Cell death induced by physical agents: morphological features

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Summary

The aim of this work is to present and discuss in vitro cell death appearing after exposure to physical conditions such as UVB radiation, static magnetic fields, hyperthermia and hypothermia. UVB radiation induces oxidative stress, leading, in most experimental models, to apoptotic death. Generally death occurs through the intrinsic apoptotic pathway, even if the extrinsic one cannot be excluded. UVB radiation also appears effective on cell systems which are normally apoptosis-resistant, such as muscle cells. Static magnetic fields mostly induce plasma membrane and microvilli alterations; occasionally apoptotic cell death appears. Hyperthermic conditions applied were mild, i.e. variable exposures to 43°C, as well as hypothermia, consisting of variable exposures to 0-6°C. Both treatments were followed by incubation at physiological conditions. Heat exposure is a powerful apoptotic inducer in a variety of cells, where it induces classical apoptotic changes and the well known biochemical pathways. The effect of hyperthermia has been described in adherent human tumor cells, which undergo cell rounding and progressively detach from the substrate, in close correlation with the down-regulation of adhesion molecules. Hypothermia, only occasionally triggers apoptosis, more frequently inducing cell necrosis. Therefore, cell death can be induced by physical agents dependently on the treatment and cell model. In particular, UVB and hyperthermia can be considered reliable and reproducible apoptotic triggers.

Key words: apoptosis, UV-B irradiations, magnetic fields, hyperthermia, hypothermia.

Introduction

Apoptosis plays a pivotal role in the deletion of unwanted, damaged, or infected cells in multicellular organisms, as well as in development and tissue homeostasis, cell differentiation, and proliferation (Ola *et al.*, 2011). It is a highly regulated form of cell death, the dysregulation of which results in pathological conditions including cancer, autoimmune disorders and neurodegenerative diseases. It is known in literature that various physical agents are possible apoptotic inducers, but, in some cases, their mechanism of action is only partially known. Some of them, however, are frequently used in clinical trials and represent a potential apoptogenic tool, which requires a major consideration both in terms of basic and applied biomedical research. Ultraviolet (UV radiations) in the middle-wavelength range, between 290 and 320 nm (UV-B), represent a relevant environmental danger because of their role in skin aging and cancer (Lei *et al.*, 2010), as well as in the exacerbation of infections (Srivastava *et al.*, 2008). UV-B radiation is a potent apoptosis inducer in many cell types (Luchetti *et al.*, 2006; Liu *et al.*, 2007; Pozzi *et al.*, 2007; Paz *et al.*, 2008) and also in muscle cells (Salucci *et al.*, 2010). It can induce both extrinsic and intrinsic apoptotic pathways, but it is still unclear how these pathways are interrelated (Sandri *et al.*, 2001). However, DNA damage is a

crucial event in UV-B-mediated apoptosis (Burattini *et al.*, 2009). Morphological observations showed that low doses of UV induced apoptosis (Caricchio *et al.*, 2003; D'Emilio *et al.*, 2010), whereas higher doses triggered both apoptosis and necrosis (Abu-Yousif *et al.*, 2008). Static magnetic fields (SMF) might cause cancer or many other human health problems, but there is very little laboratory or epidemiological evidence that connects SMF exposure and human health hazards. There have been few studies on the effects of static magnetic fields at the cellular level, compared to those of extremely low frequency magnetic fields. Past literature showed that a static magnetic field alone has no lethal effect on cell growth or survival under normal culture conditions. It has also been reported that cell cycle is not influenced by extremely strong static magnetic fields (up to a maximum of 10 T). A further area of interest is whether static magnetic fields cause DNA damage, and/or micronuclei formation (Miyakoshi *et al.*, 2005). With the advent and vast use of diagnostic instrumentations such as NMR and the generation of extremely low frequency electromagnetic fields from common electrical devices, many sources of SMF have been introduced into our living environment (Franco *et al.*, 2008).

An increasing amount of evidences indicates that SMF can induce apoptotic changes, mainly through a modulation of Ca^{2+} influx (Teodori *et al.*, 2006). Exposure of cells to hyperthermia is known to induce apoptosis, although the underlying mechanisms are only partially understood. It has become a useful strategy, as well as surgery, chemotherapy, radiation and biological therapy in the fight against cancer. It plays an important role in multidisciplinary treatments (Harima *et al.*, 2001; De Hass-Kock *et al.*, 2009), representing a very promising method that will be seriously considered in the near future (Liang *et al.*, 2007). Hyperthermia-induced apoptosis is characterized both by its distinctive ultrastructural features and by the occurrence of internucleosomal DNA cleavage (Burattini *et al.*, 2009). The scientific basis of hyperthermia treatment is relatively known and its mechanisms of action seem to be multifactorial (Aravindan *et al.*, 2009). In the treatment of malignancies, hyperthermia was shown to induce in vitro both necrosis and apoptosis in a temperature-dependent manner (Sakaguchi *et al.*, 1995). Different experimental studies have demonstrated that it is an effective apoptotic trigger, favouring, through integrin modifications, confirmed by flow citometry adherent human cancer cell rounding and detachment from the substrate. Therefore, changes in cell adhesion properties, together with apoptosis induction were discussed and a potential role of hyperthermia in the control of metastatic diffusion was hypothesized (Luchetti *et al.*, 2004). Mild hypothermia protects cardiomyocytes undergoing oxidative stress by preventing apoptosis via inhibiting mitochondrial dysfunction and DNA damage (Lin *et al.*, 2010; Diestel *et al.*, 2010). It reduces myocardial damage and dysfunction after cardiopulmonary recovery correlated to a reduced rate of apoptosis and pro-inflammatory cytokine expression (Meybohm *et al.*, 2009). It protects the functions of brain cells from ischemic impairment by attenuating apoptotic death (Saito *et al.*, 2010; Li and Wang, 2011). Cells treated for 0-90 min at 0°C exhibited an exponential survival curve: thus, even short exposures to the cold (e.g. 5 min) produced measurable cell killing. Apoptosis represents a new manifestation of cell injury after brief exposure to 0-6 °C hypothermia in different cell lines (Columbaro *et al.*, 1995). The purpose of this work is to examine and discuss the morphological apoptotic effects of UV-B radiations, static magnetic fields, hyperthermia, hypothermia.

Materials and Methods

Cell Culture

Mouse C2C12 myoblasts were grown in flasks or on coverslips (in dishes), in the presence of DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum, 2 mM glutamine at 37° C and 5% CO_2. To induce myogenic differentiation, when 80-100% cell confluence was obtained, previous medium was changed with DMEM supplemented with 1% FBS (D'Emilio *et al.*, 2010; Salucci *et al.*, 2010). Promyelocytic leukemia HL60 cells, myelomonocytic leukemia U937 cells, T-lymphoblastoid Molt-4, erythromyeloblastoid leukemia K562 cells, mastocytoma P815 cells and neuroblastoma SK-N-MC cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 25 mM HEPES pH 7.5, and 1% antibiotics. They were maintained at 37°C in humidified air with 5% CO_2 and cell viability was assessed by the trypan blue exclusion test (Mariani *et al.*, Luchetti *et al.*, 2002; Sestili *et al.*,

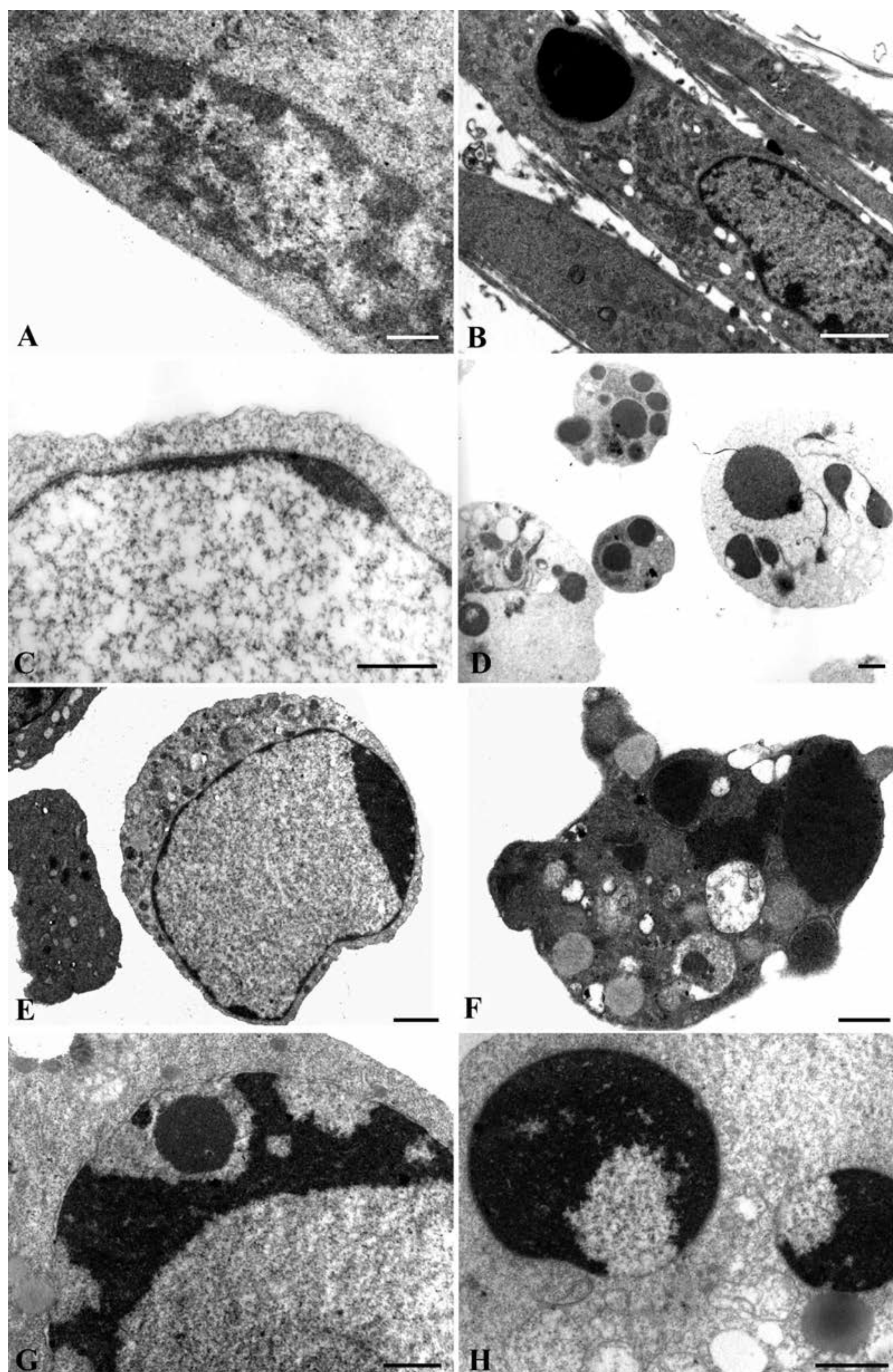

Figure 1. TEM of UVB-treated C2C12 (A, B), HL-60 (C, D), U937 (E, F) and MOLT-4 cells (G, H). All show chromatin margination (A, C, E, G), followed by nuclear splitting and micronuclei appearance (B, D, F, H). A, Bar = 1 µm; B, Bar = 10 µm; C, D, E, F, G, Bar = 0.5 µm.

2006). Glyoblastoma cells were grown in a RPMI medium supplemented with 10% FCS. For the experimental procedures, the cells were plated on glass coverslips and, at a near sub-confluence stage, were exposed to different doses of SMF (Teodori *et al.*, 2006).

Cell Treatment

The triggers used were:
- UV-B (312 nm) for 30 min, followed by 4h recovery,
- SMF 3000G, followed by 2h recovery.
- hyperthermia for 1h at 43°C, followed by 2-4 h recovery,
- hypothermia for 2h at 0 - 6°C, followed by 2-4 h at 37°C.

Transmission electron microscopy

Specimens were processed for transmission electron microscopy (TEM) according to conventional procedures (D'Emilio *et al.*, 2010). Briefly, pellets were washed and immediately fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 2h. After a gentle washing, a post-fixation was performed for 2h in 1% OsO_4 in the same buffer. Alcohol dehydration and araldite embedding were performed and thin sections, collected on nickel grids, and stained with uranyl acetate and lead citrate were analysed with a Philips CM10 electron microscope.

Scanning electron microscopy

After washing, specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 1h. They were quickly washed in 0.15 M phosphate buffer. The samples were then 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 scanning electron microscopy (SEM) stubs by means of silver glue, specimens were gold-sputtered (Sestili *et al.*, 2006). Observations were carried out with a Philips 515 scanning electron microscope.

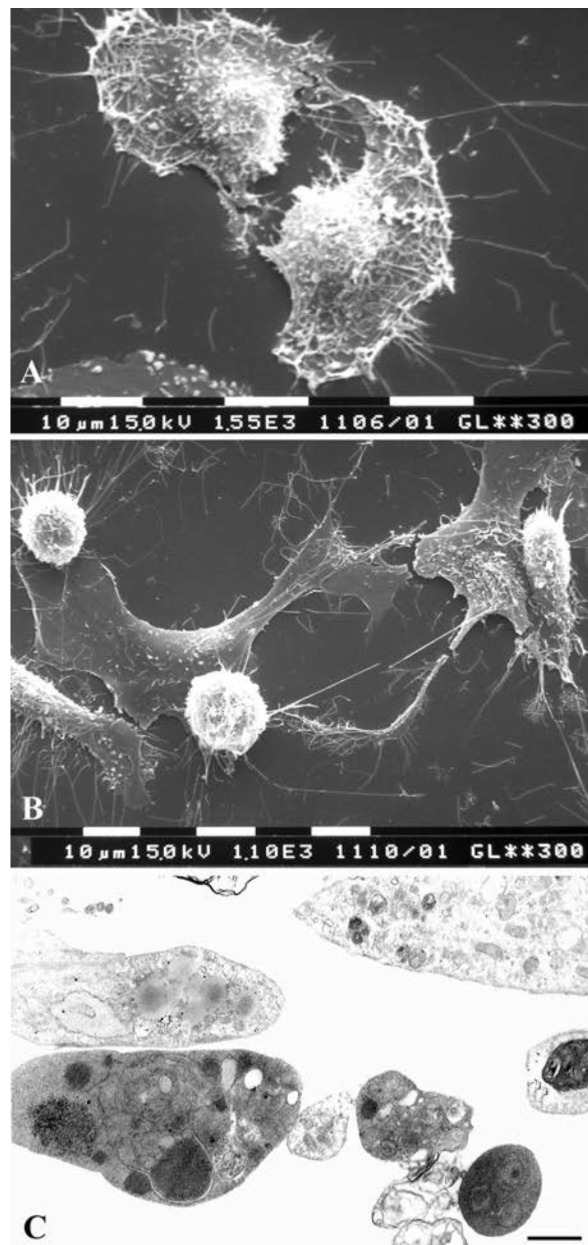

Figure 2. SEM (A, B) and TEM (C) of glyoblastoma SMF-treated cells. Progressive cell detachment, loss of the long projections (A) and occasional spherical apoptotic cells appear (B). Micronuclei and vacuoles can be also revealed (C). A, B, C, Bar = 10 µm

Results

UVB-Irradiation

UV-B- radiations induced apoptotic patterns are shown in Figure 1. C2C12 murine myoblasts and

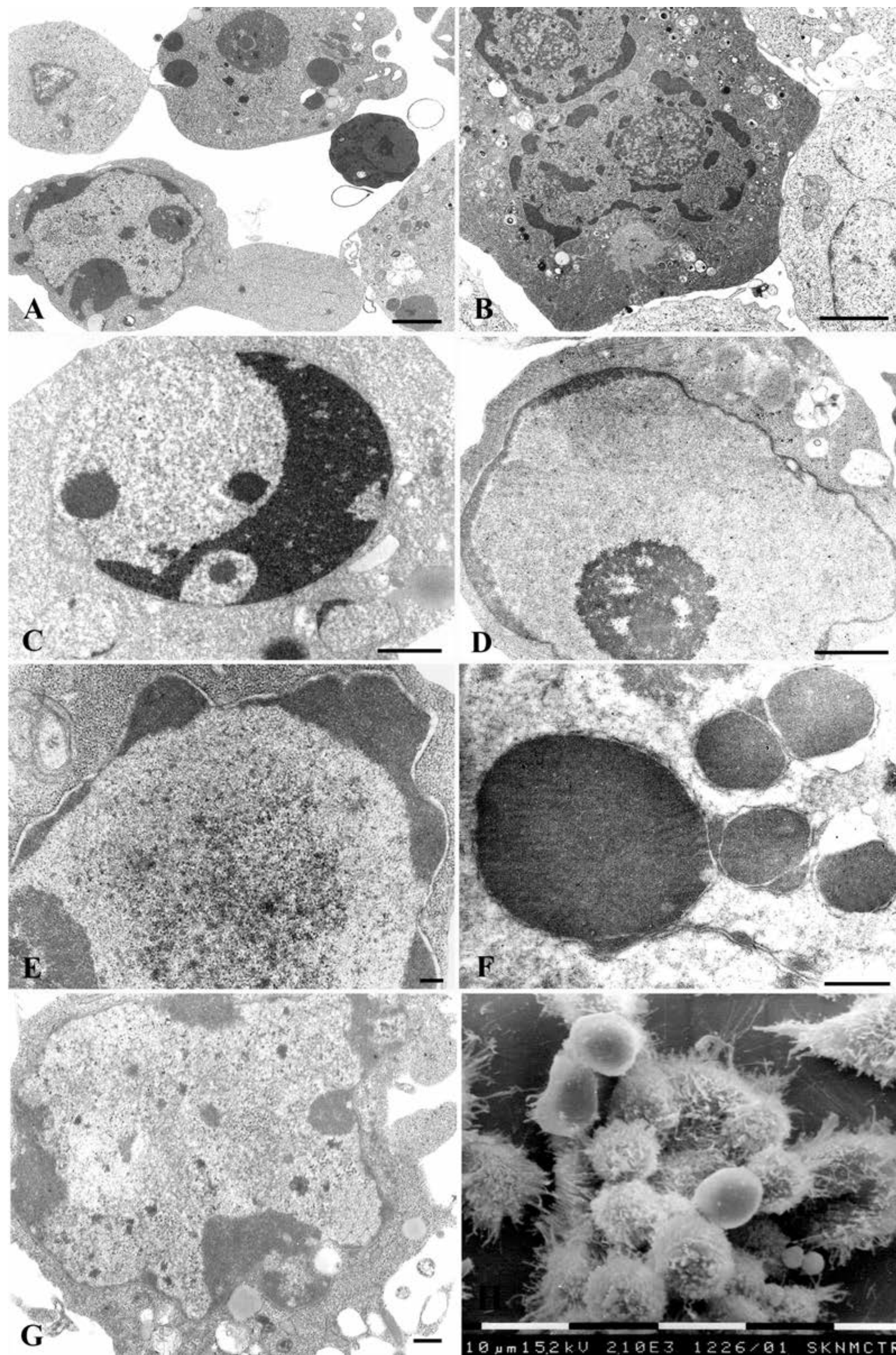

Figure 3. Hyperthermia induces nuclear changes in HL60 (A), U937 (B), MOLT-4 (C), K562 (D) and P815 (E, F) cells. Progressive chromatin condensation appears at TEM observation (A, B, C, D, E), as well as micronuclei (A, F). SK-N-MC cells show chromatin condensation (G) and progressive cell rounding with detachment from the substrate (H). A, B, C, D, E, F, G, H, Bar = 1µm

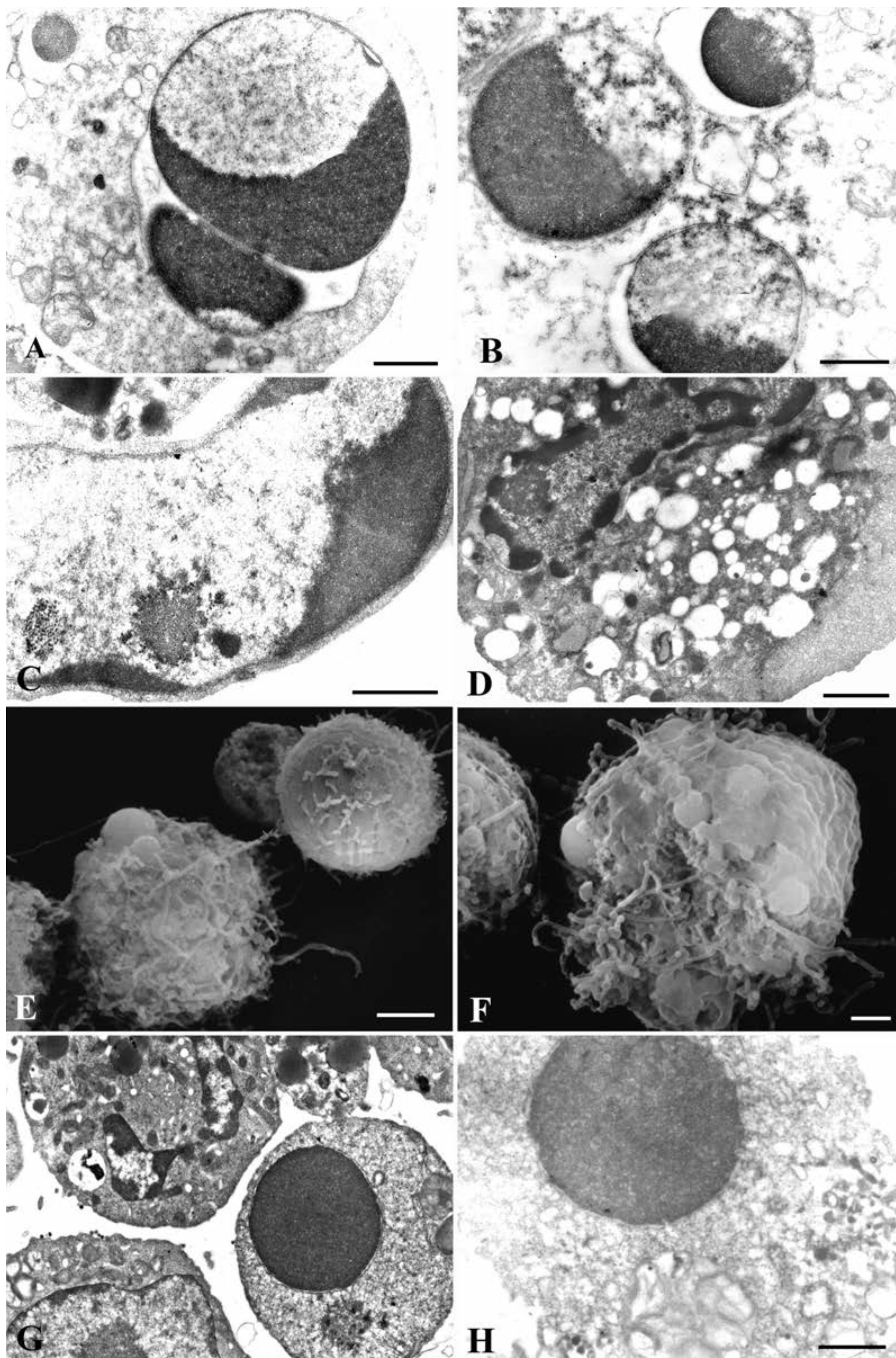

Figure 4. MOLT-4 (A, B), K562 (C, D), and P815 (E - H) cells after hypothermia treatment. MOLT¬4 cells show micronuclei formation (A, B) and K562 evidence chromatin margination (C, D). Progressive cell rounding and detachment from the substrate can be revealed in P815 cells (E, F), which also show, at TEM observation, chromatin margination (G) and micronuclei (H). A, B, C, D, E, F, G, H, Bar = 1µm

myotubes showed morphological apoptotic changes. In myoblasts, a characteristic chromatin margination at the nuclear periphery frequently appeared (A). Even if cup-shaped masses, comparable to those of more classic apoptotic models, could not be found, the evidenced nuclear features, when analysed in detail, appeared to suggest apoptosis. An intriguing behaviour was revealed in myotubes, where apoptotic and normal nuclei appeared together within the same fiber (B). HL60 showed initial chromatin margination with traslocation of nuclear pores (C). Nuclear splitting and micronuclei occurred (D). Successively UVB-induced apoptosis in U937 (E, F) or in Molt-4 (G, H) cells showed chromatin condensation (E, G) and, particularly U937, cell shrinkage and blebs formation (F), with final nuclear splitting and micronuclei formation (F, H). Occasional autophagic vacuoles appeared in U937 treated cells (F). UVB also act by producing oxidative stress through an increased generation of Reactive Oxygen Species (ROS). Consequently, a mitochondrial involvement in UVB-induced apoptosis is certain and mitochondrial changes appear (F).

SMF treatment

We investigated the biological effects of SMF, in glyoblastoma cells exposed to SMF 300 and 3000 G. Their effects are shown in Figure 2. SEM demonstrated a dose-dependent cell shape modification, with progressive cell detachment, loss of the characteristic projections (A) and occasional appearance of spherical apoptotic cells (B). At TEM observation, SMF modified cell shape changes also appeared with occasional presence of micronuclei and vacuoles (C).

Hyperthemia

Hyperthermia-induced apoptotic patterns are shown in figure 3. Hyperthermia (43°C for 1 hour) induced nuclear changes in HL60 (A), U937 (B), MOLT-4 (C), K562 (D) and P815 (E, F) cells. Progressive chromatin condensation appears at TEM analyses (A, B, C, D, E), generally followed by micronuclei formation (A, F). In SK-N-MC cells hyperthermia induces nuclear chromatin condensation (G) and a progressive cell rounding and detachment from the substrate, typical of apoptotic cell behaviour (H).

Hypothermia

Interestingly, apoptotic features characterize cell injury after brief exposure to 0-6°C hypothermia, as shown in Figure 4. In MOLT-4 cells (A, B) micronuclei formation appeared. K562 cells, after hypothermic treatment, showed chromatin margination (C) with a diffuse cytoplasmic vacuolization (D), with occasional presence of autophagic vacuoles (D). Similarly, could induced a progressive cell rounding and blebbing in P815 cells (E), which showed, at TEM observation, chromatin margination (G) and micronuclear formation (H) too.

Conclusions

When compared to the chemical induction of apoptosis, relatively little is known about cell treatment by means of physical agents. UVB radiation, which represents a recent source of interest because of its role in skin aging and cancer, is a potent apoptotic inducer in hemopoietic tumor cell lines, where its mode of action is now relatively well known. Their application to other cell models should hopefully be carried out. Intriguingly, skeletal muscle cells, known to be resistant to apoptotic death, appear sensitive to UVB-induced apoptosis both as undifferentiated myoblasts, and in form of highly differentiated myotubes. The typical chromatin condensation with progressive compacting and formation of micronuclei, appear indeed at morphological investigation. High doses of static magnetic fields generate reproducible effects on targeted cells. We can suggest, however, that the principal cytotoxic damages resulting from magnetic field exposure, only occurs at the level of the plasma membrane and its surface specializations, while it rarely induces apoptosis. Hyperthermia has been revealed to be a powerful apoptotic trigger, widely utilized *in vitro*, where it induces the typical apoptotic features. Hypothermia, even if occasionally inducing apoptotic cell death, seems to stimulate, more diffusely, cell necrosis. Taken together, our data evidence a variable, but generally significant, apoptotic response to physical agents.

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