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Research Article

Assessment of DNA Damage after Photodynamic Therapy Using a Metallophthalocyanine Photosensitizer

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Photodynamic therapy (PDT) is a chemotherapeutic approach that utilizes a bifunctional reagent, a photosensitizer (PS) that localizes to the target tissue relative to the surrounding tissue and is toxic when exposed to laser light. PDT rapidly induces cell death, inflammatory and immune reactions, and damage of the microvasculature. DNA damage results from a variety of factors including UV-light, X-rays, ionizing radiation, toxins, chemicals, or reactive oxygen species. The aim of this study was to determine the effect of PDT as well as the influence of presensitization leading to the adaptive response (AR) on the integrity of DNA. Lung (A549), breast (MCF-7), and esophageal (SNO) cancer cells and Zn sulfophthalocyanine as PS with irradiation conditions of 10 J/cm^2 at 636 nm were used. Subcellular localization of PS, cell morphology, and viability after PDT and DNA damage were determined. A significant decrease in viability and marked DNA damage was observed in all 3 cancer cell types in response to PDT while the adaptive response was demonstrated to significantly decrease the effectiveness of the PDT.

1. Introduction

Worldwide, cancer is undoubtedly a serious and potentially life-threatening illness. As is the case in South Africa and Egypt, worldwide, breast cancer is the most common cause of cancer in women while lung and esophageal cancer accounts for millions of cancer deaths annually [1, 2]. There are multiple types of cancer which can be treated effectively to eliminate, reduce, or slow the impact of the disease on patients' lives.

Phototherapy or laser irradiation is used to treat various diseases and is becoming an increasingly important tool in the management of a number of medical conditions including cancer [3]. Nonthermal damage induced by the interaction of low-power laser light and photosensitizing drugs is known as PDT. It is a developing modality used for the treatment of cancer; it is considered to be minimally invasive and can be applied repeatedly at the same site and has the added advantage that it does not preclude the use of chemotherapy, ionizing radiation, or surgery. This therapy involves the use of a photosensitizing compound that is nontoxic in its inactive form, but is activated by a specific wavelength of light that matches the absorbance spectrum of the PS. These absorbed photons excite the PS molecules to an excited singlet state that lasts for only a short period of time, after which it relaxes via intersystem crossing to a longer lasting triplet state of excitation [4]. In the triplet state, either the molecules are able to exchange electrons or hydrogen atoms with nearby molecules to form reactive oxygen species (ROS), which is known as type I photochemical reactions, or the PS molecules can transfer energy to ground-state molecular oxygen, known as type II photochemical reaction [5]. The longer time the PS molecules are able to spend in this triplet state, the more efficient the PS is considered to be as this allows more time for the transfer of the energy to the target molecules [4]. In type I photochemical reactions, electron transfer results in the formation of superoxide anions which can then be converted to hydroxyl ($^{\bullet}OH^{-}$) radicals. In type II photochemical reactions, singlet molecular oxygen ($^1O_2$) is formed [5]. During PDT, the latter reactions are more prominent, therefore the cytotoxic singlet molecular oxygen is most commonly produced [6]. This resulted oxidative stress is the most likely reason for the higher rate of DNA damage shortly after PDT treatment.

Numerous studies have shown that cells can become refractory to the detrimental effect of ionizing radiation when previously exposed to an adapting dose. This phenomenon has been termed the adaptive response (AR) to ionizing radiation. The AR may severely decrease the efficiency of various medical treatments which involve the use of radiation therapy, such as certain cancer therapies [7], and including PDT.

Indeed, the biggest challenge in effective anticancer therapeutic design and development is to achieve a high degree of antitumor effect with minimal damage to the healthy tissues. The overall objective is to identify relatively small molecules with predilection for molecular target(s) truly specific for malignant cells. It is well established, though poorly understood, that PSs used in conventional PDT exhibit a higher degree of selectivity for tumor tissues, though not entirely innocuous to normal tissues [8]. The selectivity is achieved by an increased PS accumulation within the tumor as compared to normal tissues and by the fact that illumination is limited to a specified location. Several possible mechanisms of selective PS retention within tumors include greater proliferative rates of neoplastic cells, a lack of or poor lymphatic drainage, high expression of low-density lipoprotein (LDL) receptors on tumor cells (many PSs bind to LDL), low pH (which facilitates cellular uptake), increased vascular permeability, abnormal structure of tumor stroma characterized by large interstitial space and increased production of collagen that binds porphyrins, or tumor infiltration by macrophages that are efficient traps for hydrophobic PSs [9]. The selectivity can further be enhanced by binding of PSs to molecular delivery systems such as growth factors or monoclonal antibodies that are characterized by high affinity for target tissues [10].

The recently developed Single-Cell Gel Electrophoresis (SCGE) assay, or what is known as the comet assay, is regarded as a sensitive method for detecting DNA damage [11]. The major advantage of the comet assay over other methods of measuring DNA damage, such as pulse-field gel electrophoresis, step-graded electrophoresis, and alkaline filter elution, is that information is gained about the distribution of DNA damage and/or repair among individual cells within a cell population, providing an intercellular distribution of damage. In addition, only a small number of cells are required, allowing examination of a large number of experimental conditions from a single-treated population of cells. Moreover, the use of selective enzymes can produce a greater sensitivity model of a comet assay and in addition a more specific assay can be developed. The aim of this study was evaluating cell morphology, proliferation, viability, and DNA damage after photosensitization of three cancer cell lines using Zn phthalocyanine PS. In addition the effect of presensitization with an increased irradiation dose prior to PDT was determined using the same parameters in order to identify a possible AR.

2. Materials and Methods

Five experimental groups, namely, control cells (Control), cells irradiated with laser only (Laser), cells treated with PS alone (Photosensitizer), lung, breast, and esophageal cells treated with PDT (PDT), and finally the three cell types preexposed to laser irradiation of 15 J/cm^2 before PDT (AR), were used.

2.1. Cell Culture. Human lung (A549), breast (MCF-7), and esophageal (SNO) cancer cell lines were used in this study. Cells were grown in 25–30 mL Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Celtic Molecular Diagnostics, Cape town, RSA) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen, Celtic Molecular Diagnostics, Cape town, RSA), 1% Streptomycin/Penicillin/Fungizone (Pen/Strep/Fungizone; Scientific Group, Midrand, RSA), and 1% Gentamycin (Scientific group) in T75 cell-culture flasks fitted with vented caps (Scientific Group). This supplemented DMEM was used throughout for all further cell culture. The cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere and subcultured twice a week. The cells were sub-cultured by discarding the spent media and washing the cells twice in 10 mL Hanks Balanced Solution (HBSS; pH 7.4; Invitrogen, Celtic Molecular Diagnostics, Capetown, RSA) warmed to 37°C. Three mL Tryple Express (Invitrogen, Celtic Molecular Diagnostics, Capetown, RSA) warmed to 37°C was added to the cells and incubated at 37°C, 5% CO_2 for 5 minutes. The Trypan blue (Sigma, St. Louis, MO, USA) exclusion test was used to conduct a cell count in order to determine the number of live and dead cells as well as the cell concentration and cell number. A sample of cells was diluted ten times with 0.4% Trypan blue (dissolved in saline solution) and counted on a haemocytometer (Merck, Darmstadt, Germany) using an Olympus light microscope.

2.2. Preparation of Cells for Assays. The spent media were discarded, and the cells were trypsinized. Ten mL of media was added to the flask and the cell suspension was transferred to a 50 mL Falcon tube, which was centrifuged at 2,200 rpm for 4 minutes. Following centrifugation, the supernatant was discarded and the pellet resuspended in 1 mL media. A cell count was done using the Trypan blue test and the concentration of cells per mL was determined, approximately 2×10^5 A549 and SNO cells, while 6×10^5 MCF-7 cells in 3 mL culture medium were seeded into 3.3 cm diameter culture dishes and incubated for 4 hours to allow cells to attach.

2.3. Addition of Photosensitizer. A Zn sulfophthalocyanine photosensitizer was used in this study. It was synthesized at Rhodes University, South Africa and kindly provided by Professor Tebello Nyokong. Zinc is incorporated into the macrocycle of the PS, and sulfur groups were added [12]. After 4 hours of incubation, culture media was removed and 1 mL fresh culture medium was added to each culture dish. The PS at concentrations of 10 μM, 8 μM, and 2 μM were added to A549, SNO, and MCF-7 culture dishes, respectively for the PS and the PDT experimental groups. Afterwards, the culture dishes were incubated at 37°C with 5% CO_2 and 85% humidity for 24 hours. All the culture plates were covered with aluminum foil as the PS is light sensitive. These concentrations of the PS were chosen according to the lowest

concentration that yielded 50% cell viability in order to establish cell death induction potential, but still be able to determine cellular response.

2.4. Laser Irradiation. After 24 hours of cell incubation, only laser, PDT, and AR experimental group cells were irradiated using a diode laser (LTIO00-PLT20) obtained from the National Laser Center, South Africa, with a wavelength of 636 nm and an output power of 60 mW. To obtain fluences of 10 J/cm^2 and 15 J/cm^2, an irradiation time was calculated according to the following equations:

$$\text{Power density } (\text{mW/cm}^2) = \frac{\text{Power (mW)}}{\text{Area (cm}^2)},$$
$$\text{Work } (\text{mW/cm}^2) = \frac{\text{Energy (J)}}{\text{Time (s)}}. \quad (1)$$

Culture dishes were incubated for a further 24 hours at 37°C with 5% CO_2 and 85% humidity.

2.5. Cell Morphology and Cell Viability. Changes in cellular morphology were observed using an inverted microscope, and images were taken immediately using an Olympus digital camera. The Trypan blue exclusion test was used to estimate the proportion of viable cells in a population based on the fact that viable cells do not take up the dye and remain clear since the chromophore of the dye is negatively charged and does not react with cells unless there is damage to the membranes (nonviable cells). The viability concentration is determined by the following equation:

$$\% \text{ Viability} = \left(\frac{\text{Number of viable cells}}{\text{Total cell number}}\right) \times 100. \quad (2)$$

2.6. Live Imaging. For subcellular localization of the PS, cells were grown on glass coverslips in 3.5 mm Petri dishes. Only subconfluent and well-spread cells were used. The cells were incubated with the PS for four hours prior to laser irradiation. The coverslips were washed with HBSS and incubated in the same medium for 24 hours at 37°C. After cell treatment, mitochondria were stained by incubating the cells with green MitoTracker for 15 minutes using a working concentration of 50 nM. The cell-permeant MitoTracker probes (Invitrogen, Celtic Molecular Diagnostics, Capetown, RSA) contain a mildly thiol-reactive chloromethyl moiety. For staining endoplasmic reticulum (ER), cells were incubated for approximately 15 minutes at 37°C with 100 nM ER tracker Blue-White Dapoxyle dye (DBX; Invitrogen, Celtic Molecular Diagnostics, Capetown, RSA). DPX dyes have long emission wavelengths and high extinction coefficients with working concentrations of 100 nM. For lysosomal staining, the cells were incubated with the LysoTracker probes (Invitrogen, Celtic Molecular Diagnostics, Capetown, RSA), by working concentrations of 75 nM for 15 minutes, which consist of a fluorophore linked to a weak base that is only partially protonated at neutral pH, freely permeant to cell membranes and typically concentrate in spherical organelles. Finally, the cell plasma membranes were stained by incubating the cells with wheat germ agglutinin conjugate with working concentration of 5.0 μg/mL for 10 minutes at 37°C. Acidic organelles were stained with a solution of acridine orange in normal medium for 5 minutes. The stained cells were fixed with 4% formaldehyde for 10–20 minutes at 37°C. Prior to mounting, two 5-minute washes in Phosphate Buffered Saline (PBS) were performed. Coverslips were mounted on microscope slides for viewing on a Carl Zeiss Axio Z1 Observer fluorescence inverted microscope with 20x objective lens using the appropriate fluorescence emission filters (38 HE GFP, 43 HE DS Red, and 49 DAPI). Data acquisition, processing, and analysis were performed with AxioVision software.

2.7. Comet Assay. The comet assay determines the percentage DNA damage [13]. Cells were embedded in 1% low melting temperature agarose on microscope slides and lysed for 1 hour at 4°C with 1% Triton X-100; 0.1 M EDTA; 10 mM Tris and 2.5 M NaCl pH 10, leaving the DNA stripped of protein, as nucleoids. DNA, being supercoiled, resists electrophoresis, however, breaks in loops of DNA relax supercoils in those loops, which are then free to migrate. They form a "tail" to the nucleoid "head" (hence the designation of "comet"), and the percentage of DNA that moves into the tail reflects quantitatively the frequency of DNA breaks [14]. The assay for DNA breaks is of limited use in looking for endogenous oxidative damage to DNA, since breaks can arise in a variety of ways unrelated to oxidation, and furthermore breaks are quite rapidly repaired by cells. This led to the introduction of a modification to the assay by incorporating a step, between lysis and electrophoresis, at which the DNA is digested with a bacterial repair endonuclease specific for certain kinds of DNA lesions [15]. Endonuclease III nicks DNA at sites of oxidised pyrimidines; formamidopyrimidine glycosylase (FPG) recognizes altered purines including 8-oxo-guanine and nicks the DNA alongside [16]. By estimating the percentage of DNA that moves into the tail in the presence of one of these enzymes, the amount of oxidative base damage in a sample of cells could be estimated. Enzyme digestion (FPG: 1 : 3000 for 30 minutes and EndoIII: 1 : 1000 for 45 minutes at 37°C in enzyme reaction buffer of 40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA and 0.2 mg/mL BSA; pH 8) was performed after rinsing the DNA 3 times in enzyme buffer for 5 minutes at 4°C. The slides were placed in alkaline solution (0.3 M NaOH; 1 mM EDTA) for 40 minutes at 4°C prior to electrophoresis at 25 V for 30 minutes at 4°C. Neutralization was performed in 0.4 M Tris at pH 7.5. After staining with 1 μg/mL 4′,6-Diamidino-2-Phenylindole (DAPI; a fluorescent dye which binds to DNA), relative tail intensity was assessed visually, by sorting 100 comets into classes from 0 (no detectable tail) to 4 (large tail, minimal head), giving an overall damage score of 0–400. Steady-state level represents the balance between input and repair, both of which are presumably continuously occurring.

2.8. Statistical Analysis. All experiments were independently performed three times, and differences between the assay mean values were tested using one-way ANOVA to estimate

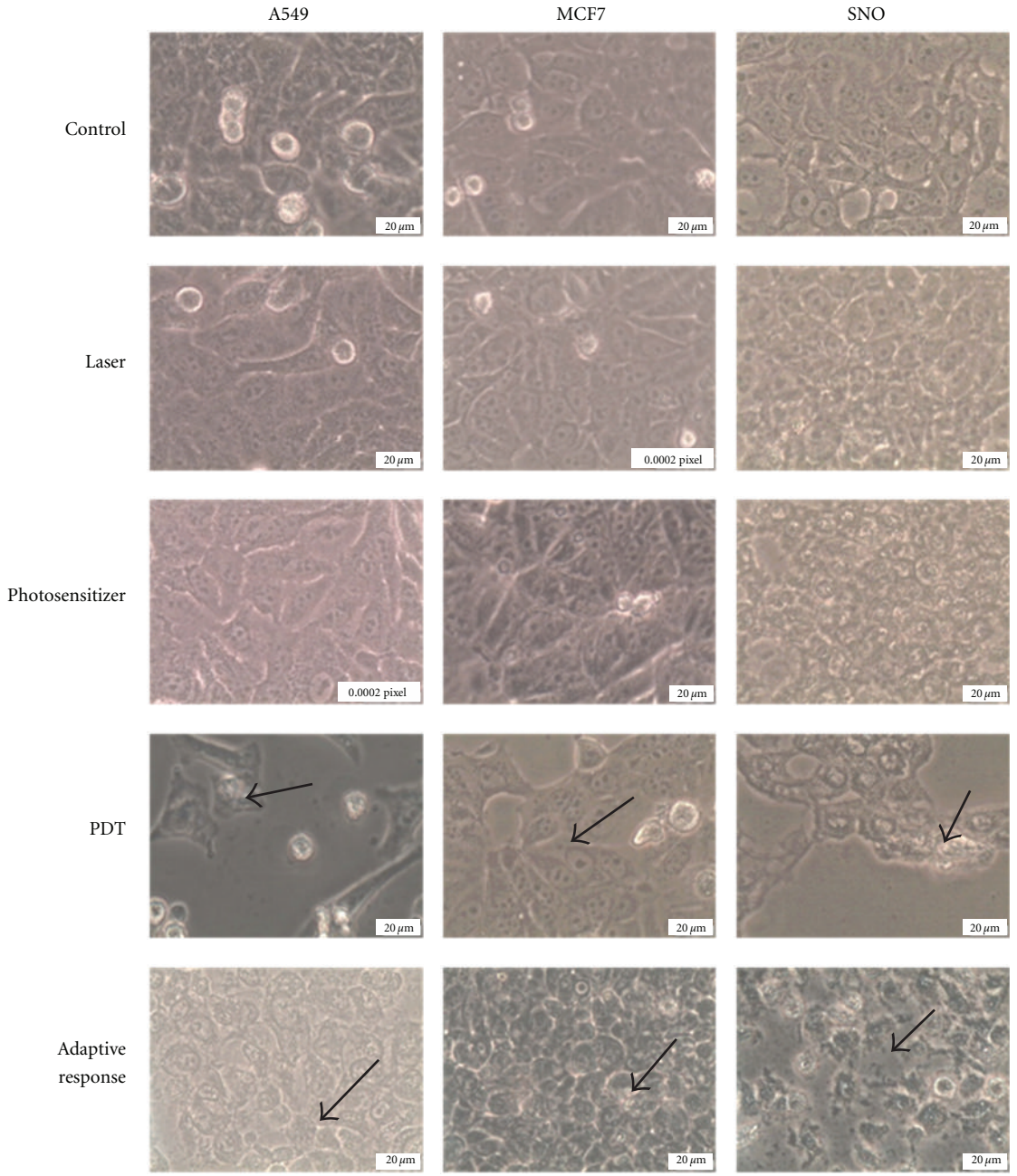


Figure 1: Human lung cancer cells (A549), Human breast cancer cell (MCF7) and Human esophageal cancer cells (SNO) under light microscope with 40X magnification showing a dramatic reduction of the cell viability in PDT groups and a less dramatic effect on cell viability in cells pre-sensitized. This is also visible when studying the morphology of the cells where PDT treated cells show cells rounding and releasing from the flask indicative of cell death.

overall significance. A probability level of 5% ($P < 0.05$) was considered significant.

3. Results and Discussion

The trypan blue exclusion test showed a decrease in cell viability in PDT and AR experimental groups for all three cell lines. In addition, these two groups showed significant morphological changes, respective to the other groups (Figure 1).

Figure 2 shows that PDT can effectively reduce the viability of all three cancer cell types (A549, MCF7 and SNO) compared to the control group that received no PS or irradiation ($P < 0.001$, $P < 0.005$, $P < 0.005$). Moreover, the AR group showed a significant reduction in cell viability compared to the control group ($P < 0.001$, $P < 0.005$, $P < 0.001$) for the same three cancer cell types respectively. Although this reduction in viability in the AR group is less than that observed in the PDT group, when compared to the PDT group there was

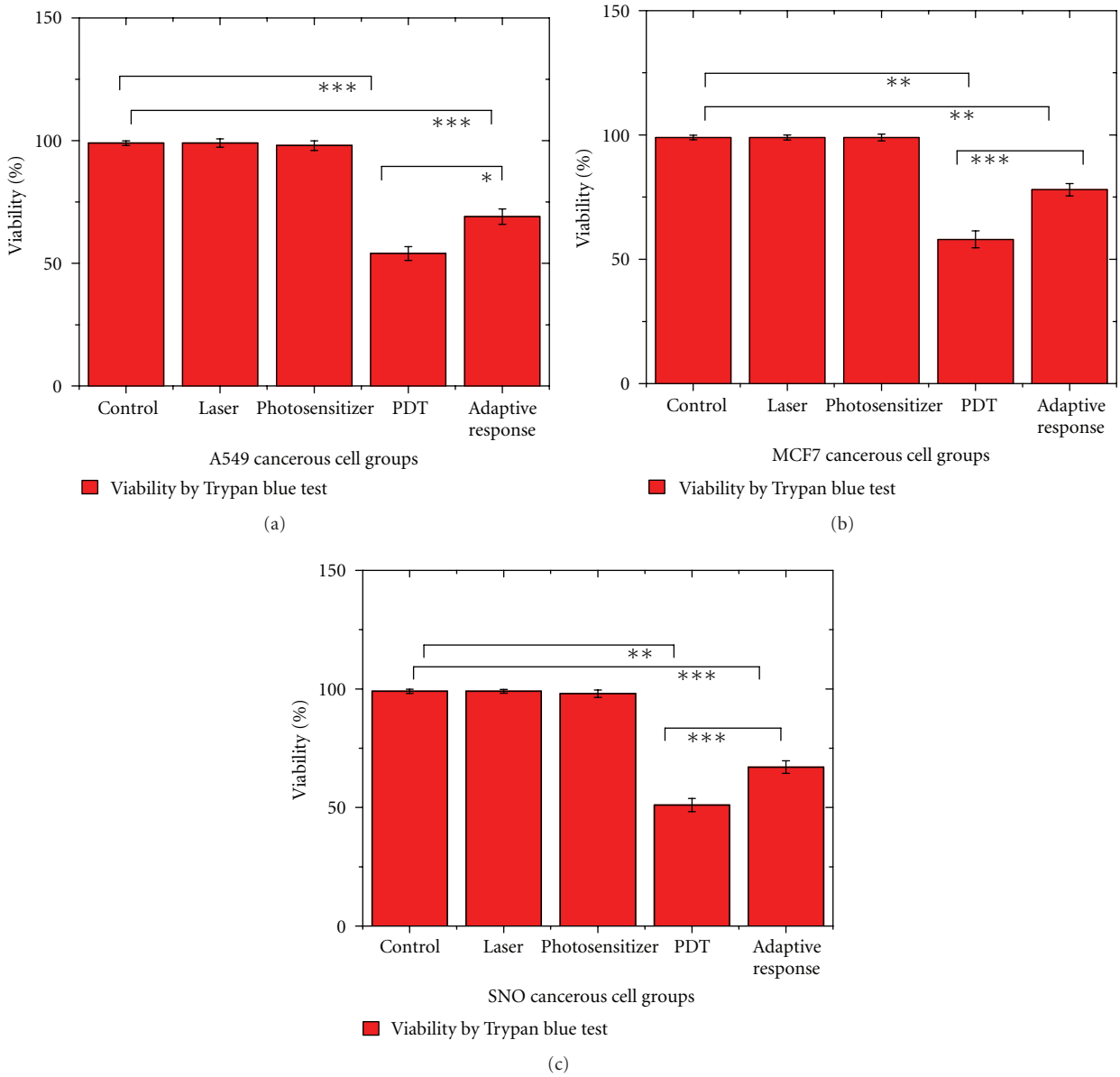
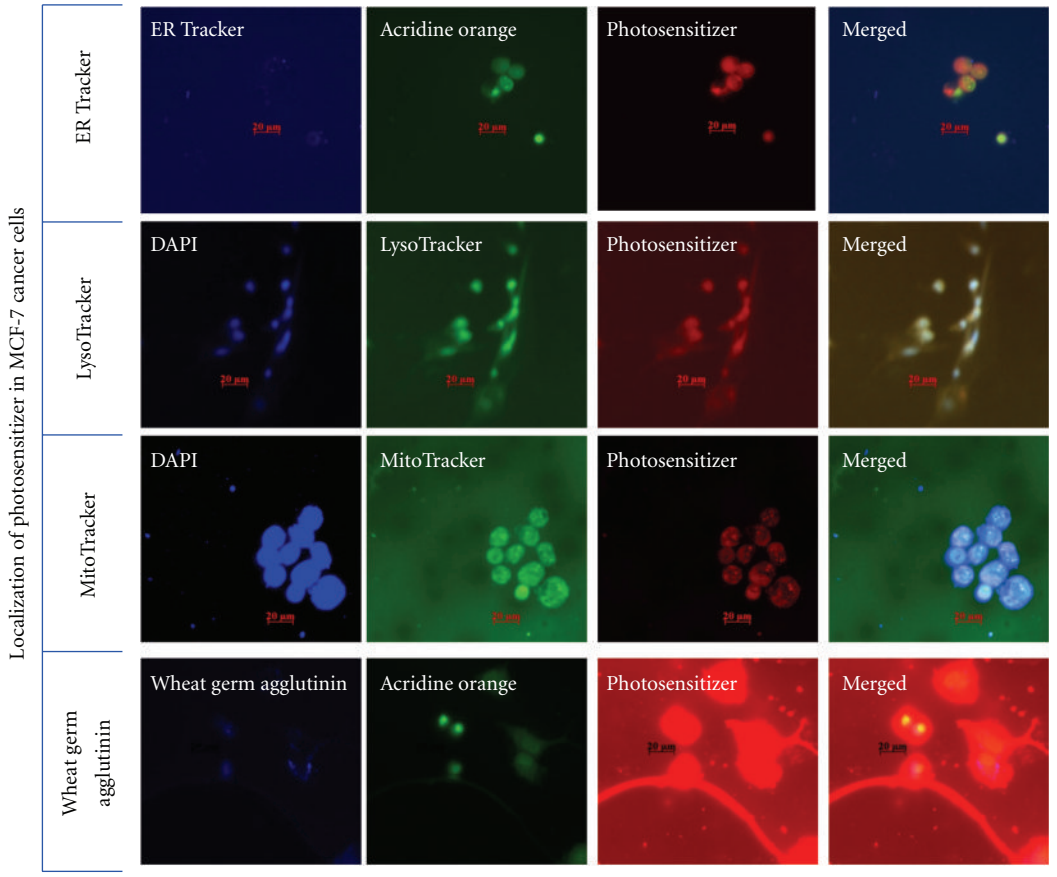


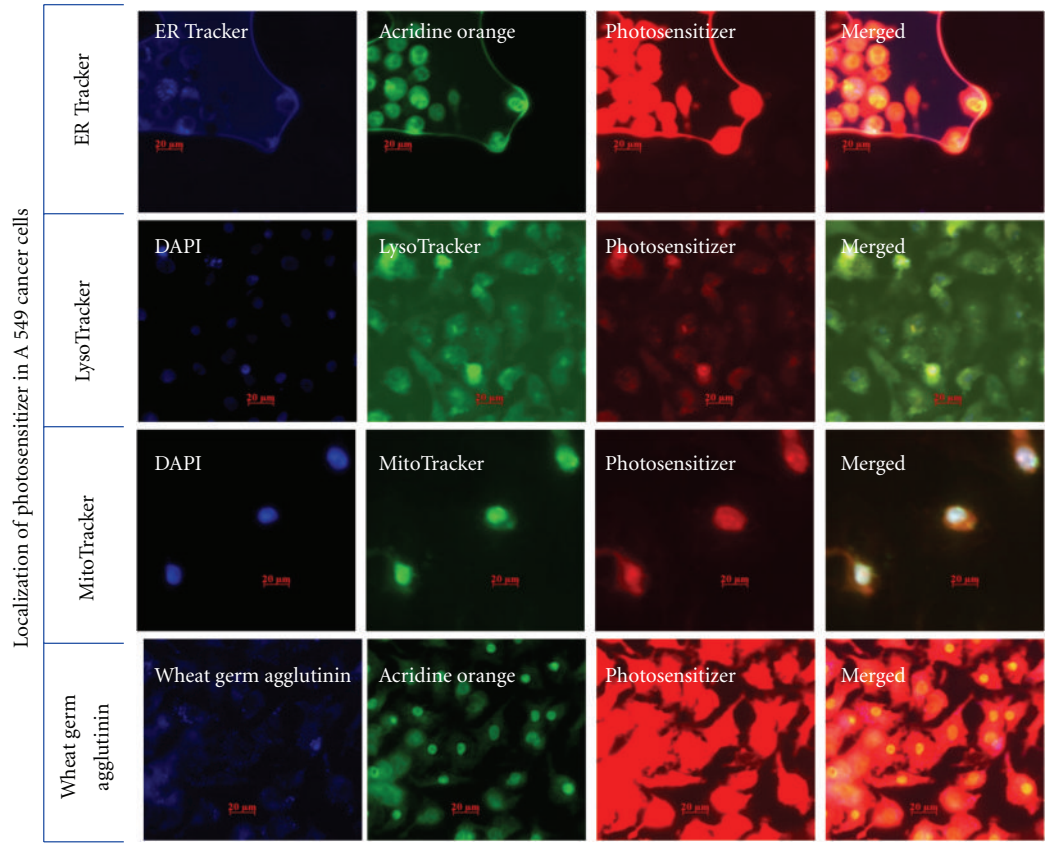
Figure 2: Cell viability expressed as a percentage for the five groups. (a) A549; (b) MCF7; (c) SNO cancer cells. In all three cells types the cells showed a significant decrease in viability after PDT treatment while cells that were pre-sensitized displayed an increase in viability compared to the PDT-treated cells. (***$P < 0.001$; **$P < 0.005$; *$P < 0.01$).

a significant difference between these two groups ($P < 0.01$, $P < 0.001$, $P < 0.001$) for A549, MCF7, and SNO cancer cells, respectively. Since the AR occurs when cells are pre-exposed to low-dose irradiation it can be described as a cellular response which may be expressed as gene mutation, chromosome aberration, and cell survival. The DNA repair ability of these cells increases, and they have higher antioxidant activity [17]. Wang (2004) suggested that some proactive proteins such as Hsp27 are induced in such cells which are resistant to PDT [18].

It was very important in the current research to investigate the localization of the PS inside the cancer cells. Figure 3 shows that the PS has been dispersed, and localized in the cells passing through the plasma membranes and in particular the ER, lysosomes and mitochondria. At a cellular level, lysosomes are the cell's first line of defense. These organelles act as the disposal system of the cell. These organelles are responsible for digesting the macromolecules that pose a threat to the cell by phagocytosis. An interesting characteristic of lysosomal function is autophagy. Autophagic cell death is a catabolic process that involves degradation of a cells' components through the lysosomal machinery leading to cell death, or autolysis. Using organelle-specific fluorophore staining, the localization of the metallophthalocyanine PS was primarily to mitochondria in all three cancer cell lines. It has been reported that most



(a)



(b)

Figure 3: Continued.

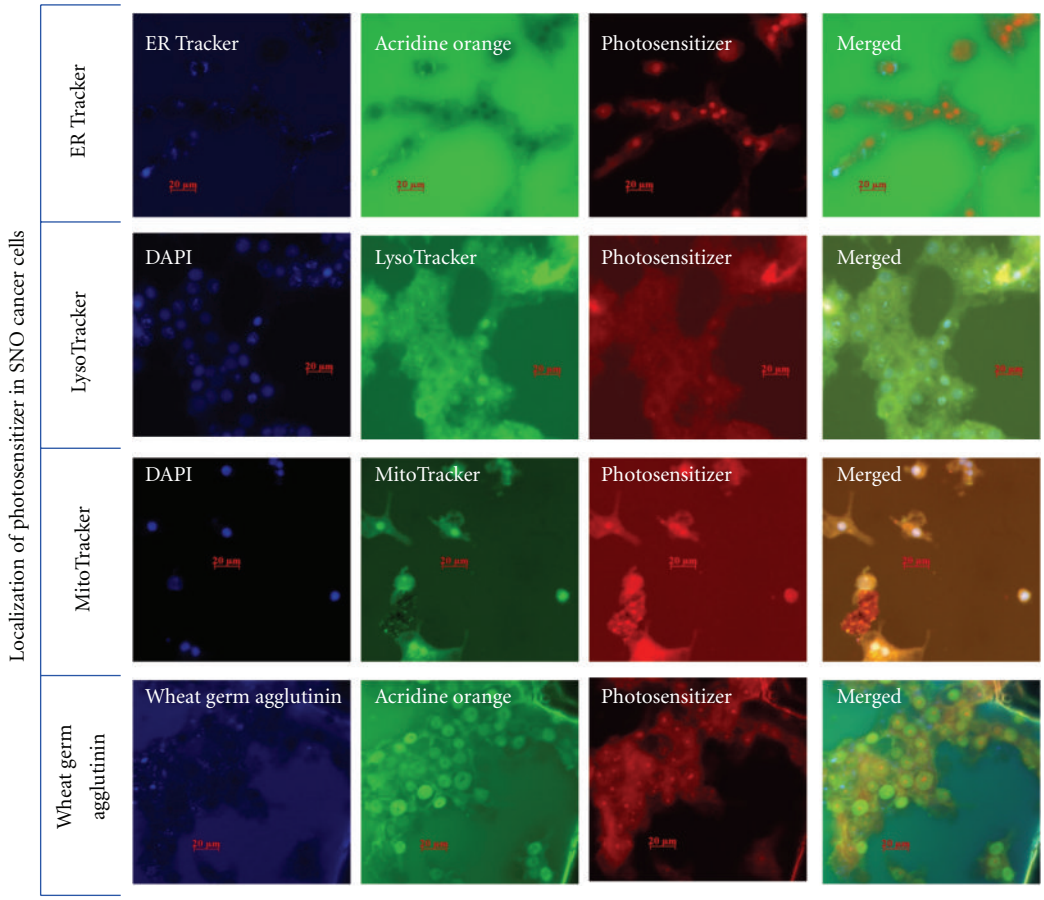


Figure 3: Live imaging of the PS in (a) A549, (b) MCF7, and (c) SNO cancer cells after PDT treatment with staining the ER, lysosomes, mitochondria, and plasma membrane, showing the intrusion of the PS through the plasma membrane and its localization in different cell organelles inside the cancerous cell groups.

ROS generation during PDT occurs within mitochondria and is mitochondrial function independent. This could be followed by inner membrane permeabilization with mitochondrial depolarization and swelling, which in turn can lead to cytochrome c release and apoptotic cell death. Intracellular ROS generated by PDT results in an increase in intracellular Ca^{2+} level, which would then activate the mitochondrial nitric oxide synthase (mtNOS) and endothelial cell nitric oxide synthase (eNOS) to produce nitric oxide (NO). The role of NO in apoptosis induced by PDT is complex and dualistic, as it is well known that NO-mediated apoptotic effects are modulated by different mechanisms in different cell types. PS accumulation in lysosomes and subsequent PDT can cause rapid disintegration of lysosomes. It is postulated that chelatable iron released from lysosomes during PDT contributes to mitochondrial damage and subsequent cell death. The reactions of $^1O_2$ and free radicals with unsaturated lipids and proteins in the membrane may directly cause alterations of membrane function.

The SCGE assay showed remarkable DNA damage in the PDT and AR groups for the three cancer cell lines. The mechanisms of DNA damage induced by PDT are not well understood. PDT can cause base oxidation, cross-linking of DNA strands or sister chromatid exchange [19, 20]. Two aspects of DNA damage in response to PDT should be distinguished: first, what kind of DNA damage PDT inflicts on tumor cells and how tumor cells deal with PDT-induced lesions and the second, the possible DNA damage to surrounding normal cells. PDT generates either ROS—such as hydroxyl ion, hydrogen peroxide, and superoxide through the PS—or singlet oxygen. The latter has a very limited range ($<0.1\,\mu$m) and life-span (less than 1 second), so the probability of singlet oxygen-induced DNA damage is low, unless it is generated in close proximity to a DNA strand. On the other hand, ROS can cause oxidative damage to DNA and are believed to be the major endogenous toxic agents, although at the physiological level they play a vital role in several cellular processes. However, several studies pointed to ROS-induced damage to cytoplasmic proteins and mitochondria, rather than specific DNA damage, as a cause of cell death after PDT. Durmuş and Nyokong (2007) proved that cytotoxic singlet oxygen, which is produced by the activation of the PS during PDT, may have directly damaged the DNA as oxidative stress causes single-strand breaks (SSBs)

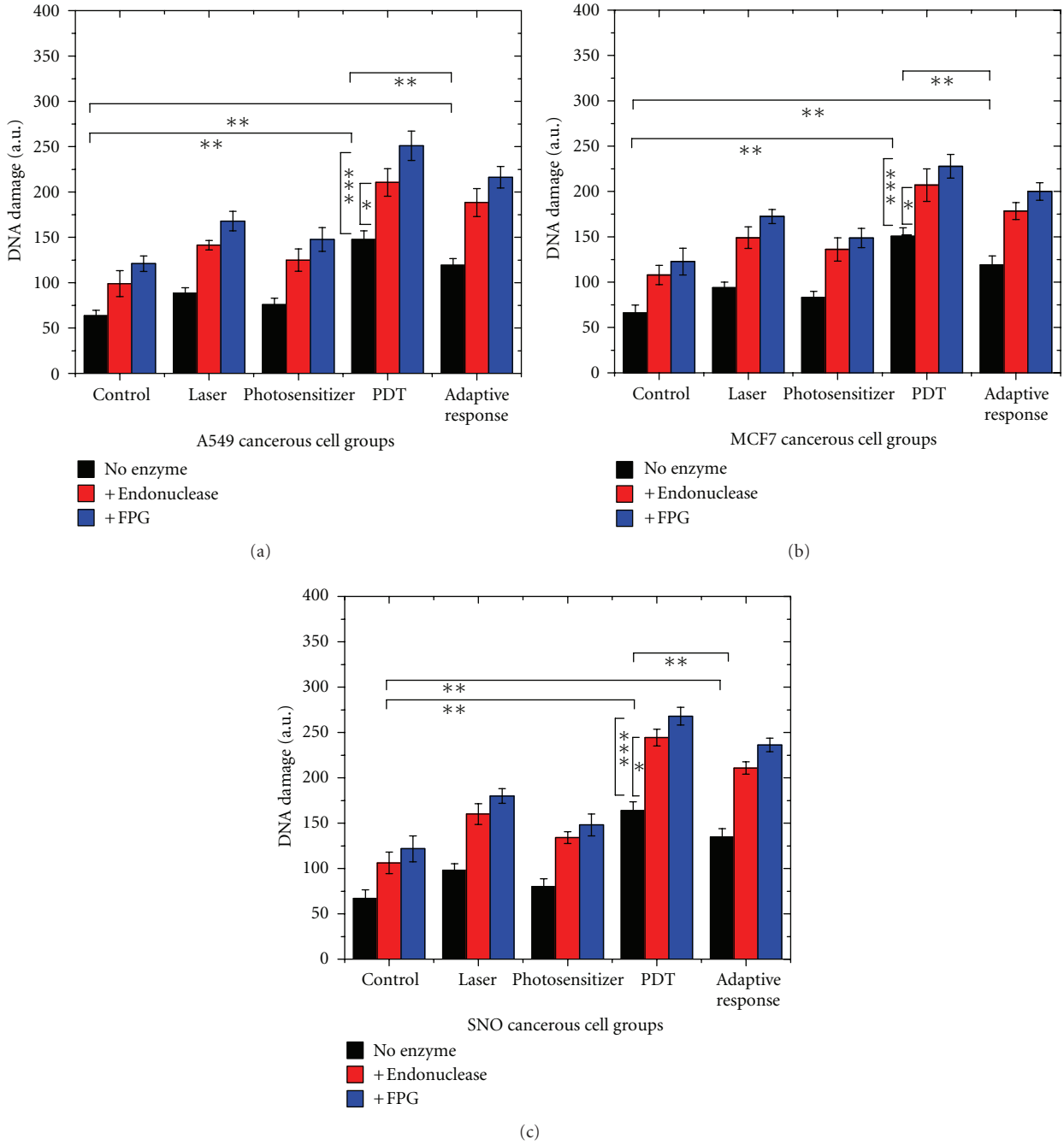


Figure 4: Comet assay measuring the DNA damage without using any enzyme, with using Endonuclease III and FPG protein for the five experimental groups of (a) A549, (b) MCF7, and (c) SNO cancer cells. In all three cell types the cells showed significant DNA damage after PDT treatment while cells that were pre-sensitized displayed less DNA damage compared to the PDT-treated cells. The sensitivity of the comet assay is enhanced significantly by using Endonuclease II and FPG proteins ($^{***}P < 0.001$; $^{**}P < 0.05$; $^{*}P < 0.01$).

[21]. Double-strand breaks (DSBs) have often been cited as the critical lesion produced by ionizing radiation [22]. If unrepaired, or incorrectly repaired, DSBs can lead to chromosome damage and cell death. Apoptosis results in the extensive formation of DSBs and is readily detected using either neutral or alkaline electrophoretic conditions. When viewed using the comet assay, only a small percentage of DNA of an apoptotic cell remains associated with the comet head.

Apoptosis has been shown to be a rapid and dominant form of cell death following PDT in multiple experimental settings utilizing various PSs and cell types [23]. Apoptosis is a tightly controlled, energy-consuming process of suicidal cell death involving activation of hydrolytic enzymes such as proteases and nucleases leading to DNA fragmentation and degradation of intracellular structures. Apoptosis was, however, the only form of cell death investigated in the majority

of studies, and it is likely that some cells may also undergo necrosis after PDT. High doses of PS, glucose starvation, and extramitochondrial localization of PS may promote necrosis [24, 25]. The cell genotype could be another influence on the form of cell death following PDT [26]. PDT induces apoptosis via two major pathways: mitochondria-mediated or intrinsic pathway and death receptor-mediated or extrinsic pathway [24].

The use of lesion-specific enzymes such as Endonuclease III and FPG protein is a useful modification of the comet assay because it increases both its sensitivity and its specificity as shown in Figure 4. It is clear that the greatest DNA damage in PDT and AR groups for all the cancer cells was observed when FPG protein was incorporated in the comet assay. These enzymes strongly enhance the DNA migration to express specific base damage as SSBs [16]. The differences between the effects in the absence and the presence of the Endonuclease III and FPG protein were statistically significant. Comparing the DNA damage of the PDT group to the control using typical comet assay with no enzymes added showed a statistical significant difference between the groups ($P < 0.05$) for A549, MCF7, and SNO cancer cells. Using Endonuclease III, significant differences were again observed ($P < 0.01$) for all three cancer cells when compared with the PDT of the typical, no enzyme comet assay. Similarly, addition of FPG protein induced significant higher DNA values ($P < 0.001$) for all the cancer cell types used when compared to the PDT group in the normal comet assay without enzyme addition (Figure 4).

Comparing the DNA damage of the AR group to the control using typical comet assay, a fine discrimination could be seen with statistical significance of ($P < 0.05$) for A549, MCF7, and SNO cancer cells. The same tendency is observed when using Endonuclease III ($P < 0.01$) for all three cancer cells as well as FPG protein ($P < 0.01$) for all the cancer cells used in the current study. An interesting observation is the statistical significance difference between the DNA damage of the PDT group and that of the AR group using typical comet assay a clear discrimination could be seen ($P < 0.05$) for A549, MCF7, and SNO cancer cells. The same difference but with higher significance could be observed using Endonuclease III ($P < 0.01$) for the three cancer cells. But this difference is more clearly seen using FPG protein ($P < 0.01$) for all cases.

FPG and Endonuclease III recognize different types of oxidative damage. FPG is specific for oxidized purines, including 8-oxo-7,8-dihydroguanine(8-oxoGua), 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and other ring-opened purines. The FPG protein is recommended for the detection of oxidative DNA base damage, in particular, 8-OH guanine [26, 27]. However, it is known that the FPG protein, besides its high sensitivity for detecting 8-OH guanine and other damaged purines, also detects abasic sites (AP sites) and ring-opened N-7 guanine adducts [28–31]. Endonuclease III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol.

The results presented here confirm that, after PDT treatment, pronounced increases in strand breaks are seen with FPG and, to a lesser extent, with Endonuclease III. This enhanced effects in the comet assay after FPG treatment should not readily be interpreted as oxidative DNA damage when the full mode of action of the inducing agent or the kind of exposure is unknown. The overall comparison shows that both FPG and Endonuclease III are capable of cleaving damage recognized in addition to oxidative DNA damage. In addition, this study demonstrated the protective effect on DNA integrity exhibited by the AR for PDT treatment on cancer cells pre-sensitized with irradiation.

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