

The Effect of Human Mesenchymal Stem Cells-Conditioned Media on Glioblastoma Cells Viability *In Vitro*

ROXANA MIHAELA FOLCUTI¹, ISABELA SILOSI², STEFANA OANA PURCARU³,
DANIELA ELISE TACHE³, ADA MARIA GEORGESCU¹,
GHAZALEH HOOSHYAR EMAMI¹, ANICA DRICU³

¹PhD Student, Biochemistry Dept., Faculty of Medicine, University of Medicine and Pharmacy of Craiova

²Immunology Dept., Faculty of Medicine, University of Medicine and Pharmacy of Craiova

³Biochemistry Dept., Faculty of Medicine, University of Medicine and Pharmacy of Craiova

ABSTRACT: A novel target for cancer treatment is based on the effects of non-tumor cells, including hMSCs on tumor growth. However, the results are controversial: some studies showed that hMSCs inhibit tumor progression, while others found they promote tumor cell proliferation. In this study, we analyse the effect of human mesenchymal cells derived from umbilical cord tissue (hUC-MSCs) and bone-marrow- mesenchymal stem cells (hBM-MSCs) on glioblastoma cells viability in vitro. GB cell cultures were established from fresh sample tissues provided by "Bagdasar-Arseni" Hospital, Bucharest, from consented GB patients. hUC-MSCs, HUC-1 and HUC-2 cell lines, were established from human umbilical cord tissue collected after delivery from natural term births at the Emergency Hospital of Craiova, Romania. hBM-MSCs cell line was purchased from Life Technologies. Conditioned media (CM) from MSCs was used to treat GB cells for 24, 48, 72 and 96 hours. To determine GB cell viability was used MTT cell proliferation assay. Statistical analyses were performed using Students t-test. hUC-MSCs CM displayed the potential to be cytotoxic to GB cells, while the treatment with hBM-MSCs CM significantly stimulated GB cell growth 24 hours after the treatment and showed minor growth cell inhibition 48, 72 and 96 hours after the treatment. This report proved that hUC-MSCsCM inhibited GB cell proliferation, while little inhibitory effect was exerted by hBM-MSCs CM.

KEYWORDS: mesenchymal stem cells, glioblastoma, conditioned media

Introduction

Despite the existing aggressive therapy, brain tumors are a group of malignant diseases difficult to treat. There is no curative treatment for this kind of malignancies, therefore they are associated with a poor prognosis and a high degree of mortality. Glioblastoma (GB) is a highly malignant neoplasm, which represents about 15,4% of all primary brain tumors (PBT). This tumor with high vascularity contains a mixed grade of cells including malignant cancer cells, cancer stem cells, brain endothelial cells, microglia cells astrocytes and different stromal cell types. Because of this mixture of cells, glioblastoma is a fast-growing tumor with a rare long-time survival, depending on the characteristics of the patient (age, sex) and of the tumor (size and position). Although the conventional treatment is multimodal, including radical surgery, radiotherapy and chemotherapy, glioblastoma has a fast progression, leading to death within the first year [1]. Recent studies have shown a strong connection between the tumors and their microenvironment [2].

A very important part of the tumor microenvironment is represented by human mesenchymal stem cells (hMSCs) derived both

from umbilical cord (hUC-MSCs) and bone marrow (hBM-MSCs) [3, 4]. The organism's response at the malignant cells is providing new stromal support for the tumor to develop. This process mimics the ones of a tissue repair, recruiting new mesenchymal stem cells due to their capacity to differentiate into multiple cell types [5].

In the past decades, many researchers have studied hMSCs as a new approach in cancer therapies. It has been suggested that they interact with tumor cells by direct contact or by secreted factors (chemokines, growth factors, cytokines) [2]. However, the reported results are controversial: some studies have indicated that mesenchymal stem cells may play a role in cancer therapy by killing malignant cells, while others found they promote cancer and metastasis. In scientific literature, there are important breakthroughs regarding mesenchymal stem cells: it was proved they are capable to inhibit breast and colon cancer proliferation, tumor cell growth in a model of Kaposi's sarcoma (KS) or metastasis of liver carcinoma [2, 6].

Concerning hBM-MSCs, Khakoo *et al.* [6] attested the fact that they were able to inhibit

tumor growth or metastasis in some forms of lung carcinoma or melanoma.

Using hBM-MSCs have many disadvantages, for example it was shown that they are age-related, adult organisms exhibit a decreased proliferation and differentiation. Also, clinical use of hBM-MSCs after transplantation revealed a higher risk of infection and tumor development. In addition, the collection of bone marrow is more invasive than that of cord blood. However, these findings need further investigation in order to establish the effects exerted by hMSCs on cancer cells.

In this study, we analyzed the effect of hUC-MSCs and hBM-MSCs GB cells using CM.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, antibiotic (penicillin-streptomycin). Human bone marrow mesenchymal stem cell line (hBM-MSC) and XenoFree Supplement medium were obtained from Life Technologies,

All other chemicals were purchased from Sigma-Aldrich

Cell lines

GB cell cultures were established from fresh sample tissues kindly provided by "Bagdasar-Arseni" Hospital, Bucharest, from consented GB patients undergoing surgery.

HUC-1 and HUC-2 cell lines were established from human umbilical cord tissue. Umbilical cord tissue was collected after delivery from term natural births at the Emergency County Hospital of Craiova, Romania. Patients with chromosomal anomalies, congenital malformations or other pathologies were excluded. The tissue has been processed within 3–5 h post-delivery. In brief, the segments of umbilical cords were washed with phosphate buffered saline containing antibiotic and then immersed in standard DMEM. The tissue was cut in small pieces (1mm), suspended in 0.1% collagenase type I, (Sigma Aldrich, St. Louis) and incubated for 15 min at 37°C. Collagenase was then inactivated by addition of standard DMEM and the samples were centrifugated, washed and transferred in polystyrene dishes in standard DMEM and incubated at a temperature of 37 degrees Celsius, in an atmosphere of 5% CO₂, allowing the cells to grow out (explant culture) for about 10 days. When sufficient cell numbers grew

from the tissue, the cells were harvested and cryopreserved.

The informed consent of the patients and the ethical approval for the project were obtained prior biological material collection.

Cell culture conditions

Cells were isolated and the culture medium was not supplemented with growth factors in order to keep the original characteristics of human stem cells.

GB cell lines were maintained in DMEM and supplemented with 10% fetal bovine serum (FBS) and a combination of antibiotics: Penicilin and Streptomycin (100U/ml).

hUC-MSCs were cultured in DMEM without FBS and hBM-MSC were cultured in XenoFree Supplement medium and supplemented with Penicilin and Streptomycin

The cultures were grown in an incubator at a temperature of 37 degrees Celsius, in an atmosphere of 5% CO₂.

Cell proliferation assay

GB cell lines cultured in 96-well plates were treated with conditioned media (CM) isolated from hUC-MSCs cell lines and hBM-MSCs for 24, 48, 72 and 96 hours. At the end of the treatments, cells proliferation was quantified by MTT assay. MTT reagent (10μl) was added to each well and then incubated for 4h at 37°C. After that, cells were lysed by addition of 100μl solubilization buffer. The assay is based upon the cleavage of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by metabolically active cells. Optical density (OD) was measured using a spectrophotometer at 595 nm and relative cell viability was expressed as percentage of that in untreated control cultures.

All experiments were performed in triplicate.

Statistical Analysis

The results were exhibited as mean ± standard deviation (SD) and for revealing statistical comparison was used Student t-test. p value lower than 0,05 was accepted as statistical significant.

Results

The outcomes of recent studies examining the effect of hUC-MSCs and hBM-MSCs on tumoral cells may vary due to the differences in experimental techniques, the characteristics of the tumor or a combination between these factors.

Effects of hUC-MSCs-CM on GB cells

HUC 1 and HUC 2 CM inhibited proliferation of all three GB cell lines as compared to untreated control cells.

As seen in Fig.1, HUC 1 and HUC 2 CM induces a decrease in GB1B cell viability by

about 30%, 24 hours after treatment. The effect of HUC 1 and HUC 2 CM on GB1B was irreversible, persisting until the end of the experiment (Fig.1).

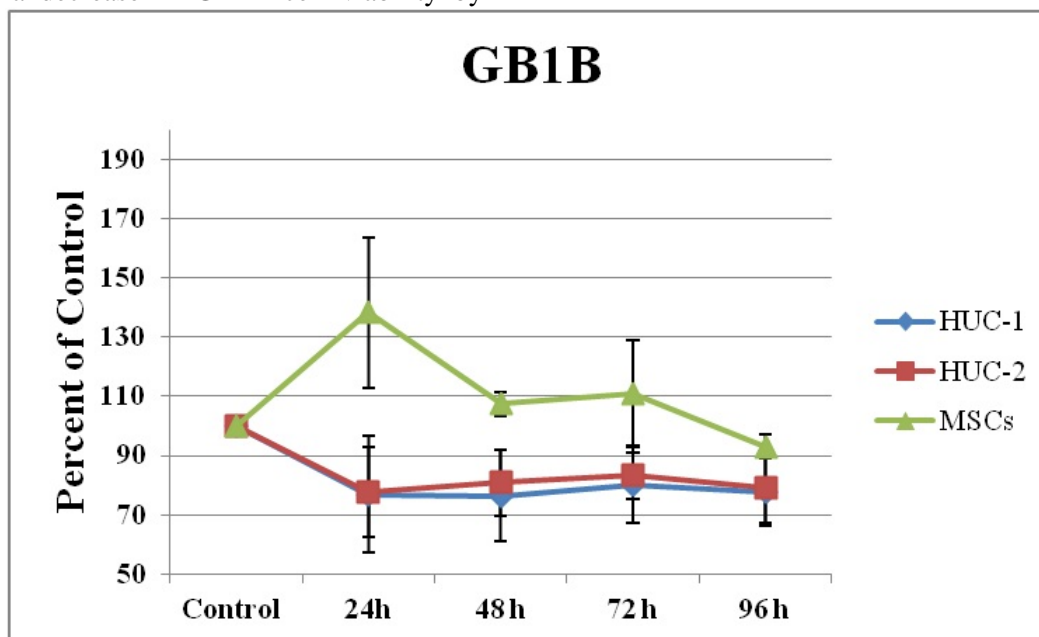


Fig.1. The effects of HUC-1, HUC-2, hBM-MSCs-CM on GB1B cell proliferation. The results obtained over four days exposure of GB1B cells to hBM-MSC-CM and hUC-CM are expressed as percent of control and the values are mean and standard deviation of three different experiments.

In GB2B cells, HUC 1 and HUC 2 CM treatment provoked a sequential decrease of viability: about 15% after 24h, about 30% after

48h and 72h and then there was a slight recovery of cell viability towards the end of the treatment (Fig.2).

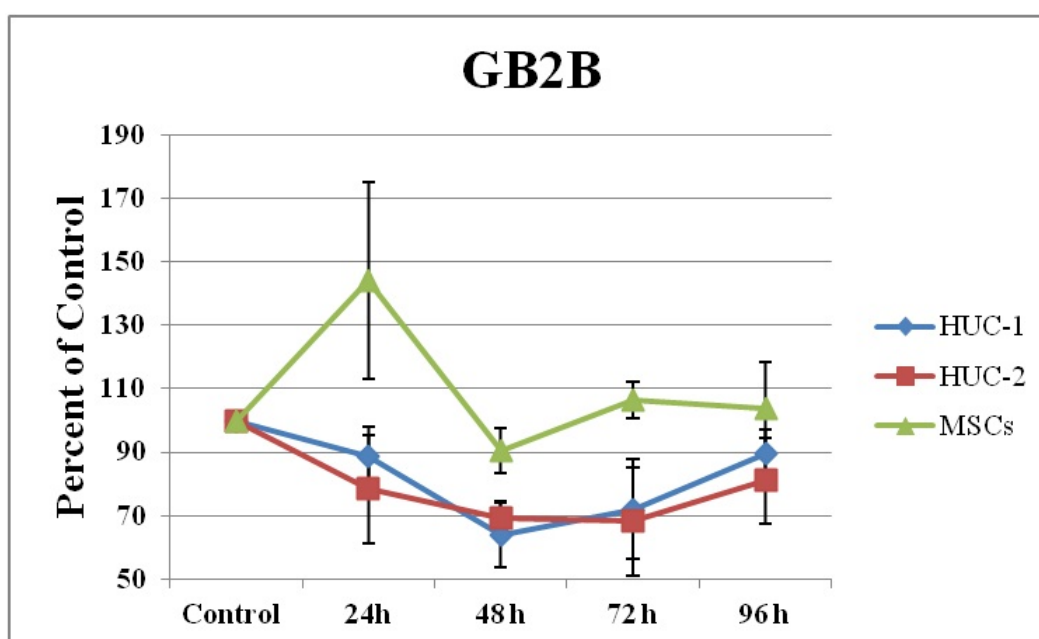
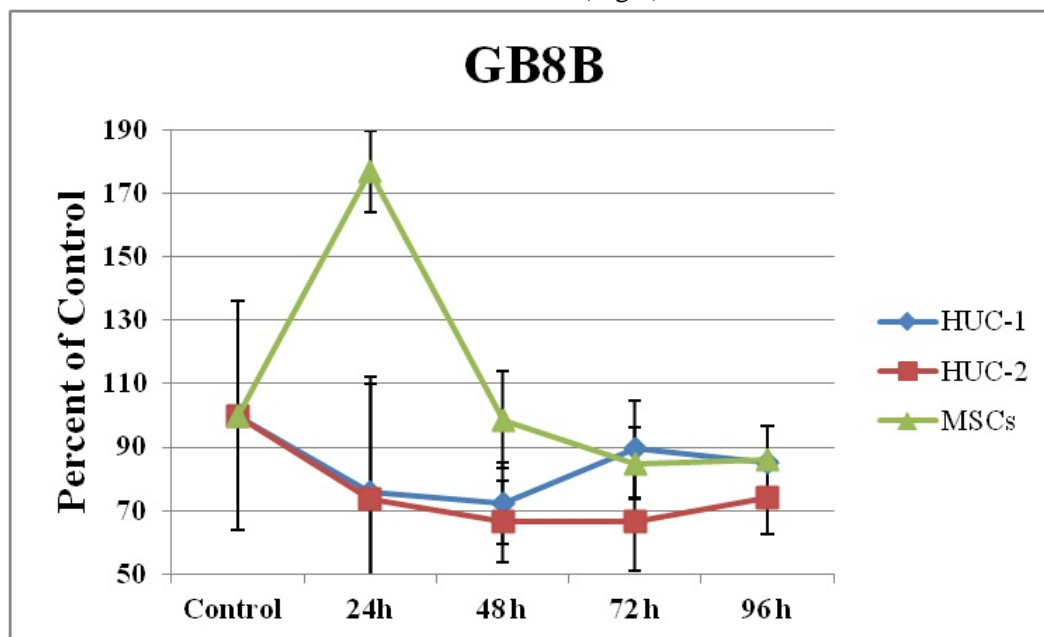


Fig.2. The effects of HUC-1, HUC-2, hBM-MSCs-CM on GB2B cell proliferation. The results obtained over four days exposure of GB2B cells to hBM-MSC-CM and hUC-CM are expressed as percent of control and the values are mean and standard deviation of three different experiments.

HUC 1 media reduced GB8B cell viability by approximately 30%, 24 and 48 hours after treatment, thereafter GB8B cells started to grow back at the end of the treatment

(Fig.3). Instead, HUC 2 CM on GB8B was irreversible, decreasing cell viability by about 30%, 24, 48, 72 and 96 hours after the treatment (Fig.3).



The effects of HUC-1, HUC-2, hBM-MSCs-CM on GB8B cell proliferation. The results obtained over four days exposure of GB8B cells to hBM-MSC-CM and hUC-CM are expressed as percent of control and the values are mean and standard deviation of three different experiments.

In conclusion, the set of values with statistical significance are found for both hUC-MSC lines at 24-48 hours of treatment. The most significant inhibition was reached at 48 hours for HUC 1 cell line (Fig.1), respectively at 72 hours for HUC 2 (Fig.2). Also, there is a tendency of stimulating cell growth at 96 hours above the values obtained after 24 hours of treatment. However, in the fourth day of the treatment, we observed an average percentage of 16% cell death in all GB cell lines.

Effects of hBM-MSC CM on GB cells

Regarding hBM-MS-CM, we noted a statistically significant enhancement of all GB cells within the first 24 hours, while the prolonged treatment for 96 hours showed a minor inhibition of GB1B (Fig.1) and GB8B (Fig.3) cell viability and stimulated proliferation for one GB2B cells (Fig.3).

Discussions

Human umbilical cord represent an ethically acceptable source of mesenchymal stem cells due to their easily extraction [7].

In this study, we found that hUC-MSCs-CM inhibited glioblastoma cells proliferation, while little inhibitory effect was exhibited by hBM-MSCs-CM.

hBM-MSCs and hUC-MSCs have common features that make them promising therapeutic vehicles [8] including the ability to diminish inflammatory reactions [9], tumor homing capacity [10, 11] and genetic stability [12]. Moreover, both hBM-MSCs and hUC-MSCs have the aptitude for differentiation into different types of cells, like osteoblasts, adipocytes [13]. Therefore, there is a large field of clinical application for hMSCs both in regenerative medicine and malignant diseases therapy. hUC-MSCs and hBM-MSCs are shown to exert in vivo and in vitro inhibitory effect on tumor cells [14]. A various number of studies have demonstrated that tumor cells are not the only components involved in tumor growth and behavior, non-tumor cells gaining field in scientific researches of novel cancer treatment [15]. Among the main non-tumor cells existing in the tumor microenvironment are macrophages, vascular endothelia cells, tumor associated fibroblasts and mesenchymal stem cells [16].

Mesenchymal stem cells are previously proved to inhibit tumor cell proliferation [17], stimulate apoptosis [18] or facilitate tumor development [19]. These results are controversial and depend on the consequence of tumor microenvironment on mesenchymal stem

cells differentiation and on the mechanisms by which they are homing to the tumor sites. These mechanisms rely upon the combined effect of chemokines, cytokines and growth factors [20]. Furthermore, tumor development can be influenced by various activated signaling pathways.

The most common source of hMSCs used in recent studies is normal tissue, like bone-marrow, umbilical cord tissue (Wharton's Jelly) and umbilical cord blood. These naïve mesenchymal stem cells carry exogenous genes and produce anti-tumor effects when co-cultured with tumor cells *in vitro*. The underlying mechanisms through which mesenchymal stem cells can inhibit tumor development are not fully known, but as we can see in scientific literature, they are related to secretion of soluble factors resulting in cell-cycle suppression [21], upregulation of an apoptosis-related protease [22] or by inhibiting angiogenesis.

The soluble factors released by mesenchymal stem cells are quoted to have anti-tumor properties, restricting hepatoma, lung and cancer cells [23]. Qiao *et al.* [24] showed that hBM-MSCs CM's soluble factors can inhibit hepatoma cells proliferation through Wnt signaling pathways.

There are also evidences that rat intracranial glioma cell growth was inhibited by amnion derived mesenchymal stromal cells [15]. Also, Khakoo *et al.* [6] reported that hBM-MSCs diminished the proliferation of Kaposi's sarcoma via direct cell contact. Nevertheless, Hou *et al.* [14] found that cell to cell contact was not essential for displaying the inhibitory effect on liver carcinoma cells –HepG₂, after treating the tumor cells with CM. Furthermore, Qiao *et al.* found that fetal bone marrow MSCs secreted factor-Dkk1- inhibit breast cancer proliferation [25]. Besides these reports, there are a few debates whether hMSCs are involved in growth and promotion of tumor progression [14].

Conclusions

Various studies suggested that tumor microenvironment is very important in malignant progression and response to treatment. The interactions between neoplastic cells and different mesenchymal cells, existing in tumour environment, were indicated to have a crucial role in brain tumors behaviour and dissemination. In the present study we showed that CM isolated from hUC-MSCs and hBM-CSM inhibit GB cells proliferation, however hUC-MSCs CM displayed more cytotoxic effect

than hBM-CSM CM. These findings support the idea that mesenchymal stem cells may be important in development of novel therapeutic methods for cancer treatment.

Acknowledgement

This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/136893.

References

1. Lefranc F, Sadeghi N, Camby I, et al. Present and potential future issues in glioblastoma treatment. *Expert Rev, Anticancer Ther* 2006; 6(5): 719-732.
2. Rhodes L, Antoon J, Muir S, et al. Effects of human mesenchymal stem cells on ERpositive human breast carcinoma cells mediated through ER-SDF-1/CXCR4 crosstalk, *Molecular Cancer* 2010; 9: 295.
3. Prockop DJ, Marrow stromal cells as stem cells for nonhematopoietic tissues, *Science* 1997; 276: 71-74.
4. Qiao C, Xu W, Zhu W, et al. Human mesenchymal stem cells isolated from the umbilical cord, *Cell Biol Int* 2008; 32: 8-15.
5. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells, *Science* 1999; 284: 143-147.
6. Khakoo AY, Anderson SA, Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma, *J Exp Med* 2006; 1235-47.
7. Hamada H, Kobune M, Nakamura K, et al. Mesenchymal stem cells as therapeutic cytoreagents for gene therapy, *Cancer Sci* 2005; 96: 149-156.
8. Benedetti S, Pirola B, Pollo B, et al. Gene therapy of experimental brain tumors using neural progenitor cells, *Nat Med* 2000; 6: 447-450.
9. Djouad F, Plence P, Bony C, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogenic animals, *Blood* 2003; 102: 3837-3844.
10. Kucerova L, Altanerova V, Matuskova M et al. Adipose tissue-derived human mesenchymal stem cells mediated produg cancer gene therapy, *Cancer Res* 2007; 67: 6304-6313.
11. Nakamizo A, Marini F, Amano T, et al. Human bone marrow derived mesenchymal stem cells in the treatment of gliomas, *Cancer Res* 2005; 65: 3307-3318.
12. Vilalta M, Degano IR, Bago J, et al. Biodistribution, long-term survival and safety of human adipose tissue-derived mesenchymal stem cells transplanted in nude mice by high sensitivity non-invasive bioluminescence imaging, *Stem Cells Dev* 2008; 17: 993-1003.
13. Yang B, Guo H, Zhang Y et al. MicroRNA-145 regulates chondrogenic differentiation of mesenchymal stem cells by targeting Sox9, *PLoS ONE* 2011; 6(7).
14. Hou L, Wang X, Zhou Y et al. Inhibitory effect and mechanism of mesenchymal stem cells on liver cancer cell, *Tumor Biol* 2014; 35: 1239-1250.

15. Rolfo A, Giuffrida D, Giuffrida MC, et al. New perspectives for prostate cancer treatment: in vitro inhibition of LNCaP and PC3 cell proliferation by amnion-derived mesenchymal stromal cells conditioned media, *The Aging Male* 2014; DOI: 10.3109/13685538.2014.896894.
16. Riss J, Khanna C, Koo S et al. Cancers as wounds that do not heal: differences and similarities between renal regeneration/repair and renal cell carcinoma, *Cancer Res* 2006; 66(14): 7216–7224.
17. D'Souza N, Burns JS, Grisendi G, et al. MSC and Tumors: Homing, Differentiation, and Secretion Influence Therapeutic Potential, *Adv Biochem Eng Biotechnol* 2012; DOI:10.1007/10_2012_150.
18. Zhu Y, Sun Z, Han Q, et al: Human mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1, *Leukemia* 2009; 23(5): 925–933.
19. Sun B, Roh KH, Park JR, et al. Therapeutic potential of mesenchymal stromal cells in a mouse breast cancer metastasis model, *Cytotherapy* 2009; 11(3): 289–298.
20. Nishimura K, Semba S, Aoyagi K, et al. Mesenchymal stem cells provide an advantageous tumor microenvironment for the restoration of cancer stem cells, *Pathobiology* 2012; 79(6): 290–306.
21. Uchibori R, Tsukahara T, Mizuguchi H, et al. NF-kappaB activity regulates mesenchymal stem cell accumulation at tumor sites, *Cancer Res* 2013; 73(1): 364–372.
22. Ramasamy R, Lam EW, Soeiro I, et al. Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth, *Leukemia* 2007; 21(2): 304–310.
23. Lu YR, Yuan Y, Wang XJ, et al. The growth inhibitory effect of mesenchymal stem cells on tumor cells in vitro and in vivo, *Cancer Biol Ther* 2008; 7(2): 245–251.
24. Khalid S, Mesenchymal stem cells engineered for cancer therapy, *Adv Drug Deliv Rev*, 2012; 64(8): 739–748.
25. Qiao L, Xu ZL, Zhao TJ, et al. Dkk-1 secreted by mesenchymal stem cells inhibits growth of breast cancer cells via depression of Wnt signaling, *Cancer Lett* 2008; 269(1): 67–77.

Corresponding author: Anica Dricu, University of Medicine and Pharmacy of Craiova, 2-4, Petru Rares St., Craiova - 200349, Romania; e-mail: anica.dricu@live.co.uk