



## The effect of amniotic membrane on growth, proliferation, and survival of the myeloma cells and examination of genes related to proliferation (BCL2), implantation (CXCR4), and cell cycle stop (P21 and P27)

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### Original Article

#### Abstract

**BACKGROUND:** The myeloma cell is not able to grow and proliferate out of bone marrow (BM) media, and in laboratory conditions its survival is low. We considered to use an environment that has the same conditions as body physiological conditions. In this study, the effect of the amniotic membrane (AM) on the growth and proliferation of myeloma cells were evaluated.

**METHODS:** This study was performed on plasma cells derived from BM aspiration (primary cells) in 3 patients with multiple myeloma (MM). Plasma cells of these patients were isolated by magnetic-activated cell sorting (MACS) technique and cultured in different environments of AM for two consecutive weeks, and then were examined by qualitative polymerase chain reaction (PCR) technique for expression of genes related to proliferation [B-cell lymphoma 2 (BCL2)], implantation [chemokine receptor type 4 (CXCR4)], and cell cycle stop (P21, P27).

**RESULTS:** Isolated plasma cells were cultured in 3 different groups for 2 weeks. The most cell proliferation was observed in the medium containing Roswell Park Memorial Institute (RPMI) medium from amniotic cultures and plasma cells [an environment without fetal bovine serum (FBS)]. All genes were expressed on day zero (on the day of isolation). On the day 4, proliferation genes (BCL2) and implantation genes (CXCR4) had an expression in the control group without FBS medium, but P21 and P27 genes had no expression.

**CONCLUSION:** The best environment for the growth and maintenance of plasma cells in vitro is the use of RPMI from the AM (without FBS) in which plasma cells can be kept alive for 10 days.

**KEYWORDS:** Amniotic Membrane, Multiple Myeloma, Cell Cycle

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### Introduction

Multiple myeloma (MM) is a type of plasma cell malignancy that accounts for about 10% of blood cancers. Patients with MM suffer from osteoporosis and severe bone pain. Other MM symptoms include the secretion of osteoclast activating factors (OAF) from malignant plasma cells, renal impairment due to increased calcium levels and Bence-Jones protein (BJP)

excretion, anemia due to replacement of malignant cells in the bone marrow (BM) and consequently hematopoiesis impairment, and recurrent infections due to inefficient production of immunoglobulins (Igs).<sup>1</sup>

MM microenvironment consists of clonal plasma cells, extracellular matrix (ECM), proteins, and stromal cells of BM.<sup>2</sup>

Myeloma cells express chemokine receptor type 4 (CXCR4), which binds to stromal cell-derived factor 1 (SDF-1), leading to the localization of myeloma cells in BM. SDF-1 is

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expressed in stromal cells of BM, and by signaling through CXCR4, it causes the migration and implantation of hematopoietic cells. SDF-1 increases the Interleukin-6 (IL-6) and, as a result, increases the proliferation of the myeloma cells.<sup>3</sup>

One of the most effective cytokines in the growth of myeloma cells is IL-6, which by phosphorylation of the Janus Kinase-Signal Transducer and Activator of Transcription protein (JAK-STAT) and Mitogen Activated Protein Kinase (MAPK) pathways, causes the growth of myeloma cells and acts as autocrine. Other cytokines that affect myeloma cell growth include insulin-like growth factor 1 (IGF-1), tumor necrosis factor (TNF), IL-10, IL-15, IL-21R, IL-21, and interferon alfa (IFN- $\alpha$ ).<sup>4,5</sup>

Myeloma cell growth is an angiogenesis-dependent process controlled by pro-angiogenic agents. One of these factors is vascular endothelial growth factor (VEGF) with a high binding affinity to VEGF receptor (VEGF-R).

For starting the process of angiogenesis, the VEGF and epidermal growth factor (EGF) are essential, and myeloma cells secrete VEGF directly.<sup>6,7</sup>

As mentioned, the myeloma cell needs proper environment and conditions to maintain its survival. The close interaction of myeloma cells with BM stromal cells, growth factors, and various signaling pathways plays a very important role in the development of myeloma. Given that outside of this environment and *in vitro*, the myeloma cell is unable to grow and reproduce and will not survive, and on the basis of many previous studies about MM done on the cell line, there is no strong data on the primary cell; so, we decided to design a suitable environment similar to physiological environment of the body for growth and reproduction of myeloma cell. As a result, the effect of amniotic membrane (AM) on growth, proliferation, and survival of myeloma cells has been investigated.

AM has been used for many years as clinical scaffolds as well as cellular scaffolds due to its many biological properties.

Finally, in order to investigate the proliferation, implantation, and apoptosis of myeloma cells, the gene expressions associated with these processes have been evaluated.

## Materials and Methods

The AM sample was taken in sterile conditions from healthy women during cesarean section with patients' satisfaction in Bahman Hospital, Tehran, Iran. The AM sample was transferred to the laboratory under sterile conditions inside a 50ml Falcon tube containing sterile phosphate-buffered saline (PBS) buffer and antibiotic at 4 °C (with ice). After clearing the AM, the curtain was cut into small pieces using a razor blade and a pencil, then placed in Roswell Park Memorial Institute (RPMI) 1640 medium flasks and cultured for 10 days at 37 °C in an incubator. The RPMI medium was then collected and stored at -80 °C.

BM aspiration samples of patients with MM, referring to the hematology department of the Army and Imam Khomeini Hospitals, Tehran City, based on the patients' satisfaction and permission of the Medical Ethics Committee, were collected to 6 cc in sterile vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and transferred to a university lab in an ice bag.

All of these patients were suspected as cases of MM. Therefore, to confirm the presence of myeloma cells, the results of flow cytometry analysis and aspiration smear were revised.

The mononuclear cells (MNCs) of the BM were separated by ficoll based on the difference in density with other blood cells.

BM aspiration specimens were diluted (1:7) in sterile PBS. Then, the diluted sample was slowly transferred into the ficoll (the same volume of the sample), so that it was not mixed with the ficoll. In the next step, the Falcon tube containing the blood and ficoll was

centrifuged for 35 minutes at 445 rpm at 20 °C without brake and acceleration. After the four-layer centrifugation in the Falcon tube consisting of 1) plasma, 2) MNCs, 3) ficoll, and 4) red blood cells (RBCs) and neutrophils, we gently separated the MNCs and accurately transferred them to a 50ml conical Falcon tube.

The cells were washed with 40 ml of PBS buffer, completely mixed, and then centrifuged at 3000 rpm for 10 minutes at 20 °C. Then, we discarded the supernatant precisely.

To remove the platelets, we remixed the cells in 50 ml buffer, and after centrifugation at about 200 g for 10-15 minutes at 20 °C, carefully removed the supernatant (this step increases the cell's purity).

We mixed the cell pellet for later use in a proper volume of buffer.

**Plasma cell isolation with Syndecan-1 (CD138) index using magnetic-activated cell sorting (MACS) technique:** At this stage, the rapid operation and keeping the cells cool prevented non-specific labeling of the cells.

At first, 8 µl of CD138 microbial antibody was added to the pellet in dark and mixed, and the falcon tube containing the sample was completely covered with foil. The specimen was placed at 2-4 °C for 15 minutes. Then, the cells were washed with sterile PBS buffer (1-2 ml) and centrifuged for 10 minutes at 20 °C with 300 g. Supernatant was discarded precisely. At the next step, we passed the cell pellet from the LS column.

**Plasma cell culture in different environments from AM:** 1. The first group, which was the control group, contained RPMI, 10% fetal bovine serum (FBS), and plasma cells.

2. The second group contained RPMI derived from AM cultures, 10% FBS, and plasma cells.

3. The third group contained RPMI derived from AM cultures and plasma cells.

**Cell count using Neubauer chamber slide:** Under sterile conditions by slow pipetting, 10 µl of the suspension was removed from the

medium and the same volume of trypan blue 0.4% solution in isotonic buffer with PH = 7 was added. So, the cells were diluted 2 times. Then, both sides of the Neubauer slide were filled with it, and we waited a few moments to settle down the cells and then studied it with a light microscope at 20 magnification.

**Preparation of cellular deposition of the isolated plasma cells for extraction of ribonucleic acid (RNA):** Some amount of the sample was poured into 1.5ml microtubes and centrifuged at 3000 g for 10 minutes at 20 °C.

**RNA extraction:** 1 ml of RNX-Plus was added to the suspended or sediment cells, the cells were transferred to a 1.5ml microtube, and vortexed for 1 minute; after the vortex, the mixture was placed at room temperature for 5 minutes.

The homogenized solution was transferred to a 1.5ml microtube and 200 µl chloroform was added to each ml of RNX-Plus; tubes were recapped firmly and mixed vigorously with hand or vortexed for 15 seconds, then incubated for 5 minutes at 4 °C. It was then centrifuged for 20 minutes at 4 °C at 12000 g. The three layers formed after centrifugation were the upper aqueous or blue phase containing RNA, the intermediate layer containing deoxyribonucleic acid (DNA), and the underlying layer or the organic phase containing proteins.

Then, the RNA-containing aqueous phase was transferred to a 1.5ml RNase-free microtube by means of RNase-free micropipette tips such precisely that there was no contact with the intermediate layer. The same as the volume of the aqueous or upper phase, 100% isopropanol was added to the microtubes, then gently mixed and placed overnight at -80 °C.

Samples were taken out of the freezer and then centrifuged at 12000 rpm for 20 minutes at 4 °C.

To completely remove chloroform, RNA extract was washed with 70% ethanol. In order to wash RNA, 1 ml of 70% ethanol per each ml

of RNX-Plus was added to each microtube, and the contents of the tubes were well mixed. Then, microtubes were centrifuged for 20 minutes at 4 °C with 12000 g, and supernatant solution was excluded.

RNA should not be completely dried because its solubility is reduced. For partial drying, the microtube tip was opened for a few minutes and before complete evaporation of ethanol, RNA was dissolved in 20 µl of RNase-free distilled water. Then, the microtube was incubated for 10 minutes at 55 to 60 °C.

**Quantitative evaluation of the extracted RNA:** Optical absorption of RNA samples was read by a biophotometer at wavelengths of 280 nm/260 nm and 260 nm/230 nm.

To read the concentration and absorption of the sample, we dissolved 1 µl of the sample in 49 µl of RNase-free water and multiplied the obtained concentration in the dilution factor (50). So, we would find out 1 µl of the dissolved RNA had how many micrograms of RNA. To determine the purity of RNA, the optical absorption in 280 nm/260 nm should be more than 1.8. If the light absorption of 280 nm/260 nm is less than 1.8, it indicates the RNA contamination with the protein, and it should be re-purified.

The optical absorption of RNA samples at a wavelength of 280 nm/260 nm was between 1.80 to 1.95, indicating the high purity of the RNA samples.

RNA was stored at -80 °C. If the RNA is

precipitated in ethanol, it will not lose its integrity for a long time at -20 °C.

**Synthesis of complementary DNA (cDNA):**

Using the cDNA kit (purchased from Yekta Tajhiz Azma Co., Tehran, Iran), the cDNA was built from RNA according to the manufacturer's kit.

To say briefly, at first, 1 µl of random hexamer was added to an RNA-containing solution, and the microtube was incubated for 5 minutes at 70 °C, the buffer mixture was prepared according to protocol:

4 µl = first standard buffer

1 µl = deoxynucleoside triphosphate (dNTP)

1 µl = moloney urine leukemia virus (M-MLV)

We poured the buffer mixture into each tube and filled the volume up to 20 µl. If the volume does not reach 20 µl, it should be filled with distilled or sterilized water to 20 µl. Then the microtubes were spinned shortly and incubated for one hour at 42 °C. The cDNA was maintained at -20 °C.

**Polymerase chain reaction (PCR)**

All the reagents and solutions used in PCR were stored at -20 °C in freezer. To carry out the PCR, the reagents and solutions were removed from the freezer and placed on the ice. After defrosting of the reagents, Master Mix was prepared according to the number of the samples.

The PCR solution contained 10 µl ampliqon Master Mix, 0.5 µl primer, 1 µl cDNA, and 8.5 µl distilled water (Table 1).

**Table 1. Specifications for utilized primers**

Name	Sequence	Products length
CDKN1A-F(P21)	CCA GCA TGA CAG ATT TCT ACC	150
CDKN1A-R(P21)	AGA CAC ACA AAC TGA GAC TAA GG	150
H CXCR4(F)	CGC CAC CAA CAG TCA GAG	176
Both variants		
H CXCR4(R)	AAC ACA ACC ACC CAC AAG TC	176
Both variants		
H-P27-F	CA ACC GAC GAT TCT TCT AC	204
H-P27-R	TGT ATA TCT TCC TTG CTT CAT C	204
H-BCL2-F	CAGGATAACGGAGGCTGGGATG	70
H-BCL2-R	AGAAATCAAACAGAGGCCGCA	70
HPRT1-F	CCT GGC GTC GTG ATT AGT G	125
HPRT1-R	TCA GTC CTG TCC ATA ATT AGT CC	125

**Electrophoresis of PCR samples on an agarose gel:** To prepare 30 ml of agarose solution, we added 0.6 g of agarose powder to 30 ml of Tris/Borate/EDTA (TBE) buffer at a concentration of 1X in a 100 ml Erlenmeyer flask, allowing it to be produced. Then, we boiled the suspension in the microwave until it was cleared and all agarose particles were solved in.

Then we placed the gel tray in a flat area and placed the comb on the gel tray. When the molten agarose temperature dropped slightly and there was no vapor, 2  $\mu$ l of the green viewer dye was added to the agarose solution and dissolved.

We poured the agarose solution in the gel tray and carefully removed the gel comb after solidifying, and placed it in the electrophoresis tank containing TBE buffer at a concentration of 1X, so that the surface of the gel was completely covered by buffer.

Then, we added 10  $\mu$ l of each PCR product in wells. A well is assigned as the DNA molecular weight index, which determines the size of the PCR product.

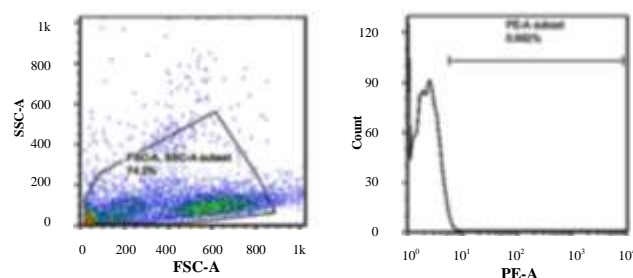
The Mini Run Gel Electrophoresis System has two potential differences between 50 V and 100 V. The device was set to 50 V. Due to the negative charge of DNA and thus moving toward the positive pole in the electric field, the gel was placed in the tank in an order that the wells were placed on the negative pole. As the green viewer dye reached the end of the gel, we turned off the device and placed the gel on the digital gel documentation device and took a photo after adjusting it (Ethic code: IR.TMU.REC.1394.65).

## Results

After separating plasma cells (with CD138 index) by MACS technique, a flow cytometric technique was used to determine the purity of the cells. The results are as follows (Figures 1 and 2):

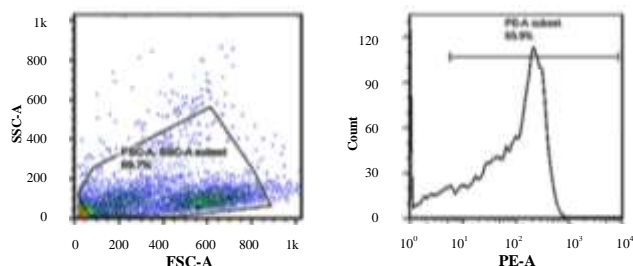
Flow cytometric evaluation results of

plasma cells after isolation



**Figure 1. Flow cytometric evaluation results after the isolation stage (first patient control group)**

The results of cell culture in the AM environment and the effect of AM on growth and maintenance of myeloma cells



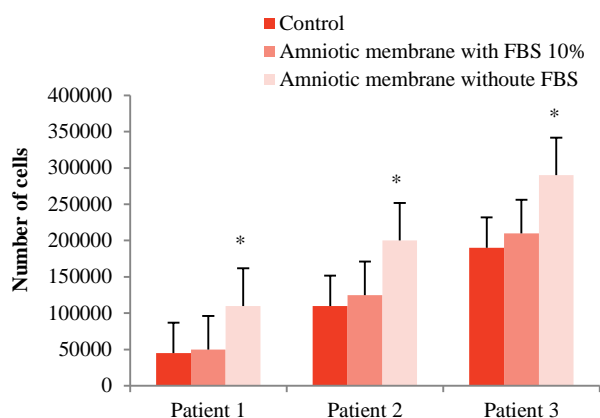
**Figure 2. Flow cytometric evaluation results after the isolation stage (first patient test group)**

After isolating the plasma cells using the MACS technique and verifying it with flow cytometry, the myeloma cells were cultured in an AM containing medium in 3 different groups for 2 weeks. By trypan blue staining, the number of cells was counted per well every other day and their growth curves were plotted.

The first group or control group contained RPMI, 10% FBS, and plasma cells. The second group contained RPMI product of AM culture, 10% FBS, and plasma cells. And the third group contained RPMI product of AM culture and plasma cells.

The results showed that the AM medium without FBS (containing RPMI derived from AM culture and plasma cells) was more effective on the growth, survival, and proliferation of myeloma cells, and they survived for 10 days. These results were

almost the same in all 3 patients (Figure 3).



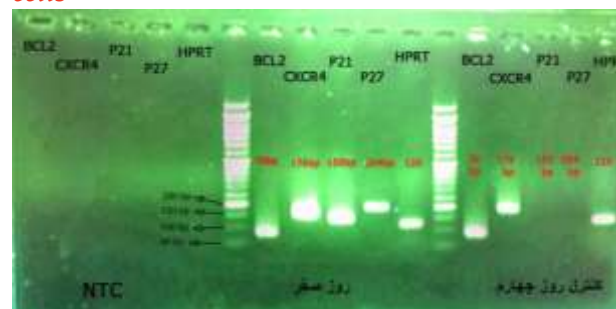
**Figure 3. Plasma cell culture results of 3 different patients in different amniotic membrane (AM) environments**

**Results of gene expression in plasma cells of individuals with MM:** In order to examine the expression of genes associated with proliferation [B-cell lymphoma 2 (BCL2)], implantation (CXCR4), and cell cycle control (P21 and P27) on day 0 (day of isolation) and on day 4 of cell culture, after preparation of cDNA from extracted RNA from the cells of the sample, PCR was performed using specific primers of each of the genes, the sequences of which were listed in the previous section. After electrophoresis of PCR products and gel photocopying with digital gel documentation, the results were as follows. The results of the expression of genes in the plasma cells of the 3 patients were similar (Figures 4-9).

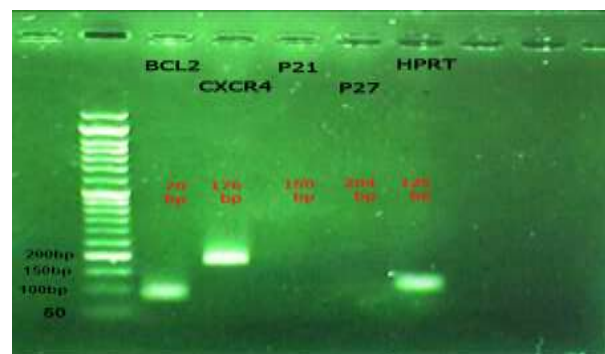
**Results of genes expressions in plasma cells of patients:** On day zero (isolation day), BCL2, CXCR4, P21 and P27, as well as Hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes had expression. On the day 4, control group cells expressed BCL2, CXCR4, and HPRT genes, but P21 and P27 genes had no expression.

Similarly, the cells in AM medium without FBS (containing RPMI produced from AM culture and plasma cells) expressed BCL2, CXCR4, and HPRT genes and did not express P21 and P27 genes.

**Results of gene expression in first-line plasma cells**

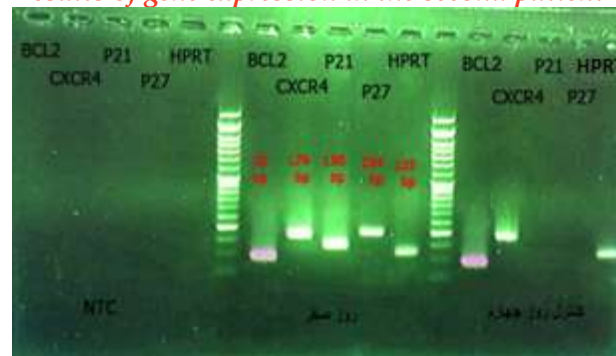


**Figure 4. Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of the patients with multiple myeloma (MM) (first patient) on the day 0 (the day of cell separation), and on the day 4 in control group**

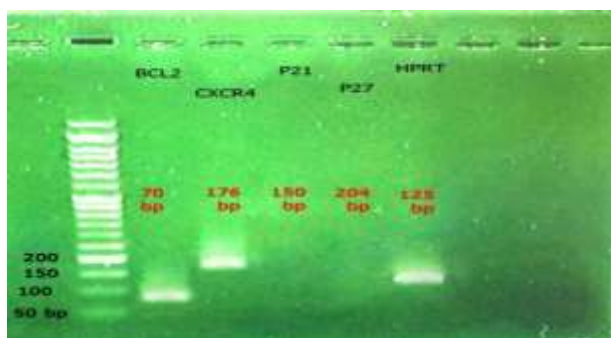


**Figure 5. Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of individuals with multiple myeloma (MM) (first patient) on fourth day of cell culture (FBS-free group)**

**Results of gene expression in the second patient**

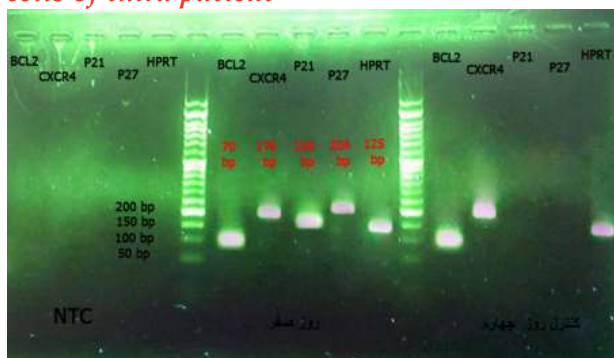


**Figure 6. Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of the patients with multiple myeloma (MM) (the second patient), on the day zero and on the fourth day of cell culture**

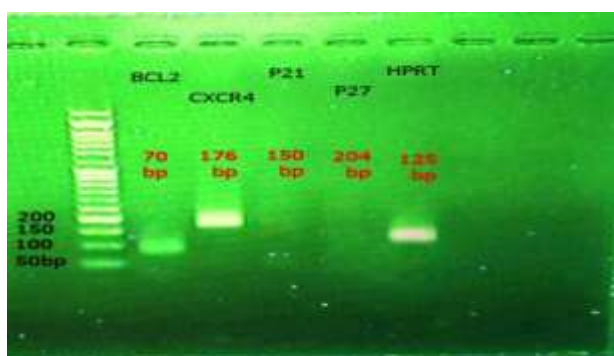


**Figure 7.** Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of individuals with multiple myeloma (MM) (the second patient) on the fourth day of cell culture (FBS-free medium)

*The results of gene expression in the plasma cells of third patient*



**Figure 8.** Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of the patients with multiple myeloma (MM) (third patient) on the day 0 and fourth day of cell culture (control group)



**Figure 9.** Expression of BCL2, CXCR4, P21 and P27, and HPRT genes in plasma cells of patients with multiple myeloma (MM) (third patient) on the fourth day of cell culture (FBS-free medium)

## Discussion

MM is a malignancy associated with plasma cells. It is believed that this disorder is caused by genetic damages such as chromosomal translocations. In addition to genetic changes, the microenvironment of the BM plays a role in the pathophysiology and malignant growth of myeloma cells. The result of the interaction between the BM and the myeloma cells is the expression of factors that indirectly increase the growth of myeloma cells by stimulating vascularisation or acting directly as growth factors for the malignant cell.<sup>8</sup>

The interaction of myeloma cells with BM stromal cells causes adhesion, drug resistance, and activation of signal transmission pathways, which lead to progression of cell cycle development and protection of cells against apoptosis.<sup>9</sup> The myeloma causes production and activation of osteoclasts, so eliminating bone regeneration. Otherwise, osteoclasts by means of intercellular direct interactions cause the myeloma cells' growth and survival.<sup>10</sup>

The most known growth factor of myeloma cell, IL-6, is thought to play a very important role in pathogenesis and malignant growth of myeloma cells.

Growth factors and cytokines such as IL-6, IGF-1, VEGF, TNF- $\alpha$ , transforming growth factor beta (TGF- $\beta$ ), SDF-1, fibroblast growth factor (FGF), and IL-21 are considered as a part of the pathogenesis of MM which promote survival, growth, and migration of the myeloma cells as well as the vascularization in microenvironments of the BM.<sup>11</sup>

Considering myeloma cell properties and the role of the microenvironments of BM in survival of the myeloma cells, due to the presence of growth factors and signaling pathways, it is suggested that in vitro growth and proliferation of myeloma cells need a suitable environment similar to body physiologic conditions, so that the myeloma cells can survive long outside the body in

order to conduct more research on primary cells and obtain more beneficial results in treating this disease.

AM has been used in cellular scaffolds for many years due to its various biological properties and also in clinical studies and specifications. AM can also be used in cell therapy, because it has the characteristics of pluripotent stem cells, the ability of differentiation and low immunogenicity, and production from the placenta, a post-partum discarded tissue. Epithelial and mesenchymal cells of AM contain a variety of regulatory mediators that promote cell growth, multiplication, differentiation, and epithelialization, and also inhibition of fibrosis, immune rejection, inflammation, and bacterial invasion.<sup>12</sup>

The specific structure and biological survival of the AM in addition to the AM matrix and its components, including growth factors, have made it an ideal scaffold in tissue engineering.<sup>13,14</sup> AM matrix contains many growth factors including EGF, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), FGF, platelet-derived growth factor (PDGF), VEGF-R3, TGF- $\beta$ , and EGF. These growth factors create a natural treatment environment that accelerates treatment and also imitates stem cell niches in growth under laboratory conditions. AM secretion of VEGF and HGF leads to a good balance between TGF-1 and TGF-3 and prevents scar formation in the wound, and this property is done by tissue regeneration instead of promoting scars and fibrosis, and in this way, it can help in wounds treatment.<sup>15</sup>

Possibly, the presence of these growth factors in the AM can play an effective role in the proliferation of myeloma cells. In this study, the growth and proliferation of plasma cells isolated from BM aspiration of 3 patients with MM (using MACS technique) were studied for two consecutive weeks in 3 different groups of AM. Because of the presence of complement

components and other invasive factors that affect cell growth, inactive FBS was used. The result of this study showed that the growth and proliferation of cells in the first week had an increasing process and the highest increase in the number of cells was observed in day 10 in the AM medium without FBS (containing RPMI produced from AM culture and plasma cells). In the second week, we saw a declining trend in the number of cells. In this study, we used the qualitative PCR method to show that plasma cells isolated from BM aspiration expressed reduction-related genes (BCL2) and implantation-related genes (CXCR4) on the fourth day of cell culture, and genes related to cell cycle control (P21 and P27) were not expressed; this was an indication of myeloma cell proliferation in the AM environment. Plasma cell culture was first launched in the 1970s with the aim of conducting research on MM disease. Plasma cell culture has since been carried out for various purposes, such as the assessment of plasma cell malignancies, autoimmune diseases, and human therapeutic antibody production.

### Conclusion

In general, the results of this study are very valuable findings applicable in designing of a suitable environment similar to the physiologic of the body, provided for growth, proliferation, and survival of the myeloma cells, and also for further assessment.

### Conflict of Interests

Authors have no conflict of interests.

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