagel, C.N., et al., 2007. Physiological death of hypertrophic chondrocytes. Osteoarthr. Cartil. 15, 575–586.
Atasoy, P., Bozdogan, O., Erekul, S., et al., 2003. Fas-mediated pathway and apoptosis in normal, hyperplastic, and neoplastic endometrium. Gynecol. Oncol. 91, 309–317.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B. Methodol. 57, 289–300.
Brun, J.-L., Cortez, A., Lesieur, B., et al., 2012. Expression of MMP-2, -7, -9, MT1-MMP and TIMP-1 and -2 has no prognostic relevance in patients with advance epithelial ovarian cancer. Oncol. Rep. 27, 1049–1057.
Carlson, C.S., Cullins, L.D., Meuten, D.J., 1995. Osteochondrosis of the articular-epiphyseal complex in young horses: evidence for a defect in cartilage canal blood supply. Vet. Pathol. 32, 641–647.
Chandra, D., Liu, J.-W., Tang, D.G., 2002. Early mitochondrial activation and cytochrome *c* up-regulation during apoptosis. J. Biol. Chem. 277, 50842–80854.
Crowley, L.C., Marfell, B.J., Scott, A.P., Waterhouse, N.J., 2016. Analysis of cytochrome *c*

release by immunohistochemistry. Cold Spring Harb Protoc 12 (pdb.prot087338). https://doi.org/10.1101/pdb.prot087338.
Desjardin, C., Chat, S., Gilles, M., et al., 2014. Involvement of mitochondrial dysfunction and ER-stress in the physiopathology of equine osteochondritis dissecans (OCD). Exp. Mol. Pathol. 96, 328–338.
Duesterdieck-Zellmer, K.F., Semevolos, S.A., Kinsley, M.A., et al., 2015. Age-related differential gene and Protein expression in postnatal cartilage canal and osteochondral junction chondrocytes. Gene Expr. Patterns 17, 1–10.
Fernandes, J.C., Martel-Pelletier, J., Pelletier, J.P., 2002. The role of cytokines in osteoarthritis pathophysiology. Biorheology 39, 237–246.
Goggs, R., Carter, S.D., Schulze-Tanzil, G., et al., 2003. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet. J. 166, 140–158.
Hall, A.K., 1995. Thymosin beta-10 accelerates apoptosis. Cell. Mol. Biol. Res. 41, 167–180.
Hashimoto, S., Setareh, M., Ochs, R.L., et al., 1997. Fas/Fas ligand expression and induction of apoptosis in chondrocytes. Arthritis Rheum. 40, 1749–1755.
Hwang, S.-G., Ryu, J.-h., Kim, I.-C., et al., 2004. Wnt-7a causes loss of differentiated phenotype and inhibits apoptosis of articular chondrocytes via different mechanisms. J. Biol. Chem. 279, 26597–26604.
JH, McDonald, 2009. Handbook of Biological Statistics, 2nd ed. Sparky House Publishing, Baltimore, Maryland.
Kawamoto, Y., Ayaki, T., Urushitani, M., et al., 2016. Activated caspase-9 immunoreactivity in glial and neuronal cytoplasmic inclusions in multiple system atrophy. Neurosci. Lett. 628, 207–212.
Kim, Y.C., Kim, B.G., Lee, J.H., 2012. Thymosin β10 expression driven by the human TERT promoter induces ovarian cancer-specific apoptosis through ROS production. PLoS One 7, e35399.
Kinkel, M.D., Yagi, R., Mcburney, D., et al., 2004. Age-related expression patterns of Bag-1 and Bcl-2 in growth plate and articular chondrocytes. Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 279A, 720–728.
Kinsley, M.A., Semevolos, S.A., Duesterdieck-Zellmer, K.F., 2015. Wnt/β-catenin signaling of cartilage canal and osteochondral junction chondrocytes and full thickness cartilage in early equine osteochondrosis. J. Orthop. Res. 33, 1433–1438.
Laverty, S., Girard, C., 2013. Pathogenesis of epiphyseal osteochondrosis. Vet. J. 197, 3–12.
Li, Q., Dashwood, W.M., Zhong, X., et al., 2004. Cloning of the rat beta-catenin gene (Ctnnb1) promoter and its functional analysis compared with the Catnb and CTNNB1 promoters. Genomics 83, 231–242.
Mirams, M., Tatarczuch, L., Ahmed, Y.A., et al., 2009. Altered gene expression in early osteochondrosis lesions. J. Orthop. Res. 27, 452–457.
Mirams, M., Avodele, B.A., Tataczuch, L., et al., 2016. Identification of novel osteochondrosis—associated genes. J. Orthop. Res. 34, 404–411.
Ning, B., Wang, P., Pei, X., et al., 2012. Dual function of β-catenin in articular cartilage growth and degeneration at different stages of postnatal cartilage development. Int. Orthop. 36, 655–664.
Olstad, K., Ytrehus, B., Ekman, S., et al., 2008. Epiphyseal cartilage canal blood supply to the tarsus of foals and relationship to osteochondrosis. Equine Vet. J. 40, 30–39.
Olstad, K., Ytrehus, B., Ekman, S., et al., 2011. Early lesions of articular osteochondrosis in the distal femur of foals. Vet. Pathol. 48, 1165–1175.
Olstad, K., Hedrickson, E.H.S., Carlson, C.S., et al., 2013. Transection of vessels in epiphyseal cartilage canals leads to osteochondrosis and osteochondrosis dissecans in the femoro-patellar joint of foals; a potential model of juvenile osteochondritis dissecans. Osteoarthr. Cartil. 21, 730–738.
Opferman, J.T., Kothari, A., 2018. Anti-apoptotic BCL-2 family members in development. Cell Death Differ. 25, 37–45.
Peng, X.X., Zhao, R.L., Song, W., et al., 2012. Selection of suitable reference genes for normalization of quantitative real-time PCR in cartilage tissue injury and repair in rabbits. Int. J. Mol. Sci. 13, 14344–14355.
Presser, J.S., Sen, P.L., Offenbacher, S., 2011. Multiple hypothesis testing for experimental gingivitis based on Wilcoxon signed rank statistics. Stat. Biopharm. Res. 3, 372–384.
Renz, A., Berdel, W.E., Kreuter, M., et al., 2001. Rapid extracellular release of cytochrome *C* is specific for apoptosis and marks cell death in vivo. Blood 98, 1542–1548.
Riddick, T.L., Duesterdieck-Zellmer, K., Semevolos, S.A., 2012. Gene and protein expression of cartilage canal and osteochondral junction chondrocytes and full-thickness cartilage in early equine osteochondrosis. Vet. J. 194, 319–325.
Roach, H.I., Erenpreisa, J., 1996. The phenotypic switch from chondrocytes to bone-forming cells involves asymmetric cell division and apoptosis. Connect. Tissue Res. 35, 85–91.
Santelli, G., Califano, D., Chiappetta, G., et al., 1999. Thymosin β-10 gene overexpression is a general event in human carcinogenesis. Am. J. Pathol. 155, 799–804.
Schatz, F., Kayisli, U.A., Vatandasiar, E., et al., 2012. Toll-like receptor 4 expression in decidual cells and interstitial trophoblasts across human pregnancy. Am. J. Reprod. Immunol. 68, 146–153.
Schultz, D.R., Harrington, W.J., 2003. Apoptosis: programmed cell death at a molecular level. Semin. Arthritis Rheum. 32, 345–369.
Shiotsuka, M., Wada, H., Kiyoshima, T., et al., 2013. The expression and function of thymosin beta 10 in tooth germ development. Int. J. Dev. Biol. 57, 873–883.
Takayama, S., Sato, T., Krajewski, S., et al., 1995. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 80, 279–284.
Thomas, C.M., Fuller, C.J., Whittles, C.E., et al., 2011. Chondrocyte death by apoptosis is associated with the initiation and severity of articular cartilage degradation. Int. J. Rheum. Dis. 14, 191–198.
Toddallen, R., Robertson, C., Harwood, F., et al., 2004. Characterization of mature vs. aged rabbit articular cartilage: analysis of cell density, apoptosis-related gene expression and mechanisms controlling chondrocyte apoptosis. Osteoarthr. Cartil. 12, 917–923.
Viglietto, G., Calitano, D., Bruni, P., et al., 1999. Regulation of thymosin β10 expression by TSH and other mitogenic signals in the thyroid gland and in cultured thyrocytes. Eur. J. Endocrinol. 140, 597–607.
Wachmann, K., Pop, C., van Raam, B.J., et al., 2010. Activation and specificity of human caspase-10. Biochemistry 49, 8307–8315.
van Weeren, PR and Barneveld, A. The effect of exercise on the distribution and manifestation of osteochondrotic lesions in the warmblood foal. Equine Vet. J. Suppl. 1999;31:16–25.
Wei, M., Duan, D., Liu, Y., et al., 2013. Increased thymosin β4 levels in the serum and SF of knee osteoarthritis patients correlate with disease severity. Regul. Pept. 185, 34–36.
Weng, L.-H., Wang, C.-J., Ko, J.-Y., et al., 2010. Control of Dkk-1 ameliorates chondrocyte apoptosis, cartilage destruction, and subchondral bone deterioration in osteoarthritis knees. Arthritis Rheum. 62, 1393–1402.
Yang, L., Tsang, K.Y., Tang, H.C., et al., 2014. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proc. Natl. Acad. Sci. 111, 12097–12102.
Yoshimura, F., Kanno, H., Uzuki, M., et al., 2006. Downregulation of inhibitor of apoptosis proteins in apoptotic human chondrocytes treated with tumor necrosis factor-alpha and actinomycin D. Osteoarthr. Cartil. 14, 435–441.