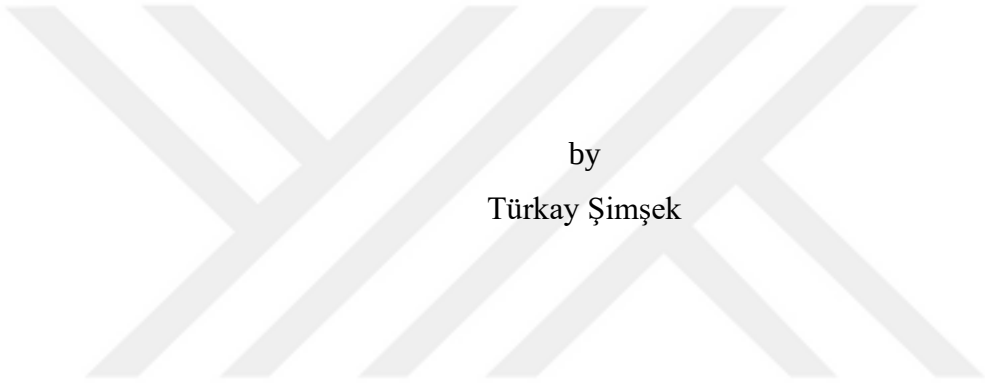


INVESTIGATION OF THE EFFECTS OF CYTOKINES OF YOUNG AND OLD
MESENCHYMAL STROMAL CELLS ON CANCER CELL LINES BY SMALL
MOLECULES



by
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ABSTRACT

INVESTIGATION OF THE EFFECTS OF CYTOKINES OF YOUNG AND OLD MESENCHYMAL STROMAL CELLS ON CANCER CELL LINES BY SMALL MOLECULES

Nowadays, stromal cell therapies are among the most important treatment methods used experimentally in many fields of medicine or clinically in some fields. Although different treatment methods are used, there is not yet a method that can cure cancer. Brain cancer is one of the difficult types of cancer to treat, and new methods are being researched for this. It has been reported in many experimental studies that Mesenchymal Stromal Cell (MSC), which has therapeutic and protective properties, suppresses cancer cells in different cancer models and stops their proliferation. However, in recent studies using stromal cells for cancer treatment; it has been shown that stromal cells trigger cancer formation, enlarge the mass, help the formation of new vessels, and invasion of cancer. It is known that communication between MSCs and cancer cells is achieved with the help of cytokines released from MSCs. The presence of these cytokines secreted by MSC and their effect on cancerous cells reduce the treatment reliability of MSC. However, it is found that MSCs are divided into two subgroups, one prevents the division and proliferation of cancer cells, while the other helps cancer cells to proliferate. Quercetin has been shown to play a positive role in many biological activities such as inflammation, oxidative stress, cancer formation and progression, platelet aggregation, atherosclerosis, and aging. This study aims to stimulate the release of cytokines and chemokines that will suppress cancer cells by stimulation of MSCs with Quercetin. The results showed that the conditioned MSC medium led to apoptosis and stopped the proliferation of the cells, giving different results for each patient for 24 and 48 hours. Different cell lines of glioblastoma such as A172 and U87 were used in this study.

ÖZET

MEZENKİMAL STROMAL HÜCRELERİN SALDIĞI SİTOKİNLERİN ÇEŞİTLİ APOPTOTİK MADDELER ARACILIĞI İLE KANSER HÜCRE HATLARI ÜZERİNDEKİ ETKİLERİNİN İNCELENMESİ

Günümüzde stromal hücre tedavileri, tıbbın birçok alanında deneysel olarak veya bazı alanlarda klinik olarak kullanılan en önemli tedavi yöntemleri arasında yer almaktadır. Farklı tedavi yöntemleri kullanılsa da henüz kanseri tedavi edebilecek bir yöntem bulunmamaktadır. Beyin kanseri, tedavisi zor kanser türlerinden biridir ve bunun için yeni yöntemler araştırılmaktadır. Terapötik ve koruyucu özelliklere sahip Mezenkimal Stromal Hücrenin (MSH) farklı kanser modellerinde kanser hücrelerini baskıladığı ve çoğalmasını durdurduğu birçok deneysel çalışmada bildirilmiştir. Bununla birlikte, kanser tedavisinde stromal hücrelerin kullanıldığı son çalışmalarda; stromal hücrelerin kanser oluşumunu tetiklediği, kitleyi büyüttüğü, yeni damar oluşumuna ve kanser istilasına yardımcı olduğu gösterilmiştir. MSH'ler ile kanser hücreleri arasındaki iletişimin, MSH'lerden salınan sitokinlerin yardımıyla sağlandığı bilinmektedir. MSH tarafından salgılanan bu sitokinlerin varlığı ve kanserli hücreler üzerindeki etkileri, MSH'nin tedavi güvenilirliğini azaltmaktadır. Bununla birlikte, MSH'lerin iki alt gruba ayrıldığı, biri kanser hücrelerinin bölünmesini ve çoğalmasını önlerken, diğerinin kanser hücrelerinin çoğalmasına yardımcı olduğu bulunmuştur. Quercetin'in iltihaplanma, oksidatif stres, kanser oluşumu ve ilerlemesi, trombosit agregasyonu, ateroskleroz ve yaşlanma gibi birçok biyolojik aktivitede olumlu bir rol oynadığı gösterilmiştir. Bu çalışmanın amacı, MSH'lerin Quercetin ile uyarılmasıyla kanser hücrelerini baskılayacak sitokinlerin ve kemokinlerin salınımını uyarmaktır. Sonuçlar, koşullandırılmış MSH besiyerinin apoptoza yol açtığını ve hücrelerin çoğalmasını durdurduğunu, her hasta için 24 ve 48 saat boyunca farklı sonuçlar verdiğini gösterdi. Bu çalışmada glioblastoma'nın A172 ve U87 gibi farklı hücre hatları kullanılmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

VEGF	Vascular endothelial growth factor
MSC	Mesenchymal stromal cell
IL-6	Interleukin 6
IL-8	Interleukin 8
SDF-1	Stromal cell-derived factor 1
CCl-5	Chemokine (c-c motif) ligand 5
TGF β 1	Transforming growth factor beta 1
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
CXCL7	Chemokine (c-x-c motif) ligand 7
CXCL5	Chemokine (c-x-c motif) ligand 5
CXCL9	Chemokine (c-x-c motif) ligand 9
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
PDGF	Platelet-derived growth factor
bFGF	Basic fibroblast growth factor
GBM	Glioblastoma
DPBS	Dulbecco's phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ATCC	American tissue and cell culture
HBSS	Hanks' balanced salt solution
7-AAD	7-aminoactinomycin
STR	Short tandem repeat
PI	Propidium iodide
EDTA	Ethylenediamine tetraacetic acid

1. INTRODUCTION

Cancer is a disease whose incidence has increased in recent years although different treatment methods are used, there is no treatment that can completely eliminate cancer yet. Cancer was ranked 7th-8th among the diseases that caused death in the early part of this century. Today, it has ranked second after cardiovascular diseases in many countries. The most common types of cancer in men are lung, prostate and colorectal, while in women, breast, colorectal and lung. Different treatment methods are used in cancer treatment to reduce death and increase survival such as surgical methods, radiotherapy, chemotherapy, hormone therapy, immunotherapy, signal transduction system inhibitors, gene therapy and angiogenesis inhibitors. Although many drugs and new treatment methods have been developed for cancer treatment in recent years, there are hardly any treatment options for some of advanced cancer patients today. This can be partially explained by the complexity of the cancer development process and the large number of points that need to be clarified.

MSCs are the most commonly used cell type in cellular treatments both in clinical studies and experimental studies. From clinical trials to animal model studies, MSCs have been promising in the treatment of many diseases. Clinical studies on MSCs are increasing day by day. In line with the recommendations of the American Food and Drug Administration, a cell to be used in cellular treatments; it must be a cell that can be easily obtained, does not create an immune response, is non-carcinogenic, divides rapidly, can be noticed by cells in the tissue it goes, and can migrate to the injured area when given through blood. MSCs have all these features [1][2]. Since 1995, MSCs have been used in many clinical studies. When we look at the clinical studies where MSCs are used for cancer, diabetes, Chron's disease, heart diseases, liver diseases, multiple sclerosis, graft versus host disease, bone and cartilage damages, spinal cord injuries and brain injuries.

At the beginning phase of MSC-cancer cell studies; anti-carcinogenic, proliferative and apoptotic effects of MSCs have been demonstrated. In later studies, MSCs have been shown to trigger cancer formation, enlarge the mass, help the formation of new vessels and invasion of cancer. These effects of MSCs on cancer cells have limited the use of MSCs as a treatment method [3].

Many cytokines that are released from MSCs and negatively affect cancer cells have been identified. Some of these cytokines are IL-6, IL-8, SDF-1, CCL-5, TGF β 1, EGF, FGF, VEGF and CXCL7[3].

1.1. MESENCHYMAL STROMAL CELL

Stromal cells are a type of cell that is found in both embryonic and adult stages with high differentiation capacity into a different cell that can remain silent for many years in adulthood but become active in cases of injury or repair. There are two common types of stromal cells in mammals. The first of these are embryonic cells that form the inner cell mass in the blastocyst stage of the embryo. These cells are called "pluripotent cells" and they have the potential to form cells and tissues located in every layer (mesodermal, endodermal and ectodermal) of a developed organism. Another type of stromal cell with a lower transformation potential are multipotent stromal cells found in every tissue of the organism. Multipotent stromal cells play a role in the repair and renewal of the tissue in which they are located. Mesenchymal stromal cells [4] are adult stromal cell types and they are undifferentiated stromal cells that can generally be found in any tissue. It can be isolated from many tissues, especially bone marrow and adipose tissue. When we look at stromal cell isolation sources, adipose tissue is one of the sources from which easy and excess cells can be isolated [5].

Although each new information starts to create differences in the definition and properties of stromal cells, a few basic criteria accepted today help us to identify stromal cells. Stromal cell characteristics can be listed as follows:

- Self-renewal,
- Differentiation ability (potency) to different cells,
- Clone creation ability (clonality).

Self-renewal: Stromal cells divide in order to maintain the continuity of the tissues and their division results in the formation of new stromal cells. This event is called stromal cell self-renewal. In these divisions, the stromal cell undergoes asymmetric and symmetrical divisions. While one of the two resulting cells has the same characteristics as the mother's stromal cell, the other differentiates by continuing to divide. The stromal cell remaining

undifferentiated ensures the continuity of the stromal cells in the tissue. A disorder in the self-renewal mechanism can cause developmental retardation and cancer in the organism. The dissolution of the self-renewal mechanism in stromal cells may explain the causes of development, cancer and aging events [6].

Pluripotency (multiple competence) or differentiation richness is used to define the capacity of the stromal cell to differentiate into a specialized cell. The cell to be called a stromal cell should be able to display this feature like the other two. This feature is the most important feature that distinguishes stromal cells from other cells. Stromal cells form a hierarchy according to their potency characteristics. While some of the stromal cells can differentiate into just one cell, some can form the whole organism [7].

Clone-forming feature: Another feature of stromal cells. The word clone can be thought of as a union of similar cells. A stromal cell divides and creates its own clone. Cells differ in shape and physiological form with the clones they form [8].

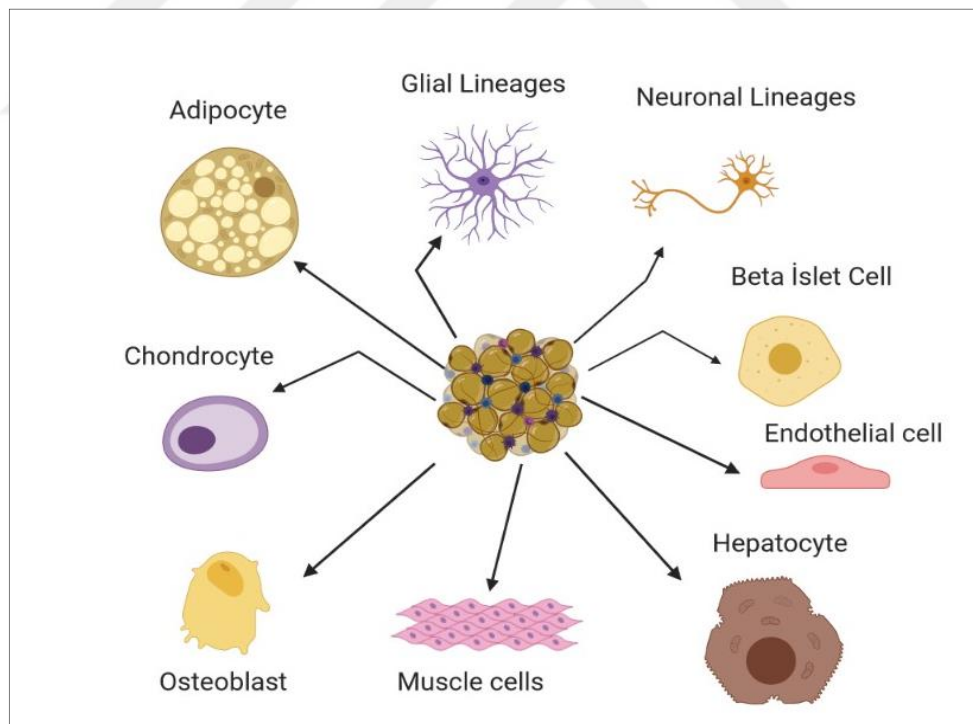


Figure 1.1. The capacity of msc's to transform into other cells.

It has been reported with many articles on differentiation capacities of MSCs and has been used in clinical applications. MSCs are of mesodermal origin and It is widely shown that

MSCs can differentiate into adipogenic, osteogenic, chondrogenic, myogenic, angiogenic, cardiomyogenic, tenogenic and periodontogenic lines due to their mesodermal origin. Although MSCs are mesodermal, they have been shown to differ in ectodermal and endodermal cells[9][10]. In vitro MSC differentiation is induced by the selective medium containing origin specific induction factors.

1.1.1. Adipose Tissue-Derived Stromal Cell

Stromal cells that can renew themselves, are long-lasting and show multiple differentiation. The multiple differentiation characteristic depends on the level of differentiation at which the stromal cell is located. Embryonic stromal cells have the highest differentiation capacity, but the experimental and clinical use of embryonic stromal cells is limited for ethical reasons. Therefore, the multipotent bone marrow derived mesenchymal stromal cell, which was obtained for the first time from bone marrow, has provided an alternative stromal cell and resource to scientific studies [11].

The fact that MSC isolation from bone marrow is very painful and costly for the patient has directed researchers to different sources. In addition, not many MSCs are produced from bone marrow aspirate. Like bone marrow, adipose tissue originates from mesenchymal. Their origination from a common source has accelerated MSC research in adipose tissue. Lipoaspirate is generally used as a source of adipose tissue. In terms of obtaining lipoaspirate, it is more painless and inexpensive than bone marrow aspirate. As a result of primary cell culture, a mesenchymal stromal cell that can be differentiated from lipoaspirates to fat, bone and cartilage tissue could be isolated. It has been shown that adipose tissue contains more stromal cells than bone marrow [12].

Stromal cells have been isolated from adult tissues (such as bone marrow, blood, synovial fluid, umbilical cord, tooth, skeletal muscle, cord blood and skin). All of these stromal cells are multipotent stromal cells. In general, these multipotent stromal cells are located around the vessels. When all MSC sources are compared, it has been shown that most stromal cells can be isolated from adipose tissue [13]. For the first time in 2001, Zuk et al. made stromal cell isolation from adipose tissue.

When we look at the isolation steps, firstly, the surgeon collects adipose tissue in a tube as aspirate under sterile conditions. Washing, chemical digestion or mechanical digestion, centrifugation, washing and purification from blood cells are followed in isolation. The fat tissue aspirate collected in the tube is incubated with collagenase enzyme for 1 hour. As a result of chemical breakdown, the layers of fat and stromal cells in the tube are separated from each other by centrifugation. After the stromal cells collected at the bottom are washed, the blood cell is treated with lysis buffer and the blood cells are separated from the stroma cells. Cells are passed through 100-150 μm filters before being planted in flasks. When the isolated stromal cells reach a certain density, they are differentiated into bone, cartilage and adipose tissue [14].

Adipose tissue is one of the most concentrated tissues in the human body. Adipose tissue amount for about 20 percent of men's body weight and 25 percent of women and is widely distributed across various body areas. We can classify the fat tissue in two parts basically; white adipose tissue (WAT) and Brown adipose tissue (BAT). These two tissue types show different functions, morphologies and distributions. The cell group, which is dominant in both white adipose tissue and brown adipose tissue, that is adipocyte and its other parts form different cell communities called stromal vascular. The mesenchymal stromal cell source of white adipose tissue is higher than brown adipose tissue. In addition, compared to 1 g of bone marrow and 1 g of adipose tissue, mesenchymal cell efficiency is 500 fold higher in adipose tissue. ADMSCs can be obtained from different sources and with different techniques [15].

Therefore, researchers have turned to adipose tissue derived mesenchymal stromal cells. The biggest reason for this is that they are both easily available and obtain high efficiency compared to other MSC sources.

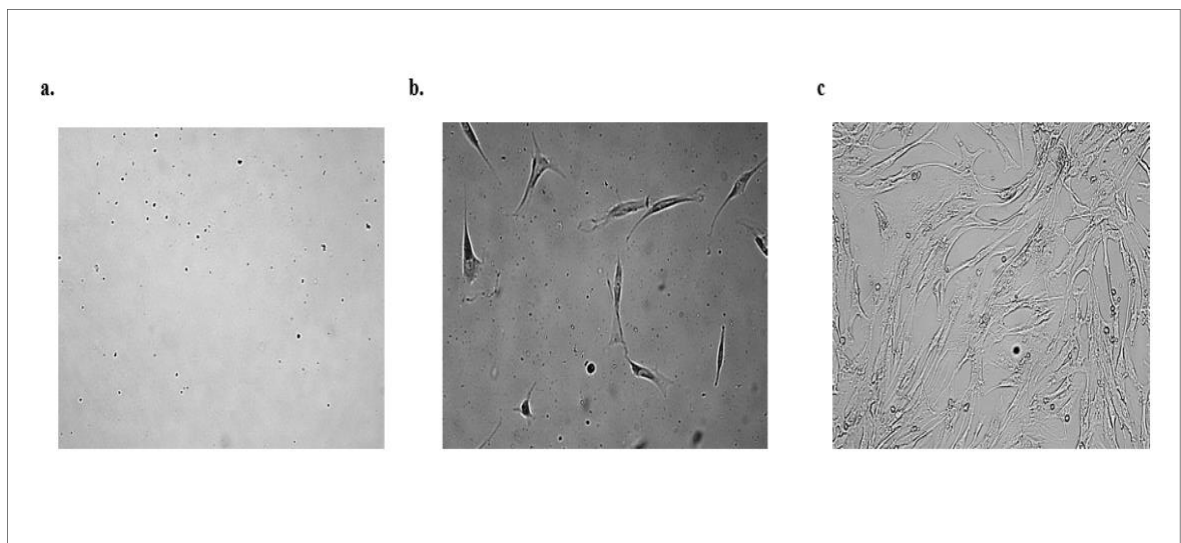


Figure 1.2 (a) Images of MSCs at 0 hours, (b) Images of MSCs after 48 hours, (c) Images of MSCs at the 3rd passage.

1.1.2. Clinical Usage of Mesenchymal Stromal Cells

In the last decade, the number of studies on cellular therapy methods in in-vitro and in-vivo preclinical studies has increased considerably. In many disease models, both embryonic stromal cells and adult stromal cells have been used as a means of cellular therapy. The more stable and limited differentiation feature of mesenchymal stromal cells compared to embryonic stromal cells has increased the use of this cell [16].

MSC was first found in 1968 by Friedenstein due to the adhesion of stromal cells obtained from bone marrow to plastic. As a result of increasing studies, it has become easier to isolate and phenotyping MSCs. The first clinical study was conducted in 1995 and has increased gradually since then. As of September 2020, there are 1119 studies on MSCs in various phase steps (Phase I, Phase II and Phase III) entered at <http://clinicaltrials.gov>.

Clinical experience with MSCs has been the most and perhaps the most successful in the treatment of graft versus host disease in the literature. This disease is a side effect of bone marrow transplantation. Other areas where MSCs are widely used; meniscus damage, cartilage and bone defects, osteoporosis, spinal cord injuries, motor neuron disease, Hurler syndrome, metachromatic leukodystrophy, cirrhosis, multiple sclerosis, Chron's disease.

Due to the immunosuppressive properties of MSCs, they are used in tissue transplantations. MSCs have been tested on humans in the treatment of many diseases and positive results have been obtained [17].

1.1.3. Paracrine Effect of Mesenchymal Stromal Cells

Paracrine signal transduction is when the signal molecule secreted by a cell acts on cells in its micro-environment. Many growth factors that regulate development, especially in multicellular organisms, are effective in short distances, so paracrine is transported by signal transmission. These signals create an intensity gradient by spreading far from the cell where the signal is generated and stimulate different cellular responses depending on the distance of the target cell. Mesenchymal stromal cells also perform many jobs thanks to the active molecules they secrete [18].

It suggests that MSCs can change tissue by secreting factors that may be important for tissue repair. For example, when human MSCs were given to immunocompromised mice with acute myocardial infarction, although no donor cells were found for 3 weeks after injection, improvements were observed in the heart functions of mice [19]. In addition, significant improvements have been found in growth rate, bone mineral density and movement of children with osteogenesis problems and receiving MSC treatment [20]. It has recently been noticed that the damaged tissue is repaired by MSCs participating in the circulation. These MSCs secrete growth factors that affect tissue-specific stromal cells in order to heal the damaged area [21]. Besides all these restorative functions of MSCs, there are some disadvantages for the organism. MSCs can provide vasculature to tumor tissue by producing growth factors that will affect tumor cell, endothelial cells and smooth muscle cells by secreting angiogenic growth factors such as VEGF, PDGF, bFGF, SDF-1.

Many soluble factors are released from MSC cells, namely CP-3, CXCL8, CXCL9, CXCL16, CCL20, CCL25, IL-6, and IL-12[22]. MSCs can cause the proliferation of cancer cells as well as take part in stopping proliferation. In a study conducted in 2012, MSCs were separated for two sub-phenotypes, which were made by TLR4 and TLR3 priming[23]. In their study, they observed that IL-6, IL-8, collagen 1, collagen 2 and T cell activation increased in MSC1 after TLR4 priming. In addition, they obtained the opposite result at the end of TLR3 priming. In another study of the same group on the same subject, they formed

three groups, MSCs, MSC1 and MSC2, and these groups were co-cultured and examined their efficacy on cancer cells. In the results obtained, while MSC1 prevented the proliferation of cancer cells, MSC2 and MSCs increased the proliferation of cancer cells, and when they measured cytokines and chemokines in the environment, they observed that CXCL9 increased in MSC1, which was not seen in MSC2. [24]

1.2. CANCER

The uncontrolled proliferation of cells as a result of damage to the mechanisms that control the growth and proliferation of cells is called cancer. Every year, 100-350 out of 100,000 people in the world die because of cancer [25].

Throughout the cell's development, complex genetic control systems provide the balance between cell formation and death. Some cell types in the organism follow the pathway of division and proliferation as a tissue regeneration strategy. These cells die and disappear after a certain period of time. Disruption of control mechanisms in cells that divide and proliferate frequently causes tumor formation [26].

Disruption of genetic control mechanisms is usually caused by factors such as chemicals, hormones, viral and ionizing radiation. If the damage to the DNA caused by such factors is not corrected by the repair mechanisms, the cells undergo neoplastic transformation. Cancerization is associated with mutations in two broad gene groups. These are proto-oncogenes and tumor suppressor genes. Proto-oncogenes are genes that normally promote cell growth and mutations occurring in these genes cause the gene to transform into an oncogene and the gene product to be expressed excessively. Tumor suppressor genes that restrict growth. Mutations that occur in these genes remove the gene's suppressor feature and pave the way for extraordinary cell division. For example; half of human tumor cells have mutated the p53 tumor suppressor gene, which encodes a protein that plays a key role for transcriptional regulation. The G1 phase is an important control point in the control of the cell cycle at this stage, cells with damaged DNA cannot pass into S phase. The p53 protein ensures that cells with damaged DNA remain in the G1 phase. Another group of genes related to cancer are protective genes [25].

In the genetic basis of cancer, there are mutations that occur in the genes we mentioned earlier. For example, conferring mutations that transform proto-oncogenes into oncogenes. Four different mechanisms are known for the formation of these mutations. The first of these mechanisms is point mutations that cause the formation of an active or hyperactive protein product, and the other is chromosomal substitutions. Another is the displacements that cause a growth regulator gene to enter under the promoter of a different gene. This mutation causes the gene to be overexpressed. The last mechanism is to duplicate these proto-oncogenes.

Some viruses contain oncogenes in their genome that can cause cancer by allowing the living thing infected by these oncogenes to enter my genome. Viruses can also activate existing proto-oncogenes[25].

Senescence and apoptosis mechanisms that prevent tumor formation prevent the deterioration of balance by eliminating hundreds of damaged cells that occur every day in the organism. In this study, it will be observed how secretions released from mesenchymal stromal cells affect cancer cells in terms of apoptosis and proliferation.

1.2.1. Glioblastoma and Cell Lines

Glioblastoma (GBM) is the most common malignant primary Glioblastoma brain tumor in all world statistics. The incidence of glial tumors varies according to the country in which the analysis is performed, the medical facilities and health policies of that country. According to American statistics, gliomas constitute 30 percent of primary brain tumors and 80 percent of them are malignant and 54 percent are diagnosed as GBM [27]. In European statistics, glial tumors constitute 34 percent of primary brain tumors and 95 percent are malignant. Gliomas are among the rare tumors of the adult age group. Central nervous system cancer statistics in Turkey constitute 2 percent of all cancers [28] and this is also compatible with world statistics and in Turkey 79.9 percent of patients entering the statistic was confirmed histologically, 11.3 percent were diagnosed only by radiological, while 2.4 percent were diagnosed with central nervous system cancer, these statics were obtained from death notices [29]. The incidence of glial tumors also varies according to age groups, and the incidence of GBM increases with age, and the median age at diagnosis is 64. The incidence of GBM is minimally higher in men. According to statistics from the Ministry of Health of the Republic of Turkey in 2018 in the central nervous system cancer incidence of

5.3 per hundred thousand men and in women group at rate in Turkey is 4.1 [28]. Its incidence is higher in developed countries and societies with high socioeconomic status [29]. The highest incidence is observed in Europe, and the incidence of white race is higher than other races [30]. The incidence of GBM in American statistics has not shown a significant difference over the years [31]. Malignant gliomas are also among the most malignant tumors seen in the body. According to world statistics, the survival rates of 1, 3 and 5 years are 35 percent, 8 percent and 4.7 percent, respectively. Five-year survival also varies depending on age, for example, five-year survival in 20-44, 45-54, 55-64 age groups is 19 percent, 8 percent and 5 percent.

Our knowledge about why GBMs occur is still limited today. Current knowledge shows that three different mechanisms may play a role in the formation of these tumors. These are: environmental factors, history of allergies or atopy, and familial inheritance.

To explore the effect of quercetin on glioblastoma cells we have used A172 (glioblastoma cell line) and U87 (Epithelial cell line) cell lines.

A172 (ATCC-American Tissue and Cell Culture)

According to data from the ATCC site, the A172 cell line is glioblastoma cells belonging to a 53 year old male. A172 cell line consists of monomorphic fibroblast-like cells with processes. In previous studies, population doubling time in the logarithmic growth phase was 33–48 h for A172.

A172 cancer cells can be grown in 10% FBS + high glucose medium + 1% Penicillin / Streptomycin nutrient medium with 5% carbon dioxide, 37°C and 95% humidity. Its medium can be changed every 3-4 days. When it reaches 70-80% density, passaging is done. In the passaging process, the old medium of the cells cultivated in the flask or petri is drawn off, washed with DPBS and PBS/EDTA solution, trypsin/EDTA enzyme is applied on the cells and the cells are removed. Removed cells are counted and planted in new flasks.

For STR Profiling, DNA Profile is as follows: Amelogenin: X,Y CSF1PO: 9,12 D13S317: 11 D16S539: 12 D5S818: 11,12 D7S820: 11 TH01: 6,9.3 TPOX: 8,11 vWA: 20

U87 (ATCC-American Tissue and Cell Culture)

U87 is one of the cancer cells used in many studies and has many variations moreover their genetic profiles are different from each other. (U87MG-ATCC, U87MG-Uppsala). The U87 cell line has epithelial morphology (likely glioblastoma) and was obtained from a male in 1966.

U87 cancer cells can be grown in 10% FBS + Eagle's minimal essential medium (High glucose) + 1% Penicillin / Streptomycin nutrient medium with 5% carbon dioxide, 37°C and 95% humidity. Its medium can be changed every 3-4 days. When it reaches 70-80% density, passaging is done. In the passaging process, the old medium of the cells cultivated in the flask or petri is drawn off, washed with DPBS and PBS / EDTA solution, trypsin / EDTA enzyme is applied on the cells and the cells are removed. Removed cells should be counted and planted in new flasks.

For STR Profiling of U87MG-ATCC, DNA Profile is as follows: Amelogenin: X,Y
CSF1PO: 10,11 D13S317: 8/11 D16S539: 12 D5S818: 11,12 D7S820: 8/9 TH01: 9.3
TPOX: 8 UWA: 15-17

For STR Profiling of U87MG-Uppsala, DNA Profile is as follows: Amelogenin: X,Y
CSF1PO: 10,11 D13S317: 8/11 D16S539: 9/13 D5S818: 11,12 D7S820: 10/13 TH01: 8/9
TPOX: 8/11 UWA: 15-19

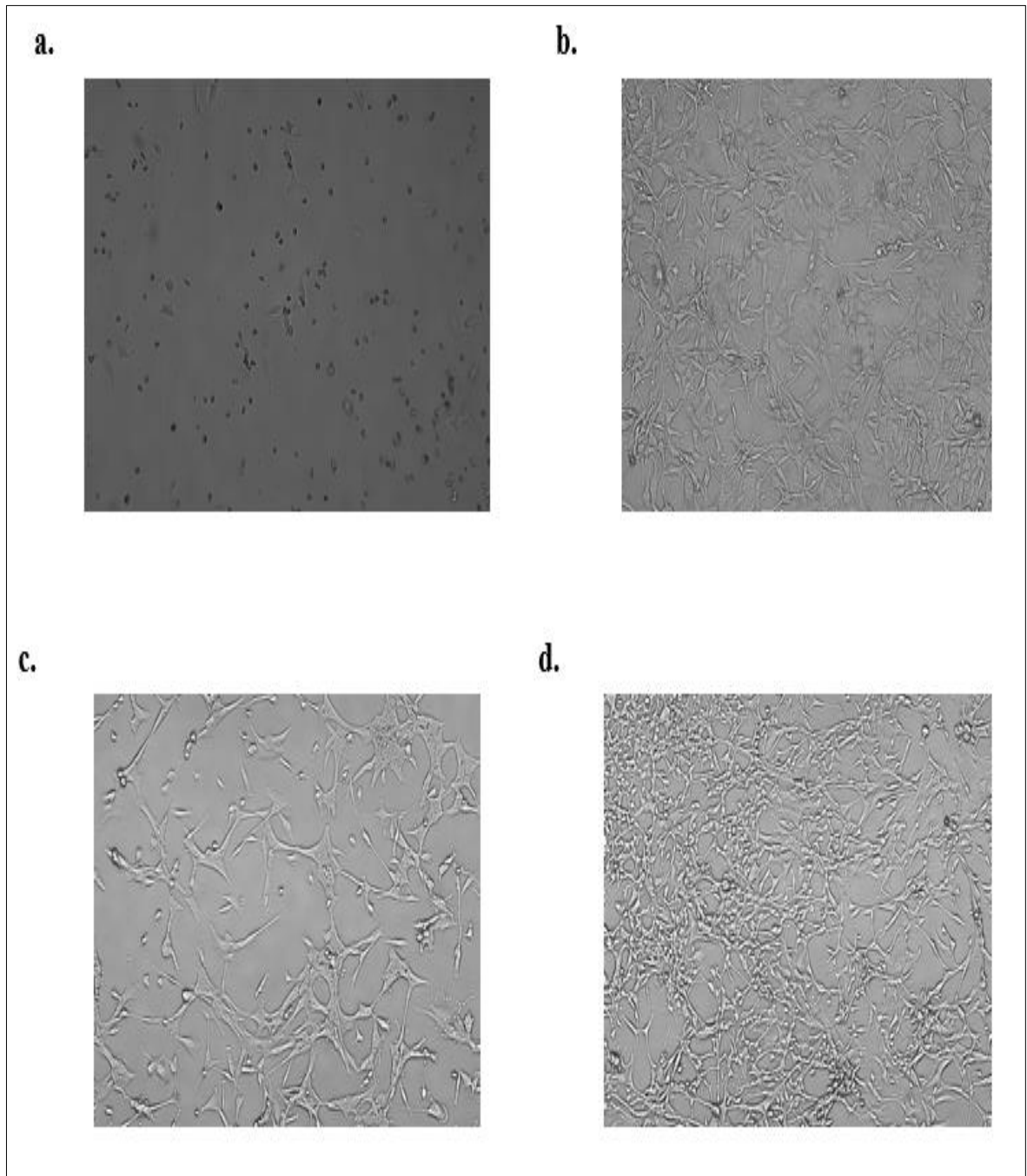


Figure 1.3. Images of A172 and U87 cell lines used in the experiment before and after proliferation. (a) A172 cell line before proliferation, (b) A172 cell line after proliferation, (c) U87 cell line before proliferation, (d) U87 after proliferation. Images were taken with JuLi Br Imaging Instrument.

1.3. FLAVONOIDS

Flavonoids are low molecular weight natural compounds found in plants with positive effects on human health [32]. They are also known as bioflavonoids because they are natural biological compounds. Flavonoids have a long and broad history. Flavonoids first attracted attention in early 1936, in terms of protecting people's heart health and improving their overall well-being. In these years, the metabolism of flavonoids found in the first citrus foods was investigated and then flavonoids in other plant foods were included in the study. Therefore, flavonoids are plant polyphenols and are present in significant amounts in the diet [33].

Flavonoid compounds are polyphenols containing broad heterogeneous groups of benzo- γ -pyron derivatives [34]. The flavonoid skeleton consists of two phenolic (A, B) rings linked by a 3-carbon chain in a heterocyclic (C) state. Sometimes these three-carbon chains can be open.

Currently, there are few studies on the bioavailability and mechanism of flavonoids. Therefore, the effects of flavonoids on human health are not clear due to the ignorance of metabolic transformations in humans. In the studies conducted, it was concluded that there are significant differences between the bioactivities of flavonoids in vitro and in vivo [34]. Therefore, it is very important to have clear absorption, bioavailability and metabolism of flavonoids [35]. In bioavailability studies, the efficiency of absorption and the metabolic use of nutrients are among the criteria to be considered [35].

The amount of consumption of flavonoids has not yet been clarified [34]. The reasons for this are light, plant genetics, environmental conditions, germination, degree of maturity, processing and storage, genus-variety, such as the presence of a number of factors that affect the formation of flavonoid in plants [36]. Furthermore, the lack of reference methods for the analysis of flavonoid compounds, the lack of standardization of analytical methods [34] and the lack of data on the amount of flavonoid in vegetable foods make it difficult. Absorption is also influenced by the dose of flavonoids, intake, nutrition, gender differences, genetic characteristics of individuals, and microbial population in the colon [35]. On the other hand, it is clear that the absorption of food-borne flavonoids is influenced by the matrix in which they are consumed together. The reason for the different absorption of different food groups is related to the difference of saccharides on the flavan core as well as other functional

groups.

Flavonoids are very potent antitumor agents and have the ability to induce apoptosis and modulate cell differentiation and cell cycle due to their antioxidant and antiproliferative functions [34]. These compounds with thermo-pathological effects are called chemical inhibitors [34]. In most studies, biological characteristics of flavonoids were analyzed and found to have antimicrobial antiproliferative and proapoptotic effects of these compounds [34]. It is also said to be antiviral, antiinflammatory. It inhibits many of the enzymes present in the oxidation system (5-lipoxygenase, cyclooxygenase, monooxygenase, xanthine oxidase) [35] and inhibits the growth of atherosclerotic plaques by inhibiting lipid peroxidation. They also protect against atherosclerosis because of their antithrombotic effect. Flavonoids capture reactive oxygen species by the following mechanism.



In a study on the protective effect of quercetin which is one of the flavonols on the oxidative stress induced by homocysteine, quercetin has been shown to have antioxidant properties that reduce toxic effects and partially oxidative damage of reactive oxygen species (ROS).

Epidemiological studies have shown the relationship between high consumption of flavonoid-containing fruits and vegetables and lower cancer risk. In a study conducted by food frequency survey in postmenopausal women who were fed with flavonoid-rich diet and smoked-nonsmokers, it was shown that the risk of developing lung cancer decreased with consumption of flavonoid-based foods [35]. In a study describing the relationship between proanthocyanidins and other flavonoids with pancreatic cancer, it was found that proanthocyanidin reduced the risk of developing pancreatic cancer by 25 percent in individuals consuming proanthocyanidine-rich fruits every day [37].

In addition, a study examining the relationship between consumption of flavonoid-rich foods and cancer risk in middle-aged women [38] showed that the effect of flavonoids on reducing the risk of developing cancer was not significant. Many epidemiological studies have suggested that flavonoids, an antioxidant, reduce the risk of developing cardiovascular disease and cancer. On the basis of the reports, various anticancer effects of flavonoids on cancer cells were found in vitro. For example, by inhibiting cell proliferation and kinase activity and inducing apoptosis. These polyphenols have been observed to inhibit proliferation in human breast cancer cells in culture medium. According to these

observations flavonoids stimulate apoptosis and inhibit cell cycle. However, some mechanisms of flavonoids, a potential anticancer, are still unclear [39]. In a study of the effect of genistein, an isoflavone, on breast cancer cells, the potential inhibitory effect on the expression of IGF-1R (Insulin-like Growth Factor Receptor 1) and the roles of specific signaling pathways in this effect were investigated. Genistein has been shown to inhibit IGF-1R expression in breast cancer cells. In a study, the cytotoxic effects of flavonoids against cancer cells were observed in the mouse venograft model. Cytotoxicity of flavonoids has been found by different mechanisms by applying flavonoid-based cytotoxic treatment to cancer cells [33].

This cytotoxic activity of flavonoids depends on the hydroxyl number and the position of the hydroxyl group. The presence of ortho hydroxylation and 4-COO- group in ring B, the presence of double bonds between C2-C3 increases cytotoxicity. In addition, orthomethylation and glucuronidation increase the cytotoxicity of flavonoid [33].

In our study, we have chosen to study effect of quercetin, a flavonol with known anti-oxidant effects, on tumor cell lines.

1.3.1 The Relationship Between Cancer and Quercetin

Quercetin (3,3',4',5,7-pentahydroxy-flavone) is a chemical pigment commonly found in the shell of many plants. This substance, which is an important flavanoid in nutrition, is found in abundance in apple peel, onion, tea and red wine. Studies in cell cultures have shown that quercetin works against certain types of cancer cells[39].

Quercetin is a type of flavonoid antioxidant found in plant foods such as greens, tomatoes, strawberries, and broccoli. Technically thought of as a "plant pigment", it is found in fully colored, nutritious fruits and vegetables.

Considered as one of the richest antioxidants in nutrition, quercetin plays an important role in combating the effects of free radical damage, aging, and inflammation. You can get plenty of quercetin from a healthy diet, but some people also take quercetin supplements for their powerful anti-inflammatory effects. Interestingly, quercetin inhibits the proliferation of a wide range of cancers such as lung, breast, liver, colon, and prostate, by inducing cell cycle arrest or promoting cell apoptosis. Recent studies also show that quercetin has various

anticancer mechanisms, which can inhibit DNA topoisomerases I and II and modulate the PI3K / AKT and NF- κ B signaling pathways[39].

In studies conducted, human subjects can absorb significant amounts of quercetin from food or supplements and elimination is rather slow and the reported half-life ranges from 11 to 28 hours. Quercetin has an average terminal half-life of 3.5 hours[40].

In study at 2010, the effect of Quercetin on mesothelioma (SPC111 and SPC112 cell lines) after combination with cisplatin was examined. it was also mentioned that it blocks colon, gastritis cancer and human leukemic T cells in the G1 / S phase, as well as breast cancer and human acute leukemia cancer in the G2 / M phase. In the study carried out, Quercetin inhibited the proliferation of these two cells, whether combined or independently, in SPC111 and SPC112 cell lines, then DNA content analysis was performed and the effect of Quercetin caused accumulation in the S phase in the SPC112 cell line, but no effect was observed in SPC111[41].

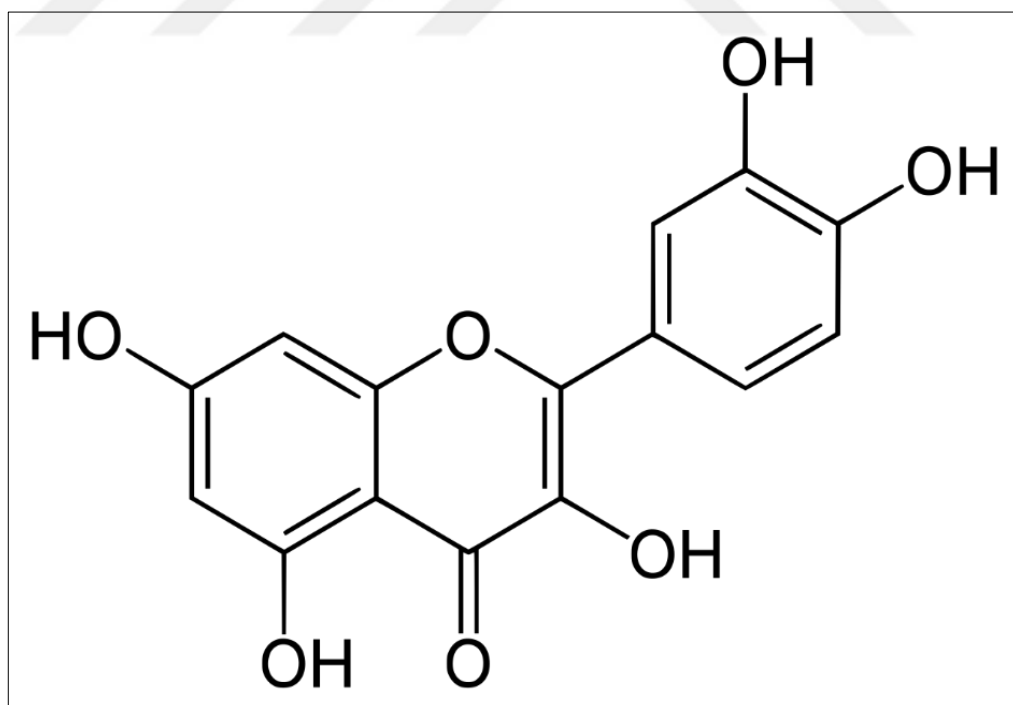


Figure 1.4 $C_{15}H_{10}O_7$ molecular formula of quercetin

1.4. AIM OF THE STUDY

Cancer is one of the biggest diseases of today. Doctors and researchers have been seeking and developing new methods for treatment moreover traditional methods such as surgical operations and radiotherapy are both expensive and long-lasting pain.

While it is stated in many articles that mesenchymal stromal cells cause the proliferation and division of cancer cells, some articles mention the opposite effect on cancer cells. Mesenchymal cells migrate to the location of cancer cells and enter into both cytokine and chemokine interaction. In our experiment, the effects of the medium of mesenchymal stromal cells stimulated with Quercetin on cancer cells will be examined.

Our expectations;

1. Bioactive substances released as a result of conditioning MSCs with Quercetin and the medium of cancer cells, may stop or reduce the proliferation of cancer cells.
2. The cancer cells may be destructed by various death pathways through these released bioactive substances.
3. To obtain MSC1 polarization as a result of using conditioning medium.
4. To observe changes in cell cycle of cancer cells.

2. MATERIALS AND METHOD

2.1. MATERIALS

2.1.1. Consumables and Reagent

- Cell cycle kit-DNA Content kit (Beckman Coulter (200 test kit) , C03551)
- Annexin V/PI kit (Beckman Coulter ,IM3546)
- Trypsin-EDTA Gibco 0,25% (Thermo Fisher,25200056)
- HBSS 500 ml 10X (SIGMA life science, H4641)
- Cryogenic Vial (NEST,607001)
- Tissue Culture Dish T75,T25 (TPP,90075-25)
- CFSE Cell proliferation kit (Thermo Fisher, C34554)
- Sterile centrifuge tube 15 ml (Isolab , LB.IS.078.02.003)
- CXCL 9 and CXCL5 Antibodies (Biolegend Inc, CXCL5: 524105- CXCL9: 357903)
- MSC Characterization markers CD45,CD34,CD105 and CD73 (Biolegend Inc - anti-CD34 PC7 : 343516, anti-CD45 Krom Orange: 304002, anti-CD105 PC5: 323216, anti -CD73 FITC : 344016 and anti-CD90 PE : 328110)
- MTS Cell proliferation colorimetric Assay kit (BioVision, K300)
- DPBS (Dulbecco's phosphate-buffered saline) - (Thermo Fisher, 14200166)
- 6-12 well cell culture plate (SPL Life Sciences)
- AISIMO syringe filter (0.22 micron) - (United Kingdom)

2.1.2. Equipment

- Galaxy 170 S incubator (New Brunswick, 272007456)
- Light microscope (Olympus,CKX41)
- Laminar cabin (ESCO, LA2-4A1)
- JuLI Br Imaging System (NanoEnTek, JULI FL)
- Centrifuge (HITACHI, CTGEL)

- Navios EX Flow Cytometry System, CytoFLEX (Beckman Coulter Inc, CYTOFLEX)
- Spectrophotometer (Epoch BioTek)

2.1.3. Cell Lines

- A172 15th passage (A172 ATCC, 30-2002)
- U87 25th passage (U87 ATCC, 30-2003)

2.1.4. Cell Culture Content

Cell culture is the separation of a tissue by mechanical, chemical and / or enzymatic methods, isolation of individual cells, and survival of these cells in vitro [42].

Cells cultured in vitro continue to show their characteristics in vivo. Thus, an answer can be given about how the cells of a living thing react to different conditions, chemicals or drugs, by experiments performed outside of living things [43]. In addition, cultured cells for cell biology research have more advantages over whole organisms, as they are controllable and allow working with a homogeneous population. In addition to its experimental importance, cell culture is also of great importance in terms of the production of cells, which are defined as stem cells and important in the treatment of disease or in the repair of damaged tissues, in high amounts other than living things and give them to the patient.

Cell culture studies originated in the early 20th century, with the development of techniques for performing experiments independent of systemic variation. In 1907, Ross Harrison cultured the tissue pieces he took from the frog embryo and also managed to keep the nerve fibers alive. Harrison probably preferred the frog as it was a cold-blooded creature and did not require incubation. In 1912, Carrel and Burrows cultured the heart muscle cells they isolated so that they could contract and relax for 3 weeks. Again, Carrel cultured fibroblastic cells in 1923. In 1952, Dulbecco started using trypsin to passage the cultured cells and in 1991, Caplan cultured adult mesenchymal stem cells. Thompson cultured human embryonic cells in 1998. In 2007, Shinya Yamanaka succeeded in obtaining induced pluripotent stem cells from a somatic cell by transfecting cells with the transcription factors specified [40].

With these studies, many studies have been conducted that make cell culture studies what they are today[42].

Cell culture cannot be done in environments where other biological experiments are performed. Cell culture should have a sterile environment and preparation area where the procedures are performed. The size of the cell culture area and the number of people working in this area are also important. On the other hand, the storage area of cell culture materials is also important.

The basic equipment of the cell culture laboratory is a sterile cabinet, a carbon dioxide incubator that provides 5% CO₂ and 37 degrees environment required for cells to survive, an inverted microscope to see the cells adhered to the bottom of the culture container, a centrifuge used during cell washing, and a long-term cell storage. Nitrogen tank, + 40 degree cooler, -20 and -80 degree freezer are the most basic cell culture laboratory equipment used for the storage of culture materials.

Cell Culture Chemicals: Medium are the nutrients necessary for the survival of cells. Cells can survive in this medium by suspending, swimming, or the cells attached to the bottom of the culture dish are covered with these medium to feed the cells. The hydrogen ion concentration of the medium is important in the cultivation of cells. Cells generally live at pH: 7.4. Phenol red is used as a pH indicator in mediums. Phenol red changes the color of the medium according to the increase and decrease of the medium pH. Medium color approaches yellow in acidic pH. In basic pH, the medium takes on a dark red, pink color [42]. Other important components in Medium are CO₂, NaHCO₃ and HEPES. Carbon dioxide dissolves in the medium while it is gaseous. As a result of the accumulation of lactic acid in the medium, the pH drops and these components are added to the medium in order to maintain the pH balance of the medium. HEPES is one of the most used buffer components. The oxygen density of the medium is another important criterion. However, the density of oxygen in the medium has different effects on different cell types. Temperature of cell culture medium is an important factor affecting cells. Mammalian cells can usually be stored frozen at -196 degrees. At + 40 degrees, they can maintain their vitality for a few days. The most suitable culture temperature for mammalian cells is 37 degrees. Since the medium used in cell culture are stored in refrigerators, it is important for the viability of the cells in the culture to approach this temperature before placing them on the cells.

Salts: Salts are components that contribute to the osmolarity of the medium. Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , PO_4^{3-} , HCO_3^- are some of the salts in the medium. Since Ca^{2+} from these salts plays an important role in cell-cell interaction, it has an important role in the growth and development of cells.

Vitamins : B group vitamins, folic acid, inositol, biotin, nicotinamide, vitamins A, D, E, K are vitamins in cell culture medium. Since these vitamins have an important role in cell metabolism, their concentration in the medium is of vital importance [42].

Glucose: As it is known, glucose is the main energy source in the cellular sense. It is found in different concentrations in the medium. Medium with different glucose concentrations are used depending on the cell type[42].

2.1.5. Other Substances Included in Medium's Content

Serum : As serums contain growth factors, they support the growth and development of cells. It is added to the medium at different rates depending on the cell type and physiological condition. Serums can generally be obtained from cattle, horses or humans [42].

Amino Acids : Amino acids are substances that play an important role in the survival and growth of cells. Amino acids can be found in basal mediums or can be added later. The most commonly used amino acid is glutamine. Since glutamine is an amino acid with a short half-life, it may be necessary to add it to mediums that have not been used for a long time [42].

Antibiotics: In in vitro cell culture, since the cells are not protected by an immune system away from their natural environment, it is important that the culture is performed under aseptic conditions and the medium is sterile. Antibiotics are added to cell culture medium to protect cells from bacterial and fungal contamination. Frequently used antibiotics; penicillin, streptomycin, gentamicin and amphotericin [42].

Hormones and Growth Factors : Growth factors are another material added to cell culture medium. They are not often used in cell culture, but can be added to the medium to support the growth and development of some cells. b-FGF and EGF are the most commonly used growth factors [42].

Pbs (Phosphate Buffered Salt Solution) : PBS is used in cell culture, often during washing procedures. It is used to remove serum residues on the cells before trypsinization and to dilute trypsin in cell solution after trypsinization. There are two types used in cell culture. The first is PBS that does not contain calcium and magnesium. This PBS is used during wash processes. The other is PBS containing calcium and magnesium. This PBS is used in conjunction with the collagenase enzyme during cell isolation. PBS containing +2 ions such as calcium and magnesium is not used in washing processes. This is because the trypsin enzyme does not work in the presence of calcium and magnesium [42].

Trypsin: It is an enzyme used to separate the cells that attach to the plastic surface from the surface to which they are attached. The time in which the cells come into contact with trypsin is important. If this period is kept too long, the protein structures on the membrane surface are broken down. This affects the cell's attachment to the plastic surface and thus its vitality. Trypsin inhibitor or serum (FBS, etc.), a component containing large amounts of protein, is used to arrest the trypsin enzyme [42].

Cancer is one of the biggest diseases of our day and is also one of the leading diseases that result in death. Researchers have been studying cancer for years and many new discoveries are being introduced. Our goal in our study is to reduce and eliminate the effect of cancer by stimulating our cells and using a completely natural active substance without taking any chemicals from outside.

According to this goal, our hypothesis is; Quercetin provides the release of cytokines that cause suppression of various cancers by creating a stimulating effect on MSCs due to its anticancer effect and affects many signal pathways, and as a result, the effect of the cancer cell decreases or results in death.

2.2. METHOD

Centrifuge (HITACHI, Japan), CO₂ incubator (New Brunswick, Canada), Laminar cabinet (Esco, UK), Automatic pipettes (receiving high volume solutions) (Thermo scientific, UK), Light Microscope (Olympus, Japan), Spectrophotometer device (for cell viability analysis) (Epoch BioTek, UK), Automatic pipette (GIBSON), Cell counter and imager JuLI Br (NanoEnTek, Korea), NAVIOS, DxFLEX, CytoFLEX (were used for Cell analysis

Annexin, DNA content etc.)(Beckman Coulter, USA), Freezer -86 (Esco, UK) were used for our experiment.

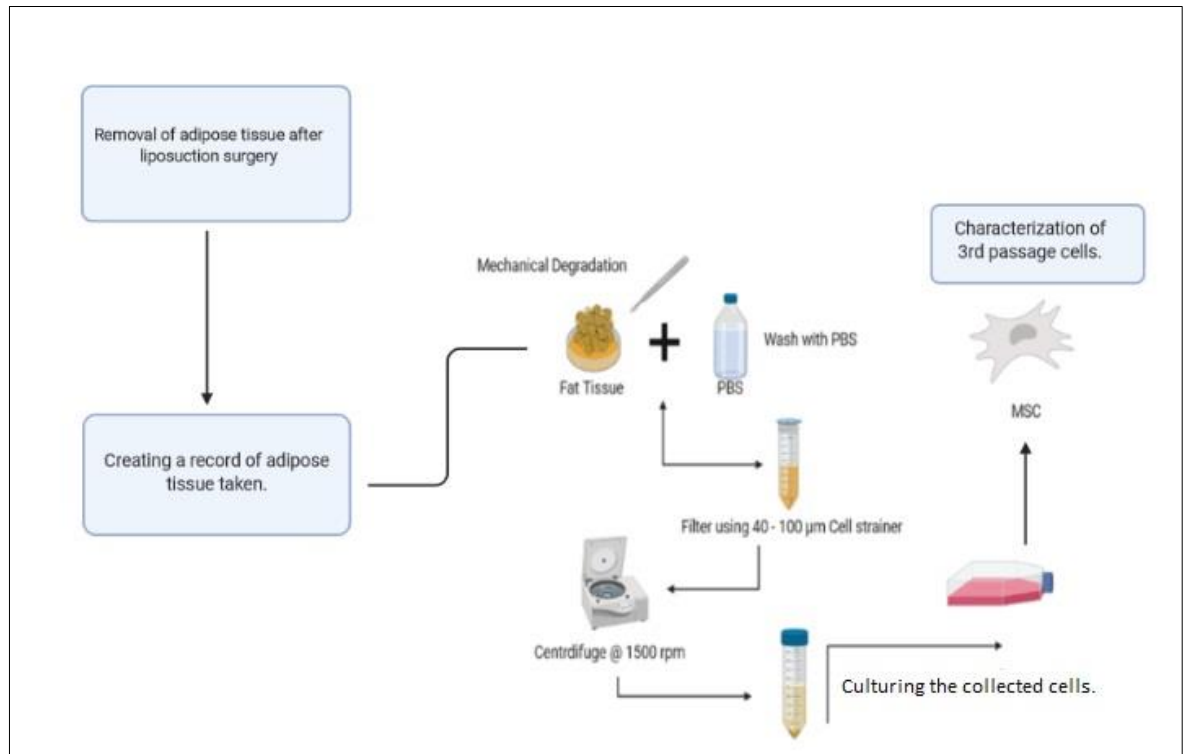


Figure 2.1. Work flow chart for obtaining and characterizing mesenchymal stromal cells.

Adipose tissue samples were collected after liposuction surgery performed by Associate Professor Dr. Uğur Anıl BİNGÖL, with the consent of the volunteers. The collected samples were kept at +4 degrees and brought to Yeditepe University Tissue Typing Laboratory. The adipose tissue sample taken was filtered through a 100 micron filter after mechanical disruption with HBSS or DPBS, and after centrifugation, mononuclear cell population was obtained. The cell population obtained was inoculated into T25 or T75 flasks depending on the density. The cells collected and characterized at the end of the third passage they were conditioned by Quercetin and cancer cell medium, and then Annexin apoptosis test, DNA content analysis and cell proliferation tests were performed on the cancer cells with the collected medium.

2.2.1. Obtaining of Mesenchymal Stromal Cells

Adipose tissue was obtained from 4 volunteers who underwent liposuction surgery at the Department of Plastic and Reconstruction Surgery of the Yeditepe University Hospital with the ethics committee decision taken on March 28, 2019 (decision number: 37068608-6100-15-1646) and the volunteer signatures of the patients. Patient data are presented on Table 2.1. Adipose tissue was kept at 4° and transferred to the Yeditepe University Hospital, HLA Typing Laboratory during the transfer, attention was paid to the confidentiality of the patients' names within the same day, and mechanically dissociated by mincing with a sterile scalpel blade in a 120mm sterile tissue culture dish. Drying was prevented by adding Hank's Balanced Salt Solution (HBSS) on cells during mechanical dissociation. Minced tissue was centrifuged (5 minutes at 300g) once to discard the fat layer and the supernatant. Mononuclear cells were seeded into either T25 or T75 flasks ($1-3 \times 10^6$ cells for T25 and $3-5 \times 10^6$ cells for T75 flasks) according to the initial cell number in mesenchymal stromal cell culture medium consisting of low glucose DMEM (1 g /L glucose) medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and antibiotic solution (100 units/mL of penicillin and 100 µg/mL of streptomycin), and will be named as complete medium hereafter[45].

The number of mononuclear cells obtained varies depending on the adipose tissue taken from the patient and where it is taken. In general, 1-2 million cells are obtained from 2cc adipose tissue. Stromal cells were observed within first 14-21 days of culture. This time varies according to the patient, mainly because of the age, weight, gender, and whether or not she/he has used heavy medication before. Once they reach 80% confluency, cells were detached by 500µL/25cm² Trypsin-EDTA (0,25%) by incubating the flask in the incubator for 1-3 minutes (depending on the passage number as the duration increases with passage number)[45].

MSCs were characterized according to their surface markers (CD34, CD45, CD73, CD90, CD105) by flow cytometry at the end of the 3rd passage[45]. Characterized cells were stored at -80°C freezer for further assays in their medium containing dimethyl sulfoxide (DMSO) at 10% final concentration. Freezing occurred when the cells reached 80% confluence and 90 percent viability. (1×10^6 cell per vial).

Table 2.1. Demographic data of donors

	Gender	Age	Weight	Height	Fasting Blood Glucose	Insulin	Other Factors
D1	Male	39	90	1,85	Normal	Normal	Normal
D2	Male	21	114	1,78	Above normal values	Above normal values	Normal
D3	Female	19	70	1,73	Normal	Normal	Normal
D4	Male	53	80	1,78	Normal	Normal	Cardiological diseases

In total, samples were taken from 14 different patients and the experiment was continued with 4 patients. The reason for this is that freeze-thawed MSCs only adhere to the flask surface and proliferation cannot be achieved after thawing some samples. In addition, some patient samples were contaminated and had to be removed from cell culture. Although more patient samples were planned to be included in the study, there were limited number of surgeries and samples could not be obtained due to the pandemic (COVID-19). To protect patient confidentiality abbreviations were used for MSC donor (D1-D4).

2.2.2. Culture of Mesenchymal Stem Cells

Cell culture was done with the medium specified in section 2.2.1. Low-glucose containing media was used because the high-glucose containing media disrupts the morphology of the MSCs as the number of passages increases[46]. Frozen cells were thawed by slowly adding the complete medium in their own vial, and then transferred into 15 mL centrifuge tubes, followed by centrifugation at 300g for 5 minutes. Supernatant was discarded and pellet was suspended in fresh complete medium. Cells were seeded in T25 flasks, and incubated in the tissue culture incubator at 37°C containing 5% CO₂ under humid environment. Cells were detached once they reach 80 percent confluency by Tyripsin-EDTA (0.25%) solution, and seeded to test plates for further analyses[45].

2.2.3. Determination of the Quercetin Dose

MSCs at the 3rd passages were seeded into 96-well tissue culture plates as 5000 cell per well in triplicates, and incubated 24 hours to allow attachment. Quercetin stock solution was prepared by dissolving Quercetin in DMSO at a final concentration of 200 mM. working solutions at 200, 100, 50, 25 and 5 μ M were prepared by diluting the stock solution in complete culture medium. Untreated and vehicle (DMSO) treated cells were used as control whereas plain culture medium was used as blank. Viability was measured by adding MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent to each well at 10% concentration, and incubating plates in the incubator for an hour. Absorbance was read with the spectrophotometer (Epoch, BioTek, USA) at 490 nm wavelength at the end of the incubation time[47].

2.2.4. Preparation of Conditioned Medium

A172 and U87 cell lines were cultured in DMEM high glucose medium (4 g /L glucose) medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and antibiotic solution (100 units/mL of penicillin and 100 μ g/mL of streptomycin). For conditioning MSCs, cancer cells' media were collected and immediately used for conditioning experiments. Experimental groups are presented on Table 2.2

Table 2.2. Experimental groups

Mesenchymal Stromal Cells	Cancer Cells
1. Control group without medication	1.Cancer cells given conditioned medium of MSC + Quercetin
2.Treated with Quercetin	2. Conditioned but drug free medium (MSC medium)
3. Only cancer cell medium has been given	3. Cells not treated with drug or conditioned medium.

5×10^4 MSCs were seeded into 6 well plates, and incubated overnight for attachment. Next day, medium was removed and fresh medium mixed with cancer cell lines' media (ratio 1:1) containing quercetin was added to each well. For the control group, either media do not containing quercetin or fresh culture media was added to wells. After 24 hours, the media were discarded, cells were washed twice with DPBS to discard residual Quercetin, and fresh culture medium were added on cells. Because the half-life of quercetin was 11 to 28 hours and the cell viability test did not differ much between 24 and 48 hours, the experiments were run over 24 hours. After an additional 24 hours, media were collected, mixed with fresh high glucose complete media at 1:1 ratio and added on cancer cells.

2.2.5. Measurement of CXCL9 And CXCL 5 Expression After Quercetin Treatment

CXCL5 and CXCL9 protein levels were evaluated by flow cytometry after incubating cells with quercetin for 24 hours. Cells detached were washed twice with DPBS, and fixed with paraformaldehyde solution (1% in PBS, pH:7.4) by incubating cells at 4°C for 30 minutes (1×10^6 - 5×10^6 cell). After discarding the fixation solution, cells were permeabilized by incubating with permeabilization solution (0.25 Triton X-100, 0.1 saponin solution and 1% bovine serum albumin (BSA) in PBS) for an hour at room temperature. Permeabilized cells were suspended in PBS containing 1% BSA, stained with APC conjugated anti-CXCL5 and PE conjugated anti-CXCL9 antibodies by incubating at room temperature for 30 minutes. CXCL 5 and CXCL 9 antibodies were titrated for use of accurate amount of antibodies. 3µl for CXCL5 and 5µl for CXCL9 were determined amounts after titration. Immunophenotyping was performed with CytoFLEX flow cytometry system. Data were evaluated with CytExpert Software (Version 2.0).

2.2.6. Analysis of Proliferation by CFSE Staining

In the study, 5 different doses of Quercetin were tried and cell viability tests were performed, and the most appropriate dose was found to be 100 µM. The proliferation of MSCs increased at 5, 25 and 50 µM, but not at 100 and 200 µM.

A172 and U87 cells were seeded into 12-well plates as 5×10^5 cell per well and incubated for 24 hours to allow attachment. CFSE dye was prepared by adding 18 µL DMSO to a

CFSE vial, and further diluted in DPBS at 0.1%. CFSE containing DPBS solution was added to each well and cells were incubated in the culture incubator for 30 minutes. At the end of the incubation, labelling solution was discarded and cells were treated with either medium of MSCs stimulated with the medium of quercetin and cancer cells or MSC medium (stimulated with cancer cell medium). Untreated cells were used as control. Proliferation was measured at end of 24 and 48 hours with Navios Cytometry System (Beckman Coulter, USA).

2.2.7. Annexin V/Propidium Iodide Staining

In total, 3 different MSC groups were used, as shown in table 2.2. 5×10^4 cells were inoculated individually for each group. For cancer cells, 5×10^5 cells were inoculated. They were incubated for 1 day for holding. After the cells are held; The medium in the culture dishes were removed and new medium added. The added new medium was made according to the groups in Table 2.2. Cells were incubated for 24 hours and washed 2 times with DPBS and new medium added and re-incubated for 1 day. Later, 50% of the incubated medium was taken and given to cancer cells as indicated in Table 2. It was incubated for 24 hours and 48 hours. Cells were harvested and centrifuged at 350g. In accordance with the manufacturer's instructions; The 10X binding buffer was diluted in 10 times distilled water, then 1ml binding buffer was transferred into 250 micrograms PI. In the next step, 1 microliter of A5-FITC and 5 microliters of PI solution were added to 100 microliter binding buffer + cell suspension (5×10^6 – 10×10^6 cells/mL) added according to the company's manual) and kept in the dark for 15 minutes. Finally, 400 microliters of 1X binding buffer were added and analyzed for 24hr and 48hr at each groups (Table 2.2. cancer cells)

2.2.8. DNA Content Analysis

It is a method based on staining the DNA of the cell with fluorochromes in living cells. Results of cellular DNA content measurements are usually presented in the form of frequency histograms. Computer analysis helps to differentiate cells at certain stages of the cell cycle based on differences in DNA content (inversion of histograms)[48]. DNA binding dyes such as Propidium Iodide (PI), 7-AAD (7-aminoactinomycin) or DAPI are used to

measure the change in the DNA content of the cell as it passes through G0, G1, S, G2 and M phase. The cell cycle kit offered by the company includes detergent, reagents such as Propidium Iodide (PI) and RNase (DNA Prep, Beckman Coulter, USA). The detergent in solution makes the cell membrane permeable, allowing PI to access DNA. RNase A helps to eliminate RNA that is also tagged by PI and may interfere with interpretation of results.

It was conducted to investigate the phases in which the cells stand and whether the applied conditioned medium has an effect on the cell phases of cancer cells. The same steps were done in the apoptosis test. The procedure continued in line with the manufacturer's instructions (Beckman Coulter, USA). After trypsin was applied, the collected cells were transferred to tubes according to the groups and centrifuged at 300g for 5 minutes. 200 microliters of cell from the cell cycle solution taken from the manufacturer was transferred into the suspension (1×10^6 - 5×10^6 cell) and left for 30 minutes. Then, 300 microliters of salt-based flow cytometry solution was added and analyzed at 24hr and 48hr for each groups.

2.2.9. Analysis of Experiment Results

Kaluza, ModFit and Graphpad prism were used for apoptosis and DNA content experimental analysis.

Kaluza: Software is used to determine the percentage of positive and negative rates depending on various factors, such as the antibody or dye bound, depending on cell scattering and to evaluate the results of related test (Annexin V / PI test, CFSE proliferation test and DNA content analysis).:

Modfit : Software program for DNA Cycle Analysis. It is one of the most used programs in research studies.

Graphpad Prism: It is one of the programs used for various analysis and graphical drawings. It can be specifically used for data analysis. It has the same features as SPSS program. Comparisons between groups within a single timepoint were done with One-factor analysis of variance (**One way ANOVA**) to determine whether there is a difference between 3 or more groups based on a particular variable.

3. RESULTS

Various effects of MSC in the body has been known for years and have been proven by many studies. MSC are known to migrate towards the tumor site, as a result, two different outcomes may be present: first, MSC can help cancer cells to proliferate and divide, or their interaction can counteract to the tumor biology, which, in turn an anti-cancer effect may be observed. Here, we investigated if the soluble mediators MSCs release upon stimulation with Quercetin have an anti-cancer effect on two different glioblastoma cell lines (U87 and A172) as well as if the treatment alter cells' CXCL9 and CXCL5 protein expression levels, which are reported to indicate the MSC type I and MSC type II transformation, respectively.

3.1. MTS ASSAY RESULTS AND DOSE DETERMINATION

We evaluated cell viability with MTS assay, and determined the ideal dose that have a minimal cytotoxic effect on MSCs. We treated MSCs (seeded as 5000 cell per well as triplicates) with varying doses of Quercetin (5, 25, 50, 100 and 200 μ M) for 24 and 48 hours. MTS reagent (BioVision Inc.) was added in each well at 10% concentration and plates were further incubated for an hour. Absorbance was read at 490 nm with a spectrophotometer (Epoch, BioTek).

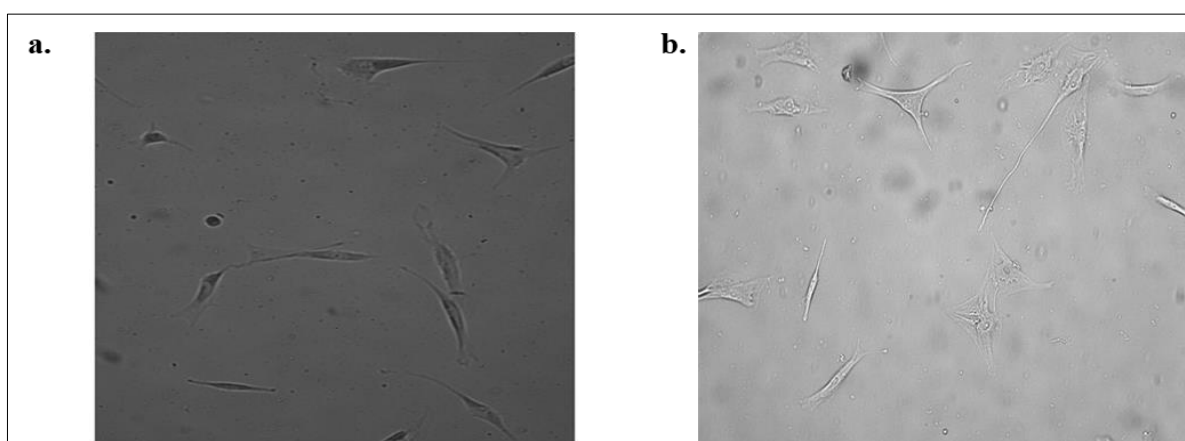


Figure 3.1. Micrographs indicating morphologies (a) before and (b) after treatment with 100 μ M Quercetin. Images were acquired with JuLi Br Imaging Instrument. An overall increase in cell size may be observed.

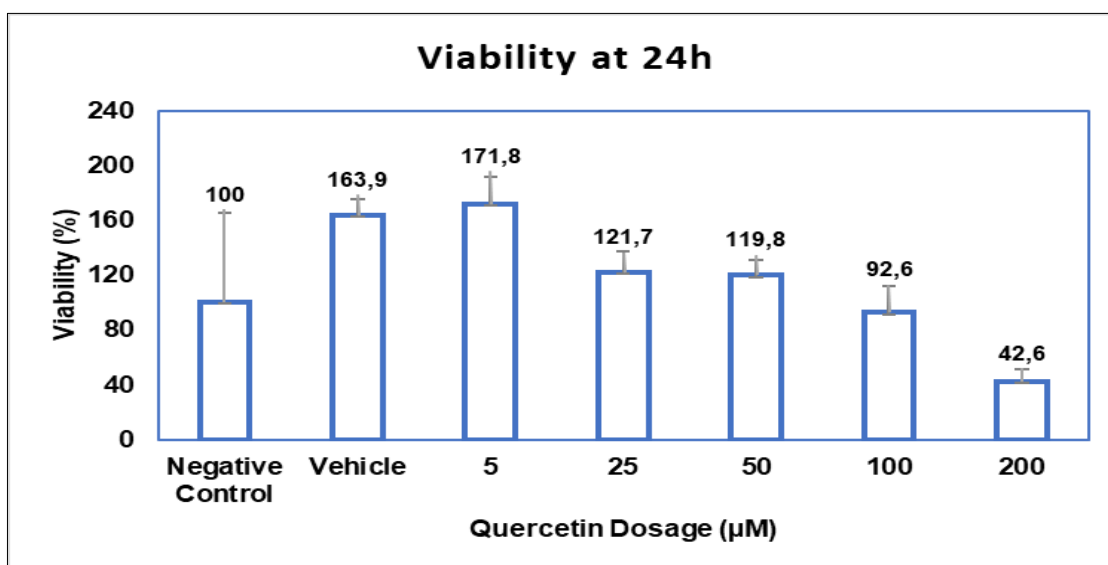


Figure 3.2. Alterations in msc's viability after treatment with various doses of quercetin for 24 hours. Viability was decreased in a dose dependent manner. Untreated cells were used as negative control. 100 µM quercetin was selected for further studies

Table 3.1. Statistics according to quercetin dosage for 6 different patients in 24 hours.

µM	NC	VEHICLE	5	25	50	100	200
AV	0,57	0,388	0,42	0,552	0,620	0,71	0,52
STDV	0,37	0,064	0,11	0,08	0,06	0,11	0,05
%AV	100,0	163,9	171,8	121,7	119,8	92,6	42,6
%STDV	65,38	11,130	19,9	15,30	11,20	19,61	9,11

According to the average percentage statistical results obtained at the end of 24 hours, Quercetin increases the proliferation of MSCs at 5µM, 25µM and 50µM. While it gave similar results to the control group at 100 micromolar, the proliferation rate decreased significantly at 200 µM. 200 µM showed a serious toxic effect.

Table 3.2. . Statistics according to quercetin dosage for 6 different patients in 48 hours.

μM	NC	VEHICLE	5	25	50	100	200
AV	0,58	0,40	0,506	0,603	0,728	0,77	0,64
STDV	0,38	0,12	0,130	0,174	0,20	0,22	0,12
%AV	100,0	169,1	204,0	132,9	140,5	100,5	52,4
%STDV	65,4	20,2	22,51	30,04	34,74	39,67	21,25

At the end of 48 hours, the increase results were obtained similar to the increases shown in Table 3.1. The toxic effect of 200 μM continues at the same rate.

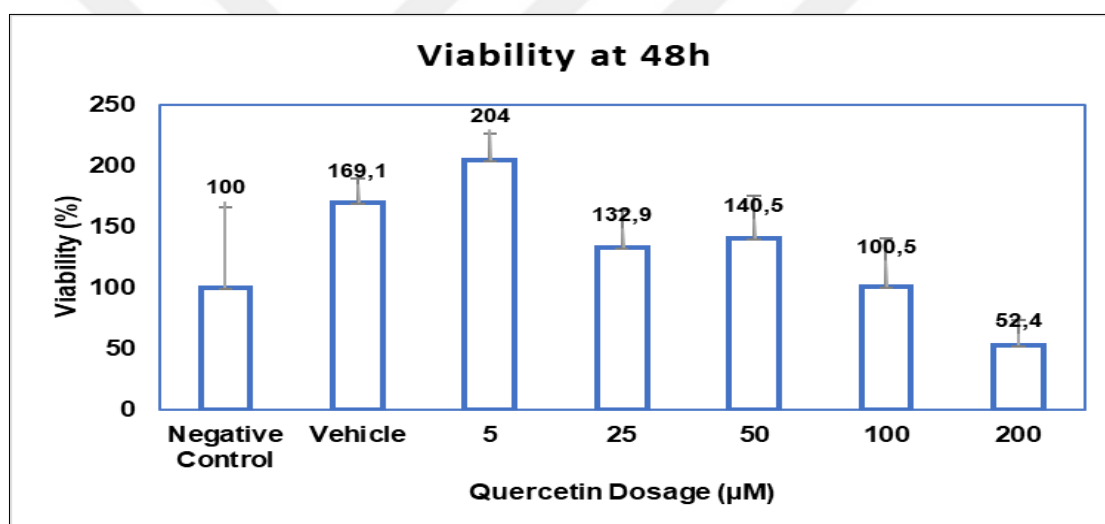


Figure 3.3. Alterations in msc's viability after treatment with various doses of quercetin for 48 hours. Viability was decreased in a dose dependent manner. Untreated cells were used as negative control. 100 μM quercetin was selected for further studies.

Our results revealed that Quercetin at relatively low (5-50 μM) concentrations induced MSC proliferation at both 24 and 48 hours. Conclusions regarding quercetin's half-life are controversial, as some studies suggest that its half-life is 3.5 hours, while other studies suggest its half-life is approximately 22 hours. Here, we chose 100 μM for further applications because this concentration is that quercetin has a minimal but not strong cytotoxic effect on MSCs.

3.2. MEASUREMENT OF CXCL5 AND CXCL9 PROTEIN LEVELS UPON QUERCETIN TREATMENT

Protein expression levels of CXCL5 and CXCL9 were evaluated by flow cytometry after treating cells with quercetin for 24 hours. Our results revealed each donor has different percentages regarding each protein, which may be dependent on patient age, weight, and other genetic factors. However, it is clear that Quercetin treatment increases CXCL 9 levels whereas CXCL 5 levels are moderately decreased.

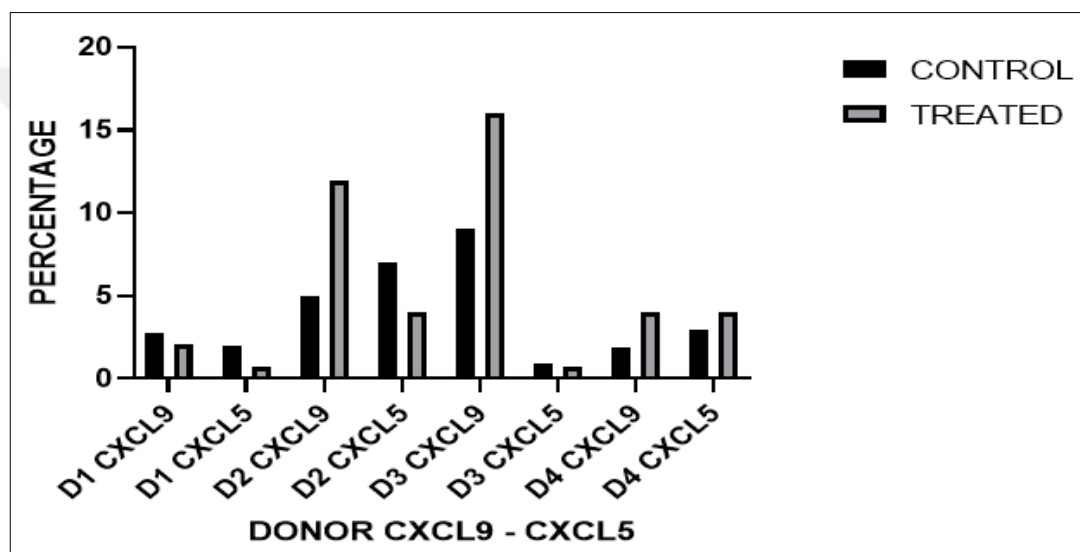


Figure 3.4. Cxcl9 and Cxcl 5 ratios of quercetin-treated mesenchymal stromal cells.

As can be seen from Figure 3.4, the percentage of CXCL9 and CXCL5 MSCs treated with Quercetin changes. CXCL9 increases after treatment of MSCs with Quercetin, while CXCL5 decreases.

3.3. STR PROFILING AND DNA CONTENT ANALYSIS RESULTS

Two different cell lines were used in the experiment, these A172 (15th passage) and U87 (25th passage) used cell lines, STR (Short Tandem Repeat) study was conducted at the Genetic Diagnosis Center of Yeditepe University to find out if there was any change in the STR profile.

3.3.1. STR Result of A172 Cell Line

For STR Profiling, DNA Profile is as follows: Amelogenin: X,Y CSF1PO: 9,12 D13S317: 11 D16S539: 12 D5S818: 11,12 D7S820: 11 TH01: 6,9.3 TPOX: 8,11 vWA: 20

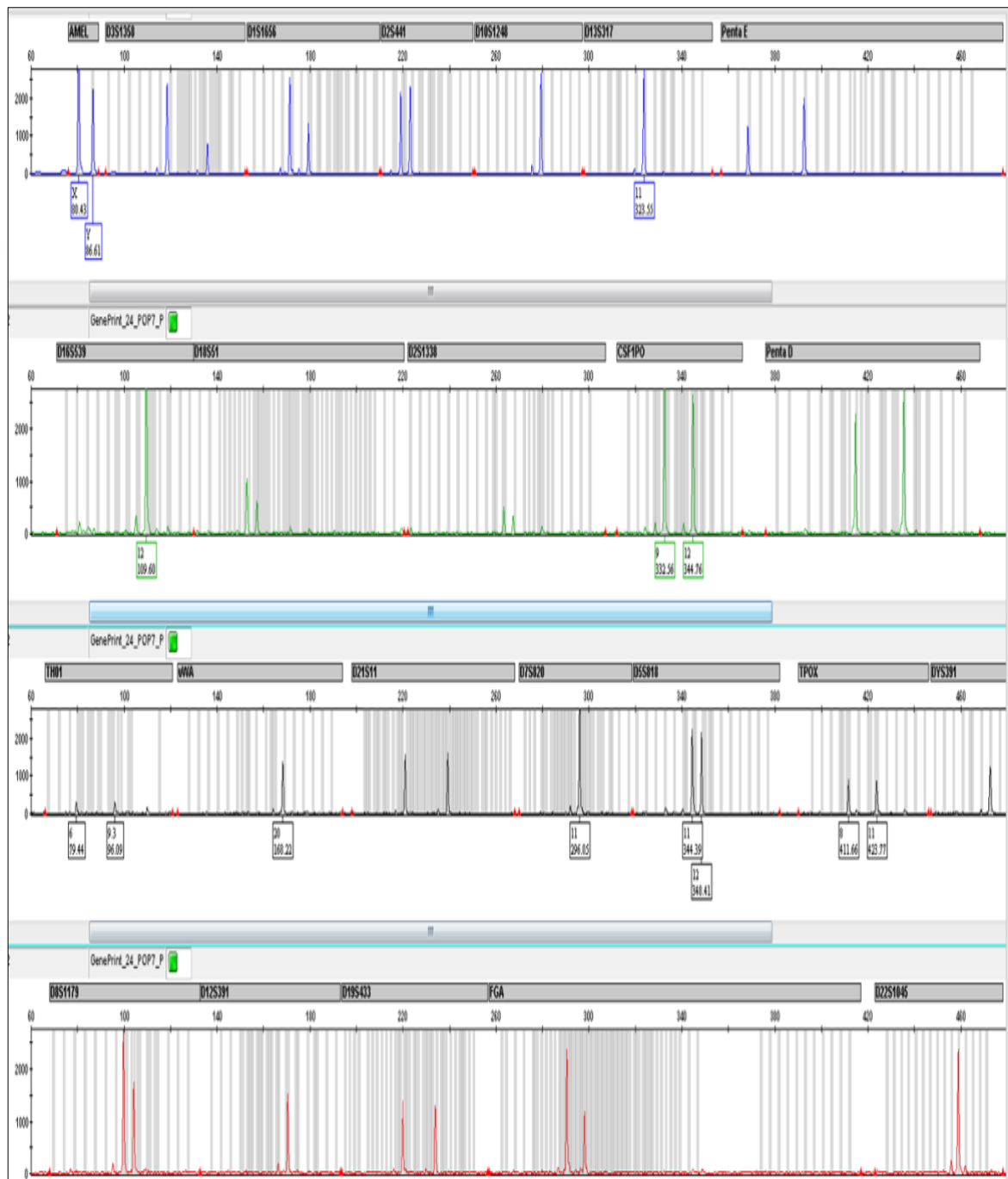


Figure 3.5. Str profiling result image for A172 cell line

3.3.2. STR Result of U87 Cell Line

For STR Profiling of U87MG-ATCC, DNA Profile is as follows: Amelogenin: X,Y
 CSF1PO: 10,11 D13S317: 8/11 D16S539: 12 D5S818: 11,12 D7S820: 8/9 TH01: 9.3
 TPOX: 8 UWA: 15-17

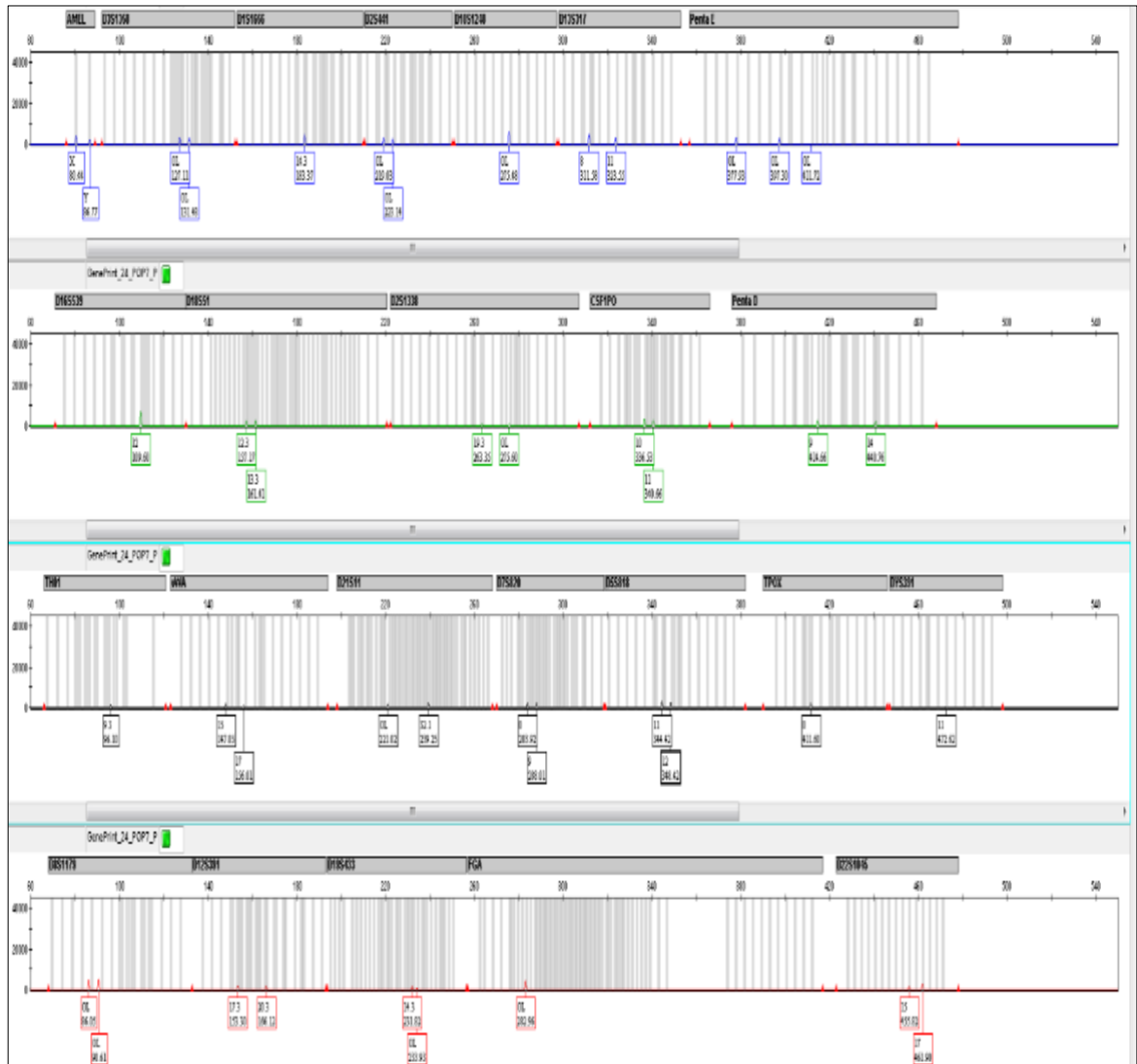


Figure 3.6. Str profiling result image for U87 cell line

As a result of the STR analysis performed, exactly the same results were obtained with the STR profile data described in section 1.2.1. taken from the ATCC site.

3.3.3. DNA Content Analysis Results Of A172 Cell Line at the end of 24 and 48 Hours.

DNA content analysis was performed to observe the effect of control, MSC and MSC + Quercetin group on cell cycle in A172 cell lines. A172 cell lines from passage 15 were used.

3.3.3.1. Comparison of A172 Cell Line Between Control Groups at 24 and 48 Hours.

In the control group, the entire A172 cell line gave similar results in the first 24 hours, but transition between phases was faster at D3. At the end of 48 hours, an increase was observed in the G1, S and G2 phases of all groups.

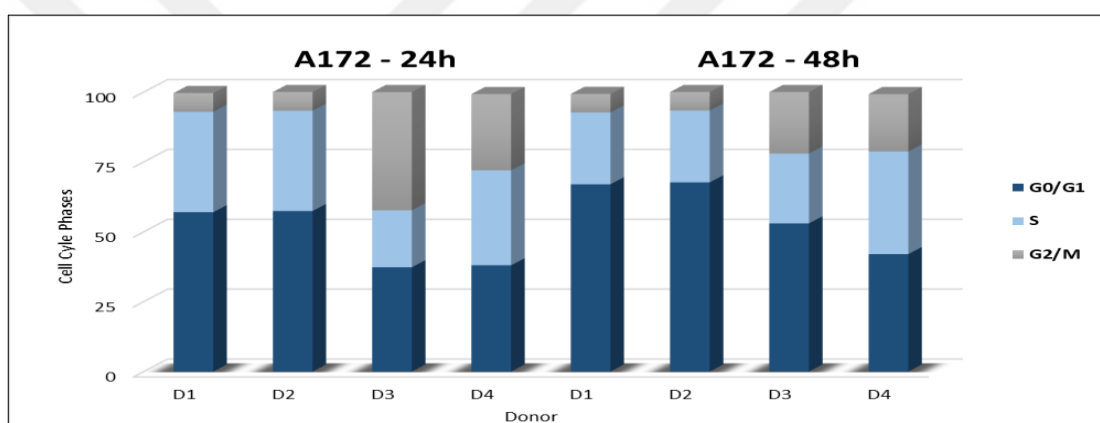


Figure 3.7. The changes between cell cycle phases of the a172 cell line relative to the control group at 24 and 48 hours.

3.3.3.2. Comparison of A172 Cell Line Between Conditioned MSC Groups at 24 and 48 Hours.

In the MSC group; D1, D3 and D4 gave almost similar results with the control group in the first 24 hours. It is clear that while there is a decrease in S phase in D2, there is an accumulation in the G2 phase. At the end of 48 hours, D1 decreased in S phase and accumulated in G2 phase. The increase in G1 and S phases in D2 is clearly seen. Although it gave similar results with the first 24 hours in D3, it was observed that the change between G2 and S phases was less. While D4 increased in G1 phase, S remained constant and G2 decreased. This may be due to division of cells that accumulate in the G2 phase after the first 24 hours.

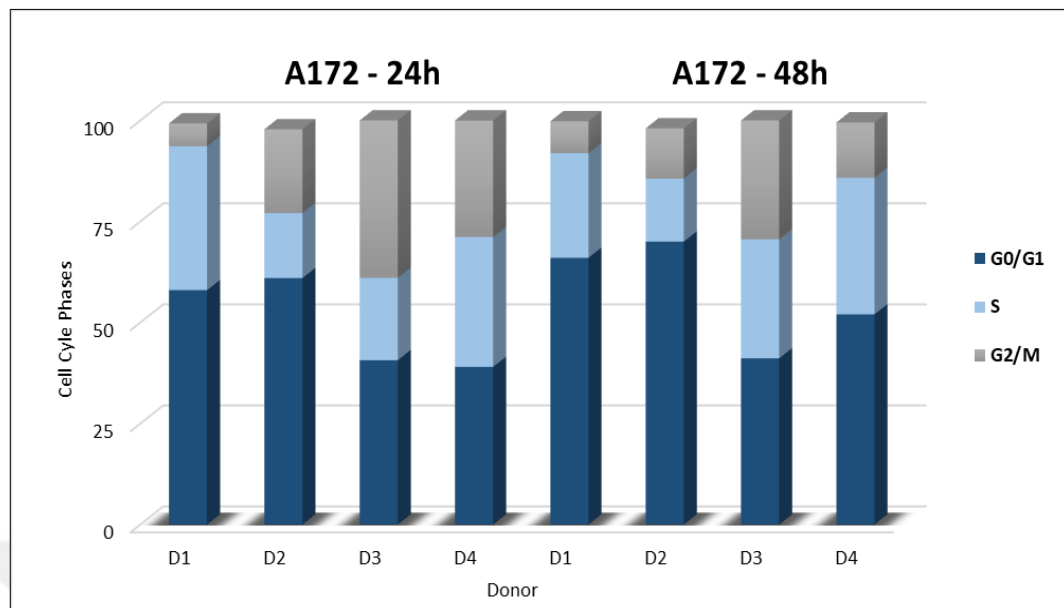


Figure 3.8. The changes between cell cycle phases of the a172 cell line at 24 and 48 hours relative to the group of conditioned msc media.

3.3.3.3. Comparison of A172 Cell Line Between Conditioned MSC+Quercetin at 24 and 48 Hours.

In the MSC + Q group, as you can see from Figure 3.9, the percentage of the G1 phase is higher in all groups except D3 in the first 24 hours compared to both the control and MSC groups. The rate of change between phases is almost the same as for the MSC group, except for D3. The MSC + Q group slightly slowed the cancer cell turnover as expected, with better results than the MSC group.

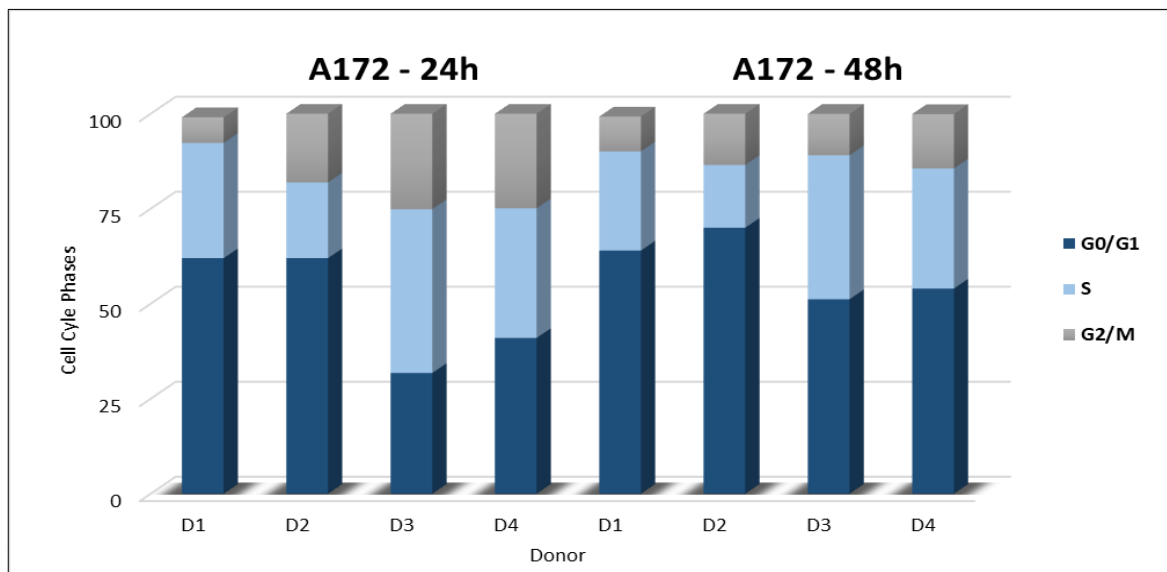


Figure 3.9. The changes between cell cycle phases of the A172 cell line at 24 and 48 hours relative to the group of conditioned msc + quercetin media.

Table 3.3. Significance values between phases of A172 cell line at 24 hours according to all groups.

A172 24 hr	SIGNIFICANT	G0/G1 P value	S - P value	G2 - P value
CONTROL vs. MSC	No	0,9953	0,9474	0,9543
CONTROL vs. MSC+QUERCETIN	No	0,9951	>0,9999	0,9902
MSC vs. MSC+QUERCETIN	No	>0,9999	0,9445	0,9863

A172 cell lines treated with control, MSC, and MSC + Quercetin were analyzed between groups, and after the first 24 hours, slight significance was seen between the control and MSC groups in all phases, control and MSC + Quercetin in the G1 and G2 phase after the first 24 hours. Due to the low number of donors, no overall significance was observed.

Table 3.4. Significance values between phases of A172 cell line at 48 hours according to all groups.

A172 48 hr	SIGNIFICANT	G0/G1- P value	S- P value	G2- P value
CONTROL vs. MSC	No	0,9946	0,9963	0,9924
CONTROL vs. MSC+QUERCETIN	No	0,9995	0,9922	>0,9999
MSC vs. MSC+QUERCETIN	No	0,9907	0,9779	0,9738

Analysis was performed between groups of A172 cell lines treated with control, MSC and MSC + Quercetin, and after 48 hours there was little significance between all phases in the control and MSC group. There is significance between the control and MSC + Quercetin groups in the G1 and S phases also there was a slight significance in the G1, S and G2 phases between MSC and MSC + Quercetin groups.

3.3.4. DNA Content Analysis Results of U87 Cell Line at the end of 24 And 48 Hours.

DNA content analysis was performed to observe the effect of control, MSC and MSC + Quercetin group on cell cycle in U87 cell lines. U87 cell lines from passage 25 were used.

Comparison between groups took place in the U87 cell line at 24 and 48 hours compared to the control group. When looking at the percentage change between MSC and MSC + Q, they gave almost similar results. According to the evaluation, a slight change was observed when MSCs were obtained from donors, conditioned and applied to cancer cells, but this change was not as important as the change in the A172 cell line. When one-way ANOVA analysis is performed, although there are few significant results among the groups, the results are not generally meaningful due to the limited number of donors.

3.3.4.1. Comparison of U87 Cell Line Between Control Groups at 24 and 48 Hours.

In the control group, the entire U87 cell line gave similar results in the first 24 hours, but transition between phases was faster at D3. At the end of 48 hours, an increase was observed in the G1, S and G2 phases of all groups.

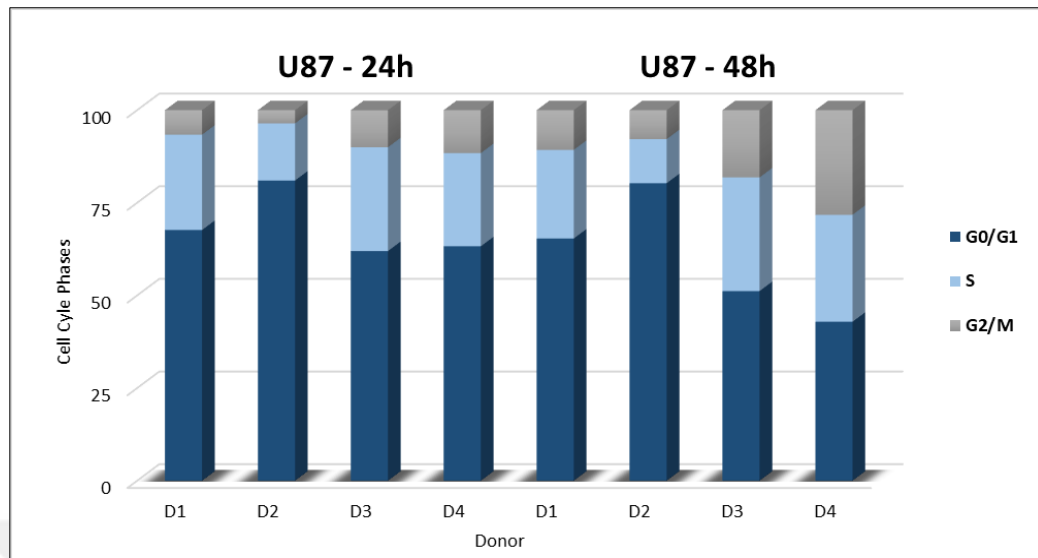


Figure 3.10. The changes between cell cycle phases of the u87 cell line relative to the control group at 24 and 48 hours

3.3.4.2. Comparison of U87 Cell Line Between Conditioned MSC Groups at 24 and 48 Hours.

MSC-treated U87 cell lines were analyzed across groups and after the first 24 hours gave slightly significant results in additional control and G1, S among MSC groups. There was a slight significance in the G1, S, and G2 phases in the G2 phase as well as in the MSC group after 24 hours.

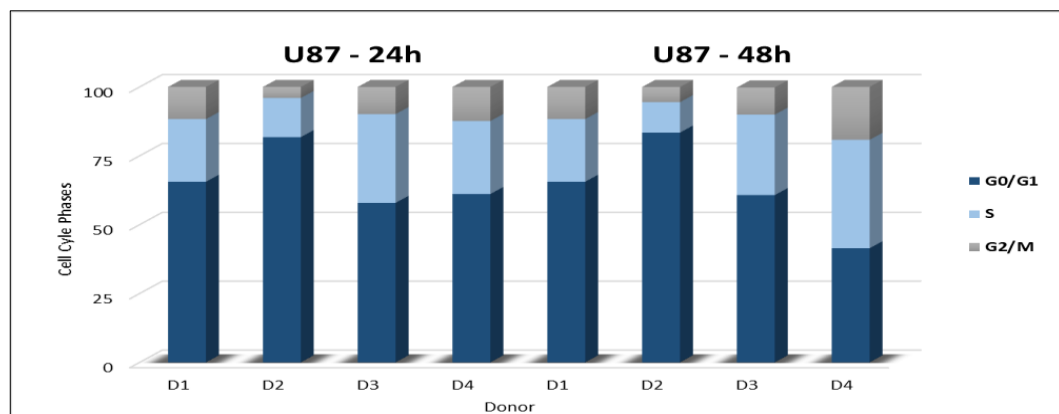


Figure 3.11. The changes between cell cycle phases of the u87 cell line at 24 and 48 hours relative to the group of conditioned msc media.

3.3.4.3. Comparison of U87 Cell Line Between Conditioned MSC+ Quercetin Groups at 24 and 48 Hours.

U87 cell lines treated with MSC + Quercetin were analyzed between groups and no significance was seen in the G1 and S phases after 24 hours. After the first 24 hours there was a slight significance in the G1, S, and G2 phases between control and MSC + Quercetin, as well as between MSC + Quercetin groups in the G1, S, and G2 phases.

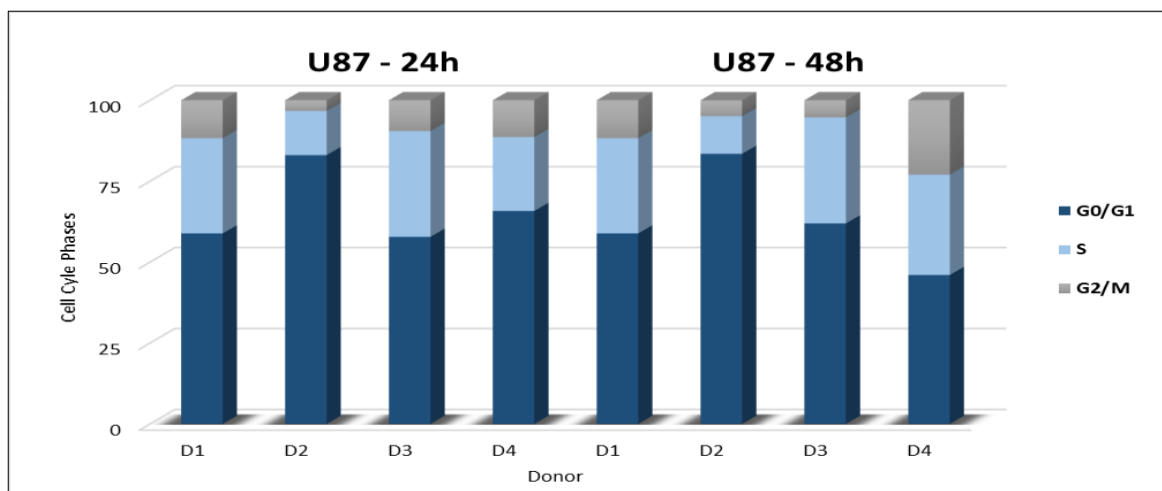


Figure 3.12. The changes between cell cycle phases of the u87 cell line at 24 and 48 hours relative to the group of conditioned msc + quercetin media.

Table 3.5. Significance values between phases of u87 cell line at 24 hours according to all groups.

U87 24 hr	SIGNIFICANT	G0/G1- P value	S- P value	G2- P value
CONTROL vs. MSC	No	0,9943	0,9974	0,8353
CONTROL vs. MSC+QUERCETIN	No	0,9555	0,9764	0,9183
MSC vs. MSC+QUERCETIN	No	0,9995	0,9893	0,9819

U87 cell lines treated with Control, MSC, and MSC + Quercetin were analyzed between groups, and after the first 24 hours, slight significance was seen between the control and

MSC groups in all phases in addition control and MSC + Quercetin in the G1,S and G2 phase after the first 24 hours also there was a slight significance in the G1, S and G2 phases between MSC and MSC + Quercetin groups.

Table 3.6. Significance values between phases of u87 cell line at 48 hours according to all groups.

U87 48 hr	SIGNIFICANT	G0/G1- P value	S- P value	G2- P value
CONTROL vs. MSC	No	0,9647	0,9675	>0,9999
CONTROL vs. MSC+QUERCETIN	No	0,9725	0,9423	0,9809
MSC vs. MSC+QUERCETIN	No	0,9998	0,9961	0,9834

U87 cell lines treated with Control, MSC, and MSC + Quercetin were analyzed between groups, and after 48 hours, slight significance was seen between the control and MSC groups in G1 and S phases. In addition between control and MSC + Quercetin in the G1,S and G2 phase after the first 48 hours also there was a slight significance in the G1, S and G2 phases between MSC and MSC + Quercetin groups.

3.4. RESULTS OF THE ANNEXIN APOPTOSIS ASSAY

Donor 1

At the end of the first 24 hours, the lowest percentage of viability in the A172 cell line was seen in the MSC + Quercetin group (44 percent), followed by the MSC (46.19 percent) and control (53.38 percent) groups, respectively. The highest percentage of necrotic cells was observed in the MSC + Quercetin and MSC groups at 17.98 percent and 15 percent, respectively, while the lowest was seen in the control group at 9 percent. Late apoptotic cells were seen the highest in the control group with a rate of 4 percent, while the early apoptotic cells were the highest in the MSC + Quercetin group with a rate of 36.55, followed by the MSC and control groups with the rates of 35.90 percent and 32.20 percent, respectively. In the U87 cell line, the highest percent viability was seen in the MSC group (87.78 percent), followed by MSC + Quercetin and control groups with 85.18 and 83.08 percent, respectively. The highest rate of necrotic cells was observed in MSC + Quercetin and the control group at

8 percent. Late apoptotic percentage gave similar results in almost all groups. The highest percentage of early Apoptotic cells was observed in the control group (8 percent). At the end of 48 hours, the lowest viability was seen in the control group (90.84 percent) in the U87 cell line, while the lowest viability was seen in the MSC group with 47.36 percent in the A172 cell line. Necrotic cells were observed at the highest rate of 4.6 percent in the control group in U87, while it was observed in the MSC group in the A172 cell line. Late apoptotic and Early apoptotic percentages in the A172 cell line were highest in the MSC group at 10 percent and 35.90 percent, respectively, followed by MSC + Quercetin and Control groups, respectively. In U87, almost similar results were obtained between the groups.

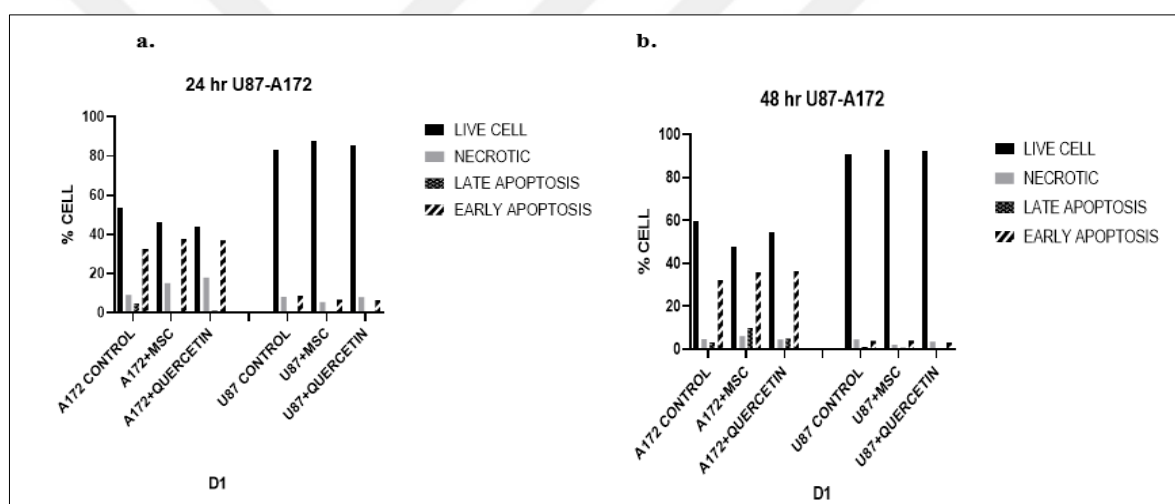


Figure 3.13. D1 result of the annexin apoptosis assay for a172 and u87 cell lines at 24 and 48 hr

Donor 2

According to the 24 hour results, in cancer cells treated with MSC and MSC + QUERCETIN medium; While the A172 cell line showed 93 percent viability in the control group, it was 89.61 percent and 85.16 percent in the MSC and QUERCETIN + MSC groups, respectively. In the U87 cell line, the control group showed 94.21 percent viability, while the MSC and

QUERCETIN + MSC groups showed 94 percent and 90.34 percent viability, respectively. According to the results of 48 hours in cancer cells treated with MSC and MSC + QUERCETIN medium; While the control group showed 97 percent viability in the A172 cell line, almost similar results were obtained in the MSC and QUERCETIN + MSC groups. In U87, although the control group was 94.55 percent, the viability in MSC and QUERCETIN + MSC groups was 95.68 percent and 96.12 percent, respectively. According to the results, while cytokines and chemokines continue to act on A172 cells, their effect on U87 cells has decreased.

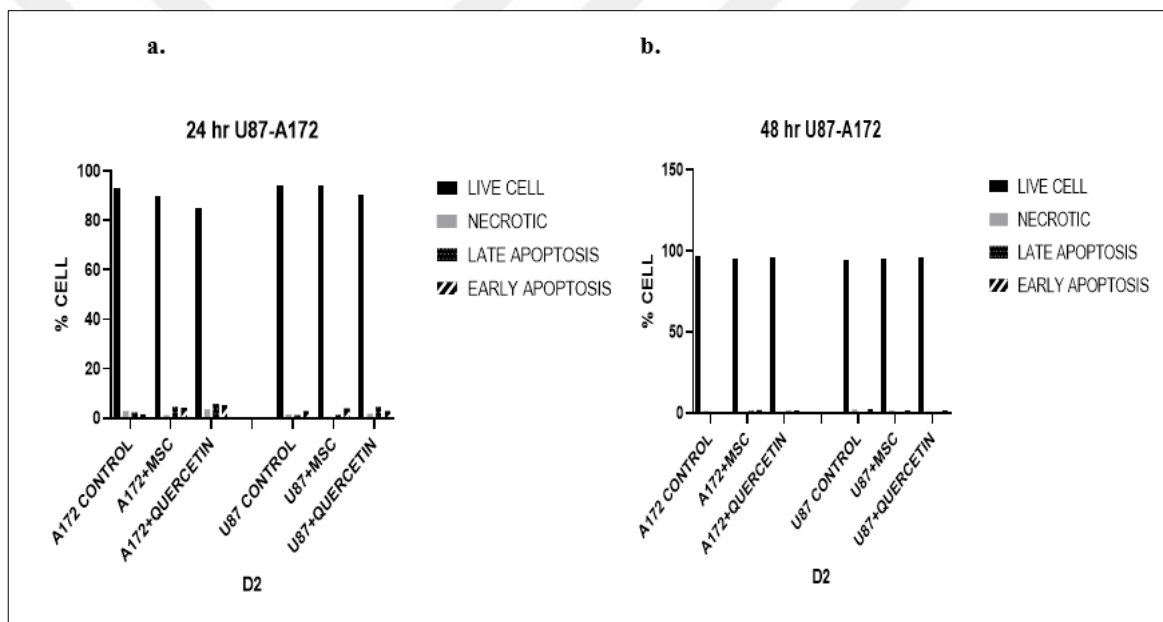


Figure 3.14. D2 result of the annexin apoptosis assay for a172 and u87 cell lines at 24 and 48 hr

Donor 3

According to the 24-hour results, cancer cells stimulated with MSC and MSC + QUERCETIN medium showed 88.26 percent viability in the control group of the A172 cell line. In the MSC and QUERCETIN + MSC groups, it showed 85.11 percent, 80.69 percent viability, respectively. In the U87 cell line, the highest viability was observed like 91.77 percent in the control group, while the MSC was 89.22 percent and the QUERCETIN + MSC was 86.69 percent. According to the results of 48 hours in cancer cells treated with MSC and

MSC + QUERCETIN medium; In the A172 cell line, the control group showed 91.84 percent viability, while the MSC and QUERCETIN + MSC survival rate was 82 percent and 88.80, respectively. Although the percentage of U87 viability in the control group was 89.84 percent, the percentage it was 86.53 percent and 89.74 percent in the MSC and QUERCETIN + MSC groups, respectively. According to the results, although cytokines and chemokines continued to show their effect on A172 and U87 cell lines, a decrease was observed in the MSC and MSC + Quercetin groups compared to the 24 hour data.

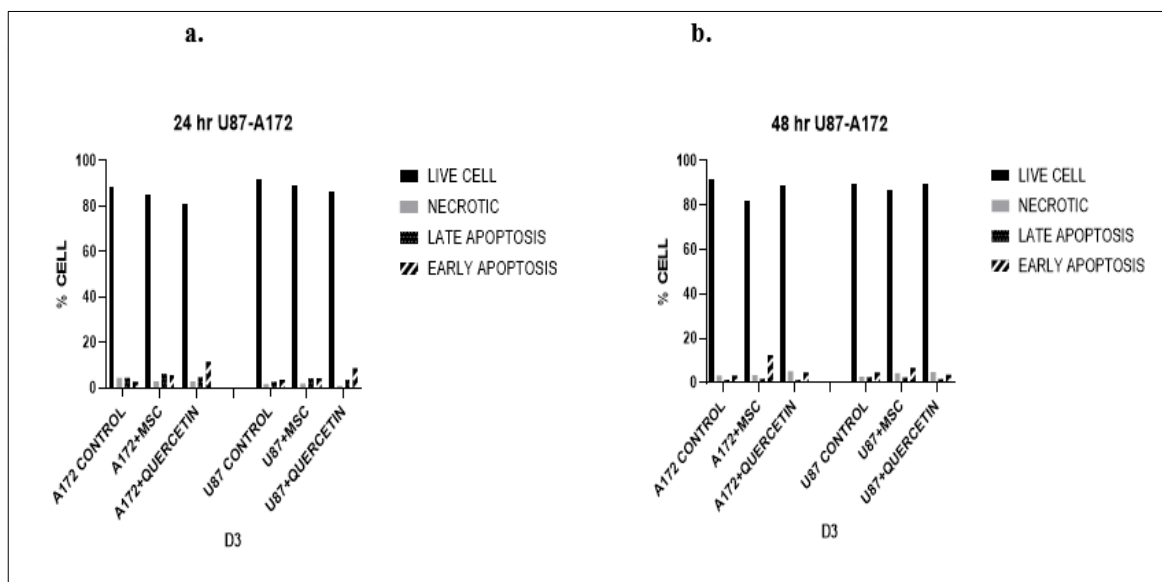


Figure 3.15. D3 result of the annexin apoptosis assay for a172 and u87 cell lines at 24 and 48 hr

Donor 4

When compared to the control group in cancer cells treated with MSC and MSC + QUERCETIN medium according to 24 hour results; While the control group showed 93.74 percent viability in the A172 cell line, 93.83 percent and 93.58 percent viability was observed in the MSC and QUERCETIN + MSC groups, respectively. In addition, while the control group showed 92.96 percent viability in the U87 cell line, it showed 92.29 percent and 94 percent vitality in the MSC and QUERCETIN + MSC groups, respectively. When comparing the groups according to the results obtained, not much change was observed in

terms of vitality. According to the results obtained at the end of 48 hours, when compared to the control group in cancer cells treated with MSC and MSC + QUERCETIN medium; In the A172 cell line, the control group showed 91.84 percent viability, while the A172 + MSC and A172 + QUERCETIN + MSC survival rates were 84 percent and 85.12 percent, respectively. In U87, although the control group is 94.55 percent, U87 + MSC and U87 + QUERCETIN + MSC are 92.34 and 92.47, respectively. In the A172 cell line, the MSC and MSC + Quercetin group showed a decrease in viability of approximately 7 percent to 6 percent, respectively. In the U87 group, there was a 2 percent decrease in the vitality of the MSC and MSC + Quercetin group compared to the control group.

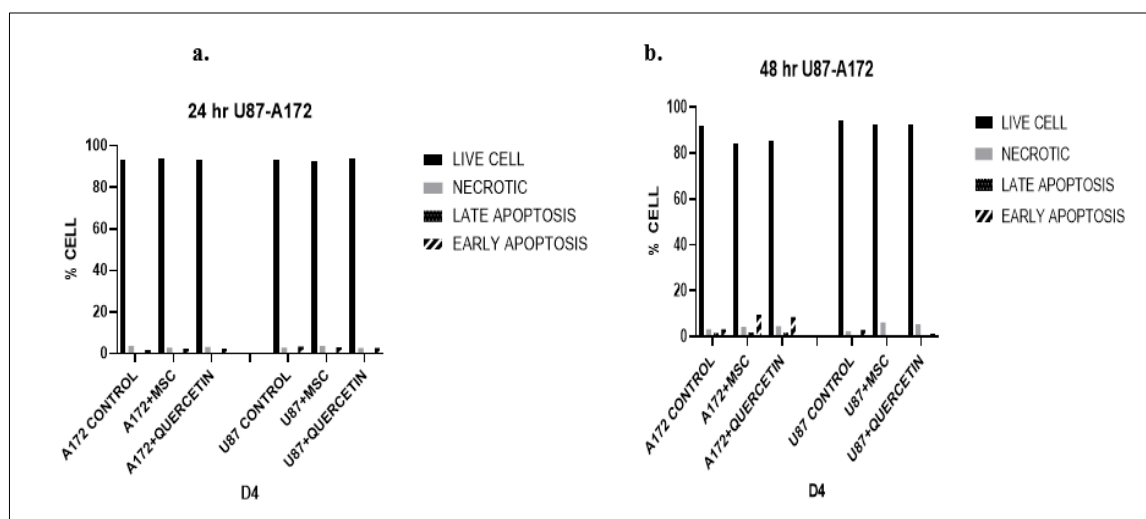


Figure 3.16. D4 result of the annexin apoptosis assay for a172 and u87 cell lines at 24 and 48 hr

Table 3.7. The significance statistical results of annexin apoptosis assay of a172 cell line at 24 hours according to all groups.

24 hr A172 Cell line	Significant	Live Cell	Necrotic Cell	Late Apoptosis	Early Apoptosis
CONTROL vs. MSC	No	0,9721	0,9908	>0,9999	0,9665
CONTROL vs. MSC+QUERCETIN	No	0,9094	0,8923	0,9997	0,9228

MSC vs. MSC+QUERCETIN	No	0,9802	0,9428	>0,9999	0,9901
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A172 cell lines treated with control, MSC and MSC + Quercetin were analyzed between groups and no significance was found first 24 hours.

Table 3.8. The significance statistical results of annexin apoptosis assay of a172 cell line at 48 hours according to all groups.

48 hr A172 Cell line	Significant	Live Cell	Necrotic Cell	Late Apoptosis	Early Apoptosis
CONTROL vs. MSC	No	0,8268	0,9280	0,5002	0,8833
CONTROL vs. MSC+QUERCETIN	No	0,9094	0,9018	0,9312	0,9578
MSC vs. MSC+QUERCETIN	No	0,9802	0,9976	0,7087	0,9788

A172 cell lines treated with control, MSC and MSC + Quercetin were analyzed between groups and no significance was found at 48 hours.

Table 3.9. The significance statistical results of annexin apoptosis assay of u87 cell line at 24 hours according to all groups.

24 hr U87 Cell line	Significant	Live Cell	Necrotic Cell	Late Apoptosis	Early Apoptosis
CONTROL vs. MSC	No	0,9933	0,9515	0,9416	0,9971
CONTROL vs. MSC+QUERCETIN	No	0,8826	0,9966	0,7076	0,9585

MSC vs. MSC+QUERCETIN	No	0,8288	0,9730	0,8783	0,9350
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U87 cell lines treated with control, MSC and MSC + Quercetin were analyzed between groups and no significance was found first 24 hours.

Table 3.10. The significance statistical results of annexin apoptosis assay of u87 cell line at 48 hours according to all groups.

48 hr U87 Cell line	Significant	Live Cell	Necrotic Cell	Late Apoptosis	Early Apoptosis
CONTROL vs. MSC	No	0,9584	0,8619	0,9933	0,9782
CONTROL vs. MSC+QUERCETIN	No	0,9915	0,8040	0,9780	0,6739
MSC vs. MSC+QUERCETIN	No	0,9152	0,9929	0,9481	0,7885

U87 cell lines treated with control, MSC and MSC + Quercetin were analyzed between groups and no significance was found after 48 hours.

According to the one-way ANOVA analysis results; No significance was found between the groups. The biggest reason for this is the low number of donors from which the cells are obtained. On the contrary, donors showed significance in themselves when analyzed individually.

3.5. CELL PROLIFERATION RESULTS

Donor 1

According to the results of the experiment; In the A172 cell line, it has been demonstrated that at the end of 24 and 48 hours, there is a decrease in cell proliferation in the 2nd, 3rd and 4th generation. At the end of 48 hour, A172 cells treated with MSC + Quercetin had 1,2 and

3 generations, while the 4th generation was not observed. The division time of the U87 cell line is indicated on the ATCC site as 1-1.25 days, accordingly, 2 generations are clearly determined in the results. After 24 hours, when compared to the control group, the ratio of cells in the 1st generation of MSC cell media and Quercetin treated groups is higher than the control group but after 48 hours, 95 percent of all groups reached equal values. As a result, it appears that the cell continues to proliferate compared to 24 hours, showing a resistance to the administered cytokine and chemokine.

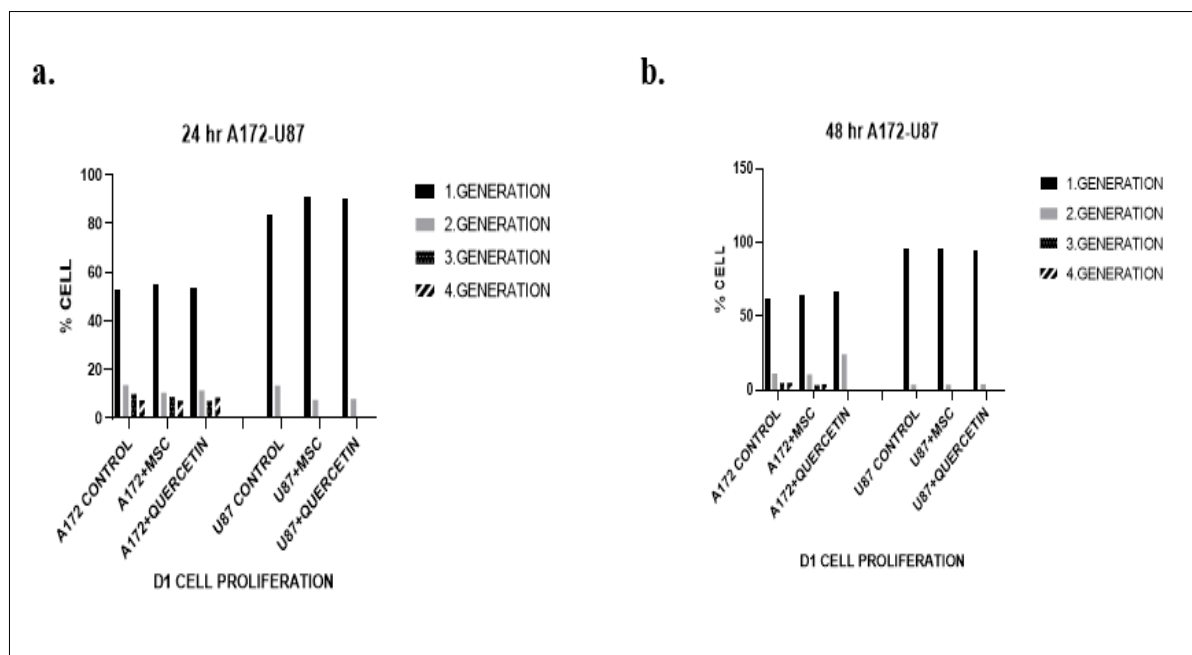


Figure 3.17. D1 cell proliferation results and generation numbers. (a) 24 hours a172-u87
(b) 48 hours a172-u87

The study was carried out with A172 and U87 brain cancer cell lines and there are 3 different groups and the difference between the groups was compared. According to this comparison; A172 and U87 cancer cells were treated with QUERCETIN + MSC and MSC medium. According to the results, a single generation was observed in A172 cells and the lowest percentage was seen in the MSC + Quercetin and MSC groups. In U87 cells, less cell percentage was observed in MSC + Quercetin and MSC groups compared to the control group. According to the results of 48 hours; The A172 cell line gave similar result like 24 hours result. In the U87 cell line, an increase was observed in cancer cells treated with MSC and MSC + QUERCETIN medium.

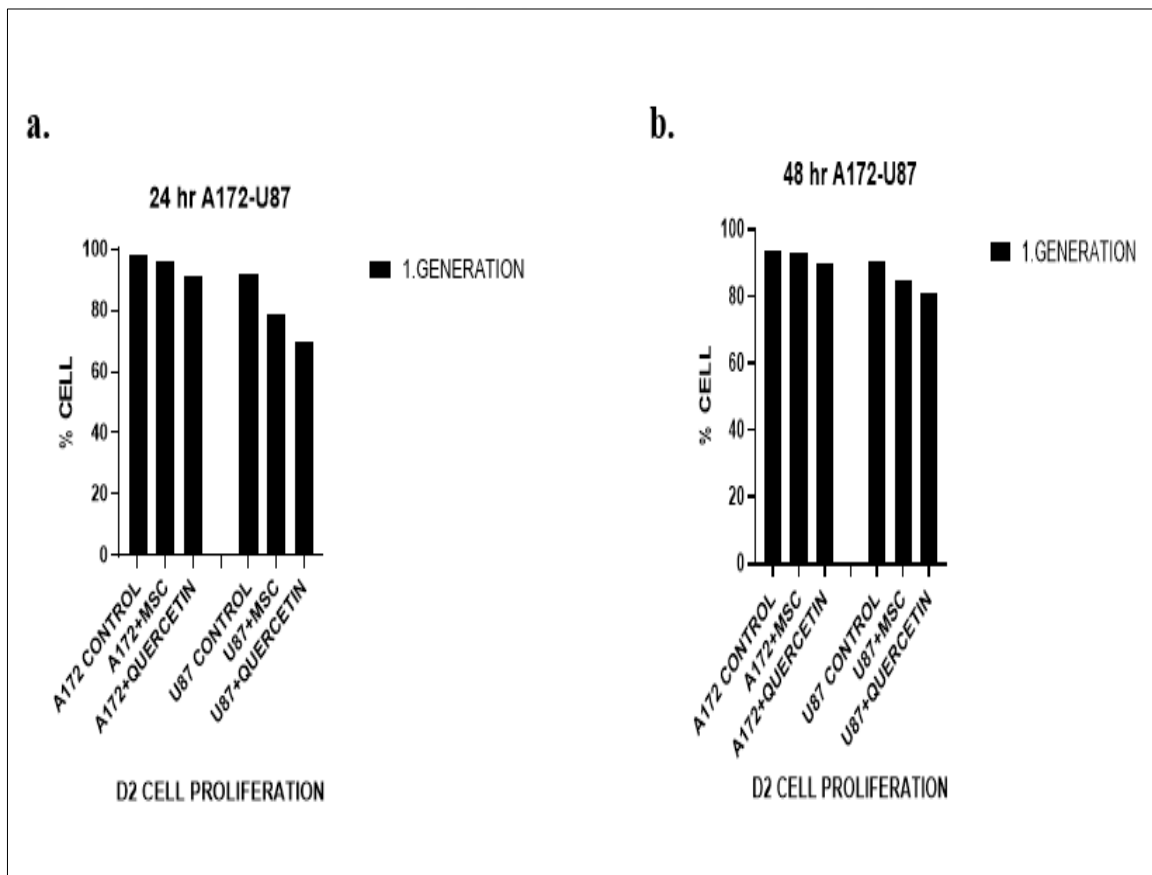


Figure 3.18. D2 cell proliferation results and generation numbers. (a) 24 hours A172-U87
(b) 48 hours A172-U87

Donor 3

The study was carried out with A172 and U87 brain cancer cell lines. As seen below, there are 3 different groups and the difference between the groups has been compared. According to this comparison; A172 and U87 cancer cells were treated with QUERCETIN + MSC and MSC medium. According to the results, a single generation was observed in all groups except MSC+ Quercetin group when the cytokines and chemokines released by Donor 3 were compared at the end of 24 hours in the A172 cell line. MSC group and MSC + Quercetin group gave almost similar results. The highest percentage of proliferation was observed in the control group. In U87 cells, the control group and MSC + Quercetin group gave almost similar results, although one generation was observed. In the MSC group, the proliferation rate went low in the first 24 hours, and reached the same point with the other groups at the end of the 48th hour.

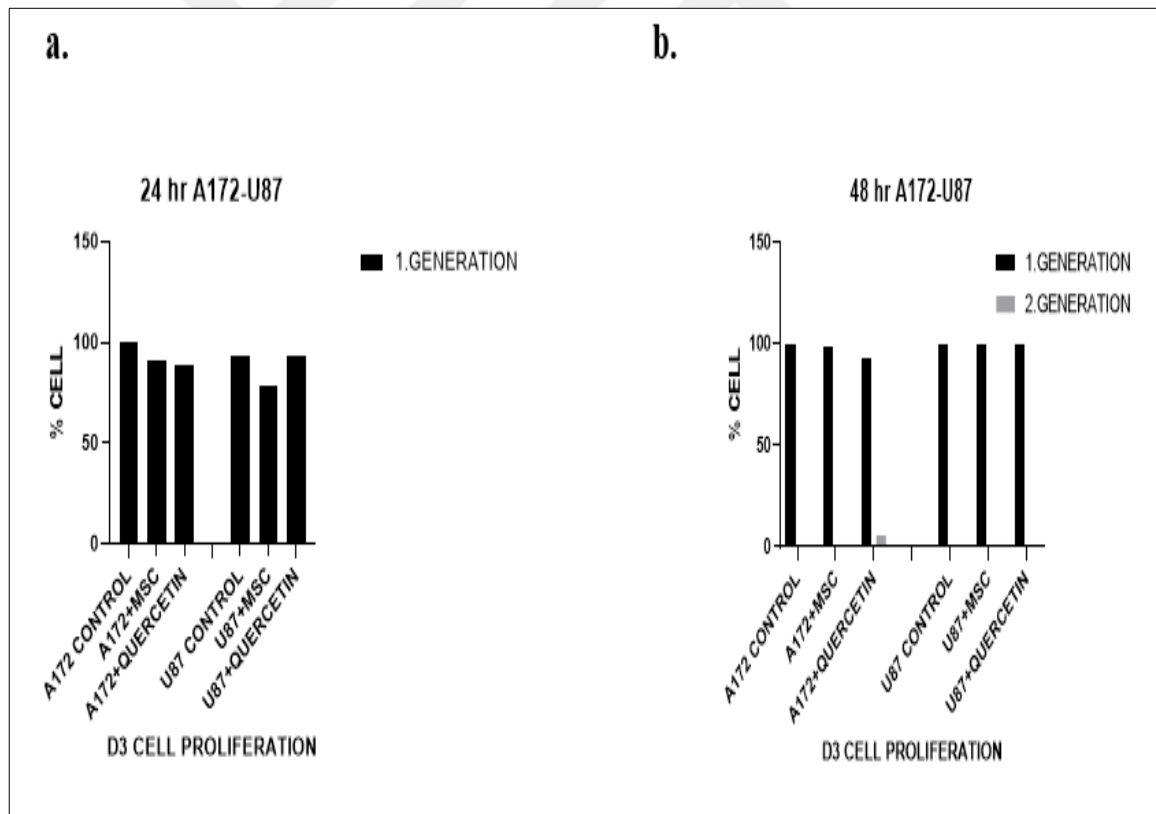


Figure 3.19. D3 cell proliferation results and generation numbers. (a) 24 hours a172-u87
(b) 48 hours a172-u87

Donor 4

At the end of 24 and 48 hours, a single generation was observed in all groups and interpretation was made on percentage proliferation. According to the results, the highest percentage of proliferation in the A172 cell line at the end of the first 24 hours is clearly seen in the control group, followed by MSC and MSC + Quercetin groups, respectively. After 48 hours, almost similar results were obtained. In the U87 cell line, the highest percentage of proliferation was observed in the control group, followed by MSC + Quercetin and MSC groups, respectively, but the increase in the MSC group at the end of 48 hours was higher than in the MSC + Quercetin group.

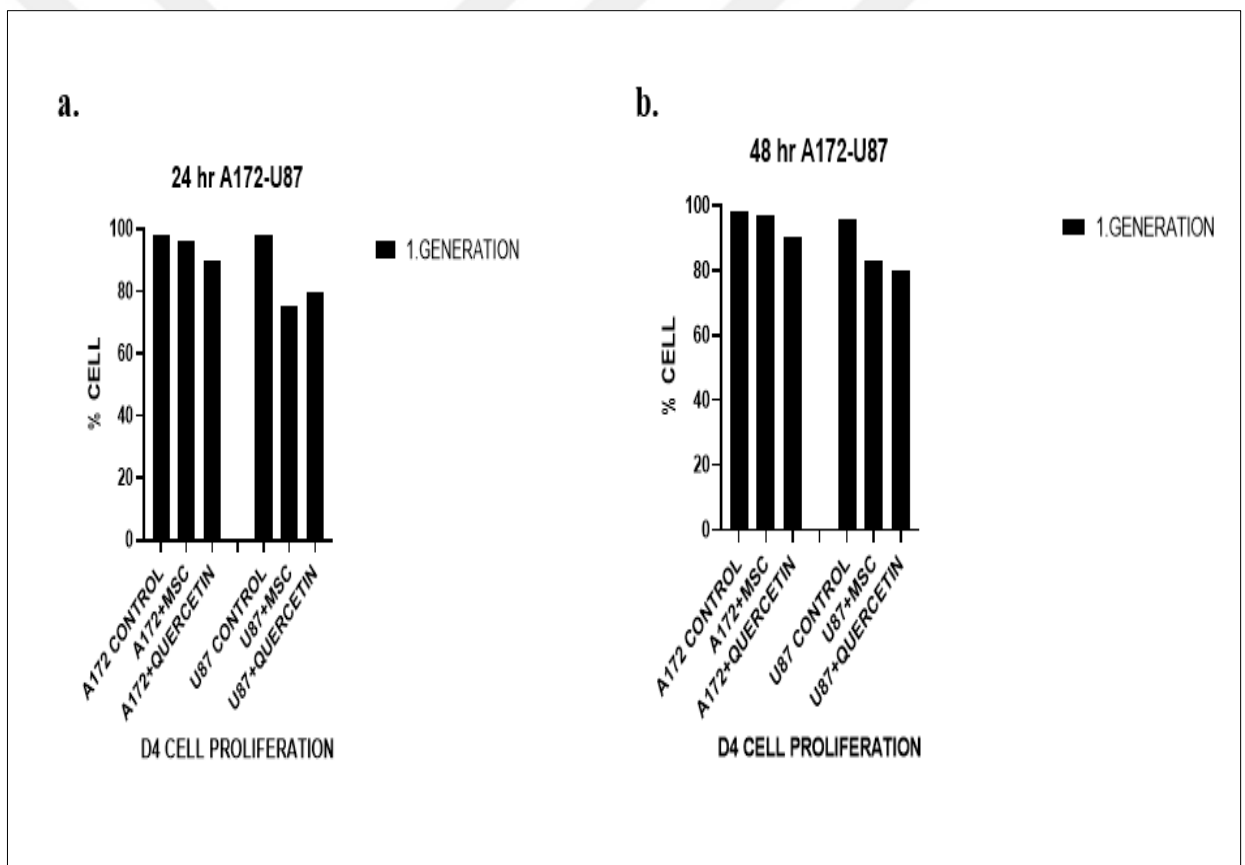


Figure 3.20. D4 cell proliferation results and generation numbers. (a) 24 hours a172-u87
(b) 48 hours a172-u87

4. DISCUSSION

The most important feature that distinguishes stromal cells from other cells is that they renew themselves for many years without differentiation and can be stimulated to differentiate into the desired cell under physiological or natural conditions. Generally, two types of stromal cells are used in stromal cell studies, the first of which are isolated from embryos that are not needed in in vitro fertilization studies and donated by their owners for scientific studies, the second is Mesenchymal stromal cells that can be isolated from both fetal and adult tissue. In addition to these two stem cells, the induced pluripotent stem cell formed from the somatic cell is also taking place in stem cell studies[49].

Mesenchymal stromal cells are multipotent stromal cells originating from the mesoderm. They can be transformed into many cells under experimental and physiological conditions. MSCs can be isolated from most adult and fetal sources. MSCs are euchromatic cells with thin extensions that emerge from the fibroblast-like cell body and have a large nucleus in the center. It was first found in the bone marrow and differentiated into 3 mesodermal tissues (fat, cartilage and bone). Subsequent studies have shown that this multipotent cell can be found in every tissue (such as fat, cord blood, placenta, placental villi, amniotic fluid, peripheral blood, and liver), and when tissue damage occurs, it takes part in tissue regeneration and migrates to the area needed with blood tissue[45].

The therapeutic and protective properties of MSCs have been shown in many experimental studies. In cancer models, it suppressed cancerous cells and stopped their proliferation. However, in recent studies using stem cells for cancer treatment; It has been shown that stem cells trigger cancer formation, enlarge the mass, help the formation of new vessels and invasion of cancer. It is known that the communication between MSCs and cancer cells is achieved with the help of cytokines released from MSCs. The presence of these cytokines and their effect on cancer cells reduce the treatment reliability of MSCs[50].

In another study, the polarization of MSCs was achieved and the stromal cells were divided into two groups, these groups were named MSC1 and MSC2. MSC1 stops the proliferation of cancer cells while MSC2 helps cancer cells proliferate. As a result, positive results were obtained in cancer cells such as SKOV3, HeLa and MOSEC in the MSC1 group. In the same article, ovarian cancer (SKOV3) and Mesenchymal stromal cells were co-cultured, and as a

result, it was observed that some cytokines and chemokines increased in the environment. We mentioned above that when MSCs are polarized, MSC1s stop the proliferation of cancer cells. While CXCL9 (MIG) increased in MSC1 medium when co-cultured, its response was not seen in MSC2[24].

Quercetin, which was used in our experiment, has been used in many areas for years and is an active ingredient in the peel of many fruits, in addition, its anti-cancer, anti-allergen and anti-oxidant effects are known. Many studies have shown that Quercetin is known to induce apoptosis and increase the release of some cytokines and chemokines[51].

As is known, MSCs migrate next to cancer cells and interact with them. This interaction may result in suppression of cancer cells or an increase in proliferation. Our experimental setup is based on this mobility of MSCs. As we mentioned above, while Quercetin causes some cytokines to increase, it also decreases the cytokines associated with cancer. According to this information, the answer to the question of whether MSCs treated with Quercetin could be effective on cancer cells was sought[51].

A172 and U87 glioblastoma cell lines were used in that experiments. In the evaluation, the cells treated with Quercetin and MSCs media showed significant differences compared to the control group. This difference varies on patient basis, but when the analysis is done collectively, no significant difference was observed, because the number of patients is small and it is thought that it will give more significant results if there are more patients. Although it gives positive results in proliferation, viability and DNA content analysis at the end of 24 hours, the difference compared to the control group has decreased with the decrease of other factors, including cytokines and chemokines, at the end of 48 hours, even in proliferation and phase transition of some cell groups (U87 MSC and U87 + Quercetin etc.) It was observed that there was an increase and this decrease or increase depends entirely on the donor.

As it is known, CXCL5 belongs to the CXC-type chemokine family. Many studies have shown that CXCL 5 promotes tumor formation and progression[52].

CXCL 9: It belongs to the CXC-type cytokine family. In previous studies, it was observed that CXCL9 increased in MSC 1, mesenchymal cells that prevent cancer formation, when co-cultured, and no response was found in MSC 2 [24]. Analysis was performed to observe whether there would be a change in these two chemokines and cytokines when Quercetin

was given to MSCs. Accordingly, the answer to the question of whether polarization will be achieved in MSCs was sought in the study. Percent analysis was performed with Beckman coulter Flow Cytometer. CXCL9 and CXCL5 cytokine and chemokine protein expression rates were examined and an increase in CXCL9 was observed in cell groups treated with Quercetin. In addition, CXCL5 ratio was generally decreased in Donor cells release. Although the results obtained according to the conditioning are consistent, it is thought that more effective results will be obtained by providing extended release when the cells are co-cultured.

In previous studies, while polarization of MSCs was performed with methods such as TLR3 and TLR4 priming[23], in our study, using Quercetin, an increase and decrease of cytokines and chemokines such as CXCL9 and CXCL5, which are effective on cancer, can be seen from Figure 6.4. Although no significant results were obtained in the results of the collective analysis due to the low number of donors, both MSCs and MSCs treated with Quercetin gave positive results in individual analysis.

Although it is thought that these two groups will have more effects on cancer cells when co-cultured, it is desired to investigate the question of whether MSCs will serve as a carrier due to easy stimulation and whether they can be used for different diseases in future studies.

If the effect of the study can be increased, it will be a solution for treating cancer and relieving the material and spiritual pain suffered after traditional treatment.

5. CONCLUSION

Within the scope of this study, the answer to the question of whether a potential treatment can be found for cancer disease, which is common today as in the past, or can it provide a different perspective to previous mesenchymal stromal cell studies, was sought. MSCs obtained after liposuction were treated with Quercetin, media of cancer cell and the effect of the treated MSCs on Glioblastoma cancer cells with cytokines and chemokines was observed. According to the results, each patient responded differently to cancer cells, but generally conditioned media slowed the division of glioblastoma cell lines (U87 and A172) and accelerated the progression of cells to apoptosis and necrosis. The increase in the percentage of CXCL9 and the decrease in the percentage of CXCL5 of the MSCs treated with quercetin are indicative of the polarization of the cells without using any chemicals. As it is known, CXCL5 is one of the cytokines responsible for the proliferation of cancer cells.

This study constitutes one of the first steps in the development of a defense mechanism by using cancer patients' own cells.

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APPENDIX A

APPENDIX A: STATISTICAL AND GRAPHICAL IMAGES OF THE RESULTS OBTAINED AT THE END OF 24 AND 48 HOURS ACCORDING TO DONORS.

Flow cytometry results between groups according to donors are as you can see below. Images of DNA content analysis, Annexin v/p1 and Cell proliferation results for 24-48 hr.

Also, the percentages of Cxcl5 and Cxcl9 of mesenchymal stromal cells treated with Quercetin are attached, as well as images of the results obtained with the CD73, CD105 and CD90 markers of the MSCs.

Donor 1:

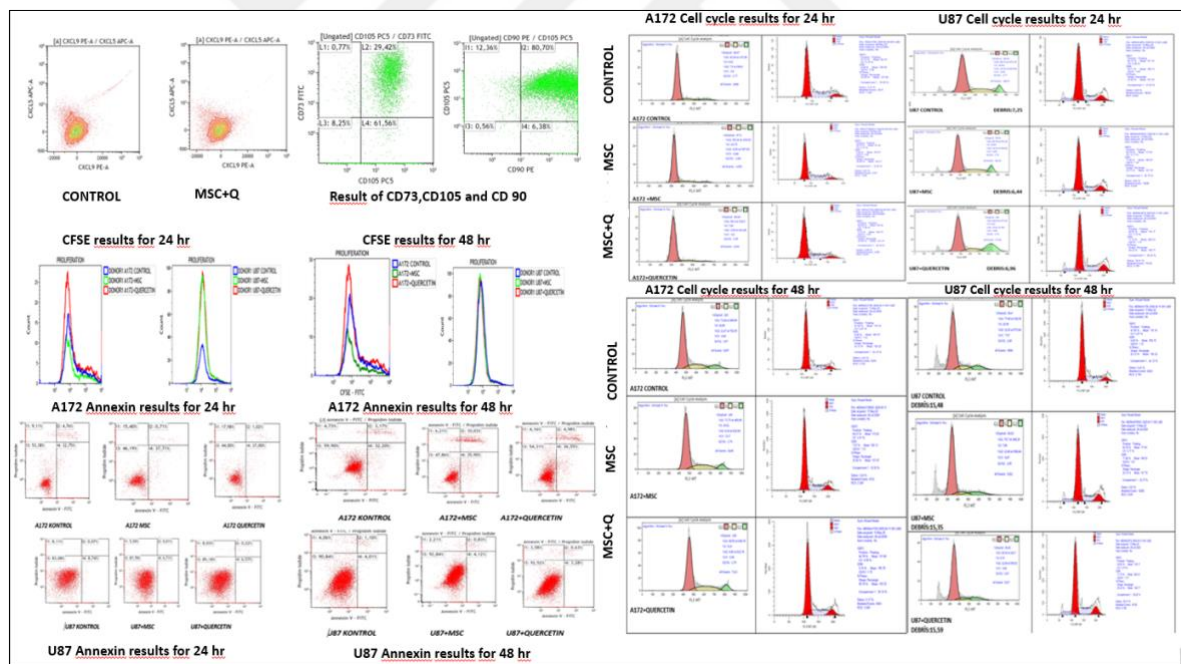


Figure A.1. Graphical images based on results from donor 1.

Donor 2:

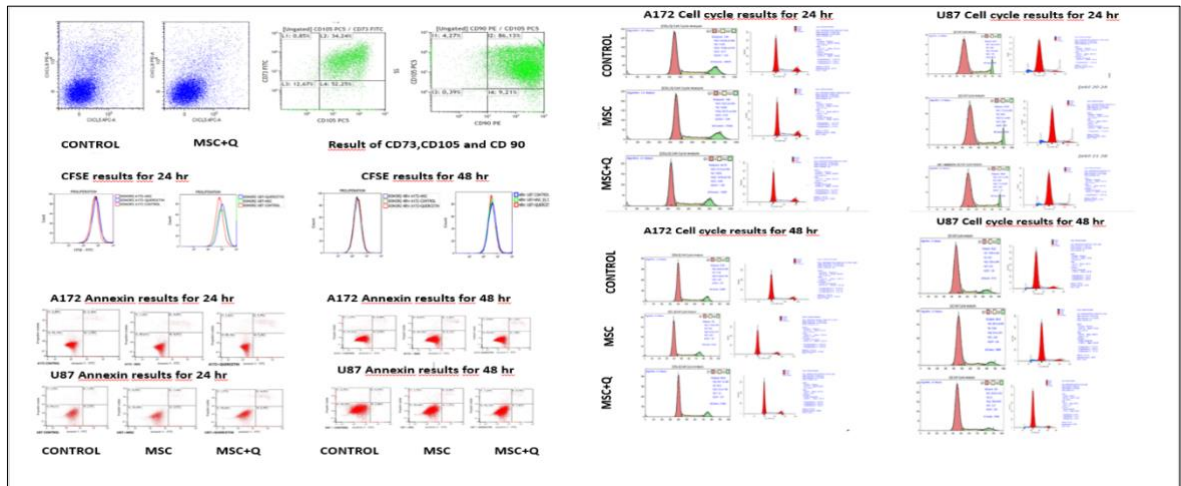


Figure A.2. Graphical images based on results from donor 2.

Donor 3:

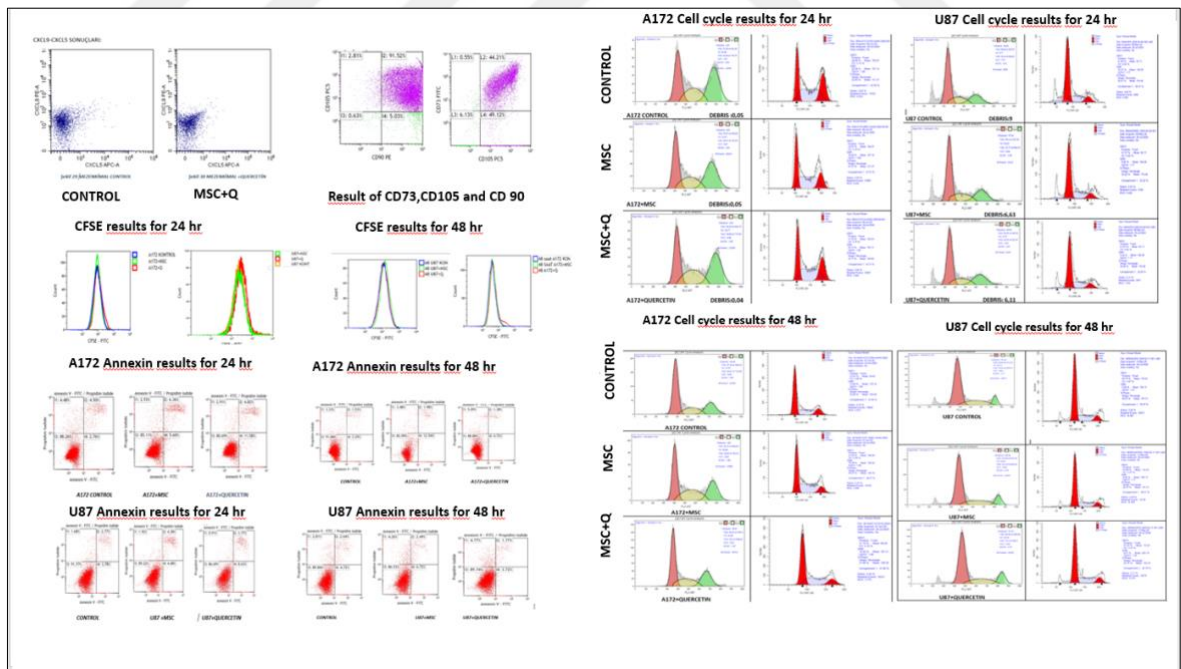


Figure A.3. Graphical images based on results from donor 3.

Donor 4:

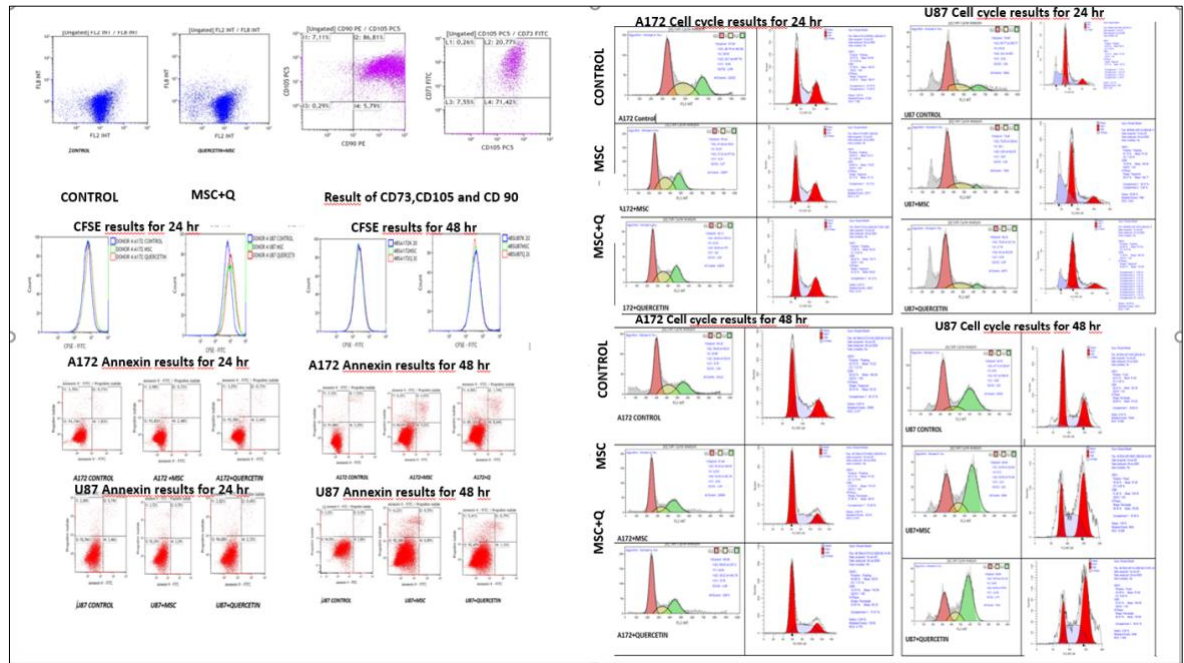


Figure A.4. Graphical images based on results from donor 4.