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Original Research

The effect of microgravity on cell death, cell growth and cell cycle on breast cancer patients

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ABSTRACT:

Breast cancer is the most common cancer among women in different societies. Because life is constructed evolutionary on the gravity of 'g', removed gravity as a variable can lead to clarify many biologic questions. Today, the weightlessness is a new method to study cellular changes. Weightlessness leads to metabolic and functional changes of the human body, and studies have shown that weightlessness leads to changes in growth and gene expression in cancer cells. The aim of this study is to evaluate the effect of weightlessness on apoptosis and cellular cycle in breast cancer cells. The tests of Annexin-V, PI-flow cytometer, and MTT have been used here. Cells of the weightlessness group are cultured on Clinostat prepared by the United Nations, and in gravity of 0.001g. The cell death and apoptosis using Annexin kit, and cell cycle using the PI and flow cytometer were investigated. Also, the amount of cell damage was determined by MTT. The apoptosis results showed that weightlessness leads to a reduction of 40% in apoptosis in cell line MCF-7, and the increase in BT-20 cell line for two times. Apoptosis in cell line MDA-MB-468 was not affected, and the results showed that the cell cycle and growth in cell line ZR-75 increased at a rate of five times (35% of weightlessness group versus 7% of control). Cell growth in the other categories showed no significant difference between two groups. Also, no significant difference was observed in the amounts of cell damage in groups of weightlessness and control.

Keywords:
Breast cancer, weightlessness, apoptosis, cell cycle.

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INTRODUCTION

Cancer is formed because of growth and uncontrolled proliferation of abnormal cells in the body. Cancer is a specific type of genetic disease. Cancer is caused by altered genes. Most of the factors that cause cancer are among the factors that lead to changes in the sequence of DNA or mutations. Finding the fact that cancer is a genetic disease is a great victory for medical sciences (King and Robins, 2006). In terms of genetics, cells are divided into two groups: germ cells and somatic cells. People who inherit cancer genes of germ line have cancer genes of germ line in all their cells, including physical and germ. Such people are carriers. Cancer genes produced in the body cells are not transferred to the next generation (Reddy and Kaelin, 2002). Any source tissue, may give cancer property and distinctive features to the specified area. Approximately, 58 percent of cancers occur in epithelial cells, and are known as carcinomas. Cancer cells with mesodermal origin, (such as bones and muscles) are called sarcoma, and cancers of lump tissue (e.g. breast) are called adenocarcinomas (Alison, 2002). Hanahan and Weinberg, 2000 suggested six main symptoms for many cancers (but not all). They suggested that gaining ability to make autonomous growth messages, avoiding growth inhibition message, and evasion of apoptosis, unlimited replication ability, angiogenesis, invasion and metastasis are essential for carcinogenesis (Hanahan and Weinberg, 2000). Cancer gene leads to the disruption of tissue homeostasis. If the gene increases the rate of cell proliferation, or limit cell maturity or death, cell containing gene is copied and the tumors are formed. This is the first stage of a tumor clonal evolution. Today, breast cancer is the most common cancer among women worldwide, and it has the highest deaths rate by cancer in women. According to statistics, the death rate caused by the disease has increased from 45.2 per hundred thousand in 2006 to 97.1 per hundred thousand in 2010 (Enayatrad *et al.*, 2015).

Also, breast cancer is the most common malignancy in women worldwide; three percent of all cancers and 15 percent of cancer-related death among women is related to it. In Iran, breast cancer forms 21.4 percent of all reported cases. Breast cancer is estimated to be in 22.4 per 100,000 women in Iran, and existing data indicates that the disease has taken an increasing trend.

Using the genes of cancer as cancer biomarkers has very common potential applications, such as determining the stage of disease. Spread of the cancer around a tissue, or metastasis, is the next warning symbol which is important in disease management. Staging, especially in the assessment of breast cancer, is important. Metastatic is the main cause of death due to breast cancer (Antonarakis *et al*., 2000).

Metastasis

Metastasis includes Growth, angiogenesis, and invasion. Tumors are not able to grow more than a few millimeters with no blood supply. Therefore, the production of angiogenic factors by tumor cells, digestion of the area around the cell, entering to bloodstream, leaving the vascular system, connecting to

Table 1. The results of metastasis in three cell lines

MDA-MB-468		20-BT		MCF7		Cell line
LOW metastasis		No Metastasis		Metastasis		
Vacuum	1G	Vacuum	1G	Vacuum	1G	
9.34	8.39	12.6	22.4	13.9	9.33	Q1
2.24	2.28	6.00	7.5	7.3	5.7	Q2
77.01	78.67	6.00	62.20	66.16	66.7	Q3
11.4	10.67	13.00	7.09	12.15	18.00	Q4M
No interpretation		Apoptosis multiplied		Apoptosis is reduced		

Table 2. The results of the cell cycles in three lines

MDA-MB-468		ZR		MCF7		Cell Line
Low metastasis		No metastasis		Metastasis		
vacuum	1G	vacuum	1G	vacuum	1G	
58.5	64.9	57.93	87.26	76.16	72.02	RN1 2n
12.3	8.8	7.38	5.86	3.71	5.07	RN2 4n
29.1	25.7	35.01	7.04	20.86	22.95	RN3 S

the basement membrane, digest basement membrane and extracellular matrix, and migration into the matrix are the steps of metastases. Cancer cells require different receptors, proteases digesting the basement membrane and extracellular matrix, and angiogenic factors. Formed vascular system does not have a uniform structure like healthy and normal vessels, and this is an important factor for further invasion into the general circulatory system (Fidler, 2003).

Microgravity

The different features of the space environment can be effective in the absence of gravity (micro gravity), the existence of cosmic rays, and the lack of circadian rhythm (24 hours). These special conditions have adverse effects on the health and efficiency of astronauts during space travel and missions. On the other hand, space has a special environment for studying biological functions in organisms with different complexity, unicellular and multicellular creatures in wide range.

Although we know some general points about the effects of gravity and weightlessness on the entire organism; but there is a lot of awareness about its impact on the surface of cells and cellular events. The results obtained from these studies could improve the quality of human life on earth, and health also. On the other hand, the study of methods of response of plants, animals and microorganisms in the ecosystems of closed life-support is useful for the development of advanced systems (Clément and Slenzka, 2006). Also, gravity affects transportation within the cell. In fact, the exothermic metabolic processes continuously cause the low density parts of the cell being warm compared with populated cellular areas. Therefore, the heat flow caused by gravity rearranges the structures of cells. Studies also suggested that cell energy efficiency increases, under the influence of gravity. Gravity causes an amorphous distribution of cells, and as a result, a torque is needed to shape and modify the cellular structure again. In fact, energy is used to maintain cell shape against gravity. In microgravity, the energy is stored and used for other

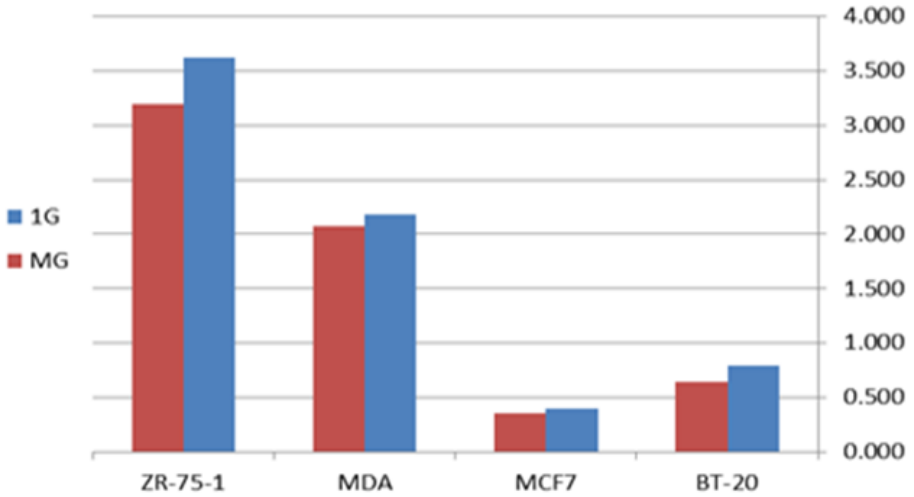
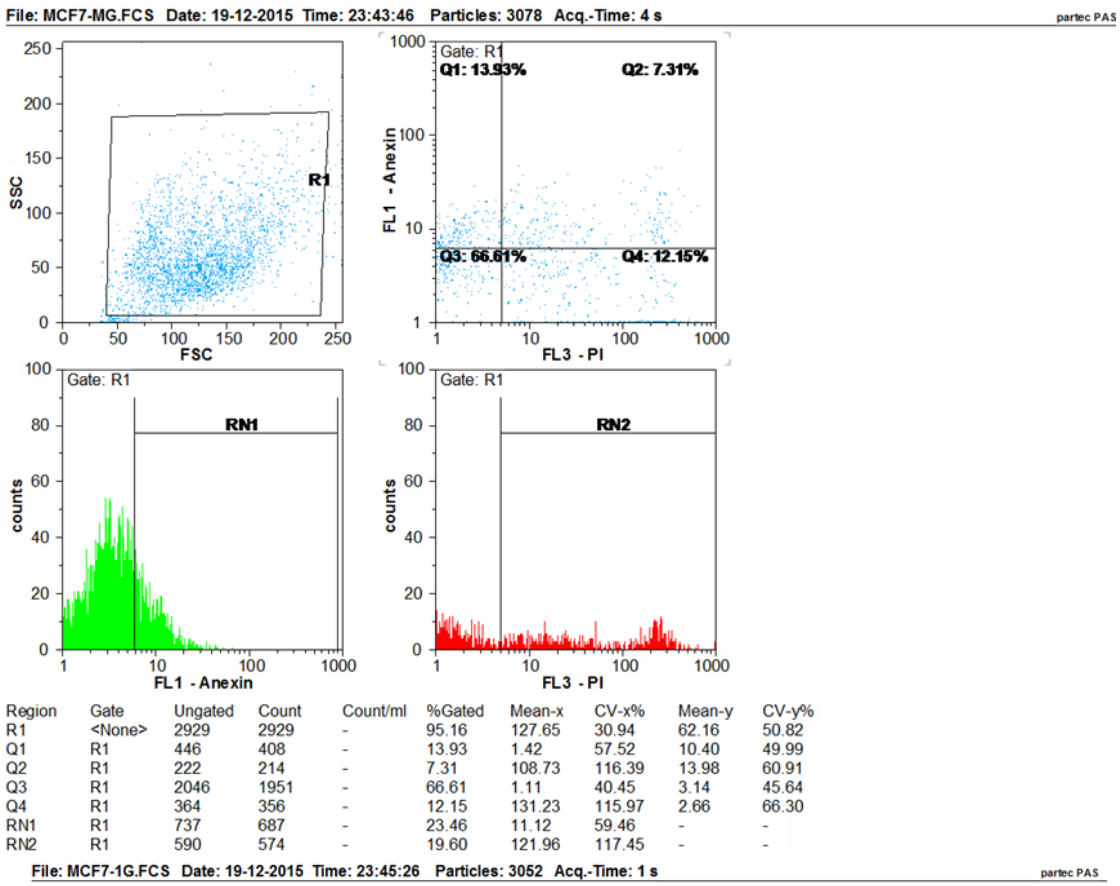
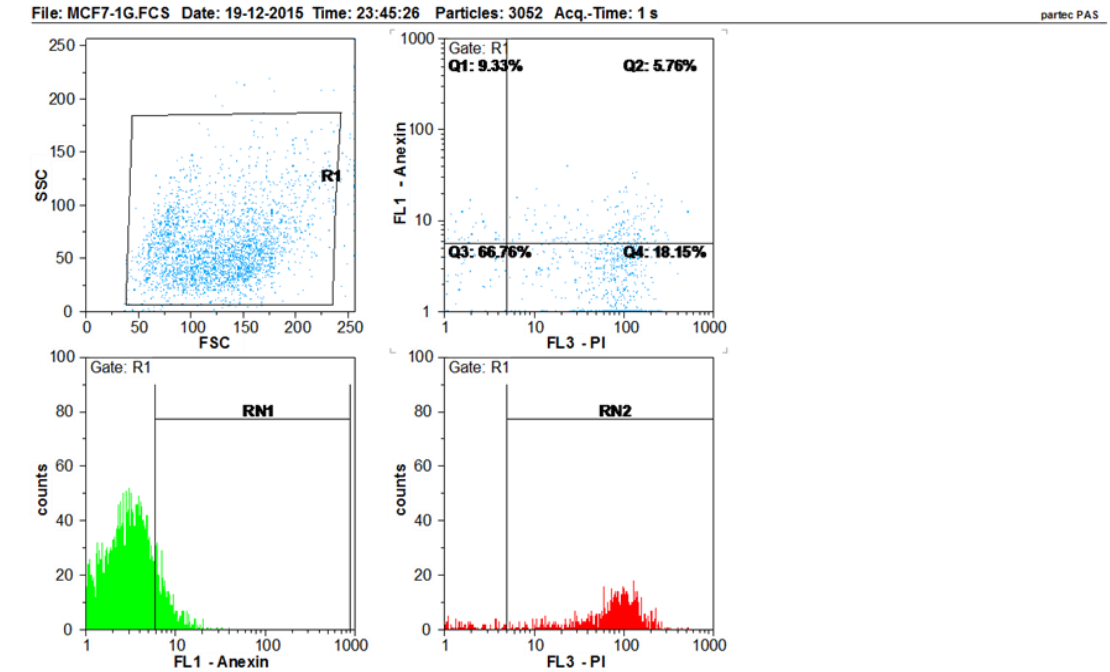


Figure 1. MTT test results; blue columns indicated 1G and red columns indicated MG

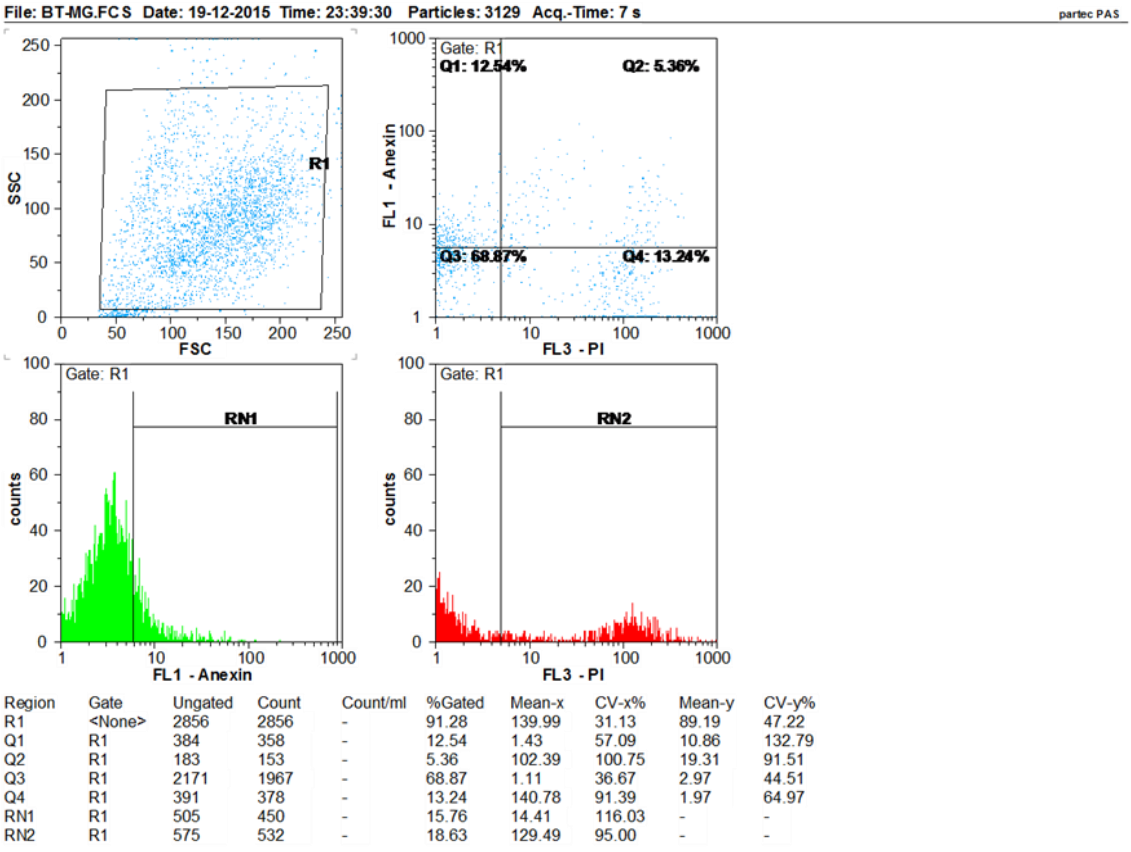


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2929	2929	-	95.16	127.65	30.94	62.16	50.82
Q1	R1	446	408	-	13.93	1.42	57.52	10.40	49.99
Q2	R1	222	214	-	7.31	108.73	116.39	13.98	60.91
Q3	R1	2046	1951	-	66.61	1.11	40.45	3.14	45.64
Q4	R1	364	356	-	12.15	131.23	115.97	2.66	66.30
RN1	R1	737	687	-	23.46	11.12	59.46	-	-
RN2	R1	590	574	-	19.60	121.96	117.45	-	-

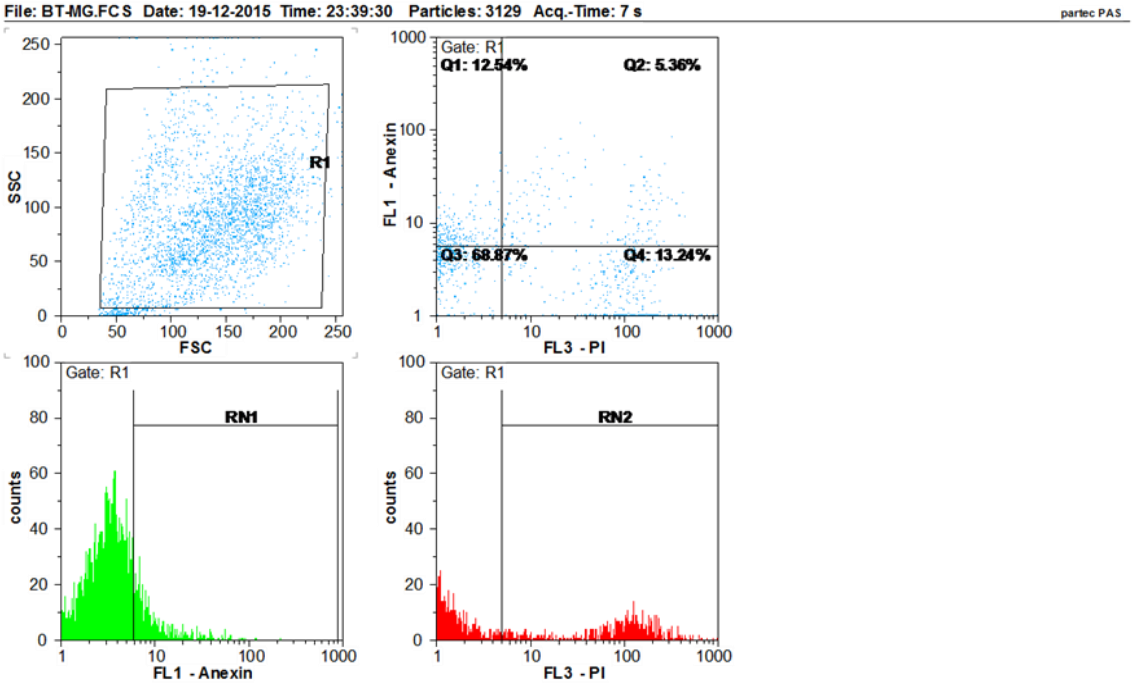


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2969	2969	-	97.28	127.13	32.18	60.36	49.31
Q1	R1	303	277	-	9.33	1.14	47.58	8.01	30.03
Q2	R1	185	171	-	5.76	102.76	80.31	10.30	53.38
Q3	R1	2021	1982	-	66.76	1.03	27.30	2.70	47.85
Q4	R1	543	539	-	18.15	89.62	60.22	2.49	59.75
RN1	R1	445	407	-	13.71	9.19	44.80	-	-
RN2	R1	728	710	-	23.91	92.79	67.09	-	-

Figure 2. Diagram of apoptosis test in MCF-7 cell line under conditions of 1G and vacuum

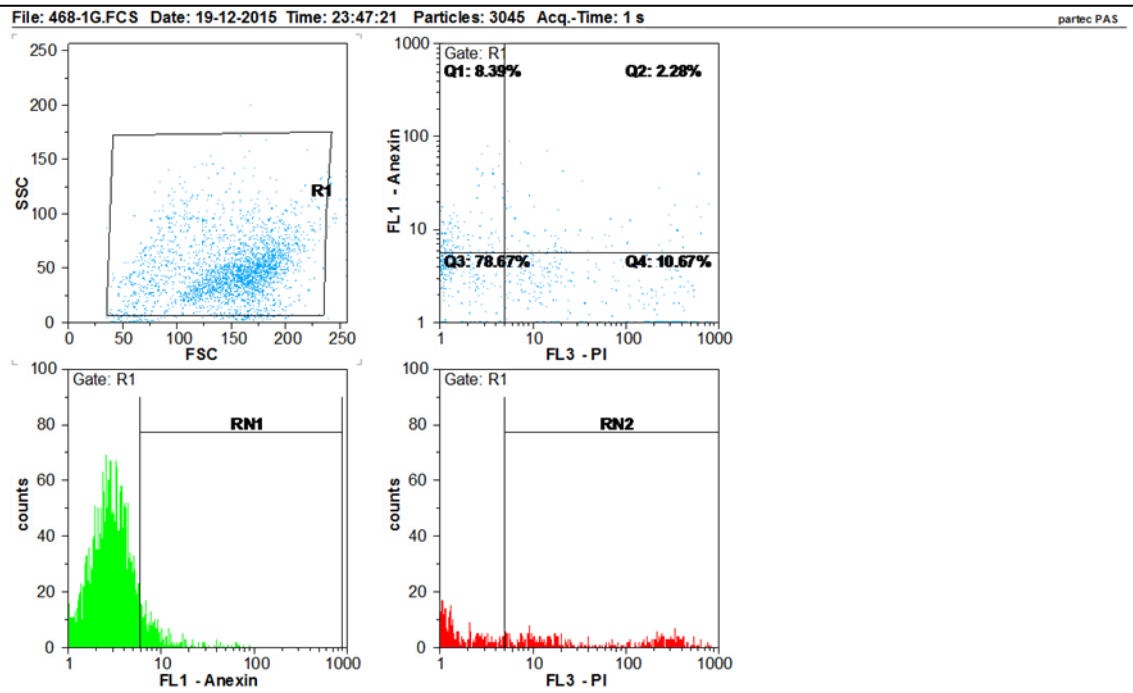


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2856	2856	-	91.28	139.99	31.13	89.19	47.22
Q1	R1	384	358	-	12.54	1.43	57.09	10.86	132.79
Q2	R1	183	153	-	5.36	102.39	100.75	19.31	91.51
Q3	R1	2171	1967	-	68.87	1.11	36.67	2.97	44.51
Q4	R1	391	378	-	13.24	140.78	91.39	1.97	64.97
RN1	R1	505	450	-	15.76	14.41	116.03	-	-
RN2	R1	575	532	-	18.63	129.49	95.00	-	-

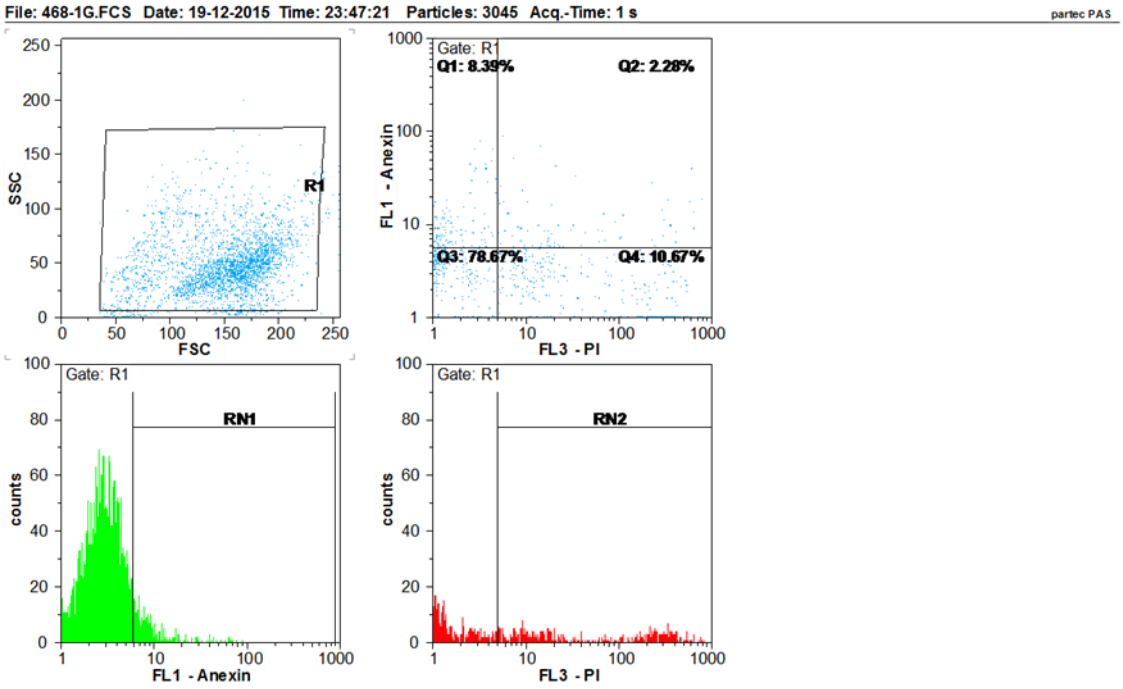


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2856	2856	-	91.28	139.99	31.13	89.19	47.22
Q1	R1	384	358	-	12.54	1.43	57.09	10.86	132.79
Q2	R1	183	153	-	5.36	102.39	100.75	19.31	91.51
Q3	R1	2171	1967	-	68.87	1.11	36.67	2.97	44.51
Q4	R1	391	378	-	13.24	140.78	91.39	1.97	64.97
RN1	R1	505	450	-	15.76	14.41	116.03	-	-
RN2	R1	575	532	-	18.63	129.49	95.00	-	-

Figure 3. Diagram of apoptosis test in cell lines BT-20 in weightlessness and 1G condition



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2897	2897	-	95.14	150.78	25.47	49.88	48.28
Q1	R1	259	243	-	8.39	1.43	61.73	11.41	96.32
Q2	R1	67	66	-	2.28	125.11	155.43	14.30	103.68
Q3	R1	2404	2279	-	78.67	1.08	35.40	2.81	42.07
Q4	R1	315	309	-	10.67	149.21	115.43	2.42	58.02
RN1	R1	294	278	-	9.60	12.71	97.60	-	-
RN2	R1	383	376	-	12.98	144.60	121.87	-	-



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	2897	2897	-	95.14	150.78	25.47	49.88	48.28
Q1	R1	259	243	-	8.39	1.43	61.73	11.41	96.32
Q2	R1	67	66	-	2.28	125.11	155.43	14.30	103.68
Q3	R1	2404	2279	-	78.67	1.08	35.40	2.81	42.07
Q4	R1	315	309	-	10.67	149.21	115.43	2.42	58.02
RN1	R1	294	278	-	9.60	12.71	97.60	-	-
RN2	R1	383	376	-	12.98	144.60	121.87	-	-

Figure 4. Diagram of apoptosis test in MDA-MB-468 cell line in vacuum and 1G condition

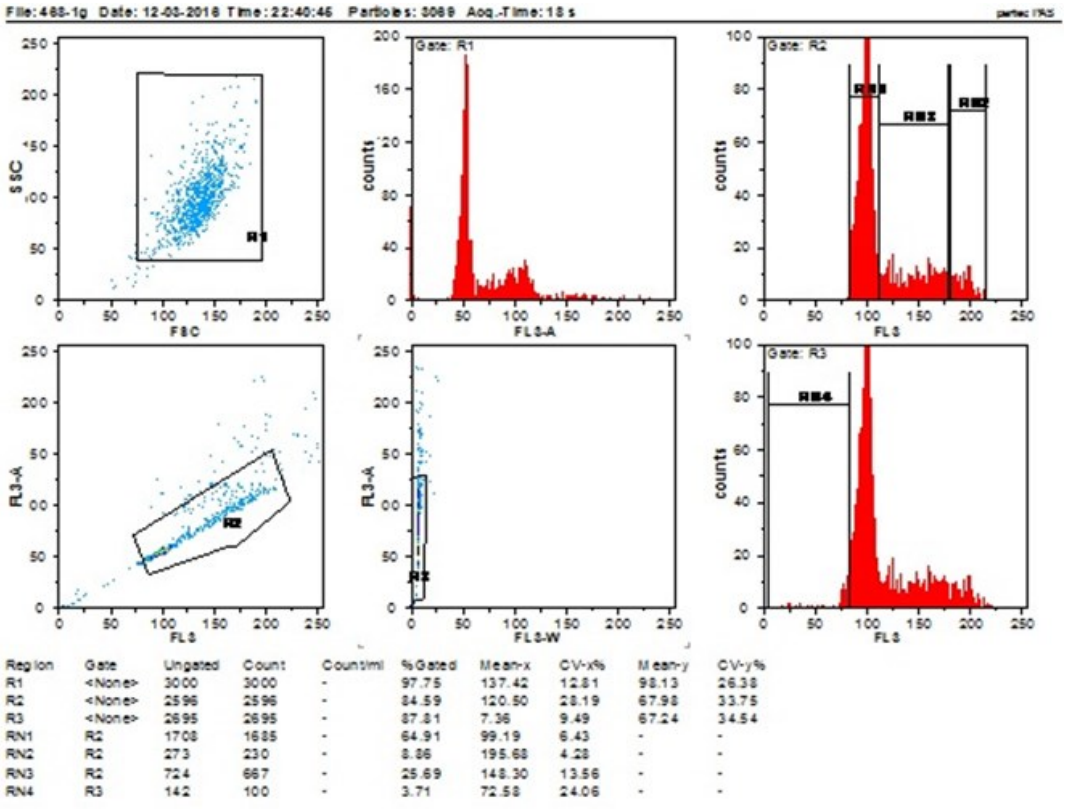
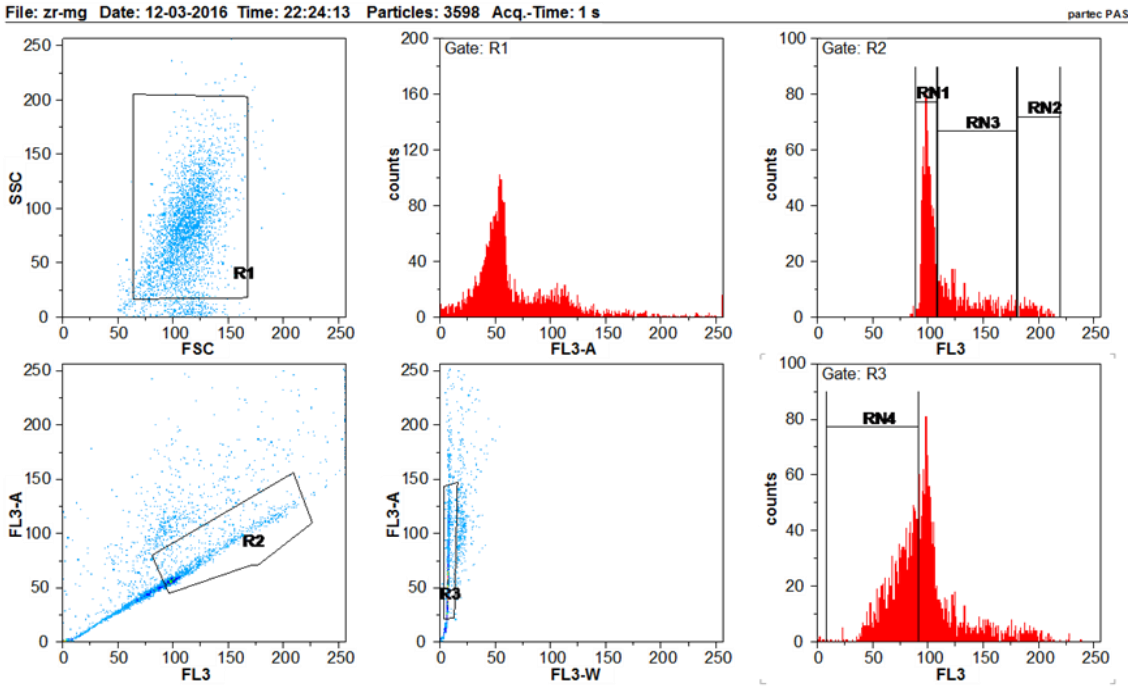
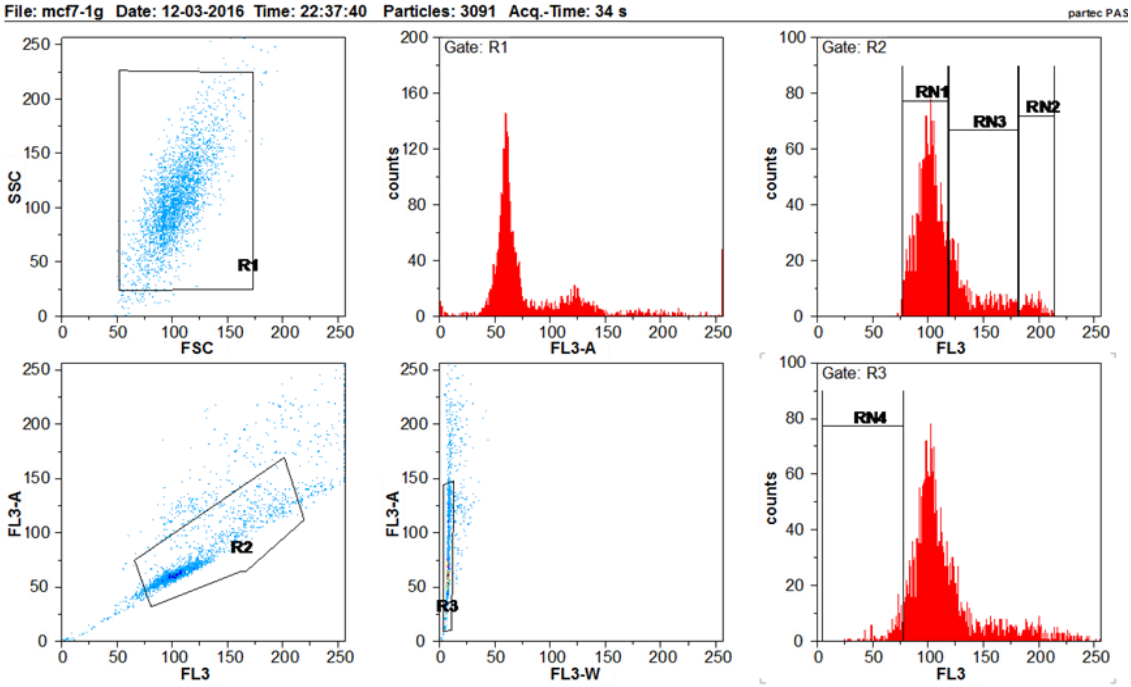


Figure 5. The diagram of cell recycle in the cell line MDA-MB-468 at vacuum and 1G condition

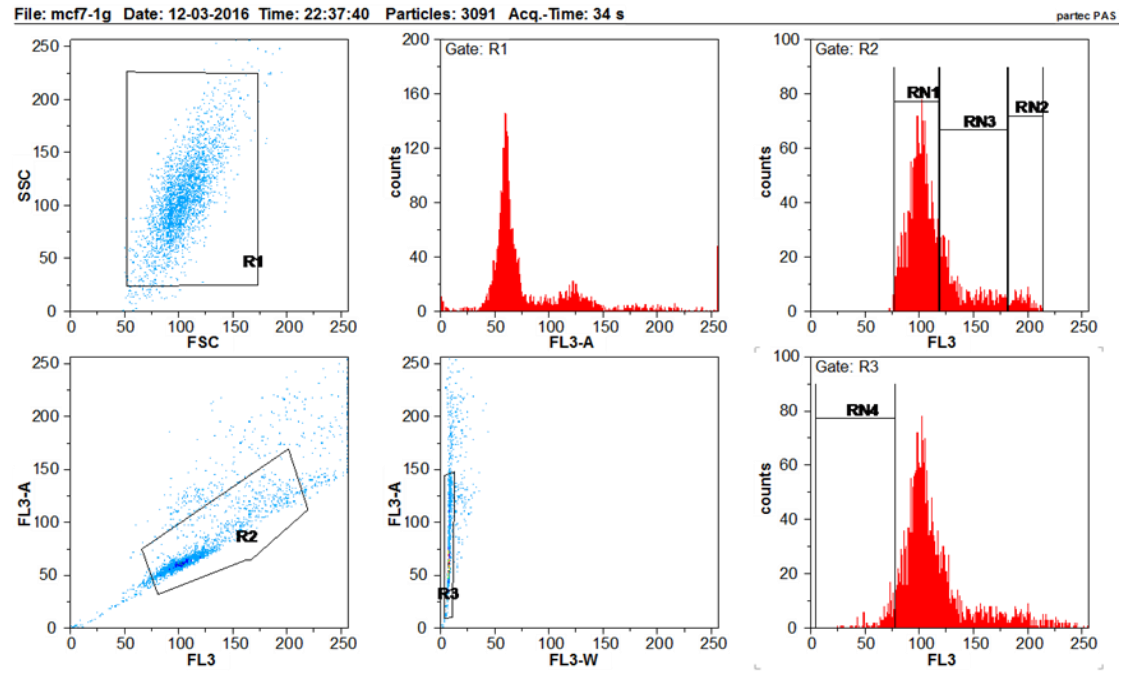


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	3238	3238	-	89.99	112.70	15.46	83.30	41.88
R2	<None>	1274	1274	-	35.41	119.82	25.40	71.74	29.51
R3	<None>	2605	2605	-	72.40	7.54	12.27	60.45	41.01
RN1	R2	1090	738	-	57.93	100.15	4.12	-	-
RN2	R2	131	94	-	7.38	194.94	4.21	-	-
RN3	R2	602	446	-	35.01	136.10	15.49	-	-
RN4	R3	1543	1174	-	45.07	72.61	19.96	-	-

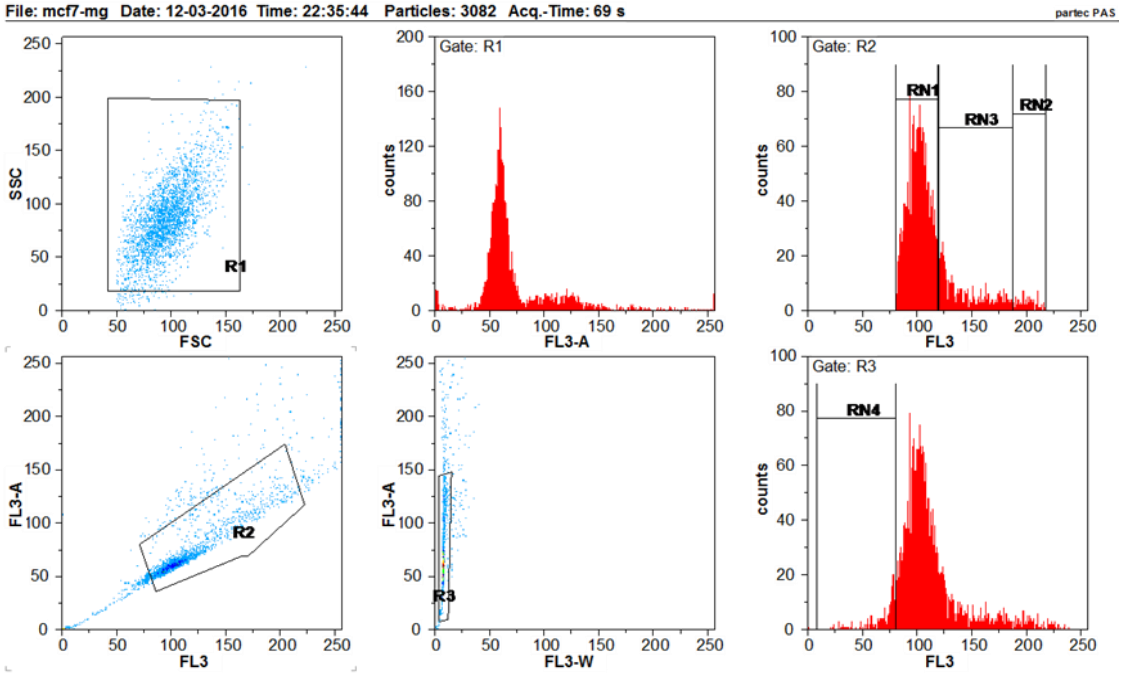


Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	3002	3002	-	97.12	104.48	17.20	107.36	31.52
R2	<None>	2327	2327	-	75.28	114.19	24.84	70.15	30.16
R3	<None>	2539	2539	-	82.14	7.94	10.02	71.03	34.95
RN1	R2	1754	1676	-	72.02	100.24	9.89	-	-
RN2	R2	174	118	-	5.07	196.14	3.72	-	-
RN3	R2	626	534	-	22.95	140.41	13.28	-	-
RN4	R3	168	143	-	5.63	66.15	19.27	-	-

Figure 6. The diagram of cell recycle test at cell line ZR in vacuum and 1G condition



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	3002	3002	-	97.12	104.48	17.20	107.36	31.52
R2	<None>	2327	2327	-	75.28	114.19	24.84	70.15	30.16
R3	<None>	2539	2539	-	82.14	7.94	10.02	71.03	34.95
RN1	R2	1754	1676	-	72.02	100.24	9.89	-	-
RN2	R2	174	118	-	5.07	196.14	3.72	-	-
RN3	R2	626	534	-	22.95	140.41	13.28	-	-
RN4	R3	168	143	-	5.63	66.15	19.27	-	-



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	3003	3003	-	97.44	96.39	20.18	86.20	34.63
R2	<None>	2315	2315	-	75.11	113.65	24.06	69.51	29.67
R3	<None>	2554	2554	-	82.87	7.91	11.34	68.60	33.57
RN1	R2	1834	1763	-	76.16	101.32	9.25	-	-
RN2	R2	107	86	-	3.71	201.63	3.79	-	-
RN3	R2	542	483	-	20.86	143.46	14.39	-	-
RN4	R3	208	178	-	6.97	67.45	22.53	-	-

Figure 7. The results of cell recycle at three cell lines

things, such as duplicating or biosynthesis of the cell. Finally, cells that are normally floated in the culture environment must consume some energy to be prevented from deposition. This energy is not necessary in the weightless environment (Lewis, 2004).

Apoptosis

Apoptosis is a common form of cell death, which in most tissues controls the number of cells during development, and also a wide range of pathological and normal conditions (Thompson, 1995). For apoptosis, other terms are also used which are: controlled cell death, cell death, physiological/ biological cell death, and programmed cell death. Molecule and cell pathologists and biologists demonstrated the main biological importance of Apoptosis just in the late 1980s (Arends *et al*., 1990).

Hosseini investigated the effects of Apoptotic (Emodine-Aloe) on the MCF-7 class cell. Increasing the concentration of Emodine- Aloe reduced the cell viability associated with the dose and time. The maximum effect of Emodin-Aloe was associated with 100 µmol concentration and 72 hours after treatment of cells. Induced apoptosis and expression of FAS were dose-dependent either. 100 µmol concentration of Emodin-Aloe showed the highest percentage of apoptosis and the highest expression of the Fas activity in MCF-7 cells. The results represented that Emodine-Aloes had anti-cancer properties which are useful in treating breast cancer (Hosseini *et al*., 2014).

However, more studies particularly *in vivo* investigations are needed. On analyzing the apoptotic effect of silibinin on MCF-7 class cells of breast cancer, the assessment of cell viability with MTT method showed that silibinin reduced the growth of MCF_7 cells associated with time and dose. Collectively, the results of this study support the effectiveness of this herbal supplement against breast cancer. Moreover, as a result of being natural product, low cost and the possibility of public access, using this herbal supplement in the diet may be effective in the treatment of breast cancer. Bond *et al*. (2002) showed that it is connected to the death of modulation receptor and activation of the apoptosis pathway type (2) (Bond *et al*., 2002).

Mohammadi and Baradaran (2015) investigated the effect of apoptotic dichloromethane extract of (Urtica dioica) on breast cancer class MDA-MB-468 cells. The results of MTT test showed that dichloromethane extract of (Urtica dioica) can destroy cancer cells. Additionally, the results showed that treatment of MDA-MB-468 cells with dichloromethane extract of (Urtica dioica) causes induction of apoptosis in these cells and this extract is useful for treating cancer (Mohammadi and Baradaran, 2015).

MATERIALS AND METHODS

Annexin-V, PI-flow cytometer, MTT tests was used in this research. Cell lines have been purchased from genetic resources.

Creating a vacuum environment

The extracted cells were grown *in vitro* to more than 15 passages, samples consisted control cells (G1 or in the presence of gravity) and samples for vacuum assays (1 day, 2 days or 3 days). The pressure of vacuum condition set at 0.001G. To create vacuum condition the clinostat two-axis device was used in Aerospace Research Institute. After sterilizing the device using UV and ethanol 70%, cells were then cultured in 96-well plates (containing 10% FCS) and incubated.

The cell cycle test (using the coloring PI)

To measure the cell ratio in each interphase steps using nucleic acid bounded by PI (Propidium Iodide) was applied.

Test MTT (using kit MTT)

To check the viability of the cells or cells metabolic activity, MTT test kit of ATOCEL Company and Cat No: ABM21-P1 was used (Gilberto *et al*., 2012).

Culturing of 50 thousand cells at 96 wells

- The powdered MTT 5 mg/ml milligrams was

deissolved and filtered. The resultant solution with a 0.2-micron filter was extracted.

- The cells under vacuum condition were taken into consideration.
- 20 µl solution of MTT to each well were added and mixed gently.
- The sample for 3 to 4 hours to find the emergence of a purple precipitate was incubated
- The medium was gently removed.
- 200 ml of DMSO was added to each well and pipetted gently.
- The absorbance was read at a 570 nm using ELISA reader.

Apoptosis test (using AnnexinV kit)

Annexin V a protein molecule binding to phospholipids in the presence of calcium ions. This material has a high affinity for molecule PS. Therefore, it is very suitable for the detection of cells in apoptosis among a cell population (and not in the tissue). Annexin-V-FlUOS kit also contains an article Annexin V and PI color that allows distinguishing apoptosis cells from necrosis cells.

- The cells with about 300 g for 5 to 10 minutes were collected.
- Cells were washed with cold PBS
- 1X binding buffer was diluted from 1 to 10 with diluted water and for each sample 400 micro-liters was prepared. The composition of binding buffer is as follows; Annexin in calibration reagent- 100UL, 10X Binding Buffer- 10UL, PI- 10UL, Annexin (v-fitc)-UL and H^2O- 79UL
- The cells were suspended in a solution prepared in the previous step (100 thousand to 1 million cells per 100 microliters) and incubated for 15 min at room temperature in the darkness.
- 400UL 1X binding buffer was added to mix cells.
- Flow cytometry was read after an hour (Darzynkiewicz *et al*., 2001).

RESULTS

MTT test was based on measurement of the amount of substance formazan. The MTT oxidized by cytochrome chain within the mitochondrial membrane and synthesis Formosan crystals. Therefore, the higher the metabolic rate of the cells leads to the higher oxidation of MTT, subsequently synthesis of formazan getting higher. Thus, the formazan production is indicators of cell health and growth.

This chart (Figure 1) is the result of MTT test. The blue columns are control samples and the red columns are the samples under vacuum condition. The higher value of the red columns represent the greater percentage of vital cells under the vacuum condition although this difference is not significant. In fact, it can be said that the vacuum condition didn't affect the cell life.

Results of metastasis with Annexin kit

Annexin test diagram was divided into two categories: 1G and MG. The green diagram indicates Annexin penetration into cells and the red graph represents the PI.

Q1: Just been painted by Annexin

Q2: Just been painted by Annexin and PI (Late Apoptosis)

Q3: Living cells

Q4: Just been painted with PI (Necrotic)

According to the tables and related diagrams (Table 1, Figure 2, Figure 3, Figure 4), apoptosis results showed that the vacuum condition leads to a reduction of 40% apoptosis in MCF-7 cell line, and the double enhancement of BT-20 cell line. Apoptosis in MDA-MB -468 cell line was not affected.

Cell recycles result

The result of cell recycle and growth (Table 2, Figure 5, Figure 6, Figure 7) showed that in ZR-75 cell line had increased to five times (35% vacuum group against 7% control group). The cell growth in other categories didn't show any significant difference. Also,

the amount of cell damage was not significant among four cell lines in both experimental and control groups.

DISCUSSION

According to the findings of research it can be concluded that:

Breast cancer is the most common malignancy in women worldwide, and its incidence is increasing in many countries including Iran.

Space biology studies increased our knowledge of the function of organisms and essential biological reactions. During the evaluation, life on earth expanded under 1g gravity. The effect of this power over life has not been studied very well yet. Since the gravity has influenced on biological processes as a variable, the omission of it can clarify some questions which exist in this field. Biological studies of Clément and Slenzka (2006) predicted that little gravity can be effective for improving the quality of life and human health on earth. For example, a study in declining bone mass in astronauts can be used for development of treatment ways of osteoporosis (Clément and Slenzka, 2006).

Apoptosis test showed that vacuum condition resulted in the reduction of 40% apoptosis and a doubled enhancement in MCF-7 BT-20 cell line. The amount of Apoptosis in MDA-MB-468 cell line didn't change. Bond *et al*. (2002) showed that it is related particularly with the death of receptor modulation and activation of Apoptosis pathway type II.

The result of the Annexin test showed that although the amount of apoptosis in cultured cells under vacuum condition increased, there wasn't any significant difference with control. The amount of Apoptosis in MCF-7 cell line and under vacuum condition was 21, 241% while under normal condition was 15, 09%. In MA-MB-468 cell line, the amount of apoptosis under vacuum simulation condition was 11.58% and under normal condition was 10.67%.

Under vacuum condition, important changes had happened like lack of sediment and sedimentation, change in convection currents, and hydrostatic pressure among cultured cells as mentioned before, vacuum condition affected the genes expression and changed the expression in relative to the normal gravity (1G).

The effect of vacuum on cancer varies at different stages so that in the early stages of tumor (BT-20) leads to increased apoptosis and in metastasis MC-7 line the death of cells have reduced. Also, it had not effect on MDA-MB-468 cell line with low metastatic ability. Therefore, vacuum condition can be used as a method to study about how cancer begins and promotes. Seemingly, in the growth and the cell recycles, the effect of vacuum condition on cancer varied at different stages so that it leads to increased cell division in tumor cells that didn't have metastatic activities. Apoptosis is a highly regulated process that plays an important role in maintaining homeostasis in multicellular organisms. It has been found that apoptosis is controlled by many intracellular and intercellular factors.

In this study, to evaluate apoptosis, Annexin V kit and flow cytometry were used. Apoptosis caused some morphological changes in cells such as loosing membrane connectors; nucleus and cytoplasm condensation and DNA break in nucleosomes parts. One of the earliest events that happen in a cell during apoptosis is displacement of the phospholipid phosphatidylcholine Serine (PS) from inner surface of the cell membrane to outer surface of the cell membrane. Consequently, the connection place of Annexin V molecules are exposed to the environment. According to Annexin V- affinity to PS, it can be labeled by materials such as biotin or fluorochromes such as FITC, PE, APC, or CYC5 in order to measure the apoptosis process in early stages by the simple technique of flow cytometry. Since PS replacement is also happening in necrosis process, Annexin V is not a dedicated marker for Apoptosis. For this reason, vital colors such as 7-ADD or PI are being used with Annexin V.

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