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Ht-29 Hücrelerinde 5-ALA Kullanılarak Tümör Floresan Görüntüleme Yöntemi

Tumor Florescence Imaging Method Using 5-ALA in Ht-29 Cells

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ÖZ

Amaç: 5-aminolevulinik asit olarak da bilinen 5-ALA, hemoglobinin önemli bir bileşeni olan heme ve vücuttaki çeşitli enzimlerin biyosentezinde çok önemli bir rol oynayan doğal olarak oluşan bir amino asittir. Mitokondride glisin ve süksinil-CoA'dan sentezlenir. Spesifik olarak, fotodinamik tanı (PDD) ve fotodinamik terapi (PDT) adı verilen teknikte bir ışığa duyarlılaştırıcı olarak kullanılır. Fotodinamik tanıda (PDD), 5-ALA tümör hücreleri veya kanser öncesi lezyonlar gibi belirli doku veya hücrelerde seçici olarak birikir. Spesifik bir dalga boyundaki ışığa maruz kaldığında, biriken 5-ALA, bu hedef alanlarda floresana neden olarak beyin cerrahisi veya üroloji gibi cerrahi prosedürler sırasında gelişmiş görselleştirme ve algılamaya olanak tanır. Bu çalışmanın amacı, kolon kanserinde 5-ALA kullanılarak optimal fotodinamik tanı koşullarını belirlemektir.
Yöntem: HT-29 hücre hattına 3 saat farklı 5-ALA (100, 200, 300, 500, 1000, 1500 µM) konsantrasyonları uygulanmıştır. Uygulanan hücrelerde hücre canlılığı, floresans şiddeti, apoptoz analizleri yapıldı.
Bulgular: Kontrol grubu ile düşük 5-ALA dozları (100,200 ve 300 µM) arasında hücre canlılığı açısından fark yokken, yüksek 5-ALA dozlarında (1000 ve 1500 µM) anlamlı fark bulundu (p<,0001) 5-ALA dozları floresan şiddeti ile paralel olarak artmış ve en yüksek floresans şiddeti 1500 µM 5-ALA'da olmuştur (p<,0001). Apoptoz/ölü oranı, floresans yoğunluğunun en yüksek olduğu 1000 µM ve 1500 µM 5-ALA'da anlamlı olarak en yüksek olduğu gösterildi (p<0.05).
Sonuç: HT-29 hücrelerinde 5-ALA konsantrasyonunun optimum dozu 500 µM olduğu belirlendi. 5-ALA'nın yüksek konsantrasyonlarının HT29 hücrelerinde apoptoza neden olduğu gösterilmiştir.

Anahtar Kelimeler: 5-aminolevulinik asit (ALA), Floresans görüntüleme, Tümör hücreleri, Apoptoz, Fotodinamik Tanı.

ABSTRACT

Objective: 5-ALA, also known as 5-aminolevulinic acid, is a naturally occurring amino acid that plays a crucial role in the biosynthesis of heme, a vital component of hemoglobin and various enzymes in the body. Specifically, it is used in technique called photodynamic diagnosis (PDD) and photodynamic therapy (PDT) as a photosensitizer. When exposed to a specific wavelength of light, the accumulated 5-ALA causes fluorescence in these target areas, allowing for enhanced visualization and detection during surgical procedures, such as in neurosurgery or urology. The purpose of this study was to evaluate the conditions for optimal photodynamic diagnosis using 5-ALA in colon cancer.
Methods: HT-29 cell line which was administered different 5-ALA (100, 200, 300, 500, 1000, 1500 µM) concentrations for 3 hours incubation time, were performed on cell viability, fluorescence intensity, apoptosis analysis.
Results: While there was no difference in cell viability between the control group and low 5-ALA doses (100,200 and 300 µM), a significant difference was found at higher 5-ALA doses (1000 and 1500 µM) (p<,0001). 5-ALA doses increased in parallel with the fluorescence intensity, and the highest fluorescence intensity was at 1500 µM 5-ALA (p<,0001). The apoptosis/dead ratio was significantly showed to be highest at 1000 µM and 1500 µM 5-ALA which had the highest fluorescence intensity (p<0.05).
Conclusion: the optimum dose of 5-ALA concentration was determined to be 500 µM in HT-29 cells. High concentrations of 5-ALA have been shown to cause apoptosis in HT29 cells.

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Key words: 5-aminolevulinic acid (ALA), Florescence Imaging, Tumor cells, Apoptosis, Photodynamic Diagnosis.

1. INTRODUCTION

Photodynamic diagnosis (PDD) is a specific application of fluorescence imaging in the field of medicine, primarily used for cancer detection. PDD combines the administration of a photosensitizing agent, such as 5-aminolevulinic acid (5-ALA) or other related compounds, with fluorescence imaging techniques (1). The photosensitizer preferentially accumulates in tumor tissue or abnormal cells. When the tissue is stimulated with light of a specific wavelength, the photosensitizer becomes activated and emits fluorescence. This fluorescence can help distinguish between healthy and diseased tissue during surgical procedures. In PDD, the goal is to enhance the visualization and identification of cancerous or abnormal tissue during surgery (2). By administering the photosensitizer prior to the procedure and using fluorescence imaging, surgeons can better identify tumor margins and potentially improve the precision of tumor removal. The fluorescence emitted by the photosensitizer provides real-time feedback to guide the surgical intervention (3–5).

5-ALA, also known as 5-aminolevulinic acid, is a chemical compound that has gained significant attention in medical and scientific applications (6–9). It is a precursor molecule in the biosynthesis of heme, a vital component of hemoglobin, myoglobin, and various enzymes involved in oxygen transport and energy production in the body (10). In the context of PDD, 5-ALA is administered to a patient, and it is preferentially taken up by certain cells or tissues, such as cancer cells or precancerous lesions. After a period, the accumulated 5-ALA is converted into a fluorescent compound called protoporphyrin IX (PpIX). When illuminated with a specific wavelength of light, PpIX emits fluorescence, allowing for the visualization and detection of abnormal cells or tissues (11).

One of the notable applications of 5-ALA is in medicine, particularly in the field of neurosurgery and dermatology. When administered as a prodrug, 5-ALA has the unique ability to selectively accumulate in certain tissues, such as malignant gliomas (a type of brain tumor), certain skin lesions, gastric and colon cancer (11). This property allows for a technique known as 5-ALA fluorescence-guided surgery. It is important to note that the specific applications and uses of 5-ALA may vary based on ongoing research and clinical developments (12). It is favored in some medicinal applications due to several advantages over other photosensitizers, including the following: (I) Selective Accumulation: In cells, 5-ALA undergoes metabolism and is changed into the photosensitive substance protoporphyrin IX (PpIX). The propensity of 5-ALA to concentrate preferentially in cancer cells and some dysfunctional organs is a benefit. The specificity of PDD may be improved by this selective accumulation, enabling more accurate differentiation between cancer and noncancer tissues. (II) Lower Systemic Toxicity: 5-ALA has a lower systemic toxicity than certain other photosensitizers. This indicates that when provided, it has fewer negative impacts on the patient's general health. It is typically well tolerated because of its metabolic route's connection to the body's normal heme production system. (III) Fast Clearance: PpIX's quick digestion and elimination usually prevent excessive accumulation in healthy or noncancer tissues, reducing the risk of harm to normal cells. This prompt approval enhances the safety profile of 5-ALA in PDD. (IV) Non-Invasive Method: PDD utilizing 5-ALA is a non-invasive method that may be carried out instantly. This helps surgeons accurately remove diseased tissue while causing the least amount of harm to healthy

tissue during surgical procedures. It is especially useful for identifying and defining tumor margins (6-7-9).

In our previous study, we have demonstrated quantitative evaluation of gastrointestinal cancer (AGS, MKN-28, and HT-116 cells line) cell motility and 5-aminolevulinic acid (5-ALA)-induced fluorescence in vitro using mathematical morphology and structural analysis methods (13). Therefore, this study has now aimed to determine the conditions required for the most effective photodynamic diagnosis performed by diagnosing fluorescence caused by 5-ALA-stimulated PpIX in the colon cancer (HT-29) cell line in vitro.

2. MATERIALS AND METHODS

Materials

Muse Caspase 3/7 Kit (MCH100108, EMD Millipore, Billerica, MA, USA) was purchased from Luminex. 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT; M5655, Tufkirchen, Germany), RPMI-1640 (R8758, Taufkirchen, Germany), fetal bovine serum (FBS; F2442, Taufkirchen, Germany), penicillin-streptomycin (P/S; P4333, Taufkirchen, Germany), l-glutamine (G7513, Taufkirchen, Germany), dimethyl sulfoxide (DMSO; D4540, Taufkirchen, Germany), trypsin/edta solution (T4049, Taufkirchen, Germany), 5-Aminolevunic Acid (5-ALA; A7793; Taufkirchen, Germany) were purchased from Sigma Aldrich. HT-29 colon cell line (Manassas, VA, USA) was purchased from ATCC respectively.

Cell Culture and Culture Methods

HT-29 cells were seed at $1x10^6$ cells/well in 24 well plate and incubated for 24 h in an atmosphere of 5% CO_2 at 37 °C. After incubation, the cells were treated with 0,100, 200, 300, 500, 1000 and 1500 μM 5-ALA for 3 h in in the dark at 37 °C in 5% CO_2. The cells were washed with PBS twice and they were examined under white light source with a filter that passes the blue light wavelength. The light source's output was 3 watts, and the light exposure time was 2 minutes. Fluorescence invert microscopy (Zeiss, Axio vert. A1 with axiocam 208 camera) was used to detect the fluorescence of 5-ALA in cells. Fluorescence images were taken twice from the same indiscriminately selected field. To detect the cell area, we took a bright-field photo. To calculate fluorescence intensity, the Image J method was used (14). Randomly 30 cells area was selected, and for the background, selected a non-fluorescence area. According to this method below, fluorescence intensity was calculated the corrected total cell fluorescence (CTCF):

CTCF = Integrated Density – (Area of selected cell x Mean fluorescence of background readings.

Cell Viability Test

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was performed to evaluate the toxicity of 5-ALA in cells. After 24 h the viability of 5-ALA-treated cells was determined. One hundred microliters of MTT were added to the solution (5 mg/ml), and the cells were incubated for a further 4 h at 37 °C in the dark. After that, 1000 μl dimethyl sulfoxide

(DMSO) was added. The absorbance at 570 nm was recorded by using a microplate reader (BioTek, Winooski, USA).

Caspase 3/7 Assay

We used the Muse Caspase-3/7 Kit to distinguish cells to produce the apoptotic ratio after 5-ALA treatment. Briefly, after HT-29 cells cultured in 12-well plates were treated with 1000 µM, 1500 µM in medium alone (control) 5-ALA for 3 h in 5% CO_2 at 37 °C, and images of cells were taken, cells were incubated for 24 h. They collected with trypsin. The kit adjusted cell concentration to $3x10^5$ cells/ml in 50 µl of 1X Assay Buffer BA. Next, 5 µl of Muse Caspase-3/7 Reagent working solution was added to the cells and incubated in 5% CO_2 at 37 °C for 30 min. 150 µl of Muse Caspase 7-AAD working solution was added and mixed. Cells were analyzed by the Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA) (15)).

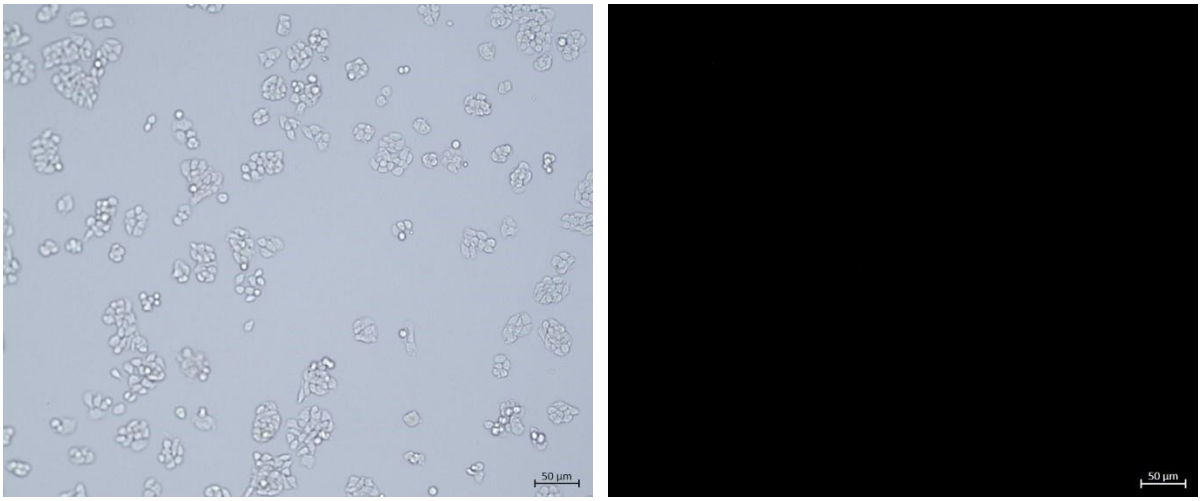
Statistical Analysis

The behavioral data were analyzed using one-way analysis of variance (ANOVA) to test whether there is any difference between at least two groups, and a significant difference between at least two groups was detected by using real data and the statistical software package SPSS (SPSS Inc., USA). Tukey test was applied as a post-doc test, and a statistical evaluation was made. The data are expressed as mean ± standard error of the mean (SEM) at a probability level (0.05).

3. RESULTS

Fluorescence Image Analysis

After cells were treated with 5-ALA (0, 100, 200, 300, 500, 1000, and 1500 µM) for 3 h, images were taken on fluorescence microscopy. To understand whether fluorescence signals in cells are in the area or not, we merged images taken with both light microscopy and fluorescence microscopy. We focused specifically on cell nuclei during taking photos of the fluorescence of 5-ALA (Fig 1) (Fig 2).



A B

Figure 1: Control group in HT-29: in vitro. Imaging was performed under white light (A) and excitation light (B) (excitation: 440 nm; emission: 575–675 nm).

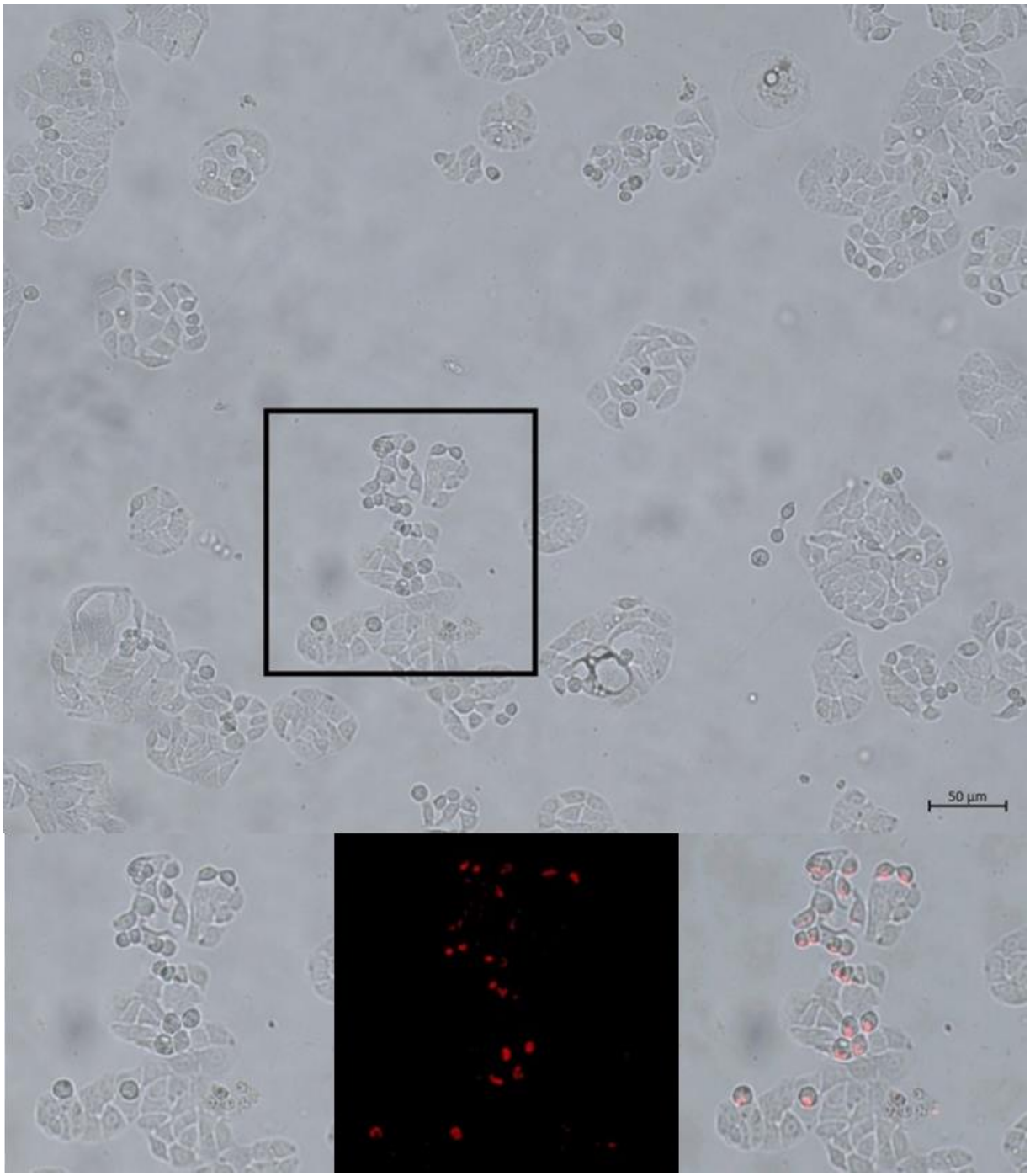


Figure 2: Accumulation of PpIX after administration of 5-ALA in HT-29, with 500 µM 5-ALA for 3 h. PpIX fluorescence was detected by fluorescence microscopy. Detection of areas covered by the cells: marked area of the bright-field image (top), selected zoom area (bottom left), fluorescence area after 5-ALA treat (middle), merged image (bottom right).

After taking the fluorescence of 5-ALA, in the ImageJ program, CTCF values from all images were analyzed as described above. According to the results, there were no significant differences between 100 µM and 200 µM, after 300 µM 5-ALA concentration, whereas all the 5-ALA concentrations' CTCF values are different (p<,0001) (Fig. 3A).

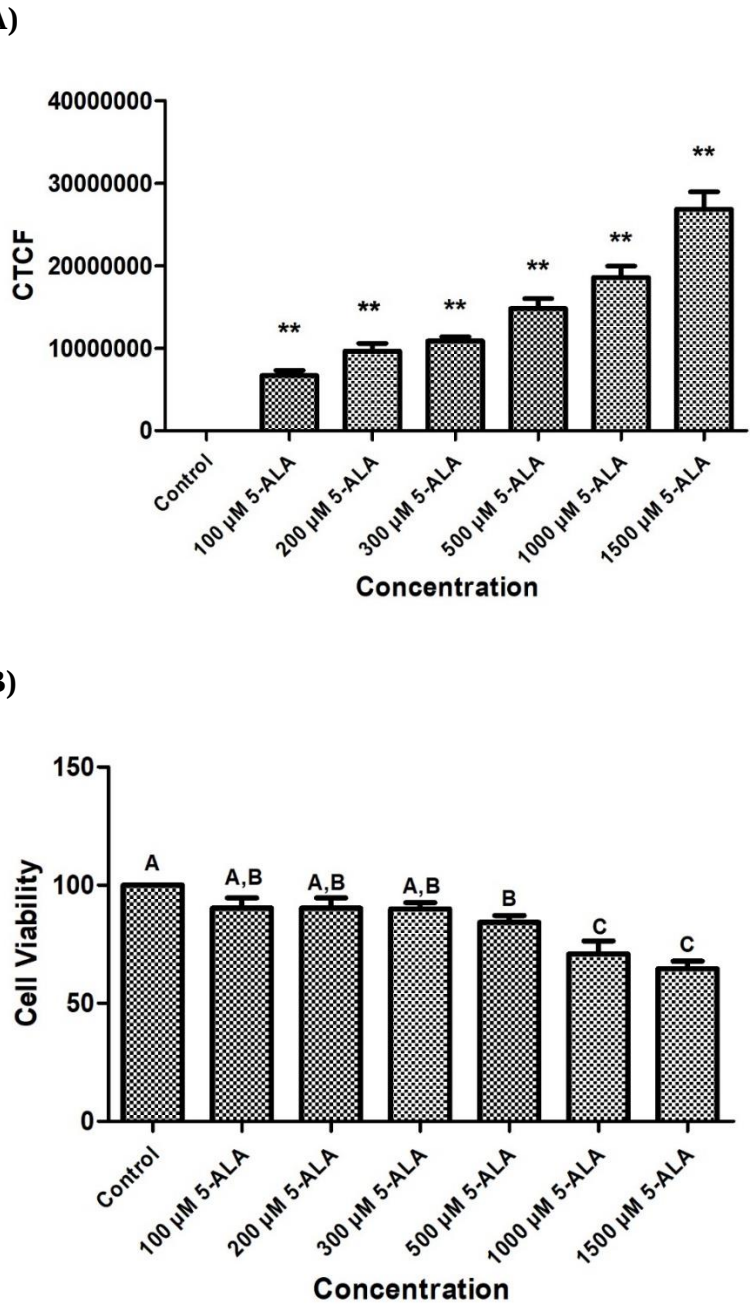


Figure 3: Corrected total cell fluorescence values of PpIX (A) and cell viability (B) in all of the 5-ALA concentrations in the HT-29 cell line. (A) CTCF values of PpIX in HT-29 cell line (**p<,0001). (B) Cells were treated with different concentrations of 5-ALA: 100 µM, 200 µM, 300 µM, 500 µM, 1000 µM, 1500 µM in serum-free medium or medium alone (control) (A, B, C: p<,0001).

Cytotoxic of 5-ALA on HT-29 Cells

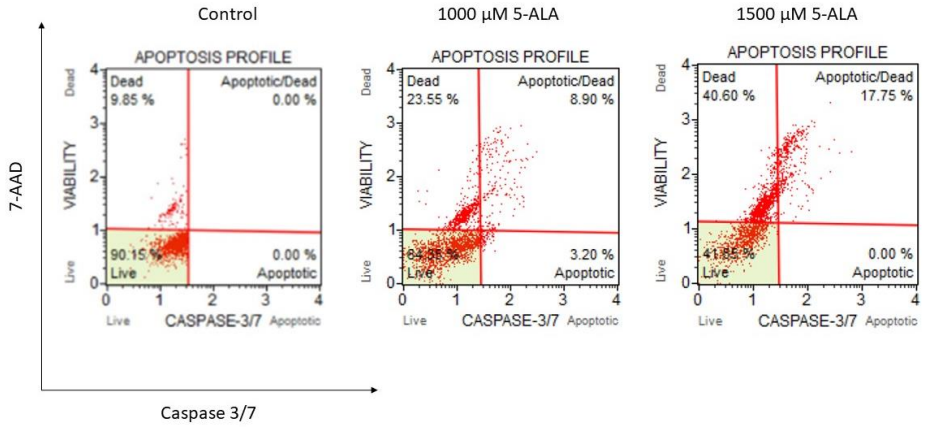
After 24 h HT-29 cells were treated with 5-ALA, and cell viability was measured by MTT (Fig. 3B). HT-29 cells showed a gradual decrease in viability dependent on the 5-ALA concentrations, up to 500 µM. Although a statistically significant change in cell viability was

not recorded among control, 100 µM, 200 µM, and 300 µM 5-ALA concentrations, a significant decrease at 1000 µM 5-ALA compared with 500 µM 5-ALA was recorded (p<,0001).

Apoptosis Measurement by Caspase 3/7 Assay

The apoptosis ratio was measured by Muse Caspase 3/7 assay. Cells were treated with 1000 µM, 1500 µM 5-ALA, and control for 3 h. Figure 4 exhibited an explicit change of apoptosis profile in HT-29 cells after 5-ALA treatment. In the control group, the apoptotic/dead ratio was 1.85%. However, the apoptotic/dead ratio of 1000 µM 5-ALA and 1500 µM 5-ALA were 8.90 % and 17.75% (p<0.05), respectively. In terms of live cells, over 90% of cells were living in the control group, whereas living of cells in 1000 µM and 1500 µM, 5-ALA was 64.35% and 41.65%, respectively. These results clearly showed that 1000 and 1500 µM 5-ALA concentrations affected the population profile of colon cancer cells.

A)



B)

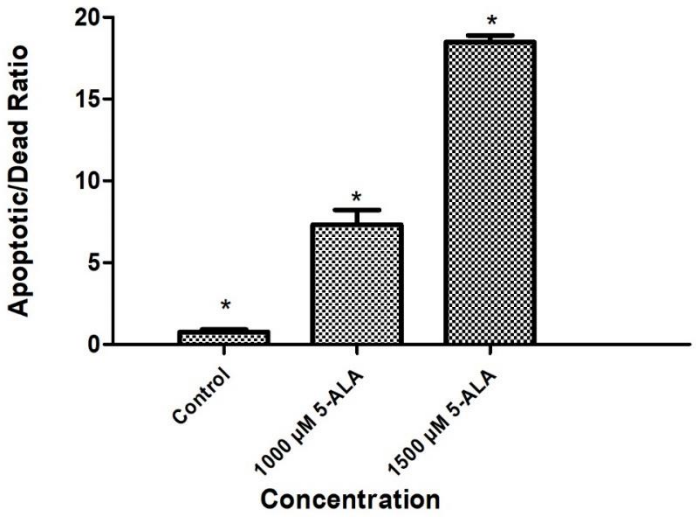


Figure 4: The cellular apoptotic ratio of HT-29 cells treated with 1000 µM and 1500 µM 5-ALA for 3 h or untreated, evaluated by detection of caspase 3/7 positive cells. Fig. 4A shows the histogram of the cellular state and Fig. 4B is the bar graph data of apoptosis and dead cell ratio. (* p<0.05).

4. DISCUSSION

Our primary goals were to assess (I) evaluate the fluorescence emission in HT-29 cell lines depending on the concentration of 5-ALA, (II) the selective cytotoxic effects of 5-ALA on HT-29 cells, and (III) the apoptosis/death rate of 5-ALA in HT-29 cells. We demonstrated that HT-29 cells successfully showed different properties at different 5-ALA concentrations under the same conditions. It was observed that the fluorescence intensities of PpIX were also different depending on the concentrations, and high doses of 5-ALA caused an increase in the apoptosis/death rate in cells in this study.

The accumulation of PpIX varies according to the applied 5-ALA concentration and cell lines (16). The reason for this is that intracellular PpIX accumulation depends on the balance between 5-ALA PpIX translation, ferrochelatase activity, and active extracellular leakage of PpIX (17). We designated that PpIX accumulation gradually increased according to 5-ALA concentration. Although many studies have demonstrated that on varied cancer cell lines the intracellular accumulation of PpIX, there have been no remarkable studies of the elaborate and simultaneous evaluation of the amount of PpIX accumulation in various types of cancer cells (18).

5-ALA influences cell viability. The common results of studies (11,12,19) indicated that the increase in concentration resulted in a decrease in cell viability but increased the toxic effects on cells at high doses. For this reason, the ideal 5-ALA concentration was different for each cell line (11). Like previous studies, it has been shown that control, 100, 200, 300 μM 5-ALA concentrations did not have a different effect on cell viability, but there was a significant difference between 500, 1000, and 1500 μM 5-ALA concentrations. Therefore, 1000 and 1500 μM 5-ALA concentrations were considered toxic doses. Parallel to our previous study (13) it was emphasized that the cell viability of MKN28, HCT116, and AGS cell lines decreased depending on 5-ALA concentrations. Also, it was revealed that the effect of 64 mM 5-ALA concentration was unfavorable on cell viability in the gli36 cell line (20).

In addition to the ideal 5-ALA concentrations for photodynamic diagnosis, the ideal incubation time is also essential due to the fluorescence intensity of PpIX. The fluorescence intensities of PpIX taken for control, 1- and 2-hours incubation after 5-ALA application is not enough for diagnosing the cancerous area. There is a remarkable decrease in fluorescence intensities of PpIX taken 6 hours after 5-ALA administration (13). Many researchers propose that 3 hours 5-ALA incubation time is suitable for optimum PpIX fluorescence (13,21). However, the fluorescence intensities of PpIX recorded after a 3-hour incubation period of 5-ALA at different doses were significantly different. Since there was no accumulation of PpIX in the control group, fluorescence intensity was not observed at all. The highest fluorescence intensity was determined in 1500 μM 5-ALA. However, a significant decrease in cell viability was observed after the 1000 μM 5-ALA application.

In PDD, high doses of 5-ALA cause apoptosis, not necrosis, in cancer cells. Apoptosis and death rates in cells increase in parallel with the concentration (22). In addition, in our study, it was confirmed that caspase 3/7 activity and apoptosis-death rate gradually increased in cancer cells after 1000 and 1500 μM 5-ALA administration. The highest apoptosis-death rate was seen in 1500 μM 5-ALA. These results suggest that an increase in 5-ALA-induced ROS generation induces apoptotic cell death in cancer cells.

Despite the data obtained in this study, there are some limitations in this study. One of the limitations of the study is the in vitro study in 5-ALA-induced photodynamic diagnosis. Another limitation is that the effect of 5-ALA on oxidative stress in the cell has not been studied.

5. CONCLUSION

Among cancer species, the mortality from colon cancer is increasing today. Many methods are used in the diagnosis of this disease. It was assumed that photodynamic diagnosis could be an alternative method in the early diagnosis of these cancers. Especially, photodynamic diagnosis mediated by 5-ALA has been used with increasing interest. In the current study, fluorescence intensity was screened in fluorescence microscopic examination 3 hours after administration of 5-ALA at concentrations of 0, 100, 200,300, 500, 1000, 1500 μM in HT-29 cancer cell line. The differences among 5-ALA concentrations were significant. Although the highest fluorescence intensity was seen at 1500 μM, mentioned concentration had the lowest cell viability. In consequence, 500 μM 5-ALA concentration and 3 hours incubation time may be suitable for photodynamic diagnosis in colon cancer. It should be recommended from the results obtained from this study that the process, 500 μM 5-ALA concentration and 3 hours incubation time, should first be studied in vivo in order to apply to cancer patients for further studies.

Ethicial Considerations

According to the Institutional Ethical Committee this study did not require ethics approval as it was conducted on cell lines and the data did not contain patient-specific information.

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Conflict of Interest

The authors have no conflicts of interest to.

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