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Article

In Vivo Fluorescent Melanoma Model: Electroporation Plus Electromagnetic Hyperthermia Significantly Reduces Tumor Size, Preliminary Results

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Abstract

Background/Objectives: Melanoma affects both sexes, and its incidence has increased in recent years. It is currently among the most common types of cancer. Standard chemotherapy, although effective, often lacks selectivity for tumor cells, resulting in dose-limiting side effects. Electrochemotherapy and electromagnetic hyperthermia have been investigated as innovative biomedical approaches. Electrochemotherapy improves drug delivery by facilitating electroporation, thereby increasing intracellular concentrations of chemotherapeutic agents and reducing associated adverse effects. Furthermore, electroporation enhances sensitivity to electromagnetic hyperthermia. However, few studies have focused on the combination of electroporation and hyperthermia in melanoma models. This study aimed to evaluate the synergistic effects of intratumoral administration of superparamagnetic iron oxide nanoparticles (SPIONs), electroporation (EP), and electromagnetic hyperthermia (EHP) on fluorescent melanoma tumors generated with the MV3-GFP cell line. **Methods:** Fluorescent melanoma tumors were generated using the MV3-GFP cell line. Treatments included SPIONs alone, SPIONs combined with hyperthermia, and SPIONs combined with electroporation and hyperthermia. Tumor size was monitored over 21 and 28 days. **Results:** SPIONs alone did not affect tumor growth (665 mm³). SPIONs plus hyperthermia reduced tumor size to 126.5 mm³ at day 21. The combination of SPIONs, electroporation, and hyperthermia produced a pronounced antitumoral effect, with tumor size decreasing to 95.5 mm³ at day 14 and 6.8 mm³ at day 21, followed by complete tumor disappearance by day 28. Electroporation significantly enhanced the antitumoral activity of the combined treatment. **Conclusions:** The combination of SPIONs, electroporation, and electromagnetic hyperthermia shows significant synergistic antitumoral activity in a melanoma model. These findings support further investigation in larger and more comprehensive *in vivo* studies to better understand the therapeutic potential of these combined approaches.

Keywords: electroporation; electromagnetic hyperthermia; SPIONs; *in vivo* assays

1. Introduction

Malignant melanoma (MM) is the most aggressive type of skin cancer, and arises from melanocytes, which are pigment-producing cells derived from the neuroectoderm with a highly polarized dendritic morphology [1]. Malignant melanoma represents a more dangerous form of skin cancer, characterized by its increasing prevalence globally and resistance to various therapeutic modalities. Melanoma cancer is the 17th most common cancer worldwide and the 22th leading cause of death in both females and males, with approximately 332,000 new cases and 58700 deaths estimated in 2022 [2].

To address the main drawbacks of chemotherapy, emerging technologies such as electrochemotherapy increase drug selectivity and effectiveness. This technique, which utilizes electroporation, facilitates the uptake of low-permeability chemotherapeutic drugs such as doxorubicin, bleomycin and cisplatin. It increases the intracellular drug concentration and cytotoxicity while minimizing side effects [3,4]. Electroporation is also used in nanomedicine to deliver nanoparticles into cells [5]. Superparamagnetic iron oxide nanoparticles (SPIONs) represent a promising avenue, leveraging their magnetic properties to induce hyperthermia when exposed to alternating magnetic fields [6]. Magnetic hyperthermia increases the temperature to 40-45°C and, in addition to inducing tumoral cell death [7,8], can potentially augment the efficacy of chemotherapy by increasing the cellular sensitivity to drugs *in vivo* and inducing apoptosis [9].

While studies have demonstrated the inhibitory effects of magnetic and laser hyperthermia on the growth of several types of cancer cells *in vitro* and *in vivo* [10–14] and its synergy with chemotherapy enhances tumor remission [15], limited research has been conducted on the effects of electroporation in combination with hyperthermia in melanoma cancer models. In this preliminary study, the aim was to assess the synergistic effects of electroporation plus hyperthermia in combination with electromagnetic hyperthermia (which uses SPION's) on the MV3-GFP cell line *in vitro*.

2. Materials and Methods

2.1. Cell Culture

The MV3-GFP cell line was obtained from Anticancer, Inc. The cells were cultured in Opti-MEM, supplemented with 10% fetal bovine serum and 1% of Penicillin Strep at Temperature: 37°C and an Atmosphere: air, 95%; carbon dioxide (CO₂), 5%.

2.2. Cytotoxicity Assays

2.2.1. MTT

To determine the cytotoxic effect of the SPIONs, an MTT cell viability assay was performed in 96-well plates. A total of 2×10^4 cells were seeded well and incubated overnight. After monolayer formation, the cells were treated with electroporation, SPIONs alone, SPIONs plus electroporation, SPIONs plus hyperthermia or SPIONs plus electroporation plus hyperthermia. Cell viability was measured after 24 hours of exposure to the treatments. Next, the Opti-MEM medium was removed, and the cells were washed with PBS. Then, 100 μ L of Opti-MEM and 10 μ L of 5 mg/mL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) were added, and the cells were incubated for 3 hours at 37°C. After the MTT solution was removed, the remaining crystals in the wells were solubilized. The absorbance was determined at a wavelength of 570 nm. Each experiment was repeated at least three times in triplicate.

2.2.2. Electrochemotherapy In Vitro Standardization

A total of 1×10^5 cells per well in a 1 ml final volume were seeded in a 24-well plate and 8 monopolar square wave pulses of 100 μ s duration were delivered immediately at a repetition

frequency of 1 Hz by an electroporation power supply (ELECTROvet EZ, Leroy Biotech) using a plate shaped contact electrode (8 mm gap) introduced into each well. For the combination of electroporation, 1×10^5 cells were collected in 500 μl of OPTI-MEM medium, then the cells were transferred to a 24-well plate and electroporation was applied. After electroporation, 100 μl of cell suspension was seeded per triplicate in a 96-well plate and incubated for 24, after which MTT assays were performed.

2.2.3. SPION's Heating Capability and Cytotoxicity

Electromagnetic Hyperthermia assays were performed using a ferrofluid of superparamagnetic iron oxide nanoparticles (SPIONs) synthesized by the co-precipitation method described by Cervantes et al., 2022 [16]. The optimal concentrations of SPIONs for hyperthermia assays were determined by Vizcarra Ramos et., al., 2024 [13].

2.2.4.. Electromagnetic Hyperthermia Standardization In Vitro

The most suitable time for electromagnetic hyperthermia was determined using the MV3-GFP cell line, considering that the irradiation time of the treatment should reduce cell viability by at least 50% [13]. After 24 h, the medium containing the SPION was discarded, and the cells were washed with D-PBS. Finally, 100 μl of fresh medium was added. Cell viability was measured using the cell proliferation reagent MTT.

2.2.5. Electrochemotherapy Combined with Electromagnetic Hyperthermia In Vitro

Once the optimal concentration of SPIONs and the optimal time of electromagnetic hyperthermia treatment were determined, the hyperthermia assay was evaluated in combination with electroporation in the MV3-GFP cell line. Treatments were applied to suspended (trypsinized) cells. For the groups of untreated cells, 3×10^4 cells per well were seeded in a volume of 250 μl into a 48-well plate. A total of 250 μl of the corresponding treatment mixture was added: Opti-MEM medium for the control group, SPIONs at a final concentration of 1 mg/ml. The electroporation variable was added to all the above groups. For this purpose, 9×10^4 cells were collected in 750 μl of Opti-MEM medium and 750 μl of the above treatments were added. Then, with plate-shaped electrodes with an 8 mm gap between them, 8 pulses of monopolar square-waves of 100 μs at 1,000 V/cm with a repetition frequency of 1 Hz were applied using an electroporator (ELECTROvet EZ, Leroy Biotech). The cells were homogenized, and 3×10^4 cells/500 μl were transferred to the 48-well plate used for the control groups and incubated for 24 h. For the experimental groups subjected to hyperthermia, 9×10^4 cells were collected in 250 μl of Opti-MEM medium in a 2 ml microtube and 250 μl of the corresponding treatment was added: 1 mg/ml of SPIONs for the hyperthermia group and electroporation plus hyperthermia. For the electroporation groups, the corresponding volume was transferred from the microtube to a 24-well plate and 8 pulses of monopolar square waves of 100 μs at 1,000 V/cm with a repetition frequency of 1 Hz were applied using a plate-shape electrode with 8 mm gap between them and an electroporator (ELECTROvet EZ, Leroy Biotech). Then, the pulses were returned to the 2 ml microtube.

All hyperthermia experimental groups were incubated at 37°C for 20 min, and a dry bath was maintained at 37°C during the hyperthermia process. Each tube was irradiated with an electromagnetic field at a heating frequency (f) of 460 kHz and an amplitude (H) of 20 kA/m for 10 min; in the first 5 minutes, a temperature of 43°C was reached, and this temperature was maintained for 5 minutes by modifying the amplitude conditions. The temperature was measured using a fiber optic probe. As described by Vizcarra Ramos, et al., 2024 [13].

Once hyperthermia was induced in all the experimental groups, 1 ml of Opti-MEM medium was added to each tube to obtain a final concentration of 1 mg/ml of SPIONs, the cells were homogenized, and 3×10^4 cells/500 μl were transferred to a 48-well plate and incubated. After 24 h, the medium supplemented with the SPIONs was discarded, and the cells were washed with D-PBS. Finally, 100

µl of fresh medium was added. Cell viability was measured using the MTT assay; 10 µl was added to each well, and the mixture was incubated for four hours.

2.2.6. In Vivo Assays

Immunodeficient nude murine models of the Nu/Nu strain, 12-week-old females, were obtained from the Bioterio Morelos and kept at the Research Bioterium of the Center for Research and Assistance in Technology and Design of the State of Jalisco (CIATEJ). The experiments were conducted in accordance with the guidelines set by the Guide for the Care and Use of Laboratory Animals and the rules established by the CICUAL of CIATEJ under the approved project number (2023-008A).

The MV3/GFP cells were cultured in Opti-MEM medium and inoculated into the murine models. Culture cells reaching approximately 85-90% confluence, were obtained by trypsinization. After dissociation, the cells were mixed with Matrigel (Sigma-Aldrich) in a 50:50 ratio, obtaining a total volume of approximately 80 µl in a microtube. This mixture was collected using a 1 ml insulin syringe 13 mm long, this inoculum was used in the murine models anesthetized previously, and the cells were administered subcutaneously once on upper right flank.

The experiment began by inoculating 5×10^6 cells per inoculation, and the appearance and development of the tumors were monitored daily. After two weeks, the tumors grew enough to begin treatments, tumors with approximate sizes of 18.5-102 mm³ were treated. Mice (n=3) were divided into three experimental treatments: a) mice treated with an intratumoural injection of ferrofluid of SPIONs, b) mice treated with an intratumoural injection of ferrofluid of SPIONs plus electromagnetic Hyperthermia, and c) mice treated with intratumoural injection of ferrofluid of SPIONs plus electroporation, plus electromagnetic Hyperthermia. Treatments were performed every 7 days until day 21. Irradiation: mice were exposed to a magnetic field with a frequency of 190 kHz and an amplitude of 50 mT for 20 min.

After the treatments were applied, the murine models were monitored using a small animal imaging system, the 'UVP iBox' from Analytik-Jena, which allows non-invasive detection of fluorescent and bioluminescent indicators.

Tumors were measured with a caliper every 7 days. Observations with the UVP iBox were performed every seven days, where, before being placed in the equipment, the tumors were measured and received the corresponding treatment. Tumor volume was calculated using the following equation: $V = 0.5 \times L \times W^2$ [17], where V is the tumor volume, L is the length of the tumor, and W is the weight of the tumor. Before tumors exceeded 18 mm in length or width the murine models were sacrificed.

2.3. Statistical Analysis

Statistical analysis was performed by one-way ANOVA and Tukey Test. A statistically significant difference was considered when the p-value was < 0.05. The results are presented as the mean ± standard error of at least three replicates.

3. Results

3.1. Electroporation Enhances Cytotoxic Effect to Hyperthermia Caused by SPION on Melanoma-Derived Cell Lines

Effects of SPIONs and Combined Treatments on Cell Viability

Determination of the optimal concentration of SPIONs for electromagnetic hyperthermia in melanoma-derived cell lines was performed as reported previously (Vizcarra Ramos et. al. 2024). Cell viability was assessed by MTT assay 24 h post-treatment across six experimental groups (n = 3 independent experiments, each performed in triplicate; N = 9 observations per group). A one-way analysis of variance (ANOVA) revealed a statistically significant effect of treatment on cell viability

($F(5, 48) = 630.81$, $p < 0.0001$, $\eta^2 = 0.985$), indicating that the experimental conditions collectively explained >99% of the variance in cell viability. Post-hoc pairwise comparisons were conducted using the Tukey HSD test (q critical = 4.05, $\alpha = 0.05$). Using SPIONs-alone as the reference condition (70.11 ± 3.72 %), three distinct response patterns were observed (Figure 1): (i) Control and electroporation-alone groups exhibited significantly higher viability than SPIONs-alone (Control: 97.56 ± 4.67 %, $\Delta = +27.44$; Electroporation: 85.44 ± 4.75 %, $\Delta = +15.33$; both $p < 0.05$, Tukey HSD), confirming that SPIONs alone produce a measurable baseline reduction in cell viability relative to untreated cells. (ii) SPIONs combined with electroporation (67.56 ± 3.13 %, $\Delta = -2.56$) did not differ significantly from SPIONs-alone ($p = \text{n.s.}$, Tukey HSD), suggesting that electroporation does not synergize with SPIONs to further reduce cell viability under the conditions tested. (iii) Hyperthermia-containing regimens produced profound and statistically significant reductions in cell viability relative to SPIONs alone. SPIONs + Hyperthermia reduced viability to 31.33 ± 2.87 % ($\Delta = -38.78$, -55% ; $p < 0.001$), while the triple combination (SPIONs + Electroporation + Hyperthermia) achieved the lowest viability recorded: 19.22 ± 1.99 % ($\Delta = -50.89$, -73% ; $p < 0.001$ vs. all other groups). These findings indicate that hyperthermia is the primary cytotoxic driver when combined with SPIONs, and that the addition of electroporation to this combination yields only marginal further reduction in viability.

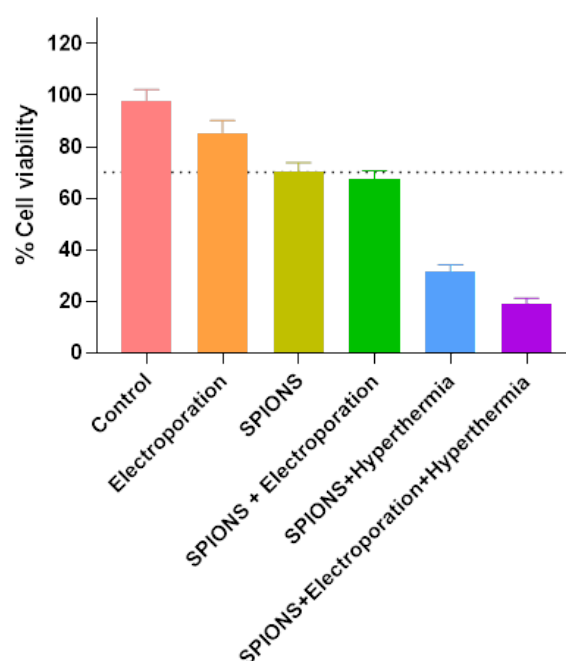


Figure 1. Cell viability (%) assessed by MTT assay 24 h after treatment. Bar graphs represent mean $\pm$ SD (N = 9 per group; three independent experiments, each performed in triplicate). The dashed horizontal line indicates the SPIONs-alone reference level (70.11 %). Groups were compared using one-way ANOVA followed by Tukey's HSD post-hoc test. Asterisks indicate statistically significant differences relative to the SPIONs group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Abbreviations: SPIONs, superparamagnetic iron oxide nanoparticles; EP, electroporation; Hyper, hyperthermia.

3.2. In Vivo Treatment by Intratumoral Injection

For intratumoral treatment, SPIONs were administered every 7 days for up to 21 days of follow-up. In the electroporation and electroporation plus electromagnetic hyperthermia groups, a solution containing SPIONs (1 mg/mL) was administered at a final volume of 100 μ L. A thermographic camera was used for real-time imaging to document temperature changes. The increase in temperature at the tumor site indicated activation of the SPIONs under electromagnetic stimulation, confirming effective and targeted photothermal conversion. Changes in tumor size were measured using a Vernier caliper

and with the IboxExplorer2/VisionWorksLS software, generating color maps based on the intensity of fluorescence emitted by viable MV3/GFP cells.

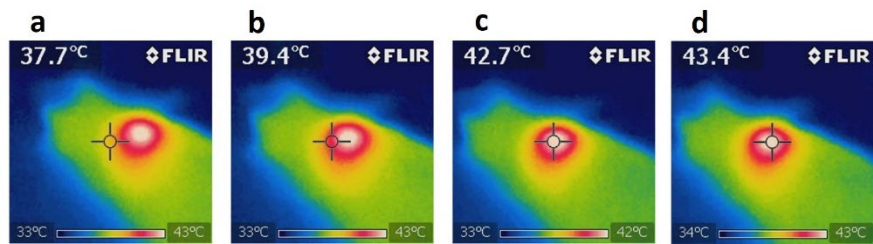


Figure 2. Thermographic images, temperature distributions during Electromagnetic Hyperthermia. a) Initial thermal image approximately 37.7 ± 0.5 °C surrounding tumor tissue. b) and c) Midpoint of irradiation, temperature was approximately 39.4 ± 0.5 and 42.7 ± 0.5 respectively and d) Final image after 10 min of irradiation, showing a peak temperature of 43.4 ± 0.5 °C.

Comparison of the Treatment and Control Groups

The measurements shown in the previous graphs for each mouse were compared in a time-course graph, where changes in tumor size for each group can be observed at day 0 and on days 7, 14, 21, and 28 in the *in vivo* experimental model.

Mice treated with SPIONs alone showed uncontrolled tumor growth, increasing from 18.45 mm^3 to a maximum size of 665 mm^3 by day 21 (Figures 3 and 4). In contrast, mice treated with SPIONs plus electromagnetic hyperthermia exhibited moderate tumor growth, suggesting an inhibitory effect, with tumor volumes of 110, 144, and 126 mm^3 on days 7, 14, and 21, respectively (Figures 3 and 5).

On the other hand, mice treated with SPIONs combined with electroporation and electromagnetic hyperthermia showed a significant reduction in tumor size at days 7 and 14, reached the smallest size by day 21, and complete tumor disappearance was observed by day 28 (Figures 3 and 6).

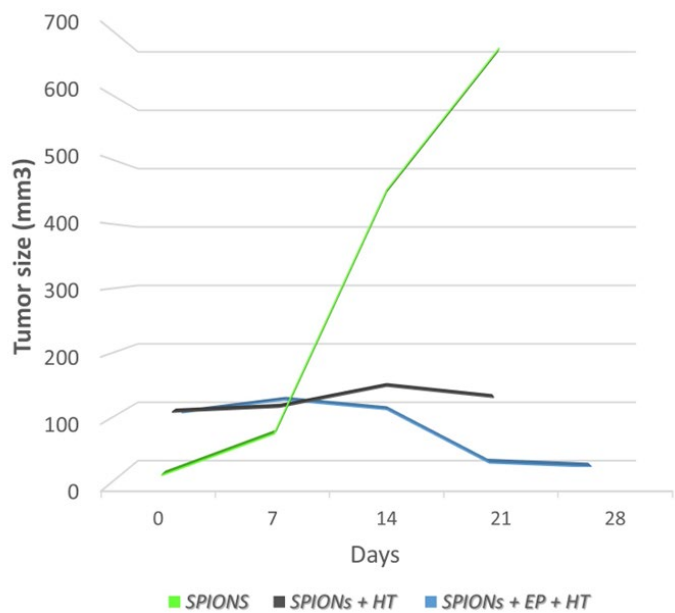


Figure 3. Effect of treatment on tumor growth. Tumor treated with just only SPIONs reach a size of 665 mm^3 ; and by other hand the SPIONs + Hyperthermia (SPIONs + HT) tumor mouse showed a stabilization in size after treatment, however, again, reaching a tumor size of 126 mm^3 . The mice treated with SPIONs + Electroporation + Hyperthermia (SPIONs + EP + HT) a significant reduction in tumor size on 21 day and disappeared on day 28 (Figure 3).

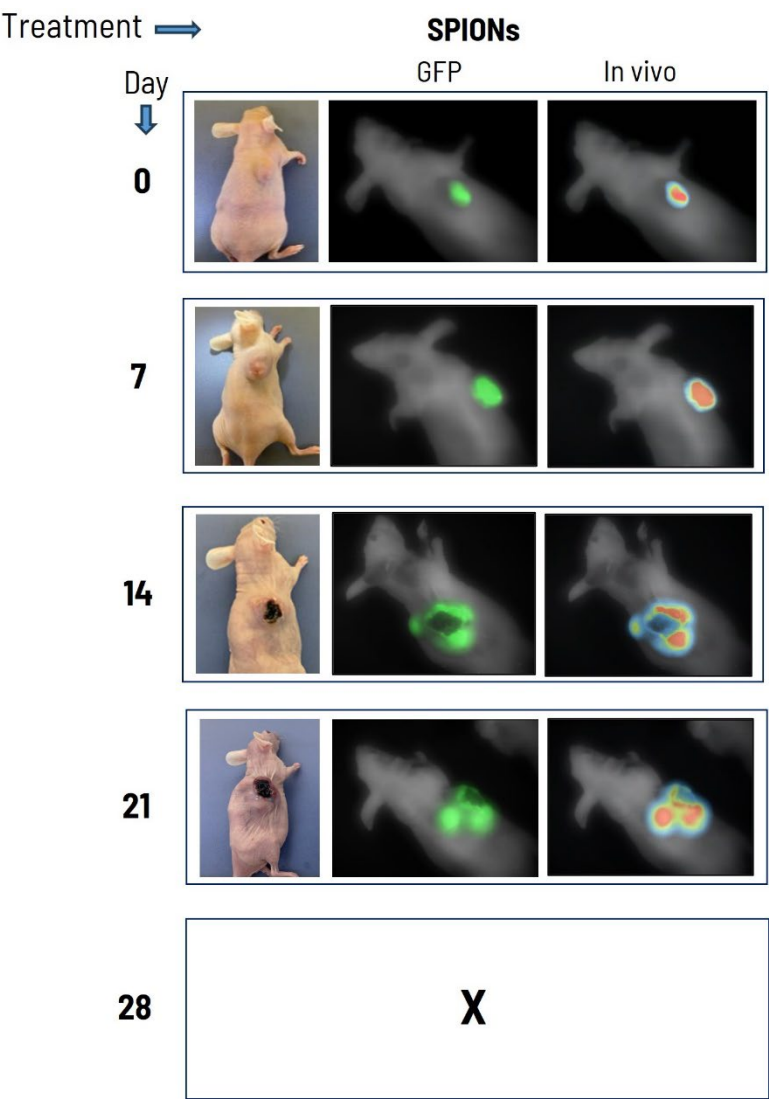


Figure 4. Effect of SPIONs on tumor growth *in vivo*. The change in tumor size and appearance. At the beginning on day 0 and on days 7, 14, 21. **GFP**; Tumor picture with Green Fluorescent Protein Filter (*In vivo*: tumor picture with “*in vivo*” (digital filter of the IboxExplorer²)).

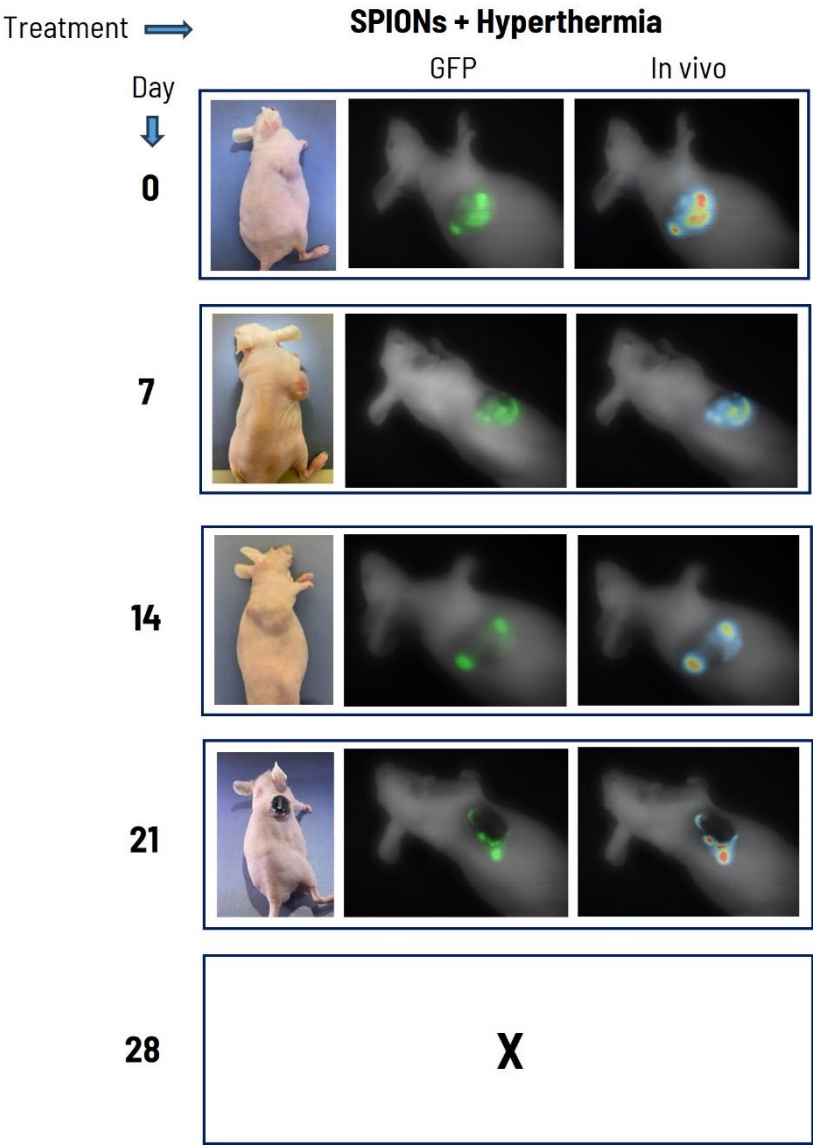


Figure 5. Effect of SPIONs *plus* electromagnetic hyperthermia on tumor growth *in vivo*. The change in tumor size is shown on days 7, 14, and 21. The reduction in tumor size and the fluorescence is evident on day 21.

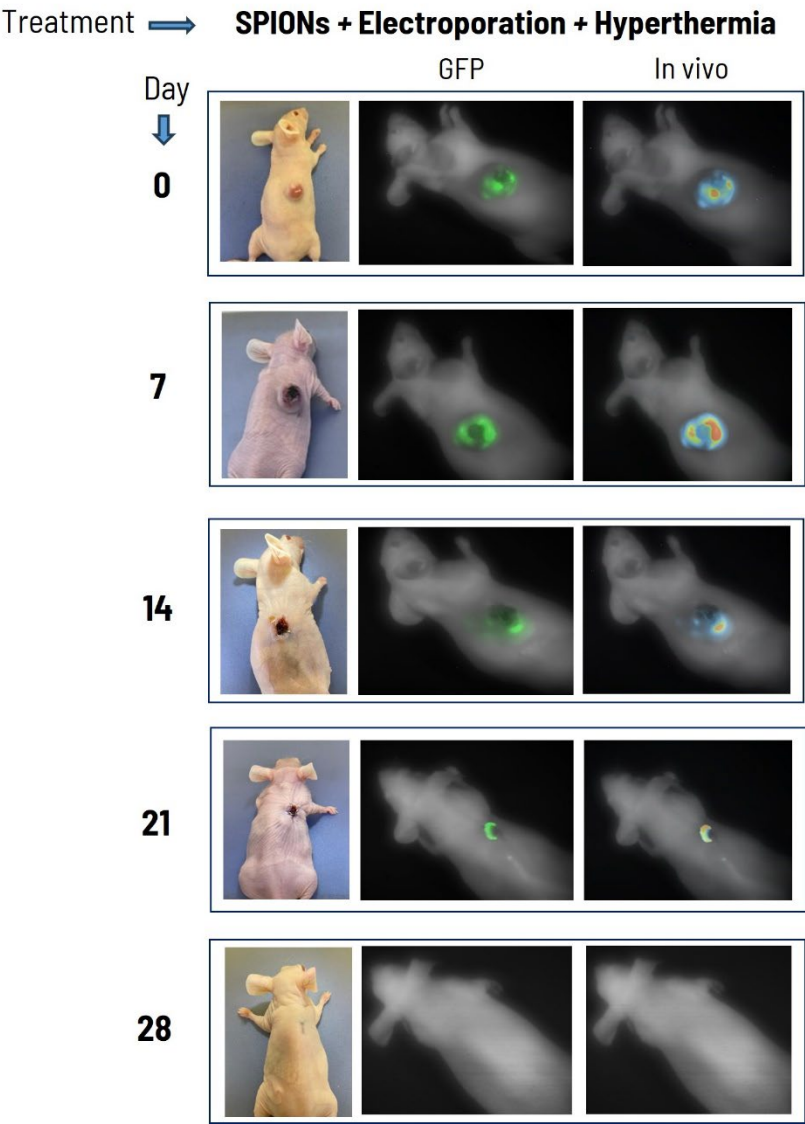


Figure 6. Effect of SPIONs *plus* Electromagnetic Hyperthermia on tumor growth *in vivo*. The change in tumor size is shown on days 7, 14, 21, and 28. Evidence of reduction of tumor size and complete elimination of the tumor at day 28.

4. Discussion

Chemotherapy remains a primary modality for managing advanced stages of melanoma. Adjuvant approaches, such as electrochemotherapy, aim to reduce the required doses of chemotherapeutic agents (e.g., bleomycin and cisplatin) while enhancing their cytotoxicity. This strategy improves therapeutic efficacy while minimizing adverse effects. Electrochemotherapy relies on electroporation to facilitate the uptake of molecules, including drugs and nanoparticles. In this context, superparamagnetic iron oxide nanoparticles (SPIONs) can be used to induce magnetic hyperthermia, thereby inhibiting tumor cell proliferation. It is hypothesized that, when combined with electroporation, increased intracellular uptake of nanoparticles will occur, leading to enhanced hyperthermia-induced cytotoxicity.

The primary objective of this study is to evaluate the effect of combining SPIONs, electroporation, and electromagnetic hyperthermia on the proliferation of melanoma-derived cell lines.

The size of the SPIONs used in this preliminary *in vivo* study is 14 nm [13] these nanoparticles enter the cell via clathrin-mediated endocytosis. But a significant increase in the cytotoxic effect of

the SPIONs is observed by adding electroporation, maybe, the increase of the cytotoxic and antitumoral effect is due to both the increase of intracellular SPIONs concentration and the irradiation with an electromagnetic field. On the other hand, the treatment with electromagnetic hyperthermia and the combination of electroporation and SPIONs allows to reduce the time of exposure to electromagnetic hyperthermia.

With respect to electromagnetic hyperthermia on this preliminary *in vivo* study, It allowed us to determine that the SPIONs concentrations of 1mg/1ml determined by Vizcarra Ramos *et al.* (13) and used *in vitro* with prostate cancer tumor cells are useful and have an important *in vitro* effect on skin cancer cells and on fluorescent melanoma tumors, Although other studies have reported different concentrations than those reported here [18,19].

Likewise, Cervantes *et al.* [16] where hyperthermia is induced at 43°C for 15 min using SPIONs coated with 1,2-benzenediol, cell viability was less than 80%. Sola-Leyva *et al.* [20] demonstrated that irradiation of SPIONs with an electromagnetic field increases ROS production compared to ROS production by unirradiated SPIONs, which agrees with the results shown in this study, since electromagnetic hyperthermia induces an increase in cell death compared to the effect of unirradiated SPIONs.

Combination of electroporation with electromagnetic hyperthermia, induce an increase the cytotoxic effect of hyperthermia; this increase could be due to an increase in the intracellular concentration of SPIONs, and Du *et al.* [21] demonstrated that administering a high concentration of SPIONs and irradiating them with an electromagnetic field exacerbates the process of cell death by apoptosis.

5. Conclusions

The preliminary *in vivo* results obtained in this study suggest that the combination of intratumoral administration of SPIONs with electroporation and electromagnetic hyperthermia significantly enhances the antitumoral effect compared to hyperthermia alone. The *in vivo* findings demonstrated notable antitumoral activity, with an initial delay in tumor growth during the first 14 days, followed by a pronounced therapeutic effect at days 21 and 28, culminating in complete tumor disappearance in the treated group.

These preliminary and brief results showed that combining electroporation plus electromagnetic hyperthermia significantly increases the cytotoxic *in vitro* assays and the antitumoral activity observed on these fluorescent tumors generated with MV3/GFP cells is an excellent model for *in vivo* evaluations.

Author Contributions: Conceptualization, R. H.-G.; data curation, R.H.-G. and A.G.-O, formal analysis, S. V.-R and A.M.-P., funding acquisition R. H.-G.; investigation, S. V.-R and A.M.-P, methodology . R.H.-G., L.F.J.-S. and A.A.-L; project administration R.H.-G, resources, R.H.-G, , L.F.J.-S. and A.A.-L; supervision, R. H.-G.; L.F.J.-S. and A.A.-L.; validation, A.M.-P.; visualization, A.G.-O; L.F.J.-S.; . and .; writing—original draft preparation, S. V.-R and A. M.-P.; writing—review and editing, A.G.-O , R.H.-G; All authors have read and agreed to the published version of the manuscript.

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