UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso em Farmácia

Isolamento e caracterização de microvesículas derivadas de gliomas

Elisa Helena Farias Jandrey

Porto Alegre, novembro de 2015.

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Profa. Dra. Ana Maria Oliveira Battastini

Orientadora

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História Verídica

Um senhor deixa cair ao chão os óculos, que fazem um barulho terrível ao bater nos ladrilhos. O senhor se abaixa aflitíssimo porque as lentes dos óculos custam muito caro, mas descobre assombrado que por milagre elas não se quebraram. Agora esse senhor sente-se profundamente grato, e compreende que o acontecimento vale por uma advertência amistosa, de maneira que se dirige a uma ótica e compra logo um estojo de couro acolchoado, com proteção dupla, como precaução. Uma hora depois deixa cair o estojo e ao abaixar-se sem maior preocupação verifica que os óculos viraram farelo. Esse senhor leva tempo para compreender que os desígnios da Providência são insondáveis e que na realidade o milagre aconteceu agora.

Histórias de Cronópios e Famas

Julio Cortázar

A todos os milagres dessa caminhada que tornaram esse sonho possível.

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Isolation and characterization of glioma-derived microvesicles

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Abstract

Background/Aim: Glioblastoma multiforme is the deadliest tumor of central-nervous-system, consisting mainly of tumor and immune cells. T-regulatory cells can modulate to a pro-tumor microenvironment. Microvesicles (MVs), among them exosomes, are responsible of cell-to-cell interaction and are involved in the modulation of tumor-microenvironment. We describe the isolation and characterization of MVs in an *in vitro* glioma model. **Materials and Methods:** We performed 4 tests to determine best conditions to isolation of MVs (size and protein content). After determine the best condition, MVs were isolated through ultracentrifugation, characterized by size (ZetaSizer/Nanosight) and protein content. **Results:** Room temperature thaw and additional centrifugation (test 3) was the test that has the best results for size and protein content. BCA is the better technique to quantify protein concentration of MVs. Nanosight analysis demonstrates minor size variations. MVs show a size increase after five days at 4°C. The treatment with MVs decreases viability of C6 cells *in vitro*. **Conclusion:** Successful isolation/characterization of MVs.

1. Introduction

Glioblastoma multiform (GBM) is the most common and malignant primary glioma in Central Nervous System (CNS) present in adults. The life expectation of the patients is lower than one year despite surgery and radio/chemotherapy. The GBM is considered the most aggressive (grade IV, referred by World Health Organization), deadliest and frequent type of glioma (1,2,3). The treatment of choice for GBM still the surgical resection of the tumor, but the complete removal of the cancer is limited due this invasiveness of the tumor mass in normal tissue, being the patients treated with chemo/radiotherapy post-operatory (4). The invasiveness, rate of proliferation, immunosuppression and chemotherapy resistance are related with the high recurrence of this cancer (5,6).

The tumor mass consist in tumor cells and other types of cells like endothelial cells, fibroblasts and immune cells such as macrophages and lymphocytes (7,8). In GBM, the presence of inflammatory infiltrate is directly correlated with the degree of tumor malignancy and evidence suggests that the presence of leukocytes in the tumor microenvironment is an indispensable component in the proliferation, migration and tumor survival (9). Lymphocytes play a central role in both cellular and humoral immune response being divided into two major subclasses, B lymphocytes and T lymphocytes, which express specific antigens, receptors and also serve as markers of adaptive immunity (10). T-lymphocytes may be divided, although not exclusively, into T-helper lymphocytes (Th, $CD3^{+}CD4^{+}$), T-cytotoxic lymphocytes (CTL, $CD3^{+}CD8^{+}$) and T-regulatory lymphocytes (Treg cells, $CD4^{+}CD25^{high}FOXP3^{+}$). The tumor

proliferation is dependent on a complex group of factors, including cytokines, chemokines and nucleosides like adenosine, which culminate in immunosuppression in tumor microenvironment, orchestrated by, among other cells, Treg cells, which are directly correlated with poor prognosis of patients with GBM (11).

T regulatory lymphocytes are phenotypically classified as $CD4^{+}FOXP3^{+}CD25^{high}CD39^{+}$ and control cell renewal in the thymus, regulating lymphocyte expansion, chronic inflammation and autoimmune processes. However, under certain conditions, such as in cancer, Treg cells contribute to an immune suppressed environment, by inhibiting the activation of T effector lymphocytes (Teffec, $CD4^{+}CD25^{negative/low}$) and NK ("natural killer") and may promote neoplastic growth (12,13). Treg lymphocytes migrate to the microenvironment of the GBM not only by chemokines secreted by cells of the immune system, but also by the tumor cells themselves (14). It has been demonstrated that once in the tumor microenvironment, Tregs that overexpress the NTPDase1/CD39 enzyme (15), in coordination with GBM cells that overexpress ecto-5'-nucleotidase/CD73(16), produce adenosine by sequentially extracellular hydrolysis of ATP. Extracellular adenosine, in turn, is a potent suppressor of immune effector cells such as CTL and NK cells, thus promoting a pro-tumor environment (17). In addition, Treg lymphocytes also secrete cytokines such as IL-10, TGF-B culminating in immunosuppression in neoplastic tissue, leading to tumor progression (10).

In addition to the factors described above, exosomes are also able to modulate tumor microenvironment (18). Exosomes (50-100nm) are microvesicles originated in physiological or pathological conditions of the

endosomal cell compartment described in the early 80 (19). Multivesicular bodies fuse with the membrane releasing the exosomes with cell characteristics originated. Thus, exosomes may contain different proteins originate from the cytoplasm, endosomes and the plasma membrane of different cell. The functions performed by these microvesicles are not yet fully known, but some are now being elucidated, for example, participation in angiogenesis, inflammation and immunomodulation (20,21). Studies suggest that exosomes can inhibit the immune system by adenosine formation via CD39 and CD73 enzymes present in such vesicles and also by stimulating the release of anti-inflammatory cytokines (22). Tumor cells-derived exosomes (TEx), but not from normal cells, have been related to the differentiation of naive T lymphocytes ($CD4^{+}CD25^{negative/low}$) to Treg cells ($CD4^{+}CD25^{high}FOXP3^{+}$) (23). Furthermore, when incubated with TEx, Treg lymphocytes secrete higher amounts of immunosuppressive cytokines like IL-10 and TGF-β, being able to inhibit effector T-lymphocytes (23,24).

Moreover, some studies suggest that dendritic cells-derived exosomes or from body fluids are able to promote the release of proinflammatory cytokines such as IL-1β, TNF-α and IL-6, leading to an antitumor immune response (25). However, despite efforts in recent years, there is still no full understanding of the functions performed by exosomes derived from GBM (GEx) (28). GEx may have an important role in the modulation of T lymphocytes in GBM. For example, Liu et al., 2013 showed that there is a decrease of cytotoxic T lymphocytes, controlling the tumor growth in mice treated with exosomes. However, it is unclear at present whether the decrease of effector cells, after treatment with exosomes, is a direct action in these cells or whether the

modulation of Treg cells may inhibit effector cells in the microenvironment of the GBM. Still, it has been demonstrated that prophylactic injection GEx promoted immune stimulation in mice and the absence of tumor growth (29).

Therefore, the role played by exosomes in tumor microenvironment appears to be concentration-dependent, management plan and cell of origin of these vesicles. As mentioned earlier, we know that the lymphocytes of the tumor microenvironment are dedicated to the growth of GBM. In addition, regulatory T cells have a central role in control of the immune system, promoting the growth of this tumor. The GEx have cellular mediators capable of modulating tumor cells to escape the immune system. However, the role of these vesicles in the modulation of regulatory T cells is not yet understood. Therefore, the possibility of this design is that exosomes generated in the tumor microenvironment can modulate regulatory T cells, thus contributing to the maintenance of a pro-tumor environment. To test this hypothesis, first it is necessary to characterize a preparation of exosomes derived from glioma cells. Therefore, in the present work, a method to isolate microvesicles derived from C6 rat glioma cell lines were characterized for further *in vivo* studies.

2. Materials and Methods

2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Fungizone®, penicillin/streptomycin, and 0.5% trypsin/EDTA solution were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). All other chemicals and solvents used were of analytical or pharmaceutical grade.

2.2 Maintenance of cell line

The C6 rat glioma cell line was obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Cells at passages 5-30 were grown and maintained in 1% DMEM containing antibiotics (0.5 U/mL penicillin/streptomycin) and supplemented with 5% (v/v) of Fetal Bovine Serum (FBS). The FBS used for the experiments has the microvesicles depleted (MV^-) by two cycles of 2 hours of ultracentrifugation in 105000x*g* at 4°C. Cells were kept at a temperature of 37 °C, minimum relative humidity of 95% and atmosphere of 5% CO_2 in air.

2.3 Isolation of microvesicles enriched with exosomes

Initially, the microvesicles were prepared using four different protocols based on studies in the literature. Characteristics of each test are described in Table I. Considering the thawing the most critical parameter for the isolation of smaller microvesicles, the chosen methodology for further experiments is described as following:

The microvesicles enriched with exosomes derived from glioma (GEx) were isolated from the supernatant of GBM C6 cell line. 75 cm^3 culture bottles were used to cultivate $7x10^6$ C6 cells for 48 hours with 11 mL of DMEM 5% of FBS (MV^-). After 48 hours, the supernatant was isolated from the culture and centrifuged at 400x*g* for 6 minutes to remove bigger particles. The resultant supernatant was than centrifuged at 2,000x*g* for 10 minutes at 4°C and 10,000x*g* for 30 minutes at 4°C, respectively. The supernatant was filtrated in a 0.22 µm micro filter and stored at -80°C. After, the supernatant was thawed at room temperature for approximately 1 hour and it was centrifuged at 17,000 x*g*

for 20 minutes at 4°C. Then, approximately 120 mL of the obtained supernatant was submitted to 2 hours of ultracentrifugation at 105,000 x *g* at 4°C. The resultant pellet from this centrifugation was suspended in sterile PBS and centrifuged at 105,000 x *g* for more 2 hours at 4°C. The final pellet was suspended in 150 µL of sterile PBS and stored at 4-8°C up to 24 hours. Alternatively, GEx was stored up to 5 days to evaluate the stability at 4°C. The total protein content was determined with BCA kit, when 10 µL of GEx suspension was analyzed in comparison to standard albumin (0.625-10.000 µg/mL). The microvesicles enriched with exosomes was characterized through the determination of the diameter of the particles and the polydispersity, using the Zetasizer Nano ZS and NanoSight equipments.

2.4 Protein determination

Total protein content was determined through three different methods in order to establish the better technique to quantify the protein amount in microvesicles samples. All experiments were performed in 96-well plates and 24 h after the microvesicles isolation. The total protein content was determined with adapted Bradford (38), Lowry (39) and BCA kit (37) techniques. For adapted Bradford, 10 µL of GEx was incubated with 250 µL of Coomassie Blue reagent for 10 minutes. The resulting color was read at 595 nm in SpectraMax M5. The protein content was analyzed in comparison to standard albumin (0.01-0.05mg) with PBS:NaOH 1:1 (v/v) as blank. To determine the protein amount through Lowry technique, 10 µL of GEx was incubated with 100 µL of Lowry reagent for 10 minutes. After, 50 µL of Folin 0,4 N was added, the plate was shaken and incubated for more 30 min. The final absorbance was determined in

750 nm in SpectraMax M5. The protein content was analyzed in comparison to standard albumin (0.5-20.0µg) with ultrapure water as blank. In BCA-kit determination, 10 µL of GEx was incubated 30 min at 37°C with 200 µL of work reagent (Solution B: Solution A; 1:50; v/v) from the kit. After cooling the plate for 2 minutes, it was read immediately at 562 nm in SpectraMax M5. The protein content was analyzed in comparison to standard albumin (0.625-10.000µg/mL) with PBS as blank.

2.5 Size characterization of microvesicles derived from glioma

Photon correlation spectroscopy (PCS) was performed using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) to determine the mean particle size (Z-average) and polydispersity index (PDI) values of the formulations. The samples were diluted 500× in ultrapure water at 25°C. Nanoparticle tracking analysis (NTA) (using a NanoSight instrument (LM10, NanoSight Ltd., UK) and NTA 2.0 (Analytical Software) was used to analyze the individual particles in the formulations after dilution (5000×) by examining Brownian motion in real time via a CCD camera, and each video clip was captured for over 60 s. All measurements were performed in triplicate batches.

2.6 *In vitro glioma viability model*

For the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, C6 glioma cells were seeded in 96-well plates and allowed to grow until semi-confluent. Cells were treated with 1.0; 5.0; 10.0; 20.0; 40.0 and 80.0 µg/mL of MVs for 96 h. At the end of the treatment, MTT (5 mg/ml) was

added to each well and the plate was incubated for 2 hours at 37°C. A total of 100 µL dimethyl sulfoxide (DMSO) was added to the wells and the level of absorbance was read at 570-630 nm in SpectraMax M5.

2.7 Statistical analysis

The data were analyzed for statistical significance by Student's t-test or one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons (Tukey test), using GraphPad Prism software®. The data are expressed as the mean ± S.D. Differences were considered significant at $p < 0.05$.

3. Results

3.1 Determination of isolation protocol

Firstly, we describe the variations tested to determinate the best isolation protocol using differential centrifugation. Initially, a detailing was made of the previously procedures reported in literature and, from that, we determined the experimental conditions which could interfere negatively in microvesicles size and protein content. We focused in situations not previously described in the literature.

Four experimental conditions were tested to determine the best protocol for isolation and protein quantification (Table I). Average size was determined by ZetaSizer Nano ZS equipment and protein amount were quantified with BCA-kit after 24 h from isolation. The main differences among the tests are: (1) filtration (0.22 µm) after overnight (4-8°C) thaw; (2) additional centrifugation

(17,000xg, 30 min, 4°C) after overnight (4-8°C) thaw; (3) filtration (0,22 µm) and additional centrifugation (17,000xg, 30 min, 4°C) after 1 h (room temperature) thaw; (4) thaw after 1 h (room temperature). In test 1 (Table II), we obtained better results for protein concentration (1.395 µg/µL); however, the average size for this isolation was the worst analyzed (277.5 nm). In test 3, we also obtained a bigger particles size (208 nm) than test 3 (145 nm) and test 4 (159 nm) both with a reliable protein content .Exosomes are described as particles with 50-100 nm, so smaller size indicates a major presence of these particles beyond the presence of other microvesicles. Considering this, condition 3 was considered the best and was used in the following experiments.

Theoretically analyzing the results showed in Table II, we figured that the filtration after the thawing was not really necessary and it does not substantially interfere with the final results. The room temperature and the additional centrifugation after the thawing were decisive to obtain the microvesicles. So, we decided to exclude the filtration after thawing step due to high cost of each filter and to get a higher yield.

3.2 Determination of protein quantification protocol

Microvesicles isolated through the chosen protocol (test 3 modified) were submitted to 3 protein analysis: Lowry (Figure 1A), Bradford Adapted (Figure 1B) and BCA-kit (Figure 1C). The standard curves of the analysis of each technique are shown in Figure 1. To verify the best protein determination technique, we compared the coefficient of correlation (r^2) obtained in each standard curve; this coefficient, when close to one, shows a better linear correlation (40). BCA analysis demonstrates the better linear correlation

(r^2=0.9992) when compared with Lowry (r^2=0.9198) and Bradford adapted (r^2=0.9792) analysis. Besides, the protein concentration determined by Lowry and Bradford assays was around the inferior limit of quantification, different from the BCA assay, where the protein concentration is around the middle of the standard curve (data not shown). Therefore, we have chosen BCA technique to determinate protein concentrations in the following experiments.

3.3 Size characterization of microvesicles derived from glioma cells

After determine the isolation and protein quantification protocols, we decided to compare the size results obtained in the two most used equipments to this finality. To determine the size and the polydispersity of the GBM-derived MVs, the final pellet resultant of the ultracentrifugations was analyzed by ZetaSizer Nano ZS and Nanosight up to 24 hours after the isolation. Analyzes from ZetaSizer demonstrate a monomodal curve representative of the MVs size (Figure 2A) which leads to a greater variation in the final size of the MVs in comparison to Nanosight analysis (Figure 2C). Nanosight assay demonstrates a polimodal curves, showing the diversity of the MVs sizes in the sample (Figure 2B). The equipment sensitivity was able to separate subpopulations of MVs, leading to more reliable results with minor variations among different analysis (Figure 2C; Table III). The Nanosight results demonstrated a presence of small subpopulations with size greater than 200 nm. These particles may be either the result of MVs aggregation or big isolated microvesicles. The indices D10, D50 and D90 are representative of the polydispersity of the results obtained in Nanosight equipment (Table III). The particles size distributions obtained in three different experiments are reproducible between themselves.

3.4 Microvesicles stability

The above mentioned experiments were performed up to 24 hours after the MVs isolation. To determine the stability of the preparation, the MVs size was also determined up to 96 hours after the MVs isolation. Figure 3A and 3B show the profile of MVs at 24 and 96 h, respectively. After 96 h of MVs isolation there was an increase in the subpopulations with bigger size than the MVs stored at 4°C for 24 h.

3.5 Effect of exosomes treatment in a rat glioma cell line

To investigate the direct interaction of glioma-derived MVs with C6 glioma cell line, MTT assay was performed. Concentrations between 1-80 µg/mL of MVs were used in cells treatment for 96 h. Analysis of MTT assay showed that 80 µg/mL of MVs were able to cause a significant reduction in cell viability when compared with PBS group (30% decrease) (Figure 4). Treatment with the vehicle PBS does not affect cell viability.

4. Discussion

GBM is the deadliest tumor of Central Nervous System (CNS) and the better understanding of this malignance is urgently required (1,2,3). Microvesicles are small particles formed through the direct bud from plasma membrane and can have different sizes (36). Exosomes are small particles (50-100nm) formed by lipid bilayer of endocytic origin and they have been referred as communicasomes, being responsible for transmitting signals between different cells (32). There have been disclosed various techniques for isolation of MVs from different biological materials such as serum and cell culture

supernatant like sucrose gradient, immune-bead isolation and ultracentrifugation (32).

In this work we opted to use the differential centrifugations and ultracentrifugations to isolate exosomes from the supernatant of rat glioma cell culture due to the applicable sample (supernatant is cleaner than serum, for example) and the lower cost of the technique. Initially, we tested some variations in the technique to determinate the best conditions to obtain GBM-derived MVs in an appropriate quantity and quality (Table I and Table II). We observed that the time of thaw and the addition of one cycle of centrifugation after thaw are decisive to have smaller particles with a good protein concentration. Differential centrifugation is considered a lower cost technique among others. We observed that the two filtration steps were not necessary so the second (after thawing) was discarded to reduce even more the technique cost and increase the yield. In relation to protein quantification, we determined that BCA assay is the best technique to quantify the protein amount when compared to Lowry and Bradford assays due its bigger correlation coefficient. Besides, the protein concentration was into de curve points only in BCA analysis rather than Lowry and Bradford. It has been described that BCA assay has the best sensitivity and lower variations in the results when compared to other protein quantification methods (34).

After setting the worth method to MVs isolation, the size and polidispersity of the isolate were analyzed in ZetaSizer and Nanosight equipments to determine which one shows the better results. The most studies published perform Nanosight analysis to measure MVs size (26, 27). However, a comparison between the different equipments was unknown so far for us. The

average size obtained in ZetaSizer is result of a monomodal curve constructed by the combination of all different particles present in the MVs suspension. These samples combination cause more variability among different analyses, since particles subpopulation may vary among isolations. Therefore, Nanosight is a more sensitive method because it can separate different subpopulations of different particles sizes present into the sample. There is a significant difference between the standard deviation obtained in both techniques for the same samples, indicating that Nanosight may be the better choice to scattered samples such as MVs, as determined by Zhang et al 2015 (31). Confirming this, the results presented in Table III show the reproducibility and the polidispersity trought indices D10, D50 and D90 that indicate the percentage of particles undersize this percentile in size distribution curve (33).

After 96 h of the isolation, the tests to measure the particle size were again conducted to check the stability of the MVs stored at 4°C. After this time, there was a significant increase in particles average size probably due to their aggregation. This result confirms the need for isolating MVs up to 24 hours prior to the experiments. Sokolova et al. also described change in size after storage at 4°C and 37°C for 96 and 48 h, respectively, indicating a change in the structure or degradation of exosomes derived from human cells (35).

Finally we test the effect of isolated MVs on C6 glioma cells viability. C6 glioma cells were exposed to increasing concentrations of MVs (from 1 to 80 µg/mL) for 96 h. The cells viability decreases with the increase of the concentration, being statistically significant between the control and the highest concentration tested (Figure 4). The MVs had citotoxic activity against glioma cells after 96 h unlike most studies that shows tumor growth of cancer cells

treated with exosomes. Setti et al. described an increase of U87MG cells proliferation when exposed to extracellular vesicles derived from the same cell (30).

MVs and exosomes are responsible to play a central role of interaction between cell to cell by endocytosis, fusion in plasmatic membrane and receptor-ligand interaction. In cancer, they are described to promote angiogenesis, growth and metastasis (32). The malignant behavior can be mediated by direct interaction between MVs and tumor cells or through the interaction of the MVs with immune cells, leading to immunosuppression. Tumor-derived MVs can block the differentiation of murine myeloid precursor cells, increase the response of Treg for Interleucin-2, expand and regulate the function of Treg cells (32). This tumor immunosuppression is, at least in part, caused by the adenosine formation through CD39 and CD73 enzymes overexpressed on T regulatory and GBM cells, respectively (15, 16). Furthermore, Treg cells in a tumor microenvironment may release cytokines such as IL-10, TGF-B (10). Together, the immunosuppressant cytokines and adenosine are potent inhibitors of Teffector and NK cells, promoting a pro-tumoral microenvironment and leading tumor progression (17).

In summary, in this work we describe an efficient method to isolate MVs derived from glioblastoma cells in quality and quantity proper to perform *in vitro* and *in vivo* assays. The apparently opposite results in relation to the *in vitro* effects of MVs on cell viability might be due to the absence of immune response *in vitro* which is present in the microenvironment of the tumor and It still needs to be confirmed. This hypothesis needs to be deeply investigated in further *in vivo* experiments.

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Table I. Experimental conditions tested to determine microvesicles isolation protocol through differential centrifugation. We performed 4 tests to determine the best condition to isolate microvesicles from C6 glioma cells supernatant. (✔) represent the presence of the step in the test and (**-**) represent the absence of the step in the test.

Table II. Average size and protein quantification of protocols tests. The tests 1-4 were performed as described in Table I. After the MVs isolation, they were submitted to ZetaSizer Nano ZS to determinate particles size and BCA assay to determinate protein concentration.

Table III. Data from Nanosight analyzes. After the determination of the isolation protocol, three samples were submitted to Nanosight analysis in triplicate. The results of size and polidispersity (D10 and D90, that represents the percentile of particles undersize 10 and 90) of each sample are demonstrated as experiment 1-3.

Figure 1. BCA method is the best technique to quantify MVs protein. We performed a standard curve with the three techniques described in Materials and Methods. It was analyzed the correlation between protein amount with the resulting absorbance in each concentration measured in Lowry (A), Bradford adapted (B) and BCA (C) assays. They are presented the curve equation and de correlation coefficient of each analysis.

Figure 2. Size determination of MVs by Nanosight and ZetaSizer. The MVs were isolated as described in Materials and Methods. After 24 hours of MVs isolation, the samples were submitted to two different size analyses: ZetaSizer and Nanosight equipments. (A) Representative graph (triplicate) from one sample analysis in ZetaSizer; (B) Representative graph (triplicate) from one sample analysis in Nanosight; (C) Average size of three independent experiments in both equipments. Values are presented as mean ± S.E.M. and were analyzed by Student's *t*-test. *Significantly different from the ZetaSizer group ($p < 0.05$).

Figure 3. Microvesicles stability. In order to check the long-term microvesicles stability at 4°C, we performed size measurement (Nanosight) up to 24 hours and 96 hours after MVs isolation. (A) Representative histogram from Nanosight analysis after 24 h of storage. (B) Representative histogram from Nanosight analysis after 96 h of storage. (C) The quantification of three independent experiments from the average size. Values are presented as mean ± S.E.M. and were analyzed by Student's *t*-test. *Significantly different from the 24 h group ($p < 0.05$).

Figure 4. MVs lead to a decrease in glioma cell viability *in vitro*. C6 glioma cells were cultured as described in Materials and Methods. A total of 2×10^3 cells were plated in 96 well and treated from 1 to 80 µg/mL of MVs. After 96 hours of treatment, MTT assay was performed to determine cell viability. The results were analyzed by one-way ANOVA followed by Tukey post-hoc test of comparisons. *Significantly different from the PBS group ($p < 0.05$).

Table I. Experimental conditions tested to determine microvesicles isolation protocol through differential centrifugation

Experimental condition \ Test	1	2	3	4
Number of cells in culture (7×10^6 cells/75 cm^3 bottle)	✔	✔	✔	✔
Centrifugation 1 (400g, 6min, room temperature)	✔	✔	✔	✔
Centrifugation 2 (2000g, 10min, 4°C)	✔	✔	✔	✔
Centrifugation 3 (10000g, 30min, 4°C)	✔	✔	✔	✔
Filtration (0,22 µm)	✔	✔	✔	✔
Freeze (-80°C)	✔	✔	✔	✔
Thaw	Overnight at 4-8°C	Overnight at 4-8°C	1 hour at room temperature	1 hour at room temperature
Filtration (0,22 µm)	✔	-	✔	-
Centrifugation 4 (17000g, 30min, 4°C)	-	✔	✔	-
Centrifugation 5 (105000g, 2h, 4°C)	✔	✔	✔	✔
Centrifugation 6 (105000g, 2h, 4°C)	✔	✔	✔	✔

Table II. Average size and protein quantification of protocols tests

Test	Average size (nm ± S.D.)	Protein quantification (µg/µL)
1	277,5	1,395
2	208,0 ± 16,1	0,930
3	145,1 ± 2,2	0,953
4	159,3 ± 4,3	1,227

Figure 1

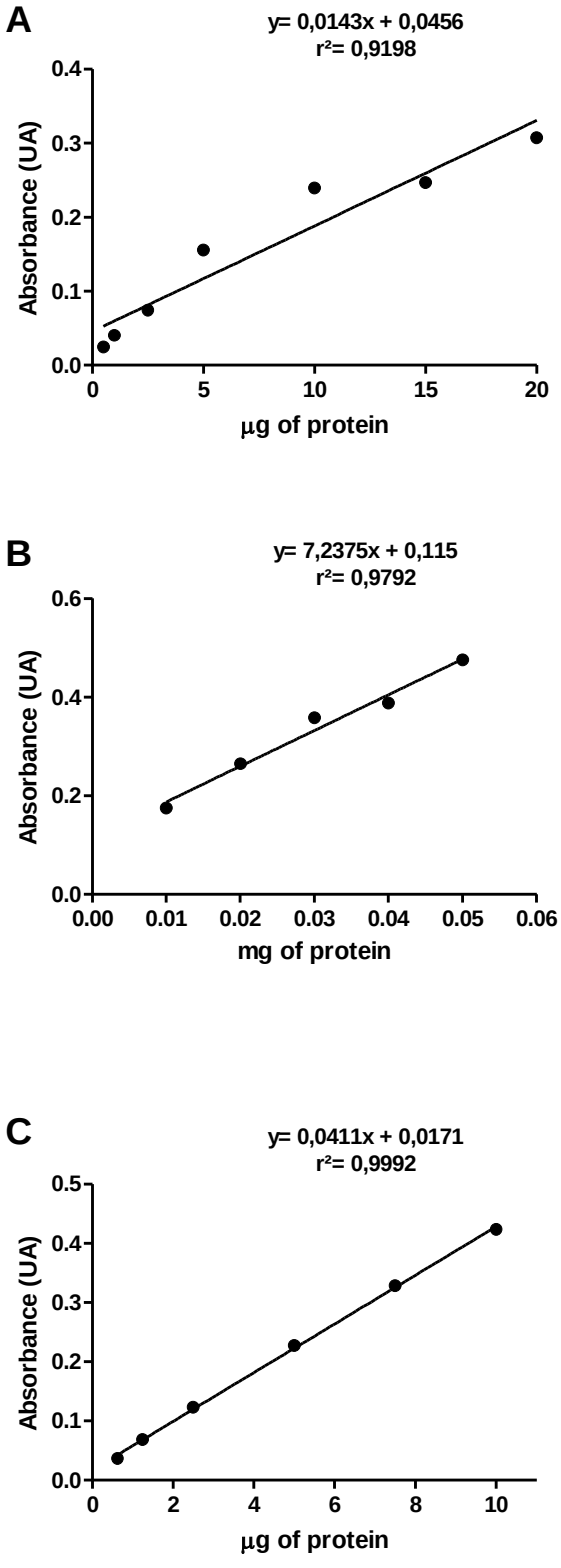

A
y= 0,0143x + 0,0456
r²= 0,9198
Absorbance (UA)
0.0
0.1
0.2
0.3
0.4
0
5
10
15
20
µg of protein
B
y= 7,2375x + 0,115
r²= 0,9792
Absorbance (UA)
0.0
0.2
0.4
0.6
0.00
0.01
0.02
0.03
0.04
0.05
0.06
mg of protein
C
y= 0,0411x + 0,0171
r²= 0,9992
Absorbance (UA)
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
µg of protein

Figure 2

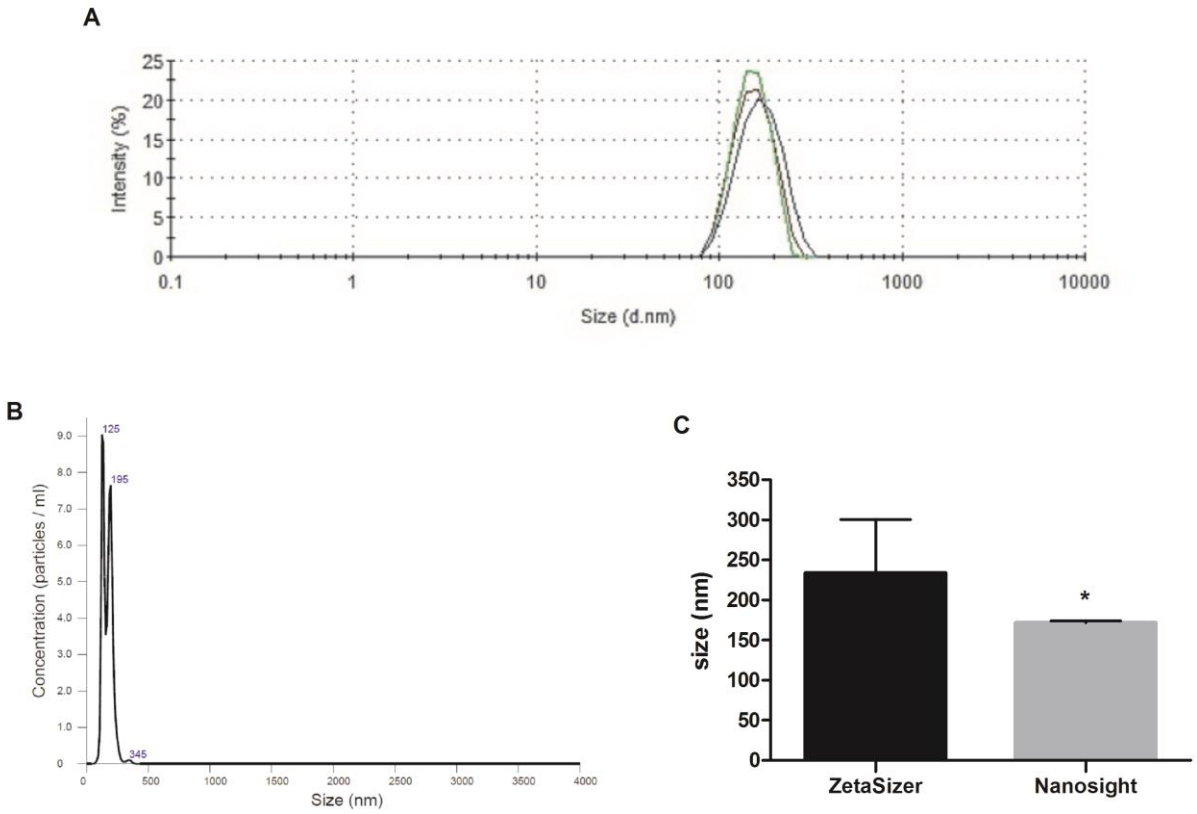

A
Intensity (%)
25
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
B
Concentration (particles / ml)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
125
195
345
0
500
1000
1500
2000
2500
3000
3500
4000
Size (nm)
C
size (nm)
350
300
250
200
150
100
50
0
*
ZetaSizer
Nanosight

Table III. Data from Nanosight analysis

Experiment	Concentration (particles/mL)	Average size (nm)	SD (nm)	D10 (nm)	D50 (nm)	D90 (nm)
1	$2,56 \times 10^8$	172,9	43,8	122,6	146,0	248,6
2	$7,39 \times 10^8$	173,2	43,0	113,9	160,1	228,3
3	$7,07 \times 10^8$	169,1	40,3	112,4	160,2	206,2

Figure 3

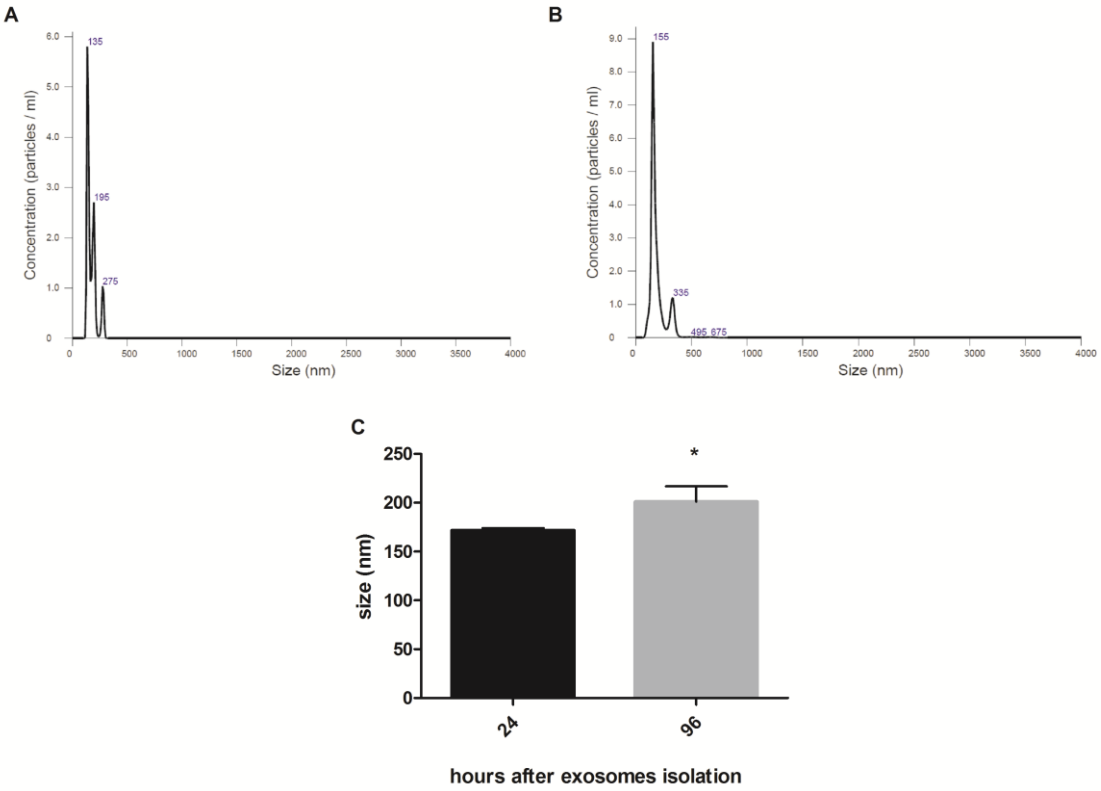
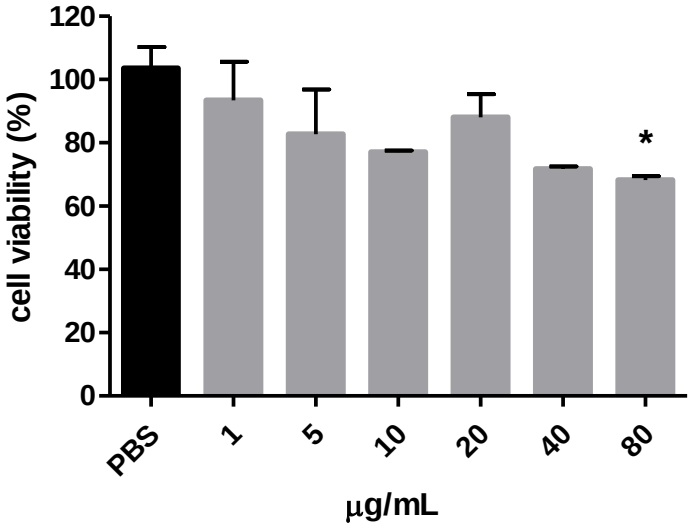

A
Concentration (particles / ml)
Size (nm)
135
195
275
B
Concentration (particles / ml)
Size (nm)
155
335
495 675
C
size (nm)
hours after exosomes isolation
24
96
*

Figure 4



ANEXO

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(a) First page including the title of the presented work [not exceeding fifteen (15) words], full names and full postal addresses of all Authors, name of the Author to whom proofs are to be sent, key words, an abbreviated running title, an indication “review”, “clinical”, “epidemiological”, or “experimental” study, and the date of submission. (Note: The order of the Authors is not necessarily indicative of their contribution to the work. Authors may note their individual contribution(s) in the appropriate section(s) of the presented work); (b) Abstract not exceeding 150 words, organized according to the following headings: Background/Aim - Materials and Methods/Patients and Methods - Results - Conclusion; (c) Introduction; (d) Materials and Methods/Patients and Methods; (e) Results; (f) Discussion; (g) Acknowledgements; (h) References. All pages must be numbered consecutively. Footnotes should be avoided. Review articles may follow a different style according to the subject matter and the Author's opinion. Review articles should not exceed 35 pages (approximately 250 words per double-spaced typed page) including all tables, figures, and references.

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double-spaced. Tables should be numbered with Roman numerals and should include a short title.

References. Authors must assume responsibility for the accuracy of the references used. Citations for the reference sections of submitted works should follow the standard form of “Index Medicus” and must be numbered consecutively. In the text, references should be cited by number. Examples: 1 Sumner AT: The nature of chromosome bands and their significance for cancer research. Anticancer Res 1: 205-216, 1981. 2 McGuire WL and Chamnes GC: Studies on the oestrogen receptor in breast cancer. In: Receptors for Reproductive Hormones

(O' Malley BW, Chamnes GC (eds.). New York, Plenum Publ Corp., pp 113-136, 1973.

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