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Modulation of Inflammation, Oxidative stress and Neuroinflammation by a short chain fatty acid: protective effects of sodium propionate in *in vitro* and *in vivo* studies

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PART I

1. CHAPTER ONE: Inflammation and oxidative stress in the pathology of inflammatory diseases

1.1 The pathophysiology of the inflammation

Inflammation is the immune system's reaction to damaging stimuli, such as pathogens, damaged cells, toxic compounds, or radiation, and acts by removing harmful stimuli and starting the restorative process (Chen 2017). Notable signs of progress have been made in studying the cellular and molecular events involved in the acute inflammatory response to infection or tissue insult. Much less is known about the causes of systemic chronic inflammation, which arises in many different diseases, including type 2 diabetes and cardiovascular diseases (Black 2002) (Calle 2012). These chronic inflammatory conditions are caused not only by the classic inflammatory process but also by failed tissues homeostasis between defense and injury (Medzhitov 2008). Different agents can stimulate the inflammatory process, such as tissue necrosis, for example, following a tissue deficiency of nutrients or oxygen, chemical or physical traumas, or bacteria/virus infections. Clinically, the main signs of inflammation are edema, caused by the transvascular flux of fluid from the intravascular compartment into the interstitium (Friedl 1989), pain, redness, and fever. The fifth cardinal sign, functio laesa (disturbance of function), was added by Rudolph Virchow in 1858 in his book Cellular pathology (Majno 1975) as a universal sign which is a common feature in all inflammatory processes (Medzhitov 2010).

1.2 The immune-inflammatory Cascade

1.2.1 A Spectrum of Inflammatory Responses

A classic inflammatory response consists of four key elements: inflammatory inducers, sensors, mediators, and targets (Figure 1). Each component comes in multiple forms and their combinations act in different inflammatory pathways. The nature of inflammatory response depends on the characteristics of inflammatory inducers: exogenous or endogenous. First, any bacteria which reaches the organism will be phagocytosed, detected, and killed by pattern-recognition receptors (PRRs), located on the cell surface (e.g. Toll-like receptors, or TLRs), that secrete inflammatory chemokines and cytokines to eradicate bacterial invasion (Arpaia 2013). On the other hand, if the dangerous signal is endogenous (intracellular products, protein covered particles, etc.), tissue-resident monocyte/macrophage cells initiate the resumption of necrotic tissues, encapsulation of foreign bodies, and triggering of immune responses. Inducers of inflammation trigger the release of several mediators, vasoactive factors, lipid mediators, cytokines, chemokines and enzymes transported to the inflamed situ. In addition, many mediators are also able to induce by themselves the production of extra mediators. In order to prevent this, protective and regenerative mechanisms are activated simultaneously with activation of sensors of inflammation, ensuring the restoration of original tissue architecture and normal metabolism once inducers are excluded (Matzinger 1994).

1.2.2 Immune System Defense

The immune system engages many mechanisms that have the ability to destroy a large range of exogenous agents. Microbial structures known as pathogen-associated molecular patterns (PAMPs) are considered dangerous exogenous agents that can activate the inflammation through the activation of PRRs, mainly expressed in immune and non-immune cells (Brusselle 2014).

Some PRRs also identify many endogenous signals activated during tissue/cell damage and are named danger-associated molecular patterns (DAMPS) (Hoebe 2004). The main classes of PRR families include the TLRs, C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Takeuchi 2010).

TLRs family is the most studied mammalian PRRs composed by a cytoplasmic portion, highly similar to that of the interleukin (IL)-1, known as Toll/IL-1 receptor (TIR) domain, and a leucine-rich repeats (LRRs) extracellular domain. TLRs participate in the activation of the inflammatory response revealing an essential role in the recognition of pathogens. Each TLR has been demonstrated to identify specific components of pathogens (Takeda 2004). TLRs can be divided into subclasses that recognize related PAMPs; TLR1, TLR2, TLR4, and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Akira 2006). The most important cells expressing TLRs constitutively or in an inducible manner are macrophages, dendritic cells (DCs), and B-lymphocytes (Miettinen 2001). Thus, their activation and cell signaling process through TLRs trigger an intracellular cascade that leads to nuclear translocation of transcription factors, such as NF-κB or interferon regulatory factor 3 (IRF3), the mitogen-activated protein kinase (MAPK), Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways.

When pathogens bypass innate immune defenses, the adaptive immune system, highly specific complex, is activated. Usually, the components of the adaptive immune system cooperate with the innate system cells to reach an effective immune response, thus eliminating the undesired pathogens. Key players of the adaptive immune response are T cells: Th1 cells are important as they eliminate intracellular pathogens, Th2, and Th17 cells act against parasites and bacteria.

However, increased activation of T cell response could lead to the early onset of inflammation by an exaggerated cytokines and chemokines production (Hoebe 2004).

1.2.3 Cytokines

The intensity and timing of the inflammatory response are correlated with different physiological events that depend on cytokines production. Cytokines are small proteins (<40 kDa) produced by different cell populations of cells as pro-inflammatory molecules and then mature, in response to the activation of inflammatory and immune processes (Schaper 2015). They are referred to as interleukins, chemokines, or growth factors that explain many effects depending on the targeted cell, and activation of different signaling pathways (Kany 2019). Among them, some cytokines stimulate inflammation and are called pro-inflammatory cytokines (IL-1, Tumuor Necrosis Factor or TNF, and chemokines), otherwise, other cytokines inhibit the inflammatory cascade and are called anti-inflammatory cytokines (IL-4, IL-10, and IL-13). During inflammation, IL-1 and TNF are released in situ stimulating gene expression for type II PLA2 (Phospholipase A2). PLA2 activation releases arachidonic acid and then, the cyclooxygenases 1 and 2 (constitutive COX-1 and inducible COX-2) convert arachidonic acid into prostaglandins (PGEs) (Dinarello 2000). Once released, PGEs exert their effects by binding with rhodopsin-like seven-transmembrane spanning G protein-coupled receptors (GPCRs) thus, leading to stimulate the inflammatory response (Ricciotti 2013).

1.3 NF-kB signaling pathway

NF-κB family refers to inducible transcription factors and, intracellular messengers, which connect various extracellular stimuli to the induction of gene expression through a cellular signaling pathway. Sen and Baltimore (Sen 1986) have been discovered NF-κB as a regulator of DNA transcription of the κ chain in B-cells (also known as B-lymphocytes) that possess a crucial role in inflammation, immune system, cell proliferation, and differentiation. (Oeckinghaus 2009). NF-κB

factors contain five members of the Rel family of proteins known as p65 (RelA), RelB, c-Rel, NF-κB1, and NF-κB2 (pro-forms p105 and p100, mature form p50 and p52) (Caamaño 2002). Each monomer can polymerize with κB factors and interact with a family of inhibitor factors commonly known as IκB proteins. The NF-κB regulation system is complicated and various as it is able to bind several DNA sequences by different preferences and approaches that reflecting in thousand of post-translational modifications (Oeckinghaus 2009). The canonical NF-κB signaling pathway is initiated by PRR binding with its ligand starting signal transduction into phosphorylation of the IKK2 and IκBα, both degraded by the proteasome system. Thus, NF-κB stops being negatively regulated and translocate to the nucleus to initiate gene transcription of cytokines (such as TNF and IL-1β), chemokines, cell adhesion molecules ((Intercellular Adhesion Molecule or ICAM and Vascular Cell Adhesion Molecule or VCAM), growth factors (GFs), and regulators of apoptosis (Hoesel 2013). The canonical NF-κB pathway is associated with inflammation and together have a controversial role in cancer, in non- and pathological conditions affecting Central Nervous System (CNS) and peripheral tissues (Disis 2010) (Engelmann 2014). Moreover, exist another NF-κB activation pathway defined as unconventional that originates from different classes of receptors including lymphotoxin β-receptor (LTβR), CD40, and receptor activator for nuclear factor kappa B (RANK) (Sun 2010).

1.4 Acute and chronic inflammation

Inflammation can be distinguished based on timing and pathological features into acute and chronic. Acute inflammation is defined as a short event (hours to days) and is characterized by vasodilatation, the exudation of protein-rich fluid (plasma), and a movement of immune system cells to the site of insult. This process has two major features: vasodilatation, resulting in increased blood flow and vascular permeability, and migration and rolling of leukocytes. Moreover, the acute

inflammatory response is divided into the initiation phase, characterized by tissue edema, inflammatory lipid mediators release in situ; and, in the post-resolution phase, that rapid response will end the acute process (Ward 1999). On the other hand, chronic inflammation is characterized by a prolonged duration (weeks to months to years) in which active inflammation, tissue destruction, and attempts at tissue repair are occurring simultaneously as in rheumatoid arthritis, diabetes, and inflammatory bowel disease (IBD) (Bian 2012).

1.5 Organ-specific inflammatory responses

1.5.1 Heart

Inflammation has been recognized as the main cause of several diseases. It is assessed that around 15% of human cancers are related to chronic infection and inflammation. Acute and chronic inflammation-mediated tissue injury is observed mostly in the heart, pancreas, liver, kidney, lung, brain, intestinal tract, and reproductive system (He1 2011). Although different categories of risk factors such as older age, male gender, and smoking are involved in hearth diseases (Ridker 2005), many of those share intermediate biological risk factors such as the circulation of inflammatory mediators. Indeed, the innate immune system was identified as the primary cardiac defense against pathogens and tissue damage. Indeed, myocardial infarction, resulting from atherosclerosis or loss of numerous myocardial cells, is the most common cause of cardiac injury in association with an unforeseen inflammatory cascade (Ptaszynska-Kopczynska 2017). Cell death provides to release intracellular content that activates innate immune mechanisms initiating an inflammatory response. Thus, endogenous ligands circulating following injury are recognized as hazard signs. (Beg 2002). NF-κB signaling is activated (Halla 2006) thus, chemokines and cytokines recruit inflammatory leukocytes to the infarct area, promoting leukocyte-endothelial cell adhesions. Pro-inflammatory cytokines such as IL-1β or TNF-α are secreted in all phases of atherosclerotic lesion progression and induce expression of "messenger" or secondary signaling cytokines such as IL-6.

Indeed, circulating levels of the inflammatory cytokines TNF-α and IL-6 independently predict the risk of Coronary heart disease (CHD) and/or coronary events (Ridker 2016). IL-6 can move from local areas of inflammation to the liver where it stimulates protein synthesis.

1.5.2 Liver

Numerous epidemiological and clinical studies have provided convincing evidence that chronic inflammation leads to carcinogenesis (Demaria 2010). Various types of cancer arise in the setting of chronic inflammation, indicating a strong link between inflammation and cancer (Mantovani 2008). It has been estimated that approximately 20 % of all human cancers are associated with inflammation and chronic infections (Pikarsky 2004). Despite the intrinsic differences among various etiological factors for types of cancer, a common denominator at the origin of this malignancy is the perpetuation of a wound-healing response activated by parenchymal cell death and the resulting inflammatory cascade. A growing number of preclinical and clinical studies have identified a plethora of inflammatory mediators and signaling pathways implicated in hepatocellular cancer (Budhu 2006). Various inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α, participate in chronic hepatic inflammation, and IL-6 is probably the most important and studied one (Park 2011).

1.5.3 Intestinal tract

Over the past decade, acute and chronic inflammation are considered a major hypothesis for the etiopathogenesis of inflammatory bowel disease (IBD). IBDs mainly includes ulcerative colitis (UC) and Crohn disease (CD), polygenetic pathologies characterized by an excessive inflammatory response to gut lumen microbial flora (McGuckin 2009). The single-layered intestinal epithelium is a physical and immunological barrier that prevents direct contact of the

intestinal mucosa with the luminal microbiota. A compromised intestinal barrier may play a crucial role in the development of IBDs, by allowing the entry of luminal antigens and microorganisms into the mucosa and initiating overwhelming inflammatory and immune responses. Innate immune cells, in particular dendritic cells and macrophages, in the gastrointestinal tract are significantly involved in both homeostasis in the normal gut and deregulated immune responses during intestinal inflammation. Activated innate immune cells are able to stimulate proliferation of primary antigen-specific responses and direct via the production of regulatory cytokines (e.g. IL-6, IL-12, IL-23, and IL-27) the differentiation of naïve T cells (Becker 2015). Once initiated, the innate immune response of intestinal tissues to components of the commensal microbiota is manifested by an exaggerated response by aggressive T cells and B cells through their production of IgG antibodies that drive a state of chronic inflammation. In addition, T cells chiefly secrete cytokines such as interferon-γ (IFN- γ) and TNF, and IL-4, IL-5, and IL-13. In the end, the inflammatory factors that derive from the initiating innate immune response and the consequential adaptive immune response result in tissue injury and the clinical symptoms that are characteristic of these disorders (Blumberg 2009). Moreover, disruption of intestinal epithelial tissue architecture, loss of epithelial permeability are due to chronic inflammatory response, which alters intestinal epithelial integrity and typical tight junctions (TJs) localization (Ding 2012).

1.5.4 Brain

In the CNS, degenerative processes are characterized by morphological, anatomical, and functional modification that lead to early, chronic, and progressive neuronal loss and increase blood-brain barrier permeability to various molecules. Chronic neurodegenerative diseases are classified as protein misfolding diseases, hereditary, sporadic, that are typically characterized by

the decline of cognitive functions such as learning and memory. These include Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD). Generally, known risk factors for most neurodegenerative diseases are genetic polymorphisms and older age but alternatives mechanisms could be involved in the pathogenesis of neurodegenerative disorders, including chronic inflammation, vascular factors, oxidative stress, and reduced availability of trophic factors in the brain (Sochocka 2017). During the CNS inflammatory response, the bactericidal and cytotoxic nitric oxide (NO) produced by the enzyme inducible Nitric Oxide Synthase (iNOS), as well as proinflammatory cytokines such as TNF, IFN-γ, IL-1, IL-6, and IL-12 play important roles. These factors belonged to the classical innate immune response and they act in association with CNS resident cells such as macrophages/microglia, astrocytes, cerebrovascular endothelial cells (CVE), natural killer cells (NK cells), and granulocytes (Stenzel 2008).

1.6 Inflammation and pain

Inflammatory responses in the peripheral and CNS play key roles in the development and resolution of many pathological pain states. Pain is defined as a sensation, an experience, and a feeling about noxious stimulation applied to the body such as heat, squeezing a skin fold, or over-rotating a joint. During evolution, living organisms develop a system to be able to sense their immediate environment in order to avoid potentially dangerous situations (Zhang 2007). It is well established that inflammatory response and pain occur by peripheral sensitization of primary sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion in association with mediators and chemokines release (Julius 2001). Indeed, cutaneous nociceptors knew as nociceptors localized in peripheral tissue respond to mechanical and thermal stimuli starting a

mechanism of signal transduction that leads inflammatory cells to release sensitizing agents such as prostaglandins PGE2, 5-HT, bradykinin, histamine, ATP, protons, nerve growth factor (NGF). These modulators activate multiple protein kinases such as PKA, PKC, ERK, and p38 MAPK, and increase the sensitivity and excitability of nociceptors through two mechanisms: post-translational regulation and translational regulation. First, occurs by phosphorylation of an ion channel regulated by inflammatory mediators such as the transient receptor vanilloid receptor 1 (TRPV1), a member of the TRP (transient receptor protein) family, and expressed by DRG cells. This ion channel is considered one of the transducers of noxious heat stimuli because it is opened by high temperature (>43°C), while the other ion channel, named TRPV2 receptor transduce stimuli from extreme heat (threshold >50°C) (Samanta 2018). Second, persistent noxious stimuli alter gene transcription and protein synthesis in DRG neurons increasing the transcription of neuropeptide substance P and calcitonin gene-related peptide (CGRP), and brain-derived neurotrophic factor (BDNF), leading to amplified the release of the neuromodulator and to enhanced pain (Clifford 1999). While substantial progress has been made in investigating nociception, the detection of intense noxious stimuli, and inflammatory pain, much still remains to be learned in targeted approaches to treating inflammatory pain.development and resolution of many pathological pain states. Pain is defined as a sensation, an experience and a feeling about noxious stimulation applied to the body such as heat, squeezing a skin fold or over-rotating a joint. During evolution, living organisms develop a system to be able to sense their immediate environment in order to avoid potentially dangerous situations (Zhang 2007). It is well established that inflammatory response and pain occurs by peripheral sensitization of primary sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion in association with mediators and chemokines release (Julius 2001). Indeed, cutaneous nociceptors known as nociceptors localized in peripheral tissue respond

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1.7 The Role of Oxidative stress during inflammatory process

The inflammation arises in response to any modification of tissue integrity, in order to restore tissue homeostasis through the induction of different repair mechanisms. Appropriate regulation of these mechanisms is crucial to prevent uncontrolled amplification of the early onset of the inflammatory response and of disease development (Goldszmid 2012). In the past, several findings have been revealed that chemical processes involving redox reactions causing cellular oxidative

stress play critical roles in the pathophysiology of inflammation (Sies 1986). Many chronic diseases such as rheumatoid arthritis (RA), Chrohn's disease, cancer, AD, and aging connected with higher production and release of reactive oxygen species (ROS) result in increased oxidative stress (Pravda 2005) (Chu 2020). Damage of oxidative stress means abundance in oxidized proteins, glycated products, nucleic acid modification, and lipid peroxidation (Araújo 2016).

1.7.1 Oxidative stress and free radicals production

A disparity between oxidants and antioxidants in favor of the oxidants, could lead into a damage condition named oxidative stress. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. Physiologically, a steady state is maintained by an intricate pattern of anti-oxidants. Usually, the antioxidant defenses are capable of adapting to changing needs (Sies 1997). Whenever an imbalance between reductants (electron donors) and oxidants (electron acceptors) develops, a generation of various oxidizing chemical species comes, leading to a disruption of redox signaling and control and/or molecular damage (Sies 2007). Oxidant species include primarily free radicals, which are molecules or fragments of molecules containing one or more unpaired electrons in their molecular orbitals, and which stabilize by removing electrons from neighboring molecules. Certain non-radical species, such as hydrogen peroxide (H_2O_2) or peroxynitrite ($ONOO^-$), also act as strong electron acceptors due to their highly positive redox potential. The two main families of relevant oxidants in biology are the ROS and the reactive nitrogen species (RNS) (Birben 2012):

Oxidant	Formula	Reaction Equation
Superoxide anion	$O_2^{-\cdot}$	$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^{-\cdot} + H^+$ $2O_2^{-\cdot} + H^+ \rightarrow O_2 + H_2O_2$
Hydrogen peroxide	H_2O_2	Hypoxanthine + H_2O + O_2 ⇋ xanthine + H_2O_2 Xanthine + H_2O + O_2 ⇋ uric acid + H_2O_2
Hydroxyl radical	●OH	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
Peroxyl radicals	$ROO^{\bullet}$	$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
Nitroxyl anion	NO−	$\bullet NO + e^- \rightarrow NO^-$
Nitrogen Dioxide	NO2	$NO + O_2 \rightarrow NO_2$
S-nitrosothiols	RSNO	$RSH + HONO \rightarrow RSNO + H_2O$
Peroxynitrite	ONOO−	$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$

Table 1. Main Oxygen and Nitrogen Oxidants

Mitochondria utilize the vast majority of O_2 taken up via inhalation in order to generate energy in the form of ATP. The electron transport chain is composed of several enzyme complexes embedded in the inner mitochondrial membrane, and a controlled transfer of electrons from one complex to the next completes the reduction of O_2 to H_2O. In general, cellular respiration results in the leakage of 1-3% of total reduced O_2, which then forms the primary ROS superoxide (O2•), particularly by the NAD(P)H oxidase complex. NAD(P)H oxidase is found in polymorphonuclear

leukocytes, monocytes, and macrophages. O2•, is converted into H_2O_2 by the action of superoxide dismutases (SODs) that easily diffuses across the plasma membrane. In a succession of reactions called Haber-Weiss and Fenton reactions, H_2O_2 can breakdown to OH- in the presence of transmission metals like Fe2+ or Cu2+ (Fenton 1984):

$Fe^{3+} + \cdot O_2 \rightarrow Fe^{2+} + O_2$ *Haber−Weiss*

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$ *Fenton reaction*

O_2^- itself can respond to H_2O_2 and generate OH^-. ●OH is the most reactive of ROS and can damage proteins, lipids, and carbohydrates and DNA. It can also start lipid peroxidation by taking an electron from polyunsaturated fatty acids (Dupuy 1991). The major RNS in biological systems is NO•, a free radical with one unpaired electron. It is formed by various isoforms (e.g. endothelial, neuronal and induced) of nitric oxide synthase (NOS). In mammals, NO• is utilized as a signaling molecule with different functions. NO• can react with O_2•-, however, to form ONOO-, particularly during inflammation (Slemmer 2008). Both of free-radical ROS and RNS at the normal low levels are considered part of cell physiology for example with antimicrobial oxidative bursts to destroy microbes, control autophagy to reuse intracellular organelles or molecules. However, at higher free-radical levels molecules such as proteins, DNA and lipids can be damaged. It has been estimated that proteins can scavenge up to 75% of free radicals, such as hydroxyl. Based on this evidence and considering that oxidized proteins have considerably long half-lives, it is reasonable to assume that proteins are likely to accumulate “evidence” of oxidative insult making those suitable markers of oxidative damage (Davies 2005). Biological effects of protein oxidative damage depend on the acting oxidant itself, the reactivity, localization, the proximity of the radical to the target, and the presence of readily oxidable amino acid residues in the protein molecule. On the other hand, the actual oxidizing capacity of an oxidant in a cell depends on the nature, function

and the ratio of radical/oxidant and available repair systems. The 20 different types of amino acids side-chains (excluding unusual amino acids and any post-translational modifications) result in a huge variety of potential reaction sites and products on reaction with oxidants. ROS cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins, and oxidation of specific amino acids and therefore lead to increased susceptibility to proteolysis by degradation by specific proteases. Cysteine and methionine residues in proteins are particularly susceptible to oxidation, as well as, oxidation of sulfhydryl groups cause conformational changes, protein unfolding, inhibited activity and at last, degradation. Enzymes that have metals on their active sites are especially more sensitive to metal catalyzed oxidation (Stadtman 1990). The •OH is one of the most reactive products: it reacts with DNA adding to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2′-deoxyribose. Another reaction of base radicals is the addition to an aromatic amino acid of proteins or combination with an amino acid radical, leading to DNA–protein cross-linking (Dizdaroglu 1998). The sites of oxidized base lesions in DNA are well studied and the number of lesions identified is still growing. For instance, it has been reported that some tumor cell lines can produce significant levels of H2O2, without exogenous stimulation, perhaps accounting for the elevated levels of oxidative DNA damage seen. Because of elevated ROS, transcription factors and their corresponding genes are perpetually activated, which, together with increased DNA damage, creates a selection pressure for a malignant phenotype seen in cancer (Toyokuni 1995). Radical interaction with DNA appears not to be the only consequence in the oxidative stress scenario. Under physiological or low lipid peroxidation rates, the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or signaling pathways activation that upregulate antioxidants proteins resulting in an adaptive stress response.

By contrast, under oxidative stress conditions free radicals will attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) rising lipid peroxidation rates that lead to a broad-spectrum of molecular cell indemnities (Ayala 2014) (Yin 2011).

1.7.2 Antioxidant defense system

To prevent oxidative stress damage, the human body is equipped with two categories of antioxidants, based on their activity, that serve to counterbalance the effect of ROS-mediated injuries: enzymatic (SOD, catalase or CAT, glutathione or GSH) and nonenzymatic (Vitamin A, C, carotenoids, flavonoids) (Birben 2012). One of the most effective intracellular enzymatic antioxidants is SOD belonged to a group of enzyme proteins containing metal ions named metalloenzymes. These proteins catalyze the dismutation of O_2^- into molecular oxygen and H_2O_2 and decrease the O_2- level that damages the cells at excessive concentration. This reaction is associated by alternate oxidation-reduction of metal ions present in the active site of SODs. Based on the metal cofactors present in the active sites, SODs are classified into Copper-Zinc-SOD (Cu, Zn-SOD), Iron SOD (Fe-SOD), and Manganese SOD (Mn-SOD) (Flora 2009). Many studies have revealed the fundamental role of SODs helping inflammatory and neuroinflammatory diseases recovery (Younus 2018). SODs overexpression inhibited O_2- production, preventing the infiltration of neutrophils at the site of damage from radiation-induced liver injury (Kang 2003), reducing hippocampal oxidative stress in AD (Massaad 2009), and attenuating diabetic renal injury (Craven 2001).

Moreover, catalase and glutathione peroxidase are associated with the enzymatic antioxidant system: catalase exerts its function breaks down two hydrogen peroxide molecules into one molecule of oxygen and two molecules of water in a two-step reaction, GPx enzymes in presence of tripeptide glutathione (GSH) add two electrons to reduce peroxides. The antioxidant features of

both enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction (Matés 1999). CAT has a prime role in regulating the cellular level of H_2O_2 and its catabolism protects the cells from oxidative assault, in fact, low catalase activities have been reported in schizophrenic patients, and in atherosclerotic patients. Moreover, catalase deficiency or malfunctioning is associated with more diseases such as diabetic renal injury, hypertension, and AD (Hwang 2012) (Gella 2009). Moreover, selenium compounds are well known for their ability to scavenge ROS, just think about the glutathione peroxidase as a selenoprotein, which acts by reducing peroxides, such as H_2O_2. If GPx activity is reduced, more H_2O_2 is present, which leads to direct tissue destruction and activation of NF-κB–related inflammatory pathways (Ishibashi 2002). Furthermore, non-enzymatic antioxidants work by interrupting free radical chain reactions. First, vitamin C (ascorbic acid) is a very important, and powerful, antioxidant that works in aqueous environments of the body. Vitamin C and E regenerate α-tocopherol55 from α-tocopherol radicals in membranes and lipoproteins. Ascorbic acid acts as an antioxidant by being ready for energetically favorable oxidation. ROS oxidize ascorbate first to monodehydroascorbate and then dehydroascorbate. The reactive oxygen products are reduced to water, while the oxidized forms of ascorbate are quite stable and not reactive, and do not cause cellular damage. Second, the antioxidant actions of carotenoids are based on their singlet oxygen reducing properties and their ability to trick peroxyl radicals, in particular the singlet oxygen (Stahl 1996). Food availability of these micronutrients in fruits and vegetables could enhance immunity by maintaining the functional and structural integrity of important immune cells and promoting antioxidant beneficial effects.

1.8 Inflammation in Central Nervous System (CNS)

1.8.1 Neuroinflammatory process

Inflammatory diseases of the CNS defined as "neuroinflammation", comprise a broad range of infection-related or putative autoimmune disorders, characterized by the presence of inflammatory infiltrates in the brain and spinal cord and immune-mediated tissue injury (Höftberger 2017). Neuroinflammation is mediated by cytokines, chemokines, ROS, and secondary messengers release. Resident CNS glia (microglia and astrocytes), endothelial cells, and peripherally derived immune cells produce these mediators. Moreover, the development of neuroinflammation depends on the context, duration, and course of the primary stimulus or insult. For instance, the recruitment of immune cells, edema formation, tissue damage, and potentially cell death of neuronal cells led to the developing many neurodegenerative diseases such as multiple sclerosis (MS), AD, Parkinson's disease (PD), narcolepsy and autism and other (Lassmann 2020). The intensity and timing of neuroinflammation may ultimately dictate the impact on CNS homeostasis. For example, an inflammatory response following CNS insult could be protective, as glial cells actioning to remove the insult and provide structural support to neurons. However, chronic inflammation, as occurs in neurodegenerative diseases, might promote neurodegeneration by generating a neurotoxic condition due to higher levels of cytokines, chemokines, pro-apoptotic molecules, and pro-inflammatory molecules (Megan 2015). For instance, lipopolysaccharide (LPS), which is an endotoxin in the outer membrane of Gram-negative bacteria, induces systemic inflammatory response through TLR signaling. LPS binding to TLR4 on the microglia surface triggers signal transduction pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), and MAPK which in the end lead to NF-κB nuclear translocation (Shabab 2017). IL-1β and TNF-α passage from peripheral district to CNS can cause blood-brain barrier (BBB) breakdown, up-

regulate adhesion-molecule expression, and stimulate the diffusion of other toxic substances such as NO (Blamire 2000). Neuroinflammatory responses are mediated by several key pro-inflammatory cytokines (IL-1β, IL-6, and TNFα), chemokines (CCL2, CCL5, CXCL1), secondary messengers (NO and PGEs) and ROS. Many of these mediators are produced by activated resident CNS cells including microglia and astrocytes Sequential activation of microglia and astrocyte cytokine expression precedes increased ionized calcium-binding adapter molecule 1 (Iba-1) or glial fibrillary acidic protein (GFAP) immunoreactivity following systemic immune challenge (Norden 2016). In addition, endothelial cells and perivascular macrophages are also important in interpreting and propagating these inflammatory signals within the CNS (Wohleb 2014). Lately, several studies have shown that CD4+ T-cells infiltrate the CNS in many neurodegenerative disorders, in which their participation has a critical impact on the outcome of microglial activation, consequent neuroinflammation and impaired memory and learning (González 2014).

1.8.2 Role of Microglia and Astroglia

Inflammation in the brain is silent since the brain has no pain fibers; however, it depends upon the synthesis of inflammatory components by local neurons and glial cells, and especially resident phagocytes which, in the brain, are known as microglia (McGeer 2004). Microglial cells deriving from blood monocytes residents in the CNS since embryonic development and are functionally involved in neuronal maintenance, injury, and repair in a similar manner to peripheral macrophages (Streit 2006). Microglia appear to be the major initial sensors of danger or stranger signals recognized by TLR4, and they secrete inflammatory mediators such as TNF-α, IL-1β and IL-6 (Saijo 2009). For instance, increased expression of IL-1β and IL-6 was found in the brain and microglia cells from aged mice. Contrariwise, anti-inflammatory factors such as IL-10 and

transforming growth factor-beta (TGF-β) are decreased in the cortex in response to lead (Cheng 2004). Despite the fact that when the severity of the immune response touches a certain extent, TGF-β initiates a feedback loop to reduce the level of IL-1, inhibiting microglial activation and resulting in suppression of inflammation in the CNS (Herrera-Molina 2005). Although the regional pattern of neuroinflammation can vary among different disorders, there are shared mechanisms by which activated glial cells sense stress and injury within the CNS and consequently transduce signals that intensify the inflammatory activity of microglia and astrocytes.

1.8.3 Neurodegenerative disorders

Etymologically, neurodegeneration definition refers to, in the case of tissues or organs, a process of losing structure or function. Thus, neurodegeneration refers to any pathological condition principally disturbing neurons. These diseases represent a large group of neurological disorders with mixed clinical and pathological signs affecting specific classes of neurons in specific functional anatomic areas (Przedborski 2003). Neurodegenerative diseases cause progressive loss of brain functions and corresponding clinical syndromes. For example, cognitive deficits occur not only in AD, but also in vascular dementia (VD), frontotemporal dementia (FTD), and dementia with Lewy bodies (LBD). In addition, the motor system is affected in amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), PD). In all of these diseases, neuronal death is a common risk factor. The main brain regions affected in neurodegenerative diseases with a consequent cell loss are the hippocampus and the substantia nigra (SN): hippocampal degeneration is characteristic of diabetic encephalopathy and AD, while SN degeneration is characteristic of PD. Apoptosis is implicits to be the major death pathway for neurons (Lossi 2003).

The apoptotic process is initiated by different signals. (Hongmei 2012). The morphologic and biochemical changes during apoptotic cell death are mediated by a family of intracellular cysteine proteases named caspases (cysteine aspartyl-specific proteases), which cleave their substrates at aspartate residues. Caspases are constitutively expressed in mammals, as inactive proenzymes (procaspase) composed of p10 and p20 subunits and an N-terminal recruitment domain, subsequently proteolytically activated to active heterotetramers, containing of two p10 and two p20 subunits (Nicotera 2008). To date, some studies indicates that there are two apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Igney 2002). Each pathway requires specific activating signals to begin an energy-dependent cascade of molecular events and activates its own initiator caspase (8, 9, and 10); both will activate the caspase-3. The activation of this pathway results in characteristic cytomorphological features including cell decline, chromatin condensation, formation of cytoplasmic vesicles and apoptotic structures and phagocytosis of the apoptotic bodies (Elmore 2007). At the end, macrophages phagocytose apoptotic bodies and the phagolysosomes degrade them.

Caspase multiplicity probably reflects the varied roles of these enzymes in pathophysiological illnesses. While, aberrant caspase activation in diseases is poor understood, in acute and chronic neurodegenerative diseases, caspase-mediated apoptotic cell death occur. Deficiency in caspase 1 or caspase 11 have significant protection from cerebral ischemic injury in interleukin-1 beta converting enzyme-deficient mice (Schielke 1998), and may possess protective effect against traumatic brain injury (Liu 2006). Another key event in neuronal apoptosis is an alteration in mitochondrial function that eventually lead to cell death are controlled and modulated by proteins of the Bcl-2 family. The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins. The Bcl-2 family of proteins directs mitochondrial membrane permeability and

can be pro-apoptotic or anti-apoptotic. Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w are considered as anti-apoptotic proteins whereas Bcl-10, Bax, Bid, Bad, and Bim, pro-apoptotic (Cory 2002). These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process (Akhtar 2004). The importance of understanding the neurodegenerative process is vital because cell death is a component of both health and disease, being initiated by various physiologic and pathologic stimuli. Moreover, the extensive involvement of cell death in the CNS urge to explore strong therapeutic targets preventing neurodegenerative diseases.

1.8.3.1 Alzheimer's disease (AD)

Numerous mechanisms for the neuronal cell death in AD have been proposed. One of these is the amyloid hypothesis, which proposes that deposition of Aβ is a chief event in the pathological cascade for AD. AD, the most common cause of dementia in aged individuals, is a complex neurodegenerative disease marked by the presence of extracellular amyloid-β (Aβ) plaques, intracellular hyperphosphorylated tau tangles, and significant synaptic loss, brain atrophy, and neuronal death. Aβ peptides of varying aspect are produced by the sequential cleavage of the amyloid precursor protein (APP). The APP belongs to a type 1 transmembrane family of glycoproteins that is ubiquitously expressed in several types of cells (Neve 2000). The N-terminal domain of APP is projected to the extracellular domain or can be localized in the lumen of intracellular vesicles, such as those of the endoplasmic reticulum, Golgi apparatus, and intracellular endosomes (Wahle 2005). On the other hand, the APP C-terminal region reaches the cytoplasmic domain. APP is susceptible to proteolysis by a set of proteases named α, β, or γ secretases. Secretases are responsible for the production of Aβ (1–40) peptide or the Aβ (1–42) variant with a higher capacity to self-accumulate. Most mutations in APP and mutations in

prenisilin 1 or 2 (PSEN1 or PSEN2), components of the γ-secretase complex, increase the relative production of an aggregation prone Aβ1−42 peptide and lead to an autosomal dominantly inherited, early onset form of AD. It has been reported that overexpression of these genes in transfected cell lines can cause apoptosis or result in an increased susceptibility to apoptosis (Guo 1996). Conversely, Bursztajn and colleagues (Bursztajn 1998) found that expression of PSEN-1 in primary neurons does not cause or enhance apoptosis; rather, it protects neurons against experimentally induced apoptosis. For that reason, the aptitude of PSEN-1 to induce apoptosis seems to be cell type specific; and this may have important consequences for the pathogenesis in AD, in which neurons are differentially affected (Wang 2006). Aβ neurotoxicity has also been associated with intraneuronal Ca^{2+} dyshomeostasis and mitochondrial dysfunction, which contribute to synaptic and neuronal damage (LaFerla 2002). Indeed, Aβ accumulation provokes mitochondrial Ca^{2+} overload in vivo leading to neuronal death Increased mitochondrial calcium levels associated with neuronal death in a mouse model of AD (Calvo-Rodriguez 2020).

This is known as chronic neuroinflammation, as is the case in AD that comprises a persistent and constant activation of microglia and production of inflammatory mediators leading to rise cytokines and chemokines levels (Stamouli 2016). Aβ itself can induce the expression of several pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ (Gitter 2017). Numerous chemokines such as CCL2 (also known as MCP-1, for monocyte chemoattractant protein-1), CXCL8 (IL-8), CXCL10 (IP-10) and CCL5 are also produced in response to Aβ peptide deposition being responsible for the recruitment of peripheral immune cells. Moreover, non-resident central nervous system cells, as peripheral immune cells can exceed the blood brain barrier and exacerbate the inflammatory response in AD brains (Domingues 2017).

1.8.3.2 Parkinson's disease (PD)

PD is a common disorder of the CNS which is clinically determined by postural instability, bradykinesia, resting tremor and muscle rigidity. The reduction of dopamine concentration in the striatum is related to the progressive death of neurons located on the Sn pars compacta (SNpc) (Casarrubea 2019). Although many theories attempted to clarify the causes of neuronal death, the precise PD etiology remains unknown. Emergent evidence highlights the correlation of the CNS and inflammatory response in PD through deregulated immune responses associated with age advancement, infectious agents (bacteria or viruses), exotoxins (e.g., pesticides or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or MPTP), or deposition of insoluble protein fibrils (e.g., alpha-synuclein) (Aloisi 2001). Neuroinflammation could be the first pathogenic mechanism of PD, and might have important repercussions for cognitive failure with a prospective for novel treatment targets. For instance, increased microglial activation is thought to lead to cell death in PD (Aarsland 2018). Numerous factors such as α-syn aggregates, LPS toxins, MPTP, pesticides, proteasome and heavy metals, leading ultimately to neuroinflammation, consequently, microglial cells produce different molecular mediators (e.g., ROS, prostanoids and cytokines) with chemotactic and immunomodulatory functions that lead to destroy dopaminergic neurons projecting to the caudate-putamen (Maccionia 2001).

1.9 Neuroinflammation in the Context of Central Nervous System Trauma

Damage to the CNS following a traumatic incident occurs in two distinct phases. First, the primary phase occurs shortly and is mainly mechanical because a physical insult causes direct structural injury to neuronal tissue and the vasculature. The secondary phase is caused by incompletely understood and complex cascade of physiologic and biochemical factors continuing for hours to

days post-injury, leading to catabolic intracellular processes that involve averabundance of free radicals, activation of cell death signaling pathways and up-regulation of inflammatory mediators. Moreover, hypoxia, cytotoxicity, ROS production, and breakdown of blood-CNS barriers, all contribute to further cell death (Chodobski 2011). Activated microglia and astrocytes, as well as infiltrating peripheral immune cells, also develops a pro-inflammatory microenvironment that is a results of the progressive destruction of CNS tissue.

1.9.1 Traumatic brain injury (TBI)

TBI is defined by a complex variety of heterogeneous physical insults of CNS, triggering in biochemical injury to the brain and associated structures. The major cause of neurodegenration following TBI is neuroinflammation characterized by microglial activation that switch into an uncontrolled process which causes, for example, an upregulation of several pro-inflammatory cytokines and a free radicals overproduction. The pro-inflammatory cytokines released TNF-α, IL-1-β, IL-6, IL-12, and IFN-δ increase the inflammatory response by abating the BBB. This notable unusual also contribute to an increased production of adhesion molecules causing a further intensified immune response (Kumar 2012) (Giunta 2013) (Ghirnikar 1998). In addition, astrocytes play key role in TBI by regulating extracellular glutamate levels. Although following TBI the astrocytes produce favorable effects, such as production and secretion of BDNF and NGF (A Zaheer 2001) (Laird 2008), excessive amounts of them often create a glial dysfunction. Actually, astrocytes produce many immunomodulatory molecules, and inflammatory mediators such as DAMPs and alarmins released by stressed, injured or dying cells. Typical DAMPs, including high-mobility group box 1 (HMGB1), heat shock proteins and S100 proteins, signal through pattern recognition receptors on phagocytic immune cells to promote clearance of cytotoxic cellular debris and decrease inflammation. Stimuli of astrocyte pattern recognition receptors by DAMPs results in NFκB signaling, in addition with proinflammtory cytokines release

such as the TNFα, α-chemokines, as well as COX-2 and matrix metalloproteinase 9 (MMP-9) mediators (Jayakumar 2014). Although the precise roles of immune and neuroinflammatory to the pathogenesis of TBI remain controversial and largely unexplored, prominent data suggest the possibility of targeting multiple pathways to limit injury and promote functional brain restoration.

1.9.2 Spinal cord injury (SCI)

Within the context of modulation of the CNS post-traumatic events, spinal cord injury (SCI) consist in a strong immune response characterized by the synthesis of cytokines and chemokines and a coordinated infiltration of the damaged site by peripheral immune cells as leucocytes. While initial damage is induced by contusion of the cord (for example, haemorrhage, membrane disruption, and vascular damage), the extent of the damage (such as excitatory amino-acid-induced intracellular Ca^{2+} increase, proteolysis, formation of free radicals and nitric oxide) is supposed to be because biochemical events leading to cellular dysfunction and neuronal cells death (Hausmann 2003). In the first phase, the gray matter at the region of impact contains disrupted cells and blood; second, after over a time-course of minutes to hours, the lesion is spread centripetally, initially by the induction of necrotic cell death that is mediated, for example, by excitatory amino-acid-induced Ca^{2+} entry and energy failure, NO production, then membrane breakdown. These events also are exacerbated by the presence of inflammatory cells and proinflammatory cytokines, such as TNFα (Yates 2019). The neurons and glial cells in the central nervous system including spinal cord are particularly disposed to oxidative due to many factors, including a high content of polyunsaturated fatty acids, a high rate of oxidative metabolic activity, intense production of reactive oxygen metabolites and relatively low antioxidant capacity as GSH activity (Azbill 1997). The CNS consumes 20% of the oxygen when the body is at rest. Oxygen production correlates with H_2O_2 formation, for that reason, superoxide production, and, in general, ROS circulation in the CNS increase markedly with aging and following trauma such as SCI. A number of studies have

demonstrated that ROS are formed following SCI, and can be initiated by excitotoxic levels of extracellular glutamate and the subsequent increase in intracellular $Ca^{2+.}$ One consequence of impaired mitochondrial function is the generation of oxygen-derived ROS, especially when respiratory chain function is reduced and molecular oxygen is present (Juurlink 2016). Since oxidative stress is considered a trademark of the secondary phase of injury of SCI, alleviating oxidative stress may be an effective way of therapeutic intervention of SCI (Hall 1992).

2. CHAPTER TWO: Regulation of inflammation and oxidative stress by Short fatty acids (SCFAs)

2.1 SCFAs: absorption, transport and metabolism

SCFAs are organic fatty acids with a variable number of carbon atoms (1 to 6) which are rpduced from bacterial metabolism of nondigestible carbohydrates entering the colon along with hydrogen gas. A large proportion of this gas volume is consumed by three bacterial reactions: (i) methanogenic bacteria that reduce CO_2 to CH_4 consuming H_2 in the process; (ii) non-methane producers that reduced SO_4 to sulphides, including H_2S; (iii) acetogenic bacteria, which reduce CO_2 to acetic acid. The most abundant SCFAs involved in mammalian physiology (≥95%) are the chain fatty acids acetate c2, propionate c3 and butyrate c4. Valerate c5, hexanoate c6, isobutyrate c4 and isovalerate c5 also remained in the colon but in smaller amounts (5–10% of total SCFAs). The different types and amounts of nondigestible carbohydrates that reach the cecum and large intestine, mainly involved in SCFAs production, depend on the daily intake and type of food. The major components of fiber that pass the upper gut are plant cell-wall polysaccharides, and oligosaccharides contained in fruit, vegetables, and legumes. Indeed, the normal concentration range of SCFAs is 70–140 mM, with the ratio of acetate, propionate and butyrate in the colon being 60:25:15. In humans, the content of fiber intake has been studied mostly by measuring the SCFA concentrations in feces followed by calculating the total rate of SCFA excretion. Fecal secretion amounts for SCFA are in the range of 10–30 mmol/day for diets with high fiber content compared with 5–15 mmol/day for control diets (Jenkins 2008). The predicted route of the SCFAs across the mucosa involves an active transport by two main receptors: the monocarboxylate transporter 1 (MCT-1) and the sodium-coupled monocarboxylate transporter 1 (SMCT-1) receptors. Both MCT-1 and SMCT-1 are highly localized on colonocytes and along the entire gastrointestinal tract up to the small intestine and the cecum. Additionally, MCT-1 is also highly

expressed on lymphocytes suggesting the importance of intracellular SCFA uptake by these cells (Iwanaga 2006).Two possible mechanisms of absorption have been proposed. (i) Diffusion of protonated SCFAs, from Na/H exchange, K+H+-ATPase, or bacterial metabolic activity, acidifying the colonic lumen promote a diffusive movement of SCFAs. (ii) Anion exchange, trough HCO_3 exchange and entry across the apical membrane (Sellin 1990). Unabsorbed SCFAs are excreted. While the majority of SCFAs are transported, absorbed by the colonocytes, and used as energy source for the local epithelial cells, a small proportion fraction can reach the liver and then the systemic circulation. Moreover, SCFAs could participate as substrate for different in different energy production processes, for example, propionate is a substrate for hepatic gluconeogenesis, and acetate participates in the synthesis of long chain fatty acids, glutamine, and glutamate (Cook 2001).

2.1.1 SCFAs receptors and signal transduction

Free fatty acid receptor 3 (FFAR3 or GPR41), FFAR2 (GPR43), GPR109A (also known as hydroxycarboxylic acid receptor 2 or HCA2)and the olfatory receptor (Olfr)-78 are expressed on cell surface and have been studied as mediators of biological effects of SCFAs. FFAR3 belongs to G protein-coupled receptors family (GPRs): GPR41 and GPR43 were activated by acetate, propionate and butyrate, GPR109A is only activates by butyrate. Following ligand binding, they reduce activity of adenylate cyclase, leading to inhibition of cyclic adenosine monophosphate (cAMP) and allowing further biological effects. Polymorphic nucleocytes blood cells (PMNs) and enteric neurons, express GPR41 (Brown 2003); intestinal leukocytes, white adipose tissue, pancreas and spleen express GPR43. Whereas GPR109A is expressed in adipose tissue, innate immune cells, intestinal epithelium, and keratinocytes. All GPCRs signaling occurs a predicted downstream via internal heterotrimeric G-proteins α, β, and γ subunits (Layden 2013).Taken together, depending on coupling of SCFAs and respective receptors, signaling response could

result in different outcomes in different cell types as well as, altered GPCR function, genetic mutations or impaired ligand activity, contributes to the pathogenesis of a number of different pathological conditions. Since GPCR and G-protein complexity, studies focused on SCFAs receptors are needed to clarify their signaling mechanism and to discover possible drug targets (Ulven 2012).

2.1.2 Physiological roles of SCFAs

Various studies reported SCFAs physiological effects in different body's districts such as gastrointestinal tract (GI), adipose tissues, immune cells, and the peripheral nerve, reporting that SCFAs by themselves influenced several physiological functions. Dietary fiber intake reduces the risk and symptoms of many metabolic and inflammatory diseases, for that reason, nondigestible carbohydrates elicit these benefits through production of SCFAs, which in addition to providing energy to colonocytes and resident bacteria, and promote bile acid excretion. SCFAs also act as secondary messengers that regulate gene expression and stimulate hormone and gut peptide synthesis [i.e., glucagon-like peptide 1 (GLP-1)], and initiate other signal transduction pathways in peripheral tissues (i.e., increased glucose utilization, reduced cholesterol synthesis) (Alexander 20198). SCFAs, particularly butyrate, are key promoters of colonic heath and integrity helping to maintain epithelial integrity and reduce luminal pH to limit pathogen growth and entry. An increased absorption of butyrate has been shown to promote and to enhance the mineral absorption of sodium, potassium, water, and electrolyte replacement that give it antidiarrheal properties (Tappenden 2010). Moreover, long-term elevation in acetate and propionate significantly reduced body weight gain and reduced liver fat storage in overweight human individuals. Thus, propionate along with acetate may also be involved in the regulation of adipogenesis and adipokine release mediated via GPRs, representing an effective inhibitor of lipid synthesis (Demigné 1995). Butyrate

also suppresses the production of pro-inflammatory cytokines and promotes the production of anti-inflammatory cytokines (Park 2007). In addition, SCFAs are able to modulate vasodilatation and induce hypotension in both rodent models and humans, (Keshaviah 1982) (Miyamoto 2016), and influences gut microbiome and energy balance and metabolism., normalizes plasma glucose and cholesterol levels (Besten 2013).

2.2 Regulation of Inflammation by SCFAs

2.2.1 Modulation of NF-kB activity by SCFAs

SCFAs are well known for their anti-inflammatory functions by influencing immune cell chemotaxis, ROS release as well as cytokine release. The SCFAs are known to alter several cellular processes including differentiation, proliferation and apoptosis. Two lines of evidence have established the link between NF-κB and gut inflammation. First, NF-κB activation has been showed in inflamed colonic tissue. Second, anti-inflammatory agents (glucocorticoids and salicylates) used to treat gut inflammatory illness are known to inhibit NF-κB function (Kopp 1198). Several evidences have been shown that SCFAs inhibit NF-kB-translocation in colonic and colonic adenocarcinoma cells (Inan 2000). Indeed, NF-kB plays an important role in acute inflammation and is involved in the pathogenesis of several diseases as well as intestinal inflammation. The nuclear translocation of NF-kB is initiated by degradation of cytosolic inhibitory proteins, named inhibitory kappa B (IkB) proteins. A variety of extracellular stimuli, including the proinflammatory cytokines IL-1 and TNF-a activate NF-kB, as described above. Therefore, the incidence of SCFAs decrease the LPS-induced production of TNF-α, IL-1β, IL-6, reinforcing at the same time the LPS-induced production of IL-10 in intestinal epithelial cells. Interestingly, it was also demonstrated that acetate is able to prevent the LPS-induced NF-kB/p65

translocation to the nucleus in macrophages cells, demonstrating that the suppression of NF-κB intracellular signaling pathway might be important for the effect of SCFAs (Corrêa-Oliveira 2016). Similar effects are demonstrated that the gene expression levels of GPR43 were increased after acetate treatment in kidney tissue induced by ischemia-reperfusion injury, and the SCFAs treatment inhibited ROS production in human kidney epithelial cells after hypoxia (Andrade-Oliveira 2015). Furthermore, SCFAs or GPR43 agonists inhibited the intracellular production of ROS and malondialdehyde (MDA), and reversed decrease of SOD in glomerular mesangial cells (GMCs) stimulated with high glucose and LPS (Huang 2017).

According to Cox et al., the presence of SCFAs also leads to anti-inflammatory effects by down regulation of cytokines and PGE2 by human monocytes following LPS stimulation (Cox 2009). Although, butyrate effects on Nf-κB activation and nuclear translocation are, by far, the mostly extensively reported, propionate action on inflammatory response mechanism are also described. The propionate-mediated inhibition of cytokine-induced ICAM-1 and VCAM-1 adhesion molecules suggested that the effects of propionate might have their origin in its influence on the on NF-κB mechanisms (Zapolska-Downar 2009).

2.2.2 SCFAs as HDAC inhibitors

One SCFAs function as signaling molecules to give rise to an extensive range of biological effects in the colonic epithelium and in other peripheral tissues is the regulation of host gene expression via histone deacetylase (HDAC) inhibition (Steliou 2012). Histone deacetylases (HDACs) and histone acetyltransferases (HATs) belonged to two large families of enzymes that controlled by the antagonistic actions the dynamic process of histone acetylation. The balance between the actions of these enzymes serves as a key regulatory mechanism for gene expression, cell growth, survival and proliferation, and their aberrant expression or activity lead to diseases development.

HDACs' predicted functions are rigorously dependent on their enzymatic activity to directly interact and inactivate specific target transcription factor (Aranda 2001). In addition to the HDACs main roles, in some circumstances HDACs have been recently shown their non-catalytic activity by interacting with different enzymes or proteins (Gallinari 2007). SCFAs negatively influence histone deacetylases (HDACs): as an example, SCFAs act on mononuclear blood cells and neutrophils through inhibition of HDAC reducing the proinflammatory TNF production and leading to inactivate the NFκB (Rooks 2016). Moreover, data on the roles of SCFAs receptors and HDACs effects on endothelial cells is occasional despite the fact that SCFAs (acetate, propionate and butyrate) attenuate TNFα- or LPS-induced endothelial activation by inhibiting the production of pro-inflammatory cytokines (IL-6 and IL-8) and GPR41 and GPR43 receptors as well as HDACs are expressed on/in endothelial cell (Li 2018).

2.2.3 Varied anti-inflammatory and antioxidant functions of SCFAs

Because SCFAs have multiple beneficial effects where they are produced hence on the gut barrier, it has been hypothesized and well reported that SCFA consumption of fermentable fibers help to relieve symptoms of IBD and overall, in GI-associated diseases. Butyrate is the preferred energy source of colonocytes and stimulates growth of the colonic epithelium, for that reason, it has been used in an effort to treat colonic inflammatory diseases: oral treatment with butyrate significantly prevented weight and tissue architecture loss following dextran sulfate sodium (DSS) administration in a murine experimental model of colitis (Lee 2017). Thus, if the SCFA butyrate is not totally used as an energy source by the colonocytes, it begins to accumulate in the cytoplasm where can exert greater effects on colon cancerous cells (Wu 2018). Moreover, it protects from lung inflammation by impairing the differentiation of Th9 cells (Vieira 2019) and suppress

proliferation and apoptosis of colorectal cancer cells (CRC) cells (Liu 2006). Propionate and butyrate impair viability of human eosinophils and butyrate alone restore injured tissue following spinal cord injury (Lanza 2018). SCFAs derived from dietary fibers also play an essential role in supporting β-cell metabolism and promoting survival under stressful conditions. A recent study has been reported how SCFAs are able to contrast ROS production. In human pancreatic β-cells acetate as well as butyrate treatments notably showed an effective anti-ROS effect (reactive oxygen species) following streptozocin (STZ) stimulation by relative ROS and NO generation measuring (Hu 2020). At the close, SCFAs showed to reduce secretion of IL-1β, MCP-1, and TNF-α by stimulated human THP-1 microglia-like cells (Wenzel 2020) and to interfere with the assembly of Aβ1-40 and Aβ1-42 peptides into neurotoxic Aβ aggregates (Lap Ho 2018).

2.3 Sodium propionate (SP)

Among the SCFAs, SP is one of the most studied together with sodium butyrate. It is chemically composed of a carboxylic acid portion and a small chain with three carbon atoms, two oxygen atoms and the hydrogen atoms. As said earlier, SCFAs are produced in the GI tract by anaerobic fermentation of water-soluble dietary fibers as well as sodium propionate, once adsorbed, it reaches systemic circulation through the hepatic portal vein (Parada Venegas 2019). The concentration of propionate in human plasma is around 3.4 – 4.9 μM even up to 13.3 μM (Chambers 2018). Lately, beneficial effects of dietary intake of SCFA were shown in different inflammatory models. Since propionate is produced in the colonic lumen, it is obviously think that the main beneficial effects of SP are exerted on intestinal and colonic barrier. Chronic inflammation as a mark of IBD, results in *in situ* generation of proinflammatory cytokines and ROS production. Thus, SP treatement inhibited the increased production of proinflammatory

factors IL-6, IL-1β, and TNF-α mRNA and increased the antioxidant activity of CAT and SOD in the colon of DSS-induced colitis mice (Tong 2016). The maintenance of gut microbiota homeostasis is considered important for cardiovascular health, for that reason, Bartolomaeus and colleagues, investigated the SCFAs importance in atherosclerosis. They found that SP increased plasma levels of the anti-inflammatory cytokine IL-10, reduced blood pressure, protects from cardiac damage, and reduces atherosclerosis in experimental hypertension (Bartolomaeus 2018).

Aim of Thesis PART I

Given the multiple SCFAs beneficial effects, the aim of this thesis (PART I) was to evaluate:

-the molecular mechanism and protective effect of SP in *in vitro* models of inflammation and oxidative stress induced by LPS and H_2O_2 using macrophage cell line J-774 A1;

- the SP anti-inflammatory and antioxidant effects in *in vivo* models of acute inflammation induced by carrageneean (CAR) injection and superoxide anion induced oxidative stress.

3. CHAPTER THREE: The Anti-Inflammatory and Antioxidant Effects of Sodium Propionate

3.1 ABSTRACT

The major end-products of dietary fiber fermentation by gut microbiota are the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate, which have been shown to modulate host metabolism via effects on metabolic pathways at different tissue sites. Several studies showed the inhibitory effects of sodium propionate (SP) on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. We carried out an in vitro model of inflammation on the J774-A1 cell line, by stimulation with lipopolysaccharide (LPS) and H2O2, followed by the pre-treatment with SP at 0.1, 1 mM and 10 mM. To evaluate the effect on acute inflammation and superoxide anion-induced pain, we performed a model of carrageenan (CAR)-induced rat paw inflammation and intraplantar injection of KO2 where rats received SP orally (10, 30, and 100 mg/kg). SP decreased in concentration-dependent-manner the expression of cicloxigenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) following LPS stimulation. SP was able to enhance anti-oxidant enzyme production such as manganese superoxide dismutase (MnSOD) and heme oxygenase-1 (HO-1) following H2O2 stimulation. In in vivo models, SP (30 and 100 mg/kg) reduced paw inflammation and tissue damage after CAR and KO2 injection. Our results demonstrated the anti-inflammatory and anti-oxidant properties of SP; therefore, we propose that SP may be an effective strategy for the treatment of inflammatory diseases.

3.2 Introduction

The Short-chain fatty acids (SCFAs) are carboxylic acids defined by the presence of an aliphatic tail of two to six carbons. Although SCFAs can be produced naturally through host metabolic pathways, the major site of production is the colon requiring the presence of specific colonic bacteria (Venegas 2019). SCFAs levels may be increased by the frequent introduction of fiber-rich products into the diet. Acetate (C2), propionate (C3), and butyrate (C4) are the major SCFAs released through fermentation of fiber and resistant starches (Tong 2016). In particular, in the intestine, SCFAs exert a trophic effect on the intestinal epithelium and play a key role in the modulation of colonic blood flow, gastrointestinal (GI) motility, and fluid and electrolyte absorption (Vinolo 2011). Several studies focused their attention on the effect of SCFAs on inflammatory signaling pathways, and it was well demonstrated that butyrate inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation, cytokines production and prevents oxidative damage in a murine model of nephropathy and colitis (Canani 2017) (Inan 2000) (Hamer 2009). Therefore, there is one report that SCFAs are able to inhibit the DNA binding and NF-κB-mediated transcription of inflammatory markers after IFN-γ-stimulation of RAW 264.7. ERK1/2 signaling pathway are involved in the potential anti-inflammatory effects of the SCFAs. Moreover, recent evidence suggested the potential therapeutic applications of butyrate in the treatment of metabolic and inflammatory diseases (Guilloteau 2010). Based on this finding, the present study aimed to investigate the mechanism of action of sodium propionate (SP), focusing our attention not only on inflammation but also on the modulation of oxidative stress and pain. The peculiarity of our study is to provide original data about the mechanisms that undergoing SP with in vitro and in vivo experimental models that has not previously been done before. The study was dividing into two steps: the first was to identify the molecular mechanism of SP by two

different in vitro models by stimulating murine macrophages cell line with liposaccharide (LPS) to induce inflammation and with hydrogen peroxide (H2O2) to induce oxidative stress. The second step was to study SP mechanism in vivo, including pain modulation, in a model of acute inflammation induced by CAR and in a model of superoxide anion-induced pain induced by of potassium peroxide (KO2).

3.3 Materials and Methods

The murine macrophage cell line J774-A1 and the culture Dulbecco's Modified Eagle's Medium (DMEM) were obtained from ATCC® (Manassas, VA, USA). Fetal bovine serum (FBS), Bio-Rad protein assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), SP, LPS, H2O2 and anti-laminin A/C antibody were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Anti-NF-κB, anti-IκBα, anti-COX-2, anti-MnSOD, anti-HO-1, and anti β-actin antibody for Western blot analysis were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Peroxidase-conjugated anti-mouse secondary antibody, peroxidase-conjugated goat and anti-rabbit IgG were obtained from Jackson Immuno Research (West Grove, PA, USA). All compounds used in in vivo study were purchased from Sigma-Aldrich Company Ltd. (Poole, United Kingdom). All solutions used for in vivo infusions were prepared using non-pyrogenic saline (0.9% wt/vol NaCl; Baxter Healthcare Ltd., Thetford, United Kingdom).

3.3.1 In Vitro Studies

3.3.1.1 Murine Macrophage Cell Cultures and Treatments

The murine macrophage cell-line J774-A1 (ATCC® TIB-67) was cultured in 75 cm2 flasks in a complete medium composed by DMEM with the addition of 10% of (FBS). Cells were maintained at 37 ◦C and 5% CO2. For the cell viability of J774-A1, 4 × 104 cells were plated (in a volume of

150 µL) in 96-well plates. Thereafter, the medium was replaced with fresh medium and cells were treated with six different concentrations (0.1, 1, 10, 100 µM and 1, 10 mM) of SP, to determine the concentrations with less toxicity. In the first set of the experiment to test inflammatory properties of SP, we pre-treated cells with SP at the concentrations of 0.1, 1, 10, 100 µM and after 2 h we stimulated cells with LPS (10 µg/mL) [25]. After 24 h, we performed mitochondria-dependent dye for live cells (tetrazolium dye; MTT) to formazan (Paterniti 2014) and Western blot analysis for NFκB, IκB-a, iNOS, and COX-2. In the second set of the experiment to test the anti-oxidant properties of SP, cells were pre-treated with SP at the concentrations of 0.1, 1, 10, 100 µM and then were stimulated with H2O2 200 µM for 10 min. After this experimental time, we performed the MTT assay and the Western blot analysis for HO-1 and MnSOD.

3.3.1.2 Western Blot Analysis

Western blot analysis was performed as previously described (Campolo 20156). J774-A1 cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested, and resuspended in Tris-HCl 20 mM pH 7.5, NaF 10 mM, 150 µl NaCl, 1% Nonidet P-40 and protease inhibitor cocktail (Roche, Switzerland). After 40 min cell lysates were centrifuged at 12,000 rpm for 15 min at 4 ◦C. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Samples were then heated at 95 ◦C for 5 min and equal amounts of protein separated on a 10–15% SDS-PAGE gel and transferred to a PVDF membrane (Immobilon-P). The following primary antibodies were used: anti-NF-κB (1:500; Santa Cruz Biotechnology, Dallas, TX, USA sc 8008), anti-IκBα (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), anti-iNOS (1:1000 BD transduction), anti-COX-2 (1:500; Santa Cruz Biotechnology, sc 376861), anti-MnSOD (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-HO-1 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). Antibody dilutions were made in PBS/5% w/v nonfat dried milk/0.1% Tween-20 (PMT) and membranes incubated overnight at 4 ◦C. Membranes were then

incubated with secondary antibody (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteins, they were also incubated in the presence of the antibody against β-actin protein (cytosolic fraction 1:500; Santa Cruz Biotechnology, Dallas, TX, USA) or lamin A/C (nuclear fraction 1:500 Sigma–Aldrich Corp.). Signals were detected with enhanced chemiluminescence (ECL) detection system reagent according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDoc TMXRS+ software and standardized to β-actin and lamin A/C levels. Data are representative of at least three replicates.

3.3.2 NOX Assay

NOX levels were measured in the J774-A1 cell line supernatant as described by Talero et al. (Talero 2012). Briefly, the supernatant was incubated with nitrate reductase (670 mU/mL) and β-nicotinamide adenine dinucleotide 3J-phosphate (NADPH) (160 µM), at room temperature for 3 h. The total nitrite concentration in the supernatant was then measured using the Griess reaction, by adding 100 µL of Griess reagent (0.1% w/v) naphthyl-ethylen-diamide-dihydrochloride in H2O and 1% (w/v) sulphanilamide in 5% (v/v) concentrated H3PO4; vol. 1:1 to the 100 µL sample. The optical density at 550 nm (OD550) was measured using a microplate reader.

3.3.3 Malondialdehyde (MDA) Assay

J774-A1 cells (1 × 105 cells/well) were seeded in poly-L-lysine-coated six-well plates. The cells were harvested to detect the levels of malondialdehyde (MDA) using the thiobarbituric acid (TBA) reactive substances assay (TBARS assay kit, Cayman Chemical, Ann Arbor, Michigan MI, USA, Item NO. 10009055). Briefly, TBA was added to samples and standard and incubated at 95 ◦C for

60 min, cooled in ice bath for 10 min. Then, samples were transferred to microplate and analyzed with microplate reader at OD of 532 nm.

3.3.4 Data Analysis

All values are expressed as mean ± standard error of the mean (SEM) of "n" observations. Each analysis was performed three times with three samples replicates for each one. The results were analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni posthoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

3.4 In Vivo Studies

3.4.1 Animals

The study was carried out by using Sprague–Dawley male rats (200–230 g, Envigo, RMS Srl Udine, Italy). Food and water were available ad libitum. This study was approved by the University of Messina Review Board for the care of animals, in compliance with Italian regulations on protection of animals (n◦ 399/2019-PR released on 05/24/2019). Animal care was in accordance with Italian regulations on the use of animals for the experiment (D.M.116192) as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

3.4.2 Carrageenan-Induced Paw Edema

Paw edema was induced by a subplantar injection of CAR (100 µL of a 1% suspension in 0.85% saline). Changes in paw volume were measured as previously described (Impellizzeri2016) using a plethysmometer (Ugo Basile, Varese, Italy) immediately before CAR injection, and, then, at hourly intervals for 6 h. Edema was expressed as the increase in paw volume (mL) after CAR injection relative to the pre-injection value for each rat. Results are reported as a paw-volume change (mL).

3.4.3 Induction of edema by O2−

Lightly anesthetized rats (CO2 (80%)/O2 (20%)) received a sub-plantar injection of O2− (1mM) or its vehicle in a total volume of 50 mL into the right hind paw as described by Salvemini et al. (Salvemini 2016).

3.4.5 Experimental Groups

Rats were divided in several groups:

Sham+ vehicle group: rats were orally administered with saline (n = 8);

Sham+ SP (10 mg/kg) group: rats were orally administered with SP at the dose of 10 mg/kg (n = 8); Sham+ SP (30 mg/kg) group: rats were orally administered with SP at the dose of 30 mg/kg (n = 8);

Sham+ SP (100 mg/kg) group: rats were orally administered with SP at the dose of 100 mg/kg (n = 8);

Carrageenan group: rats were subplantar injected with CAR (n = 10);

Carrageenan+ SP (10 mg/kg): rats were orally administered with 10 mg/kg SP 30 min before CAR subplantar injection (n = 10);

Carrageenan+ SP (30 mg/kg): rats were orally administered with 30 mg/kg SP 30 min before CAR subplantar injection (n = 10);

Carrageenan+ SP (100 mg/kg): rats were orally administered with 100 mg/kg SP 30 min before CAR subplantar injection (n = 10);

O_2^- group: rats were subplantar injected with O_2^- (n = 10);

O_2^- + SP (10 mg/kg): rats were orally administered with 10 mg/kg SP 30 min before O2− injection (n = 10);

O_2^- + SP (30 mg/kg): rats were orally administered with 30 mg/kg SP 30 min before O2− injection (n = 10);

O_2^- + SP (100 mg/kg): rats were orally administered with 100 mg/kg SP 30 min before O2− injection (n = 10);

Furthermore, data regarding groups Sham+ SP 10 mg/kg, Sham+ 30 mg/kg, and Sham+ 100 mg/kg were not showed because SP administration did not demonstrate histological changes compared to Sham group. The doses of SP were based on a previous dose-response study made in our laboratory.

3.4.6 Paw Edema Measurement

Changes in paw volume were measured as previously described (Petrosino 2017). Briefly, paw volume was measured with a plethysmometer (Ugo Basile, Comerio, Varese, Italy) immediately before the injection of O_2^- (before 30 min) and after at 1 h. Edema was expressed as the increase in paw volume (milliliters) after O2− injection relative to the pre-injection value for each animal. Results are expressed as paw volume change (milliliters).

3.4.7 Behavioral Tests

3.4.7.1 Tail-Flick Test

Tail-flick test was performed in the following experimental groups: Sham+ vehicle group; O_2^- group; O_2^- + SP 10 mg/kg; O_2^- + SP 30 mg/kg and O_2^- + SP 100 mg/kg. Nociceptive testing was performed by placing the distal portion of the tail of each animal in a water bath maintained at 52 ◦C. The time latency to withdrawal the tail (tail-flick) was measured at different time points: 30

min before O_2^- subplantar injection (pre-dose), 30 min and 60 min after O_2^- subplantar injection. The determination of antinociception was assessed between 07:00 and 10:00.

3.4.7.2 Formalin Test

Formalin test was performed in the following experimental groups: Sham+ vehicle group; O2− group; O_2^- + SP 10 mg/kg; O_2^- + SP 30 mg/kg and O_2^- + SP 100 mg/kg. To evaluate prolonged noxious stimulus produced by formalin injection, the rats were lightly anesthetized and 50 µL of 5% formalin solution was injected subcutaneously (s.c.) into the dorsal surface of the right hind paw with a 30-g needle. The rat was then placed in an open plexiglass chamber with a mirror positioned on the opposite side to allow unhindered observation of the formalin-injected paw. Pain-related behavior was quantified by counting the incidence of spontaneous flinching/shaking of the injected paw. In the first phase (phase I), the total number of flinches/shakes × min was counted starting the formalin injection until 5 min after that; in the second phase (phase II) the total number of flinches/shakes was counted every 5 min until 60 min after formalin injection. After 1 h of observation, rats were sacrificed by anesthetic overdose.

3.4.8 Myeloperoxidase Activity (MPO Activity)

MPO activity, an index of polymorphonuclear cell accumulation, was determined as previously described in the hind paw tissues of all experimental groups (Chia 2020). The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was measured as the quantity of enzyme degrading 1 mM of peroxide min-1 at 37 ◦C and was expressed in units per gram weight of wet tissue.

3.4.9 Histological Examination of the CAR-Inflamed Hind Paw

Biopsies of hind paws were taken 6 h following CAR injection. Histology was performed as previously described (Esposito 2009). The degree of paw damage was evaluated according on a

six-point score: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation and 5 = severe inflammation.

3.4.10 Histological Examination of the O2−-Inflamed Hind Paw

For histopathological examination, hind paws were taken 60 min after the intra-plantar injection of O_2^-. Tissue from the pads of rat hind paws was removed with a scalpel and processed (Petrosino 2017).

3.4.11 Western Blot Analysis for COX-2, iNOS, MnSOD and HO-1.

Cytosolic and nuclear extracts of the hind paws were performed as previously described. The levels of COX-2, iNOS, MnSOD, and HO-1 were quantified in the cytosolic fraction. The filters were blocked with 1X PBS, 5% (w/v) nonfat dried milk for 40 min at room temperature and subsequently probed with one of the following primary antibodies (all from Santa Cruz Biotechnology, Dallas, TX, USA) COX-2 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), iNOS (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), Mn-SOD (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), or HO-1 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) in 1X PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 at 4 ◦C, overnight. Membranes were incubated with secondary antibody (peroxidase conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated antirabbit IgG, 1:2000; Jackson Immuno Research, West Grove, PA1:2000) for 1 h at room temperature. Bands were detected by chemiluminescence (ECL) system (Thermo, USA), visualized with the ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) and analyzed by using Image Lab 3.0 software (Bio-Rad, Hercules, CA, USA). The expression levels of β-actin served as an internal control for protein loading. Data are representative of at least three replicates.

3.4.12 Glutathione Assay (GSH Assay)

One hour after the O_2^- intraplantar injection, rats were killed, and hind paws were collected and then suspended in 5 mL of 10 mM sodium phosphate buffer (pH 7.4) containing 8.56% (w/v) sucrose and homogenized in variable amounts of the buffer by using of an Ultra-Turax (Wilmington, NC, USA) tissue homogenizer. Reduced (GSH) and oxidized glutathione form (GSGG) ratio was measured using an enzymatic method that utilizes Ellman's Reagent (DTNB) and glutathione reductase (GR). DTNB reacts with reduced glutathione to form a yellow product. The optical density was measured at 412 nm.

3.5 Statistical Evaluation

All values in the figures and text are expressed as mean standard deviation (SD) of N observations. For in vivo studies, N represents the number of animals studied. In the experiments involving histology, the figures shown are representative of at least three experiments performed on different days. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons.

3.6 Results

3.6.1 In Vitro Studies

3.6.1.1 Effect of SP on Vitality Following LPS Stimulation

In order to choose the highest SP concentrations with the lowest toxicity, cell viability was assessed stimulating J774-A1 with different concentrations (0.1, 10, 100 µM, and 1, 10 mM) of SP. Treatment of SP at different concentration such as 100 µM, 1 mM, and 10 mM, markedly increased the basal proliferation of cells. Then, we decided to use the concentrations of 0.1–1 and 10 µM. (Figure 1A). Moreover, we assessed the viability following LPS stimulation. The J774-A1

cells pre-treated with SP showed an increased proliferation following LPS-induced cytotoxicity (Figure 1B).

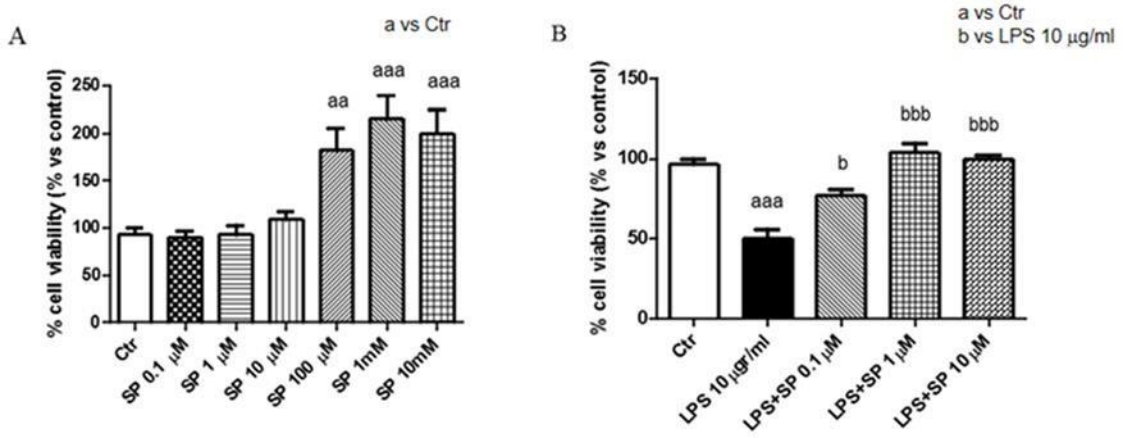

Figure 1. Anti-inflammatory effects of sodium propionate (SP) in cell-lines J774-A1 following liposaccharide (LPS) stimulation. Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) assay 24 h after treatment with SP. Cells showed an increased proliferation following pre-treatment SP of 100 µM and 1, 10 mM (A). J774 cell vitality was assessed following 24 h treatment with LPS 10 µg/mL and different concentrations (0.1, 1, 10, 100 µM). SP at 1 µM and 10 µM significantly locked damage caused by LPS 10 µg/mL more than SP 0.1 µM. (B). Data are representative of at least three independent experiments. aaa $p < 0.001$ versus Ctr; aa $p < 0.01$ versus Ctr; b $p < 0.05$ versus LPS 10 µg/mL; bbb $p < 0.001$ versus LPS 10 µg/mL.

3.6.1.2 Effect of SP on the Expression of iNOS and COX-2 Following LPS Stimulation

To evaluate the nitrosative stress and lipid peroxidation induced by LPS 10 µg/mL stimulation and the protective role of SP, we evaluated inducible nitric oxide synthase (iNOS) and cicloxigenase-2 (COX-2) expressions by western blot analysis. Basal levels of iNOS were observed in the control groups, whereas LPS stimulation induced a significant increase in iNOS expression (aaa $p < 0.001$ versus Ctr, Figure 2A,A1). Pre-treatment with SP reduced the expression of iNOS in a concentration-dependent manner, significant at 1 µM and 10 µM. COX-2 was significantly increased after LPS stimulation, whereas pre-treatment with SP, for all the concentrations, significantly reduced COX-2 expression (Figure 2B,B1).

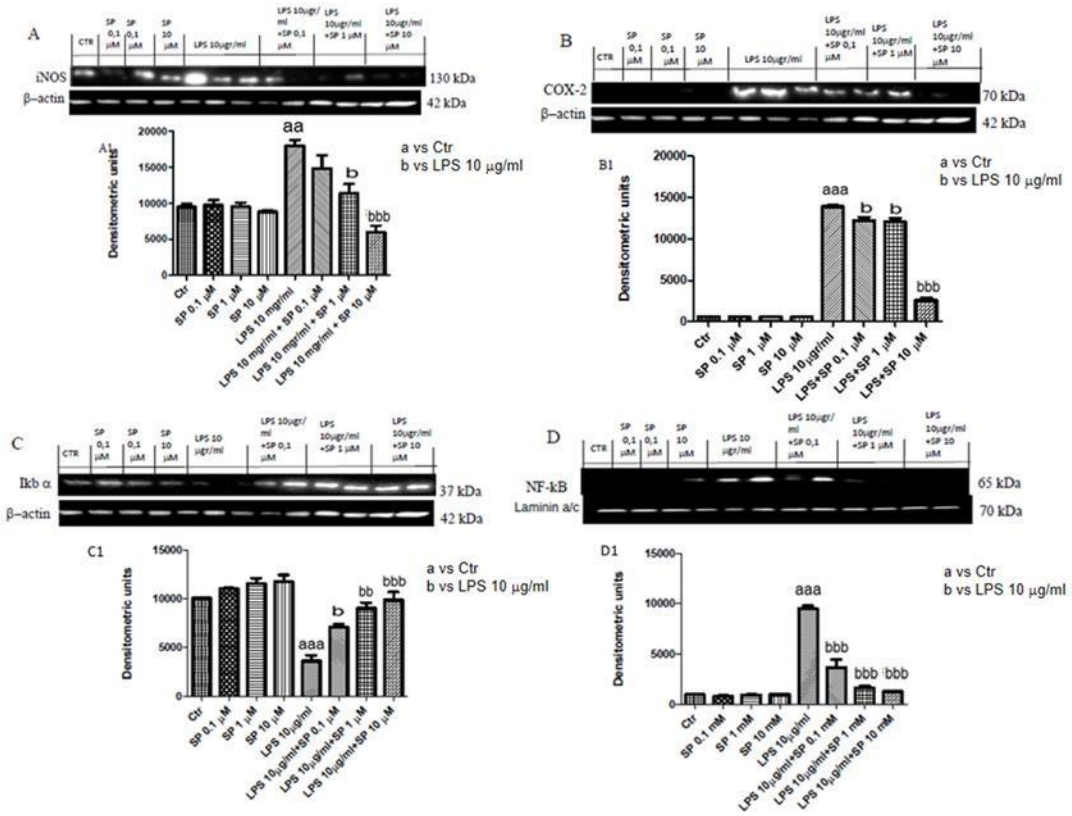

Figure 2. Effect of SP on the expression of iNOS, COX-2, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκBα), and NF-κB. iNOS and COX-2 levels were increased in LPS 10 μg/mL group, whereas pre-treatment with SP at the concentration of 10 μM significantly reduced these expressions more than SP 0.1 and 1 μM (A,A1,B,B1). Blots revealed a significant increase of NF-κb expression in LPS group meanwhile its expression was attenuated in group pre-treated with SP at concentration dependent-manner (D,D1). Therefore, IκBα level was decreased in LPS, SP restored these levels at all concentrations (C,C1). Data are representative of at least three independent experiments. aa $p < 0.01$ versus Ctr; aaa $p < 0.001$ versus Ctr; b $p < 0.05$ versus LPS 10 μg/mL; bb $p < 0.01$ versus LPS 10 μg/mL; bbb $p < 0.001$ versus LPS 10 μg/mL.

3.6.1.3 Effect of SP on the Expression of IκBα and NF-κB following LPS Stimulation

To investigate the molecular mechanism of SP against LPS-induced inflammation, we evaluated NF-κB pathway. Basal levels of IκBα was detected in control groups, while LPS stimulation induced IκBα degradation. Treatment with SP, for all three concentrations, restored IκBα expression (Figure 2C,C1). LPS stimulation induced NF-κB translocation into the nucleus, while SP treatment at all concentrations significantly reduce NF-κB translocation (Figure 2D,D1).

3.6.1.4 *Anti-Oxidant Effect of SP in J774-A1 Cell Cultures Stimulated with H2O2*

To evaluate the antioxidant effect of SP and its potential capability to induce recovery after oxidative stress, J774-A1 cells were pre-treated with SP and then stimulated with H2O2 200 µM for 10 min. We observed that cytotoxicity induced by H2O2 decreased the cell viability about 80%, while the pre-treatment with SP at the concentrations of 1 µM and 10 µM significantly restored cell viability, highlighting its potential anti-oxidant effect (Figure 3).

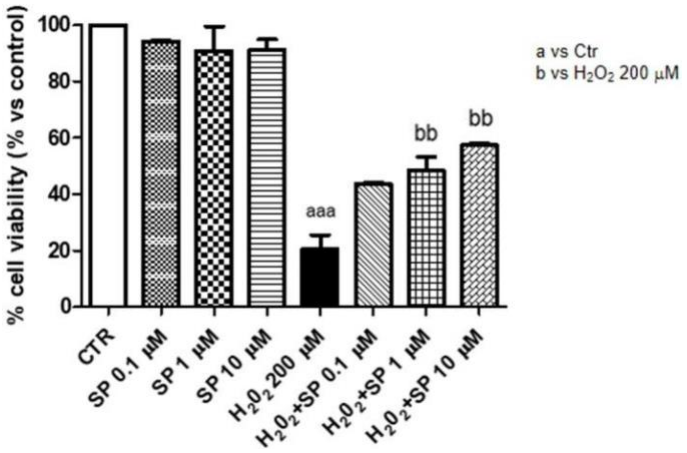

Figure 3. Anti-oxidant effect of SP in J774-A1 cells stimulated with H2O2. Cell viability was evaluated by MTT assay 24 h after stimulation with 200 µM H2O2. Cells showed an increased proliferation proliferative following treatment with 100 µM, 1 mM, and 10 mM SP. SP at 1 µM and 10 µM locked toxicity induced by 200 µM H2O2. Data are representative of at least three independent experiments. aaa $p < 0.001$ versus Ctr; bb $p < 0.01$ versus H2O2 200 µM

3.6.1.5 *SP Reduces the Nitrite Production and MDA Level in J774-A1*

We also tested lipid peroxidation through the production of malondialdehyde (MDA) in LPS-stimulatedmacrophagestoverifytheanti-inflammatoryactivityofSP; moreover,nitriteproduction was measured because NO is a toxic molecule released by the innate immune cells during disease. The control groups released low levels of NO_2^-; instead, H_2O_2 stimulation significantly increased NO_2^- production. Pre-treatment with SP in a concentration dependent-manner significantly decreased the NO_2^- levels (µM/mL) (Figure 4A). Phospholipids membrane are

susceptible to the attack of free radicals during oxidative stress; for this reason, we evaluated by MDA assay, the lipid peroxidation of the membrane. Our data showed a significant increase of MDA level after H_2O_2 stimulation while pre-treatment with SP attenuated the levels at a concentration of 1 µM and 10 µM (Figure 4B).

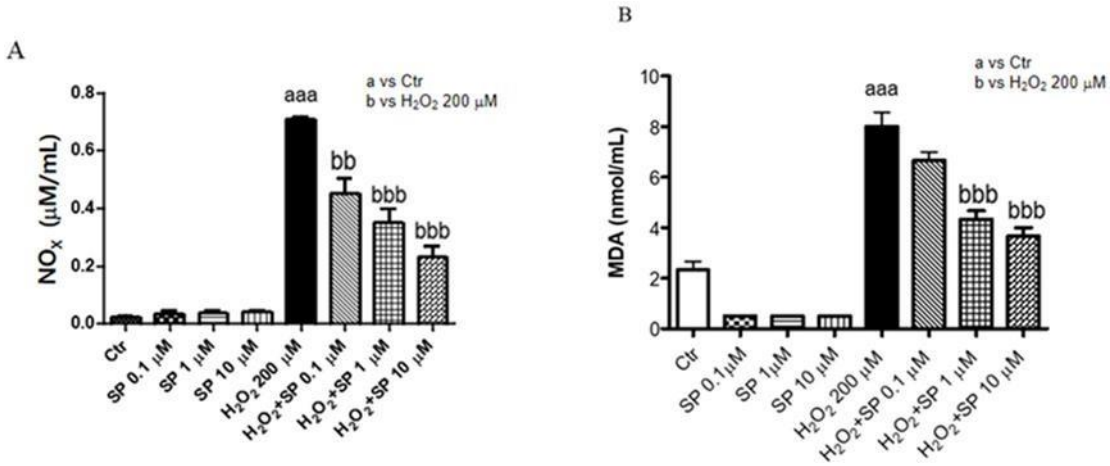

Figure 4. Effect of SP on nitrite production and, malondialdehyde (MDA) level. An increased of nitrite production and MDA level were evident in the 200 µM H2O2 groups, while the pre-treatment with SP at the concentration of 0.1, 1, 10 µM significantly decreased the oxidative stress-induced NOx (A) and MDA production (B). Data are representative of at least three independent experiments aaa $p < 0.001$ versus Ctr; bb $p < 0.01$ versus 200 µM H2O2; bbb $p < 0.001$ versus H2O2 200 µM.

3.6.1.6 Effect of SP on Anti-Oxidant Enzymes In Vitro

The stimulation with H_2O_2 caused important oxidative damage that reflected in a modified expression of anti-oxidant enzymes such as MnSOD and HO-1. By Western blot analysis, our results showed a significant increase in both enzymes following H_2O_2; however, SP at at concentration of 10 µM up-regulated their expression (Figure 5A,A1,B,B1).

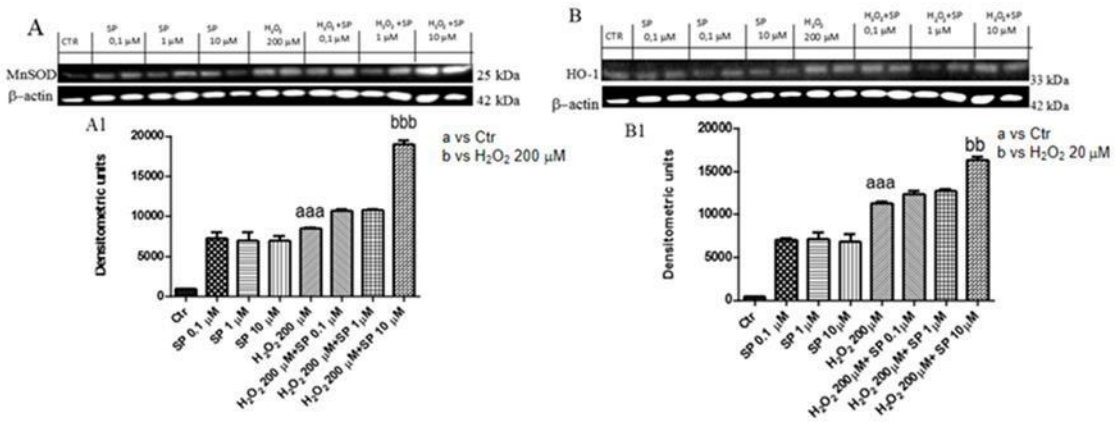

Figure 5. Effect of SP on antioxidant enzymes. Western blot analysis of cells lysates revealed a low increase in Mn-SOD (A,A1) and HO-1 (B,B1) levels in 200 µM H2O2 groups, whereas pre-treatment with SP 10 µM significantly restored antioxidant enzyme levels. Data are representative of at least three independent experiments. aaa $p < 0.001$ versus Ctr; bb $p < 0.01$ versus 200 µM H2O2; bbb $p < 0.001$ versus 200 µM H2O2.

3.6.2 In Vivo Studies

3.6.2.1 Effect of SP on Time-Course of CAR-Induced Paw Edema in Rats

Injection of CAR into the hind paw rapidly induced a clear and time-dependent increase in paw edema volume. That increase started at 3 h until 6 h (Figure 6). A significant reduction of paw edema volume was observed in rats treated with SP at 10 mg/kg, 30 mg/kg and 100 mg/kg compared to the CAR group (p-value of less than 0.05 was considered significant) (Figure 6).

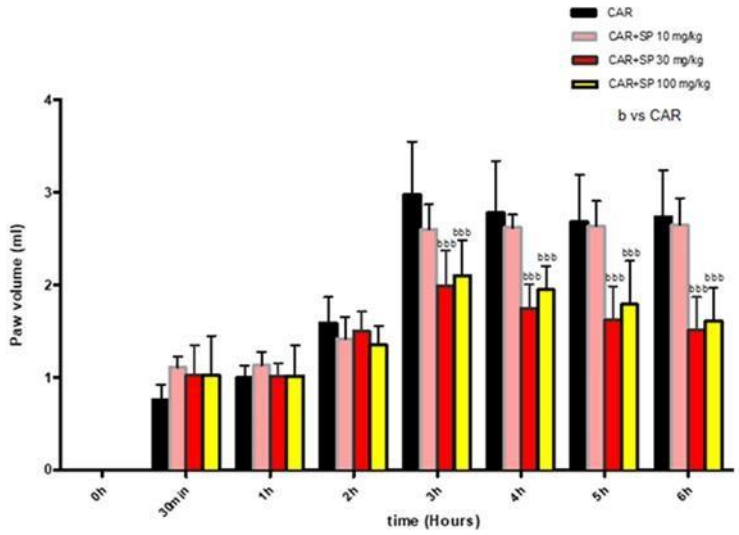

Figure 6. Effect of SP on time-course carrageenan (CAR)-induced paw edema. Paw edema volume was assessed at the time points indicated (t = 0, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h) and at different doses of SP (10, 30, and 100 mg/kg). SP groups showed significant reduction of paw volume compared to the CAR group. Values are showed as mean ± SD of 10 animals for each group. bbb $p < 0.001$ vs. CAR.

3.6.2.2 Histological Analyses of Paw Tissues and MPO Activity in CAR-Treated Rats

Histological evaluation was performed by hematoxylin and eosin (H&E) staining as described above. No histological damage was observed in control rats (Figure 7A), whereas important damage was observed 6 h after CAR injection with a marked accumulation of infiltrating inflammatory cells, edema and loss of normal muscle paw architecture (Figure 7B; see histological score Figure 7F), compared to control (Figure 7A; see histological score Figure 7F). SP treatment significantly reduced morphological alterations (Figure 7C–E). Moreover, histological damage was associated with an increased neutrophil infiltration as shown by MPO activity. SP 30 mg/kg and 100 mg/kg significantly reduced the enzyme activity (Figure 7G).

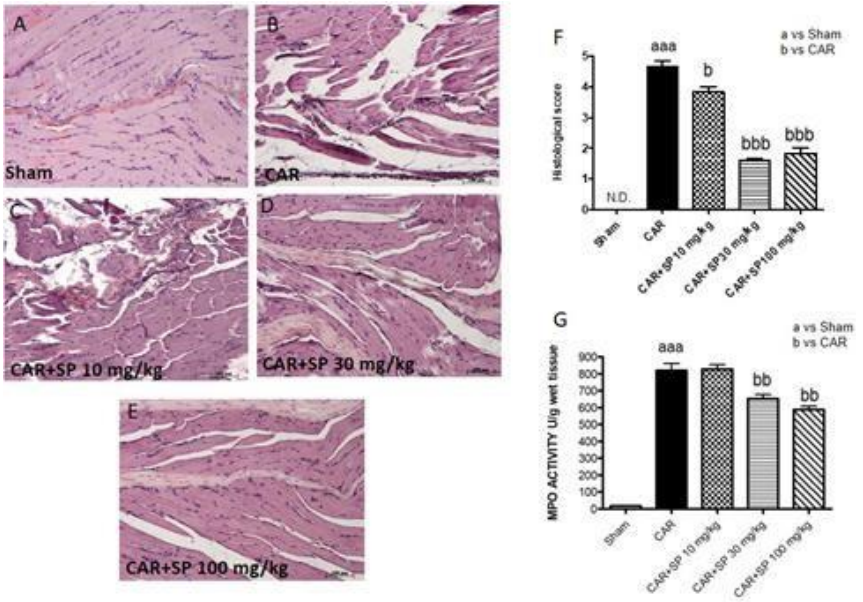

Figure 7. Histological analyses of paw tissues and myeloperoxidase (MPO) activity in CAR-treated rats. Control (A), intraplantar injection of CAR into the rat hind paw (B), intraplantar injection of CAR with SP 10 mg/kg (C), intraplantar injection of CAR with SP 30 mg/kg (D), and intraplantar injection of CAR with SP 100 mg/kg (E). Histological scores (F) MPO activity in paw tissues from the various treatment groups (G). The histological score was made by an independent observer according to this: 0 = no inflammation, 1 = mild inflammation, 2 = mild/moderate

inflammation, 3 = moderate inflammation, 4 = moderate/severe inflammation, and 5 = severe inflammation. The figure is representative of at least three experiments performed on different experimental days. Values are expressed as mean SD of 10 animals for each group. aaa $p < 0.001$ vs sham; bbb $p < 0.001$ vs. CAR; bb $p < 0.01$ vs. CAR; b $p < 0.005$ vs. CAR.

3.6.2.3 Effect of SP on the Expression of iNOS and COX-2 in Hind Paw Tissue from CAR-Treated Rats

We also determined the effect of SP (10 mg/kg, 30 mg/kg and 100 mg/kg) on pro-inflammatory enzymes as COX-2 and iNOS. COX-2 and iNOS expressions were assessed by western blot analysis paw homogenates 6 h after CAR injection. The expression of COX-2 was increased in paw tissues subjected to CAR injection compared to control rats. On the other hand, COX-2 expression was decreased by oral treatment with SP at 30 mg/kg and 100 mg/kg (Figure 8A,A1). Moreover, Figure 8B showed a significant increase in iNOS expression in the CAR group, which was significantly reduced following the treatment with SP at 30 mg/kg and 100 mg/kg (Figure 8B,B1).

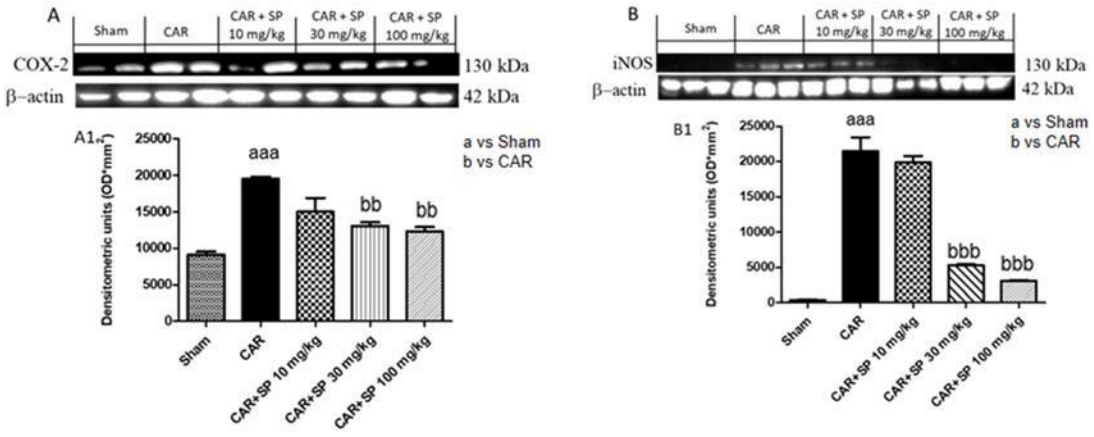

Figure 8. Effect of SP on expression of iNOS and COX-2 in paw tissue of CAR-treated rats evaluated by Western blot analysis. COX-2 levels in paw (A,A1) tissues were significantly increased after CAR induction; SP at 30 mg/kg and 100 mg/kg reduced COX-2 (A,A1). iNOS expression was low in paw tissue homogenates from control rats (B,B1), increased after CAR injection, and treatment with SP 30 and 100 mg/kg decrease significantly iNOS expression more than SP 10 mg/kg treatment. Data are representative of at least three independent experiments. Values are means ± SD of 10 animals for each group. aaa $p < 0.001$ vs. sham; bb $p < 0.01$ vs. CAR; bbb $p < 0.001$ vs. CAR.

3.6.2.4 Effect of SP on Time-Course of O2− Anion-Induced Inflammatory Pain

Intraplantar injection of O2− evoked an increase in paw edema of rapid onset (within 5 min) that reached a peak at 60 min. Oral administration of SP at 10, 30 and 100 mg/kg reduced paw edema compared to the vehicle within 1 h (Figure 9A).

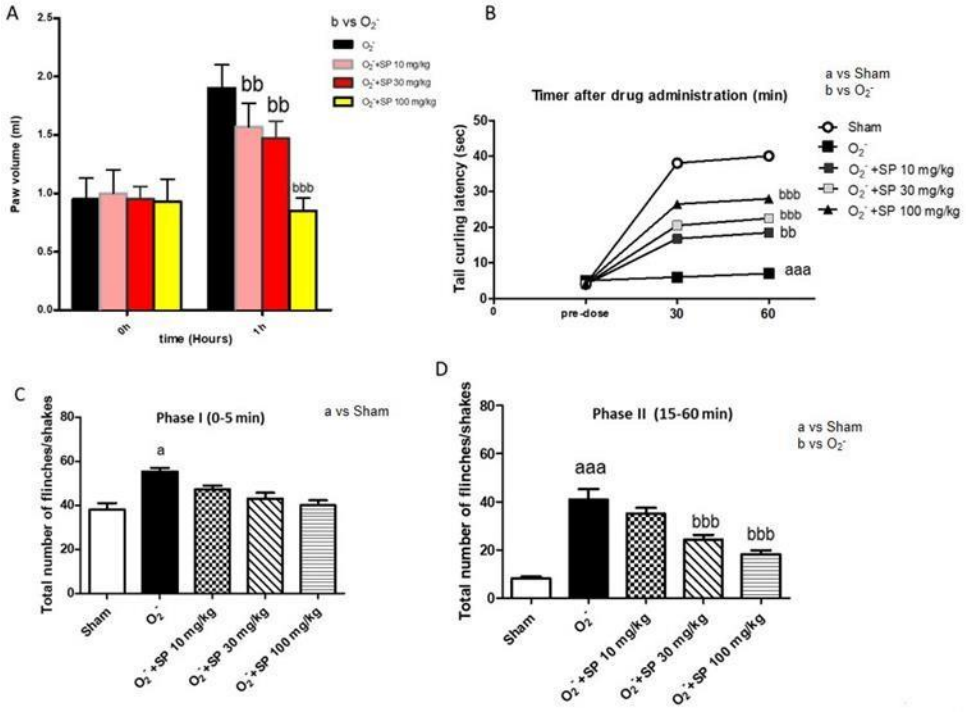

Figure 9. Analgesic profile of SP administration. SP when given orally 30 min before O2− injection inhibited the paw edema volume in a dose-dependent manner, as measured at 1 h after O2− injection (A). Effect of SP (10, 30, and 100 mg/kg) on nociceptive stimulus following the intraplantar injection of O2− (B). The effects of systemic doses of SP (30 and 100 mg/kg) on formalin-induced pain in rats (C,D). a $p < 0.005$ versus Sham; aaa $p < 0.001$ versus. sham; bb $p < 0.001$ versus O2− group; bbb $p < 0.001$ versus. O2−.

3.6.2.5 The Nociceptive Response Following O2− Intraplantar Injection

The analgesic response was measured at 30 min and 60 min after O_2^- intraplantar injection. SP administration at doses of 10, 30, and 100 mg/kg at the time point of 30 min did not give evidence of analgesic activity. Moreover, at 60 min after O2− intraplantar injection, the oral administration of SP at all doses significantly increased the latency before tail curling in rats (Figure 9B).

3.6.2.6 Analgesic Effect of SP Evaluated by Formalin Test

The early analgesic response occurs 5 min after the formalin injection (a $p < 0.005$ versus. Sham) (phase I). On this phase, oral treatment of SP (10–30 mg/kg and 100 mg/kg) did not evoke an analgesic response associated with damage induced by O_2^- intraplantar injection (Figure 9C); in phase II, formalin response following O2− injection, was significantly blocked by SP administration at the dose of 30 mg/kg and 100 mg/kg (Figure 9D).

3.6.2.7 Histological Evaluation of Paw Tissues and MPO Activity following O2− Intraplantar Injection

No histological damage was observed in control rats (Figure 10A), whereas damage was observed 1 h after O_2^- injection associated to inflammatory cells infiltration, edema, and alteration of tissue architecture (Figure 10B) compared to control group (Figure 10A). SP treatment at the all doses (10–30 mg/kg and 100 mg/kg) (Figure 10C–E; see histological score Figure 10F) significantly reduced morphological alterations. However, the administration of SP (10–30 mg/kg and 100 mg/kg) significantly reduced MPO activity in a dose-dependent manner (Figure 10G).

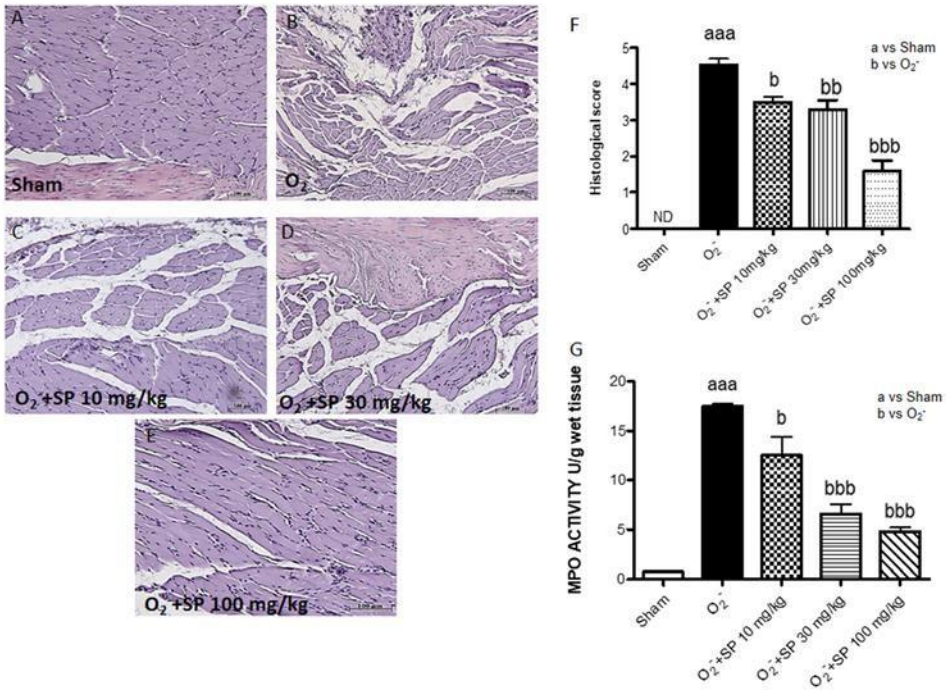

Figure 10. Histological evaluation of paw tissues and MPO activity following O2− intraplantar injection. No histological alteration was observed in control group (A). Treatment with SP (10–30 mg/kg and 100 mg/kg) significantly reduces the pathological changes and prevents the inflammatory cells infiltration (C–E) compared to O2− group (B, see histological score F). Effect of SP on the activity of MPO in the rat paws treated with O2−. Orally administration of SP (all doses) decreased the MPO levels after O2−-induced oxidative damage (G). Data is expressed as mean ± S.E.M. aaa $p < 0.001$ vs sham, b $p < 0.05$ vs. O2−; bb $p < 0.01$ vs. O2−; bbb $p < 0.001$ vs. O2−.

3.6.2.8 SP Reduce O2− Induced Oxidative Stress

It is well known that O_2^- intraplantar injection modulates the expression of anti-oxidant enzyme as MnSOD and HO-1 in rats hind paw. As shown in Figure 11, SP treatment (30 mg/kg and 100 mg/kg) significantly up regulated the expression of these enzymes compared to the control group (Figure 11A,B).

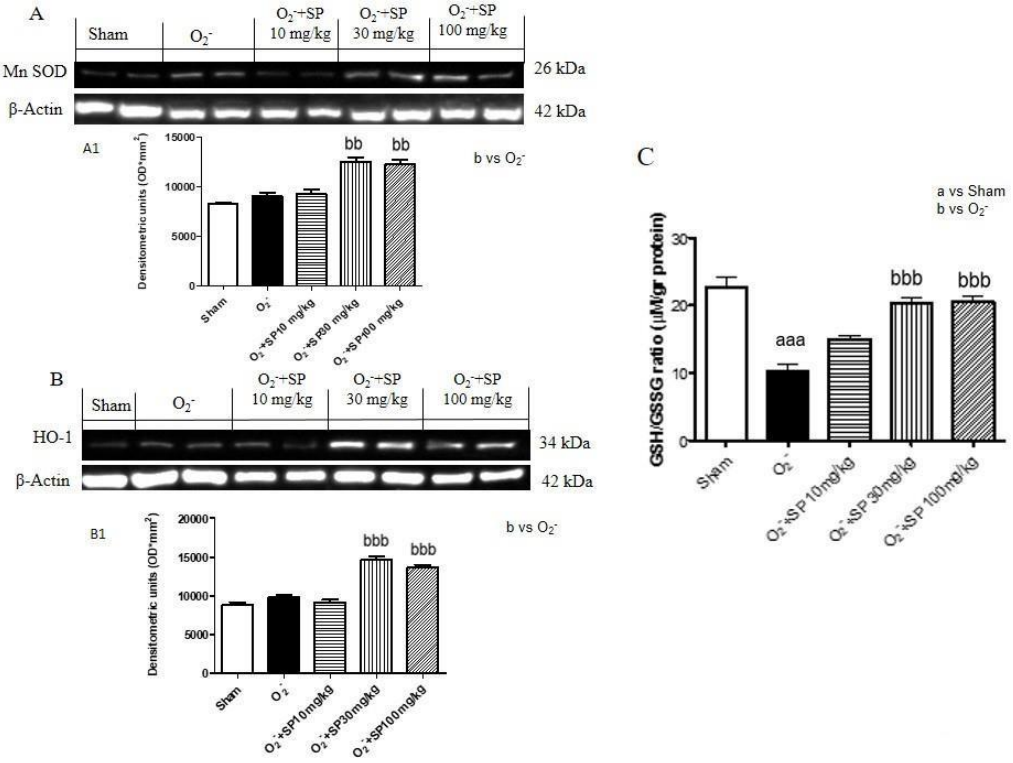

Figure 11. SP reduce O2− induced oxidative stress in rat paws following O2− intraplantar injection. Western blot analysis of hind paw tissues revealed a basal expression of MnSOD (A,A1) and HO-1 (B,B1) levels in the O2− group, whereas oral treatment with 30 mg/kg SP significantly increased levels more than SP 100 mg/kg. GSH/GSGG ratio was measured on hind paw tissues. SP treatment (30 mg/kg and 100 mg/kg) significantly reduce the GSH/GSGG ratio (C) aaa $p < 0.001$ vs. sham; bb $p < 0.01$ vs. O2−; bbb $p < 0.001$ vs. O2−.

3.6.2.9 SP Reduce GSH Levels in Rat Paws Following O2– Intraplantar Injection

GSH is produced in tissues as a result of GSH peroxidase activity and it is immediately reduced by GSH reductase, thereby maintaining constant levels of reduced glutathione in the normal tissue. Basal levels of GSH were measured in control rats, while O_2^- intraplantar injection contributes to increased GSH in levels. Oral administration of SP at concentrations of 30 mg/kg and 100 mg/kg, significantly increased GSH levels compared to the control group. However, 10 mg/kg SP did not show any significant modulation (Figure 11C).

3.7 Discussion

Inflammation and oxidative stress are linked together in a large number of pathophysiological processes (Biswas 2011). SCFAs exerted different anti-inflammatory properties, mainly derived from the inhibition of NF-κB translocation. To date, butyrate was exhaustively studied among the SCFAs, while the molecular mechanism of propionate is not well documented (Halliwell 2004). A recent study demonstrated that propionate, similarly to butyrate, may inhibit the activation of NF-κB in colon adenocarcinoma cell line (Inan 2000) with a consequent reduction of pro-inflammatory factors expression such as TNF-α and IL-1β in colon tissues. Moreover, we have previously shown the neuroprotective effect of SP in an in vitro neuroinflammatory model and in vivo model of spinal cord injury (SCI), recognizing in SP an optimal therapeutic target for neuroinflammatory disorders (Filippone 2020). In this study, we confirmed the ability of SP to contain the peripheral acute inflammation trough the down-regulation of NF-κB pathway. Moreover, we demonstrated that SP was able to reduce oxidative stress and nociceptive stimuli. It is well known that LPS stimulation induces the nuclear translocation of NF-κb (Segain 2000) and the subsequent degradation of Iκb-α, as well as the stimulation of iNOS and COX-2 expression (Sheeba 2009). Here, the pre-treatment with SP at the concentration of 1 μM and 10 μM on

macrophages cells significantly decreased the activation of inflammatory modulators induced by NF-κB activation. In recent years, many studies evidenced that oxidative stress plays a crucial role in the development and propagation of inflammation (Tan 2018) (Liu 2017). The production of various reactive oxidant species, in excess compared to an endogenous antioxidant defense mechanism, promotes the development of a state of oxidative stress with significant biological consequences (Lugrin 2014). H2O2 is a relatively stable oxidant but is also converted by neutrophils and macrophages to more reactive species such as superoxide and hydroxyl radicals. As these oxidants are detrimental to the surrounding cells, in vitro and in vivo studies tested many antioxidants compounds to improve anti-oxidant response. The in vitro study to test if SP treatment exerted antioxidant activity showed a key role in the up-regulation of the anti-oxidant enzymes Mn-SOD and HO-1 and in the reduction of MDA formation and NO_2^- production. These results highlight that SP prevents oxidative stress by scavenging free radical species and/or boosting the endogenous antioxidant system capacity by stimulating the synthesis of endogenous antioxidants. The first conclusion resulted in in vitro studies, confirmed SP effect against inflammation, and showed for the first time its potential effect to contrast oxidative stress. To better evaluate the properties of SP in a more complex system, we developed an in vivo model of acute inflammation induced by CAR in rats. CAR intraplantar injection is one of the techniques used to screen anti-inflammatory drugs because it exerted profound development of edema and cytokines release (Fehrenbacher 2012). We evidenced that SP possessed the ability to counteract peripheral inflammation following CAR-injection, in particular, decreasing paw edema, reducing histological damage, and infiltrating inflammatory cells. Moreover, in an in vivo model, SP was able to prevent the activation of NF-κB pathway. The peculiarity of this study was to go in deep into the anti-oxidant and analgesic effect of SP. Through the in vivo model, we injected a superoxide anion

donor, KO2, used as a generator of superoxide anion and considered a critical mediator in nociception (Ndengele 2008). KO2 has been used as a generator of superoxide anion in vivo. Superoxide anion is a precursor of additional reactive oxygen species (ROS) with enhanced toxicity, including hydroxyl radical, hypochlorous acid, and singlet oxygen (Taubert 2003) (Choi 2012). The role of superoxide anion is not directly related to its deleterious effects but rather to its activation of signaling molecules. For instance, the capability of neuropathy and inflammation-induced superoxide anion is to activate kinases and transcription factors such as NF-κB which guide the increase of the production of pro-inflammatory molecules (Maioli 2015) (Wang 2014). Surprisingly, we elucidated by behavioral nociceptive tests the analgesic effects of oral administration of SP showing the ability to attenuate the pain following superoxide intraplantar injection. Moreover, as described by Wang (Wang 2014), superoxide intraplantar injection modified hind paw tissue architecture and increased oxidative stress in situ. We showed that SP oral treatment was able to preserve paw tissue lost the anti-oxidant properties of SP was evidenced by the significant up regulated of anti-oxidant enzymes MnSOD and HO-1 that was accompanied by the reduction of GSH levels, highlighting the key role of SP in maintaining antioxidant defense systems.

3.8 Conclusions

Previously, it was shown that SP exerts beneficial e ects on the intestinal epithelium, inhibiting inflammation and modulating oxidative stress in a dextran sulfate sodium (DSS)-induced colitis mouse model (Cox 2016). It is common to believe that SCFAs play a key role in reducing inflammation in disorders of the gastrointestinal tract because the SCFAs are already produced in the colonic lumen by anaerobic fermentation of

carbohydrates, so the increased intake of these attenuates colon damages. Here, we elucidated that SP exerts important anti-inflammatory effects, accompanied by the ability to prevent oxidative stress and reduced pain also in peripheral tissue out from the origin area. The anti-inflammatory and anti-oxidant activities of SP are associated with its ability to down-regulate the NF-κB pathway and up-regulate antioxidant enzymes. Although there are limitations and challenges with animal models of acute pain modulation, clinically relevant models are essential for a fuller understanding of the behavioral consequences of acute and chronic pain and how they relate to the numerous complex histopathological cascades and to other inflammatory signaling pathways. This study is also unable to provide information about the inflammatory assessment of chronic pain. Our findings suggested the potential therapeutic efficacy of SP in several pathological events involving pro-inflammatory molecules and oxidants mediators recruitment; therefore, SP could represent a promising therapeutic target for the treatment of acute and chronic inflammatory diseases.

4. CHAPTER FOUR: Protective effect of sodium propionate in Aβ1-42 - induced neurotoxicity and spinal cord trauma

4.1 ABSTRACT

Sodium propionate (SP) is one of the main short chain fatty acids (SCFA) that can be produced naturally through host metabolic pathways. SP have been documented and include the reduction of pro- inflammatory mediators in an in vivo model of colitis. The aim of this study is to evaluate the neuroprotective effects of SP in reducing inflammatory process associated to neurological disorders. We performed both in vitro model of Alzheimer's disease, induced by oligomeric A□1-42 stimulation, and in in vivo model of spinal cord injury (SCI) in which neuroinflammation plays a crucial role. For in vitro model, the human neuroblastoma SH-SY5Y cell line was first differentiated with retinoic acid (100 μM) for 24 hours and then stimulated by oligomeric Aβ1-42 (1μg/ml) and treated with SP at 0.1- 1-10 μM concentrations for another 24 hours. Instead, the in vivo model of SCI was induced by extradural compression of the spinal cord at T6-T8 levels, and animals were treated with SP (10-30-100 mg/kg o.s) 1 and 6 h after SCI. Our results demonstrated that both in in vitro neuroinflammatory model and in vivo model of SCI the treatment with SP significantly reduced NF-κB nuclear translocation and IκBα degradation, as well as decreases COX-2 and iNOS expressions evaluated by Western blot analysis. Moreover, we showed that SP treatment significantly ameliorated histopathology changes and improved motor recovery in a dose-dependent manner. In conclusion, our results demonstrated that SP possesses neuroprotective effects, suggesting it could represent a target for therapeutic intervention in neuroinflammatory disorders.

4.2 Introduction

Sodium propionate (SP) is currently an authorized food additive in the European Union under Annex II of Regulation (EC) 1333/2008 for use in several food categories (bakery and cheese products) and in meat preparations, processed meat and processed fish(Banipal et al., 2016). SP is classified such as short chain fatty acid (SCFA). SP and other SCFAs are generated in the intestine by the bacterial metabolism of dietary fiber. SCFAs play beneficial effects on various aspects of gut physiology, included barrier function and metabolism (Ziegler et al., 2003). Furthermore, SCFAs can promote intestinal homeostasis and suppress intestinal inflammation (Smith et al., 2013) (Chang PV et al., 2014). Moreover, in parallel with its anti-inflammatory effect on defense processes, SCFAs are able to eliminate potentially damaging products, by counteracting free radicals, and decreasing cellular oxidative stress caused induced by inflammatory cascade. The beneficial aspects of short chain fatty acids are well studied (Tedelind et al., 2007) (Vinolo et al., 2011) (Cook et al., 1998) but a considerable attention was focused on propionate and its influence on inflammation, maybe derived from its ability to inhibit histone deacetylases (HDAC). The anti-inflammatory effects of HDAC inhibitors, such as sodium butyrate and sodium propionate, have attracted more attention for their ability to modulate the activity of the transcription factor nuclear factor kappa-light-chain- enhancer of activated B cells (NF- κB) in different cell types (Rahman et al., 2003), (Inan et al., 2000). Whereas anti-inflammatory properties of SP are well documented on peripheral tissues as colon and blood, the aim of this study was to evaluate the neuroprotective effect of SP in an in vitro and in vivo model of neuroinflammation. We tested the neuroprotective effects of SP in vitro by stimulating mature neurons with amyloid beta 1- 42 (Aβ 1-42). In vitro findings demonstrated that Aβ fragments is able to promote a marked neuroinflammatory response; in particular Aβ can attract and activate microglia leading to clustering of microglia

around sites of Aβ deposits in the brain. Exposure of neurons to Aβ leads to increase cell surface expression of MHC II along with increased secretion of pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα) as well as the chemokines and interleukin-8 (IL-8). Moreover, we tested the neuroprotective properties of SP in an in vivo model of spinal cord injury (SCI). SCI result in neurological deficits through both the primary and secondary damage. The "primary" injury involves the immediate mechanical damage to the spinal cord tissue that occurs at the moment of impact, which is irreversible and not preventable. The "secondary" injures the result of the pathological processes initiated at the time of the primary injury and continues for several days or months, characterized by leukocytes infiltration and activation of glial cells that aggravate tissue damage by releasing proteases, reactive oxygen intermediates, lysosome enzymes, and proinflammatory cytokines and chemokines (Di Sabato et al, 2016). The identification and targeting of molecules involved in the inflammatory and metabolic responses appear to have potential for use as a therapeutic approach in the treatment of neuroinflammatory diseases.

4.3 Material and methods

The human neuroblastoma cell line SH-SY5Y and the culture medium Eagle's Minimum Essential Medium and F12 Medium was obtained from ATCC® (Virginia, USA). Fetal bovine serum (FBS), Bio-Rad protein assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and anti- laminin A/C antibody were obtained from Sigma-Aldrich (Missouri, USA). Aβ 1-42 was purchased from Tocris Bioscence. SP and all materials utilized for the in vivo study were obtained from Sigma-Aldrich (Missouri, USA). Antibodies were purchased from Santa Cruz

Biotechnology (Texas, USA), BD Transduction Laboratories (CA, USA) and Jackson ImmunoResearch (Cambridge, UK).

4.3.1 In vitro study

4.3.1.1 Neuroblastoma cell line SH-SY5Y and treatment

The neuroblastoma cell line SH-SY5Y (ATCC CLR-2266) was grown in 75 cm2 flasks in complete medium composed by 1:1 mixture of Eagle's Minimum Essential Medium (EMEM) and F12 Medium supplemented to 10% fetal bovine serum. Cells were maintained at 37°C and 5% CO2. For cell viability, 4x104 cells were plated in 96-well plates (Corning Cell Culture, Corning, NY, USA) in a volume of 150 µl. Increasing concentrations of SP (0.1-1-10-100 µM and 1-10 mM) were used to determine the effective concentration with minimal toxic effects on cell viability. In another set of experiments, 2x106 cells were plated in plates in a volume of 2 ml and differentiated with RA (100 nM) for 24 h. Differentiated SH-SY5Y cells were pre-treated for 2 h with SP at the concentration of 0.1 µM, 1 µM and 10 µM, respectively. After 2 h, neurons were stimulated with Aβ 1-42 1□M for 24 h (Esposito et al. 2010) (Paillaud E et al., 2002). After 24 h, we performed Western blot and biochemical analysis. SH-SY5Y cells were divided into 8 experimental groups:

1. Ctr: cells were incubated with normal culture medium;
2. Ctr+ SP 0.1µM: cells were stimulated with SP at the concentration of 0.1 µM ;
3. Ctr+ SP 1µM: cells cultured stimulated with SP at the concentration of 1 µM;
4. Ctr+ SP 10 µM: cells were stimulated with SP at the concentration of 10 µM;
5. Aβ 1-42: cells were stimulated with Aβ 1-42;

6. Aβ 1-42+SP 0.1 µM: cells were stimulated as described, and SP at the concentration of 0.1 µM was placed in culture medium 2 h before damage;

7. Aβ 1-42+SP 1 µM: cells were stimulated as described, and SP at the concentration of 1 µM was placed in culture medium 2 h before damage;

8. Aβ 1-42+SP 10 µM: cells were stimulated as described, and SP at the concentration of 10 µM was placed in culture medium 2 h before damage.

4.3.1.2 Vital Staining (MTT assay)

Cells were pre-treated with increasing concentrations of SP (0.1 µM to 10 µM) and incubated at 37°C with MTT (0.2 mg/ml) for 1 h. The medium was removed by aspiration and the cells lysed with dimethyl sulfoxide (DMSO) (100 µl). The extent of reduction of MTT to formazan was quantified by measurement of optical density at 550 nm (OD550) with a microplate reader.

4.3.1.3 Western blot analysis for NF- -2 B p65, IκBα, iNOS and COX

SH-SY5Y lysates were used for the experiment as previous described. Membranes were incubated overnight at 4°C with the following primary antibodies: anti-NF-κB p65 (1:500; Santa Cruz Biotechnology), anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-α (IκBα) (1:500; Santa Cruz Biotechnology), anti-inducible nitric oxide synthase iNOS (1:1000 BD transduction), anti-cyclooxygenase 2 (COX-2) (1:500; Santa Cruz Biotechnology). Membranes were then incubated with peroxidase-conjugated anti-mouse secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteins they were also incubated in the presence of the antibody against β-actin protein for cytosolic fraction (1:500; Santa Cruz Biotechnology) and Lamin A/C for nuclear fraction (1:500; Santa Cruz Biotechnology). Signals were detected with enhanced chemiluminescence (ECL) detection system

reagent according to the manufacturer's instructions (Thermofisher, Massachusetts, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDocTMXRS+software and standardized to β-actin and lamin A/C levels.

4.3.1.4 NOX assay

Total nitrite levels, as an indicator of nitric oxide (NO) synthesis, were measured in the supernatant of SH-SY5Y cell line as described by Paterniti et al., 2017.

4.3.2 In vivo study

4.3.2.1 Animals

Male CD1 mice (25-30 g; 6-8 weeks of age, Envigo, Italy) were located in a controlled environment and supplied with standard rodent chow and water. Mice were housed in stainless steel cages in a room maintained at 22°C ±1°C with a 12-h light, 12-h dark cycle. The study and experimental protocols were approved by the University of Messina Review Board for the care of animals. Animal care was in conformity with regulations in Italy (D.M.116192), Europe (O.J. of E.C. L 358/1 12/18/1986), and the United States (Animal Welfare Assurance No. A5594-01, Department of Health and Human Services).

4.3.2.2. SCI model

Mice were anesthetized with xylazine and ketamine (0.16 and 2.6 mg/kg body weight, respectively). A longitudinal incision was made in the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5 to T8 vertebrae. SCI was produced by extradural compression of the spinal cord at T6 to T7 using an aneurysm clip with a closing force of 24g as previously described (La Rosa G et al., 2004). In the injured groups, the spinal cord was compressed for 1 minute. Sham-injured animals were only subjected to laminectomy. Spinal cord tissues were collected 24 hours after trauma.

4.3.2.3 Experimental groups

Mice were divided randomly in 8 groups:

1. Sham + vehicle group. Mice were subjected to the surgical procedures except for the aneurysm clip; these mice were administered with saline orally, 1 h and 6 h after laminectomy (N =20);

2. Sham + SP 10 mg/kg group. Identical to Sham + vehicle group except for the administration of SP 10 mg/kg, o.s 1 h and 6 h after laminectomy (N = 20) (data not show);

3. Sham + SP 30 mg/kg group. Identical to Sham + vehicle group except for the administration of SP 30 mg/kg, o.s 1 h and 6 h after laminectomy (N = 20) (data not show);

4. Sham + SP 100 mg/kg group. Identical to Sham + vehicle group except for the administration of SP 100 mg/kg, o.s 1 h and 6 h after laminectomy (N = 20) (data not show);

5. SCI + vehicle: mice were subjected to SCI plus administration of saline (N =20);

6. SCI +SP 10 mg/kg: mice were subjected to SCI plus orally administration of SP at the dose of 10 mg/Kg 1h and 6 h after SCI (N =20);

7. SCI +SP 30 mg/kg: mice were subjected to SCI plus orally administration of SP at the dose of 30 mg/Kg, 1h and 6 h after SCI (N =20).

8. SCI +SP 100 mg/kg: mice were subjected to SCI plus orally administration of SP at the dose of 100 mg/Kg, 1h and 6 h after SCI (N =20).

Data regarding groups Sham+ SP 10 mg/kg, Sham+ 30 mg/kg and Sham+ 100 mg/kg, were not showed because SP alone didn't demonstrate histological changes different from sham group. The doses of SP were based on a previous dose-response study in our laboratory.

4.3.2.4 Histological examination

Spinal cord tissues were collected 24 h following SCI. Histology was performed as previously described (Lanza et al 2018).

4.3.2.5 Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in spinal cord tissues as previously described (Mullane et al., 1985)

4.3.2.6 MDA assay on spinal cord tissues

Malondialdehyde (MDA) levels in the spinal cord tissue were determined as an indicator of lipid peroxidation as previously described. Spinal cord tissues were collected and then homogenized in 1.15% (w/v) KCl solution as described by Genovese T et al., 2004.

4.3.2.7 Grading of Motor Disturbance

The motor function of animals subjected to spinal cord damage was evaluated for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale (BMS) open-field score as the BMS has been shown to be a valid locomotor rating scale for mice(Basso et al., 2006). The BMS scale ranges from 0 (indicating complete paralysis) to 8 (indicating normal hindlimb function), and rates locomotion on such aspects of hindlimb function as weight support, stepping ability, coordination, and toe clearance).

4.3.2.8 Enzyme-linked immunosorbent assay (ELISA) kits

The spinal cord tissues were homogenated by a handheld homogenizer, which has connecting plastic pestle tips that homogenize the tissue through vibrating motions. The concentrations of NF-κB and IκB- α in SCI-tissue homogenates were measured using specific ELISA kits, respectively Mouse Nuclear Factor Kappa B P50 (Catalog number: MBS031656) and Mouse Inhibitory Subunit of Nuclear Factor Kappa Alpha (IκB-α) (Catalog number: MBS2024419) ELISA Kits according to the manufacturers' instructions (MyBiosource, CA, USA).

4.3.2.9 Western blotting

Levels of COX-2, iNOS, glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor protein-1 (Iba1), tumor necrosis factor-□ □TNF-□□, IL-1□ and β -actin were detected in cytosolic fraction as previously described by Cordaro et al 2016. Membranes were incubated at 4 °C overnight with primary antibody for iNOS (1:500; BD Trasduction), COX-2 (1:500; Cayman Chemicals), GFAP (Santa Cruz Biotechnology, sc 9065), Iba-1 (Santa Cruz Biotechnology, sc 32725), TNF-□ (1:500; Cayman Chemicals) and then incubated for 1 h at room temperature with a secondary antibody. Bands were detected by chemiluminescence (ECL) system (Thermo, USA), visualized with the ChemiDoc XRS (Bio-Rad, USA) and analyzed by using Image Lab 3.0 software (Bio-Rad, USA). The expression levels of β -actin was used as an internal control for protein loading.

4.4 Data analysis

All values are expressed as mean ± standard error of the mean (SEM) of 'n' observations. The results were analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni posthoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

4.5 Results

4.5.1 In vitro study

4.5.1.1 Neuroprotective effect of SP in neuronal cell lines SH-SY5Y damaged by Aβ1-42

To evaluate the effect of SP on cell viability, SHSY5Y cell line was incubated for 24 h with different concentrations of SP (0.1- 1- 10- 100 µM, 1 mM and 10 mM) using the MTT assay. All concentrations of SP resulted not cytotoxic, however concentration of 1 mM and 10 Mm resulted proliferative, thus we decided to teste only the concentrations of 0.1-1-10 µM of SP (Figure 12A).

Moreover, to identify the potential neuroprotective effect of SP we induced neuronal damage with Ab 1-42 on differentiated SHSY5Y. As showed in Figure 12B, SP treatments at the concentrations of 0.1-1 and 10 mM significantly preserved cell viability following Ab 1-42 stimulation.

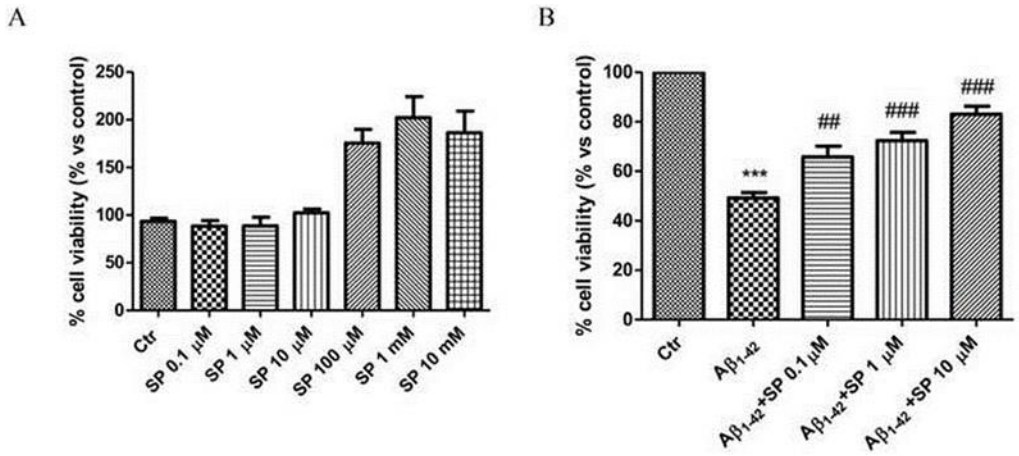

Figure 12. Neuroprotective effect of SP in SH-SY5Y neuronal cells damaged by in Aβ 1-42. Cell viability was evaluated using MTT tetrazolium dye and quantified by measurement of optical density at 550 nm (OD550). Cell death was assessed 24 h after treatment with different concentrations of SP. Cell death was significantly higher (≅ 90%) in groups treated with SP (A). Cells showed a proliferative condition following treatment at the highest concentrations of SP (1 mM and 10 mM). SP at 1-10-100 µM, locked damage caused by Aβ 1-42 (B). Data are representative of at least three independent experiments. ***P<0.001 versus Ctr; ##P<0.01 versus Aβ 1-42; ###P<0.001 versus Aβ 1-42

4.5.1.2 Neuroprotective effect of SP on NF-κB p65 nuclear translocation and IκBα degradation

Inflammation is a common condition identified in cell cultures stimulated by Aβ 1-42 1 µM, due to the activation of nuclear translocation of NF-κB (Valerio A et al., 2006). To evaluate SP effect on NF-κB pathway, we evaluated translocation of NFκ-B and degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, κ (IκBα). Cells stimulated with Aβ 1-42 for 24 h markedly showed high levels of NF-κB expression compared to Ctr and Ctr + SP groups, while the group pre- treated with SP, in a concentration dependent-manner, reduced significantly NF-κB translocation. Moreover, it was observed that pre-treatment with SP was able

to restore IκBα levels in a concentration dependent manner compared to Aβ 1-42 – stimulated group (Figure 13B, see densitometry analysis B1).

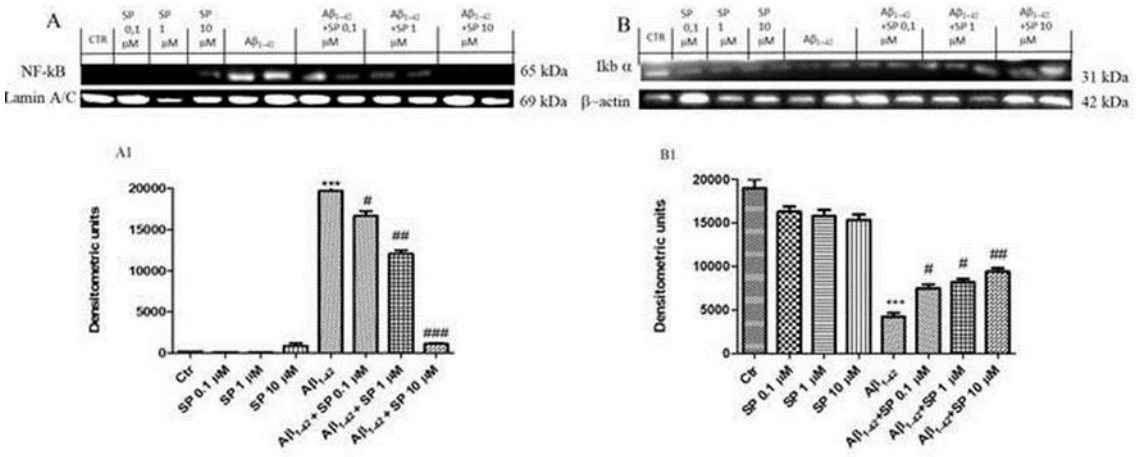

Figure 13. Neuroprotective effect of SP on NF-kB p65 pathway Western blot analysis of cells lysates revealed a significant increase in NF-□b in group Aβ 1-42 stimulated meanwhile its expression was attenuated in group pre-treated with SP at concentration dependent-manner (A). Therefore, the IκBα expression decrease in Aβ 1-42 group and was restored significantly by SP 10 μM (B). Data are representative of at least three independent experiments. ***P<0.001 versus Ctr; #P<0.05 versus Aβ 1-42; ##P<0.01 versus Aβ 1-42; ###P<0.001 versus Aβ 1-42

4.5.1.3 SP reduces the nitrite and nitric oxide production in Aβ 1-42 stimulated cells

The presence of nitrites in cell cultures identifies an alteration in the metabolic activity of neuronal cells (Paterniti et al., 2017). The levels of nitrite released into the culture medium were investigated by Griess reagent assay. The control group and the groups treated with SP alone released low levels of NO-2; instead, Aβ 1-42 1 μM stimulation over 24 h significantly increased levels of NO-2 production. SP pre-treatment significantly decreased the Aβ 1-42 induced NO-2 production in a concentration dependent-manner (Figure 14A). To corroborate the high increase of NO production after Aβ 1-4 stimulation, we evaluated iNOS expression through Western blot analysis. Our results showed a significant increase of iNOS after Aβ 1-42 stimulation compared to the control group (Figure 14B, see densitometry analysis B1). However, the treatment with SP reduced significantly

iNOS expression in a concentration dependent manner. Another important target for the modulation of inflammatory pathway is COX-2 (Chang et al., 1996). Our results showed a significant increase of the expression of COX-2 in the group stimulated with Aβ 1-42 compared to the control group. Whereas pre-treatment with SP significantly prevented COX-2 increasing especiall (Figure 3C, see densitometry analysis C1).

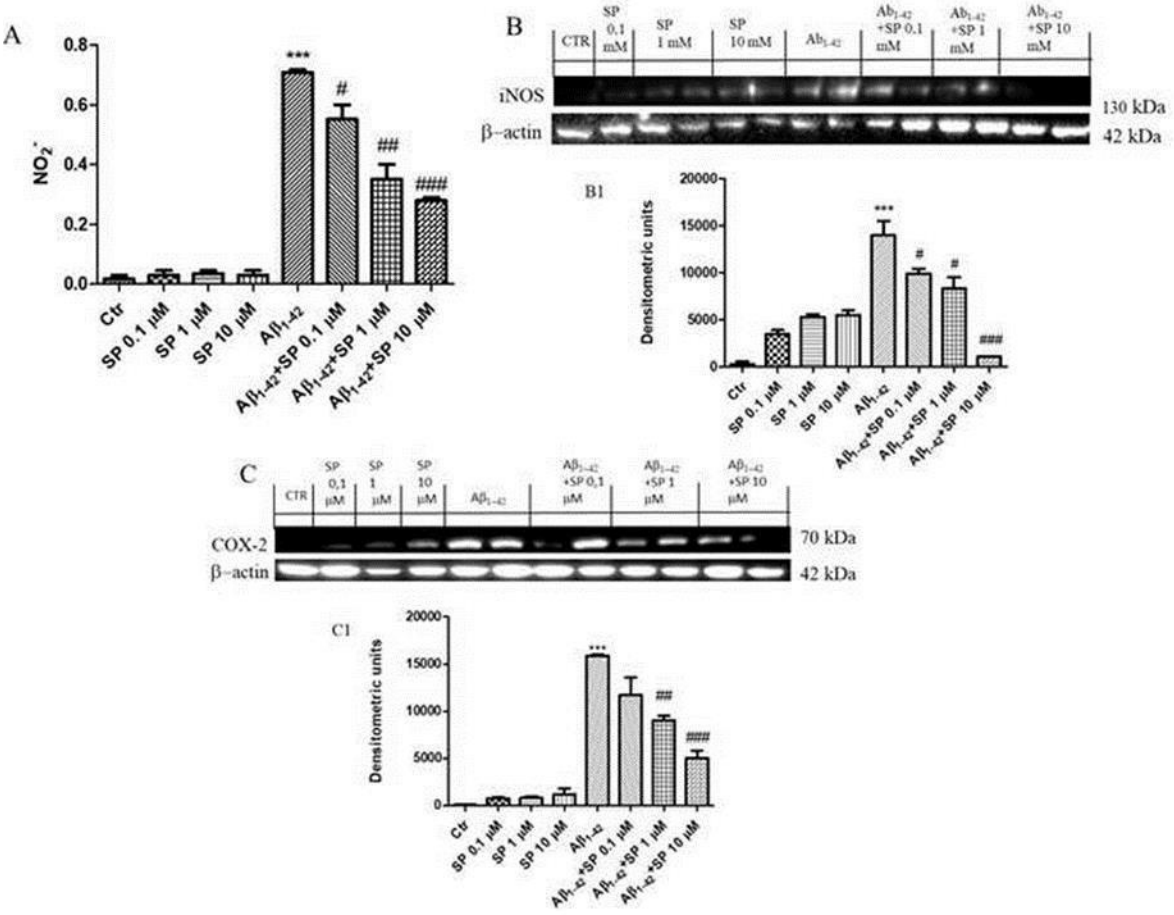

Figure 14. SP reduces the nitrite and nitric oxide production in Aβ1-42 treated human SH-SY5Y neuronal cells COX-2 levels in Aβ 1-42 -treated samples, whereas pre-treatment with SP 1 μM and 10 μM significantly restored both levels in a concentration-dependent manner. ###P<0.001 versus Aβ 1-42 (A). The iNOSexpression was evaluated by Western blot analysis in the cell lysate collected 24 hours after injury (B) compared to the control group. iNOS expression was also significantly reduced by pretreatment of SP at the three different concentrations 0.1-1-10 μM. P<0.001 versus Ctr; ###P<0.001 versus Aβ 1-42. (B). We evaluated the nitrite formation by nitrite assay on medium. An increased formation of nitrite levels was evident in the Aβ 1-42 injured group, while the pretreatment with SP at the concentration of 0.1-1- 10 μM decreased the injury-induced NO production. Data are representative of at least three independent experiments (C). ***P<0.001 versus Ctr; ###P<0.001 versus Aβ 1-42.

4.5.2 In vivo study

4.5.2.1 SP reduced the severity of SCI

The severity of trauma in the perilesional area was evaluated by hematoxylin and eosin staining 24 h after trauma. A significant tissue damage was observed in the spinal cord from mice subjected to SCI assessed by the presence of edema, as well as alterations of white matter (Figure 15B, see histological score 15F) compared with the Sham group where tissue architecture was intact (Figure 15A). Notably, a significant protection against SCI-tissue damage was shown in SP-treated mice at the doses of 30 mg/kg and 100 mg/kg (Figure 15D and E, see histological score F) more than SP treatment at the dose of 10 mg/kg (Figure 15C, see histological score F) ($F(8,80)= 32.92$). Moreover, we investigated the effect of SP treatment on the neutrophils infiltration by measuring MPO activity. MPO was significantly elevated in the spinal cord of mice subjected to SCI when compared with Sham-operated mice (Figure 15G). In SP treated mice at the doses of 30 mg/kg and 100 mg/kg, the activity of the peroxidase enzyme was significantly attenuated (Figure 15G) compared to SCI, meaning a decreasing of neutrophils infiltration ($F(5,50)= 80.31$). Furthermore, to evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the BMS open-field score was used (Figure 15H); SCI-injured mice showed significant deficits in hindlimb movement, starting with the first evaluation performed 24 h after damage, compared to control group (Figure 15H). In SP treated mice at the doses of 30 mg/kg and 100 mg/kg, motor functions were significantly improved starting by the third day after injury, compared with the SCI vehicle mice, while neuromotor recovery in animals treated with SP at 10 mg/kg was observed more slowly and it was of lesser relevance (Figure 15H) ($F(5,75)= 343.0$; 303.2; 453.4).

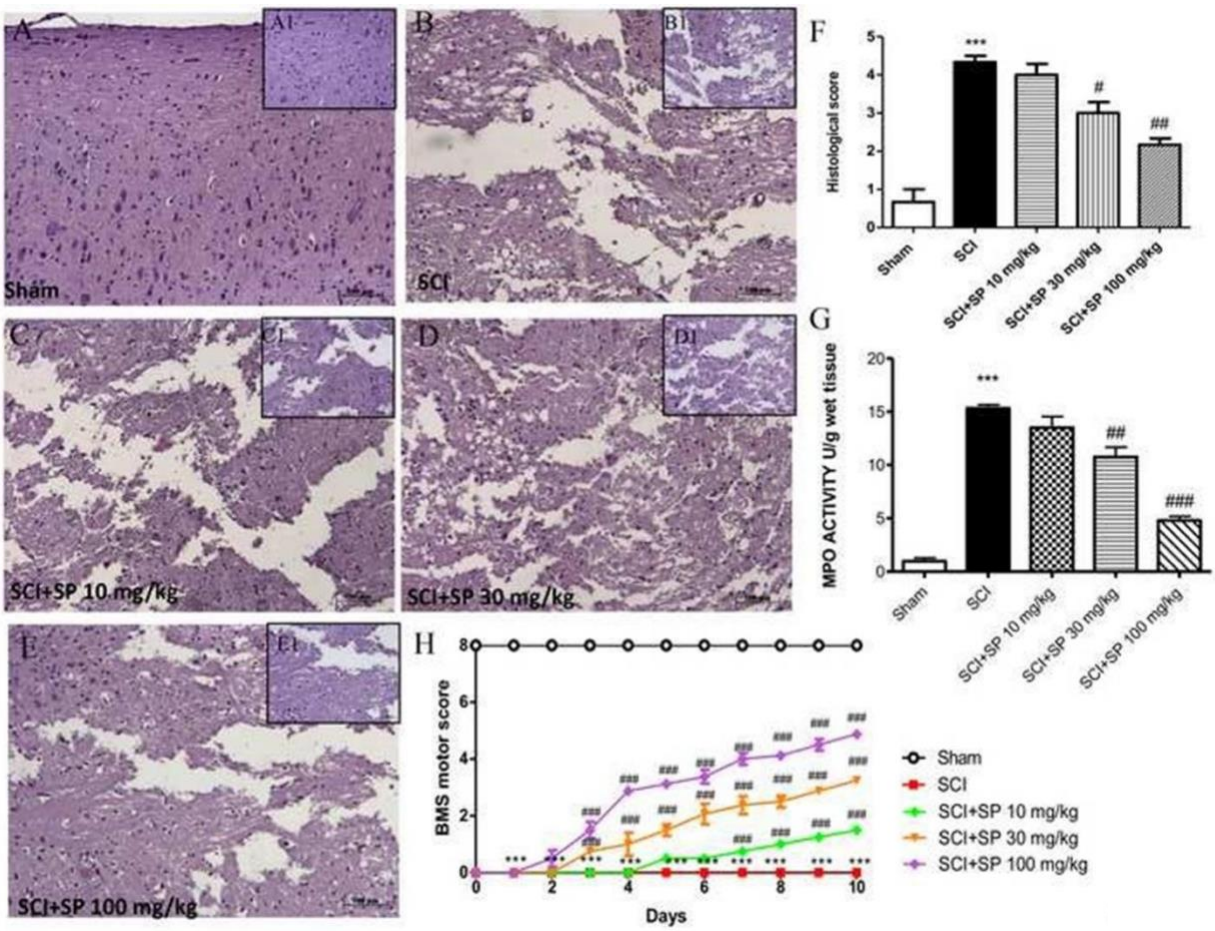

Figure 15. SP reduced the severity of SCI The severity of tissue damage following SCI is decreased in SP treatment mice. As shown in Figure 4, an extensive damage to the spinal cord was observed in SCI mice group (B) compared to shamoperated mice (A). Figure D, E and relative quantification in figure F showed a decrease in the severity of trauma in mice treated with SP 30 mg/kg and 100 mg/kg. Indeed, treatment with the SP 10 mg/kg do not reduced histological alterations. As showed in Figure G, treatment with SP 30 and 100 mg/kg significantly reduced MPO activity. The BMS score highlighted the restoration of motor activity in mice treated with SP 30 and 100 mg/kg compared to damaged animals (H). ***P < 0.001 vs sham group; #P < 0.05 vs SCI group; ##P < 0.01 vs SCI group. ###P < 0.001 vs SCI group.

4.5.2.2 SP treatment prevent lipid peroxidation

The lipid peroxidation is a molecular event that is involved in neuroinflammation included the molecular events occurring during SCI. SCI group was characterized by a significant increase of malondialdehyde (MDA) levels (Figure 16). The treatment with SP at the doses of 30 mg/kg and 100 mg/kg (1 hr and 6 hr after SCI) showed a significant reduction of MDA levels in the injured

tissue more than 10 mg/kg (Figure 5). No significant lipid peroxidation was observed in the sham-operated mice (Figure 16) (F(5,50)= 63.56).

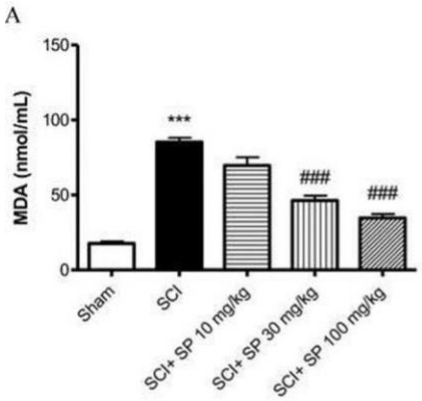

Figure 16. SP treatment prevent lipid peroxidation Effect of SP on MDA levels (A) in the spinal cord 24 hr after trauma. MDA levels and was significantly increased in the SCI group. SP 30 mg/kg and 100 mg/kg reduced the spinal cord injury□ induced increase in MDA levels. ***P<0.001 vs sham group; ###P < 0.001 vs SCI group.

4.5.2.3 Effect of SP treatment on NF-κB pathway

The anti-inflammatory properties of SP were quantified using ELISA kits for NF-κB and IκB-α 24 hr after injury. Compared with the findings from the sham group, the expression levels of NF-κB in spinal cord were significantly increased, while treatment with SP significantly reduced NF-κB quantity in dose-dependent manner (Figure 17A (F(5,50)= 92.71). Instead, the expression of IκB-α was reduced in SCI-injured mice, while the treatment with SP, mainly at the doses of 30 and 100 mg/kg, increased IκB- α expression levels (Figure 17B) (F(5,50)= 76.45). Furthermore, to evaluate if SP could modulate the inflammatory mediators in the central nervous system (CNS), we assessed Western blot analysis for COX-2 and iNOS. As showed, there was a significant increase in the expression of iNOS and COX-2 (Figure 17C and D, see densitometry analysis C1,

D1 respectively) 24 hr after SCI. Instead, treatment with SP at the dose of 100 mg/kg significantly attenuated COX-2 and iNOS expression (respectively, (F (5,50)= 64.93; (F(5,50)= 68.37).

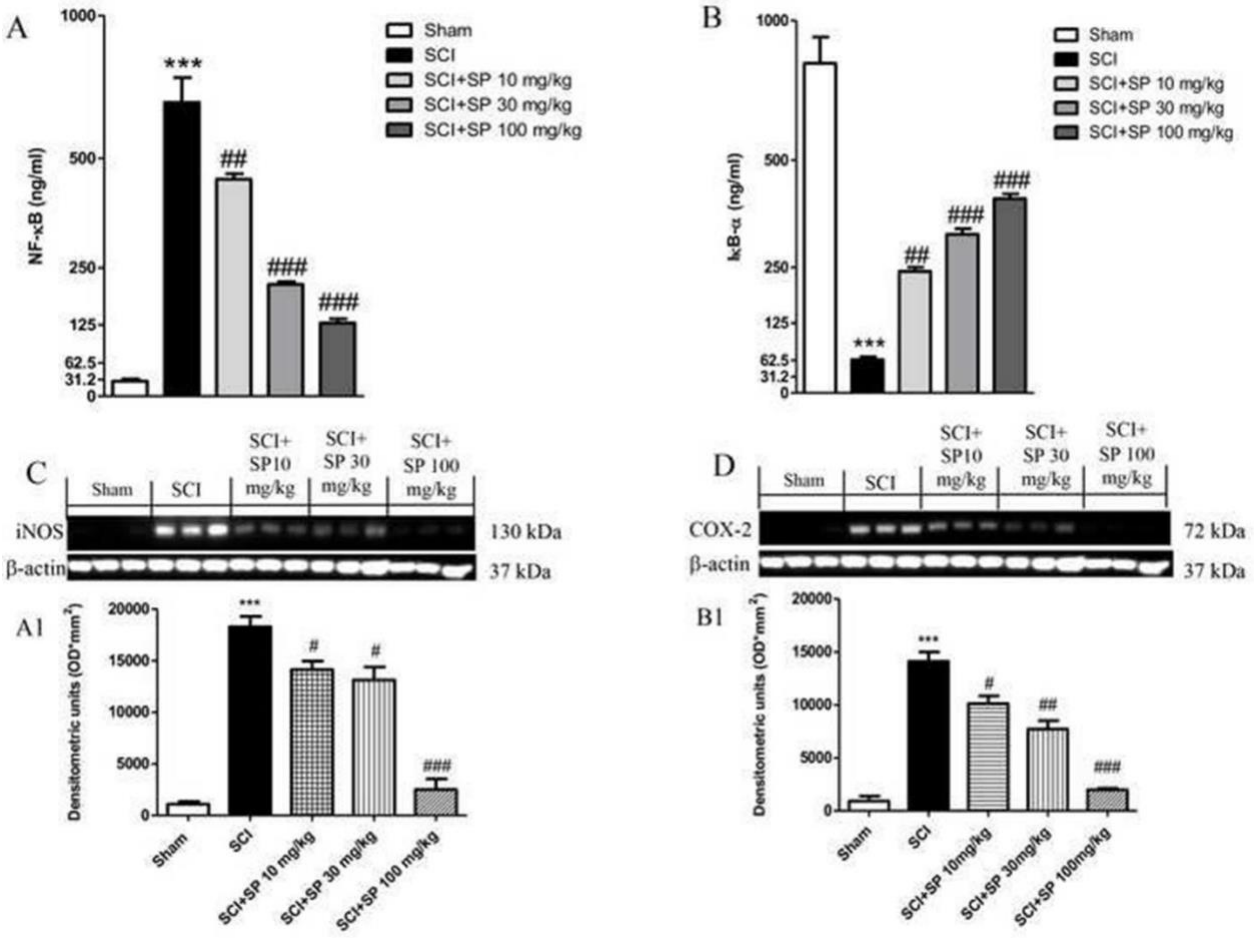

Figure 17. Effect of SP on NF-κB pathway SP modulates NF-κB, IκB-α, iNOS and COX-2 expressions after SCI (A, B, C and D respectively). A substantial increase in NF-κB (A), iNOS (C) and COX-2 (D) expression was observed in spinal cord samples obtained at 24 h after SCI, while SP treatment significantly reduced these expressions. The levels of IκB-α was significantly increased in SP treated mice, compared to injury-animals (B). ***P<0.001 vs sham group; ##P < 0.01 vs SCI group; ###P < 0.001 vs SCI group.

4.5.2.4 *Effect of SP treatment on microgliosis and astrocytosis*

Microglia represent the innate immune system of the CNS and play a key role in the neuroinflammatory processes (Streit W et al., 2004). A substantial increase in GFAP and IBA1 expressions were found in spinal cord tissues collected 24 h after SCI. Spinal activation of

astrocytes and microglia were significantly attenuated in SP 30 mg/kg and 100 mg/kg treated mice compared to SCI group. (Figure 18A and B, see densitometry analysis A1, B1 respectively) (respectively, (respectively, (F(5,50)= 236.2; (F(5,50)= 126.6). The secondary damage to the spinal cord initiates with the activation of microglia and astrocytes that release a large number of proinflammatory cytokines. Western blot analysis revealed a markedly increase of expression of IL-1β and TNF-α production in the spinal tissues collected at 24 h after SCI compared to sham group. SP treatment at the doses of 30 mg/kg and 100 mg/kg significantly decreased IL-1β and TNF-α expression (Figure 19A and B, see densitometry analysis 19A1, B1 respectively) (respectively, (F(5,50)= 409.2; (F(5,50)= 111.1).

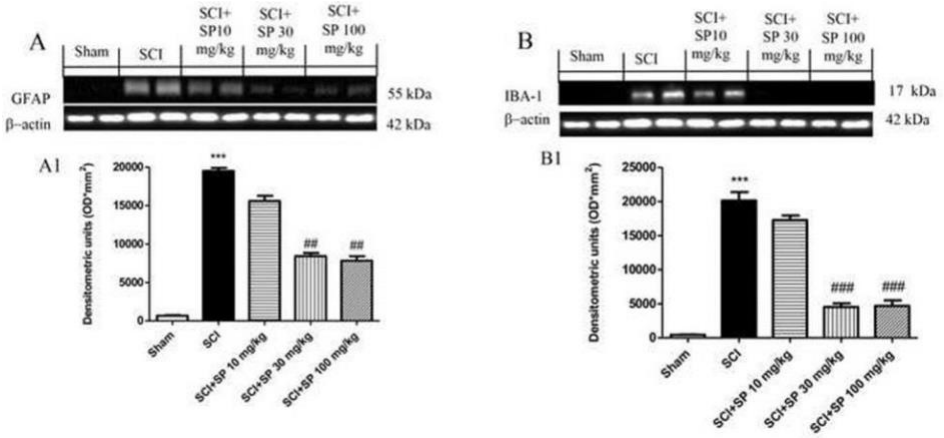

Figure 18. Effect of SP treatment on astrocyte and glial cells activation SP down-regulated the expressions of GFAP and Iba1 in the spinal cord following SCI. (A and B, respectively) Compared with the sham mice, the spinal cord trauma had increased protein expression of GFAP and Iba1. Oral treatment with SP down-regulated GFAP and Iba1 (A and B, respectively) expression levels in SCI mice but had no effect in sham mice. ***P<0.001 vs sham group; ##P < 0.01 vs SCI group; ###P < 0.001 vs SCI group.

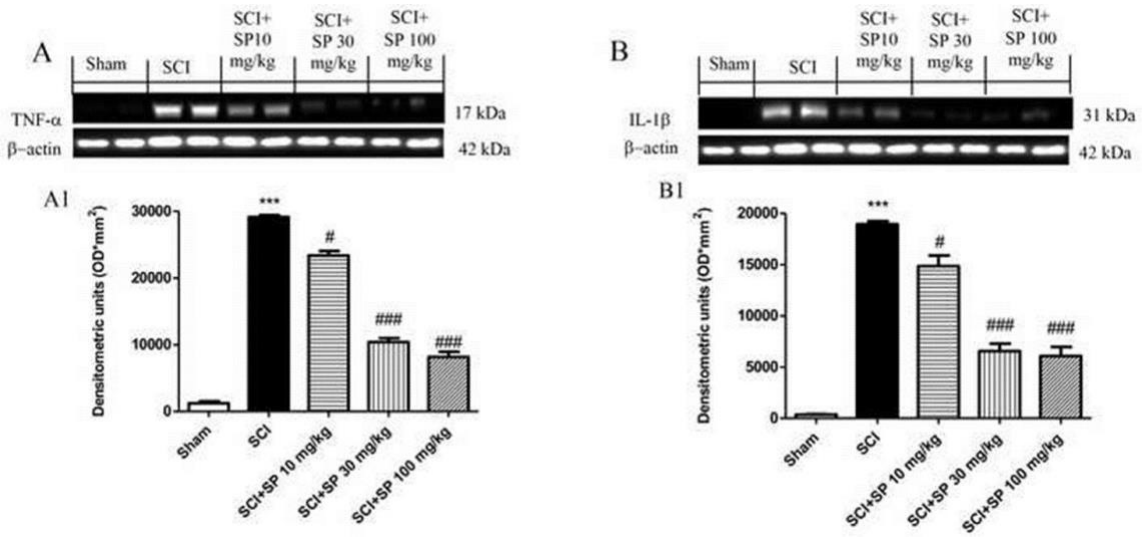

Figure 19. Effect of SP treatment on cytokines production Western blot analysis revealed a markedly an increase of expression of TNF-α and IL-1β in spinal tissues collected at 24 h after SCI compared to sham group (A and B, respectively). SP treatment at all doses significantly diminished the post-SCI expression of TNF-α and IL-1β. ***P<0.001 vs sham group; ##P < 0.01 vs SCI group; ###P < 0.001 vs SCI group.

4.6 DISCUSSION

The beneficial physiological effects of SCFAs have been well documented, which include the reduction of proinflammatory factors (Huang and Wu, 1997; Meijer et al., 2010), the enhancing of intestinal barrier function (Mariadason et al., 1997, 1999; Peng et al., 2007; Suzuki et al., 2008; Van Deun et al., 2008; Elamin et al., 2013) and the inhibition of oxidative stress (Hamer et al., 2009, 2010), in many pathologies included colon cancer (Clausen et al., 1991; Hijova and Chmelarova, 2007) in both in vitro and in in vivo studies. Propionate could readily penetrate the Blood-Brain-Barrier, in fact results of gas chromatography (GC) analysis indicated that increased concentration of propionate can be explored in hippocampus from propionate + pentylenetetrazol treated animals after oral administration of 50 mg/kg propionate (Propionate relieves pentylenetetrazol-induced seizures, consequent mitochondrial disruption, neuron necrosis and neurological deficits in mice(Cheng et al., 2019). Moreover, fifteen 0.1 nmol/mg

tissue, and the level in serum was 2.3± 0.9 mmol/L (Morland et al., 2018). SP can act as an inhibitor of HDAC. It has been reported that HDAC inhibitors modulated the activity of NF-κB in a number of different cell types including colonic epithelial cell lines and macrophages (Inan MS et al., (2000) Moreover, a study demonstrated that propionate, similarly to butyrate, may inhibit the activation of NF-κB in colon adenocarcinoma cell line (Segain et al., 2000a) (Segain et al., 2000b) (Luhrs et al., 2002) (Kannan et al. 2013), as well as the expression of pro-inflammatory factors such as TNF- and IL-1β in colon tissues. In this study, we demonstrated for the first time, that SP has neuroprotective activity modulating inflammatory process through down- regulation of NF-κB pathway, modulation of inflammatory cytokines and reduction of mediators production involved in the CNS. To evaluate the mechanism of SP to counteract inflammation in the CNS, we used an in vitro model of neuroinflammation by the stimulation of neuronal cells with Aβ 1–42. Here, the stimulation of SH-SY5Y cells with Aβ 1–42 substantially modulated NF-κB pathway, increasing NF-κB nuclear translocation and degradation of IκBα. Pre-treatment with SP at the concentration of 1 μM and 10 μM significantly prevented the activation of this pathway. This data confirmed the modulation of NF-κb by SP also at CNS level. COX-2 represents a major player in inflammatory reactions in CNS and peripheral tissues (Minghetti 2004). In fact, COX-2 expression in brain, has been associated with many inflammatory activities, playing a central role in neurodegenerative processes of several acute and chronic diseases (Bartels A et Leenders K 2010). Moreover, one of the cytotoxic mechanisms induced by proinflammatory cytokines in neuroinflammation is the activation of iNOS, which mediates the synthesis of high levels of NO, known to be toxic for cells (Wang W et al., 2015). Here, we demonstrated that the treatment with SP, especially at the concentration of 10 μM, downregulated iNOS and COX-2 expressions as well as the production of NO. Once the protective mechanism of action of SP has been established, we

wanted to investigate the protective effects of SP in an in vivo model of neuroinflammation induced by trauma in the spinal cord. It has been shown in several studies that NF-kB pathway plays a key role in the pathophysiology of SCI (Liu T et al., 2017). Different studies demonstrated a peak of NF-κB binding activity starting from the 1st day postinjury. Moreover, several studies demonstrated that after trauma, included SCI, neuroinflammatory cells expressed pro-inflammatory markers such as iNOS and COX-2 (Impellizzeri 2017). In this study, we demonstrated that SP at the dose of 100 mg/kg modulated COX-2 and iNOS expression, significantly reducing both mediators following spinal cord trauma. After trauma in CNS, reactive astrocytes and microglia contributes to the neuroinflammation within the injured tissue, denoted by increased GFAP and Iba1 expressions respectively. It has been shown that SCI to may trigger microglial and astrocytic activation, increase inflammatory cytokine generation and release as IL1-β and TNF-α and finally result in permanent neuronal injury (Liu X et al., 2018). Our results clearly demonstrate that spinal activation of astrocytes, microglia and relative cytokines were significantly attenuated in SP 30 mg/kg and 100 mg/kg treated mice compared to SCI. Microglia and macrophages display a particularly robust inflammatory response, and participate in cross-talk with probably from direct influence on glial and neuronal cells, but also on peripheral cells of the immunologic system. Further studies on target cells of sodium propionate are needed to better describe its neuroprotective action. All this data were confirmed by histological staining that demonstrated, following spinal cord trauma, a loss of tissue architecture characterized by edema and neutrophils infiltration; however SP treatment showed an important brain-protective activity restoring the traumatic tissue damage.

4.7 Conclusions

In conclusion, these results clearly demonstrated that SP exhibit important activities like reducing the pro-inflammatory molecules, decreasing the activation of glial cells and prevented tissue damage denoting its protective effect also in the CNS. Moreover, we confirmed that the activity of SP, also in the CNS, is associated with its ability to down-regulate NF-κB pathway. Therefore, SP could represent a promising therapeutic target for the treatment of more CNS pathologies.

PART II

5. CHAPTER FIVE: Protein sorting, trafficking and regulation in CNS

5.1 Endosomal protein sorting and trafficking

The compartments that comprise the endosomal system perform the essential role of sorting proteins that have been delivered from both the biosynthetic route and endocytosed from the plasma membrane. Sorting of membrane proteins occurs through a complex interplay between sorting motifs intrinsic in the membrane proteins, and the sorting machinery that recognizes the motifs to direct the membrane protein to different purpose (Seaman 2008). Sorting decisions for most proteins need to be made only once so they can exert their biological activity and then they come to the final permanent residence. A protein that has been endocytosed from the plasma membrane or sorted at the trans-Golgi network (TGN) and delivered to an endosome will be directed to the lysosomes, for example, mostly for degradation (Slagsvold 2006).

The endosomal system is a dynamic and solid "highway" machinery that transfers cargoes (as receptors and their bound ligands) between distinct vesicular compartments (200 – 500 nm in diameter) often known as multivesicular bodies (MVBs). This process is recognized as an endosomal maturation process in which through several mechanisms, early endosomes (EE) mature to late endosomes (LE) (Elkin 2016). The EE is the first endocytic structure that accepts the cargo for internalization. At the EE, rapid sorting occurs, and cargo is transported to LE for degradation, or they are recycled back to the plasma membrane. Two recycling routes have been classified: 'fast recycling pathway' and 'slow recycling pathway'. For fast recycling, vesicles containing cargo come out from the EE membrane and undergo transport directly to the plasma

membrane (Maxfield and McGraw, 2004). By contrast, slow recycling is considered a process trough cargo is transported to a perinuclear-localized tubulo-vesicular RE and from there, vesicles are released to the plasma membrane as common (Naslavsky 2018). The described sorting processes trough endosomal system require conserved machinery that is closely linked to a range of proteins, complexes and factors that entirely mediate the process (Gautreau 2014). SNARE proteins, a superfamily of small and mostly membrane-associated proteins, play a key role in intracellular membrane sorting mediating fusion of EEs and Les, (Antonin 2000), Rabs such as Rab5 and Rab 7 are involved in the endosome maturation process. Moreover, the retromer has recently been shown to be the receptor of the WASH complex at the surface of endosomes. Indeed, WASH complex tags a restricted domain of endosome, which corresponds to a location where endosomal tubules are elongated, demonstrating a key role of WASH complex into endosomal system regulation pathway. Endosomal recruitment of the WASH complex: active sequences and mutations impairing interaction with the retromer (Helfer 2013). Moreover, the WASH complex associates with the retromer via an interaction between the unstructured tail of FAM21 and the C-terminus of VPS35, and this interaction mediates the endosome-to-TGN retrieval of select cargo such as the cation-independent mannose 6-phosphate receptor (CI-MPR) (Seaman 2013).

5.1.1 The retromer complex: structure and function

Retromer is an evolutionary conserved multi-protein complex associated with the cytosolic side of endosomes, where exerts its functions such as receptors, transporters, adhesion molecules, and other proteins transpport to the trans-Golgi network (TGN) and the plasma membrane through sorting into tubular-vesicular carrier (Seaman2004). It is constituted by five proteins organized

into two sub complexes; the conserved heterotrimer formed by VPS gene products Vps26, Vps35, and Vps29 mediates cargo recognition, and will be referred to here as cargo-selective complex (CSC), whereas a dimer of phosphoinositide (PtdIns)-binding sorting nexins (SNX) determines retromer localization and deforms the membrane to ensure sorting. The SNX dimer are composed of SNX1 or SNX2, in conjunction with SNX5 or SNX6, although other SNXs can be involved. VPS35 represent the main cargo-binding subunit and together with VPS29 and VPS26 forms the cargo-loading core complex. To date, no defined structure exists for the VPS35 subunit; however, a C-terminal fragment encompassing residues 476–780 of the protein (from 796 residues total) has been crystallized in complex with the small VPS29. Indeed, X-ray crystallography identified VPS29 as a small subunit composed by an active site containing two metal ions (usually Zn2+ and Fe2+) and two other sites of VPS35 and SNX interactions (Collins 2005). In addition, VPS26, the medium retromer core component, possess a similar structure to arrestin proteins family and have an extended C-terminal tail and an unusual polar core between the N-terminal and the C-terminal domains, by which it interacts with other proteins (Collins 2008). Retromer was first described in yeast in 1998 (Seaman 1998) as an essential complex for maintaining normal cell homeostasis and function. Following from the initial studies demonstrating a function in hydrolase receptor transport, evidence is now accumulating that retromer plays a critical role in numerous other endosomal trafficking processes (Marcel Vergés 2016). These include the transcytosis of the polymeric immunoglobulin receptor (pIgR) and mostly, the sorting of receptors for amyloid precursor proteins (APPs) involved in Alzheimer's disease and neurodegenretive disease associated with protein accumulation in CNS (Li 2020). Other various functions have been suggested for the retromer complex. The well-established role of retromer complex came from a several studies of the retromer in regulating the retrograde transport from endosome to TGN.

Besides, it has recently emerged that retromer also plays a central role in endosome-to-plasma membrane recycling (Temkin 2011) influences Wnt secretion by recycling Wntless from endosomes to the trans-Golgi network (TGN) (Belenkaya 2008).

5.1.2 Retromer complex and neurodegenerative disease

Retromer is consider a 'master conductor' of endosomal sorting and trafficking dysfunction. Since it contributes to the presynaptic release of neurotransmitters and regulates receptor density in the postsynaptic membrane, a process that is crucial for neuronal plasticity, new evidences have been associated retromer defects to several neurodegenerative diseases (Carroll 2011). Deficiency or mutations in one of retromer complex components could be directly lead into the pathogenesis of neurodegenrative conditions, implicating the regulation of endosomal trafficking and protein recycling (Vagnozzi 2019).

5.1.2.1 Alzheimer's disease

Cataldo and colleagues have given essential evidence of the presence of abnormally enlarged endosomes during the earliest stages of Alzheimer's disease. They also found that the number of neuronal early endosomes was increased further, leading to an impaired endosomal system activity (Cataldo 2000). In addition, protein levels of cathepsins D and B different forms (pro and mature) were prominent in enlarged endosomes, according with increases of cation-dependent mannose 6-phosphate receptors, which mediates the transport of acid hydrolases to endosomes (Cataldo 2012). Moreover, previous studies have shown that retromer-related defects reduced trafficking of APP out of endosomes and caused an elevation in Aβ levels in vivo (Muhammad 2008). VPS35 silencing using siRNA strategy raises Aβ production, which resulted in a longer APP retention in early endosomes (Zhang 2018). Taken together, these fundings identify that endosomal

dysfunctions are a causes of a pathogenic mechanism, and validate endosomal transport as a "cell biological" target for drug discovery.

5.1.2.2 Parkinson's disease

Parkinson's disease (PD) is the second-most common neurodegenerative disorder affecting over 1% of the population older than 60. It is characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta and other monoaminergic cell groups in the brainstem. Clinically, this degeneration leads to bradykinesia, resting tremor, muscular rigidity, and postural instability as well as nonmotor symptoms. On a cellular level, PD is characterized by the subcellular formation of large, perinuclear accumulations of aggregated proteins called Lewy bodies (LBs) often abundant in α-synuclein deposits, or containing other proteins and lipids. (Auluck 2002). Although most PD cases are sporadic and hereditary, several studies demonstrated a point mutation within the VPS35 (p. D620N) and the VPS26 (p.K93E) that underling retromer complex defects and the late-onset of PD (Vilarino-Guell 2011). Therefore, other evidences have shown that retromer malfunction reduces the iterative transport of CI-M6PR, in the absence of retromer, the efficiency of CI-MPR retrieval is perturbed and an increase in endosomal localization of CI-MPR is observed, thus, leading to a perturbation of degradation route (Arighi 2004). Therefore, one idea is that retromer malfunction causes a defect on cathepsin D processing, leading to a decreased level of its activated forms in lysosomes (Cui 2017). Together, these and further studies suggest that target the retromer-dependent transport, is necessary to clarify this role in PD as well as a therapeutic strategy to contrast the pathology.

5.1.3 The degradation pathways

Proteolysis (or protein degradation) is mediated by proteases, intracellular enzyme and others regulator factors, possessing key roles in different pathways. The rapid turnover of proteins is essential to allow their correct levels in response to exterior stimuli. In addition, damaged or

unnecessary proteins are targeted and rapidly degraded by two major pathways: autophagy-lysosome system (ALS) and the ubiquitin-proteasome system (UPS).

5.1.3.1 Autophagy-lysosome system (ALS)

Autophagy is an evolutionarily conserved degradation mechanism for undesired components of the cytosol and organelles. Under physiological conditions, autophagy occurs maintaining cellular homeostatic functions such as protein turnover. When cells need to produce nutrients or energy in response at starvation, higher bioenergetics requests, during oxidative stress, infection, or protein aggregate accumulation, autophagy could be up regulated (Levine 2008). The autophagy process is distinguished in three different types: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. Macro-autophagy transport cytoplasmic material to the lysosome through a double membrane vesicle, named autophagosome that fuses with the lysosome to form an autolysosome. In micro-autophagy, cytoplasmatic cargo is directly delivered to the lysosome by invagination of itself. In chaperone-mediated autophagy (CMA), proteins are transported across the lysosomal membrane by chaperone proteins cooperation and recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A) (Glick 2010). Among these different types, macroautophagy is the most studied and it refers to the predicted autophagy process. Autophagy consists of few steps: induction, nucleation, elongation, fusion and degradation (Parzych 2014).

-In mammalian cells, the ATG1, derived from ULK1 kinase family, is necessary for the induction of autophagy.

-In the nucleation phase, ATG14 (ATG14-BECN1-PIK3C3-PIK3R4) complex is required and also regulated by antiapoptotic proteins such as Bcl-2 (Liang 1998).

- Atg12–Atg5-Atg16 complex formation during the elongation phase, allows the phagophore and autophagosome membrane fusion and expansion. Here, ATG9, LC3, and its lipidation process from LC31 to LC32 seem to be both necessary for phagophore elongation and autophagosome closure (Noda 1998).

-Once the autophagosome is completed, it fuses with a lysosome, forming an autolysosome, responsible for cargo degradation. This is a crucial step in autophagy: degradative enzymes and hydrolases runs to digest unnecessary cytoplasmic content.

Several regulators of macroautophagy have been identified: SQSTM1 is important an adaptor protein that interacts with LC3-II to target aggregates for degradation, and Ca2+ increased concentrations results to induce autophagy (Høyer-Hansen 2007). Lately, understanding autophagy modulation is growing up because it may have a key role in the treatment of major diseases including cancer and neurodegenerative disease (Egil 2011). A recent study established that in multiple tumor types, ATG5 mutations prevent ATG12 conjugation by disrupting ATG5-ATG16L1 interactions, which inhibits autophagy pathway (Wible 2019). Related with cancers, the roles of autophagy in neurodegenerative diseases appears to be poor understood even as in PD patients, altered expression of ATG5, ATG7, ATG12, and LC3B genes may contribute to PD pathogenesis (Li 2017).

5.1.3.2 The ubiquitin-proteosome system (UPS)

The ubiquitin–proteasome system is also essential for the degradation of proteins. The main players of UPS is the proteasomes, organelles responsible for degradation of short-lived proteins and soluble misfolded proteins. Most proteins are targeted for proteasomal degradation after being covalently added with ubiquitin in a process kwon as ubiqutination. Three types of enzymes are

required in this process: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) hydrolyses add the ubiquitin to the substrate (Rubinsztein 2006). The proteasome composition is fundamental for explaining of its activity: the 26S proteasome subunit is an ATP-dependent protease complex, the 20S subunit is a regulatory complex, and the 19S subunit is the proteosome cap responsible for substrate binding (Kocaturk 2018). Over the past years, few findings suggest that dysfunction of the UPS can directly causes tissue damage. In kidneys, the UPS affects TGFβ signaling which results in the development of fibrosis in nephropathy (Liu 2008), in colon, evidence accumulates that the proteasome activity is elevated in tumor cells through enhanced activation of Nrf2 pathway (Arlt 2009). Moreover, intracellular Aβ accumulation and impaired proteasome function are common features in AD and genetic mutation of PARK2 are correlated with familial PD pathogenesis (Zheng 2016).

Aim of Thesis PART II

The key aim of this thesis was to identify whether retromer complex impairments, focusing on VPS35 down-regulation, leads to compromise the protein degradation pathways in Neuro-2 A neuroblastoma cells stably expressing the Swedish mutant APP (N2A APP Swe).

6. CHAPTER SIX: VPS35 down regulation influences degradation pathways in murine neuroblastoma APP Swedish cells

6.1 ABSTRACT

The vacuolar protein sorting 35 (VPS35) is the main component of the retromer recognition core complex which regulates intracellular protein sorting and trafficking. Downregulation in VPS35 has been linked to endosomal deregulation and defects in protein degradation pathways. Here we showed that the genetic knockdown of VPS35 leads to stimulate autophagy pathway. We found by LC3B and SQSTM1/p62 immunoblotting and immunofluorescence evaluation that the autophagy flux was altered following VPS35 silencing. Moreover, down-regulation of VPS35 results in an accumulation of acidic structures into neuronal cells providing that VPS35 deficiency leads to cause alteration in autophagy as well as in the ubiquitin-proteasome pathway through the accumulation of poly-ubiquitinated proteins. Taken together, our data recognize VPS35 as a novel critical regulator in both protein degradation pathways.

6.2 Introduction

Neurodegenerative diseases are among the most devastating of human illnesses. Multiple genetic loci form the basis of risk factors for development of neurodegenerative diseases (Tsuji S, 2010). Mostly, the protein products of these loci are involved in important cell biological pathways, which have been implicated in the development of the neurodegenerative disorders. Protein sorting and trafficking mechanisms play a key role in maintaining cellular and vesicular homeostasis. One pathway widely involved in neurodegeneration is protein trafficking and dynamics through the endocytic pathway. The endocytic pathway, composed of various dynamic membrane-enclosed structures such as early endosomes, recycling endosomes, and late endosomes, drives

macromolecules and transmembrane proteins, rightly targeted, at the plasma membrane destined for degradation (Lamb, 2013). In recent years, the endosomal network, in particular, any changes in this system, has attracted interest for the understanding of neurodegenerative mechanisms, which is predominant in CNS diseases such as Alzheimer's disease (AD) (O'Brien 2011) and Parkinson-'s disease (Benjamin 2014). The main regulator in endosomal protein sorting is the retromer complex system, an evolutionarily conserved multimeric system first discovered in yeasts that operates in retrograde transport of cargo from the endosome to the trans-Golgi network (TGN) (Seaman, 2012). The system complex is a hetero-pentameric protein complex, comprised of a sorting nexin (SNX) and a cargo-recognition core composed by the vacuolar protein sorting 35 (Vps35)/ Vps26/ Vps29 trimer (Haft 2000). Besides, the cargo recognition core collaborates with WASH (Harbur 2010) and retriever complexes and with associated receptor and proteins include cation-independent mannose 6-phosphate receptor (CIMPR), Vps10-member sortilin, sortilin-related receptor 1 (SORL1; also known as SORLA), glutamate receptors, and sorting nexin-Bar (Snx-BAR) proteins family (Teasdale 2012). Deficiency or mutations in one or more protein components of the retromer complex leads to an increase in the accumulation of protein aggregates as well as in the development of PD and AD (Vagnozzi 2019). The endosome-to-TGN via of retromer complex might also be directly required from two of the most important proteolytic degradation and recycling pathways: autophagy-lysosome system (ALS) and ubiquitin-proteosome system (UPS). (Young 2006). The UPS is responsible for the degradation of most cytosolic and nuclear proteins, including short- and long-lived proteins as well as aberrant proteins (Collins 2017), whereas the ALS is specialized in protein aggregates and damaged organelles degradation. A common feature of the two processes is the attachment of polyubiquitin conjugates to specific cargo proteins that initiate these degradative processes (Bustamante 2018). Recent

studies have shown that defects in the ALD and the UPS pathways are likely to precede the formation of protein aggregates such as amyloid-beta plaques or neurofibrillary tangles (NFT) and accumulation of defective organelles (Correia 2015) (Dantuma 2014). Several studies have shown that retromer dysfunction is correlated to defects in protein sorting and trafficking. Thus, deficiency in the retromer function resulting from down-regulation of VPS35 has been reported in AD patients and aged DS individuals (Curtis 2020). Interestingly, few studies have shown a tie between VPS35 deficit and impairments in ALS and UPS pathways. In this study, we show that VPS35 genetic reduction results in a decreased expression level of retromer core components VPS26 and VPS29 in neuro-2A neuroblastoma (N2A) cells stably expressing human APP carrying the K670 N, M671 L Swedish mutation (N2A APPSwe). Moreover, VPS35 deficiency displayed aletration of autophagy as demonstrated by elevation of LC3B2 protein levels, intracellular accumulation of autophagosomes, and decreased SQSTM1/p62. Moreover, VPS35 silencing caused a dysfunction in both the lysosomal and proteasome degradation pathways, culminating in altered substrate degradation.

6.3 Material and Methods

6.3.1 Cell culture and transfection

N2A APPSwe cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL streptomycin (Mediatech, Herdon, VA) and 100 mg/mL Gentamicin (Invitrogen, Carlsbad, CA) at 37 °C in the presence of 5% CO2. For knockdown experiments, cells were cultured to 70% confluence in six-well plates and then transfected. Briefly, a mixture of optiMEM with 100 nM control siRNA (Thermo Fisher/Ambion, Waltham, MA) was prepared and incubated at RT for 5 minutes. Another mixture using optiMEM with 100 nM of VPS35 siRNA

was prepared and incubated for 5 minutes. Both of the solutions were mixed together and incubated for approximately 15 minutes. The total volume was added to one well of a 6-well plate, already containing 1 ml of optiMEM. After 6 h of incubation, fresh medium was replaced. After 72 h treatment, supernatants were collected and cells were harvested in lytic buffer for biochemistry analyses. Briefly, cell lysates were centrifuged at 10,000 × g for 10 minutes at 4 °C and the supernatant were used.

6.3.2 Cells treatments

For Bafilomycin A1 (BafA1) exposure, after 68h of transfection, cells were exposed to BafA1 (20-50 and 100 nM) for 4 h. Then, cells were collected as described above. For Borteozomib (BTZ) exposure, after 68h of transfection, cells were exposed to BTZ (10 and 100 μM) for 4 h. Then, cells were collected as described above.

6.3.3 Western blot analysis

RIPA extracts from cells were used for Western Blot analyses as previously described (Di Meco et al., 2017; Lauretti et al., 2017). Briefly, samples were electrophoresed on 10% Bis-Tris gels or 3–8% Tris-acetate gel (Bio-Rad, Richmond, CA, USA), transferred onto nitrocellulose membranes (Bio-Rad), and then incubated overnight at 4˚C with the appropriate primary antibodies LC3B, SQSTM1/p62, ATG9, ATG7,ATG5, VPS35, VPS26, VPS29, Ubiquitin. After three washings with T-TBS (pH 7.4), membranes were incubated with IRDye 800CW-labeled secondary antibodies (LI-COR Bioscience, Lincoln, NE, USA) at room temperature for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience, Lincoln, Nebraska). GAPDH was always used as internal loading control.

6.3.4 Immunofluorescence staining

The N2A APPSwe cells were cultured on glass coverslips, and treated with control siRNA or VPS35 siRNA, as early described. After PBS 1X rinsing, cells were fixed with 4%

paraformaldehyde for 20 min, and then incubated with 0.1% Triton X-100 for 30 min. The cells were treated with 10% donkey serum/PBS for 30 min, and then incubated with LC3B antibody (Cell signaling Technology 1:500), SQSTM-1/p62 (Abcam 1:250) overnight at 4°C. After rinsing with PBS 1X, cells were incubated with the Alex Fluro 488/568-conjugated secondary antibodies (1:300, Abcam) for 30 min. The nucleus was stained with DAPI (1:1000). Coverslips were mounted using Vectashield mounting medium (Vector Laboratories) and images were taken by using Olympus BX60 fluorescent microscope (Olympus, Center Valley, PA) with 100× objectives.

6.3.5 LysoTracker Red staining

The N2A APPSwe cells were cultured on glass coverslips, and treated with control siRNA or VPS35 siRNA, as early described. After PBS 1X rinsing, cells were incubated with 75 nM LysoTracker red in 10% Donkey serum for 90 min at RT followed by washing with fresh PBS 1X. Subsequently, cells were fixed as previously described. Coverslips were mounted and all images were taken by using Olympus BX60 fluorescent microscope (Olympus, Center Valley, PA) with 100× objectives.

6.4 Statistical analysis

All the data are expressed as mean ± standard error of the mean and represented with individual data values. Comparisons between two groups were made using an unpaired two-tailed t-test. Comparisons between more than two groups were made using a one-way ANOVA with Bonferoni's multiple comparisons test. The p-values for each comparison are listed in each figure legend with $p < 0.05$ considered statistically significant. All statistical tests were performed using GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA, USA).

6.5 Results

6.5.1 VPS35 gene silencing down-regulates retromer core components

VPS35 is important for regulating endosome-to-Golgi and endosome-to-cell surface retrieval of membrane proteins. To determine whether VPS35 silencing also regulates the levels of other retromer recognition core components, we down-regulated VPS35 and investigated its effect on VPS26 and VPS29 expression in N2A APPSwe. We confirmed the efficiency of the VPS35 down-regulation system using western blot since found a significant ~50% decrease in its protein levels, which was associated with a significant decrease in VPS26 levels, and VPS29 (Figure 20)

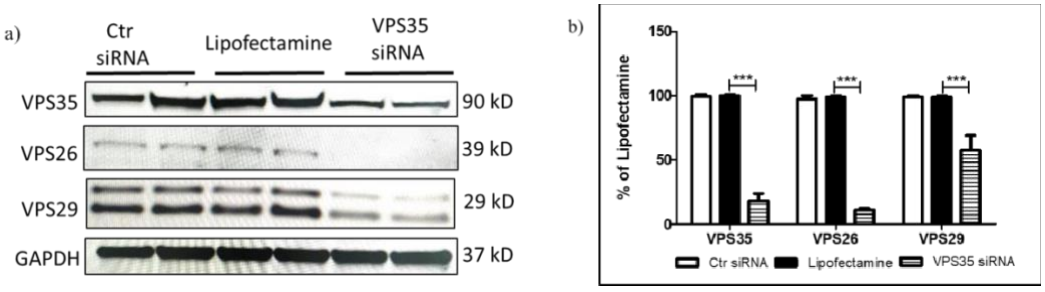

Figure 20. a) VPS35 silencing decrease expression of retromer components. N2A APPSwe cells were transfected with VPS35 siRNA or controls for 72 h, then supernatants and cell lysates were harvested and collected. a Representative western blot analysis for retromer core, VPS35, VPS26, and VPS29. b) Densitometric analyses of the immunoreactivity to the antibodies shown in panel a (***$p < 0.001$ vs Lipofectamine) N=3

6.5.2 Autophagy-related proteins levels are unchanged following VPS35 silencing

After determining that retromer recognition core was down regulated following genetic manipulation of VPS35 in vitro, we investigated whether VPS35 silencing influences autophagy process. The autophagy-related proteins (ATGs) are essentially involved in the canonical autophagosome formation (Mizushima 2011). Under our experimental conditions, We detected that the steady state protein levels of ATG5, ATG9 and ATG7 were unchanged-among Ctr, siRNA

and Lipofectamine groups since we observed no significant changes in ATGs proteins in lysate from cells down expressing VPS35 (Figure 21).

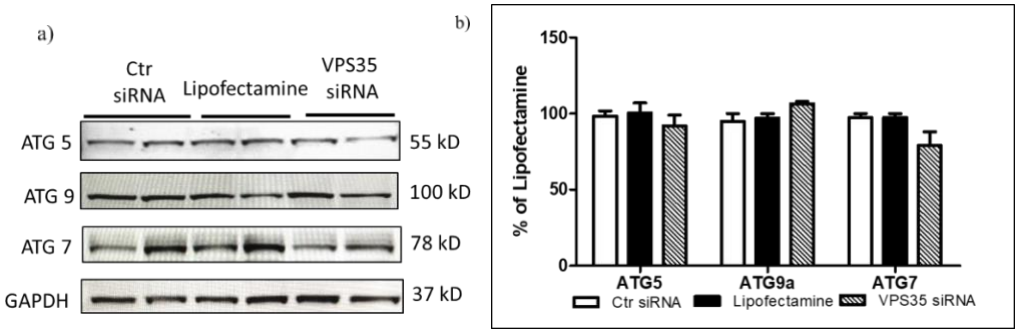

Figure 21. ATGs evaluation following VPS35 silencing. a) Representative western blot analysis for ATGs proteins ATG5, ATG9 and ATG7. b) Densitometric analyses of the immunoreactivity to the antibodies shown in panel a (* VPS35 siRNA vs Lipofectamine) N=3

6.5.3 Down-regulation of VPS35 modifies the autophagy pathway

Given that VPS35 down regulation did not result in any changes in ATGs proteins levels, we next investigated the contribution of LC3B and SQSTM1/p62, which are measures of autophagic flux. During autophagy, cytoplasmic LC31 protein is lipidated in LC3B2 and recruited to the autophagosomal membranes. SQSTM1/p62, which binds to LC3, is also used to monitor autophagic flux. (Runwal 2019). We found that VPS35 silencing in N2A APPSwe did not influenced LC31 protein levels; by contrast, as measured by immunoreactivity, LC3B2 and LC3B2/1 ratio expressions were significantly increased when compared with control group. Interestingly, N2A APPSwe VPS35 siRNA treated cells showed a reduction of SQSTM1/p62 as observed by Western blot analysis (Figure 22).

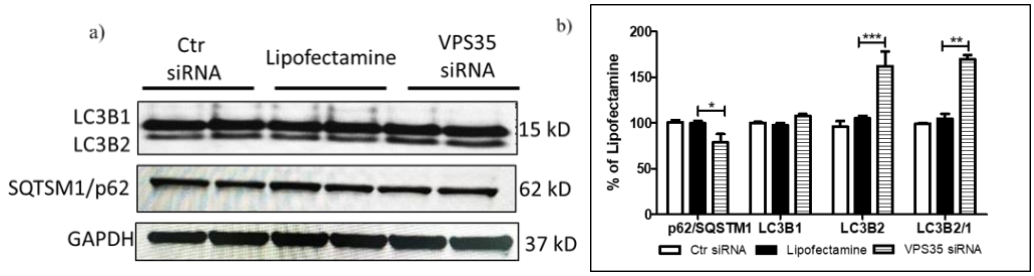

Figure 22. Representative western blot evaluation of autophagy following VPS35 silencing. (a) Representative western blot showing no changes in LC3B1 levels in cells upon VPS35 down regulation. Significant increase showing in LC3B2 with respect to levels of GAPDH upon VPS35 silencing. (***p < 0.001 vs Lipofectamine;**p < 0.01 vs Lipofectamine, *p < 0.05 vs Lipofectamine). **N=3**

6.5.4 VPS35 silencing influences LC3B and SQSTM1/p62 fluorescence immunoreactivity

To confirm our Western blot analysis observations, we performed immunocytochemistry to determine mean fluorescence intensity of LC3B and SQSTM1/p62 after VPS35 silencing. Firstly, we aimed to determine if VPS35 was down regulated in our cell line following VPS35 siRNA treatment. Next, we stained N2A APPSwe cells for LC3B and SQSTM1/p62. Compared to control cells, VPS35 silenced cells showed a significant increase in mean fluorescence intensity for LC3B (Figure 23) while there was change in intensity for SQSTM1/p62 (Figure 24).

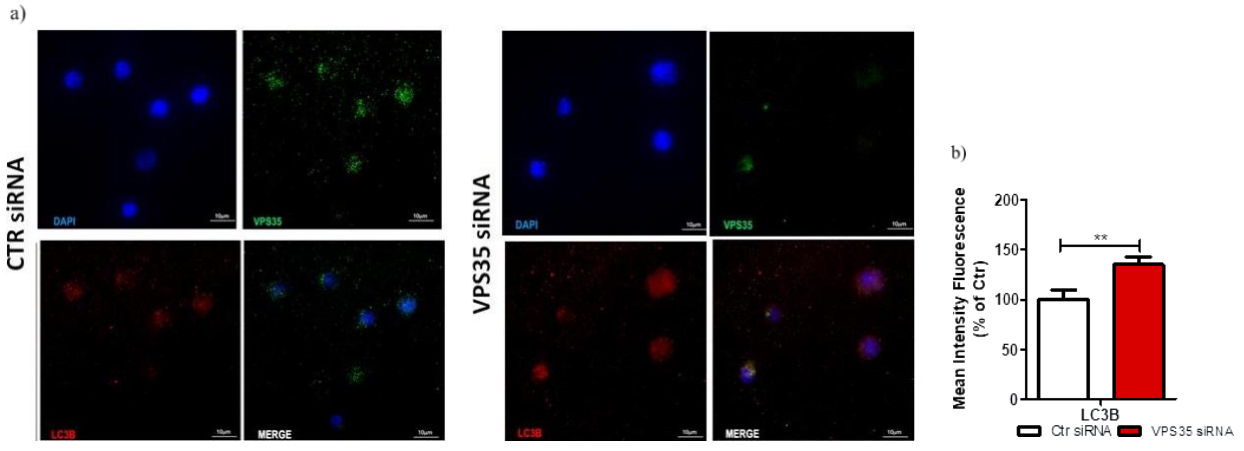

Figure 23. LC3B immunofluorescence following VPS35 silencing. a Representative microscopy images of VPS35 silenced and control cells for LC3B (TXred), VPS35 (FITCH), nuclear stain DAPI (blue); (Scale bar = 10 µm). b Mean fluorescence intensity. (** p < 0.01 vs Ctr siRNA). N=3

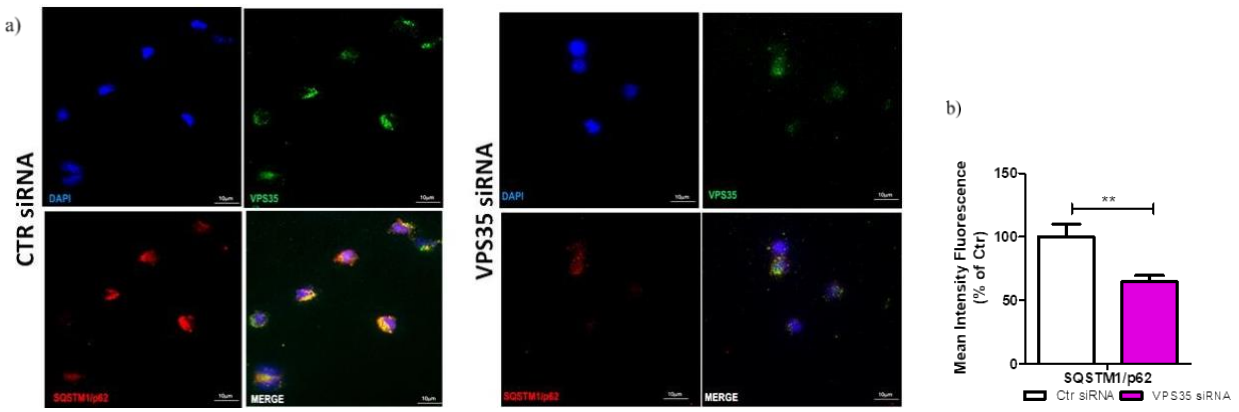

Figure 24. SQSTM1/p62 immunofluorescence following VPS35 silencing. a Representative microscopy images of VPS35 silenced and control cells for SQSTM1/p62 (TXred), VPS35 (FITCH), nuclear stain DAPI (blue); (Scale bar = 10 µm). b) Mean fluorescence intensity. (** $p < 0.01$ vs Ctr siRNA). N=3

6.5.5 Bafilomycin effects on the autophagy pathway in N2A APP Swedish cells

To test whether the above observation could be replicated under conditions of acute autophagy blockade and retromer complex defect, we-incubated N2A APPSwe cells with BafA1 (an inhibitor of autophagosome and lysosome fusion) (Redmann 2017). First, we determined what dose of BafA1 results in a significant level of autophagy inhibition, incubating N2A APPSwe cells to increasing drug concentrations. Significant increases in LC3B2 and LC3B2/1 ratio were observed at 100 nM. Once we established the concentration of BafA1 which was effective, we incubated them with BafA1 (Figure 25a). We observed no significant changes in LC3B1, but a statistically increase in LC3B2 and LC3B2/1 ratio for BafA1 exposure. The autophagy substrate SQSTM1/p62 and VPS35 were also measured and showed decreased levels for BafA1 exposure (Figure 25b).

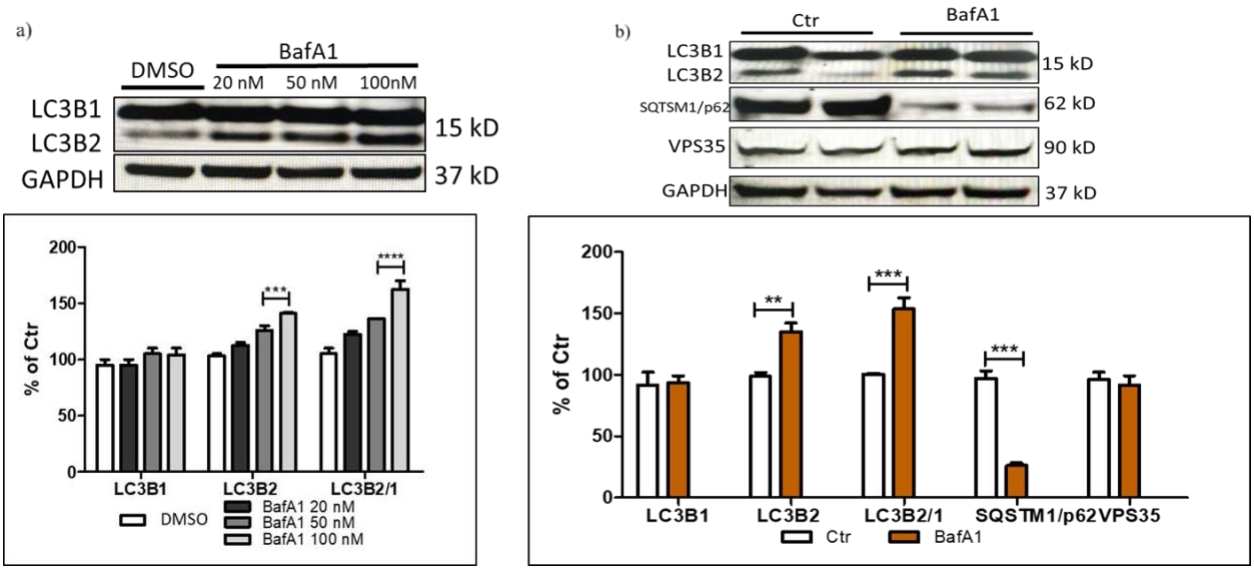

Figure 25. Representative western blot evaluation of BafA1 exposure. (a) Representative western blot showing changes in LC3B2 and LC3B2/1 ratio following BafA1 stimulation at different concentration (20-50 and 100 nM). b) BafA1 stimulation significantly increase LC3B2 protein level and LC3B2/1 ratio, while, decrease SQSTM1/p62. Expression of VPS35 is unmodified following BafA1 exposure. (***$p < 0.001$ vs Lipofectamine;**$p < 0.01$ vs Lipofectamine). N=3

6.5.6 LC3B positive autophasomes are prominent following VPS35 silencing and BafA1 stimulation

BafA1 suppresses the autophagy flux by blocking the fusion of autophagosomes with lysosomes (Yamamoto 1998). As showed in Fig above, after treatment of bafilomycin A1, the LC3B fluorescence intensity was significantly increased and indicated that autophagosomes were accumulated within the cells. To confirm this result, the fluorescence microscopy of LC3B was further applied to estimate the autophagosomes accumulation. As shown in Figure 26, BafA1 markedly increased the mean intensity fluoresce for LC3B as well as increase the number of puncta cell in VPS35 down regulated cells.

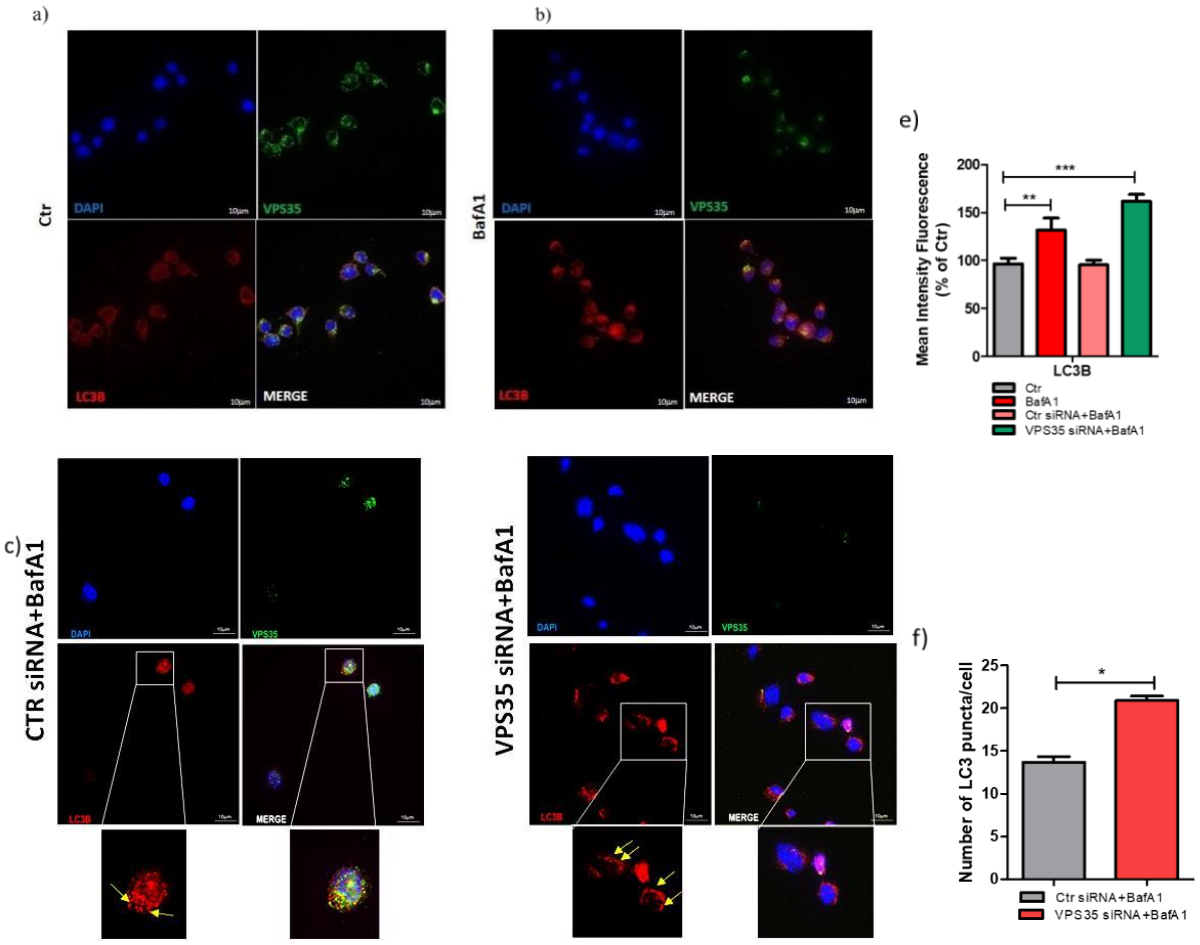

Figure 26. LC3B immunofluorescence following VPS35 silencing and BafA1 incubation. a Representative microscopy images of a) control cells b) Baf1 treated cells c) Ctr siRNA treated cells d) VPS35 silenced cells) for LC3B (TXred), VPS35 (FITCH), nuclear stain DAPI (blue); (Scale bar = 10 µm). e) Mean fluorescence intensity. f) Quantification of the LC3 puncta average number (* $p < 0.05$ vs Ctr siRNA) (** $p < 0.01$ vs Ctr) (*** $p < 0.001$ vs Ctr). N=3

6.5.7 BafA1 exposure attenuates the expression of SQSTM-1/p62 following VPS35 silencing

As BafA1 exposure following VPS35 silencing increased LC3B positive structures, we next investigated whither under this condition, SQSTM/p62 could be influenced. Our results showed that BafA1 exposure attenuates the mean intensity fluorescence for SQSTM1/p62 in VPS35 down regulating cells (Figure 27).

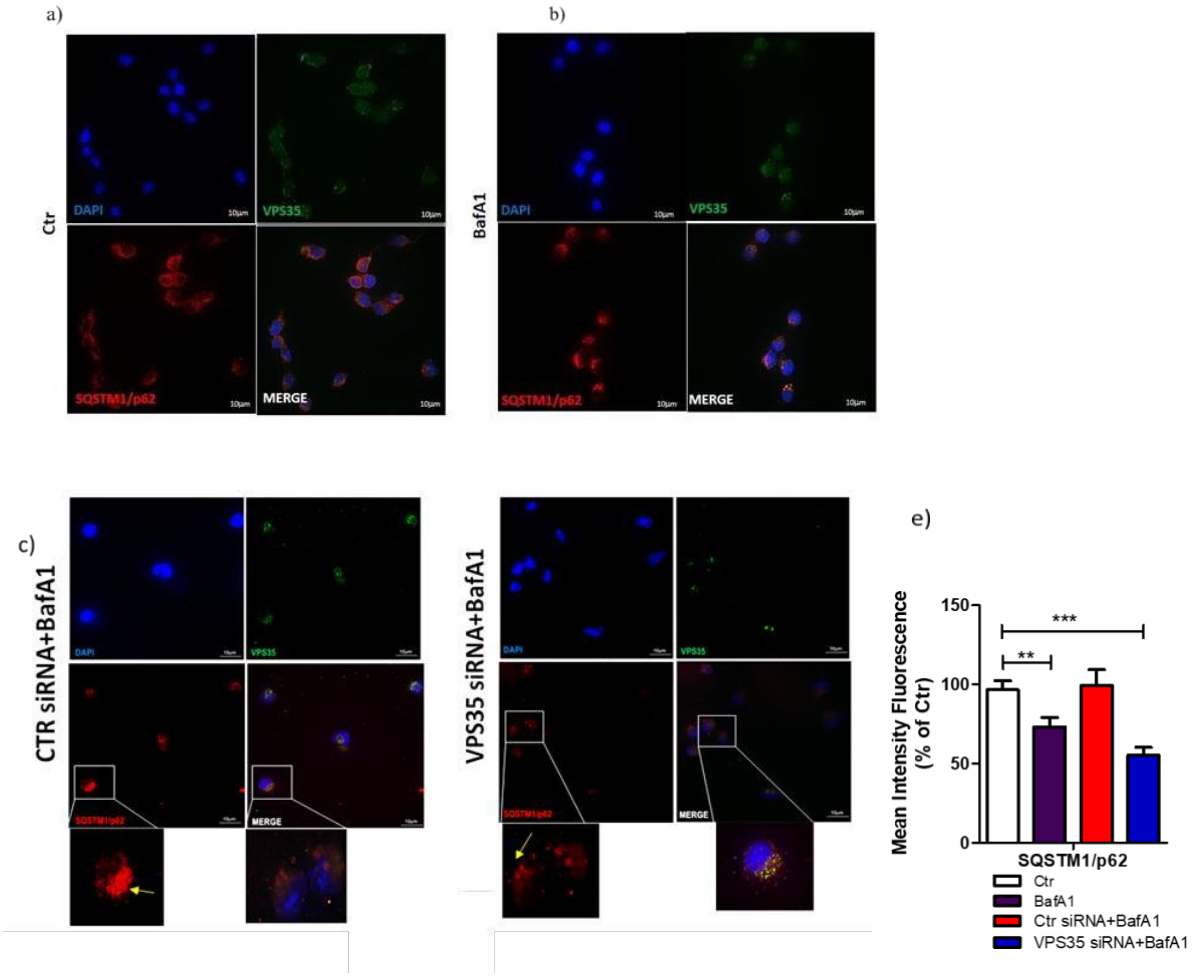

Figure 27. SQSTM1/p62 immunofluorescence following VPS35 silencing and BafA1 incubation. a Representative microscopy images of a) control cells b) BafA1 treated cells c) Ctr siRNA treated cells d) VPS35 silenced cells for SQSTm1/p62 (TXred), VPS35 (FITCH), nuclear stain DAPI (blue); (Scale bar = 10 µm). e) Mean fluorescence intensity. (*** $p < 0.001$ vs Ctr). N=3

6.5.8 VPS35 downregulation leads to an increase in acidic vescicles

The impairment of autophagic flux in N2A APPSwe cells following VPS35 silencing could reflect in accumulation or defects in acidic vesicles formation such as lysosomes, autophagosomes, and endosomes. LysoTracker Red, a deep red-fluorescent dye, was used to detect abnormalities in vesicular pH and to examine the efficiency of autophagosome/lysosome fusion in live cells (Yu

2017). As shown in Fig 28a, positive acidic structures for LysoTracker Red dye were found in control cells. However, a remarkable increase and accumulation in positive acidic structures was detected in N2A APPSwe cells following VPS35 siRNA treatment (Figure 28b). Thus, the intensity of LysoTracker Red dye can evidence that acidic vesicles were accumulated non-degraded material (Figure 28c).

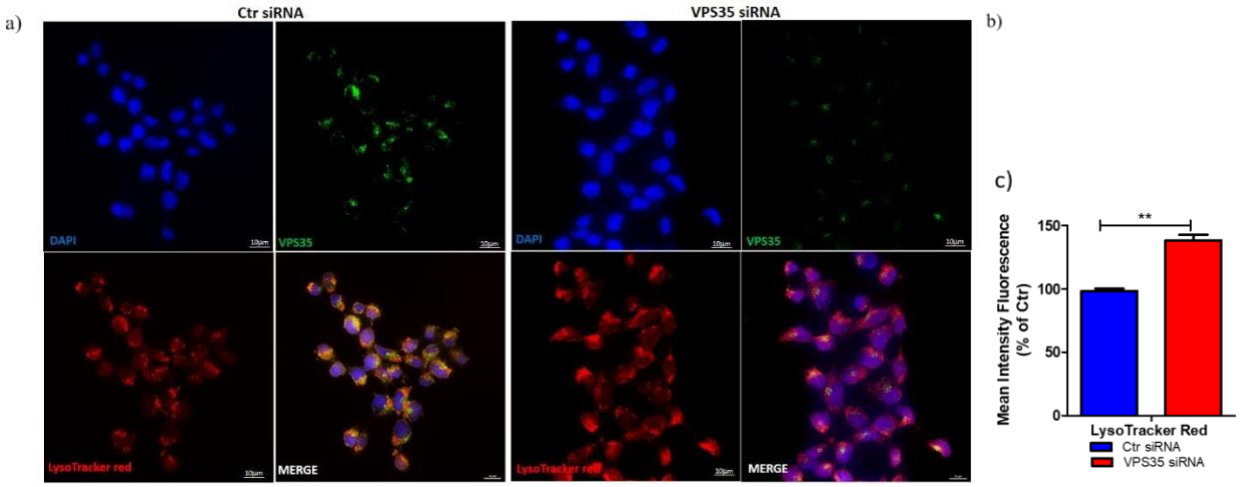

Figure 28. LysoTracker Red staining in cells downregulating VPS35. a) The SweAPP N2A cells were treated with a) Ctr siRNA (control) or b) VPS35 siRNA. The distribution of acidic vescicles was examined by LysoTracker Red staining and visualized with various channels (FITCH and TXred) using fluorescence microscopy (100 × magnification). Dapi was used to identify nuclei. Scale bar = 10 mm. c) Mean fluorescence intensity. (** $p < 0.01$ vs Ctr siRNA). N=3

6.5.9 VPS35 knockdown increases ubiquitination process

The UPS and autophagy are the two major degradation and recycling systems in mammalian cells. Interestingly, a strong interconnection between these systems has been reported therefore perturbations of one can affect the other (Kocaturk 2018). We first assessed BTZ effects as a proteasome inhibitor on N2A APPSwe. Consistent with previous studies, BTZ treatment at a concentration of 100 nM resulted in an increase in poly-Ub proteins compared with control cells (Figure 29a). As shown in figure 29b, level of poly-Ub proteins was significantly increased following VPS3 genetic suppression, suggesting that retromer complex defects results in alteration of the UPS system.

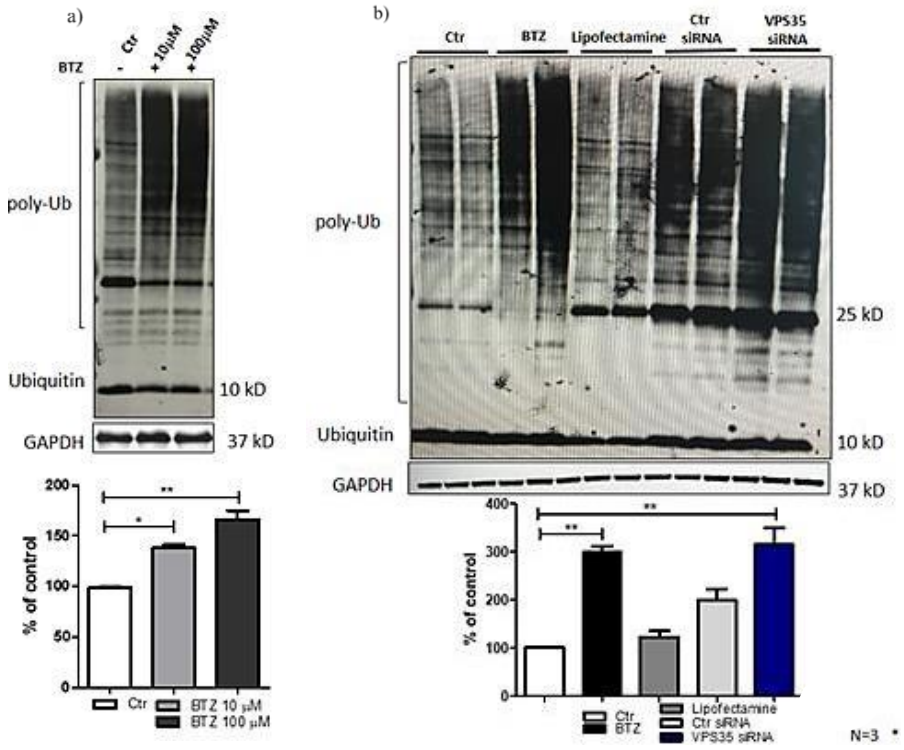

Figure 29. Evaluation of ubiquitination process. a) Western blot analysis of SweAPP N2A cells following proteasome blocking (10 and 100 nM BTZ for 4 hours b) Poly-ubiquitinated proteins levels following VPS35 silencing. (** $p < 0.001$ vs Ctr). N=3

6.6 Discussion

The cargo dynamic traffic between TGN and endosomes, well known as endosomal system, plays a key role in the sorting, recycling, or degradation proteins. The retromer complex, conserved from yeast to humans, is involved in this process and its defect could hypothetically lead to reduce degradation process or to increase the accumulation of proteins inside the cell compartments. VPS35 is known to be essential for the recruitment of the retromer core complex in association with VPS26 and VPS29. Several studies have shown that VPS35 genetic manipulation negatively influences the complex assembly, thus, influencing the expression of VPS26 and VPS29 and destabilizing the complex (Lee 2010). Moreover, recent studies showed the role of the retromer

complex in regulating the accumulation of misfolded α-synuclein protein in PD (Dhungel 2015) and amyloid-beta in AD, and tau protein in primary tauaopthy (Vagnozzi 2019) suggesting that VPS35 deficit leads to alter not only protein sorting and trafficking as well the degradation pathway. Autophagy is the main intracellular degradation pathway by which unnecessary cytosolic contents are enclosed, digested and, then delivered to lysosomes. Usually, autophagy has been viewed as a process for nonspecific degradation but it is also involved in cellular homeostasis by degrading offensive pathogens and protein aggregates (Shacka 2008). An interrelationship exists between endosomal system defects and autophagy, but the relative roles of each pathway in regulating the intracellular degradation process is still unclear (Di Meco 2019). Here we provide new experimental evidence showing that modulating VPS35 levels affects the degradation pathway with possible consequences ~~in~~ for the pathogenesis of AD. In the present work, we used an in vitro experimental method to demonstrate a correlation between the retromer complex, in particular VPS35 core component, and degradation process at different steps.

First, we assessed that in neuronal cells stably expressing Swedish mutation of amyloid precursor protein (APP), genetic knockdown of VPS35 caused a decrease of the other two components of the recognition core, VPS26, and VPS29.

Next, by looking at a correlation between the retromer complex defects and the autophagy pathway, we found that VPS35 silencing did not change ATGs proteins levels in our neuronal cells. It is interesting to notice that while we were not able to observe any changes of ATGs proteins after VPS35 down-regulation, Western blot analysis revealed substantial alterations of LC3B and SQSTM1/p62 expressions. The LC3B and SQSTM1/p62 expressions were investigated as biological players and markers of the involvement of autophagy activation during VPS35 silencing. Under our experimental conditions, we observed that VPS35 deficiency was responsible

for the LC3B lipidation process from LC3B1 to LC3B2 form and accumulation of autophagosome structures. Moreover, SQSTM1/p62 expression was decreased in VPS35 downregulating cells, suggesting an alteration of the autophagy pathway. Given these results, we performed immune-cytochemical studies and confirmed that VPS35 silencing resulted in increased fluorescence intensity for LC3B and decrease for SQSTM1p62.

In order to determine the lysosome-dependent degradation, BafA1, a potent V-ATPase inhibitor, was used. By Western blot analysis, we observed differences in LC3B2/1 ratio and SQSTM1/p62 protein levels between control cells and VPS35 downregulated cells that represented alterations of autophagic flux (Yoshii 2017). Therefore, it was essential to measure by fluorescence the autophagy "flux" using BafA1, as described above. Following VPS35 silencing and BafA1 exposure, in N2a APPSwe cells showed prominent puncta cells LC3B positives, which could be recognized as autophaghosome structures, while, VPS35 silencing and BafA1 stimulation attenuated cell positivity for SQSTM1/p62. Since fusion between autophagosomes and lysosomes is the final step of the degradation pathway, we decided to evaluate the distribution of acidic vesicles such as autophagosomes and lysosomes using LysoTracker red dye. Defects in VPS35, resulted in an increase of fluorescent intensity of LysoTracker suggesting that autophagic structures were longer accumulated non-degraded material. Regarding the protein turnover, we also looked at the UPS system, which is known to be involved in selective protein elimination and degradation, and we found that the polyubiquitinated proteins were increased following VPS35 silencing. Taken together these findings support the new hypothesis that defects in retromer complex, in particular VPS35 downregulation, directly influence two of the most important degradation pathways such as ALS and UPS.

6.7 Conclusions

Considering the rigor of the previous studies showing that dysfunction of the endosomal system is a common feature of many neurodegenerative diseases, our study by providing new insights on the molecular mechanisms involving the endosome and these diseases, establishes VPS35 as a novel therapeutic target for restoring cellular homeostasis trough adequate protein sorting, trafficking, and degradation.

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