

Magnetic nanoparticles-based therapy for malignant mesothelioma

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Abstract

This work was aimed to analyze the versatility of the chick embryo chorioallantoic membrane (CAM) as *in vivo* model for the study of the malignant pleural mesothelioma (MPM) and the therapeutic potential of Fe₃O₄/salicylic acid magnetic nanoparticles (SaMNPs) on MPM cells. The antitumor effects of SaMNPs were studied by *in vitro* and *in vivo* tests on CARM-L12 TG3 rat malignant mesothelioma cells and human MPM xenografts implanted on CAMs. In order to assess the human MPM xenograft growth characteristics, calretinin, HBME-1 (Hector Battifora mesothelial epitope-1), and cytokeratins immunohistochemical stainings were performed. The human MPM xenografts continue to develop on the CAMs and xenograft MPM cells showed highly metastatic features and a particular pattern of metastasis. The SaMNPs had a specific uptake by the MPM cells and an antiproliferative effect at therapeutic doses greater than 100 µg/mL. The results confirmed the possibility to use the CAM as *in vivo* model to study the biology of MPM and to evaluate the antitumor potential of new therapeutic agents. They highlighted a strong antitumor effect of the SaMNPs on the rat and human MPM cells and open new perspectives in the treatment of MPM.

Keywords: magnetic nanoparticles, malignant mesothelioma, salicylic acid, CAM, antitumor therapy.

Introduction

The human malignant pleural mesothelioma (MPM) is an aggressive type of cancer with increased incidence, high resistance to radiation and chemotherapy, and a poor prognosis [1]. Platinum–antifolate therapy is the standardized treatment in the first-line setting, unfortunately with no remarkable effect on patient quality of life [2]. Many therapeutic agents are currently under *investigation* as a potential *new therapy* for this disease with dismal prognosis: *Vinca* alkaloids [3], vitamin E analogue [4], ascorbic acid [5], etc.

Recent reports and the clinical observations highlighted the salicylates potential as antineoplastic agents in some types of cancer: colorectal, esophageal, breast, lung, prostate, liver and skin [6–8]. Salicylic acid is a natural compound whose plasma concentration is dependent on aspirin or vegetables intakes, and with chemotherapeutic and chemopreventive effects by cyclooxygenase (COX) dependent/independent mechanisms of action [9, 10].

Nowadays, the ferromagnetic nanoparticles are frequently studied as antitumor agents due to their high cytotoxic activity and tropism for tumor cells *versus* normal cells [11, 12]. Iron oxide nanoparticles specifically act on cancer cells and induce their apoptosis by impaired mitochondrial functions [13].

Based on these findings, we have designed and synthesized a combined nanocomposite having an iron oxide core and a salicylic acid shell (Fe₃O₄/salicylic acid nanoparticles), as a strategy to assemble their synergistic anticancer effects [14, 15].

In order to evaluate the cytotoxic effects of this nanocomposite, we have performed *in vitro* tests on rat malignant mesothelioma cells and *in vivo* tests on human MPM xenografts implanted on the chick embryo chorioallantoic membrane (CAM). We used the CAM model due to its suitability to investigate the tumoral angiogenesis and the metastatic potential of different human malignancies [16]. Another goal of this work was to investigate the tumor growth patterns and immunohistochemical features of the human MPM xenograft implanted on CAM. Ultimately, we aimed to evaluate the usefulness of the CAM as *in vivo* model for study the new therapeutic agents for treatment of malignant mesothelioma.

Materials and Methods

Fe₃O₄/salicylic acid nanoparticles dispersion

Fe₃O₄/salicylic acid magnetic nanoparticles (SaMNPs) were synthesized by modified Massart co-precipitation, and were characterized by inductively coupled plasma–optical emission spectrometry (ICP–OES), high-resolution transmission electron microscopy (HRTEM) and dynamic light scattering (DLS), according to these methods as previously reported by some of us [14, 15].

Cell culture

CARM-L12 TG3 rat malignant mesothelioma cell line was obtained from *European Collection of Authenticated Cell Cultures* (ECACC). CARM-L12 TG3 mesothelioma cell line was propagated in Dulbecco's Modified Eagle's

Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S) and 2 mM Glutamine at 37°C and 5% CO₂. The cells were seeded until confluent in a humidified atmosphere and standard cell culture protocols were used to maintain the cell line by sub-culture and passage of the cells as monolayer in 25 cm² cell culture flasks (Corning, USA). The cell culture media, FBS and cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, USA). For the experiments, cells were trypsinized and cultured at a density of 2×10^5 cells/flask. The cells were treated with two different concentrations, respectively 100 µg/mL and 150 µg/mL Fe₃O₄/salicylic acid magnetic nanoparticles and incubated at 37°C, 5% CO₂, for 24 h, 72 h and 120 h. Suitable untreated controls were also concomitantly employed. After incubation, the cells were washed with phosphate-buffered saline (PBS) and detached from the flasks with Trypsin–Ethylenediamine-tetraacetic acid (EDTA). The cell number and viability was determined by Trypan blue (Sigma Aldrich, Germany) dye exclusion assay. Each dose was tested in a replicate flask.

Fe₃O₄/salicylic acid magnetic nanoparticles uptake

Nanoparticles uptake was assessed using the specific Perl's Prussian blue staining for Fe³⁺ ions evidence. Briefly, the prepared slides or the alcohol-fixed cells smears were immersed for 20 minutes in 10% potassium ferrocyanide and 20% hydrochloric acid solutions mixed in equal parts. The counterstaining was made with the Nuclear Fast Red or Eosin for five minutes. A cell was considered Perl's Prussian blue staining positive if the intracytoplasmic blue granules could be observed with a Nikon microscope at $\times 100$ magnifications. The percentage of nanoparticle-loaded cells was determined by manual counting of stained cells on 40 microscopic fields.

Cell viability assay

After the nanoparticles treatment, the cells were gently harvested and a cell sample (200 µL) was taken from each flask. A cell suspension was made at a suitable dilution in Hanks' Balanced Salt, mixed with 200 µL of a 0.4% Trypan blue solution and allowed to stand for 5 minutes at room temperature. A small amount of the mixture was transferred to a hemocytometer and cells were counted using an inverted microscope (Olympus, CKX41). The viable cells with an intact membrane are able to exclude the dye and were counted as clear cells, whereas dead cells without an intact membrane take up the dye as blue ones. The viable cells were expressed as a percentage of the total counted cells.

Human tissue samples

Fresh samples of the tumor tissue were harvested from a patient with MPM who underwent palliative surgery (pleurectomy/decortication) in the Clinic of Thoracic Surgery, Emergency County Hospital of Craiova, Romania. Patient individual informed written consent was obtained according to the guidelines of the Ethics and University and Scientific Deontology Committee of the University

of Medicine and Pharmacy of Craiova, Romania. The samples were washed several times in isotonic saline solution then they were divided with a microtome blade to obtain pieces of about 2 mm³ for immediately implantation on the chick embryo chorioallantoic membrane. Some pieces were fixed in 4% neutral buffered formalin solution and processed for immunohistochemistry.

Chick embryo chorioallantoic membrane model

A number of 20 fertilized White Leghorn chicken eggs were incubated at 37.5°C and 70% relative humidity. On the third day of incubation, 3 mL of albumen were evacuated using a 18G needle and the chick embryo was exposed through a 2 cm diameter round window cut in the top of the egg. The window was resealed with adhesive tape and the egg was placed back in the incubator until days 7, when the CAM was sufficiently developed to offer an optimal microenvironment for human tissue xenografts growth.

Human MPM tissue samples were transplanted on the CAM on the seventh day of chick embryo development and the xenografts evolution was daily examined by a stereomicroscope with lateral illumination. Four days later (on the 11th day of incubation), half of the eggs were selected to evaluate the SaMNPs effects on the mesothelioma xenografts cells, and the rest were used to assess the development of human MPM xenografts on the CAMs. Each egg of the first group was intravenously injected with a 0.2 mL dose of the SaMNPs aqueous dispersion. Then, the nanoparticles were targeted with a 0.18 T NdFeB rod magnet that was placed for 15 minutes on the elected tumor area. The eggs were placed back in the incubator for 24 h. Another 0.2 mL dose of SaMNPs was intravenously administered and magnetically targeted to the same tumor area 24 h later (on the 12th day of incubation).

All the chick embryos were sacrificed on the 16th day of incubation. The tumor tissue with surrounding CAM was harvested and fixed in 4% neutral buffered formalin solution for further investigations.

Histology and immunohistochemistry

Formalin-fixed samples of the human MPM tissue and mesothelioma xenografts harvested with surrounded CAM were processed for paraffin embedding. Tissue sections, 5 µm thick, were cut for both classical Hematoxylin–Eosin (H&E) staining and immunohistochemistry analyses. Briefly, after antigen retrieval, sections were first incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next blocked for 30 minutes in 3% skim milk (Biorad, Medicalkit, Romania), then they were incubated with the primary antibodies overnight, at 4°C, and the next day the signal was amplified for 30 minutes utilizing a species specific human-adsorbed peroxidase polymer-based system (Nichirei–Histofine, Medicalkit, Romania). Finally, the signal was detected with 3,3'-Diaminobenzidine (DAB, Dako, Medicalkit, Romania) and the slides were coverslipped in DPX (Fluka, Medicalkit, Romania) after a Hematoxylin staining. Each experiment included negative controls obtained by omitting the

primary antibodies. Diagnostics of mesothelioma has been drawn based on the presence of immunopositive cells for calretinin, HBME-1 and low-medium molecular weight cytokeratins (CK 5/6).

Results

Fe₃O₄/salicylic acid magnetic nanoparticles synthesized by some of us and used in this work have a hydrodynamic diameter around 50 nm, and core diameter in the 10–12 nm range (Figure 1, a and b). Also, the nanoparticles dispersion is stable (*zeta* potential of +39.3 mV), has an iron concentration of 0.356 mg/mL, and it has a great maneuverability in static magnetic field [14, 15].

In vitro assay

Table 1 shows the survival rate evolution of the CARM-L12 TG3 rat malignant mesothelioma cells after addition of the aqueous dispersion of SaMNPs in the culture medium. *In vitro* test showed a significant decrease in the survival rate of malignant mesothelial cells three days after nanoparticles administration at a dose of 150 µg/mL and five days after nanoparticles administration at a dose of 100 µg/mL (Figure 1, c and d). Total number of viable cells counted per flask denoted that the 150 µg/mL dose of nanoparticles slowed down the cell proliferation even after 24 h from treatment.

Table 1 – Results of *in vitro* nanoparticles assessment

Time	Probes	Uploaded cells [%]	Total average count of viable cells ($\times 10^5$)	Survival rate [%]
24 h	control	–	30.38	95
	100 µg/mL	79.9	15.77	83
	150 µg/mL	97.5	2.8	70
72 h	control	–	63.75	94
	100 µg/mL	92.27	7.5	73
	150 µg/mL	100	4.05	27
120 h	control	–	53.4	87
	100 µg/mL	100	3.0	30
	150 µg/mL	100	0.105	3

Prussian blue staining used to assess the nanoparticles uptake by the malignant mesothelioma cells showed that three days after administration almost all the cancer cells had embedded intracytoplasmic nanoparticles deposits (Figure 1, e and f). Microscopic analysis of the cell smears highlights that the cancer cell destruction is a result of a cell progressive loading with nanoparticles until their complete blockage (Figure 1g).

In vivo assay

Histological and immunohistochemical analysis of human MPM tissue harvested from a patient (Figure 2, a and b) showed a biphasic type of mesothelioma with both epithelial and sarcomatoid cells immunopositive for calretinin, HBME-1 (Hector Battifora mesothelial epitope-1) and low-medium molecular weight cytokeratins (Figure 2, c–e).

Human MPM tissue evolution on the CAM model

All the eggs survived after human tissue implantation

(Figure 3a), and tumor cells from 18 (90%) xenografts migrated to develop metastatic tumor on the CAM surface. Two xenografts failed to develop.

The stereomicroscopic examination of the chorio-allantoic membrane revealed the metastatic features of the xenograft mesothelioma cells. One day after the human tumor tissue implantation, tumor cells clusters that broke away from xenograft, and disseminated on the chorio-allantoic membrane surface could be observed. Three days after implantation, some “fern-like” tumor growths (Figure 3b) developed on some areas of chorionic epithelium overlying the CAM veins (that carry oxygenated blood).

On the fifth day of tumor development, the eggs were randomly divided into three groups: the first group of four eggs was used to confirm by immunohistochemistry the presence of the tumor cells into tumor growths developed on CAM; the second group of four eggs was used as a control for untreated tumor development; the third group of 10 eggs was used to evaluate the effect of nanoparticles on tumor development.

First group evaluation

Histological and immunohistochemical analysis of metastatic tissue harvested from CAM of the first group of eggs showed the same biphasic features of the metastatic human MPM cells that kept only HBME-1 staining (Figure 3, d and e).

Second group evolution

On the seventh days after xenograft implantation, the tumor growths joined together to form a large tumor mass on the CAM surface that entirely covered the underlying veins (Figure 3c). It should be noted that metastatic growths do not developed on the chorionic epithelium overlying the CAM arteries. A weak angiogenic response induced by the primary tumor at the site of implantation was demonstrated by the presence of a few CAM arteries, which developed in a “spoked wheel” pattern around the xenograft.

Third group treatment

On the fifth and the sixth days from xenograft implantation, each egg from this group was treated with 0.2 mL aqueous dispersion of SaMNPs. Intravenous administration followed by the action of an external magnetic field allowed the accumulation of nanoparticles inside the arterioles that supply the xenografts (Figure 4, a and b). A second dose of nanoparticles was administered in order to achieve a concentration of nanoparticles greater than 100 µg/mL. Five days after the first intravenous administration of nanoparticles, we observed a decrease until disappearance of tumor masses both in the area exposed to the magnetic field and in surrounding areas (Figure 4c).

Immunohistochemistry and Perl’s Prussian blue staining of mesothelioma metastases treated with nanoparticles revealed only a few HBME-1 positive cells and many apoptotic bodies in the poorly developed tumor masses as a result of impaired mesothelioma cells proliferation by the nanoparticles uptake (Figure 4, d–h).

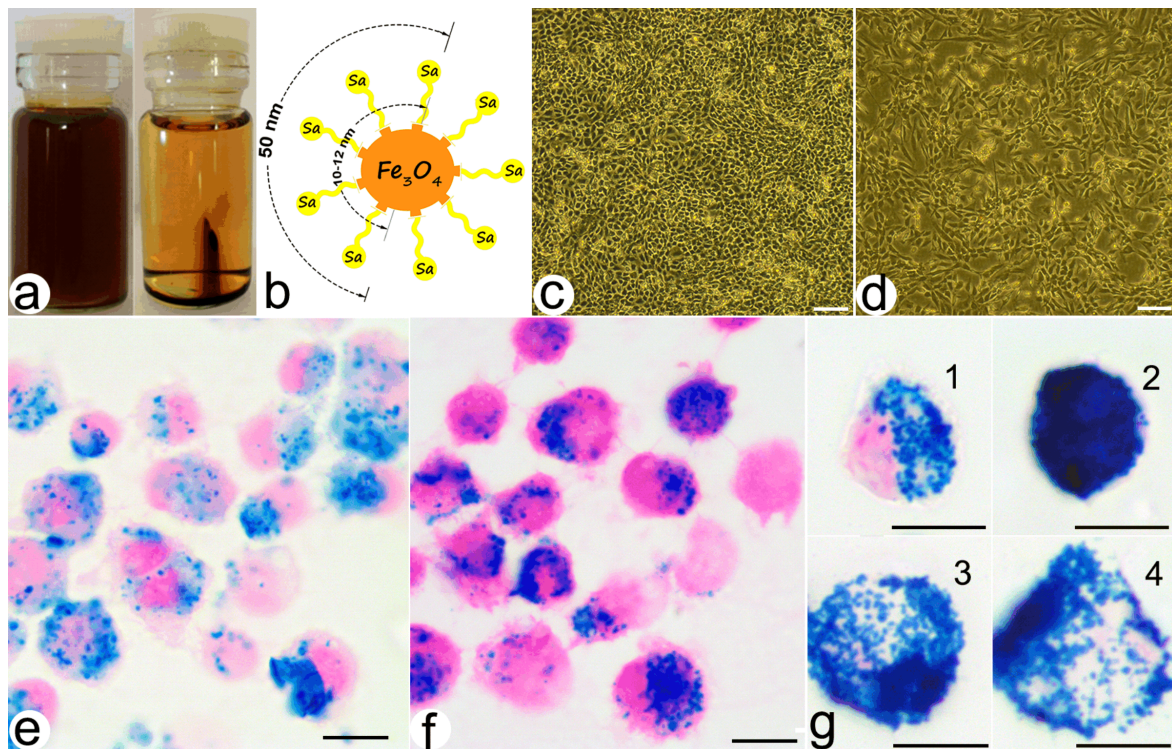


Figure 1 – *In vitro* actions of Fe_3O_4 /salicylic acid nanoparticles on CARM-L12 TG3 rat malignant mesothelioma cells: (a) Aqueous dispersion of nanoparticles before and after magnetic aggregation; (b) Fe_3O_4 /salicylic acid nanoparticles design; Day 5 – aspect of untreated (c) mesothelioma cell culture and after treatment (d) with 100 $\mu\text{g/mL}$ nanoparticles; Nanoparticles uptake assessing by Perl's Prussian blue staining with Nuclear Fast Red (e) and Eosin (f) counterstaining; (g) Stages of the mesothelioma cells evolution after nanoparticles uptake: nanoparticle's cell loading (1) is followed by complete cell filling (2), impaired cell functions (3) and apoptosis induction (4). Scale bars: (c and d) 50 μm ; (e–g) 20 μm .

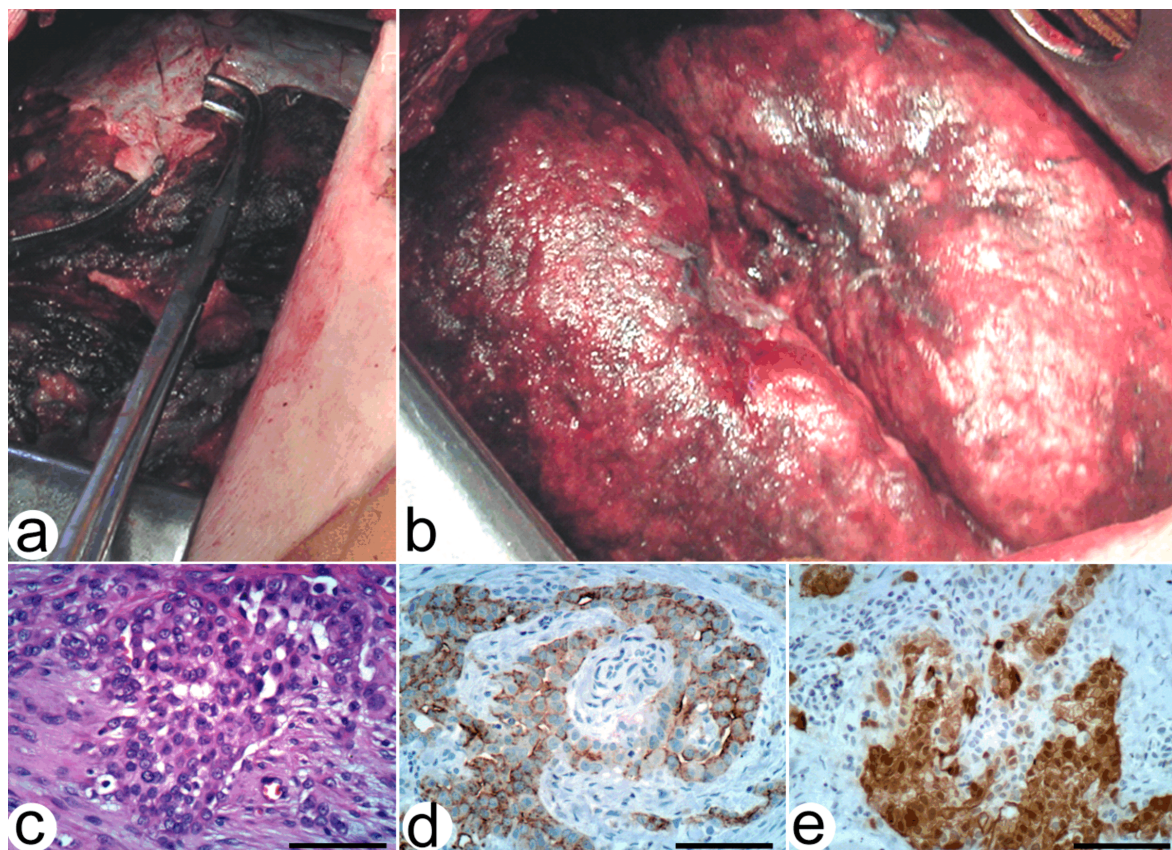


Figure 2 – Intraoperative (a) and after pleurectomy/decortication (b) lung aspect. H&E (c), HBME-1 (d) and calretinin (e) stainings revealed a biphasic type of malignant pleural mesothelioma. Scale bar: 50 μm .

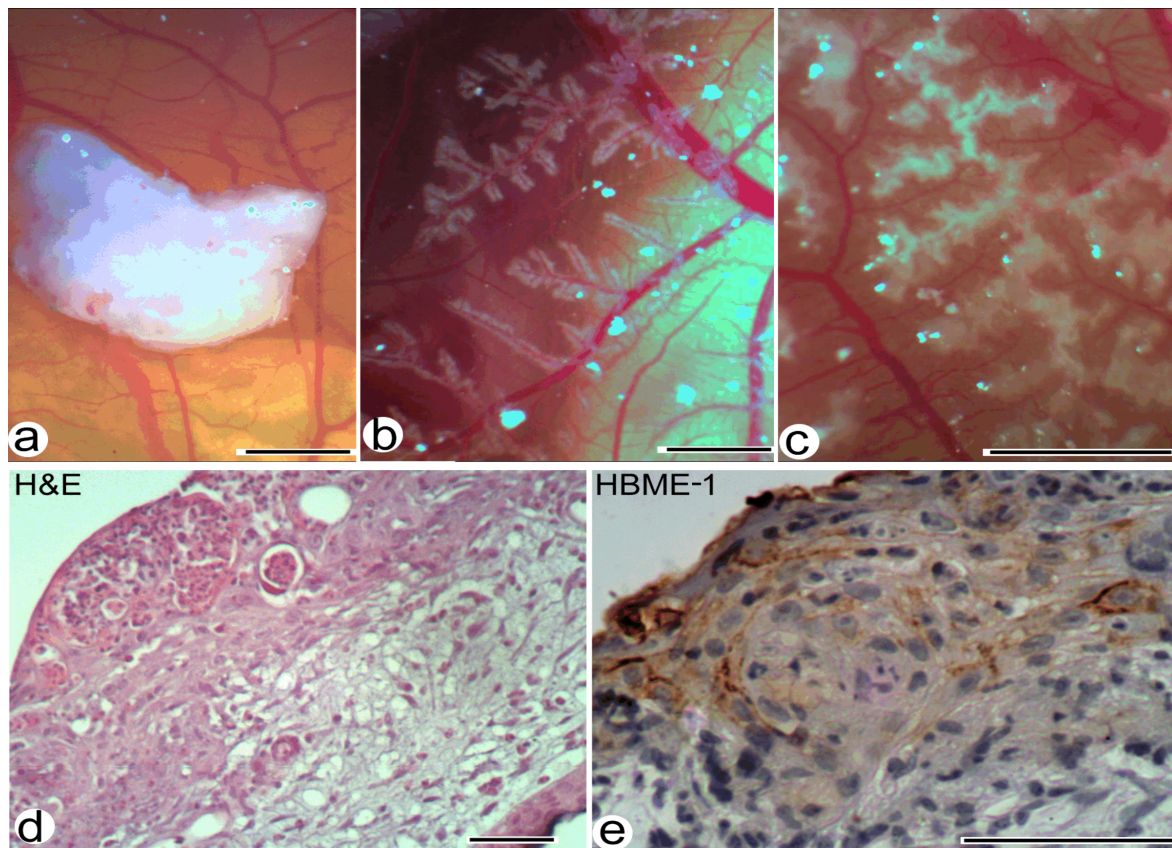


Figure 3 – Normal evolution of the human mesothelioma xenograft on the chorioallantoic membrane model: (a) Xenograft implanted on 7-day-old chorioallantoic membrane; Tumor growths aspects of mesothelioma cells migrated along the veins in the third (b) and seventh (c) day of implantation; Metastatic mesothelioma showed both structural (d) and immunohistochemical resemblance with the implanted tumor, many cells' membranes still being immunopositive for HBME-1 (e). Scale bars: (a–c) 1 mm; (d and e) 50 μ m.

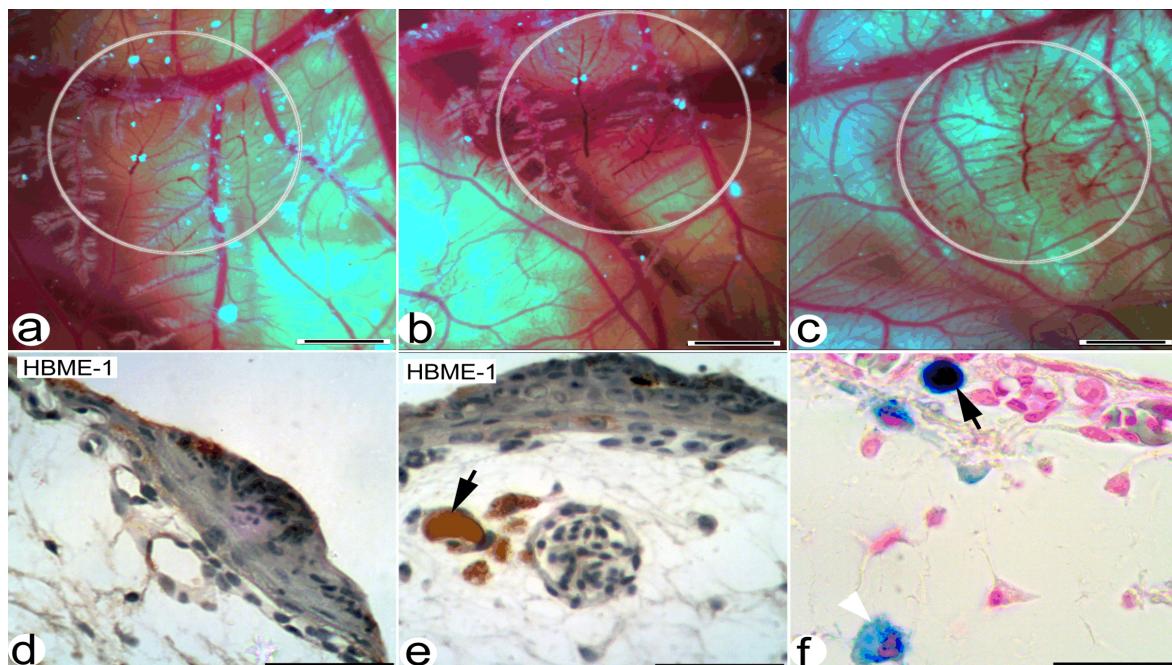


Figure 4 – Evolution of the mesothelioma metastasis treated with Fe_3O_4 /salicylic acid nanoparticles. Tumor growths visible after first (a) and second (b) intravenous administration of nanoparticles regress until the almost total disappearance (c) from the chorioallantoic membrane surface. Circles mark the exposed area to the static magnetic field action. Immunohistochemistry has shown a significant reduction in the tumor growths volume and number of HBME-1 positive staining cells both in the areas with unblocked (d) and blocked (e) arterioles by the magnetically aggregated nanoparticles. Perl's Prussian blue staining (f) highlighted the specific loading of mesothelioma cells (white arrowheads) with Fe_3O_4 /salicylic acid nanoparticles in the blocked arterioles area (black arrows). Scale bars: (a–c) 1 mm; (d–f) 50 μ m.

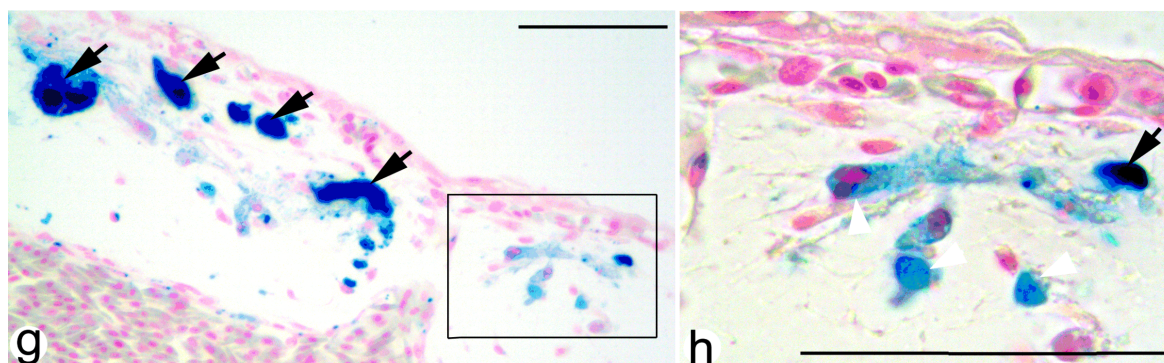


Figure 4 (continued) – Evolution of the mesothelioma metastasis treated with Fe_3O_4 /salicylic acid nanoparticles. Perl's Prussian blue staining (g and h) highlighted the specific loading of mesothelioma cells (white arrowheads) with Fe_3O_4 /salicylic acid nanoparticles in the blocked arterioles area (black arrows). (h) Detailed image of (g) inset. Scale bars: 50 μm .

Discussion

The highly vascularized CAM capable to provide optimal growing conditions for the implanted human tumor xenografts and the lack of the developed immune system are the major traits that recommend the chick embryo as an attractive alternative to assess the malignity of the tumor cells [17–21].

Until now, we did not found any reports about the use of CAM as *in vivo* model for human MPM study. The results of this study established that the human MPM xenografts continues to develop with their human features on chorioallantoic membrane and chick embryo can be a beneficial *in vivo* assessing model for the human mesothelioma.

In vitro tests on CARM-L12 TG3 rat malignant mesothelioma cells showed that the treatment with SaMNPs can induce a 70% decrease of survival rate. *In vitro* cytotoxic effects of same concentrations of uncovered ferromagnetic nanoparticles evaluated on different tumor cell lines [11, 12] showed a maximum 25% decrease of the survival rate. Previous reports highlighted the specificity of nanoparticles for cancer cells due to nanoparticles higher uptake by the cancer cell than that of the normal cells [22], and the ferromagnetic nanoparticles ability to selectively inhibit the cancer cell viability by inducing the mitochondrion-dependent apoptosis [13].

The higher cytotoxicity of SaMNPs on the CARM-L12 TG3 rat malignant mesothelioma cells can be explained by the synergistic antitumor effect of the shell. Salicylic acid appears to have anticancer effects by multiple mechanisms of action: down-regulate COX-2 [23] and c-Myc genes [8]; prevent NF- κ B activation [24]; affect mitochondrial functions [25], or inhibit the cell proliferation [26, 27] and angiogenesis [9]. Also, the rapid uptake of nanoparticles by rat malignant mesothelioma cells can be attributed to the same salicylic acid shell. The negatively charged shell provided by salicylic acid [14] increases the nanoparticle uptake [28] by the cancer cells. On the other hand, it has been shown that salicylate compounds increase the plasma membrane permeability and promote non-absorbable drug absorption [29].

The nanoparticles therapy of the human MPM metastasis developed on the chick CAM was guided by the

results from *in vitro* assessment. Therefore, *in vivo* assay on human mesothelioma cells showed the same antiproliferative effect obtained at therapeutic concentration higher than 100 $\mu\text{g/mL}$. The presence of many apoptotic bodies and the poorly developed tumor masses after nanoparticles therapy were observations that argue the *in vivo* antitumor effects of our synthesized SaMNPs. These antitumor properties can be assigned both to the cancer cell apoptosis induced by the nanoparticles core and to the inhibition of cancer cell proliferation by the nanoparticles shell. Ferromagnetic nanoparticles specific uptake by the cancer cell highlighted by the *in vitro* tests [11, 22] was also confirmed by the chick embryo CAM *in vivo* test. The nanoparticles appropriate load of the human MPM cells was sustained by the Perl's Prussian blue test. The nanoparticles' loading was low or unobservable into the CAM cells.

Conclusions

In this work, we demonstrated the versatility of the chick embryo CAM as a model for *in vivo* human MPM study. This model can be used for elucidating the mechanisms of mesothelioma cells metastasis and it has high value in evaluating the antitumor potential of new therapeutic agents. *In vitro* and *in vivo* tests on rat and human MPM cells show a specific uptake by the cancer cells and a strong antitumor effect of the SaMNPs. These results open new perspectives in the malignant mesothelioma therapeutic approaches and must be validated on larger mammalian models before clinical trials introduction.

Conflict of interests

The authors declare no conflict of interests.

Author contribution

Ion Mîndrilă, Sandra Alice Buteică and Dan Eduard Mihaiescu equally contributed to the manuscript.

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