

Targeting Glioblastoma Multiforme with intranasal delivery of milk-exosomal Mitoxantrone

Dissertation

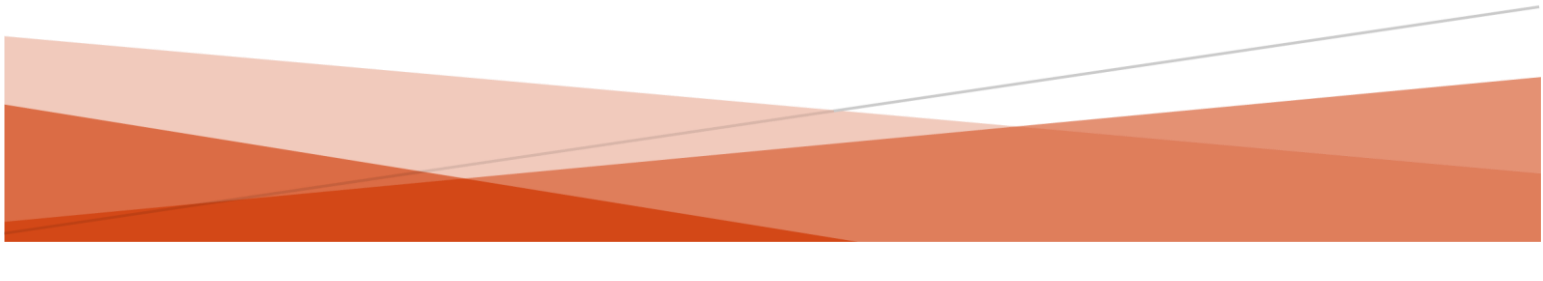
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Master's in Molecular Bioengineering

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Porto, March 2022





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Dissertation submitted to Faculdade de Engenharia da Universidade do Porto and to
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“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.”

Albert Einstein (1879-1955)

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Abstract

Glioblastoma Multiforme is the most aggressive brain tumor in adults. The current treatment options include surgical resection, radiation, and chemotherapy. The advances in the knowledge of molecular pathology and cell signaling pathways have deepened the description of mechanisms of intercellular communication that can induce tumor progression, namely the release of extracellular vesicles. Several evidences indicate that exosomes play a fundamental role in the progression of glioblastoma being advantageous tools for diagnostic and therapeutic applications. Exosomes are present in various biological fluids and released by almost all cells. The application of milk-exosomes in cancer nanomedicine can be particularly useful, as it could minimize the side effects of chemotherapeutic agents in non-target tissues. Mitoxantrone is an anti-neoplastic anthracenedione derivative with potent activity against glioblastoma multiforme. However, mitoxantrone has poor penetration through the blood-brain barrier, cardiotoxicity, and myelosuppression side effects from systemic delivery. Mitoxantrone has other side effects, such as nausea, generalized discomfort, neuropathy, nephrotoxicity, among others.

This work aimed to develop and evaluate, *in vitro*, the delivery of milk-exosomes with encapsulated mitoxantrone for glioblastoma multiforme.

Hence, milk-exosomes were obtained for encapsulation of mitoxantrone through an optimized protocol of ultracentrifugation. Milk-exosomes were successfully obtained by ultracentrifugation with effective stability of 3 months. Characterization regarding size, protein concentration and stability were performed. Subsequently, mitoxantrone was encapsulated through sonication. The results describe a formulation of mitoxantrone entrapped in the exosomal membrane, with an encapsulation efficiency of 55% and a more efficient drug release profile.

The formulation was evaluated *in vitro*, regarding cell viability and permeability for intranasal administration route in RPMI-2650 cells. The results demonstrate that milk-exosomes were not cytotoxic to RPMI-2650 cells and the cell viability for mitoxantrone was enhanced when encapsulated in milk-exosomes. Moreover, RPMI-2650 cell line formed a barrier similar to the nasal epithelium and the nanoformulation demonstrated enhanced permeability through paracellular transport.

Lastly, studies in SF-268 cell line were performed. The cell viability was lower when mitoxantrone (free or encapsulated) was applied. The confocal microscopy characterization demonstrated the faster cellular internalization of mitoxantrone encapsulated in milk-exosomes to the cytoplasm and the retarded transport of mitoxantrone to the nuclei of SF-268 cells.

Resumo

O glioblastoma multiforme corresponde ao tumor cerebral mais agressivo nos adultos. Atualmente, as opções de tratamento incluem a remoção cirúrgica, radiação e quimioterapia. Os avanços no conhecimento da patologia molecular e vias de sinalização celular aprofundaram a descrição de mecanismos de comunicação intercelular que podem induzir a progressão tumoral, nomeadamente a libertação de vesículas extracelulares. Há várias evidências que indicam que os exossomas desempenham um papel fundamental na progressão do glioblastoma, funcionando assim como ferramentas vantajosas para aplicações no diagnóstico e na terapêutica. Os exossomas estão presentes em vários fluidos biológicos e são libertados por quase todas as células. Assim, a aplicação dos exossomas do leite na nanomedicina do cancro pode ser particularmente útil, dado que pode minimizar os efeitos adversos dos agentes quimioterapêuticos em tecidos não-alvo. A mitoxantrona é um fármaco derivado das antracenedionas anti-neoplásicas com forte atividade contra o glioblastoma multiforme. No entanto, a mitoxantrona possui fraca capacidade de atravessar a barreira hematoencefálica, e efeitos adversos como a cardiotoxicidade e a mielosupressão quando aplicada por via sistémica. A mitoxantrona possui outros efeitos secundários associados como náusea, desconforto geral, neuropatia, nefrotoxicidade, entre outros.

Assim, este trabalho focou-se no desenvolvimento e avaliação, *in vitro* de um sistema de entrega de mitoxantrona encapsulada em exossomas do leite para o glioblastoma multiforme.

O isolamento de exossomas do leite fresco de vaca foi otimizado e obtido através de protocolos de ultracentrifugação, tendo-se obtido exossomas do leite, com sucesso, através da ultracentrifugação com uma estabilidade efetiva de, pelo menos, 3 meses. Por conseguinte, a mitoxantrona foi encapsulada nos exossomas do leite por sonicação. Os resultados descrevem uma formulação de mitoxantrona encapsulada na membrana dos exossomas do leite, com eficiência de encapsulamento de 55% e um perfil de libertação de fármaco mais eficiente.

A formulação foi avaliada *in vitro* para administração intranasal em células RPMI-2650, considerando a viabilidade celular e a permeabilidade da monocamada. Os resultados demonstram que os exossomas do leite não são citotóxicos para as células e que a viabilidade celular da mitoxantrona encapsulada em exossomas do leite é superior à viabilidade celular da mitoxantrona livre. Adicionalmente, os resultados demonstram a formação pelas células RPMI-2650 de uma barreira epitelial semelhante ao epitélio nasal humano e a permeabilidade aumentada da mitoxantrona quando encapsulada em exossomas do leite, através de transporte paracelular.

Por último, foram efetuados estudos para a linhagem de células SF-268. No geral, viabilidade celular na presença de mitoxantrona (encapsulada em exossomas do leite ou livre) foi inferior. A caracterização por microscopia confocal demonstrou que a acumulação de mitoxantrona encapsulada em exossomas do leite na membrana celular era superior.

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Abbreviations

ADME – Adsorption-Distribution-Metabolization-Elimination

AKT – Protein Kinase B

ALI – Air-Liquid Interface

ALIX – ALG-2-interacting Protein X

ANOVA – Two-way Analysis of Variance

ATP - Adenosine Triphosphate

BBB – Blood-Brain Barrier

BCA – Bicinchoninic Acid

BMDCs – Bone Marrow-derived Cells

BSA – Bovine Serum Albumin

BTB – Brain-Tumour Barrier

CD – Cluster of Differentiation

CDKN 2A/B – Cyclin-dependent kinase inhibitor 2 A/B

CLSM - Confocal Laser Scanning Microscopy

c-Myc – MYC proto-oncogene

CNS – Central Nervous System

CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated Protein 9

CSF – Cerebrospinal Fluid

DLS – Dynamic Light Scattering

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediamine tetraacetic acid

EGFR – Epidermal Growth Factor Receptor

EMEM – Minimum essential medium Eagle

EMT – Epithelial-Mesenchymal Transition

EPR – Enhanced Permeability and Retention

ESCRT – Endosomal Sorting Complex Machinery

EXO – Milk Exosomes

EXO-MTX – Exosomal Mitoxantrone

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FTIR – Fourier-Transform Infrared Spectroscopy

GBM – Glioblastoma Multiforme

GLUT1 – Glucose Transporter 1

GTPases – Guanine Triphosphatases

HIF-1 α - Hypoxia-inducible factor 1-alpha

HK2 - Hexokinase 2

Hsps – Heat Shock proteins

IDH – Isocitrate Dehydrogenase

IL – Interleukin

ILVs – Intraluminal vesicles

IR - Infrared

LLI – Liquid-Liquid Interface

MDM2 – Murine double Minute 2

MDSCs – Myeloid-derived Suppressor Cells

MGMT - O⁶-methylguanine-DNA-methyltransferase

miRNAs – microRNAs

MRI – Magnetic Resonance Imaging

mRNA – Messenger RNA

mTOR - Mammalian Target of Rapamycin

MTX – Mitoxantrone

MVBs – Multivesicular Bodies

MVE – Multivesicular Endosomes

NEAA – Non-Essential Amino Acids

NKT – Natural Killer T

NTA – Nanoparticle Tracking Assay

NVU – Neurovascular Unit

P_{app} – Apparent Permeability

PBS – Phosphate Buffered Saline

PDGFA/PDGFR α - Platelet-Derived Growth Factor A and Platelet-Derived Growth Factor Receptor Alpha

PDGFR - Platelet-Derived Growth Factor Receptor

PD-L1 – Programed Death-Ligand 1

PEG – Polyethylene Glycol

Pen-Strep – Penicillin-Streptomycin

PGE2 – Prostaglandin E2

Pgp – P-glycoprotein

PI3K – Phosphatidylinositol 3-kinase

PIK3CA - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PTEN - Phosphatase and Tensin Homolog

RB – Retinoblastoma

RNA - Ribonucleic Acid

RNAi – RNA Interference

RNases – Ribonucleases

ROS – Reactive Oxygen Species

SD – Standard Deviation

SEC – Size Exclusion Chromatography

TAMs – Tumour-associated Macrophages

TEER – Transepithelial Electrical Resistance

TERT – Telomerase Reverse Transcriptase

TGF- β – Transforming Growth Factor Beta

Th – T Helper

TP53 – Tumor Protein P53

Treg – T Regulatory

TSG101 – Tumor Susceptibility Gene 101

VEGF - Vascular Endothelial Growth Factor

VPS4A – Vacuolar Protein Sorting-Associated Protein 4A

WHO – World Health Organization

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Chapter I: Theoretical Background

1. General considerations of Cancer Nanomedicine

Cancer is a disease caused by somatic or germinative gene mutations followed by tumor progression, which is defined by increased proliferation and decreased apoptosis of tumor cells causing an increase of tumor cell mass, invasion of adjacent tissues, and metastatic spread from the primary tumor via blood vessels or lymphatics to distant organs. Metastatic cancer frequently causes death of the patient. Moreover, tumor cells undergo genetic and phenotypic alterations becoming resistant to many cytotoxic drugs, radiation, and developing mechanisms of escape from immune recognition (Gabizon et al., 2020).

The main conventional methods to treat localized tumors include surgery, radiotherapy, and chemotherapy. Moreover, disseminated tumors are commonly treated by drug-based therapy which includes chemotherapy, hormonal therapy, and immunotherapy. However, many of these approaches were ineffective for several types of cancer (Aftab et al., 2018).

The last two decades have been impactful for the advancement of cancer nanomedicine research. The deepening of knowledge about cancer and nanotechnology, the discovery of new biomarkers, and the development of new diagnostic and therapeutic systems are increasing the efficacy of therapies (Gabizon et al., 2020).

Nanotechnology includes the control of size and shape of a material at the nano-level to design nanomaterials that enable complex interactions with the biological milieu. In most cases, nanomedicine applies for the development of drug-delivery systems, which consists in the delivery of a nanoparticle with an associated drug, either for diagnostics or therapy. Moreover, the nanoparticle itself can be applied as an active agent, as gold nanoparticles are applied to destroy tumors by photothermal effect or as imaging agent for diagnostic by Magnetic Resonance Imaging (MRI) (Gabizon et al., 2020).

The drug delivery systems developed have several advantages in comparison with conventional drug-therapies. Nanotechnology-based systems may increase the bioavailability of bioactive molecules to cancer cells through the enhanced permeability and retention (EPR) effect, high clearance value, larger volume distribution, diagnostics at a molecular level, and transport of the therapeutic agents across biological barriers. The size and shape of nanoparticles can affect cellular uptake and the biodistribution of drugs, as nanoparticles with hydrodynamic diameters between 200 and 5 nm have extended blood circulation, per example (Zhao et al., 2019). Moreover, a study demonstrated that nanoparticles with an average size of 100 nm tended to have higher tumor uptake (Zhao et al., 2019). Additionally, nano-based technologies are efficient to protect non-target tissues and cells from serious undesirable symptoms as the ease of surface modification of nanoparticles enhances the development of targeted drug delivery systems (Aftab et al., 2018). In this way, the tissue-specific concentration of the therapeutic agent is increased, decreasing the administration doses necessary, the toxicity and adverse effects, and increasing the lifespan of the drug (Patra et al., 2018).

For an effective targeting drug-delivery system, selection of a proper route of administration is demanded. To reach a specific target for a specific disease, strategies include active, passive, inverse, dual, combination and/or triggered targeting. Regarding the drug carrier, the Food and Drug Administration (FDA) has already approved several nanoformulations for cancer treatment, which include inorganic nanoparticles and organic nanoparticles (Figure 1). Depending on the route of administration, several processes can influence the distribution of nanoformulations in the body and the absorption-distribution-metabolization-elimination (ADME) properties of nanoformulations can differ substantially from drug-based therapies (Moss & Siccardi, 2014). There are several administration routes described in preclinical and clinical studies. However, preferred routes of administration are oral, transdermal, ocular, pulmonary, intravenous, intra-arterial, intratumoral, and intranasal (Chenthamara et al., 2019; Youn & Bae, 2018).

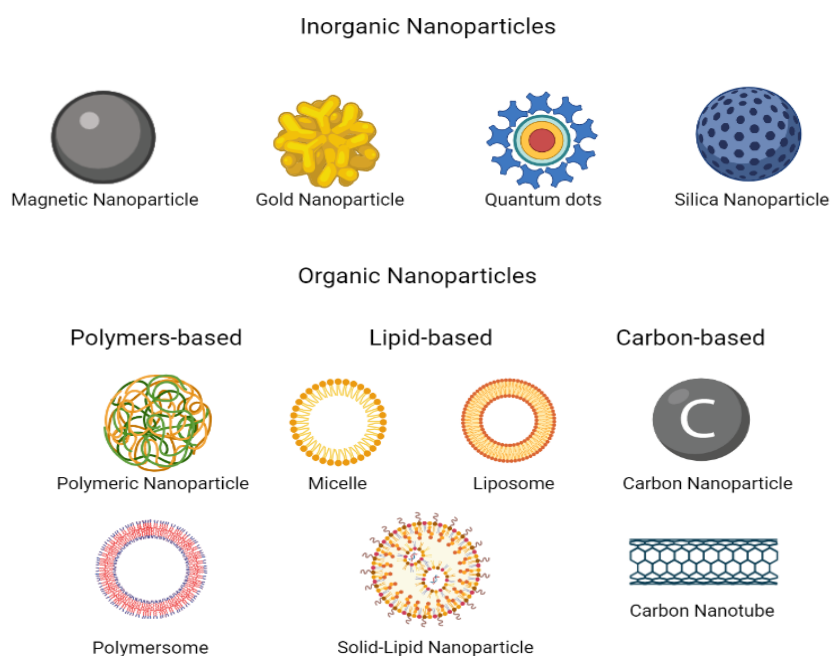


Figure 1. Examples of nanoparticles approved by the FDA for clinical use. Created with *BioRender.com*.

2. The Intranasal Route

The intranasal drug administration is a non-invasive administration route of active pharmaceutical compounds for local, systemic, and central nervous system (CNS) action (Erdő et al., 2018). The intranasal route offers higher bioavailability with avoidance of first-pass metabolism, intestinal efflux transporters, and degradation in the digestive system, as well as a large absorptive surface area (Kreft et al., 2015). Due to leaky epithelial tissue, the tightness of intercellular junctional complex of the nasal mucosa is low. Additionally to the leaky epithelial tissue, the extensive vascularization of the mucosa

and lamina propria provide an optimal absorption surface for drug delivery (Erdő et al., 2018). Moreover, active pharmaceutical compounds absorbed in the nasal mucosa can bypass the blood-brain barrier (BBB) and potentially increase CNS availability of the drug (Dufes et al., 2003).

2.1. Anatomy and Histology of the human nasal cavity

To understand the mechanisms of drug absorption and penetration to the brain, it is necessary to explain the anatomy and histology of the nasal cavity. In fact, the peculiar cellular structure of the nasal cavity has an important role in the mechanisms of absorption. Thus, the anatomical and histological characteristics of the nasal cavity will influence the design of efficient drug delivery systems for intranasal route (Gizurarson, 2012).

The nasal cavity is separated from the oral cavity by the palatine bone and divided into two separate cavities by the septum (Erdő et al., 2018). Each cavity consists of a roof, floor, medial wall, and lateral wall, lined with a layer of mucosa, and a total area of 150-160 cm² for both cavities (Lochhead & Thorne, 2012). Additionally, three different regions divide each cavity: nasal vestibule, respiratory region, and olfactory region (Sobiesk & Munakomi, 2019).

The nasal vestibule extends from the nostrils to the inferior turbinate with a total surface area of 0.6 cm² (Crowe et al., 2018). Histologically, the first half of the vestibule is covered with keratinized stratified squamous epithelium that contains coarse hairs called vibrissae responsible to filter inhaled particles while the second half is covered in pseudostratified ciliated columnar epithelium with few if any ciliated cells (Sobiesk & Munakomi, 2019). Due to the histological characteristics and small surface area, the absorption of drugs is very limited in this region (Erdő et al., 2018).

The respiratory region is located on the lateral walls of the nasal cavities, including the three projecting turbinates - lower, middle, and upper nasal turbinates – being the region with the largest surface area at approximately 130 cm² (Crowe et al., 2018). The respiratory region functions to humidify the air, filter, protect, eliminate debris, and also regulate the temperature of the air as it traverses through the nasal cavity (Sobiesk & Munakomi, 2019). The respiratory region is covered in respiratory epithelium and mucous cells, namely goblet cells. The ciliated and columnar cells present on the ciliated pseudostratified columnar epithelium possess a large surface area combined with a high degree of vascularization which could enhance the drug absorption to the systemic circulation rather than to the CNS. The goblet cells secrete mucin to create the mucus layer together with some of the nasal glands, which can be regulated by the neurovascular supply (Erdő et al., 2018). The mucus produced on the nasal cavity forms a protective barrier to inhaled particles. Therefore, to reach the surface of the epithelium and be absorbed, drug delivery systems must pass through this mucus layer. The higher viscosity of the mucus causes lower clearance rate for the drugs. However, it may also allow larger percentages of doses of drugs to be absorbed after traversing the mucus (Erdő et al., 2018).

Additionally, the olfactory region is located on the superior aspect of the nasal cavity, covering the inferior surface of the cribriform plate of the ethmoid bone (Gänger & Schindowski, 2018). The total surface area is small (10cm²) and varies according to the species. Histologically, the olfactory region is constituted by the olfactory epithelium and mucosa. The olfactory region functions to detect odorants in the inhaled air by olfactory neural cells present on the mucosa (Gänger & Schindowski, 2018). The

olfactory neural cells are unmyelinated, bipolar cells that reside in the epithelial layer between numerous supporting cells. Moreover, olfactory neural cells project axons through the cribriform foramina which are encompassed by a continuous layer of interconnected olfactory “Schwann-like” cells that span the entire distance from the olfactory epithelium to olfactory bulb (Crowe et al., 2018). This layer together, covered with additional layers of neural fibroblasts, constitutes a perineural sheath which is continuous with the subarachnoid space since the layer of neural fibroblasts is continuous with the meninges covering the brain (Y. Li et al., 2005).

Lastly, the lamina propria contains numerous blood vessels, the nasal lymphatics draining to the deep cervical nodes, and fiber for both somatic and autonomic innervation. The lamina propria is located on the basolateral of all regions of the nasal cavities and contains sentinel and secretory immunocompetent cells and two layers of glands secreting enzymes and osmotic compounds of nasal mucus (Crowe et al., 2018).

2.2. From the nasal cavity to the Brain: mechanisms of transport

The large surface area combined with the high degree of vascularity makes the respiratory region a great site for drug absorption to the systemic circulation rather than to the CNS (Arora et al., 2002). Nevertheless, the mechanisms by which a drug travels intranasally to the CNS have not been fully elucidated (Gänger & Schindowski, 2018).

The delivery of drugs in the nasal epithelium occurs through olfactory and trigeminal nerve components to the olfactory bulb and brainstem with further dispersion to other CNS areas. Therefore, to reach the brain, drugs have to cross the olfactory or respiratory epitheliums in the nasal passages, reach the brain entry near the pial brain surface in the cranial compartment, and enter to other sites within the CNS (Lochhead & Thorne, 2012). The transport across the olfactory or respiratory epithelia may occur by intracellular or extracellular pathways (Keller et al., 2021).

The intracellular pathways across the olfactory epithelium include endocytosis into olfactory sensory neurons with subsequent intraneuronal transport to the olfactory bulb, and transcytosis across sustentacular cells to the lamina propria. Olfactory sensory neurons are capable of endocytosis of a variety of molecules ranging in size from insulin (5.8kDa) to gold nanoparticles (50nm) (Crowe et al., 2018). Moreover, the transport of large molecules can occur via adsorptive endocytosis. Additionally, endocytosis into peripheral trigeminal nerve processes located near the epithelial surface may occur across the respiratory epithelium with subsequent intracellular transport to the brainstem (Gänger & Schindowski, 2018). Transcytosis through other cells from the respiratory epithelium to the lamina propria may occur. Following internalization by the olfactory neuron, the endosome is trafficked along the axon in the antero- or retrograde direction towards the olfactory bulb. Neuronal translocation is a very slow process, and thus, intracellular pathways are unlikely to be the primary mechanism of transport (Crowe et al., 2018).

Extracellular transport pathways include paracellular diffusion to the underlying lamina propria, possibly through the paracellular cleft, navigating tight-junctions to reach the olfactory bulb (Balin et al., 1986). Most of the paracellular clefts possess tight junctions that are determinant for the permeability of any molecule. Nasal epithelia tight junctions are perhaps more permeable than those

found in the intestine (Crowe et al., 2018). Although the size limit differs based on the exact protein composition of tight junctions small hydrophilic molecules can diffuse through these barriers and it is possible to diffuse larger molecules through the paracellular space, as shown for stem cells and mesenchymal stem cell-derived exosomes (Herman et al., 2021; Perets et al., 2018). In contrast, certain small lipid-soluble drugs with molecular weight (MW) smaller than 400 Da may cross the blood-brain barrier (BBB) however, the majority of the therapeutic agents are larger than the size limit, and thus cannot cross the BBB (Chen & Konofagou, 2014).

Studies have suggested that the regular turnover of cells in the nasal epithelium to replace olfactory sensory neuron cells in the olfactory epithelium and goblet cells in the respiratory epithelium, may rearrange or loose the tight junctions and compromise the paracellular permeability of a given molecule (Kerjaschki & Hörandner, 1976).

The substances that crossed the nasal epithelium into the lamina propria via paracellular diffusion may be drained within nasal lymphatic to the deep cervical lymph nodes and enter systemic circulation or absorbed by nasal blood vessels and enter the systemic circulation, and thus, must be able to cross the blood-brain-barrier to reach the brain, or diffuse in compartments associated with nerve bundles, specifically perineural or perivascular spaces, with subsequent entry into the cranial compartment. The respiratory region is rich in vasculature, however, the systemic pathway seems less impactful than the lymphatic or perineural pathways (Crowe et al., 2018).

2.3. Experimental models for intranasal route

The design and application of suitable models for intranasal route administration, as well as appropriate data interpretation, are essential to characterize the new formulation and important for the advancement to clinical trials (Brake et al., 2017).

Intranasal administration has been reported for several applications. In particular, the nose-to-brain delivery has been reported in human and animal models for brain tumors, epilepsy, pain, Parkinson, among others (Haney et al., 2015; Hashizume et al., 2008; Qian et al., 2018; Wermeling, 2009).

Models for testing intranasal delivery can be divided into *in vitro* models, *in vivo* models, and *ex vivo* models. The evaluation of efficacy and safety of new formulations includes *in vitro* and *in vivo* assays (Brake et al., 2017). *In vitro* assays are commonly used to evaluate permeation, while *in vivo* assays are more suitable for characterization of nasal absorption and also for pharmacokinetics and pharmacodynamics studies, and *ex vivo* assays are performed to evaluate the nasal perfusion (Erdő et al., 2018). Moreover, *in vivo* assays protocols to study the intranasal route are designed considering the influence of the anatomy of the nasal cavity of the animal selected (Brake et al., 2017). On the other hand, *ex vivo* models for the nasal barrier are obtained from nasal tissue explants from animals or humans, which can be applied in a horizontal Ussing chamber to perform permeability studies with an air interface and maintain viability of the tissues throughout the study by measuring electrophysiological parameters (Östh et al., 2002).

Despite nasal tissue explants representing the most physiological approach in terms of histology, transporter expression and cell type distribution, the variability between human samples is elevated

and the availability is low since most of the biopsies are used for diagnostic examinations and yield only a very limited amount of tissue (Yoo et al., 2003). Moreover, the variability between species, regarding key parameters such as variations of the anatomy, histology, genetics, among others, may cause several distinct responses in the animal and give rise to data that do not correlate to the predicted human response (Cho et al., 2010).

2.3.1.1. *In vitro* models

In vitro models are the starting point to validate new formulations for nasal drug delivery (Erdő et al., 2018). For intranasal studies of new formulations, *in vitro* models can be used to simulate a nasal barrier model to perform permeation studies focused on drug bioavailability. An ideal *in vitro* model should be easy to use, reproducible and standardized. Additionally, *in vitro* models must be able to simulate some *in vivo* features of the nasal mucosa including the presence of tight junction proteins, a specific value of physical transepithelial electrical resistance (TEER), specific permeability values of standard compounds, and the expression of drug transporters (for example glycoprotein-P) (Mercier et al., 2018). Epithelial cell line models are the most applied ones for drug delivery studies, including drug transport and absorption. Some years ago, the CaCo-2 cell line, which is derived from human colon carcinoma and differentiates to various monolayers slowly, has been used to mimic and evaluate nasal absorption ((Qian et al., 2018).

In vitro cell culture models for nasal barrier are obtained from primary nasal cell culture, immortal nasal epithelial cell lines of animal and human and nasal tissue explants - giving rise to the *ex vivo* models (Bai et al., 2008). Primary cell cultures are morphologically and physiologically close to *ex vivo* tissues, although having a short lifespan, complex isolation protocols, and high heterogeneity due to differences between donors' sampling locations. On the other hand, the immortalized cell lines have advantages such as the combined genetic stability, affordable cost, and easy culturing which provides access to unlimited cell material allowing the establishment of large screening studies with the guarantee of reproducible data. Presently, immortalized nasal cell lines that have been used in drug transport studies include the RPMI 2650 cells, among others (Bai et al., 2008).

2.3.1.1.1. The RPMI 2650 immortalized cell line

The RPMI 2650 immortalized cell line is derived from an anaplastic squamous cell carcinoma of the human nasal septum which closely resembles to the normal human nasal epithelium regarding the quasi-diploid karyotype, the expression of cytokeratin polypeptides, and the production of mucous (Bai et al., 2008). RPMI 2650 cell line can be cultured in a transwell with a liquid-liquid interface (LLI), adding medium to the basolateral side and the apical side. As an *in vitro* model, and at the beginning of its utilization, the RPMI 2650 cell line was considered to not form monolayers and thus not form tight junctions, being described as a disappointing model due to the poorly differentiation and the lack of cell polarization which is, in fact, essential for nasal drug transport studies (Bai et al., 2008). Additionally, the higher TEER value peak appeared on the second day of culture and then decreased rapidly (Erdő et al., 2018). However, it has been proposed that growing the cells at an air-liquid

interface (ALI) could play an important role in the differentiation of cells (Bai et al., 2008). The normal human nasal epithelial is exposed to ALI conditions and thus, simulating the ALI conditions could expose the cultured cells to an environment more similar to the *in vivo*. In these conditions, the ALI conditions are created by adding medium on the basolateral side of the transwell and exposing the cells on the apical side to air. In fact, previous studies have demonstrated the expression of tight junctions and an adequate permeation barrier in the RPMI 2650 model under these conditions (Reichl & Becker, 2012). Usually, the maximum TEER value appears on the fifth day, and is maintained over 10 days (Erdő et al., 2018). Additionally, other parameters may influence the differentiation of the RPMI 2650 cell line, including the culture medium and potential supplements, and pore density, surface and coating of the transwell (Mercier et al., 2018).

Lastly, a reconstructed human nasal mucosa model has been designed using a collagen matrix with fibroblasts covered by RPMI 2650 cells (Mercier et al., 2018). The three-dimensional nasal mucosa model shows comparable permeation barrier properties and faster paracellular permeation. Although is a complex model, the three-dimensional approach is a promising model to evaluate passive permeation of substances through nasal mucosa (Wengst & Reichl, 2010).

3. Exosomes

3.1. Exosomes in a nutshell

The field of exosome biology had an explosive growth of investigation on the last three decades (Harding et al., 2013). In 1986, the name “exosome” was coined for this extracellular vesicle by Rose Johnstone (Johnstone, 2005). The definition has been updated as studies of exomes evolve. Nowadays, exosomes are defined as 30-150 nm vesicles released during reticulocyte differentiation as a consequence of the fusion of multivesicular endosomes (MVE) with the plasma membrane (Doyle & Wang, 2019). Structurally, they are described as a unilamellar amphiphilic lipid bilayer that surrounds an aqueous core which allows the transport of various molecules (for example, lipids, proteins, coding and non-coding ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) fragments) of cellular origin (Georgiana et al., 2020; J. Wang et al., 2021). Exosomes can be applied as drug delivery systems, where hydrophobic drugs are distributed within the bilayer while hydrophilic drugs are distributed within the lumen (J. Wang et al., 2021).

Exosomes have properties of potential non-viral vectors, such as high biocompatibility, low immune response and cytotoxicity, long circulation time, and good cell uptake (Ou et al., 2021). The composition of exosomes depends on the cell that synthesizes them and on the organism's physiology. Generally, exosomes consist of sphingomyelin, phosphatidylserine, cholesterol, ceramide, phosphatidylethanolamine, specific proteins of multivesicular bodies such as tumor susceptibility gene 101 (TSG101) protein, glycosylphosphatidylinositol coupled to cluster of differentiation (CD) 55 and CD59, heat shock proteins (Hsps), fusion and membrane transporters such as annexins, Rab-guanine triphosphatases (Rab-GTPases) and flotilin, ALG-2-interacting protein X (ALIX), integrins and tetraspanins (CD9, CD63, CD81 and CD82) (Rincón-Riveros et al., 2021).

Several human cells can produce exosomes. These include red blood cells, platelets, lymphocytes, dendritic cells, epithelial cells, adipocytes, fibroblasts, neural cells, stem cells and tumor cells. Mesenchymal stem cells (MSCs) release immense exosomes and are a viable source. Additionally, it is possible to harvest bone marrow cells and propagate them in culture to differentiate into other cell subtypes, obtaining specific protein exosomes inherent to the cells that release (Batrakova & Kim, 2015). Finally, the exosomes released by MSCs are biocompatible in various animal models and more stable and sustainable in human plasma at -20°C (Gomari et al., 2018).

3.2. Biogenesis and Release mechanisms

The biogenesis of exosomes consists of 3 steps: membrane invaginations that give rise to endocytic vesicles, followed by inward budding of the endosomal membrane that originates the multivesicular bodies (MVBs), fusion of the MVBs with the plasma membrane and release of the vesicular contents. MVBs are characterized by the presence of the intraluminal vesicles (ILVs) which are formed by inward budding from the membrane. The biogenesis of ILVs was studied to investigate the release of exosomes (Kowal et al., 2014). The process starts with budding of late endosomes, which form MVBs that will contain intraluminal vesicles. Once formed, MVBs fuse with the cell membrane of various cell types to release the ILVs at the same time as the release of exosomes occurs (Rincón-Riveros et al., 2021). However, ILVs, and thus exosomes, can be generated at least by two mechanisms which are highly regulated by multiple signal transduction cascades (Rajagopal & Harikumar, 2018). The endosomal sorting complex machinery (ESCRT) pathway where ESCRT participates in the transport of exosomes, is fundamental to this process, and the ESCRT-independent pathway where several authors have suggested that mechanisms involving lipids, tetraspanins, or heat shock proteins, could also be involved in the biogenesis of exosomes.

The concept of an ESCRT role in exosome biogenesis is not new and seems logical that exosomes originate in multivesicular bodies with the ESCRTs machinery comprising the major functions for multivesicular bodies biogenesis (James H, 2015). The ESCRT machinery consists of five subcomplexes, the ESCRT-0 which is responsible for the ubiquitin-dependent clustering charge; ESCRT-I and ESCRT-II which induce bud formation, ESCRT-III which drives vesicle cleavage and accessory proteins like vacuolar protein sorting-associated protein 4A (VPS4A) which allow dissociation and recycling of ESCRT (Juan & Fürthauer, 2018). Several studies employing dominant-negative VPS4AA and various knockdowns reported the ESCRT-machinery and associated proteins could regulate the secretion of exosomes (James H, 2015). A study with the RNA interference (RNAi) screen targeting 23 ESCRT and ESCRT-associated proteins in HeLa cells identified 7 ESCRT proteins that affected exosome secretion (Colombo et al., 2013). A recent study showed that silencing of ALIX could modify the protein composition of exosomes, indicating that it may affect the cargo loading and reduce the release of exosomes (Hessvik & Llorente, 2018). ALIX binds to the specific adapter protein syntenin and establishes a link between syndecans and the ESCRT machinery which also facilitates the intraluminal vesicles formation. Syndecans, are heparan sulfate-presenting proteins distributed on the surface of the plasma membrane of exosomes, according to a specific adapter protein, the syntenin (Baietti et al., 2012).

As described, some mammalian cells with depleted ESCRT components and both the same lipids and proteins are able to form intraluminal vesicles (Stuffers et al., 2009). In addition, proteomic characterizations of purified extracellular vesicles suggest that mixed subpopulations of extracellular vesicles can be released together by a single cell type. Therefore the coexistence of both pathways could explain the various subpopulations of extracellular vesicles obtained in various isolation methods as the microvesicles and apoptotic bodies do not need the ESCRT machinery (Juan & Fürthauer, 2018). Studies suggested that ceramides had a significant role in MVBs formation. Moreover, a ceramide-based mechanism of intraluminal vesicles biogenesis was proposed for ESCRT-independent pathway as the inhibition of enzymes involved in ceramide synthesis may decrease the release of exosomes (Trajkovic, 2008). On the other hand, the tetraspanin CD63 was consistently observed in exosomes, suggesting that tetraspanin might constitute a separated mechanism (James H, 2015). More recently, the knockout of CD63 using clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/cas9) decreased the secretion of exosomes (Production et al., 2017).

3.3. Bovine Milk-Exosomes

Exosomes are present in various biological fluids such as breast milk, urine, saliva, plasma, synovium fluids, malignant ascites, bronchoalveolar lavae fluid, amniotic fluid and cerebrospinal fluid (B. Wang et al., 2019). Most fluids that contain exosomes are of cellular origin and, therefore, their large-scale production can be challenging. On the other hand, some biological fluids can be an interesting source of exosomes, such as milk. Several studies have tested the isolation of milk-exosomes from various mammals, from cow's milk to camel's milk, showing the potential of milk-exosomes for drug delivery systems (Adriano et al., 2021).

Milk-exosomes are potential candidates for new therapeutic approaches to treat various diseases. Moreover, milk is the only exosome-containing biological fluid that is commercialized. However, the application of milk vesicles to deliver biologically active molecules to cells is still emerging (Sedykh et al., 2020a). In cancer treatment, milk-exosomes can be applied to deliver antitumor drugs and therapeutic nucleic acids. Despite the presence of mRNA, microRNA (miRNA), and proteins that may influence the host immune response, milk-exosomes can be used as drug delivery systems (Sedykh et al., 2020a). Milk-exosomes were tested in mouse models and did not cause systemic toxicity or anaphylactic shock (Somiya et al., 2018).

3.3.1. Bioactive compounds of Bovine Milk-Exosomes

The biochemical molecules present in milk-exosomes suspensions can significantly affect therapeutic molecule delivery (for example, immunogenicity, cytotoxicity, target cells, among others), especially if the isolation is contaminated with other molecular compounds. In this regard, a detailed analysis of the content of these molecules in milk-exosomes suspensions is required. There are three principal biochemical components – proteins, lipids, and nucleic acids (Adriano et al., 2021).

Milk-exosomes contain several proteins that participate in their biogenesis, as already described, and biological functions, namely, tetraspanins (CD9, CD63, CD81) for transport of miRNA and adhesion to

target cells. Milk-exosomes also contain cytoskeleton proteins – actin, tubulin, cofilin, heat shock proteins, and molecules - involved in signal transduction. As exosomal biogenesis occurs in a specific cellular compartment, the protein composition of a pure solution is not accidental and therefore not prejudicial to drug delivery systems. However, milk-exosomes suspensions can be contaminated with natural protein compounds present in milk, such as casein, which compromise the proteomic analysis of milk-exosomes (del Pozo-Acebo et al., 2021).

Regarding nucleic acids, several noncoding RNA – miRNA, long noncoding RNA, circular RNA – were identified in bovine milk. Milk messenger RNAs (mRNAs) are mainly concentrated in exosomes while miRNAs are concentrated in exosomes and the supernatant, which was verified by microarray technology where 79 different miRNAs were identified in the exosomal fraction and 91 in the supernatant and, additionally, 39 miRNAs in both fractions (Sedykh et al., 2020a). Presently, there are up to 19,230 mRNAs characterized in bovine milk-exosomes (Sedykh et al., 2020b). Additionally, these RNA have been identified in commercial dairy products, being possible that RNA in milk have functions in the mammalian gastrointestinal tract (Izumi et al., 2015). The mechanism by which these nucleic acids can co-isolate with vesicles is not perceptible, in fact, it can be difficult to describe how thousands of mRNAs can fit a 40-100 nm exosome (Sedykh et al., 2020a).

Depending on the source and isolation methods used, the composition of exosome lipids in milk differs (Sedykh et al., 2020a). As already described, hydrophilic compounds bound to surface proteins and hydrophobic molecules are delivered as part of the lipid bilayer. Therefore, exosomal lipids play a significant role in drug delivery (Skotland et al., 2019). Usually, bovine milk-exosomes suspension is contaminated with lipid droplets, lipoproteins, and other particles and limits the characterization of intrinsic exosomal lipids (Egea-Jimenez & Zimmermann, 2020).

One of the challenges of using milk-exosomes is their non-specificity for target cells, making it necessary to perform functionalization on the surface of the exosomes to direct it to target cells or tissues (Sedykh et al., 2020a). Liposomes have been studied as a possibility of designing synthetic exosomes for targeted drug delivery, where functionalized liposomes with monoclonal antibodies, aptamers, proteins and/or small molecules, fusion with 'loaded' natural milk-exosomes (Bungulawa et al., 2018). However, natural lipid compounds of milk-exosomes are less toxic and more compatible than artificial liposomes, and thus, further studies to design efficient techniques for modification of exosomal surface for targeting cells are necessary (H. Kim et al., 2021).

3.3.2. Stability and Biological activity of milk exosomes

In terms of stability, bovine milk-exosomes are expected to be stable as milk is a food ingredient commercially available and milk-exosomes have already been studied for drug delivery systems (Ahn et al., 2021; Hata et al., 2010; Munagala et al., 2015). For exosomes, stability is defined as resistance to aggregation, loss of structure, and protein degradation. Additionally, bovine milk must be stable before and after isolation. For drug delivery systems, characterization of bovine milk-exosomes is highly important, and best evaluated by measurements of zeta potential, size distribution, and concentration (J. Wang et al., 2021a). Storage parameters influence the stability, namely, temperature, time, freeze-thaw cycles, and pH. Briefly, exosomes should be frozen at -20 °C or -80 °C

or long-term lyophilized (one week at least). Aliquots should be made to minimize the freeze-thaw cycles applied to the sample. Storage at neutral pH with buffer capability should be performed as exosomes stored at pH of 4 or 10 exhibited greater loss of exosomal protein and exosome concentration (Cheng et al., 2019). However, the stability evaluation is limited to the isolation method applied, as co-precipitate proteins and lipoproteins present in suspension may distort concentration measurements (J. Wang et al., 2021). Therefore, more studies for the stability of bovine milk-exosomes regarding storage and physiological conditions are necessary. Regarding physiological characterization, a study confirmed that miRNAs and mRNAs present in bovine milk are stable at low intestinal pH values and in the presence of ribonucleases (RNases) (H Izumi et al., 2012). Additionally, the higher ratio of sphingomyelin, cholesterol, and desaturated lipids in the lipid bilayer enhances the stability of exosomes (Ou et al., 2021).

As already described, the colloidal suspension of bovine milk-exosomes contains bioactive compounds which may influence the interactions between milk-exosomes and cells. Additionally, as the purity of the milk-exosomes suspension is highly dependent on the isolation method applied, co-precipitate proteins and lipid aggregates can modulate the cellular response to the exosomal suspension. Therefore, the biological activity of milk-exosomes has been studied over the years, focusing initially on the cellular uptake of bovine milk-exosomes by several cells. The incorporation of human exosomes was verified in THP-1 cell line due to exosomal RNAs (Izumi et al., 2015). Another study verified the presence of transforming growth factor beta (TGF- β) on the surface of bovine milk-exosomes in a dose-dependent response with increasing amounts of milk-exosomes and cellular uptake in rat macrophages and fibroblasts. As studies for biological activity of milk-exosomes emerge, the impact of bioactive compounds in cellular responses becomes more evident and important to the effective design of drug delivery systems for targeted therapies (Adriano et al., 2021b).

3.4. Exosomes Isolation

Exosomal isolation process is challenging since the isolation method used must preserve the physicochemical and biological properties of the exosomes despite high heterogeneity in size and surface biomarkers. Furthermore, other extracellular vesicles, such as apoptotic bodies and microvesicles which have similar properties, can be isolated together with the exosomes, and misinterpret the results obtained. Therefore, it is necessary to develop an isolation strategy that is specific for exosomes, efficient and that presents a good long-term perspective for clinical applications (Yuana et al., 2013).

Currently, the used methods include the ones based on particle size, density and charge, affinity between proteins or receptors on the exosome membrane and specific ligands, and isoelectric point of proteins (Ayala-Mar et al., 2019).

3.4.1. Ultracentrifugation-based techniques

Ultracentrifugation technique is the gold standard for exosome isolation with simple protocols and preservation of physicochemical properties. The principle relies on the application of a centrifugal

force in a heterogeneous suspension to sediment particles sequentially according to their density, size, and shape, and density and viscosity of the solvent (P. Li et al., 2017). Additionally, the method is optimized to generate high centrifugal forces, most of the times reaching at least 100,000 – 200,000 g. There are two types of ultracentrifugation – analytical and preparative ultracentrifugation. Analytical ultracentrifugation is used to study hydrodynamic properties of the solution as well as physicochemical properties of the particles in suspension and molecular interactions of polymeric materials while preparative ultracentrifugation is used to purify and fractionate small bioparticles such as viruses, bacteria, cell debris, and extracellular vesicles (P. Li et al., 2017). Preparative ultracentrifugation can be divided into two types – differential ultracentrifugation and density gradient ultracentrifugation. Differential ultracentrifugation is based on a gradual increase of centrifugal force and duration while density gradient ultracentrifugation depends mainly on a density gradient medium. (Cvjetkovic et al., 2014; Sidhom et al., 2020).

However, since the different types of extracellular vesicles have similar sedimentation properties, ultracentrifugation-based techniques often display unsatisfactory results, providing populations of exosomes with low purity and yields. Moreover, protocols are applied with different rotors and without accounting for the differences in viscosity of the solutions, which often leads to dissimilarity of the data obtained, being likely to observe differences in the reported proportions of exosomes and shedding vesicles (Livshits et al., 2015).

3.4.2. Size-based Filtration/Ultrafiltration

One of the most size-based method used to isolate exosomes is ultrafiltration which merely depends on size or molecular weight (Gurunathan et al., 2019). To perform isolation by filtration, the fluid must pass through commercial membranes filters (for example, polycarbonate, pore size 0.2 μm) with defined molecular weight or size exclusion limits. This process is selective, which means that vesicles with an effective diameter greater than the membrane pore are retained in the filter. There are two ways to perform filtration - direct flow filtration and tangential flow filtration (J. Wang et al., 2021a). In direct flow filtration, the fluid circulates perpendicularly to the membrane, being necessary to exert controlled pressure to cross the membrane without collapsing. However, the exosomes can be trapped in the membrane and/or excessive pressure can cause larger vesicles to readjust and cross the membrane pore, conditioning the purity of the extraction (Heinemann et al., 2014). In tangential flow filtration, the fluid flows tangentially to the filter in a dynamic way, not being pushed against the membrane (Shah et al., 2020). In this method, it is possible to keep the solutes of interest and efficiently remove the waste products. The process takes longer because the fluid that does not cross the membrane returns to cross the chamber again, increasing the amount of extracted exosomes and purity (Busatto et al., 2018).

Ultrafiltration is faster than ultracentrifugation, being possible to extract suspensions of exosomes using only this method. However, it is difficult to remove contaminating proteins from the suspension. Thus, this method usually complements other exosome isolation techniques such as ultracentrifugation, serving as an intermediate step to sieve cells and large extracellular vesicles after

which the separation of exosomes from proteins is achieved via ultracentrifugation (Gao et al., 2019; Gurunathan et al., 2019; Yamauchi et al., 2020).

3.4.3. Other Methods studied for the isolation of exosomes

One common method used to isolate exosomes is size exclusion chromatography (SEC). SEC is a size-based technique by differential passage through physical barriers, using filters or chromatography columns in the stationary phase. The various populations of particles are separated according to their size or hydrodynamic volume (Batrakova & Kim, 2015; Liangsupree et al., 2021). Several investigations have reported results suggesting that SEC is the best technique to obtain pure exosomes from human body fluids. In addition to the high degree of purity, this technique does not require large sample volumes for the isolation of exosomes (Sidhom et al., 2020). However, the chromatographic processing time can be high and, depending on the operation buffer chosen, there can be degradation of the exosomes (Ayala-Mar et al., 2019).

Moreover, precipitation methods have also been used to isolate exosomes, using polymeric materials and low speed centrifugations. Polymeric precipitation is a method in which one of the approaches can be based on the precipitation of water-excluding polymers in the solution. Different intermediates, such as polyethylene glycol (PEG), can be used, however, the polymer chosen needs to be made from innocuous and inert materials that are not immunogenic (Tarasov et al., 2021). Polymeric precipitation is scalable and easy to use and does not require any specialized equipment, allowing the easy integration into clinical usage (Gurunathan et al., 2019). However, exosomes of different sizes are mixed without any specificity for non-exosomal material, such as protein aggregates, when isolated by polymeric precipitation (Peterson et al., 2015). Another method to isolate exosomes, specifically studied for milk, is isoelectric precipitation. The technique is based on the precipitation of the casein protein using hydrochloric acid (Yamauchi et al., 2020).

Another commonly technique is immunoaffinity capture which is used to overcome the impurity of exosome preparations by selectively capturing specific exosomes from a complex population, based on certain surface markers (Gurunathan et al., 2019). Tetraspanin proteins found on the surface of exosomes are the most common targets thus, Anti-CD63, -CD9, and -CD81 antibodies are commonly used (Gurunathan et al., 2019). One of the main advantages of this method is the possibility of isolating exosomes containing specific surface markers, such as cancer-specific proteins (Liangsupree et al., 2021). However, this technique is still recent, quite expensive as it involves the design of specific antibodies and the yield of exosomes obtained in each isolation step is low (Georgiana et al., 2020).

In the last years, microfluidic chambers have been developed for the isolation of exosomes. The use of microfluidic devices to isolate exosomes proves to be an innovative approach. In addition to techniques based on the separation by size, density and immunoaffinity, it is also possible to utilize innovative mechanisms such as acoustic, electrophoretic and electromagnetic applications (P. Li et al., 2017). Moreover, this method requires reduced sample volume, less reagent consumption and has faster isolation periods. The microfluidic device preferentially traps exosomes with diameters between 40 and 100 nm with higher purity. Based on immunoaffinity, microfluidics uses specific antibodies to

target the surface of exosomes, allowing for specificity in the extracellular vesicle subtype captured on the microfluidics chip (Gurunathan et al., 2019).

3.5. Encapsulation Methods

Efficient encapsulation methods are necessary for exosomes to be profitable as drug delivery systems. Several methods of encapsulation have been studied for exosomes isolated from various sources, namely cells and biological fluids such as milk. Incubation as an encapsulation method has been the most studied and applied to hydrophobic drugs (Mehryab et al., 2020). There are two ways to carry out this technique; either the exosomes already isolated are incubated with the drug or the drug is incubated with the cells that will release the exosomes which are posteriorly isolated (Mehryab et al., 2020). This method is simple and inexpensive, however, the loading efficiency is low (M. S. Kim et al., 2016).

Another commonly used method for encapsulation of drugs and even small molecules such as nucleic acids and iron oxide nanoparticles in exosomes is electroporation (Balachandran & Yuana, 2019). Electroporation consists of the application of external electrical pulses which permeabilize the plasmatic membrane of the exosomes in a transient way that will allow the passage of these compounds (Familtseva et al., 2019). This method has been more used for hydrophilic drugs such as doxorubicin (M. S. Kim et al., 2016). Although loading capacity and efficiency of encapsulation are superior to incubation, electroporation can enhance the aggregation of extracellular vesicles and compromise encapsulation (M. S. Kim et al., 2016). For this reason, electroporation buffers such as sucrose and trehalose are used, which stabilize the exosome membrane (Balachandran & Yuana, 2019).

Several methods have been tested to overcome the low encapsulation efficiency verified for incubation as well as the difficulties inherent to electroporation. Sonication has been tested as an encapsulation method for paclitaxel with a higher loading capacity (about 30%) when compared to the loading capacity of incubation and electroporation, for the same drug (1% and 5%, respectively) (M. S. Kim et al., 2016). Sonication is based on the use of ultrasound that deregulates and permeates the exosome membrane, being a relatively recent method in the study of exosomes. In addition to sonication, a loading method based on a pH gradient has been studied. In addition to the pH gradient, the encapsulation of drugs in exosomes was also studied with freeze-thawing. After incubation, at room temperature, this technique applied 2-3 times generated transient pores in the membrane of the exosomes that allowed the incorporation of the drugs (Kalani et al., 2016).

Recently, several techniques have emerged for the encapsulation of molecules in suspensions that have more subtypes of extracellular vesicles, namely exosomes and microvesicles. Studies to test a saponin-assisted encapsulation and a hypotonic dialysis encapsulation to encapsulate porphyrins were performed (Fuhrmann et al., 2015). These techniques were easily applicable to solutions containing only exosomes and improved the cellular uptake and therapeutic effect of porphyrins *in vitro* (Fuhrmann et al., 2015).

To date, sonication appears to be the most efficient method of encapsulation for exosomes. However, alternatives to the use of exosomes of biological origin are being considered. The production of

synthetically personalized exosomal mimetics could be an alternative. The technique consists of incubating MSCs with the drug of interest and consequent cellular uptake of the drug, forming synthetic exosomes. These synthetic exosomes have been shown to be effective in the treatment of breast cancer both *in vitro* and *in vivo* (Kalimuthu et al., 2018).

4. Brain tumors - Gliomas

Gliomas are brain tumors of the central nervous system, which are classified according to the cellular origin of the tumor. This classification includes tumors originating from astrocytes - astrocytoma, anaplastic astrocytoma, and glioblastoma - oligodendrocytes (such as oligodendrogliomas), ependymal cells (giving rise to ependymomas), and more than one cellular origin (mixed gliomas) (Hanif et al., 2017).

The standard classification for gliomas is defined by the World Health Organization (WHO). Gliomas are classified according to their malignancy, determined by histopathological criteria. They are divided into 4 classes, from grade I to grade IV. Grade I and grade II gliomas have poor proliferative capacity, can be surgically removed, and there is a low probability of recession, indicating a good prognosis. Grade III and grade IV gliomas are usually invasive and grow more aggressively, and surgery is often not sufficient to completely remove the tumor mass, increasing the probability of recession (Claus et al., 2015).

Global Cancer Statistics of 2020 showed that brain tumors rank 14th among tumors with the highest incidence and mortality for both men and women in developed countries. Incidence rate per 100,000 population in developed countries was 4.7 for men and 3.6 for women while for underdeveloped countries was 2.4 for men and 1.7 for women. Additionally, mortality rates in developed countries were 3.6 for men and 2.8 for women while in underdeveloped countries were 2.1 for men and 1.4 for women (Sung et al., 2021). Global Cancer Statistics 2020 report shows that the mortality rate is relatively close to incidence rate (Sung et al., 2021).

Malignant gliomas, including glioblastomas, represent approximately 75% of malignant brain tumors (McNeill, 2016). Although Glioblastoma Multiforme (GBM) is rare, several studies indicate a rise in incidence. The rising incidence is multifactorial and possible contributing factors include an aging population, advanced diagnosis techniques, ionizing radiation, air pollution, among others (Grech et al., 2020).

Nevertheless, higher incidence rates imply higher mortality rates, unless new therapeutic and diagnostic approaches emerge for gliomas, in particular glioblastoma multiforme.

4.1. The Glioblastoma Multiforme

GBM is a grade IV glioma possibly originating from primary astrocytomas or *de novo*. The term 'multiforme' indicates pleomorphism which means tumor cells vary in size and shape. GBM is characterized by high cell and mitotic activities, vascular proliferation, necrosis and nuclear atypia (Omuro & DeAngelis, 2013). In terms of incidence, about 60% of GBM is located in the brain - frontal lobe (25%), temporal lobe (20%) parietal lobe (13%) and occipital lobe (3%) - while, the remaining 40%

may arise in the brainstem, cerebellum and spinal cord (Davis, 2016). Conventional therapy for GBM includes surgical removal of the tumor mass followed by radiotherapy in combination with chemotherapy, where the FDA-approved drugs, temozolomide and bevacizumab, bearing the commercial name Avastin, are the most chemotherapeutic agents used for GBM. Although the combination of these approaches has increased the average life expectancy of patients, the average life expectancy rarely exceeds 15 months (Cha et al., 2020).

Since 2016, the WHO has been establishing a more precise classification for brain tumors (Georgiana et al., 2020). Nowadays, the GBM is categorized into 2 types of cancer according to the genetic profile: primary or de novo tumor and secondary tumor. About 85% of cases correspond to primary tumors which typically occur in elderly patients, usually from a known cellular precursor - glia cells, neural stem cells, among others - while, secondary tumors arises from the evolution of a grade II astrocytoma or a grade III anaplastic astrocytoma and constitutes approximately 15% of all cases, usually appearing in patients with at least 45 years old (Agnihotri et al., 2013).

The prognosis of a primary or secondary GBM is dependent on the molecular and gene variations that occur. In fact, genetic mutations prevalent in each type of tumor have been identified. Primary GBMs are characterized by amplification and mutation of the epidermal growth factor receptor (EGFR); loss of heterozygosity from chromosome 10q which contains mutations on tumoral suppressor genes – phosphatase and tensin homolog (PTEN) and murine double minute 2 (MDM2) - deletion of p16 protein and cyclin-dependent kinase inhibitor 2A/B (CDKN 2 A/B) gene, mutations in telomerase reverse transcriptase (TERT) promoter and isocitrate dehydrogenase (IDH) 1, amplification of platelet-derived growth factor receptor (PDGFR) gene on chromosome 4, mutations in phosphatidylinositol 3-kinase (PIK3CA) gene, among others (Georgiana et al., 2020; Agnihotri et al., 2013; Hanif et al., 2017). Secondary GBMs are characterized by mutations of tumor protein p53 (TP53), IDH1/2, over expression of platelet-derived growth factor A and platelet-derived growth factor receptor alpha (PDGFA/PDGFR α), retinoblastoma (RB), loss of chromosome 19q, methylation of O⁶-methylguanine-DNA-methyltransferase (MGMT) promoter on chromosome 10q26, among others (Georgiana et al., 2020).

4.1.1. Neoplastic biology and strategies of tumoral progression

The GBM may arise from several cell types (for example, glial cells, stem cells, neurons, among others) with stemness ability and in different stages of differentiation with phenotypic variations due to active signaling pathways. However, the biological origin of GBM is still uncertain, although several signaling pathways have already been identified (Davis, 2016). Several patients must often cope with cancer recurrence after the failure of conventional therapy due to cancer resistance developed. Recent studies confirmed the existence of cancer stem cells in GBM and the mechanisms that enhances cancer progression and/or recurrence by developing drug resistance (Marzagalli et al., 2021). Cancer stem cells are usually defined as tumor propagating cells which constitutes a small sub-population with low proliferative activity that is responsible to generate cellular heterogeneity in the tumor at genotypic and phenotypic levels. Cancer stem cells have the ability to self-renew asymmetrically and to generate either cells with stemness phenotype or differentiated cells (Safa et al., 2015).

Brain tumors are protected by the BBB which regulates CNS homeostasis and cerebral blood flow and the passage of molecules, as it forms a tightly regulated neurovascular unit (NVU), where interactions between cells and blood vessels occur. The NVU is characterized by the presence of tight junctions formed by endothelial cells and the covering of the basal lamina by pericytes, smooth muscle cells, neural cells and extracellular matrix (Netto et al., 2018). Moreover, the BBB is regulated by intracranial signals that control several mechanisms to maintain integrity. Therefore, the BBB is regulated by various strategies, such as direct cell contact, signaling molecules, cells and even exosomes, helping the extravasation to the neuroparenchyma. Consequently, one of the criteria for the selection process of vectors and/or drugs for targeted therapies to GBM is the ability to extravasate the BBB (Arvanitis et al., 2020). During the development of primary tumors, as tumor development expands through cell proliferation or intracranial metastases, NVU acquires different properties that may compromise the functions of the BBB, and thus, form the brain tumor barrier (BTB), with a looser vasculature. BBB/BTB have a complex structure and heterogeneity in active molecular mechanisms that compromise the normal integrity of a barrier and therefore should be considered in the development of new targeted therapies (Arvanitis et al., 2020).

The molecular patterns of the GBM can explain the different outcomes of tumors with a similar histological profile and be useful to predict the patient's diagnosis and prognosis (D'Alessio et al., 2019). Moreover, there are 3 main pathways involved in the pathogenesis of GBM, which give rise to different prognoses - the TP53 pathway, the phosphatidylinositol 3-kinase / protein kinase B / mammalian target of rapamycin (PI3K/Akt/mTOR) pathway and the RB pathway. The three pathways presented have mutations that affects DNA repair and tumoral suppression capacities, leading to excessive proliferation of tumor cells by increasing their life span via inhibition of apoptosis and increased proliferation rate (Georgiana et al., 2020). Genetic variations contribute to excessive cell proliferation as well as errors in DNA repair mechanisms, dysregulation of cell cycle checkpoints, among others which promotes tumor progression.

Research in the 1920s demonstrated that cancer cells avidly utilized higher levels of glucose in the presence of oxygen, with an associated increase in lactate production, to produce energy. This phenomenon was called aerobic glycolysis, also termed the Warburg Effect, and has been identified on GBM (Potter et al., 2016). Cancer cells have active and functional mitochondria being possible in a tumor to have a dynamic interplay between oxidative metabolism and aerobic glycolysis to produce adenosine triphosphate (ATP), which is linked to environmental conditions and cancer-associated mutations.

GBM is a fast-growing tumor, which means cell proliferation can outgrow angiogenesis, vasculogenesis and nutrient supply. In this way, the oxygen supply to cancer cells is conditioned and regions of hypoxia, or lack of oxygen, are formed and detectable by magnetic resonance imaging and microscopic analysis (Kaur et al., 2005).

In chemotherapy, hypoxia confers resistance due to the lack of properly formed vasculature that difficult drug perfusion into the tumor microenvironment. Furthermore, treatment with temozolomide has been shown to increase the expression of hypoxia-inducible factor 1- α (HIF-1 α) (Fisher et al., 2007). Consequently, cancer cells can adapt to hypoxic conditions as HIF1- α interacts

with the MYC proto-oncogene (c-MYC) to alter tumor metabolism and enhances glycolysis by increased expression of the glucose transporter 1 (GLUT1), which allows for influx of glucose, and hexokinase 2 (HK2), the enzyme necessary to direct the cells towards glycolysis, thus, increasing the synthesis of ATP (Yoshida et al., 2018).

Under hypoxic conditions, the ability of reactive oxygen species (ROS) such as O₂⁻, H₂O₂, and OH⁻ to damage DNA is diminished. Therefore, the hypoxia created in solid tumors confers resistance to radiotherapy (Ali et al., 2020). Additionally, with the lack of properly formed vasculature which constrains the oxygen supply, there may be shorter oxygen perfusion periods followed by more efficient oxygenation, exposing the cells to cycles of hypoxia and reoxygenation – cycling hypoxia (Michiels et al., 2016). This phenomenon increases radioresistance by triggering the activity of HIF-1 α . Additionally, induction of HIF-1 α can enhance the influx of bone marrow-derived cells (BMDCs) into the tumor, as the BBB is disrupted, restoring the radiation damaged vasculature by vasculogenesis and, thus, the growth of survival cancer cells. Therefore, HIF-1 α can promote tumor recurrence after irradiation. The inhibition of vasculogenesis, and not angiogenesis, decreased tumor recurrence after irradiation, as the pharmacological inhibition of HIF-1 α diminished the recruitment of BMDCs to the tumor (Kioi et al., 2010).

4.1.1.1. Tumor Immune Microenvironment and Exosomes

The tumor microenvironment is constituted by both neoplastic and non-neoplastic cells in which the tumor cells grow and develop diversity within the tumor. The cellular sub-populations present in the microenvironment produce biomolecules that together with extracellular matrix constituents, forms the tumor microenvironment (Ali et al., 2020). GBM is located in the brain, an immune-privileged organ, where the infiltration of peripheral immune cells only occurs due to the disruption of the BBB by inflammation, rapid expansion of tumors, and tumor infiltration by immunosuppressive immune cells from blood circulation (Goenka et al., 2021). The cellular composition of non-neoplastic cells includes stromal cells and endothelial cells, pericytes, fibroblasts, glial cells, glioma stem cells and immune cells (such as monocytes, macrophages, and T cells) (Hambardzumyan & Bergers, 2015).

Several studies to characterize the tumor microenvironment have been published. The T cells accountability in GBM biopsies was made by flow cell analysis, showing that CD8⁺ cytotoxic T cells, account for only one-fourth of the total CD3⁺ T cell population, being less responsive to anti-CD3⁺ stimulation *in vitro* (Han et al., 2016). Cancer cells in GBM can suppress the activity of CD8⁺ cytotoxic T cells through the transport of programmed death-ligand 1 (PD-L1) by exosomes, which amplifies T regulatory (Treg) cells activation, limits T-cell reaction and restrain T-cell anti-tumor activity (Litak et al., 2019).

The characterization of 33 cancer types identified six different immune subtypes. For GBM, this study described an immunosuppressive environment with the lowest number of lymphocytes and the highest number of tumor-associated macrophages (TAMs) (Thorsson et al., 2018). Additionally, immunosuppressive cytokines and chemokines such as interleukin (IL)-10, prostaglandin E₂ (PGE₂), TGF- β , and immune cells like immunosuppressive natural killer T (NKT) cells, Treg cells, T helper (Th) 2 Cells, M2-macrophages, TAMs, and myeloid-derived suppressor cells (MDSCs) have been identified in

GBM, thus, creating an immunosuppressive microenvironment which supports pro-tumorigenic activities (Kaffes et al., 2019).

Moreover, neoplastic cells establish intercellular communication with non-neoplastic cells present in the tumor microenvironment, to induce cellular mechanisms for tumor progression. One of the several strategies to establish intercellular communication is through the release of extracellular vesicles (Georgiana et al., 2020). The extracellular vesicles contain coding and non-coding RNAs, and are often involved in regulating tumor proliferation and survival (Quezada et al., 2018). Glioblastoma-derived exosomes may contain pro-angiogenic factors (for example, vascular endothelial growth factor (VEGF), TGF- β , CD184 and plasminogen activators), anti-angiogenic factors, miRNAs and extracellular proteases that are essential for the promotion of angiogenesis, thus allowing endothelial cells to migrate, proliferate and form new blood vessels that will favor the obtaining of nutrients in the tumor microenvironment (Quezada et al., 2018). In particular, exosomes regulate the intercellular communication using three main mechanisms - activation of signaling pathways through interactions between receptor and ligand, cleavage of exosomes by extracellular proteases with binding of exosomal biomolecules to cell surface receptors, fusion with the cell membrane and release of intraluminal content and by endocytic mechanisms such as phagocytosis, endocytosis and macropinocytosis (Gomari et al., 2018).

Cancer cells release anti-inflammatory cytokines (IL-10, IL-12, TGF- β) that induce an anti-inflammatory response by recruiting Th-2 or Treg cells that also promote differentiation of macrophages in the M2 phenotype. Glioblastoma-derived exosomes can induce the production of anti-inflammatory cytokines in non-neoplastic cells. Thus, the immune response mediated by Th-1 cells is compromised (Georgiana et al., 2020). Moreover, the expression of exosomal PD-L1 possesses a strong immunosuppressive effect and accelerates tumor formation and progression (Pi et al., 2021).

Although the tumor microenvironment is mainly immunosuppressive, cancer cells can evade innate immune response mechanisms. Neoplastic cells release extracellular vesicles which induce the conversion of macrophages from M1 phenotype to M2 phenotype while decreasing the induction of monocyte differentiation and triggering actin reorganization, thus promoting tumor progression, mainly due to the loss of the pro-inflammatory phenotype of macrophages (Georgiana et al., 2020; Quezada et al., 2018).

4.1.2. Therapeutic Role of Exosomes in Glioblastoma Multiforme

Exosomes have been described as promoters of Glioblastoma Multiforme, as tumor-derived exosomes may participate in generating a protumorigenic or antitumorigenic response by manipulating host stromal responses (Kalluri & Kalluri, 2016). Although exosomes have emerged as critical mediators of immune/cancer cell communication, the role of exosomes is unclear, possibly reflecting the functional heterogeneity in the tumor microenvironment. Moreover, extracellular vesicles have been reported to participate in the regulation of pathological angiogenesis, activation of fibroblasts, regulation of tumor growth and metastasis, determination of tumorigenic potential of epithelial cells, invadopodia formation, and induction and/or maintenance of the epithelial-mesenchymal transition (EMT) in cancer cells (Kalluri & Kalluri, 2016).

Exosomes are important tools in the diagnosis and prognosis of a patient with GBM. Only one cancer cell secretes about 10,000 extracellular vesicles in 48 hours (Kowal et al., 2014). In addition to the strategies presented, cancer cells also manipulate the signaling pathways involved in cell cycle through the release of exosomes containing coding RNA and non-coding RNA – the miRNAs. The small non-coding RNAs have the capacity to inhibit gene expression after transcription. In 2013, it was found that approximately 235 miRNAs are overexpressed and 95 are downregulated in glioblastoma when compared to normal brain tissue (Møller et al., 2013). Tumor-derived exosomes carrying miRNAs have been characterized as potential diagnostic biomarkers for glioblastoma (Basu & Ghosh, 2019).

In the therapeutic context, extracellular vesicles have been tested in various approaches, including drug delivery systems for chemotherapeutic agents; delivery of small interfering RNA (siRNAs) and miRNAs that edit or silence target genes – gene therapy; and cancer vaccines for the delivery of molecules that stimulate a host-immune response against the tumor (Tarasov et al., 2021). For glioblastoma, an *ex vivo* approach has been reported, using mesenchymal stem cell-derived exosomes to deliver inhibitors for miRNAs knockdown, such as inhibition of miRNA-9, which reverses multidrug resistance to temozolomide through induction of apoptosis and downregulation of transporters such as P-glycoprotein that promote resistance to chemotherapeutic agents (Munoz et al., 2013).

As the molecular and genetic profile of GBM is being characterized and the knowledge about exosomes in the tumor microenvironment is deepened, the hypothesis of designing targeting therapies for specific tumor signaling pathways, using exosomes as nanocarriers has emerged. Understanding the mechanisms of biogenesis and cellular uptake of exosomes creates the possibility of using exosomes to create an unfavorable microenvironment for the development of GBM (Georgiana et al., 2020). Therefore, combining biological mechanisms with the delivery of molecular compounds that manipulate specific signaling pathways, causing a cytotoxic response in cancer cells, it is possible to design therapeutic strategies capable of disfavor the tumor progression. Moreover, using exosomes as a nanocarrier could be seen as ‘Trojan horse’ strategy, where cancer cells would not recognize the encapsulated molecular compounds and develop resistance mechanisms. On the other hand, knowing that tumor exosomes have a key role in tumor progression and recurrence, tumor-derived exosomes may be a therapeutic target rather than a carrier, inhibiting the secretion of these extracellular vesicles to interrupt one of the cell communication strategies in the tumor microenvironment (Basu & Ghosh, 2019).

4.1.3. Intranasal delivery of Chemotherapeutic agents to treat Glioblastoma Multiforme

Intranasal delivery of drugs has been studied in animals and humans showing high potential when applied to GBM. The nasal delivery of several compounds has been reported to reduce brain tumors and increase survival in animal models. These substances include telomerase inhibitors, methotrexate, raltitrexed and 5-fluorouracil (van Woensel et al., 2013). The oligonucleotide telomerase inhibitor GRN163 seems to selectively distribute into tumor cells, which are positive for GRN163 antisense oligonucleotide targeting telomerase, without toxic effects in normal brain tissue, whereas the

telomerase is not expressed (Djupestrand et al., 2014). The preferential distribution was more pronounced after intranasal administration, showing the possibility of tumor-tropism (Ozawa et al., 2004). Moreover, the intranasal delivery of vesicular stomatitis virus VSVrp30a, a virus which do not cross the BBB, the olfactory bulb gliomas were selectively infected and killed (Özduman et al., 2008). Regarding chemotherapeutic agents, methotrexate is an anti-tumor agent that passes through the BBB very poorly, and thus, the intranasal delivery favored the therapeutic effect. Raltitrexed is clinically used in the treatment of advanced colorectal cancer and, when applied intranasally to the brain, different concentrations of raltitrexed were found, with the highest concentration found in olfactory bulb and the lowest concentration found in cerebellum. These results showed that intranasal administration delivered significantly more raltitrexed into the CNS than intravenous administration (van Woensel et al., 2013). A similar study with 5-fluorouracil, a chemotherapeutic agent that is clinically used to treat breast cancers, melanomas, and pancreatic cancers, was performed earlier, showing that the delivery of 5-fluorouracil in the cerebrospinal fluid (CSF) is augmented by nasal drug application (Sakane et al., 1999).

As already mentioned, intranasal delivery does not seem to have a restrict size distribution as the intranasally administration of neural stem and progenitor cells was reported. Moreover, a study with intranasal administration showed that neural stem cells travel specifically to the tumor microenvironment, making neural stem cells a good candidate for the targeted delivery of biologically active gene products (Aboody et al., 2008).

Lastly, T cell-derived exosomes were studied as anti-inflammatory delivery agents, such as curcumin, via intranasal route to treat the mouse glioblastoma cell line GL26 and showed delayed brain tumor growth (Zhuang et al., 2011). However, the intranasal delivery of milk-exosomes to treat brain tumors is not reported yet.

4.1.4. Preclinical approaches to apply Mitoxantrone in Glioblastoma Multiforme

Several studies over the years have found that glioblastoma has developed strategies of resistance to temozolomide, which can develop tumor recurrence and more aggressiveness (Ali et al., 2020). Several chemotherapeutic agents, approved to other cancers, are being studied as an alternative to treat GBM, such as Mitoxantrone (Ellis et al., 2016; Lam et al., 2018).

Mitoxantrone is a cytotoxic antineoplastic agent that inhibits nucleic acid synthesis and affects cells at various stages of the cell cycle. Mitoxantrone is a dihydroxyanthracenedione derivative which as intravenous administration has proven efficacy, alone or in combination, in the treatment of breast cancer, acute leukemia and non-Hodgkins lymphoma, prostate cancer and melanoma (Faulds et al., 1995; Xin et al., 2017). As an alkylant agent, mitoxantrone causes DNA-protein crosslinks and single and double strand breaks by intercalation with DNA and interacts with topoisomerase-II which causes its cytotoxicity (Gerhard et al., 1990). Moreover, the immunosuppressive capacity directed at proliferating immune cells, namely, B cells, T cells and macrophages, may affect the effectiveness of the patient's immune system (Neuhaus et al., 2005). Mitoxantrone causes myelosuppression but it is usually less cardiotoxic than doxorubicin (Schwendener et al., 1991). Mitoxantrone has other side

effects in addition to cardiotoxicity and myelosuppression, such as nausea, generalized discomfort, neuropathy, nephrotoxicity, among others (Xin et al., 2017).

To optimize the administration of drugs such as mitoxantrone to treat GBM it is necessary to develop efficient drug delivery systems that promote their release in the target tissue and enhance their therapeutic effect, with lower toxic concentrations. For brain tumors, mitoxantrone is poorly lipid soluble, and thus, cannot cross the BBB. Moreover, systemic delivery is not feasible due to dose-limiting side effects, and poor penetration in brain tumors (Lam et al., 2018). However, mitoxantrone has proven efficacy to treat GBM *in vitro*, *in vivo* and clinical assays, showing the necessity to create a drug delivery system with an appropriate route of administration to the brain (Table 2). Moreover, the intranasal delivery of mitoxantrone has not been reported yet.

Table 1. Preclinical and Clinical assays developed to test Mitoxantrone in the treatment of Glioblastoma Multiforme regarding the drug-delivery system developed, the administration route applied, and the sample of Glioblastoma Multiforme.

Drug delivery system	Administration Route	Sample	Reference
Plant virus-based (cowpea mosaic virus) nanoparticle with encapsulated mitoxantrone	Intratumoral	<i>in vitro</i> : U87-MG cells	(Lam et al., 2018)
PLGA nanoparticles introduced in mitoxantrone-loaded ultrasound-responsive liposomes	Intratumoral	<i>In vivo</i> : Sprague-Dawley rats	(Xin et al., 2017)
Mitoxantrone in association with 90-Y radioimmunotherapy	Intratumoral	Twenty-six recurrent glioblastoma Patients treated at the National Neurological institute ‘C Besta’	(Boiardi et al., 2005)
Mitoxantrone	Intra-arterial; Intra-arterial transient cerebral hypoperfusion;	<i>In vivo</i> : Sprague Dawley rats	(Ellis et al., 2016)

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Chapter II: Hypothesis and Objectives

Considering the literature review presented, if exosomes of cellular origin have clinical importance in the diagnosis and therapy of glioblastoma having the possibility of being used as ‘Trojan Horses’, and have already been administered intranasally, then milk-derived exosomes will also be potential biological carriers with therapeutic potential and possible to administer intranasally. Moreover, knowing that milk exosomes have already been studied in drug delivery systems for other therapeutic applications and that mitoxantrone has already been applied, with success, in *in vitro* studies in gliomas, then the encapsulation of mitoxantrone in milk exosomes targeting glioblastoma multiforme is also possible.

The aim of this work is to evaluate, *in vitro*, the hypothesis of intranasally delivering milk exosomes with encapsulated mitoxantrone to reach GBM. The studies begin with the physicochemical characterization of the nanoformulation with and without drug. The studies in cell cultures to simulate and evaluate the interaction of the nanoformulation with the nasal epithelium in terms of viability and permeability are performed. Subsequently, the interaction of the nanoformulation with GBM cells is evaluated through cell viability and live cell imaging.

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Chapter III: Materials and Methods

1. Materials

Cow's milk was provided by the Vairão Institute of Agricultural Sciences of the University of Porto (ICAV-UP, Vila do Conde, Portugal). Ethylenediamine tetraacetic acid (EDTA), mitoxantrone hydrochloride and Dulbecco's phosphate buffered saline pH 7.4 (PBS) were obtained from Sigma-Aldrich® (St. Louis, MO, USA). RPMI 2650 cell line was obtained from Cell Line Service (CLS, Eppelheim, Germany). SF-268 cell line was obtained from American Type Culture Collection (ATCC, Salisbury, UK). Dulbecco's Modified Eagle's Medium (DMEM)+GlutaMAX™-1, 0.25% Trypsin-EDTA (1x), Penicillin-Streptomycin (Pen-Strep) and Heat Inactivated Fetal Bovine Serum (FBS) (origin: South America) were purchased from Gibco® by Life Technologies™ (UK). Minimum essential medium Eagle (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1.0 mM sodium pyruvate, and 10% fetal bovine serum was purchased from CLS cell line service (Eppelheim, Germany). Ultrapure water (18.2 MΩ cm) was purified by an Ultra-pure water system (Healforce, Shanghai, China).

2. Preparation of Mitoxantrone-loaded Exosomes

2.1. Optimization of isolation parameters

Centrifugation speed, time, and the use of EDTA were considered for optimization of isolation process. Initially, the centrifugation speed and time were mathematically proposed, following the equations:

$$\frac{T_1}{K_1} = \frac{T_2}{K_2}$$

Equation 1. Equation used to determine centrifugation time by the conversion of a centrifugal run with one rotor to an equivalent run applied with another rotor. T is the centrifugation time, K is the k-factor (clearance factor) for a rotor.

$$k_1 = k_2 * \left(\frac{\text{maximum RPM}}{\text{actual RPM}} \right)^2$$

Equation 2. Equation used to determine centrifugation speed by known clearance factors of rotors. K is the k-factor (clearance factor) for a rotor, RPM is the revolutions per minute, maximum RPM the maximum rotor speed, and actual RPM the pretended centrifugation speed.

Where T is the centrifugation time, K is the k-factor (clearance factor) for a rotor, RPM is the revolutions per minute, *maximum RPM* the maximum rotor speed, and *actual RPM* the pretended centrifugation speed. The first equation was used to determine centrifugation time while the second equation was used to determine centrifugation speed. The protocols were designed to optimize the following parameters: centrifugation speed, centrifugation time, and addition of calcium chelate agents, namely EDTA. For protocol 1, milk was centrifuged at 14,000 g for 30 min at 4°C to remove fat and cell debris and the supernatant was collected and centrifuged at 40,000 g for 60 min at 4°C to remove other particles and then the supernatant was collected and centrifuged at 100,000 g for 120

min at 4°C to obtain the exosomes pellet. For protocol 2, on the second centrifugation, the centrifugation speed was 50,000 *g* and all the other parameters remained equal. Protocol 1 was repeated with the addition of 0.25M EDTA to the supernatant after the second centrifugation step. The suspension was put on ice for 15 min and then centrifuged at 100,000 *g* for 120 min at 4°C. For purification methods, successive filtrations, using a filter holder set with vacuum pump, and 0.22 µm pore size filters (Durapore® PVDF membrane, Millipore), and 0.1 µm filters (Sartorius Minisart™ PES membrane) and syringe pumps were performed.

2.2. Optimized Isolation process

Milk-Exosomes (EXO) were isolated by differential centrifugation using a T29-8 x 50ml Fixed-Angle Rotor (Thermo Scientific, Waltham, MA, USA). Briefly, milk was centrifuged at 14,000 *g* for 30 min at 4°C to remove fat and cell debris and the supernatant was collected. Then, 8 mL of EDTA 25M were added and the solution was put on ice for 15 minutes to extract Ca²⁺ ions. The obtained suspension was centrifuged at 40,000 *g* for 60 min at 4°C to remove other particles and then the supernatant was collected and centrifuged at 100,000 *g* for 120 min at 4°C to obtain the exosomes pellet. The exosome's pellets were washed with PBS one time at 100,000 *g* for 20 min at room temperature, and re-suspended in PBS. The exosome's aliquots were stored at -80°C until being used. To augment the exosomal concentration, three pellets were suspended together in each aliquot. Posteriorly, milk-exosomes were purified by successive filtrations, using a filter holder set with vacuum pump, and 0.22 µm pore size filters (Durapore® PVDF membrane, Millipore), and 0.1 µm filters (Sartorius Minisart™ PES membrane) and syringe pumps.

2.3. Mitoxantrone encapsulation on milk exosomes

For mitoxantrone encapsulation, the exosomes suspension obtained from cow milk's ultracentrifugation was used. Sonication method was used for encapsulation of mitoxantrone in exosomes. Briefly, 1 mL of mitoxantrone (1 mg/mL) was added to 5 mL of exosomes. The suspension was sonicated (pulsed ultrasonication; pulse-on 40 seconds and pulse-off 60 seconds, totaling 3 minutes, at 30 intensity) using a probe sonicator (Sonics and Material Vibra-Cell™ with CV-18 probe; Newtown CT, USA). The sonication process was repeated 3 times, with a cooling period of 3-minutes between each cycle. After sonication, the suspension was incubated at 37°C for 60 minutes to restore the exosomal membrane.

The encapsulation efficiency was determined by a direct method. All suspensions were transferred to tubes with Amicon® Ultra Centrifugal Filter Devices, ultracel®-50 k (50000 NMWL) (MERK Milipore, Ltd; Cork, Ireland) and centrifuged at 3300 rpm for 3 min. Centrifugation was repeated until the entire sample was placed on the filters. The amount retained on the filter was recovered and diluted with ethanol, to destroy the exosome membrane and release the encapsulated mitoxantrone. Then, the 1 mL suspension was centrifuged at 4,500 rpm for 10 min. The supernatant was quantified by measuring the absorbance at 620 nm using the V660 Spectrophotometer (JASCO Corporation, Tokyo, Japan). A

standard curve was performed to determine the amount of drug in supernatant. The encapsulation efficiency was calculated from the following formula:

$$\text{Efficiency of Encapsulation (\%)} = \frac{\text{amount of drug in supernatant (mg)}}{\text{initial amount of drug (mg)}} \times 100$$

Equation 3. Efficiency of encapsulation defined by the amount of drug present in supernatant over the initial amount of drug.

3. Characterization Methods

3.1. Particle size measurement

EXO and exosomal mitoxantrone (EXO-MTX) were characterized by their hydrodynamic diameter and size distribution (polydispersity index) in a Particle Size Analyzer (Brookhaven Instruments Corporation; Software: Particle Sizing v.5 Brookhaven Instruments; Holtsville, NY, USA), operating at a scattering angle of 90°, at 22°C, with dust cut-off set to 30 and refractive index of the particles set to 1.33. Samples were read undiluted, and the measurements performed by 6 runs of 2 min each.

3.2. Nanoparticle Tracking Assay

Detailed nanoparticles size distribution was also determined using nanoparticle tracking analysis (NTA, NanoSight NS300, Malvern Instruments, Worcestershire, UK) equipped with a high sensitivity scientific CMOS camera. Samples were diluted at a ratio of 1:100 and injected using 1 mL sterile syringe in the viewing chamber and measured under constant flow (infusion rate of 50) at room temperature. Size measurements were obtained based on a 20 s video captured with a camera level of 10 and a detection threshold of a 5. For each sample, 5 size measurements were conducted. Data was analyzed using the NTA 3.4 software.

3.3. Bicinchoninic Acid Protein Assay

A PierceTM BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) was used to determine protein concentration of milk exosomes. A standard curve (range 0-2000 µg/mL) was derived with nine points of a serial dilution with bovine serum albumin (BSA) and PBS. All samples and standard points were replicated three times. The samples (25 µL each) were mixed with 0.2 mL working reagent, shaken for 30 s, and incubated at 37°C for 30 min. After cooling to room temperature, absorbance was measured at 562 nm with a microplate reader (BioTek Instruments Inc., Synergy HT, Software: Gen5 v1.08.4. BioTek Instruments Inc.; Winooski, USA) and the absorbance differences were converted to concentrations (mg/mL) via the standard curve.

3.4. CD81 detection

The typical exosomal surface marker CD81 was measured by enzyme linked immunosorbent assay (ELISA) respectively using Exo-ELISA-ULTRA CD81 Kit (System Biosciences, CA, USA) according to the manufacturer's instructions.

3.5. Fourier-transform infrared spectroscopy analysis

Fourier-transform infrared spectroscopy (FTIR) spectra were collected using a FTIR Spectrophotometer (Frontier™, PerkinElmer, Santa Clara, CA, USA) equipped with a diamond crystal. 0.1 mL of sample (EXO, EXO-MTX and mitoxantrone (MTX)) were transferred directly into ATR compartment and a thin dry film was obtained by slow evaporation of the solvent under a nitrogen stream. The process was repeated until 0.5 ml of dry sample was obtained. The measurements were performed at room temperature, immediately after drying the sample. Obtained spectra were a result of 32 combined scans recorded between 4,000 and 600 cm^{-1} with spectral resolution of 4 cm^{-1} .

4. *In vitro* release studies

The *in vitro* release of EXO-MTX was performed using a Slide-A-Lyzer dialysis device (Slide-A-Lyzer™ MINI Dialysis Device, MWCO 10 kDa, Thermo Scientific™, MA, USA). The release profile was assessed in acetate buffer, pH 5.5, to simulate tumor pH. EXO-MTX (0.5 mL) was added into the upper compartment of the dialysis device and 14 mL of the adequate buffer was added to the lower compartment. Devices were then placed in an incubator shaker (ES-60, Lan Technics, USA) at 125 rpm at 37°C for 23 hours. At regular intervals, aliquots of 200 μL were collected from the lower compartment and replaced with 200 μL of fresh buffer heated at 37°C. Mitoxantrone content was then analyzed by UV-Vis spectrophotometer. Absorbance was read using a microplate reader at 610 nm (BioTek Instruments Inc., Cytation 3, Software: Gen 5 v1.08.4, BioTek Instruments Inc.; Winooski, USA).

5. Cellular studies

5.1. Cell culture conditions

SF-268 cells were cultured in DMEM supplemented with 10% FBS (v/v) and 1% Pen-Strep (v/v), while RPMI 2650 cells were cultured in EMEM. Cells were maintained in a 37°C and 5% CO_2 atmosphere (Unitherm CO_2 Incubator 3503 Uniequip; Planegg, Germany). Cells were subculture at 80 to 90% confluence by chemical detachment with trypsin-EDTA and counted in a Neubauer chamber (Improved Neubauer Bright-line, Boeco; Germany) with 50% Trypan Blue solution (0.4% (w/v) in PBS).

5.2. Cell Viability assay

The cell viability was measured using the resazurin assay. RPMI 2650 and SF-268 cells were seeded in 96-well plate at a density of 5×10^4 cell/well in 100 μ L supplemented culture medium and incubated for 24h at 37°C in a 5% CO₂ atmosphere. Different concentrations of free mitoxantrone, mitoxantrone-loaded in exosomes, and correspondent amounts of exosomes and PBS were added, and cells were incubated for 24h. Positive (culture medium) and negative controls (Triton-X-100 2% (v/v) in PBS) were also included. After the incubation time, the supernatant was discarded and 100 μ L of resazurin solution (0.1 mg/mL in culture medium) were then added to each cell seeded well and incubated for 2h at 37°C in a 5% CO₂ atmosphere. Fluorescence was read using a microplate reader (BioTek Instrument Inc., Synergy HT, Software: Gen5 v1.08.4. BioTek Instruments Inc.; Winooski, USA) at 560 nm excitation and 590 nm emission filter. Cell viability was determined according to the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Experimental value} - \text{Mean negative control value}}{\text{Mean positive control value} - \text{Mean negative control value}} \times 100 \quad (4)$$

Equation 4. Percentage of cell viability. Experimental value corresponds to the samples, Negative control was performed with Triton-X-100 2% (v/v) in PBS and Positive control was performed with culture medium.

5.3. RPMI 2650 cell permeability assay

RPMI 2650 cells were seeded on Transwell devices with a density of 1×10^5 cells/insert (1.12 cm² polycarbonate membrane with 0.4 μ m porosity) and growth 7 days in EMEM at LLI conditions. After 7 days under submerged conditions, the apical volume was removed, and the cells were cultivated under ALI conditions for 14 days. For each 2 to 3 days, TEER was measured using an epithelial voltohmmeter (EVOM) from World Precision Instruments (Sarasota, FL, USA) to monitor the integrity of the cell membrane and evaluated tight junctions formation.

After 21 days Cells were then incubated with free mitoxantrone and EXO-MTX on the apical side, at a concentration of 7.65 μ g/mL of mitoxantrone using PBS as a transport media, for 24 hours at 37°C in a 5% CO₂ atmosphere. At different times, 300 μ L aliquots were collected for a 96-well plate from the receptor well and replaced by the same amount of the freshly PBS. A blank PBS solution was used as a negative control. Mitoxantone quantification was performed by UV-Vis spectroscopy using a microplate reader, at a wavelength of 608 nm.

Apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app}(\text{cm/s}) = \frac{Q}{A \times C \times t}$$

Equation 5. Apparent permeability coefficient P_{app} . Q represented the total amount of permeated mitoxantrone (μ g), A is the surface area of the insert (cm²), C is the initial mitoxantrone concentration (g/cm³) and t is the experiment time (s).

Where Q represented the total amount of permeated mitoxantrone (μ g), A is the surface area of the insert (cm²), C is the initial mitoxantrone concentration (g/cm³) and t is the experiment time (s).

5.4. Live cell imaging – SF268 cell line

Time-lapse live-cell imaging of exosomes uptake was performed using confocal laser scanning microscopy (CLSM). SF-268 cells were seeded on 35 mm μ -Dishes (polymer-coated, Ibidi, Munich, Germany) at a density of 9×10^4 cells per dish for 48h. Cell culture dishes were placed in the confocal incubator chamber, at 37°C, 5% CO₂. Exosomal mitoxantrone and free mitoxantrone (0.48 μ g/mL) were then added and immediately observed in CLSM. Images were automatically collected for up to 30 minutes with a time interval of 5 minutes between frames on a Leica Stellaris 8 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a $\lambda_{ex}/\lambda_{em}$ of 610/630 nm (mitoxantrone) with a resolution of 1024x1024 using a 63x/1.4 oil immersion objective. CLSM studies were performed at the High-Resolution Microscopy Unit, CEMUP, Portugal.

6. Statistical Analysis

Experiments were performed at least in triplicate (n=3) and the data obtained is expressed as mean $\pm$ standard deviation (SD). The statistical analysis was made with GraphPad Prism 7.00 software. Data was analysed by Two-way Analysis of Variance (ANOVA) coupled with Tukey HSD analysis to determine statistical significance for multiple comparisons between EXO-MTX, MTX, EXO, and PBS. P values <0.05 were considered statistically significant, where * indicates P<0.05, ** indicates P<0.009 and *** indicates P<0.001. The absence of asterisks means the results were not statistically significant.

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Chapter IV: Results & Discussion

1. Optimization of isolation process

Differential ultracentrifugation was applied to isolate milk-exosomes. To optimize the isolation process, three parameters were manipulated and evaluated, namely, centrifugation time, centrifugation speed, and addition of calcium chelate agents (EDTA). The parameters considered for each protocol are present in Table 2.

Table 2. Proposed protocols for optimization of isolation process. For each protocol, centrifugation speed, time and addition of calcium chelate agents (EDTA) were considered. Centrifugation time and speed for protocol 1 were obtained by mathematical conversions. For protocol 2, centrifugation time and speed were increased in second step. Protocol 1' is a repetition of protocol 1 with the addition of EDTA.

Ultracentrifugation			
Protocol	Centrifugation speeds	Centrifugation time	Addition of Calcium chelate agents (EDTA)
Protocol 1	14,000 g ; 40,000 g ; 100,000 g;	30 min; 1 hour; 2 hours;	no
Protocol 2	14,000 g; 50,000 g; 100,000 g;	30 min; 90 min; 120 min;	no
Protocol 1'	14,000 g; 40,000 g; 100,000 g;	30 min; 60 min; 120 min;	0.25M EDTA

Initially, following centrifugation protocols stipulated for other rotors, the centrifugation parameters were defined for T29-8 x 50ml Fixed-Angle Rotor mathematically. The objective of this step was to transpose the centrifugation speeds and times of optimized ultracentrifugation protocols for rotors, to the T29-8 x 50mL Fixed-Angle Rotor used for this work (Aqil et al., 2019). Therefore, protocol 1 corresponds to an optimized protocol transposed for T29-8 x 50ml Fixed-Angle Rotor. The results for protocol 1 are present in Table 3. The average size of particles for method 1 was 169.5 ± 34.7 nm with a polydispersity index of 0.279 ± 0.025 . The effective diameter of the main population was 16.1 ± 2.4 nm. For protocol 1, the suspension obtained is considered polydisperse (polydisperse index > 0.2). The average size of exosomes is defined between 30-150 nm (Doyle & Wang, 2019). For protocol 1, the average size was higher than the size expected for exosomes and the size of the main population was lower than the size of exosomes. To evaluate if the main population with an average size lower than exosomes were casein micelles, the pH of the suspension was adjusted to 4.6, which is the isoelectric point of casein, and the suspension was centrifuged to verify the existence of casein precipitates (Yamauchi et al., 2020). After centrifugation, the existence of sediments of precipitated casein was observable thus indicating the presence of casein protein in the suspension. Therefore, for method 1, milk exosomes were co-isolated with casein aggregates, compromising the purity of the suspension. Considering the results obtained for protocol 1, protocol 2 was designed to evaluate if alterations in centrifugation time and centrifugation speed could optimize the results (Table 3).

Table 3. Characterization of milk-exosomes from several protocols by sizing through dynamic light scattering. All values presented as mean $\pm$ SD for each protocol, n=5.

Ultracentrifugation	Dynamic Light Scattering		
	Effective Diameter (nm)	Polydispersity Index	Effective Diameter of main population (nm)
Protocol 1	169.5 $\pm$ 34.7	0.279 $\pm$ 0.025	16.1 $\pm$ 2.4
Protocol 2	185.2 $\pm$ 11.8	0.241 $\pm$ 0.008	35.4 $\pm$ 7.5
Protocol 1'	156.7 $\pm$ 3.5	0.106 $\pm$ 0.013	54.0 $\pm$ 3.9

As a second centrifugation is applied to sediment microvesicles/apoptotic bodies and large debris, method 2 was designed with an increase in time and centrifugation speed in the second ultracentrifugation to possibly remove concomitants that were present in the suspension of method 1. For method 2, the average size of particles was 185.2 $\pm$ 11.8 nm and the polydispersity index was 0.241 $\pm$ 0.008. The size of the main population was 35.4 $\pm$ 7.5 nm. For method 2, the average size of the particles obtained was bigger than method 1, casein aggregates were present and the suspension obtained was polydisperse. The extension of centrifugation time and speed applied in method 2, probably enhanced contamination of soluble proteins to vesicular material collected at earlier time points (Cvijetkovic et al., 2014). Therefore, increasing centrifugation time and centrifugation speed was not effective to purify the milk-exosomes suspension.

Lastly, to remove casein micelles, protocol 1 was repeated with addition of EDTA (Table 3). The average size of particles was 156.7 $\pm$ 3.5 nm, and the polydispersity index was 0.106 $\pm$ 0.013. The size of the main population was 54.0 $\pm$ 3.9 nm. The average size is correspondent with the size of exosomes, approximately between 30-150 nm, and the suspension is considered quasi-monodisperse (Doyle & Wang, 2019). The size of the main population corresponds to the size of exosomes, and the precipitation of casein was not observed in this suspension.

The suspensions were posteriorly subjected to purification techniques, namely, vacuum filtration with 0.22 μ m pore size filters and syringe pumps with 0.1 μ m filters to remove possible macromolecules and other vesicles possibly co-isolated with milk-exosomes. Filtration serves as an intermediate step to sieve cells and large extracellular vesicles after the separation of exosomes from proteins is achieved via ultracentrifugation (Gao et al., 2019; Gurunathan et al., 2019; Yamauchi et al., 2020).

The results demonstrate the challenging nature of ultracentrifugation protocols to isolate suspensions of exosomes without concomitants. The pellets obtained after ultracentrifugation contain exosomes as well as other macromolecules, vesicles and protein aggregates. Protocols differ in centrifugation speed, time and chelating agents. Moreover, protocol 1 with the addition of EDTA coupled with filtration techniques was proposed for the optimized method.

The hypothesis of the formation of casein micelles along with the existence of aggregates of casein and/or exosomes coated with casein during ultracentrifugation steps was proposed as casein micelles in milk are formed during phase separation (Farrell, 1973; Obeid et al., 2019). Casein micelles in milk consist of a phosphoprotein complex constituted by four isoforms of caseins and proteins, 93% are micelles and 7% inorganic components (Ca^{2+}). Casein micelles of bovine milk exist as polydisperse,

large, roughly spherical colloidal particles, with an average size between 50-600 nm in diameter (Hristov et al., 2016). The EDTA is a chelating agent which reacts with the calcium ions in dentine and forms soluble calcium chelates (Mohammadi et al., 2013). Therefore, by adding EDTA to the solution, the precipitation of casein micelles and exosomes coated with casein and successive sedimentation is enhanced during the isolation process, thus providing more pure suspensions of milk exosomes (Vaswani et al., 2017). However, the addition of EDTA may diminish the total protein concentration resulting in the solubilization of protein agglomerations and exosomes coated with casein, thus decreasing the exosomal abundance and the total protein concentration necessary for further experiments. Therefore, a last optimization step was performed, where 3 pellets of milk-exosomes were re-suspended in each aliquot to augment the total protein concentration which implies that the characterization of optimized milk-exosomes was performed for the suspensions containing 3 pellets per aliquot.

2. Characterization of bovine milk-Exosomes and Exosomal Mitoxantrone

2.1. Encapsulation of Mitoxantrone in optimized milk-Exosomes

Mitoxantrone was encapsulated in exosomes using the sonication technique. To load mitoxantrone into exosomes, 0.167 mg/mL mitoxantrone was used. After removal of unbound mitoxantrone by dialysis during exosome purification, where the amount retained on the filter was recovered and diluted with ethanol, to destroy the exosome membrane and release the encapsulated mitoxantrone. To determine the encapsulation efficiency, a standard calibration was performed to determine the amount of mitoxantrone in the supernatant (see annexes; Figure S 1) and then divided by the initial amount of 1 mg mitoxantrone added. The efficiency of encapsulation (%) was 55.3 ± 6.4 (n=3).

According to the literature, sonication has been the most efficient method for encapsulating exosomes, reaching at least 40% encapsulation efficiency (M. S. Kim et al., 2016; Vashisht et al., 2017).

It has been suggested that, during sonication cycles, the bubbles formed generate vibrational waves that release energy and cause disruption of the exosome membrane, allowing mitoxantrone to diffuse along the highly structured lipid bilayer during the reorganization of the exosome membrane, becoming entrapped in the exosomal membrane during incubation used to re-stabilize the exosome membrane (M. S. Kim et al., 2016).

Considering the therapeutic potential of exosomes as drug delivery systems, efficient encapsulation in milk-exosomes is desirable for clinical applications. Moreover, mitoxantrone is an alkylating agent whose interactions with the plasmatic membrane of non-neoplastic cells may result in cytotoxicity, as with cardiomyocytes when applied systemically (G. X. Wang et al., 1999). The encapsulation of mitoxantrone in exosomes, a nanoparticle-based drug delivery, may reduce the toxicity and modify the pharmacokinetics and biological distribution, thus, reducing the side effects of the drug (Odiba et al., 2016).

2.2. Sizing of optimized milk exosomes and Exosomal Mitoxantrone

EXO samples were isolated from mature cow's fresh milk by ultracentrifugation and purified by filtration. EXO and EXO-MTX samples were analyzed by particle size distribution, using Dynamic Light Scattering (DLS) and Nanoparticle tracking assay (NTA). The results are present in Tables 4 and 5, respectively. Regarding DLS, EXO samples had an average particle size of 157.1 ± 2.9 nm, a polydispersity index of 0.086 ± 0.035 and an average size in the main population of 88.7 ± 60.1 nm, while EXO-MTX had an average particle size of 255.9 ± 42.6 nm, a polydispersity index of 0.255 ± 0.127 and an average size in the main population of 140.9 ± 9.8 nm. The increased average size of EXO-MTX corroborates the presence of mitoxantrone entrapped in the exosomal membrane (Table 4).

Moreover, the average size of the main population is slightly lower for both suspensions, compared to the average particle size of the samples.

Table 4. Characterization of milk-exosomes (EXO) and exosomal mitoxantrone (EXO-MTX) by sizing through dynamic light scattering. EXO-MTX presents increased size and polydispersity index of exosomes. All values presented as mean $\pm$ SD for n=3.

Dynamic Light Scattering			
Sample	Effective Diameter (nm)	Polydispersity Index	Effective Diameter of main population (nm)
EXO	157.1 ± 2.9	0.086 ± 0.035	88.7 ± 60.1
EXO-MTX	255.9 ± 42.6	0.255 ± 0.127	140.9 ± 9.8

The DLS provides only an indicative size of the exosomes by measuring the particle size from the correlation between the light intensity in a small-time scale allowing the determination of the diffusion coefficient and the hydrodynamic radius of the exosome. The hydrodynamic radius is the radius of the hypothetical hard sphere that diffuses with the same speed as each particle assayed under DLS (Bhattacharjee, 2016). Uncontrollable aggregation of exosomes may occur, affecting the size measurements with DLS. In this case, it seems that aggregation does not occur as EXO formulation is quasi-monodisperse, thus indicating a homogeneous suspension. EXO-MTX presents polydispersion which indicates the existence of a range of particles with a range of molecular weights and thus, sizes. The measurement of EXO-MTX showed 3 populations in a range size of 50-1000 nm. Moreover, as the efficiency of encapsulation was 55%, the existence of free-mitoxantrone in solution is possible and, as the absorbance frequency of mitoxantrone is the same of the light frequency applied, instead of scattering, an absorbance phenomenon may occur and interfere with the photons reaching the detector. To evaluate this hypothesis, EXO-MTX was purified through dialysis method to remove the free mitoxantrone present in suspension. In fact, sample purification was not effective as the presence of the subpopulation in the 1000 nm range was observable. Therefore, the subpopulation in the 1000 nm range, which interferes with the average size of EXO-MTX, may arise due to an absorbance phenomenon caused by mitoxantrone entrapped in the exosomal membrane.

To confirm the indicative size of EXO and EXO-MTX obtained with DLS, NTA assay was performed (Table 5). The NTA assay is useful to verify the measurements obtained by DLS, as the NTA assay tracks the movement of individual nanoparticles to measure the diffusion coefficient. The mean size of EXO was

148.6 ± 1.1 nm, and the mode was 135.1 ± 5.9 nm while the mean size of EXO-MTX was 181.0 ± 3.4 nm and the mode was 144.3 ± 9.0 nm. Furthermore, in NTA assay, the average particle size is calculated by summing all measured diameter values divided by the total number of suspended particles. The mode represents the most frequent size value in the suspension and is then obtained by the frequency with which this value appears. The different average sizes obtained for EXO-MTX by NTA and DLS demonstrate that the sample is not perfectly monodisperse, as described by polydispersion index, while the proximity between average sizes obtained for EXO samples is indicative of a quasi-monodisperse suspension. Additionally, the results present a mean value higher than the mode. In fact, the interquartile 90 (D90) indicates that 10% of the particles in the suspension have diameters much higher than the average value which could be to possible noise or the existence of agglomerates of exosomes. Moreover, the average size of D90 demonstrates that the possible existence of agglomerates of exosomes may be interfering with the DLS measuring as particles with higher hydrodynamic radius scatter light more intensely thus having a greater contribution to the average size.

Table 5. Characterization of milk-exosomes (EXO) and exosomal mitoxantrone (EXO-MTX) by sizing through nanoparticle tracking assay. The encapsulation of mitoxantrone increased the size of milk-exosomes. All values presented as mean ± SD for n=3.

Nanoparticle Tracking Assay						
Sample	Mean (nm)	Mode (nm)	SD (nm)	D10 (nm)	D50 (nm)	D90 (nm)
EXO	148.6 ± 1.1	135.1 ± 5.9	49.9 ± 1.7	82.8 ± 6.2	142.7 ± 2.6	216.5 ± 7.6
EXO-MTX	181.0 ± 3.4	144.3 ± 9.0	64.0 ± 3.2	121.3 ± 1.3	160.5 ± 4.0	280.4 ± 8.7

Additionally, the size distribution for NTA assay, and imaging of EXO and EXO-MTX samples are shown in Figure 2. The overlap of exosomes was observed in the nanoparticle tracking assay video's performed (Figure 2B).

The size distribution for EXO (Figure 2A) is correlating with the polydispersity index, demonstrating that the suspension is quasi-monodisperse. The sizing characterization revealed that the sonication increased the size of the formulations, as the EXO-MTX size was increased. The stabilization of exosomal membranes with the incorporated MTX, after sonication, may contribute to the increased size. Additionally, the size distribution of EXO-MTX demonstrates interesting results (Figure 2C), denoting that EXO-MTX might be quasi-monodisperse and thus, the results for DLS assay might have the interference of the absorbance of mitoxantrone. Moreover, the literature reports that sonication affects the structure and surface of nanoparticles, breaking down the large agglomerates of nanoparticles into individual nanoparticles and leading to the prevention of the formation of agglomerates thus, resulting in more stable nanofluids (Asadi et al., 2019).

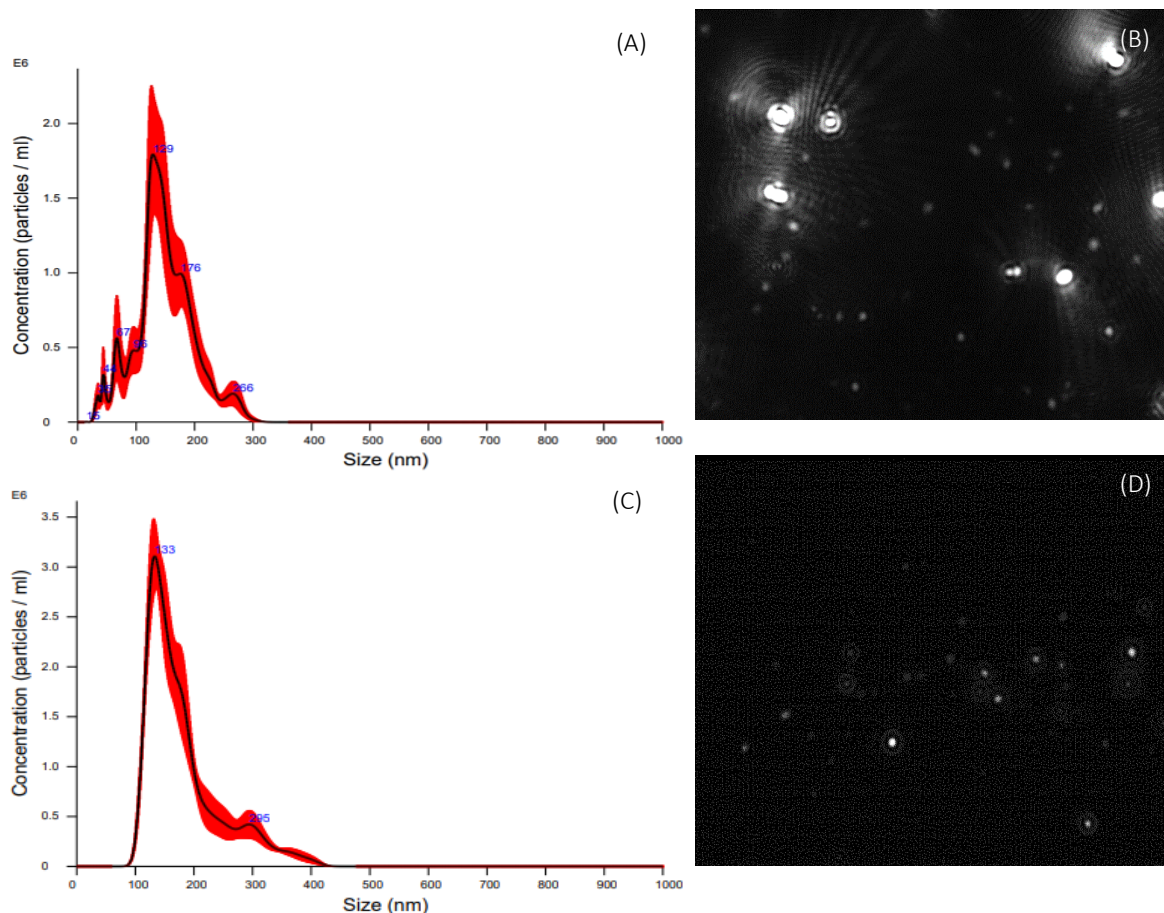


Figure 2. Characterization of milk-exosomes (EXO) and exosomal mitoxantrone (EXO-MTX) by sizing and imaging through nanoparticle tracking assay. In (A) and (C) the size distribution versus the concentration (particles/mL) for EXO and EXO-MTX, respectively. In (B) and (D) imaging made with nanoparticle tracking assay for EXO and EXO-MTX, respectively. All values presented as mean $\pm$ SD for $n=3$.

The stability test was assessed by measuring the average size variation through time, using DLS. The stability evaluation was made considering sizing measurements since co-precipitate proteins and lipoproteins present in suspension may distort the concentration measurements (J. Wang et al., 2021). For stability tests, aliquots of exosomes were performed to minimize the freeze-thaw cycles. The samples were stored at -80°C and pH 8 (due to the EDTA). The stability test was conducted for 3 months. The results are present in Table 6. The average size of EXO at day 1 was 159.7 ± 7.8 nm, the polydispersity index was 0.106 ± 0.0134 and the size of the main population was 46.6 ± 6.6 nm. After 1 month, the average size of EXO was 158.2 ± 1.3 nm, the polydispersity index was 0.108 ± 0.017 and the average size of the main population was 49.5 ± 10.3 nm. After 3 months, the average size of EXO was 172.5 ± 2.3 nm, the polydispersity index 0.120 ± 0.044 and the average size of the main population was 100.93 ± 64.13 nm. A slight increase in size and polydispersion is observed. The size of the main population increased, thus indicating the possibility of diminished resistance to aggregation of exosomes. The stability of exosomes is evaluated by resistance to aggregation, loss of structure, and protein degradation. Bovine milk exosomes are expected to be stable through time. However, storage parameters influence the stability, namely, temperature, time, freeze-thaw-cycles, and pH. Regarding the pH, some authors demonstrated a greater loss of exosomal protein and concentration at pH 10 (Cheng et al., 2019). Milk-exosomes can be stored at -80°C for long periods, however, the suspensions

obtained presented instability after 3 months, where the sizing variations were observable. Therefore, the lifetime of the aliquots of milk-exosomes used should not exceed the 3 months mark.

Table 6. Long-term stability assay. The characterization of milk-exosomes (EXO) by dynamic light scattering was made on day 1 after the isolation process. Milk-exosomes stored in aliquots and characterized after 3 months demonstrated an increase in size and polydispersity index. All values presented as mean $\pm$ SD for n=3.

Dynamic Light Scattering			
Sample	Effective Diameter (nm)	Polydispersity Index	Effective Diameter of main population (nm)
EXO (day 1)	159.8 $\pm$ 7.8	0.106 $\pm$ 0.0134	46.6 $\pm$ 6.6
EXO (1 month)	158.2 $\pm$ 1.3	0.108 $\pm$ 0.017	49.5 $\pm$ 10.3
EXO (3 months)	172.3 $\pm$ 2.33	0.120 $\pm$ 0.044	100.9 $\pm$ 64.1

2.3. Protein quantification of milk-Exosomes

The protein composition of milk exosomes was initially analyzed by bicinchoninic acid assay (BCA) to determine the total protein concentration. Subsequently, the quantification of the cluster of CD81 protein, present in the exosomal membranes, was performed by ELISA to determine the abundance of exosomes present in the solution. For BCA and ELISA, a standard curve was obtained to calculate the total protein concentration and exosome abundance, respectively (see annexes; figure S2).

The total quantification of protein was performed for 1 pellet of exosomes to determine the protein concentration obtained in 35 mL of milk and compare to the total protein concentration of the suspensions from the optimized protocol. The results are present in Table 7. For 1 pellet, the total protein concentration was 0.053 $\pm$ 0.008 mg/mL. However, for further experiments, it was necessary to optimize the isolation protocol to increase the total concentration of exosomes per aliquot. The total protein concentration was quantified by BCA, obtaining a concentration of 0.153 $\pm$ 0.006 mg/mL corresponding to the minimum concentration necessary for *in vitro* release assays.

Interestingly, according to the literature, the total protein concentration obtained per pellet by the isolation protocol (centrifugation + vacuum filtration) is congruent with the total protein concentration obtained from SEC which, as mentioned, corresponds to the isolation method with higher degree of purification (Sidhom et al., 2020; Vaswani et al., 2017). Regarding the ultracentrifugation protocols, several authors report higher protein concentrations (Hata et al., 2010; Munagala et al., 2015). The variations of total protein concentration may be due to the addition of EDTA in solution, which may solubilize protein agglomerations. Additionally, the BCA assay is used to quantify the total protein concentration of a biological sample. The BCA principle is based on the formation of Ca²⁺-protein complexes under alkaline conditions, followed by the reduction of Cu²⁺ to Cu¹⁺ thus quantifying the total protein concentration (Smith et al., 1985). In this way, the total amount of protein may correspond to milk exosomes co-isolated with other bioactive compounds such as protein aggregates (Yuana et al., 2013). Thus, proteomic analysis assays to quantify the abundance of exosomes are necessary.

Table 7. Protein quantification of milk-exosomes by Bicinchoninic acid (BCA) assay performed for milk-exosomes after the isolation process stored 1 pellet/aliquot and after the optimization to increase the total protein concentration, 3 pellets/aliquot. All values presented as mean $\pm$ SD for n=3.

Sample Total protein content (mg/mL)	Bicinchoninic acid assay	
	Aliquot with 1 pellet	Aliquot with 3 pellets
	0.053 $\pm$ 0.0080	0.153 $\pm$ 0.006

Therefore, ELISA was performed on the 3-pellets aliquots to evaluate the exosome abundance in suspension. Antibodies designed against CD81 (anti-CD81) were applied to evaluate the affinity of the particles present in solution to a specific membrane-protein of exosomes. A calibration curve was performed to quantify the exosome abundance (see annexes; Figure S 2). Moreover, NTA assay was also performed to quantify the abundance of exosomes in suspension. The results for exosome abundance obtained by both methods are present in Table 8. The exosome abundance for ELISA was $6.89\text{e}+09 \pm 1.57\text{e}+09$ particles/mL and for NTA was $1.57\text{e}+08 \pm 8.03\text{e}+06$ particles/mL. The concentration measurements for NTA may require some caution due to noise which could explain the lower values for exosome abundance when compared to ELISA.

The ELISA applied relies on the principle of the indirect method. Indirect ELISA consists of the incubation of exosomes in a microplate, with sequential incubation of a primary antibody designed to interact with the exosomal membrane, enzyme-linked secondary antibody, and a substrate to elicit a fluorescent signal on the enzymes on the secondary antibody (Koenig, 1981). Despite the inability to determine the affinity of the CD81 and the exosome, ELISA has been widely used in the clinic for the detection of several proteins (Khodashenas et al., 2019). Moreover, ELISA could be designed for the detection of specific exosomes using antibodies against conserved exosomal proteins in milk, being possible to characterize and compare milk exosomes of several species (Khodashenas et al., 2019).

The protein analysis indicates that BCA is a reliable method to quantify the total amount of protein, while ELISA can be more suitable to identify the existing milk exosomes within the isolated milk.

Table 8. Protein quantification of milk-exosomes by nanoparticle tracking assay (NTA) and enzyme linked immunosorbent assay (ELISA). The results from NTA may contain noise interfering with values. All values presented as mean $\pm$ SD for n=3.

Method	NTA	ELISA
Concentration (particles/mL)	$1.57 \text{ e}+08 \pm 8\text{e}+06$	$6.89\text{e}+09 \pm 1.57\text{e}+09$

2.4. Fourier-transform infrared spectroscopy analysis

The protein concentration of MTX, EXO, and EXO-MTX obtained was very low. Therefore, the intensities of infrared (IR) absorption bands of PBS molecules would interfere with that of the samples. Consequently, the subtraction of pure PBS spectra was required to further analysis. Representative IR spectra of MTX, EXO, and EXO-MTX are presented in Figure 3.

The bands presented in MTX spectra represent the chemical structure of the drug. The transmittance band at 3276 cm^{-1} , correspond to the O-H stretching vibrations. The small bands at 2852 cm^{-1} , and

2850 cm^{-1} , may be due to the acyl CH_2 groups while the bands at 1742 cm^{-1} , 1542 cm^{-1} , and 1478 cm^{-1} correspond to the aromatic esters and amine groups and the secondary amines, arising from N-H stretching vibrations and aromatic ring. The region of 1200 - 800 cm^{-1} arises aromatic esters, namely from the stretching vibrations of C=O.

Regarding EXO spectra, the main features are two amide bands, around 1652 cm^{-1} , originating from C=O stretching vibrations of the protein-peptide backbone, and 1538 cm^{-1} , arising from N-H bending vibrations of the peptide groups. The strong band at 3370 cm^{-1} corresponds to a superposition of -OH stretching vibrations and N-H stretching vibrations of the peptide groups of proteins. Several lipid absorptions appear around 1706 cm^{-1} , 2970 cm^{-1} , 2936 cm^{-1} , and 2874 cm^{-1} . The band at 1706 cm^{-1} , corresponds to ester groups of phospholipids, triglycerides, and cholesterol esters while the absorptions at 2970 cm^{-1} , 2936 cm^{-1} , and 2874 cm^{-1} correspond to the dominant antisymmetric and symmetric stretching vibrations of the lipid acyl CH_2 groups. The bands from the spectral region of 1200-850 cm^{-1} are originating from the stretching vibrations of the phosphodiester groups of phospholipids and to the C-O-C stretching vibrations of phospholipids, triglycerides and cholesterol esters.

The spectrum of EXO-MTX shows the main characteristic bands of EXO and MTX. The bands of the spectral region of 3500-3300 cm^{-1} , corresponds to an overlap of O-H stretching vibrations of MTX and N-H stretching vibrations of the peptide groups of proteins of milk-exosomes. The bands of the dominant antisymmetric and symmetric stretching vibrations of the lipid acyl CH_2 groups of EXO are shown in 2922 cm^{-1} spectra along with the stretching vibrations of the acyl CH_2 groups of MTX at 2850 cm^{-1} . The spectra region of 1750 – 1400 cm^{-1} shows a preferential absorption at the band 1630 cm^{-1} and 1548 cm^{-1} corresponding to the amide bands of EXO, also a preferential absorption at the band 1742 cm^{-1} and 1458 cm^{-1} corresponding to the aromatic esters and amine groups and the secondary amines of mitoxantrone.

The spectra region of 1200-850 cm^{-1} preferential absorption of phosphodiester groups of phospholipids, triglycerides and cholesterol esters of exosomes at the band 1186 cm^{-1} and overlapped bands of stretching vibrations of aromatic esters from MTX and the stretching vibrations of the phosphodiester groups of phospholipids and the C-O-C stretching vibrations of phospholipids, triglycerides and cholesterol esters of EXO. The variation of the bands verified at EXO-MTX could be associated with the transition dipole formed between the non-polar region of exosomes, namely the hydrophobic region of the membrane and the mitoxantrone, indicating the entrapment of the drug in the exosomal membrane after sonicating the sample as the sonication is a method that disrupts the exosome membrane. The possible structure of EXO-MTX indicates that after the sonication, mitoxantrone is entrapped in the membrane of the exosome and adsorbed to the surface of the exosome.

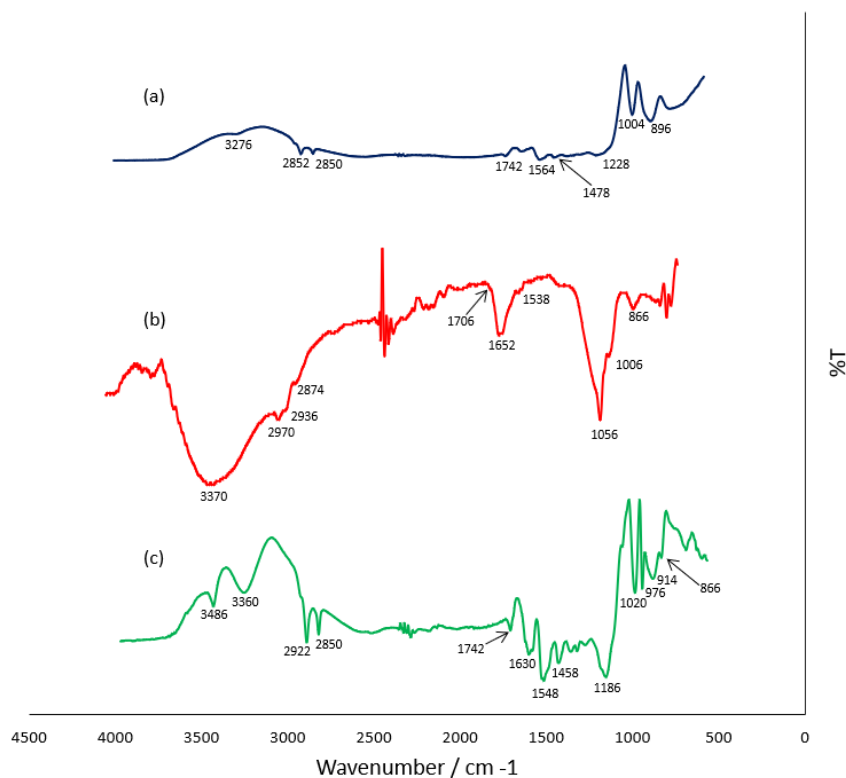


Figure 3. Representative FTIR spectra of samples after PBS buffer subtraction. In (a) mitoxantrone (MTX), (b) milk-exosomes (EXO), and (c) exosomal mitoxantrone (EXO-MTX).

3. *In vitro* release studies

In vitro release studies were performed by dialysis method, using an acetate buffer pH 5.5, to mimic the extratumoral microenvironment. According to the literature, the milk exosomes would be rapidly transported intact from the nasal cavity, making the mitoxantrone available in the tumor (Perets et al., 2018). The *in vitro* release study was performed in less than 24 hours, at defined timepoints. The results of the *in vitro* release study are shown in Figure 4. After 6 hours, all free drug was released while the total release of EXO-MTX only occurred at the end of 21 hours (the total release may have occurred between hour 7 and hour 21).

Drug release from a lipidic matrix follows two main mechanisms: diffusion of drug and degradation *in vivo* of the lipid by enzymes (Rawal & Patel, 2018). Regarding MTX, the initial burst release observed may be due to high drug solubility or high accumulation of MTX near the surface of the dialysis membrane. Moreover, for EXO-MTX the existence of an initial rapid release observed between hours 2 and 5, and hour 6 and 7, suggests that part of the drug may be adsorbed to the exosomal membrane; moreover, the sustained release observed between hour 7 and 23 may be attributed to the slow release of MTX entrapped within the membrane of exosomes. Additionally, several parameters inherent to the formulation can influence the release of MTX from exosomes, namely, physicochemical properties of the drug and exosomes, drug:lipid ratio, parameters used during sonication, bioactive compounds present in the solution of milk exosomes (Rawal & Patel, 2018).

However, regarding the method for the release assay used, despite dialysis being a simple and economical method for measuring drug release *in vitro*, the release profile obtained for the EXO-MTX

formulation may not exclusively reflect the drug release from exosomes, since the burst releases observed may be the release of bioactive compounds (proteins, nucleic acids and/or lipids) existing on the exosome membrane, making it difficult to predict whether the initial burst release was due only to drug adsorbed to the surface of the exosome membrane (J. Wang et al., 2021b).

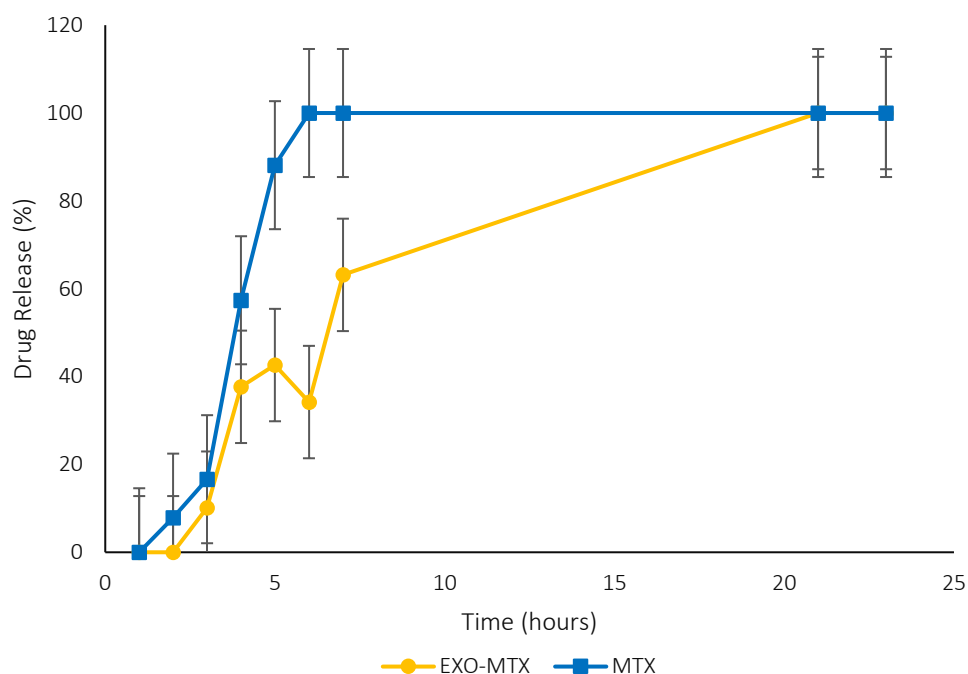


Figure 4. Release profile for mitoxantrone (MTX) and exosomal mitoxantrone (EXO-MTX) obtained by sonication. All values presented as mean $\pm$ SD for n=3.

4. Cellular studies

4.1. RPMI 2650 cell line: nasal mucosa model

The RPMI 2650 cell line is derived from an anaplastic squamous cell carcinoma of the human nasal septum which closely resembles to the normal human nasal epithelium, being able to mimic the nasal epithelium. RPMI 2650 cell line was used to mimic the nasal epithelium for applied studies of cell viability and permeation assay.

Initially, cell viability in the presence of EXO-MTX, MTX, EXO and PBS, was evaluated, through the resazurin assay for concentrations between 30.48 – 0.48 $\mu\text{g/mL}$ with a dilution factor 2. Cell viability results are shown in Figure 5. Cells were considered viable when cell viability (%) was higher than 80%. RPMI-2650 cells presented good viability regardless of the concentration of PBS, EXO and EXO-MTX. However, MTX was significantly different, as non-viable values were observable for the concentrations 30.48 $\mu\text{g/mL}$ and 15.24 $\mu\text{g/mL}$.

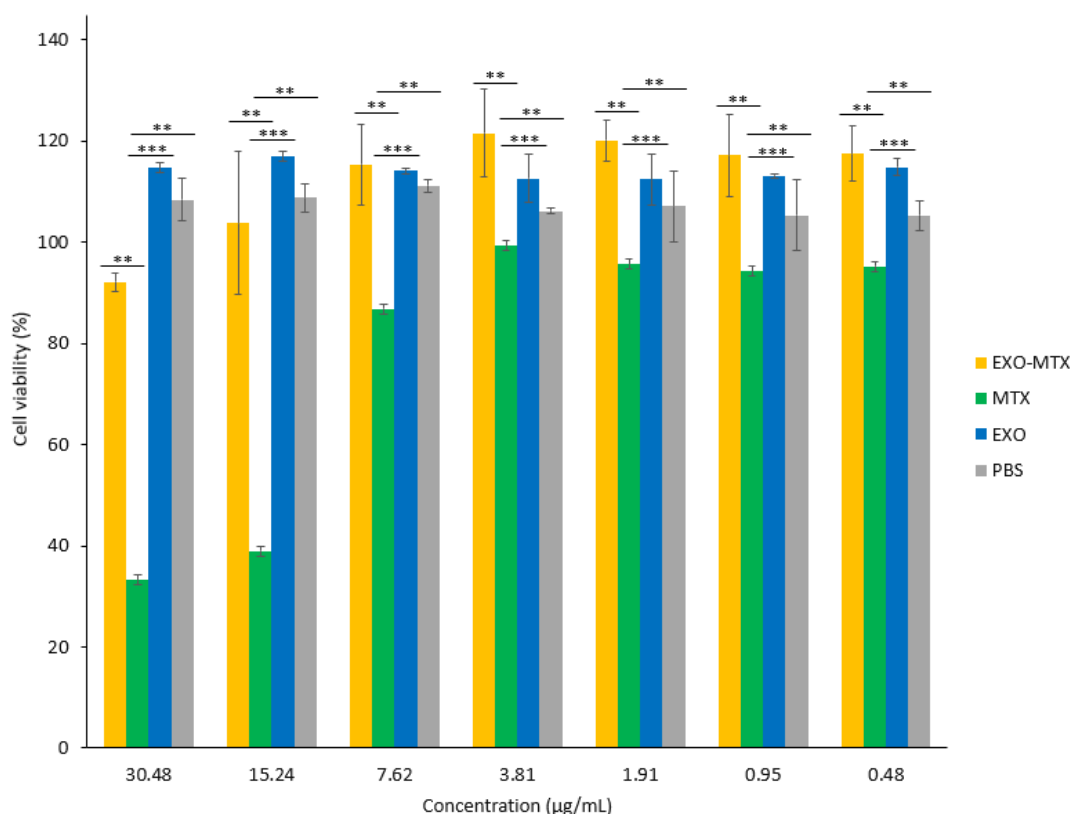


Figure 5. Cell viability analysis by resazurin assay for EXO-MTX, MTX, EXO, and PBS at concentrations between 30.48 – 0.48 µg/mL. RPMI-2650 cells remained viable after being incubated with EXO-MTX, EXO, and PBS. For MTX, RPMI-2650 cells were considered viable at 7.62 µg/mL. Results were statistically significant where * indicates $P < 0.05$, ** indicates $P < 0.009$ and *** indicates $P < 0.001$.

Additionally, the cell viability was evaluated at various concentrations to select a non-cytotoxic concentration common to the four samples to evaluate the transport efflux through cell permeability assays, as the nasal tissue is not the target tissue for the nanoformulation designed. Therefore, after selecting a non-cytotoxic concentration for both EXO-MTX and MTX (7.62 µg/mL) a RPMI 2650 permeability assay was performed.

To ensure that the RPMI 2650 cells form a cell monolayer with representative tight junctions, the cells were cultured in LLI conditions for 8 days and transferred to ALI conditions at day 8 of culture. TEER was measured throughout the culture period to determine monolayer integrity. For 12 monolayers, the TEER measurements varied between $127 \pm 5 \Omega \cdot \text{cm}^2$ to $140 \pm 3 \Omega \cdot \text{cm}^2$ (see annexes; Figure S 4). In the literature, it is described that for RPMI-2650 cells in ALI conditions, TEER values vary between 80 – 200 $\Omega \cdot \text{cm}^2$ (Charles et al., 2019). TEER values of human nasal mucosa typically range from 60 to 180 $\Omega \cdot \text{cm}^2$, which is considerably lower when compared to other barriers, namely intestinal and pulmonary mucosa, due to the leaky nature of nasal mucosa (Mercier et al., 2018). Thus, the RPMI cell cultures in ALI conditions exhibited TEER values similar to the human nasal mucosa.

Therefore, the permeability assay was performed to evaluate the transport of MTX and EXO-MTX. A standard control was made with a buffer (PBS). The transport through RPMI-2650 cell monolayer was measured through the quantification of the amount of sample present at the basal side at defined timepoints. The amount of sample quantified (permeability (%)) on the basal side is presented in Figure

6. At hour 8, 94 ± 7 % of EXO-MTX and 69 ± 25 % of MTX were present on the basal side. Moreover, for EXO-MTX the transport through RPMI-2650 cell monolayer initiated between hour 0 and 1, while for MTX the transport initiated between hour 1 and 2. For drug transport studies, the paracellular barrier formed by RPMI 2650 cell line can be evaluated using TEER measurements, where values for human nasal mucosa, for paracellular transport, varies between $75\text{-}100 \Omega\cdot\text{cm}^2$ or $90\text{-}180 \Omega\cdot\text{cm}^2$ (Reichl & Becker, 2012). TEER measurements were performed during the permeation assay, and varied between $100.0 \pm 0.00 - 131.25 \pm 11.09 \Omega\cdot\text{cm}^2$ for EXO-MTX, $100.0 \pm 0.00 - 125.00 \pm 5.77 \Omega\cdot\text{cm}^2$ for MTX and $100.0 \pm 0.00 - 121.25 \pm 2.50 \Omega\cdot\text{cm}^2$ for PBS (see annexes; Figure S 5). TEER measurements were congruent and indicated the presence of paracellular drug transport through the RPMI 2650 cell monolayer.

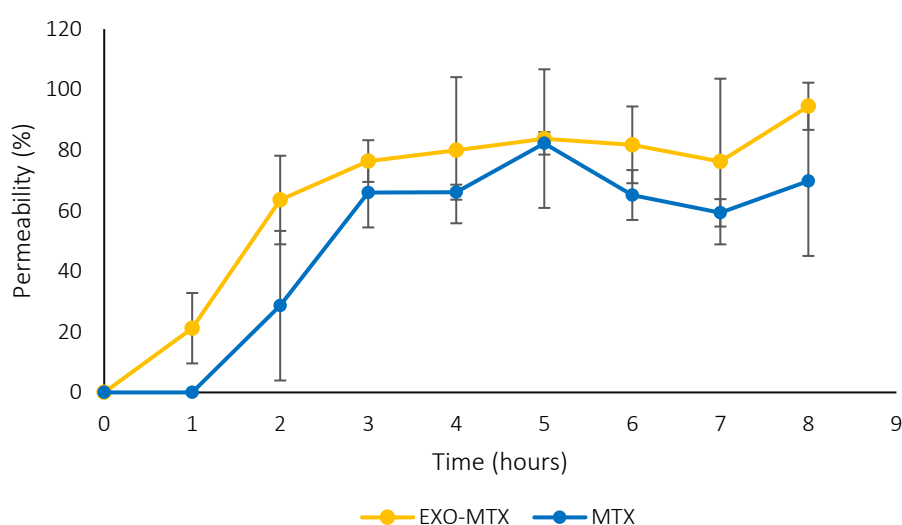


Figure 6. Permeability profile of exosomal mitoxantrone (EXO-MTX) and mitoxantrone (MTX) in RPMI-2650 cell monolayer. All values presented as mean $\pm$ SD for $n=3$.

Several authors have demonstrated that for respiratory tract drug permeability, the type of application of the test materials, such as solutions or dispersions have a great influence on the outcome of a permeability assay, and thus, a suspension of milk-exosomes could influence the permeability of RPMI-2650 cell monolayer to mitoxantrone (Gonçalves et al., 2016). The apparent permeability (P_{app}) was calculated with the concentrations of EXO-MTX and MTX at the basolateral side at hour 8. The results are present in Table 9. The apparent permeability in the apical-to-basolateral direction for EXO-MTX was $2.5 \pm 0.2 \times 10^{-6} \text{ cm/s}$ while the apparent permeability in the apical-to-basolateral direction of MTX was $0.3 \pm 0.1 \times 10^{-6} \text{ cm/s}$.

Table 9. Apparent permeability coefficient (P_{app}) of exosomal mitoxantrone (EXO-MTX) and mitoxantrone (MTX) in RPMI-2650 cell monolayer. All values presented as mean $\pm$ SD for $n=3$.

P_{app} ($\times 10^{-6} \text{ cm/s}$)	
EXO-MTX	2.5 ± 0.2
MTX	0.3 ± 0.1

The differences of permeability in Figure 6 demonstrate that EXO-MTX may have enhanced permeability due to the mucus penetration behavior of milk-exosomes, thus demonstrating the impact of applying mitoxantrone in a suspension of exosomes (Warren et al., 2021). A few studies of permeability values on RPMI 2650 model have been made. The P_{app} values for dextran FD-4 varied between 24.8 and 37.7×10^{-7} cm/s on RPMI-2650 cell model while 9400 MW dextran was shown to permeate rabbit nasal mucosa with a P_{app} of 6.77×10^{-7} cm/s (Kreft et al., 2015). The P_{app} values for propranolol, a lipophilic beta blocker, varied from 16×10^{-6} to 31×10^{-6} cm/s on RPMI-2650 model and 20×10^{-6} cm/s through an excised nasal mucosa (Mercier et al., 2018). The P_{app} of ketoprofen encapsulated into alginate:carrageenan microparticles presented the highest permeability, namely 1×10^{-5} , suggesting a better permeation of the drug (Gonçalves et al., 2016). Comparing the permeability values obtained, EXO-MTX and MTX would be considered to have an effective permeation capacity through RPMI-2650 model, congruent with results obtained by several authors. However, the results for permeability of EXO-MTX and MTX may be influenced by external factors related to RPMI-2650 cell barrier as the mucus properties, expression of tight and adherent junction proteins, drug transports expression, among others, thus explaining the standard deviations present in Figure 6.

The RPMI-2650 model still has several limitations which can interfere with the relevance of drug transport studies. Further studies to characterize the composition of secreted mucus in RPMI-2650 cell line and to standardize data concerning the drug transporter functionality and permeability of reference are necessary to better understand the type of interactions between drug delivery systems and the mucus present at the cells surface and to determine the kinetics parameter of standard compounds for nasal transport (Gonçalves et al., 2016; Mercier et al., 2018). Additionally, ALI culture RPMI-2650 cells show similar thickness as nasal mucosa being only composed of squamous cells instead of the four cell types of the human nasal epithelium – ciliated, goblet, columnar and basal cells – thus, directly impacting the physiology of RPMI 2650 model, specifically in the simulation of mucocilliary clearance present *in vivo* (Gonçalves et al., 2016; Mercier et al., 2018).

4.2. SF-268 cell line: GBM model

The SF-268 cell line corresponds to a primary glioma (high-grade astrocytoma/glioblastoma) of human origin (female, 24 years old), usually applied for the discovery and development of anticancer drugs. The SF-268 cell line was used to initialize the evaluation of the ability of milk-exosomes as nanocarriers of mitoxantrone for the treatment of glioblastoma, via intranasal administration. The resazurin assay was performed to evaluate cell viability for EXO-MTX, MTX, EXO and PBS, at several concentrations, where the initial concentration corresponds to the non-cytotoxic concentration applied for RPMI-2650 cell permeability assay. Moreover, the concentrations evaluated in cell viability assay varied between $7.62 - 0.24 \mu\text{g/mL}$. The results for cell viability are present in Figure 7. Cells were considered viable when cell viability (%) was higher than 80%.

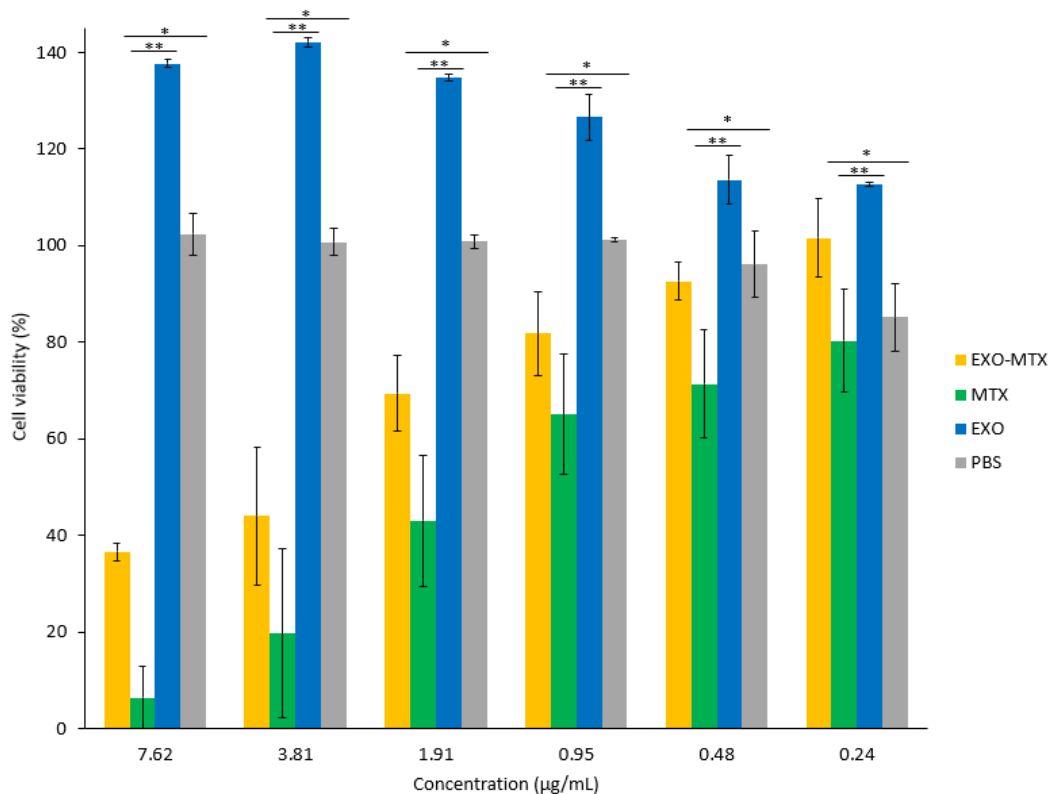


Figure 7. Cell viability analysis by resazurin assay for EXO-MTX, MTX, EXO, and PBS at concentrations between 7.62 – 0.24 µg/mL. SF-268 cells remained viable after being incubated with EXO and PBS. For EXO-MTX, SF-268 cells were considered viable at 0.95 µg/mL. For MTX, SF-268 cells were considered viable at 0.48 µg/mL. Results were statistically significant where * indicates $P < 0.05$, ** indicates $P < 0.009$.

As with the RPMI-2650 cell line, SF-268 cells presented an overall good cell viability for EXO and PBS. Additionally, for EXO-MTX, SF-268 cells were not viable between the concentrations of 7.62 – 1.91 µg/mL, while for MTX, cells were not viable between the concentrations of 7.62 – 0.95 µg/mL. Moreover, the results for MTX were significantly different from the results of EXO and PBS.

Interestingly, for RPMI 2650 and SF-268 cell line, there are significant differences in cell viability between both cell lines

The mechanism of action of mitoxantrone consists of inhibition of nucleic acid synthesis which affects cells in various stages of the cell cycle, namely the mid-to-late G1 phase and mid-to-late G2 phase, preventing the cells from entering mitosis. However, mitoxantrone is a cell cycle nonspecific anticancer drug, showing inhibitory effects on both proliferating and nonproliferating cells (G., 1990). Additionally, as described, SF-268 cell line, as a glioblastoma cell line, has numerous genetic alterations, including activation of general oncogenes - EGFR, PIK3CA, c-Myc - by several mechanisms and mutations, also by several mechanisms, of tumoral suppressor genes - PTEN, TP53, RB1, TERT, - (McLendon et al., 2008). The molecular heterogeneity of glioblastoma cells enhances the proliferation of tumor cells and resistance to certain therapeutic strategies. However, to proliferate, cells have to enter in cell cycle, and thus, if more cells are entering the cell cycle, then more cells will be sensitive to topoisomerase II inhibitor agents, like mitoxantrone. Moreover, the genetic pattern of RPMI-2650

cell line is described presenting several mutations which do not directly affect the cell proliferation like the molecular heterogeneity of glioblastoma multiforme (COSMIC Cell Line Gene Mutation Profiles, 2022). Therefore, the molecular heterogeneity could explain the differences between the cell viability of both cell lines.

The cellular internalization of MTX and EXO-MTX was evaluated in SF-268 cell line, by cell live imaging using a confocal fluorescence microscope. SF-268 cells were initially labeled with Hoechst, specifically used to stain the nuclei of living cells, being possible to identify replicating cells (see annexes; Figure S 7).

The results for the cellular internalization after exposure of SF-268 cells to 0.48 $\mu\text{g/mL}$ of EXO-MTX and MTX are shown in Figures 8 and 9, respectively. The images describe 2D qualitative distribution of EXO-MTX and MTX. Figure 8A shows the co-localization of the delivery of MTX at minute 5, while Figure 9A shows the co-localization of the delivery of EXO-MTX at minute 1. Comparing to MTX components, the interactions between EXO-MTX and cell surface, internalization and spreading through cytoplasm initialized first. Additionally, Figures 8B and 9B, show the co-localization of the delivery of MTX and EXO-MTX, at 37°C after 30 min. The accumulation of MTX and EXO-MTX into the cells was observable. Moreover, in Figure 8B MTX appears to be primarily located in the cytoplasm with initiating accumulation on the nucleus. Interestingly, EXO-MTX is predominantly located in the cytoplasm and cell membrane, with an apparent accumulation of EXO-MTX which is delimiting undistinguishably the cell membrane of SF-268 cells. Figures 8C and 9C show the co-localization of the delivery of MTX and EXO-MTX after incubation at 37°C after 90 min. MTX and EXO-MTX were both internalized in SF-268 cells, however, for MTX it is possible to visualize the formation of apoptotic bodies, and nuclear shrinkage thus, indicating higher accumulation of MTX on the nucleus and subsequent apoptosis. The remaining existence of MTX in the medium indicates that MTX was not fully internalized. In contrast, EXO-MTX appears to be fully internalized in SF-268 cells without the formation of apoptotic bodies or nuclear alterations. Moreover in Figure 9C, it is possible to observe the overlap of EXO-MTX and cellular nucleus, as the nucleus of one cell disappeared at this time-point with no further morphological changes thus possibly indicating higher accumulation on the cell cytoplasm instead of the nucleus.

Comparing the results between EXO-MTX and MTX, the differences in cell viability could be explained by the mechanisms of membrane trafficking of the milk-exosomes (Salarpour et al., 2019).

The results demonstrate that milk exosomes do not appear to induce cytotoxicity and function as nanocarriers for MTX. In addition, the characterization of milk exosomes showed a varied size range of particles which does not seem to cause cytotoxicity. Additionally, the presence of non-sterile PBS, as the pellet of milk-exosomes was resuspended under non-sterile conditions, also didn't cause cytotoxicity. Milk-exosomes are biological nanoparticles and thus, solvent-free nanoparticles which eliminate the solvent-related toxicities present in conventional solvent-delivery systems (Dikmen et al., 2011). Moreover, the incorporation of mitoxantrone into the exosomal membrane may attenuate the cytotoxic effect of mitoxantrone as demonstrated in Figure 7 (Ellis et al., 2016). As described, the continuous accumulation of MTX is accompanied by a dramatic increase of intranuclear MTX-DNA complexes, resulting in the destruction of nucleic acid conformation (Feofanov et al., 1997). However,

the accumulation of EXO-MTX in the nucleus is conditioned, as the milk-exosomes preferably accumulate on the cytoplasm and the release of MTX is slower thus, explaining the significant differences for cell viability between EXO-MTX and MTX, where EXO-MTX presents, for the same drug concentration, a controlled release and nuclear accumulation of the drug. Therefore, the fact that the cell viability of EXO-MTX differs from MTX may be due to the incorporation of the drug into the membrane of milk exosomes, since the nanocarrier used, as demonstrated in the literature and the results, is non-cytotoxic.

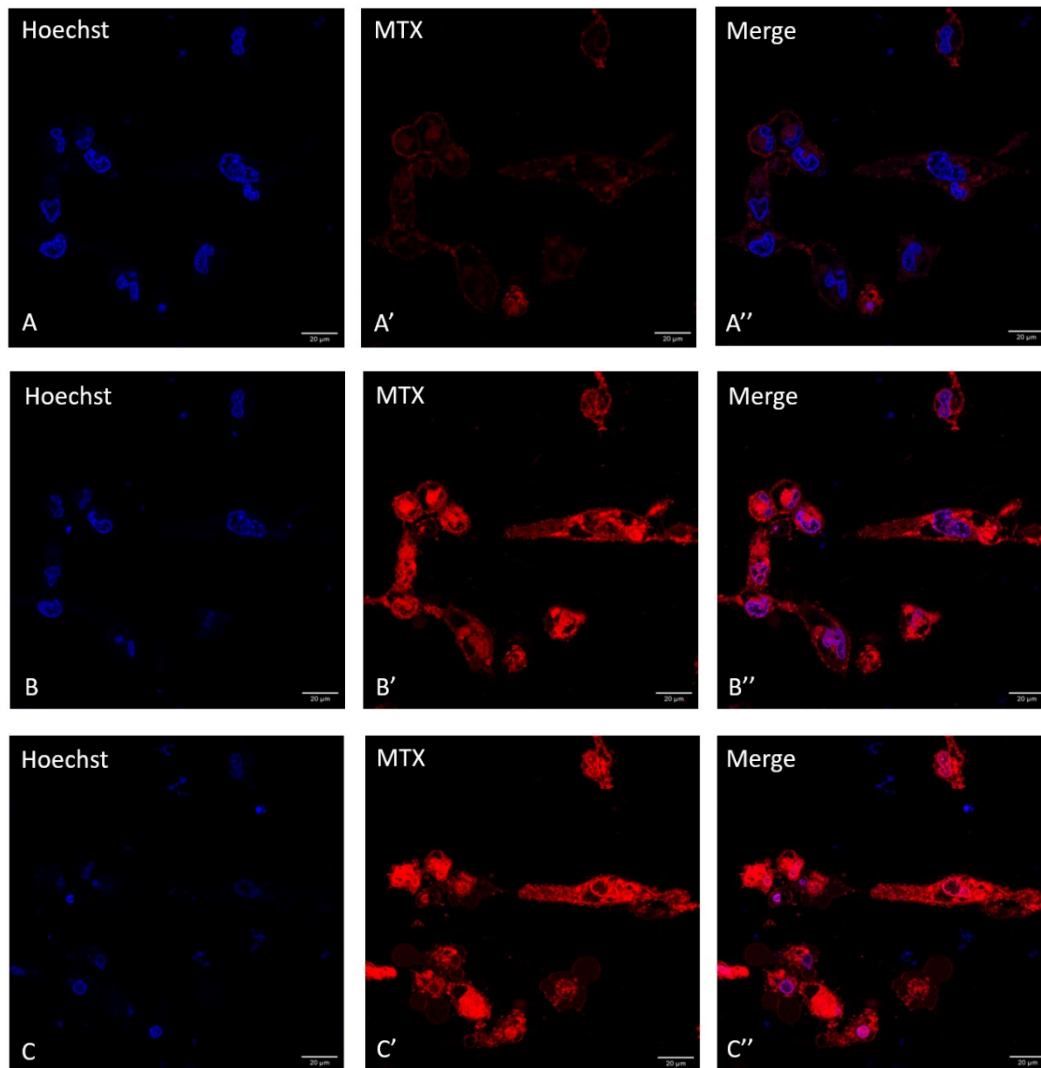


Figure 8. Confocal fluorescence microscopy of SF-268 incubated 10 minutes with Hoechst. In (A) SF-268 cells at 5 minutes of incubation at 37°C with 0.48 $\mu\text{g/mL}$ MTX. In (B) SF-268 cells at 30 minutes of incubation at 37°C with 0.48 $\mu\text{g/mL}$ MTX. In (C) SF-268 cells at 90 minutes of incubation at 37°C with 0.48 $\mu\text{g/mL}$ MTX. Blue channel: A, B, and C (nucleus labeled with Hoechst). Red Channel: A', B', and C' (λ_{ex} : 610nm; λ_{em} : 630 nm). Merge: A'', B'', and C''. Scale bar: 20 μm .

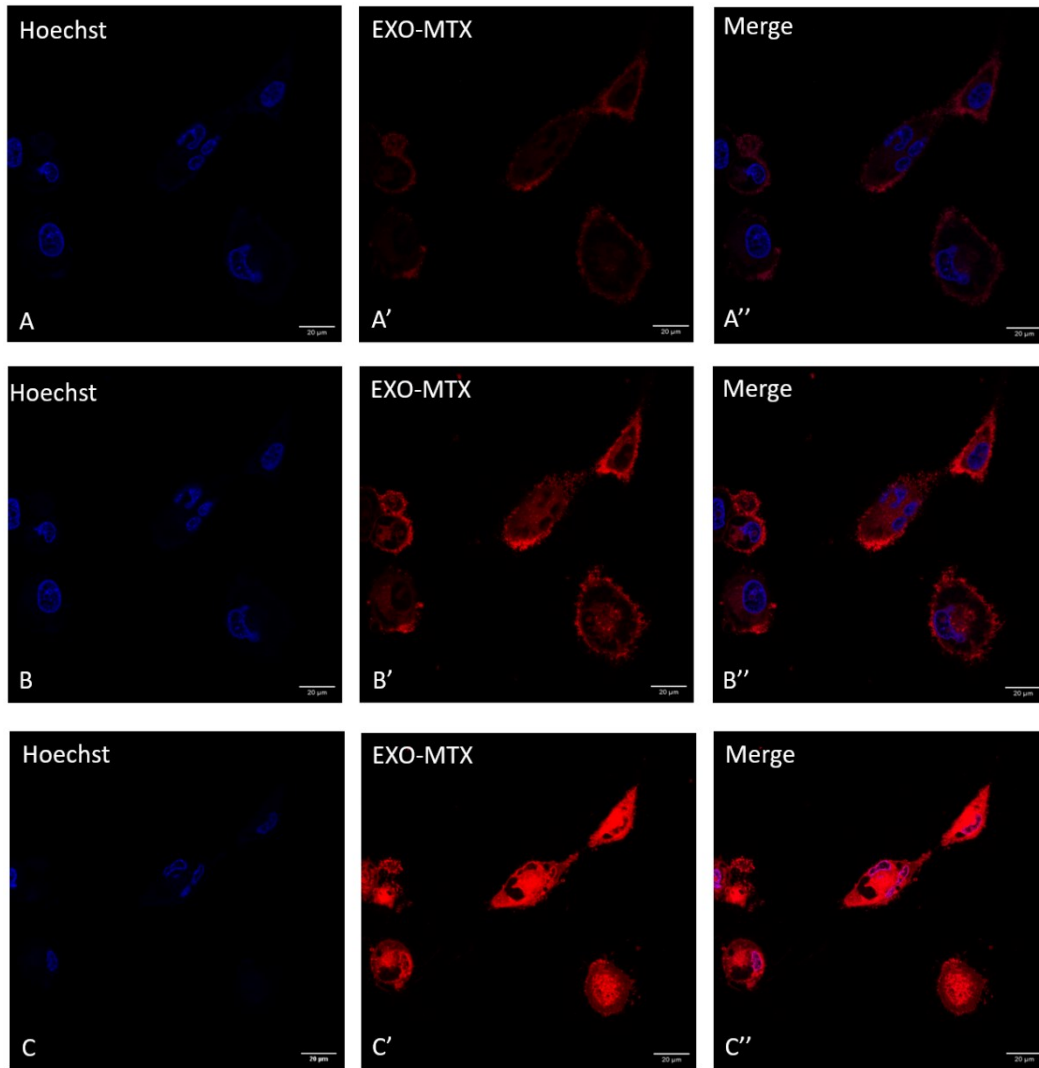


Figure 9. Confocal fluorescence microscopy of SF-268 incubated 10 minutes with Hoechst. In (A) SF-268 cells at 1 minute of incubation at 37°C with 0.48 µg/mL EXO-MTX. In (B) SF-268 cells at 30 minutes of incubation at 37°C with 0.48 µg/mL EXO-MTX. In (C) SF-268 cells at 90 minutes of incubation at 37°C with 0.48 µg/mL EXO-MTX. Blue channel: A, B, and C (nucleus labeled with Hoechst). Red Channel: A', B', and C' (λ_{ex} : 610nm; λ_{em} : 630 nm). Merge: A'', B'', and C''. Scale bar: 20 µm.

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Chapter V: Conclusion

Milk exosomes may have diagnostic and therapeutic potential, but the current challenge in exosome research is the ability to obtain purified samples (Tauro et al., 2012). Differential ultracentrifugation is still considered the 'gold standard' method to isolate exosomes due to the simplicity of protocol by a stepwise increase in centrifugation speed (Wang et al., 2021). The biochemical molecules present in milk exosomes suspension may be co-isolated with exosomes, as observed with casein micelles, and misinterpret results. The results allow concluding that the centrifugation speed and time applied influence the sedimentation of co-contaminants and the yield and purity of exosomes where the ideal protocol must not exceed the 4 hours of ultracentrifugation and at least one ultracentrifugation of 100,000 x g should be applied to obtain the exosomal pellet. The use of EDTA as a chelate agent to remove Ca^{2+} ions is effective, however, the resistance of aggregation of exosomes may slowly decrease through time as the suspension is stored at pH 8. The addition of EDTA may contribute to a reduced protein concentration, as the total protein concentration was lower than the described for ultracentrifugation techniques, however, the yield of exosomes was congruent with the literature.

Milk exosomes can provide advantages as drug delivery systems by overcoming various biological barriers. Mitoxantrone was efficiently encapsulated in milk-exosomes by the sonication method, which uniformized the size distribution of exosomes. The membrane disruption enhances the entrapment of mitoxantrone through the hydrophobic region of the exosomal membrane avoiding the burst release of mitoxantrone in an acidic environment. Therefore, with EXO-MTX would be possible to reduce the dosing frequency with a more uniform effect of the drug. Generally, exosomes possess good properties as potential nanocarriers, namely stability in circulation, biocompatibility, low immunogenicity, and toxicity (Bellingham et al., 2012).

Milk-exosomes possibly reach the brain, *in vivo*, through paracellular transport mechanisms from the nasal cavity to the underlying lamina propria, navigating through tight-junctions to reach the olfactory bulb. RPMI-2650 cells were able to establish tight junctions' complexes, provide a TEER similar to normal mucosa, being the cultivation at ALI the most critical parameter considering the barrier properties. Moreover, the permeation studies demonstrated that RPMI-2650 cell monolayer in ALI culture could be used as a first screening tool regarding cytotoxicity and permeability, however, *in vivo* studies may be necessary to validate the intranasal delivery of mitoxantrone encapsulated in milk-exosomes, as the precise involvement of drug transporters in this model is still unknown (Gonçalves et al., 2016). RPMI-2650 cell model will be considered a predictive pharmacological *in vitro* model of human nasal mucosa when the transporters functionality and influence in drugs bioavailability is characterized (Mercier et al., 2018). The encapsulation of mitoxantrone in milk-exosomes resulted in a faster internalization in the cell cytoplasm, without causing cell death, as the release of mitoxantrone from milk-exosomes is controlled.

Therefore, EXO-MTX showed controlled release time, reduced toxicity of mitoxantrone, and the possibility of delivery of a higher dose in short period of time through intranasal administration, as the initial concentrations applied for RPMI-2650 cell line were not cytotoxic. Moreover, as milk-exosomes

are natural nanomaterials, there is the possibility to deliver more than one active constituent demonstrating enhanced potential to deliver drugs straight to the cytoplasm, as visualized in live cell imaging, and posteriorly to the nucleus.

The results for EXO-MTX are promising for intranasal administration as the suspension only showed cytotoxicity for SF-268 cell line. Moreover, milk-exosomes enhanced the cellular uptake of mitoxantrone, but not the immediate nucleic uptake of mitoxantrone as the release of the drug from milk exosomes was slower.

To date, studies with the encapsulation of mitoxantrone in milk exosomes for intranasal delivery, to target glioblastoma multiforme, have not been performed. Therefore, the next step for this work would be to further characterize EXO-MTX in the pre-clinical stage. Regarding the biophysical characterization, as mitoxantrone is entrapped in the exosomal membrane, further surface characterization of EXO-MTX should be performed. In fact, for *in vivo* models it may be necessary to functionalize the surface of EXO-MTX since milk-exosomes are non-specific for target cells being necessary to characterize the surface of EXO-MTX.

The establishment of a standard permeability model for preclinical assays of intranasal delivery of exosomes is necessary to interpret results, and thus the optimization of an *ex vivo* model for permeability of milk-exosomes regarding human nasal epithelium may be advantageous. Moreover, *in vitro* studies to fully characterize SF-268 cells in the presence EXO-MTX should be performed, namely, cellular uptake and membrane transport studies for EXO-MTX. Lastly, to determine the pharmacokinetic and pharmacodynamic profile that characterize nasal absorption of EXO-MTX, *in vivo* models are essential and should be performed after the full characterization *in vitro* and *ex vivo*.

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Annexes

1. Characterization of bovine milk-Exosomes and Exosomal mitoxantrone

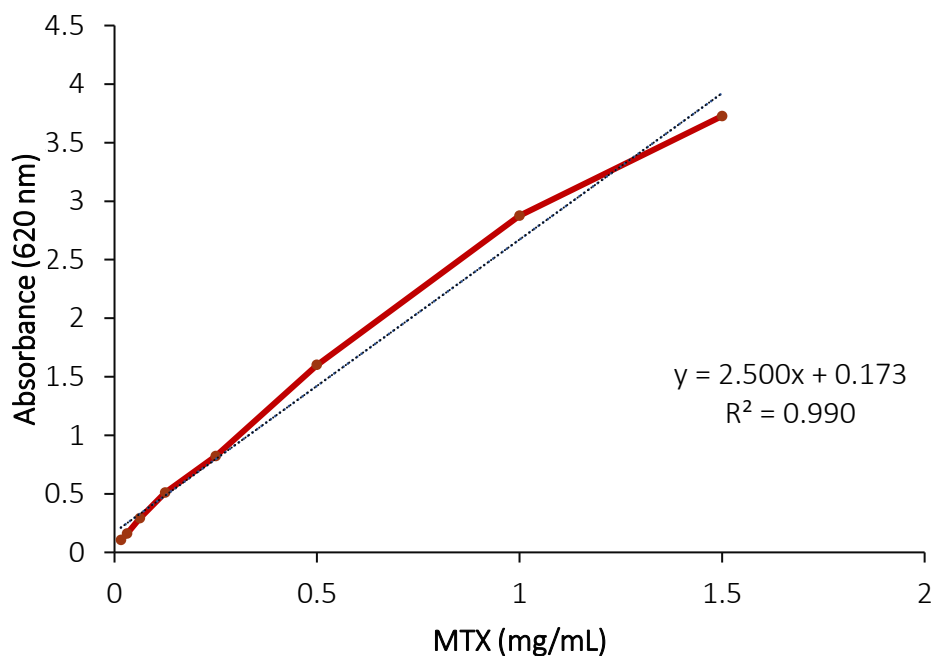


Figure S 1. Efficiency of encapsulation calibration curve. The standard curve of Concentration of free mitoxantrone versus Absorbance was used to estimate the amount of mitoxantrone in the supernatant. Coefficient of the slop $b = 2.500$, and intercept $= 0.173$ was used to calculate the concentration of mitoxantrone present in the supernatant from absorbance readings at 620 nm. $R^2=0.990$.

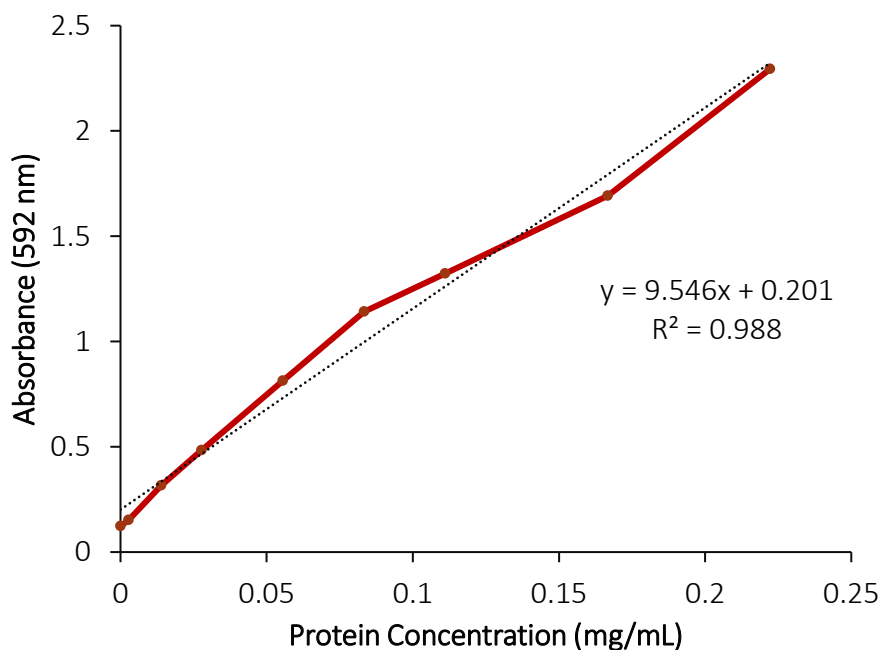


Figure S 2. BCA calibration curve. The standard curve of Protein concentration versus was used to estimate the total protein concentration of milk-exosomes. Coefficient of the slop $b = 9.546$, and intercept $= 0.201$ was used to calculate the total protein concentration in mg/mL from absorbance readings at 592 nm. $R^2=0.988$.

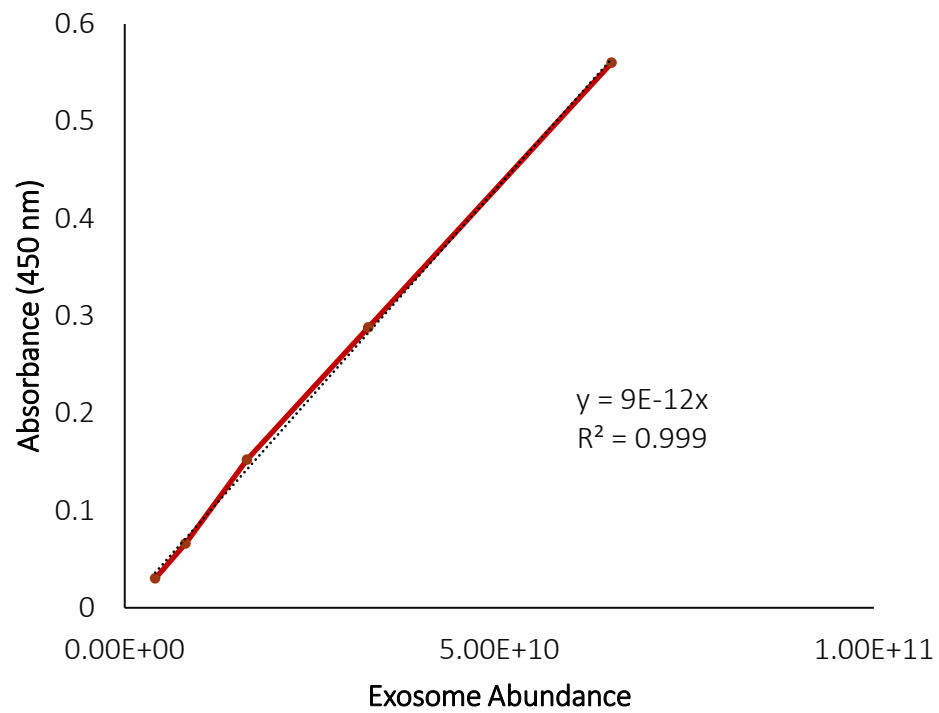


Figure S 3 ELISA calibration curve. The standard curve of Exosome Abundance versus Absorbance was used to estimate the abundance of milk-exosomes. Coefficient of the slop $b = 9.00 \text{ E-}12$, was used to calculate the exosome abundance from absorbance readings at 450 nm. $R^2=0.999$.

2. *In vitro* release studies

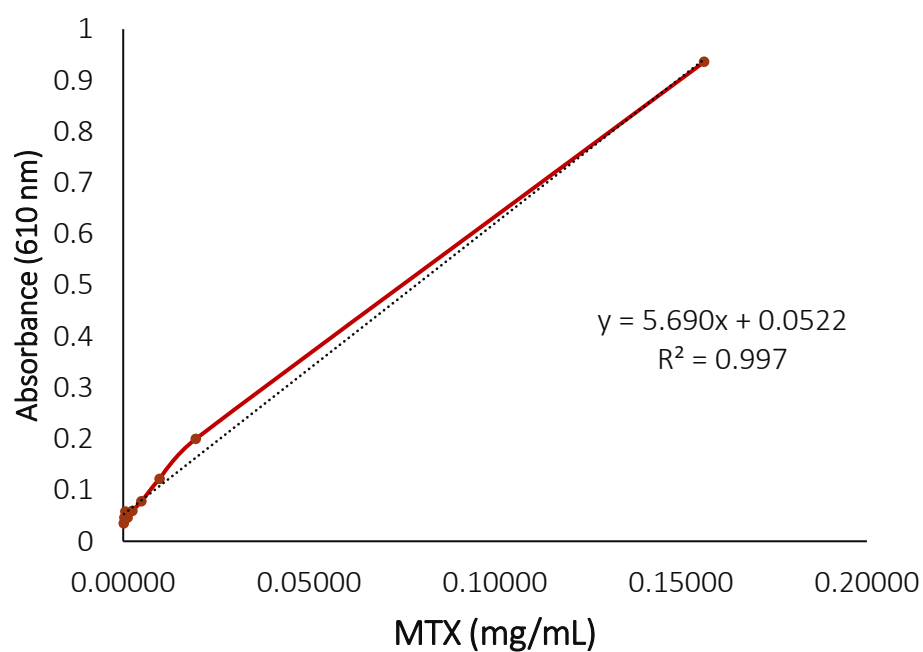


Figure S4. *In vitro* release calibration curve. The standard curve of concentration of free mitoxantrone versus Absorbance was used to estimate the amount of mitoxantrone in the the lower compartment at each timepoint. Coefficient of the slop b = 5.690, and intercept = 0.0522 was used to calculate the concentration of mitoxantrone present in the supernatant from absorbance readings at 610 nm. $R^2=0.997$.

3. Cellular studies

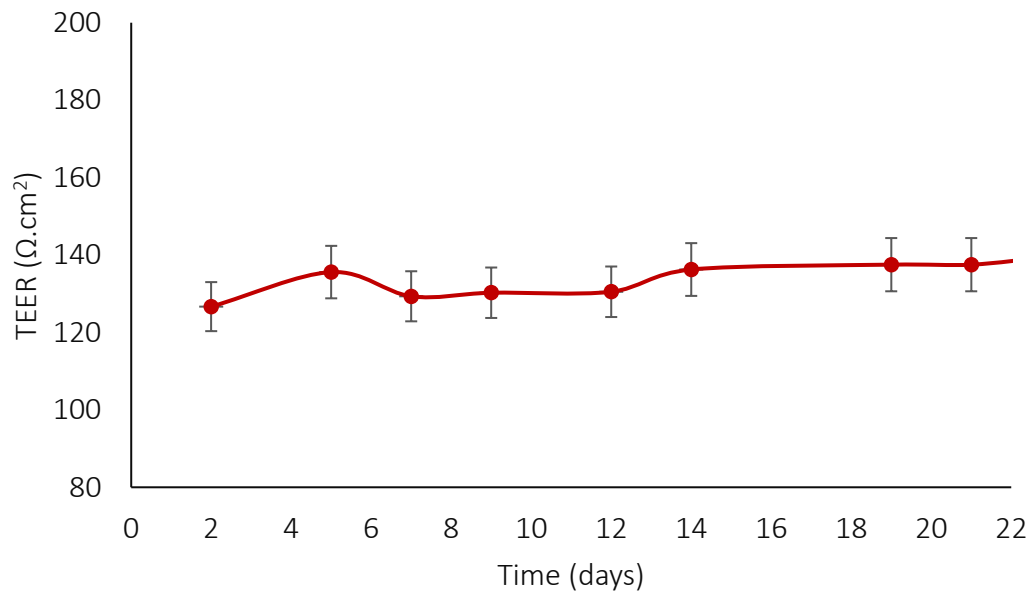


Figure S5. Transendothelial electrical resistance (TEER) measures of RPMI-2650 cell monolayers during 22 days of culture. All values presented as mean $\pm$ SD for n=3.

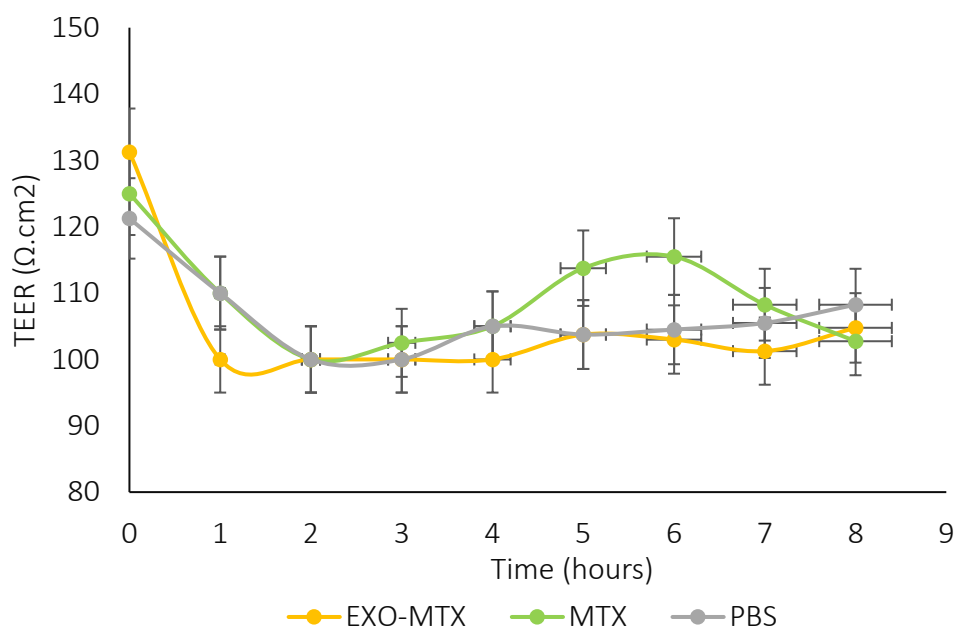


Figure S6. Transendothelial electrical resistance (TEER) measures of RPMI-2650 cell monolayers during permeability assay for exosomal mitoxantrone (EXO-MTX), mitoxantrone (MTX), and buffer (PBS). All values presented as mean $\pm$ SD for n=3.

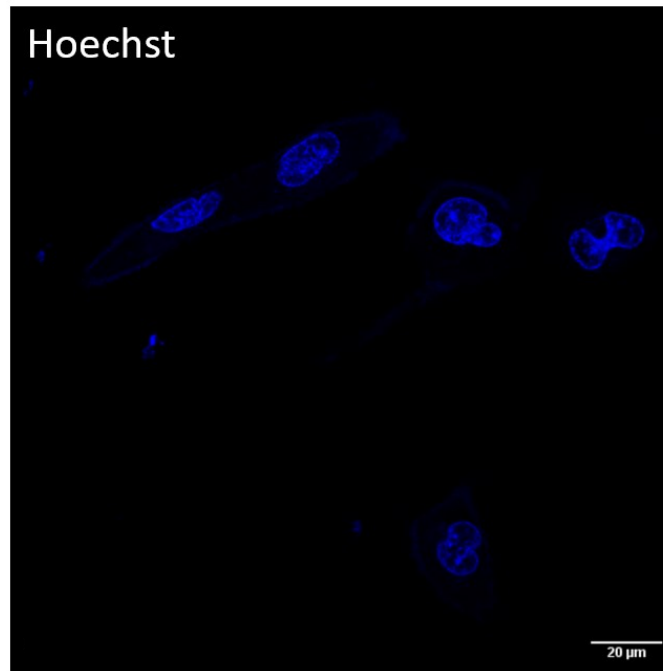


Figure S 7. Confocal fluorescence microscopy of SF-268 incubated 10 minutes with Hoechst. The confocal fluorescence microscopy shows SF-268 cells during cell cycle. Scale bar: 20 μ m.

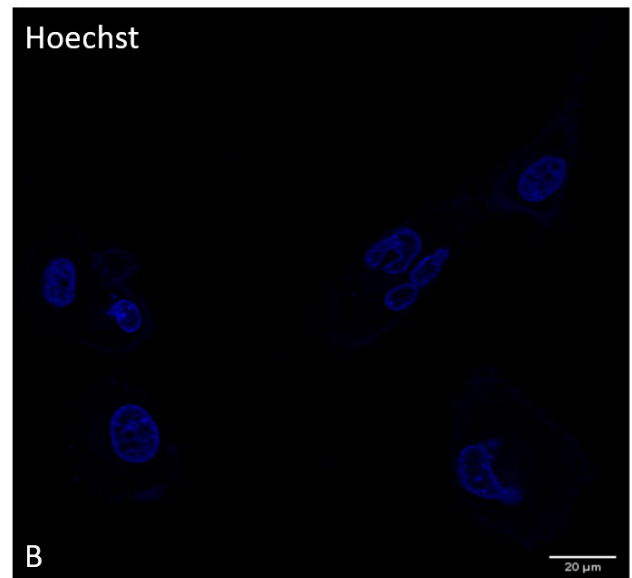
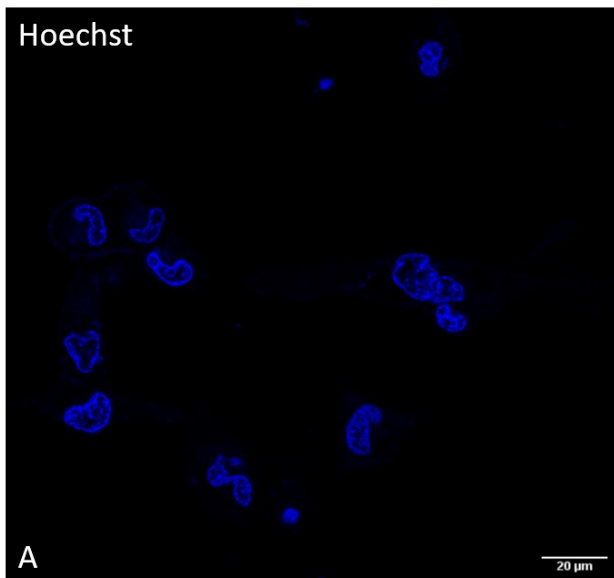


Figure S 8. Confocal fluorescence microscopy of SF-268 incubated 10 minutes with Hoechst. In (A) SF-268 cell nucleus before incubation with MTX. In (B) SF-268 cell nucleus before incubation with EXO-MTX. Scale bar: 20 μ m.

