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ARTICLE

IRON OXIDE MAGNETIC NANOPARTICLES AS DRUG DELIVERY SYSTEMS FOR BRAIN CANCER TREATMENT

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Abstract

Nanotechnology offers a new horizon for cancer drug administration and systemic safety of oncological treatments. Compared with conventional pharmaceutical forms, nanoparticles (NPs) have many advantages such as larger surface, ability to adsorb and targeted delivery of different types of drugs, providing decreased side effects and a patient customed approach in cancer treatment. Due to their diverse chemical composition, NPs offer the possibility of developing innovative therapies, which may be also applied in glioblastoma treatment. Fe_3O_4 magnetic nanoparticles (MNPs) have been previously used in cancer treatment, as targeted drug delivery systems. Helianthin is an azo dye compound that we found to induce cell death in high grade glioma (HGG) cells. In this study, we analyzed the *in vitro* effect of Fe_3O_4 magnetic nanoparticles (MNPs) or Helianthin loaded MNPs (HeMNPs) on a glioblastoma cell line (GB2B). The results shown that the administration of Helianthin coated MNPs provided cytotoxic effects on glioblastoma cells.

Keywords: *nanoparticles, glioblastoma, iron oxide*

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Introduction

Glioblastoma (GBM) or glioblastoma multiforme represents an aggressive and unpredictable form of brain cancer, with an incidence of 5-6/100.000 persons and a male to female ratio of 3:1, more frequently in middle-aged males (1). Current standard of care (SOC) consists of: surgical removal, radiotherapy accompanied by oral administration of a DNA-alkylating agent, namely temozolomide (TMZ) (2). This treatment modality presents numerous side effects such as: nerve damage, diarrhea, hair loss, skin rash, fibrosis, cardiomyopathy etc. and also confers a median survival rate of only 8 months (3). According to WHO, GBMs are categorized as Classical, Mesenchymal, Neural and Proneural, presenting anaplastic cells and regions of necrosis and angiogenesis, being overall very diverse.Their malignancy trait, heterogeneity, poor prognosis and response to treatment have determined the

need to study in depth the molecular pathways and to assess the effectiveness of the treatments. Not only GBM exhibits high abilities of rapidly spreading throughout the brain parenchyma (gliomatosis cerebri), thus presenting multiple relapses during the patient's disease course and the impossibility to completely remove the tumor via surgical resection resulting in minimal residual disease (MRD), but it also presents difficulties in establishing an effective treatment, due to its special characteristics (3, 4). Moreover, the complex interaction with the microenvironment and the ability to acquire new mutations, resulting in a drug resistant recurrence, impose the researchers to find new methods to overcome the barriers for a proper drug delivery and interaction with cancer cells. Cancerous cells are protected by the blood brain barrier (BBB) and the blood brain tumor barrier (BBTB), therefore determining the right dose of administered drug becomes especially strenuous and narrowed by the limited number of chemotherapeutic agents able to cross the BBB. The protective role of the BBB is ensured by the enzymatic barrier (capable of metabolizing neurotoxins such as chemotherapeutic agents), efflux protein membranes (ATP-binding cassette) and immunological cells (microglia, perivascular macrophages, mastocytes) (5). A series of methods to cross it have been studied, such as: convection-enhanced delivery, mechanical and chemical disruption of the BBB, peptide masking, nanoparticles (NPs) etc. (3).

Nanotechnology opened a new horizon regarding drug administration and systemic safety of oncological treatments. Due to their diversity in chemical composition, NPs offer the possibility of using innovative therapies. Also, NPs active surface is larger compared to conventional pharmaceutical forms and they also have the ability to adsorb and transport different types of drugs, crossing the usual barriers and delivering them directly to a specific site where they accumulate, sparing the healthy tissue. All these provide a more targeted and patient customed approach in cancer treatment, as well as other advantages such as decreased side effects of usual systemic chemotherapy, and improved imaging, monitoring and teranostics (6, 7). Although there is the necessity of in-depth research of nanoparticle toxicity, nanotechnologyis a promising strategy for glioblastoma treatment (3).

Materials and methods

Materials

Reagents: Helianthin, KOH, $FeCl_3$ and $FeCl_2$ (Sigma-Aldrich), DNase (Sigma-Aldrich), Pronase (Boehringer-Mannheim), Collagenase IV (Gibco/BRL), Hank's Buffered Saline Solution (Gibco/BRL), Minimum Essential Medium (Gibco/BRL), Fetal Bovine Serum (Gibco/BRL) Glutamine (Gibco/BRL), Penicillin

(Gibco/BRL) and Streptomycin (Gibco/BRL).

Cell cultures: Early passage cell cultures (GB2B) established from tissue obtained from a glioblastoma diagnosed patient with at "Bagdasar–Arseni" Emergency Hospital, Bucharest, Romania.

Devices: Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom)

Methods

Functionalized Fe_3O_4/salicylic acid magnetic nanoparticles

A method previously described in the literature (8, 9) based on Ferrite salt co-precipitation synthesis was used to obtain an aqueous solution of 60 nm MNPs characterized by well-dispersed properties and homogenous size distribution.

Synthesis of Fe_3O_4/Helianthin magnetic nanoparticles

Analytical grade reagents (Helianthin, KOH, $FeCl_3$ and $FeCl_2$) were purchased from Sigma-Aldrich. A modified Massart method was used to synthesize the Helianthin/Fe_3O_4 core–shell magnetic nanoparticles (HeMNPs) (8).

2 g $FeCl_3$ and 1.25 g $FeCl_2$ (hydrated salts) were dissolved in ultrapure water (250 ml) and then precipitated at basic pH. The excess of KOH and Helianthin were removed by several washing steps, using sonication and magnetic separation (10).

Then, the HeMNPs Zeta potential and hydrodynamic diameter were evaluated using a DLS analysis. In brief, a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom) was used to analyze the mean diameter (Z-average) and the polydispersity index (PdI) of the HeMNPs in aqueous dispersion, at an angle of 173º. All measurements were performed at room temperature (25ºC) and data represent the mean of 5 separate measurements.

The DLS analysis confirmed a good dispersion stability. The hydrodynamic diameter of magnetic dispersions was 65.9 nm, the Zeta potential value was 42.2 mV, and the polydispersity index (PdI) was 0.119, confirming that the HeMNPs are monodispersed in aqueous phase (11).

In vitro assay

GB2B cell lines were established from glioblastoma tissue. Early passage cell cultures (GB2B) used in this study were established from tissue obtained from a glioblastoma diagnosed patient at "Bagdasar–Arseni" Emergency Hospital, Bucharest, Romania.

Standard procedures were applied to establish cell lines. Briefly, after dissection into small pieces (<5 mm diameter) the samples were incubated for 30 min at 37°C followed by 30 min at 40°C in a Hank's buffered saline solution (HBSS) containing 0.4 mg/ml DNase (Sigma-Aldrich), 0.5 mg/ml pronase (Boehringer-Mannheim) and 0.25 mg/ml collagenase IV (Gibco/BRL), with gentle stirring. The tumor

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slurry was passed through a tissue culture sieve and the resulted cells suspension was plated into tissue culture flasks (12).

Cell culture and cell treatment

GBM cells were cultured in monolayers in tissue culture flasks using minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 UI/ ml penicillin and 100 UI/ml streptomycin). The cells were maintained in a humidified incubator (95% air/ 5% CO_2 atmosphere, 37°C). For experiments, cells were seeded in 6-well culture plates, at a density of 2,000-3,000 cells/cm^2. The experiments were initiated at 50% confluence of cells.

The cells were treated with Fe_3O_4 magnetic nanoparticles (MNPs) or Helianthin loaded MNPs (HeMNPs) using 3 different concentrations (0.25 µg/ml, 0.5 µg/ml, and 1 µg/ml) and then incubated for 24, 48 and 72 hours. Appropriate control groups with diluents only were included.

Cell viability

Cellular proliferation was measured by determining the number of cells attached to the surface of duplicate wells by microscopic counting of cells in ink-marked areas. Changes in the number of attached cells were followed by repeating the counting at specified time intervals.

Statistical analysis

All data are represented as mean ±SD. Data were analyzed using ANOVA two-tailed t-test. $P < 0.05$ values were considered statistically significant.

Results

The effect of iron oxide NPs on glioblastoma cells

MNPs are used to administer different therapeutic agents in the oncological field and also in other pathologies such as hemophilia (Adynovate polymer), acromegaly (Somavert) and anorexia (megestrol acetate), but the ability to induce cytotoxic effects in cancer cells is a ground for multiple studies (7, 13-15).

In the current study, GB2B glioblastoma cells were treated with a solution of 0.25 µl/ml MNPs, but after the three time points, besides a slight decrease in the MNPs group, no statistically significant change in cells viability was observed between the two groups.

Both groups presented a parallel growth during the 24h and 48h after treatment initiation with MNPs 1 µl/ml, however a slight decrease of cell numbers by roughly 18% can be observed at the 72h mark in comparison to the control group.

The increase of MNPs concentration up to 0.5 µl/ml rendered a slight decrease of tumor cells proliferative index from approximately 2% to 4%.

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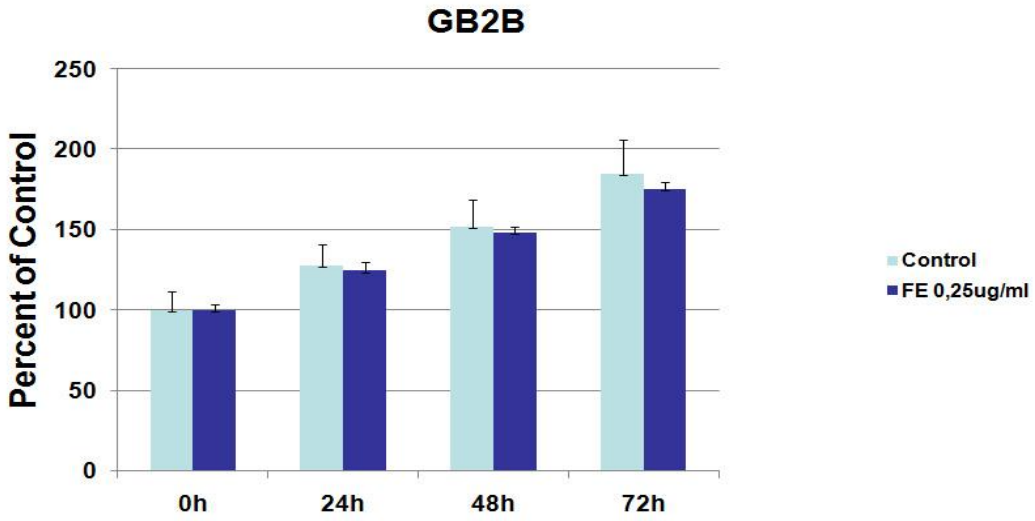

Figure 1. Effect of MNPs on GB2B glioblastoma cell line viability. 0.25 µl/ml MNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours. Results are presented as percentage of control and the tests were repeated 3 times. Data is reported as mean ± SD.*P<0.05

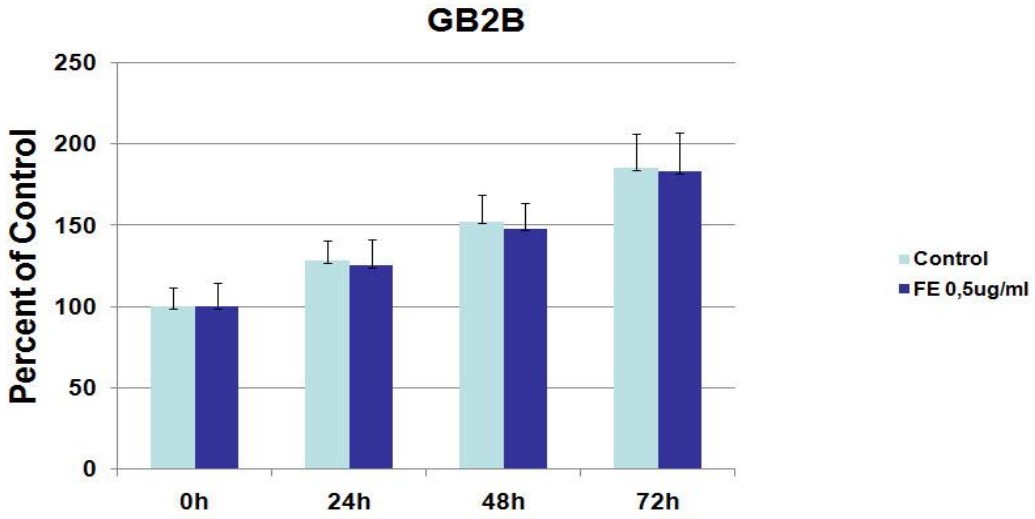

Figure 2. Effect of MNPs on GB2B glioblastoma cell line viability. 0.5 µl/ml MNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours. *P<0.05

Both groups presented a parallel growth during the 24h and 48h after treatment initiation with MNPs 1 µl/ml, however a slight decrease of cell numbers by roughly 18% can be observed at the 72h mark in comparison to the control group.

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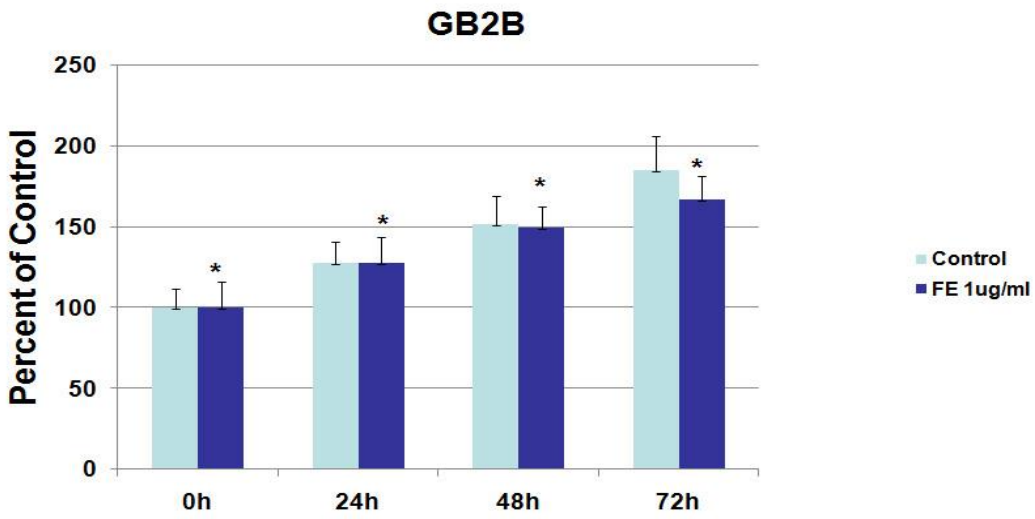

Figure 3. Effect of MNPs on GB2B glioblastoma cell line viability. 1 µl/ml MNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours.Results are presented as percentage of control and the tests were repeated 3 times. Data is reported as mean ± SD.*P<0.05

The effect of HeMNPs on glioblastoma cells

Treatment of brain cancer proves to be very complex, due to the inability to surpass the BBB and to specifically target the cancerous cells. NPs have offered a new perspective regarding this problem being extensively researched on different types of cancer and in combination with other methods such as chemotherapy, radiotherapy and hyperthermia (14-16). Metal oxide nanoparticles appear to provide selective action on cancer cells, sparing the healthy tissue (17). In this study, ferrite nanoparticles are used as carriers for Helianthin and their effectiveness is assessed.

GB2B glioblastoma cell line was treated with 0.25 µl/ml of HeMNPs and cell proliferation was analyzed at 24, 48 and 72h. The treated group exhibited a significant reduction of cell proliferation by 36%, 59% and 93% respectively, in comparison to the untreated group.

0.5 µl/ml of HeMNP presented a significant impact on cell proliferation. At 24, 48 and 72 hours the untreated cells increased their numbers by 28%, 52% and 85%, whilst the HeMNPs group decreased by approximately 6% at 24 hours and remained at a stable level the following 48 and 72 hours.

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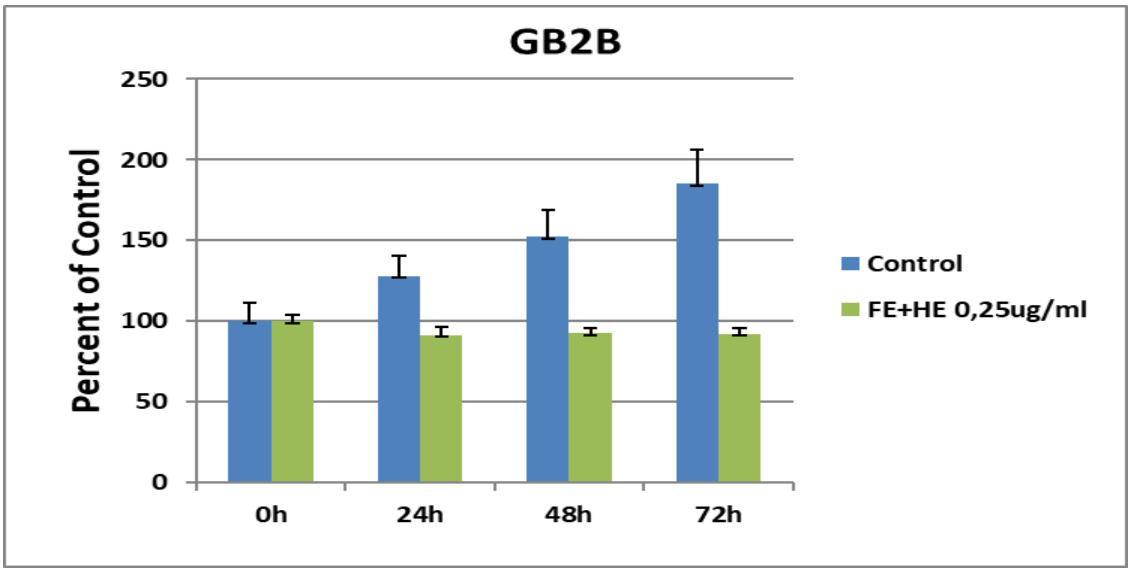

Figure 4. Effect of HeMNPs on GB2B glioblastoma cell line viability. 0.25 µl/ml HeMNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours. Results are presented as percentage of control and the tests were repeated 3 times. Data is reported as mean ± SD.*P<0.05

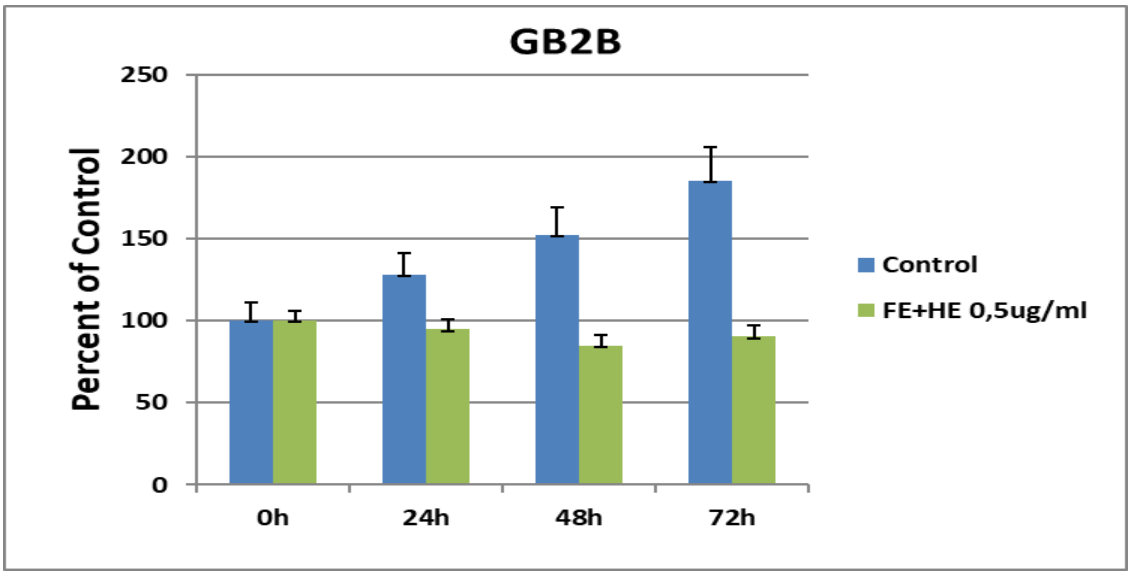

Figure 5. Effect of HeMNPs on GB2B glioblastoma cell line viability. 0.5 µl/ml HeMNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours.Results are presented as percentage of control and the tests were repeated 3 times. Data is reported as mean ± SD.*P<0.05

By initiating a treatment of 1 µl/ml HeMNPs, cell viability exhibits a downward trend of 8% at 24h, 11% at 48h and 16% at 72h, while the control group presents a raise of 28%, 52% and 85%.

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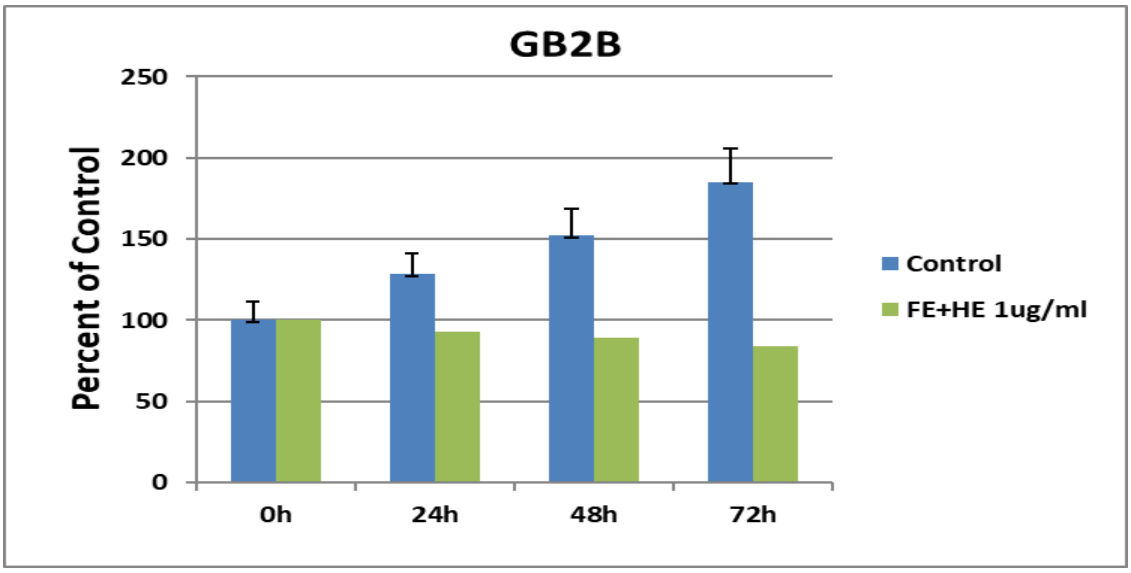

Figure 6. Effect of HeMNPs on GB2B glioblastoma cell line viability. 1 µl/ml HeMNPs solution was administered and microscopic cell count was performed at 24, 48 and 72 hours. Results are presented as percentage of control and the tests were repeated 3 times. Data is reported as mean ± SD.*P<0.05

The different effects on cell viability between loaded and unloaded MNPs are systemized in the following graphs.

Cell treatment with a solution of 0.25 µl/ml MNP induced an increase in proliferation index of 25% at 24 hours, 48% at 48 hours and 75% at 72 hours, whilst cell viability decreased when treated with a solution of 0.25 µl/ml HeMNP with 9% at 24 hours and then remained at a constant level. The difference in proliferation between the two groups is 83% at 72 hours.

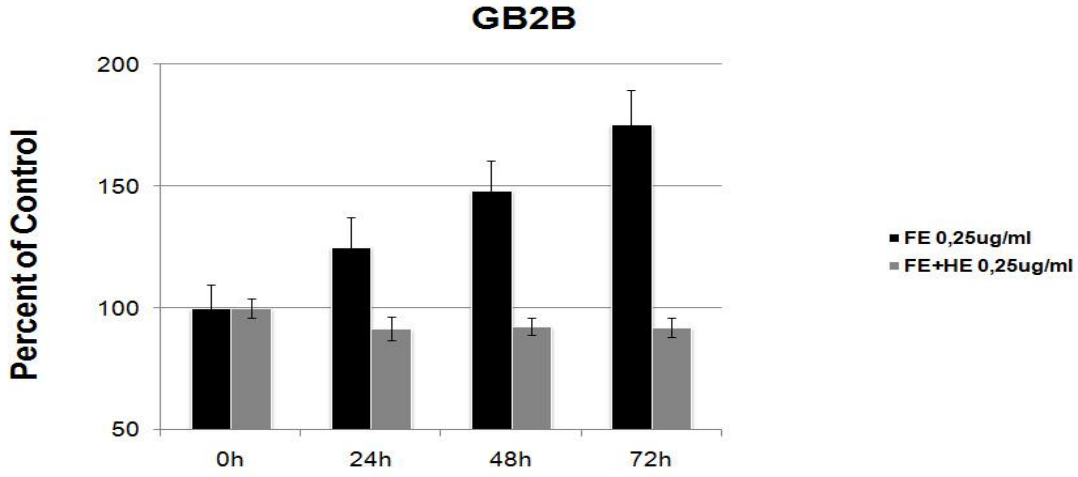

Figure 7. An increase in cell proliferation can be observed at a dosage of 0.25 µl/ml unloaded MNP of 25%, 48% and 75% during the analyzed period of time. The evolution of GB2B cell line decreases with 9% and then remains unchanged after HeMNP treatment. Results are presented on a comparative basis according to their effects on the cell culture. *P<0.05

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By adding 0.5 µg/ml MNP solution, the proliferation of glioblastoma cells increases by 25%, 48% and 83%, while GBM cells treated with 0.5 µg/ml HeMNP solution experience a decrease in viability by 6% at 24h, 16% at 48h and 10% at 72%.

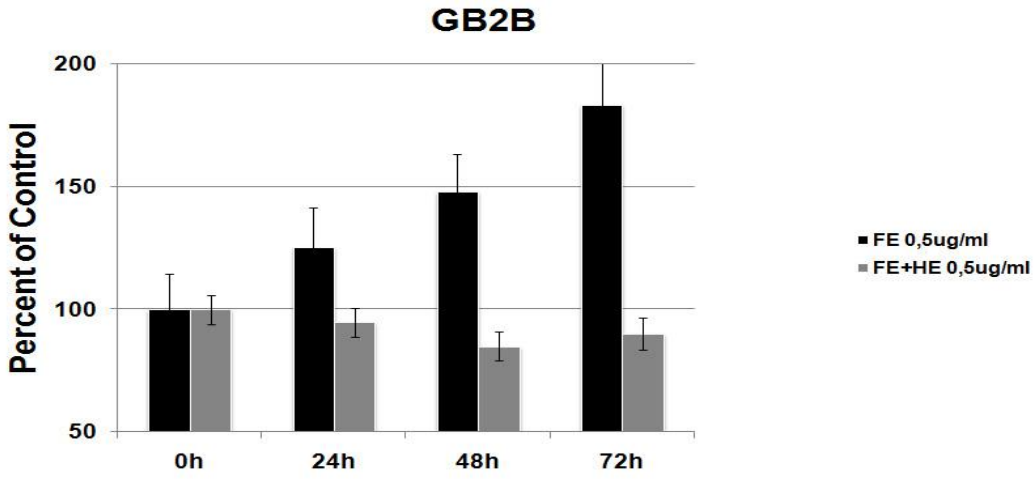

Figure 8. The effects of MNP and HeMNP on GB2B cells viability. The cell cultures were treated with 0,5 µl/ml solutions of MNP and HeMNP and the viability assessed at 24, 48 and 72 hours by hemocytometer. Results are presented on a comparative basis according to their effects on the cell culture. *P<0.05

At a concentration of maximum 1 µg/ml of MNP, cancer cells experience a steady increasing in proliferation reaching a maximum of 67% in 72h. 1 µg/ml of HeMNP solution hinders cell proliferation by 8% in 24h, 11% in 48h and 17% in 72h.

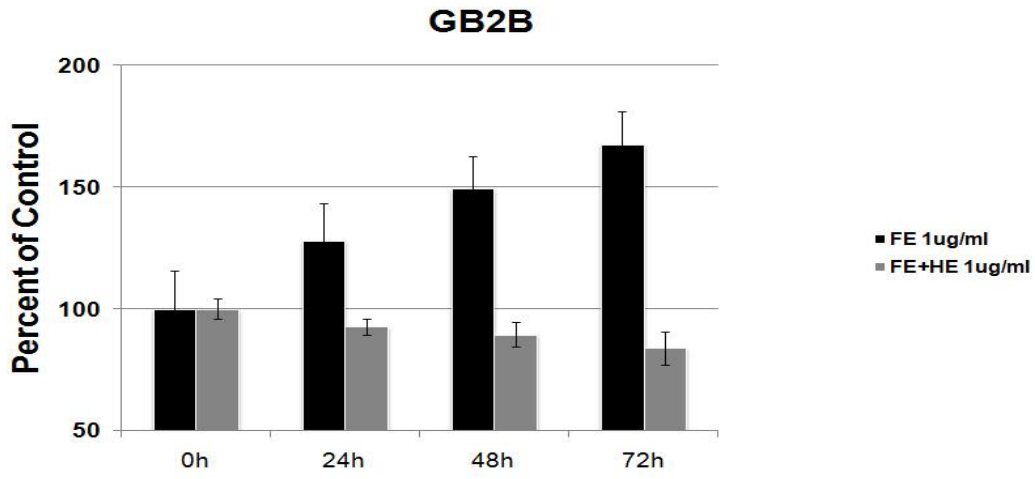

Figure 9. The effects of MNP and HeMNP on GB2B cells viability. The cell cultures were treated with 1 µl/ml solutions of MNP and HeMNP and the viability assessed at 24, 48 and 72 hours by hemocytometer. Results are presented on a comparative basis according to their effects on the cell culture. *P<0.05

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Discussions

Being one of the most aggressive types of cancers, glioblastoma is continuously subjected to new researches for improving the treatment options and patients'quality of life. Currently, GBM's treatment has a low efficiency and potentially serious side effects. Since their first use, NPs have sparked interest in GBM, offering a new possibility to approach this disease.

The antitumor effect of NPs is associated with their ability of forming reactive oxygen species (ROS) resulting in cellular apoptosis, along with their usage as co-adjuvant agents for radiation and carriers for numerous chemotherapeutic agents (13). A study showed that a magneto-sensitive complex consisting of iron oxide NPs and doxorubicin provided an increased cytotoxic effect on the cancer cells, due to the gainof free radicals, resulting in the lysis of mitochondria, DNA and other structural molecules (18). NPs are able to reduce the necessary chemotherapeutic dose, thus reducing the drug related side effects (19). There are studies that also suggest the interaction with the microenvironment hindering the tumor's progression, as well as the ability to diffuse through the neovascularization, due to the permeability and retention effect. Some disadvantages would be the inability to correctly measure the size of the NPs *in vivo*, the interaction of the corona protein with the plasma protein that can potentially affect their clearance and immune reactions (18).

In this study, ferrite NPswere used as carriers for Helianthin, for the purpose of determining if the viability of glioblastoma cell line is affected. Cell treatment with a solution of 0.25 µl/ml MNP induced an increase in proliferation index of maximum 75% at 72 hours, whilst cell viability decreased when treated with a solution of 0.25 µl/ml HeMNP with 9% between 24-72 hours. By increasing the concentration up to 0.5 µg/ml, the proliferation of glioblastoma cells increases to 83% for the MNP treated group, and decreases the cellular viability 10% at 72 hours.

At a concentration of 1 µg/ml, cancer cells increase in proliferation at 67% in 72h for the MNP treated group, and the cell proliferation is hindered by 17% at 72 hours. As it was expected, the difference in proliferation between the two groups reached its maximum (83%) at 72 hours.

The HeMNPs treated group exhibited significant reduction of cell proliferation compared to MNPs treated and untreated group. The administration of Helianthin coated MNPs provided cytotoxic effects on glioblastoma cells, with a decrease in cell proliferation at 24, 48 and 72 hours after treatment.

Our results are in concordance with previous studies performed on glioblastoma cell lines treated with different types of NPs loaded with anticancer drugs (17, 18).

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Conclusions

Nanotechnology offers a wide horizon for oncological treatments, and NPs are currently used in innovative therapies.

Our study proved that the treatment using Helianthin coated MNPs provided cytotoxic effects on glioblastoma cells, offering a decrease in cell proliferation during the 24, 48 and 72 hours check-up.

Even though nanotechnology provides a step forward towards cancer research and cancer treatment delivery, it requires extensive work in toxicology and proper ways of administration. Regarding high grade brain tumors, the aim is to assess the MNP's applicability and effectiveness in its treatment.

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Conflict of interest

The authors declare no conflicts of interests.

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