

Osteopontin expression and localization of Ca²⁺ deposits in early stages of osteoarthritis in a rat model

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Summary. Calcium deposits have been related to articular cartilage (AC) degeneration and have been observed in late stages of osteoarthritis (OA). However, the role of those deposits, whether they induce the OA pathogenesis or they appear as a consequence of such process, is still unknown. In this work, we present the kinetics of expression and tissue localisation of osteopontin (OPN), a mineralisation biomarker, and calcium deposits in samples from (normal, sham) and osteoarthritic cartilage (in a rat model). Immunohistochemical and Western blot assays for OPN, as well as Alizarin red staining for calcium deposits were performed; superficial, middle, and deep zones of AC were analysed. An increased expression of OPN and calcium deposits was found in the osteoarthritic cartilage compared with that of control groups, particularly in the superficial zone of AC in early stages of OA. In addition, the expression and localisation of OPN and calcium deposits during the OA pathogenesis suggest that the pathological AC mineralisation starts in the superficial zone during OA pathogenesis.

Key words: Osteoarthritis, Articular Cartilage, Ossification, Osteopontin, Calcium deposits

Introduction

Osteoarthritis (OA) is a chronic-degenerative disease characterised by degradation of articular cartilage (AC), accompanied by hypertrophic changes in bone and osteophyte formation that result in loss of AC and subsequent reduction of joint space (Goldring and Goldring, 2007; Martel-Pelletier and Pelletier, 2010). All the joint structures are affected by progression of OA, including the cartilage, subchondral bone, joint capsule, meniscus, and synovium (Goldring and Goldring, 2010). It has been reported that OA appears to be the result of complex interactions between mechanical, biological, and enzymatic events. However, the etiology of OA is not completely understood yet. Cartilage destruction results from an alteration in chondrocytes, the only cell type present in the cartilage, when such cells are not able to maintain the homeostatic balance between synthesis and degradation of the extracellular matrix (Martel-Pelletier and Pelletier, 2010). In particular, the presence of calcium deposits in up to 50 to 60 percent of osteoarthritic joints emphasises their pathogenic role among the mechanisms involved in cartilage degradation (McCarthy et al., 2001; Ea and Lioté, 2004; Yavorsky et al., 2008). Some studies reported that cartilage calcification occurs as an active process and has a clear degenerative effect on the AC (Ea et al., 2011). In addition, it is known that high intracellular and/or extracellular Ca²⁺ levels play a critical role in cytotoxicity and cell death (Orrenius and Nicotera, 1994; Blanco et al., 2004), while disturbed equilibrium in Ca²⁺ levels contributes to mineralisation due to precipitation of Ca_x(PO₄)_y complexes.

On the other hand, it has been suggested that pathologic mineralisation of AC emulates the hypertrophic differentiation of chondrocytes, a normal mineralisation process that occurs during endochondral ossification (EO) and results in bone formation (Aigner et al., 2007; Goldring et al., 2011). Some molecules involved in the normal process, such as WNTs, OPN, alkaline phosphatase, and type X collagen, are considered ossification biomarkers, and their presence has also been reported in AC with OA (Kouri et al., 2000; Rosenthal et al., 2007; Velasquillo et al., 2007). In this work, we study osteopontin (OPN) and calcium deposits with the aim of generating new data that help to elucidate their role in mineralisation during progression of OA.

The non-collagenous glycoprotein called osteopontin is characterised by the presence of a polyaspartic-acid sequence and Ser/Thr phosphorylation sites that mediate binding of hydroxyapatite and calcium ions (Sodek et al., 2000). It has been shown that OPN stimulates the formation of calcium pyrophosphate dihydrate (CPPD) crystals in chondrocytes *in vitro* (Rosenthal et al., 2007), and it has been found in plasma and synovial fluid in patients with OA (Gao et al., 2010). However, OPN levels in AC at different clinical stages of OA have not been reported. On the other hand, calcium is essential in the process of biomineralisation. Calcium precipitates, forming carbonates and phosphates; the insolubility of these salts makes them an excellent inorganic material with high structural definition under biological conditions. Due to the similarity between EO and pathological ossification in AC, it is important to identify the presence of key biomarkers in AC, because they could lead to mineralisation during the progression of OA. Therefore, this work presents the kinetics of expression and tissue localisation of OPN and calcium deposits, evaluated in different zones of AC, at early and late stages of OA in our experimental rat model.

Materials and methods

Animals and surgery

Wistar male rats of 130-150 g used in this work were sorted out into four groups: OA rats treated with partial meniscectomy in right back leg and exposed to high impact exercise; normal rats without surgery and without exercise; sham rats without exercise as surgery controls (SWE); and sham rats with exercise and without surgery (SE), exercised under the same conditions as those in the OA group.

The surgical procedure and exercise were followed according to protocol described elsewhere (Lozoya and Flores, 2000). Cartilage samples were collected from femoral condyles of the knee joint in the right back leg after 5, 10, 20, and 45 days. Animals were sacrificed in a CO₂ chamber, following the Guidelines of the Internal Committee for Care and Use of Laboratory Animals

(NOM-069-ZOO-1999) (Aluja, 2002).

Tissue sampling

Thirty-six femoral condyles were removed from the right back leg from Normal, SWE, SE, and OA rats at each time to study. Samples were observed in a stereoscopic microscope (Leica E24D, Heerbrugg, Switzerland) to establish the correlation between macroscopic morphology and histological changes. Subsequently, AC samples were obtained from weight bearing areas of femoral condyles. All samples were fixed in 4% PBS-paraformaldehyde at 4°C for 24 hours and cryopreserved in 10% PBS-sucrose for 24 hours. Then, samples were cryosectioned to 6 µm thickness (Cryostat Leica CM 1100, Heerbrugg, Switzerland), mounted on gelatin-coated slides, and stored at -20°C, until their further use.

Alizarin red staining

Cryosections were dehydrated with 50% alcohol for 20 seconds and washed twice for 2 minutes with distilled H₂O. Staining was performed for 10 minutes at room temperature (RT) with Alizarin red 1%, at pH 4.2, adjusted with 10% NH₄OH. Subsequently, slides were immersed in acetone for 20 seconds and afterwards in 1:1 acetone-xylene solution for 20 seconds. Slides were mounted and observed using light microscopy. Positive controls were made on 15 days rat embryos with ossification centers of right back leg. Cells with calcium deposits were stained in orange-red. Negative controls with no stain after Alizarin red treatment were performed on healthy AC from rats.

Immunohistochemistry (IHC)

Cryosections were hydrated in PBS and treated with 0.2% Tween 20 in PBS (PBS-T) for 10 minutes. Pre-incubation was performed with 0.2% albumin (A2058, Sigma Chemical, Germany) for 20 minutes at RT. Then, cryosections were incubated overnight at 4°C with a mouse monoclonal anti-OPN (1:100, AKm2A1: sc-21742, Santa Cruz Biotechnology, Dallas, TX, USA). Subsequently, samples were incubated with fluorescein isothiocyanate (FITC)-tagged Donkey anti-mouse (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at RT. Nuclei were counterstained with propidium iodide (PI) for 1 minute (1:1000; Vector Laboratories, Burlingame, CA, USA) and coverslipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Positive controls were conducted on 15 days rat embryos with ossification centers; anti-OPN antibody (FITC, green) was used, counterstained with PI (red). Similar preparations were used for negative controls where primary antibody was omitted. Samples were observed with a Leica TCS-SP5-DMI6000B confocal microscope (Heidelberg, Germany).

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Western Blot (WB)

Western blot was used as a complementary methodology to demonstrate the presence of OPN. Forty rats per group (Normal, SWE, SE, and OA) were used; the procedure was repeated in triplicate. Cartilage samples from femoral condyles were obtained after 5, 10, 20, and 45 days. Sample processing was conducted as described elsewhere (Almonte-Becerril et al., 2010). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% gels, loading 40 μ g of protein for each gel lane. Proteins were transferred by wet transfer for 90 minutes at 350 mA, to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with nonfat dry milk (Almonte-Becerril et al., 2010) and then incubated overnight at 4°C with mouse monoclonal anti-OPN (1:500; Santa Cruz Biotechnology, Dallas, TX,

USA). Immunoreactions were observed after incubation for 1 hour with horseradish-peroxidase-labelled anti-mouse secondary antibody (1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The labelling was performed using chemiluminescence ECL Plus (GE Healthcare, Buckinghamshire, UK). The expression of beta-actin (1:2,000; Santa Cruz Biotechnology, Dallas, TX, USA) was used as internal control.

Statistical analysis

Statistical analyses were performed on histological samples from 10 different rats in each experiment. The analysis included three different sections per rat and three randomly picked fields per section. Count of cells was performed on all three regions of AC superficial zone (SZ), middle zone (MZ), and deep zone (DZ) as

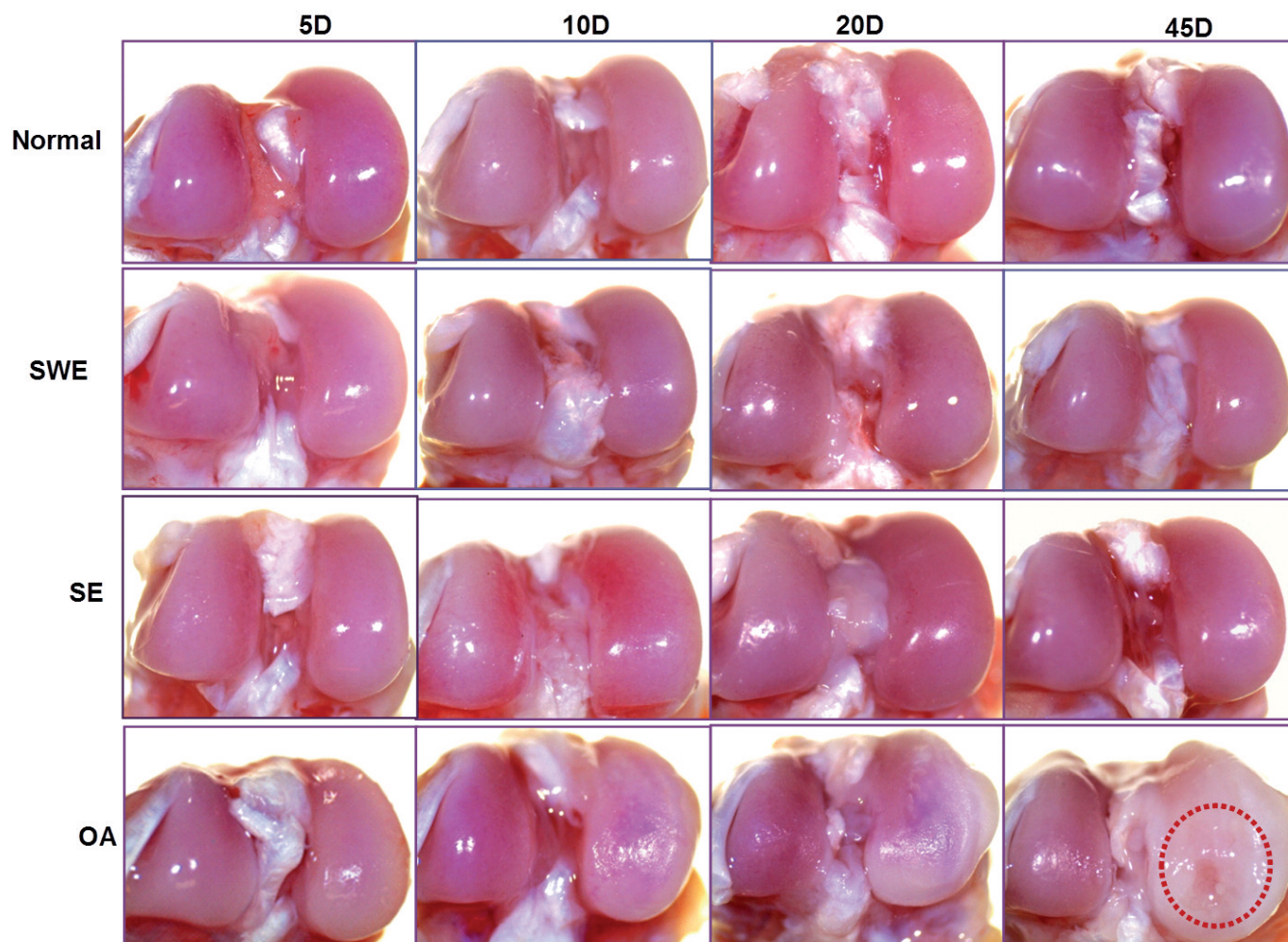


Fig. 1. Changes in the morphology of AC. Representative femoral condyles from the right back leg of Normal, SWE, SE, and OA rats; the red circle shows the loading area in the femoral condyle (45-day OA) where the cartilage exhibits more damage. Degenerative changes characteristic of AC observed at different periods after induction of OA. Images acquired with a stereoscopic microscope.

described elsewhere (Kouri et al., 2002). In this way, positive cells were counted for calcium deposits and OPN expression. To quantify the immunolabelling of OPN protein, the Leica LAS AF lite confocal program was used. Fluorescence intensities were measured in each zone by number of pixels per area (μm). Statistical analysis was performed by means of Tukey–Kramer multiple comparison tests and Dunnett’s method for comparing treatments versus control, using Graphpad Prism software v. 5.01. Osteopontin expression by WB assays was evaluated by densitometry using ImageJ software v.1.46. Values were considered statistically significant at $P < 0.05$.

Results

Macroscopic studies

The AC from femoral condyles in Normal, SWE, and SE rats appeared white, smooth, and shining, which are characteristic elements in normal cartilage, in comparison with the yellowish, rough, and opaque OA cartilage, which also showed erosion and thinning of AC in the weight-bearing areas (Fig. 1).

Calcium deposits

We found a progressive increase of calcium deposits in the OA group compared with the Normal, SWE, and SE groups (Fig. 2a). In the Normal, SWE, and SE groups, few calcium deposits in cytoplasm and chondron (pericellular matrix) of chondrocytes were observed at

late times (20 and 45 days) (Fig. 3). The AC samples from the SE group showed an increase in the mean value of calcium deposits (Fig. 2a). The statistical analysis performed to compare control groups (SE versus Normal, SE versus SWE), did not show significant differences between groups (data not shown).

Calcium deposits at SZ and MZ in different stages of OA

Cartilage samples from Normal, SWE, and SE rats reacted positive to Alizarin red staining. In early stages (5 and 10 days), calcium deposits were localised in cytoplasm and chondron of chondrocytes from DZ towards the proximity to subchondral bone. In late stages (20 and 45 days), calcium deposits were observed in all three zones of AC (Fig. 3). Compared with the OA group, positive staining for calcium deposits was observed beginning in the early stages: 5 days (SZ, $p < 0.01$; MZ, $p < 0.05$) and 10 days (SZ, $p < 0.001$; MZ, $p < 0.01$; DZ, $p < 0.05$). Positive staining increased in the late stages of 20–45 days (SZ, $p < 0.001$; MZ, $p < 0.01$; DZ, $p < 0.001$) (Fig. 2b), covering the three zones of the osteoarthritic AC and homogeneous staining inside and outside the chondrocytes (chondron, territorial, and interterritorial matrix) was detected (Fig. 3).

Osteopontin

We found a progressive increase in OPN expression (75 kDa) in the OA group beginning in the early stages of OA, compared with a basal expression observed in the Normal, SWE, and SE groups. Similar results were

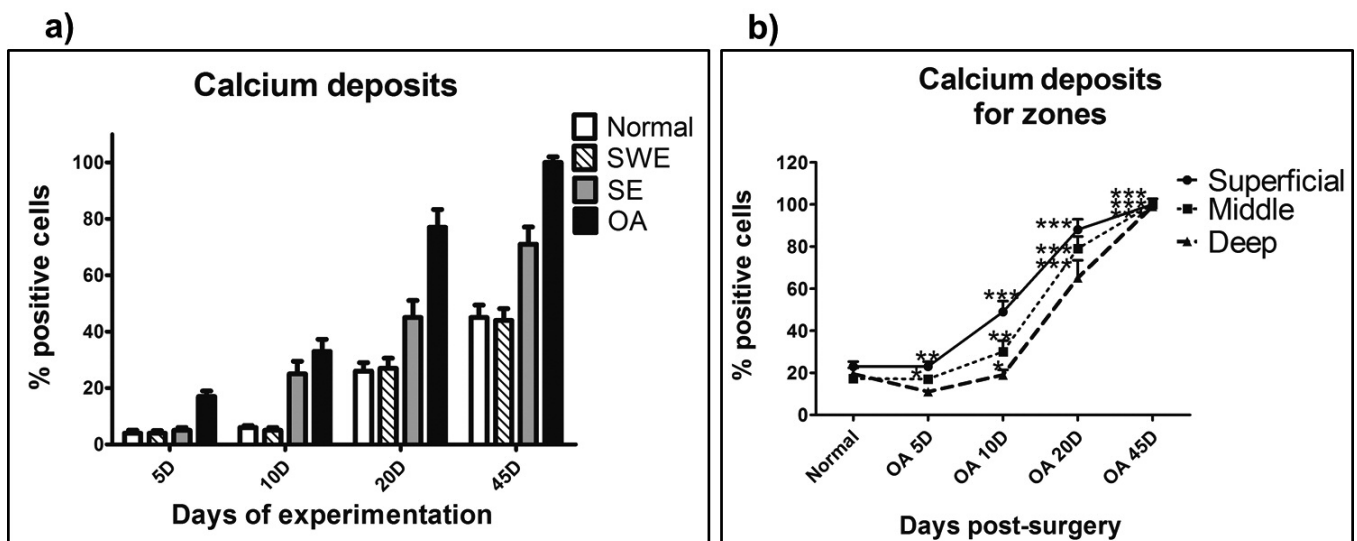


Fig. 2. Distribution of calcium deposits on AC. **a.** Percentage of positive chondrocytes to calcium deposits in the OA group compared with control groups (Normal vs. OA, $p < 0.001$; SWE vs. OA, $p < 0.001$; SE vs. OA, $p < 0.01$). **b.** Percentage of positive chondrocytes to calcium deposits from SZ, MZ, DZ (Normal vs. OA). The results show means \pm S.D from three independent experiments using Tukey-Kramer and Dunnett tests. Significant differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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observed by IHC (Fig. 4a,b) and WB assays (Fig. 4c,d) at 5, 10, 20, and 45 days.

Progressive increase of OPN expression in SZ and MZ from the early stages of OA

During the pathogenesis of OA, OPN expression was observed from the early stages, mainly in the SZ at 5 days (SZ $p < 0.05$), 10 days (SZ $p < 0.01$), 20 days (SZ, $p < 0.001$; MZ, $p < 0.01$; DZ, $p < 0.05$), and 45 days (SZ, $p < 0.001$; MZ, $p < 0.001$; DZ, $p < 0.001$) (Figs. 4b, 5); OPN was localised in the cytoplasm and the perinuclear region of chondrocytes; in 20-day OA samples, OPN expression was more evident in chondrocytes that were

forming clusters (Fig. 6). OPN basal expression was observed in samples from Normal, SWE, and SE rats; it was found in the cytoplasm and the perinuclear region of chondrocytes from the DZ in proximity to subchondral bone (Fig. 5). In later stages (20 and 45 days), OPN was expressed in all three zones of AC. A similar pattern was found with calcium deposits in control groups (Fig. 3).

Discussion

Pathological ossification observed in articular cartilage during OA is an important process that contributes to cartilage degeneration. As a result, bone replaces cartilage through cellular mechanisms similar to

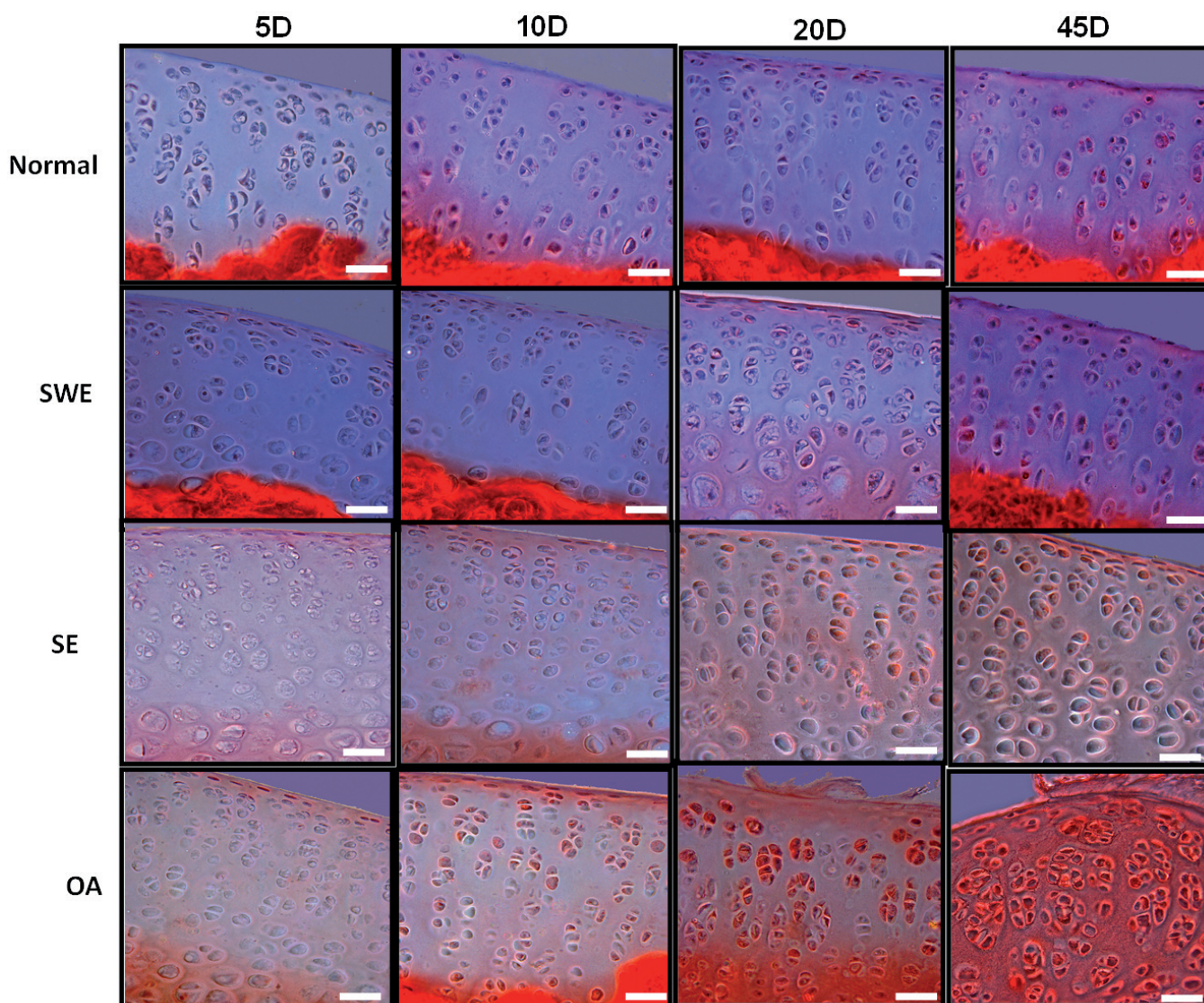


Fig. 3. Representative picture from AC stained with Alizarin red. The cells staining positive for calcium deposits were orange-red coloured. Positive staining for calcium deposits is shown in the cytoplasm and chondron of chondrocytes in early stages of OA. Homogeneous staining was observed inside and outside the chondrocyte (chondron, territorial, and interterritorial matrix) in late stages of OA. Scale bar: 50 μm .

those presented in EO during skeletogenesis (Kouri et al., 2000; Velasquillo et al., 2007; Goldring et al., 2011). The results obtained in our study complement this information. Although OPN and calcium deposits are biomineralisation markers normally involved in skeletogenesis, we have demonstrated the presence of such biomarkers in abnormal ossification during OA pathogenesis. Other studies have also reported the presence of these biomarkers in osteoarthritic AC (Rosenthal et al., 2007; Fuerst et al., 2010; Gao et al., 2010). However, this is the first report that shows the kinetics of expression and localisation of these molecules in different stages of the progression of OA. In addition, we report a gradual increase of calcium deposits and OPN expression from the early stages of OA, starting in the SZ from AC.

Calcium deposits were allocated mainly in the cytoplasm and the chondron. This information is relevant because the chondron is implicated in the initiation and progression of OA, due to pericellular

remodelling, the loss of type II and IX collagen, and the clonal chondrocyte expansion that exists during the OA. All that leads to changes associated with cartilage catabolism (Lee et al., 1997; Poole, 1997; Alexopoulos et al., 2005). On the other hand, it is known that high levels of calcium in the cytosol may cause intracellular precipitation of calcium phosphate inside chondrocytes and can be coupled with the formation of matrix vesicles that have also been involved in pathological mineralisation of AC (Wuthier, 1993; Wu et al., 1995; Kouri et al., 2000; Anderson, 2003).

Nevertheless, some studies indicate that chondrocytes may be directly involved in the acquisition and processing of calcium and inorganic phosphate before the appearance of minerals in the extracellular matrix (Wuthier, 1993; Wu et al., 1995). The high negative-charge density of proteoglycans allows its binding with large amounts of calcium, favoring mineral deposits (Kirsch et al., 1997).

The presence of calcium deposits on samples from

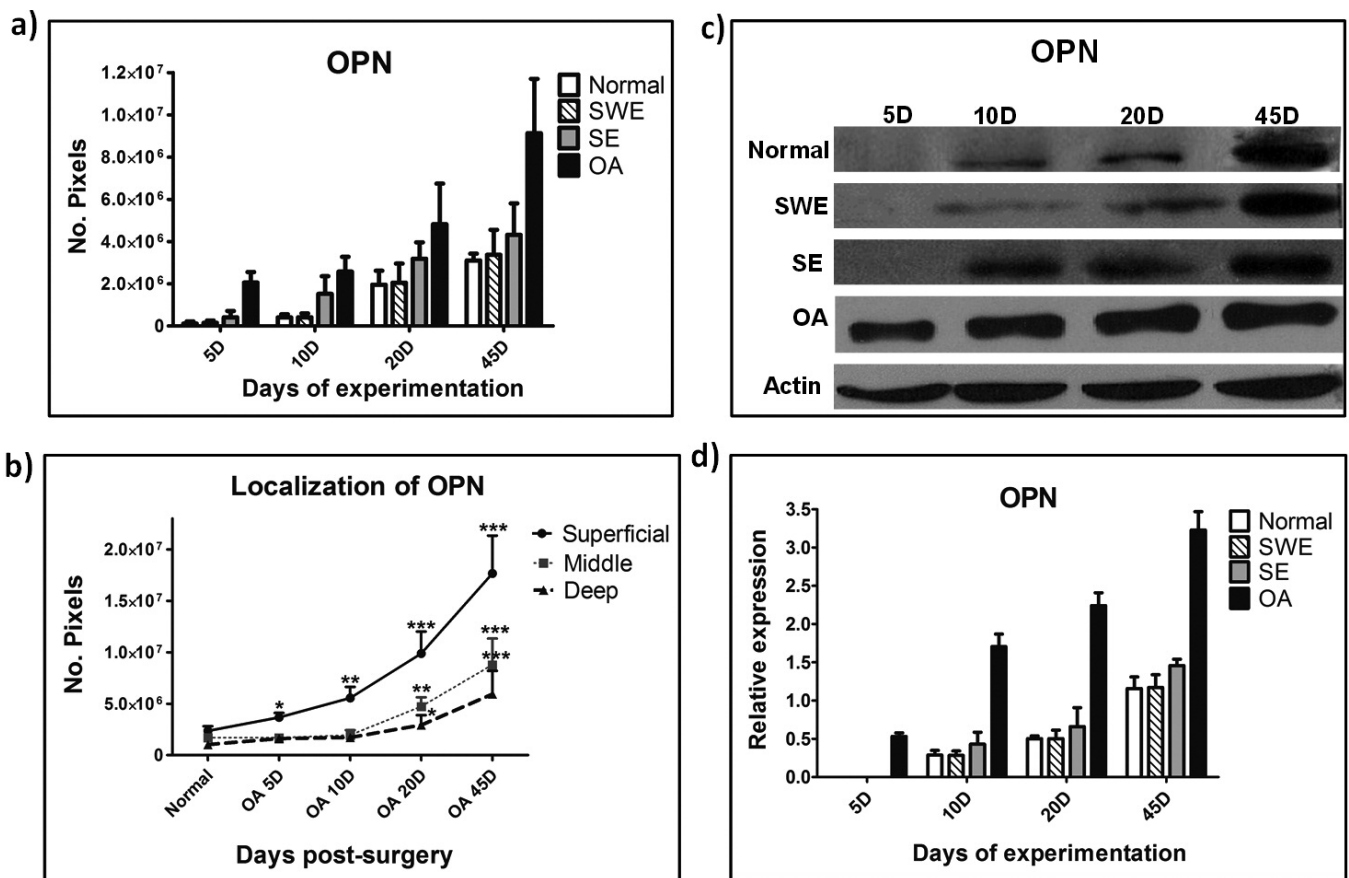


Fig. 4. Change in the expression and localisation of OPN. **a.** OPN expression to number of pixels in OA group compared with control groups (Normal vs. OA, $p < 0.01$; SWE vs. OA, $p < 0.01$; SE vs. OA, $p < 0.05$). **b.** OPN expression to number of pixels in the three zones from cartilage (Normal vs. OA). **c.** WB analysis shows the kinetics of expression of OPN (75 kDa). Beta-Actin was used as charge control. **d.** Densitometry analysis of OPN (Normal vs. OA, $p < 0.001$; SWE vs. OA, $p < 0.001$; SE vs. OA, $p < 0.01$); immunoreactive bands were expressed as a relation of OPN/actin. The results show means \pm S.D from three independent experiments using Tukey-Kramer and Dunnett tests (* $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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human osteoarthritic AC has been correlated with its degradation (Jaovisidha and Rosenthal, 2002; Fuerst et al., 2009, 2010). On the other hand, it has been described that OPN may play a significant role in the pathogenesis of OA participating in CPPD crystal formation, while its overexpression induces the release of proinflammatory cytokines and chemokines (Sodek et al., 2000; Rosenthal et al., 2007). These data also correlate with previous studies made in our group. For instance, the expression of proinflammatory cytokines and metalloproteinases

and the increased expression of apoptotic cell death markers have been reported, mainly in chondrocytes from SZ in early stages of OA (Kouri and Lavalle, 2006; Almonte-Becerril et al., 2010). Due to that, the chondrocytes from SZ are the first response to mechanical damage (Kouri and Lavalle, 2006; Almonte-Becerril et al., 2010; Thomas et al., 2011). Therefore, it is reasonable to assume that calcium deposits and OPN expression found in early stages of OA and their localisation mainly in the SZ may be involved in the

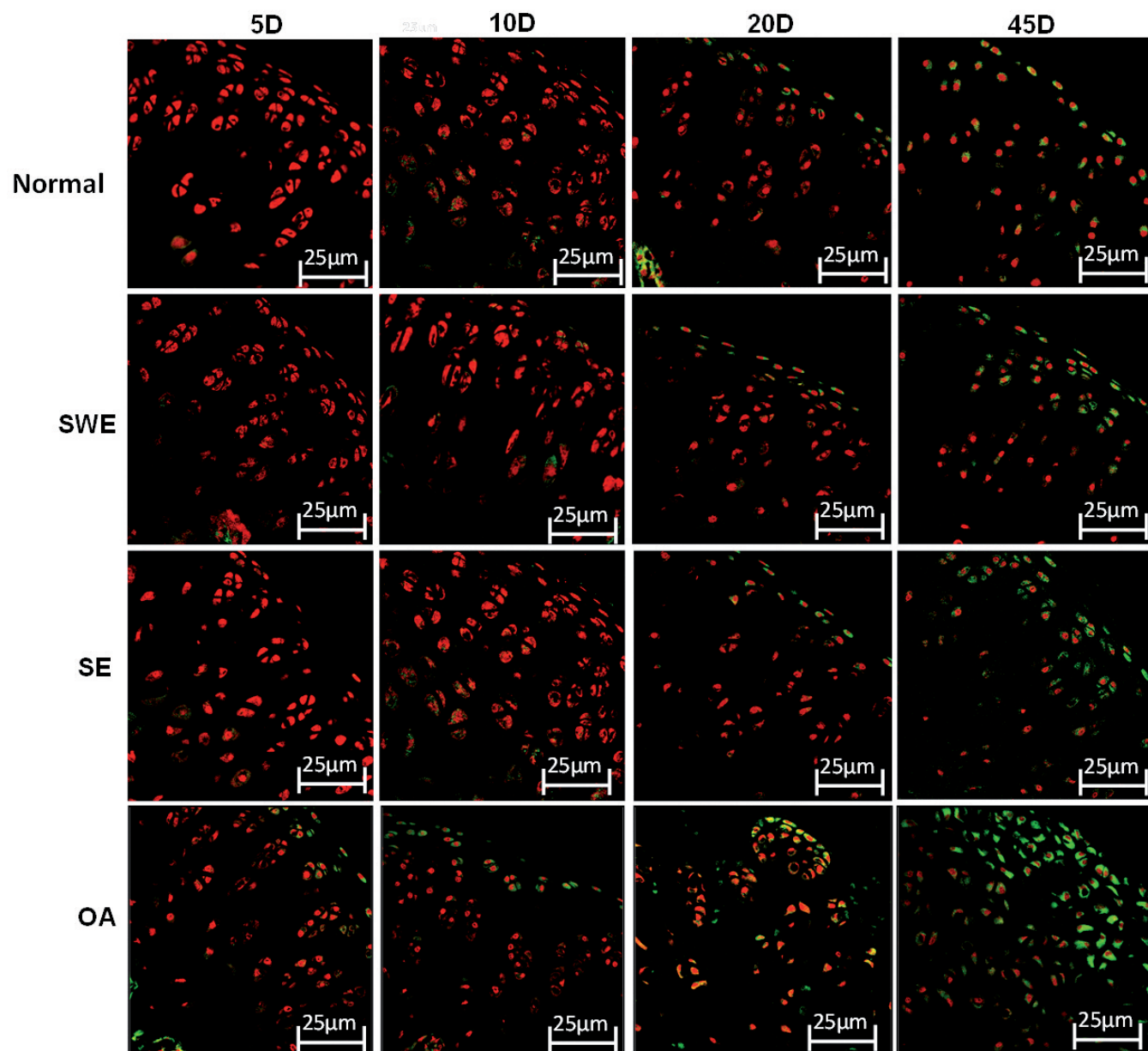


Fig. 5. IHC representative assays of OPN expression during OA pathogenesis compared with the Normal, SWE, and SE groups. In the SZ, OPN expression was increased in the early stages of 5-10 days and the highest expression was at 20 and 45 days of OA; anti-OPN, FITC (Green). Nuclei were stained with PI (red). Scale bar: 25 μ m.

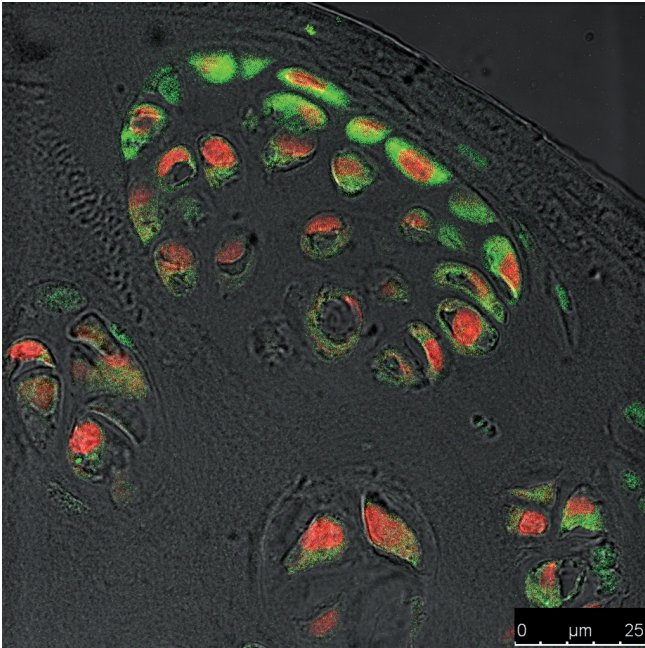


Fig. 6. Localisation of OPN in the cytoplasm and perinuclear region of chondrocytes. In 20-day OA samples, OPN expression was observed mainly in chondrocytes that were forming clusters; anti-OPN, FITC (Green). Nuclei were stained with PI (red). Scale bar: 25 μ m.

beginning of AC degeneration. The basal expression of OPN and localisation of calcium deposits found in late stages in control groups are attributed to normal aging of AC. It has been suggested that AC calcification may be a secondary effect of aging changes (Mitsuyama et al., 2007; Loeser, 2009; Grogan and D'Lima, 2010).

In addition, this study included sham rats with exercise to observe if the exercise could induce degenerative changes in the AC. However, we did not find any difference. In normal cartilage, the balance between anabolic and catabolic processes maintains the integrity (homeostasis) of AC, compared with osteoarthritic AC, where the equilibrium is altered.

In this study, individual calcium crystals were not identified by light microscope, although deposits of these crystals were visible when stained with a calcium-deposit-specific stain such as Alizarin red (Moriguchi et al., 2003; Yavorsky et al., 2008; Martínez-Castillo et al., 2010). However, the identification of this mineral component is considered for future studies using transmission electron microscopy and electron diffraction in both early and late stages of OA on all three areas of articular cartilage.

Finally, we conclude that calcification appears as an early phenomenon that occurs before evidence of cartilage breakdown, in agreement with other descriptions (Scotchford et al., 1992; Mitsuyama et al., 2007). Additional studies are required to expand our knowledge on this subject with the purpose of

elucidating new preventive treatments for the diagnosis of OA, given the fact that progression of OA is particularly difficult to follow in humans at early stages due to the slowness and subtleness of this degenerative process.

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Conflict of interest. There are no conflicts of interest from any of the authors of this manuscript that could have inappropriately influenced this work.

References

- Aigner T., Söder S., Gebhard P.M., McAlinden A. and Haag J. (2007). Mechanisms of disease: role of chondrocytes in the pathogenesis of osteoarthritis-structure, chaos and senescence. *Nat. Clin. Pract. Rheumatol.* 3, 391-399.
- Alexopoulos L.G., Williams G.M., Upton M.L., Setton L.A. and Guilak F. (2005). Osteoarthritic changes in the biphasic mechanical properties of the chondrocyte pericellular matrix in articular cartilage. *J. Biomech.* 38, 509-517.
- Almonte-Becerril M., Navarro-Garcia F., Gonzalez-Robles A., Vega-Lopez M., Lavalle C. and Kouri J. (2010). Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of Osteoarthritis within an experimental model. *Apoptosis* 15, 631-638.
- Aluja A. (2002). Laboratory animals and Official Mexican Norm.(NOM-062-ZOO-1999). *Gac. Med. Mex.* 138, 295-298.
- Anderson H.C. (2003). Matrix vesicles and calcification. *Curr. Rheumatol. Rep.* 5, 222-226.
- Blanco F.J., López-Armada M.J., Maneiro E. (2004). Mitochondrial dysfunction in osteoarthritis. *Mitochondrion* 4, 715-28.
- Ea H.K. and Lioté F. (2004). Calcium pyrophosphate dihydrate and basic calcium phosphate crystal-induced arthropathies: update on pathogenesis, clinical features, and therapy. *Curr. Rheumatol. Rep.* 6, 221-227.
- Ea H.K., Nguyen C., Bazin D., Bianchi A., Guicheux J., Reboul P., Daudon M. and Lioté F. (2011). Articular cartilage calcification in osteoarthritis: insights into crystal-induced stress. *Arthritis. Rheum.* 63, 10-18.
- Fuerst M., Bertrand J., Lammers L., Dreier R., Echtermeyer F., Nitschke Y., Rutsch F., Schäfer F.K., Niggemeyer O., Steinhagen J., Lohmann C.H., Pap T. and Rütther W. (2009). Calcification of articular cartilage in human osteoarthritis. *Arthritis. Rheum.* 60, 2694-2703.
- Fuerst M., Lammers L., Schäfer F., Niggemeyer O., Steinhagen J., Lohmann C.H. and Rütther W. (2010). Investigation of calcium crystals in OA knees. *Rheumatol. Int.* 30, 623-631.
- Gao S.G., Li K.H., Zeng K.B., Tu M., Xu M. and Lei G.H. (2010). Elevated osteopontin level of synovial fluid and articular cartilage is associated with disease severity in knee osteoarthritis patients. *Osteoarthritis Cartilage* 18, 82-87.

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- Goldring M.B. and Goldring S.R. (2007). Osteoarthritis. *J. Cell. Physiol.* 213, 626-634.
- Goldring M.B. and Goldring S.R. (2010). Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. *Ann. N.Y. Acad. Sci.* 1192, 230-237.
- Goldring M.B., Otero M., Plumb D.A., Dragomir C., Favero M., El Hachem K., Hashimoto K., Roach H.I., Olivotto E., Borzì R.M. and Marcu K.B. (2011). Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *Eur. Cell. Mater.* 21, 202-220.
- Grogan S.P. and D'Lima D.D. (2010). Joint aging and chondrocyte cell death. *Int. J. Clin. Rheumatol.* 5, 199-214.
- Jaovisidha K. and Rosenthal A.K. (2002). Calcium crystals in osteoarthritis. *Curr. Opin. Rheumatol.* 14, 298-302.
- Kirsch T., Nah H.D., Shapiro I.M. and Pacifici M. (1997). Regulated production of mineralization-competent matrix vesicles in hypertrophic chondrocytes. *J. Cell. Biol.* 137, 1149-1160.
- Kouri J.B. and Lavalle C. (2006). Do chondrocytes undergo "activation" and "transdifferentiation" during the pathogenesis of osteoarthritis? A review of the ultrastructural and immunohistochemical evidence. *Histol. Histopathol.* 21, 793-802.
- Kouri J.B., Aguilera J.M., Reyes J., Lozoya K.A. and González S. (2000). Apoptotic chondrocytes from osteoarthrotic human articular cartilage and abnormal calcification of subchondral bone. *J. Rheumatol.* 27, 1005-1019.
- Kouri J.B., Rojas L.P. Pérez E. and Abbud-Lozoya K.A. (2002). Modifications of Golgi complex in chondrocytes from osteoarthrotic (OA) rat cartilage. *J. Histochem. Cytochem.* 50, 1333-1340.
- Lee G.M., Poole C.A., Kelley S.S., Chang J. and Caterson B. (1997). Isolated chondrons: a viable alternative for studies of chondrocyte metabolism *in vitro*. *Osteoarthritis Cartilage* 5, 261-274.
- Loeser R.F. (2009). Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. *Osteoarthritis Cartilage* 17, 971-979.
- Lozoya K.A. and Flores J.B. (2000). A novel rat osteoarthrosis model to assess apoptosis and matrix degradation. *Pathol. Res. Pract.* 196, 729-745.
- Martel-Pelletier J. and Pelletier J. (2010). Is osteoarthritis a disease involving only cartilage or other articular tissues?. *Eklemler Hastalik Cerrahisi* 21, 2-14.
- Martínez-Castillo A., Núñez C. and Cabiedes J. (2010). Análisis de líquido sinovial. *Reumatol. Clin.* 6, 316-321.
- McCarthy M.G., Westfall P.R., Masuda I., Christopherson P.A., Cheung H.S. and Mitchell P.G. (2001). Basic calcium phosphate crystals activate human osteoarthrotic synovial fibroblasts and induce matrix metalloproteinase-13 (collagenase-3) in adult porcine articular chondrocytes. *Ann. Rheum. Dis.* 60, 399-406.
- Mitsuyama H., Healey R.M., Terkeltaub R.A., Coutts R.D. and Amiel D. (2007). Calcification of human articular knee cartilage is primarily an effect of aging rather than osteoarthritis. *Osteoarthritis Cartilage* 15, 559-565.
- Moriguchi T., Yano K., Nakagawa S. and Kaji F. (2003). Elucidation of adsorption mechanism of bone-staining agent alizarin red S on hydroxyapatite by FT-IR microspectroscopy. *J. Colloid Interface Sci.* 260, 19-25.
- Orrenius S. and Nicotera P. (1994). The calcium ion and cell death. *J. Neural. Transm. Suppl.* 43, 1-11.
- Poole C.A. (1997). Review. Articular cartilage chondrons: form, function and failure. *J. Anat.* 191, 1-13.
- Rosenthal A.K., Gohr C.M., Uzuki M. and Masuda I. (2007). Osteopontin promotes pathologic mineralization in articular cartilage. *Matrix. Biol.* 26, 96-105.
- Scotchford C.A., Greenwald S. and Ali S.Y. (1992). Calcium phosphate crystal distribution in the superficial zone of human femoral head articular cartilage. *J. Anat.* 181, 293-300.
- Sodek J., Ganss B. and McKee M.D. (2000). Osteopontin. *Crit. Rev. Oral. Biol. Med.* 11, 279-303.
- Thomas C.M., Fuller C.J., Whittles C.E. and Sharif M. (2011). Chondrocyte death by apoptosis is associated with the initiation and severity of articular cartilage degradation. *Int. J. Rheum. Dis.* 14, 191-198.
- Velasquillo C., Garcíadiago-Cázares D., Almonte M., Bustamante M., Ibarra C., Kouri J.B. and Chimal-Monroy J. (2007). Expression of MIG-6, WNT-9A, and WNT-7B During Osteoarthritis. *Ann. NY Acad. Sci.* 1117, 175-180.
- Wu L.N., Ishikawa Y., Sauer G.R., Genge B.R., Mwale F., Mishima H. and Wuthier R.E. (1995). Morphological and biochemical characterization of mineralizing primary cultures of avian growth plate chondrocytes: evidence for cellular processing of Ca²⁺ and Pi prior to matrix mineralization. *J. Cell. Biochem.* 57, 218-237.
- Wuthier R.E. (1993). Involvement of cellular metabolism of calcium and phosphate in calcification of avian growth plate cartilage. *J. Nutr.* 123, 301-309.
- Yavorsky A., Hernandez-Santana A., McCarthy G. and McMahon G. (2008). Detection of calcium phosphate crystals in the joint fluid of patients with osteoarthritis - analytical approaches and challenges. *Analyst* 133, 302-318.

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