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ORIGINAL ARTICLE

Evaluation of Pharmacological Effective Concentrations of Aspirin with/without Radiotherapy on MCF-7 Breast Cancer Cell Line

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Keywords:
Aspirin;
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Abstract

Objective--The aim of the present study was to evaluate pharmacological effective concentrations of aspirin with/without radiotherapy on growth rate of MCF-7 breast cancer cell line.

Design - Experimental *in vitro* study.

Procedures- The MCF-7 breast cancer cell line was prepared commercially and cultured. The cultured cells were then separated to labelled tubes and treated for 24 hours with 1, 2, 3, 4, and 5 mg aspirin plus 0.1 mg doxorubicin. Cells were then exposed to radiation. Cell proliferation and survival were measured by MTT assay, following acridine orange and propidium iodide staining methods using spectrophotometry and fluorescence microscopy.

Results- The findings showed that proliferation and survival of the cells treated with 5 mM aspirin followed by radiotherapy were significantly decreased compared to them of the control group ($P<0.05$).

Conclusion and clinical relevance- Although anti-proliferative activity of aspirin was lower than that of doxorubicin, it can be considered in combination therapy because of its affordability and cost-effectiveness.

Keywords- Aspirin, radiotherapy, MCF-7 breast cancer cell line

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1. Introduction

In the last half century, along with a reduction in mortality due to infectious diseases, cancer has become a common cause of death in industrialized countries. According to a report, cancer accounts for about 13% of human deaths in 2007. Despite the extensive worldwide researches done for understanding the causes of cancer and examining several therapeutic methods for it, the pathologic aspects of the disease are still unknown.[1] There are now several treatment methods for cancer patients that depends on some clinical factors like: the type of cancer, the condition of the disease at the beginning of treatment, the age of the patient, the general health, and the patient response to the applied treatment type. These treatments include surgery, radiation therapy, chemotherapy, gene therapy, etc. Chemotherapy was first performed in 1955[2], when there was just one anticancer drug, but todays thousands of new and effective drugs have been discovered. Many cancer cells respond initially to chemotherapy, but then develop resistance to it. In addition, chemotherapeutic drugs produce undesirable side effects.[3] Therefore, development of an inexpensive and eco-friendly method for cancer treatment is essential. Breast cancer is the most prevalent malignancy and second leading cause of death from cancer of women around the world. Breast cancer accounts for about 4.21% of all reported cases of cancers in Iran.[4] The raw incidence of breast cancer in Iran is estimated to be about 4.22 per 100,000 women, and the available data suggests that the disease has taken an increasing trend. In spite of the important advances in diagnosis and treatment, breast cancer is one of the most common problems of worldwide women. Common treatments for breast cancer often have short-term therapeutic effects, and sometimes could lead to drug resistance.[5] This is done in various ways, including induction of apoptosis, alteration in DNA structure, inhibition of topoisomerases and tyrosine kinases, inhibition of mitotic division, transcriptional inhibition, inhibition of replication, etc.[6] Aspirin (acetylsalicylic acid; ASA) is essentially an anodyne, anti-fever and anti-inflammatory drug, belongs to the family of non-steroidal anti-inflammatory drugs (NSAIDs).[7] Aspirin is mainly used as a painkiller, but its many other effects make it one of the most widely used medicines around the world with about 80 billion annually sold worldwide. Having anticoagulant effects, aspirin could also be used as a blood diluent to prevent formation of blood clots in people that have history of heart or brain stroke.[8]

Recent studies have been shown that daily intake of low-dose aspirin (70-100 for several years) reduces the risk of cancer and death from it.[9] In an study based on case-control and laboratory reports, aspirin usage reduced the incidence of colorectal cancer by 35%, gastrointestinal cancers by 30%, breast cancer and prostate cancer each by 10%, and lung cancer by 5%.[10] Aspirin inhibits cyclooxygenase 1 (which induces pain, fever, inflammation and platelet aggregation), but activates cyclooxygenase 2. Aspirin also reduces the production of prostaglandins. Prostaglandins may have activator role for cancer stem cells.[11] In breast cancer tissue, the expression levels of cyclooxygenase 1 and 2, as well as type 2 prostaglandins are higher than normal tissues. Type 2 prostaglandins causes increase in expression of BCL-2 causing a decrease in apoptosis and increase in angiogenesis.[12] Different therapies such as surgery, chemotherapy and radiotherapy could be used to treat various types of cancer. The basis of radiation therapy is explosion of malignant cells to an ionizing light which causes death of these malignant cells. The ideal goal in radiation therapy is that the target tumor receives the highest dose of radiation, while the surrounding healthy tissues take lowest.[13] Regarding to these facts, we aimed to investigate the effects of aspirin along with radiotherapy on MCF7 cell line.

2. Materials and Methods

MCF-7 Cell Culture

MCF-7 Cells were Prepared from the Iranian Cell Bank (Pasteur Institute, Tehran, Iran). Cells were cultured in RPMI-1640 medium with 10% FBS in 37 $^{\circ C}$ incubator and 5% CO_2. When cells occupy about 70% of the surface of the flask, they were removed from the flask by trypsin and transferred to a 96-well plate. Following the method applied by Abtahi Froushani et al. in 2015, the cells with concentration of 0.25 mg/ml were treated with doxorubicin 0.1 mM as positive control for 48 hours.[14]

Design of the Experiment

Aspirin and doxorubicin were bought from Sigma-Aldrich (Tauf-kirchen, Germany) and various dilutions of aspirin and dilution of 0.1 of doxorubicin were prepared. A 2G radiation access was also settled up. Categorization of study groups were performed as shown in Table 1.

Table 1. Study Grouping

Group	Treatment	Group	Treatment
1		8	Radiation
2	Doxorubicin	9	aspirin 1 mM + Radiation
3	aspirin 1 mM	10	aspirin 2 mM + Radiation
4	aspirin 2 mM	11	aspirin 3 mM + Radiation
5	aspirin 3 mM	12	aspirin 4 mM + Radiation
6	aspirin 4 mM	13	aspirin 5 mM + Radiation
7	aspirin 5 mM		

Determination of Cell killing Efficacy Using MTT Method

MTT method using tetrazolium color is an appropriate laboratory method based on colorimetry that determines cellular life style. 48 h after treatment of the cells, 20 µl of prepared MTT solution (PBS =5mg/ml) was added to each well. Plates were then incubated for 4 hours. Then, the remnants were removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to allow dissolving the created formazan. After shaking the plates for 20 minutes on shaker, the formazan light absorption (OD) of tests and standards were measured and concentration of tests were calculated using standard concentration curves .[15] The results were expressed as the "Proliferation Index" according to the ratio of OD492 of the live cells to dead cells.

Evaluation of apoptosis rate

It is believed that fluorescence staining followed by cell counting under the microscope (if done meticulously) is the easiest and fastest way to detect living cells from dead ones. In this part of the study we used the fluorescence dyes acridine orange and ethidium bromide. Acridine orange is a vital dye and is absorbed by living cells. Acridine orange enters the DNA of living cells, and under the microscope, gives a green view to the chromatin of live cells. But ethidium bromide only paints the dead cells. Ethidium bromide enters the dead cell's DNA and gives an orange color under the fluorescence microscope to their chromatins. When using ethidium bromide solution plus acridine orange as dying solution, living cells take green and dead cells take orange to brown view. For this purpose, we prepared 100 µg/ml concentrations of both acridine orange and ethidium bromide by dissolving 100 µg of acridine orange or ethidium bromide in 1 ml of PBS buffer. We then mixed 10 µl of the gained solution with 250 µl of cell suspension, and placed 10 ml of this mixture on a clean slide and cover it with lamellas. Then we counted at least 200 live or dead cells with fluorescence microscope using 40x and 60x lenses. The number of green cells (live) divided by sum of green cells (live) plus dead cells (orange) is equivalent to the percentage of live cells.[16]

3. Statistical Analysis

The collected data were analyzed by EXCEL and SPSS softwares. After calculating the central index, mean, and dispersion index, standard deviation, the student *t* and ANOVA tests were used to analyze the data. The results were reported as mean ± standard error and $p < 0.05$ was considered as significant.

4. Results

Comparing the data obtained from three tests: MTT, evaluation of the percentage of cancer cells treated with different concentrations of aspirin, and radiation, showed that the mean percentage of cell life in the treated group with 5 mM aspirin plus radiation was significantly lower than that in negative control and other treated groups except doxorubicin group. On the other hand, the mean vitality of cancer cells in treated with doxorubicin was lower than that of other treated groups (Table 2). The results of coloring of acridine orange and propidium iodide showed that the apoptosis of treated cells with 5 mM aspirin plus radiation was significantly higher than negative control group and other treatment groups in except of doxorubicin. However, the mean vitality of cancer cells in treated with doxorubicin was higher than that of other treated groups. (Table 2; Figures 1 and 2).

Table 2. Results of proliferation and apoptosis of MCF-7 cells before and after treatment with aspirin, doxorubicin, and radiation.

Study group	Proliferation Index (MTT)	Percentage of apoptosis
Negative control	0.529 ± 0.006	21.0 ± 1.000
Doxorubicin	0.235 ± 0.005	73.0 ± 3.000
Aspirin 1 mM	0.481 ± 0.003	23.0 ± 2.000
Aspirin 2 mM	0.455± 0.005	29.66 ± 1.527
Aspirin 3 mM	0.442 ± 0.005	35.0 ± 2.000
Aspirin 4 mM	0.356 ± 0.007	38.66 ± 1.527
Aspirin 5mM	0.445 ± 0.005	42.66 ± 2.081
Radiation	0.386 ± 0.010	41.0 ± 2.000
Aspirin 1 mM + Radiation	0.354 ± 0.004	39.33 ± 1.527
Aspirin 2 mM + Radiation	0.305 ± 4.018	46.33 ± 1.527
Aspirin 3 mM + Radiation	0.284 ± 0.009	53.33 ± 3.511
Aspirin 4 mM + Radiation	0.261 ± 0.003	61.0 ± 3.605
Aspirin 5 mM + Radiation	0.252 ± 0.003	65.66 ± 2.081

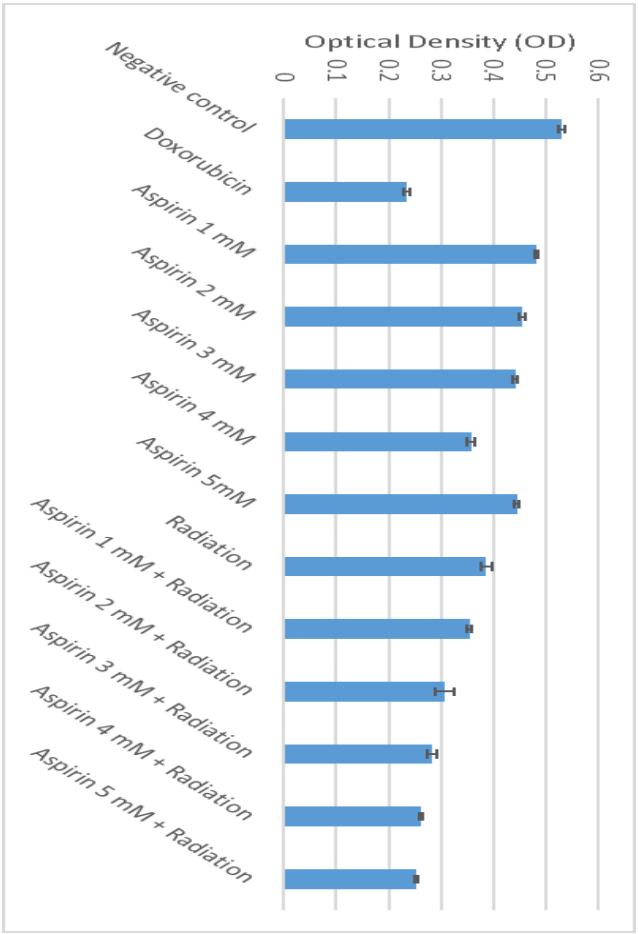

Figure 1. Results of proliferation of MCF-7 cells before and after treatment with aspirin, doxorubicin, and radiation

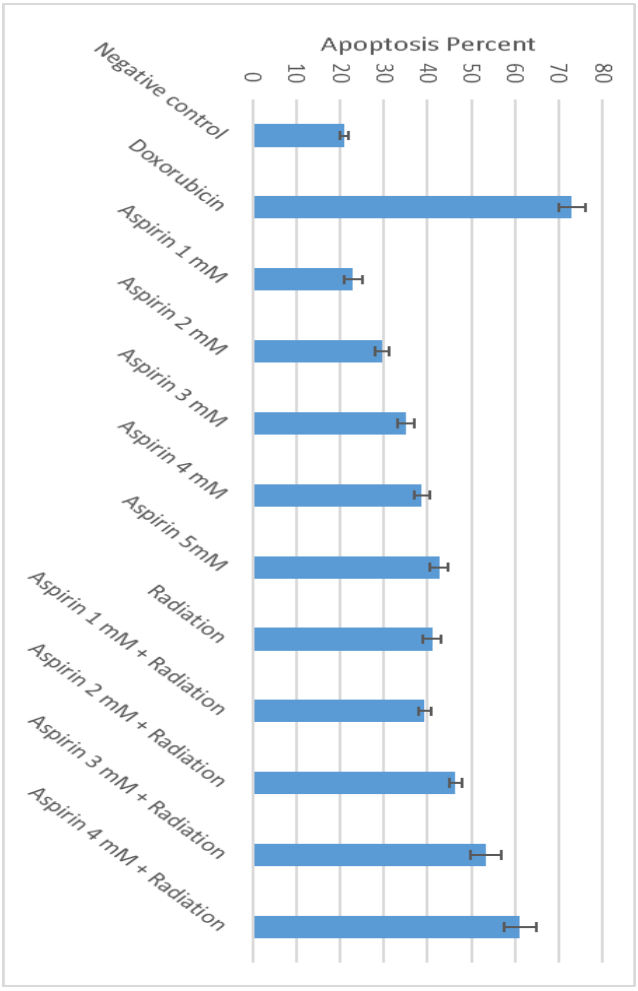

Figure 2. Results of apoptosis of MCF-7 cells before and after treatment with aspirin, doxorubicin, and radiation

5. Discussion

According to studies on the anti-proliferative effects of aspirin on cancer and taking in account that its effects are not tissue dependent, we have investigated the synergistic effects of aspirin with radiotherapy. Our findings showed that aspirin shows an anti-proliferative effect, which is in accordance with other studies. This effect was higher in patients treated with aspirin plus radiotherapy than the drug alone. Recent studies have been shown that nitro-aspirin has an anti-proliferative effect on MCF-7 cells. Placing various side chains on aspirin molecule would increase its anti-tumor effect. Studies on mechanism of its effect has been shown that aspirin induces apoptotic effects on MCF-7 cells through capturing the Wnt/b-catenin/TCF pathways. It has been shown that nitro-aspirin may reduce the proliferation and increase the percentage of apoptosis in MCF-7 cells.[17] Keramati et al. (2011) stated in their study

titled "Effect of Ketoprofen on Ovarian Cancer Induced by DMBA in Female Rat" that as cyclooxygenases cause production of E2-type prostaglandins and hereto activates an enzyme called aromatase that is a part of cytochrome P450 enzymes. This aromatase is capable of converting endogens into estrogen that may cause an increment in tumor growth. They included that ketoprofen may reduce tumor growth, inhibit angiogenesis, decrease cell proliferation, and increase apoptosis by inhibiting cyclooxygenases. They also concluded that cyclooxygenases increase EGF (Epithelial Growth Factor) level causing increase in cellular proliferation tumor growth. Therefore, by inhibiting cyclooxygenase and reducing EGF, ketoprofen decreases cell proliferation and tumor volume and increases apoptosis rate.[18] Fouad et al. In their study in 2014 demonstrated that the transcription factor NF-KB, type 2 cyclooxygenase, and JAK-STAT signaling pathway play an important and significant role in the breast tumorigenesis. Inflammatory molecules such as IL-6, IL-8, TNF-α, interferon gamma, and IL-1β play an impressive role in increasing the expression of these molecules. They prevented the proliferation and survival of breast cancer cells by using inflammatory inhibitors in their study.[19] Li-Xia et al. (2012) stated according to their study results that expression of type 2 prostaglandins was higher in breast cancer cells than normal cells, and these molecules, in turn, increases the expression of Bcl2, a molecule that diminishes apoptosis rate in breast cancer cells. Bcl2 also stimulates estrogenesis and increase in estrogen which stimulates angiogenesis of cancerous cells. The authors suggested that the use of inhibitors of prostaglandin synthesis would diminish the growth rate of breast cancer cells.[20]

Weinman et al. (2015), used Firocoxib, a non-steroid anti-inflammatory drug (NSAID), in their study to treat interstitial cell cancer. According to the results of their study, there is a strong association between this drug, cyclooxygenase and prostaglandin. This drug by inhibiting cyclooxygenase would inhibit the synthesis of prostaglandins and subsequently estrogen production. Diminish in estrogen concentration would increase apoptosis rate of cancerous cells, and the proliferation of these cells would stop. This drug and its derivatives will be more effective in chemotherapy by reducing the amount of prostaglandins.[21] In the study of Mahzouni et al. in 2014 on meningioma (central nervous system tumor), it is revealed that there is a close relationship between the expression of cyclooxygenase and the degree of disease, so that in the severe meningioma, the expression level of cyclooxygenase is highest, Consequently, the level of prostaglandin expression is also increased and body conditions become more favorable for tumor growth. The authors stated that the use of cyclooxygenase inhibitors would be helpful in meningioma, and the patient would receive an appropriate healing response.[22] Our study showed similarly that the rate of proliferation and survival of cancer cells in aspirin plus radiotherapy was lower than that of aspirin alone. According to the results of our study, it seems that the use of aspirin plus radiotherapy along with common chemical drugs used in the treatment of breast cancer, would be helpful as a combination without side complications, and given the fact that this drug is easy to access and economic in our country, It's administration and use could be of great help to breast cancer patients.

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Conflicts of interest

None

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چکیده

ارزیابی اثر فارماکولوژیکی غلظت‌های آسپرین با و یا بدون پرتودرمانی بر روی رشد رده سلولی MCF-7 سرطانی سینه

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هدف: مطالعه حاضر به‌منظور ارزیابی اثر فارماکولوژیکی غلظت‌های آسپرین با و یا بدون پرتودرمانی بر روی رشد رده سلولی MCF-7 سرطانی سینه بود .

طرح مطالعه: مطالعه تجربی برون اندامی

روش کار: رده سلولی MCF-7 سرطانی سینه به‌طور تجاری تهیه و کشت داده شد. سپس سلول‌های کشت داده‌شده به لوله‌های برچسب دار منتقل شده و به مدت ۲۴ ساعت دوزهای ۱، ۲، ۳، ۴ و ۵ میلی‌گرمی آسپرین همراه با ۰/۱ میلی‌گرم دوکسوروبیسین به آن‌ها افزوده شد. سپس سلول‌ها در معرض پرتو قرار گرفتند. تزاید و بقای سلولی با استفاده از سنجش MTT مورد بررسی قرار گرفت و متعاقب آن سلول‌ها با آکریدین اورنج و ید پروپدیوم رنگ‌آمیزی شده و با طیف‌سنج و میکروسکوپ نوری مورد بررسی قرار گرفتند.

نتایج: یافته‌های این مطالعه نشان داد که تزاید و بقای سلولی در سلول‌های درمان شده با آسپرین ۵ میلی‌گرمی متعاقب پرتودرمانی به میزان معنی‌داری در مقایسه با گروه کنترل کاهش یافت ($P<0.05$).

اهمیت بالینی: اگرچه فعالیت ضد تکثیر سلولی آسپرین کمتر از دوکسوروبیسین بود، اما می‌توان آسپرین را به علت ارزان و در دسترس بودن همراه با دوکسوروبیسین بکار برد.

کلمات کلیدی: آسپرین، پرتودرمانی، رده سلولی MCF-7 سرطانی سینه