CEFEPIME, A CEPHALOSPORIN, INDUCES THE APOPTOSIS AND OXIDATIVE STRESS IN NB2A NEUROBLASTOMA CELLS

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ABSTRACT

Neuroblastoma, heterogeneous solid children tumour, usually shows high proliferation and metastase rates. As such, it is important to find more effective therapeutic agents for the treatment of neuroblastoma. Cefepime is an antibiotic that belongs to the cephalosporins. Due to the some cephalosporins antitumour potential, this study aimed to investigate the apoptotic and oxidative stress effects of cefepime in NB2a neuroblastoma cell line using flow cytometry and immunocytochemistry analysis. The cell viability and Annexin V results demonstrated that cefepime showed antiapoptotic effects. Cefepime arrested the cells in the S and G2/M phases. In addition it increased the oxidative stress and decreased the cell proliferation. It can be concluded that the cefepime exhibits anticancer potential to neuroblastoma cell and thus warrant further studies to assess its potential effects on the other cancer types.

Keywords: Cefepime, NB2a, Cephalosporin, apoptosis, oxidative stress

INTRODUCTION

Neuroblastoma is a heterogeneous solid tumor. These tumors usually develop in the abdomen and adrenal glands (Mlakar et al, 2017; Twist et al, 2019; Zafar et al, 2021). Neuroblastoma often begins in infancy and is one of the most common types of cancer in children. Clinical treatments include chemotherapy, radiotherapy, immunotherapy and surgery. Although there are various treatment options, neuroblastoma is a tumor type whose high proliferation and migration can hardly be prevented. Although current treatment methods stimulate apoptosis, the inability to stop the invasion leads to the search for new treatments (Ün and Ugan, 2021).

Cephalosporins are β-lactam antibiotics that are toxic to bacteria by inhibiting beta-lactamase activity and cell wall peptidoglycan synthesis (He et al, 2021). Labay et al (2016) reported that cephalosporin increases DNA damage and amplifies the effects of ionizing radiation, possibly through the production of reactive oxygen species (ROS). Studying off-label uses of existing drugs and using them for new applications is the most cost-effective way to drug discovery. Antibiotics are recognized as important sources for antitumor drugs. Bleomycin, mitomycin, kinomicins, doxorubicin etc. Antitumor antibiotics have been used successfully in the treatment of cancer (He et al, 2021). Cefepime (FEP) is a member of the fourth-generation cephalosporin class with broad-spectrum activity against both Gram-positive and Gram-negative bacteria, which is widely used for approved indications such as febrile neutropenia, a major adverse event in patients receiving cancer chemotherapy with or without radiotherapy (Jandula et al, 2001; Saito et al, 2014; Zhang et al, 2015). Shi L and Fang J suggested that nasopharyngeal cancer cells have a higher basal ROS level than normal cells and that increased ROS levels are toxic to cancer cells (Shi and Fang, 2008). In this

study, we aimed to examine whether cefepime, one of the cephalosporin antibiotics, can induce cytotoxicity in neuroblastoma cells.

2. MATERIAL and METHODS

2.1 Reagents

The following reagents were used: High glucose DMEM medium (Pan Biotech, Germany), fetal bovine serum (FBS) (Pan Biotech, Germany), trypsin/EDTA (Pan Biotech, Germany), penicilin/streptomycin (Gibco, USA), Horse serum (Gibco, USA), Gentamicin (Capricorn Scientific, Germany), trypsin (Thermo scientific, USA); 3% of hydrogen peroxide (H_2O_2, Thermo scientific, USA); blocking solution, secondary antibody, strepavidine-peroxidase (Thermo scientific, USA); Diaminobenzidin (DAB, Thermo scientific, USA); Primary antibodies: nitric oxide synthase 1 (NOS, Bioorbyt, orb251481, UK), Ki-67 (Bioss, bs-23105R, USA), and caspase-3 (Bioss, bs-0081R, USA)

2.2 Cell Culture

Mouse Neuroblastoma cell line NB2a was obtained from ATCC (Manassas, VA, USA). Cells were grown in high glucose DMEM medium supplemented with %5 horse serum, %5 fetal bovine serum, %1 penicilin/streptomycin and 25 µg/ml gentamicin in a humidified incubator at 37^0C and %5 CO_2. Cells were passaged using 0.25% trypsin/EDTA and sowed in fresh medium. In addition, cells were regularly monitored for mycoplasma contamination.

2.3 Cell viability

NB2a cells were seeded at 10^4 cells/well in a 96 well-plate and treated with different concentrations of cefepime (0, 2, 4, 8, 16, 32 mM) and incubated for 24 hours. Cell viability was determined by 3-3-(4,5-D-methylthiazole-2-yl)-2,5- diphenyltetrazolium bromide, thiazolyl blue (MTT) test at 550 nm with a microplate reader (BioTek; Winooski, VT) method. The resulting colored solution was quantified by measuring absorbance at 570 nm on a multi-well spectrophotometer (BioTek; Winooski, VT). IC50 values were calculated using Graph Pad Prism 7 software. Experiments were performed in triplicate and results were expressed as mean ± standard deviation.

2.4 Detection of apoptosis and cell cycle by flow cytometer

The apoptotic cell distribution was determined using the MUSE Annexin V & Dead Cell Kit (Merck MCH100105) according to the manufacturer's instructions. Briefly, control group cells without cefepime and treatment group cells with IC50 concentration of cefepime for 24 hours were collected and diluted with PBS containing 1% bovine serum albumin (BSA) as a dilution buffer to a concentration of $5x10^5$ cells/ml. Cells were then analyzed using the flow cytometry-based Muse™ Cell Analyzer instrument. The apoptotic ratio was determined by the identification of four populations: (i) nonapoptotic cells, not undergoing detectable apoptosis: Annexin V (−) and 7-AAD (−); (ii) early apoptotic cells, Annexin V (+) and 7-AAD (−); (iii) late apoptotic cells, Annexin V (+) and 7-AAD (+); (iv) cells that have died through nonapoptotic pathway: Annexin V (−) and 7-AAD (+). The samples were determined by the Muse Cell Analyzer (Batır et al, 2019).

The cell cycle position was analysed using the MuseTM Cell Analyser. NB2a cells were seeded into a 12-well plate at 2×10^5 cells/well for 24 h before the cells were treated with only culture medium or 14,12 mM cefepime. After 24 h treatment, cells were washed with PBS, dislodged with 0.25% trypsin EDTA, harvested, resuspended in PBS and fixed with ice-cold 70% ethanol and stored at −20 °C for 24 h. Ethanol-fixed cells were then washed with PBS and stained with 200 µL of MuseTM Cell Cycle Reagent (MCH100106, Merck Millipore, Billerica, MA, USA) for 30 min at room temperature in the dark and analysed using the Muse flow cell analyser. Results were expressed as percentage of cells in G0/1, S and G2/M phases of the cell cycle.

2.5 Immunocytochemical staining

NB2a cells were seeded at $5x10^4$ cells/well in a 24 well-plate and received either no cefepime or IC50 consentration of cefepime (14,12 mM) and incubated for 24 hours. After the incubation, cells were fixed with 4% paraformaldehyde and washed with PBS and incubated with 3% H_2O_2 (Merck, USA) at room temperature to inhibit endogenous peroxidase activity. The cells washed 3 times with PBS for 5 min, then kept on ice for 15 min in % 0,1 triton-X-100 solution to increase membrane permeability. The blocking solution was added at room temperature for 10 min, and the primary antibodies caspase-3, Ki-67 and NOS1 at a dilution of 1:100 were applied and incubated overnight at 4 ° C. The next day, antibodies were removed by washing with PBS, incubated with biotinylated secondary antibody then with horseradish peroxidase-conjugated streptavidin for 15 min each. After washing three times for 5 min each time with PBS, cells were incubated in DAB for 5 min for immunolabeling and subsequently stained with Mayer's hematoxylin. Cells were covered with mounting medium and viewed using an Olympus light microscope (Kurtman et al, 2022). Quantification of staining intensity was performed using the image processing software Fiji (ImageJ version 2.0) as described by Algaidi et al (2019). Data are presented as optical density mean ± standard deviation.

2.6 Statistical Analysis

Graphpad Prism 7 program was performed to evaluate the data. Student-t-test was assessed. Descriptive analyses are presented as mean ± standard deviation (SD). Values with $p < 0.05$ were considered statistically significant (Birinci et al, 2021).

3. RESULTS

The MTT and Muse assays were used to detect the effects of cefepime on cell viability and apoptosis on NB2a mouse neuroblastoma cells. When cefepime was given at 0, 2, 4, 8, 16 and 32 mM concentrations for 24 hours, it was determined that cell viability decreased and apoptosis increased in a dose-dependent manner. The cefepime dose that inhibited 50% of the cells (IC50) for 24 hours was found to be 14.12 mM (Figure 1). According to Annexin V results, total apoptosis was found to be 4,06% (4,10% early apoptosis and 0,016% late apoptosis) in the control group (Figure 2A). In the cefepime treated group, total apoptosis increased and was found to be 48,75% (39,51% early apoptosis and 8,74% late apoptosis) (Figure 2B). To learn the mechanism(s) of interaction, further mechanistic studies were performed using muse analysis to evaluate the effect of the different treatments on the cell cycle profile of the cells. Cell cycle assay showed that the cells in the control group mostly were in G0/G1 phase (64,4% in G0/G1; 26,6% in S phase; 8,1% in G2/M phase) (Figure 2C). However in the cefepime group, the percentage of cells arrested in the S and G2/M phases increased compared to the control group (38,5% in G0/G1; 38,9% in S phase; 18% in G2/M phase) (Figure 2D).

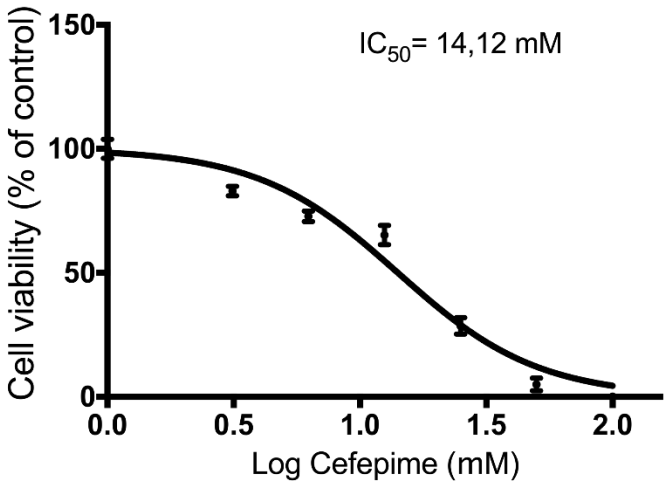

Figure 1. The cell viability effect and IC50 value of cefepime for 24 hours.

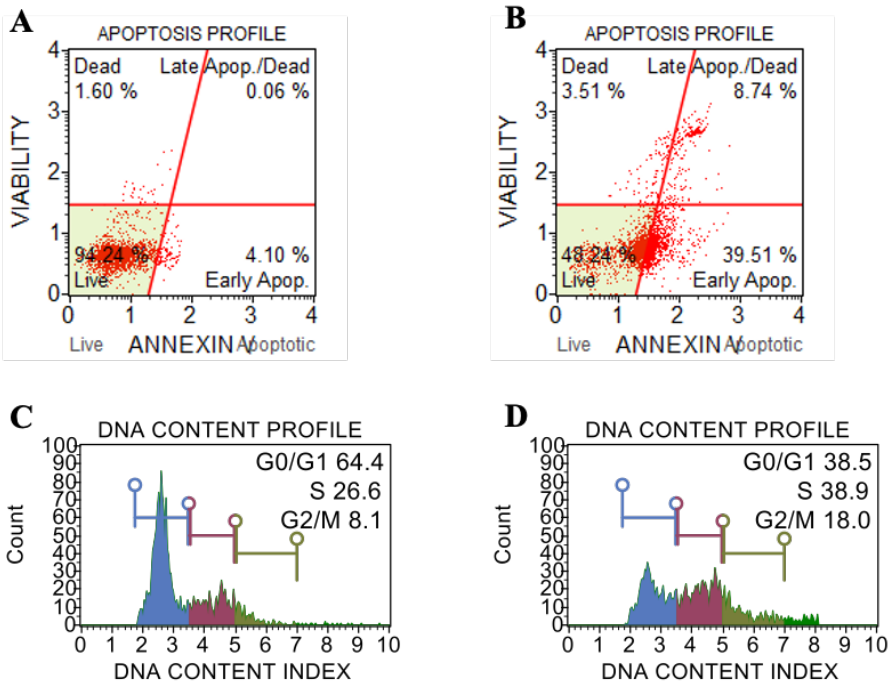

Figure 2. Muse analysis of the groups. A. Annexin V control B. Annexin V cefepime C. Cell cycle control D. Cell cycle cefepime

Caspase-3 and NOS1 immunoreactivities were significantly higher in cefepime group according to the control group ($p<0,001$) (Figure 3,4 and Table 1). Despite that Ki-67 immunoreactivity was significantly lower in cefepime group related to control group ($p<0,001$) (Figure 3,4 and Table 1).

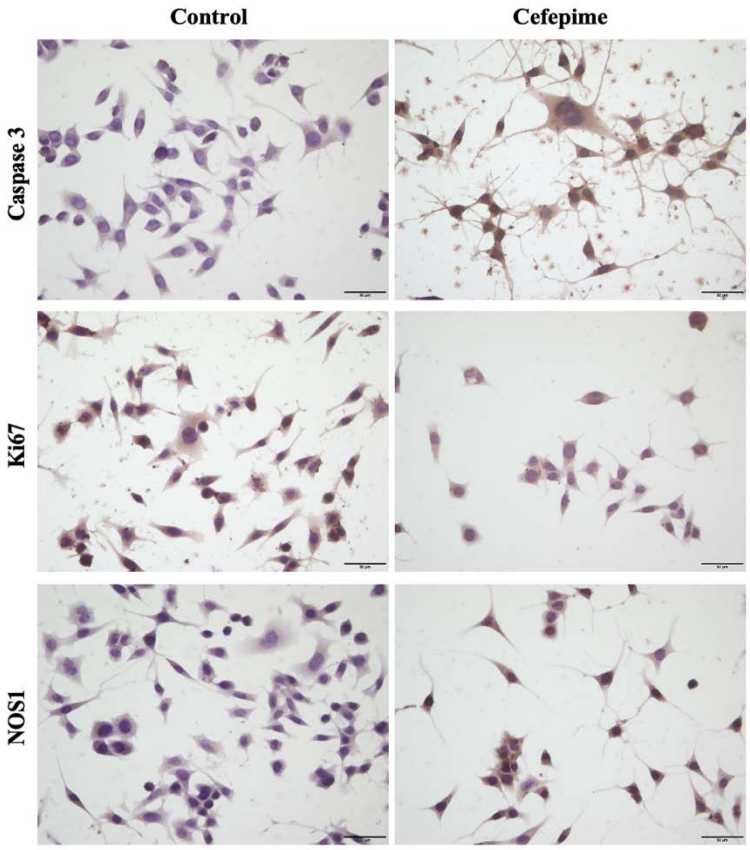

Figure 3. Immunocytochemical images of control and cefepime groups of NB2a cell line. Barr 50µm.

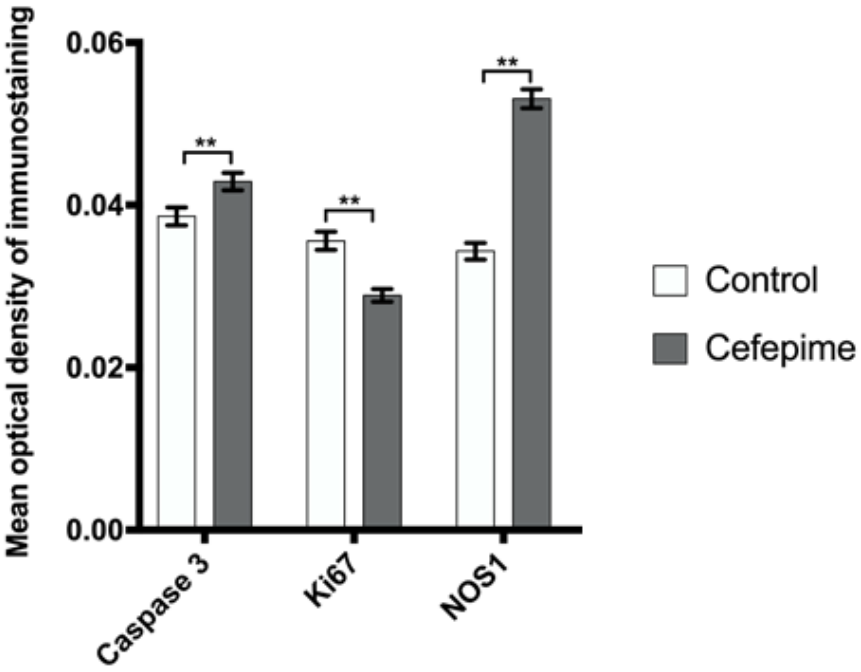

Figure 4. Graphical demonstration of mean optical density of immunostaining results of control and cefepime groups of NB2a cell line. **p<0,001.

Table 1. Mean optical density of immunostaining (mean ± std deviation)

	Control	Cefepime
Caspase-3	0,0386 ± 0,0011	0,0429 ± 0,0011
Ki-67	0,0356 ± 0,0011	0,0289 ± 0,0008
NOS1	0,0343 ± 0,0010	0,0531 ± 0,0012

4. DISCUSSION

Neuroblastoma is a complex disease that affects the sympathetic nervous system and shows high metastasis (Brodeur, 2003; Ün and Ugan, 2021). The majority of patients with neuroblastoma have advanced disease at time of diagnosis. This necessitates the use of aggressive treatment approaches including surgery and high dose chemotherapy (Shusterman et al, 2010). Because of the treatment time and cost there is an interest to new treatment options that may reduce the time, healing duration and also toxicity to normal cells.

Drug development is a time-consuming and costly process, moving from preclinical screening through to clinical trials while resulting in only a few drugs being proven effective and thereby gaining approval. More than $800 million is the estimated cost with 10–17 years required for developing a drug de novo (Li et al, 2012). A few convincing evidence shows that identifying potential 'off' targets of known drugs both helps to avoid serious side effects and supports the possibility of optimizing them for new uses (Hopkins, 2008). Beta-lactam antibiotics include cephalosporin and penicillin antibiotics, they are the most widely used class of antibiotics. They have strong antibacterial effects with minimal toxicity on human cells except for allergic reactions (He at al, 2021). Several evidence shows the potential of ß-lactam antibiotics as anticancer agents specifically targeting tumor cells (Kuhn et al, 2004; Zhang and Jia, 2020). Here we examined the anticancer effect of cefepime, a fourth generation cephalosporin, on the neuroblastoma cells in vitro.

According to our study results, it was determined that cefepime decreased neuroblastoma cell viability over time and the IC50 was found at 14,12 mM. Limited study showing the IC50 values of cefepime was found about the cancer cells in the literature, but there were anticancer studies with different cephalosporins. Pfab et al (2022) applied 50µg/ml cefepime to HT-116 colon cancer cells. He et al (2021) evaluated cell inhibition of some cephalosporins in nasopharyngeal carcinomas and found Ic50 values of 73.10 (cefuroxime sodium), 110.64 (ceftazidime), 111.30 (cefotaxime sodium) and 194.69 (cefmatozele) µg/ml, respectively. Li et al (2012) studied the effect of ceftriaxone on Mouse epidermal JB6 P+ cells and they showed that ceftriaxone had no cytotoxicity toward JB6 P+ cells up to 2000 µM at either 24 or 48h.

The IC50 values and cytotoxic effects of pharmacological agents may vary depending on the cell line used and the method of administrations. In the light of these data, it can be argued that human nerve cells are more resistant to the cytotoxic effects of ACE.

Then we tested the apoptotic rates of the groups after the cefepime administration via the muse analysis and it was shown that the cefepime decreased the cell viability and increased the apoptotic cell numbers. Also according to cell cycle analysis as showing the percentage of cells in the different phases of the cell cycle (pre-G1, G0/G1, S, and G2/M) phases, it was detected that by affecting the cell cycle, cefepime slowed down the rate of cell division and caused the cells to arrest in the S and G2/M phases. Pfab et al (2022) showed that the cefepime alone increased slightly the cells in pre-G1 in HCT-116 cells up to 72 h post-treatment. The IC50 values and cytotoxic effects of pharmacological agents may change depending on the cell line used, the method of administration and the laboratory condition (Cortes et al, 2001). In the light of these data, it can be argued that Mouse neuroblastoma NB2a cells are more resistant to the cytotoxic effects of cefepime.

It was purposed to identify the alterations in the expression level of apoptotic marker caspase-3, cell cycle marker Ki-67 and oxidative stress marker NOS1 proteins related to cefepime administration by

immunocytochemistry. Our data showed that cefepime increased the caspase-3 and NOS1 expressions and decreased the Ki-67 expression in cells. These results revealed that cefepime increased apoptosis and oxidative stress. Elbaz et al (2020) conducted an animal study and they found that all of the ALT, AST, and ALP serum activities as well the serum levels of total, direct, and indirect bilirubin were dramatically reduced upon the treatment with cefepime in the E.coli infected male albino rats. They also found that the serum IL-10, Nitric oxyde and TNF-α levels improved with the cefepime treatment after E.coli infection. Our results demonstrated neuroblastoma toxicity of cefepime in line with existing studies.

As a result, in our study, cefepime suppressed proliferation in Mouse neuroblastoma NB2a cells, induced apoptosis, and caused cell toxicity by increasing oxidative stress. It is important to increase efficiency analyzes in order to reduce the costs in cancer treatment and to use existing drugs more efficiently. We think that larger studies are needed to investigate the toxic effects and mechanisms of cefepime in neural cells.

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