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Magnetic hyperthermia enhances cell toxicity with respect to exogenous heating

Beatriz Sanz [a, 1], M. Pilar Calatayud [a, b], Teobaldo E. Torres [a, b, c], Mónica L. Fanarraga [d], M. Ricardo Ibarra [a, b], Gerardo F. Goya [a, b, *]

[a] Instituto de Nanociencia de Aragón (INA), Universidad de Zaragoza, C/Mariano Esquillor S/N, CP 50018, Zaragoza, Spain
[b] Departamento de Física de la Materia Condensada, Facultad de Ciencias, C/ Pedro Cerbuna 12, 50009, Zaragoza, Spain
[c] Laboratorio de Microscopias Avanzadas (LMA), Universidad de Zaragoza, C/Mariano Esquillor S/N, CP 50018, Zaragoza, Spain
[d] Grupo de Nanomedicina-IDIVAL, Universidad de Cantabria, Herrera Oria s/n, CP 39011 Santander, Spain

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ABSTRACT

Magnetic hyperthermia is a new type of cancer treatment designed for overcoming resistance to chemotherapy during the treatment of solid, inaccessible human tumors. The main challenge of this technology is increasing the local tumoral temperature with minimal side effects on the surrounding healthy tissue. This work consists of an *in vitro* study that compared the effect of hyperthermia in response to the application of exogenous heating (EHT) sources with the corresponding effect produced by magnetic hyperthermia (MHT) at the same target temperatures. Human neuroblastoma SH-SY5Y cells were loaded with magnetic nanoparticles (MNPs) and packed into dense pellets to generate an environment that is crudely similar to that expected in solid micro-tumors, and the above-mentioned protocols were applied to these cells. These experiments showed that for the same target temperatures, MHT induces a decrease in cell viability that is larger than the corresponding EHT, up to a maximum difference of approximately 45% at T = 46 °C. An analysis of the data in terms of temperature efficiency demonstrated that MHT requires an average temperature that is 6 °C lower than that required with EHT to produce a similar cytotoxic effect. An analysis of electron microscopy images of the cells after the EHT and MHT treatments indicated that the enhanced effectiveness observed with MHT is associated with local cell destruction triggered by the magnetic nano-heaters. The present study is an essential step toward the development of innovative adjuvant anti-cancer therapies based on local hyperthermia treatments using magnetic particles as nano-heaters.

The use of heat as a therapeutic tool in oncology dates back to the beginning of the previous century [1]. The biological effects of hyperthermia are two-fold: concomitant with the sensitizing effect of heat on tumor cells, which can improve the therapeutic effect of radiation, hyperthermia also produces a direct cytotoxic effect. As a result, an adequate heat treatment can practically kill tumor cells within a nutritionally deficient, hypoxic and acidic environment [2]. Although the therapeutic uses of heat on tumor cells have remained essentially unchanged, the methods for heat generation and delivery have experienced impressive advances. From the first protocols using water baths, heat delivery has evolved to precise, non-contact methods, including radiofrequency, microwaves, and focused ultrasound waves. The use of magnetic nanoparticles (MNPs) as nano-heaters is the most recent of such developments in the delivery of heat to tumor cells and is known as magnetic hyperthermia (MHT) or thermotherapy. The MHT protocol is based on the application of an alternating magnetic field (AMF) to the applied MNPs, which results in the coupling of the magnetic moments of the MNPs with the oscillating field and the conversion of the absorbed energy into heat within the target tumor. The remote, contactless action of the AMF to produce heat locally in the body makes MHT an interesting treatment for eliminating non-accessible, deep tumors that often cannot be treated with surgery. The sublethal heating of cancer cells has been well recognized for many years as a coadjutant therapy to radio- or chemotherapy [3–5].

* Corresponding author. Instituto de Nanociencia de Aragón (INA), C/Mariano Esquillor S/N, CP 50018, Universidad de Zaragoza, Zaragoza, Spain.
E-mail address: goya@unizar.es (G.F. Goya).
[1] Currently at nB Nanoscale Biomagnetics S.L., Zaragoza, Spain.

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The frequencies used for MHT are in the low radiofrequency range, i.e., at the approximately 100-kHz range. The physical mechanism underlying the observed heat generation is the interaction between the magnetic moments of the MNPs and the magnetic component of the applied electromagnetic waves, and the direct interaction of both components with living matter is almost negligible; thus, heat production is only observed in the area where the nano-heaters are delivered in the target region of interest [6]. The rationale behind the use of hyperthermia as a coadjutant protocol for radio- or chemo-therapy is that cancer cells are more sensitive to radiation when they have been previously subjected to high temperatures of 42–45 °C, and thus, the use of these two therapies results in a synergistic effect [2,7].

The physiological effects of temperature on living organisms are well understood [8]. The exposure of mammalian cells to elevated temperatures produces a dose-dependent effect that triggers a cascade of cellular events that compromise and/or damage the cells [9]. In cancer cells, high temperatures also interfere with the regulation of biological processes, such as proliferation and metabolism functions [10], and the exposure of tissues to temperatures above 43–45 °C induces necrosis or apoptosis [11]. Hyperthermia also triggers protein denaturation, which affects the function of most proteins involved in signaling, membrane, cytoskeleton or DNA maintenance, among other functions, and causes cell-growth inhibition and cell apoptosis [12]. Mammalian cells have short-term thermotolerance mechanisms that could be overridden by resistant cancer cells [13], such as the expression of a heterogeneous family of proteins labeled heat shock proteins (HSPs). These HSPs participate in the process of polypeptide refolding after protein denaturation caused by heat stress.

The neuroblastoma-derived SH-SY5Y cell line is a model of human malignant metastatic neuroblastoma characterized by a high resistance to oxidative [14] and thermal [15] stresses through the overexpression of high levels of HSPs. SH-SY5Y cells therefore exhibit a notable resistance to hyperthermic stress [16]. For this reason, this oncogenic thermo-tolerant cell line was selected as an *in vitro* model for analyzing the effects of magnetic hyperthermia [17].

This manuscript reports the results of a comparative study of two different heating treatments: heating using an exogenous source, specifically immersion in a water bath (EHT), and local intra-tumoral heating produced by magnetic hyperthermia (MHT). An empirical analysis of the different cellular responses to these two treatments, which focused on the comparison of the cytotoxic effects triggered by MNP-based hyperthermia with the effects induced by exogenous hyperthermia, is then presented.

1. Results and discussion

1.1. Heating treatments: exogenous hyperthermia (EHT) and magnetic hyperthermia (MHT)

This experiment aimed to compare the effects of EHT- and MHT-induced hyperthermia on human neuroblastoma cells. To determine the latent mechanisms of cell death induced by hyperthermia, we produced MHT using in-house synthesized PEI-MNPs as nano-heaters (see Fig. 1). Dense cell pellets (see Methods), which roughly simulate a "*micro-tumor*" environment and thus present conditions that resemble some of the intra-tumoral conditions observed during *in vivo* experiments, were used in two comparative analyses, namely immediate (t_o) and long-term (t = 6 h) cell viability assays.

1.2. Exogenous hyperthermia (EHT)

To investigate the effect of hyperthermia produced by 'exogenous heating' on a set of compacted neuroblastoma cell pellets, these pellets were exogenously heated in a water bath at different target temperatures up to 56 °C for 30 min and were then allowed to recover in cultures plates immediately after each treatment for 6 h before their cell viability was measured. The latter analysis aimed to assess any long-term heat-induced cytotoxic effects. These hyperthermia-treated cells were compared with (i) cells treated with AMF, (ii) cells incubated with MNPs but not treated with AMF, and (iii) cells maintained at sub-hyperthermic temperatures (experimental controls).

The control cell samples were maintained at sub-hyperthermic temperatures of 37 °C and 40 °C and presented viability levels of 97 and 93%, respectively. These results confirmed that sample manipulation induced minor or no effects on cell viability (SI, Fig. S1). The viability of cells exposed to mild-temperature hyperthermia (T = 42 °C) and then maintained for 6 h at 37 °C in CO_2 conditions was also tested, and no changes in cell viability or proliferation were detected. These data allowed us to discard the existence of any 'latent' effect on cell physiology. Incubation of the cells at 43 °C resulted in a significant decrease in cell viability to 87% (normalized to the control samples and measured immediately after the hyperthermia treatment), and a viability of 78% was obtained 6 h after the heating experiment. These divergences between t_o and t_{6h} increased with increasing temperature and reached a maximum difference of 22% with a temperature of 52 °C, as shown in the inset of Fig. S1 in the SI. For target temperatures above 52 °C, a massive cytotoxic effect, with an overall cell viability less than 20%, was observed; thus, no further differences were detected between the early and long-term cytotoxic effects.

In all cases, the viability data as a function of temperature could be fitted by a sigmoidal-type equation:

$$\mathbf{C}(\boldsymbol{T}) = \frac{\boldsymbol{A}}{1 + e^{\boldsymbol{B}\cdot(\boldsymbol{T}-\boldsymbol{T_o})}} \quad (1)$$

which is a simplified expression based on the two-state model of cell damage developed by Feng et al. [18] for constant exposure times. A more in-depth analysis of Eq. (1) within the context of the two-state model is beyond the scope of the present work, but it is important to note that the phenomenological approach using Eq. (1) allows a straightforward comparison of the parameters that characterize the temperature behaviors obtained with both the EHT and MHT protocols used in this study. The parameter A represents the viability percentage of the control cells (~98%), and B quantifies the temperature width for a given decrease in cell viability. Finally, the parameter T_0 (in °C) determines the temperature at which the cell viability function *C*(T) decreases to 50% of the maximum value. This 50% lethal dose (LD50%) represents the exposure required to kill half of the original cell population. We used Eq. (1) to comparatively analyze the LD50% of both EHT and MHT as a function of increasing target temperatures. The results for EHT showed that it was necessary to reach a temperature of 47.7 °C to achieve the long-term effect (t_{6h}) of killing 50% of the cells.

1.3. Magnetic hyperthermia (MHT)

Using MNPs as nano-heaters to increase the temperature in 'micro-tumor-phantoms' requires not only detailed knowledge of the power absorption profile of the MNPs but also thermodynamic considerations regarding heat loss in small-scale biological environments [12,19]. To determine the minimum number of cells required for attaining the target temperatures in the hyperthermia region (T < 42 °C), we compared several cell pellets containing various numbers of cells (from 2×10^6 to 2×10^7 cells in a 100-μL pellet) that were incubated with different concentrations (from 10