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Effects of Copper and Zinc on Cathepsin B Activity in Equine Articular Chondrocytes

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Abstract: The effects of copper and zinc on cathepsin B activity, chondrocytes behaviour and matrix turnover by articular cartilage were investigated *in vitro* using normal horse cartilage co-cultured with synovial tissue. Proteoglycan (PG) depletion was assessed by toluidine blue staining. Co-culture cartilage expressed elevated levels and activity of the PG-degrading enzyme cathepsin B. Copper but not zinc was able to reduce the depletion of PG and the enzyme activity induced by the synovial tissue.

Key words: Horse, cathepsin B, enzymatic activity, copper, zinc, chondrocytes, proteoglycan, articular cartilage and dyschondroplasia

INTRODUCTION

One of the orthopaedic problems that can present itself during equine development is dyschondroplasia or the early stage of Osteochondrosis (OC) (Lillich *et al*., 1997). In the horse, OC is due to a focal failure of endochondral ossification (process by which cartilage in substituted by bone) at predilection sites in articular/epiphyseal cartilage (Jeffcott and Henson, 1998). The first lesions are commonly generated in the deep or hypertrophic articular cartilage zone, thus causing retention of the demineralized cartilage (Savage *et al*., 1993; Henson *et al*., 1997).

The pathogenesis of osteochondrosis has not yet been precisely defined. It is believed to be a multifactorial problem that can be caused by growth rate, genetic influences, nutrition and mineral imbalances, endocrine alterations and biomechanical trauma (Jeffcott, 1991).

During the normal ossification process chondrocytes synthesized and release several enzymes capable of degrading the collagen and the proteoglycans such as metalloproteinases (Sires *et al*., 1995), cysteine proteinases and Adenosine Triphosphatases (ATPases) (Sasaki and Ueno-Matsuda, 1993). It has been suggested, that dischondroplastic cartilage may be due to alterations in the matrix-degrading enzymes produced and regulated by chondrocytes (Hernandez-Vidal *et al*., 1996). High levels of cathepsin B are secreted by chondrocytes under pathological condition such as dyscondroplasia (Hernandez-Vidal *et al*., 1998) and after its phenotypical modulation by subculture (Hernandez-Vidal *et al*., 2008).

Based on the knowledge that chondrocytes can be stimulated by cytokines to produce cartilage-degrading enzymes (Jasin and Dingle, 1981) it was decided to study, the effects of cytokines produced by synovial tissue on the production, release and activity of cathepsin B. *In vitro* studies suggest that cathepsin B plays an important role in the degradation of the cartilage proteoglycans in the presence of a predominant cytokine produced by synovial tissue, Inteleukin 1 (IL-1) (Buttle *et al*., 1992, 1993; Buttle and Saklatvala, 1992). Based on *in vitro* cellular culture (Davies *et al*., 1996), demonstrated that copper can reversed the proteoglycan depletion induced by synovial tissue co-cultured with

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articular cartilage explants. Since, it has already been suggested (Pasqualicchio *et al*., 1996) that copper indirectly inhibits interleukin 1, its effect, together with that of zinc should also be examined on the enzyme activity.

It has been reported that copper and zinc affect cartilage matrix remodelling (Bridges and Moffitt, 1990; Knight *et al*., 1990; Hurtig *et al*., 1993) and there is evidence to suggest that copper deficiency results in defective collagen cross-linking in cartilage lesions in foals with osteochondrosis (Hurtig *et al*., 1993). Copper was first suggested as a potential cause of OC by Bridges *et al*. (1984), who observed OC-like lesions in copper-deficient foals.

There are reports of experimentally inducing OC-like lesions by feeding low copper diets to foals (Bridges and Harris, 1988). Knight *et al*. (1990) and Hurtig *et al*. (1993) performed dose-response studies where both demonstrated a reduction in OC-lesions in foals fed high copper levels. Pearce *et al*. (1998) showed that copper supplementation of pregnant mares reduced the prevalence of developmental orthopaedic disease in theirs foals at 5 months of age. Additionally, Weeren *et al*. (2003) suggested that OC lesions in foals with high-level copper status at birth are significantly less in number and severity than those in foals with low-level copper status at birth.

Although, these findings offer some indirect explanations for the skeletal lesions instigated by copper deficiency and for the anti-arthritic efficacy of copper and zinc (Sorenson, 1989; Whitehouse *et al*., 1990) the objective of this study was to identify the effects of these essential metals on cathepsin B activity in equine articular cartilage.

MATERIALS AND METHODS

Preparation of femorotibial synovial tissue: Equine synovial tissue was removed under sterile conditions from the lateral trochlear ridge of femorotibial joints of horses in the age range of 12-15 months. Immediately prior to synovial excision the joints were inmmersed in 70% ethanol for 5 min. Aseptic techniques and sterile equipment were used throughout the procedure. The joint was opened and the synovium removed using Noyes curved scissors. The synovium was finely minced using the Noyes scissors and washed extensively in Dulbecco's- Modified Eagle's Medium (DMEM) by repeated centrifugation in a Mistral MSE 2000 bench centrifuge at 2500 rpm for 5 min to separate cells from fat tissue, then immediately used for co-culture experiments.

Co-culture of articular cartilage and synovium: Macroscopically normal articular cartilage and synovial tissue were removed under sterile conditions from the lateral trochlear ridge of femorotibial joints of horses in the age range of 12-15 months. The samples were collected during the first 3-6 h post-mortem. Cartilage pieces were cultured alone or were co-cultured in 1 mL of DMEM (Dulbecco's-modified Eagle's medium (Gibco, UK) containing 10% (v v^{-1}) of heat-inactivated Foetal Calf Serum (FCS), 200 UI mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 2.5 μg mL^{-1} of fungizone at 37°C, 95% air and 5% CO_2), with minced synovial tissue as previously described (Davies *et al*., 1991). Co-cultures were arranged in square 25-well plastic Petri dishes (Sterilin, UK) by placing the synovial mince on a Millipore membrane on a metal grid with the full depth of the cartilage directly in contact with the synovium.

Cartilage explants and co-cultures were maintained for 48 h in the presence or absence of 0.01 mM copper sulphate or 0.01 mM zinc sulphate.

After 48 h some of the cartilage explants were removed, snap-frozen in OCT embedding compound and stored at -20°C prior to sectioning for toluiding blue histology and enzyme activity localization. The frozen samples were cut perpendicular to the articular surface, (8 μm thick) at -30°C with a cryogenic microtome. The samples included the three articular cartilage zones (superficial, mid and deep zone).

Eight cartilage explants were used in all experimental groups. The experiment was performed 7 times.

Preparation of cartilage sections: Slides were coated with poly-L-lysine (0.1% v v^{-1} SIGMA) for 5 min and dried at 60°C for 1 h, then stored at room temperature for up to 2 weeks to ensure adherence of cartilage sections to slides. Frozen articular cartilage explants were cut on a motor driven Bright cryostat at a cabinet temperature of -30°C. Sections (thickness 8 μm) were transferred onto poly-L-lysine coated slides and air-dried for 5 min at room temperature.

Toluiding blue histology: Frozen sections were stained with 5% (v v^{-1}) ethanol containing 0.5 % (w v^{-1}) toluidine blue and examined for the appearance of chondrocyte clusters, disruption in the normal transition of chondrocytes and proteoglycan depletion (Loss of sulphated PG from cartilage Extracellular Matrix (ECM) is associated with a corresponding reduction in the intensity of metachromasia).

Detection of cathepsin B activity: Detection of cathepsin B enzymatic activity in unfixed cartilage explants and co-

cultured with synovial tissue, copper and zinc was performed essentially according the method of Van Noorden and Vogels (1986), Van Noorden *et al.* (1987) and Hernandez-Vidal *et al.* (2008).

N-carbobenzoxyl-L-alanyl-L-arginyl-L-arginine-4-methoxy-β-naphthyl-amide (Z-Ala-Arg-Arg-4MβNA) was used at a concentration of 0.01% as specific substrate for cathepsin B, 1mM 2-hydroxy-5-nitrobenzaldehyde (5-nitrosalicylaldehyde) as coupling reagent and E-64 (10 μM) as specific inhibitor of cathepsin B.

The articular cartilage explants were rinsed in 100 mM phosphate buffered saline, pH 6.0, for 2-5 min and dried during 10 min at 25°C. Later, they were coated with 50 μL of enzymatic incubation medium. The incubation medium for the detection of cathepsin B enzymatic activity consists of 1.3 mM of EDTA, 1 mM of (Dithiothreitol (DDT), 2.65 mM of L-cysteine, 1 mM of 2-hydroxy-5- nitrobenzaldehyde and 1 mg mL of Z-Ala-Arg-Arg-4 MβNA in 100 mM of phosphate buffered solution (pH 6.0).

The tissue sections were incubated at room temperature for up to 4 h, in this way, the product reaction bound to 5-nitrosalicylaldehyde could be continually monitored. The specificity of the enzymatic reaction was confirmed during the incubation period in the presence of inhibitor E-64 or in the absence of the specific substrate.

The slides were examined and photographed during the incubation period using a Nikon Diaphot microscope fitted with epifluorescent illumination and photographed during the time-course (5, 25, 45, 60, 80 and 150 min) on Kodak Ektachrome P1600 X film.

RESULTS

In this study, co-cultures of articular cartilage with synovial tissue were use as *in vitro* model to study the effects of copper and zinc on cathepsin B activity in equine articular chondrocytes.

There was a correlation between the increased levels of expression and the activity of the lysosomal enzyme cathepsin B observed in co-culture experiments.

Cathepsin B activity was detected by the fluorescence method using Z-Ala-Arg-Arg-4MβNA as specific substrate for cathepsin B (McDonald and Ellis, 1975; Van Noorden *et al.*, 1987; Baici *et al.*, 1988; Shuja and Murnane, 1996) and 5-nitrosalicylaldehyde (Dolbeare and Vanderlaan, 1979; Van Noorden *et al.*, 1987) as coupling reagent providing a fluorescent end-product. Specificity, of the enzymatic reaction was confirmed by use of the specific cathepsin B inhibitor E-64 (Barrett *et al.*, 1982; Baici and Lang, 1990; Kawada *et al.*, 1995; Aisa *et al.*, 1996).

Effects of copper and zinc on cathepsin B activity: In order to investigate *in vitro* correlation of the expression of cathepsin B and its activity by chondrocytes, cartilage explants were co-cultured with synovial tissue, copper and zinc (0.01 mM). A total of 120 unfixed frozen cryostat sections of full thickness articular cartilage explants were stained for cathepsin B activity using a coupling reagent (5-nitrosalicylaldehyde) (Dolbeare and Vanderlaan, 1979; Graf *et al.*, 1979; Van Noorden *et al.*, 1987) and specific inhibitor (E-64) (Barrett *et al.*, 1982; Baici and Lang, 1990; Kawada *et al.*, 1995; Baici *et al.*, 1995a; Aisa *et al.*, 1996) and Z-Ala-Arg-Arg-4MbNA as specific substrate for cathepsin B (McDonald and Ellis, 1975; Van Noorden *et al.*, 1987; Baici *et al.*, 1988, 1995a; Shuja and Murnane, 1996) as described by Hernandez-Vidal *et al.* (2008).

All cartilage samples were carefully examined by light microscopy. The outstanding peculiarity of cartilage explants co-cultured with synovial tissue, was the presence of chondrocytes clusters in the articular zone in association with extensive loss of sulphate PG (Fig. 1a) in areas with high enzyme activity for cathepsin B (Fig. 1b).

Figure 1b shows strong intracellular activity to cathepsin B, the yellow fluorescent lysosomal particles became visible after 30 min of incubation, during this time there was no indication of extracellular activity. It was interesting to observe that the addition of copper sulphate (0.01 mM) to the co-culture considerably reduced not only the PG depletion (Fig. 2a) but also, the cathepsin B activity (Fig. 2b). The activity of this enzyme was not confined to chondrocytes in the deep zone of the articular cartilage but extended through the intermediate or mid zone to the articular surface where it was also detected at reasonable high levels. Figure 3a-c shows the variation in levels of cathepsin B activity within the different cartilage specimens (control, co-culture and co-culture + Cu) in the intermediate or mid-zone. Interestingly, the different levels of cathepsin B activity observed with the different explant specimens in the articular and intermediate or mid-zone resemble that observed in the deep-zone. These findings suggest a lack of heterogeneity in cathepsin B activity in co-cultured explants in the presence of copper (0.01 mM). Normal horse cartilage showed strong metachromatic staining after 48 h in culture (Fig. 4a). No positive activity to cathepsin B was detected in normal equine cartilage where no chondrocytes clusters and extensive depletion of PG were present in Fig. 4b.

It was noteworthy, that fluorescent particles were observed in the extracellular matrix after 45 min of incubation. The bright yellow fluorescing particles indicate the sites of cathepsin B activity at the

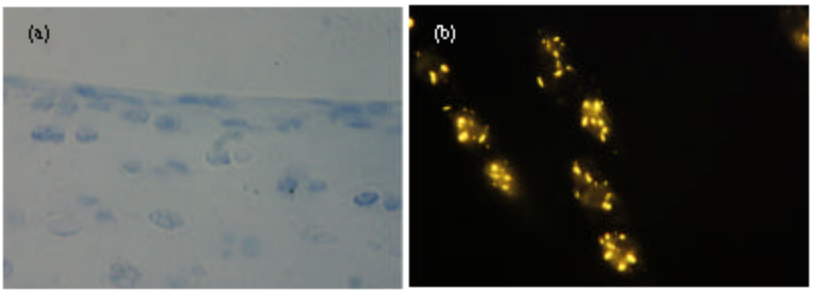

Fig. 1: Normal equine articular cartilage from lateral trochlear ridge co-cultured for 48 h with synovial tissue. a): Full thickness toluidine blue-stained section of equine articular cartilage showing a great loss of metachromasia of cartilage matrix. b): Cathepsin B activity in normal equine unfixed articular cartilage incubated (30 min) with enzyme activity medium containing Z-Ala-Arg-Arg-4MßNA and 5-nitrosalicylaldehyde (×40)

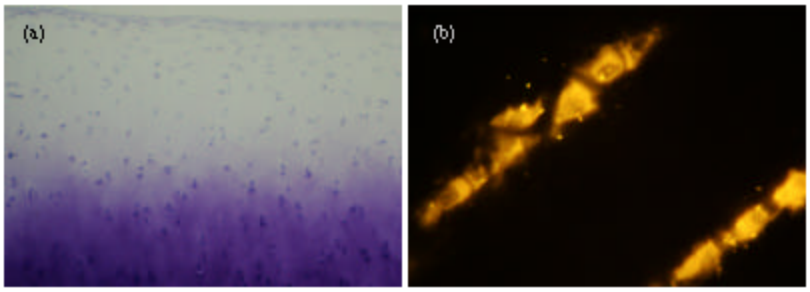

Fig. 2: Normal equine articular cartilage from lateral trochlear ridge co-cultured for 48 h with synovial tissue and copper sulphate (0.01 mM). a): Full thickness toluidine blue-stained section of equine articular cartilage showing copper-induced reversal of the loss of metachromasia of cartilage matrix. b): Cathepsin B activity in normal equine unfixed asrticular cartilage incubated (30 min) with enzyme activity medium containing Z-Ala-Arg-Arg-4MßNA and 5-nitrosalicylaldehyde (×40)

intracellular and extracellular level (Fig. 5a). Extracellular activity was a regular characteristic in all samples that had been co-cultured with synovial tissue alone and in the presence of zinc sulphate (0.01 mM).

No extracellular activity was observed in either copper-treated samples or controls. Changes in the extracellular activity were followed up to 4 h in all specimens to reduce the possibility of a time-dependent activity.

Recrystallization (Van Noorden *et al*., 1987; Hernandez-Vidal *et al*., 2008) of the fluorescent particles was observed in sections incubated longer than 60 min. Samples co-cultured with synovial tissue were incubated in the presence of inhibitor solution containing 10 μM of E-64, a specific inhibitor to cathepsin B, in 100 mM of phosphate buffer solution (pH 6.0) and used as a controls (Fig. 5b).

Zinc sulphate (0.01 mM) was unable to reverse the PG-depleting effects of synovial tissue and had no influence on the activity reaction of the enzyme cathepsin B (Table 1).

Histological examination of toluidine blue stained cartilage: The metachromatic stain of toluidine blue assesses the levels of sulphate proteoglycan histologically. PG depletion is associated with a reduction in the intensity of metachromasia. In 7 separate experiments loss of metachromatic staining resulted from the extensive depletion of PG was observed in cartilage co-cultured with synovial tissue (Fig. 1a). The loss of PG was accompanied by clusters formations in the articular zone (Fig. 1a). Davies *et al*. (1996), demonstrated that copper sulphate at concentrations ranging from 0.001 mM to 1 mM annulled the proteoglycan depletion caused by synovial tissue in a dose-dependent manner. The results in this investigation agrees with those of Davies *et al*. (1996) where, a reversal depletion of PG was observed by the intensity and distribution of metachromatic staning at a copper concentration of 0.01 mM (Fig. 2a). Around individuals chondrocytes of cartilage co-cultured with copper at 0.01 mM, distinct halos of staining were observed, probably indicating attempts to remodel by switching on PG synthesis. On the other hand, zinc

Table 1: Effects of synovial tissue and copper and zinc on the activity of cathepsin B in equine articular cartilage

	Cathepsinb			
Cartilage zones	Controls cartilage alone	Co-culture synovial tissue	Co-culture + Cu (0.01mM)	Co-culture + Zn (0.01 mM)
Articular-zone activity	-	+++++	+	+++++
Mid-zone activity	-	+++++	+	+++++
Deep-zone activity	-	+++++	+	+++++
ECM activity (>45 min)	No	Yes	No	Yes

Age range 12 to 15 months old. - = 0% no activity; +/- = <10%, weak activity; + = 10-30%, weak activity, ++ = 40-50%, moderate activity; +++ = 50-70%, strong activity; ++++ = 70-90%, strong activity; +++++ = >90% strong activity. Extracellular Matrix (ECM)

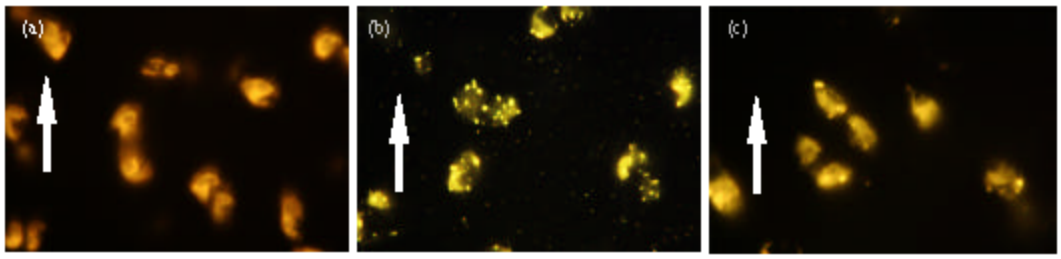

Fig. 3: Cathepsin B activity in the intermediate or mid-zone of normal equine articular cartilage from lateral trochlear ridge (×40). a): Control specimen of equine cartilage cultured alone for 48 h. b): Co-culture with synovial tissue for 48 h. c): Co-culture with synovial tissue and copper sulphate (0.01 mM) for 48 h. Arrows point towards articular surface. Unfixed articular cartilage incubated (30 min) with enzyme activity medium containing Z-Ala-Arg-Arg-4 MßNA and 5-nitrosalicylaldehyde

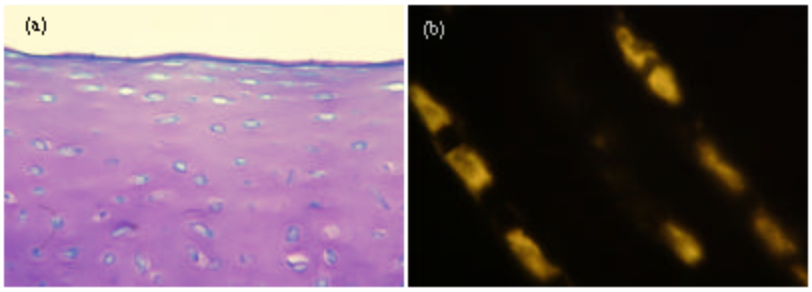

Fig. 4: Normal equine articular cartilage from lateral trochlear ridge cultured for 48 h in the absence of synovial tissue (control). a): Full thickness toluidine blue-stained section of equine articular cartilage showing normal metachromasia of cartilage matrix. b): Unfixed articular cartilage incubated (30 min) with enzyme activity medium containing Z-Ala-Arg-Arg-4MßNA and 5-nitrosalicylaldehyde (×40)

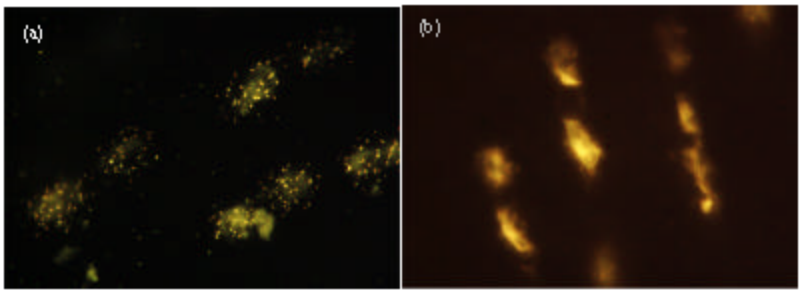

Fig. 5: Extracellular cathepsin B activity in normal equine articular cartilage co-cultured with synovial tissue and E-64 (×40). a): Unfixed articular cartilage incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MßNA and 5-nitrosalicylaldehyde for 45 min. b): Unfixed articular cartilage incubated with enzyme activity medium containing Z-Ala-Arg-Arg-4MßNA, 5-nitrosalicylaldehyde and E-64 inhibitor solution for 4 h

sulphate (0.01 mM) was unable to reverse the PG-degrading effects of synovial tissue. Normal equine articular cartilage showed deep metachromatic staining after 48 h in culture (Fig. 4a).

DISCUSSION

The present study set out to examine the effects of copper and zinc on cathepsin B activity in equine articular chondrocytes. It was demonstrated that cartilage co-cultured with synovial tissue showed an increased activity of the lysosomal enzyme cathepsin B. Histological abnormalities such as chondrocyte clusters and PG depletion were observed in cartilage co-cultured with synovial tissue alone and in the presence of zinc sulphate (0.01 mM). Copper but not zinc was found to protect the cartilage against the PG-degrading action of synovial tissue as assessed by histological staining and by the reduce levels of cathepsin B activity.

Previous studies (Hernandez-Vidal *et al*., 1998, 2008) reported that levels of cathepsin B are elevated in articular chondrocytes from joints of horses with dyschondroplasia, compared with normal equine articular chondrocytes. Phenotypically modulated normal chondrocytes showed morphologic and biochemical characteristics similar to the ones observed in dyschondroplastic chondrocytes.

Expressions of cathepsin B activity were particularly strong in chondrocyte clusters, which were evident in co-cultured cartilage with synovial tissue. Although, these cathepsin B-containing clusters were predominantly associated with areas of abnormal cartilage in the deep zone, their presence also, in the intermediate or mid-zone and at the articular surface was remarkable. Chondrocyte clusters are a common pathological indicator of osteoarthritic degeneration, but little is known of their formation or function (Poole *et al*., 1991).

At present, the importance of the increase expression of cathepsin B in the chondrocyte clusters is unknown (Hernandez-Vidal *et al*., 1998).

Since, cluster formation is considered a characteristic feature of equine dyschondroplastic lesions and has been used as one of several histological features for confirmation of diagnosis (Savage *et al*., 1993), it is not unreasonable to suggest that the presence of cathepsin B in these structures may have some pathogenic significance. Chondrocyte clusters, which differ in morphology and distribution from hypertrophic chondrocytes (Poole *et al*., 1991; Henson *et al*., 1997) are present in other pathologies, particularly Osteoarthritis (OA) (Pool and Meagher, 1990; Poole *et al*., 1991; Baici *et al*., 1995a, b). However, despite many descriptions of their occurrence in both natural and experimental OA cartilage, little is known of the mechanism of their formation or of their function. As early as Mankin and Lippiello (1970) suggested that the reduced levels of proteoglycan observed in the cellular microenvironment trigger chondrocyte metabolism and proliferation, resulting in cluster formation. Poole *et al*. (1991) proposed a role for these hyperplastic chondrocytes in progressive remodelling resulting in gradual depletion of pericellular proteoglycan.

Alternatively, the suggestion that the clusters are involved in local repair processes at sites of OA cartilage damage was made by Baici *et al*. (1995a, b). It is certain that enzymes are involved in these ECM remodelling events in OA but it has proved difficult to identify the enzyme (s) and define the initial steps. Attempts have been made to demonstrate enzyme-induced degradation of type II collagen in the cellular microenvironment (Dodge and Poole, 1989) and changes in the organization of pericellular collagens VI and IX have also been reported (McDevitt and Miller, 1989).

Based on these observations, this study suggest that the cathepsin B, present in increased amounts in equine dyschondroplastic cartilage clusters, could play some as yet unknown part in ECM turnover in this disease (Hernandez-Vidal *et al*., 1998). It is of interest that Baici *et al*. (1995a, b) also found high levels of cathepsin B in human OA clusters and proposed a role for the enzyme in cartilage regeneration.

Although, able to degrade proteoglycan, it is unlikely that cathepsin B is directly responsible for the low levels of this matrix component, as reflected by the reduced metachromatic staining often seen in the ECM adjacent to dyschondroplastic clusters.

Depletion of proteoglycan by other degradative enzymes such as serine and metalloproteinases and aggrecanase (Henderson and Blake, 1994; Buttle *et al*., 1995) cannot be excluded or alternatively, the loss of metachromasia may indicate sites of localized reduction in proteoglycan synthesis.

The technique used to identify the presence of the enzymes in this study exploits its proteolytic activity. A great advantage of the activity method for localizing cathepsin B is its high resolution to cells and tissues for examination in light microscopy. The sensitivity of the fluorescence activity method is greater than that of immunofluorescence staining. Furthermore, this method offers faster direct localization of the cathepsin B in the cartilage and isolated cells (Hernandez-Vidal *et al*., 2008) and was demonstrated to be useful tool for further investigations of the role of cathepsin B in normal and pathological protein turnover.

The activity of cathepsin B has been demonstrated in phenotypically modulated and dyschondroplastic isolated chondrocytes (Hernandez-Vidal *et al*., 2008) and now in cartilage explants co-cultured with synovial tissue.

Ultrastructural studies, provided evidence that chondrocytes, in the advanced phases of OA, undergo consecutive enlargement, contain more intracellular organelles and are capable of replication (Weiss and Mirow, 1972; Weiss, 1973). These findings indicated a potentiation of the secretory activities of chondrocytes, which can be mimicked *in vitro* by serial sub culturing (Hernandez-Vidal *et al*., 2008). Comparable morphological variations have also been observed in co-cultures of pig cartilage with synovium (Barratt, 1975; Fell *et al*., 1976).

In this study, it was demonstrated that co-cultures of articular cartilage with synovial tissue provided a useful *in vitro* model to investigate the effects of copper and zinc on pro-inflammatory cytokine-induced changes in proteoglycan turnover and cathepsin B activity in the extracellular matrix.

Chondrocytes may be stimulated to secrete cartilage-degrading enzymes under the influence of cytokines (Jasin and Dingle, 1981). It is known that normal synovial tissue promotes loss of PG from cartilage (Fell and Jubb, 1977) and secretes significant amounts of the pro-inflammatory cytokine Interleukin 1 (IL-1) (Fell and Jubb, 1977; Davies *et al*., 1991), which is one of the synovial mediators indirectly responsible for this degradative activity (Saklatvala *et al*., 1984; Shingu *et al*., 1995). This cytokine plays a major role in the regulation of cartilage matrix turnover (Smith *et al*., 1989; Platt and Bayliss, 1994).

Scuderi (1990) showed that copper is able to inhibit IL-1 secretion and thus it can be speculated that by inhibiting IL-1 copper protects cartilage from synovial-induced damage. Inhibition of IL-1 would also explain the observations on the ability of copper to reduce the expression, by chondrocytes, of the PG-degrading enzyme cathepsin B.

In this co-culture study, a proportional correlation between PG degradation and proteolytic activity in equine cartilage has been demonstrated for cathepsin B. Histochemical studies demonstrated intracellular and extracellular cathepsin B activity in cartilage that had been co-cultured with synovial tissue, suggesting that chondrocytes under normal conditions do not usually, secrete this enzyme and thus implicating the synovial tissue as one of the probable stimuli for PG-depletion and cathepsin B activity induction by chondrocytes *in vitro*. Synovium is a major source of IL-1 (Fell and Jubb, 1977; Davies *et al*., 1991) therefore, one may assume that the chondrocytes in cartilage co-cultured adjacent to the synovium would be exposed to the highest concentration of cytokine, which may account for the high levels of cathepsin B activity expressed by these chondrocytes. The activity of cathepsin B observed at extracellular sites in the co-culture experiments may be determined to some extent by the degree of proteoglycan depletion and tissue damage, since normal tissue did not show any activity and copper-treated explants abrogated the PG-degrading effects of synovial tissue. In view of this it is noteworthy, that in a previous study (Hernandez-Vidal *et al*., 2008) the extracellular medium around isolated dyschondroplastic chondrocytes showed cathepsin B activity, whereas none was see in the medium of chondrocytes from normal tissue.

Zonal distribution of cathepsin B activity correlated with tissue degradation in co-culture explants as determined histochemically. This is in agreement with Baici *et al*. (1995b) who reported that the zonal distribution correlated not only with the hypercellularity or cloning of chondrocytes but with tissue degeneration. Addition of copper sulphate (0.01 mM) to the culture resulted in a significant decrease in intracellular activity but also in a decreased release of enzyme extracellularly.

These findings offer a possible explanation for the anti-arthritic action of copper and may be of significance for the treatment of degenerative diseases such as rheumatoid and osteoarthritis. But is this proposed mechanism of action of copper relevant to equine dyschondroplasia? If copper is able to inhibit nitric oxide and IL-1, then its ability to influence chondrocyte behaviour must also be considered. Chondrocytes have sole responsibility for maintaining the integrity of their surrounding extracellular matrix which is achieved by controlled secretion of a complex network of mediators and cytokines, including IL-1 and nitric oxide (Rath *et al*., 1988; Davies *et al*., 1991; Frean *et al*., 1997). It is clear that any disturbance of this finely balanced microenvironment e.g., inhibition of nitric oxide or IL-1 by copper, could result in production of abnormal or defective cartilage, which is characteristic of some of the equine Developmental Orthopaedic Diseases (DOD).

It is of interest, in this respect that abnormalities such as lameness, angular limb deformities; bone fragility, thickened cartilage and cartilage erosion have been linked to copper deficiency in growing foals (Cupps and Howell, 1949). Egan and Murrin (1973) and Carberry (1978), have established some correlation between copper status and DOD and Bridges and Harris (1988) (who speculate an action of copper in the defective collagen structure in copper-deficient foals) reported an association between copper deficiency and physeal dysplasia and osteochondrosis. Hurtig *et al*. (1990, 1993) concluded

that, in osteochondrosis-affected foals, copper deficiency results in imperfect cross-linking which disrupts the fibrillar collagen network causing biomechanically weakened cartilage and microfractures in the metaphyseal and subchondral bone.

Although, these findings offer a convincing explanation of the skeletal lesions instigated by copper deficiency, they fail to provide any information about the direct effects of copper on the extracellular matrix components. However, this present study, offers an insight into the direct effects of copper at the cellular level in horse joint tissues.

CONCLUSION

The results so far have indicated potential pathophysiological role for cathepsin B in ECM turnover in equine cartilage. However, their mechanisms are far from understood particularly in dyschondroplasia for which the specific aetiology (undoubtedly multifactorial) remains unknown.

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