Journal of Medical Case Reports and Reviews

Received 10 Jun2019 | Revised 14 Jun 2019 | Accepted 14 Aug 2019 | Published Online 18 Aug 2019

JMCRR 02 (08), 336-341 (2019) ISSN (O) 2589-8655 | (P) 2589-8647

JMCRR

INVESTIGATION OF APOPTOTIC EFFECTS OF ASPIRIN USE ON HUMAN NEUROBLASTOMA SK-N-MC CELL LINE

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Abstract:

Objective: Apoptosis, which is a programmed cell death, is an event in which cells destroy themselves without inflammation, as well as requiring energy and homeostasis is preserved. In cases where apoptosis slows down, autoimmune diseases and cancer occur. Aspirin is a salicylic ester of acetic acid and has analgesic, anti-inflammatory, antipyretic and antithrombotic effects. In this study, we investigated the effects of aspirin on apoptosis in human neuroblastoma SK-N-MC cell line *in vitro.*

Methods: In our study, trypan blue viability test (Tyripan Blue Exclusion) method was used for aspirin doses to be given to the cells. The apoptotic effect of aspirin in the SK-N-MC cell line was determined by flow cytometry imaging using APC Anexin V antibody.

Results: The aspirin was incubated in SK-N-MC cells at concentrations of 10-100µM, and measurements at 24 and 48 hours were recorded. According to trypan blue results, cell density decreased proportionally with increasing aspirin concentration compared to the control group. The cytotoxic dose was found to be between 50-80 µM at the 24th hour and 70 µM at the 48th hour. Anexin V flow cytometry measurement results showed that 60 and 70 µM aspirin given to SK-N-MC cells increased early apoptosis compared to control group.

Conclusion: The results of our study showed that aspirin induced apoptosis in SK-N-MC cells *in vitro* depending on dose and time.

Keywords: Apoptosis, SK-N-MC Cell Line, Aspirin, Flow Cytometry

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Introduction:

Cancer is a lethal disease with metastasis. There are many types of cancer and can affect all parts of the body. It is caused by the deterioration of the proliferation and differentiation mechanisms of the cells. In a cancer cell, the mechanism of apoptosis is disrupted and the cell has become immortal [1,2]. Apoptosis is programmed cell death. Examples of diseases where apoptosis is unnecessary or accelerated include neurodegenerative diseases, AIDS, myocardial infarction and atherosclerosis; autoimmune diseases and cancer can be given as an example of slowing down diseases [3,4].

Early treatment is important in cancer without spreading to other parts of the body. The earlier the diagnosis is made in cancer, the sooner the treatment starts and the higher the chance of success with early treatment [5]. Although some of the cancer types have very important responses in treatment, it is known that treatment is successful in less than half of the

individuals who have cancer. From this perspective, it is clear that other different solutions to cancer, a global problem, are needed.

Neuroblastoma, which is one of the most common solid tumors in children, originates from primordial neural crest cells normally found in adrenal medulla or sympathetic ganglia and constitutes 8-10% of childhood cancers. Its incidence is similar worldwide and is 1 in 7000 live births annually. The 5-year survival rate is 95% in patients under 1 year of age and 68% in patients between 1 and 14 years of age [6,7]. The etiology is unknown, and there is no environmental factor that has proven to play a role in the etiology. In a group of patients, familial neuroblastoma with autosomal dominant inheritance has been shown, and genetic predisposition in these cases has been shown to be due to germinal mutation [7,8].

Chemotherapy is the most preferred treatment for pediatric tumors. The main source of success in the last 40 years is the addition of chemotherapy to surgical and radiotherapy. The basic principle in chemotherapy is not a single drug, but combined treatment with multiple drugs. Approximately 40 drugs used in different types of cancer are combined according to the results of preclinical studies. The basic principles of the combination are the active substances used at different points of the cell cycle, the cytotoxic/antiproliferative effect of that tumor type on the cell lines, and the non-cumulative toxicity. In addition, the identification of mutations and epigenetic changes in genes thought to be involved in carcinogenesis and targets for this are thought to change the course of treatment [9,10].

Aspirin, also known as acetyl salicylic acid, is the salicylic ester of acetic acid and entered clinical use in 1899 for the first time. Aspirin has analgesic, antipyretic, anti-inflammatory, antipyretic and antithrombotic effects. When it enters the body, aspirin is hydrolyzed and converted to salicylate. It is generally equivalent to most nonsteroidal anti-inflammatory drugs (NSAIDs) in terms of anti-inflammatory and analgesic effects. It is used in the treatment of most inflammatory and autoimmune diseases such as juvenile arthritis, rheumatoid arthritis and osteoarthritis. Due to its antithrombotic effect, it is useful in reducing the risk of myocardial infarction and preventing recurrent transient ischemic attacks. Despite this diversity of effects, it has been used only for anti-inflammatory and analgesic effects for most of the past century. After the determination of the ability to inhibit platelet aggregation in the 1980s, its importance as an antithrombotic drug increased [11]. In recent years, it has been shown that the risk of developing colorectal cancer can be reduced with regular use [12,13].

Based on the fact that cancer is still a serious health problem all over the world, we aimed to investigate the effects of aspirin on apoptosis in vitro, especially in neuroblastoma, one of childhood cancers.

Materials and Methods:

Cell culture

In this study, human neuroblastoma cell line SK-N-MC cells obtained from Ankara Şap Institute Cell Culture Collection (HÜKÜK) were used. Cells were cultured in Dulbecco's Minimum Essential Medium (DMEM- Biochrom, F-0445, Germany) containing 15% fetal calf serum (Biochrom, S0115, Germany), 10,000 U/ml penicillin-10 mg/ml streptomycin-0.025 mg/ml amphotericin-B (Biological Industries 03–033–1C, Israel) and 200 MM L-Glutamine (Biochrom, AG K0282, Germany) at 37◦C, 5% carbon dioxide and 1 atmosphere pressure. Cells were routinely passaged twice a week and used in the experiments when the cells occupied 75% of the culture vessel in density.

In vitro Cytotoxicity Assay

Three culture dishes were prepared for each dose of aspirin. Aspirin (Sigma-Aldrich, A2093, USA) was added at different dilutions to $1x10^5$ cells/1ml in 24-well plates; toxicity was determined by trypan blue stain (Sigma-Aldrich, T8154, USA) for 12, 24, 36 and 48 hours.

No aspirin dose was administered to the control group. The concentration showing a cytotoxic effect of 50% compared to the control was considered as the cytotoxic dose. Concentrations of 60 µM and 70 µM with a viability of 70-80% were used in flow cytometer experiments.

Flow Cytometry Analysis

Aspirin (Sigma-Aldrich, A2093, USA) $1x10^6$ cells/1ml was added to 24-well plates at 60-70 µM concentrations and incubated for 24 and 48 hours. At the end of the period, the cells were treated with Annexin-V FITC Apoptosis Detection Kit (BD Pharmingen, 556547, USA). All experimental steps were performed on ice. The protocol was performed as follows: cells were removed from the culture dish and 2 ml of cold phosphate buffer was suspended. Following centrifugation, the supernatant was discarded and 150 µl of anexin buffer was added to the cell pellet. 5 µl of anexin dye was added to each sample and vortexed for 40 min at room temperature and incubated in the dark. At the end of the incubation, 300 µl of cold anexin

buffer was added. 5 µg/ml propodium iodide was added and left for another 15 minutes. At the end of time, cell samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA).

Statistical Analysis

Flow cytometric analyzes were repeated three times. Data were expressed as mean ± standard error and Dunnet _t test was used. In the statistical study, α = 0.05 was determined as the first type error amount and compared with the significance values obtained from each study (p). Statistica v7.0 package program was used in the analysis.

Results:

In our study, three serial cultures were performed for each dose of aspirin. No application was made to the control group. Cell viability, early apoptosis, late apoptosis and necrosis rates at 24th and 48th hours were evaluated by flow cytometry (Figure 1).

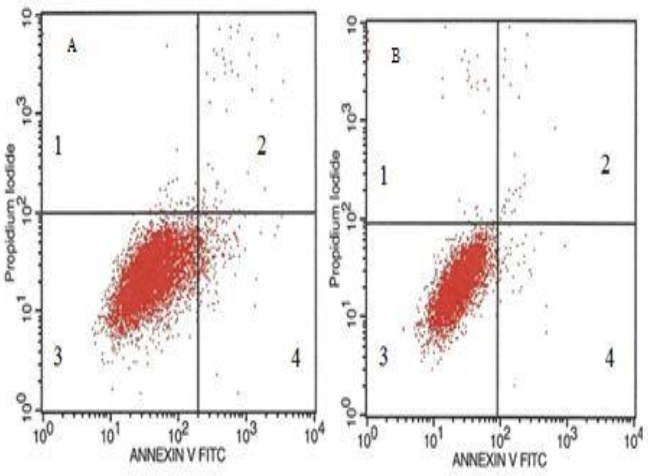

Figure1: Flow cytometry evaluation of viability and apoptosis in SK-N-MC cells.

A) Negative Control / 24 hours; B) Negative Control / 48 hours. Area 1 shows necrosis, area 2 shows late apoptosis, area 3 shows live cells and area 4 shows early apoptosis.

The mean percentage of viable cells in the SK-N-MC cell line at 24 h was compared to the negative control, although there was a decrease in the number of live cells in the 60 µM aspirin group, it was not statistically significant. In the 70 µM aspirin group, there was a statistically significant decrease in the number of viable cells. On the other hand, there was a decrease in the percentage of live cells at 48 hours in all groups (Table 1).

Table 1. Live cell average at 24th and 48th hours in the SK-N-MC cell line.

	Group Name	Number of Samples (N)	Average Cell Percentage		Standard Deviation		Average Difference		p Value	
			24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour
Live Cells	Aspirin 70 µM	3	94.02	86.21	0.098	1.05	-3.11	-12.64	0.027*	0.000*
	Aspirin 60 µM	3	95.02	91.19	0.325	0.67	-2.11	-7.66	0.193	0.007*
	Negative Control	3	97.13	98.85	1.43	0.24				

The mean percentage of early apoptotic cells in the SK-N-MC cell line at 24 h was compared to the negative control and a statistically significant increase was detected in the 70 µM aspirin-treated group. The mean early apoptotic cell percentage at 48th hour was significantly higher in all groups (Table 2).

Table 2. Early apoptotic cells average at 24th and 48th hours in the SK-N-MC cell line.

	Group Name	Number of Samples (N)	Average Cell Percentage		Standard Deviation		Average Difference		p Value	
			24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour
Early Apoptotic Cells	Aspirin 70 µM	3	6.69	12.25	0.41	0.91	4.84	11.84	0.000*	0.000*
	Aspirin 60 µM	3	1.03	6.19	0.48	0.44	-0.82	5.78	0.783	0.007*
	Negative Control	3	1.85	0.41	1.38	0.13				

The mean percentage of late apoptotic cells in the SK-N-MC cell line at 24 h was compared to the negative control and a statistically significant increase was detected in the 70 µM aspirin-treated group. Although the mean late apoptotic cell percentage at 48th hour was increased in all groups, this increase was not statistically significant (Table 3).

Table 3. Late apoptotic cells average at 24th and 48th hours in the SK-N-MC cell line.

	Group Name	Number of Samples (N)	Average Cell Percentage		Standard Deviation		Average Difference		p Value	
			24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour
Late Apoptotic Cells	Aspirin 70 µM	3	2.55	1.19	0.35	0.04	1.98	0.79	0.008*	1.000
	Aspirin 60 µM	3	1.08	1.46	0.16	0.09	0.51	1.07	0.913	1.000
	Negative Control	3	0.57	0.39	0.07	0.04				

The mean percentage of necrotic cells in the SK-N-MC cell line at 24 h was compared to the negative control and a statistically significant

increase was detected in the 70 µM aspirin-treated group. Although the mean percentage of necrotic cells at 48th hour was increased in all groups, this increase was not statistically significant (Table 4). Flow cytometric evaluation of all studied groups is shown in Figure 2.

Table 4. Necrotic cells average at 24th and 48th hours in the SK-N-MC cell line.

	Group Name	Number of Samples (N)	Average Cell Percentage		Standard Deviation		Average Difference		p Value	
			24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour	24 Hour	48 Hour
Necrotic Cells	Aspirin 70 µM	3	2.37	0.68	0.62	0.53	1.91	0.31	0.0034*	1.00
	Aspirin 60 µM	3	0.44	1.46	0.09	1.06	-0.02	1.09	1.000	0.99
	Negative Control	3	0.46	0.37	0.02	0.16				

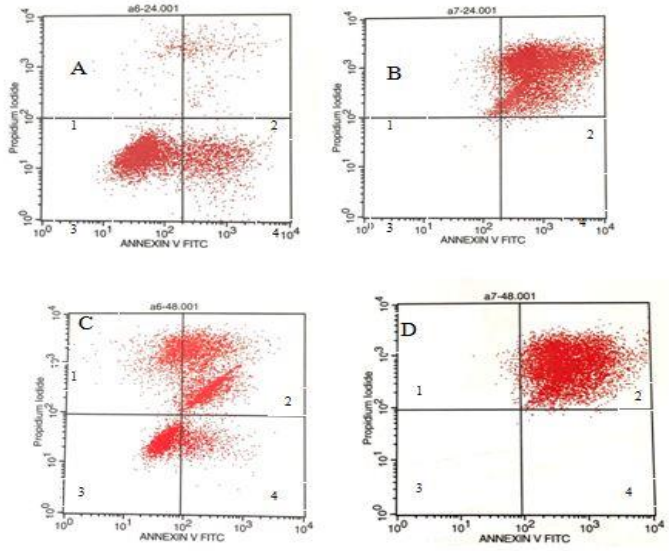

Figure 2: Flow cytometry evaluation of viability and apoptosis in SK-N-MC cells. A) 24-hour group with 60µM aspirin; B) 24-hour group with 70 µM aspirin; C) 48-hour group with 60 µM aspirin; D) 48-hour group with 70 µM aspirin. Area 1 shows necrosis, area 2 shows late apoptosis, area 3 shows live cells and area 4 shows early apoptosis.

Discussion:

In the process of diagnosis and treatment of cancer, new drug development studies are very important because of the psychological and physiological side effects experienced by the patients. Although its role has not been fully elucidated, the expression of cyclooxygenase (COX) inhibitors is known to be quite high in many types of cancer. With the introduction of NSAIDs that suppress COX expression in the treatment of cancer, a new perspective has been introduced to the treatment process. Aspirin inhibits COX enzyme by irreversible acetylation and thereby inhibits prostaglandin synthesis. In recent years, *in vitro* and animal modeling studies have shown that aspirin has effects in reducing the risk of various cancers. In this context, we aimed to investigate whether aspirin use has effects on apoptosis, especially in neuroblastoma, which is one of the childhood cancers.

Aspirin and non-steroidal antiinflammatory drugs have been found to reduce the risks associated with many types of cancer, prostate cancer patients have been found to have life-saving benefits. 1206 patients undergoing local radiation therapy and 232 patients who had normally used aspirin prior to treatment were studied in Fox Chase Cancer Center. In patients who had used aspirin and other NSAIDs before the disease, the rate of spread of the disease was scientifically decreased and the risk of a second cancer was reduced. Laboratory research also found that these drugs prevent the formation of blood vessels that feed tumors [14].

In animal models and various in vitro epidemiological studies have shown that aspirin and other non-steroidal anti-inflammatory drugs have anticancer effects in various organs including colorectum, esophagus, stomach, breast, lung and ovarium. The biochemical mechanism of these effects of NSAIDs has been linked to providing inhibition of COX activity and ultimately reducing prostaglandin levels [15].

Zhong et al. found that regular aspirin intake reduces the risk of breast cancer [16]. In particular, aspirin has been shown to be more protective in breast cancer individuals carrying the PIK3CA mutation [17]. The use of aspirin in breast cancer patients has been found to inhibit various pathways associated with cell growth, invasion, motility and metastasis [18].

Another study showed that routine but low-dose aspirin use can significantly reduce the risk of prostate cancer [19]. Barton et al. showed that the use of aspirin in individuals with prostate cancer may reduce mortality [20]. There are several studies suggesting that aspirin use also reduces the risk of cancer in gastric cancers. Yang et al. found that aspirin in human gastric carcinoma inhibits survivin gene expression and reduces cell growth [21]. Tahara et al. showed that routine aspirin use inhibits CDH1 methylation in human gastric mucosa [22]. Aspirin has been shown to induce caspase-independent pathway of mucosal

cell death in human gastric cells [23]. People with routine aspirin and other nonsteroidal anti-inflammatory drugs have a low risk of colorectal cancer [24, 25]. Routine aspirin use increases the risk of colorectal cancer by 22%; NSAID use has been shown to reduce by 30-40% [26]. The American Cancer Society (ACS) does not recommend the use of aspirin and other NSAIDs to prevent colorectal cancer because of possible side effects, such as gastric bleeding. However, they suggested that individuals taking aspirin may have a low risk for colorectal cancer in various special disease states (such as chronic arthritis) [27].

Mc Menamin et al. reported that the use of low-dose routine aspirin reduces mortality in a study of patients with lung cancer [28]. Hochmuth et al. reported that aspirin use reduces the risk of non-small lung cancer in their case control study [29].

In this study, in accordance with the literature, aspirin use has been shown to increase especially early apoptosis. One of the aims of cancer treatment is to enable cancer cells to go to apoptosis. Aspirin cause an increase in early and late apoptotic levels in accordance with this aim supports this thesis. According to the results of our study, the effects of aspirin in cancer were determined in an in vitro study which is a guide for cancer treatment. Thus, with this preclinical study, the benefits of this aspirin in cancer treatment were evaluated before application to the patient.

In conclusion, the findings of this study suggest that the use of aspirin in neuroblastoma may induce apoptosis in part and contribute to tumor regression. In recent years, the use of aspirin and other NSAIDs in various cancers has emerged as alternative therapeutic strategies. The molecular mechanisms of these strategies will shed light on the development of new treatment modalities in cancer.

Acknowledgements: The authors were grateful for their support in this study PhD. Ata Özçimen, MD. PhD. Burak Çimen and Chemist Cemil Gülüm.

Conflict of Interest: The authors declared that this article has not had any conflict of interest during the preparation and publication of this manuscript.

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