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Resveratrol-loaded nanocapsules treatment reduces *in vitro* and *in vivo* glioma growth

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Abstract

Glioblastoma multiforme is a devastating cerebral tumor that submits to an awful prognostic. Because of this, improvements in the treatments are necessary to decrease the side effects and to increase the survival of the patients. Resveratrol, a constituent of the red wine, has been described to inhibit carcinogenesis at multiple stages. Biodegradable nanoparticles are emerging as promissory drug delivery system for specific tissues, and because of its useful uptake by tumor cells. The main aim of the current study was to evaluate the antiproliferative effect of *trans*-resveratrol-loaded nanocapsules treatment against C6 glioma cell line in culture and in a rat implanted glioma model. Our results showed that *trans*-resveratrol-loaded nanocapsules decreased the viability/proliferation of glioma cells. This is preceded by an arrest of the cell cycle progression in G2/M and subsequent cell death, represented by sub-G1 phase and propidium iodide stained. In the *in vivo* glioma model, we observed a significant decrease in the tumor size and reduction in some malignant characteristics, typical of the glioblastomas, in the treated rats with this formulation. These effects were more pronounced with the treatment with *trans*-resveratrol-loaded nanocapsules when compared to the treatment with *trans*-resveratrol in solution. Furthermore, *trans*-resveratrol-loaded nanocapsules treatment was not toxic to organotypic cultures, a model of healthy neural cells. The present results suggest that this formulation can be a potential useful chemotherapeutic agent for the treatment of brain tumors. More intensive researches are necessary in order to evaluate its clinical feasibility, though.

Keywords: Glioblastoma multiforme, *trans*-resveratrol, nanocapsules, drug delivery

1. Introduction

Malignant gliomas are the most common primary central nervous system (CNS) tumors in adults accounting for 78% of all primary malignant CNS tumors. The World Health Organization (WHO) classified astrocytomas into 4 grades according to histopathology being the glioblastoma multiforme (GBM), referred to grade IV, the most malignant with median survival of only 9-12 months [1]. This poor prognosis for the GBM is the result of the high capacity of proliferation and variability in the tumor histopathology and resistance to radiation and chemotherapy. Additionally, the diffusely infiltrative behaviors inside the regions of the normal brain give rise to a recurrent tumor in a greater number of cases [2, 3]. Moreover, the localization of these tumors disfavors the treatment, because the potential linking of the chemotherapics to plasma proteins, solute molecular weight, and multiple drug-resistance (MDR) that are intimately related to blood-brain barrier (BBB) and its transposition [4]. About the difficulty on the treatment of these malignancies, new drugs need to be discovered in order to increase the survival of the patients.

Resveratrol (*trans*-3,4,5-trihydroxystilbene) is a polyphenolic naturally abundant component found in grapes, berries, peanuts, including food and various herbs. This promising dietary phytochemical exhibits pleiotropic health beneficial effects including anti-oxidant, anti-inflammatory, cardioprotective and anti-tumor activities [5]. The activity against tumor was first reported in 1997 by Pezzuto and collaborators, which showed the resveratrol capacity in inhibiting the three major steps of carcinogenesis, initiation, promotion and progression in preclinical studies [6, 7]. Resveratrol has been reported to elicit many cellular responses including cell cycle arrest, differentiation and apoptosis through modulation of many different pathways [8, 9]. Nevertheless, animal and human studies indicated a poor bioavailability of resveratrol that is inadequate in permitting the circulating concentrations consistent with *in vitro* antitumor activity [10, 11]. Because of this, new technologies that improve its delivery are necessary, such as the use of a drug delivery system, as the nanoparticles [12].

Nanoparticles have received great interest because of its effective drug deliver to a target site and, thus, increase lately the therapeutic benefit, while minimizing the side effects, principally. Nanoparticles are subdivided into nanoemulsion, nanospheres or nanocapsules, that can be obtained according to the different preparation methods and generally vary in size from 10 to 1000 nm [13]. Nanoparticles are normally composed by a

hydrophobic core and, in this way, hydrophobic drugs can be incorporated, permitting a sustained release of the drug [14], and also protecting the drug degradation [12]. The most promising application of polymeric nanoparticles is their use as carriers for anticancer drugs [15], particularly in solid tumors [16]. It has seen that the conjugated polymer with anticancer drugs were more accumulated in the tumor tissues than in the normal tissues due to the enhanced permeability in endothelium of the tumor vasculature, that characterizes the blood brain barrier and retention effect [12, 17]. Another strategy suggested to delivering the anticancer drugs and the MDR overcoming is the association with nanocarriers, in comparison to competitive P-glycoprotein (PGP) inhibitors as verapamil that causes serious adverse effects [18, 19]. Recently, our group showed that polymeric nanocapsules are able to increase the intratumoral bioavailability of indomethacin and to reduce the growth of implanted gliomas in rats [20].

Earlier studies have shown that resveratrol has excellent ability to inhibit different types of cancer as breast, prostate and liver [21]. In the present work, we have evaluated the anti-glioma efficiency of *trans*-resveratrol-loaded nanocapsules (RSV-NC), its nontoxic effects in healthy cells in equivalent concentrations and its arresting cell cycle effects, in culture. Considering these experiments we have performed *in vivo* assays, through the pathological analysis and tumor volume quantification in a rat glioma experimental model. These results, as a whole, have evaluated the role of resveratrol as chemotherapeutic agent against gliomas, as well as the success of the nanocapsules as drug delivery system.

2. Materials and methods

2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), Hank's balanced salt solution (HBSS), horse serum, fetal bovine serum (FBS), Fungizone®, penicillin/streptomycin, 0.5% trypsin/EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Gentamicin was obtained from Schering do Brazil (Rio de Janeiro, RJ, Brazil). Propidium iodide (PI), dimethylsulphoxide (DMSO) and ([3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide]) (MTT) were obtained from Sigma (St. Louis, USA). *trans*-reveratrol was obtained from Gerbras (Anápolis, GO, Brazil). Poly(Є-caprolactone) (PCL) (M_w=65,000) was supplied by

Aldrich (Strasbourg, France). Caprilic/capric triglyceride mixture was delivered from Brasquim (Porto Alegre, Brazil). Span 60® (sorbitan monostearate) and Tween 80® (polysorbate 80) were obtained from Delaware (Porto Alegre, Brazil). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2. Preparation of nanocapsules

Nanocapsule suspensions were prepared by interfacial deposition of the polymer as previously described [22]. At 40 °C, *trans*-resveratrol (0.010 g), poly(ε-caprolactone) (0.100 g), capric/caprylic triglyceride (0.33 mL) and sorbitan monostearate were dissolved in acetone (27 mL). In a separate flask, polysorbate 80 (0.038 g) was added to 53 mL of water (MilliQ®). The organic solution was injected into the aqueous phase under magnetic stirring at room temperature. After 10 min, the acetone was evaporated and the suspensions concentrated under reduced pressure. The final volume was adjusted to 10 mL. Control formulation (drug-unloaded nanocapsules) was prepared without *trans*-resveratrol.

2.3. Characterization of nanocapsules

The formulation was characterized as described by Frozza and collaborators [23]. Briefly, *trans*-Resveratrol was determined by high-performance liquid chromatography (HPLC) at 306 nm. The system consisted of a UV-Vis detector, pump and auto-injector S200 Perkin-Elmer (PerkinElmer Instruments, Norwalk, CT) and a Shim-pack CLC-C8 (M) column (150 mm, 4.6 mm, 5 μm, Shimadzu Corporation, Japan) with a guard-column. The mobile phase was prepared by using Milli-Q® water and HPLC grade acetonitrile, and consisted of acetonitrile/water (40:60 v/v). The isocratic flow rate of the mobile phase was 1.2 ml/min and the retention time of *trans*-resveratrol was 3.45 min. The pH values of the suspensions were determined using a potentiometer B-474 (Micronal, Brazil). Mean diameters (*z*-average), polydispersity and zeta potentials were measured at 25°C using a Zetasizer®nano-ZS ZEN 3600 model (Nanoseries, Malvern, UK), previously diluting the samples with MilliQ® water or with 0.01 $mol.L^{-1}$ NaCl aqueous solution. To avoid any sample selection only the dilution media were filtered (Millipore 0.45 μm) before analysis. The measurements were carried out with 3 different batches for each formulation in triplicate. The samples were

maintained at 25°C. Calculation of the size and polydispersity indices was achieved by the software (Total Chrom Navigator v6.2.0.0.1) provided by the manufacturer.

2.4. Maintenance of cell lines

The C6 rat glioma cell line was obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Cells at 5-15 passages were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) 1%, containing antibiotics penicillin/streptomycin 0.5 U/ml, and supplemented with 5% (v/v) fetal bovine serum (FBS). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. All the experiments throughout this study were conducted in serum supplemented DMEM.

2.5. Drug exposure

trans-resveratrol (RSV) was dissolved in cell culture-grade dimethylsulphoxide (DMSO). The *trans*-resveratrol-loaded nanocapsules (RSV-NC) and drug-unloaded nanocapsules (NC) were prepared as described above. The glioma cells were seeded according each experiment and after reaching semi-confluence the cultures were exposed for 48 h to formulations: RSV and RSV-NC (1, 5, 10, 15, 25 or 50 μM) for viability cells assay (MTT); with respective IC_{50} (34.39 μM to RSV-NC and 39.61 μM to RSV) for proliferation assay and with RSV-NC (5, 10, 25 or 50 μM) for Propidium iodide assay. Control cells were treated with the highest volume of the vehicle DMSO or drug-unloaded nanocapsules.

2.6. Assessment of glioma cell viability

Glioma cells were plated in a 96-well plate at 10^3 per well and, after reaching semi-confluence, the cultures were treated as described above. After 48 h of treatment, each culture medium containing the drug was removed and the cells were washed twice with 100 μl of phosphate buffered saline (PBS) (pH 7.4). After removing the PBS, 90 μl of culture medium and 10 μl of MTT (5mg/mL) were added to each well. MTT provides a quantitative measure of metabolically active mitochondria and it is based on the

mitochondrial reduction of a tetrazolium bromide salt to a chromophore, formazan product. The cells were incubated for 2 h and the solution was then removed from the precipitate. A total of 100 µl of DMSO were added to the wells and the level of absorbance was read at 490 nm. This absorbance was linearly proportional to the number of living cells with active mitochondria. The cell viability was calculated using Eq. (1):

$$\text{Cell viability (\%)} = (abs_s / abs_{control})\ 100 \quad (1)$$

where Abs_s is the absorbance of cells treated with different formulations and $Abs_{control}$ is the absorbance of control cells (incubated with cell culture medium only).

2.7. Cell counting (Proliferation)

The rat glioma cells were seeded at 5×10^3 cells per well in DMEM 5% FBS in 24-well plates, and allowed to grown until reaching semi-confluence, glioma cells were treated with the respective IC_{50} value, as describe above, for 48 h. At the end of the treatment, the medium was removed. Cells were washed twice with 200 µl (PBS) and 100 µl of 0.05% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a haemocytometer.

2.8. Cell cycle analysis

Cells were plated in 12-well plates and, after 2 days, were treated with 10 and 50 µM of RSV or RSV-NC for 24 h. After the supernatant was removed, the cells were detached, spun down twice and suspended, firstly with PBS pH 7.4 and subsequently with 500 µl staining solution (Tris-HCl 3.4 mM pH 7.6 ; NaCl 10 mM ; NP 40 0.1% (v/v) ; RNAse 700 U/L ; PI 0.075 mM). After 10–30 min, data was collected using The BD FACSCalibur multicolor flow cytometer. Results were analyzed using Winmdi. Control cultures were treated with DMSO or drug-unloaded nanocapsules.

2.9. Propidium iodide assay

Glioma cells were treated as described in drug exposure. After the 48 h of treatment, the cell cultures were incubated with 5 μM of propidium iodide (PI) for 2 h. PI is excluded from healthy cells, but following loss of membrane integrity this molecule enters into the cells, binds to DNA and becomes highly fluorescent. PI fluorescence was excited at 515–560 nm using an inverted microscope (Nikon Eclipse TE300) fitted by a standard rhodamine filter. Images were captured using a digital camera connected to the microscope.

2.10. Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared by method of Stoppini and collaborators with modifications [24]. Briefly, 400- μm-thick hippocampal slices were prepared from 6- to 8-day-old male *Wistar* rats using a Mcllwain tissue chopper and separated in ice-cold HBSS, pH 7.2. The slices were placed on Millicell culture insert (Millicell®-CM, 0.4 μm, Millipore®, Bedford, MA, USA) and the inserts were transferred to a 6-well culture plate. Each well contained 1 ml of tissue culture medium consisting of 50% MEM, 25% HBSS, 25% heat inactivated horse serum, pH 7.3. Organotypic cultures were maintained in a humidified incubator gasified with 5% CO_2 atmosphere at 37°C. The medium was changed every 3 days and experiments were carried out after 14 days *in vitro* when the cells were treated for 48 h with 5, 10, 25 or 50 μM of RSV-NC. Quantification of cellular death in organotypic hippocampal cultures was assessed by fluorescent image analysis of PI uptake [25]. After 46 h of treatments, 5 μM PI was added to the cultures and incubated for 2 h. Cultures were observed with an inverted microscope (Nikon Eclipse TE300) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was determined using the "density slice" option of Scion Image software and compared to the total hippocampus area to obtain the percentage of damage [25]. All animal use procedures were approved by local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.11. Glioma implantation

Glioma implantation was performed according previous works in our group [20, 26, 27]. Briefly, rat C6 glioma cells at around 80% confluence were trypsinized, washed once in DMEM/5% FBS, spun down and ressuspended in the same medium. A total of one million cells in a volume of 3 µL were injected using a 5 µL Hamilton microsyringe coupled in the infusion pump (0.1µL/min) at a depth of 6.0 mm into the right striatum (coordinates with regard to bregma: 0.5 mm posterior and 3.0 mm lateral) of adult *Wistar* male rats (9 weeks old, 220-260 g) anesthetized by intraperitoneal (i.p.) administration of ketamine/xilazine [28]. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from National Institutes of Health (NIH) and were approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre.

2.12. Treatment of animals

After 10 days of glioma cell implantation, the animals were randomly divided into five groups as follows: (1) untreated (Control group); (2) treated with 33% DMSO in NaCl 0.9% (Vehicle group); (3) treated with drug-unloaded nanocapsules (NC group); (4) treated with 5 mg.Kg/day of free *trans*-resveratrol (RSV group); (5) treated with 5 mg.Kg/day of *trans*-resveratrol-loaded nanocapsules (RSV-NC group). The drugs were administered to the animals intraperitonealy (i.p.) for 10 consecutive days. After 20 days (10 days for glioma implantation + 10 days for treatment), the rats were decapitated and the entire brain was removed, sectioned and fixed with 4% paraformaldehyde. For analysis of body weight, the experimental groups were compared with the control group.

2.13. Pathological analysis and tumor volume quantification

Pathological analysis was performed at least three Haematoxylin and Eosin (H&E) sections (3-6 µm thick, paraffin embedded) of each animal. For tumor size quantification, images were captured using a digital camera connected to the microscope (Nikon Eclipse TE300) and tumor area (mm^2) was determined using Image Tool Software^{TM}. The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by summing the segmented areas [20, 26, 27].

2.14. Statistical analysis

Data are expressed as mean ± S.D. and analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by post-hoc for multiple comparisons (Tukey test) using a *GraphPad Prism Software*. Differences between mean values were considered significant when $p<0.05$.

3. Results

3.1. Physicochemical characterization of nanocapsule formulations

The nanocapsule formulations were prepared by interfacial deposition of polymer without the need of any subsequent step of purification. *trans*-Resveratrol-loaded nanocapsules and drug-unloaded nanocapsules presented a macroscopic homogeneous aspect, such as white bluish opalescent liquids. After preparation, the particle sizes were 240 nm (NC) and 241 nm (RSV-NC), conforming previously described for the same formulations [23]. The suspensions showed stable monomodal size distributions and polydispersity indexes lower than 0.3, indicating narrow size distributions. The HPLC method was validated presenting linearity between 2.5 and 17.5 μg/ml, ($r > 0.9999$), inter- and intraday variability lower than 2.0%, and accuracy from 96.19% to 97.21%. The *trans*-resveratrol content was 0.964 ± 0.037 mg/ml and the encapsulation efficiency was 99.89 ± 1.3%.

3.2. trans-Resveratrol-loaded nanocapsules decrease cell viability/proliferation in rat glioma cells

The MTT assay was used to investigate whether RSV and RSV-NC affects the cell viability. Cultured cells were treated with several concentrations as described in Materials and Methods. Analysis of MTT assay showed that 25 and 50 μM RSV caused a significant reduction in cell viability compared with the control culture cells (35.28 ± 9.58% and 62.23 ± 8.30%; respectively) (Fig. 1). Importantly, 15, 25 and 50 μM RSV-

NC (28.26 ± 5.39%, 39.49 ± 2.38% and 71.06 ± 7.67%, respectively) were already enough to decrease the cell viability (Fig. 1). The IC_{50} values showed that RSV-NC increases 1.15 fold the cytotoxicity for glioma cells when compared to RSV. In order to reinforce the effect of RSV-NC on cell growth, we studied the effect of these formulations on the proliferation of glioma cell line. The treatment of the glioma cells with the respective IC_{50} value, as described above, for 48 h, resulted in significant decrease in the cell number when compared to the control cultures (69.8 ± 4.45% to RSV-NC and 53.95 ± 1.5% to RSV) (Fig. 2). These effects were more pronounced than observed in MTT assay (Fig. 1). The DMSO and drug-unloaded nanocapsules treatments did not show significant alterations in the cell viability/proliferation.

3.3. Cell cycle arrest in G2/M and sub-G1 phases induced by trans-resveratrol-loaded nanocapsules in glioma cells.

DMSO and drug-unloaded nanocapsules neither cause G2/M cycle arrest (21% and 24%, respectively) nor Sub-G1 phase arrest (2% to both) (Fig. 3A and 3B). RSV also has not effect with 10 μM following its treatment for 24 h (24% G2/M and 5% sub-G1) (Fig. 3C). However, RSV-NC showed G2/M arrest of the cell cycle progression (41%), which had a strong decrease in G1-phase cell population in this condition (Fig. 3D). In cell cycle and sub-G1 studies assessing whether RSV-NC also causes apoptotic cell death, as shown in Fig. 2F, the treatment with 50 μM for 24 h resulted in an apoptotic cell death representing by 21% sub-G1 arrest versus just 13% of the compost in solution (Fig. 2E).

3.4. Cell death induced by trans-resveratrol-loaded nanocapsules in glioma cell lines

To verify whether RSV-NC could induce necrosis cell death, glioma cells were treated with several concentrations of this formulation, as described above, for 48 h. The treatment of drug-unloaded nanocapsules did not show significant alterations in PI incorporation (Fig. 4A). As observed, the glioma cells treated with 5 μM of RSV-NC did not present reduction in the cell number with no PI incorporation (Fig. 4B). Similar results were obtained with 10 μM (Fig 4C). However, when the cells were treated with 25 or 50 μM of RSV-NC, it was observed a significant reduction in the cell number and

an increase of the PI incorporation, suggesting there is some necrotic cell death (Fig. 4D and E).

3.5. trans-Resveratrol-loaded nanocapsules did not cause cytotoxic effect on organotypic hippocampal slice cultures

To evaluate the effect of RSV-NC on healthy neural cells, organotypic hippocampal slice cultures were used as a model. These cultures provide an excellent *in vitro* model system to study physiological factors, cellular and molecular mechanisms of neural death, and pharmacological compounds to neural survival [29]. After 14 days in culture, the organotypic hippocampal was treated and analyzed as described above. As a positive control of cell damage we used organotypic hippocampal slice cultures exposed to oxygen and glucose deprivation (OGD), which showed significant cell damage (approximately 65%) (Fig. 5). It is important to note that RSV-NC did not cause organotypic hippocampal culture damage in any concentration suggesting its safety for healthy neural cells (Fig. 5).

3.6. trans-Resveratrol-loaded nanocapsules treatment reduced tumor size in the rat glioma model

Implanted gliomas were performed by the injection of C6 glioma cells in the *striatum* of adult *Wistar* rats and after 10 days of glioma implantation, the animals were randomicaly separated in five groups and treated for 10 days as described in Material and methods. The results showed that the animals treated with RSV-NC had a significant reduction in the tumor size (20.45 ± 6.76 mm^3) when compared to untreated (239.9 ± 59.39 mm^3), drug-unloaded nanocapsules (263.6 ± 58.15 mm^3), vehicle group (289.0 ± 6.00 mm^3) and versus 167.5 ± 63.66 mm^3 of the RSV group (Fig. 6).

3.7. Histopathological analysis

Implanted tumors presented characteristics that are close to those human glioblastoma multiforme (Grade IV) with the cells growing in the intracerebral,

intraventricular and intraparenchymal spaces (data not shown). A representative figure of H&E analysis showed that the control group presented coagulative necrosis, intratumoral hemorrhage, peripheric pseudopalisading and vascular proliferation (Fig. 7A and 7B). Moreover, pathological analysis identified pallisading cells and lymphocytic infiltration, with formation of edema fluid and neovascularization, which are characteristics of glioblastoma multiforme (Fig. 7, Table 1). It is important to note that in addition to the observed reduction in the tumor volume, the RSV-NC treatment produced glioma with less tumor coagulative necrosis, peritumoral edema and peripheric pseudopallisading that indicate a less invasive/proliferative tumor (Fig. 7C and 7D, Table 1).

4. Discussion

Despite the difficulty of the treatment and the low survival of the patients with glioblastoma multiforme, we have researched a promising dietary phytochemicals with chemopreventive and chemotherapeutic potential, resveratrol [5] and its better delivery to overcome the glioma cell [14]. In the present work it was found the great ability of the RSV-NC as potential antitumoral agent against glioblastoma multiforme**.** Firstly, we have evaluated the decrease on the viability and cell proliferation of the gliomas. After, we have showed that this cytotoxicity is selective to tumoral cells through the PI incorporation analysis. We have also showed that this death in glioma cell line has been preceded by cell cycle arrest in G2/M phase with subsequent sub-G1 arrest. Additionally, we have studied the RSV-NC role on the decreasing the volume as well as some malignant characteristics of the implanted tumors. Moreover, even being cytotoxic to the tumoral cell RSV-NC neither alters hepatic enzymes nor the body weight of the treated rats (data not shown). Because of this, RSV-NC appears like a promising component against gliomas.

Previous studies have showed the ability of RSV in decreasing the proliferation in glioma cells [30]. Within this context, we verify whether RSV-NC decreases the viability of glioma cells by MTT assay. Our results have denoted that in 15 μM RSV-NC had significant effect in the cell viability versus 25 μM of RSV (Fig. 1). The difference in the cytotoxic effect of nanocapsules against free drug in glioma cells may

be explained by a higher potential cell division with an increased endocytotic activity resulting in enhanced nanocarrier uptake [19]. After this first experiment, we calculated the IC_{50} value for respective formulations and performed the proliferation assay that caused 69.8% of inhibition in RSV-NC treatment (Fig. 2). Probably, this decrease bigger than IC_{50} value was the consequence of the nonlinear dose-response of this formulation. Our preliminary results have showed that this death of glioma cells is after-effect the cell cycle arrest in G2/M phase (cell with duplicated DNA) with just 10 μM of RSV-NC for 24 h (Fig. 3D) and we have not observed it with this compost in solution under these conditions (Fig. 3C). Corroborating, the cell cycle analysis has detected the formation of a sub-G1 cell population with 50 μM of RSV-NC for 24 h (cells with hypodiploid DNA content), confirming DNA fragmentation (Fig. 3F). Collectively, these preliminary results show the cytotoxic effects of RSV-NC on C6 cell growth and the death through the cell cycle arrest and possible induction of apoptosis. Nevertheless, for an exposure time of 48 h, RSV-NC caused some PI incorporation with 25 and 50 μM being an indicative of necrotic cell death (Fig. 4D and 4E). Necrosis is an event that occurs when cells are exposed to a serious physical or chemical insult. This fact is according to literature, which has described necrosis when the glioma cells were treated with high concentration of resveratrol [31].

The results of the molecular therapy against glioma have not been universally successful and the main obstacle targeted molecular drugs is its toxicity [32]. Thinking on that, we have performed a treatment with RSV-NC in organotypic hippocampal slice cultures and the RSV-NC showed safety for these healthy neural cells (Fig. 5). As it has been mentioned above, the *in vitro* cytotoxicity in glioma line may be triggered by a higher potential cell division activity with an increased endocytotic resulting in enhanced nanocarrier uptake by glioma cells, that also explain, at least in part, the nontoxicity for neural cells that own low capability of division. Moreover, the uptake is just one explanation for the superior ability of the nanocapsules containing resveratrol against free drug in decrease tumor size and malignancy in glioma implanted in rats. Indeed, the beneficial nanoparticles can be a consequence of the drug plasma protein unbound, the hyperpermeable BBB transposition, the ability of these polymeric nanoparticles to circumvent MDR mechanisms and for the additional retention of the colloidal particles in the tumor interstitium with lymphatic system compromised [4, 12, 17]. Consequently, the nanoparticles enhance the drug antitumor efficacy to

successfully increase its concentration of resveratrol in the brain tissue in compare the free drug [23].

Although most *in vivo* studies strongly support a chemotherapeutic effect of resveratrol, there are notable exceptions in which no benefit has been observed. For example, the administration of 5 mg/Kg/day for 23 days of resveratrol failed to affect the growth or metastasis of breast cancer in mice, in spite of the promising *in vitro* results [33]. The literature has also showed that it is necessary a dose of 40 mg/Kg/day for 28 days to decrease the size tumor in mice with neuroblastoma [34]. We administered low doses of 5 mg/Kg/day for 10 days of *trans*-resveratrol-loaded nanocapsules and observed the significant reduction in the tumor size of implanted glioma in rats (Fig 6). These notable differences in the efficacy of resveratrol treatment may be due to variations in the dosage, the route of administration, the tumor origin, the presence of other dietary components [5], and in the present study, mainly by the use of the nanocapsules, as it was already shown by our group, acting as a drug deliver system [20]. The other important finding showed here is that RSV-NC treatment reduced some important malignant characteristics typical of the glioblastomas in the tumors of rats treated with this formulation, as coagulative necrosis, peritumoral edema and peripheric pseudopalisading (Fig. 7, table 1).

The inhibition of ciclooxigenase, with consequent reduction in its enzymatic products, mainly prostaglandin, can explain, at least in part, the resveratrol effect against cancer that own pharmacological anti-inflammatory propriety [35, 36]. There are many other mechanisms that may explain the reduction in the glioblastoma growth by resveratrol. The alteration in regulator genes expression and signaling pathway in glioma could suppress the cell proliferation, induce cell cycle arrest and promote apoptosis [37]. These mechanisms include tumor suppressors; cell cycle regulators; transcription factors; angiogenic and metastatic factors; and apoptotic and survival regulators [38]. Several pathways could permit this compost to overcome chemoresistance-inducing mutations in cancer cells. Therefore, the data from this study not only confirm the potential of resveratrol in treating malignant glioma but also offers an effective and safe way to improve the anticancer efficiency by nano-drug delivery systems. Because of this, *trans*-resveratrol-loaded nanocapsules appear as a promissory treatment for gliomas.

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Legends to the figures

Fig. 1. Effect of trans-resveratrol (RSV) or in nanocapsule formulations (RSV-NC) on viability of C6 rat glioma cell line.

C6 glioma cells were treated with *trans*-resveratrol (RSV) or *trans*-resveratrol-loaded nanocapsules (RSV-NC), cultures were also treated with DMSO or drug-unloaded nanocapsules (NC). After 48 h of treatment, the cell viability was evaluated by MTT assay as described in Materials and methods. The cell viability was represented in relation to control cells (100% of cell viability). The values are represented as means ± S.D. of tree independent experiments made in triplicate. Data were analyzed by one-

way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the control group (P<0.001); **significantly different from the control group (P<0.01).

Fig. 2. Effect of trans-resveratrol (RSV) or nanocapsule formulations (RSV-NC) on glioma cell proliferation.

Semi-confluent cultures of glioma cells were treated for 48 h as describe in Materials and methods. Control cultures were treated with DMSO or drug-unloaded nanocapsules. After 48 h of treatment, the cells were detached with 0.05% trypsin-EDTA and counted in haemocytometer. The values are represented as means ± S.D. of tree independent experiments made in duplicate. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the control group (P<0.001); #Significantly different from the RSV group (P<0.05).

Fig. 3. trans-Resveratrol-loaded nanocapsules cause the cell cycle arrest in G2/M and sub-G1 group of C6 cells.

C6 glioma cells were plated in a 12-well plate and treated as described above for 24 h and the data were collected using BD FACS Calibur multicolor flow cytometer. Pictures represent the percentage of cell phase distribution: (A) DMSO; (B) NC; (C) 10 μM of RSV; (D) 10 μM of RSV-NC; (E) 50 μM of RSV and (F) 50 μM of RSV-NC.

Fig. 4. Effect of trans-resveratrol-loaded nanocapsules on cell death in C6 glioma cells by propidium iodide incorporation.

Representative pictures of cells treated with drug-unloaded nanocapsules (NC) (A); cells treated with 5 μM (B); 10 μM (C); 25 μM (D) or 50 μM (E) of *trans*-resveratrol-loaded nanocapsules (RSV-NC). After 46 h of treatment, glioma cells were incubated with 5 μM of propidium iodide (PI). Cellular death was analyzed by PI incorporation that was visualized using a Nikon inverted microscope (at 40× magnification). Panels right side A, B, C, D and E are correspondent contrast phase photomicrographs and to the left represent fluorescence photomicrographs.

Fig. 5. Effect of trans-resveratrol-loaded nanocapsules on organotypic hippocampal slices cultures.

(A) Representative pictures of organotypic hippocampal cultures. (B) Quantitative analysis of hippocampus damage after treatment of formulations. Organotypic

hippocampal slices at 14 days were treated as described above for 48 h. Control: organotypic cultures not exposed to formulations; OGD: cultures had been exposed to oxygen glucose deprivation and then used as a positive control of cell damage. Cellular death was analyzed by propidium iodide (PI) incorporation, which was visualized using a Nikon inverted microscope (at 40× magnification). Data represent the means.±.S.D. of three animals. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey). *Significantly different from the control group ($P<0.001$).

Fig. 6. Tumor size of implanted gliomas.

Animals were treated as described in Materials and methods. Tumor size was measured 20 days after implantation of C6 cells through three haematoxylin and eosin (H&E) sections of each tumor. For tumor size quantification, images were captured using a digital camera connected to the microscope and total volume (mm^3) was determined using Image Tool Software™. The values are represented as means ± S.E.M of for animals per group. Data were analyzed by one-way ANOVA followed by *post-hoc* comparisons (Tukey). * Significantly different of the control group ($P<0.001$).

Fig. 7. Histological analysis of implanted gliomas.

The sections of implanted rat glioma were stained with hematoxylin and eosin (H&E) as described in Materials and methods. Representative pictures of histological characteristics that define glioblastoma multiform as seen in rats implanted with gliomas: control group (A,B) and resveratrol-loaded nanocapsule (C,D). Necrosis (N), microvascular proliferation (V), hemorrhages (H) and peripheric pseudopalisading (P). Scale bars= 100 μM (A,C) and 20 μM (B,D).

Figure 1

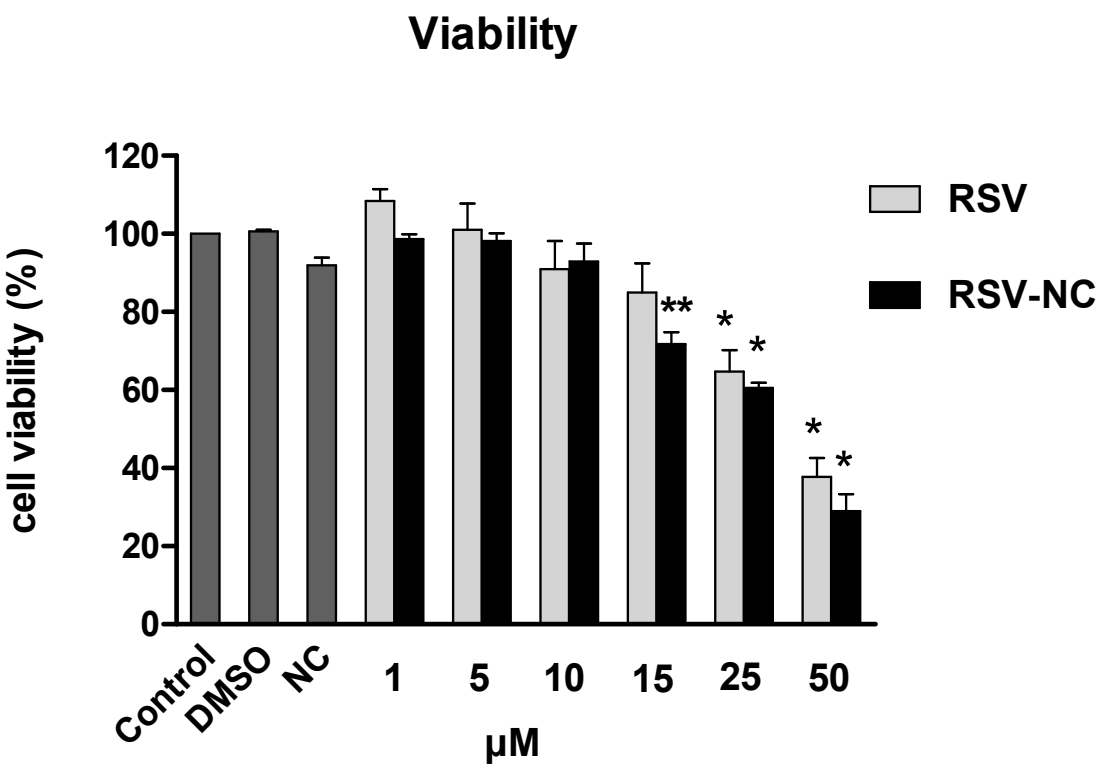

Viability
cell viability (%)
120
100
80
60
40
20
0
RSV
RSV-NC
**
*
*
*
*
Control
DMSO
NC
1
5
10
15
25
50
μM

Figure 2

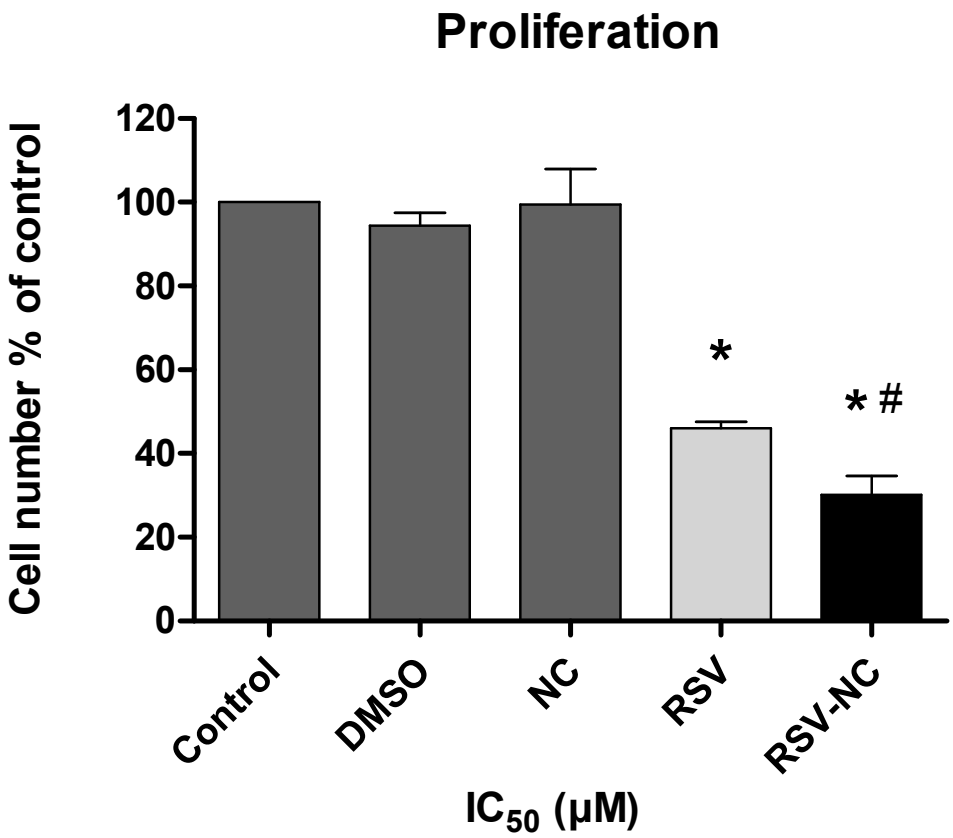
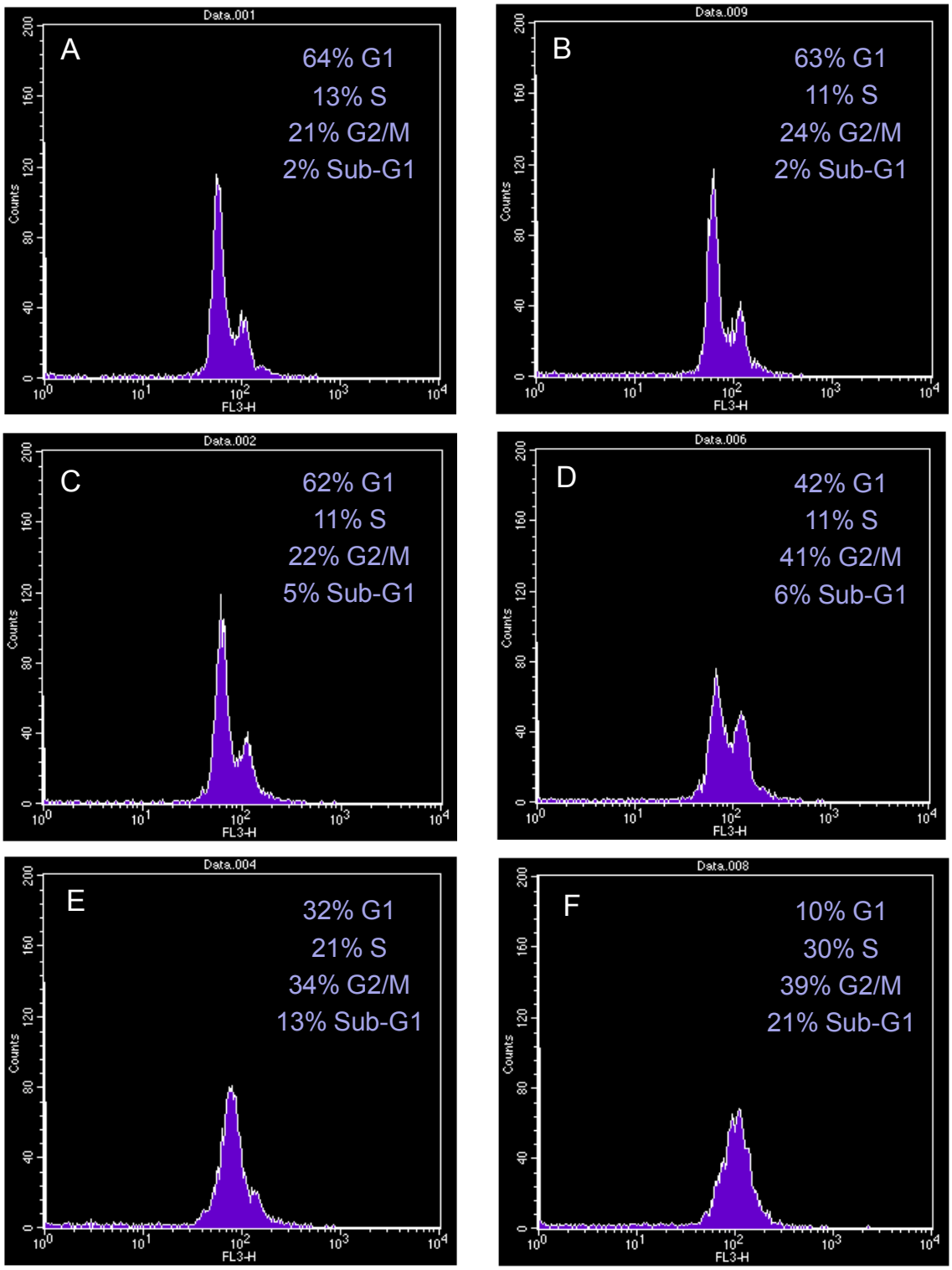

Proliferation
Cell number % of control
120
100
80
60
40
20
0
*
* #
Control
DMSO
NC
RSV
RSV-NC
IC50 (µM)

Figure 3


Data.001
A
64% G1
13% S
21% G2/M
2% Sub-G1
Counts
FL3-H
Data.009
B
63% G1
11% S
24% G2/M
2% Sub-G1
Counts
FL3-H
Data.002
C
62% G1
11% S
22% G2/M
5% Sub-G1
Counts
FL3-H
Data.006
D
42% G1
11% S
41% G2/M
6% Sub-G1
Counts
FL3-H
Data.004
E
32% G1
21% S
34% G2/M
13% Sub-G1
Counts
FL3-H
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F
10% G1
30% S
39% G2/M
21% Sub-G1
Counts
FL3-H

Figure 4

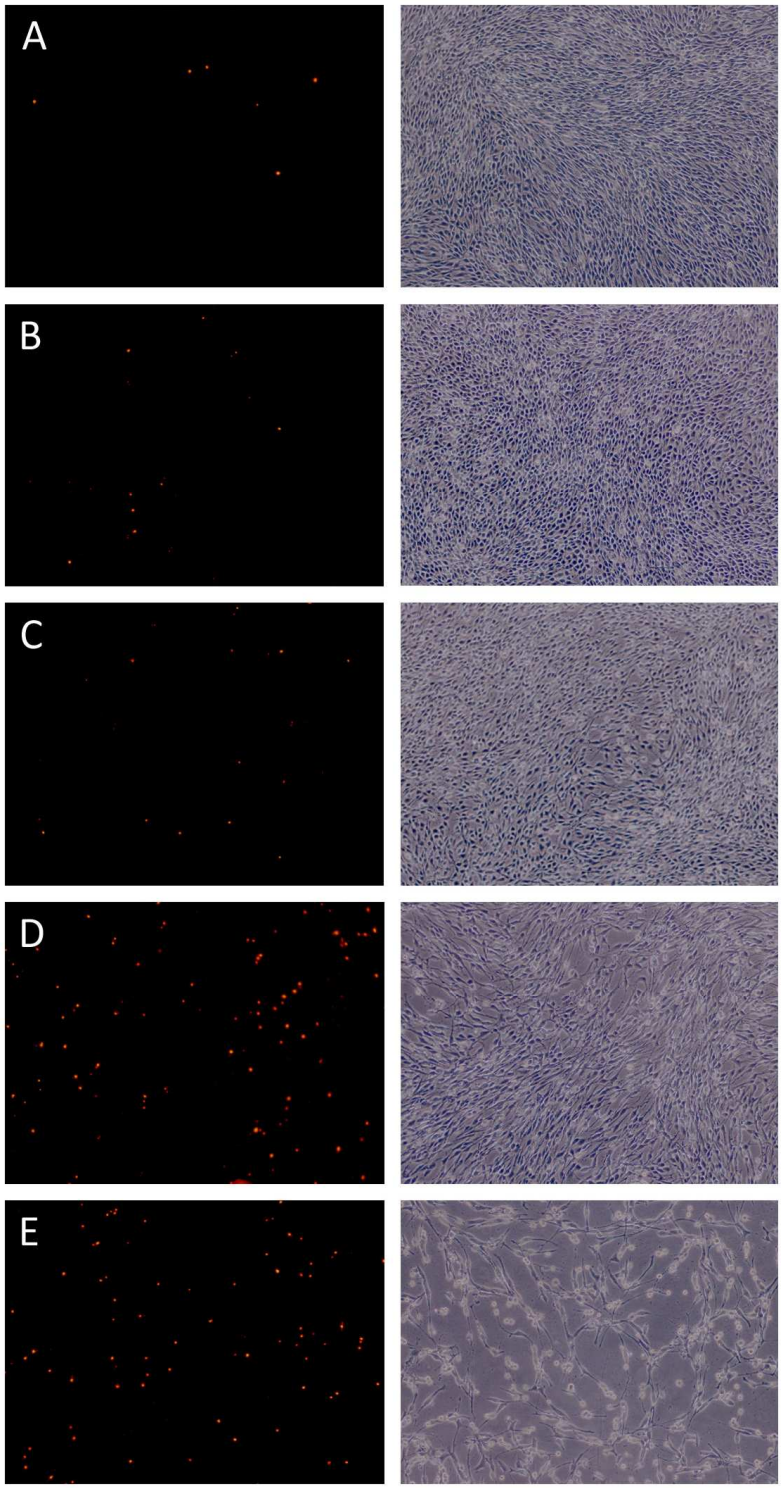

Figure 5

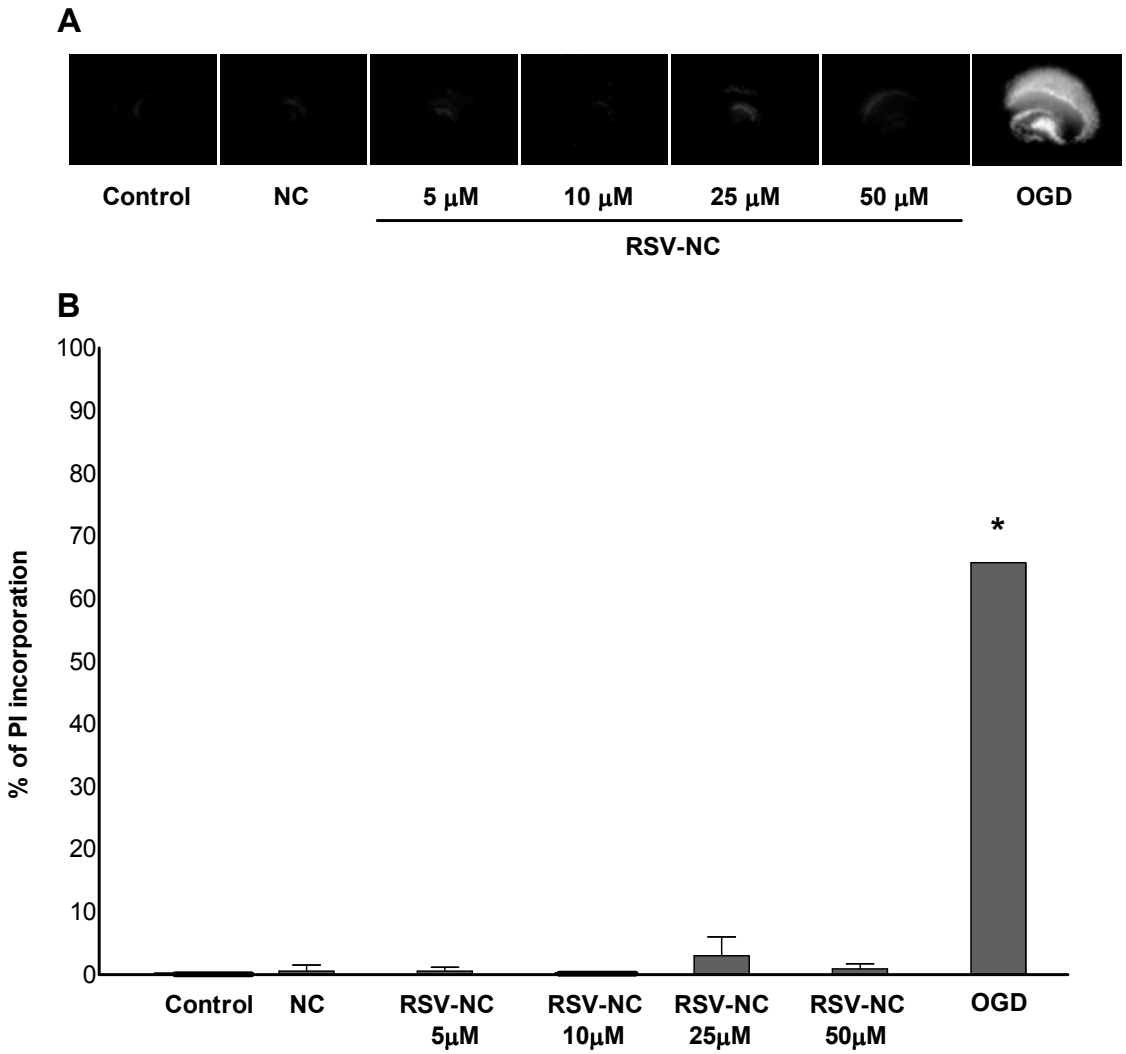
A
Control
NC
5 μM
10 μM
25 μM
50 μM
OGD
RSV-NC
B
% of PI incorporation
100
90
80
70
60
50
40
30
20
10
0
*
Control
NC
RSV-NC 5μM
RSV-NC 10μM
RSV-NC 25μM
RSV-NC 50μM
OGD

Figure 6

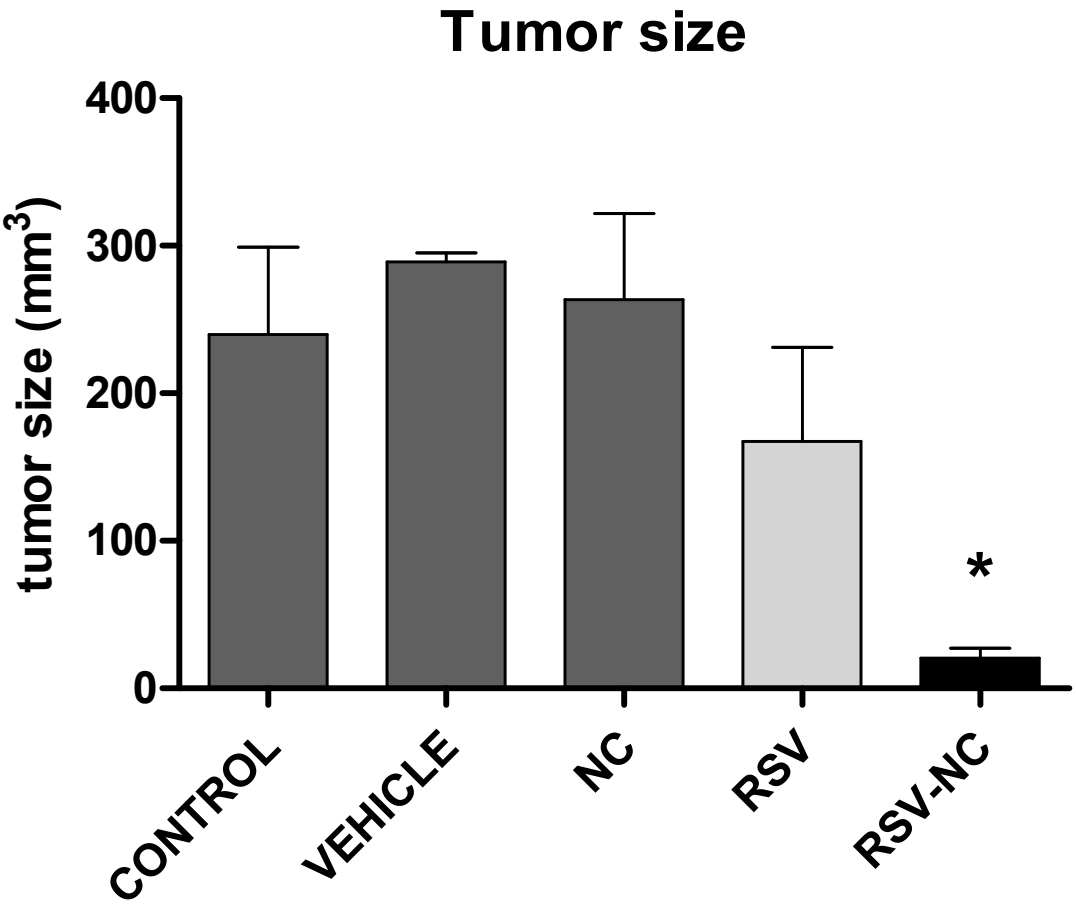
Tumor size
tumor size (mm3)
400
300
200
100
0
*
CONTROL
VEHICLE
NC
RSV
RSV-NC

Figure 7

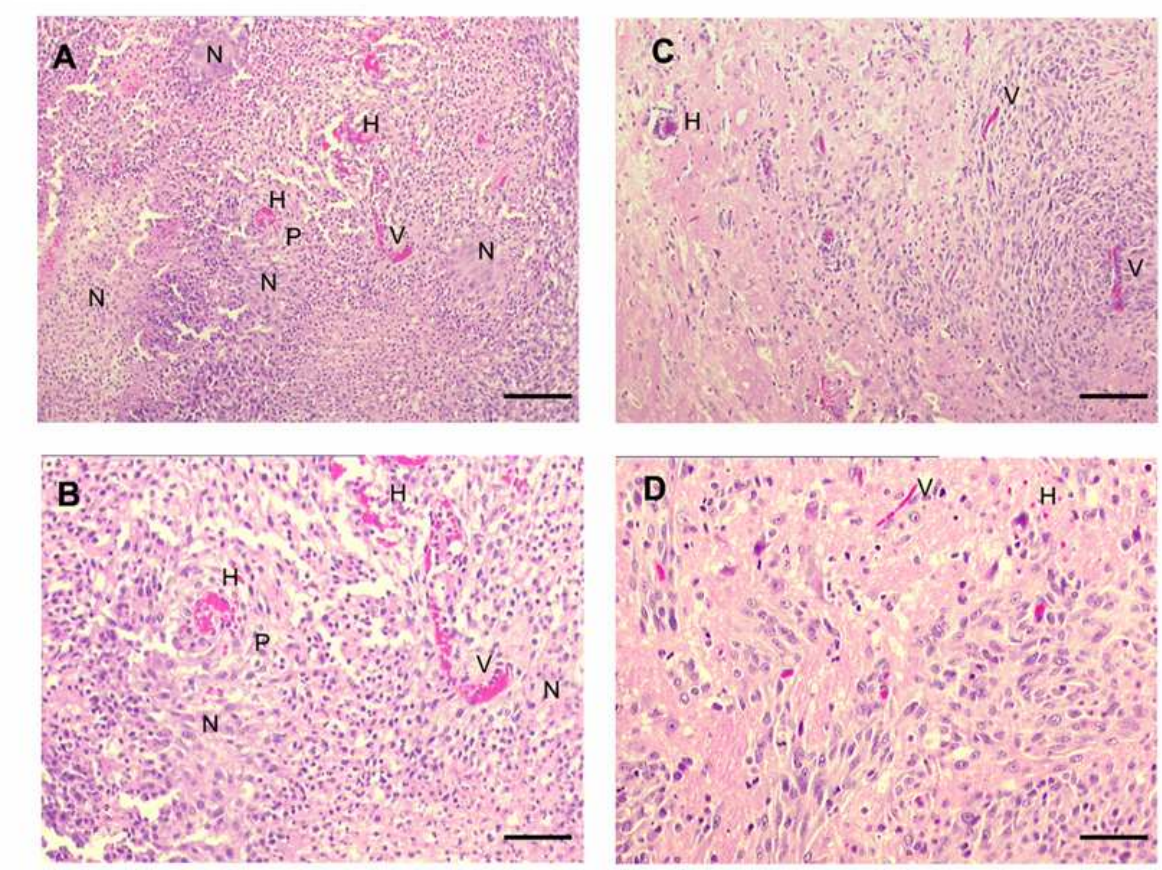

Table 1 - Histological characteristics of implanted gliomas

	Control (n=4)	Vehicle (n=2)	NC (n=4)	RSV (n=4)	RSV-NC (n=4)
Coagulative necrosis	3/4 (75%)	2/2 (100%)	3/4 (75%)	3/4 (75%)	0/4 (0%)
Intratumoral hemorrhage	4/4 (100%)	2/2 (100%)	4/4 (100%)	3/4 (75%)	3/4 (75%)
Lymphocytic infiltration	4/4 (100%)	2/2 (100%)	4/4 (100%)	4/4 (100%)	4/4 (100%)
Peritumoral edema	4/4 (100%)	2/2 (100%)	3/4 (75%)	2/4 (50%)	0/4 (0%)
Peripheric palisading	3/4 (75%)	2/2 (100%)	3/4 (75%)	3/4 (75%)	1/4 (25%)
Vascular proliferation	4/4 (100%)	2/2 (100%)	3/4 (75%)	4/4 (100%)	3/4 (75%)

The histological variables (coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, peritumoral edema, peripheric pseudopalisading and vascular proliferation) were regarded as present or absent. The groups are control, vehicle, drugunloaded nanocapsules, resveratrol and resveratrol-loaded nanocapsules, respectively.