

ROLE OF L-GLUTAMINE IN THE *IN-VITRO* GROWTH OF HCT-8 AND HT-29 CELL LINES

(Peranan L-Glutamin dalam Pertumbuhan Sel HCT-8 dan HT-29 *In-Vitro*)

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Abstract

L-glutamine is one of the essential supplements of *in-vitro* growth medium for cancer cells. The amino acid L-glutamine is well known as the vital source of nutrition in cancer cell growth for its ability to provide carbon and nitrogen. A common phenomenon of cancer cell is the rapid production of lactic acid through aerobic glycolysis. Apart from nutritional value, the released ammonia from L-glutamine may neutralize the acidic environment to ensure continuous cell growth. The current study is to observe the effect of L-glutamine concentration in culture media for cancer cell lines. Detection of L-glutamine consumption by the cells was carried out after 8 hours of incubation period. Numerous culture media were prepared adding L-glutamine concentration of 0 mM, 5 mM and 10 mM with different pH range. The cell density was calculated after 8 hours of incubation using trypan blue staining method. UV-Vis spectrophotometer was used to detect the concentration of L-glutamine consumption. The result shows that the cell density did not increase significantly in the media without L-glutamine supplement whereas, a rapid increase was observed in L-glutamine supplemented growth media in HCT-8 and HT-29 cell lines. The L-glutamine consumption was found higher in the media with low pH, but a relatively low L-glutamine consumption was observed in media with higher pH condition. The result confirms the necessity of L-glutamine in cancer cell growth. In addition, higher L-glutamine uptake in acidic condition supports the role of L-glutamine in acid resistance activity in cancer cell growth.

Keywords: L-glutamine, cancer cell growth, aerobic glycolysis, acid resistance

Abstrak

L-glutamin adalah salah satu tambahan penting dalam pertumbuhan medium *in-vitro* untuk sel-sel kanser. Asid amino L-glutamin terkenal sebagai sumber pemakanan penting dalam pertumbuhan sel kanser kerana keupayaannya menyediakan karbon dan nitrogen. Fenomena sel kanser yang biasa adalah pengeluaran asid laktik melalui glikolisis aerobik. Selain daripada nilai pemakanan, ammonia yang dikeluarkan dari L-glutamin dapat meneutralkan persekitaran asid untuk memastikan pertumbuhan sel berterusan. Kajian semasa adalah untuk melihat kesan kepekatan L-glutamin dalam media kultur untuk sel-sel kanser. Pengesanan penggunaan L-glutamin oleh sel-sel telah dilakukan selepas 8 jam tempoh inkubasi. Sejumlah media kultur telah disediakan dengan menambah kepekatan L-glutamin sebanyak 0 mM, 5 mM dan 10 mM dengan pelbagai pH yang berlainan. Ketumpatan sel dikira selepas 8 jam inkubasi menggunakan kaedah pewarnaan trypan biru. UV-Vis spektrofotometer digunakan untuk mengesan kepekatan penggunaan L-glutamin. Hasilnya menunjukkan bahawa ketumpatan sel tidak meningkat dengan ketara dalam media tanpa tambahan L-glutamin sedangkan, peningkatan pesat diperhatikan dalam media pertumbuhan L-glutamin ditambah dalam sel-sel HCT-8 dan HT-29. Penggunaan L-glutamin didapati lebih tinggi dalam media dengan pH rendah, tetapi penggunaan L-glutamin yang agak rendah diperhatikan dalam media dengan keadaan pH yang lebih tinggi. Hasilnya mengesahkan keperluan L-glutamin dalam pertumbuhan sel kanser. Di samping itu, penyerapan L-glutamin yang lebih tinggi dalam keadaan berasid menyokong peranan L-glutamin dalam aktiviti rintangan asid dalam pertumbuhan sel kanser.

Kata kunci: L-glutamin, pertumbuhan sel kanser, glikolisis aerobik, rintangan asid

Introduction

Glutamine is a non-essential amino acid which is produced abundantly in the human body. It is considered as one of the essential nutrients in different physiological functions and especially in the cell growth [1]. Glutamine supplies high energy to the cells in the process of cell proliferation which involve in vast protein and nucleic acid synthesis [2].

Glutamine directly involves in numerous regulatory processes of the cancer cell growth. Nitrogen is one of the essential atoms required in amino acids, nucleotides, amino sugars and in case of rapidly dividing cancer cells the nitrogen demand is very high. Glutamine supplies nitrogen atom by breaking down into glutamate and ammonia [3]. The glutamate produced due to the dissociation of glutamine is assumed to participate actively in tricarboxylic acid (TCA) Cycle in cancer cell [4]. However, apart from the energy supply glutamine possess a function of acid resistance [5, 6, 7].

Warburg effect describes the phenomenon of energy production by cancer cells where the cells undergo aerobic glycolysis that results accumulation of huge amount of lactic acid [8]. The excess production of lactic acid decreases the extracellular pH value [5]. According to Griffiths, the biological reactions linked to cancer cell growth are mostly pH dependant and cell growth is greatly affected if the extracellular pH value is below 5.6. Hence, it is detrimental to maintain a continuous acid resistance in order to sustain cancer cell growth [9]. Huang et al. proposed that glutamine plays a key role in extracellular acid resistance [5]. In all cell culture media glutamine is an essential component to supply energy [10]. It was also observed that the glutamine uptake is higher in cancer cells than in normal cells [11].

Therefore, the study was performed to prevail the role of glutamine in extracellular acid neutralization in *in-vitro* cancer cell culture. The quantitative detection of ammonia in the cell culture media and the cell growth quantification is essential in order to understand the role of ammonia in acid resistance.

Materials and Methods

Experimental cell lines

Two colorectal cancer cell lines namely HCT8 and HT29 were used as the experimental cell lines. HCT-8 cells was purchased from AddexBio Technologies, Inc., San Diego, United States and HT29 from American Type Culture Collection, Virginia, United States.

Preparation of culture media

A set of four different Dulbecco's Modified Eagle's Medium (DMEM) cell culture media with varying concentration of L-glutamine was prepared where the L-glutamine concentrations were 0, 5 and 10 mM. All the culture media were supplemented with 10% fetal bovine serum (FBS, heat inactivated) and 1% Antibiotics-Antimycotic mixed stock solution (Sigma Aldrich) consisting of penicillin, streptomycin and amphotericin. The composition of the supplemented DMEM medium is shown in Table 1.

Table 1. Composition of 100 mL of supplemented DMEM culture medium

Ingredient	Percentage (%)	Volume (mL)
DMEM	89	89
Fetal bovine serum (FBS, heat activated)	10	10
Antibiotics-antimycotic mixed stock solution	1	1
Total	100	100

Cell growth assay

HCT-8 and HT-29 cell lines were seeded into t_{25} cell culture flask containing media supplemented with 0, 5 and 10 mM of L-glutamine, respectively. In each flask approximately 1×10^5 cells were seeded. After seeding the cell lines, the flasks were incubated at 37 °C and 5% CO₂ in air. After 8 hours of incubation, the cells were counted staining with 0.4% trypan blue solution. The cells were diluted with 0.4% trypan blue solution and loaded onto the haemocytometer. The number of cells in each of the five red outlined squares (Figure 1) were counted, and the total number of cells were determined using following equation [12].

$$\begin{aligned} \text{Total Cells} &= \text{Mean cell count in 5 squares} \times \text{dilution factor} \times 10^4 \times \text{volume of the cells} \\ &= \text{Mean cell count} \times \text{dilution factor} \times 10^4 \times \text{volume of the cells} \end{aligned} \quad (1)$$

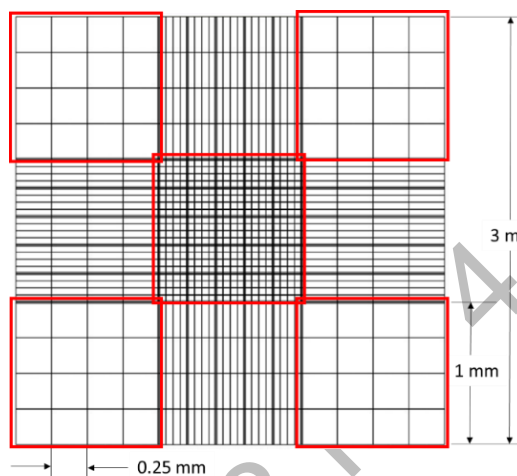


Figure 1. Grid pattern of the haemocytometer chamber. The number of the cells in red outlined squares to be calculated

UV-Vis spectrophotometric observation

Approximately 1×10^5 cell of both HCT-8 and HT-29 cell lines were seeded into t_{25} cell culture flask separately with complete DMEM media supplemented with 5 mM of L-glutamine. The cell culture flasks were incubated at 37 °C and 5% CO₂ in air. After 8 hours of incubation the media were collected into previously labelled 10 mL falcon tubes. The cells were counted in haemocytometer with 0.4% trypan blue staining. The L-glutamine and glutamate concentration were determined in UV-Vis spectroscopy using glutamine/glutamate determination kit (GLN-1; Sigma-Aldrich). Each of the samples was divided into two parts, first part of was subjected to measure glutamate directly and the second part was to measure glutamine plus glutamate as directed by the glutamine/glutamate determination kit protocol. The L-glutamine concentration in the media was calculated by subtracting the value of part one from the value of part two. The glutamine was transferred to glutamate by the addition of glutaminase, and the glutamate was dehydrogenized to α -ketoglutarate where NAD⁺ reduces to NADH. The glutamate amount is directly proportional to the amount of NADH and hence NADH concentration was taken into consideration. The NADH concentration was measured in UV-Vis spectrophotometer at 340 nm. A standard curve was prepared following the protocol described in glutamine/glutamate determination kit to calculate the concentration of glutamate in the experimental samples.

Data analysis

Data collected was analysed into graph by using Microsoft Excel 2013. The glutamate concentration was determined by plotting a concentration vs absorbance standard curve. The L-glutamine consumption data was normalized with the cell numbers.

Results and Discussion

The L-glutamine concentration is very crucial in the growth of cancer cell. The study observed that higher glutamine concentration promotes the growth dramatically. Beside L-glutamine concentration, the pH condition is also important especially in cancer cell growth. HCT-8 and HT-29 cells maintained their growth even with no L-glutamine supplement in the media with higher pH range (pH 7.3) (Figure 2). But in non-glutamine supplemented media with pH 6.5, there was no notable growth in both HT-29 and HCT-8 cell lines. However, L-glutamine supplement stimulates cell growth in both pH condition but the growth is relatively higher in pH 7.3.

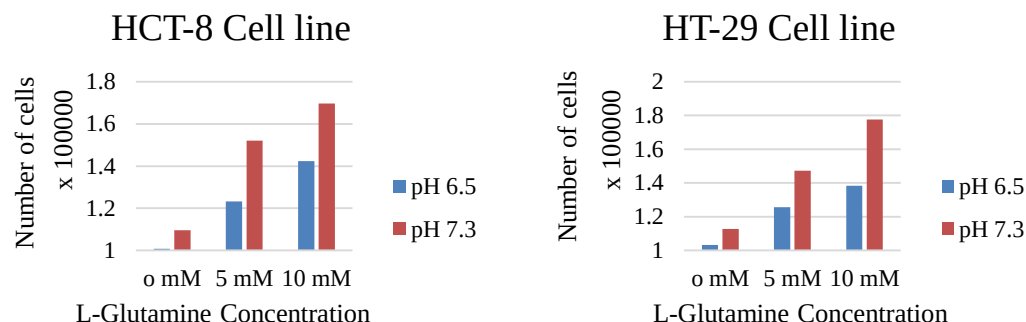


Figure 2. Cell growth after 8h of incubation. The media were supplemented with 0, 5 and 10 mM of L-glutamine in pH 6.5 and pH 7.3

The study evaluated the comparative growth of two different cancer cells in different pH condition and well as with various concentration of L-glutamine in growth media. The result shows that in pH 7.3, the HCT-8 cell growth had been increased up to 1.7-fold with 10 mM L-glutamine supplement in the culture media. HCT-8 cells grow moderately in pH 7.3 with 5 mM L-glutamine supplement to reach 1.52×10^5 cells where the initial number of cells was 1×10^5 . The HT-29 cell line also follows the similar trend with the increase of L-glutamine concentration in the media with pH 7.3. After 8 hours of inoculation, the number of cells were obtained 1.13×10^5 , 1.47×10^5 and 1.78×10^5 in the media supplemented with 0 mM, 5mM and 10 mM L-glutamine respectively. The result also shows that HT-29 cell line growth rate is comparatively higher than that of HCT-8 cell lines at pH 7.3. At pH 6.5, the number of HCT-8 cells reached as high as 1.42×10^5 with 10 mM of L-glutamine while HT-29 cell growth was comparatively lower with same concentration of L-glutamine. These observations showed cancer cell proliferation is influenced by the glutamine [11]. Other study by Huang et al. [5] have stated the same condition in which HeLa (cervical cancer cells) and MCF-7 cells (breast cancer cells) growth are reduced due to glutaminase activity is inhibited under acidic environment. HeLa and MCF-7 cells growth increased at pH 7.3 compared at pH 6.3 even in the glutamine absence. In such case, alkaline pH has been recognized as an important role increasing cell proliferation [13].

To investigate the L-glutamine consumption from the media a UV-Vis spectroscopy assay was carried out using glutamine/glutamate determination kit. HCT-8 and HT-29 cells were supplemented with 5 mM L-glutamine, incubated for 8 hours and then L-glutamine consumption was determined. Figure 3 shows that both HCT-8 and HT-29 cells exhibited higher L-glutamine uptake in pH 6.5 than in pH 7.3, although the trend is opposite in case of cell growth (Figure 2). The glutamine consumption normalized for cell density was relatively lower in media with pH 7.3 than in media pH 6.5. Glutamine consumption was 55% and 52% more in HCT-8 and HT-29 cells at pH 6.5 compared to pH 7.3.

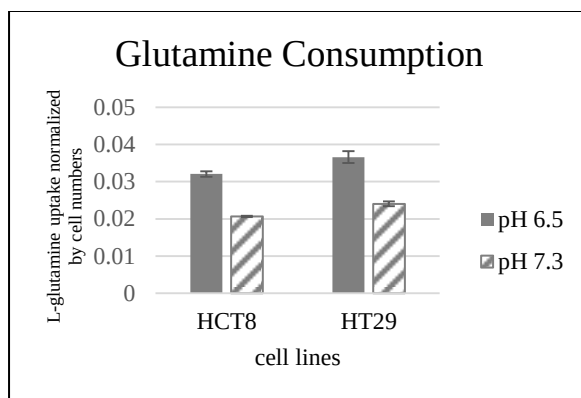


Figure 3. Normalized glutamine consumption from the media after 8 hours of incubation at pH 6.5 and pH 7.3. Bars represents the mean $\pm$ SD (Standard Deviation).

It is well known that glutamine metabolism promotes cancer cell proliferation by fuelling TCA cycle [4]. Due to the rapid cell growth cancer cells go through a distinctive metabolism characteristic that requires extensive biosynthesis and elevated energy to maintain rapid proliferation [14]. Two primary components, glucose and glutamine are the major energy provider as well as mediators of macromolecular synthesis in cell proliferation. In normal cells, proliferation takes place in lower energy environment which may be carried out by glucose metabolism, and hence the glutamine consumption is lower [15].

Apart from elevated energy requirement, cancer cell growth is also dependent on intracellular pH condition [5]. The increased aerobic glycolysis in cancer cells results decrease in extracellular pH as low as 5.6 [9, 16]. But several biological processes involved in cancer cell proliferation sternly depend on pH. And hence it is essential to continuously maintain a higher extracellular pH in order to promote proliferation. The findings of the study recommend the role of glutamine in extracellular acid resistance. Higher pH contributes in neutralizing abundant lactic acid produced by aerobic glycolysis and maintain the pH higher. Whereas, at pH 6.5, lactic acid lowers the pH of the media and slow down the cell growth. Overall, acid resistance is a must in cancer cell proliferation.

Higher concentration of L-glutamine and high pH confirms the optimum environment for cancer cell proliferation, thus there was robust growth of both of the cell lines. On the other hand, at higher pH state the glutamine consumption is lower compared to lower pH state. This could be due to the less need of acid resistance activity in higher pH state. In the media glutamine is reduced to glutamate and ammonia. The basic ammonia neutralizes lactic acid and maintain a constant extracellular pH. In the same way, lower pH state requires more acid to be eliminated and thus glutamine uptake is higher. Overall, the obtained data suggests that besides providing nutrients, glutamine conversion is essential to resist continuous acidic stress in extracellular environment of the cancer cells.

Conclusion

The study was performed to support the hypotheses that the highly glutamine dependency of cancer cells is not only because of nutrition but also to fight against acid stress. The experimental findings of higher glutamine consumption in lower pH supports the assumption of the possible acid resistance activity. Elevated cancer cell growth at higher pH state also recommends the importance of acid resistance in cancer cell proliferation. Further studies related to glutamine and acid resistance would help to understand homeostasis and anticancer therapeutic design.

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