Apoptosis 2005; **10**: 1105–1110

Chondroptosis: An immunohistochemical study of apoptosis and Golgi complex in chondrocytes from human osteoarthritic cartilage

E. Pérez H, J. Luna M, L. Rojas M, J. B. Kouri

Departamento de Patología Experimental (E. Pérez H, J. B. Kouri), Unidad de Microscopia Confocal (J. Luna M), Unidad de Microscopia Electrónica (L. Rojas M), Centro de Investigación y de Estudios Avanzados (CINVESTAV-IPN), México D.F., México

The Golgi complex is thought to play an important role in the apoptotic process of osteoarthritic (OA) chondrocytes. However, the exact relationship between modifications of the Golgi complex and apoptosis in human OA cartilage requires to be established. We compared the patterns and immunolabeling intensities for anti-Golgi 58 K protein with apoptosis markers such as TUNEL and caspase-2L in OA cartilage removed from patients during knee total replacement surgery. We observed important modifications in labeling of the Golgi 58 K protein in OA chondrocytes compared with normal cell. Immunohistochemical analysis revealed co-localization between 58 K protein and caspase-2L, suggesting that this enzyme was localized in Golgi complex of OA chondrocytes. In addition, these cells labeled positive with the TUNEL technique, but in different proportions to caspase-2L. Our results support the concept, previously reported, that apoptosis in OA cartilage (chondroptosis) might be a variant of the classical apoptosis.

Keywords: caspase-2L; chondrocytes; Golgi; osteoarthritis; TUNEL.

Introduction

The Golgi complex plays a central role in processing, sorting and/or trafficking of different proteins implicated in the secretion from the endoplasmic reticulum (ER) to the plasma membrane or other post-Golgi compartments. The organelle consists of the stacked cisterns located in the yuxtanuclear region of the cell.[1,2]

A substantial change of this organelle has been related to programmed cell death by apoptosis. Lane *et al*. reported that the Golgi ribbon is fragmented into clusters of tubulo-vesicular membranes and that fragmentation is caspase dependent. He further identified a 65 kDa stacking protein (GRASP65) as a substrate of caspase.[3] Mancini demonstrated that caspase-2L was localized at the Golgi complex, in addition to its nuclear distribution.[4] Modification of the Golgi complex has been reported earlier in chondrocytes from an osteoarthritis induced rat model.[5] Additionally, apoptosis is known to be involved in the pathogenesis in human osteoarthritis.[6–9] However, changes in the Golgi distribution pattern and direct evidence of its association with apoptotic cell death markers have not yet been described in human OA cartilage. Recently, it has been suggested that apoptosis in chondrocytes might be a variant of the classical apoptosis and the term chondroptosis has been proposed.[10] This report was mainly based on morphological studies. Here, we co-localized the immunolabeling of Golgi complex protein 58 K with caspase-2L immunolabeling, in apoptotic TUNEL positive chondrocytes from human OA cartilage. We speculate that an increase in Golgi is part of the mechanism of apoptotic cell death (chondroptosis) within the OA chondrocytes.

Correspondence to: J. B. Kouri, Centro de Investigación y de Estudios Avanzados (CINVESTAV-IPN), Ave. Politécnico Nacional 2508, Col. San Pedro Zacatenco, C.P. 07360, México, D.F. e-mail: bkouri@mail.cinvestav.mx

Material and methods

Tissue sampling

Condyles of OA cartilage were removed from seven patients during knee total replacement surgery. Five women and two men aged between 63 and 76 years were included in this study. All displayed grade IV osteoarthritis[11] without prior trauma or any metabolic disease and without steroid treatment. Condyles from three non-osteoarthritic donor cadavers (25–45 years old) were used as controls. Samples were obtained according to the guidelines approved by the appropriate Committee on Medical Ethics.

Immunofluorescence

Full-thickness samples from the weight bearing areas of the cartilage, were fixed with 4% PBS-paraformaldehyde

at 4°C, cryosectioned (Leica cryostat CM 1100; Heerbrugg, Switzerland), and mounted on gelatine-coated slides. The sections were incubated overnight at 4°C with monoclonal anti-Golgi 58 K (1:350; Sigma-Aldrich, Inc., G2404, Saint Louis, Missouri, USA) or polyclonal antibody anti-caspase-2L (1:50; Santa Cruz Biotechnology, Inc., sc-625, Santa Cruz, California, USA), followed by FITC-tagged anti-mouse IgG (1:250; Vector Laboratories, Burlingame CA) or FITC-tagged anti-rabbit IgG (1:60; Zymed Laboratories Inc. S. San Francisco, California, USA) for 1hr at room temperature. Nuclei were counterstained with propidium iodide for 5–10 min (1:1200; Vector Laboratories, Burlingame, CA). For negative controls the primary antibody was omitted. When performing double labeling, sections were incubated overnight with a mixture of both primary antibodies, followed by a mixture of both secondary. To avoid propidium iodide cytoplasmic RNA staining, sections were pre-treated with ammonium chloride (50 mM) for 10 min and hydrochloric acid (1 M) for 2 hr.[5]

Tunel

For double labeling and DNA fragmentation, sections were stained first for immunofluorescence, simultaneously, with a mixture of anti-Golgi 58 K (1:250) and anti-caspase-2L (1:25), with CY5-tagged anti-mouse IgG (1:100, Zymed Laboratories Inc. S. San Francisco, California, USA) and TRITC-tagged anti-rabbit IgG, (1:40, Zymed Laboratories Inc. S. San Francisco, California, USA), respectively. This was followed by the histoenzimatic TUNEL technique for DNA fragmentation, using an *in situ* cell death detention kit with fluorescein (Promega Corporation, Madison, WI, USA). Sections were incubated with 50 μl of TUNEL (90 μl equilibration buffer, 10 μl nucleotide mix and 2 μl rTdT enzyme) for 60 min at 37°C. Two controls were performed; the first was omitting the enzyme deoxynucleotidyl transferase (negative control) and the second adding DNase I (Roche, Indianapolis, USA) as positive control. All samples were cover-slipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Confocal microscopy

Samples were observed under a 60 and 100 × objective on a Nikon Microscope with attached Confocal System (Bio-Rad MRC 600; Watford, UK); and 100 × objective on a Confocal Leica Microscope (TCPSP2 Leica, Heidelberg, Germany). From each area, ten to twelve serial optical sections (0.7–1 μm thick) were collected.

Quantification of immunofluorescence intensity

To quantify Golgi 58 K and caspase-2L labeling intensity, all samples were collected using the same magnification and microscopy setting: thirty projections, ten for each of normal cartilage. Regarding OA cartilage the labelings were achieved in cluster of chondrocytes from the fibrillated zone of the tissue, where most labelings were observed.

We measured the average of the specific fluorescence intensity of each projection. Since the cartilage damage is focal, most labeling were present only within the damaged region of the tissue, therefore we only measured the area occupied by the immunostaining. Immunofluorescence intensity scoring was possible by means of COMOS/MRC 600 BIO RAD software used with the confocal microscope.

Statistical analysis

Graph Pad Prism computation program version 4.0 was used to compare statistics value. Analysis of variance (ANOVA) and the Tukey multiple comparisons test were used for differences between labeling intensity of 58 K and caspase-2L proteins for each area of cartilage. The Student's t-test was used to compare differences between labeling intensity for OA and normal cartilage. $P < 0.05$ was considered significant.

Results

Immunofluorescence detection of anti-Golgi 58 K in cryosections

In control specimens, labeling with the 58 K Golgi protein was scarce and compact, located in the paranuclear region of the cells. Labeling was observed in some chondrocytes, predominantly in the superficial zone of the cartilage (Figure 1A). In contrast, in OA chondrocytes the Golgi labeling appeared as numerous granules finely dispersed in great part of the cytoplasm, predominantly within the upper zones clustered chondrocytes (Figure 1B). Quantification of the Golgi labeling intensity from normal and OA cartilage showed a remarkable difference between both groups (Figure 2).

Immunofluorescence detection of anti-caspase-2L in cryosections

In normal cartilage, caspase-2L appeared as finely punctuate immunolabeling restricted to the yuxtanuclear region cytoplasm of some chondrocytes. Labeling was

Figure 1. Golgi complex and apoptosis by immunofluorescence microscopy. Chondrocytes of normal (A) and OA cartilage (B) stained with anti-Golgi 58 K (green); normal (C) and OA cartilage (D) stained with anti-caspase-2L (green). Nuclei were counterstained with propidium iodide (red). Double immunolabeling (E, F). Chondrocytes of OA cartilage were stained antibodies against 58 K (red) and caspase-2L (green). Regions of overlap between both proteins are shown in the merge yellow. Double immunolabeling and DNA fragmentation (G, H). TUNEL-positive chondrocytes were stained with antibodies against caspase-2L and 58 K. A merge image showing TUNEL (green), caspase-2L (red) and 58 K (blue) is on the right.

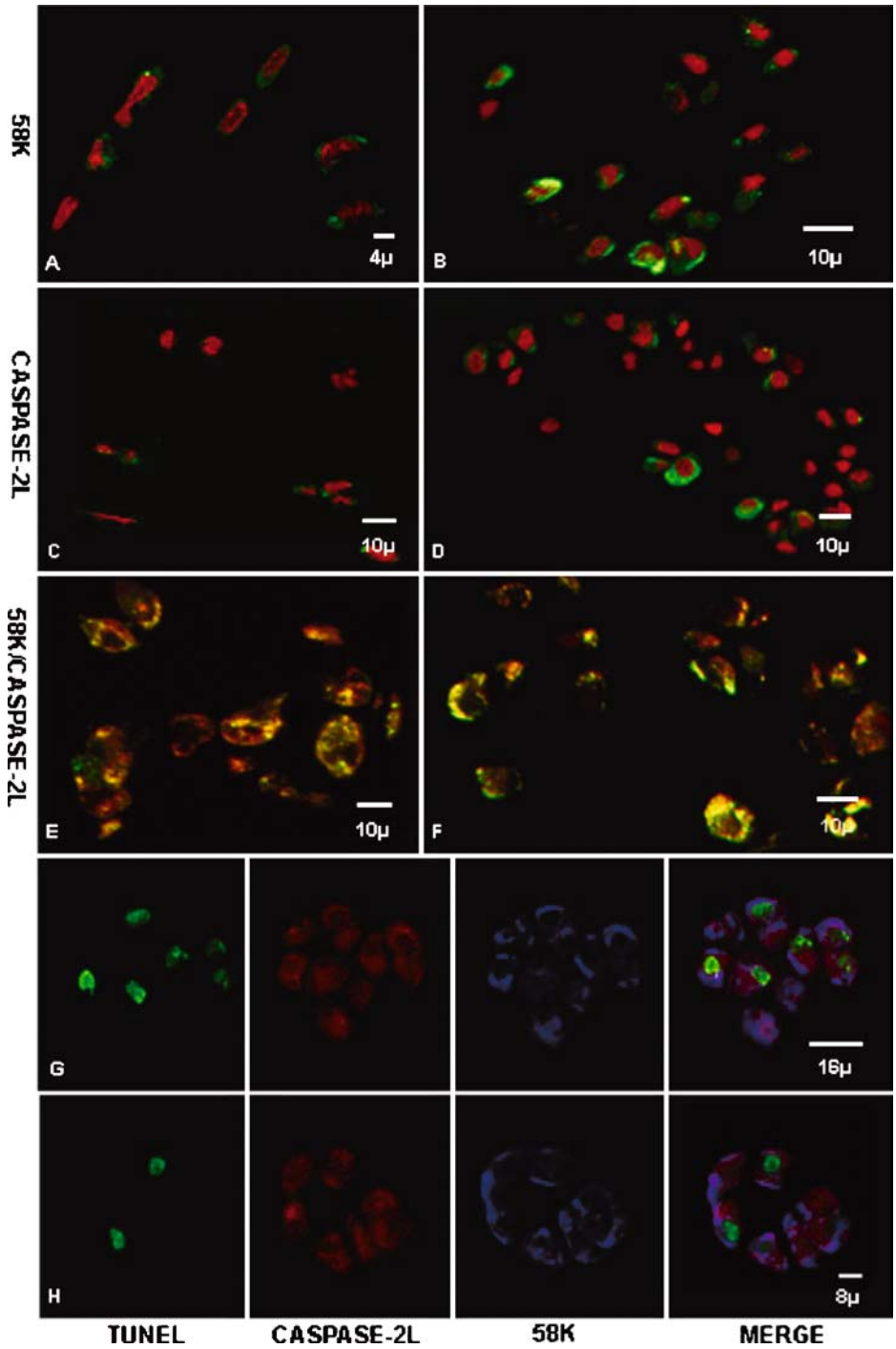


Figure 2. Histogram from the fluorescence intensity values of 58 K protein between normal and OA cartilage. The average specific fluorescence intensity was assigned by pixels number/area μm^2. SEM are shown.

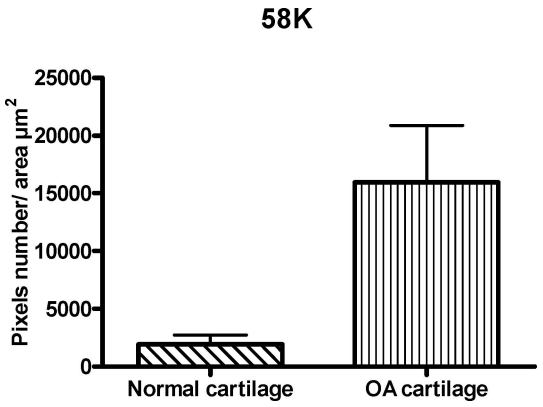
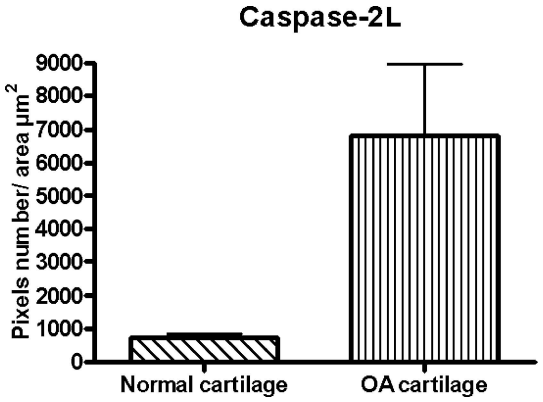


Figure 3. Histogram from the fluorescence intensity values of caspase-2L between normal and OA cartilage. The average specific fluorescence intensity was assigned by pixels number/area μm^2. SEM are shown.



extremely scarce and located mainly in the superficial zone of the tissue (Figure 1C). By contrast, OA chondrocytes display abundant caspase-2L labeling, which showed a granular pattern throughout the cytoplasm, also located predominantly within the upper zones clustered chondrocytes (Figure 1D). Furthermore, when the average fluorescence intensities were assessed, a significant difference was found between the normal and OA cartilage (Figure 3). Co-localization between caspase-2L with 58 K immunolabeling was observed in most clustered OA chondrocytes, suggesting an association between caspases-2L and the Golgi complex (Figure 1E and F).

Co-localization of Golgi/caspase-2L, and DNA fragmentation

Nearly half of clustered chondrocytes displayed Golgi 58 K/caspase-2L co-localization, within cells with positive TUNEL nuclei. However, others showed chondrocytes that displayed only 58 K labeling, or caspase-2L labeling, or TUNEL labeling alone Also different combinations of these three markers were observed (Figure 1G and H). The non-clustered chondrocytes displayed only a small amount of labeling compared to clustered cells.

Discussion

Our results show a significant increase in Golgi, caspase-2L and TUNEL labeling in OA cartilage compared to normal, mainly in clustered chondrocytes from the fibrillated OA cartilage. Furthermore, we demonstrated a correlation between TUNEL positive cells, caspase-2L and the Golgi 58 K protein predominantly in chondrocytes from the upper zones of OA cartilage.

Various studies suggested that the Golgi can play an important role in apoptosis due to the presence of proapoptotic molecules associated with the organelle such as caspase-2L,[4] and several receptors of death cell such as Fas,[12] TRAIL R1 and R2.[13] Classical apoptosis has been reported as regulate by two major pathways; via death-signal receptors such as Fas and TNF, and via mitochondria by means of cytochrome c release.[14] However, more recently a cellular secretory pathway (ER and Golgi complex) has been highlighted.[15]

The receptor/ligand interaction in the death receptor pathway, promote the recruitment adaptor proteins and initiator caspases. Active initiator caspases such as caspases-2L trigger effector caspases, such as caspase-3, to induce apoptosis. Two distinct caspase-2 isoforms has been identified: caspase 2L and caspase-2S. Overexpression of caspase-2L induces cell death, whereas overexpression of caspase-2S can antagonize cell death.[16] In addition, in the mitochondrial pathway the death signals stimulate mitochondria resulting in the release of cytochrome c that binds to an adapter protein Apaf-1 and recruit initiator caspase-9. Active caspase-9 activates effector caspases to induce apoptosis.[14] Caspase-2L induces the translocation of Bid from cytosol to mitochondria, suggesting that caspase-2L may invoke the mitochondrial pathway by processing this protein.[16]

Cleavage of ER or Golgi-resident proteins can signal repair or apoptosis and promote organelle disassembly during apoptosis. The Golgi complex becomes fragmented and key proteins (*e.g.*, GRASP65 and p115) are targets for caspase cleavage. GM130, an integral membrane protein, contributes to the maintenance of Golgi structure and facilitates membrane fusion with secretory vesicles. GM130 is diminished during Fas-mediated apoptosis associated with Golgi fragmentation.[17]

Previous reports described two localizations for caspase-2L, nuclear and within Golgi membranes;[4] however other authors have proposed that the localization of

this enzyme depended on the cell type.[18] The present study showed that caspase-2L was not located in the nucleus of OA chondrocytes, only within Golgi membranes.

Members of BCL-2 family have been localized within the ER membranes. In addition, the ER contributes in a way to Fas-mediated apoptosis and to p53 dependent pathways resulting from DNA damage and oncogene expression. Mobilization of ER calcium stores can initiate the activation of cytoplasmic death pathways as well as trigger mitochondria to direct pro-apoptotic stimuli.[19]

The convergence of two markers of apoptosis with Golgi complex in chondrocytes from OA cartilage, suggests that this organelle might be associated with chondrocytes cell death via a secretory pathway. These findings lined up with the previous reported concept of chondroptosis.[10]

Furthermore, prominent Golgi has been previously reported in the experimental osteoarthritis model using 58 K immunolabeling, which displayed intense immunostaining in most chondrocytes from some regions of 20 days osteoarthritis-induced cartilage.[5] There, we described that Golgi labeling from the whole tissue diminished as damaged progressed probably related to cell death, in a model where the cartilage was completely absent after 60 days of experimental induction. In human OA cartilage damage shows a long evolution process.

Our results made us speculate that within the damaged region of OA cartilage most cells develop the secretory pathway, which might first trigger a supposed recovery mechanisms followed by chondroptosis since repair was unsuccessful. The different combinations of the three labelings (58 K, caspase-2L and TUNEL) described here, might well be associated to dissimilar stages of the catabolic metabolism from clustered chondrocytes during the progression of cartilage degradation. Cellular and matrix degradation is not a synchronic event, as suggested at the ultrastructural level, on previous studies on the kinetic (from 0 to 45 days after induction) of the morphological changes of chondrocytes from osteoarthritis induced model.[20]

Further studies are been carried out to determine the involvement of chondrocyte apoptosis (chondroptosis) and matrix degradation, as has been suggested by several authors.[21–23]

We can conclude that the prominent development of the Golgi complex in human OA chondrocytes might be associated with chondroptosis, corroborating the concept that apoptosis in cartilage may well be a variant of the classical apoptosis. In addition, the implications of modifications of a secretory pathway in chondrocytes during the pathogenesis of osteoarthritis needs to been establish.

Acknowledgments

We thank Dr. H. I. Roach, from Bone & Joint Research Unit, University of Southampton, Southampton, U.K. for the critical review of the article; Dr. Francisco A. Vidal Rodriguez (Hospital Ortopedia Dr. Victorio de la Fuente Narváez-IMSS) for his collaboration in the obtaining of OA cartilage samples; Dr. Carlos de la Garza Páez, Dr. Med. Eduardo Álvarez Lozano, Dr. Rafael Briceño Navarro and Lic. Enfermería Alejandro Rentería Cano (Banco de Hueso y Tejidos, Hospital Universitario Dr. José E. González UANL, México) for supplying normal cartilage samples; and María Magdalena Miranda Sánchez, research assistant, David Pérez Rangel and Clara Castelán Domínguez for technical support.

References

1. Farquhar MG, Palade GE. The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol* 1998; **8**: 2–10.
2. Rios RM, Bornens M. The Golgi apparatus at the cell centre. *Curr Opin Cell Biol* 2003; **15**: 60–66.
3. Lane JD, Lucocq J, Pryde J, Barr FA, Woodman PG, Allan VJ, Lowe M: Caspase-mediated cleavage of the stacking protein GRASP65 is required for Golgi fragmentation during apoptosis. *J Cell Biol* 2002; **156**: 495–509.
4. Mancini M, Machamer CE, Roy S, Nicholson DW, Thornberry NA, Casciola-Rosen LA. Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. *J Cell Biol* 2000; **149**: 603–612.
5. Kouri JB, Rojas L, Perez E, Abbud-Lozoya KA. Modifications of Golgi complex in chondrocytes from osteoarthrotic (OA) rat cartilage. *J Histochem Cytochem* 2002; **50**: 1333–1340.
6. Kouri JB, Rosales-Encina JL, Chaudhuri PP, Luna J, Mena R. Apoptosis in human osteoarthritic cartilage: A microscopy report. *Med Sci Res* 1997; **25**: 245–248.
7. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol* 1995; **146**: 75–85.
8. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. *Arthritis Rheum* 1998; **41**: 1632–1638.
9. Kim HA, Lee YJ, Seong SC, Choe KW, Song YW. Apoptotic chondrocyte death in human osteoarthritis. *J Rheumatol* 2000; **27**: 455–462.
10. Roach HI, Aigner T, Kouri JB. Chondroptosis: A variant of apoptotic cell death in chondrocytes? *Apoptosis* 2004; **9**: 265–277.
11. Kellgren JH, Lawrence JS. Radiological assessment of osteoarthrosis. *Ann Rheum Dis* 1957; **16**: 494–502.
12. Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: A rapid mechanism of p53-mediated apoptosis. *Science* 1998; 282: 290–293.
13. Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P. Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J Immunol* 2000; **164**: 3961–3970.
14. Gupta S. Molecular signalling in death receptor and mitochondrial pathways of apoptosis (Review). *Int J Oncol* 2003; **22**: 15–20.

15. Maag RS, Hicks SW, Machamer CE. Death from within: Apoptosis and the secretory pathway. *Curr Opin Cell Biol* 2003; **15**: 456–461.
16. Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T, Alnemri ES. Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J Cell Biol* 2002; **159**: 739–745.
17. Walker A, Ward C, Sheldrake TA, Dransfield I, Rossai AG, Pryde JG, Haslett C. Golgi fragmentation during Fas-mediated apoptosis is associated with the rapid loss of GM130. *Biochem Biophys Res Commun* 2004; **316**: 6–11.
18. Shikama Y, UM, Miyashita T, Yamada M. Comprehensive studies on sub cellular localizations and cell death-inducing activities of eight GFP-tagged apoptosis-related caspases. *Exp Cell Res* 2001; **264**: 315–325.
19. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* 2003; **22**: 8608–8618.
20. Kouri JB, Abbud-Lozoya KA, Rojas-Morales L. Kinetics of the ultrastructural changes in apoptotic chondrocytes from an osteoarthrosis rat model: A window of comparison to the cellular mechanism of apoptosis in human chondrocytes. *Ultrastruct Pathol* 2002; **26**: 33–40.
21. Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasheri A. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. *Vet J* 2003; **166**: 140–158.
22. Kim HA, Suh DI, Song YW. Relationship between chondrocyte apoptosis and matrix depletion in human articular cartilage. *J Rheumatol* 2001; **28**: 2038–2045.
23. Hashimoto S, Ochs RL, Rosen F, Quach J, McCabe G, Solan J, Seegmiller JE, Terkeltaub R, Lotz M. Chondrocyte-derived apoptotic bodies and calcification of articular cartilage. *Proc Natl Acad Sci USA*. 1998; **95**: 3094–3099.