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Cytotoxic effect of *N*-acetyl cysteine in DU145 human prostate cancer cells

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ABSTRACT: Prostate cancer is one of the most common types of cancer in men all over the world. Chemotherapy, radiotherapy and surgery are conventional cancer treatment modalities. However, these modalities have many complications and side effects. Therefore, it is very important to support the treatment with compounds that will reduce the side effects and increase the effectiveness of the treatment. The aim of this study is to investigate the cytotoxic effects of N-acetyl cysteine (NAC), an antioxidant, on human prostate cancer cells. This study was performed on DU145 human prostate cancer cells. The cells were divided into 7 groups (control and 0.2, 0.5, 1, 2, 5 and 10 mM NAC groups). Cell index was determined using real time monitoring of electrical impedance in all groups. Apoptotic activity was determined using flow cytometry. Apoptotic activity was measured only 5 and 10 mM NAC groups. The cell index increased significantly at lower doses of NAC (0.2, 0.5, 1, 2 mM) but decreased at higher doses (5 and 10 mM). NAC significantly increased percentage of early and late apoptotic cells at 10 mM concentration. Percentage of necrotic cell significantly increased at 5 and 10 mM concentrations. Our data showed that NAC could significantly inhibit the proliferation of DU145 human prostate cancer cells in high doses. In addition, in this study for the first time, we report that NAC enhances apoptotic activity of human prostate DU145 cancer cells. These findings denote the antitumor properties of NAC.

KEYWORDS: N-acetyl cysteine, DU145, cell index, apoptosis, prostate cancer.

1. INTRODUCTION

Cancer has taken its place among the main health problems in today's. According to statistics from 2015, cancer is responsible for the second most mortalities immediately after cardiovascular diseases [1]. Prostate cancer is a serious health problem, especially in the elderly population with its increasing incidence. its mortality cannot be underestimated since it is known that prostate cancer is the second most mortal cancer after lung cancer in male population [1-5]. Furthermore, despite being considered as a low risk cancer, complications in elderly patients due to chemotherapeutic agents used for the treatment of prostate cancer, such as diabetes mellitus, cardiovascular diseases and hypertension cannot be overlooked. It is argued that there is an increasing need for the reduction of side effects and complications created by the current drug usage. Therefore, new treatment modalities must be found [6]. One of the supplementary compounds used for this purpose is the antioxidants. Several studies have examined the relationship between cancer and antioxidants; however, the results of these studies are controversial. Many studies in the literature have highlighted antioxidant agents as a chief potential replacement for chemotherapeutic therapy, this class of drugs has demonstrated efficacy in both in vitro and in vivo animal experiments, thus proving its vital role in the pathogenesis of cancer proliferation and metastasis [7-12]. Moreover, many publications in the literature have evidently shown reactive oxygen species (ROS) related pathways and their roles in cancer pathogenesis [13-17]. In contrast, some publications and articles have supported theories in the other direction, it is argued that antioxidants drugs may play a role in the prevention of the killing mechanism of the oxidative stress created by chemotherapeutic agents and may accelerate the cancer progression [18-22]. Whereas, other articles have advocated that the inherent ROS may lead to the formation of an aggressive phenotype of prostate cancer [23]. The dilemma created by this controversy could be solved if there is more specific research conducted on the antioxidant agent's effects on cancer cells [16, 24].

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N-acetyl cysteine (NAC) is an acetylated cysteine residue. It is a cheap and easily accessible drug with few side effects. N-acetyl cysteine's anti-inflammatory and antioxidant effects are well known [25]. Due to its antioxidant properties, this substance has led to controversy in the scientific world as to whether it has a preventing effect on prostate cancer cells [16]. In a study highlighting the antimetastatic effects of NAC has shown its role as a suppressant of prostate cancer cells and its potential contribution to the therapy of prostate cancer in the future [18]. A different study has found that the cytotoxic effects of chemotherapeutics agents had gradually increased in NAC coated DU145 prostate cancer cells. Moreover, NAC had inhibitory effects on harmful metabolites that had formed during the experiment [7]. A similar result had been observed in another study, where NAC had inhibitory effects on free radicals secreted during treatment of prostate cancer and as a result of abstinence syndrome of androgen [8]. Another study has demonstrated both antiproliferative and tumor suppressor effects of NAC on prostate cancer cells [9]. However, the effect of NAC on cancer cell development is not yet fully understood and it needs to be illuminated. The aim of this study was to investigate dose dependent cytotoxic effects of NAC on the DU145 prostate cancer cells.

2. RESULTS

2.1. Monitoring cell proliferation in real time

Cell growth curves were automatically recorded by dynamic monitoring of cell adhesion and spreading process using xCELLigance electrical impedance sensing platform. DU145 cells were treated with 0.2, 0.5, 1, 2, 5 and 10 mM NAC. Cell index values of control and treated groups were 0.50±0.02, 0.50±0.01, 0.51±0.06, 0.48±0.02, 0.49±0.02, 0.50±0.02, 0.49±0.09, respectively for 0 hours (h) (before NAC addition); after 24 h, the cell index values were 0.63±0.07, 0.61±0.02, 0.61±0.05, 0.59±0.04, 0.57±0.02, 0.461±0.09, 0.30±0.1 respectively; after treatment for 48 h, the cell index values were 0.81±0.06, 0.82±0.03, 0.82±0.05, 0.83±0.06, 0.72±0.01, 0. 44±0.16, 0.22±0.09, respectively; after treatment 72 h, the cell index values were 1.12±0.17, 1.12±0.08, 1.13±0.09, 1.19±0.08, 1.04±0.04, 0.36±0.16, 0.17±0.08, respectively and after treatment 96 h, the cell index values 0.97±0.15, 0.94±0.10, 0.92±0.10, 0.84±0.11, 0,75±0.04, 0.24±0.09, 0.12±0.08, respectively. In the cell index value, there was significant decrease in 5 and 10 mM NAC groups as compared to control group ($p<0.05$). After 24, 48, 72 and 96 h, a marked decrease of the cell index as compared to the 0 h data had been observed in 5 mM and 10 mM concentrations of NAC ($p<0.05$). Whereas, concentrations of 0.5, 1 and 2 mM had demonstrated a significant increase of the cell index when compared to the 0 h data ($p<0.05$). The cell index curve was shown in Figure 1.

2.2. Determination of the effect of NAC on the apoptosis of DU145 cells with flow cytometry

To determine apoptosis, we used 5 and 10 mM NAC concentrations that significantly inhibited cell proliferation. The results of apoptosis were shown in Figure 2. Mean percentage of early apoptotic cells in control, 5 mM NAC and 10 mM NAC was measured as 0.53±0.24, 0.66±0.33, and 5.26±0.74, respectively. The increase in the percentage of early apoptotic cells of 5 mM and 10 mM groups were found to be statistically significant ($p<0.05$) when compared to control group. There was a significant difference ($p<0.05$) between 5 mM and 10 mM NAC groups (Figure 3). Mean percentage of late apoptotic cells in control, 5 mM NAC and 10 mM NAC was measured as 0.13±0.09, 1.09±0.27, and 3.23±0.74, respectively. The increase in the percentage of late apoptotic cells of 5 mM and 10 mM groups were found to be statistically significant ($p<0.05$) when compared to control group (Figure 4). There was a significant difference ($p<0.05$) between 5 mM and 10 mM NAC groups (Figure 4). Mean percentage of necrotic cells in control, 5 mM NAC and 10 mM NAC was measured 3.30±0.50, 10.26±1.41 and 11.83±2.37, respectively. In 5 mM and 10 mM NAC groups, mean percentage of necrotic cells was significantly lower ($p<0.05$) than the control group (Figure 5). There was no significant difference ($p>0.05$) between 5 mM and 10 mM NAC groups (Figure 5).

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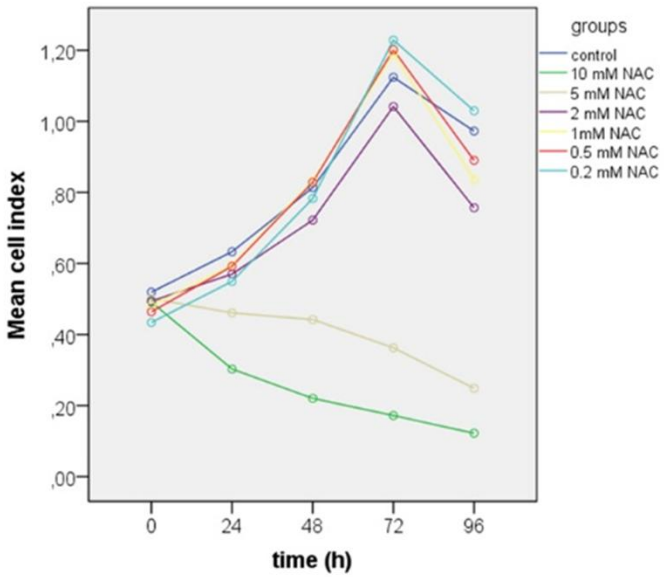

Figure 1. Dose and time dependent effect of NAC on the proliferation of DU145 cells.

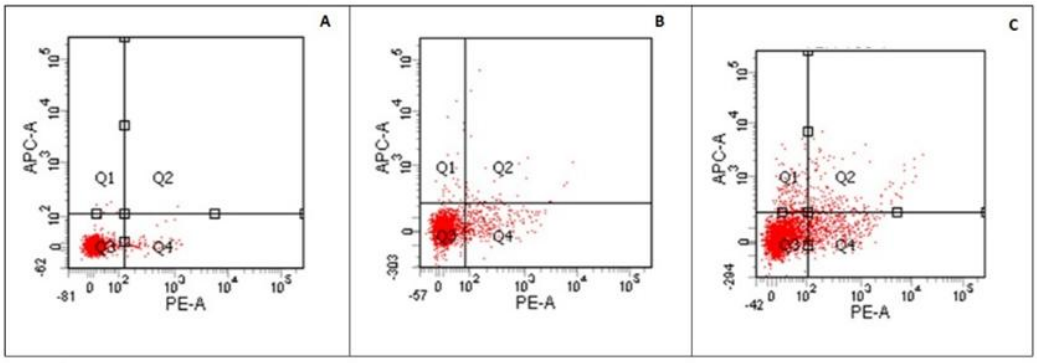

Figure 2. Effects of NAC on the apoptosis of DU145 cells. A: control, B: 5 mM NAC, C:10 mM NAC. Q1: early apoptosis; Q2: late apoptosis; Q3:viable cells; Q4: necrotic cells.

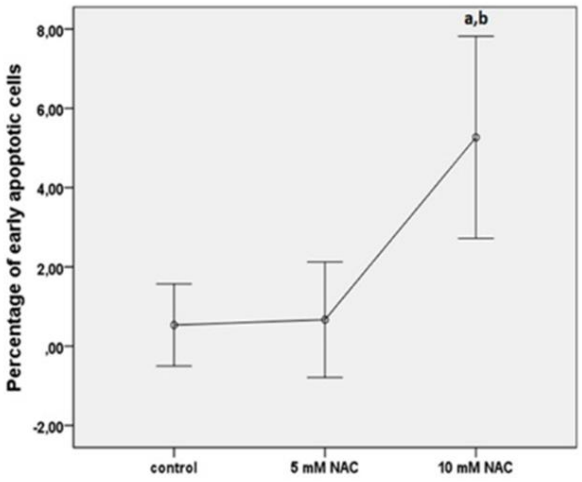

Figure 3. Percentage of early apoptotic cells. [a] Significantly difference from control ($p<0.05$). [b] Significantly difference from 5 mM NAC group.

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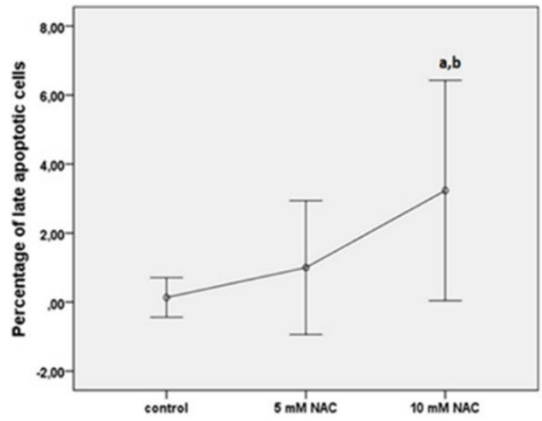

Figure 4. Percentage of early apoptotic cells. [a] Significantly difference from control ($p<0.05$). [b] Significantly difference from 5 mM NAC group.

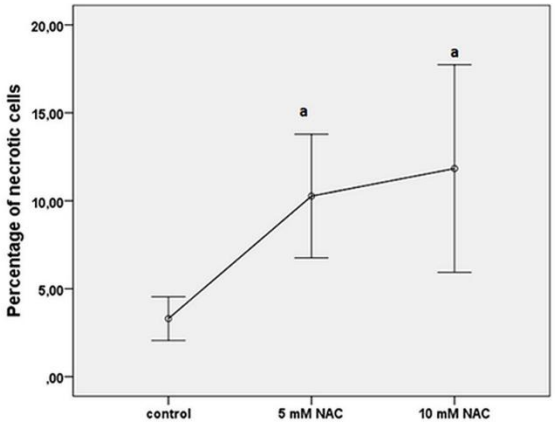

Figure 5. Percentage of necrotic cells. [a]Significantly difference from control ($p<0.05$).

3. DISCUSSION

In this study, we investigated the cytotoxic effect of NAC, an antiinflammatory and antioxidan agent, on the DU145 prostate cancer cell line. Cytotoxic effect in DU145 cells were assessed at different concentrations of NAC through 24, 48, 72 and 96 hours. In the present study, to determine the action mechanism for inhibited cell proliferation we also examined the effect of NAC on apoptosis of DU145 prostat cancer cells. Our findings showed that NAC may be effective agent for the treatment of prostate cancer.

In the present study, DU145 prostate cancer cells were treated with different concentrations of NAC, then analyzed according to cell index. As a result, the two major dosage used during the experiment 5 mM, 10 mM respectively have both shown efficacy with respect to suppressive effects on prostate cancer proliferation. (noting that the suppressive effects had started in the 24th hour of the experiment and carried out until the 96th hour). However, effects of lower concentrations (0.2, 0.5, 1 and 2 mM) of NAC were dismissed, demonstrating little to no significant effects on prostate cancer cells proliferation. In a previously conducted experiment in the literature, combination of NAC with chemotherapeutic agents had shown positive outcomes by strengthening treatments efficacy [7]. In their study, 0.2, 1 and 5 mM doses of NAC have been used. In a more recent study [18] proliferation, migration, adhesion and invasion inhibitory characteristics of NAC on DU145 prostate cancer cells have been reported. All concentrations of NAC (5, 10, 15, 20, 25, 30, 35 and 40 mM) had significantly inhibited cell growth in a dose dependent manner in DU145 and PC3 cells. In another study, 10 and 20 mM concentrations of NAC had suppressed human prostate cancer PC-3 cell proliferation [9]. Consistent with these studies, our study demonstrates that NAC has the potential to reduce prostate cancer cell proliferation.

Previous studies have shown that NAC is able to induce apoptosis in vascular smooth muscle cells, in colorectal carcinoma cell lines, in cardiomyocyte cells and murine embryonic fibroblasts [26-28]. In our study,

we determined the apoptotic effect of NAC on DU145 prostate cancer cells. In this study, for the first time, we report that NAC enhances apoptotic activity of human prostate DU145 cancer cells. We found that NAC significantly increased the percentage of early and late apoptotic cells at 10 mM NAC concentration when compared to control group. Percentage of necrotic cell significantly increased at 5 and 10 mM concentrations when compared to control group. Early stage apoptosis is represented by disruption of mitochondrial membrane potential, but cells maintain membrane integrity [29]. Since mitochondrial dysfunction is an early event in the process of apoptosis, early apoptotic cells arise from a reduced mitochondrial transmembrane potential that may precede the release of cytochrome c from the mitochondria. On the other hand, the late stages of apoptosis are characterized by DNA fragmentation and loss of cell membrane permeability [29]. Thus, a feature of late apoptosis is large fragmentation of genomic DNA that produces multiple DNA double-strand breaks with accessible 3′-hydroxyl groups. In this study, we did not investigate the effect of NAC on apoptotic signaling pathways in DU145 prostate cancer cells and possible DNA fragmentation resulting from these pathways. These are important limitations of our study.

4. CONCLUSION

In the present study, our data showed that NAC could significantly inhibit the proliferation of DU145 human prostate cancer cells. In addition, for the first time, we have been able to report that NAC has a role in the enhancement of apoptotic activity of human prostate DU145 cancer cells. These findings indicate the antitumor properties of NAC and may imply that NAC is a potential adjuvant drug for the treatment of prostate cancer. However, further research is needed to determine underlying molecular mechanisms that driving this potential effect of NAC. Also, clinical researches should be conducted to ascertain the applicability of the NAC treatment on prostate cancer patients.

5. MATERIALS AND METHODS

5.1. Chemicals, drugs and antibodies

NAC was provided as a pure dry powder (Bilim İlaç Sanayi, İstanbul, Turkey). Other chemicals and antibodies were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co, MO, USA).

5.2. Cell culture

5.2.1. Medium preparation

The human DU145 prostate cancer cells were provided from the American Type Culture Collection (ATCC; Manassas, VA, USA). For DU145 prostate cancer cells, RPMI medium (2,5 mM L-Glutamine+) had been used as the basal medium. In addition, 10% of inactivated fetal bovine serum (FBS), 1% penicillin of streptomycin and 1% amphotericin has been added to the medium, then the prepared medium had been preserved at +4°C.

5.2.2. Cell lysis

Firstly, the designated temperature for the present cell lines (37°C in bath water or ambient temperature) was obtained. Thawed and 70% ethanol wiped cryovial has been placed in laminar flow cabinet. Cryovial was opened once a sterile environment had been obtained and cells were poured into a 15 mL falcon tube with the 37°C medium added and then washed. With completion of the washing process, falcon tube (150xg in 5 min) was centrifuged at room temperature and RPMI-1640 medium had been added, the culture's bottle cap was sealed and left 24 hours for incubation at 37°C and %5 CO_2. The culture medium has been renewed after 24 hours had passed. Renewal of incubated medium cells continued once every 2-3 days until 80% confluence level had been obtained.

5.2.3. Passaging the cells and cell count

The outer most medium of the 80% proliferating cells was aspirated using a sterile needle. Phosphate buffered saline (PBS) had been added to remove the residual medium overlaying the cell lines. Tamponade was also removed afterwards to be replaced with a 0,25% trypsin/PBS covering the cells surface. Then trypsin coated cells had been incubated for approximately 5 minutes at 37°C. Inspection for the separation of the surface cell layer was conducted under a light microscope, trypsin was retracted when separation had been completed. A supplemented medium had been added to the cells proportional to the retracted amount. 1mL of the cells had been placed in a microfuge tube and a cell count under the microscope had been performed.

A final decision was made regarding the intended concentration levels of dilution and inoculation of the cells, the cells were then placed in a new flask (culture bottle). Preheated at 37°C, a new medium had been added to the cells, the flask had been shaken gently to establish homogenous distribution and then the cells were incubated. Renewal of incubated medium cells continued once every 2-3 days until 80% confluence level had been obtained. This process was repeated until there was a sufficient cell count for this experiment. For cell count Cedex XS (Roche, Mannheim, Germany) machine had been used. This semi-automatic technology and the use of a trypsin blue staining technique provides highly sensitive information regarding concentration and viability of the cells. The cells were centrifuged (for 5 min at 130xg) after the trypsinization process had been completed. After disposal of the supernatant that formed above the pellets, the cells were re-suspended with addition of 1mL medium. 20 µL of the re-suspended cells had been transferred into 0,2 mL of Eppendorf, when a 1:1 ratio of sterile distilled water and 20 µL trypsin blue (Roche, Mannheim, Germany) had been obtained, the entering of the above substances into the cell had allowed differentiation between living and dead cells. 20 µL of the acquired homogenous mixture had been added to the machine's special Smart slides (Roche, Mannheim, Germany) tank. A specimen was placed in a microscope slide and then into a Cedex XS machine for cell count using trypsin, cells that reached 80% of confluence had been removed from the medium in order to determine the sufficient cell amount to be used for the cytotoxicity experiment, then the cell count was conducted.

5.2.4. Real time monitoring of cell proliferation

Real-time monitoring of electrical impedance (which depends on cell number, degree of adhesion, spreading, and proliferation of the cells) to determine cytotoxic effects of N-acetyl cysteine was performed using the xCELLigance DP system (ACEA Biosciences, San Diego, CA, USA).

On the day of the experiment, the medium had been removed and then cells were washed three times with the use 1X Dulbecco's Phosphate-Buffered Saline (DPBS). After retraction of DPBS 2,5 mL %0,05 of trypsin-EDTA had been added to the cells, then left for incubation for 5 min at 37 ^{0} C. For ensuring inactivation of Trypsin, a FBS supplemented RPMI medium had been added to the flask. The suspended cells in the flask were then transferred into a 15 mL conic culture tube. The supernatant had been removed after centrifuging (130xg in 5 min), the pellet was then re-suspended after the addition of a 1mL of supplemented medium. A cell viability count had been conducted by Cedex machine. Overall, a sum total of 3X10^{6} cells were obtained from the T25 flasks. 30.000 cells had been planned for inoculation into each well of the 16 welded e-plate. Thus, a cell suspension had been calculated. RTCA program had been initiated. Groups were prepared by determining concentrations as control, 0.2 mM, 0.5 mM, 1 mM 2 mM, 5 mM and 10 mM. 100 µL of supplemented medium was added to each well of the e-plates, then they were placed in the incubators system afterwards. The first reading (blind-reading) was then obtained after initiation. After completion of the blind-reading, the plates were removed from the incubator and then transferred into a laminar cabinet. The cell suspension containing 30.000 cells in a volume 90 µL was added to all wells to reach a final volume of 190 µL cells, which had been resituated into the incubator and cell proliferation curve had been observed over 24hours. The following day, necessary concentration of the applied substances had been prepared. After the passing of 24 hours, the E-plates were transferred from the incubator to a laminar flow cabinet. According to the pre-specified well codes of the E-plates, 10 µL of the appropriate concentrations of vitamin and drug stocks had been used. Thus, the size of the wells had reached a sum of 200 µL. The spaces of the E-plate system between the wells were filled with 1X D-PBS. With the placement of E-plates into the incubator, the compound activity phase had been initiated. Enabled by the applied substance and the cell line, this phase can be sustained between 48-100 h from each group. Cell-sensor impedance was expressed as an arbitrary unit called the Cell Index. The Cell Index at each time point is defined as (Rn-Rb)/15, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the media alone.

5.3. Determination of the apoptotic cells using flow cytometry

The apoptosis was determined with flow cytometry [30, 31]. Cell apoptosis ratio was assessed using Annexin V-FITC apoptosis detection kit (Bio Legend 640932) according to the manufacturer's instructions. After being treated with N-acetyl cysteine, both the adherent and floating cells were harvested with trypsinization free of EDTA and washed with precooling PBS. Then the cells were incubated with staining solution (195 µL Annexin V-APC binding buffer, 5 µL Annexin V- APC and 10 µL PI) for 20 minutes at room temperature in the dark. The cell apoptosis was analyzed with a BD FACS ARIA III within 1 h and calculated by dot plot analysis with the built-in software. Annexin V- APC positive cells were considered to be

undergoing apoptosis, including: early apoptotic cells (Annexin V+/PI−) and necrotic or late apoptotic cells. (Annexin V+/PI+), and those negative for APC were considered to be alive.

5.4. Statistical analysis

Data were analyzed using a statistical package program (SPSS v.11.5, SPSS Inc., Chicago IL, USA). The checks of normality of variables are tested with Kolmogorov-Smirnov test. Descriptive statistics of variables are expressed as the mean and standard deviation (SD). One-way ANOVA was used to compare groups for normal distribution variables. LSD test was used for multiple comparison tests. *P* values less than 0.05 were considered as statistically significant.

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