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Chondrocyte death by apoptosis is associated with cartilage matrix degradation[1]

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Summary

Objectives: To investigate the frequency of chondrocyte apoptosis in equine articular cartilage (AC) specimens and to examine the relationship between the process of cell death and the degree of cartilage degradation using a direct quantification of numbers of apoptotic cells and expression of active caspase-3.

Methods: AC from equine metacarpophalangeal (MCP), proximal interphalangeal (PIP) and distal interphalangeal (DIP) joints was used and each joint was graded macroscopically for cartilage degradation (macroscopic osteoarthritis (OA) score). Cartilage sections were graded using a 'modified' Mankin scoring system. Apoptosis of chondrocytes in cartilage sections was assessed morphologically by appearance of apoptotic features (direct method) and by expression of active caspase-3 using indirect immunohistochemistry.

Results: The extent of apoptosis assessed by the direct method did not show any relationship with increasing severity of OA ($P = 0.72$). However, there was a significant positive correlation between 'modified' Mankin score and apoptosis determined by caspase-3, with the extent of apoptosis found to increase linearly with increasing severity of OA ($r = 0.44$, $P = 0.0043$). Caspase-3 expression was found to be significantly higher in the superficial and middle zones than in the deep zone ($P < 0.001$). In the superficial, middle and deep zones, expression of caspase-3 was significantly higher in the MCP joint than in the PIP joint ($P = 0.013$, $P = 0.0018$ and $P = 0.029$, respectively). Within the MCP joints, apoptosis was higher in the lateral compartment compared to the medial ($P = 0.053$).

Conclusions: The data presented in this study demonstrate that chondrocyte apoptosis is positively associated with degree of cartilage matrix damage and that the extent of apoptosis varies with cartilage zones and mechanical loading environment of the joint.

Key words: Cartilage, Chondrocytes, Apoptosis, Caspase-3, Histological grading.

Abbreviations: AC articular cartilage, DIP distal interphalangeal, ECM extracellular matrix, GAG glycosaminoglycan, IL-1β interleukin-1β, MCP metacarpophalangeal, OA osteoarthritis, PBS phosphate buffered saline, PIP proximal interphalangeal, S.E.M. standard error of mean, TNF-α tumour necrosis factor-α, TUNEL terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling.

Introduction

Chondrocytes are the only cell type resident in articular cartilage (AC). As a result, they are solely responsible for the synthesis and maintenance of AC extracellular matrix (ECM). Furthermore, chondrocytes regulate the enzymatic breakdown of the ECM, hence maintaining the equilibrium between synthetic and degradative processes. Loss of homeostasis in favour of catabolic activities leads to the destruction of AC characteristic of the slowly progressive, degenerative joint disease, osteoarthritis (OA). Consequently, chondrocyte death has been postulated to be a crucial event in the pathogenesis of OA[1,2]. The mechanism of cell death in OA has received considerable attention, particularly in the last 10 years. Necrosis was originally postulated as the predominant pathway; however, more recent studies intimate that cell death occurs primarily by apoptosis or apoptosis-like programmed cell death[1–6].

There is a wide range of reported frequencies of apoptotic cells in osteoarthritic cartilage and results are not in good agreement. For example, Heraud *et al.*[4] reported a mean apoptosis of 18.1% in human osteoarthritic hip cartilage, whilst a more recent study found a considerably reduced rate of apoptosis in similar cartilage specimens[6]. Numerous methods exist and are employed for the detection of apoptotic cells, this being the most likely cause of discrepancies and inconsistencies in data recorded. Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) is perhaps the most commonly used. Problematically, it has been reported that the TUNEL assay is prone to technical errors and is highly dependent on various technical aspects such as TdT enzyme concentration and proteinase K digestion[7]. Furthermore, it appears that TUNEL is not specific for apoptosis and has been shown to label non-apoptotic cells[8–10]. In summary, accurate quantification of numbers of apoptotic cells using a robust method is required to determine the precise role apoptosis plays in the pathogenesis of OA.

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Activation of a group of cysteine proteases called caspases is the central component of the apoptotic machinery. Indeed, Roach *et al.*[11] defines caspases as 'the executioners of cell death'. The 14 caspases identified in mammals thus far can be divided into three groups; the apoptotic initiators (caspases 2, 8, 9 and 10), the apoptotic executioners (caspases 3, 6 and 7) and the cytokine processors (caspases 1, 4, 5, 11, 12, 13, 14). There are three pathways that signal a cell to undergo apoptosis, each of which are expressed by chondrocytes. Caspase-3 is a downstream caspase which is activated by each of the cell death pathways, and in its active form is one of the key mediators of apoptosis in its execution phase. Caspase-3 immunohistochemistry has been used to identify apoptotic cells in several recent studies[6,12] and may represent a means by which apoptotic chondrocytes may be correctly identified and allows distinction between cells that have died by other mechanisms.

The aim of this study was to investigate the incidence of chondrocyte apoptosis in equine AC specimens and examine the relationship between the process of cell death and the degree of cartilage degradation using direct quantification of numbers of apoptotic cells and expression of active caspase-3 (hereafter referred to as caspase-3) as methods of apoptosis detection.

Materials and methods

AC SAMPLING

The study comprised two populations of equine AC. No information was available regarding the ages and genders of the horses in population 1. Of the nine horses in population 2 the mean age was approximately 10.4 ($\pm$5.7) years, of which five were male and four were female.

Population 1 consisted of equine left metacarpophalangeal (MCP), proximal interphalangeal (PIP) and distal interphalangeal (DIP) joints obtained from horses ($n = 10$) slaughtered at a local abattoir. It should be noted that for each horse, cartilage from each of the three joints was not always available. Cartilage from the PIP joint was available in all but one case, while MCP and DIP joints cartilage were available in half the cases. The joints were opened and the cartilage visually graded using a macroscopic OA grading system proposed by Fuller *et al.*[13].

Full depth cartilage samples, approximately 1 cm^2, were taken from weight-bearing areas of the joints. Samples were placed in chilled isopentane and snap frozen immediately in liquid nitrogen, then stored at $-70\,^{\circ}$C until required for sectioning. Frozen cartilage samples were then embedded in Tissue Tek OCT compound (Raymond A Lamb, UK) and 7 μm thick sections cut on a cryostat (Bright Instruction Company Ltd, Huntingdon, England), placed on Superfrost Plus microscope slides (VWR International, UK) before being left to air dry overnight.

Population 2 consisted solely of equine left MCP joints ($n = 9$) also obtained from the local abattoir. The articular surface was macroscopically graded as previously described[13]. Cartilage was taken from six different regions of the articular surface, as illustrated in Fig. 1. However, cartilage from each of the six regions for each horse was not always available. Regions A, D and E were available in most cases while regions B, C and F were only available in two to three cases. Cartilage samples were then frozen, stored and sectioned as before. The time interval between slaughter and cartilage harvest was approximately 3 h for both populations.

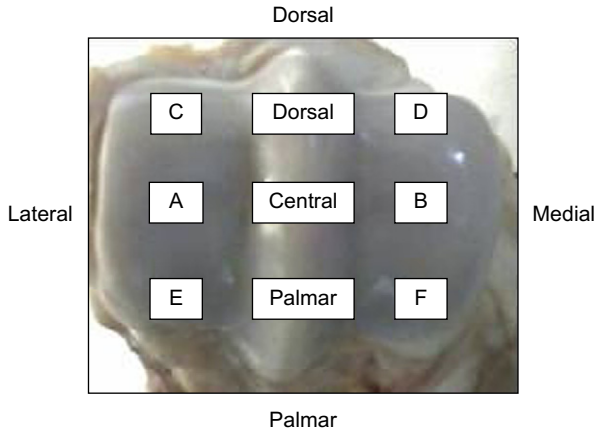

Fig. 1. AC surface of a MCP joint showing the six different sampling sites: centrolateral (A), centromedial (B), dorsolateral (C), dorsomedial (D), palmarolateral (E) and palmaromedial (F) regions.

HISTOLOGY

Haematoxylin and eosin/Safranin-O stained sections were used to score samples for features of cartilage pathology including changes in cellularity, structural abnormalities and uptake of Safranin-O as a measure of glycosaminoglycan distribution and loss, using a scoring system adapted from Mankin *et al.*[14] and Ostergaard *et al.*[15] (Table I). The calcified cartilage and subchondral bone were not included in the cartilage specimens; therefore, the evaluation criteria 'Tidemark integrity' was omitted. This gave a maximum total score of 11 per cartilage specimen. Each specimen was scored twice and the mean of the two scores used in any analysis performed.

PHYSICAL METHOD OF APOPTOSIS DETECTION

Haematoxylin and eosin stained sections were used in order to directly estimate numbers of apoptotic cells as a 'gold' standard in this study. Apoptotic cells were classed as those with morphology indicative of apoptosis including membrane blebbing, nuclear condensation and cell

Table I
'Modified' Mankin scoring criteria

	Score
Structure	
Intact surface	0
Surface fissures	1
Surface fissures to mid zone	2
Surface fissures to deep zone	3
Complete disorganisation	4
Cells	
Normal	0
Clusters/cloning	1
Some hypercellularity	2
Hypocellularity	3
Stain: haematoxylin and eosin with Safranin-O	
Normal	0
Slight reduction	1
Moderate reduction	2
Severe reduction	3
No dye noted	4

shrinkage using a Nikon Eclipse Microscope at 1000× magnification. Due to constraints on the availability of cartilage sections this method was only performed on about half of the total number of samples ($n = 24$).

IMMUNOHISTOCHEMISTRY

Indirect immunohistochemical staining was used to detect expression of active caspase-3. After fixation in acetone and blocking in goat serum diluted 1:5 with phosphate buffered saline (PBS), the primary antibody (polyclonal rabbit anti-human active caspase-3, AF835, R&D Systems, UK) was applied at a 1:100 dilution for 1 h. This was followed by application of biotin-labelled secondary antibody (goat anti-rabbit, Sigma) and ExtrAvidin Alkaline Phosphatase Conjugate (Sigma) at 1:100 dilutions for 30 min and Fast Red (Sigma) for 10 min. Haematoxylin (Ehrlich's) was used for counterstaining. For a negative control, the primary antibody was omitted and PBS alone applied. Sections were assessed by a 'blinded' observer using a Nikon Eclipse Microscope at 400× and 1000× magnification. The percentage of chondrocytes staining positive for caspase-3 was calculated by counting the number of positively and negatively stained cells in each zone. Where possible every chondrocyte per sample was counted, when this was not possible, at least 350 cells per specimen were assessed.

STATISTICAL METHODS

Linear regression analysis was used to assess the relationship between severity of OA and chondrocyte apoptosis. Pearson's correlation was used to compare apoptosis as determined by the two different methods. One-way Analysis of Variance (ANOVA) with Tukey's multiple comparison test was used to compare differences in capsase-3 expression between cartilage zones, to compare expression in different joints and to analyse expression in the different sampling sites of the MCP joint. A two tailed un-paired *t* test was used to compare caspase-3 expression in the lateral and medial sides of the MCP joint surface.

Results

Figure 2 shows typical images of cartilage sections corresponding to different modified Mankin grades. Table II shows numbers of joints assigned to each macroscopic OA score group and the equivalent mean modified Mankin score of each macroscopic OA score group. It shows that the macroscopic OA grades assigned to each joint did not correlate with the severity of AC destruction as given by modified Mankin score.

Macroscopic OA changes were largely confined to the central regions of the MCP joint. Because of this, the data were analysed to see if there was a relationship between macroscopic OA score and modified Mankin score in these regions alone (population 2), but no significant relationship was found. Furthermore, analysis carried out on cartilage taken from all regions excluding the central areas (on which the macroscopic OA scores were based) revealed no significant association. For population 2, for which demographic data were available, association between ages, modified Mankin score and apoptosis was investigated but none of the correlations were significant.

Twenty-four cartilage specimens were assessed for apoptosis by the direct physical method. Of these, data from all three cartilage zones were obtained for 13 samples. Overall, the extent of apoptosis assessed by the direct method did not show any relationship with increasing severity of OA ($r = 0.11$, $P = 0.72$). When the data were analysed for each zone of cartilage, there were similarly no trends evident. Furthermore, there was no significant association between the extent of apoptosis determined by this direct method and expression of caspase-3 ($r = 0.5$, $P = 0.096$).

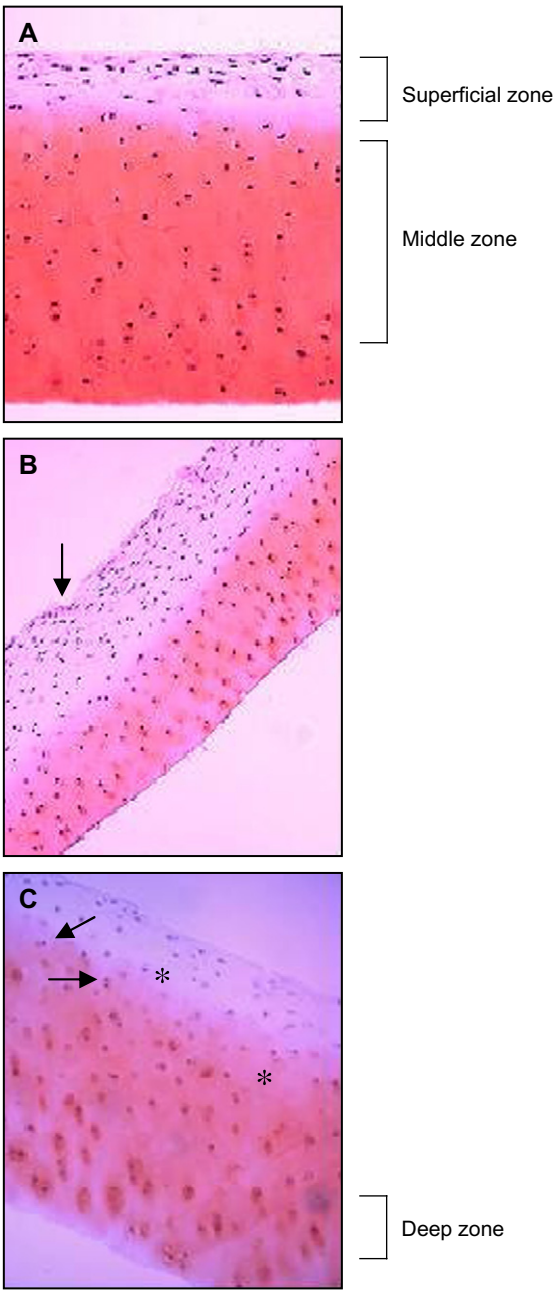

Fig. 2. Images of cartilage sections of different modified Mankin grades. (A) Microscopically normal cartilage (histological grade = 0), (B) some loss of Safranin-O stain, slight disruption to articular surface (arrow) (histological grade = 2), and (C) some loss of Safranin-O stain, hypocellularity (*), some cloning (arrow) (histological grade = 5).

Table II
Numbers of each type of joint assigned to each macroscopic OA score group and the equivalent mean modified Mankin score of each macroscopic OA score group

		Macroscopic OA score				
		0	1	2	3	
	Population 1					
Joint	DIP	3	1	1	0	
	PIP	7	2	0	0	
	MCP	1	2	1	0	Number of joints
	Population 2					
Joint	MCP	2	1	4	2	
Equivalent mean modified Mankin score		3.85	6.17	4.97	4.38	

However, there was a highly significant positive correlation between 'modified' Mankin score and apoptosis determined by caspase-3. A typical example of a caspase-3 positive chondrocyte is illustrated in Fig. 3. Of the 41 cartilage specimens assessed for expression of caspase-3, data from each zone of AC were available for 38 specimens. Overall, the extent of apoptosis (caspase-3 expressing chondrocytes) was found to increase linearly with increasing severity of OA (Fig. 4) ($r = 0.44$, $P = 0.0043$). This relationship is consistent and significant for the superficial, middle and deep zones of AC ($r = 0.36$, $P = 0.019$; $r = 0.49$, $P = 0.0011$ and $r = 0.37$, $P = 0.023$, respectively).

There were marked differences in caspase-3 expression between the three cartilage zones, with the lowest number of apoptotic chondrocytes detected in the deep zone (Fig. 5). Caspase-3 expression was significantly higher in the superficial and middle zones than in the deep zone ($P < 0.001$). There was no significant difference in expression between the superficial and middle zones.

Figure 6 demonstrates a marked difference in overall caspase-3 expression with joint type in the order MCP > DIP > PIP. In the superficial, middle and deep zones the expression of caspase-3 was significantly higher in the MCP joint than in the PIP joint ($P = 0.013$, $P = 0.0018$ and $P = 0.029$, respectively).

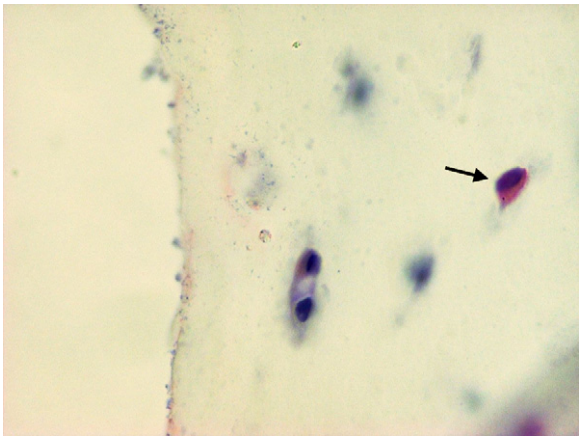

Fig. 3. Image of a typical cartilage section stained for active caspase-3 by indirect immunohistochemistry (magnification 1000×). The arrow head indicates a cell positive for caspase-3.

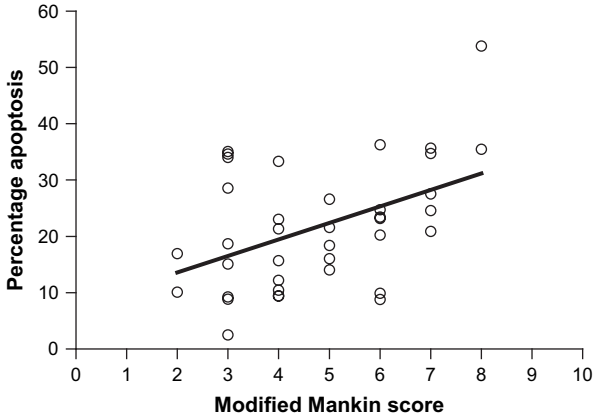

Fig. 4. Overall relationship (——) between severity of Mankin score and expression of caspase-3. The extent of apoptosis increased linearly with increasing severity of OA ($r = 0.44$, $P = 0.0043$).

To determine the variations of apoptosis within different anatomical locations of the MCP joint, expression of caspase-3 in different regions of the joint was analysed (Fig. 7). Caspase-3 expression was highest in the centrolateral and centromedial group and lowest in the dorsolateral and dorsomedial group. The differences, however, were not significant. This relationship was consistent for the middle and deep zones. However, in the superficial zone caspase-3 expression was highest in the palmarolateral and palmaromedial group and lowest in the centrolateral and centromedial group.

Comparison of caspase-3 expression in the lateral and medial regions of the MCP joint showed that overall expression was highest in the lateral side of the joint (Fig. 8). The lateral group comprises all of the results for the dorsolateral, centrolateral and palmarolateral regions and the medial group comprises the dorsomedial, centromedial and palmaromedial regions. The relationship was not quite significant ($P = 0.053$) and was consistent for each zone of AC.

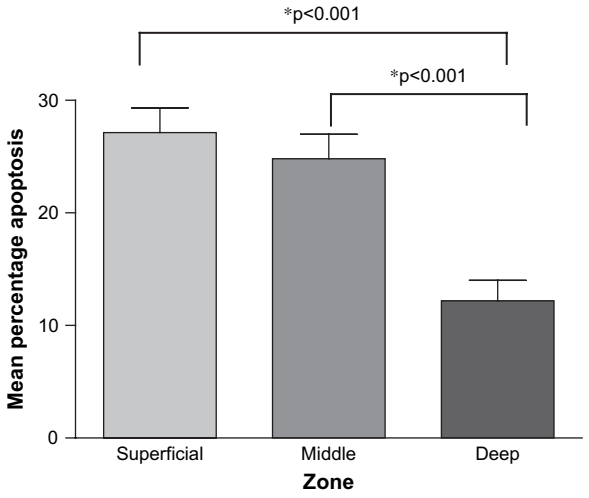

Fig. 5. Zonal variation in apoptosis (error bars indicate standard error of mean (S.E.M.)).

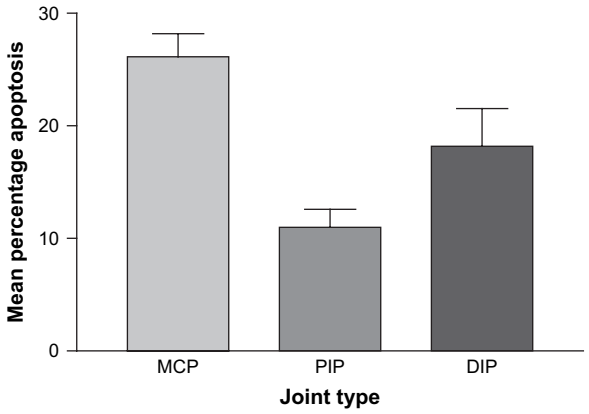

Fig. 6. Variation in apoptosis levels in the MCP, PIP and DIP joints (error bars indicate S.E.M.).

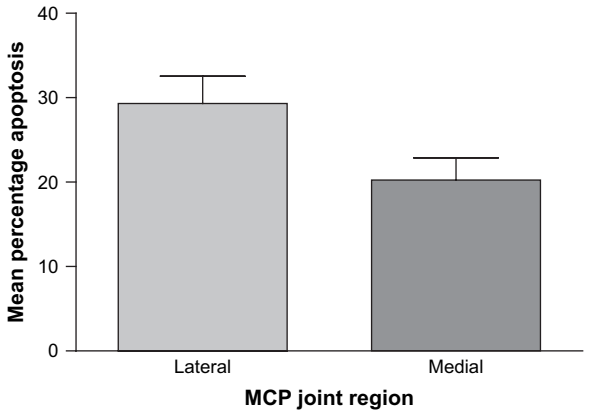

Fig. 8. Variation in caspase-3 expression in the lateral and medial regions of the MCP (error bars indicate S.E.M.). The difference was of borderline significance ($P = 0.053$).

Discussion

The data presented in this study demonstrate that the extent of apoptosis determined using active caspase-3 expression and degradation of AC is closely correlated, with a high incidence of chondrocyte apoptosis noted in the severely degenerated cartilage matrix. The extent of apoptosis is also related to the mechanical loading environment of the joint.

The results using the direct physical method of apoptosis detection did not show any association with severity of OA as determined by the 'modified' Mankin score. There may be a number of possible explanations for this, but the most obvious being that the direct method is very subjective and so a large number of samples would need to be analysed to see any significant association. Moreover, it was consistently noted that apoptosis values as quantified by the direct method were lower than those determined by caspase-3 immunohistochemistry, suggesting that the two methods differ with regard to the stage of chondrocyte apoptosis they measure. For example, expression of caspase-3 may occur prior to some of the features of apoptosis that were used to identify apoptotic chondrocytes using the physical method. Thus, the discussion below is based on apoptosis as determined by caspase-3 expression, and severity of cartilage damage determined the modified Mankin score.

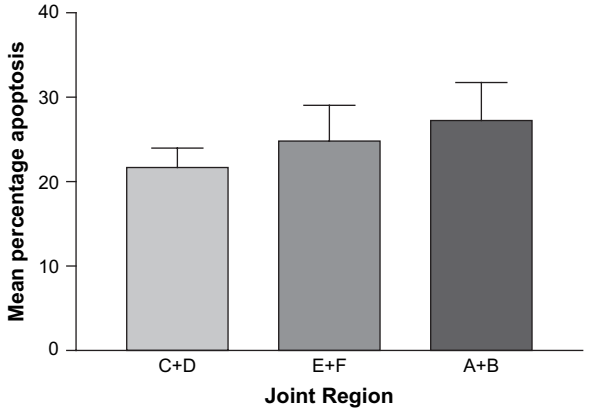

Fig. 7. Variation in apoptosis in the centrolateral (A), centromedial (B), dorsolateral (C), dorsomedial (D), palmarolateral (E) and palmaromedial (F) regions of the MCP joint (error bars indicate S.E.M.). None of the variations between the different regions were statistically significant ($P > 0.05$).

The macroscopic OA scores did not show any significant associations with either the modified Mankin scores or caspase-3 expression. This may be because the macroscopic OA score described the gross appearance of the joint as a whole, rather than the specific cartilage sample. In order to address this in future studies, individual scores could be assigned to a specific particular cartilage site, with the total joint score being a product of these individual site scores. However, even when the data were analysed for the central regions alone, where the cartilage damage tended to occur, no associations were found with OA score. It is difficult to draw any firm conclusions here since this involved a relatively low number of samples, most of which were of the same OA grade.

Chondrocyte apoptosis in osteoarthritic cartilage has been reported in dogs, humans, and horses[1–6] and is considered to be one of the major factors in the pathogenesis of the OA disease process. The detection of apoptosis has received widespread attention and the specificity of TUNEL; one of the main methods of detection currently used has come into serious question, as studies suggest it is not specific for apoptosis and is prone to labelling non-apoptotic cells[8–10]. This study demonstrates, using active caspase-3 immunohistochemistry as a previously characterised and reliable method of apoptosis detection[6,12] that there is close correlation between degradation of AC and chondrocyte apoptosis. Indeed, the incidence of apoptosis in the most severely degenerated cartilage was approximately 44%; however, it remains unclear whether chondrocyte apoptosis is a cause of, or the result of, cartilage matrix breakdown. The phenomenon of 'anchorage dependence' dictates that cells require attachment to the ECM or to each other for survival, function and growth; otherwise they are committed to apoptosis. As loss of proteoglycan and disruption of the collagen network are two of the main pathologic features of cartilage degradation, this provides an ample environment for the disturbance of chondrocyte anchorage to the ECM[16]. In short, chondrocyte apoptosis may exacerbate existing cartilage breakdown or indeed be at its cause, as chondrocytes are the sole source of matrix renewal and maintenance,

a decreased cell population would likely lead to an inferior ECM.

Apoptosis in the superficial and middle zones of AC was found to be significantly higher than that observed in the deep zone. This is in agreement with Sharif *et al.*[6] who found the highest expression of caspase-3 in chondrocytes of the superficial zone. This process may be the result of cells in the superficial zone undergoing greater deformation than those found in deeper zones[17,18] or the fact that the parallel alignment of collagen fibrils in the superficial zone may not confer the same protection afforded to cells of the deep zone in which the collagen network is arranged in a perpendicular configuration. Another potential cause is the likely heightened exposure of cells of the superficial zone when compared to those of the deep zone, to pro-apoptotic cytokines produced by the synovium that reach the cartilage via the synovial fluid.

The principal causes of lameness in the equine population are joint injury and joint disease, with a large proportion of these being attributed to OA[19,20]. Of all the joints, the MCP joint is the most common site of OA, having the largest number of site-specific traumatic and degenerative lesions of all limb joints[19].

An interesting and perhaps significant finding of this study was that the MCP joint had the highest incidence of apoptosis across each zone of cartilage when compared to the PIP and DIP joints, each of which is less commonly affected by OA. Significant differences across all zones were noted between the MCP and PIP joint. The basis for the increased incidence of OA in the MCP joint may arise from the biomechanical loading during impact and in the early part of the stance phase it is subjected to. At fast speeds horses transmit all of their body weight through the MCP joint during one phase of the stride[19]. It is reported to act as a propulsive strut 'bouncing' at impact[21].

Mechanical stimuli are key regulators of chondrocyte function, with certain types of mechanical injury known to induce chondrocyte apoptosis. OA lesions are often localised to weight-bearing cartilage or sites of trauma; therefore repetitive mechanical overload is likely to be a key signal for the initiation and progression of OA. Indeed, an increased risk of OA is found in the obese, those with occupations involving heavy lifting, and individuals who have suffered from previous joint injury[22,23]. Our findings would suggest that mechanical load of the joint is intrinsically linked to the extent of apoptosis and cartilage breakdown.

The second population of cartilage samples taken from six different regions of the MCP joint articular surface was used to examine joint site-related differences. Overall, apoptosis was found to be highest in the central region (across the transverse ridge) and lowest in the dorsal aspect of the joint. This is notable as the central regions (A and B) were those where any osteoarthritic lesions were normally observed. Furthermore, the central areas of the articular surface of the MCP joint are the most common site of OA lesions, a pathological entity termed 'transverse ridge OA'. Various degenerative changes are observed, ranging from mild fibrillation to deep ulcerations that extend into the subchondral bone[19]. None of the differences were, however, significant, possibly due to the limited availability of cartilage samples in each group.

Further studies utilising a larger number of macroscopically normal joints are required to determine whether regional differences in chondrocyte apoptosis exist and whether this is linked to differences in composition, mechanical load and ultimately susceptibility to OA that exists within the MCP joint.

Interestingly, Todhunter *et al.*[24] determined that the central regions of the MCP had the greatest intensity of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), followed by the palmar and then dorsal regions. Caspase-3 expression showed similar regional differences. IL-1β and TNF-α are pro-apoptotic molecules and have been shown to induce apoptosis in AC chondrocytes[25,26]. Likewise, when a selection of the cartilage specimens used in this study was analysed for expression of various cytokines it was noted that there was significant difference in the distribution of IL-1β ($P < 0.003$), TNF-α ($P < 0.006$), TNF p55 receptor ($P < 0.001$) and IL-18 receptor ($P < 0.02$) between articular surface regions, with the highest percentage expression being in the central regions[27].

Apoptosis in each zone of AC was found to be higher in the lateral compared to the medial side of the MCP joint, but the differences were not significant. Brommer *et al.*[28] deduced that cartilage degradation was considerably more pronounced at the medial than lateral side of the articular surface of the MCP joint. However, a later study reported no difference in cartilage degradation between the medial and lateral aspects of the MCP joint[21]. Further studies are required to fully assess whether apoptosis predominates in the lateral or medial side of the joint, and whether this is linked to differences in cartilage degradation in these regions.

The study has a number of limitations; firstly, the primary antibody used in this study was raised against human active caspase-3 as anti-equine antibodies were unavailable. Hence, the specificity of binding of anti-human antibodies to equine antigens may be questionable. However, positive results were observed and the pattern of staining was comparable to that noted in studies that have used the same antibody[12]. Secondly, limited information was available regarding the ages, genders and training backgrounds of the horses used in the study and where such data were available (e.g., population 2), lack of information regarding the training/exercise background of the horses meant that any significant results between age, 'modified' Mankin score and apoptosis could not still be fully explained.

Finally, although the vast majority of studies in the area have reported significant reduction in chondrocyte apoptosis following specific caspase-3 inhibition[29–31], several recent studies have shown that general caspase inhibitors were more effective in reducing apoptosis in human chondrocytes than caspase-3 specific inhibitors[32–34]. The latter studies imply that chondrocyte death by apoptosis does not always involve caspase-3 expression and that different pathways of chondrocyte apoptosis may exist, some of which involve expression of caspase-3 and others which do not. However, direct comparison of results from these studies is difficult as the cell type, detection techniques and agents used to induce apoptosis were different in each study. Further studies are therefore required to confirm whether chondrocyte apoptosis always involves expression of caspase-3.

In conclusion, we have demonstrated that there is a significant positive correlation between severity of OA and apoptosis, suggesting that this process plays an important role in the pathogenesis of the disease process. We have also shown that joint and indeed joint region differences in the extent of apoptosis exist, which may be linked to biomechanical load and susceptibility to OA. Further studies are required to confirm this.

Acknowledgements

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