TURKISH REPUBLIC OF NORTHERN CYPRUS

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDENT

THE EFFECT OF L-ASPARAGINASE OBTAINED FROM *ESCHERICHIA COLI* ON THE PROLIFERATION OF MCF-7 AND MDA-MB-231 BREAST CANCER CELLS

By

BAHEZ AHMED ABDALLA

DEPARTMENT OF MEDICAL BIOLOGY AND PATHOLOGY

MOLECULAR MEDICINE MASTER'S PROGRAM

THESIS SUPERVISOR

Assoc. Prof. Pinar Tulay

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APPROVAL

Thesis submitted to the Institute of Graduate Studies of Near East University in partial fulfillment of the requirement for the degree of Master of Science in Molecular Medicine.

Thesis Committee;

Chair of the committee:	Prof. Gamze Mocan Near East University ……………….
Supervisors/ Member:	Assoc. Prof. Pinar Tulay Near East University ………..........
Supervisors:	Assoc. Prof. Mahmut Cerkez Ergoren Near East University ……..............
Member:	Assist. Prof. Ozel Yuruker Girne University ………..........
Approved by:	Prof. Kemal Husnu Can Baser Director of Institute of Graduate Studies Near East University ....

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name:

Bahez Abdalla

Signature:

Date:

COMPLIANCE AND APPROVAL

His master thesis "Bahez Ahmed Abdalla" was written in accordance with the NEU Postgraduate thesis proposal and thesis writing directive

Prepared by Thesis	**Supervisors**
Bahez Ahmed Abdalla	**Assoc. Prof. Pinar Tulay** **Assoc. Prof. Mahmut C. Ergoren**

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Abstract:

Aim: The aim was to investigate the sensitivity of tumor cells seeded at different concentrations to L_asparaginases.

Background: The nutritionally dependent proliferation of acute lymphoblastic leukemic cells needs to rely on the external supply of asparagine. The enzyme L-asparaginase which is hydrolysed in the blood, is used for the therapy of acute lymphoblastic leukemia and other associated cancers in the blood. Asparaginase mainly hydrolyzes asparagine into aspartate and ammonia, which causes apoptosis of the cancer cells. The dose and time-dependent antitumor and cytotoxic effects of L_asparaginases from *Escherichia coli* have been investigated using breast cancer cell lines. These included MCF-7 and MDA-MB-231 breast cancer cell lines.

Materials and Methods: The growth of cell lines treated for 24,48, and 72 hours with L-asparagenase, Cell cycle analysis by flow cytofluorimetry and detected of apoptotic cells before and after treatment by TUNNEL stain. Evaluation both cell lines number after treatment with L_asparaginases for 24, 48 and 72 h demonstrated that asparagine deficiency then using nonlinear regression, normalized values are analyzed and IC50 values (95% Cl) and R2 are determined.

Results: These findings show the effected of L-asparagenase on MCF-7 and MDA-MB-231 cell lines. It did not recover the cells ability to proliferate du to cell death triggered by L-asparaginase.

Conclusion: L-asparaginase was observed to have more effect on the MDA-MB-231 cell line than on the MCF-7 cell line

Keywords: L-Asparaginase, Tumor, asparagine, aspartate, ammonia, *Escherichia coli*, MCF-7, MDA-MB-231

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ABBREVIATIONS

ALL: Acute Lymphoblastic Leukemia

BPRIP: BRCA 1 Interacting Protein C-Terminal Helicase

BRCA1: Breast Cancer Associated Gene 1

BRCA2: Breast Cancer Associated Gene 2

BSE: Breast Self-Examination

CSCs: Cancer Stem Cells

DCIS: Ductal Carcinoma *In Situ*

DMEM: Dulbecco'sodified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

E. coli: Escherichia Coli

EGFR: Epidermal Growth Factor Receptor

ESR1: Estrogen Receptor

HER2: Human Epidermal Growth Factor Receptor-2

IGF-1R: Insulin-Like Growth Factor Type I Receptor

IHC: Immunohistochemistry

ITC: Invasive Tubular Carcinoma

NST: No Special Type Invasive Carcinoma

P53: Tumor Protein 53

PBS: Phosphate-Buffered Saline

PR: Progesterone Receptor

PRLR: Prolactin Receptor

RNA: Ribonucleic Acid

TNBC: Triple-Negative Breast Cancer

WHO: World Health Organization

1.1. INTRODUCTION

1.1. GENERAL INFORMATIONS

1.1.1. What Is A Cancer

Cancer is defined when the cells are growing abnormally that can be developed from any organ of the body. Also, it is generated from those cells that lost the ability to stop developing. However, there are special cases like in cancer of the blood and bone marrow which do not give rise to a mass but are diagnosed with laboratory tests. Cancer cells are very dangerous because the immune cells are unable to identify and destroy them when they are still very few in number (Hanahan & Weinberg, 2011). Risks of cancer are higher in an individual with immunologic disabilities, and this can be due to chronic stress, old age, chronic diseases, chemotherapy and drug abuse. Cancers have well defined features with regards to their different origins. About 85% of cancer originates from epithelial cells and they are called carcinomas, others originating from mesodermal cells are called sarcomas while glandular tissue cancers (example breast) are called adenocarcinomas (Ferlay *et al.*, 2012).

1.1.2. Cancer Incidence

Cancer can be counted as the prime cause of death in Europe and among the world in general. There are more than two hundred different malignant growths that have been grouped and classified. In 2012, about 14 million new cases of cancer were reported and in 2015, about 8.8million cancer deaths were announced which in 2018 increased to 9.8 million deaths worldwide. The most common cases of cancer deaths are breast, lungs, liver, colorectal and stomach cancer, respectively According to the World Health Organization (WHO), 23% of all cancer's diagnoses in women are breast cancer cases which affect each year on 2.1 million women and about 627 thousand women died suffering from breast cancer per year especially those in developed countries. One in eight

deaths around the world caused by cancer (Garcia *et al.*, 2007). It relates with over 100 different diseases along various risk-factors and epidemiology.

1.1.3. Cancer Risk Factors

The exact cause of cancer is still unknown well, but investigation has identified many factors that are correlated with cancer development. Those possible risk factors includes alcohol use, endogenous and exogenous exposure to hormones, ageing, lack of physical activity, obesity, radiation, sunlight, tobacco smoke, chronic inflammation, and several dietary factors (Mena *et al.*, 2009)

1.1.4. Hereditary Cancer Syndrome

Hereditary cancer syndrome is the genetic mutation inherited by a person's parents which makes the person vulnerable to early onset of cancer (Clause *et al*., 1996). Those variations in genes result in cancers that affect various tissues and its manifest autosomal dominant inheritance. Several gene variants have been incriminated in more than 50 types of hereditary cancer syndromes such as breast and ovarian cancer syndrome. Hereditary variations in some tumour suppressor genes, for instance *BRCA1* and *BRCA2* have given rise to about 7% of the breast malignancies and 10% of ovarian malignancies (Clause et al., 1996). These malignancies were initiated by genes that act distinctively which categorized as oncogenes and tumour suppressor genes (Weinberg, 1996). Mutations in oncogenes induce a growth advantage to a cell (gain of function) while tumour suppressor gene mutation induces loss of function of a gene. A loss of function mutation needs a two mutated copy of the gene of each chromosome. (Claus *et al*., 2001).

1.2. Breast Cancer

Breast cancer is the neoplastic developed of a breast tissue. It is occurs in different types which is dependent upon which cells in the breast that turns cancerous (Gøtzsche & Jørgensen, 2013). The breast consists of three parts; connective tissues that surrounds and holds the breast, the ducts where the tubes carrying milk and nipple and milk production part is the lobules where known as the glands. Most of breast cancers originate in the

lobules or ducts and metastasize exterior of breast via lymph vessels and blood vessels. Usually, it is felt as a lump when forms a tumor and can be seen using X-ray and it majorly occurs in female but rarely in males (Merck 2003). However, it is vital to considering some breast lumps are not cancerous (malignant) and are benign. The non-cancerous tumors in the breast are only growths abnormally that not life threatening and do not metastasize outside of the breast, but some types might increase an individual's risk of having breast cancer.

1.2.1. Morphology Breast Cancer Classification:

Breast cancer is morphologically classified according to whether they have penetrated the membrane in the basement. Those that remain within this boundary are called in situ-carcinomas, and those that have continue to spread beyond it are designated as invasive carcinomas. We can classification of breast cancer by morphology is divided for Noninvasive such as (ductal carcinoma in situ, lobular carcinoma in situ) and invasive such as (invasive-ductal carcinoma, invasive-lobular carcinoma, carcinoma with medullary features, mucinous carcinoma, tubular carcinoma)(Kumar *et al.*, 2017).

1.2.2. Non-invasive (*in situ*) Carcinoma

ductal-carcinoma *in situ* (DCIS) and lobular-carcinoma *in situ* (LCIS) are two morphological types of non-invasive breast carcinoma. These are thought that both kinds of CIS derive from cells in the terminal ductal that contribute to lobules (Kumar *et al.*, 2017). DCIS is a disorder affecting breast and it could bring about breast cancer. Here, cancerous cells are found mainly in duct linings and not metastasized into more breast tissues. From the name, we can find that ductal means the cancer originates from the ducts of milk and carcinoma stands for any cancer that originates in the tissues that cover internal organs, then in situ refers to it's in original place. It is not life-threatening and is non-invasive but it can increase the chances later of developing an invasive breast cancer in life various clinical and biological feature differentiate (DCIS) from usual breast tissue and other benign breast tumors. Chromosomal disbalances arise in multiple location for gain or loss function, while hyperplastic lesions lead to in-vasive breast cancer through in

ductal-carcinoma in situ. Approximately 25 percent of all ductal-carcinoma in situ lesion mutate in the P53-gene is a tumor-suppressor but is uncommon in normal or benign breast proliferative tissue mutation. Genomic and proteomic analyses have identified many variations in healthy or hyperplastic breast tissue and ductal cancer in different patterns of gene-expression and protein-expression. Common factors correlate with cell growth and division have been studied, including cytoskeletal activity, intra-cellular cell membrane transport and microenvironment activity. During in the transformation from normal cell to ductal-carcinoma in situ, the most dramatic change occurs throughout breast tumorigenesis (Burstein *et al.*, 2004). There is no risk of developing invasive carcinoma for any morphological classification systems (DeVita *et al.*, 2018). Also, LCIS has differentiated pathological properties defined in the terminal duct and lobular apparatus by the proliferation of bland, homogeneous malignant cells. The lobular structure and investment membrane in the basement remain intact without evidence of penetration of the surrounding-stoma. The LCIS are cancer cells look more like normal cells and tend to grow and spread more slowly (low histologic) and nuclear grade, have a highly positive receptor for ostrogen and have indolent growth and good prognosis marker characteristics. This differs greatly from it is noninvasive-ductal counterpart, DCIS, that has more aggressive cytological and biological properties (Frykberg, 1999).

1.2.3. Pathology of Invasive Breast Cancer

Depending on the development pattern and cytological features of invasive cancer cells, the most widely established description is the WHO, while invasive-ductal and invasive-lobular carcinoma are known in the classification system, this histologist does not indicate that the first is in the ducts and second in the lobules of breast. Also, most invasive breast-tumors occur in the terminal ductal lobular unit, regardless of the Invasive ductal carcinoma, is one of the most known type of breast cancer and it makes up to 80% of all breast cancers. From the name, as the word invasive means the carcinoma has advanced to or “invaded” local tissues of the breast. While ductal meaning cancer originates from the ducts of milk. Carcinoma referring to any cancer that originates in the tissues that covers internal organs. In general, it is a carcinoma that start to invade tissues of the breast

and has breached the wall of the milk duct. By time passing, it can metastasize to the lymph nodes and other parts of the body. It can affect at any age on women, but it is risk factors increase as women grow older (Siu, 2016). Invasive Tubular Carcinoma (ITC) is a subtype of invasive ductal carcinoma. Usually smaller where it's about 1cm and have a structure of tube-shaped form which are called tubules. The tumor's cells look somehow like to normal healthy cells and also grow very slowly (Kleer *et al.*, 2000).

1.2.4. Incidence of Breast Cancer World-Wide

According to the groups of people and regions the rates of breast cancer are differ. Also, it differs between men and women and between different societies and ages. In 2018, there were approximately more than two million new breast cancer cases that was diagnosed worldwide among men and women, (WHO, 2018) generally in developed countries such as U.S, Australia etc have higher rates of breast cancer than developing countries like North Cyprus, Cambodia etc.

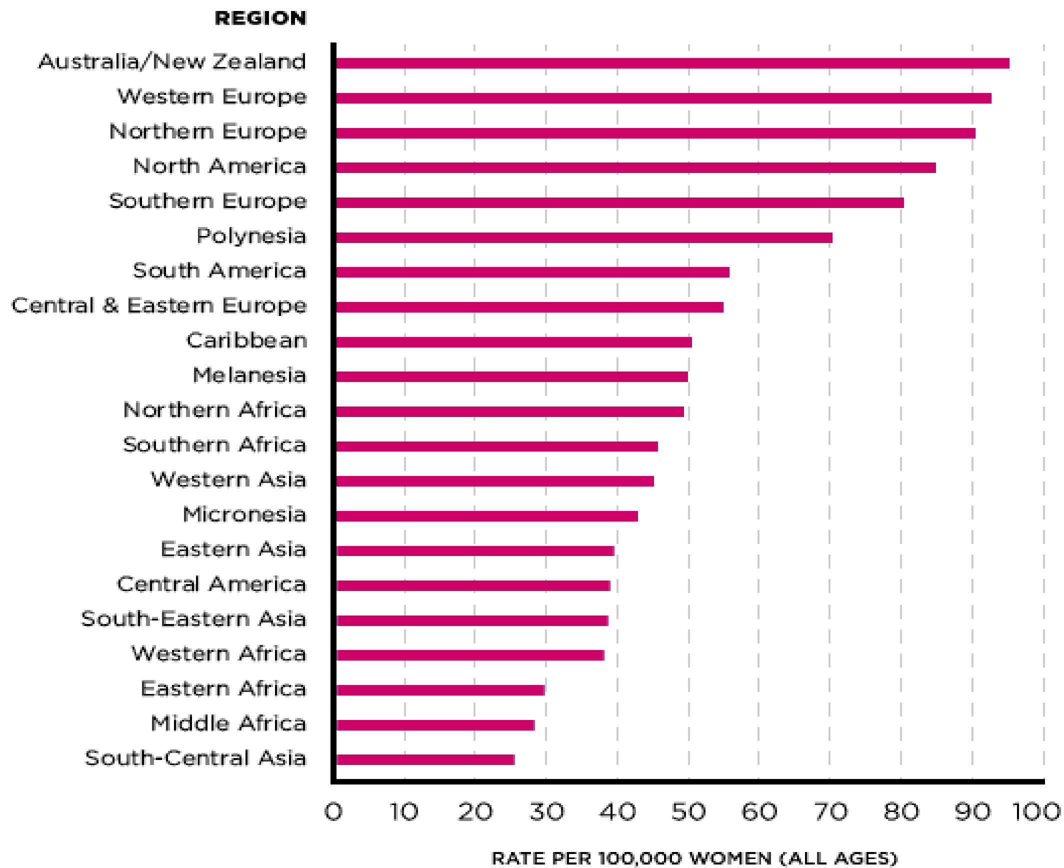

Figure1.1. Breast cancer incidence rates worldwide (adapted WHO 2018).

1.2.5. Risk Factors for Breast Cancer

Risk factor can be identified as anything that would increases the chances to develop a disease. it does not necessarily signify that it is absolutely sure for one to get the disease (Kelsey & Horn-Ross, 1993). There are some breast cancer risk factors older age; as age increases the risk for breast cancer increase. Mostly it is diagnosed beyond the age of 50 in sporadic cases generally (Colditz *et al.*, 2012). Genetic mutations; women whom inherited certain mutated genes like *BRCA1* and *BRCA2* are at a very high chance of getting ovarian and/ or breast cancer (Iodice *et al.*, 2010). Reproductive history, starting menopause or early menstrual periods starting before 12 years-old in women might give

rise to higher risk of getting breast cancer (Kahlenborn *et al.*, 2006) Having dense breasts; there are more connective tissues than fatty tissue in dense breasts and growths in their breast cannot be detected early enough (Tian *et al.*, 2018). Physical activity, many evidences has shown that constant physical activities reduces breast cancer risk especially in post-menopausal women (P. S. Kim & Reicin, 2005). There is a confusion in how much activity that is needed but it has been discovered that as little as a couple of hours a week is very helpful. Family history, A person's chances for getting breast cancer increases when a daughter, sister, mother or other members of family has been diagnosed of breast cancer in past (Gaffield *et al.*, 2009). Previous treatments; Persons who has had radiation therapy for their breasts or chest prior the age of 30 is said to have chance of having breast cancer in their life time (Hendrick, 2010)

1.2.6. Breast Cancer Classifications by Molecular Subtypes

Historically, the classification schemes for breast cancer have been focused on histopathological assessment including the histological type and grade. Breast cancer is divided into both invasive and in situ (ductal and lobular). The fourth edition of the WHO classification of breast tumors defines more than 21 subtypes of invasive breast carcinoma. The most common is No Special Type invasive carcinoma (NST), however recognized as (NST) invasive ductal carcinoma, comprising 70-80% of cases (DeVita et al., 2018). also, it was classified for three major groups: ER positive (HER2 negative: 50-60% of cancers), HER2 positive (ER positive or negative: 10-20% of cancers) and triple negative (ER, PR and HER2 negative; 10-20% of cancers) (Kumar *et al*., 2017).These three classes show remarkable variations in patient properties, pathological symptoms responsible to therapy, metastatic patterns time to relapse and result. An alternate classification system of significant overlap is focused on analysis of the gene expression. This system is currently used primarily in clinical research. Breast cancer is divided into four different types. Those include: luminal A, Luminal B, HER2, Tripal negative or basal-like

1.3. Asparaginase and *Escherichia Coli*

E. coli which is derivative from *Escherichia Coli* is a Gram-negative bacterium. They are coliform, rod-shaped and facultative anaerobes bacteria. They are majorly seen in intestine (lower part) of warm-blooded organisms (Singleton, 2004). Majority strains of *E. coli* are not harmful, but some strains are incriminated in poisoning and contamination of food (Vogt & Dippold, 2005). Those strains are harmless and take a part of the normal bacteria flora of the gut and they are helping the host in production of vitamin K2 (Bentley & Meganathan, 1982) and protection against pathogenic bacteria. It is excreted into the environment together with fecal matter (Russell *et al.*, 2001).

On the other hand, Asparaginase is a catalyzing enzyme that breakdown l-asparagine to l-aspartic acid and ammonia. It can obtain from different kinds of bacteria like *Escherichia coli* and *Erwinia chrysanthemi*. The asparaginase can be obtained and has the high-molecular-weight enzyme called l-asparaginase amidohydrolase, type EC-2 from the *E.coli* and clinically it has shown to have antitumor qualities (Roberts *et al.*, 1966). L-asparaginase obtained from E. coli is marketed under numerous trade names to use as a treatment for acute lymphoblastic leukemia (ALL) beside as part of a multi-agent chemotherapeutic regimen. Therapeutic L-asparaginase obtained from E. coli acts by draining the levels of asparagine in lymphoblastic leukemia cells, thereby causing death of the cell (Asselin *et al.*, 2015).

1.4. History

The development and discovery of asparaginase as an anti-cancer therapy kicked off as early as 1953. This was the year some scientists first observed that lymphomas found in rat and those in mice relapsed after treatment with serum of guinea pig (Kidd, 1953). Afterwards, it was observed with more evidence that it was not the serum itself which caused the tumour reversion but it was the enzyme asparaginase (Broome, 1963). Further studies which involved comparison of different kinds of asparaginases were carried out and the results showed that the asparaginase derived from *Erwinia chrysanthemi* and

Escherichia coli have the best anti-cancer qualities. Since then and due to the fact that it is very easy to produce asparaginase in large quantities *E. coli* has become the main source of it. However, In 1978 Asparaginase was approved for medical use (Salzer *et al.*, 2014) and today it is on the WHO List of Essential Medicines(WHO., 2019).

1.4.1. Mechanism of Action

Aparagine is a non-essential amino acid that maintains DNA, RNA and maintains protein synthesis. Asparagine also promotes cell growth. Normal healthy cells are able to derive asparagine via dietary intake or they can manufacture it from aspartate via the actions of asparagine synthetase. On the other hand, lymphoblastic leukemia cells do not have enough asparagine synthetase enzyme needed to synthesize asparagine and as a result they are unable to produce asparagine *de novo* (Asselin et al., 2015). Hence, leukemic cells rely solely on external sources of asparagine for protein synthesis and cell survival (Asselin et al., 2015). L-asparaginase from *E. coli* acts in draining plasma levels of asparagine in leukemic cells by transforming the L-asparagine to L-aspartic acid and ammonia (Asselin et al., 2015). This activity leads to reduced DNA, RNA, reduced protein synthesis, inhibition of cell growth and finally cell-death (Asselin et al., 2015). However, normal healthy cells can produce asparagine and their growth are not hindered by draining of asparagine caused by the treatment with asparaginase from *E coli*.

1.4.2. Side effects of Asparaginase

Asparaginase mainly causes allergic reaction, hypersensitivity reaction and anaphylaxis (BNF 57, 2009). The rates of these side effects of Asparaginase are high when compared to other chemotherapeutic agents. The rate of those side effects largely depends on the route of administration, the specific agent used and other accompanying therapies. The risk of side effect can be up to 8% for each administered dose and 33% by 4th dose (Larson et al. 1998: Evans et al. 1982) and from 2–75% in general (Wacker et al. 2007).

1.5. Breast Cancer Treatment and Prevention

Early diagnosis is very important in that case appropriate type of treatment should be started after identification of the stage of the disease (Agrawal, 2014). Tumors having amplified HER-2 can be treated with chemicals/ agents which inhibit HER-2. Triple-negative breast cancer can be treated with chemotherapy (Agrawal, 2014). Triple-negative breast cancer is an aggressive form of breast cancer with limited treatment options. Strategies used to treat breast cancer are surgery, radiation therapy, hormone therapy and radiotherapy plus adjuvant chemotherapy, (Arı 2010). Basis of breast cancer treatment is chemotherapy. Usually, combination of drugs used in chemotherapy is more effective to treat breast cancer. Metotreksat(M) 5-fluorourasil (F), siklofosfamid (C), epirubisin (E), adriamisin (A) are main anti-cancer drugs that are used in combinations like CMF, FAC, FEC, AC. Vinerelbin, dosetaksel and paklitaksel are also used on secondary stage. Mitomisin C and prednizon are the anti-cancer agents that are used in late stage of this disease (Mavroudis et al., 2009). However, these therapies have many side effects on body. For example, radiation therapy may cause rib fracture, second non-breast infield malignancies, tissue necrosis, brachial plexopathy. Cancer stem cells are very resistant to theraphies. Due to these side effects, there has been a growing interest in alternative treatment modalities. Finding alternative therapies with less or no side effects are essential. Many plant extracts have been used as alternative to treat cancer for many years. For many studies, use of cell lines have proven to be the ideal initiation of the experiments. For breast cancer studies, two cell lines; MCF7 and MDA-MB-271; are the main ones used in the studies.

1.6. MCF-7 Breast Cancer Cell Line

In 1970 MCF-7 cell line was obtained from 69-year-old woman (Lee *et al.*, 2015). MCF-7 stands for Michigan Cancer Foundation-7, where the cell line was established in an institute by Herbert Soule and co-workers in 1973 in Detroit (Brooks *et al.*, 1973). Although in origin the cell line is non-invasive even when it's from the metastases of an advanced tumour. Because of the estrogen dependence for growth and presence of functional ER both *in vivo* and *in vitro* it represents the model for disease in early stage.

Before the isolation of MCF-7, it was unimaginable for the researchers of cancer to gain a breast cell line which can survive more than a couple of months (Sharma, 2016).

1.6.1. Uses For MCF-7 Cell Line

They have been utilized widely for investigation of breast cancer pathobiology, for the screening and characterization of new therapeutics. In vitro breast cancer studies MCF-7 cells were utilized due to the cell line holding on too many unique characteristics as a result specific to the breast epithelium. Such characteristics are the potential for MCF-7 cells processing estrogen in sort of estradiol through the receptors of estrogen in cytoplasm of cell (Levenson & Jordan, 1997). In addition, MCF-7 cell line is progesterone receptor (PR) positive and HER2 negative

1.6.2 Characteristics of MCF-7 Cell Lines

MCF-7 belongs to the luminal A molecular subtype (Done, 2011) and are ER-positive and progesterone receptor (PR)-positive. Also, it is useful in the evaluation of the functional impact of PRLR variants in a mammary cell context, unresponsive to vimentin, very sensitive to cytokeratin, GAP, desmin and endothelin. Via cytoplasmic estrogen receptors the cell line is able to process estradiol and forming domes when grown in vitro. MCF-7 cancer cell growth can be subdued by anti-estrogens treatments, that has an automate impact on cell growth reduction. MCF-7 cells are in general a slow population growing but easy to propagate. It has 30-40 hours doubling time with typical cell size of 20-25 microns, that are fairly large adherent cells. Recommended medium is for MCF-7 cell line is EMEM which contains fatal bovine serum (10%), 0.01 mg/mL bovine insulin (90%),2 mM L-glutamine and Earle's BSS (Mansara *et al.*, 2015).

1.6.3. Molecular Profile of MCF-7 Cell Lines

MCF-7 cell line is used in breast tumor research for the study of the estrogen receptor (ER) alpha. MCF-7 cell lines depend on E2 in order to proliferate and are estrogen (E2)-sensitive cells (Perrot-Applanat *et al.*, 2012). They show low levels of ERβ and high levels of ERα transcripts (Buteau-Lozano *et al.*, 2002). In parallel experiments MCF-7 is used

as a parental control to its derivative cell lines and it's also the parental line from which many tamoxifen-resistant cell lines have been derived (Leung *et al.*, 2014). In the absence of estrogens, ER expression is increased by MCF-7 cell line and estrogen deprivation for short period of time causes unique responses of MCF-7 cells when compared to a long period (more thansix months) estrogen privation. Proliferation reduction rate lasts approximately a month after estrogen absence and this shows that MCF-7 has not established counterbalance mechanisms of growth yet during that period (Sweeney *et al.*, 2012). In reaction to E2 treatment of MCF-7 strains, the growth changes are not as a result of differences in ER functionality or expression level. Activating the IGF-IR by secretion of autocrine factor is what E2 receptiveness of MCF-7 depends upon (Hamelers *et al.*, 2003). Evidence exists concerning the contribution of IGF-1 signaling in the management of miRNAs in MCF-7 (Martin *et al.*, 2012). In addition, plasma membrane-associated growth factor receptors can control breast tumor cell growth. They include HER2, and EGFR activated by epidermal growth factor (EGF), both are seen in MCF-7 (Leung et al., 2014). MCF-7 cell lines moderately express EGFR-cell lines (Done, 2011). In modelling the progression and genesis of ER-positive breast cancer, as well as explaining how ER functions MCF-7 cells will continue to be an important tool.

1.6.4. MDA-MB-231 Breast Cancer Cell Line

This cell line originally was first obtained from a woman aged 51years with pleural effusion and metastatic mammary adenocarcinoma and it's an epithelial human breast cancer cell line (Cailleau *et al.*, 1978). It is comprehensively using breast cancer cell lines in laboratories for medical research today.

1.6.5. Characteristics of MDA-MB-231 Breast Cancer Cell Line

These cells are characterized by its more aggressive, hormone-independence, invasiveness and unwell differentiation of TNBC cell line. Additionally, they do not have oestrogen receptor (ER), progesterone receptor (PR) expression and also lacks HER2 amplification (Liu *et al.*, 2003) hence it was classed initially as a ‘basal’ breast cancer cell line. CTH and Globo side are the major neutral glycolipids in MDA-MB-231 line (Holliday &

Speirs, 2011). Comparing MDA-MB-231 to MCF-7 the ganglioside content in MDA-MB-231 was four times greater than that in MCF-7. GM3, GM2, GM1, and GD1a were abundant gangliosides found in both cell lines. The cell line shows endothelial-like morphology in 3D culture (Harrell *et al.*, 2014) and it is differentiated by stellate projections which sometimes link many cell colonies with its invasive phenotype (Kenny *et al.*, 2007).

1.7. The aim of study

Aim of this study is to evaluate the effect of asparaginase obtained from Escherichia coli on the growth of MCF-7 and MDA-MB-231 breast cancer.

1.8. Importance of the study

Breast tumor is causing many deaths among women in the world and its treatment methods like radiotherapy, chemotherapy, radiation therapy, hormone therapy and surgery have a lot of side effects on the patient's body. Hence, finding alternative treatment methods with less or no side effects is vita such as using *E. coli* asparagnase as an alternative treatment method. Since the need of cancer cells for amino acids is already proven possible target in the therapy development and to utilize the knowledge is essential for treatment. Acute lymphoblastic leukemia cells (ALLs) have very little number or even no asparagine synthetase, as a result their cell multiplication greatly relies on the external sources of asparagine unlike in normal healthy cells that has enough asparagine synthetase. l-asparaginase from *Escherichia coli* hydrolyzes extracellular l-asparagine into l-aspartate and this activity results in deprivation of asparagine from cancer cells that depends on external supply of asparagine for proliferation.

CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals

Penicillin-Streptomycin (+10000 µg/mL streptomycin) (Sigma-Aldrich, St. Louis, Missouri, United States), Human insulin (Sigma-Aldrich, St. Louis, Missouri, United States); Fetal Bovine Serum (Sigma-Aldrich, St. Louis, Missouri, United States) (DMEM /F-12(1:1)(1x) (Dulbecco's Modified Eagle medium F-12, +L-glutamate, +15mM HEPES (Sigma-Aldrich, St. Louis, Missouri United States) Nutrient Mixture (ham), (Sigma-Aldrich, St. Louis, Missouri United States) Trypsin/EDTA Solution, 0.25% (Sigma-Aldrich, St. Louis, Missouri United States). DMSO-Dethyl Sulfoxide (Sigma-Aldrich, St. Louis, Missouri United States)

2.1.2. Kits

Cell viability/cytotoxicity: TEBU-BIO cell counting kit 8 (CCK8) (Sigma-Aldrich, St. Louis, Missouri United States). Nonradioactive CCK-8 kit is used for sensitive colorimetric assays to countnumberof viable cells in the cytotoxicity assays and cell proliferation. This one-bottle solutiondoes not require premixing of components. It utilizes tetrazolium salt WST-8 [2-(2-methoxy4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Sigma, Germany), monosodium salt]. WST-8 is lowered by dehydrogenases to produce water-soluble formazan dye (orange colored) in cells.The quantity of formazan to the number of viable cells is straightly proportional.

2.1.3. Instrumentation

Utilized instruments

Instruments	Brand	Manufacturers
Safety Cabinet	ESCO class II type A2 biological	Germany
Incubator	Binder	Germany

centrifuge	Sigma 3-18k	Germany
Inverted microscope	Olmpus IX53	Germany
External computer		
Freezer	Panasonic, Tweenguard	UK
Water bath	Huber combatible control, cc1	Germany
Falcon tubes	14 mL* 17x100 mm	USA
cryotubes	Thermo fisher	Germany
Pipette,	Thermo fisher	Germany
eppendorf	Size 1.5 ml	Germany
hemocytometer	Sigma-Aldrich	Germany
Cell culture T-7 flasks	Thermo fisher	Germany
96-well plates	Sigma-Aldrich	Germany
microplate reader	Versa max tunable	USA

Table1.1.: Instrument with company

2.1.4. Bacteria Extract

Asparaginase from *Escherichia coli* bulk pack in a 100-300 units/mg protein (biuret), lyophilized powder, product number: A3809, CAS Number: 9015-68-3 was obtained from Sigma-Aldrich, Turkey.

2.1.5. Cell Lines

Breast cancer luminal A molecular subtype MCF-7 and MDA-MB-231 cell lines were gotten from American Type Culture Collection (ATCC), Rockvilles, Maryland, USA.

2.2. METHODS

2.2.1. Freezing and Thawing Cell Lines

Cell lines were stored in cryotubes containing DMSO (Dimethyl sulfoxide (%5) in a freezer at (-80°C). This prevents formation of ice crystals. Frozen cells were thaw in a water bath at 37 °C and then transferred into a centrifuge tube containing 15ml culture medium. Contents in tube were centrifuged (speed 1000 RPM, RCF 192, time 5min., temperature 22°C). Then supernatant was discarded from the tube.

2.2.2. Cell Culture

MCF-7 and MDA-MB-231 cell lines are grown in T75 flasks with the supplement of 45ml

DMEM/F-12 (1:1) (1X) containing F-12 Nutrient Mixture (Ham) (+) L-Glutamate and (+) 15mM HEPES, 5ml Fetal Bovine Serum (%10), 0.5ml penicillin streptomycin (%1), 125ml insulin human (at 4mg/ml), and incubated at 37˚C and in a 5%CO2 containing humidified chamber. The medium was refreshed every other day every week.

2.2.3. Sub Culturing

T-75 flasks were taken out from the incubator and examined under inverted microscope at low power. Cells were sub-cultured if there were no floating cells and they were 80-100% confluent. Culture medium was taken away from the flasks. Then cell layer was rinsed briefly with 2ml, 0.25% (w/v) Trypsin to remove traces of serum having trypsin inhibitor. 7ml trypsin was added to flask and placed in incubator and left for 10 minutes at 37°C. Then under inverted microscope cells were examined to be sure that all the cells are floating and detached. A growth medium with 6.0 to 8.0 mL was put into flask and by pipetting gently the cells are suck out. After transfer of cell suspension into the centrifuge tube with medium and cells, this tube was centrifuged for 5 minutes (RCF:192, temperature: 24°C, speed: 1000 RPM). Then supernatant is thrown out. 1ml (1000 µL) medium was put into the centrifuge tube containing only cells. 100 µl of suspension is placed on hemocytometer (counting chamber) and number of cells were counted. 3 x 106 cells were seeded into a new sterile flask containing 14ml medium. Then flask was placed in incubator and incubated at 37°C, 5% CO_2 containing humidified chamber.

2.2.4. Preparation of Stock Solution and Various Concentrations of Asparaginase

200 U/mg of L-asparaginase was dissolve in 1000 µl of distilled water. Then stock solution containing 100 µl L-asparaginase and 900 µl of color less medium was prepare. This stock solution used to prepare various concentrations of as 0.05 µM, 0.1 µM,0.2 µM,1 µM,5 µM

2.2.5. Measuring Cell Viability/Cytotoxicity

Cell viability/Cytotoxicity effects of Asparaginase was evaluated by using TEBUBIO cell counting kit 8 (Code: 277CK04-11) = QTY: X1 on breast cancer cell lines MCF-7 and MDA-MB231. Following procedure was applied recommended by the company. 100 µl

of cell suspension was dispensed (3000 cells/ well) in each of 3 x 96-well plate. In a humidified incubator plates are pre-incubated for 24hours (at 37°C, 5%CO_2). Then plates were investigated under inverted microscope to be sure that they were attached. Then medium in each well was removed. 10 µl of various concentrations of Asparaginase (0.05 µM, 0.1 µM,0.2 µM,1 µM,5 µM) were added to be tested to the plates. Each of the three plates was labeled as duration of exposure, name of cells and date. First plate was incubated for 24hours, the second plate for 48 hours was incubated and the third one was incubated for 72 hours in the incubator. 10 µl of CCK-8 is put into every well of the plates with attention. The plates were incubated for 4 hours in the incubator. The absorbance was measured at 450nm by using Versa max tunable microplate reader connected to an external computer.

2.2.6. Cell Staining Procedure

It was carried out Using 4 µl stock solution with 396 µl of color less media for both cell lines and prepared negative control for each cell lines then incubated for 24 hours. Fixation of cells after 24 hours incubation. Then the media was removed and was washed by PBS. The formaldehyde 400 ul was added on each well and wait for 30 minutes. formaldehyde was removed and washed by PBS (Phosphate Buffered saline). Then, it was stored at 4°C

2.2.7. Tunnel Staining Protocol

PBS 25% 9 ml was diluted with 300 ml distil water, then 50 ml of diluted PBS and 15 ml PBS -15 µL triton-x, 50 ml PBS -50 µL triton-x was added. The PBS was first removed, and PBS was put -kept for 10 minutes plates was put in the ice and It was washed by PBS three times. Hydrogenperoxide was added for five minutes to inhibit the hydrogen peroxide enzyme and, it was washed three times by PBS then added PBS. Equilibrate buffer was added for five minutes and added cover slip. 55 µL of TdT was added for each well and TdT was kept for 1 hour. Stopped wash buffer ten minutes. It was washed three times by PBS. Anti-digoxigenin peroxidase conjugate is put into the wells 65 µL and incubated in humidified chamber for 30 minutes. The anti-DiG was removed and was washed three times wash PBS. The DAB substrate was diluted with DAB dilution buffer

(16 µl of DAB with µL 150 DAB dilution) and 17- 50 µl of DAB substrat DAB dilution was added on each well for 5 minutes. It was washed by distil water three times. Hematoxylin stain was added and washed after five minutes. Aqeous-mont medium was added over the slid (just one drop) to put cover slip.

CHAPTER 3: RESULTS

3.1.1. Cytotoxicity results for MCF-7 cell line

The different concentrations of Asparaginase 0.05 µM, 0.1 µM, 0.2 µM, 1 µM, and 5µM were incubate with MCF-7 breast cancer cells and the absorbance values were measured at 450nm using Versa max tunable microplate reader. The absorbance values were normalized against the negative control (max.100-min.0). Normalized values were analyzed by using nonlinear regression and IC50 (%95 CI) values were calculated (Table 3.1).

Exposure Time to Asparaginase	R^2	IC50 (µM)
24 hours	0.2046	0.02473
48 hours	0.8780	0.002519
72 hours	0.3417	0.05034

Table 3.1: IC50 values obtained in molar, R2 values and IC50 values in µM for MCF-7 cell line.

Figure 3.1 shown below is the Nonlinear regression analysis result for MCF-7 cell for 24Hours, 48Hours and 72Hours.

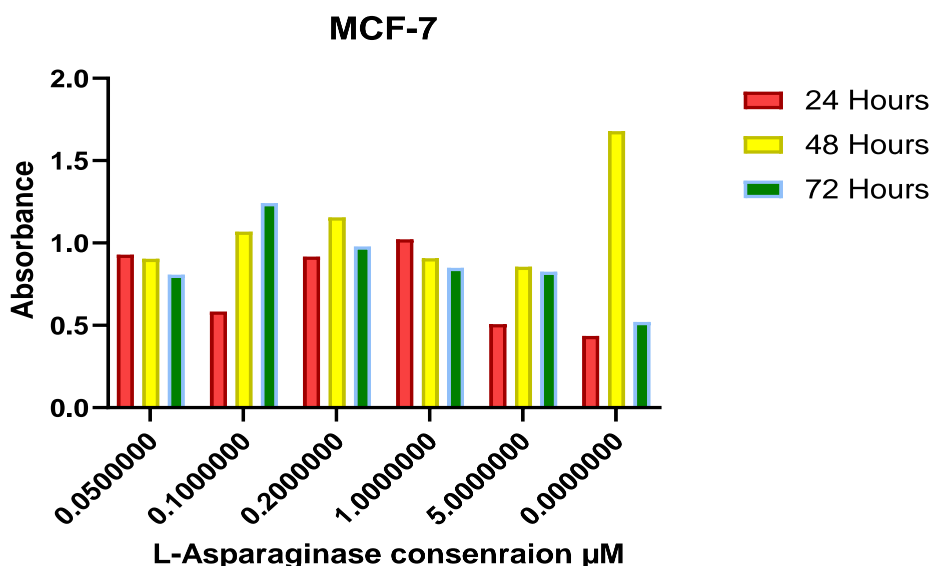

Figure 3.1: Nonlinear regression analysis results for MCF-7 cell line for 24hours, 48hours and 72hours.

According to our research, we have found that the time and amount of the L-Asparaginase are effective on MCF-7. Further statistical investigation was conducted to investigate the significance of time and the mean difference within each group of concentration of L-Asparaginase for MCF-7 cell line and means obtained after 24Hours, 48Hours and 72Hours were compared. There were significant differences between the concentrations of the Asparaginase means at 24 Hours -48 Hours, 24h-72 Hours, 48 Hours -72 Hours values compared in control group. The best concentration that significantly affected the MCF-7 cells proliferation was 5 μM at 48 hours, R^2 values=0.8780 with IC50 values of 0.002519 μM.

In addition, two-way Anova Multiple Comparison Test was employed to ascertain significance in mean difference between the control sample and different concentrations of L-Asparaginase on MCF-7 cell line for 24 Hours, 48 Hours and 72 Hours, respectively. After the exposure of the MCF- 7 cell line to different concentrations of Asparaginase for 24 hours, 48 hours and 72 hours, the IC50 values were obtained as follows 0.02473 μM, 0.002519μM and 0.05034μM respectively and the R^2 values for 24 hours, 48 hours and 72 hours exposure to L-Asparaginase were calculated as follows 0.2046, 0.8780 and 0.3417, respectively. The results show that the mean difference is significant at 95% confidence level between control group and 0.05μM group and between control group and 0.1μM group after 48 hours of exposure to L-Asparaginase. When the values in 24 hours-48 hours, 24 hours-72 hours, 48 hours -72 hours were compared to the control group, there were significant differences between the means. Figure 3.2 shown below is the Two-way Anova multiple comparisons test results for MCF-7 cell line for 24 hours, 48 hours and 72 hours exposure of L-asparaginase.

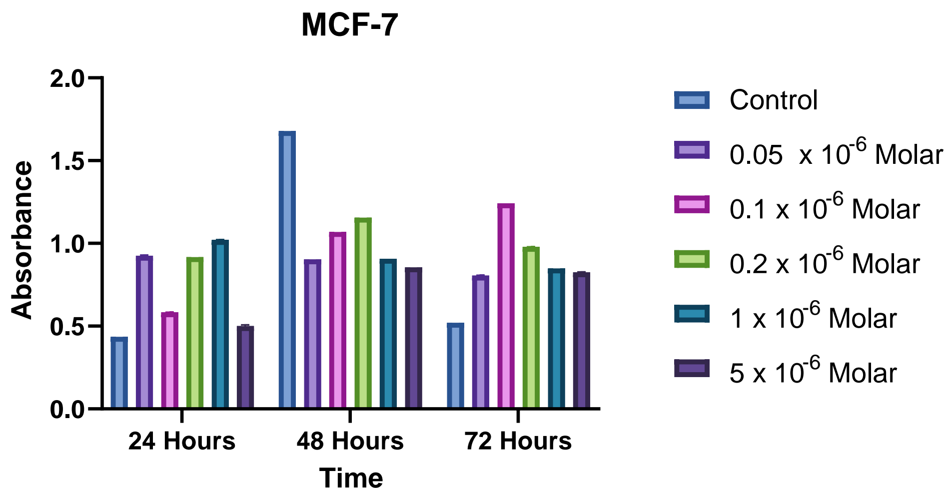

Figure 3.2 is the Two-way Anova multiple comparisons test results for MCF-7 cell for 24 hours,48 hours & 72 hours exposure of Asparaginase.

3.1.2. Cytotoxicity results for MDA-MB 231 cell line

Different concentrations of L-Asparaginase 0.05, 0.1, 0.2, 1, and 5µM were incubated with MDA-MB 231 cells and absorbance values measured at 450nm using Versa max tunable microplate reader. The absorbance values were normalized against the control samples (max.100-min.0). Normalized values were analyzed by using nonlinear regression and IC50 (%95 CI) values were calculated. (Table 3.2).

Exposure Time to Asparaginase	R^2	IC50 (µM)
24hours	0.7838	0.9165
48hours	0.7886	0.3847
72hours	0.9895	0.6933

Table 3.2: IC50 values obtained in molar, R2 values and IC50 values in µM for MDA-MB 231 cell line.

Figure 3.3 shown below is the Nonlinear regression analysis result for MDA-MB-231 cell for 24Hours, 48Hours and 72Hours

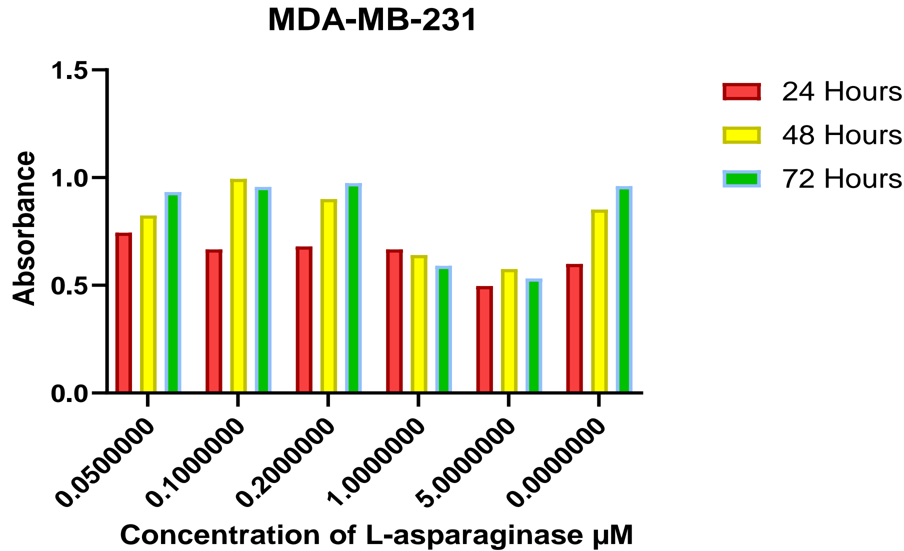

Figure3.3 Nonlinear regression analysis results for MDA-MB 231 cell for 24hours, 48hours and 72hours.

Further statistical investigation was performed to determine the significance of mean difference within each group of concentration of L-Asparaginase for MDA-MB 231 cell line and means obtained after 24 hours, 48 hours and 72 hours were compared. There were significant differences between means when 24 hours -48 hours, 24 hours -72 hours, 48 hours -72 hours values when compared in control group. There was no effect of L-Asparaginase at 1 μM after 24 hours exposure but at the same concentrations they had a significant effect at 48 hours. The best concentration that significantly affected the MDA-MB-231 cell proliferation was 5 μM at 72 hours, R^2 values= 0.9895 with IC50 values= 0. 6933μM. Two-way Anova Multiple Comparisons Test was conducted to analyze the significance of mean difference between control and different concentrations of L-Asparaginase on MDA-MB 231 cell line for 24 hours, 48 hours and 72 hours respectively. After the exposure of the MDA-MB-231 cell line to varying concentrations of L-Asparaginase for 24 hours, 48 hours and 72 hours, the IC50 values were obtained as follows; 0.9165 μM, 0.3847 μM and 0.6933μM, respectively and the R^2 values for 24hours, 48hours and 72hours exposure to L-Asparaginase were calculated as follows;

0.7838, 0.7886 and 0.9895, respectively. The results show that the mean difference is significant at 95 % confidence level between control group and 0.05µM group and between control group and 5µM group after 24 hours of exposure to L-Asparaginase. When the values in 24 hours-48 hours, 24 hours-72 hours, 48 hours-72 hours were compared to the control group there were significant differences between the means. Figure 3.4 shown below is the Two-way Anova multiple comparisons test results for MDA-MB-231 cell line for 24 hours, 48 hours and 72 hours exposure of L-Asparaginase.

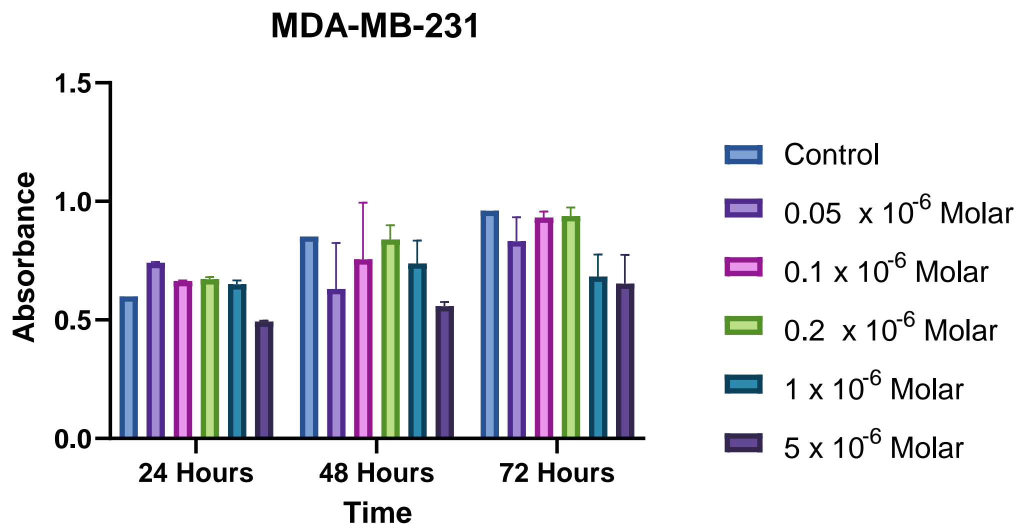

Figure 3.4:is the Two-way Anova multiple comparisons test results for MDA-MB 231 cell line for 24 hours,48 hours and 72 hours exposure of Asparaginase.

3.2 Detection of apoptosis *via* tunnel assay in MCF-7 AND MDA-MB-231

The apoptotic effect of 1µM of L-asparaginase on MCF-7 and MDA-MB-231 cell lines following 24hour exposure was investigated. The slides examined under light microscope were the apoptotic cells stained dark with Tunnel counted, and the results were evaluated using KRUSKAL-WELLIS TEST by SPSS program. The results showed that there was

no significant difference compared with negative control group (p-value for MCF-7 cells 0.225, and 0.500 for MDA-MB-231 cells, respectively). However, the apoptotic cells were detected and thus the effects of L-asparaginase on both cell lines were clearly visible even though it was not statistically significant.

4. Discussion and conclusion

About 1.2 million people, mostly women, are affected yearly by breast cancer around the World. Widespread presence and increasing frequency of breast cancer makes it very important for early diagnosis and treatment. In spite of advances in treatment in the early stage of breast cancer, many women experience recurrence and metastasis. Treatment for breast cancer patients include radiation therapy, chemotherapy, surgery, hormone therapy, and targeted therapy. Currently cancer treatments have major side effects which include heart damage, hair loss, diarrhea, dry skin, and kidney damage (Khan *et al.*, 2014). Chemotherapy destroys cancer cells and damages normal cells with lots of adverse effects, reduces white and red blood cells, increases risk of having infection and anaemia, causes diarrhea, fatigue, hair loss, sore throat, ulcers, nausea, constipation, loss of appetite and change in colour of the skin, radiation therapy may cause rib fracture, second non-breast infield malignancies, tissue necrosis, brachial plexopathy. Cancer stem cells are very resistant to therapies. Some of the examples of complications associated with adjuvant treatment are skin changes, cardiac toxicity, neuropathy, reproductive dysfunction, arm lymph edema and pneumonitis (Agrawal, 2014).

Improvement in our understanding of the physiology of breast cancer using cell lines has been made over past several decades. Culturing of tumours cells *in vitro* has been the major method of understanding the mode of actions of therapeutic agents within a regulated environment and condition. However, it has been acknowledged that many changes occur in physiological conditions, such as cell cycle kinetics are different and the biochemical parameters may affect the cell's responses to the drug treatment (Benz et al., 1981).

In this study, we demonstrated that L-Asparaginase induces cytotoxicity by targeting the Asparagine addiction phenotype in breast cancer cell lines. A clinically acceptable intermediate dose of L-Asparaginase exerts enormous apoptosis in breast cancer cell lines. L-Asparaginase treatment together with glutamine deprivation extremely impede the refilling of the tricarboxylic acid cycle by intracellular glutamate.

MCF-7 and MDA-MB-231 cell lines were used for this study because of their unique attributes. This research was designed to to examine the effect of asparaginase obtained from *Escherichia coli* on the proliferation of MCF-7 and MDA-MB-231 cell line. Asparaginase is a good anticancer agent against acute lymphoblastic leukaemia and strong tumour malignancies (Ghasemi *et al.*, 2017). The use of asparaginase depends on the fact that cancer cells are not capable of producing asparagine synthase, as a result of this, a high amount of asparagine from serum and cerebrospinal fluid is required (Knott *et al.*, 2018). Asparaginase mainly hydrolyzes asparagine into aspartate and ammonia, which causes apoptosis of the cancer cells. Formation of secondary tumors *via* metastasis of breast cancer cells is the reason for most cases of death. The regulation of metastatic headway is greatly affected by the change in asparagine accessibility (Knott et al., 2018). Furthermore, L-asparaginase used as a potential anticancer agent for malignancy, is obtained from *Erwinia chrysanthemi* and *Escherichia coli* (Duval *et al.*, 2002).
This study evaluated the effects of L-Asparaginase obtained from *Escherichia coli* on the proliferation of MCF-7 *in vitro.* After the exposure of the MCF- 7 cell line to different concentrations of Asparaginase for 24 hours, 48 hours and 72 hours, the IC50 values were obtained as follows 0.02473 µM, 0.002519µM and 0.05034µM respectively and the R^2 values for 24 hours, 48 hours and 72 hours exposure to L-Asparaginase were calculated as follows 0.2046, 0.8780 and 0.3417, respectively. In addition, a two-way Anova multiple comparisons test was carried out for MCF-7 cell line to determine the significance mean difference between control and other concentrations of L-Asparaginase for 24 hours, 48 hours and 72 hours respectively. Mean difference is significant at 95 % confidence level between control group and 0.05µM group and between control group and 0.1µM group after 24hours of exposure to L-Asparaginase, respectively. When the values in 24hours-48hours, 24hours-72hours, 48hours-72hours were compared to the control group there were significant differences between the means.

Furthermore, in this study we evaluated the effect of asparaginase obtained from *Escherichia coli* on the proliferation of MDA-MB-231 breast cancer cell line *in vitro*. After the exposure of the MDA-MB-231 cell line to varying concentrations of L-

Asparaginase for 24 hours, 48 hours and 72 hours, the IC50 values were obtained as follows; 0.9165 µM, 0.3847 µM and 0.6933µM espectively and the R^2 values for 24 hours, 48 hours and 72 hours exposure to L-Asparaginase were calculated as follows; 0.7838, 0.7886 and 0.9895, respectively. In addition, a two-way Anova multiple comparisons test was carried out for MDA-MB-231 cell line to determine the significance mean difference between control and varying concentrations of L-Asparaginase for 24 hours, 48hours and 72 hours, respectively. Mean difference is significant at 95% confidence level between control group and 1µM group and between the control group and 5µM group after 24 hours of exposure to L-Asparaginase. When the values in 24 hours-48 hours, 24 hours-72 hours, 48 hours-72 hours were compared to the control group there are significant differences between the means.

The evaluation of cell lines after treatment with L-asparaginases for 24 hours, 48 hours and 72 hours showed that asparagine deficiency inhibited normal cell division, proliferation and reduction in the L-Asparaginase concentration led to reduced cell death in all the cell lines tested. The limitation of L-Asparaginase treatment is in its short biological half-life (T50 = 2.88 h) and this gives need to increase the dosage at intervals, but this limitation can be defeated by the use of drug carriers, such as liposomes. This will increase L-Asparaginase potency and decrease its toxicity (Do *et al.*, 2019).

When comparing the effects of Asparaginase from *Escherichia coli* with the L-Asparaginase from other sources like *Erwinia chrysanthemi* in breast cancer cell lines, it is established that L-Asparaginase from Escherichia coli has superior clinical efficacy than L-Asparaginase from *Erwinia chrysanthemi* and this is because the serum half-life of L-Asparaginase activity from *Erwinia chrysanthemi* is significantly shorter (0.65day) as against (1.24days) for L-Asparaginase from *Escherichia coli*. However, L-asparaginase obtained from *Erwinia chrysanthemi* has less toxicity than that of *Escherichia coli* (Duval et al., 2002). There is no difference between the L-Asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* in terms of allergy rates, insulin-requiring diabetes, infections, and risk of pancreatitis. As a result, the L-asparaginase obtained from *Escherichia coli* is recommended for first-line therapy and the asparaginase from *Erwinia chrysanthemi* is

reserved for allergic patients because some patients who are allergic to L-Asparaginase obtained from *Escherichia coli* are not immediately allergic to the L-Asparaginase from *Erwinia chrysanthemi* (Egler *et al.*, 2016).

Further studies compared the effects of L-Asparaginase from *Escherichia coli* with Pegylated Asparaginase in breast cancer cell lines. It is observed that Pegylated Asparaginase is less immunogenic than L-Asparaginase from *Escherichia coli* and it has a longer half-life. This allows one dose of Pegylated asparaginases to be administered instead of multiple doses of L-Asparaginase from *Escherichia coli*. Pegylated Asparaginase has similar toxicity and efficacy to L-Asparaginase from *Escherichia coli*. The most common toxicity for both L-asparaginase from *Escherichia coli* and Pegylated Asparaginase is hepatoxicity (H. J. Kim *et al.*, 2018).

In conclusion, L-asparaginase was observed to have more effect on the MDA-MB-231 cell line than on the MCF-7 cell line. There is an urgent need to evaluate new sources of Asparaginase, such as from different plant sources with more effective therapeutic properties and less side effects due to the side effects of commercially available bacteria Asparaginase.

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