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In vitro effects of photodynamic therapy induced by chloroaluminum phthalocyanine nanoemulsion

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ABSTRACT

Background: The photodynamic therapy (PDT) has been used to treat cancer mainly by inducing oxidative stress. Our aim was to evaluate the effect of PDT and its combination with methoxyamine (MX), a blocker of base excision repair (BER), in cells expressing high levels of the APE1 protein, which is involved in cell oxidative damage response.
Methods: The HeLa and A549 cells were treated for 3 h with chloroaluminum phthalocyanine incorporated into a well-designed nanoemulsion (ClAlPc/NE); and then irradiated by visible light (@670 nm) with doses of 0.1, 0.5 and 1.0 J/cm^2. A simultaneous combination of MX + ClAlPc/NE was performed and then irradiated with the selected dose of 0.5 J/cm^2. The treatments were evaluated in terms of viability, clonogenicity, DNA fragmentation, and cell death mechanism by apoptosis and/or necrosis.
Results: The APE1 protein expression observed was higher in HeLa than in A549. Both cell lines exhibited substantial differences in cell cytotoxicity. The PDT decreased the clonogenicity of HeLa by inducing apoptosis (sub-G1 and annexin detection). Additionaly, the MX potentiates the PDT-effects in HeLa. Otherwise, low cytotoxicity was observed in A549 cells.
Conclusion: The PDT induced apoptosis in high APE1 expressive HeLa cells, and the blockage of BER by MX increased its effects.

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1. Background

The photodynamic therapy (PDT) has become an emerging promise to anticancer therapy [1,2]. The chloroaluminum phthalocyanine (ClAlPc) are molecules excited by visible light (@ 670 nm) which generate reactive oxygen species (ROS) causing oxidative stress [3]. It may be incorporated into a nanoemulsion as a drug delivery system (ClAlPc/NE) and used as photosensitizer in PDT [4]. The oxidative DNA damage is repaired by Base Excision Repair (BER), being its main component, APE1 (apurinic/apyrimidinic endonuclease 1), overexpressed in numerous solid cancers [5]. Methoxyamine (MX) is an organic amine that interrupts BER by tightly interacting with the DNA apurinic/apyrimidinic sites [6], which are processed by APE1.

Thus, this study aimed to compare the responses of cell lines with high APE1 protein basal levels (A549 and HeLa) to PDT exposure, as well as, evaluate the influence of BER blockage (PDT plus MX), in order to potentiate the cytotoxicity of this therapy.

2. Materials and methods

The GM07492 (human fibroblast cells, Coriell Institute for Medical Research); the A549 (lung adenocarcinoma) and HeLa (cervical adenocarcinoma) cells (both from American Type Culture Collection) were cultured in Ham-F12 + DMEM 1:1 medium, 10% fetal bovine serum (Cultilab, Brazil), antibiotics (1%) at 37 °C in a humidified 5% CO_2-incubator.

The nanoemulsion of ClAlPc is type oil-in-water (o/a) obtained by spontaneous emulsification process and quantified as described by Siqueira-Moura et al. [7]. Briefly, the organic phase with acetone was prepared containing natural soy phospholipids and ClAlPc at 55 °C. Subsequently, this organic solution was added into the

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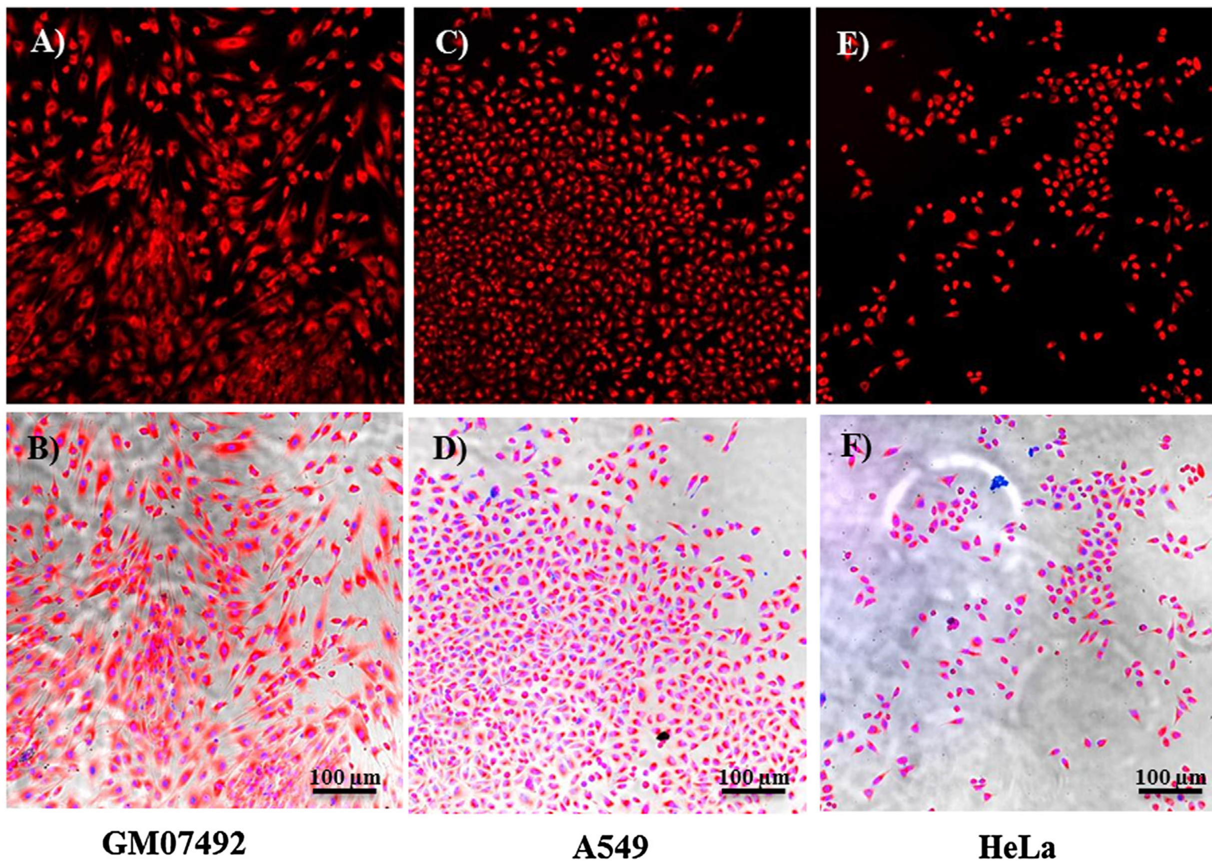

Fig. 1. The ClAlPc nanoemulsion uptake. Fluorescent photomicrographs of GM07492 (A–B), A549 (C–D) and HeLa (E–F) cells exposed to 600 nM of ClAlPc/NE for 3 h. The red fluorescence indicates the ClAlPc presence inside the cells (A1–C1). The overlay with bright field images are depicted in B–D–F. Scale bar: 100 nm.

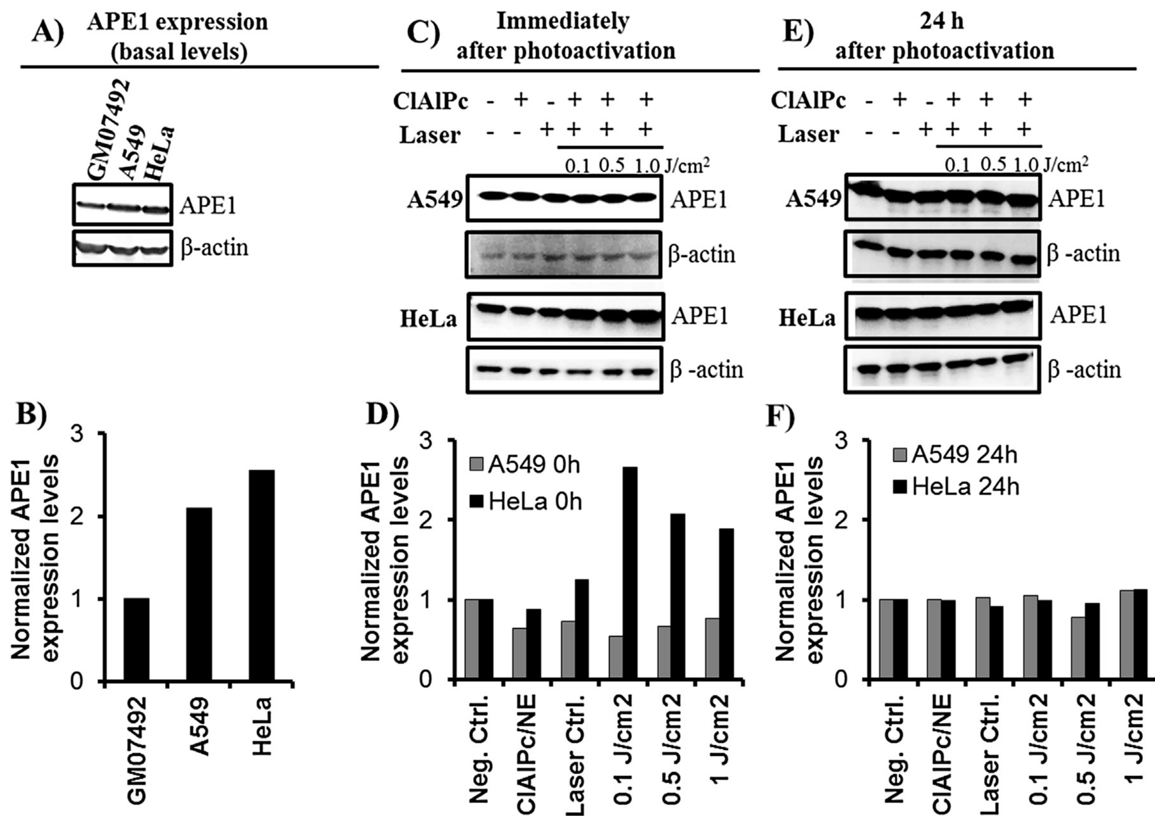

Fig. 2. Protein expression assessed by Western blotting using anti-APE1 (40 kDa – Santa Cruz) and anti-β-actin (42 kDa – Cell Signaling), which was used as endogenous control. The APE1 protein basal levels in GM07492, A549 and HeLa cells (A–B). The modulation of APE1 expression immediately (C–D) and 24 h (E–F) after PDT in A549 and HeLa cells.

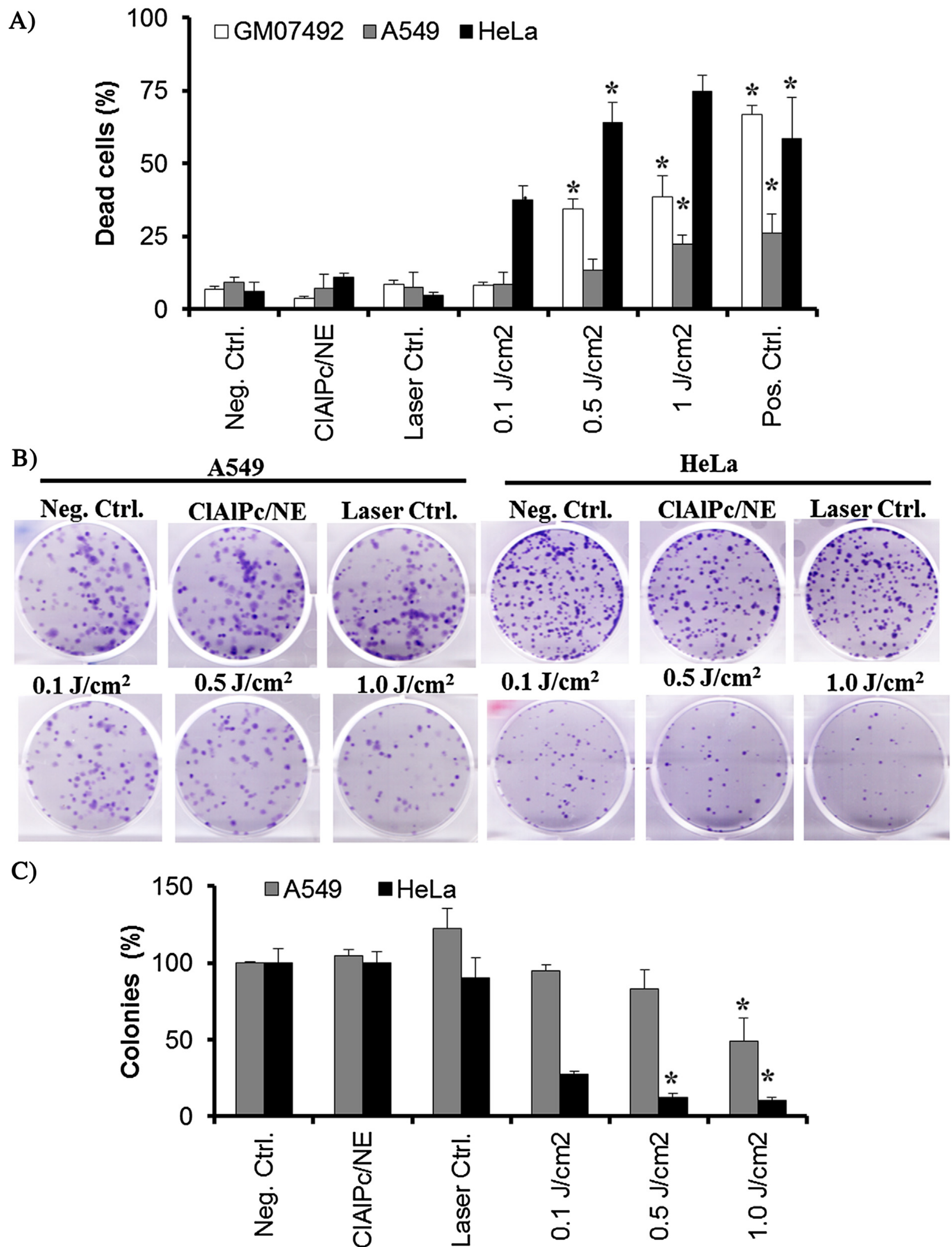

Fig. 3. Cytotoxicity evaluation of PDT: (A) Cell death induction in GM07492, A549 and HeLa cells observed at 24 h after PDT exposure (0.1, 0.5 and 1.0 J/cm^2); Positive control ($KBrO_3$: potassium bromate–20 mM – 3 h-treatment). The assay was performed by using the ViaCount assay. (B) Representative images of the clonogenic assay of A549 and HeLa cells observed 10 days after PDT exposure (0.1, 0.5 and 1.0 J/cm^2) (C). The values represent the mean ± standard deviation obtained in three independent experiments. Data from treated groups were compared to the negative control. Statistical analysis (SigmaStat for Windows v. 3.5, Jandel Corporation) was performed using the analysis of variance (One way ANOVA) test and the *p* value < 0.05 (*) was considered significant using Bonferroni test.

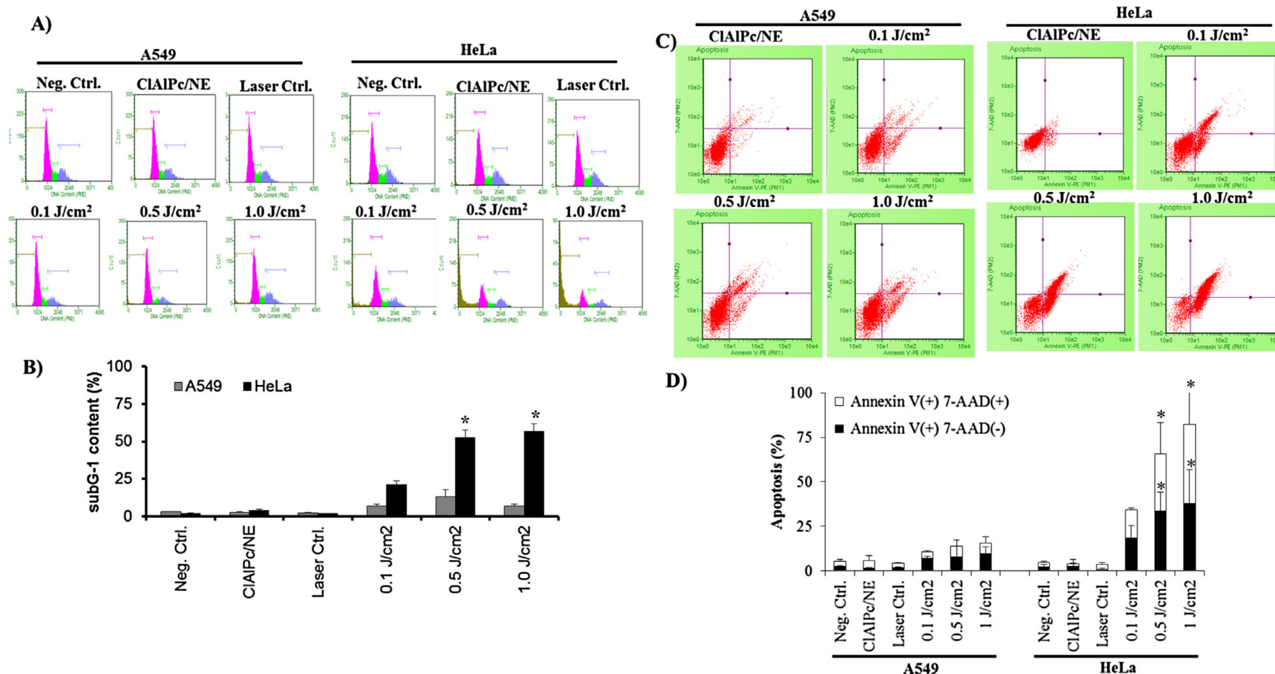

Fig. 4. The sub-G1 content (DNA fragmentation) in A549 and HeLa cells observed 24 h after PDT (0.1, 0.5 and 1.0 J/cm^2): Histogram (A) and percentages (B) of cell populations at sub-G1 fraction. Cell death evaluation in A549 and HeLa cells observed 24 h after PDT exposure (0.1, 0.5 and 1.0 J/cm^2): (C) Dotplot and (D) percentage of apoptotic cells (Annexin-V positive cells: annexin-V-PE (+) 7-AAD(+) –white bars; and annexin-V-PE (+) 7-AAD(−) – black bars). The values represent the mean ± standard deviation obtained in three independent experiments. Data from treated groups were compared to the negative control. Statistical analysis (SigmaStat for Windows v. 3.5, Jandel Corporation) was performed using the analysis of variance (One way ANOVA) test and the p value < 0.05 (*) was considered significant using Bonferroni test.

aqueous phase containing, poloxamer 188 as an anionic surfactant under magnetic stirring. Organic solvent was removed by evaporation under reduced pressure at 60 °C. The volume of the NE was then concentrated to the aqueous phase [4,8].

We used fluorescence microscopy (High Content Screening, Operetta, PerkinElmer) to detect the uptake of ClAlPc/NE identified as a red fluorescence inside the three cell lines studied (600 nM, 3 h-treatment) (Fig. 1). The APE1 protein expression was detected by Western Blot (Life technologies) as an indicative of BER modulation in response to PDT. And GM07492 cells were used for APE1 expression normalization.The cellular responses after ClAlPc photoactivation, with visible light at 0.1, 0.5 and 1.0 J/cm^2 doses using a diode laser at 670 nm (Eagle Quantum Tech, Brazil), were evaluated in terns of: cell death by using the cell ViaCount® assay; the ability of cells to form colonies evaluated by clonogenic assay; DNA fragmentation (sub-G1 phase) measured by propidium iodide staining; and detection of necrotic and apoptotic cell death by using the dyes annexin V-PE and 7-AAD, following the methods described elsewhere [9,10]. The combined treatments were performed simultaneously (3 h-incubation, MX–40 mM plus ClAlPc/NE at 0.5 J/cm^2) (Reagents from Sigma-Aldrich; flow cytometry assays from Merck-Millipore).

3. Results and conclusions

The HeLa and A549 cells were chosen because show high levels of basal APE1 protein. In A549 cells, we detect a 2.10-fold higher APE1 protein levels compared to fibroblasts (GM07492), which did not change after ClAlPc-photostimulation (Fig. 2). After PDT exposure we observed in this cell line that only the highest dose (1.0 J/cm^2; $p < 0.05$) increased cell death (Fig. 3), the clonogenic capacity was reduced only at the highest dose (49% survival rate at 1.0 J/cm^2; $p < 0.05$), and the sub-G1 content (DNA fragmentation) was not increased. In order to distinguish between apoptosis and necrosis, cells were stained with annexin-V/7-AAD. Apoptosis and necrosis were not significantly increased in this cell line.

In relation to HeLa cells, it was detected the highest APE1 protein levels among the cell lines; 2.56-fold compared to GM07492 cells. Moreover, its levels were clearly increased immediately after PDT exposure (2.67, 2.08, and 1.89-fold at 0.1, 0.5 and 1.0 J/cm^2 doses, respectively) (Fig. 2). A dose-dependent cell death induction was observed in HeLa (37, 63, and 74% at 0.1, 0.5, and 1.0 J/cm^2, respectively; $p < 0.05$) (Fig. 3). The clonogenic survival rates were reduced at all doses tested (27, 13, and 10% at 0.1, 0.5, and 1.0 J/cm^2, respectively; $p < 0.05$) (Fig. 3). All doses tested also resulted in sub-G1 accumulation (21, 52, and 57% at 0.1, 0.5 and 1.0 J/cm^2, respectively; $p < 0.05$). Additionally, apoptosis induction was seen as the main cell death pathway in HeLa cells (total annexin-V positive: 65 and 80% at 0.5 and 1.0 J/cm^2; respectively; $p < 0.05$) (Fig. 4).

We also evaluated the BER influence in responses to PDT. The dose 0.5 J/cm^2 was chosen to detect the different responses in the cell lines studied. Moreover, this dose induced ~30% of cell death in GM07492 showing low cytotoxicity to healthy cells (Fig. 3). In A549 cells, only the combination (MX plus PDT) was effective in reducing the clonogenic rate (37%; $p < 0.05$) (Fig. 5), however, not induced cell death (Fig. 5), sub-G1 accumulation and apoptosis/necrosis rates (Fig. 6).

On the other hand, in HeLa cells the clonogenic rates decreased from 12 to 6% in combined treatment. Interesting, the MX tested alone decreased survival rates (33%; $p < 0.05$) showing that BER is an important repair pathway in HeLa cells (Fig. 5). Additionally, the combined treatment increased cell death at 23% (60 vs. 83%; $p < 0.05$) (Fig. 5), elevated the sub-G1 content (46% to 62%; $p < 0.05$), which was confirmed by an increment of apoptosis from 45 to 68% (Fig. 6).

We could observe that PDT did not modify the A549-APE1 protein levels, considering that this cell line was more resistant than HeLa cells, which showed high protein levels after PDT exposure. The NF-E2-related factor 2 (Nrf2), a key transcription regulator for

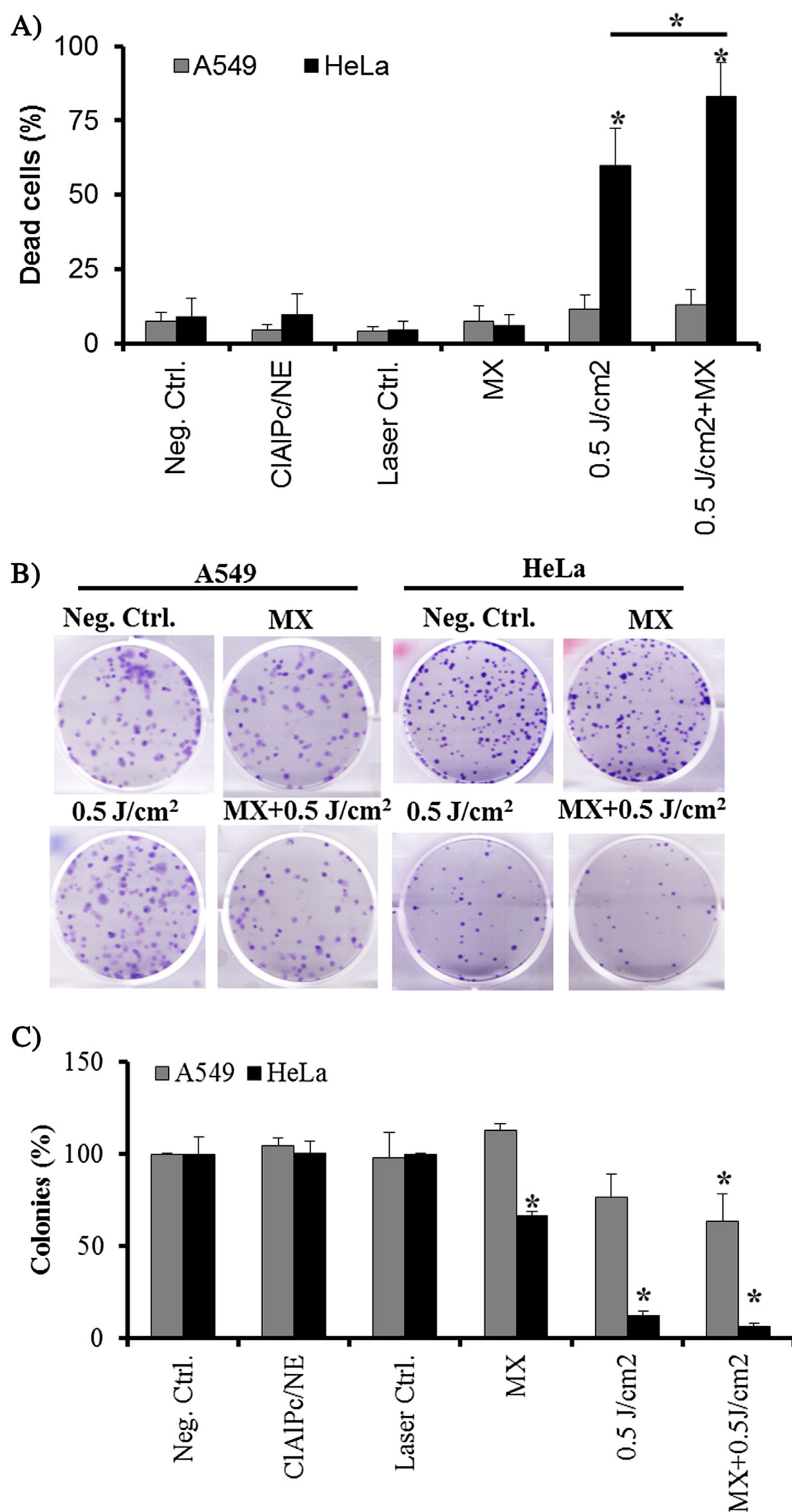

Fig. 5. Cytotoxicity evaluation of combined or isolated treatments (MX–40 mM and ClAlPc/NE at 0.5 J/cm^2): (A) Cell death induction in A549 and HeLa cells observed 24 h after treatments. The assay was performed the ViaCount Reagent. (B) Representative images of the clonogenic assay of A549 and HeLa cells observed 10 days after treatments (C). The values represent the mean ± standard deviation obtained in three independent experiments. Data from treated groups were compared to the negative control; and 0.5 J/cm^2 vs. MX + 0.5 J/cm^2. Statistical analysis (SigmaStat for Windows v. 3.5, Jandel Corporation) was performed using the analysis of variance (One way ANOVA) test and the *p* value < 0.05 (*) was considered significant using Bonferroni test.

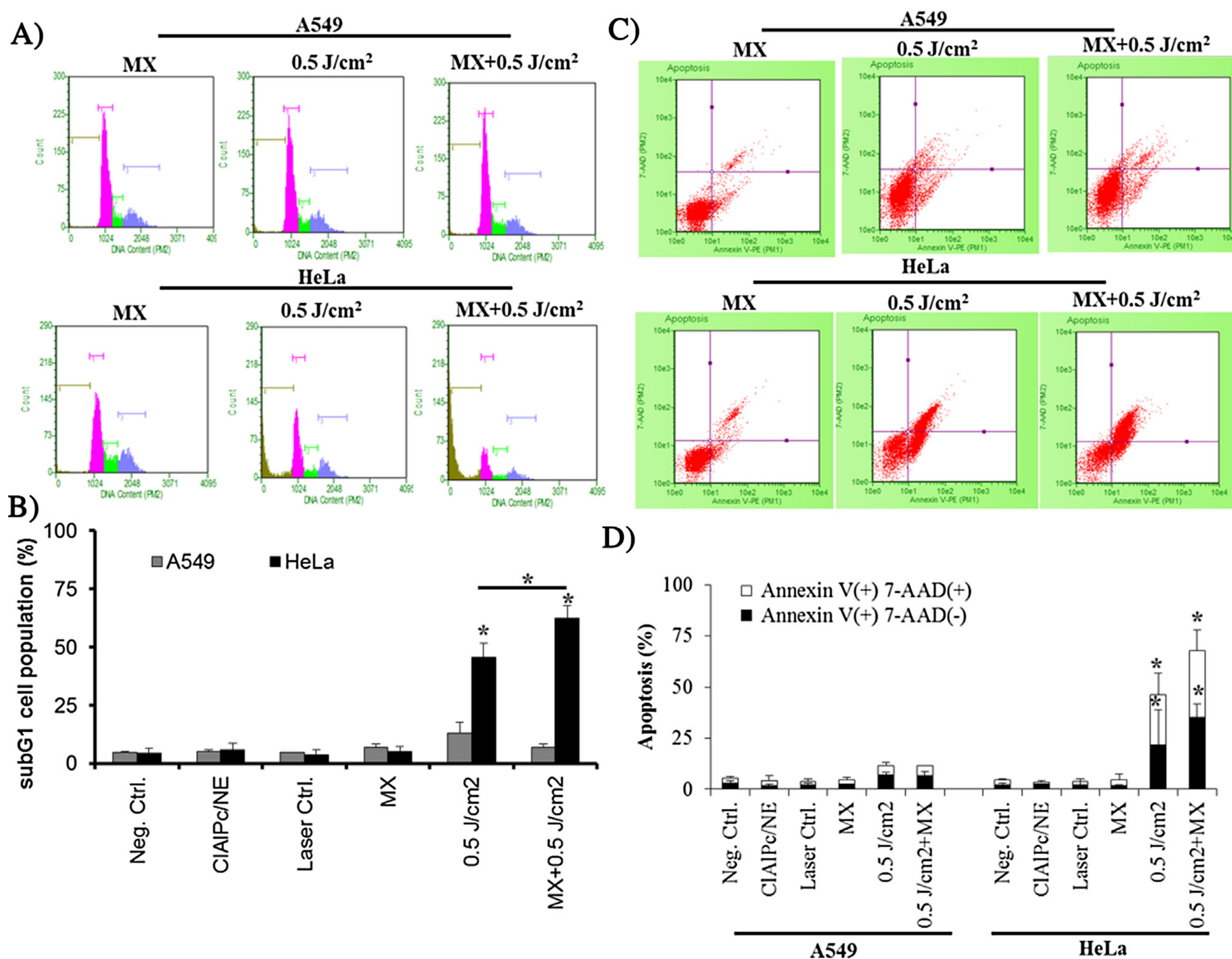

Fig. 6. The sub-G1 content (DNA fragmentation) in A549 and HeLa cells observed 24 h after combined or isolated treatments (MX–40 mM and ClAlPc/NE at 0.5 J/cm^2): Histogram (A) and percentage (B) of cell populations at sub-G1 fraction .Cell death evaluation in A549 and HeLa cells: (C) Dotplot analysis and (D) percentage of apoptotic cells (Annexin-V positive cells: annexin V-PE (+) 7-AAD (+) – white bars; and annexin V-PE (+) 7-AAD (−) – black bars). The values represent the mean ± standard deviation obtained in three independent experiments. Data from treated groups were compared to the negative control; and 0.5 J/cm^2 vs. MX + 0.5 J/cm^2. Statistical analysis (SigmaStat for Windows v. 3.5, Jandel Corporation) was performed using the analysis of variance (One way ANOVA) test and the p value < 0.05 (*) was considered significant using Bonferroni test.

antioxidant and detoxification enzymes, is abundantly expressed in A549 cells and it may be involved in PDT-resistance of this cell line [11].

Nevertheless, MX increased PDT effects in HeLa cells. Therefore, the BER impairment can be considered as a strategy to improve the efficacy of ClAlPc/NE-induced PDT.

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