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ORIGINAL RESEARCH

Metastatic Melanoma: A Preclinical Model Standardization and Development of a Chitosan-Coated Nanoemulsion Containing Temozolomide to Treat Brain Metastasis

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Abstract

Melanoma is the most aggressive type of skin cancer. Brain metastasis is the worst scenario in metastatic melanoma and the treatment options for these patients are limited. Temozolomide (TMZ) is a chemotherapy agent used to treat primary central nervous system tumors. Our objective was to develop chitosan-coated nanoemulsion containing temozolomide (CNE-TMZ) for nasal route administration to melanoma brain metastasis treatment. A preclinical model of metastatic brain melanoma was standardized, and the efficiency of the developed formulation was further determined *in vitro* and *in vivo*. The nanoemulsion was done by spontaneous emulsification method and the formulation was characterized by size, pH, polydispersity index, and zeta potential. Culture assessments to determine cell viability were done in the A375 human melanoma cell line. To determine the safety of formulation, healthy C57/BL6 mice were treated with a nanoemulsion without TMZ. The model *in vivo* used B16-F10 cells implanted by stereotaxic surgery in C57/BL6 mice brains. The results demonstrate that the preclinical model used showed to be useful to analyze the efficiency of new candidate drugs to treat melanoma brain metastasis. The chitosan-coated nanoemulsions with TMZ showed the expected physicochemical characteristics and demonstrated safety and efficacy, reducing around 70% the tumor size compared to control mice, and presenting a tendency in mitotic index reduction, becoming an interesting approach to treat melanoma brain metastasis.

Keywords Melanoma · Brain metastasis · Nanoemulsion · Temozolomide · Chitosan

Introduction

Skin cancers are the most common cancer type in the world according to the World Health Organization (WHO 2022). Melanoma accounts for 3% of all skin cancers with 132,000 new cases every year worldwide. Although melanoma accounts for merely 3% of all skin cancers, it is responsible for the majority of skin cancer-related fatalities (Linos et al. 2009; Erdei and Torres 2010; Tas 2012). Metastasis to distant organs and particularly to the brain still represents the most serious obstacle in melanoma therapies (Tas 2012). Brain metastasis is the worst scenario in metastatic melanoma: more than 40% of patients with metastatic melanoma have this condition and a median survival of 6–10 months (Korn et al. 2008; Davies et al. 2011). The therapeutic strategies for these patients are limited, and the most usual treatment is

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surgery followed by radiotherapy or stereotactic radiosurgery (SRS). However, only 5% of patients are eligible for surgery due to tumor location and size (Lee et al. 2000; McDonald et al. 2018). Further progress toward developing new therapeutic strategies for metastatic melanoma will require a more specific approach for brain metastasis.

Chemotherapy is not the first option in melanoma because of its limited efficacy, being used most as a palliative care with two regimens, which include dacarbazine (DTIC) with intravenous administration or temozolomide (TMZ) by oral administration (Li et al. 2015; Young et al. 2017). TMZ and DTIC do not show significant differences in the efficacy for these patients, however, TMZ is most well tolerated, and its oral administration is an advantage when compared with intravenous administration of other chemotherapy agents or even immunotherapy (Chiarion-Sileni et al. 2011; Teimouri et al. 2013; Li et al. 2015). Another challenge involving the treatment of central nervous system (CNS) disorders is the blood–brain barrier (BBB), a specific layer of protection that impair the drug release, challenging the development of new strategies in treatments (Miyake and Bleier 2015). TMZ crosses BBB but presents a short half-life. High doses are required to reach therapeutic brain levels and, due to that, the high systemic levels induce severe side effects including headache, nausea, vomiting, myelosuppression, and oral ulcerations (Trinh et al. 2009; Portnow et al. 2009). An interesting approach to overcome these challenges is the use of modified release systems based on nanotechnology such as nanoemulsions, which are composed of about 10–20% of oil, stabilized by a surfactant (Calderó et al. 2010). The rationale for using nanoemulsions is to increase drug brain targeting and reduce the peripheral side effects associated with TMZ oral administration. These systems can be combined with the nasal route that promotes rapid absorption, biodegradability, ability to escape the BBB, being safe, non-invasive, and convenient (Intakhab Alam et al. 2011; Kim et al. 2015; Khan et al. 2016; Ho et al. 2017; Jiang et al. 2017). However, the rapid mucociliary clearance and enzyme degradation are obstacles in the administration of drugs through the nose to the brain (Fan et al. 2018). A strategy that appears to be useful to avoid this issue is the use of surface modifiers such as chitosan, lectin, and cyclodextrin-cross-linker complex that improve transport and bioavailability (Suri et al. 2007; Ho et al. 2017). Chitosan has been extensively explored in recent years for presenting interesting biological characteristics, such as biocompatibility, biodegradability, and mucoadhesion (Ishak et al. 2013).

The aim of this research was to develop a chitosan-coated nanoemulsion containing temozolomide (CNE-TMZ) for nasal route administration to treat melanoma metastasis. A preclinical model of metastatic brain melanoma was standardized, and the efficiency of the developed formulation was further determined *in vitro* and *in vivo* melanoma growth.

Material and Methods

Materials

Temozolomide (TMZ) (≥ 99% purity) and polysorbate 80 (Tween 80) were obtained from Sigma-Aldrich Ltd (São Paulo, Brazil). Ethanol was acquired from Tedia® (Fairfield, USA). Medium chain triglycerides (MCT) and egg-lecithin (Lipoid E-80®) were acquired from Lipoid GmbH (Ludwigshafen, Germany). Low molecular weight chitosan (50,000–190,000 Da, 75–85% deacetylated) was purchased from Sigma-Aldrich Co. (St. Louis, US).

Preparation of Nanoemulsions

The nanoemulsions were prepared by spontaneous emulsification method as described by Fachel et al. (2018). First, the nanoemulsions phases were prepared. The aqueous phase was composed of ultrapure water and Tween-80® (1% w/v), while the organic phase was composed of egg lecithin (2.5% w/v), medium chain triglycerides (6% w/v), and ethanol and TMZ (final concentration 2 mg/mL). The organic phase was dissolved in ethanol and further dropped into the aqueous phase under stirring. Then, the organic solvent was evaporated at 40 °C on a rotary evaporator under reduced pressure. The obtained nanoemulsions were further coated with chitosan aqueous solution (0.2% w/v) in 1:1 ratio and shaken at room temperature for 15 min. The TMZ concentration following chitosan coating was 1 mg/mL (CNE-TMZ) and the chitosan coating 0.1% w/v. Nanoemulsions prepared at the same conditions in the absence of TMZ were identified as nanoemulsion blank (CNE-B) and were applied as control.

Characterization of Nanoemulsions

Droplet size and polydispersity index (PDI) were measured by photon correlation spectroscopy after appropriate dilution of samples in water. The ζ-potentials were determined by electrophoretic mobility at 25 °C after appropriate dilution with 1 mM NaCl solution. The measurements were achieved using a Zetasizer Nano-ZS90® (Malvern Instruments, England, GB) equipment. The pH of formulations was measured at room temperature using a calibrated digital pH meter (Digimed®, Brazil). TMZ content in the nanoemulsions was determined by diluting an appropriate aliquot of CNE-TMZ in ACN: H_2O (50: 50, v/v) in triplicate and by analyzing the samples through a HPLC method previously developed and validated (Michels et al. 2019).

Mucoadhesive Properties of Nanoemulsions

Mucoadhesive properties of nanoemulsions were evaluated by using a texture analyzer TAXT plus® (Stable Micro Systems, GB) and porcine nasal mucosa obtained from a local slaughterhouse following a protocol previously described by Fachel et al. (2018). Briefly, nasal mucosa hydrated with artificial nasal mucus was attached onto the texture analyzer probe base and formulations were placed in the lower surface of equipment. The measurements were performed with a triggered force of 2 mN, and no force was applied for 60 s. The maximal force (mN) required for detachment of samples ($n = 3$) from the nasal mucosa model was used to compare mucoadhesive properties from nanoemulsions before and after coating with chitosan.

Cell Lines

The human A375 and mouse B16-F10 melanoma cell lines were obtained from American Type Culture Collection (ATCC, USA). The A375 and B16-F10 were cultured in RPMI or DMEM, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (0.5%), and fungizone (0.1%) (Gibco, USA). Cells were allowed to grow in a 5% CO_2 and 95% humidity atmosphere at 37 °C.

Cell Treatment and Determination of Viability Assay

A375 cells were seeded in a 96-well plate (5×10^3 cells/well) and treated with increasing concentrations of TMZ (31.5, 62.5, 125, 250, and 500 µM) in a free solution (TMZ free) or CNE-TMZ. TMZ-free solution was prepared in DMSO (stock solution 51 mM), and it was further diluted in the working concentrations in RPMI/10% FBS. Cultures exposed to equivalent volumes of CNE-B or 0.1% DMSO were considered controls since they have the same components of the CNE-TMZ or TMZ free, respectively, without TMZ. Following 48 and 72 h of treatment, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, USA) (Riss et al. 2004).

Animals

C57/BL6 mice, male, 8 weeks old, 15–25 g weight were used here. The animals were obtained from the Federal University of Health Sciences of Porto Alegre facility. The current work was approved by the Universidade Federal de Ciências da Saúde de Porto Alegre ethics committee (approval number 245/18). All the animals had free access to food and water and followed the sanitary recommendations.

In Vivo Toxicity of CNE-B

To test the toxicity of CNE-B, healthy C57/BL6 mice ($n = 5$) were treated twice a day via nasal route with 40 µL of CNE-B (~ 20 µL per nostril) or physiological solution (0.9% NaCl). After 5 days of treatment, the mice were euthanized and the hepatic and renal parameters of transaminases (AST and ALT), creatinine and urea were measured in serum following manufacturer's instructions (LabTest, Minas Gerais, Brazil). The histology of the organs (brain, lungs, heart, liver, spleen, inguinal lymph node, and kidney) was evaluated by hematoxylin–eosin (HE) standard staining by a pathologist in a blinded manner.

TMZ Brain Distribution Protocol

Male Wistar rats (250–300 g, 8 weeks old, $n = 3$) received about 100 µL of treatment (CNE-TMZ and TMZ free) per nostril (total of 200 µg of TMZ) in alternate administrations with a micropipette. After 1.5 h of the nasal administration of treatments, the brain tissue was promptly removed, was immediately processed, and TMZ was quantified in the brains of the animals through HPLC as earlier validated and described (Michels et al. 2019).

Standardization of In Vivo Metastatic Melanoma Preclinical Model

To mimetize the *in vivo* brain metastatic melanoma, we have adapted the method described by Kong et al. (2011). The animals ($n = 12$) were anesthetized using a ketamine (0.1%) and xylazine (0.05%) solution and submitted to a stereotaxic surgery. Mouse B16-F10 melanoma cells (8×10^4) were resuspended in a final volume of 3 µL (DMEM/10% FBS) and were injected from the bregma 2 mm to the right and 3 mm deep. The euthanasia was done on days 5, 10, and 13 following the surgery to assess the brain tumor volume as described by Azambuja et al. (2020) or to detect the presence of metastasis in the peripheral organs (Azambuja et al. 2020). Pathological analyses were performed by two pathologists in a blinded manner.

Immunohistochemistry (IHC)

Paraffin-embedded specimens were sectioned into 3 µm slices, deparaffinized and rehydrated. Antigen retrieval was performed in a water bath for 40 min with Tris–EDTA 10 mM/1 mM (pH9.0) for melanosome and Sodium Citrate 10 mM for GFAP (pH 6.0). Endogenous peroxidases were blocked with 5% hydrogen peroxide 30 V in methanol

three times of 10 min. To avoid non-specific binding sites, the sections were incubated for one hour with 1% bovine serum albumin Fraction V (BSA) (Sigma® ref: A9647) in PBS at room temperature. Primary antibodies included (a) Monoclonal mouse anti-human melanosome, clone HMB45 (Ref.: M0634, Agilent Dako, CA, USA), 1:20 dilution, to assess the presence of melanosomes; (b) polyclonal rabbit anti-S100 (Ref.: Z0311, Agilent Dako, CA, USA), 1:1000 dilution, to assess the presence of S100 and (c) Anti- GFAP (Reg.: G9269, Sigma-Aldrich, MI, USA); overnight incubation. All primary antibodies were tested for dilution and reactivity before usage. After incubation with primary antibodies, HRP-labeled polymer conjugated (Envision + Dual Link system-HRP kit, Dako ref: K4061) was added and incubated for 30 min at room temperature. Diaminobenzidine (Liquid DAB + Substrate Chromogen System, Dako ref: K3468) was used to visualize the reactions staining. Negative controls were obtained performing the same protocol described above, with the omission of primary antibody, which was replaced by BSA.

Warthin–Starry Staining

The samples were fixed in formalin and embedded in paraffin. The 5-µm paraffin-embedded sections were deparaffinized and stained with Warthin–Starry silver-plating staining as previously described (Propet 1994).

Treatment of Brain Melanoma-Bearing Mice

The animals were submitted to a stereotaxic surgery as described above ($n = 20$). After 5 days of surgery, the animals were randomly divided in four groups and treated with PBS ($n = 5$), CNE-B ($n = 5$), TMZ (2 mg/kg; $n = 5$), and CNE-TMZ (2 mg/kg; $n = 5$). They were treated twice a day via nasal route, with 40 µL of the formulation. On the 11th day after surgery, the animals were euthanized and the blood, brain, lung, heart, liver, spleen, kidney, and inguinal lymph nodes were removed to analyze. For tumor size quantification, images were captured using a digital camera connected to a microscope (2 × magnification; Nikon Eclipse TE300) and the tumor area (mm^2) was determined using ImageJ software. The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by summing the segmented areas (Azambuja et al. 2020). At least three HE sections from each animal were analyzed by a pathologist in a blinded manner. The histology was compared among groups by HE staining of tissue sections. The renal and hepatic parameters were assessed in the blood as shown in the toxicity test explained above.

Statistical Analysis

Statistics were performed using software Graphpad Prism 9 and data were expressed as mean ± standard deviation (SD) and were subjected to one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test (for multiple comparisons) or student's t test when appropriate. Differences were considered significant for a p value of $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, respectively (Prism GraphPad Software, San Diego, USA).

Results

Physicochemical Characteristics and Mucoadhesive Properties of Chitosan-Coated Nanoemulsions Aiming Nasal Delivery

The results obtained from the physicochemical characterization of chitosan-coated nanoemulsions are summarized in Table 1 and demonstrate that both formulations yielded a nanometric diameter around 250 nm and PDI below 0.2. Chitosan-coated nanoemulsions also presented a ζ-potential of approximate + 20 mV and a TMZ content was over than 96% for CNE-TMZ. In addition, the mucoadhesive force of CNE-TMZ and NE-TMZ (TMZ-loaded nanoemulsions, before coating with chitosan) was found to be 15.73 ± 0.35 mN and 4.12 ± 0.33 mN, respectively.

Chitosan-Coated Nanoemulsion Reduces In Vitro A375 Cell Viability

We have treated the A375 human melanoma cell line with increasing concentrations of TMZ free or TMZ-loaded nanoemulsion (31–500 µM) (Fig. 1a, b). We find out that the

Table 1 Physicochemical characteristics of the developed nanoemulsions

Physicochemical characteristics	Droplet Size (nm) ± SD	PDI ± SD	pH ± SD	ZP (mV) ± SD	TMZ content ± SD
CNE-B	246.93 ± 5.35	0.15 ± 0.03	4.12 ± 0.03	19.20 ± 0.82	–
CNE-TMZ	260.93 ± 0.97	0.13 ± 0.02	4.36 ± 0.02	20.50 ± 0.79	96.20 ± 2.89

The results are expressed as mean ± SD (standard deviation) of three independent experiments

TMZ Temozolomide, *CNE-B* chitosan-coated nanoemulsion blank (unloaded drug), *CNE-TMZ* chitosan-coated nanoemulsion containing TMZ, *PDI* polydispersity index, *ZP* ζ-potential

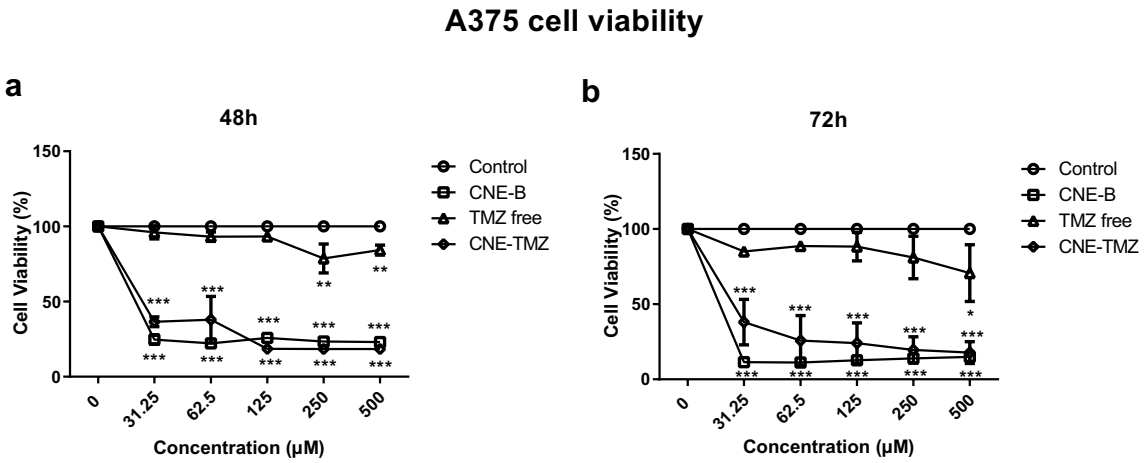


Fig. 1 Chitosan-coated nanoemulsions reduces A375 human melanoma cell viability. A375 cells were exposed to a TMZ-free solution, nanoemulsion blank or nanoemulsion containing TMZ and the cell viability was determined by MTT assay following 48 h (**a**) or 72 h (**b**) of treatment. Untreated cells were considered control of experiment (100% of cell viability). Results were expressed as mean ± standard deviation which were obtained from at least three independent experiments and analyzed by one-way ANOVA following Tukey post hoc (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). *CNE-B* chitosan-coated nanoemulsion blank, *CNE-TMZ* chitosan-coated nanoemulsion containing TMZ, *TMZ free* temozolomide-diluted DMSO

TMZ free (500 μM) reduced 20 and 30% melanoma cell viability following 48 and 72 h of treatment, respectively, when compared to control. Meanwhile, the CNE-TMZ reduced around 60–80% the melanoma cell viability after 48 and 72 h of treatment in all tested concentrations. Similarly, the exposure of melanoma cells to unloaded-drug nanoemulsion (CNE-B) also reduced the cell viability, suggesting that the toxicity observed was also related to the formulation.

B16-F10 Cell Line is Suitable to Form a Tumor When Injected in the Mouse Brain

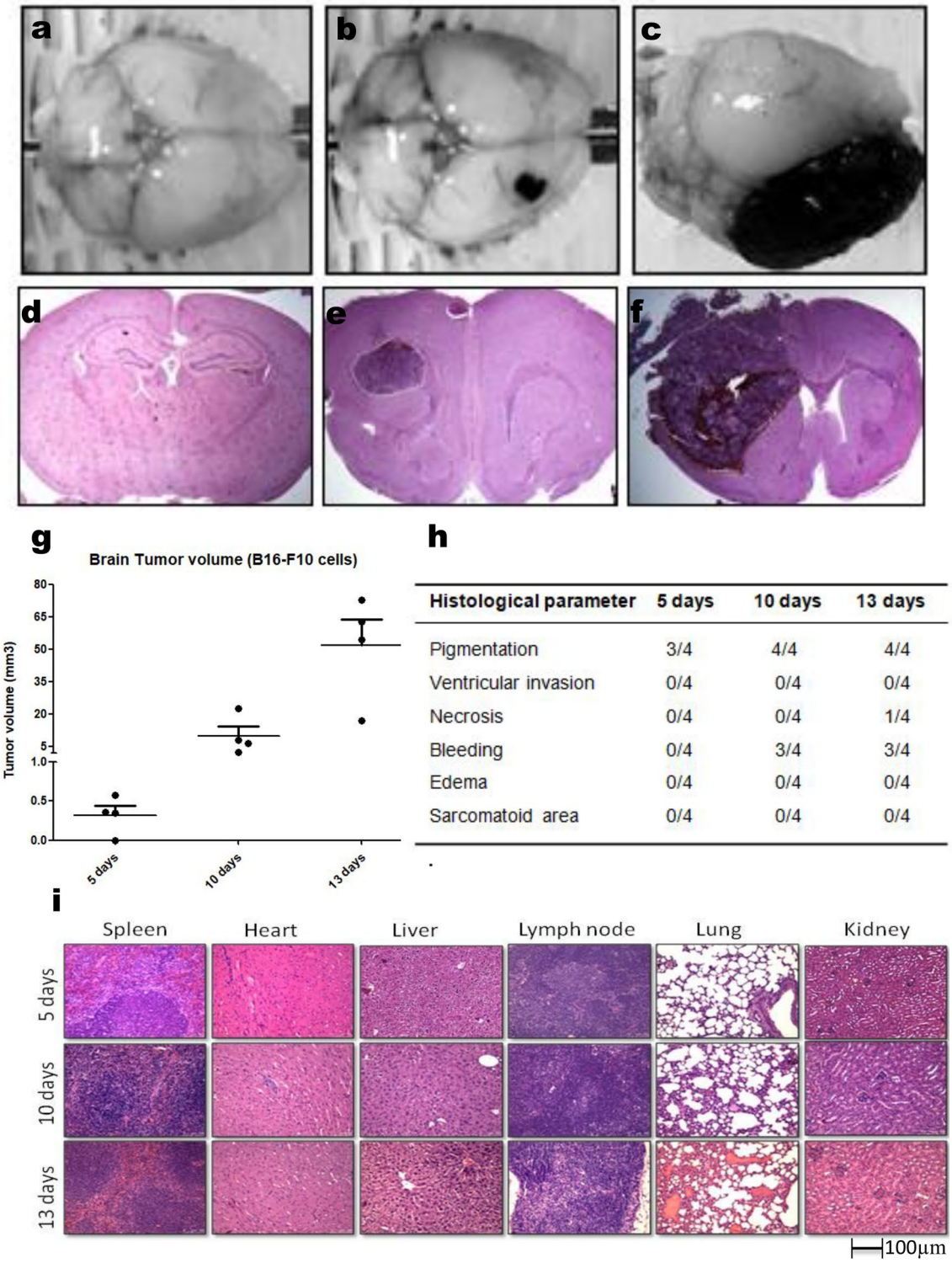
To standardize the melanoma brain metastatic model, the mouse B16-F10 melanoma cell line was implanted in the brain of C57/BL6 mice via stereotaxic surgery as described in material and methods ($n = 12$). As shown in Fig. 2, an exponential and time-course increase of tumor volume 0.4, 5, and 50 mm^3 was observed following 5, 10, and 13 days of cell implant, respectively (Fig. 2a–g). The histological analysis revealed that most tumors have presented pigmentation and hemorrhage. Other malignity parameters such as edema, ventricular invasion, and presence of sarcomatoid areas were not observed (Fig. 2h). No differences were observed in the histology of peripheral organs (spleen, heart, liver, lymph node, lung, and kidney) (Fig. 2i). In order to further characterize the melanoma model, an IHC panel was performed. GFAP was used to identify astrocytes from normal glia in contrast to the tumor area. It was possible to visualize the tumor edge and non-stained cells into the tumor (Fig. 3a, b), as expected. Melanosome, a vesicle produced by melanocytes and, S100, a marker of choice for immunohistochemical identification of malignant melanoma, were positive in the implanted tumor (Fig. 3c–f, respectively). In addition, Warthin–Starry was performed to demonstrate melanin, showing the presence of this pigment into the tumor area (Fig. 3g, h).

CNE-B Formulations Do Not Provoke Toxicity in Health Animals

To evaluate the toxicity, healthy animals were treated with CNE-B and compared with physiological solution. The hepatic and renal functions were assessed by the AST, ALT, creatinine, and urea measures in the blood serum of control (physiological solution) or CNE-B-treated animals via nasal route (Fig. 4a). No differences were observed through the groups. The histology of the organs (liver, heart, lungs, spleen, and kidney) was analyzed by a pathologist in a blinded manner and it was not observed damage in the organs in both groups (CNE-B and physiological solution) (Fig. 4b). Also, the brains of treated animals were analyzed in a blinded manner by a pathologist. There was no damage or cell changes in the CNE-B group compared with the brains of animals that received only physiological solution through nasal via. Taken together, these results indicate that CNE-B does not promote tissue toxicity at this experimental condition, suggesting safety for *in vivo* delivery.

CNE-TMZ Nasal Administration Facilitates the Brain Reach of TMZ

TMZ ability to reach the brain tumor was studied through TMZ quantification in the brain after CNE-TMZ and



Histological parameter	5 days	10 days	13 days
Pigmentation	3/4	4/4	4/4
Ventricular invasion	0/4	0/4	0/4
Necrosis	0/4	0/4	1/4
Bleeding	0/4	3/4	3/4
Edema	0/4	0/4	0/4
Sarcomatoid area	0/4	0/4	0/4

Fig. 2 Standardization of melanoma brain metastatic preclinical model. B16-F10 cells were injected in the C57/BL6 mouse brain by stereotaxic surgery. Following 5, 10, and 13 days of cell implantation, the animals were euthanized, and the tumors were evaluated. **a**–**c** Representative pictures of brain-bearing tumors on the days 5, 10, and 13, respectively. **d**–**f** The HE histology of the brain. The area circulated indicates the tumor. **g** Demonstrates graphically the quantification of the tumor volume (data were expressed as media and they were analyzed by one-way ANOVA following Tukey post hoc [$*p < 0.05$, $**p < 0.01$]). **h** Demonstrates the histological parameters assessed in HE slides and **i** demonstrates representative photos of organs histology (magnification 10×)

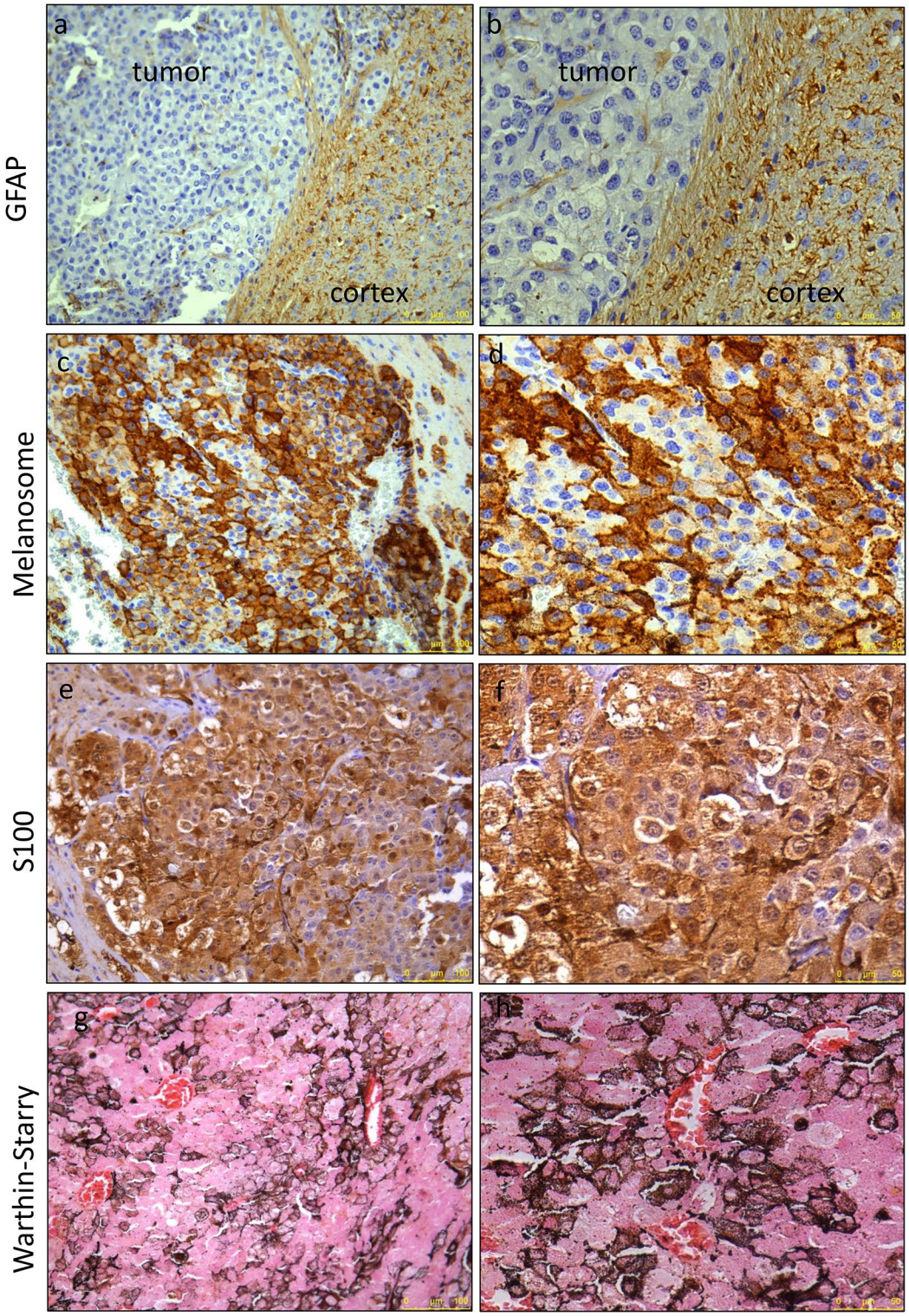


Fig. 3 Molecular characterization of melanoma brain metastatic preclinical model. B16-F10 cells were injected in the C57/BL6 mouse brain by stereotaxic surgery. Following 13 days of cell implantation, the animals were euthanized, and the tumors were evaluated. Representative pictures of IHC are shown to GFAP (**a**, **b**), melanosome (**c**, **d**), and S100 (**e**, **f**). Warthin–Starry was used to stain melanin (**g**, **h**). Magnification 20× (left) and 40× (right). Positive cells are considered the ones maroon (**a**–**f**) or black (**g**, **h**)

a

Parameters		AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Control	#1	252	56	70	0.35
	#2	152	40	64	0.96
CNE-B	#1	104	44	44	0.60
	#2	91	56	56	0.28
	#3	168	44	44	0.28

b

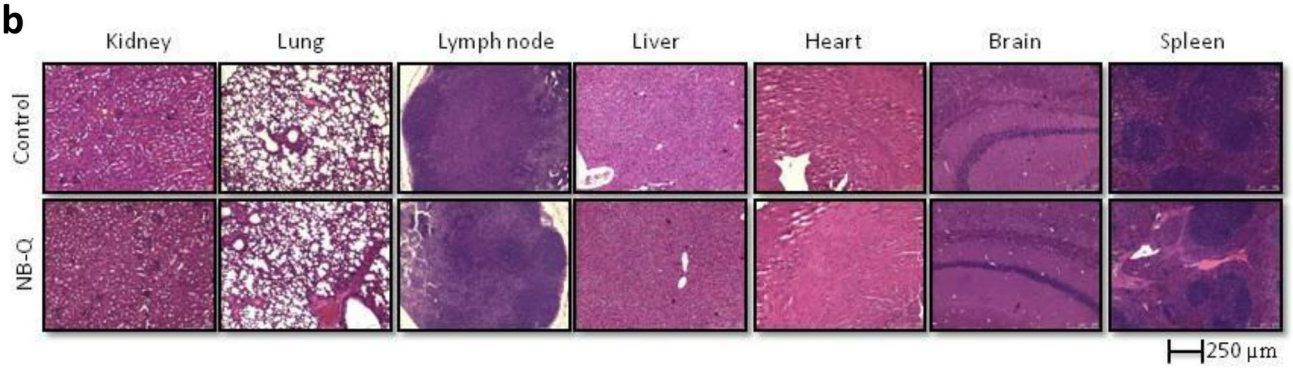


Fig. 4 Toxicity assessments in healthy mice. Health C57/BL6 mice were treated for 5 days, twice a day (final volume 40 µL) with physiological solution (control) or CNE-B via nasal route. **a** Mice were euthanized on the 5th day and the biochemical markers were assessed in the blood serum of control and treated animals. **b** Representative pictures of organ histology (HE) from control and treated mice (magnification 10×)

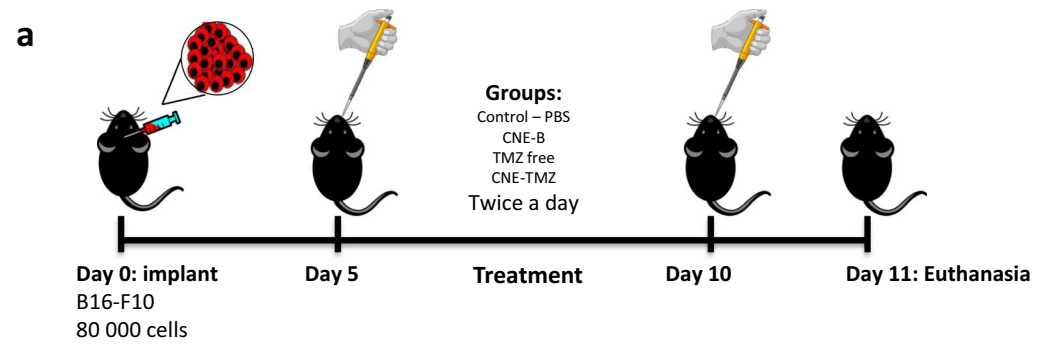
TMZ-free nasal administration. Overall, the results demonstrated that after 1.5 h of CNE-TMZ and TMZ-free nasal administration (100 µL per nostril) the TMZ brain concentration was 0.8095 ± 0.0014 and 0.1958 ± 0.0037 µg/g, respectively.

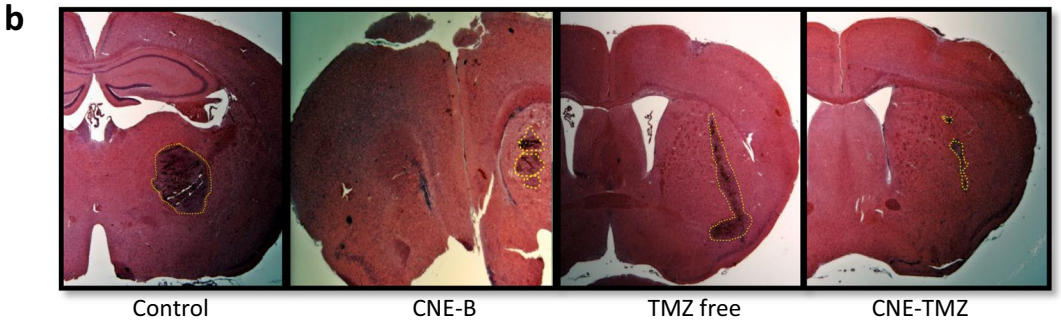
Comparative Analysis of TMZ-Free and CNE-TMZ Antitumor Effect in a Preclinical Model of Melanoma Brain Metastasis

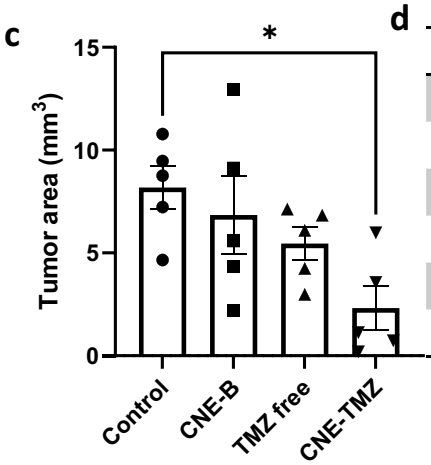
The animals were randomly divided and treated via nasal route with PBS (control), CNE-B, TMZ free,or CNE-TMZ for 5 days (Fig. 5a). After the treatment it is possible to see a reduction of 71.6% of tumor size in the animals treated with CNE-TMZ (2.32 ± 2.42 mm^3) when compared with control (8.18 ± 2.34 mm^3) (Fig. 5b, c). The pathological analysis shows that the treatments with TMZ free or CNE-TMZ have a tendency to reduce the mitotic index (no statistical significance) (Fig. 5d). Regarding the biochemical markers evaluated in the blood serum, it was observed an impairment in creatinine levels in CNE-B group when compared to the control. However, when the formulation was loaded with TMZ (CNE-TMZ) this difference disappeared. In the same way, CNE-B reduced the urea levels when compared to TMZ free. Again, when the nanoemulsion was loaded with the treatment, no effect was observed (Fig. 5e). These results suggest the safety of CNE-TMZ, complementing the results observed in healthy animals in the experiments performed previously.

Discussion

Melanoma is the most aggressive type of skin cancer. Its ability to form metastasis and the low efficacy of treatment decreases the prognosis of the patients (Sabit et al. 2020). TMZ is a chemotherapy already used to treat tumors that occur in CNS, such as glioblastoma, due to its ability to cross the BBB. In a review of clinical trials, Zhu et al. (2014) have analyzed the efficacy of TMZ in brain metastasis and found that it alone has a limited efficacy. Nevertheless, with the advance of science, new strategies to enhance drug delivery, such as nanosystems, have demonstrated anticancer activity in preclinical models of lung, ovarian, glioma, breast, and even in melanoma cancer (Clemente et al. 2018; Azambuja et al. 2020; Ferraro et al. 2020; Pantshwa et al.







d

	Control	CNE-B	TMZ free	CNE-TMZ
Pigmentation	5 (100%)	5 (100%)	5 (100%)	5 (100%)
Invasion	3 (60%)	3 (60%)	1 (20%)	1 (20%)
Necrosis	0 (0%)	1 (20%)	0 (0%)	0 (0%
Bleeding	2 (40%)	4 (80%)	1 (20%)	2 (40%)
Edema	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Mitotic Index	3.25	6.20	2.09	2.40

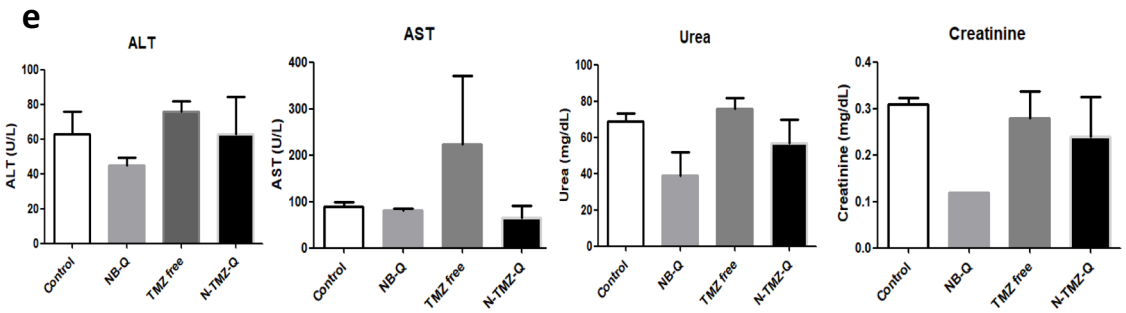


Fig. 5 In vivo treatment with formulations. **a** Representative schema of the experimental protocol applied for the treatment in the preclinical model of brain metastatic melanoma. C57/BL6 were submitted to a stereotaxic surgery for implantation of B16-F10 cells ($n=5$ for each group). Following 5 days of surgery, the animals were randomly allocated into four groups of treatment: control (PBS-treated), *CNE-B* chitosan-coated nanoemulsion blank, *TMZ free* temozolomide diluted in saline solution, *CNE-TMZ* chitosan-coated nanoemulsion containing TMZ. The administration was through the nasal route, twice a day, ~ 20 µL per rostrum (final volume per animal 40 µL). On the 11th day, euthanasia was performed. **b** HE slides of representative tumor volume of the treated groups (magnification 2×). **c** Graphs containing the tumor volume data of each group. **d** Pathological analysis of the implanted tumors. **e** Results of biochemistry assessments in blood serum

2020). Another way to improve brain-delivery is the nasal route administration. Bruinsmann et al. (2019) have analyzed nasal drug delivery to treat glioblastoma and found an improved efficacy and a better biodistribution of anticancer compounds after nasal delivery (Bruinsmann et al. 2019). Based on these findings, in this study was proposed the use of a chitosan-coated nanoemulsion combined with TMZ to treat melanoma brain metastasis via nasal route.

For therapeutic application, an adequate physicochemical characterization of formulations is important to obtain well-defined nanostructures. Our research group has previously developed, optimized, and fully characterized chitosan-coated nanoemulsions by using a Box–Behnken design and confirmed to be a suitable carrier for nasal drug delivery (Fachel et al. 2018). In this context, in the present study first the physicochemical characterization from developed CNE-TMZ and CNE-B was performed. Overall, the results showed an adequate physicochemical characterization from chitosan-coated nanoemulsions, while demonstrating that the spontaneous emulsification procedure yielded nanometric diameter (~ 250 nm) and monodisperse nanoemulsions (PDI < 0.2). In addition, both formulations showed a slightly acid pH, around 4, and ζ-potential ~ + 20 mV, suggesting physical stability of formulations. Furthermore, chitosan-coated nanoemulsions exhibited a positive charge, which could be attributed to the presence of the cationic polysaccharide chitosan on the surface of nanoemulsions, suggesting the efficiency in coating the nanodroplets and in accordance with other chitosan-coated systems reported in literature aiming nasal route delivery (Prego et al. 2005; Fachel et al. 2018; Adnet et al. 2020).

Mucoadhesive properties from TMZ nanoemulsions before and after chitosan coating were also investigated. In summary, the CNE-TMZ presents a mucoadhesive force significantly higher ($p < 0.001$) than NE-TMZ, which demonstrates its mucoadhesive potential. These results are in accordance with the previous literature, since the positively-charged surface can favor the mucoadhesion through a possible electrostatic interaction with the negatively charged sialic groups from mucin present on mucosal surfaces, and for that reason chitosan has been widely used as a mucoadhesive polymer in the development of delivery systems for nasal route, aiming to long lasting nasal uptake increasing the residence time and contact with the mucosa (Prego et al. 2005; Fachel et al. 2018; Adnet et al. 2020).

After the characterization, the anti-melanoma effect of formulations was tested in A375 cell cultures. The cytotoxic effect could not be evaluated because the formulation without TMZ (CNE-B) decreased the melanoma cell viability in the same or higher proportion when compared to CNE-TMZ. The non-specific cytotoxic effect targeted by nanostructures could be related to the chitosan's coat used in the formulation. De Matteis et al. (2016) and Fachel et al. (2018) have found that nanoformulations containing chitosan induced toxicity in high concentrations in cultures of Vero cells (kidney epithelial cells) and astrocytes, respectively. On the other hand, by the previous experience of our group, the human cell line used is very sensible when exposed to treatment with other types of drug vehicles in culture. These factors together could explain the results obtained of cell cultures assays. However, more studies are necessary to better understand the response of A375 cells in culture.

Even with toxicity results in culture, several studies demonstrate the safety of formulations containing chitosan in *in vivo* studies and human administration (Tapola et al. 2008; Baldrick 2010; Lebre et al. 2016; Matica et al. 2017). Supported by these publications, we have tested the *in vivo* toxicity of CNE-B to determine if further use of this formulation might be safe. We have found no differences between groups treated with PBS or CNE-B in histological analysis and renal and hepatic functions suggesting that in the *in vivo* scenario the formulation is safe.

A significantly higher ($p < 0.05$) TMZ brain distribution was found after 1.5 h of nasal administration of CNE-TMZ compared with the TMZ free. These results suggest that the addition of chitosan as a mucoadhesive biopolymer in nanoemulsions may be a favorable role in the enhancement of TMZ brain uptake, probably associated with the prolonged contact time with nasal mucosa that may be advantageous to TMZ bypass the BBB. The results are also in accordance with the previous study developed by Fachel et al. (2020) that demonstrates that the CNE nasal administration also facilitates rosmarinic acid bioavailability in the brain (Fachel et al. 2020).

With the formulations already characterized and tested for toxicity, we would like to know if the proposed anti-melanoma treatment would succeed in a complete *in vivo* system. To test our formulation, we searched for a preclinical model of melanoma brain metastasis. The results found were not applicable to our conditions due to the necessity of using an extracellular matrix in the implant. Therefore, in this study, we have adapted a model of melanoma based on a glioma *in vivo* model and the melanoma metastatic model described by Kong et al. (2011). We have implanted the wild-type B16-F10 mouse cell line directly on the brain and it was able to form tumors with pigmentation and the mitotic index had increased through the time—characteristics of this type of tumor. Melanoma brain metastasis presents a distinct molecular profile compared to primary tumors (In et al. 2020). Here, we chose B16-F10 cell line, a highly metastatic subclone of melanoma that was already characterized by single-cell analysis (Kim et al. 2022) showing upregulated genes related to metastasis, a higher amount of cycling cells, compared to other melanoma cell line (B16-F0) and, genomic alterations that include *Braf*, *Pten*, and *Trp53*. In addition, the implanted

tumor showed positive cells to S100, a sensitive marker for melanocytic lesions, the presence of melanosomes and melanin, expected characteristics of melanoma.

The most used model is the syngeneic transplantation model, where cells are transplanted into the foot pad, intradermally or subcutaneously. In this way, to form metastasis in most cases the primary tumor is removed and usually brain metastasis is not formed (Bobek et al. 2010). Other ways include implant of cells in the tail vein or use of genetic modified cells (Bobek et al. 2010; Kircher et al. 2016). The implant in the tail vein is used to mimetize lung metastasis (Timmons et al. 2016). The main difference of our model is the use of just cells, without matrix or genetic modifications and the injection of cancer cells directly on the brain. To the best of our knowledge, there is no other study using this technique for metastatic melanoma preclinical models. The advantage of our model is the preservation of the brain sites characteristics such as BBB mimetizing the real condition. Besides that, the tumor grows fast decreasing the time needed and how the cells used are wild type it decreases the costs of the in vivo experiments. However, the limitations are that, in this case, there is no metastasis in other sites and the tumor looks superficial.

Finally, with a model standardized, the treatment with the formulation has been tested. Based on the tumor volume and health condition of the animals in standardization, 10 days have been chosen as an endpoint to the following experiments. In the control group, we found a tumor growth like that in standardization 10 days, corroborating the efficiency of the model proposed. In the treated groups, a reduction in tumor size has been observed in the group treated with CNE-TMZ and no differences were observed in renal and hepatic functions between CNE-TMZ treated and control groups. In the pathological analysis we could see a tendency of reduction in mitotic index in the treated groups with free TMZ and CNE-TMZ. These results are interesting because in humans, the low mitotic index is a predictor for patient survival, with a better prognostic (Bogunovic et al. 2009; Azimi et al. 2012).

Other authors have also demonstrated TMZ combined delivery strategies to increase its effectiveness. In the context of glioblastoma, Michels et al (2023) showed that the nasal administration of a temozolomide-loaded thermoresponsive nanoemulsion reduced tumor growth in a preclinical glioblastoma model. Soni et al (2021) have tested the combination therapy of cold atmospheric plasma and TMZ demonstrating a sensitization of glioma cells to TMZ-induce cell death. In melanoma context, Dianzani et al. (2020) have tested a nanotechnology-based polychemotherapy that included TMZ was able to reduce tumor size in a preclinical model.

In conclusion, we have developed chitosan-coated nanoemulsions containing TMZ aiming to treat melanoma brain metastasis that is able to reduce tumor size and mitotic index, becoming an interesting approach for treatment. Further investigations are needed to investigate the mechanisms involved with this reduction. In addition, we have adapted a preclinical model to melanoma brain metastasis that showed to be useful to analyze treatments to melanoma in CNS.

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Data Availability Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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