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Original Article

Evaluation of cell damage and modulation of cytokines TNF-α, IL-6 and IL-10 in macrophages exposed to PpIX-mediated photodynamic therapy

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(With 6 figures)

Abstract

Little is known regarding whether photodynamic therapy (PDT)-induced cell death can substantially compromise macrophages (MΦ), which are important cells in PDT-induced immune responses. Here, parameters of PDT-mediated MΦ cytotoxicity and cytokine production in response to protoporphyrin IX (PpIX) were evaluated. Peritoneal MΦ from BALB/c mice were stimulated in vitro with PDT, light, PpIX, or lipopolysaccharide (LPS). After that, cell viability, lipid peroxidation, Nitric Oxide (NO), DNA damage, TNF-α, IL-6 and IL-10 were evaluated. Short PDT exposure reduced cell viability by 10–30%. There was a two-fold increase in NO and DNA degradation, despite the non-increase in lipoperoxidation. PDT increased TNF-α and IL-10, particularly in the presence of LPS, and decreased the production of IL-6 to 10-fold. PDT causes cellular stress, induces NO radicals and leads to DNA degradation, generating a cytotoxic microenvironment. Furthermore, PDT modulates pro- and anti-inflammatory cytokines in MΦ.

Keywords: photodynamic therapy, macrophage, DNA damage, cytokines, oxidative stress.

Avaliação do dano celular e da modulação das citocinas TNF-α, IL-6 e IL-10 em macrófagos expostos à terapia fotodinâmica mediada pela PpIX

Resumo

Pouco se sabe se a morte celular induzida pela terapia fotodinâmica (PDT) compromete os macrófagos (MΦ), envolvidos nas respostas imunes induzidas pela PDT. Neste estudo, foram avaliados parâmetros de citotoxicidade dos MΦ mediada pela PDT e a produção de citocinas, frente à protoporfirina IX (PpIX). MΦ peritoneais de camundongos BALB/c foram estimulados *in vitro* com PDT, luz, PpIX ou lipopolissacarídeo (LPS). Após isto, a viabilidade celular (VC), a lipoperoxidação, os níveis de óxido nítrico (NO), de DNA degradado, de TNF-α, IL-6 e IL-10 foram avaliados. A exposição curta à PDT reduziu a VC em 10-30%. Os níveis de NO e de DNA degradado duplicaram, sem aumento da lipoperoxidação. Houve aumento de TNF-α e IL-10, sendo maior na presença de LPS. Já a produção de IL-6 reduziu em dez vezes. A PDT induz estresse celular, gera radicais NO e causa dano ao DNA, tornando o microambiente citotóxico. Ainda, modula citocinas pró e anti-inflamatórias em MΦ.

Palavras-chave: fotoquimioterapia, macrófago, dano ao DNA, citocinas, estresse oxidativo.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality specially used in the management of cancer, but it can be also indicated for the treatment of infections, as it induces pathogen inactivation, allowing for improved performance of the immune system. PDT involves a chemical photosensitizer and an irradiation source at a wavelength that transfers

energy to this compound in order to produce toxic reactive oxygen (ROS) and nitrogen (RNS) species in the tumor microenvironment (Agostinis et al., 2011; Abdel-Kader, 2014). In both external and intracellularly, these reactive species can participate in non-enzymatic lipid peroxidation of cell membrane, producing alkyl, alkoxyl and peroxyl radicals and leading to exchange mechanism failure due to the destruction of the plasma membrane (Ayala et al., 2014). In the nucleus, ROS can react with DNA bases, damaging genetic material, as observed in cancer cells (El-Hussein et al., 2012) thus leading to cell death.

PDT-mediated tumor cell death is accompanied by damage to the local microvasculature, induction of local inflammation, and development of systemic immunity with an important role of macrophages (MΦ) (Agostinis et al., 2011). MΦ are differentiated from monocytes or embryonic precursors for specific immune tasks in various tissues (Ginhoux and Jung, 2014), being able to participate in the homeostasis or in the active immune response against pathogens (Marques et al., 2003), cancer and metabolic diseases. MΦ are the most important phagocytic cells of the immune system and, among other functions, they release inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), pro-resolving cytokines, such as interleukin 10 (IL-10) (Colotta et al., 2009; Burian et al., 2017). The immune response is independent of the aggressive agent; it depends on the pro- or anti-inflammatory mechanisms triggered by these cells. Chronic inflammatory conditions can increase the risk of cancer in several organs due to some chemokines and immune response products (Colotta et al., 2009).

PDT increases the recognition of damage-associated molecular patterns (DAMPs) by MΦ or by other immune cells in cancer (Plaetzer et al., 2013), whether there is lipopolysaccharide (LPS) stimulation or not (Zheng et al., 2016). Nonetheless, high levels of ROS and RNS are triggered by PDT, hence leading to increased levels of non-enzymatic NO, as well as cytotoxicity and apoptosis of adjacent non-tumor cells, especially those carrying the accumulated photosensitizer (PS) (Yamamoto et al., 2007). This may lead to necrotic rather than apoptotic events. As current findings clearly show a higher responsiveness and a better prognosis for PDT-mediated tumor apoptosis, recent studies have focused on triggering systemic immunity with optimal PDT conditions so as to avoid necrosis (Garg et al., 2010).

However, little is known about whether PDT-derived necrotic events and even other cell death mechanisms are triggered on immune system cells, for instance, on MΦ. Cell death state can substantially lead these cells to directly or indirectly compromise the proper activation of immune system against tumor cells and pathogens, as well as cause possible damage to the MΦ involved in the immune response by altering their cytokine expression profile. This incomplete understanding has so far limited how immune therapies can be modulated, but new approaches could refine our understanding of immune cells such as MΦ on PDT (Ginhoux and Jung, 2014). Thus, the aim of this study was to evaluate events associated with rapid PDT cell exposure in the presence of protoporphyrin IX (PpIX), assessed by cell viability, MΦ lipid peroxidation, generation of RNS with production of non-enzymatic NO radical and deoxyribonucleic acid (DNA) damage, as well as by the impact of PDT on TNF-α, IL-6 and IL-10 cytokine production by MΦ from mice.

2. Materials and Methods

2.1. Peritoneal macrophages, experimental group design and drug exposures

This experimental study was conducted after approval of the Ethics Committee on Animal Use of the Federal University of Triângulo Mineiro (CEUA-UFTM), protocol no. 223/2013. MΦ migrated to peritoneal cavity and were obtained from nine-week old male BALB/c mice after three days of intraperitoneal injection of 3 mL of 3.5% thioglycolate.

The mice were anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine followed by cervical dislocation. Then, 8 mL of RPMI 1640 medium (HiMedia Laboratories, Mumbai, India) was administered with 10% fetal bovine serum (FBS) (Nutricell, Campinas, SP, Brazil), 1% penicillin (Gibco® Thermo Fisher Scientific, Grand Island, NY, United States), 1% streptomycin (Gibco®), and 8 µL heparin (HEPAMAX-S, Blausiegel, Cotia, SP, Brazil) inside the cavity, with only the external layer having been exposed by surgical procedure at first. The abdomen was intensively massaged 30 times to ensure that more MΦ would be collected.

The entire content was aspirated and centrifuged at 500 x g for 10 min. After that, 1 x 10^5 cells were placed in each well and incubated at 37 °C for 1 h and 30 min in a 5% CO_2 incubator for MΦ adhesion to plates (Zhang et al., 2008). After this period, the wells were washed three times with phosphate-buffered saline (PBS) (DPBS, Sigma Aldrich Chemical Co., St. Louis, MO, United States) to remove non-adherent cells.

Four experimental groups were analyzed: (1) MΦ not exposed to any drug or light (negative control), (2) cells exposed to light only, (3) cells exposed to PpIX only (Sigma Aldrich Chemical Co.), and (4) MΦ exposed to light and PpIX (PDT group). First, 2 M PpIX was prepared in 1M HCL and methanol (1:9 v/v), and diluted in RPMI-1640 (10% FBS, 1% penicillin, 1% streptomycin) at a final concentration of 60 µg mL^{-1} (106 µM, pH 7,0).

After PBS washing, PpIX was added in predefined groups for 2 h, a rapid cell exposure, the minimum time required for drug penetration (Xu et al., 2014). The other groups just received RPMI. MΦ cytokine levels of the predefined groups were compared to those produced by MΦ treated with lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma Aldrich Chemical Co.). The LPS groups (either LPS alone or in combination with PDT) received 20 µg mL^{-1} (14 µM) LPS with RPMI for 1 h.

All experiments were performed in triplicate, as described below. For each one, nine mice were used. However, nine mice were used for both assessment of lipid peroxidation and NO quantification.

2.2. LED-based photodynamic therapy and positive control groups

As described before, after that, specific groups received 20 J cm^{-2} of basal light dose (410-490 nm wavelength) using a light-emitting diode (LED)-based dental polymerization device (Optilight Max™, Gnatus, Ribeirão Preto, SP, Brazil). All the wells were washed three times with PBS and RPMI was added again, returning to the incubator (37°C, 5% CO_2). The plates were kept in the incubator for 1 h to evaluate cell viability, lipid peroxidation, production of non-enzymatic NO and DNA damage. Cytokine production was evaluated after 21 h of incubation (Mosser and Zhang, 2008).

Positive control groups for DNA damage and lipid peroxidation were designed exposing MΦ to RPMI with 50% H_2O_2 and 2 mM $FeSO_4$ during the incubation (1 h, 37°C, 5% CO_2). Positive control group for the presence of NO radical was designed exposing MΦ to RPMI with 10 µM $NaNO_2$ and 10 µM KNO_3 during the incubation (1h).

2.3. Cell viability

MΦ were treated with 0.25% trypsin (Gibco®) for 3 min., and the reaction was then neutralized with RPMI-1640 medium (supplemented with 10% FBS). The wells were scraped to remove adherent MΦ, and the entire medium of each well was collected, put into a new tube and mixed gently. Then, 10 µL of cell medium was collected and mixed with 10 µL of 0.7% trypan blue (Invitrogen, Carlsbad, CA, United States). Finally, 10 µL of this solution was pipetted on a special slide for automatic cell counting (Countess™ automated cell counter, Invitrogen, Carlsbad, CA, United States). The remaining cell medium was used to quantify NO and lipid peroxidation, cytokines or DNA damage by Comet assay.

2.4. Cellular lipid peroxidation

Cells from the positive control tube and from tubes used to evaluate cell viability were centrifuged at 1,500 x g for 10 min. Supernatants were used to measure NO radical levels. Each pellet was mixed with 500 µL of a solution containing 69.4 mM thiobarbituric acid and 50 mM NaOH. Then, 250 µL of 7% H_3PO_4 was added, mixed, incubated at room temperature for 10 min., and at 95 °C for 15 min. The concentration of malondialdehyde produced by lipid peroxidation in the final solutions was detected at 532 nm, using a single beam spectrophotometer (UV1600 UV/VIS; E-Chrom Tech, Taipei, Taiwan) (Paulino, 2006).

2.5. Direct non-enzymatic nitric oxide generation in macrophages

Each supernatant was mixed with 400 µL of solution A (3.9 mM n-(1-Naphthyl)ethylenediamine and 5% H_3PO_4) and with 400 µL of solution B (58.1 mM sulphanilamide and 5% H_3PO_4) in a Griess reaction. Final mixtures were incubated at room temperature for 10 min. and the absorbance was read at 550 nm in the aforementioned spectrophotometer (Archer, 1993). Absorbance values were converted to molarity using a calibration curve containing decreasing concentrations of NO_2 and NO_3 solutions.

2.6. Comet assay for quantification of DNA damage

Cells derived from MΦ viability assay were centrifuged at 1,500 x g for 10 min., and the supernatants were discarded. After that, 100 µL of 0.5% low-melting-point agarose at 37 °C was added to the pellets and homogenized. The entire volume of each tube was poured over glass slides coated with 1.5% multi-use agarose and covered with glass coverslips.

The slides were incubated at 4 °C until the low-melting-point agarose solidified. The coverslips were removed and the glass slides were immersed in a lysis buffer – 10% DMSO, 1:100 Triton X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris base at pH 10 – overnight at 4°C. After that, the slides were immersed in a running solution (200 mM EDTA at pH 10, 10 M NaOH) and incubated at 4 °C for 20 min. Electrophoresis occurred at 25 V, 30 W for 30 min. After this period, the slides were immersed in a neutralizing solution (400 mM Tris base at pH 7.5) for 20 min, dried at room temperature, and stained with 50 µL of 0.4 µg mL^{-1} DAPI (Sigma Aldrich Chemical Co.). A fluorescence microscope with an excitation wavelength of 358 nm was used to capture images of 100 cells on each slide. The images were analyzed using CometScore v1.5 software (TriTek Corp., Sumerduck, VA, United States).

2.7. Quantification of cytokines TNF-α, IL-6 and IL-10

After 21 h in the incubator, the entire medium of each well was collected into new tubes and mixed gently for new analysis of cell viability using trypan blue, as previously described. Finally, the cells were centrifuged at 1,500 x g at 4 °C for 10 min., and the supernatants were collected for quantification of TNF-α, IL-6 and IL-10 using ELISA kits (BD OptEIA™ Mouse; BD Bioscience, San Jose, CA, United States) according to the manufacturer's instructions. Concentration values were based on the standard curves generated for each cytokine.

2.8. Statistical analysis

Cell viability percentages, malondialdehyde absorbances determined due to lipid peroxidation, NO concentrations, levels of cytokines, and the comet tail lengths of the experimental groups were compared using SPSS v22.0 statistical software (International Business Machines Corp., Armonk, North Castle, NY, United States). Analysis of variance with Tukey's post-hoc test was applied to compare groups with normal distribution according to the normality test (Kolmogorov-Smirnov test) or with homogeneous variances according to the test of homoscedasticity (Bartlett's test). Non-parametric tests, such as Kruskal-Wallis followed by Mann-Whitney test, were applied to all other cases which did not meet these criteria. For all analyses, a p-value of < 0.05 was considered statistically significant. Prism v7.0 software (GraphPad Inc., San Diego, CA, United States) was used for graphical design.

3. Results

3.1. Macrophage cytotoxicity in PDT, light, PpIX and LPS exposures

In order to verify the impact of PDT, light/PpIX or LPS on cell viability, MΦ were automatically counted by exclusion using trypan blue staining. As shown in Figure 1, irradiation with 20 J cm^{-2} or just the exposure to 60 µg mL^{-1} PpIX for 2 h did not decrease MΦ viability in comparison with the control group. This result shows that light or PpIX alone do not cause cell death when used in such dose and concentration. However, PDT decreased cell viability by 10-30% when 60 µg mL^{-1} PpIX and 20 J cm^{-2} were combined, as it could be expected for a rapid dose of exposure. Interestingly, when MΦ were exposed to 20 µg mL^{-1} LPS for 1 h, cell viability also decreased by 10-30% compared to the control group, as well as when LPS and PDT were used together. These results showed that the levels of LPS did not significantly contribute to counterbalance cell death by PDT when expecting to activate immune response on MΦ.

3.2. Impact of PDT on macrophage lipid peroxidation

In order to check whether PDT or light/PpIX alone are capable of increasing MΦ lipid peroxidation, the amount of malondialdehyde in MΦ was measured, as it is one of the main secondary reaction products that results from lipid peroxidation of cell membranes and is reactive to thiobarbituric acid (Ayala et al., 2014). As depicted in Figure 2, none of the exposure conditions could significantly increase MΦ lipid peroxidation, thus suggesting that another cytotoxicity mechanism occurs in the first hour after PDT treatment with 20 J cm^{-2} of light and 60 µg mL^{-1} PpIX.

3.3. PDT-induced production of non-enzymatic nitric oxide as RNS on macrophages

The quantity of NO produced and diffused from intra-to extracellular compartments was measured after 1 h of treatment for determining whether PDT was involved in the production of radical nitrogen species in exposed MΦ. In Figure 3, PDT with 20 J cm^{-2} of light and 60 µg mL^{-1} PpIX is capable of generating approximately two times more NO than negative control group. This result shows that MΦ are quickly oxidized by PDT when non-enzymatic NO is generated instantaneously and this effect cannot be reached by light or PpIX alone.

3.4. PDT-induced DNA damage in macrophages

Considering that PDT induces the production of nuclear diffusible free radicals, as well as non-enzymatic NO in cells, it is essential to estimate how widespread the DNA damage within a number of cells is. Figure 4 shows that 60 µg mL^{-1} PpIX or 20 J cm^{-2} light alone could not compromise DNA. Nevertheless, MΦ treated with PDT have approximately two times more DNA damage than the negative group when evaluated after 1 h of treatment. In accordance with Figure 1, these findings suggest that mechanisms related to DNA damage play a key role in MΦ death. Figure 5A and B shows a representative comet image found in the negative control and PDT groups respectively.

3.5. Macrophages exposed to PDT change protein expression of cytokines

A balance between cell death, inflammation and restoration of homeostasis are important considerations when MΦ are exposed to drug and PDT cytotoxicity. In order to understand what general cell behavior will be

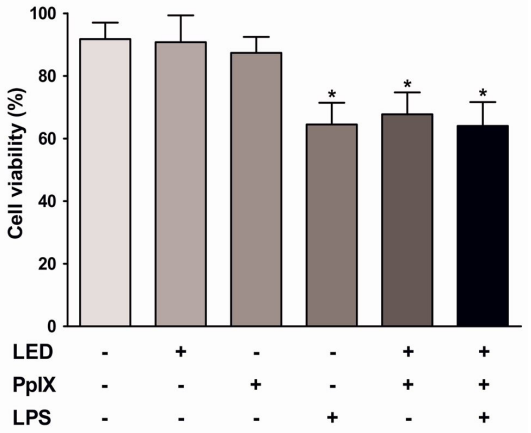

Figure 1. Evaluation of cell viability exposing MΦ to LED light only (20 J cm^{-2}), to PpIX only (60 µg mL^{-1} for 2 h), and to PDT (20 J cm^{-2} + 60 µg mL^{-1} PpIX for 2 h), as well as cell viability in LPS (20 µg mL^{-1} for 1 h with or without PDT). X-axis indicates presence (+) or absence (-) of light, PpIX and LPS. Data are presented as mean + standard deviation (ANOVA, Tukey's post-hoc test). * represents statistically significant results compared to the negative control group ($p < 0.05$).

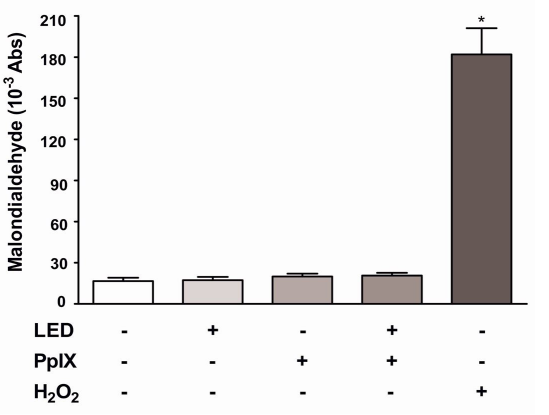

Figure 2. Evaluation of malondialdehyde induced by lipid peroxidation in MΦ exposed to LED light only (20 J cm^{-2}), to PpIX only (60 µg mL^{-1} for 2 h), and to PDT (20 J cm^{-2} + 60 µg mL^{-1} PpIX for 2 h), as well as malondialdehyde in the H_2O_2 positive control group (50% H_2O_2 + 2 mM $FeSO_4$ for 1 h). X-axis indicates presence (+) or absence (-) of light, PpIX and H_2O_2. Data are presented as mean + standard deviation (ANOVA, Tukey's post-hoc test).*Represents statistically significant results compared to the negative control group ($p < 0.05$).

like before and after a specific treatment, Figure 6 shows the quantification values of TNF-α (A), IL-10 (B) and IL-6 (C) between the experimental groups.

As can be seen in Figure 6A and B, MΦ in the negative control group do not produce TNF-α or IL-10. This was expected as the cells had not been exposed to known pro-inflammation conditions for developing an active immune response. However, MΦ treated with PpIX/LPS alone or PDT produced approximately 50-75 pg mL^{-1} of TNF-α, and 50-145 pg mL^{-1} of IL-10. These levels were also high when LPS and PDT were combined (60-115 pg mL^{-1} of TNF-α, and 200-250 pg mL^{-1} of IL-10), suggesting that PDT+LPS can be used to enhance inflammation followed by targeting homeostasis in a MΦ microenvironment.

Surprisingly, MΦ produced about five times more IL-6 (370-425 pg mL^{-1}) in the negative control group than when in contact with PpIX alone (0-75 pg mL^{-1}), LPS alone (0 pg mL^{-1}), and PDT or PDT+LPS treatment (0-70 pg mL^{-1}). This suggests that chemicals in the medium can induce activation signals for IL-6 production, and their effects can be suppressed or counterbalanced when 60 μg mL^{-1} PpIX or 20 μg mL^{-1} LPS are added to the solution.

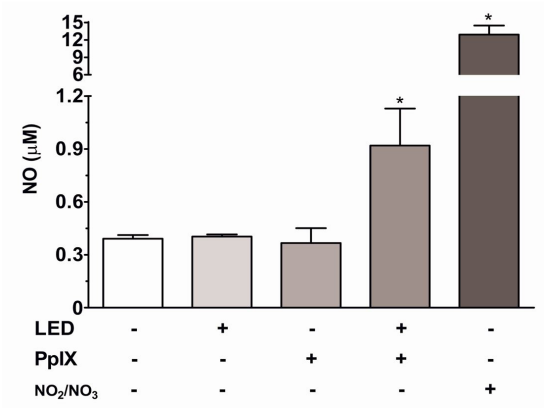

Figure 3. Quantification of NO produced after 1 h of LED light only (20 J cm^{-2}), PpIX only (60 μg mL^{-1} for 2 h), and PDT (20 J cm^{-2} + 60 μg mL^{-1} PpIX for 2 h), and also oxi-reduction of 10 μM NO_2 + 10 μM NO_3 on medium for 1 h (positive control). X-axis indicates presence (+) or absence (-) of light, PpIX and NO_2 with NO_3. Data are presented as mean + standard deviation (ANOVA, Tukey's post-hoc test). * represents statistically significant results compared to the negative control group ($p < 0.05$).

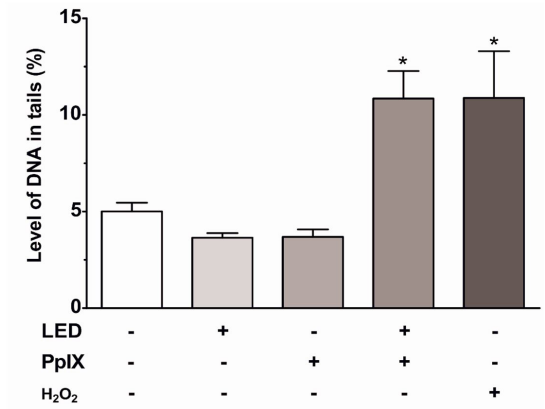

Figure 4. Quantification (%) of DNA degradation in comet tails when MΦ were exposed to LED light only (20 J cm^{-2}), to PpIX only (60 μg mL^{-1} for 2 h) and to PDT (20 J cm^{-2} + 60 μg mL^{-1} PpIX for 2 h), as well as DNA degradation in comet tails in the H_2O_2 positive control group (50% H_2O_2 + 2 mM $FeSO_4$ for 1 h). X-axis indicates presence (+) or absence (-) of light, PpIX and H_2O_2. Data are presented as mean + standard deviation (ANOVA, Tukey's post-hoc test). * represents statistically significant results compared to the negative control group ($p < 0.05$).

4. Discussion

ProtoporphyrinIX is an endogenous porphyrin derivative, a flat tetrapyrrole structure formed by methyl groups, initially synthesized from the combination of eight molecules of delta-aminolevulinic acid (ALA) (Wachowska et al., 2011). Photosensitive agents (PSs), most of which contain a tetrapyrrole structure, have been used in PDT-based cancer therapy (Agostinis et al., 2011). In order to stimulate PpIX, it suffices that a source light irradiates energy at a wavelength that is specifically absorbed by the drug. The higher the absorption of light energy at this wavelength, the greater the photophysical effects will be in terms of generation of ROS and RNS (Jacques, 1992). LED sources can be a good choice due to their low cost, easy transportation and maintenance, small thermal additive effect, high fluency rate, and a narrow range of wavelength variance (20-50 nm) (Brancaleon and Moseley, 2002).

Several studies have obtained peritoneal MΦ after induction of leukocyte extravasation following 3-4 days of thioglycolate administration, using concentrations from 2.9 to 4.5% (Evans et al., 1990; Zampronio et al., 1994; Soudi et al., 2013; Miranda et al., 2013). These studies used the trypan blue exclusion technique to analyze cell viability. This good cost-benefit technique provides stable values in comparison with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which itself has the disadvantage of interfering in the process of cellular metabolism (Śliwka et al., 2016).

Our results showed a decrease in MΦ viability in the PpIX-mediated PDT group. Two other research groups showed the same results with MΦ and explained that cytotoxicity by PDT using ALA and Photofrin II can increase programming or no-programming of cell death caused by ROS and RNS, although the MΦ were exposed to light dosages, drug concentrations or drug concentration per unit of time at levels much higher than those used in our light-drug study (Evans et al., 1990; Kawczyk-Krupka et al., 2011). Evans et al. (1990) showed that a higher dose of PDT exposure prevents MΦ from producing TNF-α. This did not occur in our study, as a lower dose was used.

With regard to cell viability, there was no difference between the group quickly exposed to 106 µM PpIX for 2 h and the negative control group in this study. This is not in keeping with the study by Xu et al. (2014) with respect to the following results. They found that MΦ viability was decreased by 50-78% after application of 10 µM PpIX for 12 h, which seems to be a long time of exposure. Nonetheless, when using flow cytometry, even after application of 10 µM PpIX for 3 h and 10 µM PpIX for 6 h, viability decreased to 64-77% and 48-68%, respectively. These results are

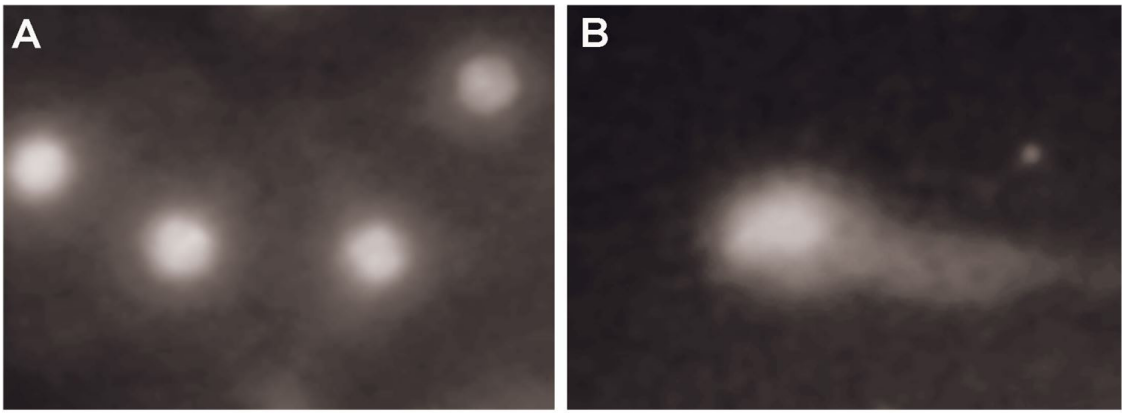

Figure 5. Representative microscopy images of comets found in the negative control group (A), showing preserved genetic material, and in the PDT group (B), showing genetic material degradation that forms a comet tail due to the lower weight of DNA fragments.

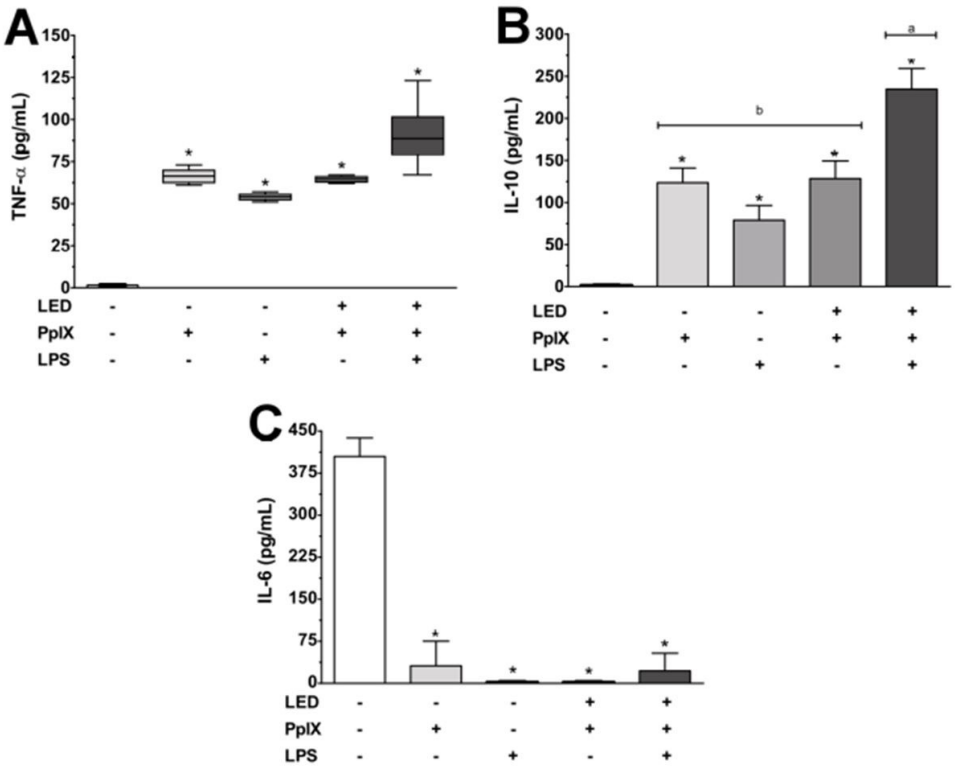

Figure 6. Quantification of cytokines released 21 h after application of PpIX alone (60 µg mL^{-1} for 2h), PDT (20 J cm^{-2} + 60 µg mL^{-1} PpIX for 2 h), LPS alone (20 µg mL^{-1} for 1 h) and PDT + LPS (20 J cm^{-2} + 60 µg mL^{-1} PpIX for 2 h + LPS for 1 h). (A) TNF-α data are presented as median + standard deviation (Kruskal–Wallis; 2×2 comparisons by Mann-Whitney test). X-axis indicates presence (+) or absence (-) of light, PpIX and LPS. Data of IL-10 (B) and IL-6 (C) are presented as mean + standard deviation (ANOVA, Tukey's post-hoc test). * represents statistically significant results compared to negative control group ($p < 0.05$). Letter "a" represents statistically significant results in comparison with groups represented by letter "b".

inconsistent with their MTT results, which showed more than 78% of MΦ viability after exposure to 10 μM PpIX for 6 h. It was expected that 10 μM PpIX for 3 h would achieve a viability of 90-95% using the MTT technique. Therefore, our cell viability results are in agreement with the MTT results of Xu et al. (2014), even though our drug concentration was 10 times higher, but for a short time of cell exposure (2 h).

Even though there was no difference in cell viability between the PpIX and the negative groups in our study, it cannot be ruled out that initial cell death signals could occur during the first two hours of exposure to PpIX. It is also possible that the measurement of cell viability becomes more relevant after 2-3 h of PpIX exposure. Thus, cell viability was rescued when MΦ-PpIX interaction was stopped after 2 h. The fact that it is possible that cell death signals could have been initiated is corroborated by the fact that no difference was observed in lipid peroxidation after 2 h. However, if the PpIX group behaves like the PDT group (which had statistical difference in NO production and DNA damage in relation to the negative control group) with respect to being cytotoxic, after this time it becomes evident that lipid peroxidation may occur later. From another perspective, PpIX has a molecular structure that could be recognized by mouse MΦ even before the activation of cell death mechanisms, eliciting active immunity followed by homeostatic immune response, because TNF-a and IL-10 levels are higher than in the negative control group.

On the other hand, the focus was on investigating PDT cytotoxicity, and it showed a greater decrease in cell viability, a two-fold increase both in non-enzymatic NO generation and in DNA damage, i.e. these effects only happened in the simultaneous presence of light and PS. Xaus et al. (2000) reported that exogenous NO is more cytotoxic than endogenous enzymatic nitric oxide. NO can increase retention of PSs in tumour cells (Yamamoto et al., 2007). Our results suggest that the cellular mechanisms involved in the rapid increase of NO and DNA damage are strongly associated with MΦ cell death (Xaus et al., 2000).

Our study also showed decreased cell viability, with 20 $\mu g\ mL^{-1}$ LPS for 1 h, and increased TNF-α production. Xaus et al. (2000) also reported that LPS can induce MΦ apoptosis with stimulation of TNF-α production, with apoptotic cells reaching a plateau at around 6 h. This also supports the observation of why there are less viable cells as a result of the stressful effect of PDT+LPS as well as an increase in the production of inflammatory TNF-α in some replicates, even though the statistical test did not show significance in comparison with PDT or LPS groups.

It is known that LPS concentrations above 1 $ng\ mL^{-1}$ in cell medium can inhibit IL-6 production by peritoneal MΦ (Meng and Lowell, 1997), and our results of LPS and PDT+LPS groups are in agreement with this explanation. On the other hand, results of negative control groups showed an uncommonly high expression of IL-6. The same was observed by Brummer et al. (2007) when comparing MΦ in the presence and absence of 10% FBS. Nevertheless, components and mechanisms involved in the stimulation of this cytokine production by FBS in MΦ are not fully clear and need to be further determined. Not only does IL-6 contribute to pro-inflammatory effects, but it is speculated to play a role in potential anti-inflammatory modulation in immune cells, as is well-known in epithelial tumor cells of the gastrointestinal tract (Scheller et al., 2011).

MΦ lacking the suppressor of cytokine signaling 3 (SOCS3) gene are more susceptible to inhibition of TNF-α production by IL-6 than by IL-10 (Yasukawa et al., 2003). So, considering TNF-α produced by wild-type MΦ can still be inhibited by IL-6, this can explain why there were higher levels of IL-6 in the negative control, which may have occurred to avoid high levels of TNF-α expression for a homeostatic *in vitro* state. Higher levels of IL-10 in the other groups may compensate TNF-α expression at the same level. However, it is still unclear which mechanisms are involved in PpIX or PDT and in IL-6 inhibition. Given the results of PpIX and PDT groups, further studies are necessary.

LPS can still induce IL-10 production during the first hours of incubation (Kontoyiannis et al., 2001), causing negative feedback on TNF-α production (Mantovani and Sica, 2010). This can explain the fact that TNF-α production in LPS and PDT+LPS groups is accompanied by IL-10 expression, and that it has low values due to the modulation of inflammatory response. However, this feedback can also be observed in the PDT group, in which relevant TNF-α production is probably stimulated by cell death signals and also has independent participation of LPS effects. This shows a flexible response by MΦ, which also have some active immune response with no cell viability decrease in the chemical presence of this photosensitizer, as observed in the PpIX group (Niziolek et al., 2003).

Malondialdehyde, which is an analyte of indirect lipid peroxidation, did not increase in any of the exposure conditions. This was expected for PpIX/light-only groups but not for PDT group. The cells sustained only the level of physiological and endogenous lipid peroxidation. Interestingly, the plasma membrane of MΦ is composed of a high amount of saturated rather than unsaturated fatty acids. This facilitates the occurrence of lipid peroxidation, which is also observed in other oxidative compounds (Ayala et al., 2014). Moreover, PpIX accumulation in non-tumorous tissues leads to a higher heme group production or to the output of the drug from the cytoplasmic to the extracellular space, making the cell membrane a target for PDT. However, in a study treating COH-BR1 breast tumor cells with PDT, 20 μM NO derived from a donor species had an anti-peroxidative effect and binded to alkoxyls and epoxides, hence interrupting the cycle of lipid peroxidation reactions (Mantovani and Sica, 2010). This may explain our lipoperoxidation results compared to NO levels, and it is a hypothesis that needs further investigation.

Comet assay results are consistent with cell viability values and with the majority of PDT studies concluding that the expressive cytotoxic effect should only depend on the joint interaction of light and PS (Juzenas et al., 2008). Therefore, analysis of proteins involved in

apoptotic pathways would provide better support for the understanding of genetic material degradation and of PDT-generated cytotoxicity. It is also important to know that apoptosis and necrosis can be caused by membrane and mitochondrial damage, causing DNA strand break (Olive and Banáth, 2006). One starting point has been proposed by Xu et al. (2014), revealing that PpIX induces necrotic cell death of MΦ through ROS production, activation of c-Jun N-terminal protein kinase (JNK) and mitochondrial permeability transition pore (mPTP) opening. Thus, it can be hypothesized that PDT generation of ROS and RNS directly triggers DNA damage with a cellular PpIX accumulation process, thereby activating JNK and mPTP pathways involved in mitochondrial dysfunction.

Studies on uptake kinetics, intracellular localization and M1/M2 MΦ activation may clarify some open questions. Other cytokines involved in other immune cells may also be investigated, since TNF-α, IL-6 and IL-10 can interact with leukocyte extravasation and with profile responses of type 1 T helper cells and regulatory T cells. The study of ROS is necessary to investigate more specific lipid peroxidation reactions in the presence of NO, for which generation may be triggered and also verified over a larger time interval in order to analyze the development of later cellular response. Variations in drug/light doses may be applied to investigate the same parameters discussed and the unknown mechanisms between PDT and cytotoxicity, as well as to redefine new discursive aspects, thus helping to improve strategies to modulate immune response against chronic diseases.

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