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Research Article

The Investigation of Efficiency of Pheophorbide-A Mediated Sonodynamic Therapy on Prostate Cancer 3d Cell Culture Model

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Abstract: The aim of this study is to investigate the effectiveness of fjorbid-a-mediated sonodynamic therapy in a 3D prostate cancer cell model. The effect of fjorbid-a-mediated sonodynamic therapy was examined by crystal violet staining in a 3D cell culture model created using human PC3 cells. Furthermore, apoptosis mechanisms were analyzed using Hoechst and propidium iodide staining (HOPI), and the levels of total oxidant (TOS) and total antioxidant (TAS) were assessed biochemically using corresponding kits. Crystal violet staining was employed to assess the effectiveness of sonodynamic therapy facilitated by pheophorbide-a, revealing a substantial 75% reduction in the viability of cancer cells. HOPI staining results indicated that there was no noteworthy increment in the count of apoptotic cells in the control, drug-only, or ultrasound-only groups. However, a remarkable 80% increase in apoptotic cell count was observed following pheophorbide-a-mediated sonodynamic treatment. Additionally, biochemical measurements demonstrated elevated levels of Total Oxidant Status (TOS) and decreased levels of Total Antioxidant Status (TAS) in the treatment groups in comparison to the control groups. Based on the acquired data, it was established that pheophorbide-a-induced sonodynamic therapy for prostate cancer treatment diminishes cell viability by inducing apoptosis through oxidative stress in a 3D cell culture system.

Keywords: 3D cell culture model; Pheophorbide-a; sonodynamic therapy; prostate cancer; PC3

3 Boyutlu Prostat Kanseri Hücre Modelinde Fiyorbid-A Aracili Sonodinamik Tedavinin Etkinliğinin Araştırılması

Özet: Bu çalışmanın amacı 3 boyutlu prostat kanseri hücre modelinde fiyorbid-a aracılı sonodinamik tedavinin etkinliğinin araştırılmasıdır. İnsan PC3 hücreleri kullanılarak oluşturulmuş 3D hücre kültürü modelinde fiyorbid-a aracılı sonodinamik tedavinin etkisi kristal violet boyama ile incelenmiştir. Ayrıca apoptoz mekanizmaları hoechst ve propidiyum iyodid boyama (HOPI) ile gerçekleştirilmiş, biyokimyasal değerlendirmeler ise total oksidan (TOS) ve total antioksidan (TAS) seviyeleri aynı isimli kitler

kullanılarak gerçekleştirilmiştir. Kristal violet boyama sonucunda fiyorbid-a aracılı sonodinamik tedavinin etkinliği incelenmiş ve kanser hücrelerin canlılığında %75 oranında azalma olduğu gözlenmiştir. HOPI boyama sonuçlarına göre kontrol ve sadece ilaç ya da sadece ultrases gruplarında apoptotik hücre sayısında anlamlı bir artış gözlenmemişken, fiyorbid-a aracılı sonodinamik tedavi sonrası apoptotik hücrelerin sayısında %80 oranında artış görülmüştür. Son olarak biyokimyasal ölçümler sonucunda kontrol gruplarına kıyasla tedavi gruplarında TOS seviyelerinde artış, TAS seviyesinde azalma gözlenmiştir. Elde edilen verilerden, prostat kanseri tedavisinde fiyorbid-a aracılı sonodinamik tedavinin hücre canlılığını oksidatif stres üzerinden apoptoza yol açarak azalttığı belirlenmiştir.

Anahtar Kelimeler: 3D hücre modeli; Fiyorbid-a; Sonodinamik Tedavi; Prostat kanser; PC3

1. Introduction

Prostate cancer ranks as the second most prevalent cancer among males, resulting in approximately 1.3 million deaths worldwide [1]. Despite its high incidence, our understanding of its metastasis and pathogenesis remains limited, necessitating the utilization of various investigative approaches. In recent years, alongside traditional two-dimensional (2D) in vitro and in vivo studies, three-dimensional (3D) cell culture methods have emerged and gained attention as they offer a more comprehensive representation of the disease. The incorporation of 3D cell culture models in cancer research has garnered increasing interest, primarily due to their ability to recreate tissue architecture and the extracellular matrix (ECM), which play significant roles in shaping tumor cell responses to microenvironmental cues [2]. These models hold particular importance in generating scientifically robust data, as they closely mimic in vivo conditions. Specifically, 3D cell culture models emulate the ECM of tissues through various techniques, enabling improved representation of cell-cell and cell-matrix interactions compared to conventional 2D cell culture models [3].

The management of prostate cancer typically involves the utilization of treatment modalities such as surgical interventions, radiation therapy, and hormone therapy. Nevertheless, the side effects associated with these interventions have a substantial impact on the patients' quality of life. For this reason, there is a need for different, non-invasive and less toxic alternative treatments to be an alternative to prostate cancer treatment [4,5]. Sonodynamic therapy (SDT) is an emerging and evolving therapeutic approach among alternative modalities, aiming to induce cellular demise through the activation of an ultrasound-responsive agent at specific frequencies [6]. SDT has emerged as a therapeutic strategy that draws its foundation from the fundamental principles of photodynamic therapy (PDT). SDT aims to provoke cell death and/or apoptosis by leveraging the generation of reactive oxygen species (ROS), with a specific emphasis on singlet oxygen. This process occurs through the interaction between photosensitizing agents and light, thereby facilitating the production of ROS [7]. The principal mechanism underlying SDT can be attributed to the non-thermal effect, specifically the phenomenon known as cavitation. Cavitation arises from the generation, expansion, and subsequent collapse of microbubbles within an environment stimulated by ultrasound. Essentially, under the influence of ultrasonic irradiation, certain microbubbles within the liquid medium experience growth and contraction, leading to ultrasonic cavitation. This process ultimately results in the production of free radicals (including H, OH, H2O2, and superoxide) through the breakdown of water molecules [8]. On the other hand, produced free radicals induce apoptotic pathways and cause cells via apoptosis pathway [9–11]. In summary, the aim of this study was to evaluate the efficacy and elucidate the underlying mechanisms of pheophorbide-a-mediated sonodynamic therapy in a three-dimensional (3D) cell culture model of prostate cancer. This study stands as a pioneering investigation in the field, as it represents the first exploration of sonodynamic therapy specifically within the framework of 3D cell culture models.

2. Material and Methods

Propidium iodide, Pheophorbide-a and Hoechst 33342 dye were procured from Sigma Aldrich (Taufkirchen, Germany) for experimental purposes. The PC3 cell line, derived from human prostate cancer, was procured from Biolegend (San Diego, CA) and ATCC (Manassas, VA) for the purpose of this study.

2.1. 2D Cell Culture

In order to examine the influence of pheophorbide-a-mediated sonodynamic therapy on 3D cell culture, RPMI 1640 medium (Sigma-Aldrich, Germany) was used to employed and cultured on prostate cells. The growth medium was composed of 10% FBS (fetal bovine serum, Sigma-Aldrich, Germany), 1% L-glutamine, and 1% streptomycin- penicillin (Sigma-Aldrich, Germany). The cells were initially seeded in a 75 cm^2 flask and maintained at a temperature of 37°C in a humidified incubator with 5% CO_2 in air until they reached an approximate confluence of 80%. Upon reaching this confluence, the cells were harvested to facilitate subsequent experimental procedures.

2.2. 3D Cell Culture

Once 1% agarose gel was prepared for 3D cell culture model. For this, 1 gram of agarose was weighed and placed in a 250 mL Erlenmeyer flask. Then, 100 mL of distilled water was added and this mixture was heated in a microwave oven until the agarose dissolved. After the gel-like mixture reached 40 °C, 6-well microplates were covered with agarose, with 2 mL of agarose in each well. Then, the prepared PC3 cells were seeded into the wells as drops with 5000 cells in each well. Commercially available ECM was then seeded according to the kit descriptions.

2.3. Pheophorbide-a-mediated Sonodynamic Treatment

Following the preparation of the 3D cell model, the cells were randomly allocated to different experimental groups, including a control group, a pheophorbide-a (Pha) alone group, an ultrasound alone (US) group, and a group receiving combined treatment with Pha and ultrasound (SDT). Subsequently, varying concentrations of Pha (0, 0.1, 0.2, 0.5, 1, and 5 µM) were added to the respective groups, and the cells were incubated for a duration of 4 hours. Following the designated incubation period, the cells underwent ultrasound treatment utilizing a Sonidel SP100 sonoporator (Sonidel Ltd., Ireland) that operated at an intensity of 0.5 W/cm2 and frequency of 1.0 MHz. The ultrasound treatment lasted for 60 seconds, resulting in the formation of the SDT and ultrasound-alone groups. Following the treatment, the cells were cultured in fresh medium and allowed to incubate for an additional 24 hours.

2.4. Cytotoxicity Tests

The cytotoxicity of Pha-mediated SDT was evaluated using crystal violet staining. Following the treatment period, each well was supplemented with 40 µl of MTT dye and incubated for a duration of 4 hours. Subsequently, the cell medium was aspirated, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The spectral absorbance of the specimens was assessed within the 560-620 nm wavelength spectrum employing a plate-reading apparatus (Thermo Multiskan SPECTRUM, Waltham, MA). All trials were iterated thrice, and the mean absorbance value from the triplicate measurements was employed for determining cell viability through the subsequent equation (Equation 1.):

Equation 1:

$$\% \text{ Cell Viability: } (OD_{control} - OD_{sample}) / (OD_{control} - OD_{blank})$$

2.5. Detection of Cell Apoptosis

The assessment of the apoptotic pathway involved the utilization of staining techniques such as Hoechst 33342 and propidium iodide. Subsequent to the treatments, the cells were collected, and a 50 µl cell suspension was combined with a mixture of Hoechst 33342 and propidium iodide (5 µl). The mixture was gently mixed and incubated for 1 hour to enable accurate measurement of apoptosis. Following this, a volume of 30 µl from the cellular solution was applied onto a slide and examined using a fluorescent microscope (Olympus BX51, Tokyo, Japan) for imaging objectives. The quantification of viable, apoptotic, and necrotic cells was performed utilizing ImageJ software (NIH, MD, USA).

2.6. Biochemical Tests

Biochemical analyses were performed to evaluate the Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) utilizing specific assay methods. The TAS level in cell lysates was determined using a commercially available kit from Rel Assay (Rel Assay Kit Diagnostics). Trolox, a water-soluble analog of vitamin E, was utilized as the calibration standard for quantification. The results were expressed as millimoles of Trolox equivalents per liter (mmol Trolox equiv./L). The TOS level in cell lysates was measured using another commercial kit from Rel Assay (Rel Assay Kit Diagnostics), employing hydrogen peroxide as the calibration standard. The results were expressed as micromoles of H2O2 equivalents per liter (µmol H2O2 equiv./L).

2.7 Statistical analysis

Statistical analysis was carried out employing GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Disparities in variances were evaluated through one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as a post-hoc analysis. Statistical significance was defined as p-values equal to or less than 0.05. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. Cytotoxic Effects of Pheophorbide-a-Mediated Treatments on Prostate Cancer Cells

The cellular morphological changes in control, drug-only, and Pha-mediated SDT groups were examined under light microscopy (Figure-1). The statistically significant reduction in cell count observed within the treatment group is consistent with the results obtained from 3D models (Figure-1b). The cytotoxic impact of pheophorbide-a (Pha)-mediated sonodynamic therapy was evaluated using the MTT assay, and the results showed that the cytotoxic effects of Pha alone and ultrasound alone did not elicit significant outcomes on prostate cells (Figure 2a-2b). Conversely, the combined application of Pha and sonodynamic therapy (Pha-SDT) exhibited a substantial reduction in cell viability, specifically targeting prostate cancer cells (Figure-2c) ($p < 0.0001$).

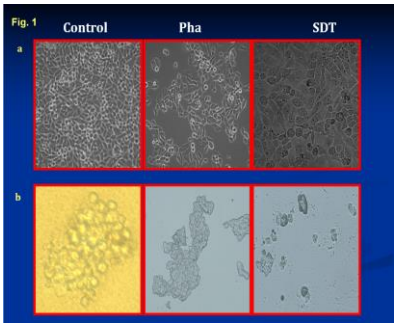

Figure 1. 2D and 3D morphological images of PC3.

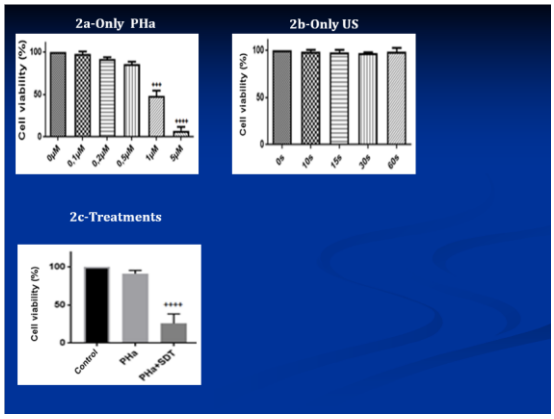

Figure 2. The cytotoxic efficacy of a) drug alone, b) US alone, and c) SDT treatments on PC3 cells was evaluated.

The data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the One-Way ANOVA test, followed by Dunnett's multiple comparison test as a post-hoc analysis. P-values equal to or less than 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$) compared to the untreated control group.

3.2. Induction of Apoptosis in PC3 Cells Following Treatment

Hoechst 33258 and propidium iodide dyes were utilized for the investigation of the effects of treatments on the apoptosis mechanism and the results are presented in Figure 3. The results illustrate that the groups subjected to SDT, PDT, and Simultaneous Sonodynamic and Photodynamic Therapy (SPDT) exhibited a notable elevation in apoptotic cell populations compared to the other groups ($p=0.0381$) (Figure-3). Remarkably, the SDT group demonstrated the highest increase in apoptotic cells, aligning with the findings obtained from the MTT assay.

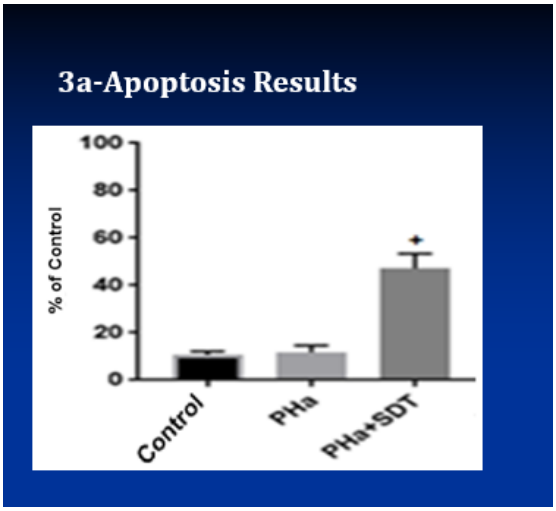

Figure 3. Apoptosis assessment was conducted by determining the percentage of apoptotic cells in PC3 cells.

The data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the One-Way ANOVA test, followed by Dunnett's multiple comparison test as a post-hoc analysis. P-values equal to or less than 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$) compared to the untreated control group.

3.3. Results of Biochemical Analysis Following Treatments

Consistent with prior research, it is well-established that the generation of oxygen species during SDT can elicit necrosis or apoptosis in PC3 cells. The degree of membrane lipid oxidation can be evaluated by measuring the levels of Total Antioxidant Status (TAS) and Total Oxidant Status (TOS). In our study, we observed a decrease in TAS levels across all drug and SDT groups, accompanied by an increase in TOS levels. Notably, cells treated with 1 µM Pha and 1 µM Pha-mediated SDT exhibited a substantial reduction in TAS levels and a pronounced elevation in TOS levels (Table-1).

Table 1. *The biochemical findings of the control, drug-alone, and treatment groups were assessed.*

	TAS (mmol Trolox Equicv./L)	**TOS (**µmol H_2O_2 Equiv/L)
Control	1.66	2.34
Ultrasound	1.54	6.4
Drug 0.25µM	0.97	8.19
Drug 1µM	1.1	7.8
Drug 0.25µM + Ultrasound	1.49	9.18
Drug 1µM + Ultrasound	0.68	9.42

4. Discussion

This study represents the inaugural investigation, elucidating the comparative antitumor effect and potential underlying mechanism of Pheo-mediated SDT using a 3D cell model of prostate cancer. In the last days, three-dimensional (3D) cell culture models has a great attention due to its ability to better mimic the in vivo and microenvironment of in vitro cultured cancer models [3,12]. 3D models better than 2D models to show basic properties of cell morphology, cell proliferation, cell differantion and others [13]. Additionally, 3D models are important for ethical problems of animals using in cancer studies [14]. 3D cancer models are obtain for many cancer types as prostate cancer by using with different procedures [15]. Grayson et al. established that three-dimensional (3D) cultures outperform 2D cell culture models due to their ability to physiologically mimic the intricate cell-cell and cell-matrix interactions observed in solid tumors. Moreover, this approach exhibits high reproducibility, enabling more efficient characterization of drug testing and potentially closing the disparity between in vitro and in vivo experiments. Consequently, these advancements hold promise for the development of patient-specific therapies [16]. Safari et all suggested that human amniotic mesenchymal stromal cells (hAMSCs) were good candite for inhibitation prostate cancer cells in 3D cell culture [17]. In another study, it was shown in

3D cell culture prostate cancer model that androjen receptor and filamin A complex a new suitable marker for prostate diagnosis and treatment [18]. As a result, 3D models are suitable and great potential

for cancer researches. As our knowledge, this is the first study of investigation sonodynamic therapy of 3D cell culture model.

Because of prostate cancer heterogeneous a wide range of treatment models are using in prostate cancer treatment. In clinical settings, localized prostate cancer is typically managed through surgical intervention, brachytherapy, or external beam radiotherapy. Nevertheless, these therapeutic interventions come with diverse adverse effects, including urinary incontinence, erectile dysfunction, and the risk of collateral damage to adjacent organs and healthy tissues [19,20]. Consequently, there has been an increasing focus on the development of alternative therapies, such as sonodynamic therapy, in recent years, aiming to address the limitations associated with the existing treatment modalities for prostate cancer [21–24]. Sonodynamic therapy is a new and minimally invasive anti-cancer therapy that uses synergetic efficiency of therapeutic ultrasound and a chemical agent called sonosensitizer [24]. In addition, using therapeutic ultrasound in sonodynamic therapy provide an advantage in deeper tumor like prostate cancer [25]. The scientific literature contains a limited number of studies exploring the efficacy of SDT mediated by various sonosensitive agents for specific cancer types [26,27]. Pha is used in some SDT studies [6,23,28], However, this study represents the first instance of demonstrating the effectiveness of pheophorbide-a (Pha)-mediated SDT on a three-dimensional (3D) cell model of prostate cancer. Umemura et al. conducted investigations under both in vivo and in vitro conditions, proposing Pha as a promising sensitizer for SDT in cancer treatment. The results of their investigations provided additional evidence demonstrating that pheophorbide-a-mediated SDT induced cellular damage in both in vitro and in vivo sarcoma cell models, thereby underscoring its superior efficacy when compared to hematoporphyrin [7]. In addition, Jin investigated Pha-mediated SDT on mouse squamous cell carcinoma (SCC) model and they found that this modality could be very useful for treatment of non-superficial or nodular tumors [29]. The administration of pheophorbide-a (Pheo) at a concentration of 50 μg/mL has been shown to lack notable cytotoxic effects on normal human umbilical vein endothelial cells (HUVEC) [30]. However, when Pheo concentrations range from 3 to 5 μM, it has been observed to hinder the growth of LNCaP cells [31]. Moreover, Xu et al. [32] have proposed that Pheo exhibits toxicity on LNCaP cell lines at concentrations exceeding 4.8 μM. Consistent with these observations, our assessments of cell viability demonstrate that pheophorbide-a-mediated SDT hinders cell proliferation in PC3 prostate cancer cell lines. This outcome aligns with earlier SDT investigations that utilized distinct cell lines and sensitizers [7,29].

5. Conclusion

The primary objective of this study was to explore the potential of pheophorbide-a (Pha)-mediated sonodynamic therapy in a three-dimensional (3D) cell model of prostate cancer. PC3 prostate cancer cell lines were utilized in this investigation, known for their aggressive nature and androgen independence. The findings indicate that Pha-mediated SDT significantly reduced the viability of cancer cells. This effect may be attributed to the generation of oxygen species, which potentially induce apoptosis and influence apoptotic pathways, thereby contributing to the production of apoptotic cells following the treatments. In conclusion, this study highlights the potential of Pha as a promising therapeutic approach when combined with sonodynamic therapy.

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Conflict Of Interest Declaration

As the authors of this article, we declare that there is no conflict of interest. Therefore, the content of this study has been presented in a neutral and objective manner.

Research And Publication Ethics Statement

The authors affirm that this study adheres to the principles of research and publication ethics.

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