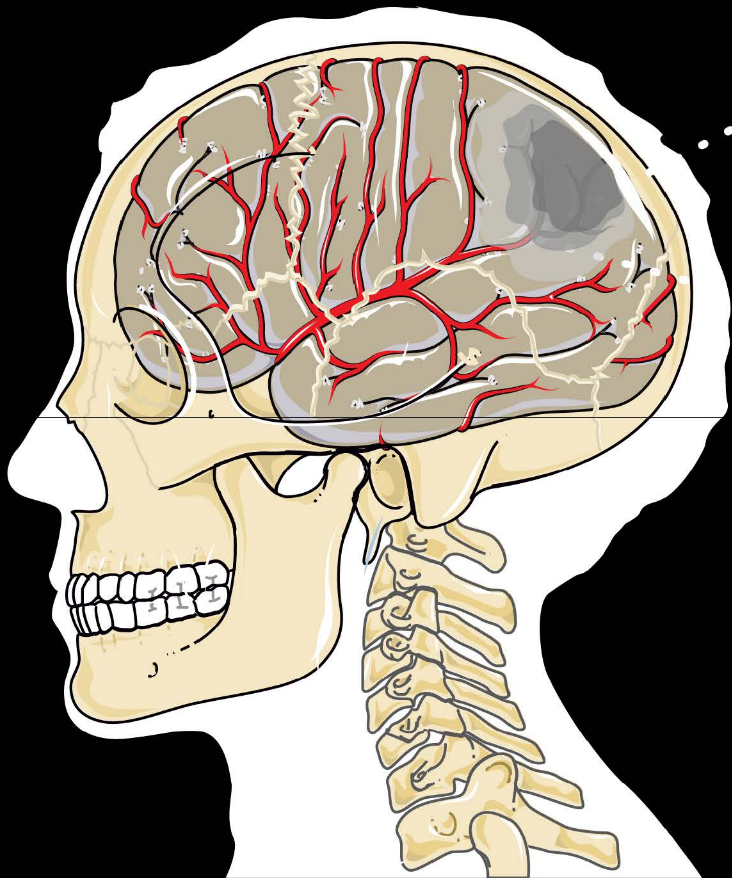
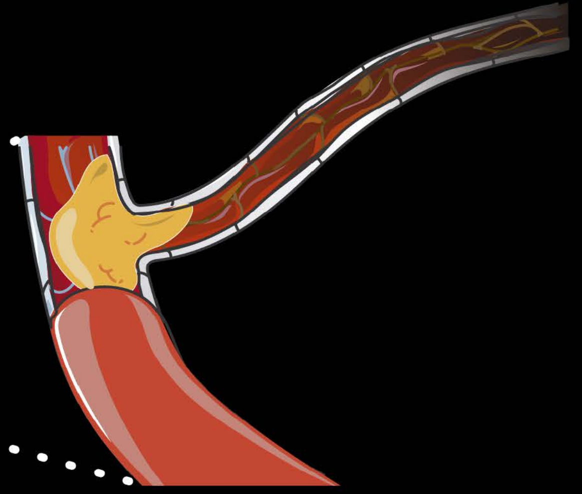
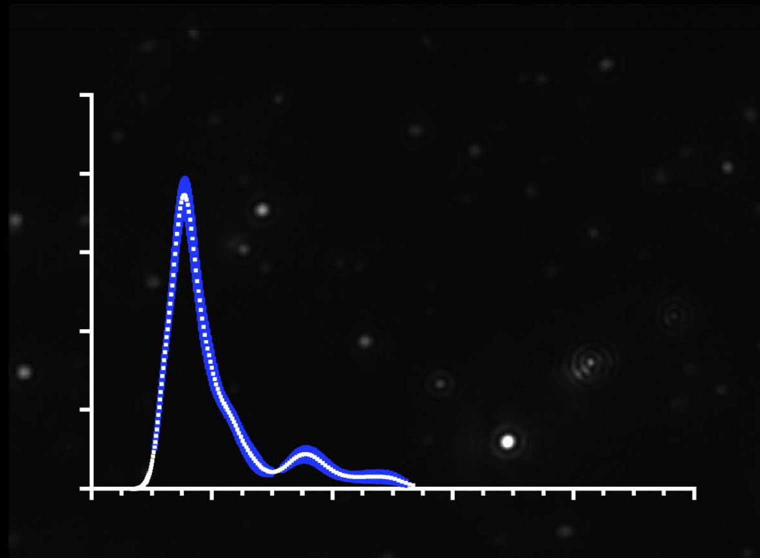

António José da Silva Santinha

Exosomes-Based Therapy for Stroke

Dissertação de Mestrado em Biologia Celular e Molecular

Junho 2017

UNIVERSIDADE DE COIMBRA

António José da Silva Santinha

Exosomes-Based Therapy for Stroke

Terapia Baseada em Exossomas para o AVC

Dissertação de Mestrado em Biologia Celular e Molecular

Junho de 2017

This work was developed in the Biomaterials and Stem Cell-Based Therapeutics Laboratory (UC-Biotech, Biocant Park, Cantanhede, Portugal), under the supervision of Dr. Lino Ferreira and Dr. Alessandra Zonari. Part of the work was performed in the group of Synapse Biology (CNC, Coimbra, Portugal) under the supervision of Dr. Ana Luísa Carvalho and Dr. Dominique Fernandes and in the Brain Repair group, under the supervision of Dr. Liliana Bernardino and Dr. Tiago Santos. It also had the collaboration of Crioestaminal (Biocant Park, Cantanhede, Portugal).

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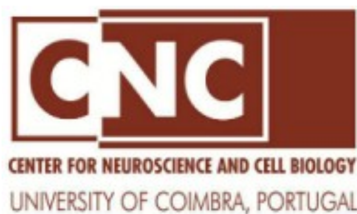

Abstract

Stroke is defined as a neurological deficit attributed to an acute focal injury of the central nervous system. Despite the advances in prevention and treatment, stroke is the second cause of mortality around the world and the patients that survive have high morbidity. Currently, there is only one FDA-approved drug available for the treatment of acute ischemic stroke, the tissue plasminogen activator (tPA). So far, there is no available treatment to increase the survival of neural cells and to rapidly stimulate angiogenesis and neurogenesis in the ischemic brain. Among the researched therapies, cell-based therapies have shown a recovery potential of central nervous system by promoting neuroplasticity, angiogenesis and immunomodulation. These effects are not directly related to the transplanted cells but with the paracrine effect of these cells. Thus, interests in exosomes have emerged recently since they are considered the major active factor that mediates restorative effect.

This work can be divided in two parts, mainly the isolation of exosomes from MNCs and WJ-MSCs, and the assessment of their bioactive role over three important cell types in ischemic stroke recovery.

We properly isolated exosomes with a circular shape and median size of 130 nm (MNC-exosomes) and 150 nm (WJ-MSC-exosomes). Moreover, both were positive for exosomal-enriched proteins CD9 and CD63 and also for specific marker from progenitor cells, proving not only their essence, but also their origin. In HUVECs and cortical neurons, WJ-MSC-exosomes are internalized at a higher rate than MNC-exosomes, with 100% of uptake after 24 hours contrasting with around 70% for MNC-exosomes. Both exosomes were able to induce survival of HUVECs subjected to oxygen and glucose deprivation, whereas, just MNC-exosomes could increase the resistance of neurons to the same stimuli. Although we demonstrate an increased amount of the neuroprotective miR-223-3pin MNC-exosomes (35 times more than in WJ-MSC-exosomes), we were not able to prove a reduction in GluN2B expression, displacing the hypothesis that this micro RNA could be increasing neuronal survival through GluN2B signaling pathways. Both exosomes were able to induce phenotypic changes over neural stem cells (NSC), however only MNC-exosomes induced a

meaningful production of new neurons. Taking together, these results reveal the potential of MNC- and WJ-MSC-exosomes over three important steps for stroke recovery, mainly neurovascularization, neuroprotection and neuroregeneration.

Keywords

Ischemic stroke, Exosomes, Characterization, Bioactivity, Neuronal recovery.

Resumo

Acidente vascular cerebral (AVC) é definido como uma patologia neurológica atribuída a um incidente no sistema nervoso central. Mesmo com todos os avanços na prevenção e tratamento, AVC ainda é mundialmente a segunda maior causa de morte e pacientes que sobrevivem apresentam alta taxa de morbilidade. Atualmente, só o ativador de plasminogênio tecidual (tPA) está aprovado pela FDA como tratamento para o AVC isquémico. Neste momento, não existem tratamentos clinicamente disponíveis para aumentar a sobrevivência de células neuronais ou estimular a formação de novos vasos sanguíneos na região afetada. De entre as várias tentativas para encontrar um tratamento, terapias baseadas em células mostraram potencial na indução de neuroplasticidade, formação de novos vasos sanguíneos e modulação do sistema imune. Os efeitos observados parecem não estar diretamente relacionadas com a transplantação celular, mas sim com o seu efeito parácrino. Desta forma, o interesse sobre o uso de exossomas tem crescido exponencialmente, pois estes são considerados um dos fatores mais importantes na mediação dos efeitos terapêuticos observados.

O trabalho aqui apresentado dividiu-se em duas etapas, o isolamento e caracterização de exossomas a partir de células mononucleares (MNC-exosomes) e de células estaminais mesenquimais (WJ-MSC-exosomes), ambas provenientes do sangue e cordão umbilical, respectivamente.

Foi possível isolar apropriadamente exossomas que apresentam uma forma circular e diâmetro com mediana de 130 nm (MNC-exosomes) e 150 nm (WJ-MSC-exosomes). Além disso, ambos apresentaram positividade para CD9 e CD63, proteínas enriquecidas em exossomas, e de forma interessante, também se mostraram positivos para marcadores específicos das células progenitores, provando não só a sua essência, mas também a sua origem. Em HUVECs e neurónios do córtex, WJ-MSC-exosomes foram internalizados mais rapidamente do que os MNC-exosomes, com cerca de 100% de internalização no final de 24 horas, contrastando com em torno de 70% observado para a segunda população. Ambos os exossomas são capazes de promover a sobrevivência de HUVECs sujeitas a

privação de oxigénio e glucose, no entanto apenas exossomas de MNCs foram eficazes a aumentar a resistência dos neurónios após o estímulo. Embora tenhamos provado que o miR-223-3p considerado neuroprotector está 35 vezes mais presente em MNC-exosomes do que em WJ-MSC-exosomes, não observámos uma redução na expressão de GluN2B, colocando de parte a hipótese que este micro RNA poderia ser o mecanismo de ação para as diferenças observadas entre os tratamentos. Ambas as populações de exossomas foram capazes de induzir alterações no fenótipo de células estaminais neurais (NSC), no entanto apenas exossomas provenientes de MNC conseguiram aumentar notoriamente a quantidade de novos neurónios formados. Os dados aqui presentes revelam o potencial de exossomas provenientes de MNC e de WJ-MSC sobre três importantes pilares da recuperação de um AVC.

Palavras-chave

AVC isquémico, Exossomas, Caracterização, Bioatividade, Recuperação neuronal.

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Glossary

B

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

BBB: Blood brain barrier

C

CNS: Central Nervous System

CVD: Cardiovascular diseases

CT: Computational tomography

CD: Cluster of differentiation

cDNA: Complementary deoxyribonucleic acid

D

DMSO: Dimethyl sulfoxide

DLL4: Angiogenic inhibitor delta like 4

DMEM: Dulbecco's modified eagle medium

DNAse: Deoxyribonucleic acid nuclease

DLS: Dynamic light scattering

E

ESCRT: Endosomal sorting complex for transport

EV: Extracellular vesicle

EDTA: Ethylenediamine tetraacetic acid

EGM: Endothelial growth medium

EBM: Endothelial basal medium

EGF: Epidermal growth factor

F

FDA: Food and drug administration

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FLT-3: Fms-related tyrosine kinase 3 ligand

FC: Flow cytometry

G

GABA: Gamma-aminobutyric acid

GDNF: Glial-derived neurotrophic factor

GAPDH: Glyceraldehyde 3-phophate dehydrogenase

GluN2B: NMDA receptor 2B

H

HSP: Heat shock proteins

HPC: Hematopoietic progenitor cells

HBSS: Hank's balanced salt solution

HUVEC: Human umbilical vein endothelial cells

HRP: Horseradish peroxidase

HLA-DR: Human leukocyte antigen D-related

I

ICH: Intracerebral hemorrhage

IS: Ischemic stroke

IGF-1: Insulin growth factor

ILV: Intraluminal vesicle

IL6: Interleukin 6

IL10: Interleukin 10

IMDM: Iscove's modified dulbecco's medium

M

MRI: Magnetic resonance imaging

MVB: Multivesicular bodies

MNC: Mononuclear blood cell

MSC: Mesenchymal stem cell

mRNA: Messenger ribonucleic acid

MEM: Minimum essential medium

miR: Micro ribonucleic acid

MACS: Magnetic associated cell sorting

N

NMDA: N-methyl-D-aspartate

NPC: Neural progenitor cells

NSC: Neural stem cells

O

OGD: Oxygen and glucose deprivation

P

PSD-95: Postsynaptic density-95 protein

ILV: Intraluminal vesicle

PBS: Phosphate-buffered saline

PFA: Paraformaldehyde

PAGE: Polyacrylamide gel electrophoresis

PI: Propidium iodide

Q

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

R

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RIPA: Radioimmunoprecipitation buffer

S

SAH: Subarachnoid hemorrhages

SVZ: Subventricular zone

siRNA: Small interfering RNA

SCF: Stem cell factor

SDS: Sodium dodecyl sulphate

SEM: Standard error of the mean

T

tPA: Tissue plasminogen activator

TIA: Transient ischemic attack

TSG: Tumor susceptibility gene

TEM: Transmission electronic microscopy

U

UCB: Umbilical cord blood cells

UCB-MNC: Mononuclear cells from UCB

V

VEGF: Vascular endothelial growth factor

W

WJ-MSC: Wharton's jelly mesenchymal stem cells

1. Introduction

1.1. Stroke

Hippocrates, 400 years BC, was the first to observe disorders related to nontraumatic brain injuries. Limited to the technology available at that time, he could not describe exactly the type of injury, naming this kind of damages as “apoplexy” (Greek for “being struck down”)[1]. The term was applied until 1689 when William Cole first introduced the word stroke in the book *A Physico-Medical Essay Concerning the Late Frequencies of Apoplexies*. At that time, a violent stroke was related to people losing their ability to declare their perceptions, while a less severe situation was characterized by people complaining about vertigos or a great oppression and pain in the head. As the diagnosis was mostly visual at that time, Cole observed that individuals suffering a stroke presented stupidness, somnolence, dazzling of the eyes and relaxation of all parts of the body[2].

Over the centuries, the knowledge about brain and its anatomy, functions and blood supply has advanced substantially. Moreover, the methods available to analyze patients affected by stroke have become more robust and accessible, resulting in the classification of stroke as a neurological deficit attributed to an acute focal injury of the central nervous system (CNS) by a vascular cause, including cerebral infarction, intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). However, the definition of stroke was not consistently accepted in clinical practice and clinical research, leading the American Stroke Association to join efforts in order to find the 21st century definition for stroke. The writing group composed by experts in neurosciences, clinical researcher, epidemiology and global public health proposed that stroke should have a different classification depending on the origin of the attack[1].

1.1.1. Stroke Types

Stroke, also called cerebrovascular accident, cerebrovascular insult or brain attack, is directly related to a situation where the brain can’t receive nutrients properly due to poor blood flow, resulting in cellular death[3]. There are two main classifications for the diseases, the hemorrhagic and the ischemic, but there is also a third condition, similar to ischemic, called transient ischemic attack (TIA). In contrast with the first two types, TIA occurs if blood flow is blocked only for a short period of time, less than 24 hours

(figure 1)[4]. Each one of them has distinct signatures, but leading to similar symptoms. The introduction of computational tomography (CT) and magnetic resonance imaging (MRI) enabled to distinguish between the two types, allowing a correct management of the illness[5].

1.1.1.1. Hemorrhagic Stroke

Hemorrhagic stroke, also called non-traumatic intracerebral hemorrhage, is a type of hemorrhage that occurs within the brain tissue. It happens when an artery in the brain bursts and consequently leaks blood. There are two kinds of stroke related to vessels rupture: intracerebral and subarachnoid hemorrhages[6].

Intracerebral hemorrhage is described as a breakdown of a blood vessel, leading to leaks into surrounding brain tissue. The bleeding promotes brain cells death, neurodegeneration and consequent incorrect working of the affected area[7]. Thus, this kind of stroke may present as a sudden focal neurological deficit or a reduced level of consciousness[8]. The risk factors for ICH are not still well established, however hypertension, excessive alcohol consumption, male sex, increasing age and smoking are all aspects that have been related with the disease[9]. In fact, about two-thirds of the patients have either pre-existing or newly diagnosed hypertension[10]. Furthermore, postmortem studies have shown that especially deep brain hemorrhages are caused by deep perforating artery lipohyalinosis attributable to chronic hypertension[9]. Other patients might present intracranial vascular malformations, cerebral angiopathy or infarcts into which secondary hemorrhage has occurred[10].

Subarachnoid hemorrhages are caused by rupture of saccular aneurysms within the subarachnoid space (area between the brain and the tissue covering it)[11]. The symptoms of SAH are less severe than those of ICH, including intense headaches with a rapid onset, vomiting, confusion and sometimes seizures[11,12]. The most important risk factors to SAH are hypertension, high alcohol intake and smoking. Interestingly, individuals with hypercholesterolemia, diabetes and from white ethnicity seem to be more resistant to the illness[13].

1.1.1.2. *Ischemic Stroke*

Ischemic stroke (IS) is caused when a vessel is blocked by a clot, leading to a decrease in blood flow, reduced oxygenation and consequently brain tissue damage. This type of stroke can currently be confused with TIA. In fact, their origin is similar, an occlusion of a blood vessel. Nevertheless, in the first the blockage is permanent and typically requires medical intervention, while in the second, the interruption of the flow is just temporary, lasting no longer than 24 hours[14]. Similarly to hemorrhagic stroke, high blood pressure is the most important risk factor for this type of attack that can occur in two main ways, thrombosis and embolism.

Thrombotic stroke happens when a blood clot is formed inside an artery that supplies blood to the brain. This kind of clot, called thrombus, is commonly related to high cholesterol levels and atherosclerosis. Depending on the vessel where the clot is formed, two types of thrombosis can be described. While the large vessel thrombosis is the most common, occurring in the brain's larger arteries and usually caused by long-term atherosclerosis in combination with rapid clot formation, the small vessel disease is characterized by the blockage of very small arterial vessel and it is closely linked to high blood pressure[14–16].

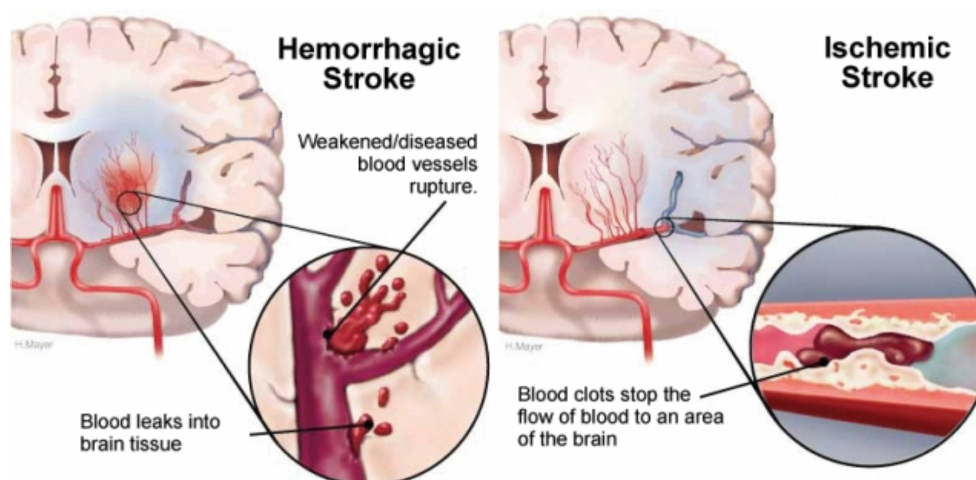


Figure 1-Hemorrhagic and ischemic as the two main types of stroke. Another type, called transient ischemic stroke can also occur, although with less neurological deficits. Adapted from http://heart.arizona.edu/heart-health/preventing-stroke/lowering-risks-stroke, accessed in June 10, 2017.

Embolic stroke happens when a blood clot is formed anywhere in the body (usually in the heart) and travels to the brain until it finds a vessel small enough to get stuck

and block the blood flow[15,17]. This type of IS has been related with people suffering from atrial fibrillation, a condition where the heart has an abnormal rhythm, characterized by rapid and irregular beating[18].

1.1.2. Pathophysiology

When a vessel blocks, initiating an IS, the tissue surrounding the ischemic core stays functionally compromised, but structurally intact[19]. This area is called ischemic penumbra and has shown to maintain its structure until 24 hours after the insult[20]. The blood flow suspension triggers a complex sequence of pathophysiological events that evolve over time and space[21]. At the begging of the stroke research, people believed that interruption of substrate delivery to neurons was responsible for a decrease in ATP production, leading to depolarization of neurons and subsequent necrosis. However, with the evolution of biochemical methods for neurological research became evident that disturbances of ion homeostasis, namely Ca^{2+}, and excitotoxicity are the main responsible for neuronal death after an ischemic situation[22]. The brain is especially vulnerable to this kind of insult because of its high intrinsic metabolic activity and a large concentration of glutamate, which acts concomitantly as an important neurotransmitter and an excitotoxin. Right after an attack, glutamate accumulates into the extracellular space due to voltage sensitive Ca^{2+} channels and failure of reuptake mechanisms, leading to prolonged stimulation of glutamate receptors in the post-synaptic membrane[23-24]. Thus, there is an enhanced influx of calcium, sodium and water into neurons, disrupting the activity of enzymes involved in catabolic processes (figure 2)[25]. Furthermore, some proteins related to reactive oxygen species (ROS) are also activated leading to the formation of nitric oxide, arachidonic acid metabolites and superoxide[26]. Sensing the above events, inflammatory cells such as astrocytes, microglia and hematogenous cells are recruited to the damaged area and communicate through a complex network of paracrine and autocrine mediators. In early stage, inflammation amplifies the ischemic lesion, but also mediate tissue repair in the late post-ischemic period[27].

Depending on the localization of obstruction and extension of brain tissue affected, an ischemic attack can have different prognosis. As one side of the brain commands the opposite side of the body, a stroke affecting the brain's right side will express

effects on the left side of the body and vice-versa. Thus, some outcomes of the disease are paralysis of one side of the body, altered behavior styles and vision, memory and speech problems.

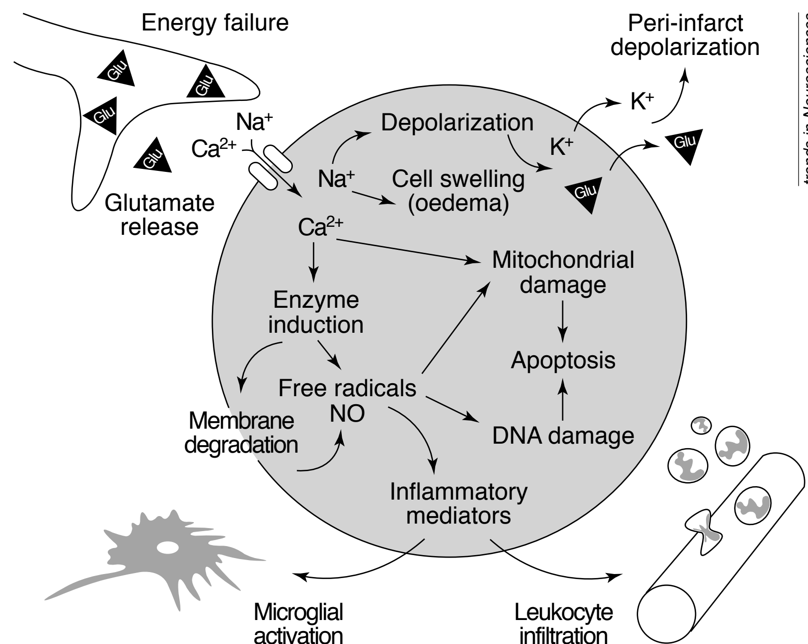


Figure 2- Simplified overview of pathophysiological mechanisms in the focally ischemic brain. Adapted from Dirnagl, Ulrich,. et al, Trends in neurosciences (1999).

1.1.3. Epidemiology

Cardiovascular diseases (CVD), where stroke is included, are the major cause of death worldwide. Data from the *European Cardiovascular diseases statistics 2017*, presented by the European Heart Network, showed that CVD is responsible for 45% of total deaths in Europe, killing near 3.4 million people each year. In 2013, stroke alone was responsible for 6.4 million deaths (11.8% of all deaths), remaining the third-leading cause of years-of-potential-life lost[28]. Between 1990 and 2010, the incidence of ischemic and hemorrhagic stroke increased by 37% and 47%, respectively. However, the number of deaths attributed to stroke only grew 20%, proving that the rate of recovery after an attack is increasing. Is expected that by 2030, may exist as many as 12 million stroke deaths and 70 million survivors may exist[29].

Economically, stroke consumes about 2-4% of total health-care costs. The total costs to society have been variously estimated to be £7.6 billion in the UK and US$40.6 billion in the USA, which represents about US$100 per head of population

per year[15]. Thus, research for easier and robust therapeutic techniques to reduce the number of deaths and increase quality of life for those who survive to a stroke should be of great interest, not only for patients but also for governments.

1.2. Stroke Therapies

Over the past 15 years, approaches in the management of stroke have evolved remarkably[15]. With a brain attack, there is the activation of complex injury pathways, disrupting the cortical maps that form the neural representation of our body and leaving sensorimotor defects. As the brain presents neuroplastic properties, some weeks after the injury it is observed an increased spine formation and axonal sprouting in the peri-infarct area, in attempt to reorganize the injured zone[30]. Increased neuroplasticity compensates the damaged networks, leading to brain remapping that has shown to result in sensorimotor and behavior progresses[31]. Thus, current therapies are approaching the diseases from three different, but yet interconnected, ways related with the restoration of blood flow, protection of the surviving tissue and renewal of dead cells (figure 3).

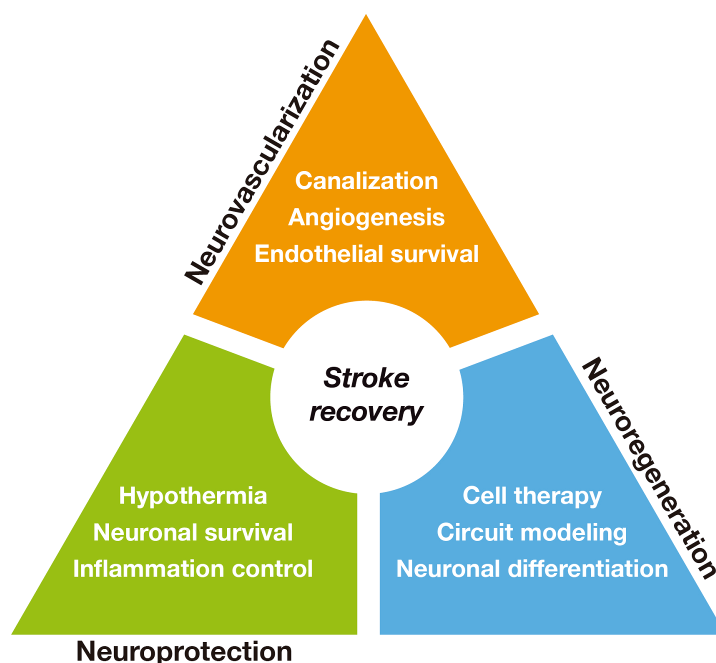


Figure 3- The 3 N's of stroke recovery: neurovascularization, neurodegeneration and neuroprotection. The blood flow reestablishment allows nutrients and drugs to reach the damaged area. Treatments should induce neuronal survival from the population already present and recruit new cells in order to maintain neurological functions.

1.2.1. Avoiding the diseases: Prevention

Effective prevention remains the best attitude for reducing the burden of stroke. Evidences have shown that a major reduction in stroke mortality mortality can be attributable to preventive treatments, particularly with the use of blood-pressure-lowering-agents for hypertension[32]. However, the targeting of other risk factors linked to the illness, such as socioeconomic status, atrial fibrillation and smoking might also present some positive effects. Treatments with warfarin for patients with atrial fibrillation, lipid lowering in patients with pre-existing ischemic heart diseases and aspirin have been used and showed promising results in the avoidance of the disease[33–35].

1.2.2. Restoring circulation

The first step that must be taken after an IS is the restoration of the blood flow to the penumbral tissue. In fact, this is the treatment currently executed in hospitals. If the bloodstream is rapidly reestablished, the severity of nutrients deprivation is much lower leading to minimal damages[36]. Moreover, breaking the clot allows other therapeutic agents with a protective and repair role to be able to reach the injured tissue and execute their functions.

The only Food and Drug administration (FDA) therapy approved for IS is tissue plasminogen activator (tPA). tPA is administrated intravenously and works by dissolving the clot, thus allowing the restoration of the blood flow to the brain region affected. Even with proved positive results when applied up to 4.5 hours after stroke, tPA is only used within 3 hours, excluding a significant number of candidates that do not get to the hospital in time for the treatment, therefore, increasing the importance of a rapid stroke identification[37 38].

In a clinical trial, mechanical endovascular interventions (thrombectomy) were applied to patients with proximal intracranial occlusion. Up to 6 hours, the intervention proved to be efficient and safe, improving the functional outcome, but not the mortality rate[39]. Another type of therapy that is being studied is sonothrombolysis (in combination with tPA) where the occluded area is exposed to pulsed-wave

ultrasounds, which promote motion around the thrombus and consequently higher tPA delivery to the area, leading to a higher rate of recanalization[40].

In an attempt to restore blood flow and normal supplement of nutrient to the injured area, therapeutic angiogenesis has also been applied with good outcomes[41]. This approach relies on use of angiogenic factors that enhance the intrinsic capacity of cells to create new vessels or on transplantation of endothelial progenitor cells[42,43].

1.2.3. Disruption of Injury Pathways and Neuroprotection

Neuroplasticity seems to be enhanced after a stroke attack, promoting cell growth and axonal sprouting in the area near to the infarct. Angiogenic and neurotrophic factors and metalloproteinases appear to be the endogenous contributors to this process, acting in an acute phase of the disease[44,45]. Besides, exogenous players in neuroprotection, such as ROS scavengers and blockers of ion transporters and glutamate signaling could have a significant role in recovery after an attack[46].

Since multiple pathways promote neurodegeneration after an ischemic situation, it is necessary the development of approaches targeting multiple mechanisms. The gold standard method for acute neuroprotection in animal models is mild brain hypothermia (33ºC)[47]. Not only for stroke, hypothermia can also be used for cardiac arrest and neonatal hypoxic-ischemic encephalopathy, presenting good neurologic outcomes[48]. This therapy is being investigated, with trials proving its feasibility[49].

One of the most important inducers of cell death during a hypoxia episode is excitotoxicity. With the oxygen deprivation, the release of glutamate from glutamatergic neurons increases, leading to the enhancement of the extracellular concentration of this neurotransmitter. In the postsynaptic membrane, sensing the high levels of glutamate, N-methyl-D-aspartate (NMDA) receptors become overstimulated, activating signaling pathways responsible for the excitotoxic cascade. One strategy in development nowadays is the targeting of PSD-95, a protein responsible for maintaining NMDA receptors anchored in the membrane and to connect the receptor to the signaling cascade. Reduction of PSD-95 levels has shown therapeutic proprieties in primates[50] and in patients undergoing endovascular intracranial aneurysm repair[51].

1.2.4. Modeling Circuits

Excitatory-inhibitory balance has been associated with some brain disorders, such as autism spectrum disorders, Alzheimer's diseases and stroke[52–54]. Balance alterations are caused by a reduction of inhibitory signals mediated by gamma-aminobutyric acid (GABA) receptors and an increase of positive signals, usually glutamate. In an IS model, it was observed that four weeks after the attack the levels of glutamate neurotransmission were augmented and several weeks later a diminished GABA neurotransmission was still present. Thus, targeting the tonic inhibition regulated by GABA receptors is portrayed as a good therapeutic window for stroke situations[55].

Among the available resources to induce circuits modeling, stimulation techniques are the most popular. Stimulation can be invasive or noninvasive, relying on electrical fields applied across vast areas of neural tissue[56]. The majority of therapies aim to restore the excitatory balance between the two brain hemispheres in order to improve recovery[57]. Currently, some trials are trying to assess the long-term potential of these treatments[58]. Another emerging approach that has evolved recently is optogenetics. With this technique it is possible to modulate neuronal circuitry activity, either by stimulating or inhibiting circuit activity. In fact, optogenetics has been used to mitigate seizures and modulate specific pathways in particular brain regions to promote recovery[59,60]. However, a small understanding of the exact regions affected after an ischemic attack and the requirement of gene alteration for optogenetics limit its use in clinical applications. Therapies such as magnetic resonance-guided focused ultrasound and stereotactic radiosurgeries are currently being investigated. Magnetic resonance-guided focused ultrasound uses advanced magnetic resonance imaging and ultrasound technology to apply ultrasonic energies in specific localizations of the brain[61]. Stereotactic surgery aims to modulate circuits and has already shown beneficial outcomes in diseases such as Parkinson, mood disorders, chronic epilepsy and essential tremor[62].

1.2.5. Cell-Based Therapies

Cell-based therapies have been under large focus over the past few years, having already multiple ongoing clinical trials. These approaches, when applied to stroke, are based whether on stimulation of endogenous neural stem/precursors cells (NSC/NPC), in the subventricular zone (SVZ) and dentate gyrus (hippocampus) or in the injection/transplantation of exogenous cells.

During an insult, endogenous NPC seem to migrate, through the rostral migratory system, to the damaged area and may differentiate into the predominant cell type of the injured region[63]. Augmenting brain's normal endogenous reaction to injury has been applied as a potential therapy. There are some proved pathways that can induce neurogenesis, such as those triggered by neurotrophic and growth factors, namely glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and insulin growth factor (IGF-1)[64]. Other mechanisms targeting the endogenous cells include anti-inflammatory drugs, hormones and manipulation of extracellular matrix[65–67]. Until the moment, these researches are still on preclinical stage.

The exogenous cells used for stroke recovery are divided into three broad categories: (i) immortalized cell lines, (ii) NPC or NSC and (iii) hematopoietic/endothelial progenitors and stromal cells[68]. Immortalized cell lines are generated from tumor cells, can be differentiated into neurons using retinoic acid and have shown good outcomes in IS models[69]. NPCs are able to differentiate into astrocytes, neurons and oligodendrocytes. When administered to stroke models they have shown a similar behavior compared with endogenous NPCs, also migrating to the damaged area, enhancing structural and functional recovery[70,71]. Lastly, cells derived from bone marrow, umbilical cord blood and adipose tissue have also shown improved recovery in brain attack models, although it is not entirely clear which mechanisms are responsible for the beneficial effect of these cells[72].

Several studies have demonstrated that the mechanism of action of these cells is not directly related to the replacement of damaged cells instead, cell-based therapy provides therapeutic benefit by remodeling of the central nervous system, i.e., by promoting neuroplasticity, angiogenesis and immunomodulation[73–76]. Although a lot

of effort has been done in this area, cell-based therapies still present some limitations, including (i) low cell survival after transplantation, (ii) risk of small vessel occlusion by cells, and (iii) malignant cell transformation[77–79]. Therefore, the success of cell-based therapies is dependent of developing strategies to overcome these challenges.

1.3. Exosomes as a Novel and Innovative Cell-Free-Based Therapy

The restorative stem cell effect is due mainly to paracrine secretion, demonstrating that cell-to-cell communication is an important process to be considered when formulating new treatments for stroke. Through the secretion of soluble factors or by direct interaction, cells can exchange information either with neighbor or distant cells[80]. Extracellular vesicles (EV), group in which exosomes are included, are a major player on this communication.

The ability of cells to release membranous vesicles was already described nearly 50 years ago, but at that time this process seemed to be related with garbage elimination (figure 4). It was in 1983, with the discovery that these vesicles are involved in mammalian reticulocytes (immature red blood cells) maturation, that EVs research started to regain attention[81–83]. However, these small vesicles were just called exosomes in 1987 and their research just started to grow significantly (with a tenfold increase) in the past decade[84]. In 1996 and 1998, the Portuguese researcher Graça Rapouso and her college Laurance Zitvogel proved that exosomes can also be released by B lymphocytes and dendritic cells[85,86]. Since then, several other cell types, either *in vitro* and *in vivo,* showed to be able to release membranous vesicles through similar mechanisms[87]. Excitingly, in 2007 messenger RNA and microRNA were discovered inside exosomes, and shown to be capable of integrate into the normal pathways related with RNA processing. It was the first time ever that genetic information was proved to be transferred between human cells, opening a new world of opportunities regarding exosomes research[88].

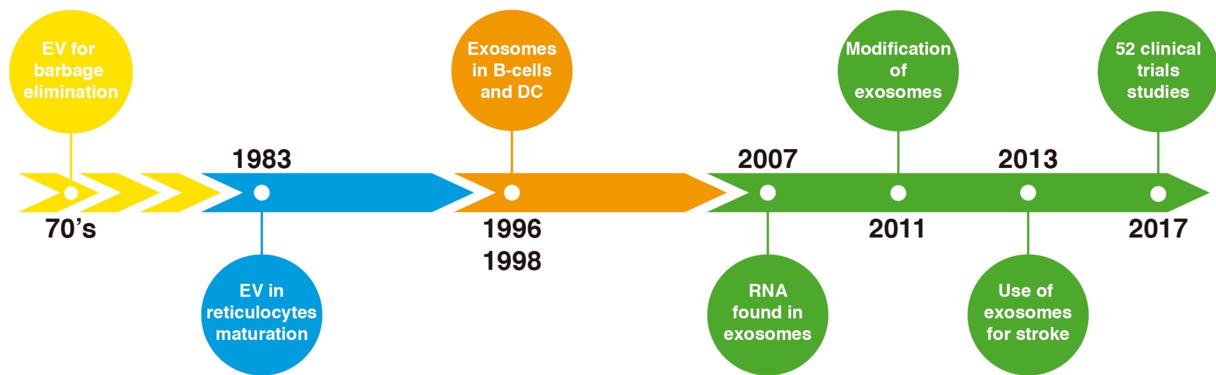


Figure 4-Extracellular vesicles research over the last 50 years. In the last 10 years, exosomes research has grown exponentially, from 80 publications in 2007 to 1143 in 2016. Relatively to 2017, in June 801 publications were already present in pubmed database and 52 clinical trials with the word "exosomes" were found in clinicaltrials.gov.

1.3.1. Exosomes Biochemistry

Biogenesis: Exosomes are membranous nanovesicles, ranging from 30 nm to 200 nm that are released by almost all cell types, with important cell-to-cell communication functions. They diverge from microvesicles essentially due to their size and mechanisms of generation[89]. While microvesicles are larger (more than 200 nm) and formed from the plasma membrane by shedding or budding, exosomes have an endocytic origin and are released by fusion of multivesicular bodies (MVB) with the cellular membrane[90].

The biogenesis of exosomes can be endosomal sorting complexes required for transport (ESCRT) dependent or independent, however both trigger the budding of the endosomal membrane, which allows direct access of RNAs and proteins to the forming vesicle interior (pre-intraluminal vesicle (ILV)), followed by its closure and consequent production of MVBs. The ILVs can later be degraded or released into the extracellular space, depending whether the MVB fuses with the lysosome or the cellular membrane, respectively (figure 5)[91]. Evidences showed that MVBs fate can be decide by its tetraspanin and lipid content, suggesting that a high content in tetraspanin (CD9) and cholesterol is related with exosome secretory pathway, while low tetraspanin and cholesterol levels drive multivesicular bodies to the lysosomal pathway[87,90].

Content: Once on the extracellular space, exosomes can be rapidly internalized by neighboring cells or travel through body fluids to distant tissues where using its lipid, protein and nucleic acid content, they can act as signalosomes, multifunctional signaling complexes for controlling fundamental cellular and biological functions[89].

Exosomes composition depends on cellular origin and also conditions of these cells. Exocarta (www.exocarta.org), a database specifically created to accumulate information regarding exosomes content, has 9769 proteins, 1116 lipids, 3408 mRNAs and 2838 miRNAs entrances, exposing the variety that exosomes can have between them[92,93]. Among the 9769 proteins found to be present in exosomes, some are transversally enriched in near all studies, therefore being used as exosomes markers. Molecules related with targeting (tetrapanins CD9, CD63 and CD81), chaperones (heat sock proteins (Hsp) 60, 70, and 90), cytoplasmatic enzymes as GAPDH and proteins involved in MVBs formation (programmed cell death 6-

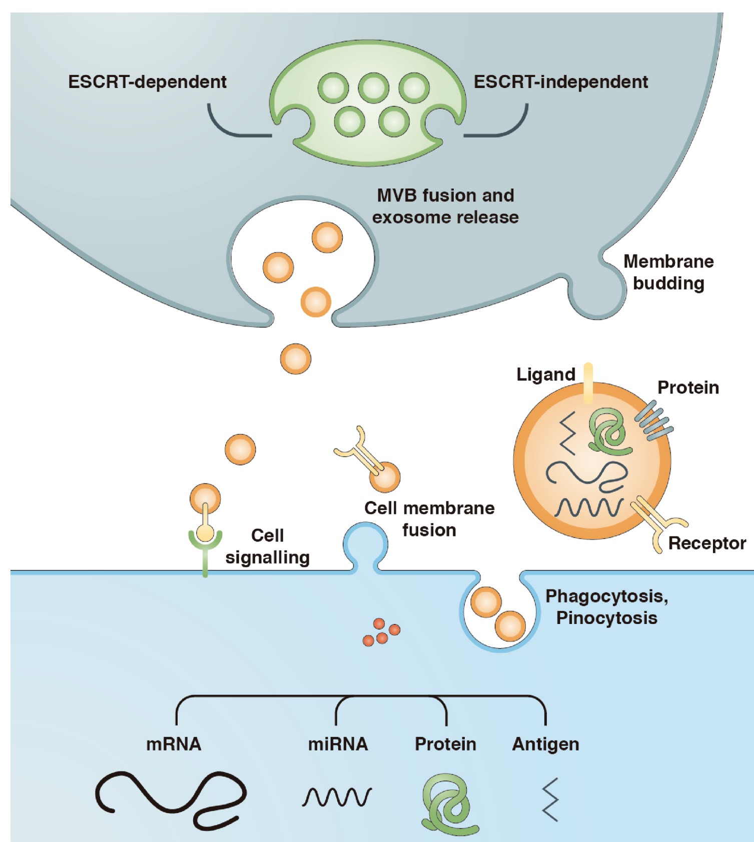


Figure 5- Exosomes secretion and composition. Exosomes can be formed and packed inside MVB through ESCRT dependent mechanisms, which involves proteins such as TSG101 and Alix or ESCRT independent way. Exosomes content can either be selectively or passively incorporated. Once in the recipient cell, vesicles are able to release mRNA, miRNA, proteins and antigen prepared to promote phenotypic alterations. Adapted from S. Andaloussi et al, Nat. Drug. Rev, 2013[89].

interacting protein (Alix) and tumor growth suppressor 101 (TGS101)) are some of the most used targets to characterize exosomes.

1.3.2. Exosomes as a promising player in biological diseases

Nowadays, exosomes are being associated with a wide list of diseases, both as (i) markers and as (ii) therapeutic tools.

Biomarkers: In physiologic conditions, cell-to-cell communication is a crucial step to keep body homeostasis. It occurs inside every tissue and also allows distant regions with distinct origins to regulate each other. However, in pathological conditions, injured tissues are altered, presenting different genetic and proteomic profiles that are reflected in exosomes content. It is thought that altered exosomes can induce disorders in other tissues, such as the case of neurodegenerative diseases and cancer metastasis[94,95]. Finding a pattern in exosomes content alterations is then critical to start using exosomes as biomarkers for diseases. In fact, there are already some preclinical and clinical trials based on using exosomes as biomarkers for breast, lung, ovarian and prostate cancer, cardiovascular and metabolic disorders and neurodegenerative diseases[94,96–98].

Therapeutic tools: Diverse studies have shown that exosomes can not only be internalized by cells, but also induce phenotypic changes[99]. Due to this propriety, tissue regeneration and immune system modulation are the two most promising subjects where exogenous exosomes can take an essential place.

Increasing evidences indicate that treatment with exosomes exhibit similar regenerative potential to cellular therapies in several diseases, including myocardial infarction, kidney injury, skeletal muscle repair and stroke[100]. This effect is due to the ability of exosomes to modulate inflammatory processes, suppress apoptosis and stimulate proliferation. Moreover, as exosomes are natural carriers, the probability of being recognized by the immune system is flat and they appear to have the capability to cross biological barriers, which are two drawbacks in therapies based on artificial nanoparticles[101].

Taking all these characteristics together, exosomes become an attractive source to small molecule delivery purpose. Therefore, pre and post-isolation modification are being explored to load exosomes with different bioactive molecules, including microRNAs. [102]. Moreover, modification of exosomes surface is being performed in order to enhance their stability and accumulation in target tissues. This can also be achieved by modification of producer cells or by direct engineering of exosomes surface, using for instance, click chemistry, fusion with liposomes or simply direct incubation with a desired ligand[103–105].

1.3.3. Exosomes Secreting Cell Source

Exosomes mediate a wide spectrum of effects on recipient cells, which could indicate that they are either highly multifunctional vesicles or that the secreting cells have an important role on their composition[106]. Different cell types, including mononuclear blood cells (MNCs) and mesenchymal stem cells (MSCs) have demonstrated beneficial paracrine effect in the treatment of ischemic diseases, therefore representing valuable sources of exosomes.

1.3.3.1. Mononuclear Blood Cells

Mononuclear cells from umbilical cord blood (UCB) are relatively easy to obtain from public and private cord blood banks. Until now, most of the preclinical studies assessing their role in the damaged CNS have transplanted the UCB mononuclear fraction (UCB-MNC). This fraction is composed of different cell types that release a mix of exosomes with distinctive content[107].

$CD34^+$ hematopoietic progenitor cells (HPCs) are at high number in UCB and present less mature and more proliferative phenotype[108]. These cells have proven to play a role in neuroprotection and immunomodulatory processes in animal models of stroke, decreasing infarcted area and the number of microglial cells[109]. Comparatively with adult peripheral blood, UCB-derived lymphocytes are phenotypically and functionally immature[110]. These cells have a role in the suppression of immune responses, and it has been suggested that they can exert a significant neuroprotective position after stroke through interleukin 10 (IL-10) release[111]. Monocytes are usually divided into two main groups, depending on the expression of CD14 or CD16.

Interestingly, $CD14^{+}CD16^{-}$ monocytes have shown poor outcome after stroke, whereas $CD14^{-}CD16^{+}$ are associated with excellent results in reducing infarcted size[112]. Compared with peripheral blood, UCB-derived monocytes can produce more tumor necrosis factor-α, interleukin 6 (IL-6) and have a higher Toll-like receptor-4 expression[113]. Interestingly, IL-6 is a neurotrophic cytokine that enhances the expansion of neural progenitor cells, which may suggest that those cells exert their therapeutic action through the secretome[114].

Numerous studies have tried to assess the neurological role of UCB-MNCs. *In vitro*, they protect neurons from oxygen-glucose deprivation (OGD), glutamate-induced apoptosis and prevent the suppression of axonal growth. [115]. After transplantation *in vivo,* UCB-MNCs are able to attenuate hypoxic-ischemic encephalopathy and perinatal asphyxia, surprisingly mainly through paracrine factors that regulate neuronal death, microglial activation and neuronal impairments[107,116].

Information about the bioactivity of MNC-exosomes for tissue regeneration is scarce so far. Nevertheless, MNC secretome has been widely used in diseases such as myocardial infarction, wound healing, stroke and spinal cord injury[117]. Moreover, the culture supernatant of UCB mononuclear cells contains VEGF, BDNF, neurotrophin 4 and 5 that are synergetic and have a role in recanalization and neuroprotection in experimental models of hypoxia[118,119]. VEGF is a major factor required for angiogenesis and has also shown to be active in neuronal plasticity, improving hippocampal-dependent contextual memory and the generation of new neurons in dentate gyrus and SVZ[120,121]. Likewise, BDNF protects the brain after an ischemic situation, decreasing neuronal death, enhancing neurogenesis through the endogenous regenerative response of the brain, and facilitating synaptic function and neural plasticity[122]. Recently, UCB plasma injected intravenously in a middle cerebral artery occlusion model of stroke as proved to decrease brain area affected and more importantly, promote behavior improvement[117].

1.3.3.2. Mesenchymal Stem Cells

MSCs are multipotent cells with the capacity for self-renewal that were first isolated from the bone marrow, but that can be found in several tissues including adipose

tissue and umbilical cord. They have the ability to differentiate into chondrocytes, osteoblasts and adipocytes, depending on signals from their microenvironment and present immunomodulatory properties. MSCs have been considered for the treatment of human brain disorders, such as Parkinson's and Huntington's disease, traumatic brain injury and stroke[123–125]. The beneficial outcomes of MSCs transplantation in traumatic brain injury and adult ischemic conditions are strongly supported by preclinical data. However, these results cannot be fully attributed directly to cells, but must also be related to factors secreted by them. In fact, through their paracrine activity they are able to stimulate proliferation of neural stem cells, as well as increase the number of mature astrocytes[126]. *In vitro*, MSCs can activate NPCs, inducing their expansion and differentiation into neurons, astrocytes and oligodendrocytes. The use of MSCs *in vivo* has shown an increase in new neuronal cells formation, myelination, synaptogenesis and axon growth in the ischemic hemisphere, as well as decreased apoptotic cell death and infarcted area[127,128]. Moreover, MSCs are also able to promote angiogenesis and vascular remodeling. Importantly, mesenchymal cells have the capability to secret molecules related to those regenerative processes, such as BDNF, GDNF, VEGF, fibroblast growth factor-2 and 7, fibronectin, heparin binding-epidermal growth factor, hepatocyte growth factor, IL-6, monocyte chemoattractant protein-1 and platelet-derived growth factor. The release of these trophic factors appears to be enhanced as a response to injury clues[129].

Another important characteristic of MSCs is the ability to communicate with immune cells. In the ischemic brain, MSCs can reduce the number of inflammatory cells, regulate the secretory profile of microglia and macrophages and change the expression of pro and anti-inflammatory cytokines. In fact, a shift in macrophages from M1 pro-inflammatory phenotypes to an M2 anti-inflammatory state has been observed, resulting in the promotion of protective and regenerative responses[130].

Based on evidences showing the paracrine effects of MSC, they became the most used progenitor cell type for exosomes production. MSC-exosomes have already shown good results over a wide range of disease. In the heart, exosomes released from MSCs stimulated by hypoxia and serum deprivation, were able to prevent apoptosis and cardiac fibrosis[131]. Moreover, in a mouse model of acute myocardial

infarction, exosomes enhanced cardiac function[132]. In the kidney, MSC-exosomes have promoted morphological and function recovery after acute kidney injury caused by cisplatin[133]. Related with CNS diseases, the administration of these exosomes in stroke mouse models has promoted neurite outgrowth, angiogenesis and neurogenesis, ultimately leading to function recovery[134–136].

1.4. Exosomes for Brain-Related Disorders

Advances in the therapeutic outcome of exosomes-based therapies have increased the interest for developing new strategies for neuronal disorder. In the central nervous system, exosomes from dendritic cells have already proved to be able to modulate neuroinflammatory responses *in vitro* and *in vivo*, mainly because they are internalized by microglial cells[137]. Exosomes from adipose-derived stem cells are being tested as a treatment for amyotrophic lateral sclerosis, mainly through reduction of SOD1 aggregation and mitochondrial dysfunction[138].

Exosomes isolated from bone marrow MSC have shown a beneficial role in traumatic brain injury and stroke, however, it is not yet known the responsible mechanism of action. Back in 2013, the group of Michael Chopp proved that MSCs exosomes are internalized by astrocytes and neurons, and mainly through the presence of miR-133b, they are able to promote axonal plasticity and neurite remodeling. These exosomes seem to be also capable of inducing angiogenesis through miR-125a, by repressing the angiogenic inhibitor delta like 4 (DLL4)[139]. In fact, nucleic acids are being pointed as responsible for the exosomes beneficial proprieties, although it is unlikely that microRNAs are the only molecules responsible for the observed effects[140].

Remarkably, the treatment of animals subjected to transient cerebral ischemia with exosomes or their progenitor cells showed to have similar therapeutic outcomes, reinforcing a paracrine mechanism. Interestingly, exosomes can exert their role either through direct phenotype remodeling of damaged cells or by induction of different paracrine pattern from supporting cells. Exosomes internalized by astrocytes induce these cells to release bioactive factors able to stimulate neural plasticity, ultimately leading to improved functional recovery after stroke[141,142].

An intense exercise is also being done in order to use loaded-exosomes to reach brain tissue. MSCs expressing miRNA-17-92 cluster have shown to be able to release exosomes containing this genetic material, enhancing their bioactivity in a stroke model through stimulation of neurite remodeling, neurogenesis and oligodendrogenesis[143]. When isolated from brain endothelial cells and loaded with siRNA to inhibit vascular endothelial growth factor (VEGF), exosomes can reduce the number of brain cancer cells[144]. Another group, demonstrated that exosomes from embryonic stem cells loaded with curcumin can restore neurovasculature after ischemia-reperfusion injury[145] and prevent oxidative and inflammatory processes[146].

The efficiency of increasing the exosome targeting to the CNS was demonstrated by modifying nanovesicles with a neuron-specific peptide. Using this methodology, it was possible to deliver GAPDH siRNA to neurons, microglia and oligodendrocytes, without significant distribution to other organs[147].

1.5. Aims and Work Plan

Exosomes have emerged as a new and promising therapeutic tool for tissue regeneration. A large amount of information is already known about the role of MSC-exosomes for cell-free-based approaches, however, beyond the use of conditioned medium, very few studies about MNC-exosomes are known.

Our aim was to reveal the therapeutic role of umbilical cord blood MNC-exosomes over three distinct, but yet interconnected intervenient in ischemic stroke recovery, endothelial cells, neurons and neural stem cells (figure 3). Furthermore, we evaluated in parallel Wharton Jelly's MSC-exosomes in other to compare the bioactivity results and assess which type of exosomes promote better outcomes.

This work is divided in two main sections: (i) MNC- and WJ-MSC-exosomes isolation and characterization; (ii) Evaluation of both exosomes bioactivity over distinct players involved in ischemic stroke recovery.

(i) We isolated and characterized both cells, using specific surface markers for each population. Cells were incubated under hypoxia for 18 hours and exosomes were isolated from cell culture medium. To prove their nature, vesicles were subjected

to size, zeta potential and specific marker analysis, before following with the bioactivity experiments.

(ii) In order to first prove that exosomes are internalized by recipient cells, they were labeled with a fluorescent dye and incubated with human umbilical vein endothelial cells (HUVECs) and cortical neurons. As our work was performed *in vitro*, we chose a well described ischemic stroke mimicking model[148], were we incubated HUVECs and cortical neurons under oxygen and glucose deprivation conditions for 2 hours, before treatment with 3 different exosomes concentrations. 24 hours later, cell survival was evaluated by nuclear Hoechst staining. Regarding the bioactivity over NSC, exosomes were also added in 3 different concentrations, without any initial stimuli. Toxicity, proliferation and differentiation were evaluated by immunocytochemistry after 2 (toxicity and proliferation) and 7 days (differentiation).

2. Materials and Methods

2.1. Cell Cultures

2.1.1. Isolation of mononuclear cells from Human Umbilical Cord Blood

All human umbilical cord blood samples were collected from patients of Dr. Daniel de Matos and Bissaya Barreto maternities in Coimbra. All donors signed an informed consent form according with the Portuguese legislation. The samples were stored in single blood bag systems containing anticoagulant citrate phosphate adenine solution. Mononuclear cells were isolated within 48 hours after collection using a density gradient solution (Lymphoprep™, STEMCELL™ technologies, Canada). Briefly, blood samples were two times diluted in dilution buffer (2mM EDTA Ca^{2+}/Mg^{2+} free PBS, pH 7.2)[149]. Carefully, 30 mL of diluted blood were layered over 15 mL of Lymphoprep™ solution and centrifuged at 400 g for 45 min at 20ºC without brake. Mononuclear cells layer was collected from the interphase and diluted with MACS buffer (2 mM EDTA, 0.5% BSA Ca^{2+}/Mg^{2+} free PBS). Cells were first centrifuged at 300 g for 10 min at 20ºC, followed by two times 200xg for 10 min in order to remove platelets. Before the last centrifugation, an aliquot of suspension was collected in order to count cells, using a hemocytometer (Synmex XE2200, Synmex Medical Instrument Co, USA). Pelleted cells resulting from the last centrifugation were ressuspended in freezing medium (10% IMDM, 5%DMSO, 85%FBS) and conserved in liquid nitrogen.

2.1.2. Isolation of mesenchymal stem cells from Human Umbilical Cord Wharton's jelly (WJ-MSC)

Human Umbilical Cord Wharton's jelly was provided by Crioestaminal (Cantanhede, Portugal). Pieces of tissue were minced and cultured in α-MEM (Genaxxon biosciences) supplemented with 10% FBS (Invitrogen) and 100 U/mL of Pen/Strep (Invitrogen) at 37ºC with 5% CO_2. Once cells spread out through the disk, pieces of tissue were removed and cells were allowed to grow[150]. The medium was changed every 2-3 days. The mesenchymal population was selected based on its ability to adhere to the culture plate. At 80–90% confluence, cells were detached using 0.5% trypsin–EDTA (Gibco) and replated at a ratio of 1:4. Cells were used at the fourth passage in all experiments.

2.1.3. Isolation of rat cortical neurons

Primary cortical neuronal cultures were prepared from the cortex of embryonic day 18-19 (E18-E19) Wistar embryos [151]. Briefly, the brain was exposed and placed on calcium- and magnesium-free hank's balanced salt solution (HBSS). Meninges and hippocampus were removed and the cortices were treated with 0.06% trypsin (Gibco Invitrogen) for 15 min at 37ºC. Tissue was then thoroughly washed with HBSS to stop trypsin activity and mechanically dissociated with a pipette. Cells were plated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid) onto poly-D-lysine coated 6-well plates at a density of 850 000 cells per well (for western blot) or poly-D-lysine coated 24 well plates at a density of 180 000 (OGD) of 90 000 (internalization) cells per well. After 2-4 hours, culture medium was changed to neurobasal medium (Gibco, Life Technologies) supplemented with 1:50 of SM1 (Stem Cell Technologies), 0.5 mM glutamine (Invitrogen) and 0.12 mg/mL gentamycin (Sigma-Aldrich). Cultures were kept in a humidified incubator with 5% CO_2, at 37ºC for 14 days.

2.1.4. Human umbilical vein endothelial cells

Human umbilical vein endothelial cells were purchased from Lonza and cultured in endothelial growth facor-2 media (EGM-2) (Lonza). The medium was changed every 2-3 days. HUVEC cells under passage 6 were used for all experiments.

2.1.5. Isolation of neural stem cells from mouse subventricular zone

Neural stem cells were isolated from the SVZ of 1-3 day old C57BL/6 mice. Animals were decapitated and the brains removed to calcium- and magnesium-free HBSS supplemented with 100 U/mL Pen/Strep. Once inside solution, meninges were pull off and the brains were cut in 450 μm thick coronal sections using a Mcilwain tissue chopper (Ted Pella, INC). SVZ fragments were dissected and posteriorly digested in 0.025% trypsin, 0.265 mM EDTA for 10 min at 37ºC, followed by mechanical dissociation with a pipette. Cells were immediately seeded on uncoated petri dishes at a density of 63000 cells per 60 mm culture dish in Dulbecco's modified eagle medium [(DMEM]/F12 + GlutaMAX-I)] with 100 U/mL Pen/Strep, 1% B27, 10 ng/mL

epidermal growth factor (EGF) and 5 ng/mL basic fibroblast growth factor-2 (FGF-2) (all from Invitrogen) and allowed to grow for at least 6 days.

2.2. Exosomes isolation and characterization

2.2.1. Exosomes isolation from MNCs and WJ-MSCs

For exosome isolation from MNCs, cells were rapidly taken out of liquid nitrogen and thawed in a warm bath at 37ºC. Each 1 mL of cells were diluted into 9 mL of X-VIVO15™ (Lonza, Switzerland) supplemented with 50 μg/mL of DNAse I (Sigma Aldrich Co). Before centrifugation at 300 g for 10 min, an aliquot was taken in order to count the number of cells. Pelleted cells were resuspended in X-VIVO15™ medium supplemented with 50 ng/mL of stem cell factor (SCF, Petrotech) and 50 ng/mL of fms like tyrosine kinase 3 (FLT-3, Petrotech). Eight million cells (2 million cells per mL) were seeded into each well of a 6-well plate (Costar) and incubated in a hypoxia chamber (BioSpherix culture chamber, BCA Scientific) under humidified atmosphere with 0.5% O_2 and 5% CO_2, at 37ºC. After 18 hours, cell culture medium was collected and exosomes isolated by differential ultracentrifugation[152]. First, conditioned medium was centrifuged at 300 g for 10 min at 4 ºC to pellet cells and supernatant was again centrifuged at 2000 g for 20 min, also at 4ºC to pellet cell debris. Using an Optima™ XPN 100K ultracentrifuge with a swinging bucket rotor SW 32 Ti (Beckham Coulter), supernatant was two times centrifuged at 10000 g for 30 min at 4 ºC in order to pellet microvesicles. Exosomes were then pelleted with a 100000 g centrifugation for 120 min at 4ºC. Pelleted exosomes were washed with cold PBS, recentrifuged at the same speed, resuspended in 200 μL of cold sterile PBS and conserved at -80ºC (figure 6).

To isolate exosomes from WJ-MSC, 1 million cells at passage 4 were seeded into each 100 mm petri dish (Falcon) and cultured in α-MEM with 10% FBS in 5% CO_2 at 37ºC. One day later, cells were washed two times with warm PBS and conventional medium was replaced by α-MEM with 10% exo-depleted FBS. In order to deplete exosomes, medium was subjected to a 100000 g overnight centrifugation at 4ºC. Cells were incubated under hypoxia conditions (0.5% O_2 and 5% CO_2), for 18 hours at 37ºC. Afterwards, cell culture medium was collected and centrifuged as described before, except for the first 300 g centrifugation.

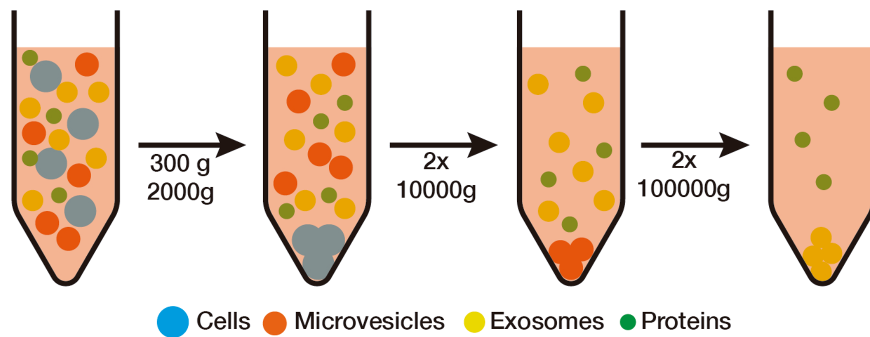


Figure 6-Schematic representation from the exosomes centrifugation and ultracentrifugation isolation processes. Once isolated, exosomes are kept at -80ºC up to one year.

2.2.2. Exosomes proteins quantification

Micro BCA™ protein assay kit (Thermo Fisher Scientific) was used to quantify the total protein content of exosomes. Briefly, standard curve BSA and exosomes were diluted in 2% sodium dodecyl sulphate (SDS) in order to disrupt exosomes membrane. Dilutions and reaction solution were prepared following manufacturer's instructions. Absorbance values were read in a microplate reader at 562 nm after 2 hours incubation at 37°C.

2.2.3. Dynamic light scattering (DLS) and zeta potential

Exosomes particle size and surface charge were determined using light scattering via Zeta PLAS potential analyzer and ZetaPlus Particle Sizing analyzer software, v2.27 (Brookhaven Instruments Corporation). From the total amount of exosomes, 10 µL were diluted into 240 µL biological grade ultrapure water (Fisher Scientific) filtered two times through a 0.2uM and introduced on a 400 µL quartz cuvette (FisherScientific). Average particle size was obtained at 25ºC with a detector angle of 90º and equilibration time of 5 min. A total of 5 individual runs of 60 sec were performed in each analysis. For the surface charge measurements, exosomes used in the last step were diluted in 1 mL of ultrapure water and placed in contact with zeta potential electrode. Five runs were done for each sample, at 25ºC with a relative residual value (measure of data fit quality) of 0.03[153].

2.2.4. Nanoparticle tracking analysis

The concentration and particle distribution of exosomes were measured by nanoparticle tracking analysis - NTA[154] (NanoSight LM 10, Nanosight Ltd). For that purpose, 1.5 µg of exosomes were diluted in 1 mL of MilliQ water, in order to achieve a final concentration between 5×10^8 and 1×10^9 particles/mL, which represents between 30 and 60 particles per frame[155]. Temperature was monitored across all samples. All samples were recorded 5 times (60 sec each)using a syringe pump speed of 10 µL to maintain a slow fluid. Camera level was set at 11 or 12 and detection threshold at 4. Videos were processed using NTA 2.2 analytical software (Nanosight Ltd).

2.2.5. Transmission electronic microscopy (TEM)

TEM was performed in exosomes isolated from MNCs and WJ-MSCs as previously described[152,154]. Briefly, 10 µg of exosomes were fixed with 2% paraformaldehyde (PFA) and deposited on Formvar-carbon coated grids (TAAB Technologies) for 20 min at room temperature. After washing 4 times with PBS, grids were placed on a drop of 1% glutaraldehyde for 5 min, followed of 7 water washes. In a dark environment, grids were incubated with uranyl-oxalate solution pH 7 for 5 min, and then placed on ice in contact with a solution of methyl cellulose (9:1) for 10 min. Exosomes imaging were performed using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 80 kV.

2.2.6. Flow cytometry

For the analysis of vesicles surface markers by flow cytometry (FC), 10 µg of exosomes quantified by Micro BCA™ were incubated with 5 µL of 3.8 µm latex beads (Life Technologies). After 15 min, PBS was added to achieve a final volume of 1 mL and the mixture was incubated in a rotative shaker overnight at 4ºC. This step will allow exosomes protein adsorption to the beads. On the next day, 110 µL from a solution of 1 M glycine was added for 30 min to saturate all the free biding sites on the beads. Following, exosomes and beads were centrifuged at 1300 g for 3 min. Pelleted beads were resuspended in 1 mL of 0.5% BSA/PBS and centrifugation step was repeated 3 times. After the final centrifugation, beads were resuspended in 0.5 mL of 0.5% BSA/PBS. For each antibody, 10 µL of exosome-coated beads were

mixture with 50 µL of antibody diluted 5 times in 0.5% BSA/PBS for 30 min at 4ºC. Labeled exosomes were washed three times with 150 µL 0.5% BSA/PBS and resuspended in 200 µL 0.5% BSA/PBS.

Cells were also subjected to flow cytometry analysis. For that, 500 000 cells in a final volume of 40 µL of MACS buffer, were incubated with 10 µL of desired antibody at 4ºC. After 30 min, cells were centrifuged at 300 g for 10 min at 4ºC and pellet washed 3 times with 500 µL of MACS buffer. Cells were resuspended in 300 µL of MACS buffer.

Both exosomes and cells measurements were performed using a BD Accuri 6 (BD Bioscience) and posteriorly analyzed in the cytometry data analysis Flowjo™ (v10, FlowJo, LLC).

Commercial antibodies used for flow cytometry were the following: CD9-FITC (RD Systems, FAB1880F), CD14-FITC (MACS-Milteney Biotec, 130-080-701), CD31-FITC (Thermo Scientific, Biosciences, E11797-1634), CD34-APC (MACS-Milteney Biotec, 130-080-002), CD45-FITC ([MACS-Milteney Biotec, 130-080-202), CD45-PE (BD Pharmigen, 555483), CD73-PE (BD Pharmigen, 550257), CD90-PE (BD Pharmigen, 561969), CD105-PE (BD Pharmigen, 560839), HLA-DR-FITC (Exbio, 1F-474-T025) and TSG101-FITC (Santa Cruz, SC-7964).

2.2.7. Preparation of protein extracts

The expression of exosomes specific markers was analyzed also by western blot. To do so, exosomes and exosomes-progenitor cells were resuspended in radioimmunoprecipitation (RIPA) buffer containing 1% phosphatase inhibitors cocktail (Invitrogen). Eveyr 5 min samples were vortex and left on ice for up to 30 min, followed by sonication in a water bath sonicator (Branson 2510, Marshall Scientific) during 10 min. Finally, the mixtures were centrifuged at 14000 g for 10 min at 4ºC and the supernatant kept at -80ºC.

To extract protein from cortical neurons, previously RIPA treated-frozen cells were taken out from the -80ºC and placed on ice. After defrosting, cells were scraped from the 6 well plate and centrifuged at 14000 xg for 10 min at 4ºC. Proteins were quantified

using Pierce™ BCA protein assay kit (Thermo Fisher) using BSA to generate the standard curve, following the manufacturer's instructions.

Equal amount of protein was mixture with Laemmli buffer (1M Tris-HCl, 5% SDS, 12% Glicerol, 13% β-mercaptoethanol, 0.025% bromophenol blue) and boiled for 5 min at 95ºC. The protein extracts were immediately used or storage at -80°C.

2.2.8. Polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot

Proteins were resolved by SDS-PAGE in 10% (exosomes and exosomes-progenitor cells) and 8% (cortical neurons) polyacrylamide gels. Resolving gels (8 or 10% acrylamide, 25% 1.5 M Tris-HCl pH 8, 0.1% SDS, 0.1% ammonium persulfate, 0.07% tetramethylethylenediamine) and 4% stacking gel (4% acrylamide, 25% 0.5 M Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, 0.07% tetramethylethylenediamine) were made inside 1.5 mm western blot glass apparatus (Bio-Rad). Runs were performed during the first 20 min at 60 V and at 90 V for 90 min in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After, gels were removed from the glass and placed on transference apparatus (Bio-Rad) in contact with previously methanol-activated PVDF membrane (Milipore) and proteins allowed to transfer at 100 V for 90 min in appropriate buffer (25 mM Tris, 200 mM glycine, 0.5% SDS, 20% methanol). Membranes were washed 2 times with TBS-T (140 mM NaCl, 2.5 mM KCl, 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.5% Tween-20), block with 5% BSA in TBS-T for 60 min and immediately incubated with desired primary antibody diluted in 1% BSA in TBS-T overnight at 4ºC. Membranes were afterwards washed 5 times with TBS-T and put together with secondary antibodies conjugated with horseradish peroxidase (HRP) diluted 1:10000 in 1% BSA in TBS-T for 1h at RT, followed by more 5 times washes. In order to read the results, 400 μL of WesternBright™ Quantum (Advansta) were put over the membrane and images acquired on a VWR imager (VWR). Bands density was analyzed using ImageJ software.

Commercial antibodies used for western blot were: primary antibodies 1:1000 GluN2B (Alomone, AGC-003), 1:15000 GAPDH (Cell Signal, 2118), 1:500 CD63 (Abcam, ab68418); secondary antibodies HRP conjugated anti-mouse (Cell Signal, 7076) and anti-rabbit (Cell Signal, 7074).

2.3. Biological assays with neurons and endothelial cells

2.3.1. Internalization kinetics

The ability of vesicles to be internalized by cells were analyzed by labeling exosomes with PKH 67 green fluorescent cell linker kit (Sigma-Aldrich Co, LLC)[156]. Briefly, 4 x 10^9 particles resuspended in 10 µL PBS were diluted with 40 µL of Diluent C (provided with the kit). On a different eppendorf, 0.5 µL of PKH 67 were diluted in 49.5 µL of Diluent C. Exosomes were afterwards placed on top of PKH 67, incubated during 3 min and purified from free dye using exosome spin columns MW3000 (Invitrogen, Life technologies).

HUVECs in 96 well plate and cortical neurons in 24 well plate seed at a density of 7500 and 90000 cells per well, respectively, were treated with 6000 particles per cells[157]. Internalization was measured after 4, 8, 12 and 24 hours. Cells were treated in triplicate with exosomes from three different donors. In each time point, cells were fixed with 4% PFA/4% sucrose and the nuclei labeled with Hoechst. Images were analyzed using InCell analyzer software. The nuclei were defined using the Hoechst signal, and a mask was created around the nuclei to determine the fluorescence intensity in the cytoplasm. If the intensity was superior to the background, that cell was considered positive. The result is presented as the percentage of cells with internalized exosomes.

2.3.2. Oxygen and glucose deprivation

The bioactivity of exosomes isolated from MNC and WJ-MSC was evaluated on HUVECs and rat cortex neurons subjected to OGD. HUVECs at passage 4 were seeded with EGM-2 in a 96 well plate at a density of 10000 cells per well and allowed to grow for 24 hours before the insult. Cortical neurons were used at DIV 15[151,158].

In the day of the insult, inside an anaerobic chamber (OGD chamber, Thermo Forma 1029, Thermo Fisher Scientific, Waltham, MA), cells were placed in a glucose-free deoxygenated buffer medium (10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 25 mM sodium bicarbonate, 25 mM sucrose, 1.8 mM $CaCl_2$, 0.04% phenol red, pH 7.1). Normoxia-control cells, called sham along this work, were

incubated in oxygenated conditions with a similar medium, containing 25 mM glucose instead of sucrose. Two hours later, cells were removed from oxygen deprivation conditions. For neurons, medium was immediately changed for their conditioned medium and exosomes added at 1.5, 3 or 4.5 x 10^9 particles per mL. In the case of HUVECs, insult medium was replaced by endothelial basal medium (EBM-2) (Lonza) supplemented with the same concentrations of exosomes as for neurons. Cells were treated with exosomes from three different donors.

2.3.3. Nuclear morphology analysis

Cellular viability was analyzed by nuclear staining. Twenty four hours (neurons) or 48 hours (HUVECs) after OGD insult, cells were washed with PBS, fixed with a solution of 4% PFA/4% sucrose for 15 min, washed again 5 times with PBS and the nuclei were labeled with 1 µg/mL Hoechst dye for 10 min in a dark environment. Next, cells were washed 3 times with PBS to remove the excess dye. Using the InCell analyzer (GE Healthcare Life Sciences), images were taken at 9 fields per well using a 20x objective and total nuclei count was measured using InCell analyzer software (GE Healthcare Life Sciences). Results are presented as percentage of live cells relatively to the non-treated-OGD-exposed control, using the following formula: % of live cells= (number of nuclei in the treated group-number of total nuclei in the non-treated-OGD-exposed control)*100/ number of total nuclei in the non-treated-OGD-exposed control.

2.3.4. GluN2B expression

To assess the expression of the GluN2B subunit of NMDA receptors (GluN2B) after insult, OGD affected neurons, in a 6 well plate, were immediately treated with 3 x 10^9 particles/mL diluted in neuronal conditioned medium. Twenty four hours later, cells were placed on ice and washed 2 times with cold PBS before adding 150 µL of RIPA buffer (50 mM Tris-HCl ph 7.4, 150 mM NaCl), 1% Triton X-100, 0.5% Deoxycholate, 0.1% SDS) containing 1% phosphatase inhibitors cocktail (Thermo Scientific). Cells were frozen at -80°C until used for protein isolation and western blot analysis.

2.3.5. Exosomes miRNA extraction and cDNA synthesis

Total RNA's were extracted from both MNC and WJ-MSC exosomes using miRCURY™ RNA isolation kit (Exiqon) following the manufacturer recommendations for cultured cells. Briefly, each sample received 700 µL of lysis buffer supplemented with 1% β-mercaptoethanol and was vortex for 1 min, followed by the addiction of 400 µL absolute ethanol. Sample RNAs were adsorbed to the silica matrix present on the provided columns and washed 3 times with a 14000 g centrifugation for 1 min using wash solution. Finally, 25 µL of water were added to the column and the RNA eluted with a 14000 g centrifugation for 2 min. Total RNA content was quantified using Nanodrop 2000 (ThermoFisher Scientific).

The synthesis of cDNA for miRNA quantification was done using the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR kit (Invitrogen). For a first step of poly-A extension, a total of 30 ng of RNA were mixture with provided reagents in a final volume of 25 µL and incubated for 15 min at 37ºC. To further produce cDNA, 4 µL from the miRNA poly-A reaction were incubated with a reverse transcriptase universal primer and incubated for 5 min a 65ºC. Next, 12 µL of reaction mix with reverse transcriptase enzyme were added and the total volume subjected to the follow protocol: 50ºC for 50 min and 85ºC for 5 min. cDNA samples were kept at -20ºC.

2.3.6. qRT-PCR quantification of miRNA expression

miRNA 223-3p expression was assessed by real time quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). For this purpose, 10 µL of SYBR green 2x (NZYTech) were mixture with 0.4 µL of universal reverse primer (Invitrogen), 0.4 µL of specific forward primer and 2 µL of 1:10 diluted cDNA sequence, in a final volume of 20 µL. U6 was used as endogenous loading control. The chain elongation reaction was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems) with the following protocol: 95ºC for 2 min and 40 cycles of 95ºC for 10 sec (denaturation) and 60ºC for 30 sec (annealing and elongation). Forward primers used were: has-miRNA-223-3p: 5'- ACAGTCAAACAGTTTATGGGGT -3' and RNU6: 5'- ACACGCAAATTCGTGAAG -3'.

2.4. Biological assays with neural stem cells

2.4.1. Neural stem cells treatment

Neural stem cells isolated from mice were allowed to grow and form neurospheres until DIV 6. In medium supplemented with growth factos [(DMEM/F12) + GlutaMAX-I] with 100 U/mL Pen/Strep, 1% B27, 10 ng/mL EGF and 5 ng/mL FGF. Neurospheres were then plated onto 10 mm coverslips coated with poly-D-lysine. In order to allow differentiation, no growth factos were given to cells (EGF and FGF). After 2 days, medium was replaced and exosomes added at a final concentration of 1.5, 3 and 4.5 x 10^9 particles per mL. For the toxicity and proliferation assays, cells were fixed after 48h, while for differentiation assays cells were fixed 7 days after the beginning of treatment beginning. In both cases, cells were first washed with PBS, followed by 15 min fixation with 4% PFA. Finally, coverslips were washed again 2x with PBS and kept at 4ºC until the immunocytochemistry procedure. Cells were treated in triplicate with exosomes from three different donors.

2.4.2. PI incorporation assay

The toxicity of exosomes was evaluated 2 days after the beginning of the treatment. Briefly, before fixation cells were incubated with 3 µg/mL of propidium iodide (PI) for 10 min and washed 2 times with PBS. After fixation, nuclei were labeled using a solution of 2 µg/mL of Hoechst for 10 min and finally washed 2 times with PBS. Coverslips were mounted with Dako fluorescent medium (Dakocytomation, Inc) and 10 images were acquired using a confocal microscope with 40x oil objective (LSM 710, Carl Zeiss). Cells were considered dead when positive for PI.

2.4.3. Immunocytochemistry for proliferation and differentiation markers

The treatment of neural stem cells with exosomes was analyzed by immunocytochemistry. Fixed cells were permeabilized using 3% BSA/0.3% Triton X-100 for 30 min at room temperature and directly incubated overnight at 4ºC with primary antibodies diluted 1:100 in 0.3% BSA/0.03% Triton X-100. On the following day, cells were washed 3 times with PBS and incubated for 1 hour at RT with respective secondary antibodies (1:200) and Hoechst (1 µg/mL) in 0.3% BSA/0.03%

Triton X-100, followed by 3 finally washes. Coverslips were mounted with Dako fluorescent medium and 10 images per condition were acquired using a confocal microscope with 40x oil objective.

Overall proliferation and proliferation of committed cells was assessed by the quantification of positive cells stained with Ki67 (Abcam, Ab15580) colocalized with Nestin ([self-renewal], Abcam, ab6142) or Doublecortin ([neuroblasts], Santa Cruz, SC-8066). Differentiation was evaluated with NeuN (Cell Signal, D3531) for neurons or with Sox-2 (Santa Cruz, Sc-17320) for stemness. Quantifications were perfomed using ImageJ software.

Secondary antibodies used (all produced in donkey) were: Alexa 647-linked anti-goat (Life Technologies, A21447), Alexa 594-linked anti-mouse (Abcam, Ab150108) and Alexa 488-linked anti-rabbit (Life Technologies, A21206).

2.5. Statistical analysis

All quantitative data is presented as an average of the number of samples indicated for each methodology and standard error of the mean (SEM). In vitro biological data was obtained from three independent experiments with at least three replicates for each condition. Statistical analysis was performed using GraphPad Prism software v7.0a (GraphPadSoftware).

Firstly, a Shapiro-Wilk test was used to ascertain about the data normality. All results showed a normal distribution and were analyzed by oneway ANOVA or two tailed Welch's t-test. For oneway ANOVA, Turkey's multiple comparisons test was used as a post-hoc pairwise comparison test. Values were considered statistically significant for $p<0.05$.

3. Results

3.1. Exosomes characterization

3.1.1. Characterization of progenitor cells

Exosomes are able to carry information from progenitor cells to other cells types, allowing modulation of distinct tissues. Moreover, nanovesicles released by the same cell type, in different culture conditions, seem to have distinct constituents, indicating that these particles can transport progenitor-state-specific material. Based on that premise, and also on results obtained in our laboratory (data not published), we isolated exosomes from hypoxia conditioned cells in order to overload hypoxia/survival precursors inside exosomes. With the focus of producing exosomes from MNC and WJ-MSC we first isolate cells from umbilical cord blood and Wharton's jelly tissue, respectively (figure 7). After hypoxia stimulus for 18 hours, conditioned medium was harvested for further exosomes collection, while the cells were prepared for flow cytometry analysis of specific markers from each type of cells, in order to characterize the phenotypes of progenitor cells.

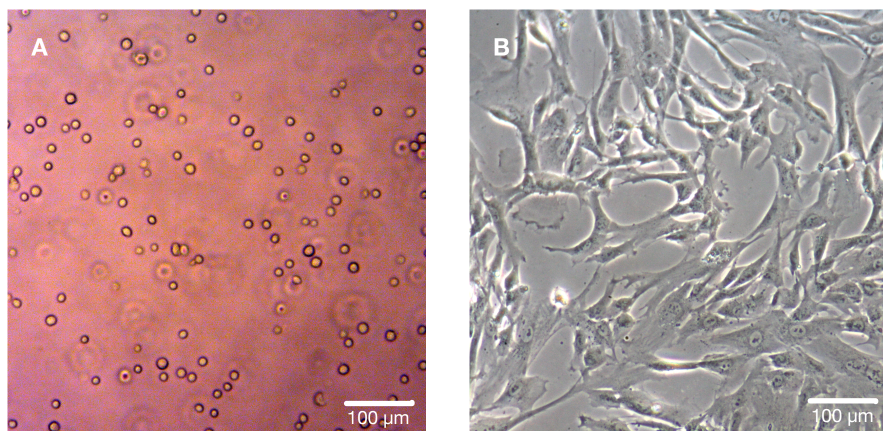


Figure 7- Brightfield images of mononuclear and mesenchymal cells morphology. A) MNCs are cultured in suspension and have a round shape. B) MSCs are plastic adherent cells and present a fusiform morphology.

MNC cells were composed mostly by small round non-adherent cells (figure 7). After hypoxia, most MNC cells (95.15%±0.03) have shown to be positive for CD45 (leukocyte common antigen) known as a marker of hematolymphoid cells (figure 8A)[159]. The appearance of CD14 positive cells (figure 8C) showed that 9.20%±0.12 of our progenitor cells are monocytes. Interestingly, CD34 (figure 8B) was present in 8.54%±0.01 of all cells, indicating that hematopoietic stem cells are enriched after

hypoxia treatment. Moreover, 2.72%±0.01 of the cells expressed CD31, an endothelial cell marker, confirming the heterogeneous phenotype of mononuclear cells population (figure 8D)[159]. Human leukocyte antigen D related (HLA-DR), normally present in B lymphocytes is present in 67.75%±0.13 of population. Moreover, the presence of exosomal markers was evaluated demonstrating that CD9 is expressed on the surface of 5.84%±0.06 cells and TSG101 only on 1.72%±0.00 (figure 8F, G).

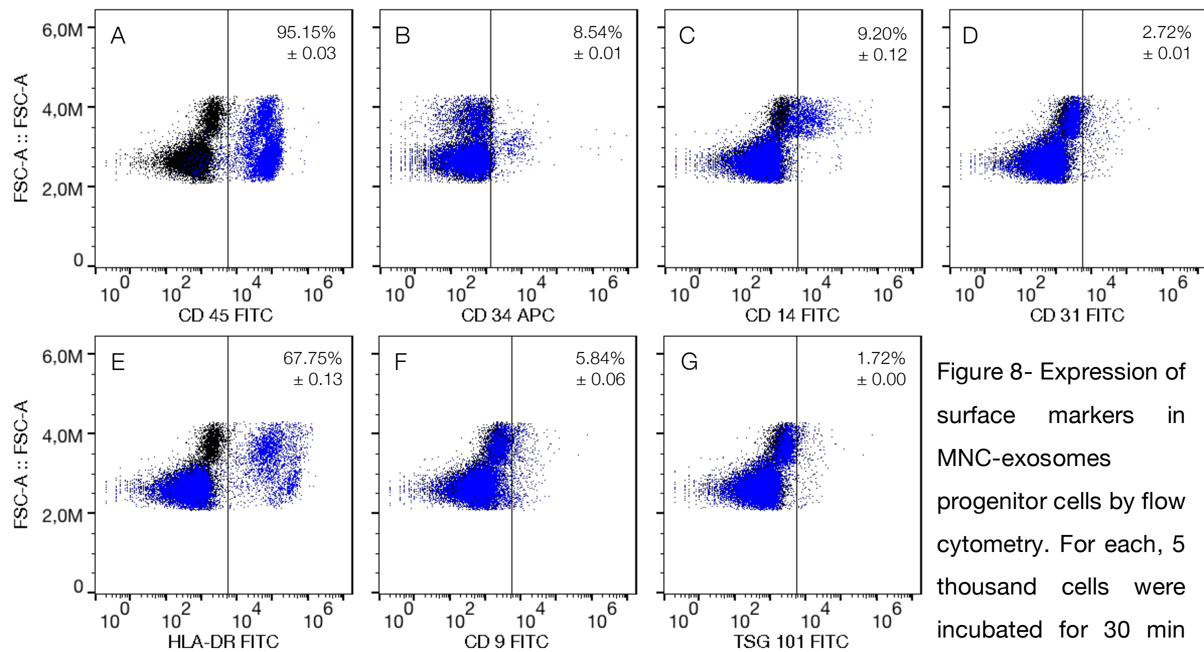


Figure 8- Expression of surface markers in MNC-exosomes progenitor cells by flow cytometry. For each, 5 thousand cells were incubated for 30 min with 10 µL of specific antibody. Black dots represent negative IgG control, while blue dots show the fluorescence intensity of cells with indicated antibodies. Data were collected from 4 individual donors (n=4).

The mesenchymal nature of isolated WJ-MSCs was also evaluated following the criteria established by The International Society for Cellular Therapy[160]. The WJ-MSCs population, isolated by plastic-adherence, presented a fusiform morphology and an immunophenotype consistent with MSC (figure 7B). Flow cytometry analysis show that WJ-MSC population was nearly 100% positive for CD73 (figure 9A), CD90 (figure 9B) and CD105 (figure 9C) and negative for CD45 (figure 9D) and HLA-DR (figure 9E). The expression of exosomal markers revealed that 28.65% of WJ-MSCs have CD9 (figure 9F), while only 4.92% express superficial TSG101 (figure 9E).

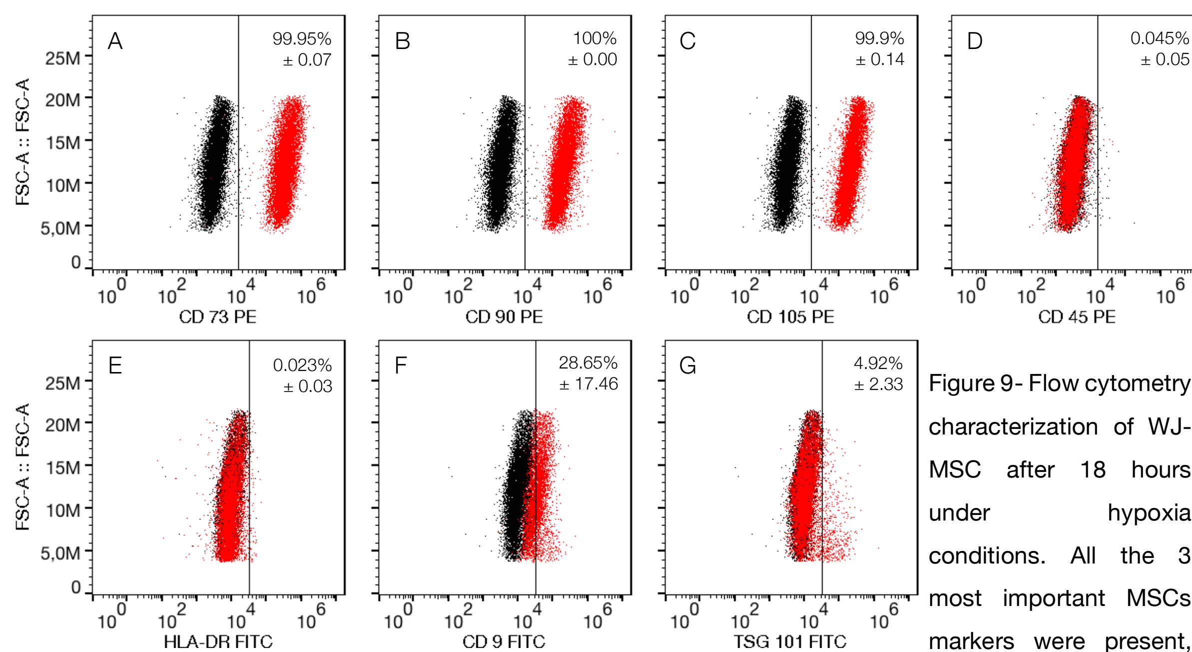


Figure 9- Flow cytometry characterization of WJ-MSC after 18 hours under hypoxia conditions. All the 3 most important MSCs markers were present, whereas CD45 and HLA-DR were absent, supporting the mesenchymal phenotype of our progenitor cells. Black dots represent negative IgG control. Red dots indicate fluorescent cells with the specific antibody. Results were obtained from 4 individual donors (n=4).

3.1.2. Exosomes morphological analysis

Exosomes were isolated from the conditioned medium of the two progenitor cell types by ultracentrifugation[152].

Following isolation, exosomes were biophysically characterized for the size and zeta potential. The size of exosomes was explored by DLS and NTA. Results from DLS show that MNC-exosomes are slightly smaller than WJ-MSC-exosomes, with a mode size of 117±14.45 nm and 137±11.51 nm, respectively (figure 10A). Both types of exosomes have strong negative zeta potentials of -31.13±2.10 mV for MNC-exosomes and -34.95±2.12 mV for WJ-MSC-exosomes, due to negatively charged phospholipid membrane. NTA analyses (figure 10C) presented different results regarding the size from those obtained by DLS, but yet with consistent variations. MNC-exosomes proved to be again smaller (150±2.5 nm) when compared with WJ-MSC-exosomes (170±12.5 nm) (figure 10D, 3E).

The morphological characterization of exosomes was performed using a transmission electronic microscope that allowed the observation of single vesicles. Exosomes from both sources had a round shape and presented bilayer membrane,

as indicated by the white arrows (figure 10F, G). Moreover, size measurements performed during image acquisition shown to be coherent with those obtained by DLS analyzes.

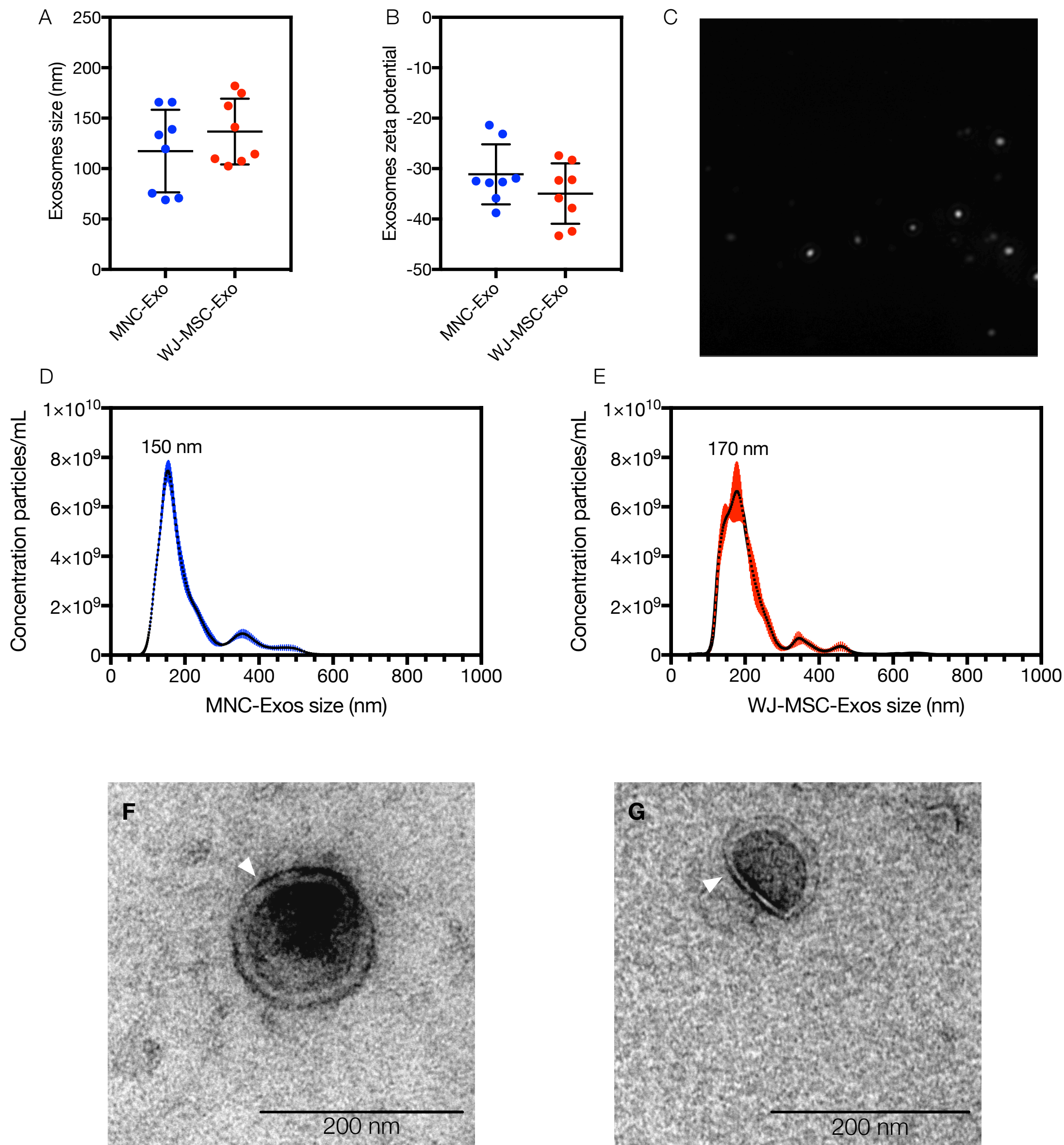


Figure 10- Size (A) and zeta potential (B) analysis of MNC- and WJ-MSC-exosomes obtained by DLS (n=8). C) Representative image of nanoparticle tracking analysis of MNC-exosomes. D and E) NTA (n=8) size distribution reported sizes with slightly difference, however the variances were consistent between the two exosomes types. TEM imaging of (F) MNC- and (G) WJ-MSC-exosomes. Both vesicles have shown a circular shape and bilayer phospholipid membrane, as indicated by the white arrows.

3.1.3. Exosomal and progenitor cells specific markers expression

The expression of exosomes specific markers was performed in accordance with the guidelines of The International Society of Extracellular Vesicles[161]. Exosomes-enriched markers and progenitor cells specific surface markers were analyzed using flow cytometry and western blot.

The majority of MNC-exosomes (96.06%±3) expressed the most well documented exosome marker CD9 (figure 11F) and 12.04%±4 were positive for TSG101 (figure 11G), which represents an enrichment compared to the expression on progenitor cells (5.84%±6 and 1.72%±0, respectively figure 8F, G). MNC-exosomes also presented a high level expression of CD45 (55.88%±27), which supports their leukocyte origin (figure 11A). CD31 was present in 27.54%±18 of the exosomes, while CD34 was only present in 2.37%±1 and CD14 in 0.83%±0 (figure 11B, C, D). The expression of HLA-DR in MNC-exosomes, 69.63%±19 (figure 11E) was similar to the progenitor cells (figure 8E).

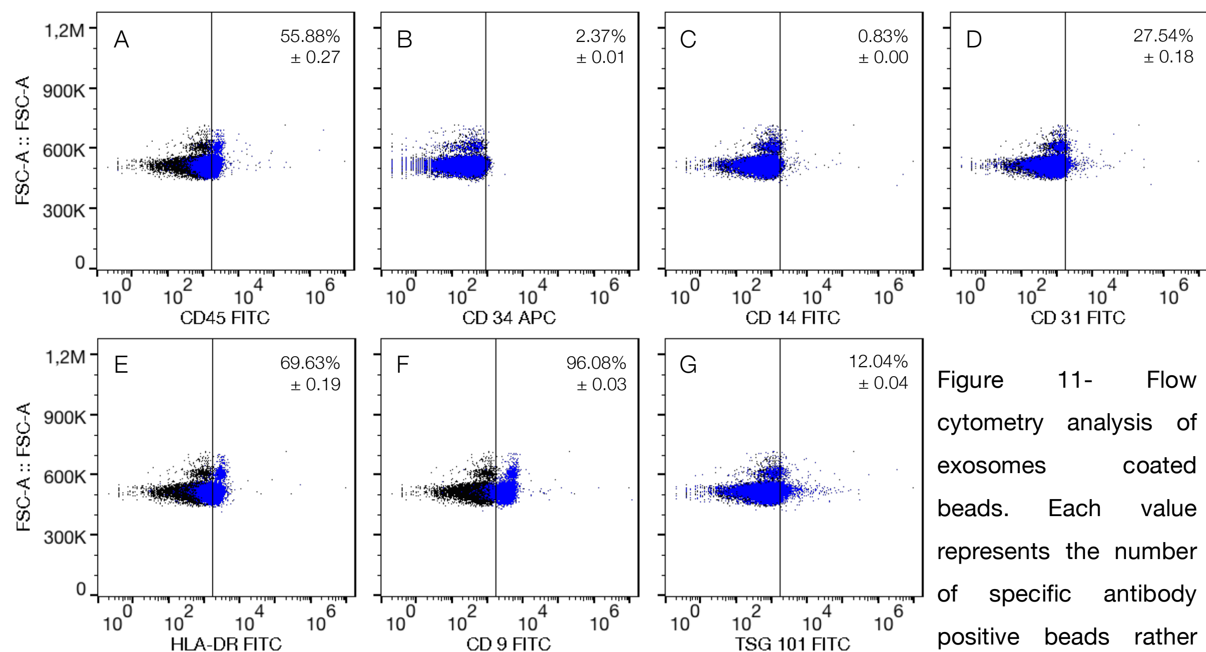


Figure 11- Flow cytometry analysis of exosomes coated beads. Each value represents the number of specific antibody positive beads rather than the number of positive exosomes. Black dots represent negative IgG control. Blue dots illustrate the fluorescence of beads for each antibody. Data were collected from 4 different batches of exosomes prevenient from different donors.

The same analysis was performed for WJ-MSC-exosomes. Specific markers CD9 and TSG101 showed to be enriched, when compared with WJ-MSCs, with 59.5%±29

and 22%±13.96 positive beads, respectively. Exosomes exhibited mesenchymal cells specific markers CD73 (8.27%±5.02), CD90 (88.5%±7.56) and CD105 (25.95%±21.8) and lack the presence of leukocytes marker CD45 (0.55%±0.42) and HLA-DR (0.40%±0.2), confirming the phenotype origin of the exosomes (figure 12).

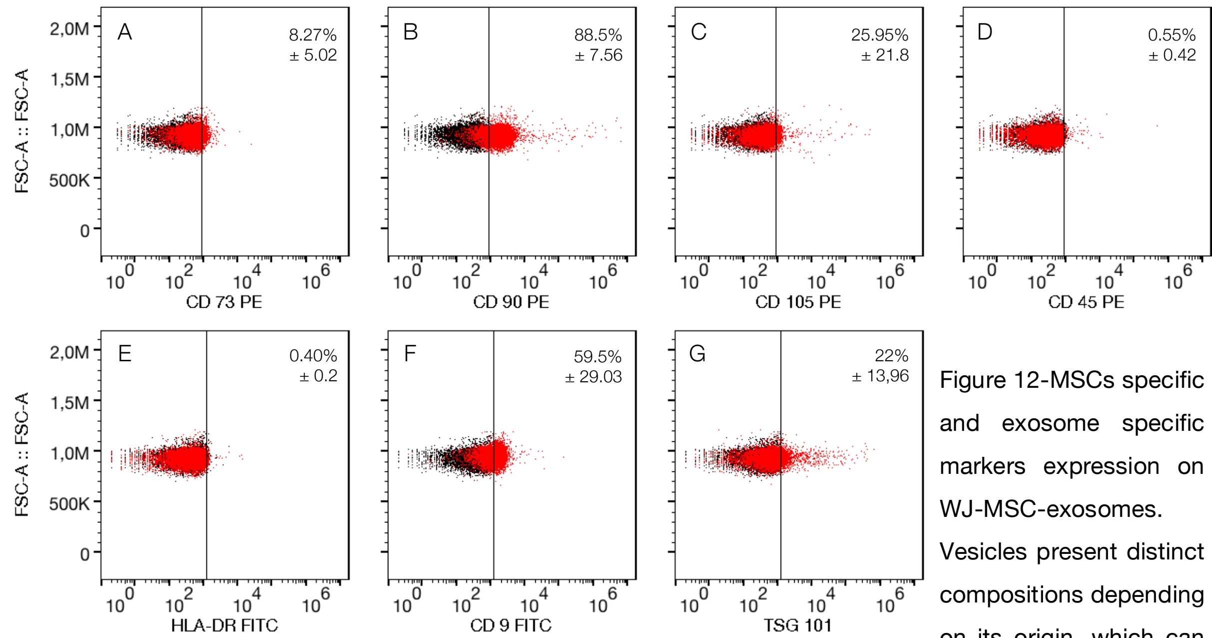


Figure 12-MSCs specific and exosome specific markers expression on WJ-MSC-exosomes. Vesicles present distinct compositions depending on its origin, which can transduce in different bioactivities. Back dots represent negative IgG control. Red dots represent exosomes coated beads fluorescence for specific antibodies. Data were obtained from exosomes isolated from 4 different donors.

The expression of another well describe exosome marker, CD63, was assessed by western blot. Loading equal amounts of protein in the gel, both cells and exosomes were positive for CD63, however the marker is clearly enriched on both exosomes relatively to their progenitor cells. GAPDH is also present in exosomes, although in less quantity (figure 13).

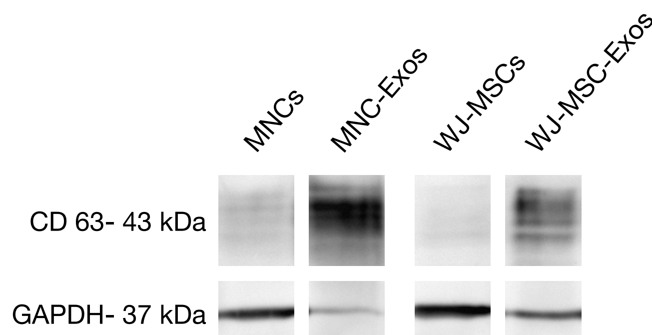


Figure 13- Western blot analysis of CD63 expression in exosomes and their progenitor cells. Both are positive for the marker, with exosomes showing an enrichment relatively to progenitor cells. Exosomes also presented GAPDH, although in less quantity than cells.

3.2. Assessment of exosomes bioactivity

3.2.1. WJ-MSC-exosomes have a quicker internalization kinetics than MNC-exosomes

One of the most promising features from exosomes is their ability to easily be internalized by cells. Since the exosomes used in this work were isolated from 2 different progenitor cells, their internalization kinetics was analyzed and compared. Exosomes were labeled with the green fluorescent dye PKH 67 and incubated in a ratio of 6000 particles per cells[157] with HUVECs and cortical neurons. At each time point, cells were fixed and their nuclei stained with Hoechst (figure 14A). An aliquot of labeled exosomes was used to measure the fluorescence intensity after labeling, which revealed that WJ-MSC-exosomes were able to retain more dye, presenting higher fluorescence intensity (figure 14B). WJ-MSC exosomes showed to be rapidly internalized in both cell types, exhibiting faster uptake kinetics than MNC-exosomes.

In HUVECs, after 4 hours almost 45% of the cells were WJ-MSC exosomes positive, against 5% positivity for MNC-exosomes. After 12 hours, around 90% of the HUVECs contained WJ-MSC-exosomes and nearly 100% were positive after 24 hours. Distinctively, the number of MNC-exosomes-containing cells had a small increase of 15% in the first 12 hours and 70% of the HUVECs were positive 24 hours after the incubation started (figure 14C). The uptake ratio of WJ-MSC-exosomes in neurons were also higher than the one showed by MNC-exosomes, and fascinatingly just after 2 hours those vesicles were already visible in almost 85% of neurons, leading to just a small increases of positivity until the 24 hours, when almost all cells contained exosomes. MNC-exosomes presented a similar kinetics in neurons as in HUVECs but with a faster initial internalization at 4 hours, presenting 35% of positive neurons. At the final observation, MNC-exosomes were present in nearly 60% of neurons (figure 14D).

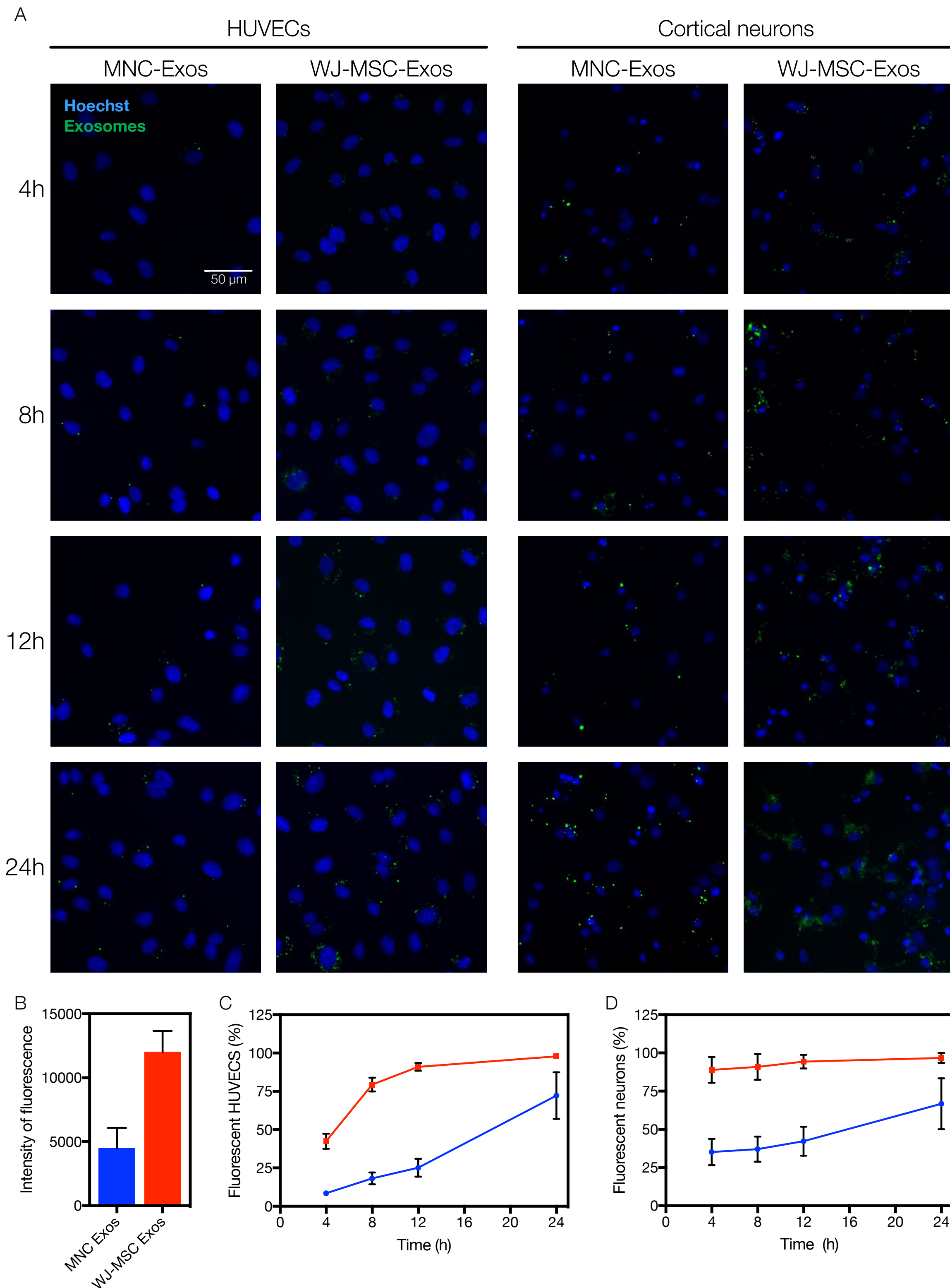


Figure 14- Exosomes form both progenitor cells are internalized by HUVECs and neurons with different kinetics. A) InCell analyzer images obtained at the different reading points for both cells treated with MNC- and WJ-MSC-exosomes, exposing distinct internalization patterns. B) Fluorescence intensity of exosomes after incubation with PKH67. MNC-exosomes presented less intensity signal than WJ-MSC-exosomes. C and D) Internalization kinetics of both exosomes in HUVECs and neurons. WJ-MSC-exosomes showed on the two cases to have higher uptake ratio and able to reach almost 100% of cells. MNC-exosomes have a slower internalization ratio and also could just be found in less than 75% of cells at 24 hours.

3.2.2. Both MNC- and WJ-MSC-exosomes promote HUVECs survival after OGD insult

After an ischemic stroke, the brain region affected by the blocked vessel gets on starvation of oxygen and nutrients, leading to cellular death. Therefore, restoring the blood vessels supply can help in recovering the neurological functions. To mimic what happens during a IS, we used the OGD *in vitro* assay. Basically, cells were placed under anoxia conditions with a glucose free medium during 2 hours, being afterwards treated with exosomes (in respective culture medium) and incubated in regular normoxia conditions (humidified atmosphere with 5% CO_2 at 37°C). To prove that cellular death was being induced by nutrients deprivation, positive control cells (sham) were incubated with glucose-containing-OGD medium under normoxia conditions during the 2 hours.

To evaluate whether exosomes were able to induce HUVECs survival after OGD procedure, cells were treated with 1.5, 3 and 4.5 x 10^9 particles per mL either with MNC-exosomes or WJ-MSC-exosomes and counted 48 hours after the treatment (figure 15A). Using Hoechst staining we considered dead cells the ones with a smaller nucleus diameter and more bright (figure 15B, white arrow), whereas live cells presented larger nuclei and low fluorescence intensity (figure 15B, black arrow).

Both vesicles showed bioactivity effect by protecting cells from death, with an increase on activity directly related with the increase of exosomes concentration. However, MNC-exosomes treatment was just statistically relevant for the higher concentration with 30% more live cells relatively to OGD non-treatment control (figure 15C). WJ-MSC-exosomes showed to be slightly more bioactive, presenting an increase in live cell percentage with the intermediate concentration and more than 40% viable cell increase for the higher concentration (figure 15D).

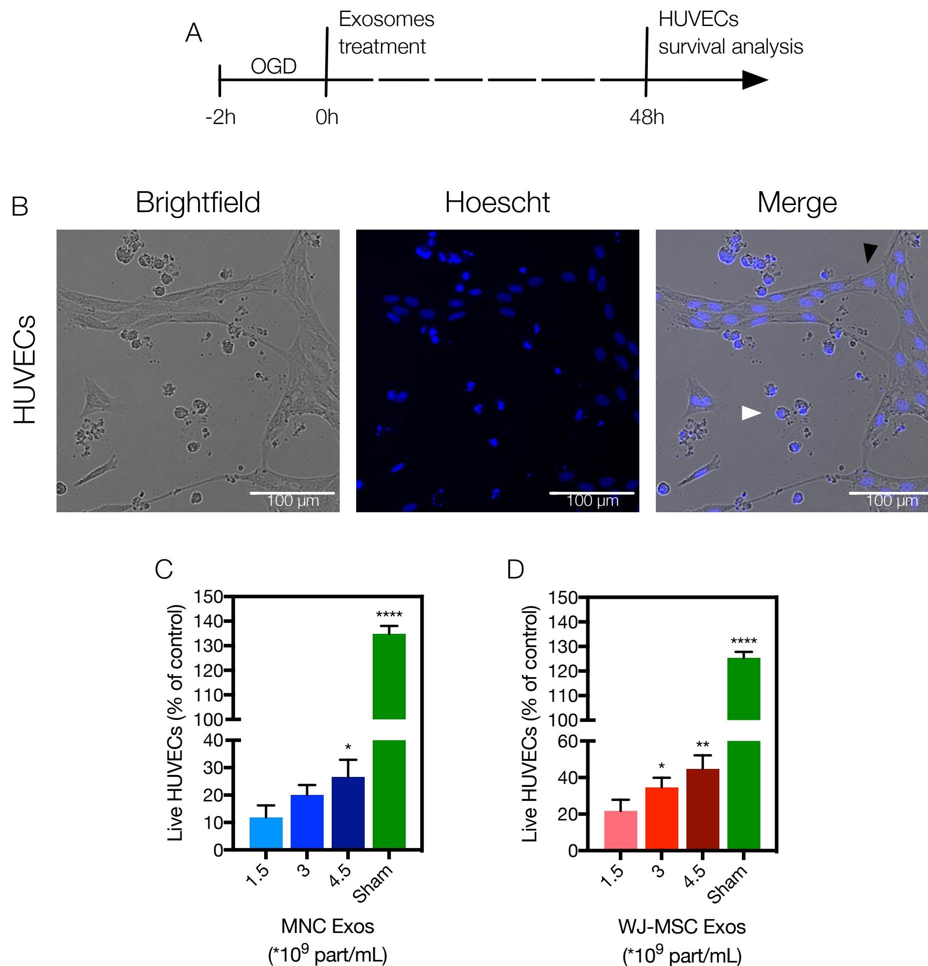


Figure 15- Exosomes treatment of HUVECs after OGD insult. A) Schematic representation of the procedure, cells were subjected to 2 hours of nutrients deprivation and treated immediately after with whether MNC- or WJ-MSC-exosomes. B) Nuclei staining by Hoechst revealing live cells with bigger nuclei/low fluorescence intensity and dead cells having smaller nuclei/strong intensity. C and D) Treatment with both exosomes showed to be efficient in protecting cells from OGD induced death. Results and expressed as percentage of live cells relatively to non-treated-OGD-exposed control. *p<0.05. **p<0.005, ****p<0.0001. (n=3).

3.2.3. MNC-exosomes, but not WJ-MSC-exosomes, are able to protect cortical neurons from death after OGD insult

The most important cell type that needs to be protected after an IS are neurons, not only because they are the functional cognitive blocks from the brain but also because they are very fragile and susceptible to external stimuli that can lead to cellular death. The reduction of neuronal death on the area affected by IS can help to attenuate the functional disabilities perceived in patients after an attack. Therefore, it was investigated the potential protective effect of both exosomes over cortical

neurons. Similar to HUVECs, cortical neurons were subjected to OGD, incubated with 3 distinct exosomes concentrations and analyzed for nuclear morphology 24 hours after the treatment (figure 16A, B). Interestingly, contrary to what we observed in HUVECs, where both exosomes were able to induce survival (figure 15C, D), just exosomes from MNCs had bioactive effect to protect neurons from OGD induced cell death. Moreover, the treatment was dose-dependent, showing an increased survival effect when cells were treated with 3×10^9 particles/mL of MNC-exosomes. The percentage of live cells in this concentration was similar to the sham control group (figure 16C). Controversially, the treatment with the higher concentration presented less live cells. In any concentration used, WJ-MSC-exosomes were not able to protect neurons against OGD-induced cell death (figure 16D).

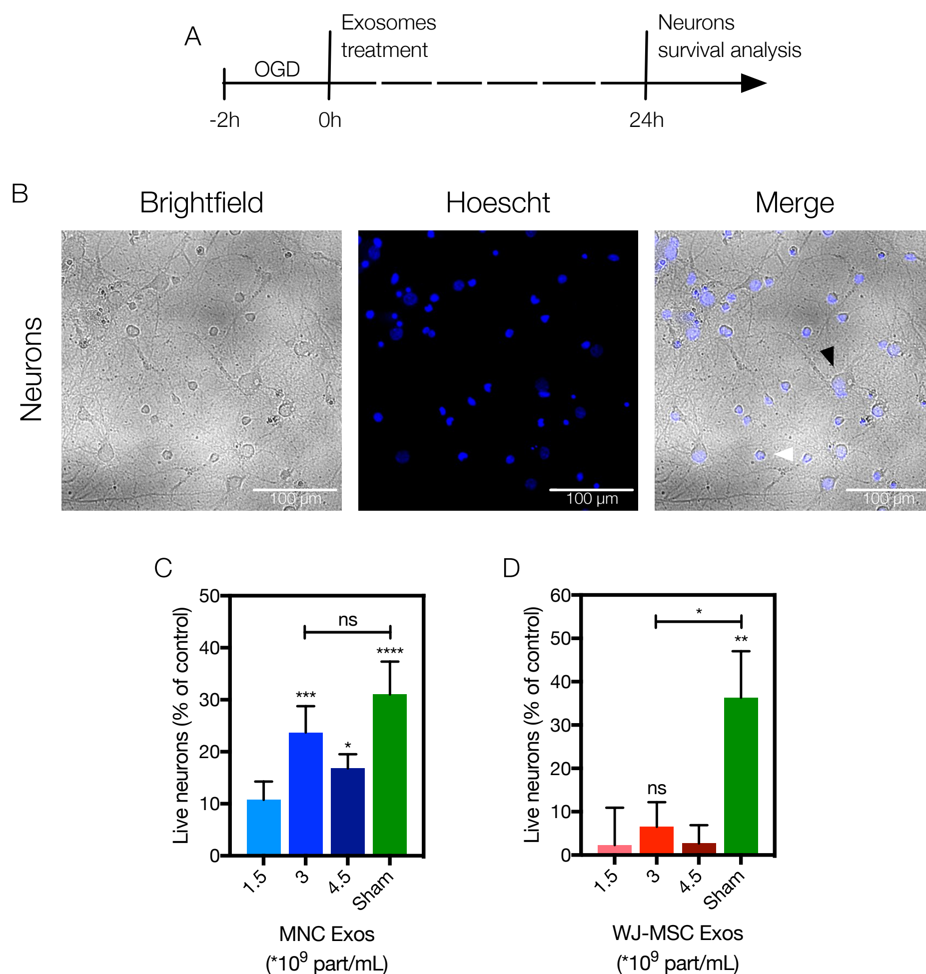


Figure 16- OGD survival assay performed in cortical neurons. A) Cells at DIV 15 were placed 2 hours under nutrients and oxygen deprivation and immediately treated with MNC- and WJ-MSC-exosomes. B) Cellular viability was assessed by nuclear morphology. C) MNC-exosomes were able to provide resistance against OGD to neurons with higher impact on live cells being observed after treatment with 3 x 10 particles/mL. D) Neurons survival was not positively affected by any concentration used of WJ-MSC-exosomes. Results and expressed as percentage of live cells relatively to non-treated-OGD-exposed control. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001 (n=3).

3.2.4. Treatment of cortical neurons with exosomes does not affect GluN2B expression

The different outcomes observed on the neuronal survival after treatment with the two exosomes populations took us to further try to explore the potential responsible mechanism. It was already reported that miRNA-223-3p has neuroprotective effects by modulating the expression of the GluN2B subunit of NMDA receptors [162]. Previous studies performed on our laboratory, demonstrated that MNC-exosomes present high levels of miR-223-3p (data not published). Therefore, we evaluated the expression level of miRNA-223-3p in both exosomes population, discovering that WJ-MSC exosomes have around 35 times less miR-223-3p than MNC-exosomes (figure 17A).

Next, we measure the protein level of GluN2B 24 hours after the treatment with both exosomes (figure 17B). Contrarily to what we expected, no significant difference was observed among the experimental groups, suggesting that the bioactive effect of MNC-exosomes is not associated with changes in the expression of GluN2B (figure 17C,D).

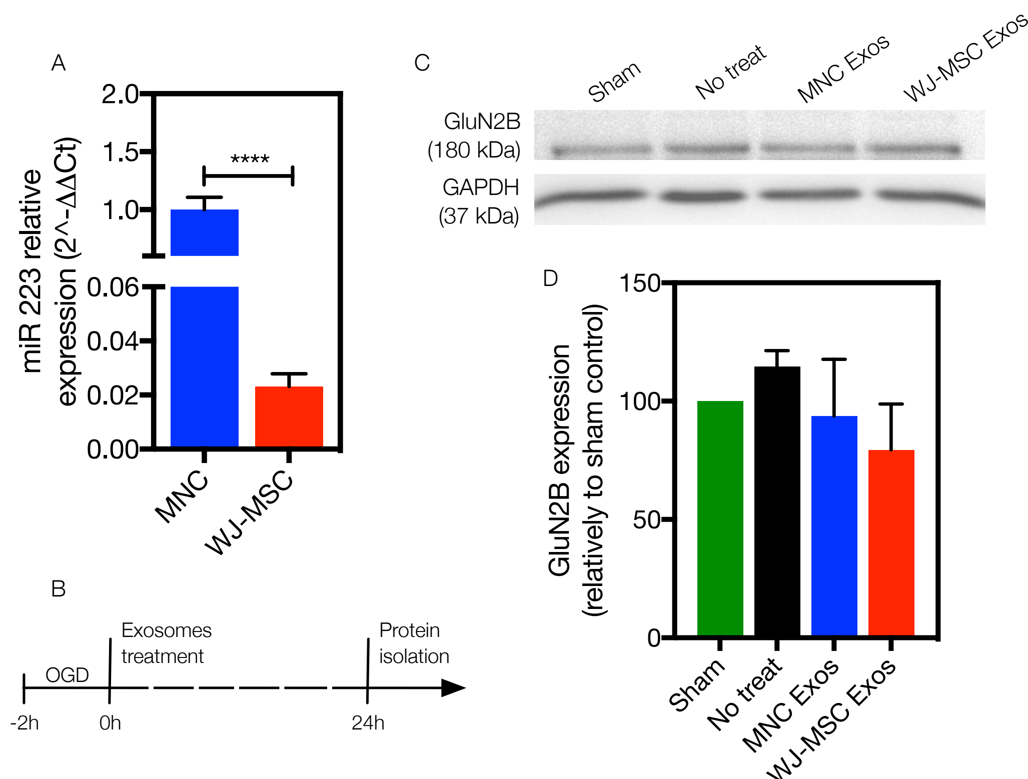


Figure 17- Treatment with exosomes did not affect the expression level of GluN2B receptor. A) qRT- PCR revealed substantial higher level of miR223-3p inside MNC-exosomes than in WJ-MSC-exosomes (n=6, ****p<0.001). B) Schematic representation of the experimental procedure C and D) Western Blot analysis using protein extracted 24 hours after treatment (n=3) showed similar amount of GluN2B in all experimental conditions.

3.2.5. MNC-exosomes protect NSCs from cell death

Neural stem cells modulation is an important factor on the regeneration of injured brain tissue. Therefore, we intended to evaluate the activity of exosomes over differentiation patterns alterations, rather than induction of survival phenotype. First, to prove that our exosomes are not harmful to cells, we performed a simple toxicity assay. After 2 days plated in factors-free medium, cells were incubated for 48 h with 1.5, 3 or 4.5 x10^9 particles per mL, from both types of exosomes (figure 18A). PI incorporation revealed that both populations did not induce any cellular death (figure 18B, C, D). Excitingly, MNC-exosomes not only showed to be non-toxic for NSC, but also to reduce in a large extend the basal cellular death that is normally observed in cell culture, indicating that these exosomes can promote cell survival (figure 18C).

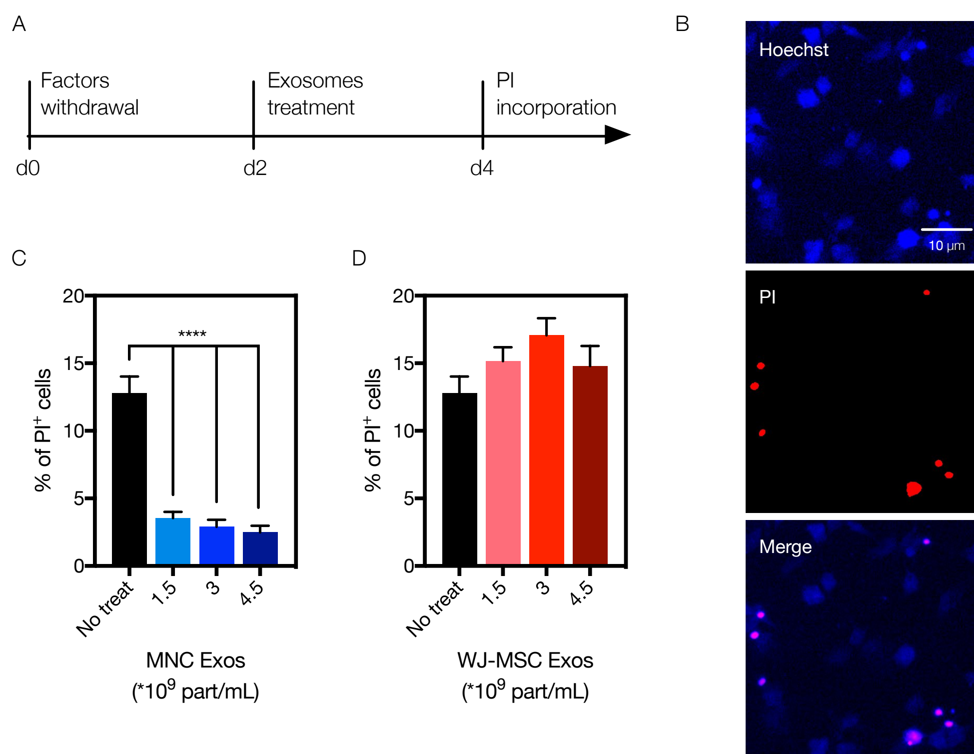
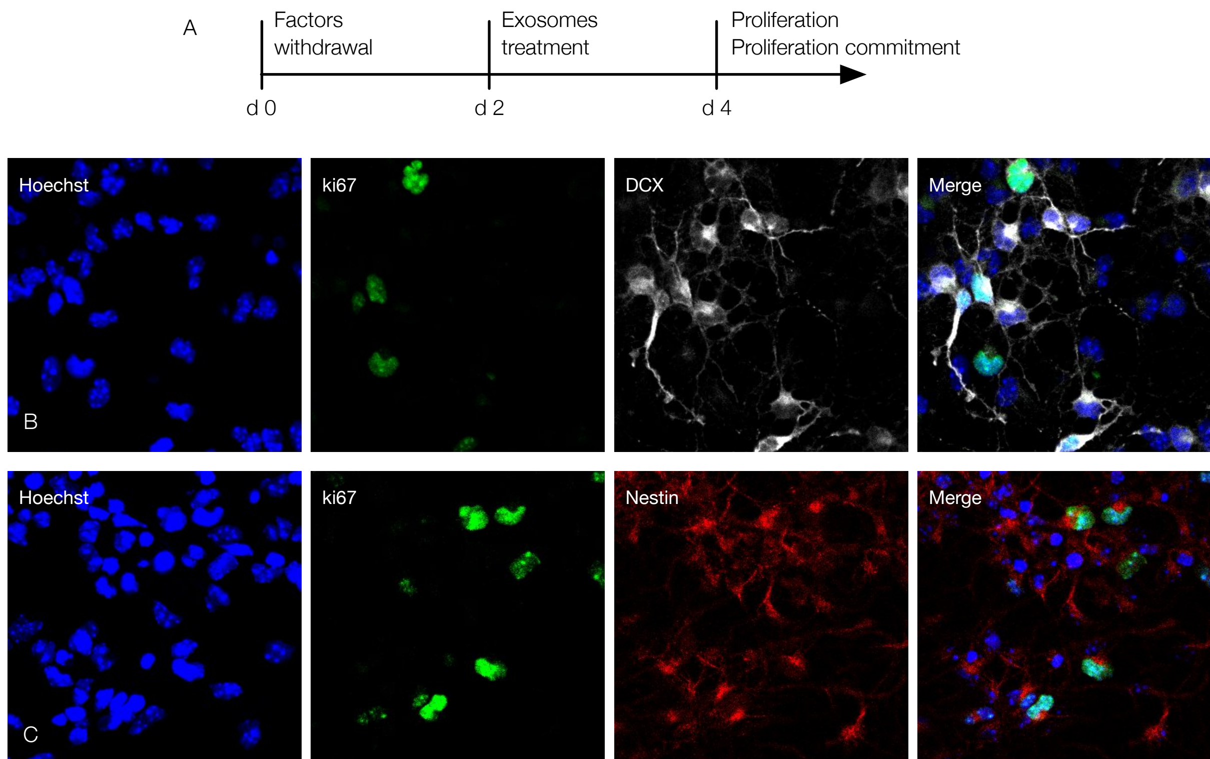


Figure 18- Exosomes toxicity over NSC. A) Cells were incubated for 2 days with exosomes and afterwards incubated with PI for 10 min. B) Nuclei were labeled with Hoechst and cells were considered dead when double positive for PI and nuclear marker. C and D) Surprisingly, MNC-exosomes reduced the levels of basal cell death, whereas WJ-MSC-exosomes showed to not promote cytotoxicity (n=3).****$p<0.0001$

3.2.6. MNC and WJ-MSC exosomes induced different proliferation commitments

We examined the effect of exosomes in cell proliferation by analyzing Ki67 expression 48 hours after treatment (figure 19 A). Ki67 is a protein associated with cell cycle, present in phases G1, S, G2 and M, therefore labeling proliferative cells (figure 19 B, C). No differences were observed on the NSC proliferation activity after treatment with both exosomes (figure 19 D, E). Further, we investigated which cell type was more proliferative, staining cells for DCX (neuroblasts) and Nestin (neural stem cells). We detected that the treatment with MNC-exosomes increased the number of proliferating neuroblasts (ki67$^+$ and DCX$^+$) and decreased the number of self-renewing cells (ki67$^+$ and nestin$^+$), in a significant level with 4.5 x 10^9 particles/mL treatment (figure 19 F, H). WJ-MSC-exosomes induced the opposite effect, exhibiting less proliferating neuroblasts and more self-renewal with the higher concentration of exosomes (figure 19 G, I).



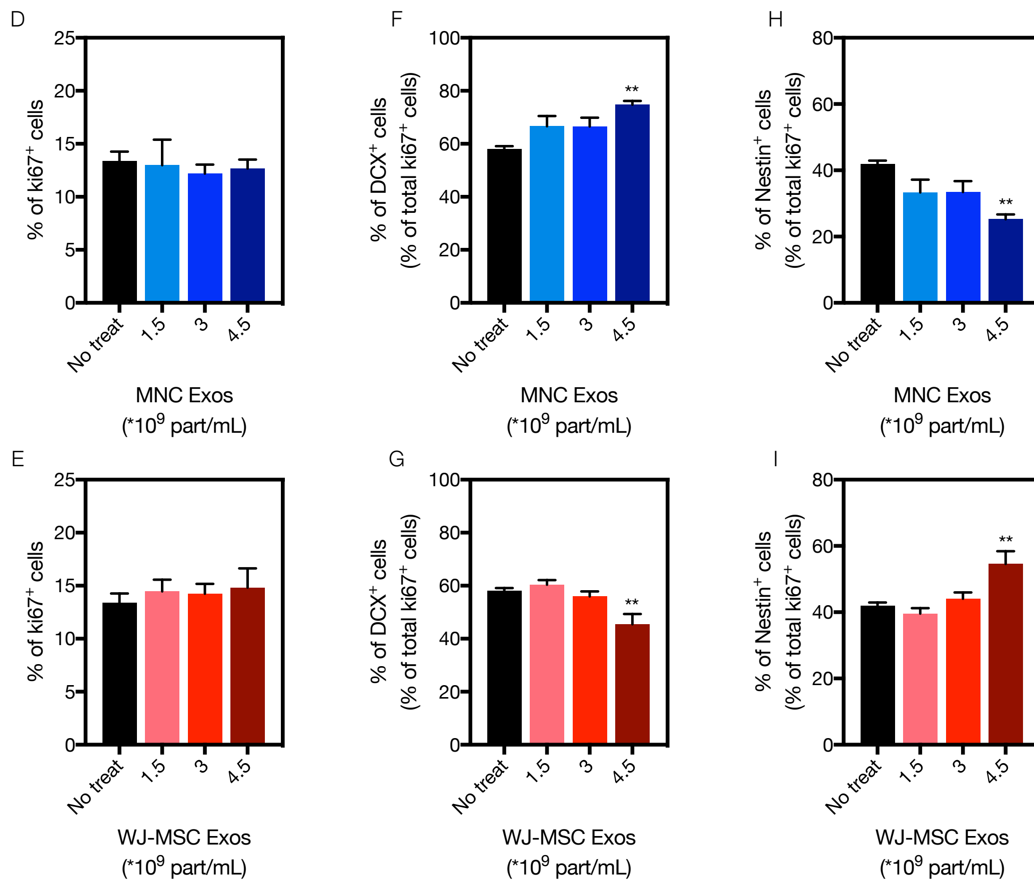


Figure 19-Proliferation of NSC.A) Cells were treated during 2 days with either MNC-exosomes or WJ-MSC exosomes. B and C) Immunocytochemistry analysis using ki67 indicating the cell cycle activity required for cell division, DCX as a marker of neuroblasts and nestin to label neural stem cells. D and E) Both exosomes were not able to enhance proliferation of NSC. F and G) Opposite effects were observed on proliferative neuroblasts, with MNC-exosomes increasing the number of DCX positive cells, while WJ-MSC-exosomes induced a decrease. H and I) Contrarily to MNC-exosomes, WJ-MSC-exosomes increased the self-renewal. n=3, **p<0.005

3.2.7. Cellular differentiation is differently affected by MNC and WJ-MSC exosomes

Moreover, we assessed whether the exosomes were able to favor NSC differentiation to neurons, or induce the maintenance of stemness. After 2 days of incubation with factor lacking medium, cells were treated either with MNC-exosomes or WJ-MSC-exosomes for further 7 days to allow differentiation processes to occur (figure 20 A). Phenotype was characterized using specific markers Sox 2 (stem cells) and NeuN (mature neurons) (figure 20 B, C).

All the three concentrations of MNC-exosomes were able to increase in nearly 15% the amount of Sox-2 positive cells, indicating an increase on the number of neural stem cells present on the culture (figure 20 D). The same was observed for the treatment with 3 x 10^9 particles/mL of WJ-MSC-exosomes (figure 20 E). No difference was observed when treating with the lower and higher concentration of WJ-MSC, indicating a dose-dependent effect.

The analysis of NeuN positive cells in each condition revealed that using the intermediate concentration, MNC exosomes were able to significantly increase the number of mature neurons, whereas WJ-MSC exosomes, even with a slightly increase in the quantity of neurons, did not presented significant difference (figure 20 F, H).

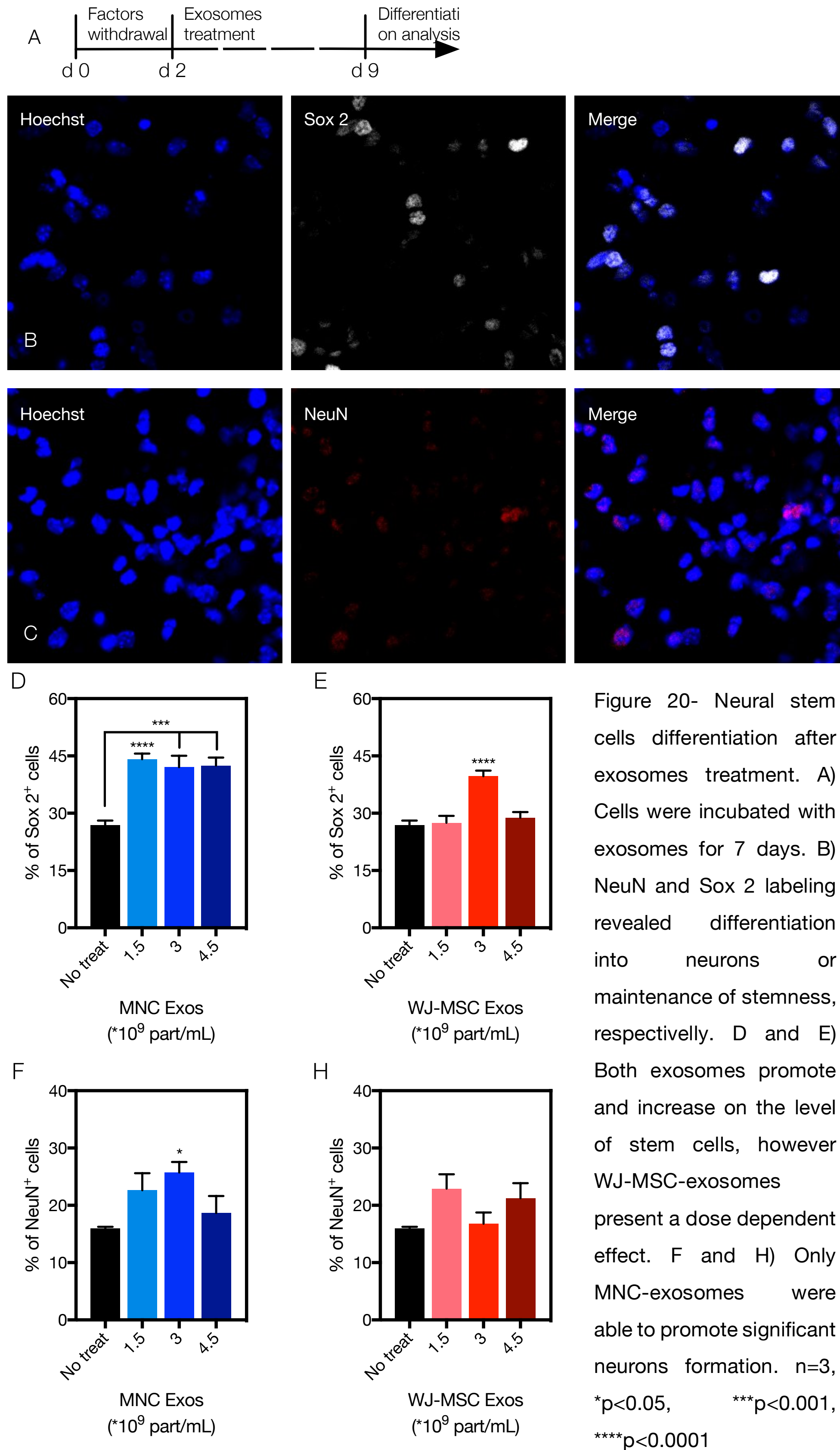


Figure 20- Neural stem cells differentiation after exosomes treatment. A) Cells were incubated with exosomes for 7 days. B) NeuN and Sox 2 labeling revealed differentiation into neurons or maintenance of stemness, respectivelly. D and E) Both exosomes promote and increase on the level of stem cells, however WJ-MSC-exosomes present a dose dependent effect. F and H) Only MNC-exosomes were able to promote significant neurons formation. n=3, *p<0.05, ***p<0.001, ****p<0.0001

4. Discussion

Exosomes research has undergone an exponential growth over the last years. This technology is being applied to a wide range of pathological conditions, not only as diseases marker, but also as therapeutic tool[87]. In fact, for both applications, results have been very promising, which encourage further investigation of these natural nanovesicles. Specifically for therapeutic application, exosomes have gained a place under the spotlight after researchers started to understand that during a cell transplantation, the beneficial outcomes observed were not only caused by cellular replacement, i.e, engraftment of transplanted cells to the injury site, but also due to paracrine effects released by these cells that can induce phenotypic alterations over the immune system or cells in the lesion area[163].

Extracellular vesicles have been isolated from almost all type of cells. Since MSCs were already widely used for cell transplantation, they logically became an important progenitor cell type. On the other hand, there are no reports on the direct isolation of exosomes from MNCs. In fact, the most closely related source of these types of exosomes already explored is blood plasm[117]. However, when isolating vesicles from plasm it is impossible to control their purity, since blood is able to carry not only exosomes produced by its constituent cells, but also from almost all type of cells that uses the blood flow to transport their exosomes and modulate distant regions.

The choice to use progenitor cells with umbilical cord origins was due to evidences showing that these cells have distinct genetic and protein expression than their similar adult ones[164]. Moreover, the distinct phenotype seems to favor the presence of genes and proteins related with plasticity and development, which can help to enhance the therapeutic effect of their exosomes. We decided to collect exosomes from hypoxia-stimulated cells due to recent evidences claiming that cells release exosomes with distinct content, depending on the culture conditions, i.e, stressful conditions can induce cells to release exosomes containing factors that can be protective for cells suffering the same insult[165,166]. In fact, data not published from our group show that cells under hypoxia are able not only to produce exosomes with different content, but also release a higher quantity of vesicles.

We pretended to characterize progenitor cells and exosomes using specific surface markers. Using CD9, the most reported exosomal marker, we proved that in fact we

were able to properly isolate exosomes (figure 11F). MNC-exosomes showed high expression of the leukocyte specific marker CD45 (figure 11A)[159], indicating not only their origin but also their ability to carry specific proteins present on progenitor cells. Moreover, the higher presence of CD31 in vesicles than in cell population take us to believe that cells expressing this marker, such as hematopoietic stem cells, monocytes and T lymphocytes, are highly committed on the process of exosomes release (figure 8D)[167]. CD31 is well known to facilitate the endothelial layer cross of leukocytes, which can indicate that CD31-exosomes can be able to cross endothelial barriers, such as the blood-brain-barrier (BBB) and get into the central nervous system[168]. This ability would be very advantageous for us, once it can allow an easily deliver of material to the damaged brain. Also, both cells and exosomes have HLA-DR, demonstrating that a high quantity of exosomes are being produced by B lymphocytes (figure 11A)[169]. Exosomes are thought to not be immunogenic, still it is also true that not all types have HLA related molecules (figure 11E). Previous experiments performed in our laboratory (data not published) did not reveal any response from the immune system after *in vivo* treatments with exosomes, however further tests need to be executed to prove that MNC-exosomes do not trigger undesirable immune responses. We applied the same approach to WJ-MSC and their exosomes, using the exosomes specific and MSCs specific epitopes. Both cells and their descendant exosomes proved to be positive for CD73, CD90 and CD105 (nearly 100% in cells) and negative for CD45 and HLA-DR, proving not only the mesenchymal phenotype of cells, but also the origin of exosomes. Interestingly, both MNC-exosomes and WJ-MSC have distinct CD45 composition, clearly evidencing that exosomes should be different depending on their progenitor cell type (figure 11, 12)[160]. However, CD9 and TSG101 can be found in both vesicle types, which prove them to be strong exosomal markers.

Nevertheless, only the phenotypic profile of exosome is not enough to fully characterize these nanovesicles, since there are not yet specific markers that can distinguish exosomes from other extracellular vesicles[161]. Thus, a morphological analysis is commonly used together with the known markers in order to better define a vesicle population.

Normally, exosomes are spherical shape vesicles, delimited by a bilayer phospholipid membrane with a size ranging between 30 nm and 200 nm[152]. Our DLS and NTA analysis revealed that in fact both our vesicles have appropriated size, and the negative zeta potential indicates the presence of negatively charged phospholipids. The conclusions from the zeta analysis are supported by TEM imaging, that allowed us to observe the round shape and bilayer membrane characteristic of exosomes (figure 10).

The physiological role of exosomes is mainly related with cell-to-cell communication, whereby is expected that they can be rapidly and easily internalized by cells. Our results indicate that exosomes origin rather than the target cell type have impact over internalization kinetics. Unpredictably, in both cell types, MNC- and WJ-MSC-exosomes presented to have major internalization differences, with WJ-MSC-exosomes showing not only a quicker uptake, but also to be present in nearly 100% of cells after 24 hours of incubation. On the other hand, MNC-exosomes are internalized at slow rhythm and at the end were visible in 70% of cells (figure 14).

Considering that our populations have been isolated from distinct cells, and the results from flow cytometry analysis, it is accepted that exosomes have different surface composition. These membrane differences can help to explain the better retention of PKH67 dye observed in WJ-MSC than in MNC-exosomes. After labeling, PKH67-exosomes are unable to be imaged, they just start to be visible after some accumulation inside target cells. Because WJ-MSC-exosomes retained more PKH67, they have higher fluorescence intensity, meaning that it is necessary a smaller quantity of exosomes to have the same detectable intensity, when compare with less bright MNC-exosomes. This means that the differences observed on the internalization measurements of both exosomes can be influenced by the fluorescence retaining and not totally by the different surface proteins that can facilitate or complicate the internalization process[170].

Exosomes isolated from different cell types have already shown positive therapeutic outcomes over stroke, cardiac disorders, wound healing, cancer and liver diseases[143,171–174]. Specially, bone marrow MSCs derived exosomes are being widely used in ischemic stroke, with already good results in animal models. These exosomes

appear to be bioactive through the enhancement of axonal plasticity and neurite remodeling and outgrowth[175,176]. On the other hand, mononuclear cells exosomes have never been used for stroke treatment, which enhanced our curiosity whether they can have a positive effect or not. MNCs cells isolated from umbilical cord blood and plasm proved already to play a role on the recovery of stroke and ischemic neonatal animal models, interfering with NSC differentiation, axonal growth and glial scar formation[177,178]. During an ischemic stroke recovery, a broad set of factors should work together in order to reestablish the physiologic conditions. Thus, we decided to explore both exosomes activity over distinct important players on recovery, mainly vasculature maintenance, neuronal protection and regeneration. During an ischemic stroke the brain region supplied by the blocked vessel suffers from oxygen and nutrients deprivation, leading to cellular impairment. In order to mimic as more as possible what happens *in vivo*, we performed an oxygen and glucose deprivation, where we incubated cells under a anoxia environment and with a glucose-lacking medium. Other *in vitro* models could be used, such as glutamate induced death[179]. However, this model represents only one form of death induced by ischemic, as opposite to OGD that can induce different kind of cellular death, thus allowing us to observe an overall exosomes effect rather than just the effect over glutamate receptors agonists induced injury.

Both exosomes were able to give endothelial cells resistance to nutrients deprivation (figure 15), however when treating cortical neurons, just MNC-exosomes could enhance cell survival after OGD (figure 16). Our findings support the differential exosomes content, dependent on the progenitor cell. Moreover, the large combination of miRNA inside each population can regulate distinct pathways, leading to different outcomes over our models.

Perhaps one of the most describe triggers of cell death is glutamate. Following an ischemic insult, an over activation of voltage sensitive Ca^{2+} channels initiates an enormous release of glutamate. Once on the synaptic space, this important neurotransmitter over activate NMDA receptors, depolarizing postsynaptic cells. As the release of glutamate is massive, it leaks from the synaptic zone to the extrasynaptic area, where NMDA receptors containing GluN2B subunity are more

expressed. After activation, these receptors allow an Ca^{2+} influx to cells that will further modulate JNK signaling, calpains and cathepsins, ROS production, impaired energy production and activation of several enzymes, ultimately inducing cellular death[181,180]. A literature review shown that miRNA-223-3p, already known to be present in our MNC-exosomes, is responsible for the modulation of GluN2B expression, demonstrating a neuroprotective effect[162]. In fact, we were able to prove that MNC-exosomes have 35x more miRNA-223-3p than WJ-MSC-exosomes, however no significant differences were observed on the expression of GluN2B, indicating either that miRNA-223-3p acts further downstream on the signaling pathway modulated by the receptor, or that there are other factors responsible by the observed effects.

Nevertheless, while mesenchymal exosomes are thought to induce plasticity phenotypes, MNC-exosomes are able to promote survival to neurons, which could indicate that a IS treatment using both populations should have a synergetic effect.

It has already been reported that functional improvement after stroke may be achieved through neural replacement by endogenous NSCs residing in the adult brain, such as in the SVZ[182]. Therefore, we intended to analyze the bioactive effect of exosomes over these cells. We started evaluating the cytotoxic effect of the treatment with exosomes using PI incorporation. Surprisingly, our results demonstrated that MNC-exosomes are able to reduce significantly the basal cellular death that normally occurs during the culture process, especially when removing the growth factors from the media (figure 17). Further studies should be performed to better understand the protective role of MNC-exosomes. On the other side, WJ-MSC did not alter the cell death pattern, reinforcing the highly diversified content of exosomes when they are release from different cell sources Together with the previous results from HUVECs and cortical neurons survival, MNC-exosomes seem to be capable of activate survival pathways, underlining their therapeutic activity.

Following an ischemic stroke situation, cellular proliferation and differentiation is activated in brain neurogenic niches. Despite the innumerous reports claiming that mononuclear and mesenchymal stem cells transplantation are able to induce neurogenesis[183–187], very few information is found about the effect of their exosomes[188,189]. The works published with MSC-exosomes so far focus on

neuroplasticity and neurite remodeling, rather than the neurogenic niche. Moreover, some articles talk about neurogenesis, however they do not prove their occurrence from the neurogenic niche[190,141]. Our results from *in vitro* treatment of NSC from SVZ showed that both exosomes were not able to enhance cellular proliferation, nevertheless MNC-exosomes could increase the number of proliferating neuroblasts, indicating their ability to promote neuronal phenotype (figure 19), which can later migrate to the damage area and increase the quantity of new neurons. Distinctly, WJ-MSC-exosomes did not increase the number of neuroblasts, but increased the percentage of self-renewing cells. This result is in accordance with the literature, since it was already reported that MSC can promote NSC self-renewal[191].

In response to factors released by damage cells presented in the ischemic area, neuroblasts can migrate towards that zone and ultimately differentiate[192]. However, not enough new neurons are produced; instead a huge quantity of reactive astrocytes is generated. Our results proved that MNC-exosome, but not WJ-MSC-exosomes, promoted a significant increase on the number of new mature neurons. In fact, WJ-MSC also showed to induce some increase, but not statistically different, mainly because of variance between donors bioactivity. Results from the group of Michael Chopp, well known by his research in stroke treatment and since 2013 also in exosomes either natural from MSC or modified with specific miRNAs, claimed that exosomes isolated from bone marrow MSC are able to induce neurogenesis *in vivo* after ischemic insult. However, in their report, it is just proved an higher number of neuroblasts around the damaged area and not the presence of fully differentiated neurons[190]. Furthermore, it was not demonstrated whether exosomes are directly inducing phenotype changes over NSC or they are modulating other cell types that will further release factors that can, as second messengers, modify NSC behavior. Our results can indicate that MNC-exosomes treatment is able to enhance the production of neurons that further can increase the probability of having new neurons integrated into circuits disrupted by the injury.

Moreover, we also assessed the quantity of Sox 2-positive cells after 7 days of exosomes treatment, which allow identifying the percentage of stem cells present on the culture that did not differentiate. All the concentrations used of MNC-exosomes

increased nearly 15% the number of Sox 2-positive cells, whereas just the concentration of 3 x 10^9 particles/mL of WJ-MSC-exosomes was able to increase the number of stem cells. The increase in stem cells can indicate that exosomes are changing the natural differentiation pattern, or enhancing proliferation of stem cells after 7 days (figure 20). Moreover, NSC differentiates in large scale, whether in normal as in pathological conditions, in astrocytes. This became a problem in stroke due to the formation of an astrocyte scar in the damage area that hinders the integration of new active neurons[193]. Therefore, future analysis of the level of astrocytes after the treatment with exosomes should be performed.

5. Conclusion and Perspectives

The field of exosome has expanded exponentially, and our understanding of possible applications, whether as biomarker or therapeutic tool, has advanced within the last few years. They became an attractive source of natural drug delivery system as they are expected to be less toxic, and more compatible with the host immune system than liposomes and other nanoparticles with synthetic origin.

Studies have highlighted the possibility of using exosomes for the treatment of ischemic stroke. Nevertheless, the mechanisms relaying this effect is still not yet completely disclosure and is limited to the use of bone-marrow derived exosomes.

In this work we gave a step forward in order to understand both MNC and WJ-MSC-exosomes isolated from umbilical cord sources. In an opposite way from MSC-exosomes, that are already very described, we were able to report the isolation of exosomes from MNC supernatant. The extracellular vesicles isolated from the condition medium were fully characterized for the size, shape, zeta potential and marker expression, corresponding to exosomes.

Importantly, we demonstrate the bioactivity of exosomes over endothelial cells, cortical neurons and neural stem cells. Especially MNC- exosomes were able to not only promote cell survival but also increase the number of differentiated neurons. These features can significantly contribute to a better recover after an ischemic brain injury by promoting neurovascularization, neuroprotection and neuroregeneration (3N´s).

Further studies should be performed to understand the mechanisms behind these effects. We hypothesized that the excess of the neuroprotective micro RNA, miR-223-3p, could be responsible for promoting neurons survival by reducing the expression of GluN2B. Nevertheless, we were not able to demonstrate a down-regulation of GluN2B, suggesting that other mechanisms can be involved in this process. Understanding the RNA and protein content of the different sources can help to unravel the active factors to promote the 3N's of stroke recovery. This knowledge can not only help us to reveal new pathways and miRNAs interaction, but also to look for ways to enhance the bioactivity of extracellular vesicles. In fact, diverse efforts are

being done in order to augment the natural ability of exosomes by modification of their content or surface.

However, it is essential to test *in vivo* our hypothesis that exosomes from umbilical cord can promote recovery after brain ischemic injury.

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