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Influence of Hydrocortisone in Chemotherapy and Photodynamic Therapy in HEp-2 Cells

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Abstract

Aim: Cancer cells exhibit resistance to the immune response by regulating and altering the expression of mediators responsible for immune cell recruitment and disease progression. Cortisol is a natural hormone that may be associated with diseases such as cancer by stimulating stress and altering the cellular environment, favoring uncontrolled division and contributing to the inhibition of the immune response. In contrast, current therapeutic strategies do not present significant concerns about stress as a variable in cancer diagnosis and prognosis. The response of HEp-2 cells to stress induced by hydrocortisone and to treatment with Cyclophosphamide (CP) and Photodynamic Therapy (PDT) was analyzed.

Methods: One mM of hydrocortisone induced stress in the cells. Cells were treated with 200 μg/mL of cyclophosphamide or Aluminum Phthalocyanine Tetrasulfonate (AlPcS4) photosensitizer, LED irradiation (660 nm wavelength), intensity of 25 mW/cm^2, power of 70 mW, fluence of 5 J/cm^2, characterizing the PDT. All groups were evaluated after 24 h and 48 h.

Results: Assessment of stress-inducing mitochondrial activity and cell viability were performed, and the results demonstrated that hydrocortisone significantly altered the rate of cell death, compromising the effects of CP.

Conclusion: However, hydrocortisone did not change the cell death rates caused by PDT, indicating the possibility of this hormone as an alternative therapy.

Keywords: Cancer; GRP78; Stress; MTT assay; Stress

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Introduction

Clinical studies indicate that pathological conditions of patients with cancer are strictly related to the release of hormones, such as cortisol. So, in a patient with cancer, the disease is accompanied by metabolic stress, mainly detected by the increase in cortisol levels in the blood. Consequently, cancer cells grow in a stressed environment resulting from the organism's response to the pathology [1,2]. Therefore, impaired levels of cortisol, commonly associated with stress conditions, have also been considered a contributor to cancer severity and mortality, which plays an essential role in antineoplastic therapy [3,4].

The current cancer treatments are surgery, radiotherapy, immunotherapy, and chemotherapy. The effectiveness and success of these therapies are highly dependent on the location and extent of the tumor [2]. Chemotherapy is a very effective treatment, but it also has several limitations. Among the drugs used in this therapy, cyclophosphamide is considered a prominent clinical drug used for treating lupus and cancer due to its cell cycle regulatory property. However, studies have shown the second action of cyclophosphamide, in which the drug is activated only in neoplastic cells capable of expressing high concentrations of phosphamidase, an enzyme capable of breaking the phosphorus-nitrogen bond, converting the drug into its most toxic form [5,6].

In addition to the mentioned treatments, Photodynamic Therapy (PDT) is a therapeutic strategy for neoplastic and non-neoplastic diseases, with fewer adverse reactions to the patients [7]. This treatment is defined as a reaction between a non-toxic photosensitizer (FS) and visible light, generating a cytotoxic effect through oxidative reactions with oxygen, causing cell death mainly by apoptosis [8,9]. Different FS are used in PDT, such as photofrin, Aminolevulinic Acid (ALA), methylene blue, and Aluminum Phthalocyanine Tetrasulfonate (AlPcS4), among others [10]. PDT uses the principle of specific wavelength light combined with FS interacting with cellular oxygen,

producing ROS (Reactive Oxygen Species) capable of inducing cell death. The action of singlet oxygen on the cancer cell death process has been evidenced in several ways, such as damage to the cell membrane, mitochondria, lysosomes, and DNA, compromising cellular integrity [8,9,11].

However, although PDT causes the death of many cancer cells, it does not eliminate cells that undergo the treatment. Further studies are therefore needed to find alternatives and therapeutic interactions to potentiate PDT, to eliminate all the target cells [12].

Based on this information, we hypothesize that the combined action of PDT and cyclophosphamide under simulated stress conditions may increase the cancer cell death rate, potentiating PDT's effects. The present study aims to evaluate *in vitro* the combined action of PDT and CP, simulating stress using hydrocortisone as analogous to the hormone cortisol, to verify if the presence of this hormone as a stressor agent changes the rate of cell death through mitochondrial activity, cell viability, and clonogenic assay.

Materials and Methods

Cell culture

HEp-2 (human laryngeal carcinoma) cells provided by Paul Ehrlich Technical Scientific Association Cell Bank (Rio de Janeiro-Brazil) were cultured in 25 cm^2 flasks at 37°C under 5% CO_2 in DMEM (Dulbecco's modified Eagles medium - Thermo Fisher Scientific, Gibco, Waltham, Massachusetts, EUA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Gibco, Waltham, MA, EUA) and 1% penicillin and streptomycin (Thermo Fisher Scientific, Invitrogen, Waltham, MA, EUA).

Hydrocortisone concentration curve

HEp-2 cells were cultivated in 96-well plates (1×10^4 cells/well). After 24 h of plating, cells were incubated for 24 h or 48 h with different concentrations of hydrocortisone (hydrocortisone sodium succinate - Sigma-Aldrich) (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM, diluted in DMEM), to establish the optimum concentration capable of inducing stress, not causing cell death.

Cyclophosphamide concentration curve

HEp-2 cells were seeded in 96-well plates (1×10^4 cells/well) for 24 h. Cells were then treated with different concentrations of Cyclophosphamide Monohydrate (Fluka, Honeywell, Charlotte, NC, USA) (1.0 µg/mL, 2.5 µg/mL, 6.0 µg/ 12 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and 250 µg/mL), diluted in DMEM, to establish the most efficient concentration to reduce cell viability.

Hydrocortisone treatment

After the hydrocortisone concentration was established, cells were cultivated at the density of 1×10^5 cells/well, in 24-well plates. Twenty-four hours after plating, the culture medium was discarded, and cells were washed twice with Phosphate-Buffered Saline (PBS). Then, the wells were added to 500 µL of the 1.0 mM/mL hydrocortisone solution (diluted in DMEM) and incubated for 24 h and 48 h at 37°C and 5% CO_2. Adapted from Zhou, 2014 [13].

Experimental groups

Cyclophosphamide treatment: After the cyclophosphamide concentration was established, cells were plated at the density of 1×10^5 cells/well in 24-well plates. Twenty-four hours after plating, the medium was discarded, and cells were washed twice with PBS. Five hundred µL cyclophosphamide solution in DMEM was added to the wells and incubated for 24 and 48 h at 37°C and 5% CO_2. Adapted from Zhou, 2014 [13].

Photosensitizer

Aluminum Phthalocyanine Tetrasulfonate (AlPcS4) (Porphyrin Frontier Scientific, Logan, UT, USA) was diluted in PBS at a concentration of 5.0 µM/mL and kept in the dark at 4°C until use [11,14].

Photodynamic therapy

Cells were plated at the density of 1×10^5 cells/well in 24-well plates containing round cover slips. Twenty-four hours after plating, the medium was discarded, and cells were washed twice with PBS. 200 µL of AlPcS4 solution (5 µM/mL) was added to the wells and incubated for 1 h at 37°C and 5% CO_2. Then, the solution was discarded, and cells were washed twice with PBS and irradiated. The LED device, Biopdi/IRRAD-LED 660 nm (Biopdi, São Carlos, São Paulo, Brazil), used for PDT irradiation consists of 54 LEDs; each LED has 70 mW of power, emitting in 660 ± 5 nm and covering an area of 150 cm^2. The power density of the delivered light was 25 mW/cm^2, and the exposure time was 3 min and 20 sec, totalizing the fluence of 5 J/cm^2. The power density was calculated according to the following formula: $(54 \times 70)/A=I$, and the fluence was calculated according to the following one:

$$I\ (W/cm^2) \times t\ (s) = F\ (J/cm^2)$$

After irradiation, PBS was removed, and 500 µL of cell culture medium was added to each well. Cells were incubated at 37°C, 5% CO_2 [11,15].

Clonogenic assay

No-adhered cells were discarded from the culture plate by washing with PBS at 37°C. Adhered cells were incubated with 100 µL of crystal violet solution for 4 min at room temperature. The plate was washed with water to remove excess dye, and 200 µL of elution solution (SDS - Sodium Dodecyl Sulfate) were added for incubation for 1 h before reading using a SpectraCount – Packard 570 nm Packard BioScience Company BS10001, Packard Instrument Company Inc). The whole process was carried out in the dark. Data collected were statistically analyzed [16,17].

Mitochondrial activity assay

Mitochondrial activity was evaluated by the colorimetric MTT [(bromide 3-4,5-dimethylthiazol-2-yl)-2,5-difeniltetrazoliol] (Sigma) assay. After PDT, 100 µL of MTT solution (5 mg/mL) was added to the wells and incubated for 2 h at 37°C in 5% CO_2. After this period, cells were incubated with 200 µL of DMSO (Dimethyl Sulfoxide) for 30 min under stirring. The absorbance was measured at 570 nm (A Packard BioScience Company BS10001, Packard Instrument Company Inc). The whole process was carried out in the dark. Data collected were statistically analyzed [15].

Immunostaining GRP-78

Cells were immunostained with GRP-78 Rabbit Polyclonal Antibody (PA5-34941 Molecular Probes, Thermo Fischer Scientific), detecting the expression of this Heat Shock Protein (HSP). After treatments, the medium was discarded. Cells were washed twice with PBS and fixed for 10 min in a solution containing paraformaldehyde and Triton X-100 (4.0% and 0.1% in PBS, respectively) at room temperature. Cells were washed with 0.1% bovine serum albumin (BSA – Sigma-Aldrich) in PBS to block non-specific binding sites. Then, cells were incubated with anti-GRP78 (1:1000, diluted in PBS)

for 1 h 30 min, at room temperature, protected from light. They were washed with PBS, incubated with the respective secondary antibody anti- rabbit FITC conjugated (1:1000, diluted in PBS) for 1 h 30 min, and washed again with PBS. Then, each well was added 10 µL ProLong TM Gold antifade reagents with DAPI (P36931, Sigma-Aldrich). Fluorescent staining was viewed under a fluorescence microscope (Leica Epifluorescence Microscope DMLB with a Leica DFC310FX model camera) [17,18].

Statistical analysis

Data are presented as mean with standard deviation compared by ANOVA two-way and confirmed by the Tukey test. Statistical significance was accepted at P<0.05 and no statistical adjustment was applied to the samples. Statistical analysis and graphs were performed using GraphPad Prism 6® software (GraphPad Inc., La Jolla, CA). All experiments were performed with sample number 9 being repeated three times separately. Thus, confirming the results and ensuring more excellent reproducibility.

Results

Hydrocortisone concentration curve

In Figure 1, in the 24 h, an increase in cell viability at 1.0 mM concentration can be observed compared to the control group with p=0.0351. However, the other concentrations do not present a statistical difference compared to the control group. After 48 h, it can be observed that all groups except the 2.5 mM concentration present a statistical difference when compared to the control group with p<0.0001. Thus, the concentration of 1.0 mM was defined as a stressor.

Cyclophosphamide concentration curve

In Figure 2, over the 24 h, an increase in cell viability at a concentration of 1.0 µg/mL can be observed with a statistical difference of p=0.0001 compared to the control group and at concentrations of 3.0 µg/mL to 25 µg/mL with p<0.0001, relative to the control group. There is no statistical difference at a 50 µg/mL concentration compared to the control group. Already at 100 µg/mL to 250 µg/mL concentrations, a reduction in cell viability with p<0.0001 can be verified compared to the control group.

In the 48 h, an increase in cell viability can be observed at a concentration of 1.0 µg/mL with a statistical difference p=0.0055, at concentrations of 3.0 µg/mL to 12 µg/mL with p<0.0001, and a concentration of 25 µg/mL there was an increase in cell viability with p=0.0055. There is no statistical difference at a 50 µg/mL concentration compared to the control group. Already at 100 to 250 µg/mL concentrations, a reduction in cell viability with p<0.0001 can be verified compared to the control group.

Thus, the concentration of 200 µg/mL was chosen for the other experiments since it is the concentration with the best efficiency in reducing cellular viability.

Cell density (clonogenic assay)

Analyzing Figure 3, after 24 and 48 h of treatment, a reduction in cell density can be observed in the CP, hydrocortisone + CP, PDT, hydrocortisone + PDT, CP + PDT groups, hydrocortisone + CP + PDT with p<0.0001 compared to the control group. However, the hydrocortisone group does not present a statistical difference compared to the control group.

Mitochondrial activity

After 24 h (Figure 4), an increase in mitochondrial activity can be observed in the hydrocortisone groups with p=0.0351, CP with p<0.0001, and hydrocortisone + CP with p=0.0290 compared to the control group. However, the PDT, hydrocortisone + PDT, CP+PDT, hydrocortisone + CP + PDT groups have reduced mitochondrial activity with p<0.0001 compared to the control group.

In the 48 h, mitochondrial activity increase can be observed in the hydrocortisone group with p<0.0001, compared to the control group. However, the hydrocortisone + cyclophosphamide, PDT, hydrocortisone + PDT, cyclophosphamide + PDT, hydrocortisone + cyclophosphamide + PDT groups have reduced mitochondrial activity with p<0.0001 compared to the control group. Except for the cyclophosphamide group, which did not present a statistical difference compared to the control group.

Immunostaining for GRP 78 protein

In Figure 5, after 24 h, it is possible to observe the absence of staining for GRP78 in the control, PCP and PDT+CP groups, with

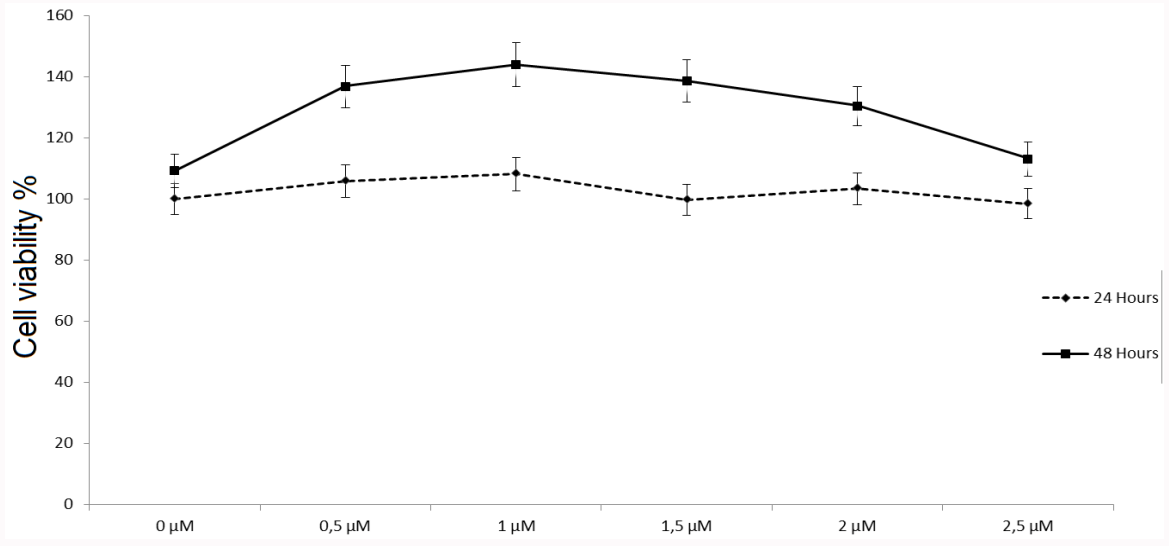

Figure 1: Hydrocortisone concentration curve on cell viability after an incubation period of 24 h and 48 h in HEp-2 cell culture; comparing the concentrations with the control group, the one that stands out corresponds to 1.0 mM with P<0.0001.

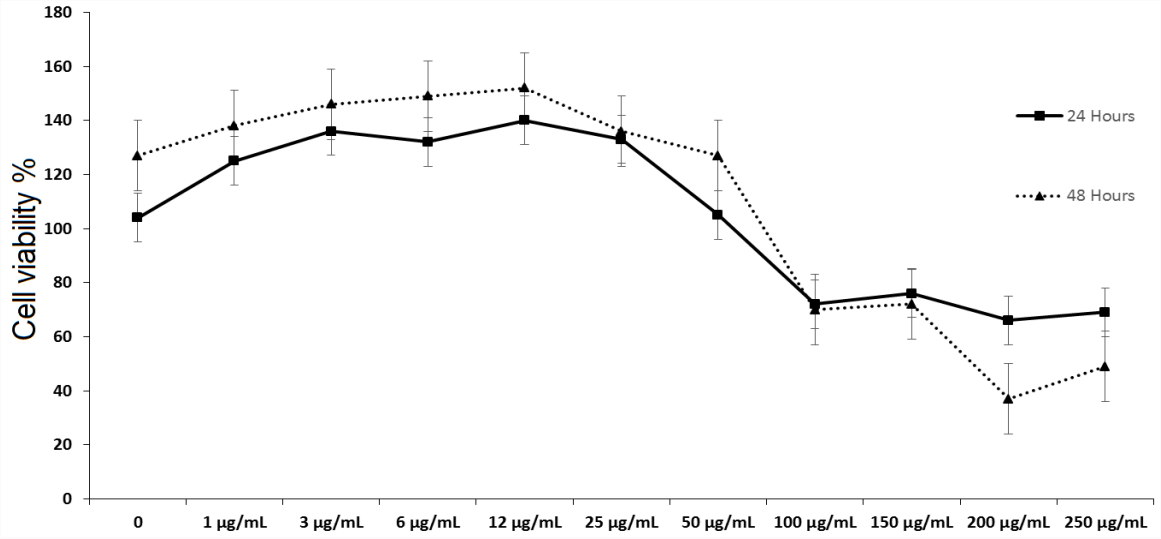

Figure 2: Cyclophosphamide concentration curve and cell viability after an incubation period of 24 h and 48 h in HEp-2 cell culture; the concentration of 200 µg/mL was chosen because it presented the best efficiency in reducing cellular viability.

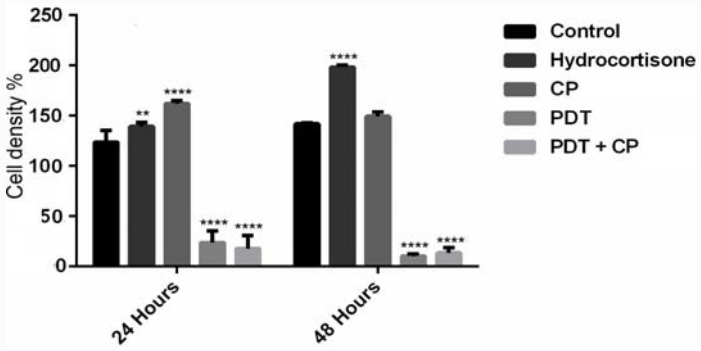

Figure 3: Cell density using the crystal violet staining clonogenic assay. In the 24 h, compared to the control group, they presented a statistical difference of P<0.0001. In the 48 h, the cyclophosphamide, PDT, and PDT + CP groups demonstrated a significant difference (P<0.0001) compared to the control group. However, the hydrocortisone group does not show statistical differences because it has similarities to the control group in both periods.

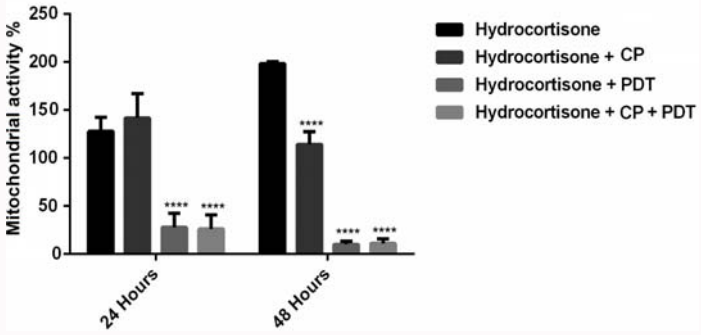

Figure 4: Evaluation of treatments in HEp-2 cell culture after 24 and 48 h; compared to the hydrocortisone group, the hydrocortisone + PDT and hydrocortisone + CP + PDT groups present a statistical difference of P<0.0001. However, the hydrocortisone + CP group did not demonstrate harmful activity. In the period and 48 h, all groups, when compared to the hydrocortisone group, showed a statistical difference of P<0.0001.

slight staining in the Hydrocortisone group. After 48 h, the control group remains negative for GRP78 staining, while the other groups show slight staining (Figure 6).

Discussion

Commonly associated with stress conditions, dysregulation of cortisol levels has also been considered a contributing factor associated with morbidity, severity, and mortality in disease processes, including a range of oncologic diseases, such as progression in breast tumors [2,20].

Thus, there is a strict interaction between the hormone cortisol, the onset of cancer, and its progression [1]. This interaction could be evidenced in this study, evaluating HEp-2 cells in the presence of hydrocortisone by the MTT assay (Figure 4); an increase in mitochondrial activity was observed after 24 and 48 h of treatment

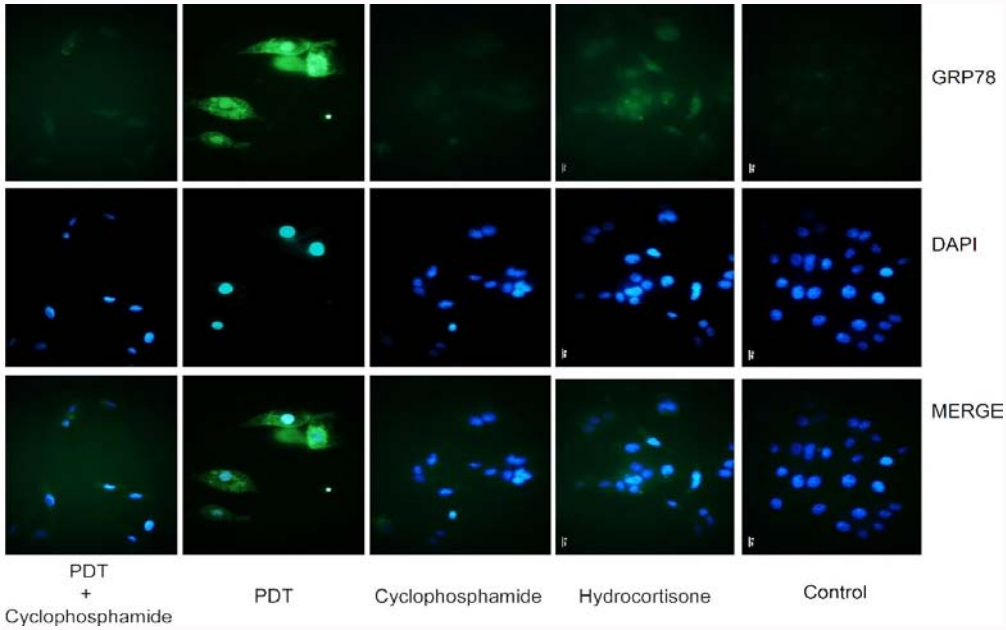

Figure 5: Immunofluorescence with antibody anti-GRP78 after 24 h. It can be observed that the control group does not show labels for GRP78, as well PDT+CP, and CP, while the Hydrocortisone group showed slight staining.

when compared to the control group. According to Bowin, 2015 the increase in energetic cellular requirement can be attributed to metabolic stress, occurring the regulation of the Nuclear Factor κB (NFkB), which will regulate the cellular response to the first stimulus, causing the production of chaperones proteins to restore the cellular protein apparatus, as well as the production of protein immunoglobulin (BiP), resulting in autophagy of subcellular structures; at the end of the process, the maintenance of cell occurs [21]. Moreover, the presence of cortisol analog favors tumor growth, confirmed by our crystal violet clonogenic assay results for 24 and 48 h (Figure 3). This data corroborates with Liu, 2015, who attested that stressed cells have altered tumor growth, modulating this pathology's classic pathways [22].

Metabolic stress induced by hydrocortisone in HEp-2 cells was verified by GRP-78 positive staining (Figure 5, 6), suggesting its great evolutional importance. However, it is not only a critical component of a complex defense mechanism but also plays a crucial role in the synthesis, folding, and degradation of proteins, providing preservation and cell survival under adverse conditions of cellular stress [23,24]. GRP-78, one of the HSPs of the chaperones class, plays a role in the regulation of calcium homeostasis, acting as a stress indicator for the endoplasmic reticulum, being important in the cryoprotection of cancer cells against toxic agents, free radicals, metabolic stress and especially the Reactive Oxygen Species (ROS) [25,26]. It also contributes to the inhibition of cell death by apoptosis, regulating cell repair, being associated with the pathological condition, recurrent disease, metastatic potential, and the survival of some clones in many types of cancers [25,27].

Consequently, it is essential the effective use methodologies against tumor cells. As demonstrated in Figure 3, CP efficiently kills cancer cells exposed to its action, decreasing cell viability within 24 and 48 h. This result corroborates with EMADI, 2009, which verified the potential of cyclophosphamide in eliminating cancer cells [6]. In agreement with the studies by Hertz, 2015 that demonstrated the viability of breast cancer cells, the effect on drug proliferation is cell-dependent, with Paclitaxel and CP being the chemotherapists that most significantly inhibited MCF-7 cell proliferation (>70%) compared to untreated control cells and cells treated by the other five agents, demonstrating the cytotoxic effect of CP on cell viability [28].

Thus, CP has the potential for cancer treatment. This compound regulates the cell cycle (limiting cell division and avoiding DNA duplication) and inactivates essential proteins necessary for DNA replication and RNA transcription [29]. Our results suggest that HEp-2 cells have phosphamidase enzymes due to the increased metabolic need of HEp-2 cells, measured by mitochondrial activity (Figure 4). According to Emadi, 2009 initial metabolic increase of the tumors is sensitive to the action of cyclophosphamide, producing at the end of the process a more toxic version of cyclophosphamide, phosphamide mustard, which also causes oxidative damage to the cellular structure [6].

It can be confirmed as cyclophosphamide can self-induce its activation by cytochrome P-450 (i.e., increased self-hydroxylation) in mitochondria, thereby increasing the cell's energy requirement and consequently increasing the evaluated cellular metabolism using the MTT test [30]. Also, it's observed that cyclophosphamide needs 48 h to be eliminated, thus explaining the normalization of mitochondrial activity in our results [6].

Studies by Trebunova, 2012 show that cyclophosphamide potentiates the anticancer effects of chemotherapeutic drugs such as docetaxel and doxorubicin, presenting higher cellular toxicity, decreasing cell viability (Figure 3), as well as altering the metabolic activity of cellular (Figure 4), thus regulating the tumor growth [31]. Sano, 2012 found that the combination of anthracyclines with CP has been a critical regimen in neoadjuvant chemotherapy for breast cancer and is the most effective treatment even for high-resistance cancer, as it demonstrates predictor markers of response as well as a putative model of regime response prediction using expression data, increasing treatment efficiency [32].

It is noteworthy that CP is cytotoxic to cancer cells but can also cause

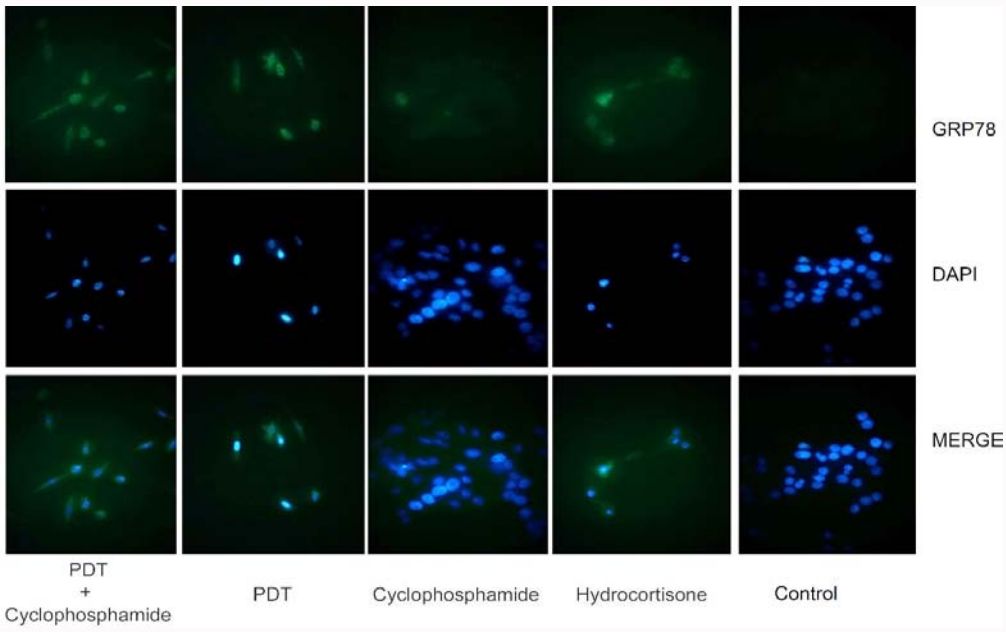

Figure 6: Immunofluorescence with antibody anti-GRP78 after 48 h. It can be observed that the control group does not show labels for GRP78, while the PDT+CP, and CP, Hydrocortisone group showed slight staining.

changes to cells with high cell division rates, so mature hematopoietic progenitors and all lymphocyte subgroups expressing low levels of ALDH1A1 can be affected by the action of CP. Thus, although leukopenia is common after treatment with cyclophosphamide, no dose of CP has been found to cause irreversible bone marrow aplasia.

Currently, chemotherapy treatments do not achieve the ultimate desired long-term effects. For this reason, developing and enhancing alternative therapeutic strategies are highly necessary. Thus, PDT is highlighted as a viable option for cancer treatment since it has a significant performance in the elimination of cancer cells, as seen in our mitochondrial activity (Figure 4) and cell viability assays (Figure 3). Moreover, Moraes, 2019 evidenced the ability of PDT to eliminate cancer cells, depleting the energetic capacity of the cell by decreasing mitochondrial activity and also by cell death, corroborating our violet crystal assay [11].

The combined treatment of cyclophosphamide with PDT showed exciting results. A reduction in mitochondrial activity was observed (Figure 4), and cell viability was reduced after the combined treatment (Figure 3), evidencing the potential of this joint strategy as a future alternative therapeutic model.

However, the possible influence of hydrocortisone, which causes cellular metabolic stress, has led to HEp-2 cell resistance to the treatments, mainly to the CP (Figure 4). According to Krishnamurthy, 2010, this occurs for modifying the tumor microenvironment after exposure to a stressor agent, such as hydrocortisone. However, HEp-2 cells may adapt to the stressed environment, resistant to treatment, especially to the chemotherapeutic agent (Figure 3) [33].

On the other hand, PDT is not influenced by hydrocortisone, as PDT and PDT + hydrocortisone results are statistically the same for both mitochondrial activity (Figure 4) and cell viability (Figure 3), demonstrating that PDT is effective in cancer cells.

Finally, the combination of CP + PDT in a stress environment (Hydrocortisone + CP + PDT) is statistically equal to PDT only group for both MTT (Figure 4) and crystal violet assays (Figure 3), highlighting the efficacy of PDT treatment in these evaluated parameters. However, cyclophosphamide also plays an important therapeutic role, being essential in the post-treatment, serving as a cell cycle regulator, limiting cell division, and prolonging the treatment efficiency [29].

Conclusion

Hydrocortisone alters the rate of cell death. In a stress-free environment, the efficiency of the chemotherapeutic CP is higher than in the environment containing hydrocortisone as a stressor agent. However, PDT changes its efficiency in none of the environments, constituting a practical methodology to eliminate tumor cells.

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