Journal of Magnetism and Magnetic Materials 380 (2015) 372–376

Contents lists available at ScienceDirect

Journal of Magnetism and Magnetic Materials

journal homepage: www.elsevier.com/locate/jmmm

Combination of hyperthermia and photodynamic therapy on mesenchymal stem cell line treated with chloroaluminum phthalocyanine magnetic-nanoemulsion

Leonardo B. de Paula [a,b], Fernando L. Primo [a,c], Marcelo R. Pinto [d], Paulo C. Morais [e,f], Antonio C. Tedesco [a,c,*]

[a] Departamento de Química, Centro de Nanotecnologia e Engenharia Tecidual, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14040-901, Brazil
[b] Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14049-900, Brazil
[c] Nanophoton® Company, SUPERA Innovation and Technology Park, Av. Doutora Nadir de Aguiar, 1805, Universidade de São Paulo, Ribeirão Preto, P 14056-680, Brazil
[d] Departamento de Química, Laboratório de Enzimologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14040-901, Brazil
[e] Instituto de Física, Universidade de Brasília, Brasília, DF 70910-900, Brazil
[f] School of Automation, Huazhong University of Science and Technology, Wuhan 430074, China

ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form
30 September 2014
Accepted 4 October 2014
Available online 5 November 2014

Keywords:
Nanobiotechnology
Magnetic nanoparticle
Phthalocyanines
Stem cell

ABSTRACT

The present study reports on the preparation and the cell viability assay of two nanoemulsions loaded with magnetic nanoparticle and chloroaluminum phthalocyanine. The preparations contain equal amount of chloroaluminum phthalocyanine (0.05 mg/mL) but different contents of magnetic nanoparticle (0.15×10^{13} or 1.50×10^{13} particle/mL). The human bone marrow mesenchymal stem cell line was used as the model to assess the cell viability and this type of cell can be used as a model to mimic cancer stem cells. The cell viability assays were performed in isolated as well as under combined magnetic hyperthermia and photodynamic therapy treatments. We found from the cell viability assay that under the hyperthermia treatment (1 MHz and 40 Oe magnetic field amplitude) the cell viability reduction was about 10%, regardless the magnetic nanoparticle content within the magnetic nanoparticle/chloroaluminum phthalocyanine formulation. However, cell viability reduction of about 50% and 60% were found while applying the photodynamic therapy treatment using the magnetic nanoparticle/chloroaluminum phthalocyanine formulation containing 0.15×10^{13} or 1.50×10^{13} magnetic particle/mL, respectively. Finally, an average reduction in cell viability of about 66% was found while combining the hyperthermia and photodynamic therapy treatments.

1. Introduction

Adult stem cells can be found in different organs or tissues such as brain, bone marrow, blood vessels, skin and liver [1], with the purpose to maintain tissue integrity through remodeling and injury repair [2]. Among the several types of adult stem cells, mesenchymal stem cells (MSCs) have attracted great interest. Their multipotentiality, easy isolation and in vitro expansion have made them an important therapeutic alternative with a wide range of clinical applications within the framework of cell therapy [3]. The attention devoted to this cell type has increased in recent years, because they can potentially regenerate tissues and repair injured organs, as demonstrated in clinical and pre-clinical studies [1].

Studies have shown that a sub-population of stem-like cells within tumors, known as cancer stem cells (CSCs), exhibits characteristics of both stem cells and cancer cells. In addition to self-renewal and differentiation capabilities, CSCs also seed tumors when transplanted into a host animal. The CSCs can be distinguished from other cells within the tumor by evaluating the symmetry of its cell division and changes in gene expression [4,5].

Hyperthermia (HPT) is an approach that promotes a rise in temperature at the biological site. It is probably one of the oldest

* Corresponding author at: Avenida Bandeirantes, 3900 Departamento de Química - Bloco: B17 - Centro de Nanotecnologia e Engenharia Tecidual - Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto - Universidade de São Paulo - 14040-901 – Ribeirão Preto/SP - Brazil.
E-mail address: atedesco@usp.br (A.C. Tedesco).

http://dx.doi.org/10.1016/j.jmmm.2014.10.098
0304-8853/

methods used to treat tumors in patients [6,8]. Nowadays, HPT has been successfully achieved by exposing tumors previously treated with magnetic nanoparticles to externally applied AC magnetic fields. Likewise, photodynamic therapy (PDT) is a promising strategy used to treat a variety of oncological diseases including skin cancer as well as other non-malignant diseases. The PDT is a well-established technology that has found application as anticancer therapy, which is based on selective absorption and retention of a photosensitizer by tumor cells. Cancer cell death can be therefore achieved by activating the drug (photosensitizer) with the appropriated wavelength using visible light [6,7]. The studies on glioblastoma have shown that the combined HPT and PDT approach is quite effective to treat this type of cancer cells [6].

Although several strategies to treat cancer currently available and based on well-established technologies the design of functional nanomaterials have paved the way for innovative types of cancer treatment, such as the combination of HPT and PDT therapies. Actually, both HPT and PDT therapies can be designed to act synergistically with minimal side effects [6–8]. Additionally, functional nanocarriers can efficiently overcome issues regarding multiple drug delivery, which, in the past, had been impossible to solve in an effective way. Indeed these, the functional nanocarriers can help to overcome the phenomenon of multidrug resistance and pervasive cellular barriers that limit access to the intended targets, such as the blood brain barrier among others [9]. One important issue in this field that remains to be bridged in the coming years is how to combine HPT and PDT therapies within the framework of a multipurpose nanomaterial platform, which can be supported by reliable protocols. Accordingly, our present work contributes towards that objective with the combination of therapies that aims to be more successful in treating this disease.

2. Experimental details

Synthesis of magnetic nanoemulsions (MNEs) via the process of spontaneous emulsification using the oil-in-water (o/w) approach has been successfully achieved in a well-controlled way as described by Primo et al. [11]. Drug-loaded MNEs have been investigated using the glioblastoma biological model in vitro assays and have provided excellent results [6]. In short, the oil phase (Edenor oil EV 85 KR Cosmoquímica Co., Jandira, SP, Brazil) consisted of acetone (Mallinckrodt – Baker Co., Dublin, Ireland) or a mix of organic solvents (organic phase) containing medium-chain-triglycerides, natural soy phospholipids (Lipoid S100, Lipid Co., Ribeirão Preto, SP, Brazil), and the PDT agent chloroaluminum phthalocyanine (ClAlPc) at 0.5 mg/mL (Sigma-Aldrich Co., St. Louis, MO, USA). The water phase contains surface-functionalized magnetic iron oxide nanoparticles with a monolayer of citrate at 1.50×10^{13} particle/mL, as previously described by de Paula et al. [6]. The drug-loaded magnetic nanoemulsion (MNE/ClAlPc) incorporated both the PDT agent (ClAlPc) plus the citrate-coated magnetic iron oxide nanoparticle.

The morphological characteristic of the resulting functional nanomaterial (MNE/ClAlPc) was evaluated by transmission electron microscopy (TEM). To this end, aliquots of the sample were submitted to ultracentrifugation (20,000 rpm at 60 min.) and the pellets were treated with cacodylate buffer at 1.0 mmol/L. Then, the samples were centrifuged again at 4000 rpm and re-suspended in glutaraldehyde 2% and osmium 2%. Finally, the samples were fixed in spurs and fractionated into microcuts for TEM analysis [12]. The laminar material was deposited directly onto microplates. The TEM equipment used in the analysis was the Morgagni 268E FEI operating at 70 kV.

Human bone marrow stromal MSC (BM-MSC) cell line used as the cell model was cultivated in α-modified Minimum Essential Medium (MEM; Gibco BRL, USA) and supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.3 μg/mL fungizone and 10% fetal bovine serum (FBS; all from Sigma) at 37 °C and with 5% CO_2. Cell micrography obtained using a Carl Zeiss microscope Axiovert 40-CFL coupled with a digital high-resolution camera Axiocam MRC.

For subcellular localization experiments, about 2×10^4 cells were carefully added under glass coverslips (13 mm) previously added to the bottom of each well. The cells remained in culture for 24 h (37 °C and with 5% CO_2) before being treated with the magnetic nanoemulsion (MNE/ClAlPc). Magnetically and optically untreated cells were used as control in the MNE/ClAlPc. The incubation time was set to three hours. After this period the cells were washed twice with Hank's buffer fixed with 2% paraformaldehyde diluted in PBS (Phosphate buffered saline) for 20 min. After fixation the cells were washed five times (three minutes each) with PBS containing 100 mM glycine and permeabilized with 0.1% Triton X-100 diluted in PBS over a period of 10 min. After the permeabilization step the cells were washed five times (five minutes each) with PBS and incubated with DAPI (4′,6-diamidino-2-phenylindole) diluted in PBS (1:200) for 20 min. After incubation this second incubation step the cells were washed five times (for five minutes each) with PBS and the coverslips containing the adhered cells were removed from the wells and mounted onto glass slides containing the Fluoromount G mounting medium. The slides were observed under the confocal microscope Leica SP5. A diode laser was used to detect the ClAlPc, whereas the nuclei were stained with DAPI (excitation/emission at 358/461 nm, respectively).

The equipment used to apply the external AC magnetic field operated at 1 MHz with 40 Oe amplitude. The HPT system was developed at the Institute of Biological Sciences, University of Brasilia (Brasilia, Brazil). The PDT treatment protocol used a typical diode laser operating at 700 mJ/cm^2. For different treatments the cell viability was assessed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), as previously described by Mosmann [10]. The MTT is a yellow salt that is reduced to formazan purple crystals in the presence of mitochondrial proteinases that are only active in viable cells [10]. TEM results revealed the spherical shape of the as prepared MNEs as well as their homogeneous particle size distribution (< 250 nm) for MNE/ClAlPc at 0.025 mg/mL (Fig. 3).

3. Results and discussions

We prepared MNEs of samples displayed enhanced thermodynamic stability. The oily core provides an optimal chemical environment that can host phthalocyanine-based compounds with lipophilic characteristics. Primo et al. [11] have described the physicochemical properties of the as produced MNEs. The in vitro studies herein described using the magnetic nanoemulsion/chloroaluminium phthalocyanine (MNE/ClAlPc) relied on the human BM-MSC biological model. Fig. 1 illustrates the BM-MSC cells in the typical confluent stage. The BM-MSC cells internalized MNE/ClAlPc after incubation for three hours at 37 °C (Fig. 2). The results suggested that the cell uptake was kept at the cytoplasmic level only once, because the MNE/ClAlPc distribution was homogeneous after three hours of incubation. Muehlmann et al. also found that ClAlPc internalization was concentrated in the cytosol of cancerous and non-cancerous cells [14].

To assess cell viability the cytotoxicity assay (MTT assay) used MNE/ClAlPc at two distinct concentrations of magnetic nanoparticles (0.15×10^{13} and 1.50×10^{13} particle/mL), at a fixed ClAlPc concentration (0.05 mg/mL). The results showed that MNE/ClAlPc was fully biocompatible in the used magnetic nanoparticle

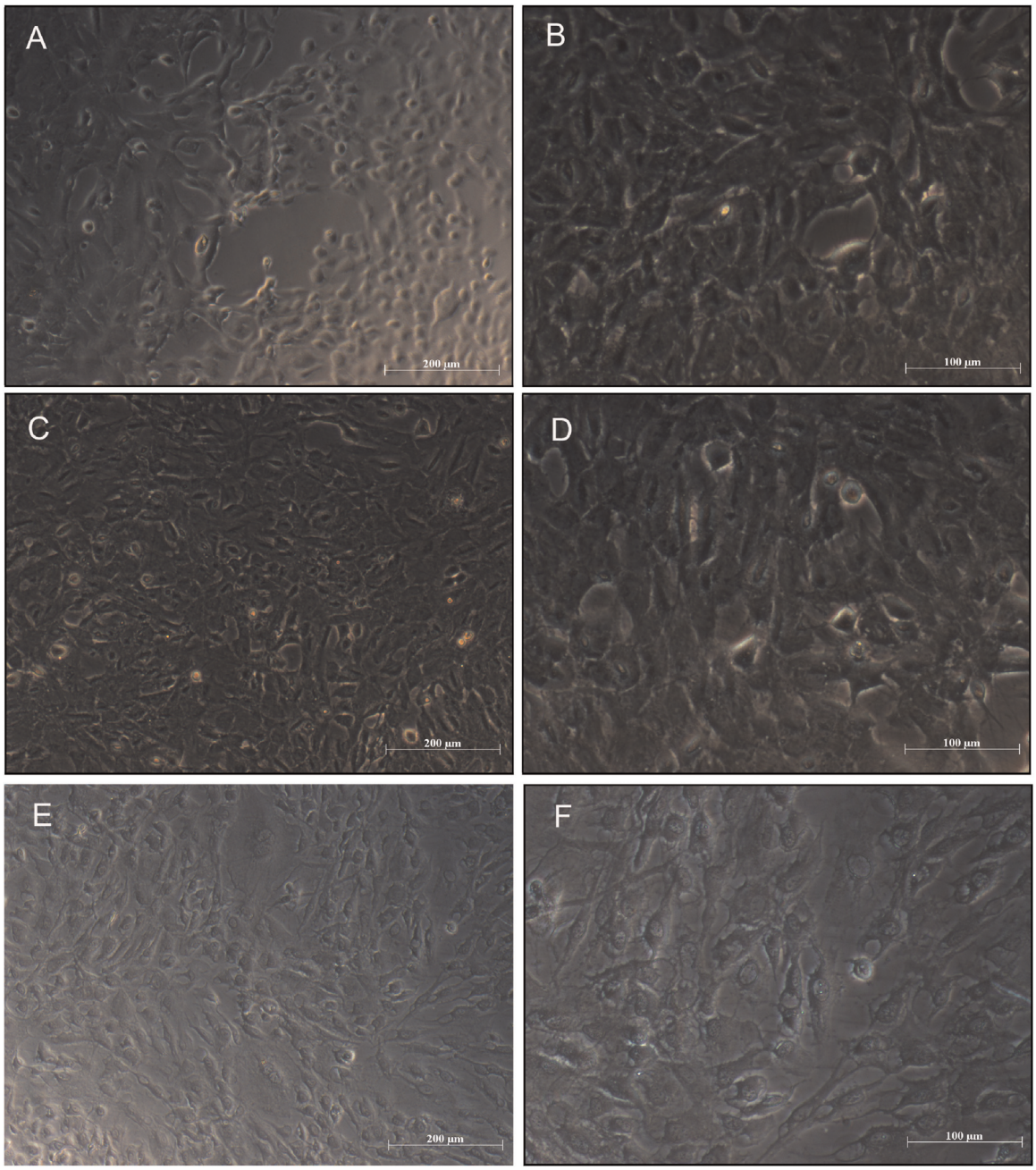

Fig. 1. BM-MSC cells control in α-MEM/FBS 10% at 48 h stage (A) 10× and (B) 20×. BM-MSC cells incubated with MNE/ClAlPc containing 0.05 mg/mL ClAlPc plus 0.15×10^{13} magnetic nanoparticle/mL in α-MEM/FBS 10% treated with the AC magnetic field (1 MHz and 40 Oe amplitude) in dark (C) 10× and (D) 20×. BM-MSC cells incubated with the MNE/ClAlPc containing 0.05 mg/mL ClAlPc plus 0.15×10^{13} magnetic nanoparticle/mL in α-MEM/FBS 10% treated with the AC magnetic field (1 MHz and 40 Oe amplitude) a diode laser at 700 mJ/cm^2 (E) 10× and (F) 20×.

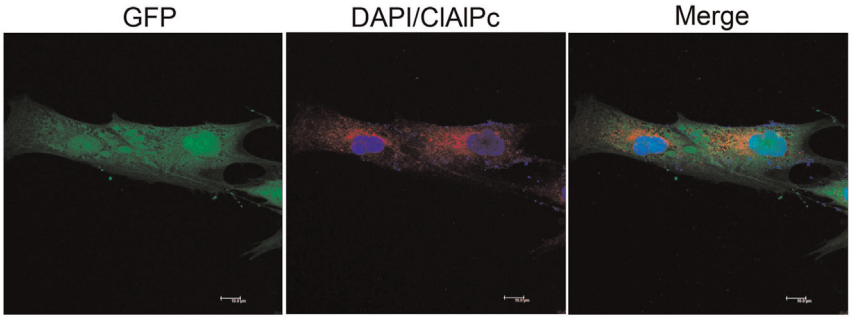

Fig. 2. Confocal laser scanning microscopy of in vitro uptake of the nanocarried photosensitive drug (MNE/ClAlPc). BM-MSC: GFP (green fluorescent protein)-expressed constitutively; DAPI (blue) – tagging nuclear; ClAlPc (red) – tagging the nanoemulsion/chloroaluminum phthalocyanine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

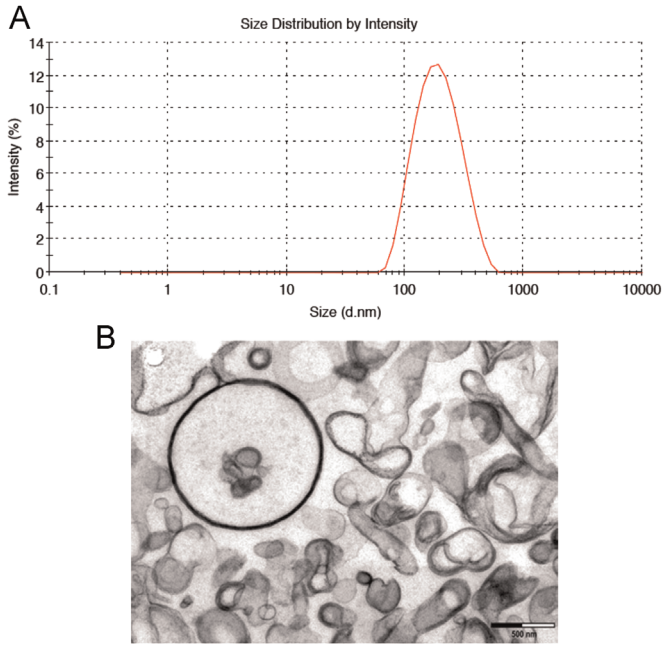

Fig. 3. (A) Size distribution of the MNE/ClAlPc sample assessed by photon intensity scattering analysis revealing average size of 173.6 nm and polydispersity index of 0.174. (B) Morphological analysis of the nanoemulsion/chloroaluminum phthalocyanine sample at 0.025 mg/mL using high-resolution TEM images recorded at 70 kV (44,000 ×).

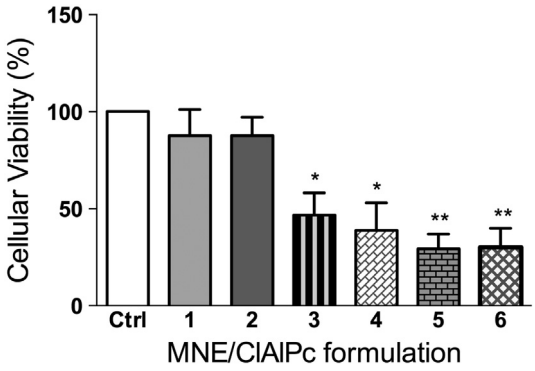

Fig. 4. Viability of BM-MSC cells incubated with different MNE/ClAlPc formulations all encapsulating equal ClAlPc contents (0.05 mg/mL). Ctrl = control (cells in medium at 3% serum); 1 = 1 MHz/40 Oe + sample containing 0.15×10^{13} magnetic nanoparticle/mL; 2 = 1 MHz/40 Oe + sample containing 1.50×10^{13} magnetic nanoparticle/mL; 3 = 700 mJ/cm^2 + sample containing 0.15×10^{13} magnetic nanoparticle/mL; and 4 = 700 mJ/cm^2 + sample containing 1.50×10^{13} magnetic nanoparticle/mL; 5 = 700 mJ/cm^2 + 1 MHz/40 Oe + sample containing 0.15×10^{13} magnetic nanoparticle/mL; and 6 = 700 mJ/cm^2 + 1 MHz/40 Oe + sample containing 1.50×10^{13} magnetic nanoparticle/mL. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey test. All the data were expressed as the mean ± SEM of three independent experiments. Statistical significance for this study was set at *; **$p < 0.05$.

concentration range from 0.15×10^{13} to 1.50×10^{13} particle/mL and loaded with 0.05 mg/mL ClAlPc, which resulted in cellular viability higher than 90%. This finding has been also demonstrated for glioblastoma cells [6].

The TEM results revealed the spherical shape of the MNEs as well as their homogeneous particle size distribution < 250 nm for MNE/ClAlPc at 0.025 mg/mL (Fig. 3).

Magnetic targeting found a wide range of applications that can be used in various in vitro applications, including cell separation, gene transfection, and sample enrichment in detection assays. Recent studies have shown that in vivo magnetic targeting can be a valuable approach to deliver therapeutic agents. The magnetic mobility and heating capability of magnetic nanoparticles can also help to trigger cellular events in vivo [13].

Fig. 4 collects the results of assays regarding the application of hyperthermia and photodynamic therapies. We found that while applying only the HPT treatment the cell viability is slightly as magnetic nanoparticle content within the MNE/ClAlPc sample increases by one order of magnitude (columns 1 and 2). Differently, when we applied only the PDT treatment (columns 3 and 4) using MNE/ClAlPc samples containing 0.15×10^{13} and 1.50×10^{13} magnetic particle/mL we found (compared to the control group) 54% and 62% in cell death, respectively. Finally, the data shown in Fig. 4 clearly evidenced the synergism of the combined HPT and PDT treatments as seen from the comparison of columns 1/2 and 3/4 with columns 5/6, revealing an increase of up to 70% in cell death.

Despite the excellent synergism achieved by combining HPT and PDT treatments we found that in the range of our experiment the therapeutic outcome did not depend strongly upon the magnetic nanoparticle content within MNE/ClAlPc samples (columns 3 and 4). Nevertheless, the cell death increases with respect to the basal level of the control (BM-MSCs in α-MEM 3%) while increasing the magnetic nanoparticle within the MNE/ClAlPc sample from 0.15×10^{13} to 1.50×10^{13} particle/mL. Compared with the control (column Ctrl) the cell viability data using the combined HPT and PDT treatments revealed that the mitochondrial activity is strongly correlated with the activation of cell death mechanisms. Our findings support the efficacy of combining both treatments (HPT and PDT) to act against potentially tumorigenic cells. In particular, association of HPT with PDT enhances the cell death activation mechanisms in adult stem cells and may represent a new strategy to treat a sub-population of stem-like cells within tumors (CSCs cells), because they exhibit features of both stem cells and cancer cells. Additionally, the combined approach presented herein may impact specific cancer treatments, for instance glioblastoma [6] and skin cancer [7].

4. Conclusions

In conclusion, stable magnetic nanoemulsion containing chloroaluminum phthalocyanine (MNE/ClAlPc) were successfully prepared and subsequently tested for cell viability using the human bone marrow mesenchymal stem cell (BM-MSC) line. The MTT assay was used to assess the cell viability under magnetic hyperthermia (HPT) and photodynamic therapy (PDT) treatments performed using isolated as well as combined protocols. The BM-MSCs were treated with two MNE/ClAlPc formulations containing equal amount of chloroaluminum phthalocyanine (ClAlPc) but different magnetic nanoparticle (MNP) contents (0.15×10^{13} or 1.50×10^{13} particle/mL). Under the HPT treatment the MTT assays showed no difference in cell viability for the two MNE/ClAlPc formulations. Differently, the MTT assays showed significant cell viability reduction while applying the PDT treatment at increasing MNP content. Moreover, further reduction in cell viability was observed while applying the HPT treatment combined with the PDT treatment. Our findings indicated that advances in clinical oncology can be envisaged by the synergy achieved while using combined HPT and PDT treatments.

Acknowledgment

L.B.P. was funded by the CNPq-PhD project 140998/2011-0 and CNPq-RHAE project 350223/2012-2. F.L.P. was funded by the PIPE-FAPESP project 2014/14231-7 (F.L.P.). We thank the Brazilian

agencies for financial support and we acknowledge the FAPESP thematic project 2008/53719-4.

References

[1] F.P. Barry, J.M. Murphy, Mesenchymal stem cells: clinical applications and biological characterization, Int. J. Biochem. Cell Biol. 36 (2004) 568–584.
[2] J.J. Mao, Stem-cell-driven regeneration of synovial joints, Biol. Cell 97 (2005) 289–301.
[3] J.J. Minguell, A. Erices, P. Conget, Mesenchymal stem cells, Exp. Biol. Med. 226 (2001) 507–520.
[4] J.M. Rosen, C.T. Jordan, The increasing complexity of the cancer stem cell paradigm, Science 324 (2009) 1670–1673.
[5] Z. Yu, T.G. Pestell, M.P. Lisanti, et al., Cancer stem cells, Int. J. Biochem. Cell Biol. 44 (2012) 2144–2151.
[6] L.B. de Paula, F.L. Primo, D.R. Jardim, et al., Development, characterization, and in vitro trials of chloroaluminum phthalocyanine-magnetic nanoemulsion to hyperthermia and photodynamic therapies on glioblastoma as a biological model, J. Appl. Phys. 111 (2012) 201–207.
[7] G.C. Bolfarini, M.P. Siqueira-Moura, G.J.F. Demets, et al., In vitro evaluation of combined hyperthermia and photodynamic effects using magnetoliposomes loaded with cucurbit[7]uril zinc phthalocyanine complex on melanoma, J. Photochem. Photobiol. B: Biol. 115 (2012) 1–4.
[8] R. Tietze, S. Lyer, S. Dürr, et al., Nanoparticles for cancer therapy using magnetic forces, Nanomedicine 7 (2012) 447–457.
[9] W.C. Zamboni, V. Torchilin, A.K. Patri, et al., Best practices in cancer nanotechnology: perspective from NCI nanotechnology alliance, Clin. Cancer Res. 18 (2012) 3229–3241.
[10] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[11] F.L. Primo, M.A. Rodrigues, A.R. Simioni, et al., Photosensitizer-loaded magnetic nanoemulsion for use in synergic photodynamic and magnetohyperthermia therapies of neoplastic cells, J. Nanosci. Nanotechnol. 8 (2008) 5873–5877.
[12] F.L. Primo, M.B.C. Reis, M.A. Porcionatto, et al., In vitro evaluation of chloroaluminum phthalocyanine nanoemulsion and low- level laser therapy on human skin dermal equivalents and bone marrow mesenchymal stem cells, Curr. Med. Chem. 18 (2011) 3376–3381.
[13] G. Bao, S. Mitragotri, S. Tong, Multifunctional nanoparticles for drug delivery and molecular imaging, Annu. Rev. Biomed. Eng. 15 (2013) 253–282.
[14] L.A. Muehlmann, B.C. Ma, J.P.F. Longo, et al., Aluminum–phthalocyanine chloride associated to poly(methyl vinyl ether-co-maleic anhydride) nanoparticles as a new third-generation photosensitizer for anticancer photodynamic therapy, Int. J. Nanomed. 9 (2014) 1199–1213.