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NIR laser pointer for *in vivo* photothermal therapy of murine LM3 tumor using intratumoral China ink as a photothermal agent

Abstract

The photothermal effect is one of the most promising photonic procedures currently under development to successfully treat several clinical disorders, none the least some kinds of cancer. At present this field is undergoing a renewed interest due to advances in both photothermal materials and better-suited light sources. However, scientific studies in this area are sometimes hampered by the relative unavailability of state-of-art materials or the complexity of setting up a dedicated optical facility. Here we present a simple and affordable approach to do research in the photothermal field that relies on a commercial NIR laser pointer and a readily available everyday pigment: China ink. A proof-of-concept study is presented in which mice bearing intradermal LM3 mammary adenocarcinoma tumors were successfully treated *in vivo* employing China ink and the laser pointer. TUNEL and Ki-67 post-treatment tissue assessment clearly indicates the deleterious action of the photothermal treatment on the tumor. Therefore, the feasibility of this simple approach has been demonstrated, which may inspire other groups to implement simple procedures to further explore the photothermal effect.

Keywords: Photothermal therapy; photothermal effect; laser pointer; China ink, NIR laser; biological window

Introduction

Near infrared (NIR) laser irradiation currently finds many applications in different fields, like cosmetics (mainly hair removal through a photothermal mechanism), but also skin resurfacing and photo-treatment of vascular-related disorders [1]. Ruby (694 nm), alexandrite (755 nm), diode (800-810 nm), and Nd:YAG (1064 nm) lasers are commonly used devices for photothermal melanin-based hair removal [2, 3]. Photodynamic (PDT) and photothermal therapy (PTT) of cancer are other very relevant applications of NIR irradiation [4, 5]. An advantage of NIR irradiation is that an optical window of very low light absorption and moderate scattering occurs between 800 and 850 nm in biological tissues (lowest absorption at 825 nm) [6, 7], whereupon photons have deep tissue penetration.

PTT and photothermal action have the advantage that they do not depend on O_2 availability at the treated sample for them to work. Only an efficient mechanism of light-to-heat conversion is necessary to induce a biological response. It is well known that thermal energy dissipation occurs from an excited molecule by relaxation of vibrational energy levels (Fig. 1 a). For an efficient photothermal action, a fast conversion of electronic excitation to vibrational excitation is primordial. Then, decay to the ground vibrational level leads the excess energy to dissipate as translational molecular energy: in other words, heat evolution. In thermal equilibrium, a sample emits as much thermal energy as it absorbs from the environment (Fig.1 b). However, under continuous light absorption the target dissipates more thermal energy than it absorbs. In consequence, it heats its surroundings [8, 9].

As it follows from the previous argument, a key element in the photothermal effect is efficient conversion of electronic excitation energy to vibrational energy and

heat. Therefore, a suitable photothermal agent (i.e. dye or nanoparticle) should (1) strongly absorb light at the desired wavelength, and (2) quickly transform any absorbed photon into vibrational excitation. Carbocyanines and macrocyclic dyes (mainly phthalocyanines [10]) at least initially fulfil these criteria, and have been applied both for PDT and PTT under NIR excitation. In particular, the carbocyanine dye indocyanine green (ICG) shows excitation and emission maxima at about 780 and 830 nm, respectively, and is widely used in studies on blood plasma volume, hepatic function, angiography, lymphography, and detection of sentinel lymph nodes [11-13], as well as for NIR-mediated PDT and PTT of tumors and metastasis [14-17]. However, doubts arise in many instances with these molecular compounds as to the real cause of biological action: photothermal or photodynamic [13].

A much better approach seems to use nanoparticles with very high absorption cross sections and extremely fast (~picoseconds) electronic-to-vibrational transformation rates, which adequately comply with the required features of an efficient photothermal agent (see above). Indeed, optimal results have been reported with noble metals nanoparticles (in particular with gold) as photothermal transducers [9, 18]. Carbon-based nanoparticles of graphene-like structure (e.g. carbon nanotubes, fullerenes) also work nicely as photothermal agents. Photothermal responses in cultured tumor cells have been described using single- or multiwalled carbon nanotubes coupled with 808-nm or 1064-nm laser irradiation [19, 20]. On the premise that not all research groups interested in the photothermal effect may have the resources to acquire carbon-based nanoparticles, we decided to search for an easier-to-obtain and cheaper alternative to those nanoparticles.

China (Indian) ink is a colloidal aqueous solution of particles composed of a hydrophobic carbon black core surrounded by a hydrophilic shell (formerly gelatin and now customarily Arabic gum). This dense and permanent ink is most used in drawing, calligraphy, and skin tattooing. The pigment carbon black (CI 77266) is formed by lampblack or finely ground charcoal, with a quasi-graphitic carbon structure of very small particle size [21]. According to the commercial manufacturer of Pelikan China ink, the typical size for carbon black is below 1 µm (private communication Pelikan PBS-Produktionsgesellschaft mbH & Co. KG, Peine, Germany). Regarding biomedical applications after *in vivo* administration in animals, China ink marks phagocytic cells from the reticulo-endothelial system, allows to show branching blood vessels within organs, and is used by surgeons to detect sentinel lymph nodes [21, 22]. China ink has also found applications as a blot staining agent after electrophoresis of proteins [23, 24] and nucleic acids [25]. Most encouraging, it has been recently reported that carbon black micro- and nanoparticles can be as efficient photothermal agents as gold nanoparticles on a mass-to-mass based comparison [26].

In this work, proof-of-concept demonstrations were designed as simple model systems to show the direct photothermal effect using China ink and NIR irradiation (808-nm commercial laser pointer). The goal is to offer proof of an easy-to-implement setup for photothermal research, without having to rely on expensive and difficult to maintain equipment and resources (laboratory lasers and state-of-art nanoparticles). Previous reports exist on the successful use of laser pointers as research tools [27]. We have chosen a wavelength of 808-nm because it lies close to the best point in the biological window [9], and there are recent reports of very efficient photothermal tumor treatment at this wavelength with continuous wave (cw) lasers [28]. Although the

optical absorption peak of China ink particles corresponds to wavelengths in the UV (see Results below), nevertheless their absorption in the NIR is high enough to guarantee a robust biological response under our experimental conditions. Our aim is to prove that these particles are efficient within the optical biological window (700-900 nm). Likewise, the mouse mammary adenocarcinoma LM3 was used for successful studies *in vivo* under our experimental conditions. The resulting findings strongly support the occurrence of a photothermal effect on model systems *in vitro* and experimental murine tumors *in vivo* after application of China ink followed by NIR irradiation using an 808-nm laser pointer.

Material and methods

China ink (Pelikan black drawing ink Z, Pelikan AG, Hannover, Germany) was used for model experiments *in vitro* or after injection into murine tumors *in vivo*, either undiluted (as received) or diluted in distilled water or 0.9% NaCl (saline) solution as required. For comparative purposes, samples of commercial black toner cartridges (Hewlett Packard CB540A-AD and Toshiba T-FC50E-K) and other black inks were also employed *in silico* to test their possible photothermal response. The absorption spectrum of China ink in distilled water is shown in Fig. 2 a. The spectrum was obtained with a UV-VIS spectrophotometer (Shimadzu UV-VIS 1604, USA). The appearance of a typical China ink solution is shown ~~in the inset~~ to the right of the absorption spectrum. The spectrum is normalized respect to the absorption at 200 nm.

A portable NIR commercial laser pointer (model GLP-808, Changchun New Industries Optoelectronics Technology Co., Ltd., P.R. China) was used for irradiation. Emission wavelength as provided by the supplier is 808±3 nm. The emission is cw and the emitted power is ~200 mW as measured with a thermopile (LM-10, Coherent Inc., USA). The beam diameter is ~1.2 mm which translates into an irradiance of ~17.68 Wcm^{-2}. In all cases, the distance between the laser output and the different irradiated targets was kept constant at 10 cm. At this distance, the spot area of the laser beam was ~1.1 mm^2.

Paper stripes were employed as test substrates to evaluate the photothermal effect of the different inks under study before the biological experiments. Ink droplets were applied on the paper, air dried, and then irradiated with the 808-nm laser for different times. Ignition point (temperature) of paper is between 218 and 246 ºC (average: 232 ºC), and it can be considered as a clear and recordable end point of the photothermal reaction. In addition, Eppendorf tubes (1.5 mL) containing either water (control) or 50 % China ink in water were subjected to 808-nm irradiation for different times and the temperature changes were recorded. In order to do so a common mercury thermometer was employed to assess the samples temperature.

In vivo biological experiments were carried out with inbred female Balb/c mice, 2–4 months old, from the Breeding Area of the Institute of Oncology, kept in a temperature- and light-controlled room with free access to water and dietary chow. Animal care was provided in full compliance with regulations for protection of animals at the Institute. LM3 cells (from a Balb/c mammary adenocarcinoma [29]) were grown at 37 °C in plastic flasks (Falcon) in a humidified 5% CO_2 atmosphere, using minimum essential medium (MEM, Gibco) with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 80 µg/mL gentamycin. For inoculation into mice, cells were harvested

from subconfluent cultures with trypsin-EDTA, and after trypsin neutralization with MEM+FBS, cells were washed thoroughly with MEM, and resuspended in MEM.

An amount of 10^6 LM3 cells in a 0.1 mL volume were inoculated intradermally into the shaved left flank of non-anesthetized animals, and 10 days later, LM3 tumors were subjected to control and experimental protocols (a: no treatment, n= 2; b: only China ink, n= 2; c: only NIR irradiation, n= 3; d: China ink followed by NIR irradiation, n= 8). The overlying hair on tumors and surrounding skin regions was clipped, and intradermal tumors were injected with 0.1 mL of undiluted (n= 6) or diluted China ink (50 % and 25 % in saline solution, n= 1 and 1, respectively). Before laser treatment, anesthesia was administrated by intraperitoneal injection of 0.2-0.3 mL of a solution of xylazine (20 mg/kg) and ketamine (50 mg/kg) in saline. The tumor area was irradiated with the 808-nm laser for 4 min. To reduce the light scattering by keratin, a droplet of glycerol was placed on the shaved skin covering the tumor area. After 24 h of the treatment, control and experimental tumors were resected, fixed with 10% buffered formalin for 24 h, embedded in paraffin, and stained with Gill's II hematoxylin and eosin Y (H&E), for histopathological study. The sections were assessed under a light microscope (Zeiss Corp., Germany).

In order to study cell proliferation and cell death after the treatment, tumor tissue sections were assessed with Ki-67 or Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, respectively. Sections of formalin-fixed, paraffin-embedded tissue from LM3 control tumors and tumors treated with undiluted or diluted China ink and laser-exposed were used for immunohistochemical detection of Ki-67 and for TUNEL technique. In brief, for Ki-67, endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Blocking solution (2% normal goat serum) was used before the specific antibody. Rabbit polyclonal anti Ki-67 antibody (ab15580, Abcam Laboratories) was used at 1:500 dilution and incubated overnight at room temperature. Microwave antigen retrieval (4 cycles of 5 min each in 0.1 M citrate buffer) using a 750 W Philips M902 microwave oven was used before primary antibody. Detection was performed using avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The reaction was developed with 3, 3′-diaminobenzidine, 0.30 mg % in PBS with H_2O_2 to a final concentration of 0.5%, under microscopic control. Specimens were lightly counterstained with Gill's hematoxylin 10%, dehydrated and mounted. With TUNEL the samples were also reacted using the deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling (TUNEL) method according to the manufacturer's instructions (Apoptag Plus Peroxidase *In Situ* kit, EMD Millipore& Co, USA). Permanent Mounting Medium (VectaMount, Vector Labs Burlingame, CA) was used in all cases.

Results

The results of experiments regarding both ignition of paper stripes with China ink (Fig. ~~3 a~~ 2 b) and heating of aqueous China ink solutions (Fig. ~~3 b~~ 2 c) clearly showed that 808-nm irradiation induces a photothermal effect. In the case of other dark pigments like dried drawing inks, black photocopy samples, blots of permanent black markers (Edding 500 and 3000, Staedtler), black ballpoint pens and pencils, all impregnated paper stripes reached ignition before 3-4 seconds, confirming its photothermal nature (data not shown). No ignition occurred in the absence of China ink (Fig. ~~3 a~~ 2 b, squares). China ink solutions subjected to NIR irradiation led to an

increase of temperature with time (Fig. ~~3 b~~ 2 c, circles), well above the control values from water alone (squares).

The solubility of toners in ethanol, olive oil, commercial detergent and liquid soap was only transient and within few hours black sediments of the toner particles were clearly visible. Under 808-nm irradiation, dried droplets of toners in ethanol on paper stripes also showed rapid ignition. Safety data sheets from Hewlett Packard and Toshiba toners indicate that the amount of carbon black is lower than 7%. Likewise, on account of the presence of several additives (styrene acrylate copolymer, polyester resin, wax), toners were considered to be inappropriate for further testing along this study.

The absorption spectrum of a diluted China ink solution (Fig. 2 a) reveals that the highest absorption occurs in the far ultraviolet region, and then it decreases continuously along the visible and infrared region. This spectrum agrees very well with a previously published absorption spectrum of carbon black particles of sub-micrometric size [26]. The absorption at 800 nm is about ~20 % of that at 200 nm. Nevertheless, 808-nm irradiation induces a rapid and strong photothermal action at our tested power levels (see below), a relevant response given that we aimed at obtaining results within the biological window.

The experimental setup for *in vivo* tumor irradiation is shown in Fig. ~~4 a~~ 3 a. The laser pointer (shown in the inset) was fixed to a metal frame to keep a constant distance to the targets. An anesthetized mouse can be seen being exposed to the laser beam. A close up image of the tumor area being irradiated with 808-nm light after China ink injection is shown in Fig. ~~4 b~~ 3 b. Immediately after the laser exposure it can be observed a clearly damaged tumoral area denoted by a very dark skin spot (Fig. ~~4 c~~ 3 c).

Regarding tissue histology of the experimental protocols *in vivo*, tumor controls either without treatment, treated with laser alone or only China ink displayed normal skin and preserved tumor tissue (Fig ~~5 a, b~~ 4 a, b). On the contrary, after China ink plus laser treatment animals showed dermal-epidermal necrosis and tumors with a variable amount of necrotic areas and abundant infiltration of inflammatory cells. The extension of necrosis induced by treatment and the amount of viable tumor areas were dependent on the different China ink dilutions used. Damaged tumor cell numbers increased progressively at 25% (Fig ~~5 c~~ 4 c), 50% and 100% (Fig. ~~5 d~~ 4 d) China ink concentrations, keeping the laser dose constant. China ink was not found as intracellular inclusions, but some intra- and peritumoral macrophages showed uptake of carbon particles. The areas with interstitial storage of China ink were associated to extensive tumor necrosis (black arrows) and vascular lesions (white arrows), leukocyte infiltration, edema and extravasated erythrocytes (see Fig. 4 d). Hemorrhagic necrosis induced by the treatment encompassed almost 90% of the tumor tissue when 100% China ink was used (Fig ~~5 d~~ 4 d), and scarcely isolated viable cells were observed only in the depth of tumors.

To further assess the photothermal effect at the microscopic tissue level we have performed two immunohistochemical assays: TUNEL to evaluate the level of apoptosis within the tumor tissues [30, 31], and Ki-67 to measure the cell proliferation activity after the treatment [32]. The results are shown in Fig. 5. In regards to TUNEL, the untreated control tumor displayed no TUNEL signal (Fig. 5 a). On the other hand,

photothermally-treated tumors showed some TUNEL signal for some cells (Fig. 5 b, white arrows). However, most cells showed a low-level or not at all TUNEL signal, and clear morphological signs of necrosis (black arrows). These results, in connection with those shown in Fig. 4 c and d, point that the main cell death process following the photothermal treatment is cell necrosis.

As for Ki-67, a widely employed cell proliferation marker, the control tumor shows a large cell subpopulation undergoing proliferative activity at the moment of fixing the tissue (Fig. 5 c, white arrows). This was expected, as the tumor is a highly proliferative tissue. In sharp contrast, a photothermally-treated tumor evaluated 24 h after the treatment shows no Ki-67 signal at all (Fig. 5 d). Therefore, a complete proliferative arrest is observed in the tissue that, at least, spans the first 24 h after the experimental treatment. Again, we found necrotic morphologies throughout the tumor sample (black arrows). Taken together, results from Fig. 4 and 5 indicate a massive cell death by necrosis, a very minor contribution from apoptosis, a severe hemorrhagic process taking place in the tumor stroma, and a complete cell proliferation arrest after the photothermal treatment with China ink and the 808-nm laser pointer.

Discussion

In this work we have introduced a simple and inexpensive model system very adequate to test possible photothermal effects. Carbon particles deposited on paper tissue, air-dried, and subjected to NIR irradiation showed almost immediate ignition. Therefore, the present results from these model experiments strongly support the conclusion that the biological response observed after tumor laser treatment with China ink plus NIR is in fact due to a photothermal process. In addition to the strong temperature increase induced in a solid phase (dried China ink and 808-nm irradiation), a considerable increase also occurs in aqueous media. Direct heating of water by NIR absorption is weak, slow and rapidly dissipated to the surrounding environment. However, an adequate infrared chromophore such as carbon black, here in the form of China ink, is able to substantially increase the temperature of water by a NIR-based photothermal action [26].

In agreement with these results, the use of China ink followed by NIR irradiation induced a clear tissue damage and cell death in LM3 tumors *in vivo*. No effects were observed in control tumors subjected only to laser irradiation or China ink. The advantage of using 808-nm is that, excepting melanin, the absorption of endogenous chromophores and water is negligible, and thus NIR penetrates deeply within tissues. The intratumoral injection of photoactive agents has been successfully used previously in experimental PDT or PTT treatments [29, 33, 34].

The immunohistochemistry evaluation of the different samples, control and photothermal, shed light on the cell death processes taking place after the treatment. The main cell death mechanism seen 24 h post-treatment is necrosis. This comes as no surprise given the high temperatures most likely induced within the treated area during the laser irradiation. Assuming a direct comparison with the water heating results displayed in Fig. 2 c (water volume of 1.5 mL), temperatures in the range of 50-60 ºC can be expected to be achievable in the tumor. These temperatures, sustained for several minutes, lead to necrotic processes [9]. There is some general low-intensity TUNEL staining in Fig. 5 b, but we deem it is due to the non-specific terminal transferase action

on necrotic cell remnants present in the sample. Apoptosis detection was negative in tumor samples after 25% China ink plus laser treatment (data not shown). The observed hemorrhage can be also considered indirect evidence of necrosis and gross tissue damage, as apoptosis does not precede this kind of vascular damage.

That some apoptotic cells are observed within the treated area under 100% China ink plus laser treatment is, in our opinion, more than reasonable. Given the large thermal stress to which the tumor has been subjected, it can be expected that some cells would survive in the first place, but having sustained damage beyond repair initiate and complete the apoptotic program. Therefore, a mix of necrotic and apoptotic cell populations should be expected under very stressful conditions [35], in particular some time (few hours) after the noxious treatment. Probably one can find a kind of apoptotic "halo" or "shell" surrounding most severe necrotic lesions. This can be a topic to deal with in future research.

Of particular interest, in our opinion, is the fact that there is a very strong antiproliferative action within the treated tumor after the photothermal exposure. As shown in Fig. 5 d any Ki-67 signal is completely absent from the sample. Therefore, although the tumor is not completely destroyed by the treatment, all cells sustain a more or less large insult which leads to an overall halting of proliferative activity for at least 24 h. This result, in combination with the massive necrosis already discussed, leads us to foresee a very strong tumor regression trend after a single treatment. In this sense, we will start new experiments where tumor size will be compared among control and experimental groups in the near future.

It is known that tumor cells are more sensitive to a temperature increase than normal cells [36, 37]. Currently, a temperature of 42-43 ºC is considered lethal for tumor cells, and the slower heat dissipation by reduced blood flow in tumors as compared with normal tissues contributes to the thermal deleterious effect [13]. Therefore, hyperthermia is a therapeutic procedure used to raise the temperature and achieve a selective tumor killing action [38]. Presently, it seems logical to assume that in our case, a photothermal effect is the factor responsible for the tumor damage after experimental treatments.

Although other mechanisms could be invoked to explain the response of tumor tissue to China ink and 808-nm irradiation (e.g., photodynamic effect, multiphoton-pumped carbon black anti-Stokes photoluminescence), it seems that the photothermal effect is the simplest option to explain our results using these model systems and experimental tumor procedures, and taking into account the low irradiance values employed with our cw source.

Several future experiments are necessary to improve the described results and to obtain better conclusions as to the action mechanisms. First, a higher power and larger spot diameter of the NIR laser would be more adequate to increase the photothermal response of tumors. Likewise, as the uptake of Chinese ink particles occurs in macrophages but not in non-phagocytic cells, delivery of liposomal carbon nanoparticles after encapsulation into phospholipid layers could be an effective strategy for photothermal therapy using systemic administration. Taking into account present results, liposomal, biocompatible, and intravenously injectable forms of carbon black could greatly expand *in vivo* applications of PTT. In conclusion, it has been our goal in

this work to present a proof-of-concept procedure to enable an easy, manageable and cheap approach for photothermal research. We hope that the presented methodology and results will encourage other groups to implement similar procedures to further advance in the field of photothermal therapy.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

Ethics approval This study was conducted according with the Committee of Animal Ethics of the Institute of Oncology Angel H. Roffo, University of Buenos Aires.

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Legend of figures

Fig. 1 a Scheme of the events taking place at the molecular level leading to the photothermal effect. An optical photon (NIR in this example) is absorbed by a photothermal molecule or nanoparticle. The electronic (thick lines) and vibrational levels (dashed lines) are highly coupled in these agents, therefore the initial optical excitation energy becomes vibrational energy very fast (wiggly arrows). This is followed by quick transformation of molecular vibrations into thermal energy, poured into the environment. **Fig. 1 b** Under dark conditions (left) the photothermal agent is in thermal equilibrium with its environment (thin lines in and out of the structure). No net thermal transfer takes place. Under NIR irradiation (thick wiggly arrows) the photothermal effect takes place (right), and the photothermal agent increases significantly its temperature. Now, the system is no longer in thermal equilibrium and there is a net transfer (thick arrows) of thermal energy from the photothermal agent to the environment.

Fig. 2 a Absorption spectrum of China ink in distilled water. The spectrum is normalized to the absorption at 200 nm. The absorption curve decreases monotonically going from the deep UV to the NIR, a typical feature of semiconductor-like chromophores like graphitic materials. The laser wavelength employed in the experiments (808-nm) is highlighted in the graph. ~~Inset~~ On the right: Colloidal China ink (50% v/v) suspension in distilled water. **Fig. 2 b** Ignition time (seconds) of paper stripes impregnated either with China ink at different % concentrations (circles) or water (squares), air dried, and then irradiated with 808-nm for different times. The higher the China ink content, the faster the paper ignition. Note that even very low China ink contents (~1%) lead to ignition at 10 s, proof of its very high photothermal efficiency. **Fig. 2 c** Temperature increase (in ºC) of water (squares) and 50 % China ink in water (circles) during 808-nm irradiation for different times (min). Particular temperature increases for China ink solution at time points 1.5 and 4 min are indicated. The temperature increases significantly for the China ink solution (57 ºC at 4 min) while the increase is negligible for water alone (33 ºC at 4 min).

Fig. 3 a Experimental setup for assessment of the *in vivo* photothermal action of China ink plus laser exposure. The NIR laser pointer is held fixed by a metal frame to keep irradiation distance constant. A tumor-bearing mouse is in the process of being irradiated. Inset: NIR laser pointer employed in the experiments. **Fig. 3 b** Close up image of the irradiation spot during laser exposure. The hairless tumor area is highlighted by scattered laser light (bright spot). **Fig. 3 c** Detail of the irradiated tumoral area 5 min after laser treatment. The area shows clear features of scarred tissue

corresponding to the skin over the tumor. The perimeter shows no sign of thermal damage.

Fig. 4 a and **b** Control tumors receiving only undiluted China ink (**a**) or only laser irradiation (**b**) showing no tissue damage 24 h post-treatment. Black areas are China ink deposits. **Fig. 4 c** Tumor section 24 h after treatment with 25% China ink dilution plus 808-nm laser irradiation (4 min) where numerous necrotic cells can be observed (black arrows). **Fig. 4 d** Tumor section 24 h after being treated with 100% China ink plus laser irradiation, where extensive necrotic areas (black arrows), surrounded by leukocytes, erythrocytes and edema (white arrows), are present.

Fig. 5 a and **b** Immunohistochemical apoptosis assessment (TUNEL assay). Control tumors (**a**) or tumors injected with 100% China ink plus 808-nm laser irradiation (**b**) were analyzed with TUNEL. In the control (**a**) there are no apoptotic cells. In the tumor undergoing photothermal treatment (**b**) there are a few apoptotic cells (white arrows). However, the bulk of the cell death observed are necrotic cells (black arrows), which can appear lightly blue or lightly brown in the picture. **Fig. 5 c** and **d** Immunohistochemical evaluation of cell proliferation (Ki-67 marker). In the control tumor (**c**) there is very intense cell proliferation as indicated by the dark brown-stained cells (white arrows), as expected in a growing tumor. In the experimental tumor (**d**) there is no Ki-67 signal at all. However, widespread necrosis (black arrows) is patent across the tumor stroma. All pictures were taken 24 h post-treatment.

NIR laser pointer for *in vivo* photothermal therapy of murine LM3 tumor using intratumoral China ink as a photothermal agent

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Figure 1

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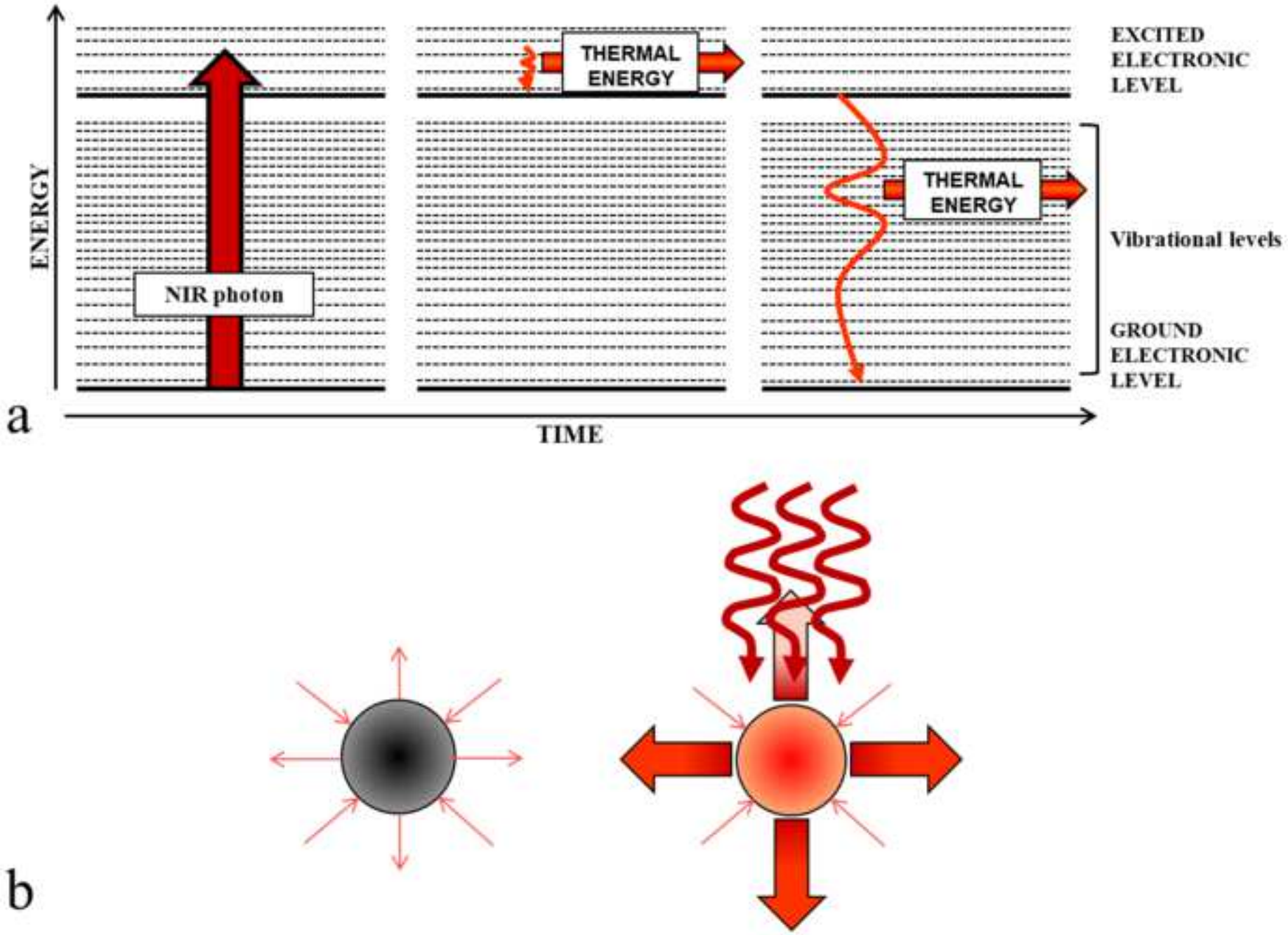

Figure 2

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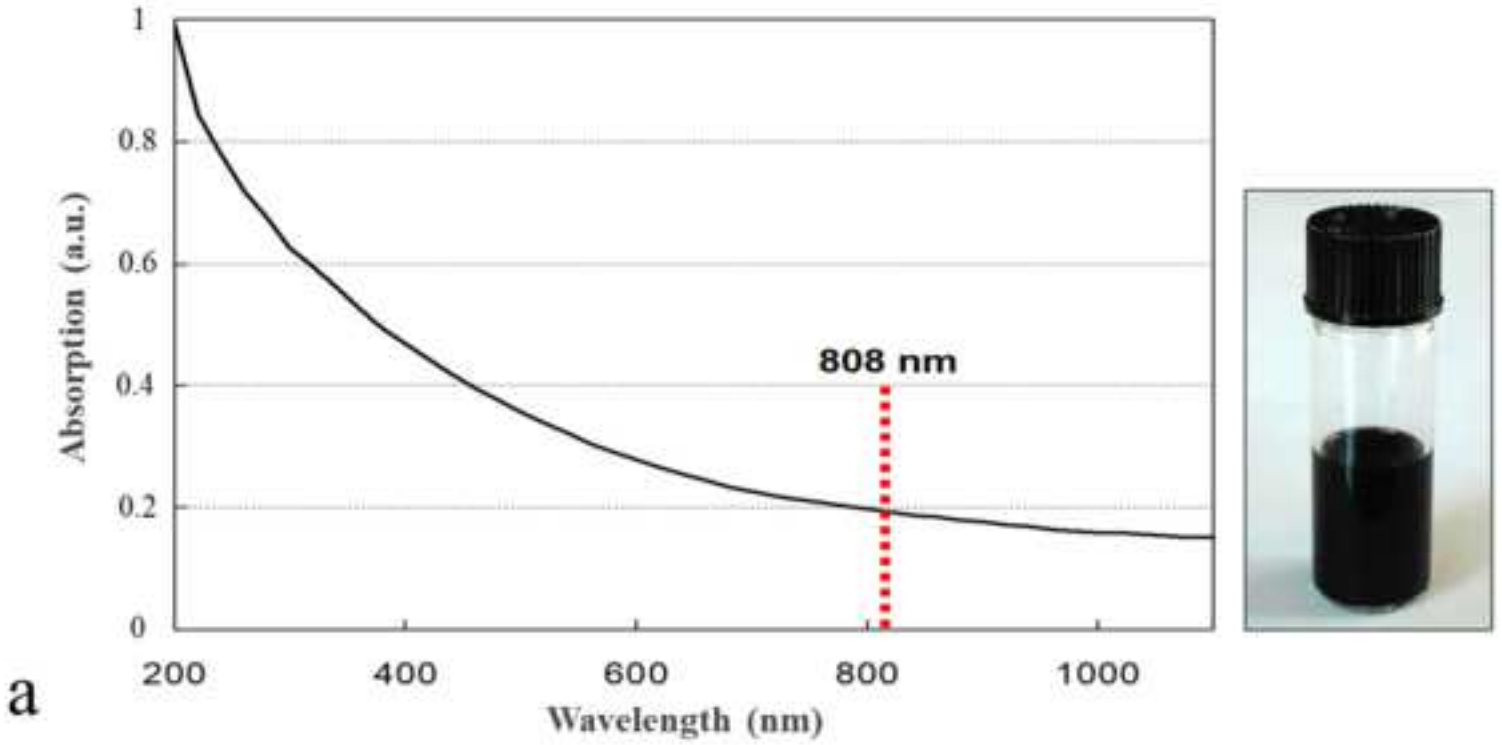

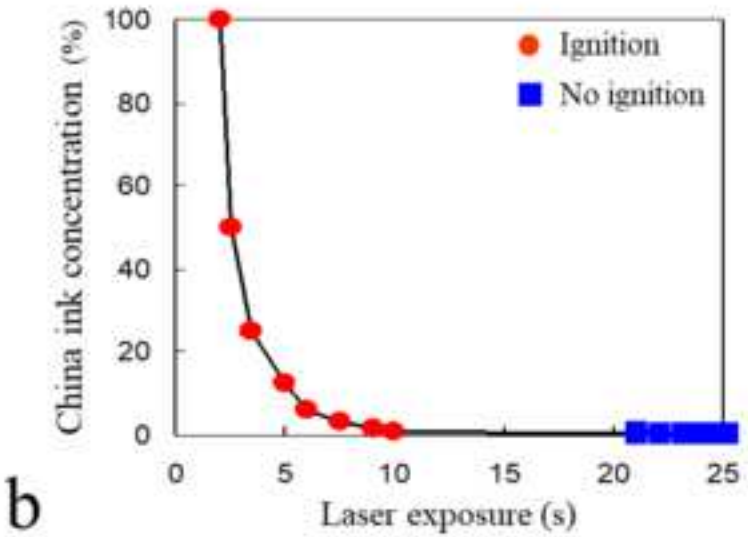

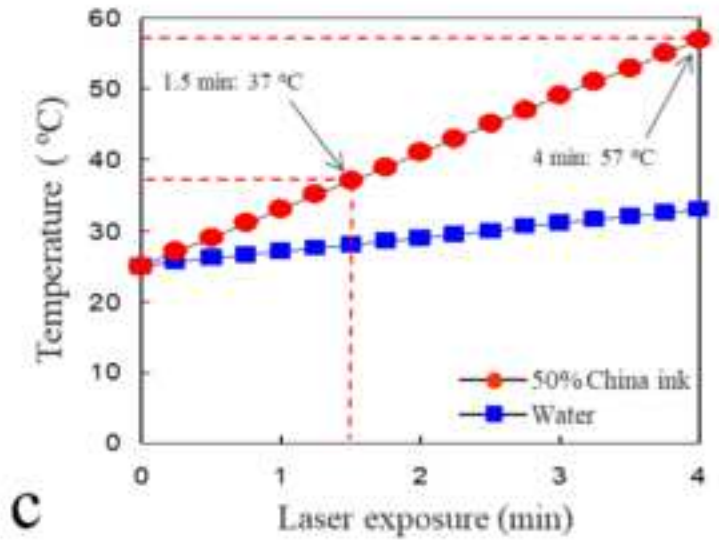

Figure 3

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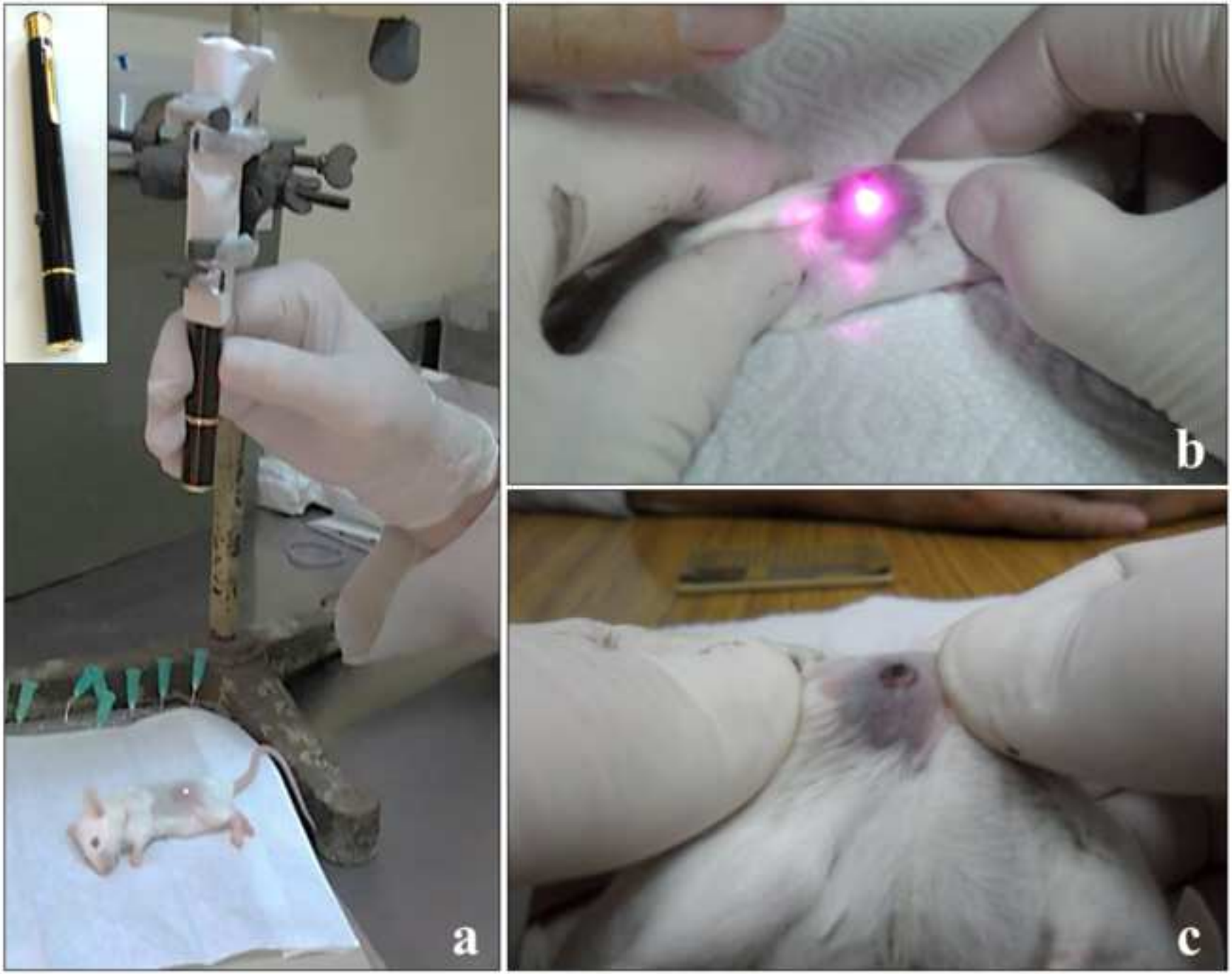

Figure 4

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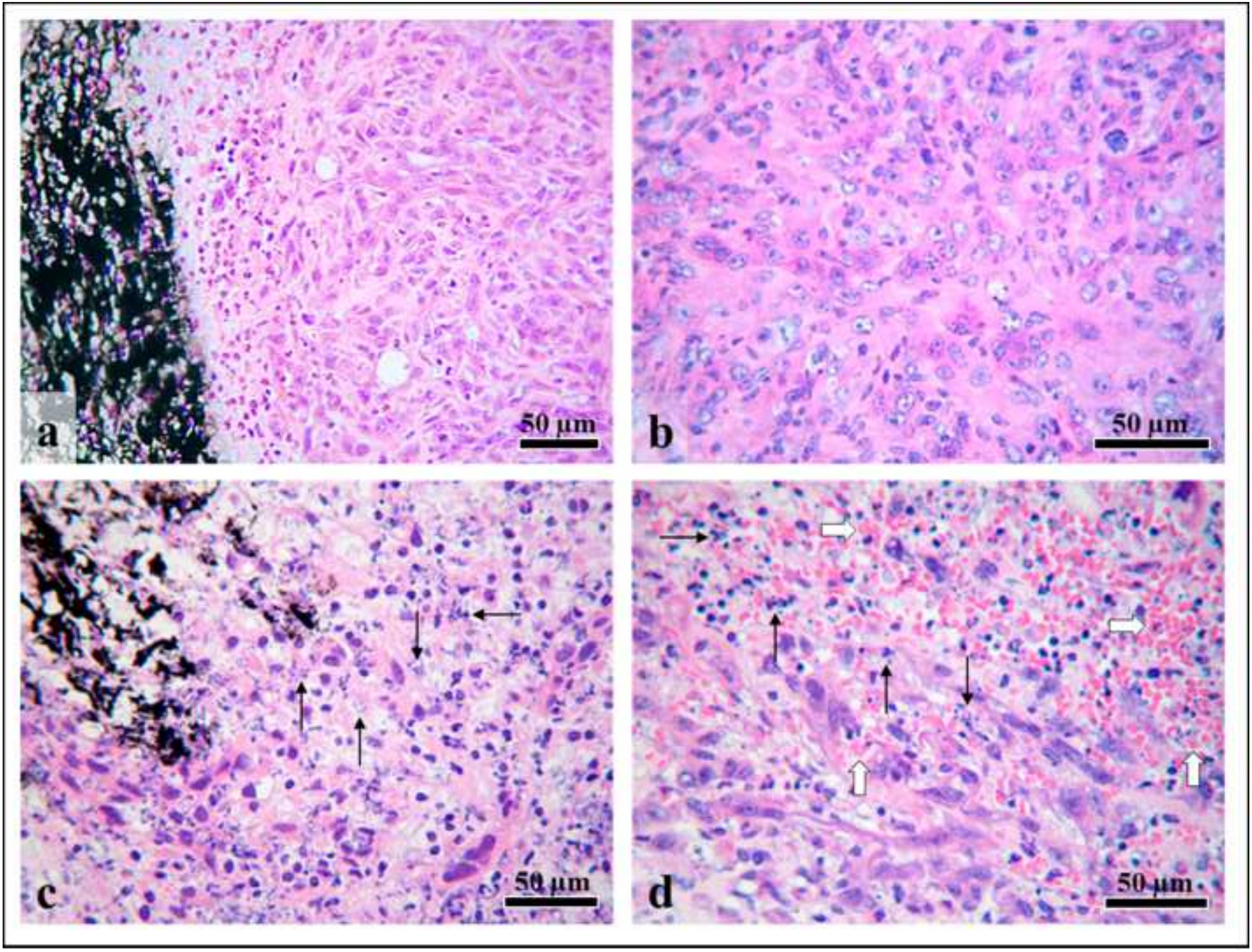

Figure 5

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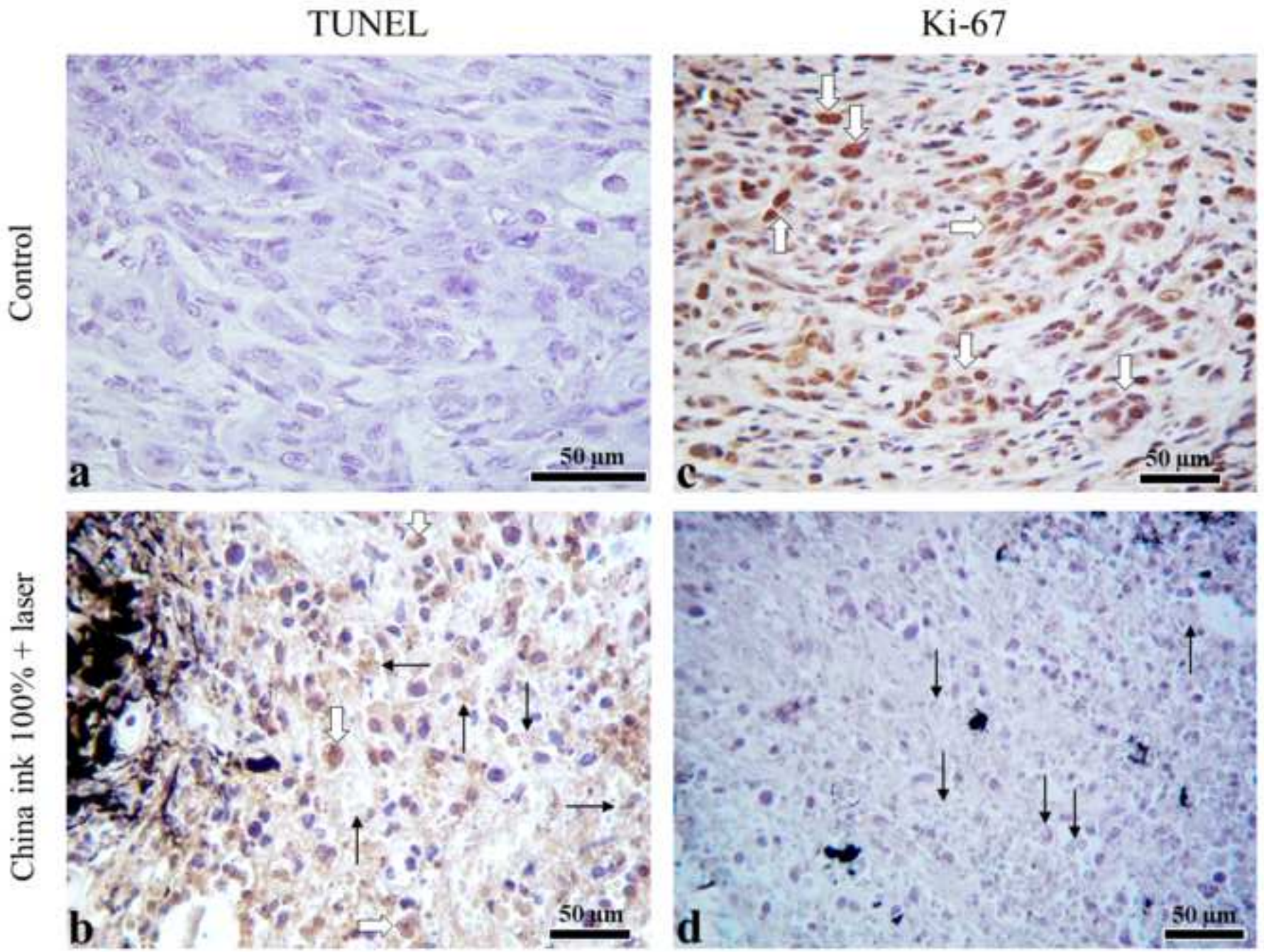

Reply to reviewer´s comments:

Reviewer #1:
The manuscript entitled "NIR laser pointer for in vivo photothermal therapy of murine LM3 tumor using intratumoral China ink as a photothermal agent" is a well written manuscript. However, it is based on just proof of concept.

We are thankful to the reviewer for his/her comments on the overall quality of the work, and his/her critical suggestions which have helped us to improve this version of the manuscript. The remark that "*it is based on just proof of concept*" is absolutely correct and, in fact, it has been our aim when presenting these results. This work is envisioned as a first in a series of publications that will make use of a NIR laser pointer as the exciting light source and different biological models to carry out photothermal research. Here, we are looking for a fast publication of the first qualitative results so far obtained. But our group is already obtaining more quantitative and systematic results with a very similar methodology, which we hope will be ready for submission within a few months.

I have following major comments:
1) For PDT, a chemical agent and laser (wavelength) is selected based its absorption peak. However, in the presented proof of concept manuscript, absorption peak of china ink is quite different from NIR laser. It's good to choose NIR laser for good penetration, however authors must have selected a compound which absorbs near IR.

We agree with the reviewer in that, customarily, the absorbing compound and the light source are chosen to fit each other for an optimum photobiological outcome. In our case, we wanted to have definitive proof that China ink, a very available chromophore, could indeed be successfully employed to induce a photothermal response with an affordable laser source within the biological window. It is true that the absorption peak for this ink is in the far UV, but nevertheless the absorption tail in the NIR is still so high, in absolute value, that it is more than enough to produce the desired effect.

Anyhow, two new sentences have been added to the last paragraph of the Introduction to better address this point:

"Although the optical absorption peak of China ink particles corresponds to wavelengths in the UV (see Results below), nevertheless their absorption in the NIR is high enough to guarantee a robust biological response under our experimental conditions. Our aim is to prove that these particles are efficient within the optical biological window (700-900 nm)."

And a new sentence has been added to the 3rd paragraph of the Results to discuss this:

"Nevertheless, 808-nm irradiation induces a rapid and strong photothermal action at our tested power levels (see below), a relevant response given that we aimed at obtaining results within the biological window."

2) No shrinkage in volume of tumor is plotted after laser and ink treatment.

We agree with the referee. However, we have submitted the present manuscript as a proof-of-concept for photothermal damage of tumors employing "over the desk" materials. Our aim is to introduce the photobiological community to these tools, not to undertake a full-fledged tumor regression study in animals.

Nevertheless, we inform here that we are now undertaking additional research using this methodology with mice bearing melanotic tumors. The ongoing research will soon be submitted for publication, and comparison among tumor sizes and mice survival plots are to be included in this upcoming manuscript.

3) No apoptosis staining is done on tissues to confirm that death is by apoptosis. A TUNEL staining can be done on tissues to confirm apoptosis.

We totally agree with the reviewer in this point. Therefore, following his/her suggestions, immunohistochemical analysis for apoptotic DNA degradation (TUNEL assay) and proliferation marker (Ki-67 assay) has been done in order to better assess the biological response to our experimental treatment. We have found a very minor apoptotic cell sub-population 24 h after the treatment. Necrosis is the widespread cell death mechanism observed in the treated tumors. In compliance with this, and a relevant result, is that Ki-67 signal in the treated tumors is practically null. This we interpret as a total mitotic arrest in the tumors. We are undertaking additional experiments to do a follow-up of tumors size after photothermal treatment (see response to point #2 above for additional comments).

Several major changes have been introduced in the manuscript following the obtained results on TUNEL and Ki-67:

- In the **Abstract**, the following sentence has been introduced: "TUNEL and Ki-67 post-treatment tissue assessment clearly indicate the deleterious action of the photothermal treatment on the tumor."
- In the **Materials and Methods** section a whole new paragraph, last paragraph of that section, has been included describing the TUNEL and Ki-67 tumor tissue immunohistochemistry: "In order to study cell proliferation and cell death after the treatment, tumor tissue sections were assessed with Ki-67 or Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, respectively. Sections of formalin-fixed, paraffin-embedded tissue from LM3 control tumors and tumors treated with undiluted or diluted China ink and laser-exposed were used for immunohistochemical detection of Ki-67 and for TUNEL technique. In brief, for Ki-67, endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Blocking solution (2% normal goat serum) was used before the specific antibody. Rabbit polyclonal anti Ki-67 antibody (ab15580, Abcam Laboratories) was used at 1:500 dilution and incubated overnight at room temperature. Microwave antigen retrieval (4 cycles of 5 min each in 0.1 M citrate

buffer) using a 750 W Philips M902 microwave oven was used before primary antibody. Detection was performed using avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The reaction was developed with 3, 3′-diaminobenzidine, 0.30 mg % in PBS with H_2O_2 to a final concentration of 0.5%, under microscopic control. Specimens were lightly counterstained with Gill's hematoxylin 10%, dehydrated and mounted. With TUNEL the samples were also reacted using the deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling (TUNEL) method according to the manufacturer's instructions (Apoptag Plus Peroxidase *In Situ* kit, EMD Millipore& Co, USA). Permanent Mounting Medium (VectaMount, Vector Labs Burlingame, CA) was used in all cases."

- In the **Results** section 2 new paragraphs and a new figure (FIGURE 5 plus figure caption) have been included to present the obtained results in regards to TUNEL (apoptosis assessment) and Ki-67 (cell proliferation). The two paragraphs are reproduced here: "To further assess the photothermal effect at the microscopic tissue level we have performed two immunohistochemical assays: TUNEL to evaluate the level of apoptosis within the tumor tissues [30, 31], and Ki-67 to measure the cell proliferation activity after the treatment [32]. The results are shown in Fig. 5. In regards to TUNEL, the untreated control tumor displayed no TUNEL signal (Fig. 5 a). On the other hand, photothermally-treated tumors showed some TUNEL signal for some cells (Fig. 5 b, white arrows). However, most cells showed a low-level or not at all TUNEL signal, and clear morphological signs of necrosis (black arrows). These results, in connection with those shown in Fig. 4 c and d, point that the main cell death process following the photothermal treatment is cell necrosis.

  As for Ki-67, a widely employed cell proliferation marker, the control tumor shows a large cell subpopulation undergoing proliferative activity at the moment of fixing the tissue (Fig. 5 c, white arrows). This was expected, as the tumor is a highly proliferative tissue. In sharp contrast, a photothermally-treated tumor evaluated 24 h after the treatment shows no Ki-67 signal at all (Fig. 5 d). Therefore, a complete proliferative arrest is observed in the tissue that, at least, spans the first 24 h after the experimental treatment. Again, we found necrotic morphologies throughout the tumor sample (black arrows). Taken together, results from Fig. 4 and 5 indicate a massive cell death by necrosis, a very minor contribution from apoptosis, a severe hemorrhagic process taking place in the tumor stroma, and a complete cell proliferation arrest after the photothermal treatment with China ink and the 808-nm laser pointer."
- In the **Discussion** section 3 new paragraphs have been included to discuss the obtained results in regards to TUNEL (apoptosis assessment) and Ki-67 (cell proliferation). The three paragraphs are reproduced here: "The immunohistochemistry evaluation of the different samples, control and photothermal, shed light on the cell death processes taking place after the treatment. The main cell death mechanism seen 24 h post-treatment is necrosis. This comes as

no surprise given the high temperatures most likely induced within the treated area during the laser irradiation. Assuming a direct comparison with the water heating results displayed in Fig. 2 c (water volume of 1.5 mL), temperatures in the range of 50-60 ºC can be expected to be achievable in the tumor. These temperatures, sustained for several minutes, lead to necrotic processes [9]. There is some general low-intensity TUNEL staining in Fig. 5 b, but we deem it is due to the non-specific terminal transferase action on necrotic cell remnants present in the sample. Apoptosis detection was negative in tumor samples after 25% China ink plus laser treatment (data not shown). The observed hemorrhage can be also considered indirect evidence of necrosis and gross tissue damage, as apoptosis does not precede this kind of vascular damage.

That some apoptotic cells are observed within the treated area under 100% China ink plus laser treatment is, in our opinion, more than reasonable. Given the large thermal stress to which the tumor has been subjected, it can be expected that some cells would survive in the first place, but having sustained damage beyond repair initiate and complete the apoptotic program. Therefore, a mix of necrotic and apoptotic cell populations should be expected under very stressful conditions [35], in particular some time (few hours) after the noxious treatment. Probably one can find a kind of apoptotic "halo" or "shell" surrounding most severe necrotic lesions. This can be a topic to deal with in future research.

Of particular interest, in our opinion, is the fact that there is a very strong antiproliferative action within the treated tumor after the photothermal exposure. As shown in Fig. 5 d any Ki-67 signal is completely absent from the sample. Therefore, although the tumor is not completely destroyed by the treatment, all cells sustain a more or less large insult which leads to an overall halting of proliferative activity for at least 24 h. This result, in combination with the massive necrosis already discussed, leads us to foresee a very strong tumor regression trend after a single treatment. In this sense, we will start new experiments where tumor size will be compared among control and experimental groups in the near future."

- Four additional references have been included [30-32] and [35], increasing the total number of references to 38.

Additional changes in the revised manuscript are briefly mentioned below:

- Former Figures 2 and 3 have been merged into a **NEW FIGURE** (**new Figure 2**), with corresponding changes in the main text and the figure caption.
- Former Figure 4 is now **NEW Figure 3**, with corresponding changes in the main text and the figure caption.
- Former Figure 5 is now **NEW Figure 4**, with corresponding changes in the main text and the figure caption.
- As mentioned in the reply to the 3rd question by the reviewer, a **NEW Figure 5** has been included in the manuscript.
- Additional information has been included in the **Acknowledgements** section.