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Real-Time Detection of Doxorubicin-Induced Apoptosis in Breast Cancer Cells Using the Back Reflection Spectroscopy

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ABSTRACT

Apoptosis detection methods cause the cell culture medium to be physically or chemically affected. In addition, these methods have disadvantages such as cost and cell losses. The aim of this study is to develop a method to assess apoptosis in real-time using the back-reflection spectroscopy (BRS) in breast cancer cells.

BRS experiment set up consists of a spectrometer, a tungsten-halogen light source and a fiber optic probe. In order to assess the changes associated with apoptosis, the intensity of the reflected light was measured with a fiber-optic probe from MCF7 breast cancer cell samples in which apoptosis induced doxorubicin. Spectroscopic measurements were performed 6 hours after incubation from control and doxorubicin groups. Cell viability measurement with MTT method, imaging of cell morphology with toluidine blue staining method, ROS measurement with DCFH-DA method were performed for the evaluation of apoptosis.

We analyzed the back reflection spectrum and determined the signal difference in apoptotic cell samples compared to the control. A significant difference was found in ROS measurements in all groups compared to the control. We did not observe any morphological change in the cells imaged with the toluidine blue method for 6 hours. Cell viability decreased 50% in all groups compared to the control for 24 hours.

BRS is a new approach that may detect apoptosis in real time without interfering cell culture conditions. This method has the potential to be developed as a non-invasive, objective and reproducible technique that enables time-dependent monitoring of apoptosis.

Key words: Apoptosis, Back Reflection Spectroscopy, Breast Cancer, Doxorubicin

Introduction

Apoptosis is a form of cell death that is essential for the development and maintenance of the multicellular organism's homeostasis and involves mechanisms that lead to cell self-destruction once cells are activated. As the cell undergoes apoptosis it is very critical to identify changes in the cell, monitoring apoptosis can provide diagnostic and prognostic information (1). It has a key role in developing new techniques, various diseases and drug development to detect and evaluate apoptosis (2). In recent years, many studies have been conducted on the role of apoptosis in cancer (3-11). The first response to successful cancer treatment in a cell is usually apoptosis, and apoptosis needs to be well understood for the development of successful treatments (12).

Apoptosis measurements are the most used method for evaluation of cancer drugs in cell culture studies. Apoptotic processes can develop within a few hours and may be difficult to detect depending on the method and sampling time used. Multiple color fluorescence microscopy and flow cytometry are available "gold standard" methods for quantitation of apoptosis (13). Microscopy is used for a qualitative observation of morphological features and to confirm the occurrence of apoptosis, while Flow cytometry can measure the percentage of apoptotic cells in the sample at a given time after the administration of apoptotic agents. All current methods for detecting apoptosis require the addition of an exogenous agent and/or physical or chemical exposure to the cell culture medium to fix and stain cells. These methods have disadvantages

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such as cost and cell losses, as well as limited to the evaluation at a certain time point such as incubation time. The ideal way to determine apoptosis is to evaluate the apoptotic process without interfering with the culture environment of the cells or creating any confounding effects. In addition, measurements should be fast, without causing significant pH or temperature changes.

The optical properties of the measured sample can be determined by the method of back reflection spectroscopy. In this method, light is sent to the sample with a fiber optic probe, then the back reflected light from the sample is collected by the fiber optic probe and sent to the spectrometer. Thus, the light intensity graph against wavelength is obtained as the 'spectrum' (14). This Spectrum is a signal based on the scattering and absorption of light that provides information about the sample being measured (15, 16). Light sent to the sample is scattered at certain angles due to factors such as the size, shape, refractive index of the particles in the sample (17). Back Reflectance Spectroscopy (BRS) is based on the back reflection signal generated by elastically scattered photons due to the refractive index change in cells (18).

Both biochemical and morphological changes related to apoptosis are different from other cell death mechanisms. Apoptotic cells undergo a series of subcellular changes that lead to cell shrinkage and cell destruction. BRS is sensitive to these subcellular micro changes. Thus, the signal associated with apoptosis in cell culture can be obtained by measuring BRS. The aim of this study is to develop a method in which apoptosis can be determined in real time by using back reflection measurements in breast cancer cells.

Materials and Methods

Cell Culture: MCF-7 Breast Cancer cells were grown with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units / mL penicillin, 100 µg / mL streptomycin and 1 mM glutamine. Cells were grown by standard cell culture technique in a carbon dioxide incubator at 37 ° C, 5% CO_2, and 95% humidified air. Doxorubicin (5-100 µM) was used at the dose range to induce apoptosis. After discarding the medium of the cells seeded in petri dishes, 5, 10, 25, 50, and 100 µM dose of Dox (five group of Dox) was applied to the medium well. The control is the group containing only MCF-7 Cells and no Dox application. Cells were incubated in a carbon dioxide incubator containing 5% CO_2 and 95% humidified air at 37 ° C for the incubation times in accordance with the determined doses of Dox and petri dishes.

MTT Cell Viability: In the method of MTT [3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide], MTT is reduced to formazan, and the color formed by this reaction is measured colorimetrically. It is based on the formation of formazan by living cells with tetrazolium, then the color change is determined by absorbance measurement. Briefly, MCF-7 cells were seeded at $1x10^4$ cells per 96-well plate and allowed to adhere overnight. After the cells were incubated with the determined doses of Dox in a 96-well plate for 24 hours, 0.5 mg / mL MTT was added to each well of the plate. Then, the cells were incubated for 4 hours at 37 ° C in a carbon dioxide incubator containing 5% CO_2 and 95% humidified air. At the end of the incubation period, formazan crystals were mixed with 200 µL of dimethyl sulfoxide at the bottom of the wells. Then, measurement was made on an multi microplate reader at wavelength of 570 nm and reference wavelength of 690 nm within 1 hour. Percent cell viability was calculated by comparing the absorbance values of the control group with the absorbance values of the cells incubated with the drug. Results are expressed as % viability. The experiments were repeated three times and three samples from each group were studied.

Determination of Intracellular ROS: Intracellular ROS was determined by fluorescence measurement using the DCFH-DA (2'7'-dichlorodihydro fluorescein diacetate) method. DCFH-DA is a cell-permeable lipophilic compound that can be deacetylated in the presence of cellular esterases, and the occurring DCFH is oxidized to fluorescent DCF in the cytoplasm. The amount of ROS formed at the end of Dox incubation in the cell was calculated by using the fluorescence property of DCF (2 ', 7'-dichlorofluorescein). Briefly, MCF-7 cells were seeded at 25 x 10^4 in 6-well plate and left overnight for cells to adhere. After the cells were incubated with the determined doses of Dox for 6 hours in the 6-well plate, the media was discarded, and the cells were washed in PBS. DCFH2-DA (2 ', 7'-Dichlorofluorescin diacetate) dye was added to a final concentration of 50 µM and incubated at 37 ° C in the dark for 30 minutes. After washing the cells with PBS, the DCF formed in the cells by ROS was examined under a fluorescence microscope at 20X magnification. This measurement was made at least 3 times in all groups. Then, the fluorescence intensity resulting

from ROS of the photographed cells was analysed by Image j program. The results were given in proportion to the control group without medication.

Cell Morphology Analysis: Toluidine blue (TB) marks acidic groups of cellular components such as the cell nucleus region and has an affinity for mucopolysaccharides found in the extracellular matrix, such as proteoglycans and glycosaminoglycans. So, this staining method is often used to evaluate the morphology of cells. Briefly, MCF-7 cells were seeded at 25 x 10^4 in a 6-well plate and left overnight for cells to adhere. After the cells were incubated with the determined doses of Dox in a 6-well plate for 6 hours, we applied the TB staining protocol. The cells were washed twice with PBS and fixed with 4% formaldehyde for at least 30 minutes. After fixation it was washed twice and then 0.1% TB solution was added for 30 minutes. The plates were washed with distilled water for 5 minutes. Washing was repeated three times until the TB was cleared. Cells were examined at 10X magnification under the microscope. Then the cell viability of the photographed cells was analyzed with the help of Image j program.

Spectroscopic Measurements: We performed the back reflection spectrum measurements with an experimental setup consisting of a spectrometer, fiber optic probe, light source and a laptop computer (Figure 1). We used tungsten-halogen lamp as the light source, and USB2000 model, spectrometer (Ocean Optics, Inc. Florida, USA), sensitive to wavelengths between 400 and 850 nm for spectroscopic data. We analyzed the data from the spectrometer with software on the computer (OOIBase32 Platinum, Ocean Optics, Inc, Florida, USA). We used a circular geometry fiber optic probe to send and collect light into the sample. In the optical probe, six fibers are arranged as a detector, in a symmetrical and circular structure around one source fiber. All fibers are 400 μm in diameter and are positioned adjacent to each other at the tip of the probe. Spectra were processed with Igor Pro 8.04 technical graphics and data analysis program.

Before the spectroscopic measurements, calibration was performed to remove the effect of the reflections at the interfaces and the spectral distribution of the tungsten halogen lamp (19). After calibration, we made measurements on a black background by immersing the probe in the prepared cell culture sample tube to a depth of 2-3 mm. After each measurement, we calculated the calibrated spectrum as described in previous studies (20, 21). Reflectance spectra were measured and recorded 8 times from each of the Dox solutions (5-100 μM) prepared in PBS. Then, we normalized these solutions to their own groups. Thus, interference of Dox absorption into the spectra was eliminated. Light intensity ratios at 550 and 800 nm in the control and Dox spectra were calculated. We named this ratio "Spectroscopic Apoptosis Index (SAI)". Also, any Dox absorption effect that might remain after normalization was ruled out by evaluating the spectrum from 550nm. SAI is the quantitative expression of the change in spectra due to apoptosis.

Statistical Analysis: Statistical analysis was performed using Graphpad Prism Version 5.03 package program. $p < 0.05$ was considered statistically significant. Experiments were done in 3 replicates for each group. Quantitative data were given as mean ± SD. Kruskal-Wallis test was used to determine significant differences among groups. If a significant difference was found in Kruskal-Wallis test, Dunn's test was used to assess comparisons between the two groups. For the results of all analyses, $p < 0.05$ and $p < 0.001$ were considered as statistically significant.

Results

Determination of Cell Viability with MTT: According to MTT results, percent viability was shown relative to control after Dox doses were applied to MCF-7 cells (5-100 μM) after 24 hour incubation (Figure 2). When the Dox groups were compared with the control group, it was found that the viability decreased more than 50% ($p < 0.05$). This was the result we expected, as cells could not survive after Dox-induced apoptosis. However, if we had made the incubation period for 6 hours, we would not see any difference between different groups. Because mitochondria take longer to be affected by apoptosis.

Determination of Intracellular ROS: ROS is one of the most important stimuli of apoptosis. The irreversible increase in ROS causes cells to undergo apoptosis. So, we measured the amount of ROS in the cells after 6 hours of incubation with Dox doses (5-100 μM). Images of the control and Dox groups are shown in figure 3. We observed an increase in the amount of ROS in all Dox groups compared to the control. This increase was not found to be significant for the 5 and 10 μM Dox groups (Figure 4). However, it was found that the amount of ROS increased

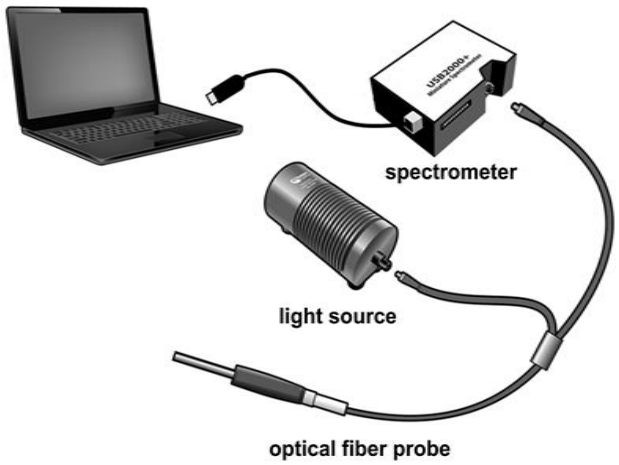

Fig. 1. Back reflection spectroscopy experimental setup

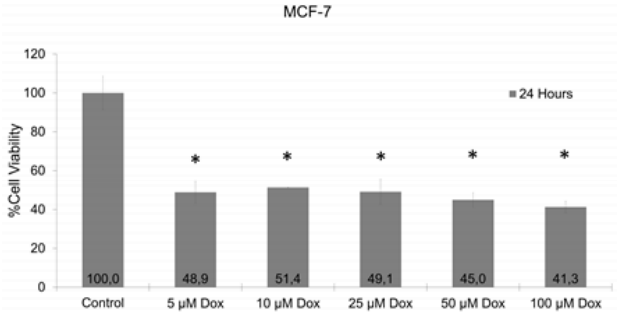

Fig. 2. Effect of different doses of Dox on viability of MCF-7 cells compared to control for 24 hours. * Values differ significantly from control groups ($p < 0.0001$)

statistically significantly in 25, 50, and 100 µM Dox dose groups ($p < 0.05$).

Cell Morphology Analysis: After 6 hours of incubation with Dox doses (5-100 µM), the cells were stained with TB and cell morphologies were examined. The microscope images obtained by 10X magnification of Toluidine blue staining of the control and Dox groups appear (figure 5). At low doses of Dox for 6 hours incubation, cellular integrity does not change significantly, but cells begin to undergo apoptosis with increasing doses of Dox. In the analysis performed with the image j program, there was no significant difference morphologically between the Dox groups at 6 hours. However, it appears that cellular integrity begins to deteriorate.

Spectroscopic Results: The back reflection spectra of the experimental groups consisting of cells incubated with Dox at control and doses of (5-100 µM) and Dox solutions prepared in PBS (5-100 µM) were measured. Measurements taken from experimental groups consisting of cells; It was normalized by dividing the measurements taken from Dox solutions (5-100 µM) prepared in PBS. We show the measured spectra depending on

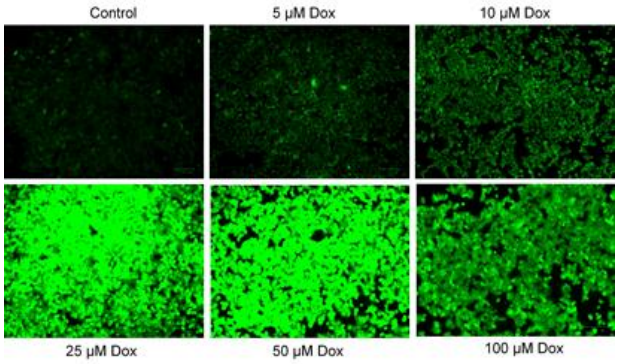

Fig. 3. Microscope image obtained by 20X magnification of the fluorescence of the control and Dox groups depending on the amount of ROS for 6 hours

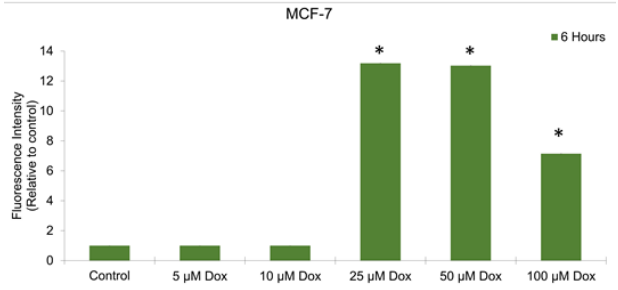

Fig. 4. Fluorescence intensities of the control and Dox groups depending on the amount of ROS for 6 hours. * Values differ significantly from control groups ($p < 0.0001$)

the wavelength with which the control and each Dox group are compared (Figure 6). We have shown that as the Dox concentration increases in the spectra, the back reflection spectra also change considerably compared to the control.

The spectrum of the control shows a uniform monotonic decrease between 500-800 nm. But it is observed that as the dose increases, the slope of the spectra decreases, and the graphs gradually flatten in all Dox Groups. We calculated the SAI to quantify this change in the control and Dox spectra (Figure 7). We found the SAI value as 2.4 in Control. We noticed that this value decreases as the cell goes to apoptosis. It is seen that this value decreased in a statistically significant way in the Dox groups compared to the control ($p < 0.05$). While the SAI value was 1.37 in the 5 µM Dox group, it declined below half of the control value in the 10 µM Dox group. It is seen that it diminished to 0.45 in the 100 µM Dox group and it decreased by 80% compared to the control.

Discussion

Apoptosis is a form of cell death necessary for the evolvement and homeostasis of the organism. Current apoptosis detection methods require

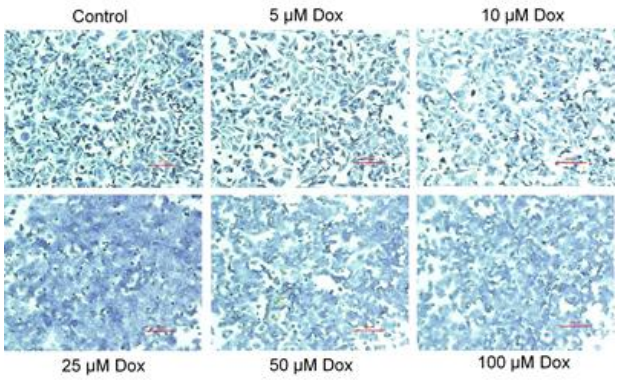

Fig. 5. Microscope image obtained by 10X magnification of Toluidine blue staining of Control and Dox groups for 6 hours

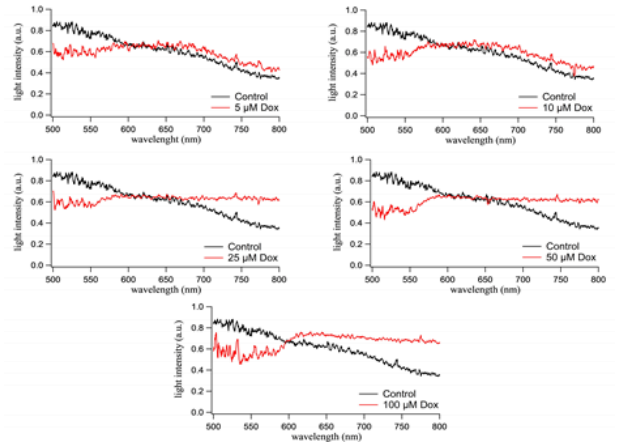

Fig. 6. Back reflection spectra of the measurements made from the control and Dox groups for 6 hours

intervention in the cell culture medium (22). It is important to assess the apoptotic process quickly, in real-time, without causing any interfering effects in cell culture. It is necessary to develop new techniques that ensure accurate and practical monitoring of apoptosis, the main target of drug development research in many diseases (23). In this study, we developed a new method for real-time monitoring of apoptosis using back reflection spectroscopy without interfering with cell culture conditions.

Apoptotic processes can progress rapidly, and it can be difficult to monitor at an early stage depending on the various protocols. The gold standard methods for monitoring apoptosis are multiple colour fluorescence microscopy and flow cytometry.(13). Fluorescence-based methods are widely used in many methods such as fluorescence microscopy, confocal laser scanning microscopy (24), flow cytometry (25), and microarray (26). Although these methods provide an accurate detection of apoptosis, they are time consuming, costly, and require expertise for application and

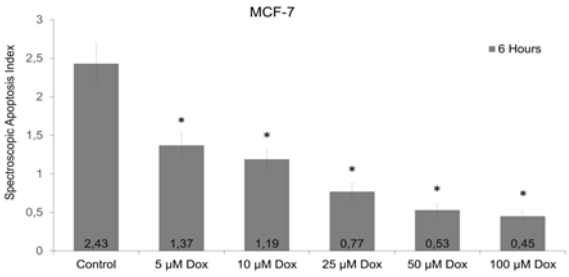

Fig. 7. Comparison of SAI of control and Dox groups for 6 hours. * Values differ significantly from control groups ($p < 0.0001$)

analysis of results (23). It is also limited to the assessment at a specific time point.

Apoptosis measurements are the primary method for evaluating cancer drugs in cell culture studies. In this study, MCF-7 breast cancer cell line was used and Doxorubicin, the apoptosis-inducing agent in breast cancer treatment. After the MCF-7 cells were incubated with Dox, the viability of the cells was determined by MTT method. It was found that in MCF-7 cells, Dox reduced viability by approximately 50% compared to control at all doses for 24 hours. On the other hand, ROS is one of the effective stimuli of apoptosis. Doxorubicin is known to cause ROS formation and oxidative stress (27). The cell undergoes apoptosis due to ROS that increases in the cell and cannot be detoxified. So, we also saw that the amount of intracellular ROS increased in ROS images compared to the control for all Dox groups (Figure 3). However, this increase was not found to be statistically significant for the 5 and 10 µM Dox groups (Figure 4). In addition, we no found statistically significant difference at 5 and 10µM concentrations, so that ROS did not occur in the early stage (6th hour) at low Dox concentrations. However, it is understood that the cell will undergo apoptosis according to the SAI value calculated from the graphs in Figure 6. We showed that SAI values decreased with the increase of apoptosis (Figure 7). The decrease for 24 hours viability to 50% in MTT experiments proves the relationship between spectroscopic data and viability. We found the amount of ROS increased significantly in 25, 50 and 100 µM Dox groups ($p < 0.05$). The staining with toluidine blue marks acidic groups of cellular components, such as the region of the cell nucleus. Cellular integrity began to deteriorate with the entry of Dox into the cell and later into the nucleus (Figure 5). However, since increasing Dox concentration resulted in cell death, we found no statistically significant difference between Dox groups.

BRS was determined to be sensitive to the apoptotic process. We found that the spectra measured from the control and Dox groups were different (Figure 6). We calculated the SAI to quantitatively express the change in spectra due to apoptosis. We evaluated the wavelengths where the difference between the spectra can be determined best are 550 and 800 nm from the analysis of the measured spectra. Therefore, we calculated the SAI value with the ratio of light intensity at 550 and 800 nm in the spectra. It is seen that the SAI value was statistically significantly decreased in Dox groups compared to the control ($p < 0.05$). The reason for the change in spectroscopic signal at the early stage may be the decrease in volume in the cell leading to apoptosis (28). The volume reduction caused by the loss of water in the apoptotic cell and the deterioration of the cytoskeleton through caspases cause increase in the refractive index of the cytoplasm. Thus, it may be the source of the difference compared to control in the refractive signal during apoptosis since the refractive index difference reduced between the organelles and the cytoplasm (29-31). Further molecular studies are needed to explain the reasons for the change in the Back Reflection signal. It should be considered that this method has the potential to detect apoptotic changes earlier than other analysis methods, practically and non-invasively. In this study, we have shown that the BRS method may be a new evaluation method for determining apoptosis. To our knowledge, this study is one of the very few studies using BRS measurements to monitor apoptosis in vitro. Further studies will be conducted to transform the BRS Method into a reliable quantitative technique such as flow cytometry.

In this study, a novel approach was developed that demonstrates apoptosis with minimal interference with cell culture conditions and without adding an exogenous agent by BRS. This method has no potential to be developed as a non-invasive, reproducible and objective method that can monitor apoptosis over time.

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