Inhibition of Invasion of Breast Cancer Cells by Glutaminase Extracted from Acinetobacter Baumannii

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Abstract

Breast cancer is the second leading cause of death for women in Iraq after cardiovascular disease. Many studies have been conducted on the activities of plant and bacterial secondary metabolites and their role in inhibiting proliferation of cancer cells. However, there is no study has dealt with inhibiting the invasion of cancer cells. The invasion potential of cancer cells is the most dangerous criteria of cancer, as the ability of cancer cells to migrate from the place where the cancer originated to the rest of the body hinders the ability to recover from cancer and hinders the application of therapeutic and excisional techniques, which leads to death. Therefore, the current study aims to inhibit the migration of breast cancer cells and restrict their movement to facilitate their eradication or treatment using the enzyme glutaminase extracted from Acinetobacter sp. Ninety five samples of bacteria were collected and diagnosed using phenotypic and biochemical tests in addition to the diagnosis by (Vitec kit). The enzyme glutaminase was extracted, purified, and characterized from the diagnosed bacteria, and the enzyme's effect on inhibiting the migration of breast cancer cells (MCF-7) and (MDA-231) was examined using a scratch assay, in addition to its effect on tumor cell proliferation using MTT assay. The results demonstrated the ability of the enzyme glutaminase to inhibit the migration and proliferation of breast cancer cells. It can be concluded that glutaminase has an antitumor effect in two different ways, the first is by inhibiting the migration of cancer cells and the second is by inhibiting the proliferation of cancer cells, making it a potential target that can be used as an antitumor therapy.

Keywords: glutaminase, Acinetobacter, breast cancer, cancer invasion, MCF-7, MDA-231.

1. Introduction

Breast cancer is one of the most prevalent malignant tumors that kills women, and hematogenous spread is common in the early stages, posing a major threat to women's health. Surgery, chemotherapy, radiotherapy, targeted biotherapy, and other treatments for breast cancer are currently available, all of which are guaranteed to kill cancer cells and heal wounds (1). After 10-15 years of follow-up, however, 25-30% of early breast cancer patients still have distant metastases. At the same time, tumor cell drug resistance and chemotherapeutic medication side effects are another significant difficulty in the cancer therapy process. As a result, natural chemicals derived from animals and plants have become the primary focus of anticancer medication research (2). Additionally, women's breast cancer stands on top of other cancer types such as breast, cervix, ovary, colorectum, and non-Hodgkin's lymphoma recorded in the Eastern Mediterranean Region. According to World Health Organization (WHO), breast cancer affected 2.3 million people worldwide in 2020, with 685 000 fatalities. Breast cancer had been diagnosed in 7.8 million women in the previous 5 years as of the end of 2020, making it the most common cancer in the world (WHO). Alwan (2016) has reported that the deaths of Iraqi women are mainly caused by breast cancer, beyond cardiovascular disease (3). It has been well documented that metastasis is the first and most threatening step of cancer, represented by the migration of tumor cells from the original tissue towards intact organs and colonizing intact tissues (4). The rapid spread of cancer around the world has led to the search for new drugs that are toxic to cancer cells while not having a harmful effect on normal cells. Previously used anticancer drugs showed relatively high toxicity not only to cancer cells, but also to normal cells in the part of the body where the cancer arose. Currently, new anti-cancer agents are being searched for among microorganisms, including bacteria (5). Among the bacterial products used as anticancer agents such as enzymes; arginine deiminase from Mycoplasma hominis (6) and L-asparaginase from Escherichia coli or Erwinia species (7). Bacteriocins such as colicins from Escherichia coli (8), laterosporulin 10 isolated from Brevibacillus sp (9), Microcin E492 from Klebsiella pneumoniae (10), Nisin from Lactococcus lactis (11), Pediocin CP2 from Pediococcus acidilactici (Pediococcus acidilactici) (12), Plantaricin A extracted from Lactobacillus plantarum (13) and Pyocins extracted from Pseudomonas aeruginosa (14). Proteins and peptides such as azurin produced by Pseudomonas aeruginosa (15) and entap produced by Enterococcus genus (16). Antibiotics such as actinomycin D produced from Actinomyces antibioticus (17), bleomycin isolated from Streptomyces verticillus (18), doxorubicin (DOX) extracted from Streptomyces peucetius (19). To the best of our knowledge, there is no study has dealt with antitumor activity of the enzyme glutaminase isolated

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from Acinetobacter baumannii. Also, there are no studies in Iraq has dealt with inhibiting the migration of cancer cells, instead, they were limited to inhibiting the proliferation of cancer cells. Therefore, the current study aimed to extract glutaminase from Acinetobacter baumannii and test its antitumor activity by inhibiting both invasion and proliferation of breast cancer cells.

2. Methods

Isolation and characterization of Acinetobacter baumannii

Acinetobacter baumannii was isolated from clinical specimens collected from four hospitals in Baghdad; Pediatric Teaching Hospital, Baghdad Teaching Hospital, Al-Shahid Gazi Al-Hariry Hospital, and The Burn Specialist Hospital. The specimen's collection included the inpatients, which usually with underlying diseases and their immune system was suppressed that made them an easy target for nosocomial infections from the pathogens colonized in the hospital. Nosocomial infections were suspected clinically by specialist physicians according to standard criteria. The collected specimens were streaked directly on blood agar and MacConkey agar and isolated bacteria were further identified as A. baumannii after further identification tests include the morphological characteristics and biochemical tests were carried out depending on (20) and confirmed by Vitek2 system.

Screening for L-glutaminase using the plate method assay (Semi Quantitative Screening)

Locally four isolates of A. baumannii were subjected to rapid screening for L-glutaminase production by agar plate assay. According to Karim and Thalji, bacterial isolates were grown on minimal salt medium with 2.5% phenol red dye and incubated for 48 hours at 37°C. Change in pH caused a change in the color of plate from yellow to pink, which indicated a positive result (21). Quantitative assay was carried according to the method described by Mannan and colleagues to determine the enzyme activity in each isolate as described by Manna and colleagues. Briefly, a reaction mixture containing µl of 0.04 M glutamine µl of 0.5 M Tris buffer, 10 µl of enzyme, and distilled water to a final volume of 40 µl. The mixture was incubated in a water bath for 30 min at 37°C and the reaction stopped by adding 10 µl of 10% trichloroacetic acid (TCA) Blank was prepared by adding TCA before the addition of enzyme. Mixture reaction (25 µl) was taken and the volume was completed to 1ml using D.W, then µl of Nessler's reagent was added. The mixture was kept for 20 min at 25°C, then the absorbance at 450 nm was measured, and the amount of released ammonia was determined. International Unit (IU) is defining glutaminase amount of enzyme which liberates µmol of ammonia per minute per ml [µmole/ml/min] (22).

Production and purification of L-glutaminase

Two sets of flasks containing sterile 50 mL of minimal salt medium supplemented with 5% glutamine (production media) were prepared. The pH of flasks was adjusted to 7.0 and two milliliters of the seed or inoculum culture was inoculated into each flask containing 50 mL production media. The flasks were incubated in an orbital shaker incubator at 37°C and 150 rpm for 48 h. Flasks with uninoculated medium served as negative controls (21). The efficiencies of the crude enzyme were analyzed. All the purification procedures were carried out at 4°C. The crude enzyme was subjected to 80% saturated ammonium sulphate precipitation. The precipitate was collected by centrifugation at 6,000rpm for 15 minutes and dissolved in minimal amount of 0.5M Tris Hydrochloric acid buffer of pH8.0 and then dialyzed extensively against the same. The enzyme activities in the dialysate were assayed. The dialyzed fraction was applied to a Diethylaminoethyl cellulose (DEAE-C) column, an anion exchanger, pre-equilibrated with TrisHydrochloric acid buffer, pH 8. The enzyme was eluted (1 ml/min) with sodium chloride gradient (0.1-0.5 M) and 0.025 M Tris buffer, pH 8.0 then the last step of purification by gel chromatography; the high activity fractions from anion exchanger were collected and applied on sephadex G-200.

Cell culture

All cell culture experiments were performed in class II cabinets to reduce the risk of infection and contamination. Then, all the cells were incubated at standard culture conditions (SCC), which were provided by a humidified incubator adjusted to 37oC, 20% O2 and 5% CO2. Unless otherwise stated, all culture conditions set out in this study for cell culture and cell experiments were SCC. The MCF-7 and MDA-231 and rabbit embryo fibroblast (REF) cell lines were kindly provided by the Biotechnology Research Center, Al-Nahrain University, and Baghdad, Iraq. These cell lines were grown at SCC in Dulbecco's Modified Eagle Medium (DMEM) (Sigma/UK). This medium is composed of 500 ml of DMEM supplemented with 10% fetal calf serum (FCS) (Sigma/UK), 4% of 200 mM L-Glutamine, and 1% of 100 IU/ml penicillin and 100 µg/ml streptomycin (PS) (Sigma/UK). To split cells, old media was aspirated, and cells were rinsed with phosphate buffered saline (PBS) (Sigma/UK), trypsinised with trypsin 1X, and incubated for 10 minutes. A quantity of 10 ml of DMEM-S10 was added to prepare the cell suspension and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and cells were counted and a cell number of 5×104 was seeded into a T75 flask. A quantity of 15-20 ml of fresh DMEM- S10 was added to the flask and incubated at SCC. Trypan blue stain was used to assess the viability of all cells. A volume of 10 µl trypan blue was mixed with 10 µl of cell suspension. A quantity of 10 µl of this mixture was transferred to a Neubauer chamber and covered with a cover slip prior to counting under the microscope (23).

$$\textbf{Total Cell Count} = \textbf{No of cells in 25 small square} \times \textbf{Dilution Fcator} \times 10^4 \quad \text{Equation 1}$$

$$\textbf{Cell Viability} = \frac{\textbf{No of viable cells in 25 small square}}{\textbf{Total cell count (Live + dead cells)}} \times 100\ \% \quad \text{Equation 2}$$

Assessment of the effect of glutaminase on breast cancer cell invasion

Scratch assay was applied to assess the invasion of breast cancer cells by measuring the area of the scratch during different time points utilizing the protocol described by Rodriguez et al. (2005) (24) with minor modifications. In brief, MCF-7, MDA-231 and REF cells were separately seeded in 48-well plates at a density of 0.05×106 cells per well and incubated at standard culture conditions (SCC) "in a humidified incubator, 37°C and 5% CO2" for 24 hours to allow cell adhesion. Each confluent monolayer was then scratched with a sterile yellow tip, making a scratch of 0.4–0.5 mm in width along the diameter of each well. The medium was removed and replaced with fresh DMEM-S10 containing 50 µg/ml glutaminase or only fresh DMEM-S10 for control cells. The plates were re-incubated at SCC to monitor wound closure by imaging at time zero, followed by imaging every until 72 hours. All conditions were achieved in triplicates. The area of each single scratch was measured at the given time points using image J software.

Assessment of the effect of glutaminase on breast cancer cell proliferation

The effect of glutaminase on proliferation of breast cancer cells MCF-7 and MDA231 and normal cells REF was achieved using (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium (MTS) (Promega / UK). According to manufacturing company, MCF-7, MDA-231 and REF cells were separately seeded at a density of 0.01×106 cell / well into the 96-well plates in 1 ml of DMEM-S10 and incubated for 4 hours at SCC to allow cell adherence. After the incubation time, cells were washed twice in serum free medium, followed by the addition of 1 ml of DMEM-S10 containing 50 µg/ml glutaminase to the cells and DMEM-S10 to the control cells, then incubated at SCC. A volume of 20 µl MTS was added to the wells after 24, 48 and 72 hours. The plate was then incubated for 3 hours to allow the viable cells to reduce the tetrazolium and release the colored formazan compound into the culture media, giving a color depending on cell viability, which was then read at a wavelength of 490 nm using an ELISA reader. Each condition was tested in triplicates.

3. Statistical Analysis

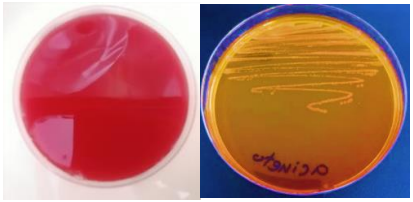
The data from the research was analysed by using means ± standard error of the mean (SEM) and a Two-way RM ANOVA test to determine significant differences between samples when P values were less than 0.05. Multiple comparisons were required to find differences between pairs of means with appropriate adjustment for multiple testing in every single condition during different time points in each separate experiment. Tukey's multiple comparisons test was used to detect variations, which were considered significant when the P value was less than 0.05. All types of analyses were achieved by GraphPad Prism software version 6.0.

4. Results

A total of 49 Gram negative bacilli isolates were collected from the 156 clinical samples. Based on the morphological characterization and biochemical behavior, four isolates were identified as suspected A. baumannii, which detected as non-lactose fermenter and oxidase negative coccobacilli (appeared as pale or beige colonies on MacConkey agar. After identification by biochemical tests and vitek 2 system, four isolates were identified as A. baumannii.

Screening the ability of Acinetobacter baumannii for producing glutaminase

Semiquantitative screening method showed that all four isolates were glutaminase producer as illustrated in figure (1).



(a) (b)

Figure (1) Primary screening of glutaminase production by A. baumannii isolate A8 grown on glutamine agar medium incubated at 37°C for 24 hours.

(a) Blank plate uncultured and incubated at 37°C for 24 hours. (b) The plate was inoculated with A. baumannii (A8) and incubated at 37°C for 24 hours.

While quantitative screening explained in table (1) all the isolates were glutaminase producers with variable degrees of production. The isolate of Acinetobacter (A8) produced the highest specific activity (2.1 U/mg protein). Moreover, specimens A10 showed the lowest specific activity (0.9 U/mg protein). According to these results, the specimens A8 was selected to be used in glutaminase production.

Table (1) Specific activity of glutaminase produced by local isolated of Acinetobacter baumannii after 48 hours of incubation at 37°C.

Acinetobacter baumannii	Specific activity (U/ mg protein)	Source of Specimens
A6	1.3	Wounds
A8	2.1	Wounds
A10	0.9	Wounds
A19	1.2	Burns

Production and purification of L-Glutaminase

Enzyme activity and specificity was calculated for selected producer isolate. The maximum enzyme activity and specific activity was observed in A. baumannii (A8) using L-glutamine as substrate showed in (Table 2).

Table (2) Specific activity and fold purification for l-glutaminase from A. baumannii (A8)

Purification step	Volume(ml)	Enzyme activity (U/ml)	Protein concentration (mg / ml)	Specific activity (U/mg)	Total activity (folds)	Purification (folds)	Yield (%)
Crude enzyme	250	800	160	5	200	1	100
Ammonium sulfate precipitation	75	730	57	13	55	2.6	55
Ion exchange chromatography DEAE cellulose	60	490	16.4	30	29	6	15
Gel filtration chromatography sephadex G-200	33	427	4.5	95	14	19	7

According to this work the enzyme l-glutaminase was purified with 95 u/mg specific activity, 19 folds with 7% yield.

Glutaminase inhibits invasion of breast cancer

Results of scratch assay showed that both MCF-7 (Figure 2a) and MDA-231 (Figure 2c) treated with glutaminase had slower migration than MCF-7 (Figure 2b) and MDA-231 (Figure 2d) treated with DMEM-S10. After 30 hours, cells treated with DMEM-S10 closed scratch are completely while cells treated with glutaminase failed to close the scratch area even at time point 72 hours. On the other hand, REF treated with glutaminase (Figure 2e) and REF treated with DMEM-S10 (Figure 2f) had same migration rate and closed the scratch area after 30 hours.

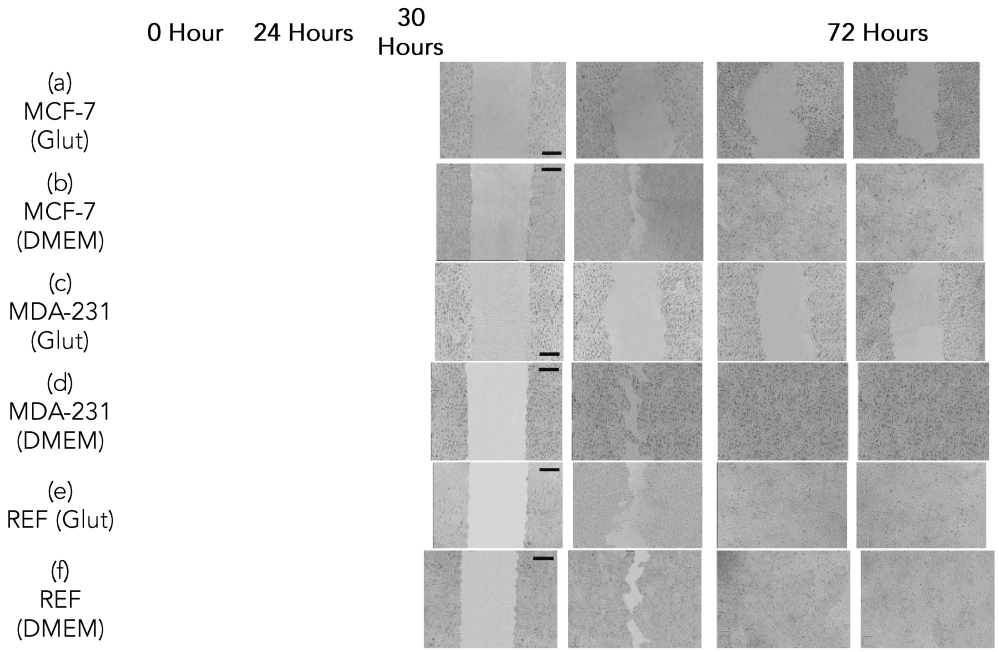


Figure 2. Invasion of MCF-7 and MDA-231 cells during scratch assay

Representative micrographs show monolayers of MCF-7, MDA-231 and REF cells were scratched and incubated at SCC for 48 hours. (a) MCF-7 treated with glutaminase had slower migration rate than MCF-7 treated with DMEM-S10 (b). (c) MDA-231 treated with glutaminase had slower migration rate than MDA-231 treated with DMEM-S10 (d). (e) REF treated with glutaminase had the same migration rate of REF treated with DMEM-S10 (f). MCF-7 and MDA-231cells were used at passage 8, REF cells were used at passage 10, scale bar=50 µm.

Two-way ANOVA showed that glutaminase inhibited the invasion of both MCF-7 and MDA-231 compared to invasion of MCF-7 and MDA-231 treated with DMEM-S10. As shown in (Figure 4a), scratch areas of MCF-7 cells treated with glutaminase were reduced from 131873±12127 µm2 at time zero to 122705±7731 µm2 at time 48 hours which represent non-significant differences (P>0.05). However, the scratch areas at 72 hours were reduced to 115833±9718 µm2 with slight significant variation (P=0.01) compared to time zero. On the other hand, MCF-7 cells treated with DMEM-S10 have faster migration rate since the scratch areas were significantly reduced (P=0.001) from 131873±12127 µm2 at time zero to 22342±14029 µm2 at 24 hours with increased significant reduction of the scratch area within time until full closure at 72 hours (P=0.0001). The same effect was observed in case of

MDA-231 cells (Figure 4b). Scratch areas of MDA-231 cells treated with glutaminase were reduced from 131873±12127 µm2 at time zero to 130981±5274 µm2 at time 48 hours which represent non-significant differences (P>0.05). However, the scratch areas at 72 hours were reduced to 116500±9716 µm2 with slight significant variation (P=0.01) compared to time zero. On the other hand, MDA-231 cells treated with DMEM-S10 have faster migration rate since the scratch areas were significantly reduced (P=0.0001) from 131873±12127 µm2 at time zero to 24633±4893 µm2 at 24 hours with increased significant reduction of the scratch area within time until full closure at 72 hours (P=0.0001). In case of REF cells treated with glutaminase, scratch areas were significantly reduced (P=0.0001) from 131873±12127 µm2 at time zero to 53474±9677 µm2 at 48 hours with increased reduction in the scratch area within time to completely close the scratch areas at 72 hours (P=0.0001).

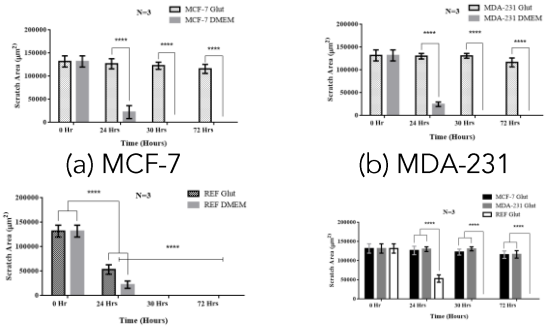


Figure 2. Statistical analysis of invasion of MCF-7 and MDA-231 cells during scratch assay

Two-way ANOVA revealed that cancer cells had nonsignificant variation in scratch area. (a) MCF-7 treated with glutaminase had nonsignificant differences (P>0.05) in scratch area within time compared to MCF-7 treated with DMEM-S10. (B) MDA-231 treated with glutaminase had nonsignificant differences (P>0.05) in scratch area within time compared to MDA-231 treated with DMEM-S10. (c) REF treated with glutaminase had significant differences (P=0.0001) in scratch area within time compared to REF treated with DMEM-S10. (d) Migration of both MCF-7 and MDA-231 treated with glutaminase was significantly less than migration of REF treated with glutaminase (P=0.0001). Columns = mean of scratch areas (N=3 technical replicates), Error bars= standard deviation (SD). (**** represents P<0.0001).

Effect of glutaminase on proliferation of breast cancer

Results of cell proliferation assay revealed that glutaminase has no inhibitory effect of proliferation rate of breast cancer cells MCF-7 and MDA-231. Statistical analysis in figure (3a) demonstrated that MCF-7 cells treated with glutamines and control cells treated with standard culture media (DMEM-S10) have significantly increased in number between time points zero and 48 hours (P=0.0001) with non-significant differences in cell number (P>0.05) of both conditions at these same time points. However, there was significant inhibition (P=0.0001) in proliferation of MCF-7 treated glutamines compared to MCF-7 cells of the control between time points 48 and 72 hours. figure (3b) illustrated that MDA231 cells treated with glutamines and control cells treated with standard culture media (DMEMS-10) have significantly increased in number between time points zero and 48 hours (P=0.0001) with non-significant differences in cell number (P>0.05) of both conditions at these same time points. However, there was significant inhibition (P=0.0001) in proliferation of MDA-231 treated glutamines compared to MDA-231 cells of the control between time points 48 and 72 hours. On the other hand, glutaminase had no inhibitory effect on REF cells as shown in figure (3c), there was significant increase in cell numbers between time zero and 72 hours (P=0.0001) with non-significant variation between numbers of cells treated with glutaminase and control cells treated with standard growth media (DMEM-S10) at the different time points of the assay (0, 24, 48 and 72) hours. Additionally, there was no significant differences between proliferation of breast cancer cells MCF-7 and MDA-231 at the different time points of the assay (P>0.05). Whereas proliferation of REF cells was significantly higher than proliferation of MCF-7 and MDA-231 at the different time points of the assay (P=0.0001) as shown in figure (3d).

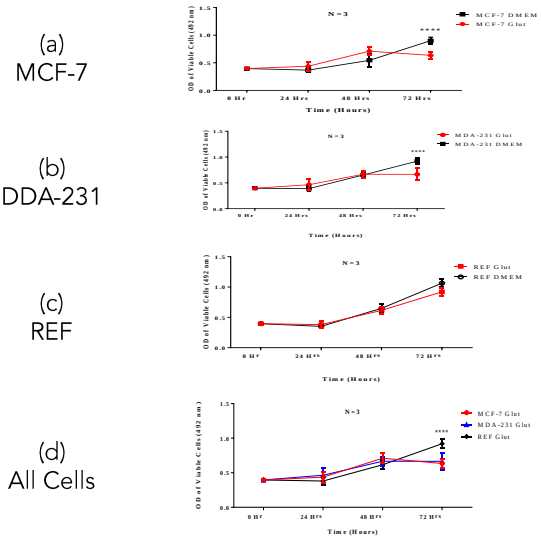


Figure (4) Statistical analysis of the proliferation of cancerous and normal cells treated with glutaminase.

(a) Statistical analysis showing the significant difference (P = 0.001) for the effect of glutaminase enzyme on inhibiting the proliferation of breast cancer cells (MCF-7) compared to cells treated with culture medium (DMEM-S10), where the effect appeared after 48 hours. (b) A statistical analysis showing the significant difference (P = 0.0001) for the effect of glutaminase enzyme on inhibiting the proliferation of breast cancer cells (MDA-231) compared to cells treated with culture medium (DMEM-S10), where the effect appeared after 48 hours. (c) Statistical analysis shows no significant difference (P>0.05) for the effect of glutaminase on the proliferation of REF cells compared to cells treated with culture medium (DMEM-S10). (d) A statistical comparison showing a significant difference (P=0.0001) between the proliferation of cancer cells (MCF-7) and (MDA-231) compared to the proliferation of normal cells (REF) starting from 48 hours.

5. Discussion

Clinical and experimental studies have reported that tumor microenvironment is quite complex and the mechanism of metastasis of breast cancer is still mysterious and requires more research and investigation; however, it could be represented by minimal component that significantly impact tumor progression in two-dimensional cell culture (2DCC) (25). In vitro cell-line proliferation screens are commonly used to assess the efficacy of potential cancer treatments. It is uncertain whether the proliferative or migratory abilities of cancer cells have a greater impact on tumor aggressiveness and patient survival (26). Previous intensive studies have reported that glutaminase plays a role in supporting proliferation of tumor cells by providing energy for the cells and promoting their proliferation by converting glutamine to glutamate via the glutaminolysis (27). However, cancer cells have other energy sources such as glucose from glycolysis (28), and fatty acids are also recognized as being crucial to cancer cells because they act as a significant energy source under metabolically stressful situations (29). Therefore, reducing energy sources of cancer cells by glutaminase inhibition represent useless approach for cancer therapy as long as the cancer cells have other energy sources. Additionally, tumor invasion is the most threating step in tumor progression; therefore, the current study investigated the invasion and migratory characteristics of breast cancer cells. To the best of our knowledge, there is no previous study has investigated the effect of glutaminase on invasion of cancer cells including breast cancer. Therefore, interpretation of our data is based on conclusions we extracted from previous studies. Glutaminase has been shown to inhibit migration of breast cancer cells within time and had no inhibitory effect on proliferation of breast cancer cells within 48 hours; however, it retarded proliferation after 72 hours of treatment. The most consensus explanation for inhibiting migration of cancer cells by glutaminase could be explained by the fact that glutaminase is upregulating the synthesis of collagen protein in fibroblasts (30). As well as, glutaminase has been reported to promote collagen translation and stability secreted by fibroblasts and mayofibroblasts (31). A collagen rich, stromal ECM has been shown to influence tumour growth and metastasis in many cancers (32). It has been reported that production of collagen and formation of extracellular matrix (ECM) are key mediators in cancer metastasis since ECM is required to enhance cells-cell contact while degradation of the ECM enhance cell dissociation as a first step in metastasis (33, 34, 35). As well established, enhancement of collagen production will provide collagenous matrix formation (36). The collagenous matrix represents the challenge for cancer cell invasion. Another possible explanation is that glutaminase enhances proline synthesis by converting glutamine to glutamate (37), the proline in turn supports collagen synthesis and maturation, thereby generating collagenous environment that retards migration and invasion of cancer cells (38). Additional possible explanation is that glutaminase enhances cancer cells to switch response from proliferation to migration when cancer cells obtain their energy from other sources such as glucose and fatty acids. This interpretation is supported by the fact that glutaminase provides more glutamate for cancer cells which is an essential molecule for the cell cycle and proliferation. Importantly, the present study reveals that glutaminase retarded cancer cells invasion indicating its importance as a target for cancer therapy. However, more research is required to investigate role of confirm this issue.

6. Conclusion

This study has investigated a new approach for cancer therapy by using glutaminase as anti-cancer substance that inhibits cancer by retarding cells migration. Hence, it could be concluded that inhibition of glutaminase may retard proliferation of cancer cells but it enhances their migration and invasion. So, inhibition of glutaminase is a two-edged sword and therefore, it is recommended that more studies should be achieved to investigate the correlation between cell migration and proliferation with and without glutaminase inhibition.

Conflict of interest

No conflict of interest.

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