Cell death in human articular chondrocytes: an ultrastructural study in micromass model

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Summary

Chondrocyte apoptosis is known to contribute to articular cartilage damage in osteoarthritis (OA) and is correlated to a number of cartilage disorders. Micromass cultures represent a convenient means for studying chondrocyte biology, and, in particular, their death. In this study, we present the first ultrastructural analysis on chondrocyte death experimentally induced by different agents, all known to be powerful apoptotic triggers.

Chondrocytes were obtained from subjects undergoing joint arthroplasty. At the end of the maturation, they were treated as follows: 10 or 30 μM etoposide for 24h, UV-B for 30 min followed by 4h recovery, 200 or 500 nM staurosporine for 24h, hyperthermia for 1h at 43°C followed by 4h recovery. They were processed for TEM and immunofluorescence.

Control chondrocyte morphology appears similar to that of human articular cartilage. Proteoglycans and collagen fibers are present in the intercellular space, indicating a good extracellular matrix (ECM) production. Etoposide, when effective, induces necrosis. UV-B treated cells show chromatin condensation and pore clustering, typical of apoptotic nuclei. Hyperthermia seems to induce apoptosis in the presence of abundant ECM, giving, differently, a general necrosis when ECM is scarse. Staurosporine has no effect at 200 nM concentration, but frequently, at 500 nM, chromatin condensation appears. Chromatin clumping appears different from that of classical apoptotic models: Roach *et al.* proposed the term "chondroptosis" to indicate this type of cell death. Cells appear shrunk and the nucleus contains condensed chromatin, not marginated into large solid masses, but in small patches, mainly at nuclear periphery. Chondrocytes seem so to undergo apoptosis in a peculiar typical manner.

Keywords: micromass, chondrocyte, apoptosis, osteoarthritis.

Introduction

Osteoarthritis (OA) is a slow progression degenerative disorder, characterized by pain, stiffness and joint functional limitation. It is the most common rheumatic disease with the highest economic impact (Attur *et al.*, 2002), generally affecting both sexes: before 45 years it mainly affects men, while it is more frequent in woman after 45 years. The risk factors, of this pathology are obesity, smoke as well as metabolic, mechanical and genetic factors.

The inflammatory factors activate chondrocytes, the unique cell component in articular cartilage, which are then no longer able to maintain tissue homeostasis. They undergo a number of cell reaction patterns including hypertrophy and terminal differentiation, as it occurs in the hypertrophic zone of the growth plate (Sandell and Aigner, 2001). A crucial role in OA pathogenesis is played by the extracellular matrix (ECM), which seems to loose its remodelling properties, thereby contributing to chondrocyte apoptosis (Kim and Blanco, 2007; Del Carlo and Loeser, 2008).

This type of cell death is known to contribute to articular cartilage damage in OA (Aigner *et al.*, 2001; Olivotto *et al.*, 2007; Scher *et al.*, 2007; Johnson *et al.*, 2008) and is correlated to a number of cartilage disorders. Roach proposed the term *chondroptosis* to evidence the fact that such cells undergo apoptosis in a non-classical manner that

appears to be typical of chondrocyte programmed death *in vivo* (Roach *et al.*, 2004). Nevertheless, its study *in vitro* has been long hampered by the difficulty to reproduce the *in vivo* cell conditions. Human chondrocyte primary monolayer culture does not recover a proper microenvironment comparable with *in vivo* ECM, and so it cannot be considered a reliable experimental model (Kavalkovich *et al.*, 2002). Differently, micromass cultures represent a convenient means for studying chondrocyte biology, pathology and death. In particular, they can be considered a useful experimental model to investigate cartilage response to physical or chemical agents (Kafienah *et al.*, 2007). In this study, we present the first morpho-functional analysis on human chondrocyte death experimentally induced by a number of agents, all known, in most common cell systems, to be powerful apoptotic triggers.

Materials and Methods

Chondrocyte cultures

Articular cartilage tissue specimens were withdrawn from the tibial plateau of OA patients undergoing joint arthroplasty. Samples were processed with a sequential enzymatic digestion (12). Chondrocytes (500,000), at first passage (p1), were pelleted (by centrifugation for 20 min, at 4°C, at 740 g) in Sarstedt cryotubes (Numbrecht, Germany) and then differentiated in 10% serum medium in standard oxygen pressure, in the presence of 50 μg/mL ascorbic acid, with medium change every second day. Immediately after centrifugation, the cells appeared as a flattened pellet at the bottom of the tube. One day later, the pellet had a thickened lip and, in a week, it became spherical without any increase in size. In 2-3 weeks, it changed from white and opaque to a glistening transparent structure (Zhang *et al.*, 2004; Olivotto *et al.*, 2008).

At the end of the maturation, chondrocyte micromasses were treated as follows:

- UV-B (312 nm) for 30 min, followed by 4h recovery;
- 200 or 500 nM staurosporine for 24h;
- hyperthermia for 1h at 43°C, followed by 2 or 4h recovery;
- 10 or 30 μM etoposide for 24h;
- 0.3 or 0.5 mM H_2O_2 for 1h;
- 60 or 90 μM cisplatin for 24h.

SEM

After washing, control micromasses, were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h. They were quickly washed and post-fixed with 1% OsO_4 in the same buffer for 1h. A progressive alcohol dehydration was performed, followed by specimen critical point drying. After mounting on conventional SEM stubs by means of silver glue, specimens were gold-sputtered (Battistelli *et al.*, 2005). Observations were carried out with a Philips 515 scanning electron microscope.

TEM

Control and treated chondrocyte micromasses were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h, post fixed with 1% OsO_4 in the same buffer for 1 h, alcohol dehydrated, and embedded in araldite, as previously reported (Luchetti *et al.*, 2006). Thin sections were collected on 400 mesh nickel grids and stained with uranyl acetate and lead citrate (Battistelli *et al.*, 2005). The observations were carried out with a Philips CM 10 electron microscope at 80 kV.

Immunofluorescence

IF staining of the main ECM components (aggrecan, chondroitin-4-sulphate and chondroitin-6-sulphate, collagen type II and type I) was performed, to assess the chondrocyte differentiation degree. Five micrometers sections of OCT embedded, snap frozen (liquid nitrogen) micromasses were fixed with 4% paraformaldehyde in PBS for 30 min. After antigen unmasking for 30 min at 37°C with 2 mg/mL hyaluronidase (required for collagen type II and collagen type I) or with 0.02 U/mL ABC chondroitinase (required for chondroitin-6-sulfate, chondroitin-4-sulfate, and aggrecan), a step to block nonspecific binding was performed with 2% BSA, 0.1% Triton, and 5% normal goat serum in TBS for 45 min at room temperature. Next, a panel of mouse monoclonal antibodies (Chemicon International, Temecula, CA), including antibodies to the different components of the branch- shaped complex named aggrecan (core protein and sulphated GAG), was applied (Battistelli *et al.*, 2005). Then, the slides were mounted with antifading (1,4-diazabicyclo octane; Sigma, St Louis, MO) and analyzed with a Nikon Eclipse E 600 fluorescence microscope. Sulphated GAG may immobilize soluble factors bearing heparin-binding domain, such as chemokines.

Results

SEM shows inner and outer micromass features: on the surface cells appear closed one another (Figure 1 A, B) while inner structure reveals wide spaces containing ECM (Figure 1 A, C).

Chondrocyte morphology in control condition appears very similar to that of human articular cartilage (Figure 1 D, E). Cells are rounding or slightly elongated with plurilobated nucleus and diffusely dispersed chromatin. The nucleus frequently, appears eccentrically located, due to a relevant amount of lipid vacuoles., We can often observe large amount of glycogen masses and iso-

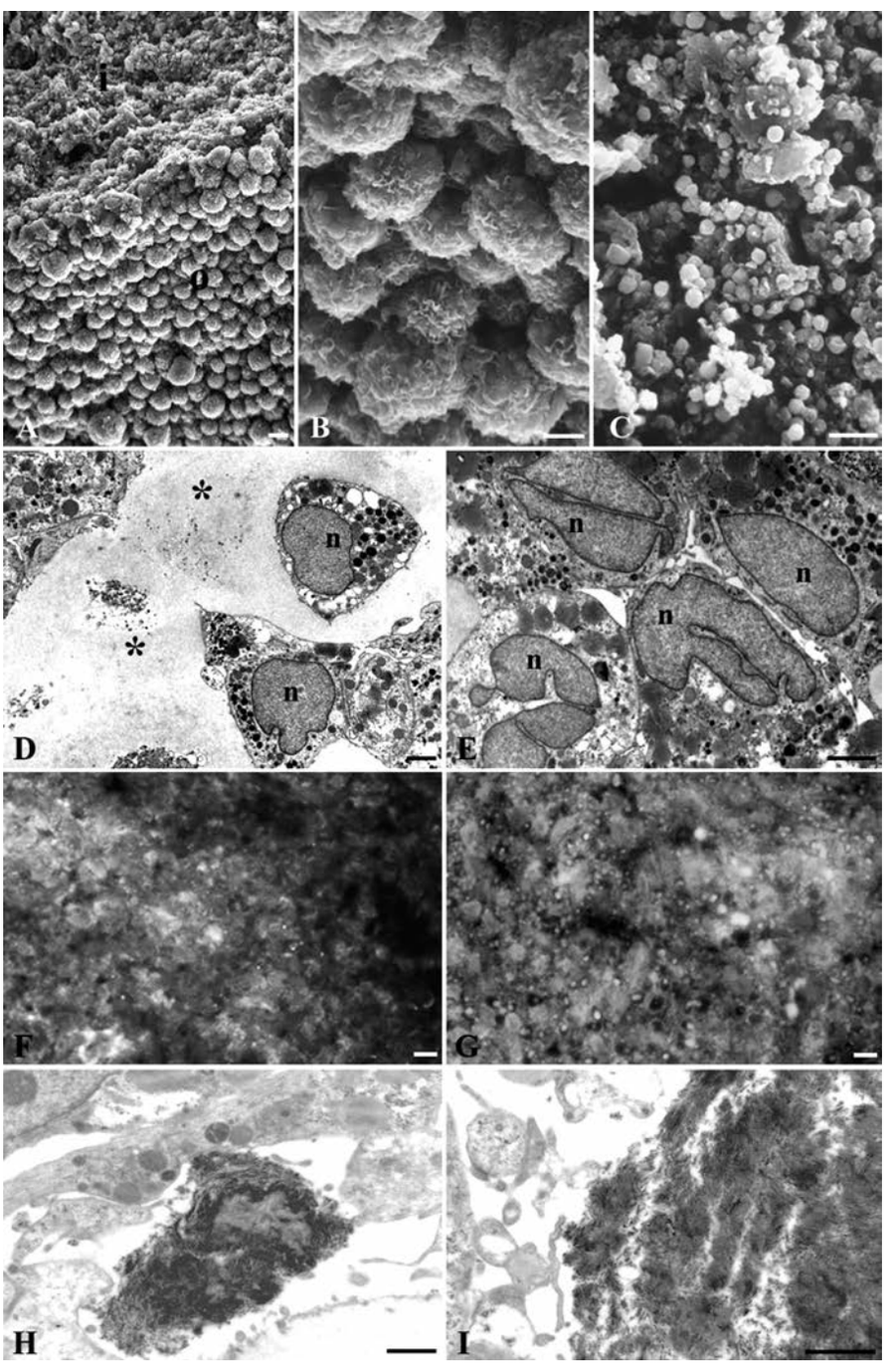

Figure 1. SEM (A, B, C), TEM (D, E, H, I) and IF (F, G) of control micromasses. SEM shows inner (A, i) and outer (A, o) micromass cells, better shown in B and C, respectively. TEM observation shows ECM (*) and nuclei (n) with diffuse chromatin (D, E). At IF staining, proteoglycan (F) and collagen (G) presence appears. In H and I calcification areas appear in the intercellular spaces. A, bar = 0,1 μm; B, C, bar = 5 μm; D, E, F, G, H, I, bar = 1 μm.

lated granules scattered throughout the cytoplasm. Proteoglycans and collagen fibers are present in the intercellular space, indicating a good ECM production (Figure 1 F, G). Occasionally it is possible to observe an initial periodic collagen organization, although less recognizable than that of mature collagen (*not shown*). Sometimes calcification areas appear (Figure 1 H, I).

UV-B, a frequently utilized apoptotic trigger, but with a partially unknown mechanism of action, induces chromatin condensation and pore clustering, typical features of apoptotic nuclei (Figure 2 A, B). Occasionally, a characteristic swelling - suggestive of necrotic progression - can be revealed

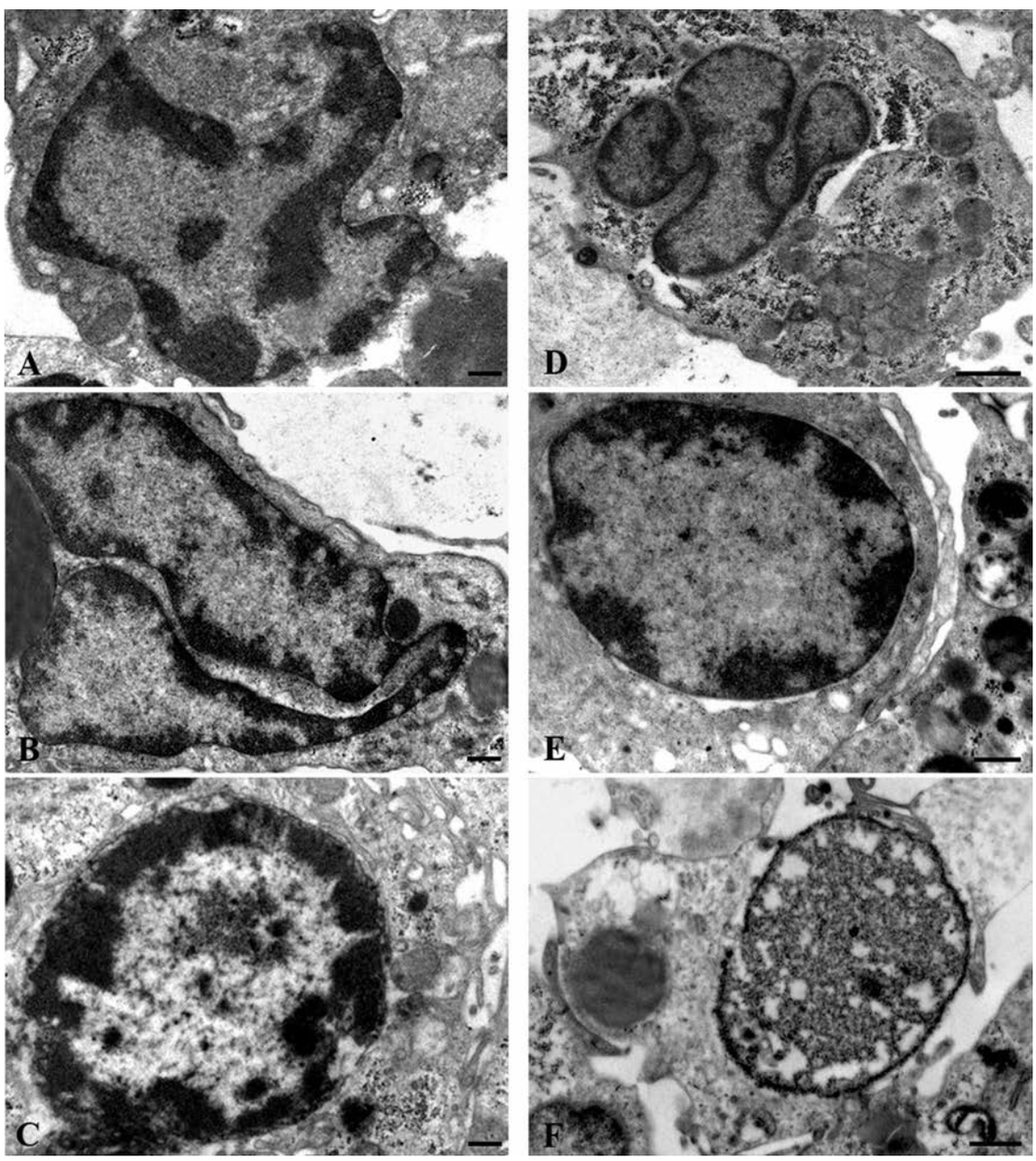

Figure 2. Chondrocytes after UV-B (A, B, C) and staurosporine (D, E, F) treatment. UV-B induces chromatin condensation and nuclear pore clustering (A, B). Occasio-nally inititial necrotic effects can be revealed (C). 200 nM staurosporine has no effect (D), but 500nM induces chromatin condensation (E) and sometimes, necrosis (F). A, B, C, D, E, F, bar = 1 μm.

(Figure 2 C). Staurosporine, a PKC inhibitor, has no effect at 200 nM concentration (Figure 2 D), but frequently, at 500 nM, chromatin condensation (Figure 2 E) and a certain necrotic effect (Figure 2 F) appear.

Hyperthermia, generally considered a powerful apoptotic trigger, induces nuclear modifications suggestive of chondroptosis after 2h post-incubation (Figure 3 A, B, C). Moreover, after 4h post-incubation, it seems to induce apoptosis in the presence of abundant ECM (Figure 3 D, E), showing, differently, a general necrosis when ECM is

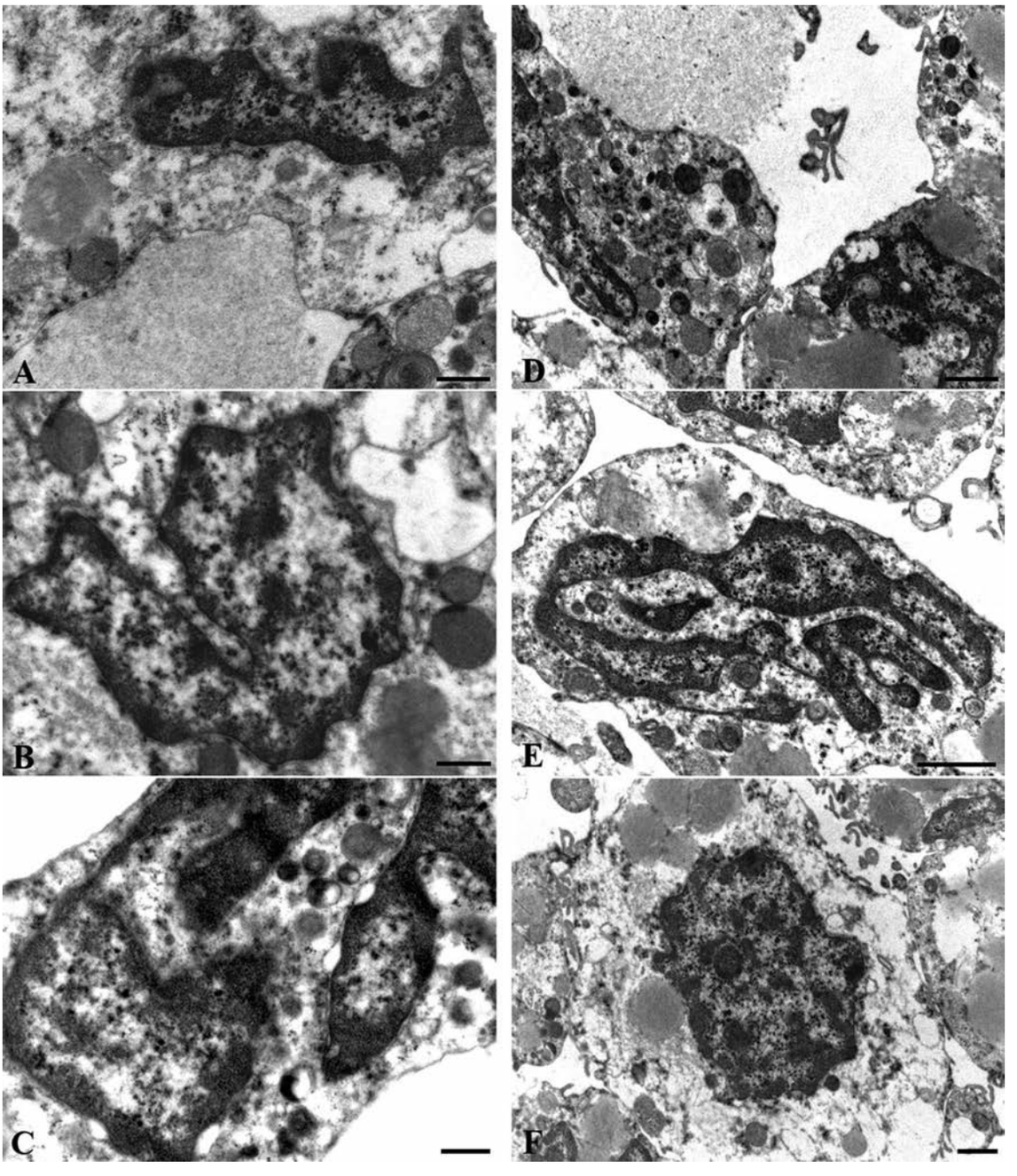

Figure 3. Hyperthermia induces nuclear changes suggestive of chondroptosis after 2h post-incubation (A, B, C). After 4h post-incubation, hyperthermia causes apoptosis in the presence of abundant ECM (D, E), and a general necrosis when ECM is scarce (F). A, B, C, D, E, F, bar = 1 μm.

scarce (Figure 3 F).

Etoposide, a topoisomerase I inhibitor, is poorly effective (Figure 4 A, C, D), occasionally inducing necrosis (Figure 4 B).

H_2O_2 and cisplatin, have not effect at low concentration but, at high ones, they induce a weak apoptotic response (*not shown*).

Therefore, chondrocytes show a heterogeneous behaviour in response to different treatments, and dependently on cartilage micromass conditions.

Extracellular matrix seems to influence chondrocyte response, being micromasses with abundant ECM more resistant to the damage, and directed to apoptotic rather that to necrotic death.

Moreover, the extent of ECM does not seem to decrease during treatments.

In all conditions in which we observed chromatin condensation, it appears different from that of classical apoptotic models. Roach *et al.* (Roach and Clarke, 2000) proposed the term "chondroptosis" to indicate this type of cell death, which was observed in the majority of articular hypertrophic chondrocytes *in vivo* (Blanco *et al.*, 1998).

In our experimental system chondrocytes seem to undergo apoptosis in a non-classical manner, but in a way that seems typical for them, which can be correlated to chondroptosis. As in commonly reported apoptosis, cells appear shrunk and the nucleus contains clumped chromatin. However, the chromatin is not marginated into large dense masses, mostly cup-shaped, but in small patches, mainly located at nuclear periphery (Figure 5 A, B, C, D, E). Chondroptosis involves an initial increase in the endoplasmic reticulum and Golgi apparatus, reflecting an increase in protein synthesis.

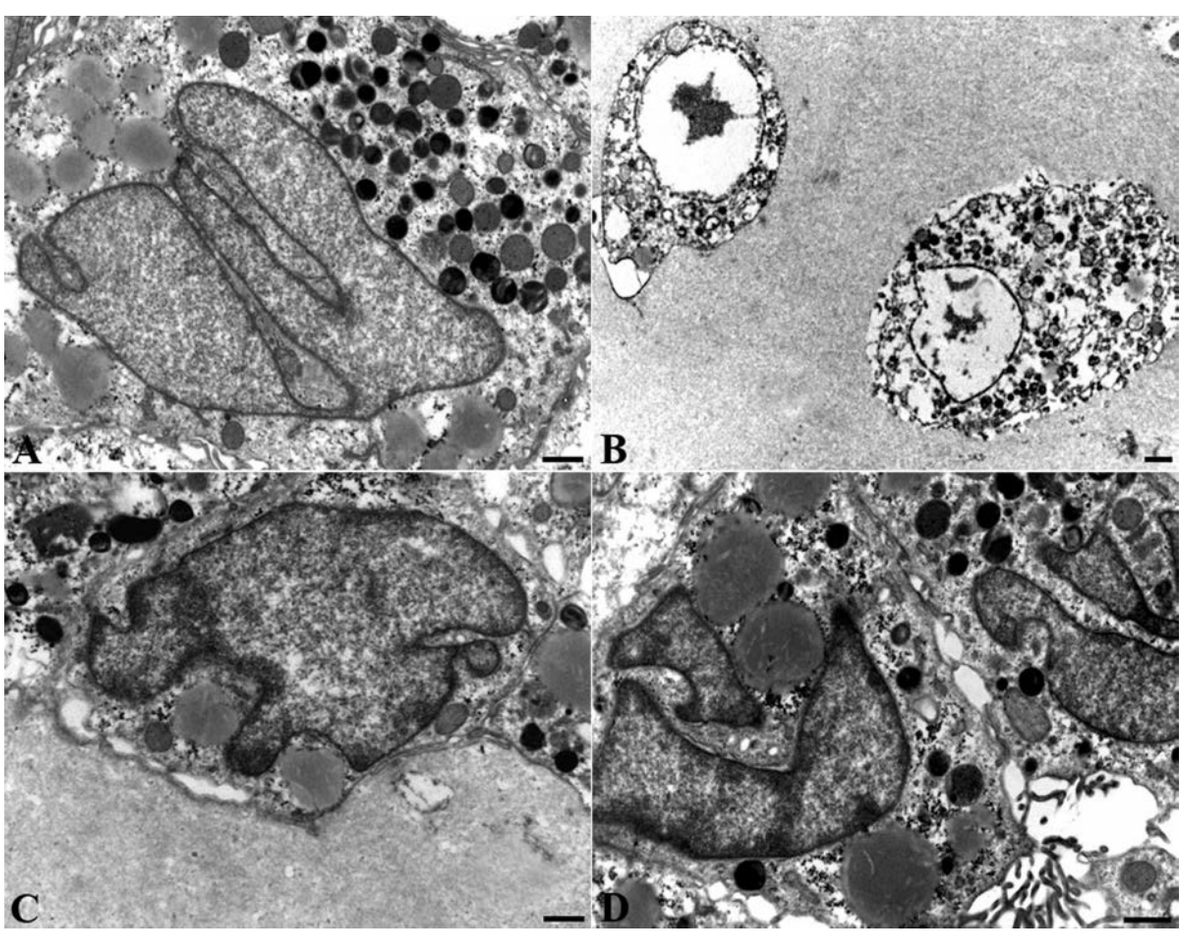

Figure 4. Necrosis, occasionally appears in etoposide-treated specimens (B), even if the trigger has generally an irrelevant effect (A, C, D). A, B, C, D, bar = 1 μm.

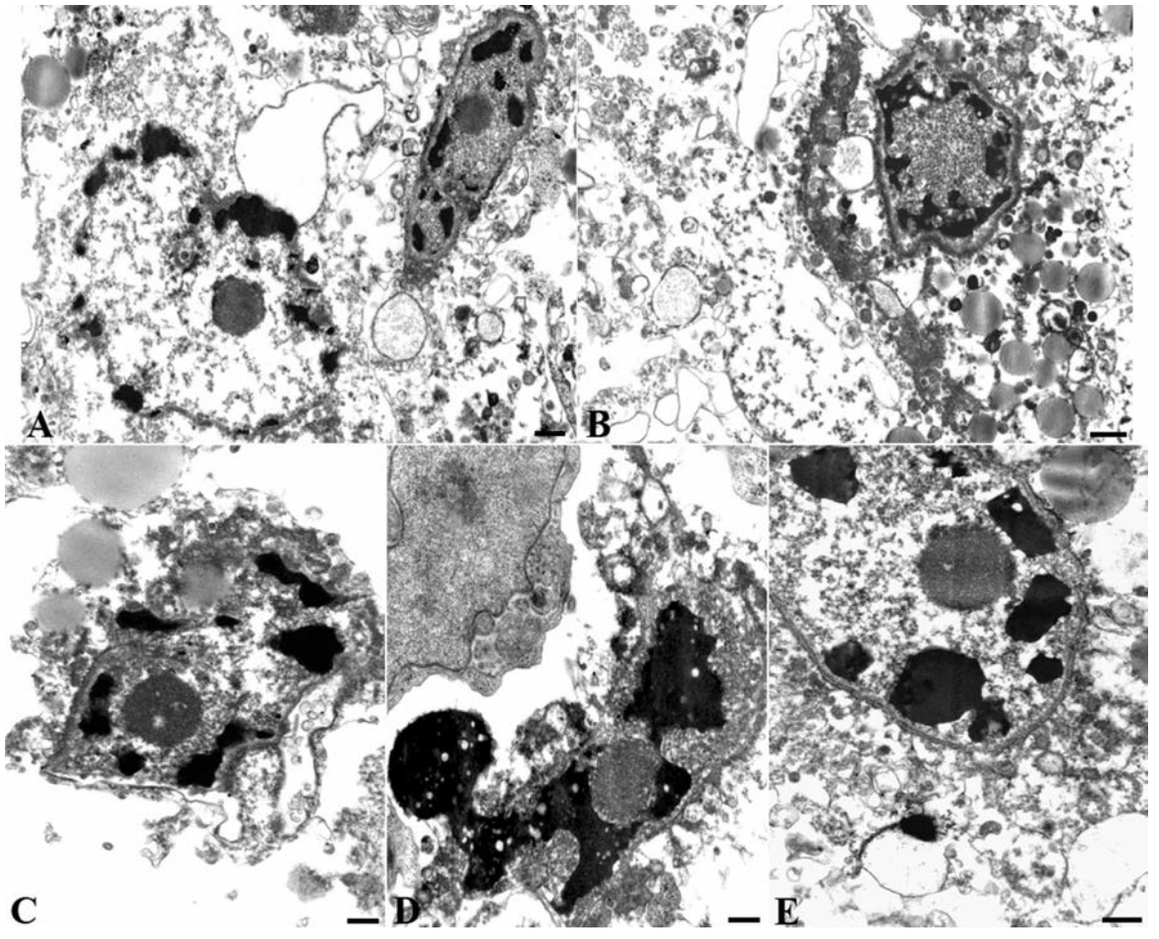

Figure 5. General ultrastructural features of chondroptosis. A, B, C, D, E, bar = 1 μm.

Table 1.

	Apoptosis	*Condroptosis*	*Necrosis*	*ECM*
Control	-	-	-	+
UV-B (312 nm)	+	+	-	+
500 nm Staurosporine	+-	+	+	+
Hyperthemia 2h post-incubation	-	++	+	+
Hyperthemia 4h post-incubation	-	++	++	+
10 μm Etoposide	-	-	-	+
30 μm Etoposide	-	-	+	+
0.3 or 0.5 mM H_2O_2	-	-	+	+
60 or 90 μM Cisplatin	-	-	-	+

Conclusion

Our observations, summarized in Table 1, demonstrate that:

- human articular chondrocytes appear able to undergo apoptosis after UV-B treatment;
- staurosporine and hyperthermia apparently induce chondroptosis;
- hyperthermia effect seems to depend on ECM quantity:
- etoposide is mostly a necrotic trigger.

Chondrocytes cultured in the tridimensional micromass model seem to evidence variable responses, when stimulated by apoptotic triggers. If compared to the classical and more widely described monolayer culturemodel, they present the peculiar characteristic to live in close contact with an ECM microenvironment, which, evidently, regulates also their reaction to external stimuli. Apoptotic/necrotic/chondroptotic responses, shown by micromass chondrocytes, represent howewer, the expression of their death *in vitro*, which can be considered an important model to understand mechanisms underlying articular cartilage disorders.

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