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Cartilage canals in the distal intermediate ridge of the tibia of fetuses and foals are surrounded by different types of collagen

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Abstract

Some epiphyseal growth cartilage canals are surrounded by a ring of hypereosinophilic matrix consisting of collagen type I. Absence of the collagen type I ring may predispose canal vessels to failure and osteochondrosis, which can lead to fragments in joints (osteochondrosis dissecans). It is not known whether the ring develops in response to programming or biomechanical force. The distribution that may reveal the function of the ring has only been described in the distal femur of a limited number of foals. It is also not known which cells are responsible for producing the collagen ring. The aims of the current study were to examine fetuses and foals to infer whether the ring forms in response to biomechanical force or programming, to describe distribution and to investigate which cell type produces the ring. The material consisted of 46 fetuses and foals from 293 days of gestation to 142 days old, of both sexes and different breeds, divided into three groups, designated the naïve group up to and including the day of birth, the adapting group from 2 days up to and including 14 days old, and the loaded group from 15 days and older. The distal tibia was sawn into parasagittal slabs and the cranial half of the central slab from the intermediate ridge was examined by light microscopy and immunohistochemical staining for collagen type I. Presence, completeness and location of the collagen ring was compared, as was the quantity of perivascular mesenchymal cells. An eosinophilic ring present on HE-stained sections was seen in every single fetus and foal examined, which corresponded to collagen type I in immunostained sections. A higher proportion of cartilage canals were surrounded by an eosinophilic ring in the naïve and adapting groups at 73 and 76%, respectively, compared with the loaded group at 51%. When considering only patent canals, the proportion of canals with an eosinophilic ring was higher in the adapting and loaded than the naïve group of foals. The ring was present around 90 and 81% of patent canals in the deep and middle layers, respectively, compared with 58% in the superficial layer, and the ring was more often complete around deep compared with superficial canals. The ring was absent or partial around chondrifying canals. When an eosinophilic ring was present around patent canals, it was more common for the canal to contain one or more layers of perivascular mesenchymal cells rather than few to no layers. It was also more common for the collagen ring to be more complete around canals that contained many as opposed to few mesenchymal cells. In conclusion, the proportion of cartilage canals that had an eosinophilic ring was similar in all three groups of fetuses and foals, indicating that the presence of the collagen ring was mostly programmed, although some adaptation was evident. The ring was more often present around deep, compared with superficial canals, indicating a role in preparation for ossification. The collagen ring appeared to be produced by perivascular mesenchymal cells.

Key words: cartilage canal; collagen type I; epiphyseal growth cartilage; foal; histology; immunohistochemical staining; osteochondrosis; tibia.

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Introduction

Most bones grow by endochondral ossification which occurs in specialised growth cartilage located at each end of the long bones (Banks, 1993). The metaphyseal growth plate, or physis, is located between the secondary

ossification centre of the epiphysis and the primary ossification centre of the diaphysis The epiphyseal growth cartilage is located between the non-vascularised articular cartilage and the secondary ossification centre of the epiphysis (Banks, 1993). Together, the articular cartilage and epiphyseal growth cartilage are known as the articular-epiphyseal cartilage complex (Carlson et al. 1986). During early skeletal development, specialised channels carry blood vessels from the perichondrium to the growth cartilage and supply it with oxygen, nutrients and mesenchymal progenitor cells (Blumer et al. 2008). As the animal grows, the layer of epiphyseal growth cartilage becomes progressively thinner. The associated loss of vascularity occurs through two distinct physiological processes. First, there is the process of chondrification (Haines, 1974), which starts in the distal end of the cartilage canal and is characterised by endothelial cell degeneration and gradual filling of the canal lumen with chondrocytes and cartilage matrix (Ytrehus et al. 2004a; Olstad et al. 2007). The second process is the incorporation of the vessels into the advancing ossification front (Ytrehus et al. 2004b; Olstad et al. 2008b). During the later stages of joint development, the cartilage is avascular and is nourished by diffusion from the nearby synovial fluid and subchondral bone.

Osteochondrosis is a disease that affects several animal species and humans (for review see Ytrehus et al. 2007; Olstad et al. 2015). It is defined as a disturbance of endochondral ossification that occurs multi-focally at predilection sites and can develop both in the physis and in the epiphyseal growth cartilage (Ytrehus et al. 2007). The term osteochondrosis dissecans (OCD) is used when the overlying articular cartilage breaks off, leading to mineralised fragments within the joint (Ytrehus et al. 2007). Heritability has been estimated in horses (Grøndahl & Dolvik, 1993) and pigs (Reiland et al. 1978). The described incorporation of vessels into the ossification front means that the arterial source must shift from perichondrially to subchondrally located vessels (Ytrehus et al. 2004b; Olstad et al. 2008b). This latter process appears to be vulnerable to failure, which then leads to ischaemic chondronecrosis (osteochondrosis latens) at intermediate depth, i.e. the deep resting and proliferative zones of the growth cartilage, which can further develop into clinically significant osteochondrosis manifesta and OCD (Ytrehus et al. 2004b; Olstad et al. 2008b). It is not currently known which step of the incorporation process fails in osteochondrosis (Olstad et al. 2015). However, it has become clear that in osteochondrosis, some canals fail while neighbouring canals at the same stage of incorporation remain intact (Olstad et al. 2008a, 2015). It is therefore necessary to study differences between individual, adjacent canals. It has been proposed that cartilage matrix changes predispose to osteochondrosis, for example, changes in cartilage matrix composition near the ossification front were suggested to affect the stability of the cartilage canals (Lecocq et al. 2008). However, that study did not report differences between neighbouring canals and therefore could not explain why only some canals fail in osteochondrosis (Lecocq et al. 2008). Recently, a study using transmission electron microscopy (TEM) and immunohistochemical staining demonstrated that the matrix immediately surrounding many cartilage canals contained tightly packed collagen type I fibres, which corresponded on light microscopy to an intensely eosinophilic ring in haematoxylin and eosin (HE)-stained sections (Hellings et al. 2016). It was also shown that some of the cartilage canals lacked the collagen ring and, instead, the vascular tissue of the canals were in direct contact with the surrounding hyaline cartilage, mainly collagen type II (Hellings et al. 2016). It was suggested that the collagen type I structure might add support to blood vessels within cartilage canals and that absence of the collagen ring could predispose individual canals to vascular failure (Hellings et al. 2016). This type of difference between individual canals warrants further study because it has the potential to explain why only some canals fail in osteochondrosis.

Growth cartilage comprising chondrocytes and their extracellular matrix (ECM) also contains cartilage canals (Lutfi, 1970a). The cartilage canals include vessels outlined by endothelial cells and perivascular mesenchymal cells. These connective tissue cells produce extracellular matrix depending on what the cell is programmed to produce (Heinegard, 2009) but also in response to various stimuli, including biomechanical force (Carver & Goldsmith, 2013). Biomechanical force has been suggested to be involved in the development of osteochondrosis and is undoubtedly involved in the progression from osteochondrosis latens and manifesta to OCD (Ytrehus et al. 2007; Olstad et al. 2015). It remains to be determined whether biomechanical force can cause the blood supply to fail in the first place (Olstad et al. 2015). It has been suggested that trabecular micro-fractures caused by biomechanical force might predispose vessels to fail at the point of incorporation into the bone (Ytrehus et al. 2004b). However, micro-fractures were not detected in a population of foals examined by micro-computed tomography with sufficient resolution to detect them (Olstad et al. 2008a).

It is not known whether the collagen ring around some cartilage canals is the result of cell programming or a response to biomechanical force. If absence of the ring predisposes to failure, it is important to discover whether the absence is due to faulty cell programming or inappropriate response to force. While *in utero*, fetuses are exposed to a minimum of gravitational force; however, movement is essential for normal joint development (Nowlan et al. 2010). In equine fetuses, tensional forces caused by extension of the legs have been considered important for functional adaptation of the musculoskeletal system, although substantial compressive forces will not be experienced until the foal is on its feet and subject to gravitational force (Lin et al. 2005; Hyttinen et al. 2009). It is not known how long

it takes for the collagen type I/eosinophilic ring to become visible in histological section after induction of collagen production from the appropriate cell. However, small amounts of collagen type I may be visible histologically immediately adjacent to fibroblasts after a few days (Oostendorp et al. 2016), and become more abundant (Kumar et al. 2003) and visible further away from the fibroblast (Oostendorp et al. 2016) by week 2. Also, maturation of collagen type I prior to mineralisation in bone formation is reported to take 10–14 days (Komarova et al. 2015). If the ring of collagen type I surrounding cartilage canals forms predominantly in response to gravitational force, it may therefore take up to 14 days from birth and the start of limb-loading until fibrils have formed and become detectable in histological sections.

The first aim of the current study was to compare fetuses, foals up to 14 days, and foals older than 14 days to infer whether the collagen ring around cartilage canals forms in response to biomechanical force or cell programming. The second aim was to describe the distribution of the ring in a larger group of foals than previously examined. The third aim was to investigate which cell type was located immediately adjacent to the rings and therefore was most likely responsible to produce the collagen.

Material and methods

Material was selected from a population of fetuses and foals submitted for routine postmortem examination collected for a previously published study (Olstad et al. 2007). A maximum age of 5 months was imposed (Table 1). The age of fetuses was based on records of breeding dates obtained from owners and on an average gestation length of 340 days. If records of breeding dates were missing, age was calculated based on crown–rump length according to Platt (1978). Included individuals were divided into three groups based on age: fetuses and foals up to and including the day of birth were designated the naïve group; foals from day 2 to up to and including day 14 after birth were designated the adapting group; and foals 15 days and older were designated the loaded group (Table 1). Both sexes and any breed of pony or horse were included (Table 1). Cause of death or euthanasia was recorded (Table 1). Foals with pathological lesions in the distal intermediate ridge of the tibia were excluded.

Collection protocol

The tibia was exposed and removed from one or both hind limbs. The distal intermediate ridge was separated from the tibia and slabs were sawn parallel to the ridge in a slightly oblique parasagittal plane (Fig. 1A). The number of slabs available from each animal varied from one to five. The most centrally positioned slab from each distal intermediate ridge was selected for further analysis. The cranial half (Fig. 1A) of the selected slab was submitted for histology. Slabs were fixed in 4% phosphate-buffered formaldehyde for 24 h and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) or formic acid. Sections 5 μm thick were cut from each slab and stained with haematoxylin and eosin (HE). Selected sections were immunostained with polyclonal antibodies against equine collagen type I. A full immunohistochemical staining protocol is included in Supporting Information Data S1.

Histological evaluation of cartilage canals and their surrounding matrix

The criteria for histological evaluation were identical to those previously described in Olstad et al. (2007) and Hellings et al. (2016). Individual cartilage canals as seen cut in transverse section were categorised as patent, early chondrifying or late chondrifying. In early chondrifying canals, vessel remnants were still visible, whereas late chondrifying canals contained only chondrocyte hypercellularity in association with hyper-intense basophilic matrix (Olstad et al. 2007).

For each cartilage canal, the eosinophilic ring around the cartilage canal seen in HE-stained sections was evaluated semi-quantitatively: when an eosinophilic ring was absent or present at less than ¼ of the peripheral margin of the cartilage canal, it was described as absent, when the eosinophilic ring was weak-staining and

Table 1 Age, breed and cause of death or euthanasia of included foals.

n	Age	*n*	Breed	*n*	Cause of death or euthanasia
8	293–300 days of gestation	20	Standardbred	11	Abortion
8	300–340 days of gestation	8	Pony breed	9	Gastro-intestinal disease
3	0–1 day	6	Warmblood riding horse	6	Non-septic orthopaedic disease
14	2–14 days	4	Thoroughbred	4	Maternal disease
1	15–28 days	4	Coldblooded trotting horse	2	CNS disease
4	29–43 days	4	Miscellaneous	2	Urinary disease
2	44–68 days			2	Cardiac disease
1	69–83 days			1	Muscle disease
2	84–98 days			1	Ocular disease
0	99–113 days			1	Prematurity
2	114–128 days			1	Thoracic disease
1	129–142 days			1	Weakness
				1	Congenital malformation
				4	Unknown
46		46		46	Total

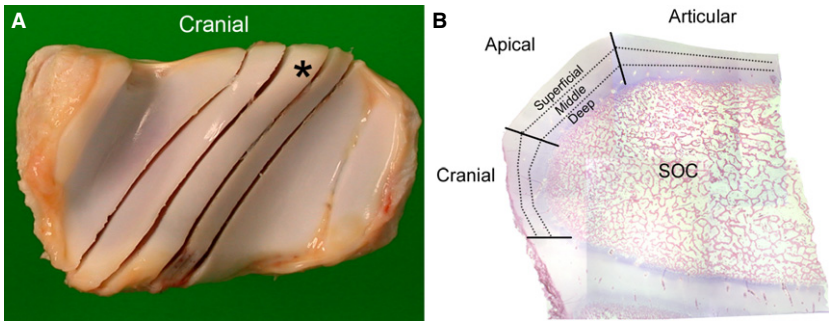

Fig. 1 Collection method. (A) The distal tibia was sawn parallel to the ridge in a slightly oblique parasagittal plane. The cranial half of the most centrally positioned slab (asterisk) was selected for further analysis. (B) For descriptive purposes, the tissue in each section was divided into cranial, apical and articular aspects and the thickness of the articular-epiphyseal cartilage complex was divided into three equally thick layers of superficial, middle and deep. SOC, secondary centre of ossification.

Table 2 Number of foals, hind limbs and cartilage canals examined.

	Naïve group ≤ 1 day old	Adapting group 2–14 days old	Loaded group ≥ 15 days old	Total
Number of foals	19	14	13	46
Number of hind limbs	22	16	13	51
Total number of cartilage canals examined	1130	492	87	1709
Average number of canals per section	51	31	7	34
Patent canals	1118 (99%)	421 (86%)	33 (38%)	1572 (92%)
Early chondrifying canals	6 (0.5%)	26 (5%)	9 (10%)	41 (2.5%)
Late chondrifying canals	6 (0.5%)	45 (9%)	45 (52%)	96 (5.5%)

present at ¼–¾ of the canal margin, it was described as partial, and when it was intensely staining and present at more than ¾ of the canal margin, it was described as complete.

The tissue in each section was divided into cranial, apical and articular aspects and the thickness of the articular-epiphyseal cartilage complex was divided into three equally thick layers of superficial, middle and deep, where deep referred to the layer closest to the subchondral bone (Fig. 1B).

In addition, the quantity of mesenchymal cells within cartilage canals was assessed subjectively, ranging from canals with few to no cells where the blood vessel endothelium was in direct contact with the surrounding cartilage matrix, to canals where blood vessels were separated from the surrounding cartilage matrix by a single layer of mesenchymal cells, to canals where there were two or more layers of mesenchymal cells separating the blood vessels from the cartilage matrix.

Results

The study sample consisted of 46 fetuses and foals, ranging from 293 days of gestation to 142 days of age (Table 1). There were 27 males, 17 females and two foals with sex unrecorded. Six breed categories were represented (Table 1). Causes of death or euthanasia are listed in Table 1. Six foals had non-septic orthopaedic disease including five foals with fractures and one foal with contracted tendons.

From the 46 foals, one section from 51 left and/or right hind limbs was analysed. The naïve group comprised 19 foals and 22 limbs, the adapting group 14 foals and 16 limbs, and the loaded group 13 foals and 13 limbs (Table 2). In the 51 sections, 1709 cartilage canals cut in transverse section were analysed (Table 2). The number of cartilage canals per section decreased after birth and the epiphyseal growth cartilage was completely avascular in the oldest foal at 142 days. The average number of canals per section therefore decreased between groups (Table 2). The categories of cartilage canals also changed with age: in the naïve group, the majority of canals were patent and chondrifying canals were only occasionally present including in the youngest foal at 293 days of gestation (Table 2). The proportion of canals that were chondrifying increased and the stage of chondrification advanced with increasing age (Table 2).

Presence of eosinophilic (collagen) rings

An eosinophilic ring in HE-stained sections was present around one or more cartilage canals in every fetus and foal examined. Overall, 463 of 1709 (27%) of all examined cartilage canals did not have an eosinophilic ring (Fig. 2A), 625 of 1709 (37%) canals were surrounded by a partial eosinophilic ring, and 621 of 1709 (36%) canals were surrounded by a complete eosinophilic ring (Table 3; Fig. 2B). The eosinophilic ring in HE–stained sections stained positive for collagen type I (Figs. 2B–D).

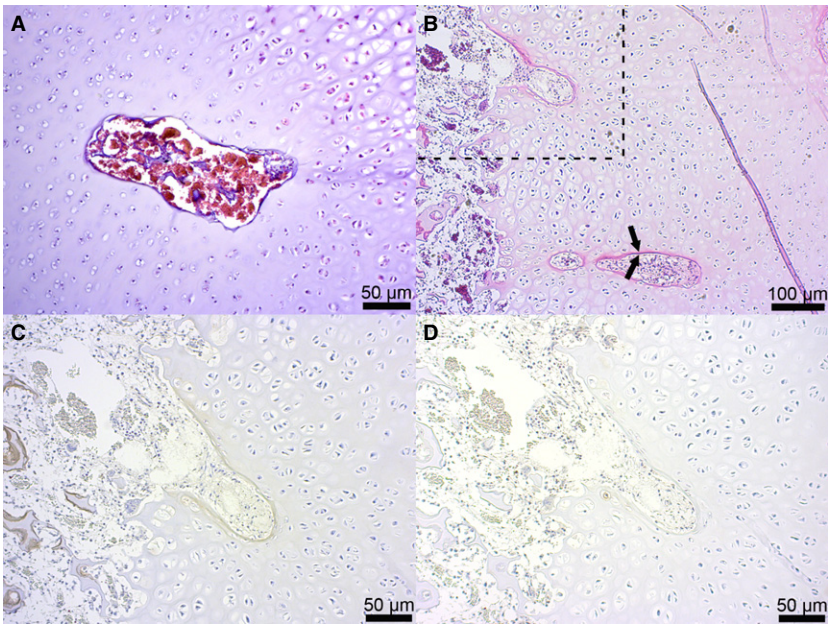

Fig. 2 Patent cartilage canals in epiphyseal growth cartilage from the distal intermediate ridge of the tibia. (A) Three-day-old male Standardbred, HE, 100×. Patent canal without an eosinophilic ring. (B) Fetus at 295 days of gestation, female Standardbred, HE, 50×. Patent cartilage canals surrounded by a complete eosinophilic ring (between arrows), articular aspect, deep layer. (C) Same foal and area inside stippled box in (B), immunostained for collagen type I, 100×. The eosinophilic ring in HE-stained sections corresponded to extracellular matrix, immunostained with polyclonal antibodies against equine collagen type I. (D) The same section as (D), control-stained with non-immune serum (isotype).

Table 3 Presence of eosinophilic ring around cartilage canals of the epiphyseal growth cartilage on HE-stained histologic sections in foal groups.

Eosinophilic ring	All foals; *n*/total *n* canals	Naïve group; *n*/total *n* canals	Adapting group; *n*/total *n* canals	Loaded group; *n*/total *n* canals
All canals				
Absent	463/1709 (27%)	304/1130 (27%)	116/492 (24%)	43/87 (49%)
Partial	625/1709 (37%)	408/1130 (36%)	187/492 (38%)	30/87 (35%)
Complete	621/1709 (36%)	418/1130 (37%)	189/492 (38%)	14/87 (16%)
Patent				
Absent	374/1572 (24%)	300/1118 (27%)	68/421 (16%)	6/33 (18%)
Partial	577/1572 (37%)	400/1118 (36%)	164/421 (39%)	13/33 (39%)
Complete	621/1572 (39%)	418/1118 (37%)	189/421 (45%)	14/33 (43%)
Early chondrifying				
Absent	8/41 (20%)	0/6	7/26 (27%)	1/9 (11%)
Partial	33/41 (80%)	6/6 (100%)	19/26 (73%)	8/9 (89%)
Complete	0/41	0/6	0/26	0/9
Late chondrifying				
Absent	81/96 (84%)	4/6 (67%)	41/45 (91%)	36/45 (80%)
Partial	15/96 (16%)	2/6 (33%)	4/45 (9%)	9/45 (20%)
Complete	0/96	0/6	0/45	0/45

When comparing the three different groups, a higher proportion of cartilage canals were surrounded by an eosinophilic ring in the naïve and adapting groups (73 and 76%, respectively), compared with the loaded group (51%; Table 3). The reverse was true when comparing only patent cartilage canals: the proportion of patent canals surrounded by an eosinophilic ring was lowest in the naïve group (73%) compared with 84% in the adapting and 82% in the loaded groups of foals (Table 3). The percentage of patent canals surrounded by a complete as opposed to a partial eosinophilic ring was also slightly higher in the adapting and loaded groups (45 and 43%, respectively) compared with the naïve group at (37%; Table 3).

Fewer canals were surrounded by an eosinophilic ring in the loaded group than in the naïve and adapting groups of foals (Table 3), which most likely is a reflection of the fact that the proportion of chondrifying canals was higher in older foals (Table 2). Whereas 39% of all patent canals were surrounded by a complete eosinophilic ring, none of the chondrifying canals were (Table 3). When an eosinophilic ring was present around chondrifying canals, it was always partial (Table 3; Fig. 3A). A higher proportion (80%) of early chondrifying canals were surrounded by a partial eosinophilic ring compared with late chondrifying canals (16%), i.e. the eosinophilic ring became weaker or disappeared with advancing stages of chondrification (Table 3; Fig. 3B).

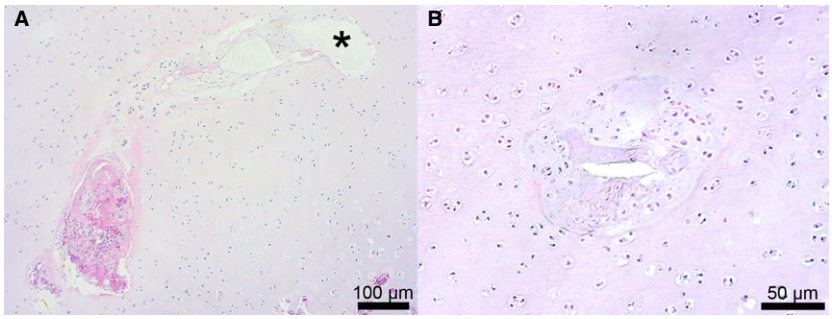

Fig. 3 Chondrifying canals in epiphyseal growth cartilage from the distal intermediate ridge of the tibia. (A) Thirty-eight day-old male Warmblood, HE, 50×. Partial eosinophilic ring (arrows) in the late chondrifying part of the canal (asterisk) in the middle layer of the cartilage does not have an eosinophilic ring, whereas the early chondrifying part in the deep layer of the cartilage is surrounded by a weak-staining eosinophilic ring (arrowheads). (B) Forty-five day-old male Quarter Horse, HE, 100×. This late chondrifying canal does not have an eosinophilic ring.

Table 4 Presence of eosinophilic ring around patent canals on HE-stained histologic sections. Presented in different depths in the articular-epiphyseal cartilage complex.

Eosinophilic ring	All foals; *n*/total *n* canals	Naïve group; *n*/total *n* canals	Adapting group; *n*/total *n* canals	Loaded group; *n*/total *n* canals
Superficial layer				
Absent	218/521 (42%)	195/437 (45%)	22/82 (27%)	1/2 (50%)
Partial	218/521 (42%)	178/437 (41%)	39/82 (48%)	1/2 (50%)
Complete	85/521 (16%)	64/437 (14%)	21/82 (26%)	0/0
Middle layer				
Absent	110/572 (19%)	84/397 (21%)	23/164 (14%)	3/11 (27%)
Partial	234/572 (41%)	158/397 (40%)	71/164 (43%)	5/11 (45%)
Complete	228/572 (40%)	155/397 (39%)	70/164 (42%)	3/11 (27%)
Deep layer				
Absent	46/479 (10%)	21/284 (7%)	23/175 (13%)	2/20 (10%)
Partial	125/479 (26%)	64/284 (23%)	54/175 (31%)	7/20 (35%)
Complete	308/479 (64%)	199/284 (70%)	98/175 (56%)	11/20 (55%)

Distribution of the eosinophilic (collagen) rings

Chondrification occurs towards the articular before the cranial and apical aspects of the distal intermediate ridge, thus if the eosinophilic ring was associated with physiological chondrification, older foals could have had stronger rings around more canals towards the articular, followed by cranial and then apical aspects of the ridge, compared with younger foals. However, when presence was evaluated, chondrifying canals were less commonly surrounded by eosinophilic rings and, when present, rings were partial, thus a relationship between chondrification and eosinophilic rings was considered unlikely and not examined further.

The apical aspect had the lowest number of canals surrounded by a complete eosinophilic ring (35%), followed by the articular and cranial aspects (40 and 43%, respectively) (Supporting Information Table S1).

An eosinophilic ring was present around 90 and 81% of patent canals in the deep and middle layers of the articular-epiphyseal cartilage complex, respectively, compared with around 58% of canals in the superficial layer (Table 4). When present, the eosinophilic ring was also more often complete around canals in the deep and middle layers (64 and 40%, respectively) than in the superficial layer of the articular-epiphyseal cartilage complex (16%) (Table 4).

Cell type adjacent to eosinophilic (collagen) rings

The eosinophilic ring was absent or partial around chondrifying canals, which contained no viable endothelial cells and where perivascular mesenchymal cells had differentiated into chondrocytes.

When an eosinophilic ring was present around patent cartilage canals, it was more common for the canal to contain one or more layers (Fig. 4A) than few to no perivascular mesenchymal cells separating the blood vessels from the surrounding cartilage (Table 5). Of the 621 patent canals with a complete eosinophilic ring, 602 (97%) contained one or more layers of mesenchymal cells and 285 of the 621 (46%) canals contained two or more layers of mesenchymal cells (Table 5). In contrast, of the 374 patent canals that did not have an eosinophilic ring, two or more layers of

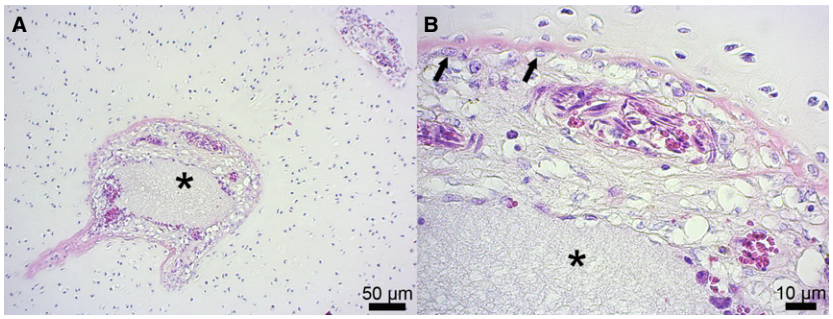

Fig. 4 Perivascular mesenchymal cells. (A) Female Warmblood Riding Horse fetus at 293 days of gestation, HE, 100×. When an eosinophilic ring was present around patent cartilage canals (asterisk), it was more common for the canal to contain one or more layers than few to no layers of perivascular mesenchymal cells separating the blood vessels from the surrounding cartilage. (B) Higher power magnification of (A), HE, 400×, showing perivascular mesenchymal cells (arrows) immediately adjacent to eosinophilic ring.

Table 5 Relationship between eosinophilic ring and mesenchymal cells on HE-stained histologic sections in patent canals.

Eosinophilic ring	Few to no mesenchymal cells	Single layer of mesenchymal cells	Two or more layers of mesenchymal cells
Absent	88/374 (24%)	203/374 (54%)	83/374 (22%)
Partial	70/577 (12%)	368/577 (64%)	139/577 (24%)
Complete	19/621 (3%)	317/621 (51%)	285/621 (46%)
Eosinophilic ring	Absent	Partial	Complete
Few to no mesenchymal cells	88/177 (50%)	70/177 (39%)	19/177 (11%)
Single layer of mesenchymal cells	203/888 (23%)	368/888 (41%)	317/888 (36%)
Two or more layers of mesenchymal cells	83/507 (16%)	139/507 (27%)	285/507 (56%)

mesenchymal cells were present in only 83 (22%) of the canals (Table 5).

It was also more common for eosinophilic rings to be more complete around 285 of 507 (56%) canals that contained many mesenchymal cells, compared with 317 of 888 (36%) canals that contained a single layer and 19 of 177 (11%) of canals that contained few to no mesenchymal cells (Table 5).

Discussion

The main finding of the study was that an eosinophilic ring consisting of collagen type I surrounding the cartilage canals was present in all fetuses and foals examined.

Does the collagen ring form in response to programming or adaptation to load?

A collagen type I ring, identified on HE-stained sections as an eosinophilic ring surrounding the cartilage canals, was present in the naïve, adapting and loaded groups of fetuses and foals. The proportion of cartilage canals with an eosinophilic ring was similar to that in a study of the femur of foals (Hellings et al. 2016), thus the current study in the tarsus of a larger group of foals agrees with and confirms the previous observation found in the femur. A similar collagen ring was also found around epiphyseal cartilage canals on transmission electron microscopy (TEM) of sheep fetuses (Stockwell, 1971) and chicken embryos (Blumer et al. 2004), and supports the conclusion that weight-bearing is not essential for development of the collagen type I ring. The presence of similar proportions in naïve fetuses as in foals that have loaded their limbs also indicates that the collagen ring around cartilage canals of the epiphyseal growth cartilage is mainly programmed in horses.

In the current study, when comparing the presence of an eosinophilic ring around patent canals between the naïve group and the adapting and loaded groups, there were fewer canals without an eosinophilic ring and rings were larger and more prominent in the two oldest groups compared with the naïve group, suggesting that some adaptation to load did occur. Articular cartilage collagen structure of type II is reported to be isotropic at birth and to develop an anisotropic structure in response to biomechanical load after birth (Brama et al. 2000a; van Turnhout et al. 2010). However, when collagen structure was examined by polarised light microscopy a zone-dependent collagen structure was shown to develop between 6 and 8 months' gestation in equine fetuses (Lecocq et al. 2008). The contents of both collagen type I in subchondral bone and collagen type II in articular cartilage differed between groups receiving different exercise regimes (Brama et al. 2002a,b),

demonstrating that mechanical load influences production of both types of collagen in equine joints. The current results augment those observations by indicating that load influences the production of collagen type I around cartilage canals within epiphyseal growth cartilage. The results of the present study indicated that the eosinophilic ring was mainly programmed, although adaptation to load also appeared to have some influence on development.

In the current study, the number of patent canals declined rapidly after birth due to chondrification, and the time available for adaptation was short compared with previous studies (Brama et al. 2000b, 2002b). In the future, the collagen ring could be studied further in joints that are vascularised for a longer period after birth, for example the femoro-patellar joint, which is vascularized for up to 7 months (Carlson et al. 1995) compared with the distal tibia with vascularisation only up to 5 months (Olstad et al. 2008b). Collagen fibril structure (Finnøy et al. 2016) and cross-linking (Hurtig et al. 1993) within the surrounding matrix of the cartilage canals could also be elucidated.

Distribution of the collagen type I eosinophilic ring

The distribution of the eosinophilic ring within the epiphyseal growth cartilage was examined more closely in order to better understand the likely function of the ring. When present around chondrifying canals, the ring was always partial, indicating that the presence of an eosinophilic ring was not associated with the physiological process of chondrification. The predilection site of osteochondrosis in the cranial distal intermediate ridge of the tibia was always located in the articular-apical aspects, distal to the point of incorporation of vessels into bone at the apex (Olstad et al. 2008b). If absence of the ring predisposed for failure, it would be expected that fewer canals would be surrounded by an eosinophilic ring in the apical aspect. However, eosinophilic rings were present in similar proportions in the three aspects examined; however, there was a tendency towards fewer and less developed eosinophilic rings in the apical rather than the articular and cranial aspects of the distal intermediate ridge. In the current study, only the central slab was examined, and it would be interesting to compare distribution of the eosinophilic ring between medial and lateral canals, as lesions were more often seen in lateral than medial slabs (Olstad et al. 2008b).

The most striking finding with respect to the distribution of the eosinophilic rings was that they were most often complete around the deep cartilage canals close to the ossification front. They were also more often complete around deep canals in the fetuses than in the two older groups, corresponding to a phase of highly active ossification. This is consistent with studies in sheep (Stockwell, 1971) and chickens (Blumer et al. 2004) and suggests that the collagen ring has a physiological role in preparation for ossification. Micro-computed tomography of young foals showed that cartilage canals in the ossification front of the talus were surrounded by tubes extending from the subchondral bone plate, possibly representing mineralized osteoid (Olstad et al. 2008a). Osteoid is composed of collagen type I and is the non-mineralized precursor of bone extracellular matrix laid down by osteoblasts on spicules of calcified cartilage between hypertrophied chondrocytes (Banks, 1993). Blumer et al. (2006) showed that mesenchymal cells within cartilage canals close to the ossification front in chickens contained osteo-progenitor cells and concluded that the collagen type I around canals was a reflection of the role of cells within the cartilage canals in the establishment of the secondary ossification centre. The distribution of the collagen rings, both in the current and previous studies (Blumer et al. 2005, 2006), support that it plays a role in preparing the growth cartilage for ossification also in foals.

Which cell type produces the collagen ring?

In the current study, chondrifying canals where mesenchymal cells had differentiated into chondrocytes were less commonly surrounded by an eosinophilic ring than were patent canals. In patent canals, the eosinophilic ring was most often complete around cartilage canals that contained two or more layers of perivascular mesenchymal cells. These results indicate that the ability to produce collagen type I is associated with the presence of mesenchymal cells.

In a previous TEM study of growth cartilage in foals, the flat, spindle-shaped mesenchymal cells close to the exterior of the canals appeared to differentiate into chondroblasts and contribute to growth of the cartilage model (Hellings et al. 2016). Identical flat cells were identified on two-photon-excited fluorescent microscopy (TPEF) of porcine cartilage canals, and when combined with second harmonic generation (SHG) microscopy, it was possible to visualise production of collagen fibres by the flat cells (Finnøy et al. 2016). The combined findings of the current, TEM (Hellings et al. 2016) and TPEF/SHG microscopy studies (Finnøy et al. 2016) suggest that the perivascular mesenchymal cells go through a fibroblast-like stage while still located within cartilage canals, during which they may be capable of producing dense and tightly packed collagen consisting of collagen type I. Mesenchymal cells within cartilage canals have been shown to have chondro-progenitor (Lutfi, 1970b) and osteo-progenitor potential (Blumer et al. 2006). As far as the authors are aware, a fibroblast-like functional stage has not previously been suggested for cartilage canal mesenchymal cells. Cartilage canals do, however, originally develop as extensions of the perichondrium (Blumer et al. 2008). Fibroblast-like cells reside on the interface between the fibrous perichondrium and the underlying growth cartilage, and give rise to chondroblasts that contribute to appositional growth of the cartilage during skeletal development (Ross & Pawlina, 2012). *In vitro*, mesenchymal stem

cells and fibroblasts had a similar morphology and expressed several of the same cell surface markers, both deposit collagen type I, and both were capable of differentiating into chondrocytes and osteoblasts (Denu et al. 2016). Although this cannot be assumed to be the case *in vivo*, these studies tentatively support the suggestion that mesenchymal cells within cartilage canals may have fibroblast-like properties and be capable of producing collagen type I, *en route* to becoming chondrocytes.

In the current study, the presence of the eosinophilic ring varied between canals at different stages of development, in different locations and at different depths. This may suggest that the functions of the mesenchymal cells differ between individual canals and, in theory, this could dictate whether the collagenous ring develops or not. In terms of adaptation, the number of studies of biomechanical forces acting on mesenchymal cells within cartilage canals is limited, but in an *in vitro* study using bovine nasal discs it was documented that when compressed, solutes moved in and out of canals in the discs, i.e. fluid shear forces are present within cartilage canals (Albro et al. 2011).

Relationship between the collagen ring and osteochondrosis

Approximately 30% of canals do not have an eosinophilic ring, which is approximately the same proportion of canals observed to fail in arterial barium perfusion studies of osteochondrosis, i.e. one out of four canals (Olstad et al. 2008a). Osteochondrosis is a disease with a considerable genetic component (Grøndahl & Dolvik, 1993), and biomechanical factors also influence disease phenotype (Lepeule et al. 2009; Etterlin et al. 2014). It is possible to speculate that if mesenchymal cells produce collagen type I mostly as a result of programming, and canals without collagen type I are more predisposed to vascular failure, genes responsible for mesenchymal cell differentiation could potentially play a role in the development of osteochondrosis.

The results of the current study show that individual neighbouring canals at the same depth and aspect of the growth cartilage differ with respect to the type of collagen they are surrounded by. Collagen type I, which was present around approximately 70% of canal cross-sections, has different biomechanical properties to collagen type II (Montes, 1996; Wang & Thampatty, 2006; Ross & Pawlina, 2012). The biomechanical properties of canals with an eosinophilic ring compared with those without are therefore likely to differ. The biomechanical properties of canals with an eosinophilic ring compared with those without are therefore likely to differ. If absence of an eosinophilic ring predisposes canals to vascular failure, the processes involved in the synthesis of the collagen could in theory be at fault. For cells to respond and adapt to different cues in the cell environment they must have mechanisms to sense stimuli and respond appropriately, as reviewed by Wang & Thampatty (2006). Mesenchymal cells and chondrocytes possess primary cilia that sense and transmit biochemical forces through the cytoskeleton (Panadero et al. 2016). Mechano-sensing in chondrocytes *in vivo* has been shown to be mediated via stretch-activated ion channels, and voltage-gated calcium channels and calcium signalling have been found to participate in mechano-transduction pathways in chondrogenic differentiation of mesenchymal stem cells and phenotype-regulation of mature chondrocytes (Panadero et al. 2016). Recently, osteochondrosis in pigs was found to be highly significantly associated with a region on sus scrofa chromosome 13 (Grindflek et al. 2014). The region contains genes coding for proteins involved in matrix synthesis, including procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), pro-collagen carboxy-terminal endopeptidase enhancer 2 (PCOLCE2) and carbohydrate sulphotransferase 2 (CHST2), and mechano-sensing, including transient receptor potential canonical 1 (TRPC1). The presence of the latter, TRPC1, was demonstrated in chondrocytes and mesenchymal cells of cartilage canals by immunohistochemical staining of experimental osteochondrosis lesions of 1–21 days' duration in foals (Hellings, I R).

The potential role of mesenchymal cells and the collagen ring in relation to osteochondrosis is currently being explored further.

Conclusion

The proportion of cartilage canals that had a ring of collagen type I was similar in all three groups of fetuses and foals, indicating that differences between individual canals are mostly programmed, although some adaptation was evident. The eosinophilic ring was more often present around deep than superficial canals, indicating a role for the collagen ring in preparation for ossification. The collagen of the eosinophilic ring appeared to be produced by perivascular mesenchymal cells.

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Author contributions

I.R.H. contributed to the study design, study execution, performed the data analysis and interpretation and drafted the manuscript. N.I.D., S.E. and K.O. contributed to the study design, acquisition of data and data interpretation. All authors contributed to the preparation of the manuscript,

critically revised the manuscript and approved the final version of the manuscript.

Conflict of interests

The authors declare that they have no competing interests.

References

Albro MB, Banerjee RE, Li R, et al. (2011) Dynamic loading of immature epiphyseal cartilage pumps nutrients out of vascular canals. *J Biomech* **44**, 1654–1659.

Banks WJ (1993) *Applied Veterinary Histology*. St. Louis: Mosby-Year Book.

Blumer MJ, Fritsch H, Pfaller K, et al. (2004) Cartilage canals in the chicken embryo: ultrastructure and function. *Anat Embryol (Berl)* **207**, 453–462.

Blumer MJ, Longato S, Richter E, et al. (2005) The role of cartilage canals in endochondral and perichondral bone formation: are there similarities between these two processes? *J Anat* **206**, 359–372.

Blumer MJ, Schwarzer C, Perez MT, et al. (2006) Identification and location of bone-forming cells within cartilage canals on their course into the secondary ossification centre. *J Anat* **208**, 695–707.

Blumer MJ, Longato S, Fritsch H (2008) Structure, formation and role of cartilage canals in the developing bone. *Ann Anat* **190**, 305–315.

Brama PA, Tekoppele JM, Bank RA, et al. (2000a) Functional adaptation of equine articular cartilage: the formation of regional biochemical characteristics up to age one year. *Equine Vet J* **32**, 217–221.

Brama PAJ, Tekoppele JM, Bank RA, et al. (2000b) The influence of strenuous exercise on collagen characteristics of articular cartilage in Thoroughbreds age 2 years. *Equine Vet J* **32**, 551–554.

Brama P, TeKoppele J, Bank R, et al. (2002a) Development of biochemical heterogeneity of articular cartilage: influences of age and exercise. *Equine Vet J* **34**, 265–269.

Brama PAJ, TeKoppele JM, Bank RA, et al. (2002b) Biochemical development of subchondral bone from birth until age eleven months and the influence of physical activity. *Equine Vet J* **34**, 143–149.

Carlson CS, Hilley HD, Henrikson CK, et al. (1986) The ultrastructure of osteochondrosis of the articular-epiphyseal cartilage complex in growing swine. *Calcif Tissue Int* **38**, 44–51.

Carlson CS, Cullins LD, Meuten DJ (1995) Osteochondrosis of the articular-epiphyseal cartilage complex in young horses: evidence for a defect in cartilage canal blood supply. *Vet Pathol* **32**, 641–647.

Carver W, Goldsmith EC (2013) Regulation of tissue fibrosis by the biomechanical environment. *Biomed Res Int* **2013**, 101979.

Denu RA, Nemcek S, Bloom DD, et al. (2016) Fibroblasts and mesenchymal stromal/stem cells are phenotypically indistinguishable. *Acta Haematol* **136**, 85–97.

Etterlin P, Ytrehus B, Lundeheim N, et al. (2014) Effects of free-range and confined housing on joint health in a herd of fattening pigs. *BMC Vet Res* **10**, 208.

Finnøy A, Olstad K, Lilledahl K (2016) Second harmonic generation imaging reveals a distinct organization of collagen fibrils in locations associated with cartilage growth. *Connect Tissue Res* **57**, 374–387.

Grindflek E, Hamland H, Aasmundstad T (2014) Genome-wide association study for conformation traits and osteochondrosis in pigs. In 10th World Congress on Genetics Applied to Livestock Production). Vancouver.

Grøndahl AM, Dolvik NI (1993) Heritability estimations of osteochondrosis in the tibiotarsal joint and of bony fragments in the palmar/plantar portion of the metacarpo- and metatarsophalangeal joints of horses. *J Am Vet Med Assoc* **203**, 101–104.

Haines RW (1974) The pseudoepiphysis of the first metacarpal of man. *J Anat* **117**, 145–158.

Heinegard D (2009) Proteoglycans and more – from molecules to biology. *Int J Exp Pathol* **90**, 575–586.

Hellings IR, Ekman S, Hultenby K, et al. (2016) Discontinuities in the endothelium of epiphyseal cartilage canals and relevance to joint disease in foals. *J Anat* **228**, 162–175.

Hurtig M, Green SL, Dobson H, et al. (1993) Correlative study of defective cartilage and bone growth in foals fed a low-copper diet. *Equine Vet J Suppl* **16**, 66–73.

Hyttinen MM, Holopainen J, van Weeren PR, et al. (2009) Changes in collagen fibril network organization and proteoglycan distribution in equine articular cartilage during maturation and growth. *J Anat* **215**, 584–591.

Komarova SV, Safranek L, Gopalakrishnan J, et al. (2015) Mathematical model for bone mineralization. *Front Cell Dev Biol* **3**, 51.

Kumar V, Cotran RS, Robbins SL (2003) *Robbins Basic Pathology*. Philadelphia: Saunders Press.

Lecocq M, Girard C, Fogarty U, et al. (2008) Cartilage matrix changes in the developing epiphysis: early events on the pathway to equine osteochondrosis? *Equine Vet J* **40**, 442–454.

Lepeule J, Bareille N, Robert C, et al. (2009) Association of growth, feeding practices and exercise conditions with the prevalence of Developmental orthopaedic disease in limbs of French foals at weaning. *Prev Vet Med* **89**, 167–177.

Lin YL, Brama PA, Kiers GH, et al. (2005) Functional adaptation through changes in regional biochemical characteristics during maturation of equine superficial digital flexor tendons. *Am J Vet Res* **66**, 1623–1629.

Lutfi AM (1970a) Mode of growth, fate and function of cartilage canals. *J Anat* **106**, 135–145.

Lutfi AM (1970b) Study of cell multiplication in the cartilaginous upper end of the tibia of the domestic fowl by tritiated thymidine autoradiography. *Acta Anat* **76**, 454–463.

Montes GS (1996) Structural biology of the fibres of the collagenous and elastic systems. *Cell Biol Int* **20**, 15–27.

Nowlan NC, Sharpe J, Roddy KA, et al. (2010) Mechanobiology of embryonic skeletal development: insights from animal models. *Birth Defects Res C Embryo Today* **90**, 203–213.

Olstad K, Ytrehus B, Ekman S, et al. (2007) Early lesions of osteochondrosis in the distal tibia of foals. *J Orthop Res* **25**, 1094–1105.

Olstad K, Cnudde V, Masschaele B, et al. (2008a) Micro-computed tomography of early lesions of osteochondrosis in the tarsus of foals. *Bone* **43**, 574–583.

Olstad K, Ytrehus B, Ekman S, et al. (2008b) Epiphyseal cartilage canal blood supply to the tarsus of foals and relationship to osteochondrosis. *Equine Vet J* **40**, 30–39.

Olstad K, Ekman S, Carlson CS (2015) An update on the pathogenesis of osteochondrosis. *Vet Pathol* **52**, 785–802.

Oostendorp C, Uijtdewilligen PJ, Versteeg EM, et al. (2016) Visualisation of newly synthesised collagen *in vitro* and *in vivo*. *Sci Rep* **6**, 18780.

Panadero JA, Lanceros-Mendez S, Ribelles JL (2016) Differentiation of mesenchymal stem cells for cartilage tissue engineering: individual and synergetic effects of three-dimensional environment and mechanical loading. *Acta Biomater* **33**, 1–12.

Platt H (1978) Growth and maturity in the equine fetus. *J R Soc Med* **71**, 658–661.

Reiland S, Ordell N, Lundeheim N, et al. (1978) Heredity of osteochondrosis, body constitution and leg weakness in the pig. A correlative investigation using progeny testing. *Acta Radiol Suppl* **358**, 123–137.

Ross MH, Pawlina W (2012) *Histology. A Text and Atlas. With Correlated Cell and Molecular Biology Baltimore*. Hagerstown: Lippincott Williams & Wilkins.

Stockwell RA (1971) The ultrastructure of cartilage canals and the surrounding cartilage in the sheep fetus. *J Anat* **109**, 397–410.

van Turnhout MC, Schipper H, van Lagen B, et al. (2010) Postnatal development of depth-dependent collagen density in ovine articular cartilage. *BMC Dev Biol* **10**, 108.

Wang JH-C, Thampatty BP (2006) An introductory review of cell mechanobiology. *Biomech Model Mechanobiol* **5**, 1–16.

Ytrehus B, Carlson CS, Lundeheim N, et al. (2004a) Vascularisation and osteochondrosis of the epiphyseal growth cartilage of the distal femur in pigs – development with age, growth rate, weight and joint shape. *Bone* **34**, 454–465.

Ytrehus B, Ekman S, Carlson CS, et al. (2004b) Focal changes in blood supply during normal epiphyseal growth are central in the pathogenesis of osteochondrosis in pigs. *Bone* **35**, 1294–1306.

Ytrehus B, Carlson CS, Ekman S (2007) Etiology and pathogenesis of osteochondrosis. *Vet Pathol* **44**, 429–448.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Immunohistochemistry.

Table S1. Presence of eosinophilic ring around patent canals by aspect in the articular-epiphyseal cartilage complex.