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Research Paper

Investigating Spleen-cell-conditioned Medium Treated with Different Concentrations of Adenosine on 4T1 Breast Cancer Cells

Maryam Talebi Lili[1], Nowruz Delirezh[2*], Seyed Meysam Abtahi Foroushani[3]

1. Master's degree of Immunology, Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
2. Professor of Immunology, Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
3. Associate Professor of Immunology, Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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Key words:
Adenosine, Breast cancer, Spleen-cell-conditioned medium

ABSTRACT

Aims The present study aimed to assess the effects of spleen-cell-conditioned medium treated with different concentrations of adenosine on the 4T1 breast cancer cell line.

Materials & Methods For this purpose, mouse spleens were isolated in completely sterile conditions, and splenocytes were then isolated for culture. Different concentrations of adenosine (25, 50, and 100 µM doses), phytohemagglutinin solution, and culture medium containing Fetal Bovine Serum (FBS) were added to splenocytes and incubated for 72 h. Thereafter, splenocytes were incubated by adding fresh culture medium without serum for 24 h. This fluid was collected and stored in a freezer at -80 for further assessment. The conditioned medium was mixed with an equal volume of the culture medium containing 4T1 cancer. The studied groups included control containing untreated 4T1 cancer cells, spleen-cell-conditioned medium untreated with adenosine, spleen-cell-conditioned medium treated with adenosine ~25 µM, spleen-cell-conditioned medium treated with adenosine ~5 µM, and the fifth group: spleen-cell-conditioned medium treated with adenosine ~10 µM. After 48 h, the viability of 4T1 cells was measured by MTT and NR tests, and the amount of apoptosis in the cells was evaluated by vital dye staining.

Findings The spleen-cell-conditioned medium is able to damage cancer cells and causes a significant reduction in neutral red uptake or damage to the cancer cell membrane. In the MTT test, a decrease was observed in the mitochondrial power; that is to say, a decrease in cell viability of cancer cells compared to the control group. In the apoptosis test, a percentage of cancer cells changed color from green to red. This function of the conditioned medium was strengthened by adding adenosine ~25 µM to the spleen cell conditioned medium so that the amount of neutral red uptake was markedly reduced and caused a further decrease in the mitochondrial power of cancer cells. It also increased apoptosis in cancer cells so that most cancer cells changed color to red. The beneficial effect of the spleen-cell-conditioned medium decreased by increasing the concentration of adenosine to 5 µM so that there was no statistically significant difference with the untreated spleen-cell-conditioned medium. When we increased the concentration of adenosine to 10 µM, the killing efficacy disappeared, and there was no significant difference with the control group.

Conclusion Based on the results, the spleen-cell-conditioned medium was able to kill a percentage of cancer cells. When we treat the spleen-cell-conditioned medium with low concentrations of adenosine, we observe a marked improvement in the killing efficacy of cancer cells, as well as an interaction between immune cells and cancer cells. Nonetheless, when the concentration of adenosine increases, the killing efficacy of cancer cells is lost and completely reversed, resulting in the growth of cancer cells.

* ***Corresponding Author:***
Nowruz Delirezh, PhD.
Address: Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
Tel: +98 9144432650
Email: n.delirezh@urmia.ac.ir

مقاله پژوهشی

بررسی اثرات محیط شرطی شده سلول‌های طحالی تیمار شده با غلظت‌های مختلف آدنوزین بر روی سلول‌های سرطان پستان رده سلولی 4T1

مریم طالبی لیلی[۱]، نوروز دلیرژ[۲*]، سید میثم ابطحی فروشانی[۳]

۱. کارشناسی ارشد ایمنی شناسی، گروه میکروبیولوژی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.
۲. استاد، بخش ایمنی شناسی، گروه میکروبیولوژی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.
۳. دانشیار، بخش ایمنی شناسی، گروه میکروبیولوژی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.

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چکیده

هدف: مطالعه بررسی اثرات محیط شرطی شده حاصل از سلول‌های طحالی تیمار شده با غلظت‌های مختلف آدنوزین بر روی سلول‌های سرطان پستان رده سلولی 4T1 است.

مواد و روش ها: برای این منظور طحال موش سوری در شرایط کاملا استریل جداسازی و سپس اسپلنوسیت‌ها برای کشت جداسازی شدند. غلظت‌های ۲۵ µM، ۵۰ µM و ۱۰۰ µM از آدنوزین به همراه محلول فیتوهماگلوتینین، محیط کشت حاوی FBS به اسپلنوسیت‌ها اضافه وبه مدت ۷۲ ساعت انکوبه شد.سپس اسپلنوسیت‌ها را با اضافه کردن محیط کشت تازه بدون سرم به مدت ۲۴ ساعت انکوبه شد. این بارمایع‌رویی جمع‌آوری و برای مطالعات در فیریز 80- نگهداری شد. محیط شرطی شده به مقدار ۵۰٪ به ۵۰٪ در محیط کشت حاوی سلول‌های سرطانی رده 4T1 استفاده شد. گروه‌های مورد مطالعه شامل: کنترل حاوی سلول‌های سرطانی 4T1 بدون تیمار، محیط شرطی شده حاصل ازسلول‌های طحالی بدون تیمار با آدنوزین، محیط شرطی شده حاصل از سلول‌های طحالی تیمارشده با غلظت ۲/۵ µM آدنوزین، محیط شرطی شده حاصل از سلول‌های طحالی تیمارشده با غلظت ۵ µM آدنوزین و گروه پنجم محیط شرطی شده حاصل از سلول‌های طحالی تیمارشده با غلظت ۱۰ µM آدنوزین. پس از گذشت ۴۸ ساعت زنده‌مانی سلول‌های 4T1، توسط تست MTT (3-(4,5-dimethyltrhiazol-2-yl)-2,5-diphenylterazolium bromide) و NR (Neutral Red) سنجیده شد و میزان آپپتوز در سلول‌ها به وسیله‌ی رنگ آمیزی با رنگ‌های حیاتی ارزیابی گردید.

یافته‌ها: محیط شرطی شده سلول‌های طحالی خود قدرت آسیب زدن به سلول‌های سرطانی را دارند و موجب کاهش معنادار در برداشت نوترال رد یا باعث آسیب غشاء سلول‌های سرطانی گردید، در تست MTT شاهد کاهش قدرت میتوکندریایی و به عبارتی کاهش قدرت حیاتی سلول‌های سرطانی در مقایسه با گروه کنترل شد، در تست آپپتوز درصدی از سلول‌های سرطانی از رنگ سبز به رنگ قرمز درآمدند. با اضافه کردن آدنوزین در غلظت ۲/۵ µM به محیط شرطی شده سلول‌های طحالی این عملکرد محیط شرطی شده تقویت شد طوری که میزان برداشت رنگ نوترال رد به شدت کاهش و موجب کاهش بیشتر قدرت میتوکنریایی سلول‌های سرطانی شد. همچنین موجب افزایش آپپتوزسلول‌های سرطانی شد، طوری که اکثر سلول‌های سرطانی به رنگ قرمز درآمدند. با افزایش غلظت آدنوزین به ۵ µM اثر مفید محیط شرطی شده سلول‌های طحالی کاهش یافت، طوری که از نظر آماری اختلاف معناداری با گروه محیط شرطی شده سلول‌های طحالی بدون تیمارنداشت. زمانی که غلظت آدنوزین را به۱۰ µM افزایش دادیم عملکرد کشتار ازبین رفت وبا گروه کنترل تفاوت معناداری نداشت.

نتیجه گیری: براساس نتایج محیط شرطی شده سلول‌های طحالی خود به تنهایی قادر به کشتار درصدی از سلول‌های سرطانی می‌باشد. زمانی که محیط شرطی شده سلول‌های طحالی را با غلظت‌های پایینی از آدنوزین تیمار کنیم شاهد افزایش بهبود عملکرد کشتار در سلول‌های سرطانی و بهبودعملکرد سلول‌های ایمنی در اندرکنش با سلول‌های سرطانی می‌باشیم. اما زمانی که میزان غلظت آدنوزین افزایش یابد عملکرد کشتار در سلول‌های سرطانی ازبین رفته و کاملا عکس می‌شود به عبارتی موجب رشد سلول‌های سرطانی می‌شود.

***نویسنده مسئول:**
دکتر نوروز دلیرژ
نشانی: بخش ایمنی شناسی، گروه میکروبیولوژی، دانشکده دامپزشکی، دانشگاه ارومیه، ارومیه، ایران.
تلفن: ۰۹۱۴۴۴۳۲۶۵۰ ۹۸+
پست الکترونیکی: n.delirezh@urmia.ac.ir

Introduction

In a natural state, the death of human body cells and their replacement with young cells follows regular programs that happen regularly and periodically [1]. Most normal cells grow and multiply or die in response to internal or external stimuli. If this process happens correctly, the cells maintain their normal function, and the person remains healthy. Nonetheless, a mutation that occurs in the genes of a normal cell and disrupts the reproduction of the cell causes a cell to become cancerous [2, 3, 4].

Cancer is a disease resulting from the uncontrollable proliferation of abnormal cells [5, 6, 7]. Cancer is caused by the disruption of the uncontrollable growth of cells and proliferation control mechanisms. If these mechanisms are not controlled and regulated, they cause the cells to grow endlessly. The majority of cancers can be attributed to defects in the cell cycle. Therefore, finding ways to prevent and deal with cancer is one of the necessities of medical science [8]. Breast cancer is the second leading cause of cancer death in women, occurring in solid organs and tissues of the body [9, 10].

Breast cancer originates from mammary cells and is considered one of the malignant tumors [11, 12]. In recent decades, the correct diagnosis and timely treatment of breast cancer has decreased the mortality from this type of cancer; nonetheless, metastasis may still occur years after treatment [13]. 4T1 mouse breast cancer cell line has been known as a poorly immunogenic and highly metastatic cell line, which is derived from the mammary gland tissue of a mouse BALB/c strain [14]. This breast cancer cell line is highly tumorigenic and metastatic and can be transplanted and induced [15]. This breast cancer cell line has the ability to metastasize to blood, lung, bone marrow, and lymph nodes; moreover, it can induce primary tumors [15, 16].

The effects of adenosine and its receptors in the regulation of inflammation and immune responses are well established. Adenosine is released by hypoxic or damaged tissues or following treatment with such drugs as methotrexate, suppressing inflammation and immune responses by stimulating its receptors on inflammatory and immune cells. Although this mechanism of action is beneficial for the treatment of diseases such as rheumatoid arthritis, immunosuppression may be harmful due to the condition of tumors [17]. The concentration of extracellular adenosine in non-stressed tissues is less than 1 mM, while the level of adenosine in inflamed or ischemic tissues can reach more than 100 mM. For instance, in a recently reported study, the level of systemic adenosine (plasma) in patients with sepsis reaches 10-4 mM in both ischemic and inflammatory conditions, while adenosine concentration in healthy individuals is less than 1 mM [18].

In general, adenosine level of 1 mM has little effect on immune processes. In ischemic and inflammatory conditions, when endogenous adenosine levels rise high enough, it exerts immunomodulatory and immunosuppressive effects. It has been well established that adenine nucleotides have strong immunomodulatory effects, which are mediated by purine receptors [19]. According to the above explanations, the present research aimed to evaluate the effect of spleen-cell-conditioned medium treated with appropriate concentrations of adenosine based on previous studies and find useful, effective, cost-effective, and less complicated methods for the treatment of breast cancer patients.

Materials and Methods

Laboratory Animals

In order to isolate 2-4 million spleen cell suspensions, four Syrian female mice aged 6-8 weeks and weighing 16-23 grams were purchased from the animal house of the Faculty of Veterinary Medicine of Urmia University. They were maintained under standard laboratory animal maintenance conditions (12/12 Light/dark, standard pellet food, temperature 24 degrees, and humidity 52). In addition, since the mice were from the Animal House of Urmia Veterinary Faculty, they did not need to adapt to new conditions.

Studied cell line

4T1 breast cancer cell line manufactured by Pasteur Institute was used.

Study groups

The control group consisted of 4T1 cell line cancer cells without treatment. The second group consisted of 4T1 cells adjacent to the spleen-cell-conditioned medium without adenosine treatment, the third group was 4T1 cells adjacent to conditioned medium treated with 2.5 µM adenosine, the fourth group was 4T1 cells adjacent to the spleen-cell-conditioned medium treated with adenosine 5 µM, and the fifth group was 4T1 cells adjacent to the spleen-cell-conditioned medium treated with adenosine 10 µM.

Culture of spleen cells and preparation of conditioned medium

To extract spleen cells from Syrian mice, they were anesthetized by **inhalation** of diethyl ether, and their spinal cords were cut. Spleens of mice were removed under sterile conditions and crushed completely in 5 ml of RPMI-1640 culture medium containing 10% FBS. The resulting tissue was passed through a wire mesh with a diameter of 0.2 mm to prepare cell suspension. After going through the steps described above, a

suspension containing 2 x 106 cells/ml was prepared and transferred to culture flasks following cell counting. These cells were cultured for 72 hours in the vicinity of phytohemagglutinin solution (1 mg/ml), RPMI-1640 culture medium containing 10% FBS, and different concentrations of adenosine (0, 2.5, 5, and 10 µM).

After 72 hours, the culture medium was removed along with the spleen cells, and it was then transferred into the Falcon and centrifuged at 10000 rpm for 15 minutes in order to remove possible cell residues in the supernatant, and the supernatant was discarded. Thereafter, the resulting cell sediment was incubated with culture medium without serum for 24 hours. After 24 hours, the culture medium and splenocytes were transferred into the Falcon and centrifuged at 10000 rpm for 15 minutes to remove possible cell residues in the supernatant. The supernatant was separated, and this time the cell sediment was discarded. The conditioned medium obtained from the culture of spleen cells was kept in a freezer at -80°C until it was used to perform study tests (MTT, NR, and apoptosis-necrosis) on the 4T1 cancer cell line.

Thawing of cells

The 4T1 cell line, which was previously stored in the nitrogen tank of the cell culture laboratory of the Faculty of Veterinary Medicine as a cell bank, was taken out to be used in this research and thawed according to the following steps to perform the cell culture process. A cryotube was taken out and cleaned with sterile gauze dipped in 70% alcohol. Thereafter, the lid of the cryotube containing the cells was immediately opened under the laminar hood to release nitrogen. Following that, the lid was fastened, and the end of the cryotube was transferred to bain-marie at 37°C. Under the laminar hood, the contents of the cryotube were slowly poured into a 15 ml falcon previously filled with 6-7 ml of serum-free medium (to neutralize Dimethyl Sulfoxide). Thereafter, it was centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the cell sediment was floated in sufficient culture medium. Cells were counted, and the percentage of live cells was determined. After counting, a complete culture medium containing 20% FBS was added, and the prepared suspension was poured into a T-25 flask. The flasks were placed in an incubator containing 5% CO2 at 37°C so that the cells start the process of growing and multiplying inside the flask.

Culture of 4T1 cell line

Firstly, the cells were thawed for cell culture, and the flask was then changed by changing the color of the culture medium, considering the use of the culture medium by the cells, checking the proliferation rate of the cells, and filling more than 70% of the flask space. According to the proliferation of cells and the placement of 80% of the flask space, cells were passaged according to the following steps: After sterilizing the hood and the equipment needed for passaging, the flask containing 4T1 cells was transferred under the laminar hood. The supernatant was slowly removed, and the flask containing 4T1 cells was washed with PBS solution to remove the remaining culture medium.

Trypsin was spread all over the surface of the flask containing cells attached to the bottom of the flask, and the flask was then incubated in the incubator at 37°C for 2-3 minutes. The flask was gently shaken to float the cells attached to the bottom of the flask, and to ensure the floating of the cells, the flask was hit a few times. An inverted microscope was used to check the effect of trypsin and the complete scrape of cells from the bottom of the flask. According to the number of cells, 90% RPMI-1640 culture medium, 10% FBS, and cell suspension were transferred to each new flask and incubated at 37°C, 5% CO2, and 95% humidity.

Freezing of 4T1 cells

After culturing the cells until the last step of the logarithmic phase and preparing a cell suspension containing cells with high density, it was checked for health and non-contamination. The cell suspension (~$5x10^6$ total cells), 90% FBS, and 10% DMSO were transferred to the transcribed cryotube (including the name of the cell line, number of cells, viability rate, and date of freezing). It was frozen at minus 20 degrees Celsius for 4-5 h and then minus 80 degrees Celsius for 24 h. Finally, it was stored in a nitrogen tank. Thereafter, the viability of cells was investigated.

Measuring the percentage of live to dead cells using trypan blue dye

In cell cultures, trypan blue dye is used to measure the ratio of live to dead cells. Trypan blue dye penetrates the membrane of dead cells and stains them; nonetheless, it cannot penetrate the membrane of dead cells, and living cells are seen as colorless.

Counting cells using trypan blue and Neubauer slide

1 mL Cell suspension was prepared; thereafter, 20 µl of cell suspension and 20 µl of trypan blue dye were slowly mixed and pipetted in the well of a 96-well microplate. 20 µl of the resulting solution was transferred to a Neubauer slide on which a stone slide was placed. Cells were counted under the 40x microscope.

The following formula was also used to determine the rate of living cells:

10^4 × dilution factor × average number of cells counted

in 25 wells = number of cells
100 × total number of live and dead cells / total number of live cells = rate of live cells
Cell viability assay (MTT or 3-(4,5-dimethyltrhiazol-2-yl)-2,5-diphenylterazolium bromide)

This method is based on the mitochondrial activity of the cell. Mitochondrial activity in living cells is stable and therefore the increase or decrease in the number of living cells is linearly related to mitochondrial activity. Methylthiazol tetrazolium (MTT) is revived in (metabolically) active cells. Mitochondrial dehydrogenases in living cells break the tetrazolium ring and lead to the formation of an insoluble purple precipitate called formazan with the production of NADH and NADPH. This precipitate can be dissolved by isopropanol or dimethyl sulfoxide. On the other hand, dead cells do not have this ability and, therefore, do not show a signal. In this method, a color change is used as an indicator of living cells.

The intensity of the produced color is measured at the wavelength of 540-630 nm and is directly proportional to the number of living cells. In this study, the cytotoxic effect of adenosine on cancer cells was investigated in terms of the proliferation and viability of 4T1 breast cancer cells. The cells were cultured until the end of the logarithmic phase, and a high-density suspension was then prepared from 4T1 cells. 1x10 cells were transferred to each well of 96-well plates and incubated for 24 h at 37°c, 5% CO2, and 95% humidity, and the cells were given time to stick to the bottom of the plate.

Fresh culture medium and adenosine-treated spleen cell supernatant were added to each well containing 4T1 cells in a one-to-one ratio and incubated for 48 h at 37°c, 5% CO2, and 95% humidity. The medium inside the wells was emptied, and 100 µl of MTT solution (5 mg/ml dissolved in PBS) was added to each well, and the cells were incubated for four h at 37°C. 800 µl of DMSO was added to the medium containing MTT, and after several times of pipetting, the rate of light absorption at the wavelength of 570 and 492 nm was read by ELIZA reader. Five repetitions were considered for each concentration. Neutral red (neutral red) or toluylene red (C15H17CLN4) is a green chemical compound that changes color to red when dissolved in water and alcohol.

Neutral red can be used as a vital stain, determining the cell viability rate by binding to lysosomes. During this process, the cells are mixed with a dye molecule and then washed and fixed. Thereafter, the dye is released from the cell using an ethanol solution. Changes in the amount of color composition by cultured cells due to the increase or decrease of living cells indicate the toxicity effect of the measured drug. In this test, fresh culture medium and supernatant of spleen cells treated with adenosine (50 ml of culture medium and 50 ml of spleen cell supernatant) were added to each well containing 4T1 cells (1x104). They were incubated at room temperature at 37°C, 5% CO2, and 95% humidity. After 48 h of incubation, 10 µL neutral red solution was added to each well of the 96-well plate, and the cells were incubated at 37°C for 2 h. The supernatant was removed, and the cells were washed three times with PBS.

The PBS was added to the surface of the cells and pipetted several times until the dye was completely removed. The same volume of culture medium from the lysis buffer solution (50% 96% alcohol, 4% acetic acid, and 46% distilled water) was added to the surface of the cells. After several times of pipetting, the rate of light absorption created at a wavelength of 540 nm was read by Eliza Reader. A minimum of five repetitions were considered for this test.

Assessment of apoptosis-necrosis assay using a fluorescent microscope

The compounds of acridine orange and propidium iodide are considered two fluorescent dyes that can differentiate between living and dead cells with their specific binding. Acridine orange easily passes through the cell membrane, penetrates into living cells, and finally binds to DNA. Propidium iodide also has the ability to bind to nucleic acids; nonetheless, it cannot penetrate into living cells. This dye is absorbed by non-viable cells and cells with membrane fragmentation. Upon binding to DNA, propidium iodide will experience a 20-30-fold increase in fluorescence. Furthermore, the propidium iodide dye absorbs the fluorescence of acridine orange and does not allow dead cells to be re-emitted by the acridine orange, resulting in the occurrence of fewer errors.

In this experiment, in order to investigate the occurrence of apoptosis and necrosis in 4T1 cells after treatment, several separate T25 flasks were considered for each group. One million 4T1 cells were placed in each flask and treated with 2.5, 5, and 10 µM adenosine. One of the flasks was regarded as control without treatment. After incubation of the cells for 48 h, the culture medium was removed from the flasks, and the cells in the flask were washed several times with the new culture medium. Finally, 1 ml of culture medium and 10 µL of prepared acridine orange solution was added to the flasks and incubated in the dark for 20 min. Thereafter, the flasks were washed again with the culture medium, and the solution containing 1 ml of the culture medium and 10 µL of propidium iodide was added to them and placed in the dark for 5 min. Finally, it was observed and examined under a fluorescent microscope.

Results

As evidenced by the obtained results, it can be concluded that the use of a spleen-cell-conditioned medium led to a marked decrease in the mitochondrial and viability of cancer cells compared to the control group. This function of the spleen-cell-conditioned medium was improved by the addition of 2.5 µM adenosine. Nevertheless, an increase in the concentration of adenosine to 5 µM or 10 µM reduced its beneficial effect. In this regard, the concentration of 5 µM was comparable to the spleen-cell-conditioned medium without any significant difference, and at the concentration of 10 µM, the density measurement (OD) was practically equal to that of the control group (Figure 1).

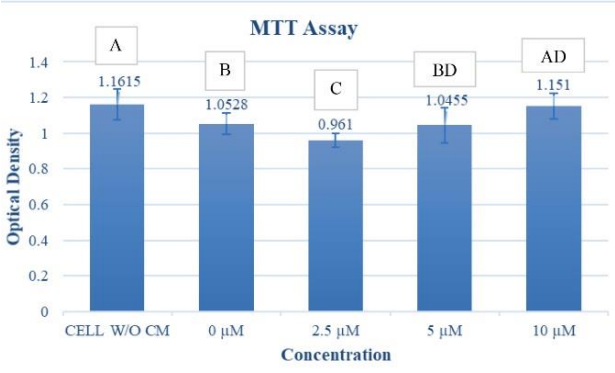

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Figure 1. Comparison of the mitochondrial activity of 4T1 cells in dye uptake in the control group with 4T1 cells adjacent to spleen-cell-conditioned medium treated with concentrations of 0, 2, 5, and 10 µM adenosine in the MTT test

The values presented in the graph are the mean of five independent replicates ± standard deviation.

(English letters indicate significant differences at the P<0.05 level).

A: 4T1 cells were placed in a normal culture medium and did not receive a conditioned medium (control group). B: 4T1 cells adjacent to spleen-cell-conditioned medium without receiving any treatment (which had a cytotoxic effect on the 4T1 cell line). C: 4T1 cells incubated with spleen-cell-conditioned medium treated with 2.5 µM adenosine (which had a stronger cytotoxic effect on the 4T1 cell line). BD: 4T1 cells adjacent to the spleen-cell-conditioned medium treated with adenosine 5 µM (which is not significantly different from group B in its cytotoxic effect on the 4T1 cell line). A: 4T1 cells adjacent to the spleen-cell-conditioned medium with a concentration of 10 µM (the increase in the viability of most 4T1 cells and the decrease in cytotoxic effect on 4T1 cells is not significantly different from the control group, i.e., A).

(Cells placed in normal culture medium and not receiving conditioned medium are CELL W/O CM)

0 µM means the spleen-cell-conditioned medium without receiving any treatment.

Based on the obtained results, it was found that the use of a spleen-cell-conditioned medium caused a significant reduction in neutral red; that is, damage to the cancer cell membrane. The conditioned medium of cancer cells treated with 2.5 µM adenosine strengthened this function of the conditioned medium, resulting in decreased levels of neutral red uptake. An increase in adenosine concentration mitigated this beneficial effect of the spleen cell supernatant so that the function of the spleen-cell-conditioned medium is not statistically significantly different from that of the control group.

An increase in the concentration of adenosine to 10 µM also caused a further reversal of this function of the conditioned medium. Finally, the viability rate of the cells in the neutral red test was statistically equal to that of the control group (Figure 2). Based on the obtained results, it is quite clear that the conditioned medium obtained from spleen cells treated with 2.5 µM adenosine when adjacent to cancer cells induced apoptosis in cancer cells. As the result of an increase in the concentration of adenosine to 10 µM, a spleen-cell-conditioned medium treated at this concentration decreased apoptosis in cancer cells.

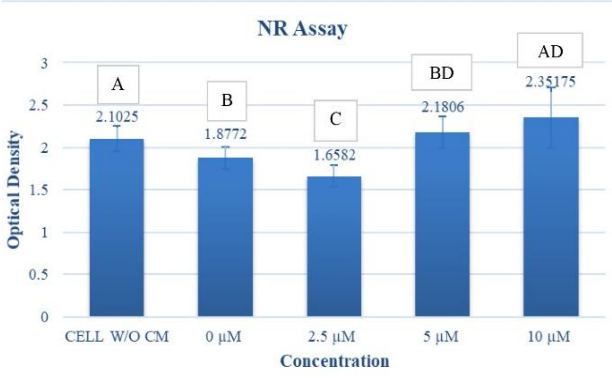

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Figure 2. Comparison of the lysosomal power of 4T1 cells in neutral red uptake in the control group with 4T1 cells adjacent to spleen-cell-conditioned medium treated with 2, 5, and 10 µM adenosine in the NR test. The values presented in the figure are the mean of five independent replicates ± standard deviation.

(English letters indicate significant differences at the P<0.05 level)

A: 4T1 cells were placed in a normal culture medium and did not receive a conditioned medium (control group). B: 4T1 cells adjacent to spleen-cell-conditioned medium without receiving any treatment (which had a cytotoxic effect on 4T1 cell line) C: 4T1 cells incubated with spleen-cell-conditioned medium treated with adenosine ~ 2.5 µM (which had a stronger cytotoxic effect on 4T1 cell line). BD: 4T1 cells adjacent to the spleen-cell-conditioned medium treated with adenosine ~ 5 µM (which is not significantly different from group B in its cytotoxic effect on the 4T1 cell line). A: 4T1 cells adjacent to the spleen-cell-conditioned medium with a concentration of 10 µM (the increase in the viability of most 4T1 cells and the decrease in cytotoxic effect on 4T1 cells is not significantly different from the control group, i.e., A).

(Cells placed in normal culture medium and not receiving conditioned medium are CELL W/O CM). 0 µM means the spleen-cell-conditioned medium without receiving any treatment

4T1 cells without any treatment emitted green fluorescence under a microscope. When we placed 4T1 in the vicinity of untreated spleen cell conditioned medium, a percentage of 4T1 cells changed from green to red. This demonstrates that the spleen-cell-conditioned medium is able to damage cancer cells. When these cells were exposed to the supernatant of spleen cells treated with a concentration of 2.5 µM, it increased the percentage of red color in 4T1 cells. In other words, it strengthened the killing efficacy of the conditioned medium. When we increased the concentration of adenosine to 5 µM, no increase was observed in the killing efficacy of 4T1 cells. In other words, it has almost the same function as the conditioned medium of untreated spleen cells. And when we increased the concentration of adenosine to 10 µM, the killing efficacy was completely reversed. That is, the killing efficacy of the spleen-cell-conditioned medium treated with a concentration of 10 µM was practically destroyed (Figure 3).

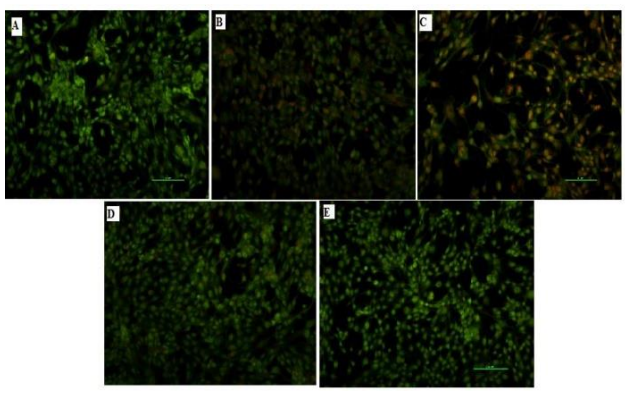

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Figure 3. Observing the morphology of apoptotic and necrotic cells by fluorescent microscope (×20)
A: 4T1 cells without receiving any conditioned medium from cultured adenosine-treated spleen cells show green cells without apoptosis. B: 4T1 cells adjacent to spleen-cell-conditioned medium without receiving any adenosine treatment, increased red color indicating apoptotic cells. C: 4T1 cells adjacent to spleen-cell-conditioned medium treated with adenosine ~ 2.5 µM showed a greater than 50% increase in red color, indicating more apoptotic cells. D: Cells adjacent to spleen-cell-conditioned medium treated with adenosine ~ 5 µM, apoptotic cells, and the penetration of red color is less than group C and equal to group B. E: cells adjacent to spleen-cell-conditioned medium treated with adenosine ~ 10 µM show an increase in green color and a decrease in red color penetration due to a decrease in apoptosis of 4T1 cells, which is not significantly different from control group A.

Discussion

The present study aimed to assess the effect of supernatant of spleen cells treated with adenosine on the growth of 4T1 cells. According to the results obtained from MTT, trypan blue, and apoptosis-necrosis tests, it was found that the treated spleen cell medium adjacent to adenosine ~25 µM causes a significant decrease in the viability and growth of 4T1 cells, compared to the untreated spleen cell medium. It is noteworthy that this cytotoxicity decreases with an increase in adenosine concentration. Cancer cells inhibit the immune system in the tumor microenvironment by active pumping of adenosine from the inside of cancer cells to the outside, i.e., the tumor microenvironment.

Cancer cells and tissues contain a series of enzymes, including ectonucleotidase (CD39, CD73), which convert adenosine into constituent nucleoporins. Consequently, adenosine first affects cancer cells, increasing the rate of metastasis and making them more malignant. Secondly, adenosine affects immune system cells in the tumor microenvironment, weakening and modifying them. On the other hand, ATP is a very important DAMP. Therefore, a low level of adenosine is useful for the immune system and activates immunocytes. Whereas, when the concentration of adenosine increases, such as in tumor, injury, and inflammation conditions, it weakens and inhibits the immune system.

For tumor immunotherapy by the patient's own lymphocytes, it is possible to activate immune cells with adenosine in a low concentration outside the body and then inject the activated cells into the patient's body since when lymphocytes interact with tumor cells, they are exposed to a high concentration of adenosine in the tumor microenvironment, which is produced by the tumor itself and pumped into the tumor microenvironment. This can weaken the inhibitory effect of the tumor microenvironment on the immune system cells. Through interaction with its four receptor subtypes, adenosine is a ubiquitous and powerful modulator of inflammatory processes. It affects almost all physiological and pathophysiological functions. All immune cells express the adenosine receptor and respond to it differently, with their concentrations varying greatly in stressful situations, including inflammation.

In fact, several clinical trials are currently being conducted on adenosine receptor ligands. As a result, the adenosinergic system has a significant therapeutic potential in all pathological conditions, especially in diseases that require inflammation regulation and immune system modulation [20]. Among the currently investigated antitumor strategies, the CD73-ADO has been considered a new immune checkpoint for cancer treatment. The potential of CD73- Adenosine Receptor to control tumor growth, metastasis, and immune system activation raises interest in targeting the ADO pathway for cancer therapy [21].

Based on a review study, A2B signaling is a major pathway that contributes to cancer cell proliferation,

solid tumor growth, angiogenesis, metastasis, and immunosuppression. Therefore, A2B antagonists, either in combination with other anticancer drugs or as a monotherapy, are potentially a new anticancer treatment [22]. Another study found that the growth and development of solid tumors are strongly affected by metabolic changes of adenosine and the interaction with the tumor microenvironment (TME), leading to tumor development and immune escape. Adenosinergic pathway plays a major role in the occurrence and development of gastric cancer (GC), especially CD73. The components of the adenosinergic pathway in GC cells and immune cells maintain the immunosuppressive TME by influencing different aspects of the immune response. In addition, some emerging adenosinergic pathway antagonists show therapeutic potential in early studies of other malignancies.

These findings revealed the mechanism by which the immunosuppressive adenosine participates in GC immunotolerance, suggesting the potential of the adenosinergic pathway as a therapeutic target or predictive marker for GC. Nevertheless, based on the available evidence so far, careful clinical evaluation is necessary to confirm whether targeted adenosinergic therapies are appropriate for GC patients [23, 24]. Today, many efforts are underway to identify new and more selective adenosine receptor ligands, as well as adenosine regulatory agents capable of modulating endogenous adenosine concentrations. In fact, a wide array of clinical trials is currently being conducted on adenosine receptor ligands. Consequently, the adenosinergic system has a significant therapeutic potential in all pathological conditions, especially in diseases that require inflammation regulation and immune system modulation [20].

Conclusion

The current study assessed the effect of spleen-cell-conditioned medium treated with adenosine at increasing concentrations (1, 10, 50, 100, and 500 µM) and untreated spleen cell medium adjacent to 4T1 cells on the growth and viability of 4T1 cells. According to the results obtained from MTT, trypan blue, and apoptosis-necrosis tests, it was found that the treated spleen cell medium adjacent to adenosine ~25 µM causes a significant decrease in the viability and growth of 4T1 cells, compared to the untreated spleen cell medium. It is noteworthy that this cytotoxicity decreases with an increase in adenosine concentration. As a result, the increase in killing efficacy depends on selecting appropriate concentrations.

Ethical Considerations

Compliance with ethical guidelines

In this research, all the experimental procedures on laboratory animals (keeping, anesthetizing, and sacrificing the animal) were carried out according to the rules of the ethics committee. This research was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Urmia University (IR-UU-AEC-3/6).

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Authors' contributions

All authors contributed to the preparation of this article.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

[1] Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics. CA: A Cancer Journal for Clinicians. 2022; 72(1): 7-33. [DOI: 10.3322/caac.21708] [PMID]

[2] Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE open medicine. 2021; 9: 20503121211034366. [DOI: 10.1177/20503121211034366] [PMID] [PMCID]

[3] Wang H, He X. Nanoparticles for targeted drug delivery to cancer stem cells and tumor. Targeted Drug Delivery: Methods and Protocols. 2022; 1831: 59-67. [DOI: 10.1007/978-1-4939-8661-3_6] [PMID] [PMCID]

[4] Zhao Z, Zheng L, Chen W, Weng W, Song J, Ji J. Delivery strategies of cancer immunotherapy: recent advances and future perspectives. Journal of hematology & oncology. 2019; 12(1): 126. [DOI: 10.1186/s13045-019-0817-3] [PMID] [PMCID]

[5] Abbas Z, Rehman S. An overview of cancer treatment modalities. Neoplasm. 2018; 1: 139-57. [DOI: 10.5772/intechopen.76558]

[6] Valter K, Chen L, Kruspig B, Maximchik P, Cui H, Zhivotovsky B, et al. Contrasting effects of glutamine deprivation on apoptosis induced by conventionally used anticancer drugs. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2017; 1864(3): 498-506. [DOI: 10.1016/j.bbamcr.2016.12.016] [PMID]

[7] Xu CF, Wang J. Delivery systems for siRNA drug development in cancer therapy. Asian Journal of Pharmaceutical Sciences. 2015; 10(1): 1-2. [DOI: 10.1016/j.ajps.2014.08.011]

[8] Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994; 266(5192): 1821-8. [DOI: 10.1126/science.7997877] [PMID]

[9] Freedman DM, Dosemeci M, McGlynn K. Sunlight and mortality from breast, ovarian, colon, prostate, and non-melanoma skin

cancer: a composite death certificate based case-control study. Occupational and Environmental Medicine. 2002; 59(4): 257-62. [DOI: 10.1136/oem.59.4.257] [PMID] [PMCID]

[10] Lodish M. Genetics of adrenocortical development and tumors. Endocrinology and Metabolism Clinics. 2017; 46(2): 419-33. [DOI: 10.1016/j.ecl.2017.01.007] [PMID] [PMCID]

[11] Balic M, Schwarzenbacher D, Stanzer S, Heitzer E, Auer M, Geigl JB, et al. Genetic and epigenetic analysis of putative breast cancer stem cell models. BMC cancer. 2013; 13(1): 1-15. [DOI: 10.1186/1471-2407-13-358] [PMID] [PMCID: PMC3727963]

[12] Liu S, Clouthier SG, Wicha MS. Role of microRNAs in the regulation of breast cancer stem cells. Journal of mammary gland biology and neoplasia. 2012; 17: 15-21. [DOI: https://doi.org/10.1007/s10911-012-9242-8]

[13] Kaur P, Nagaraja GM, Zheng H, Gizachew D, Galukande M, Krishnan S, et al. A mouse model for triple-negative breast cancer tumor-initiating cells (TNBC-TICs) exhibits similar aggressive phenotype to the human disease. BMC cancer. 2012; 12(1): 1-12. [DOI:10.1186/1471-2407-12-120] [PMID] [PMCID]

[14] Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. Current protocols in immunology. 2000; 39(1): 20-. [DOI: 10.1002/0471142735.im2002s39] [PMID]

[15] Ta K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. BMC cancer. 2008; 8(1): 1-19. [DOI: 10.1186/1471-2407-8-228] [PMID] [PMCID]

[16] Cho HJ, Jung JI, Lim DY, Kwon GT, Her S, Park JH, et al. Bone marrow-derived, alternatively activated macrophages enhance solid tumor growth and lung metastasis of mammary carcinoma cells in a Balb/C mouse orthotopic model. Breast cancer research. 2012; 14(3): 1-12. [DOI: 10.1186/bcr3195] [PMID] [PMCID: PMC3446344]

[17] Cronstein BN, Sitkovsky M. Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. Nature Reviews Rheumatology. 2017; 13(1): 41-51. [DOI: 10.1038/nrrheum.2016.178] [PMID] [PMCID]

[18] Martin C, Leone M, Viviand X, Ayem ML, Guieu R. High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock. Critical care medicine. 2000; 28(9): 3198-202. [DOI: 10.1097/00003246-200009000-00014]

[19] Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood, The Journal of the American Society of Hematology. 2001; 97(3): 587-600. [DOI: https://doi.org/10.1182/blood.V97.3.587]

[20] Gao ZG. Jacobson KA. A2B adenosine receptor and cancer. International journal of molecular sciences. 2019; 20(20): 5139. [DOI: 10.3390/ijms20205139] [PMID] [PMCID]

[21] Azambuja JH, Ludwig N, Braganhol E, Whiteside TL. Inhibition of the adenosinergic pathway in cancer rejuvenates innate and adaptive immunity. International journal of molecular sciences. 2019; 20(22): 5698. [DOI: 10.3390/ijms20225698] [PMID] [PMCID]

[22] Pasquini S, Contri C, Borea PA, Vincenzi F, Varani K. Adenosine and inflammation: here, there and everywhere. International journal of molecular sciences. 2021; 22(14): 7685. [DOI:10.3390/ijms22147685]

[23] Wang J, Du L, Chen X. Adenosine signaling: Optimal target for gastric cancer immunotherapy. Frontiers in Immunology. 2022; 13: 1027838. [DOI: 10.3389/fimmu.2022.1027838] [PMID] [PMCID]

[24] Wang L, Tang S, Wang Y, Xu S, Yu J, Zhi X, et al. Ecto-5′-nucleotidase (CD73) promotes tumor angiogenesis. Clinical & experimental metastasis. 2013; 30: 671-80. [DOI: 10.1007/s10585-013-9571-z] [PMID]