Magnetic Nanoparticles (MNPs) Influence on SK-BR3 Breast Cancer Cell Line - *in vitro* Study

VLAD IMAN*, ALINA TACULESCU, CRISTINA DEHELEAN, VIRGIL PAUNESCU
Victor Babes University of Medicine and Pharmacy Timisoara, 2A Eftimie Murgu Sq., 300041, Timisoara, Romania

Nanotechnologies involving the use of magnetic nanoparticles made of nickel, iron or cobalt are nowadays developing in anti-tumor therapy and diagnostic. The most important potential of MNPs is for drug delivery, meaning they are functionalized by binding to chemotherapeutic agents, nucleic acids, antibodies, etc. The present study is questioning the possibility of oleic acid-coated iron MNPs to induce cell death per se, selectively in tumor cells (breast cancer adenocarcinoma SK-BR3 cell line) without previous coupling with effector substances.

Keywords: magnetic nanoparticles (MNPs), tumor cells, SK-BR3 cell line, cell death

In past decade, the scientific community made important advances in understanding the mechanisms underlying the tumor cells behaviour, both *in vitro* and *in vivo*. The tools used for revealing the mechanism such as adherence, viability, and cell death are dependent on different types of labels for the cell, and an end-point analysis [1]. The RTCA (real-time cell analysis) platform is a label-free method for investigation of cell behavior, observing the cells in physiological conditions *in vitro*, based on real-time kinetic data related to adhesion to the culture plate [2]. The continuous monitoring by the xCELLigence system creates the possibility to distinguish between different perturbations of the cellular development, such as invasion, proliferation, migration [3].

The influence of magnetite nanoparticles (MNPs) on different cell types *in vitro* and *in vivo* is mainly studied in conjunction with their ability to be coupled with other chemical substance (liposomes, chemotherapeutic drugs, etc.), for precise delivery into the target cells [4]. MNPs drug delivery offers the possibility to locally enhance the effect by additional methods, so that the drug is able to accumulate in a precise area [5]. Few studies are approaching the use of MNPs *per se* for therapeutic or diagnostic procedures [6].

The present study is investigating the effects of magnetite nanoparticles (MNPs) on SK-BR3 tumor cell line, using optic and fluorescence microscopy and real-time cell analysis (RTCA) platform (xCELLigence), under *in vitro* culture conditions.

Experimental part

Cell culture

SK-BR-3 breast cancer cell line was procured from American Type Culture Collection (ATCC, Manassas, VA, USA) and the cells were expanded in McCoy's 5A medium (Gibco BRL, Invitrogen, Carlsbad, CA, USA) enriched with 10% fetal calf serum (FCS; Gibco) and 1 % Penicillin/ Streptomycin mixture (Pen/Strep, 10000 IU/mL; PromoCell, Heidelberg, Germany). Cellular maintenance was in standard incubator conditions at 37°C, 5 % CO_2, and humid atmosphere. After expansion, 5000 cells/cm^2 were seeded in 6-well plates for microscopic investigations, while for viability testing, the cells were plated in specific 16-well E-plate (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Prior to MNPs addition, the cells were left overnight (24h) to attach.

MNPs synthesis

The magnetic nanoparticles (MNPs) were synthetized by combustion method, generating Fe_3O_4 nanopowders, which were further double-layer coated with oleic acid, as described by Ianos R et al. and dispersed in Phosphate Buffer Saline (PBS, St. Louis, Missouri, USA) [7].

Real time viability assay using xCELLigence System Real-Time Cell Analyzer

This method is based on measurement of electronic impedance at the level of gold microelectrodes placed on the bottom of specific culture plates (E-plates, Roche Diagnostics). xCELLigence System Real-Time Cell Analyzer (Roche Diagnostics) continuously, non-invasive, and real-time monitors the biological changes in living cells, under optimum conditions (celular incubator), on the entire duration of the experiment.

We used the 16-well E-plates and the RTCA DP device for monitoring the cellular viability over more that 96 h. The cells were seeded in each well at a cellular density of 7000 cells/well in 150 µL of culture media, and were left to adhere for 24h. The recording was stopped after 24h and various concentrations of MNPs were added in the culture media of SK-BR3 cells as follows: 1 µL/mL, 2 µL/mL, 3 µL/mL, 4 µl/mL, 5 µL/mL, and 10 µL/ml. Additional 48h were recorded for evaluation of SK-BR3 behavior. For negative control we used dimethyl sulfoxide (DMSO, Sigma-Aldrich Company) solution and we added 10 µL in each well. The experiments were performed in duplicate and the results were expressed as mean value ± sd.

Results and discussions

Microscopic evaluation

In optic microscopy, the untreated cells had a polygonal shape, were adherent to plastic culture plate, with large nuclei and visible nucleoli (overlay with Hoechst staining) (fig. 1A). The SK-BR3 cells treated with 10 mM MNPs lost their adherence ability after 24h in contact with MNPs, and we were able to visualize the nuclei pushed to a peripheral part of the cell, close to cell membrane, while the cell had a round morphology (fig. 1B). After 48h in contact with the

* email: vlad.iman@yahoo.com, Phone: +40720543143

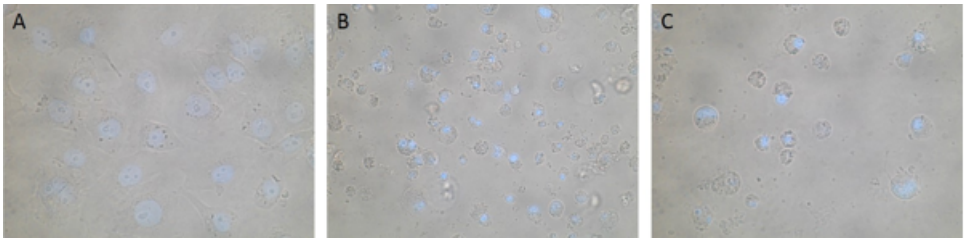


Fig. 1. Optic microscopy of SK-BR3 cell line, Hoechst staining (blue) of the nuclei overlay. A. Control cells, adherent, polygonal morphology and large nuclei; B. 24 h MNPs-treated SK-BR3 cells, detached from the culture plate, with marginalized nuclei; C. 48h treated SK-BR3 cells, floating, expulsing the nuclei (arrow) (Ob. 60x)

MNPs, on average 90% of SK-BR3 cells exhibited the loss of nucleus (results obtained by quantitation on 10 MO fields) (fig. 1C). The influence of combustion-obtained MNPs was previously discussed, and the loss of nuclei was described as enucleation [8].

xCELLigence real-time analysis

Representative graph from xCELLigence system is comparing the growth curve of all 16 wells of the E-plate during the first 24 h of SK-BR3 tumor cells to adhere and grow (n=3) (fig. 2).

The rate of proliferation is determined by analysis of doubling time and slope between 0-24h interval, showing there is no significant difference between the 16-well E-plate cultivated with SK-BR3 tumor cells (n=3) (fig. 3).

Regarding the effect of MNPs on SK-BR3 tumor cell line, xCELLigence system analysis showed that concentrations of 5 (arrow 1 in fig. 4) and 10 µL/ml MNPs (arrow 2 in fig. 4) induce a decrease of cellular index in 2-5 hours after their addition to the culture E-plate (n=3) (fig. 4). The lower concentrations did not induce any effect on the cellular index, the plateau phase being maintained with a similar pattern as the untreated, control cells.

Master AM *et al.*, 2016 [9] suggested a mechanism by which the superparamagnetic nanoparticles (SMNP) of small size (7-8 nm in diameter) can enter the tumor cells and accumulate and form clusters into the lysosomes, thus being targeted with low frequencies magnetic fields in order to disrupt cellular architecture. We can speculate that our combustion-synthesized MNPs (10 -15 nm in diameter) can also accumulate into the tumor cells and induce *per se* cytoskeletal disruption.

Due to their small size and physiochemical properties, the MNPs are considered a promising technology for applications in anti-tumor interventions [10-13]. Nanoparticles pass through physiological barriers (cell

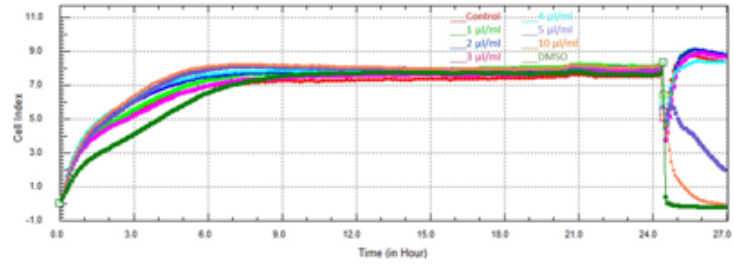


Fig. 2. Real-time 24h cell index measurement. During the first 24h of cell culture in 16-well E-plate on xCELLigence system all wells presented a progressive increase of cell index

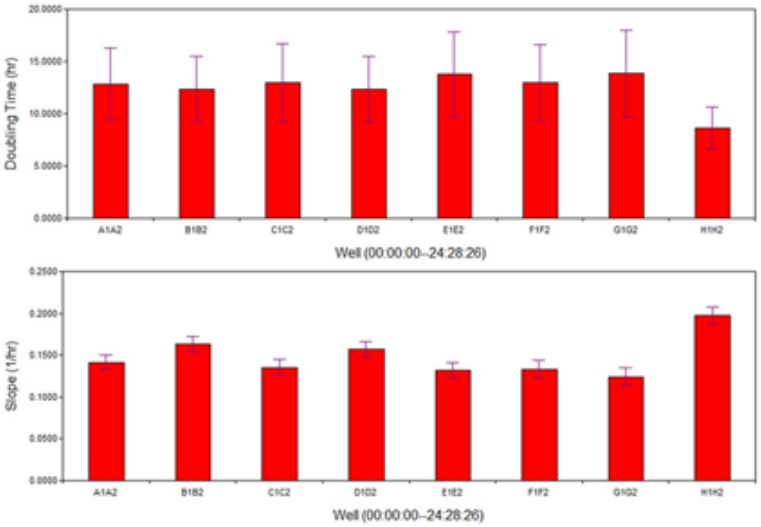


Fig. 3. Proliferation of SK-BR3 tumor cells evaluated by xCELLigence system. Doubling time and slope of SK-BR3 cells during 24h of cells culture on E-plate and analysis of these parameters using the RTCA platform

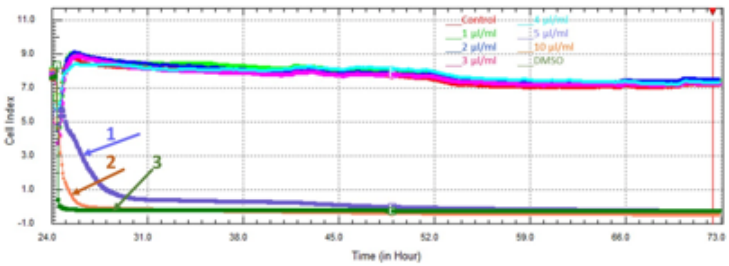
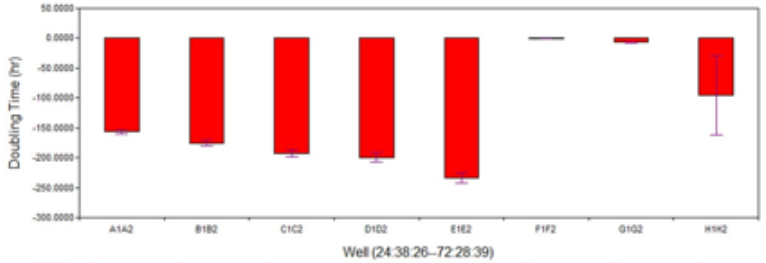


Fig. 4. Real-time 48h xCELLigence analysis of cell index. Rapidly decreasing cell index of SK-BR3 in cells treated with 5 µL/mL and 10 µL/mL MNPs (arrow 1 and 2, respectively), similar to negative control cells (treated with DMSO in concentration of 10 µL/well) (arrow 3)



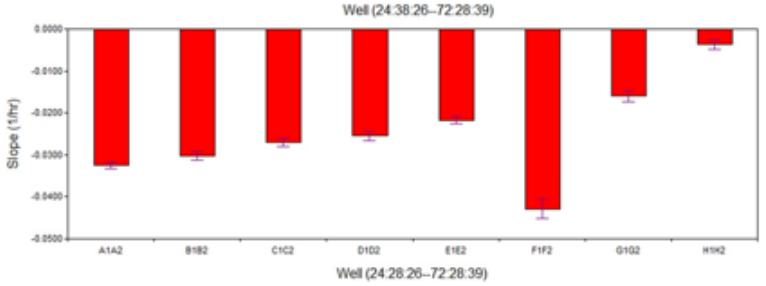
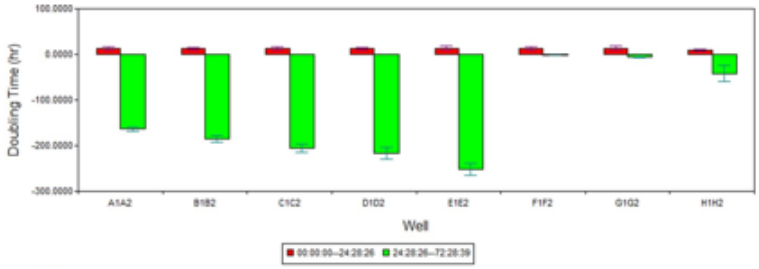


Fig. 5. Doubling time and slope of SK-BR3 cells during 48h following the addition of MNPs in different concentrations. Cells monitored on E-plate and the doubling time and slope were analyzed using the RTCA platform



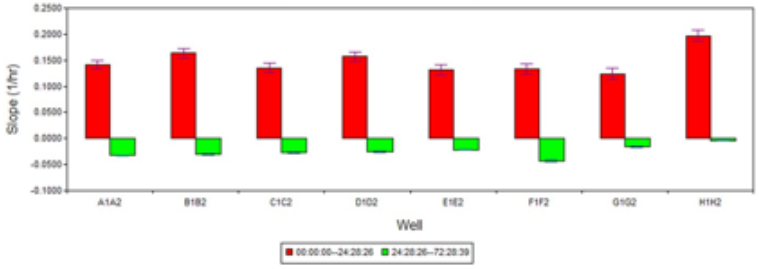


Fig. 6. Comparative analysis of doubling time and slope before and after the addition of MNPs in culture media. After addition of MNPs, there is a significant difference ($p < 0.05$, $n=3$) of doubling time and slope for SK-BR3 tumor cells.

membrane) and penetrate into cells [14], but the extent of their intracellular damage is dependent upon concentration. Here we showed that relatively low concentrations of MNPs (5 and 10 µL/mL) can induce cellular damage in such a manner that the cells are no longer able to proliferate or maintain their morphology *in vitro* conditions. Figure 5 shows significant decrease of doubling time and slope for MNPs-treated SK-BR3 during 48h monitoring using xCELLigence system and RTCA analysis platform ($p<0.05$, $n=3$).

The MNPs currently used in medicine can be used in conjugations with different compounds, such as drugs, enzymes, proteins, antibodies, nucleotides, thus being functionalized to target tumor sites using magnetic field or hyperthermia, in a theranostic approach for cancer [15]. Here we demonstrated the properties of unconjugated combustion-synthesized MNPs to target and impair the function of tumor cells *in vitro*.

Up to date, this is the first study evaluating the effect of combustion synthesized MNPs on SK-BR3 tumor cell line using the xCELLigence system as real-time analysis platform under *in vitro* conditions.

Conclusions

The combustion synthesized magnetite nanoparticles (MNPs) double layer coated with oleic acid can induce perturbations in tumor cells homeostasis, disruption of the cytoskeletal structure and loss of adhesion on the culture plate, resulting in dramatic decrease of cellular index of SK-BR3 tumor cells. Concentrations of 5 and 10 µL/mL MNPs are also exhibiting a cytotoxic effect *per se* on SK-BR3 tumor cells.

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