

Ultrastructural effects of two phthalocyanines in CHO-K1 and HeLa cells after laser irradiation

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ABSTRACT: The effects of Photodynamic Therapy using 2nd generation photosensitizers have been widely investigated aiming clinical application treatment of solid neoplasms. In this work, ultrastructure changes caused by the action of two 2nd generation photosensitizers and laser irradiation on CHO-K1 and HeLa (neoplastic) cells were analyzed by transmission electron microscopy. Aluminum phthalocyanine chloride, aluminum phthalocyanine tetrasulfonate chloride and radiation from a semiconductor laser at a fluency of 0.5 J/cm² (Power=26mW; λ =670nm) were used. The results showed induction of apoptosis. Such alterations were observed in HeLa but not in CHO-K1 cells after Aluminum phthalocyanine tetrasulfonate chloride (AlPcS₄) photodynamic treatment. The Aluminum phthalocyanine chloride (AlPc) photodynamic treatment induced necrosis on the neoplastic cell line, and cytoplasm and nuclear alterations on the normal cell line.

Introduction

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-cancerous diseases that are generally characterized by overgrowth of unwanted or abnormal cells (Dougherty *et al.*, 1998). The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with light of the appropriate wavelength, usually in the red or near-infrared region compatible with the absorption spectrum of the drug (Dougherty *et al.*, 1998; Weishaupt *et al.*, 1976).

In PDT, photosensitizers are used to absorb energy from a light source after its administration to tumour cells, producing reactive oxygen species that will cause cell death (Wilson and Jeeves, 1987; Gomer *et al.*, 1989).

Apoptosis, a process first described by Kerr *et al.* (1972) and also known as "programmed cell death", is a physiological process of cellular deletion occurring during embryogenesis, metamorphose, tissue atrophy and tumour regression (Wyllie and Currie, 1980; Walker *et al.*, 1988). The morphological characteristics of apoptosis are: chromatin condensation, nuclear membrane blebbing and the formation of apoptotic bodies (Cotran *et al.*, 2000). Since the introduction of this term, cell killing mechanisms are generally classified as occurring through apoptosis or necrosis (Ashkenzi and Dixit, 1998). The apoptotic process limits leakage of intracellular material to the immediate environment, and thereby prevents tissue inflammation (Evan and

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Littlewood, 1998; Kroemer and Reed, 2000; Reed, 2000). In contrast, necrosis results from high levels of cell damage, in which plasma membrane integrity is lost, causing cell lyses and thus tissue inflammation (Kroemer *et al.*, 1998). Although PDT can produce apoptosis or necrosis, or a combination of the two mechanism, in many cases it is highly efficient in inducing apoptosis (Oleinick, 1998).

Agarwal *et al.* (1991) first reported that cells undergo apoptosis after photosensitization with aluminum phthalocyanine chloride. The phthalocyanines are 2nd generation photosensitizers used in PDT and can be easily synthesized with a variety of different side groups that alter the molecule charge and solubility. These characteristics affect the photosensitizer uptake by tumour cells (Ali *et al.*, 1988).

The sulfonation of the side chain will render them soluble in water (Ali *et al.*, 1988). Thus, the uptake kinetics and cell retention are different for hydrophilic and for hydrophobic phthalocyanines (Ben-Hur and Rosenthal, 1986). This difference will direct the mode of cell death as a response to photodynamic therapy at a same light fluency used. Photosensitizers with highly negative charges are internalized by endocytic mechanisms due to the repulsive forces between it and the

plasma membrane, which is negatively charged (Berg *et al.*, 1994).

Many of the sensitizers used in the experimental or clinical PDT, localize in the plasma membrane, mitochondria, endoplasm reticulum and lysosomes (Santus *et al.*, 1991; Moan and Berg, 1992). PDT with most of the sensitizers tested, acts *via* singlet oxygen production. Because of the short half live of this excited species in cells (<0.1 μ s) and short radius of action (<0.02 μ m) (Moan and Berg, 1991), damage will occur mainly next to the region the sensitizer is concentrated. Incubation time of cells with photosensitizer, is another parameter that will affect the mode of cell death during exposure to light. For short period of incubation, the plasma membrane is an important site of damage (Christensen *et al.*, 1985). Prolonged incubation with Photofrin[®], the first PDT photosensitizer to win approval by regulatory agencies in several countries (Lipson *et al.*, 1961), tends to localize in the mitochondria membrane (Berns *et al.*, 1982; Hisazumi *et al.*, 1984).

The aim of the present study, was to describe the ultrastructural changes caused in a normal and a neoplastic cell line after PDT with two phthalocyanines (hydrophobic and hydrophilic photosensitizers), to determine the mode of cell death caused by the treatment.

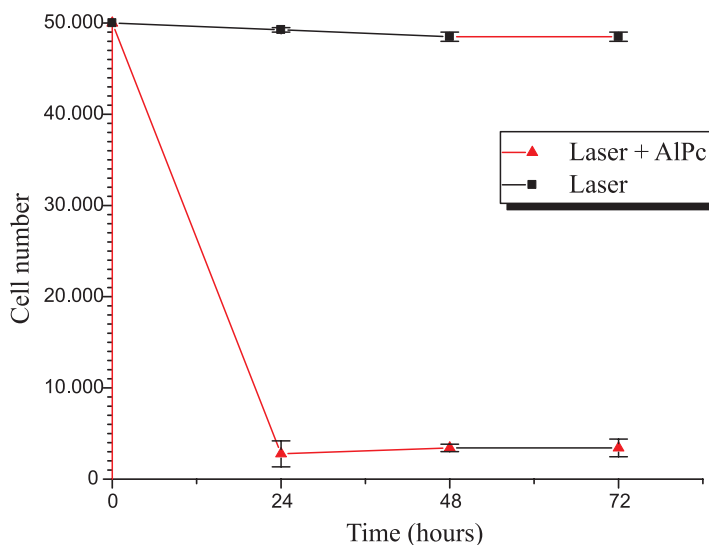


FIGURE 1. Number of living cells (HeLa) after photodynamic treatment with AlPc at different times. A decrease to ~5% on the number of living cells at 24 h after treatment can be observed (mean \pm se, n=4).

Time (h)	Number of cells (Laser treatment)				Mean \pm SE	Number of cells (Laser + AlPc treatment)				Mean \pm SE
0	50000	50000	50000	50000	50000 \pm 0	50000	50000	50000	50000	50000 \pm 0
24	49000	49000	50000	49000	49250 \pm 250	1200	1900	1000	7000	2775 \pm 1421.5
48	47000	49000	49000	49000	48500 \pm 500	4200	3000	4000	2500	3425 \pm 404.9
72	49000	49000	49000	47000	48500 \pm 500	2500	1500	6000	3700	3425 \pm 968.9

Material and Methods

Photosensitizers.

Aluminum phthalocyanine chloride (AlPc) (Sigma, USA) was kindly provided by Msc. Maria Angélica Gargione Cardoso (Univap - São José dos Campos, SP, Brazil) and Aluminum phthalocyanine tetrasulfonate chloride (AlPcS₄) was purchased from Porphyrin Products, INC.

Cell Culture.

Epithelial-like cells derived from Chinese hamster ovary (CHO-K1) (ATCC CCL-61; American Type Culture Collection, Rockville, MD) and cells from a epitheloid carcinoma of human cervix (HeLa) (ATCC CCL-2; American Type Culture Collection, Rockville, MD), were kindly provided by Dr. Técia Maria Ulisses de Carvalho (UFRJ - Rio de Janeiro, RJ, Brazil). These cells were routinely cultured in Ham-F12 and MEM media (GibcoBRL - Grand Island, NY), respectively. They were supplemented with 10% fetal bovine serum

(FBS) (GibcoBRL), 1% antibiotic-antimycotic (Gibco) and kept in a humidified 5% CO₂ atmosphere at 37°C.

Cell phototoxicity after irradiation.

The cells were plated at a number of 5x10⁴ cells/ml in each well of a 24 wells plate (Nunc, Denmark) as follows: six wells for light and photosensitizer and, six wells for light only (control). After 24 h of culture, cells were incubated with 10 μM of AlPc or AlPcS₄ in culture medium without serum for 60 min. Cells were washed twice with phosphate buffered saline (PBS) and 200 μl of fresh PBS was added for irradiation. Dark barriers were placed between wells to avoid scattered light during irradiation. Another dark barrier with an orifice of the diameter of the well, was placed on the top of the 24 wells plate for the same purpose. The irradiation was done in the dark with a semiconductor laser of InGaAlP as active medium (Thera Lase - DMC Equipamentos LTDA, São Carlos, SP, Brazil) operating at 670 nm in a continuous wave mode. An irradiation time of 35 sec was used to deliver 0.5J/cm² (Power = 26 mW). After irradiation, PBS was removed and culture

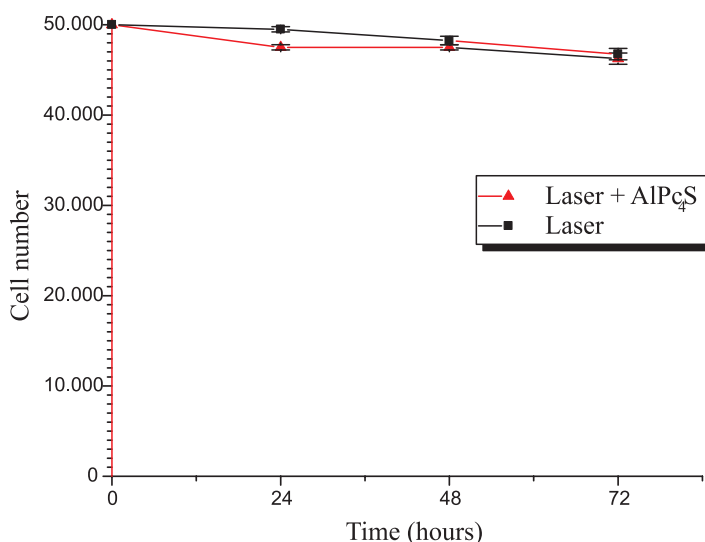


FIGURE 2. Number of living cells (HeLa) after photodynamic treatment with AlPcS₄ at different times. It can be observed a decrease to 78% of living cells within 24 h (mean ± se, n=4).

Time (h)	Number of cells (Laser treatment)				Mean ± SE	Number of cells (Laser + AlPcS ₄ treatment)				Mean ± SE
0	50000	50000	50000	50000	50000±0	50000	50000	50000	50000	50000±0
24	50000	49000	50000	49000	49500±288.7	47000	48000	48000	47000	47500±288.7
48	47000	49000	48000	49000	48250±478.7	48000	47000	47000	48000	47500±288.7
72	47000	47000	48000	45000	46750±629.1	48000	46000	45000	46000	46250±629.1

medium with 10% FBS was added to the cells for 24, 48 and 72 h of culture in a humidified 5% CO₂ at 37°C. After each period of incubation, the number of living cells were counted by the Trypan blue exclusion test.

Photodynamic treatment.

Cells were plated at a number of 5x10⁴ cells/ml in 10 mm diameter Petri dish (Nunc, Denmark). Following an overnight culture, cells were incubated with 10 μM of AIPc or AIPcS₄ in culture medium without serum for 60 min. Following washing and replacement with PBS, cells were irradiated in the dark with a semiconductor laser of InGaAlP as active medium (Thera Lase - DMC Equipamentos LTDA, São Carlos, SP, Brazil) operating at 670 nm in a continuous wave mode. An irradiation time of 183 sec was used to deliver 0.5J/cm² (Power = 26 mW). Control cells were performed without photosensitizer and laser irradiation.

Electron microscopy.

After irradiation, PBS was replaced by medium with serum. After 24 h of culture, cells were washed twice with PBS and fixed with 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in phosphate buffer

0.1M (pH 7.2) for at least 2 h at 4°C. Cells were detached from the dish with a cell scraper, centrifuged three times (1500g, 10 min) with 0.1M fresh phosphate buffer and post fixed in 1% osmium tetroxide in phosphate buffer for 30 min. Finally, cells were washed again and dehydrated in acetone and embedded in Epon. After sectioning, cells were contrasted with uranyl acetate for 30 min and lead citrate for five min. Transmission electron microscopy was performed using a Zeiss 900 and a Zeiss EM10 microscope.

Results

Cell phototoxicity after irradiation.

As can be observed in Fig.1, photodynamic treatment of HeLa cells with AIPc, caused a steep decrease in the number of living cells after 24 h of culture, with damage to approximately 95% of the cell population. Such fall in the number of living cells was not observed after phototreatment of HeLa cells with AIPcS₄ (Fig. 2). In both cases, the control treatments with light only present no reduction of living cells.

The photodynamic treatment of CHO-K1 cells with AIPc present a steeper decrease, reaching approximately

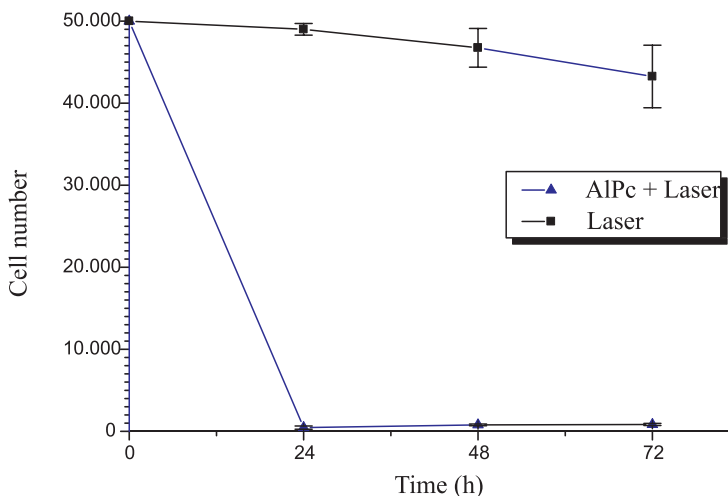


FIGURE 3. Number of living cells (CHO-K1) after photodynamic treatment with AIPc at different times. Within 24 h of incubation, about 99% of cells are dead (mean ± se, n=4).

Time (h)	Number of cells (Laser treatment)				Mean ± SE	Number of cells (Laser + AIPc treatment)				Mean ± SE
0	50000	50000	50000	50000	50000±0	50000	50000	50000	50000	50000±0
24	50000	50000	47000	49000	49000±707.1	0	900	600	300	450±193.6
48	50000	50000	47000	40000	46750±2358.5	750	500	900	1000	787.5±108.7
72	49000	50000	34000	40000	43250±816.1	450	1000	900	1000	837.5±131.3

1% of living cells after 24 h of treatment (Fig. 3). The irradiation of this cell line after AlPcS₄ incubation, caused a low toxic effect (Fig. 4). The effect of this treatment was observed on 10% of the cells population within 24 h (Fig. 4). As expected, no alteration was observed in control CHO-K1 cells.

Electron Microscopy

After photodynamic treatment with AlPc, HeLa cells presented condensed chromatin, cell elongation, cytoplasm condensation and cisterns under the plasma membrane (Fig. 5). Such features were not observed in control cells (Fig. 6) where intact cytoplasm, mitochondria and homogeneous chromatin dispersed in the nucleus were seen. In Figure 7 condensation of chromatin in the periphery of the nucleus (piknotic nucleus), a characteristic feature of apoptosis can be observed. Extensive cytoplasm vacuolization and leakage of the nuclear chromatin can also be observed.

The control group of CHO-K1 cells presents normal mitochondria with evident cristae and intact plasma membrane (Figs. 8 and 9). Cytoplasm condensation, organelle swelling and loss of microvilli, was observed

on CHO-K1 cells that were phototreated with AlPc (Fig.10). The mitochondria of AlPcS₄ phototreated CHO-K1 cells presented lower electron-density when compared to control cells and there was also mitochondrial cristae disruption (Fig. 11).

Discussion

PDT is a very promising form of treatment specially for cancer. Our analysis show that most of the cells from both cell lines died after 24h of PDT. This effect was much more distinct with AlPc than with AlPcS₄. This result confirms previously work by Kolárová *et al.* (1999) performed with MCF7 cell line.

It is known that laser radiation can stimulate cell proliferation, a mechanism dependent on the fluency applied. In this work, the fluency used (0.5 J/cm²) has an inhibitory effect rather than a stimulatory effect according to Al-Watban and Andrés (2000), who observed a bioinhibitory effect of He-Ne laser (632.8) in the region of 300 to 600 mJ/cm² in CHO cells.

It is widely accepted that non-ionized species can cross the plasm membrane more easily than charged

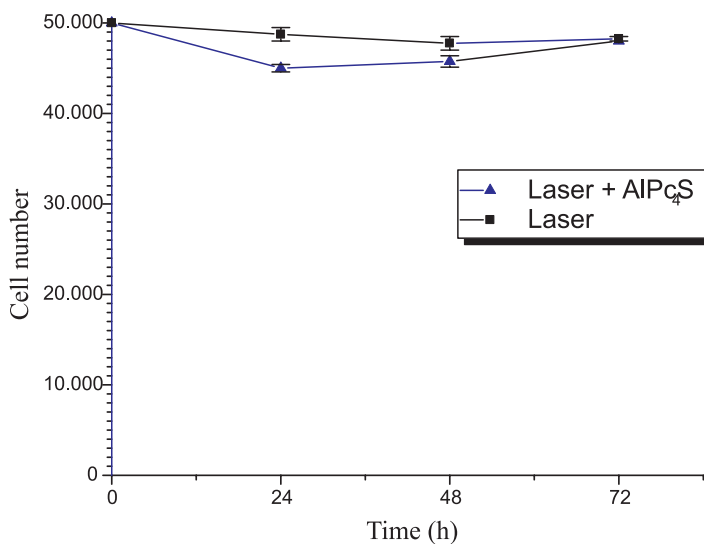


FIGURE 4. Number of living cells (CHO-K1) after photodynamic treatment with AlPcS₄ at different times. Within 24 h it can be observed a decrease on the number of living cells to 90% followed by a subsequent increase on the number of living cells next to control (mean ± se, n=4).

Time (h)	Number of cells (Laser treatment)				Mean ± SE	Number of cells (Laser + AlPcS ₄ treatment)				Mean ± SE
0	50000	50000	50000	50000	50000±0	50000	50000	50000	50000	50000±0
24	50000	50000	47000	48000	48750±750	45000	46000	45000	44000	45000±408.2
48	47000	49000	49000	46000	47750±750	46000	46000	47000	44000	45750±629.1
72	48000	48000	48000	49000	48250±250	48000	48000	48000	48000	48000±0

compounds (Mazière *et al.*, 1991; Pottier and Kennedy, 1990). Thus, AlPcS₄ that is soluble in water (Zhorina *et al.*, 1994) is not capable of penetrating into the cell by means of passive diffusion. It is endocytosed (Roberts and Berns, 1989; Moan and Anholt, 1990) by the cell and therefore localized in endosomes and lysosomes. Many authors consider that the cell uptake of the sensitizer is more efficient for lipophilic photosensitizers due to the better penetration through the cell membranes (Horobin and Radish, 1990).

The ultrastructural morphologic aspect of the HeLa cells phototreated with AlPc, reinforces the results obtained with Trypan blue exclusion test, since, a great number of cells with plasma membrane damage, characteristics of irreversible cellular lesion, and karyorrhexis nucleus were observed. These are characteristics of the cell necrosis process.

Despite the knowledge that AlPc localizes preferentially in the mitochondrial membrane and that after laser irradiation activation of proteins leads to the apoptotic process, the initial localization of the photosensitizer in the plasma membrane and the interac-

tion time in our experiments suggest a process of cell death with necrosis like characteristics. This was probably due to the free radicals generated after PDT, leading to oxidation of the side chains of the amino acid residues that promote proteins cross-linking and oxidation of the protein structure that results in its fragmentation (Bertlett and Stadtman, 1997). The oxidative modification enhances the degradation of critical enzymes by the multicatalytic proteasome complex (Mitch and Goldberg, 1996), devastating the whole cell. The subcellular location of a photosensitizer has a strong influence on whether and to what extent cells undergo apoptosis in response to photoinactivation (Oleinick *et al.*, 2002). Dellinger (1996) describes apoptosis in photoirradiated CV-1 cells after 24 h of Photofrin incubation. When the photosensitizer was incubated for only 1 h, plasma membrane as the primary localization site of Photofrin, necrosis was the predominant form of cell death. Luo *et al.* (1996) suggest that photosensitizers that deviate to membrane *loci* and mediate the membrane photodamage, can evoke a necrotic response rather than an apoptotic response. Meanwhile the sensitizers

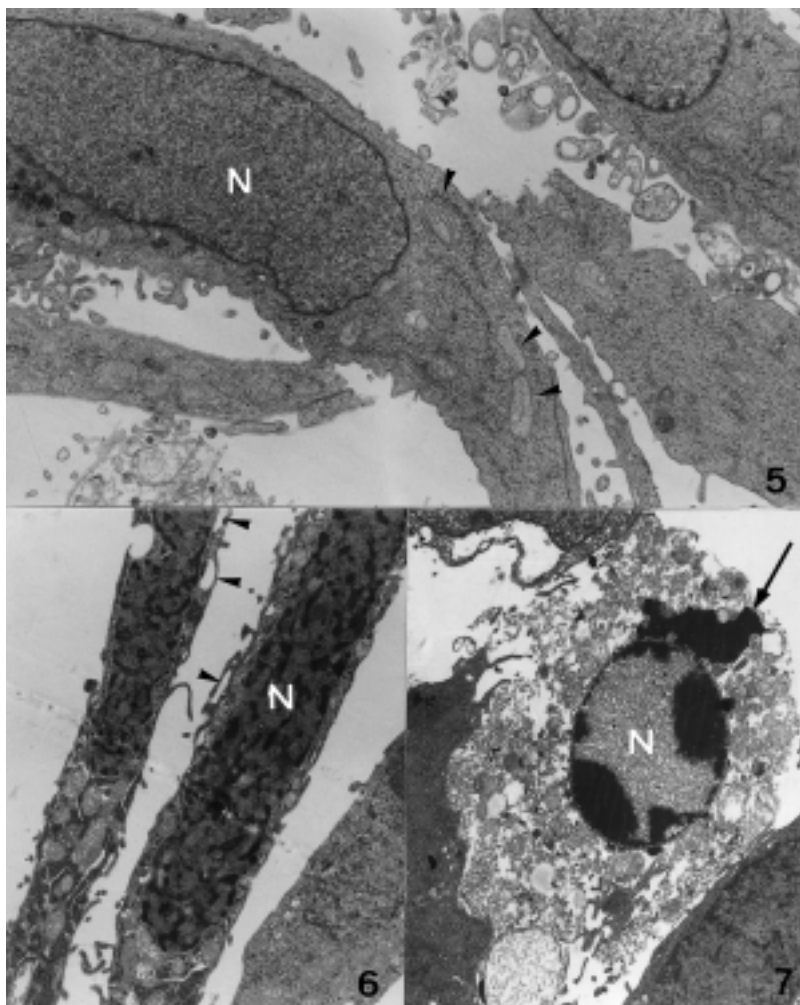


FIGURE 5. Control HeLa cells (no light, no photosensitizer). Nuclear chromatin (N) homogeneously dispersed, intact mitochondrial (arrowhead) and plasma membrane. X 6,670.

FIGURE 6. HeLa cells 24 h after photodynamic treatment with AlPc. The cells presents cisterns below plasma membrane (arrowhead) and random fragmentation of the condensed chromatin (N). X 6,670.

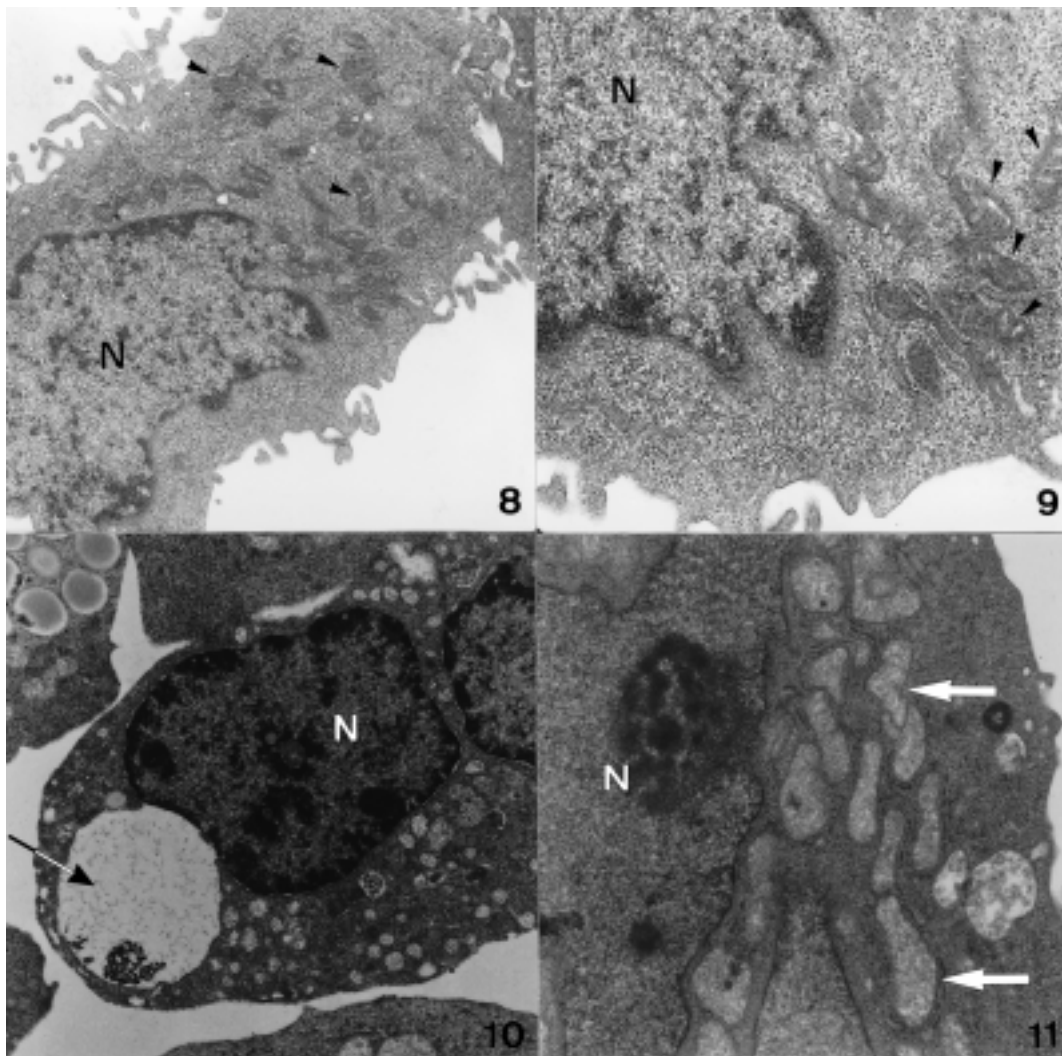
FIGURE 7. HeLa cell 24 h after photodynamic treatment with AlPcS₄. The nucleus presents condensed chromatin at its periphery (N). Extensive cytoplasm vacuolization and leakage of the nuclear chromatin (arrow) can be observed. X 6,670.

that locate in mitochondria like Photofrin[®], are more likely to induce apoptosis during exposure to light. The CHO-K1 cells showed the same plasma membrane integrity observed with the neoplastic cell line. Damage to the plasma and nuclear membrane, and a condensation of the chromatin after the photodynamic treatment with AIPc were seen. In this work, we can observe damage to the membrane in both cell lines, as evidenced with the phototoxicity test with AIPc.

Malignant cells often exhibit resistance to undergo apoptosis, an effect associated with the ability to survive to chemotherapy (Vaux and Strasser, 1996; Hickman *et al.*, 1994). PDT of HeLa cells with AIPcS₄,

shows ultrastructure features that suggest apoptotic cell death. A characteristic of this kind of death is the conservation of membrane integrity.

According to Zhang *et al.* (1998), the relation between the mode of cell death (apoptosis or necrosis) and the dose of PDT, may be dependent mainly on the cell line and the photosensitizer used. As cited, being AIPcS₄ hydrophilic, it is located in endosomes and lysosomes due to its uptake by the cells. This photoactivation would lead to a necrotic cell death. However, our results demonstrate cell apoptosis occurring in the neoplastic cell line. This may be related to the fact that lesion to the lysosomes membrane are followed by enzyme leakage



FIGURES 8-9. Control CHO-K1 cells (no light, no photosensitizer). Intact mitochondrial (arrowhead) and plasmatic membrane. N, nucleus. Fig. 8, X 12,000. Fig. 9, X 30,000.

FIGURE 10. CHO-K1 cell 24 h after photodynamic treatment with AIPc. Swelling of the nuclear membrane cistern (arrow). Chromatin condensation can be also observed in the nucleus (N). X 6,670.

FIGURE 11. CHO-K1 cell 24 h after photodynamic treatment with AIPcS₄. Cell with intact plasmatic membrane but with disruption of mitochondrial cristae (arrows). N, nucleus. X 13,000.

to the cytoplasm. The activation of these enzymes causes enzymatic digestion of cellular components evidenced by nuclear alterations (Cotran *et al.*, 2000) like picnotic nuclei and the characteristic ladder pattern of DNA fragmentation. Such process was confirmed by Arends *et al.* (1990) who propose the concept that apoptosis is marked by internucleosomal cleavage of DNA, a process probably dependent of gene expression and mediated by Ca^{2+} -dependent endonucleases. In the same way, the photosensitizer LuTex has been shown to bind to lysosomes of EMT6 cells *in vitro* and to induce apoptosis upon photoactivation (Woodburn *et al.*, 1997). To further evaluate the role of the subcellular localization of photosensitizers in the killing of V79 cells by PDT, Noodt *et al.* (1999) compared two lipophilic porphyrins (3 - THPP and Photofrin) that localize to intracellular membranes, including mitochondria, and two hydrophilic sulfonated porphyrins (TPPS_{2a} and TPPS₄), that are taken up into lysosomes by endocytosis. PDT with either of the membrane-localizing photosensitizers resulted in increasing numbers of cells becoming apoptotic (TUNEL positive) during the first 12 h, but apoptotic bodies were not observed. In contrast, after photoactivation of the lysosome-localized photosensitizers, apoptotic cells were not detected until after 12 h but extensive fragmentation of the cells into apoptotic bodies was found. These data provide evidence for at least two distinct pathways by which PDT can induce apoptosis.

CHO-K1 cells present a differentiated morphological aspect as compared to HeLa cells, where only mitochondrial alteration is observed. However, maintaining the cell membrane integrity as a whole. This resistance to therapy was explained by Penning *et al.* (1992) who reported that CHO-K1 treated cell survives PDT, due to an increase on Ca^{2+} that may be activating a cell rescue response.

The kinetics of the process depend upon a variety of factors, including the dose or the inducing agent and the ability of different cell types to carry out steps in the process (Oleinick *et al.*, 2002).

Many authors have tested PDT with ALA in several types of normal and neoplastic cells *in vitro* and demonstrated a significant response to the therapy by

malignant cells and a low response by normal cells (Rossi *et al.*, 1996).

The oxygen free radicals partially reduced are highly toxic molecules that cause lesion to cell membranes and other cell constituents (Cotran *et al.*, 2000). Mitochondria and lysosomes have been identified as key components in the induction of apoptosis (Kessel, 1997; Kessel *et al.*, 1997).

In photodynamic therapy not only the response to the treatment but also the mode of cell death expressed as apoptosis or necrosis, will vary according to structural characteristics of the photosensitizer molecule together with the interaction time of the photosensitizer with the cells, yonder the intensity and duration of the stimulus.

We can conclude that the time of cell exposure to the photosensitizer determinates its localization in the cell. The parameters of incubation and radiation used, lead the neoplastic cells phototreated with AlPcS₄ to an apoptosis cell death. Thus, the photodynamic induction of apoptosis by AlPcS₄, render this photosensitizer more advantageous in relation to AlPc for clinic application of photodynamic therapy since, this cell death does not induce acute inflammation in the adjacent tissue. Furthermore, the effects of the therapy in the cell ultrastructure with the parameters used, are more intense in the neoplastic cells than in the normal cell line used in this work indicating higher susceptibility of tumorous cells to PDT.

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