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Fumaric Acid Esters and Inflammatory Responses

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Summary

Introduction

Inflammation is a pervasive phenomenon that operates during severe homeostasis perturbation, such as infection, injury, and exposure to contaminants. The inflammatory cascade is a complex network of immunological, physiological, and behavioral events that are coordinated by cytokines, immune signaling molecules (Ashley et al., 2012). Acute and chronic inflammation can result from a failure to remove or resolve the initiating insult or from the dysregulated injury response of either the affected tissue or the recruited immune system (Lo D et al, 1999). Although the central nervous system (CNS) is an immune privileged site, inflammatory reactions can and do occur within the CNS (Bechmann, 2005). Indeed, neuroinflammation is now recognized to be a prominent feature of many classic neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, Parkinson's disease, narcolepsy and even autism (Melchior et al, 2006).

Recent studies have shown the many beneficial actions through the use of fumaric acid esters (FAEs) such as dimethyl fumarate (DMF), monomethyl fumarate (MMF) and monoethyl fumarate (MEF) (Miglio et al, 2015). They represent a class of molecules that have anti-inflammatory and anti-oxidative activities in a variety of tissues and cells types. The efficacy of FAEs has been demonstrated in psoriasis, through immunomodulatory mechanisms, which might also play a role in the treatment of other inflammatory diseases. Several anti-inflammatory effects of FAEs include suppression of adhesion molecules and inhibition of cytokines production (Miglio et al, 2015). The most pharmacologically effective molecule among the FAEs is DMF: it is a new orally available disease-modifying agent that was recently approved by the US FDA and the EMA for the management of relapsing forms of multiple sclerosis (Dubey et al, 2015). *In vitro*, DMF and its primary metabolite MMF raised the survival rate of astrocytes and neurons in oxidative stress conditions (Albrecht et al., 2012). Data from human and animal studies suggests that this compound has both anti-inflammatory and antioxidant properties (Duffy S et al, 1998). In addition, it has been demonstrated the neuroprotection and immunomodulation of DMF is mediated by Nuclear factor

erythroid 2- related factor 2 (Nrf-2) (Wang et al, 2015). Nrf-2 regulates the expression of antioxidant response element (ARE) genes such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO-1) and glutathione synthesizing enzymes. The principal current hypothesis for the pharmacodynamic effect of DMF is based on the concept that it influences proinflammatory signal transduction pathways by modulating the intracellular redox system (Scannevin et al, 2012). There is evidence that changes in this system contribute to a decreased translocation of NF-κB, leading to an inhibition of the expression of proinflammatory cytokines including tumor necrosis factor α (TNF-α). DMF directly sustained endothelial tight junctions, inhibited inflammatory cytokine expression and attenuated leukocyte transmigration. It also knows that DMF protect endothelial cells from TNFα-induced apoptosis (Oh et al, 2014). Moreover, DMF improve lifespan, reduce behavior deficits and preserve striatal and motor cortex neurons in two different genetic models of Huntington's disease (Ellrichmann et al., 2011), indicating that it may have similar neuroprotective properties of MS.

Here, we investigate the effects of DMF on the inflammatory response colitis caused by intra-colonic administration of DNBS in mice and on Parkinson's disease caused through administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice.

Crohn's disease (CD) and ulcerative colitis (UC) collectively referred as inflammatory bowel disease (IBD), are relatively common diseases of gastrointestinal tract. IBDs are characterized by dysfunction of mucosal immune response, abnormal cytokines production with increase in TNF-α and IL-1β, augmentation in adhesion molecules expression and cell infiltrate that ultimately lead to epithelial cell apoptosis and mucosal damage (Impellizzeri et al., 2015). Although the etiology of IBD remains unknown, there is circumstantial evidence supporting a central role for dis-regulation of mucosal CD4+ T helpher-1 (Th1) effectors cell responses to the normal enteric bacterial flora as a common disease mechanism (Cannarile et al., 2009).

While PD is a neurodegenerative disorder, characterized by the loss of dopaminergic (DA) neurons, cytoplasmic inclusions of aggregated proteins called Lewy bodies and neuroinflammation (Moore et al., 2005). The neuroinflammation is individuated by the presence of activated microglia and reactive astrocytes in the parenchyma of the central nervous system (CNS) inducing the production of inflammatory mediators and reactive oxygen and nitrogen species (ROS/RNS) (Ransohoff et al., 2009). Several studies focused their attention on possible intrinsic factors that cause vulnerability of dopaminergic cells (Choi et al, 2006). In this context, it has been proposed that the presence of dopamine, tyrosine hydroxylase (TH), monoamine oxidase, iron, and/or neuromelanin play an important role in death of dopaminergic cells (Choi et al, 2006). As explained, oxidative stress and inflammation played an important role in the pathogenesis of more diseases; FAEs thanks to their beneficial effects could represent a therapeutic target for many inflammatory pathologies.

Chapter 1

Fumaric acid esters

Fumaric acid (FA) was isolated for the first time from the plant *Fumaria officinalis* (Roa Engel et al, 2008), a well known for its medicinal uses. It is good source of bitter principles and alkaloids, such as fumarine and protoppine and, recently, this plant has been positively tested in rats for hepatoprotective activity. In this study, crude ethanolic extract prepared from aerial parts of the plant was administered to rats in which liver injury was induced by CCl4. The hepatoprotective activity might be caused by the presence of FA in the plant extract (Das et al, 2015). Furthermore, FA is intermediate product of Tricarboxylic Acid Cycle (TCA) or Krebs cycle in all aerobic organisms. As a chemical substance, FA has many applications in food and resin industries, dairy and poultry sector and in green chemistry (Yang, et al 2011; Goldberg, et al 2006; Rohokale, et al 2014). FA has recently regained value as a molecule for the synthesis of high value derivatives for biomedical applications. Recent studies have shown the advantage of FA and fumaric acid esters (FAEs) such as dimethyl fumarate (DMF), monomethyl fumarate (MMF) and monoethyl fumarate (MEF) in different fields, such as tissue engineering, drug delivery, cancer research, neurology, cardiology and immunology. FA and FAEs have been used in the formulations of many drug delivery vehicles and biomaterial based scaffolds for bone tissue engineering applications (Temenoff, et al 2007) (Fig.1). Moreover, the recent recognition of FAEs formulation 'Tecfidera' (DMF) to treat human adults with relapsing forms of multiple sclerosis by the U.S. Food and Drug Administration (FDA, US) is a significant achievement (Scavennin et al., 2012). With the growing interest of experimenting FA and FAEs for different purposes, many studies are being carried out to test new applications.

Fig.1: Acid Fumaric and Fumaric Acid Esters applications (Das et al, 2015).

These studies have helped in establishing that FAEs can be used as drug molecules with high efficacy and safety. Extensive research has been carried out on many FAEs based drug formulations for application in the remedies of human diseases. Some studies have been accomplished after successful human trials, while others are in vitro or in vivo stages (Das et al., 2015).

1.1 Pharmacokinetics of FAEs

There have been many efforts to discover the fate of FAEs such as DMF, MMF and MEF in human body; obtained data give strong evidence of metabolism of FAEs. In 1999, Mrowietz et al. mentioned about in vivo study of pharmacokinetics of FAEs (Mrowietz et al, 1999). They set out from the awareness that FA is a dicarbonic acid, and is a polar compound with a low bioavailability (Fig.2). A common prodrug principle to increase bioavailability is the formation of carbonic acid esters and the “masking” of carbonic acids that leads to decreased side effects. The study indicated

that DMF was rapidly (half-life of 12 min) hydrolyzed into MMF by the action of cellular esterase enzymes (Fig.2) and MMF was further metabolized into FA and later into H_2O and CO_2 by Krebs cycle. The study also confirmed no binding behavior of DMF and 50% binding of MMF with serum proteins and the half-life of MMF was found to be 36 h with a Tmax of 5–6 h. In an *in vitro* study on the first-pass effect and presystemic metabolism and intestinal absorption of FAEs, DMF was found to be completely metabolized in the intestinal tissue (Werdenberg et al, 2003). Studies conducted on porcine intestinal mucosa provided the evidence that DMF did not penetrate through this layer (Werdenberg et al, 2003).

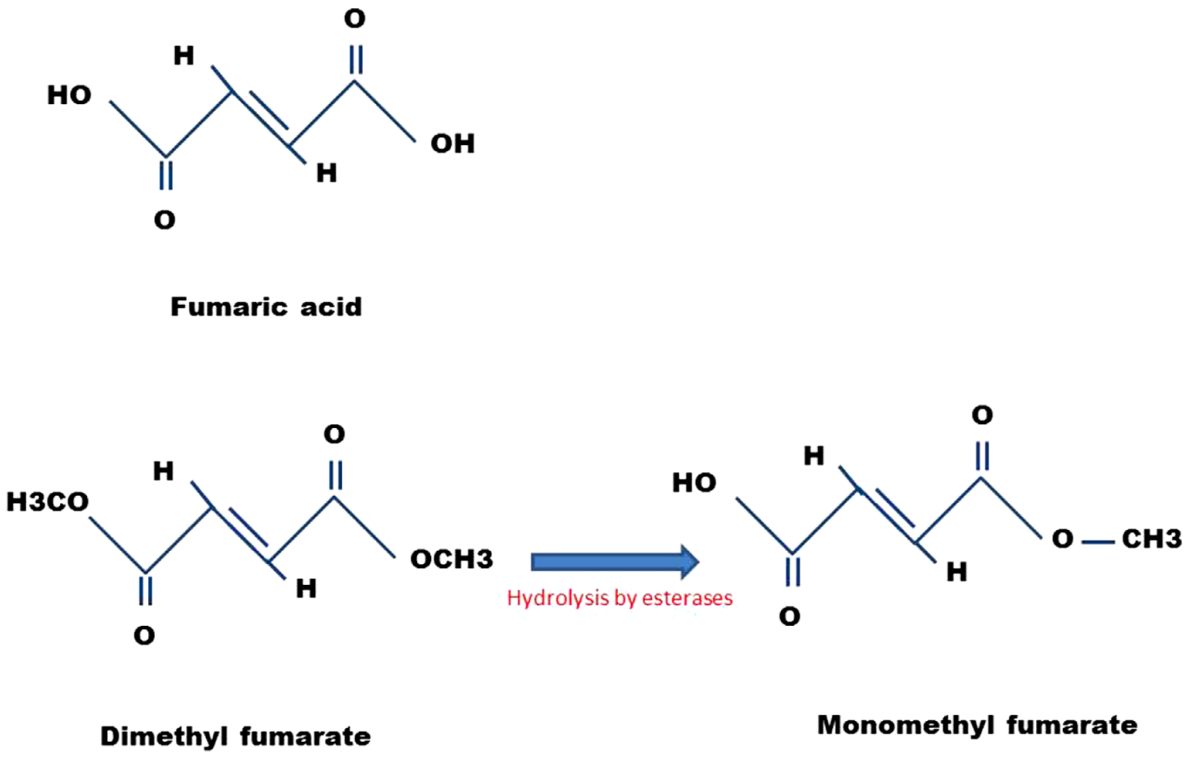


Fig.2. Acid Fumaric and FAE (Al-Jaderi et al, 2016).

These findings clearly demonstrated that the mucosa and intestinal alkaline environment represent a strong pre-systemic metabolic barrier for DMF on its way into the systemic circulation. In case of penetration of DMF, which was eventually not metabolized at this "barrier," it would be transported via the blood of the portal vein to pass the liver first. This may explain why DMF's main metabolite MMF, but no DMF, has been detected in the plasma of human beings after the oral intake of DMF

along with calcium MEF in the form of enteric-coated tablets (Litjens et al, 2004). A further degradation of systemic MMF to FA, resulting in higher plasma levels of FA, was not observed (Litjens et al, 2004). A study considered a homologous series of mono- and diesters of FA that exhibited lipophilicity dependent intestinal permeability; more lipophilic FAEs exhibited increased intestinal permeability as compared to less lipophilic ones. Interesting, presystemic metabolism rate of the FAEs was enhanced by the ester chain length and this finding led to the conclusion that increased intestinal permeability of the more lipophilic FAEs were counterbalanced by the pre-systemic metabolism. The study confirmed that DMF hydrolysis occurs in small intestine and the metabolite MMF is no more hydrolyzed and thus easily detectable in blood circulation after oral administration of FAEs (Litjens et al, 2004). MMF and MEF were found to be tolerant to both acidic (pH 1) and alkaline (pH 8) simulated fluids; however, at 7.4 pH, DMF was not hydrolyzed to MMF. Analysis of the serum and whole blood samples confirmed the involvement of lymphocytes/monocytes in the metabolism of DMF into MMF. (Litjens et al, 2004).

1.2 Mechanism of action

Despite numerous in vitro and ex vivo studies, the mechanism of action of FAE is not fully understood and novel aspects are emerging (R.A. Linker et al., 2011). Four mechanisms of action have been described. First, DMF stimulates cytoprotective and anti-inflammatory responses via activation of the Nuclear factor erythroid 2- related factor 2 Nrf2 -dependent anti-oxidant response pathway (Linker et al., 2011; Scannevin et al., 2012). Second, DMF inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-driven processes (Peng et al., 2012), resulting in downstream reduction in inflammatory cytokine production, altered maturation and function of antigen-presenting cells, and immune deviation of T helper cells (Th) from the Th1 and Th17 profile to a Th2 phenotype (Peng et al., 2012). Third, as a α, β carboxylic acid ester, DMF can bind thiol groups and modulate glutathione availability and production, which impacts cellular responses to oxidative stress (Dibbert et al., 2013; Scannevin et al., 2012). Fourth, agonism of G-protein

coupled receptor 109A (GPR109A, also known as the hydroxycarboxylic acid receptor 2 (HCA2)) by DMF and MMF reduces neutrophil adhesion, migration, and recruitment to the CNS during EAE (Chen et al., 2014). The most important mechanisms of action of FAEs are Nrf2 and NF-κB pathway; regarding Nrf2's activity, it is regulated at the molecular level by KEAP1. KEAP1 sequesters and poly-ubiquitinates Nrf2 in the cytosol, leading to constitutive degradation (Nguyen et al., 2009). Electrophilic and oxidative stress changes the interaction between Nrf2 and KEAP1, resulting in the stabilization and translocation of Nrf2 to the nucleus. Similarly, DMF interaction with KEAP-1 results in the stabilization and translocation of Nrf2 that regulate cytoprotective genes associated with the phase II antioxidant response such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO-1) and glutathione synthesizing enzymes. Instead the activation of NF-κB is mediated by an IκB kinase (IKK) complex consisting of IKKα and IKKβ that phosphorylate the inhibitor IκB, resulting in the release of p65/p50 dimers that translocate to the nucleus and stimulate gene transcription at target sites (Shih et al., 2011). Alternatively, activation of NF-κB-inducing kinase (NIK) caused processing of p100, resulting in the release and nuclear translocation of p52/relB dimers (Sun, 2012). Different published studies suggested that DMF and MMF inhibit the NF-κB pathway at the level of the NF-κB/IκB complex (Loewe et al., 2001). It has been also demonstrated an evidence of a cross-talk between the KEAP1-Nrf2 pathway and NF-κB signaling. Lee et al. demonstrated that KEAP1 binds IKKβ and mediates ubiquitination and subsequent degradation (Lee et al., 2009). Depletion of KEAP1 led to an increase in NF-κB signaling. A separate study demonstrated that genetic knockdown of KEAP1 led to enhanced IL-6 production, an NF-κB target gene, in LPS stimulated macrophages. Interestingly, it has been shown in a study that DMF treatment inhibited pro-inflammatory cytokine production by cultured WT and $Nrf2^{-/-}$ splenocytes with equal efficiency, demonstrating that the ability of DMF to suppress NF-κB dependent responses was independent of Nrf2 activity (Gillard et al., 2015).

1.3 Effect of FAE on T-cells and B-cells

One of the first observations during the application of Fumaderm® in psoriasis was the effect on T-cells where was evident a decreasing this immune cells in nearly all treated patients (Altmeyer et al 1996). This finding was further supported by an immunohistochemical study that showed a significant reduction of CD4+ cells (Bacharach-Buhles et al., 1994) in the epidermal inflammatory infiltrate. Subsequent *in vitro* studies revealed that DMF could induce apoptosis in human T-cells (Treumer et al., 2003). Upon analysis of the cytokine production in more detail, de Jong et al. (R. de Jong et al., 1996) reported that MMF increased the production of the "Th2" interleukin (IL)-4 and IL-5 in stimulated T-cells while it didn't have any effect on "Th1" cytokine interferon gamma (IFN-γ), the IL-2 production or associated proliferation of T-cells. In another study, stimulated $CD4^{+}CD45RO^{+}$ memory T-cells, showed an increased secretion of IL-4 and IL-5 after treatment with MMF. These findings suggest that FAE in psoriasis may shift the cytokine profile from a "Th1" to a "Th2" profile. This effect of FAE, on cytokines expression could be apply to other immune cell types. Although there weren't any studies on direct effects of FAE on B-cells; there was evidence that the downregulation of NF-κB in turn inhibited the anti-apoptotic protein Bcl-2, thus leading to apoptosis in B-cells (Mrowietz et al., 2005). It might speculate that FAE mediated inhibition of NF- κB activity affecting B cell functions including B cell apoptosis.

1.4 FAE and dendritic cells

Dendritic cells (DC) play a major role in regulating inflammatory responses in autoimmune diseases like psoriasis and MS by expressing cytokines and co-stimulatory molecules. Basically pro-inflammatory myeloid DCs have to be differentiated from plasmacytoid DC (pDC). These pDCs regulate immune functions typically by IFN-beta mediated response mechanisms. In monocyte derived DC, FAE induced apoptosis, lowered secretion of IL-12 and prevented appropriate cell differentiation (Zhu et al., 2001). This was reflected by an inhibition of CD1a, CD40, CD80, CD86, and HLA-DR expression as well as by a reduced capacity of FAE-treated

monocyte-derived dendritic cells to stimulate allogeneic mixed lymphocyte reaction. In another study, MMF treated monocyte-derived dendritic cells downregulated Th1 lymphocyte responses by significantly by decreasing the production of IFN-gamma after stimulation by lipopolysaccharide (LPS) (N.H. Litjens et al., 2004).

1.5 FAE effects on endothelial and glial cells

In experiments with human umbilical vein endothelial cell cultures, Asadullah et al. and Vandermeeren et al. showed that DMF inhibited TNF-α inducing the expression of ICAM-1, E selectin, and the vascular cell adhesions molecule-1 (VCAM-1) (K. Asadullah et al., 1997, Vandermeeren et al, 1997). This might correlated with the described activity of FAE on NFκB. A potential detoxification effect was found when DMF was tested in astrocytes and microglia; indeed, it has shown that DMF attenuated the LPS-induced production and release of TNFalpha, IL-1beta, IL-6 and NO, in addition, DMF increased both mRNA level and activity of NAD(P)H:quinone reductase (NQO-1), a detoxication enzyme, as well as the cellular glutathione content. Therefore, DMF can reduce mediators of inflammation and enhance detoxication enzymes in LPS stimulated co-cultures of astroglial and microglial cells. (Wierinckx et al., 2005). Moreover, it has been showed that DMF decreased the LPS induced production of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and nitric oxide. Moreover, DMF increased the expression of the NAD(P) H:quinine reductase (NQO-1) and the content of cellular glutathione. All these functions are in accord with Nrf-2 mediated anti-oxidative mechanisms (R.A. Linker et al., 2011).

Chapter 2

Clinical use of Fumaric Acid Esters as treatment of psoriasis and in multiple sclerosis

2.1 Psoriasis

Psoriasis is a type-1 cytokine-mediated chronic autoimmune skin disease aided by the infiltration of Th1/Th17 cells into the skin (Gudjonsson JE et al., 2004). DMF has been utilized to treat psoriasis in European countries for more than 30 years. FA was first used for treatment of psoriasis by the German chemist Walter Schweckendiek in 1959. In 1994, DMF was approved in Germany under the trade name Fumaderm for the treatment of psoriasis. DMF inhibited Janus kinas (JAK) signaling and interfered with intracellular proteins trafficking and consequently, inhibited the release of pro-inflammatory cytokines, such as IL-12, IL-23, and TNF, whereas the release of anti-inflammatory cytokines, such as IL-10, was increased. DMF also inhibited the production of IFN-γ and enhanced the production of IL-10 in the culture of psoriatic keratinocytes (Ockenfels HM, 1998). Previous experimental and clinical studies were focused on the mechanism of action of DMF that could affect the immune system. The immunohistochemical studies of psoriatic plaques indicated that DMF has several anti-inflammatory effects via a number of pathways, such as activation of the Nrf2-dependent anti-oxidant response pathway and NF-κB-driven processes, leading to reduction in the levels of several inflammatory T cell subsets (Basavaraj KH et al., 2010) and decreased recruitment of inflammatory cells (Rubant SA, 2008). The ability of DMF or MMF to induce apoptosis of CD4+ and CD8+ T cells and in vitro switching the immune system toward a Th2 anti-inflammatory type response in psoriasis patients could be through impaired DCs maturation and induction of apoptosis. In addition, DMF inhibited the formation of new blood vessels, a process that is involved in the formation of psoriatic plaques (Garcia-Caballero M et al., 2011). Clinical studies demonstrated that DMF reduced CD4+ T cells and CD8+ T cells by

inducing apoptotic cell death (Treumer F et al., 2003). *In vivo* studies indicated that DMF inhibited T cell mediated organ rejection in a rat model (Lehmann M et al., 2002).

2.2 Multiple sclerosis

Multiple sclerosis is a chronic inflammatory autoimmune disease of the CNS in which the insulating myelin sheaths of nerve cell axons in the brain and spinal cord are attacked by the immune system (Hestvik ALK, 2010). The principal mechanism responsible for this disease is still incompletely understood. The consensus is that activated T cells attack oligodendrocytes, leading to destruction of myelin sheaths (demyelination). Furthermore, the presence of inflammatory T cells in the CNS triggers recruitment of more T cells, B cells, dendritic cells, microglia cells, and NK cells (Hoglund RA et al., 2014). Due to the progressive neurodegenerative nature of MS, therapeutic modalities that exhibit direct neuroprotective effects are needed. *In vitro* study indicates that DMF increased the frequency of the multipotent neurospheres resulting in the survival of mouse and rat neural stem progenitor cells (NPCs) following oxidative stress with hydrogen peroxide (H_2O_2) (Wang Q et al., 2015); by using motor neuron survival assay, DMF significantly promoted survival of motor neurons under oxidative stress. Furthermore, DMF increased the expression of Nrf2 at both RNA and protein levels in the NPC cultures (Wang Q et al., 2015). *In vivo* animal studies have shown that DMF or MMF inhibited the disease course in the EAE model (Schilling S et al., 2006). Importantly, it was demonstrated that MMF was able to cross the blood-brain barrier, indicating it might have a direct cytoprotective function in the CNS (Parodi B et al., 2015). The detoxification capabilities of DMF or MMF reduced the production and the release of inflammatory molecules, such as TNF-α, IL-1β, and IL-6 as well as nitric oxide from microglia and astrocytes activated with LPS *in vitro* (Loewe R et al., 2002). Furthermore, DMF or MMF increased the production of detoxification enzymes, such as nicotinamide adenine dinucleotide phosphate quinone reductase 1 (NQO-1), HO-1, and cellular glutathione, abolishing NF-kB translocation into the nucleus (Wierinckx A et al., 2005). Consequently, the reduction of NF-kB-dependent genes expressions

such us inflammatory cytokines, chemokines, and adhesion molecules, reduced the damage to CNS cells (Scannevin RH et al., 2012). These immunomodulatory activities of DMF or MMF, like the inhibition of cytokine production and nitric oxide synthesis, has been shown to be important for the protection of oligodendrocytes against ROS-induced cytotoxicity and consequently, oligodendrocytes survival during an oxidative attack is augmented; in fact, EAE mice treated with DMF showed a significant reduction of macrophage-induced inflammation in the spinal cord (Schilling S et al., 2006).

Another showed properties of DMF was to suppress Th1 and Th17 cell differentiation as well as the expression of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 (Schimrigk S et al., 2006). The drug also promoted Th2 cells that produce IL-4, IL-5, and IL-10 (de Jong R et al., 1996). In chronic MS, microglia cells are activated and released pro-inflammatory cytokines and stress-associated molecules leading to neurodegeneration and alteration of synaptic transmission (Lull ME et al., 2010): the study on EAE demonstrates that exposure to MMF switched the molecular and functional phenotype of activated microglia from pro-inflammatory type to neuroprotective effect (Parodi B et al., 2015).

In 2008, a phase II human trial study was carried out with a second generation FA derivative known as BG-12. Clinically, the study was a multicenter, double-blind, placebo-controlled that included 257 patients in 10 countries and was designed to evaluate the safety, efficacy, and dose-ranging. This new generation FAE contained DMF as an enteric-coated and developed by Fumapharm AG and Biogen Idec Inc. The MRI analysis of the Gd^{+} enhanced T1-weighted scans and T2-weighted scans revealed a statistically significant reduction of 69% in the mean number of lesions compared to the placebo group. The overall outcome of the study was the promising short-term efficacy and safety profile of BG-12 (Rubant SA et al., 2008; Garcia-Caballero M et al., 2011). To further confirm the long-term safety and efficacy of the application of BG-12, a large scale phase III study program was launched in Europe and North America in 2008. Named as DEFINE (determination of

the efficacy and safety of oral fumarate in relapsing-remitting MS) and CONFIRM (comparator and an oral fumarate in relapsing-remitting MS), the proposed massive study included international, multicenter, two-year randomized, double-blind, placebo-controlled, dose comparison studies (Moharregh-Khiabani et al, 2009). Recently, Biogen Idec Inc. have announced that their FAE, DMF (BG-12), has met the primary and secondary end points of a global Phase III trial under the DEFINE program for treating RRMS. On March 27, 2013 the Food and Drug Administration of US (FDA, US) announced that BG-12 was approved for the treatment of MS and it was marketed under the brand name Tecfidera™ (www.fda.gov).

Chapter 3

Role of inflammation in the peripheral and central level

3.1 Inflammation and neuroinflammation

Inflammation is a protective response by the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue (Medzhitov, 2008). Inflammation is caused by various factors such as microbial infection, tissue injury, and cardiac infarction. It can be classified as either *acute* or *chronic*. In the acute inflammatory response there is a complex orchestration of events involving leakage of water, salt and proteins from the vascular compartment, activation of endotelial cells, recruitment of leukocytes, activation of tissue macrophages and release proteases and oxidants from phagocytic cells all of which may assist in coping with the state injury. Chronic inflammation is defined by the nature of inflammatory cells that appear in the tissues. The definition of chronic inflammation is not related with to the duration of inflammatory response. Reversal or resolution of the inflammatory response implies that leukocytes will be removed either via lymphatics or by apoptosis and that the ongoing acute inflammatory response is terminated. Classically, inflammation is characterized by five symptoms: redness, swelling, heat, pain, and loss of tissue function. These macroscopic symptoms reflect increased permeability of the vascular endothelium allowing leakage of serum components and extravasation of immune cells. The inflammatory response is then rapidly terminated and damaged tissues are repaired. However, overproduction of cytokines by immune cells to overwhelm pathogens can be fatal. The inflammatory response is orchestrated by proinflammatory cytokines such as TNF, IL-1, and IL-6. These cytokines are pleiotropic proteins that regulate the cell death of inflammatory tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins. Examples of disorders associated with inflammation include: Acne

vulgaris, Asthma, Autoinflammatory diseases, Celiac disease, Inflammatory bowel diseases, Rheumatoid arthritis etc.

Inflammatory responses are also critical for the pathogenesis of autoimmune diseases (figure 3). The innate immune system is the major contributor to acute inflammation induced by microbial infection or tissue damage (Akira et al., 2006; Beutler et al., 2006). Furthermore, innate immunity is also important for the activation of acquired immunity. Although innate immune cells including macrophages and DCs play important roles, nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to innate immunity. The CNS is an immunologically privileged site and circulating immune cells normally do not have access to it in the absence of inflammation or injury. (Bechmann, 2005). Indeed, neuroinflammation is now recognized to be a prominent feature of many classic neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, Parkinson's disease, narcolepsy and even autism (Melchior et al, 2006). Activation of innate immune cells in the CNS, such as microglia and astrocytes, is one of the universal components of neuroinflammation Furthermore, in chronic or remitting/relapsing neurodegenerative disorders, inflammation is unlikely to be playing a purely beneficial versus detrimental function (Melchior et al, 2006). Although inflammation may not typically represent an initiating factor in neurodegenerative disease, there is emerging evidence in animal models that sustained inflammatory responses involving microglia and astrocytes contribute to disease progression. Microglia are the major resident immune cells in the brain, where they constantly survey the microenvironment and produce factors that influence surrounding astrocytes, another type of glial cell with support functions, and neurons. Under physiological conditions, microglia exhibit a deactivated phenotype that is associated with the production of anti-inflammatory and neurotrophic factors (Streit, 2002). Microglia switch to an activated phenotype in response to pathogen invasion or tissue damage and thereby promote an inflammatory response that serves to further engage the immune system and initiate tissue repair. In most cases, this response is self-

limiting, resolving once infection has been eradicated or the tissue damage has been repaired. A major unresolved question is whether inhibition of these responses will be a safe and effective means of reversing or slowing the course of disease.

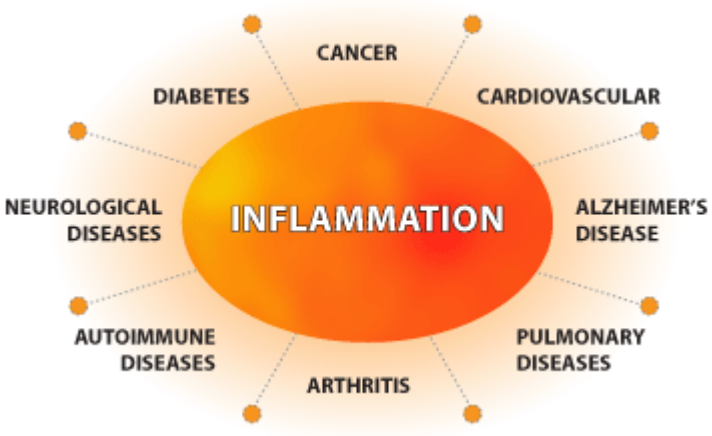


Figure 3. Inflammation (http://altered-states.net).

3.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is still a global healthcare problem with a sustained increasing incidence (Xavier RJ et al., 2007). IBDs are an immune mediated chronic or relapsing disorders of the gastrointestinal (GI) tract and consist mainly of Crohn's disease (CD) and Ulcerative colitis (UC). Although the etiology of IBD remains largely unknown, recent research indicated that the individual's genetic susceptibility, external environment, intestinal microbial flora and immune responses are all involved and functionally integrated in the pathogenesis of IBD (Danese S et al., 2006). There is no doubt that environmental factors play an important role in the pathogenesis of IBD. A large number of environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography, social stress, and psychological element (Loftus EV et al., 2004). Recent literature data suggest that vitamin D, stress and air pollution may contribute to risk of CD and UC (Mawdsley JE et al., 2006 and Thia KT et al., 2008). IBD is characterized by a chronic intestinal inflammatory process with various components contributing to the pathogenesis of the disease including environmental factors (such as smoking or non-steroidal anti-inflammatory agents

(NSAIDS), genetic background, host intestinal flora and the host immune system (Fordtman S.A. et al., 2006). CD is a transmural disease characterized macroscopically by skipped lesions with aphtae, ulcers of various sorts and at times the classical cobble-stone appearance. Microscopically a transmural chronic inflammation is present with or without granuloma formation. The chronic inflammation of UC is confined to the mucosa and is continuous in nature. Macroscopically a granular appearance or an edematous hemorrhagic mucosa, in more severe cases, is typical. Microscopically crypt architectural distortion and chronic inflammation are typical (Fordtman S.A. et al., 2006). Since CD can affect any part of the GI tract, the clinical spectrum of the disease varies widely from epigastric pain and helicobacter pylori negative gastritis, due to upper GI involvement, to diarrhea, weight loss, abdominal pain and nutritional deficiencies in patient with small bowel disease. Hematochezia, bloody diarrhea, tenesmus and occasional fever are indicative of colonic involvement in CD or the presence of UC. Up to a third of IBD patients suffer from extra intestinal manifestations of their disease. These include dermatologic manifestations such as erythema nodosum, ocular complications such as conjunctivitis and uveitis, hepatobiliary complications including cholelithiasis, steatosis and primary sclerosing cholangitis and urologic complications mainly nephrolitiasis. Finally, two very important and prevalent extra intestinal complications are rheumatic manifestations such as arthralgias, peripheral arthritis or ankylosing spondilitis and osteoporosis with increased risk of fractures secondary to vitamin D and calcium deficiency and prolonged steroid use (Loftus E.V., 2005). The investigation of IBD pathogenesis has been dominated for a long time by the studies of mucosal immunity, especially the T cell response. Available evidence suggests that the dysfunctions of innate and adaptive immune pathways contribute to the aberrant intestinal inflammatory response in patients with IBD. The focus on the adaptive immune response has ultimately led at the distinction of two main types of IBD represent clearly distinct forms of gut inflammation: CD drove by a Th1 response and UC associated with a non-conventional Th2 response (Targan SR et al., 2005). It has been shown that the newly

described Th17 cells are also involved in the gut inflammatory response in IBD (Geremia A et al., 2012).

3.3 Epidemiology

The prevalence of IBD has gradually increased in recent years and varies according to geographical location including urban vs. rural areas (Colombel J.F et al., 2013). In the United States alone, one to two million people has IBD while several million have it worldwide (Molodecky et al., 2012). The prevalence of UC varies from 4.9 to 505 per 100,000 inhabitants in Europe, 4.9 to 168.3 per 100,000 inhabitants in Asia and the Middle East, and 37.5 to 248.6 per 100,000 inhabitants in North America. CD estimates range from 0.6 to 322 per 100,000 in Europe, 0.88 to 67.9 per 100,000 in Asia and the Middle East, and 16.7 to 318.5 per 100,000 in North America (Molodecky et al., 2012). The prevalence of UC seems to increase with age (Ward et al., 2010). In the last few decades, an increase in incidence occurred in industrialized zones which earlier had low incidence of IBD such as South Korea, China, India, Iran, Lebanon, Thailand, the French West Indies, North Africa and Japan, has been reported (Burisch, J et al., 2013). Thus, IBD is emerging as an important health problem worldwide. This condition mostly affects young people of both sexes in the age group between 15 and 35 years (Hommes, D et al., 2012). Furthermore, IBD is associated with considerable healthcare costs (Kappelman, et al., 2010). Whereas childhood onset, IBD represents only 10% to 25% of all IBD cases, genetic research of pediatric IBD has contributed new knowledge and revealed unsuspected pathways. A substantial portion of patients with monogenic diseases present with very early onset intestinal inflammation (at less than 10 years of age) that is reminiscent of very early onset IBD. There is also considerable overlap with primary immune-deficiencies and very early onset IBD, a topic reviewed recently but still unknown (Okou et al., 2014).

3.4 Inflammation and oxidative stress in IBD

The intestinal inflammation has as major players the cytokines IFNγ and TNFα that able to damage epithelial barrier functions independently of their pro-apoptotic action. It has been shown that these cytokines disassemble tight junctions (TJs) of apical junctional complex and enhance paracellular permeability, by promoting internalization and cellular redistribution of their junctional adhesion molecule (JAM)-A, occludin, and claudin-1, but not of zonula occludens-1 or adherens junction proteins in T-84 cells (Bruewer M et al., 2003). It is still unclear whether damage to the transmembrane proteins, that are components of junctions, should be considered like a cause or a consequence of intestinal inflammation. Under inflammatory conditions endocytosis of junctional molecules increases, and intracellular redistribution may cause a breakdown of the protective intestinal barrier. This process may enhance production of inflammatory infiltrate and cytokines, which further contribute to amplify epithelial barrier damage. IBD development comprises alternate phases of tissue damage and tissue repair. The latter process implies neoangiogenesis, and the synthesis of pro-angiogenic factors, such as vascular endothelial growth factor and fibroblast growth factor. An important contributor of angiogenic factor production are endothelial cells of the inflamed capillaries; these intestinal cells express CD40 and CD40L proteins, which belong to the TNF receptor superfamily, having co-stimulatory activity for immune cells, such as T cells, and for non-immune cells. In both UC and CD, CD40 and CD40L are overexpressed, especially in severely inflamed mucosa (Danese S et al., 2004). Expansion of the microvasculature in inflamed intestinal tissue stimulates the recruitment of pro-inflammatory mediators, amplifying inflammation and tissue damage. The main hypothesis on the development and progression of IBD is based on impairment of immune tolerance to the gut commensal microbiota, thought to be due to a genetic predisposition of the host, which leads to chronic intestinal inflammation and mucosal damage. The chronic inflammation leads to massive gut infiltration by granulocytes and macrophages, with different features in CD and UC. These cells produce large amounts of pro-inflammatory cytokines, chemokines, and ROS/RNS intermediates, which the anti-inflammatory cytokines and antioxidant

molecules are unable to counteract. Immune cells play an important role, and they also mediate inflammatory reactions through the expression and synthesis of different cytokines. In IBD patients, the inflamed mucosa shows active infiltration of $CD4^+$ Th lymphocytes, which are responsible for maintaining chronic inflammation. In general, naïve $CD4^+$ Th cells proliferate and differentiate into different subsets on stimulation by specific cytokines: three different Th, that is, Th1, Th2, and Th17, and a subpopulation of regulatory T lymphocytes (Tregs) are formed (Figure 4).

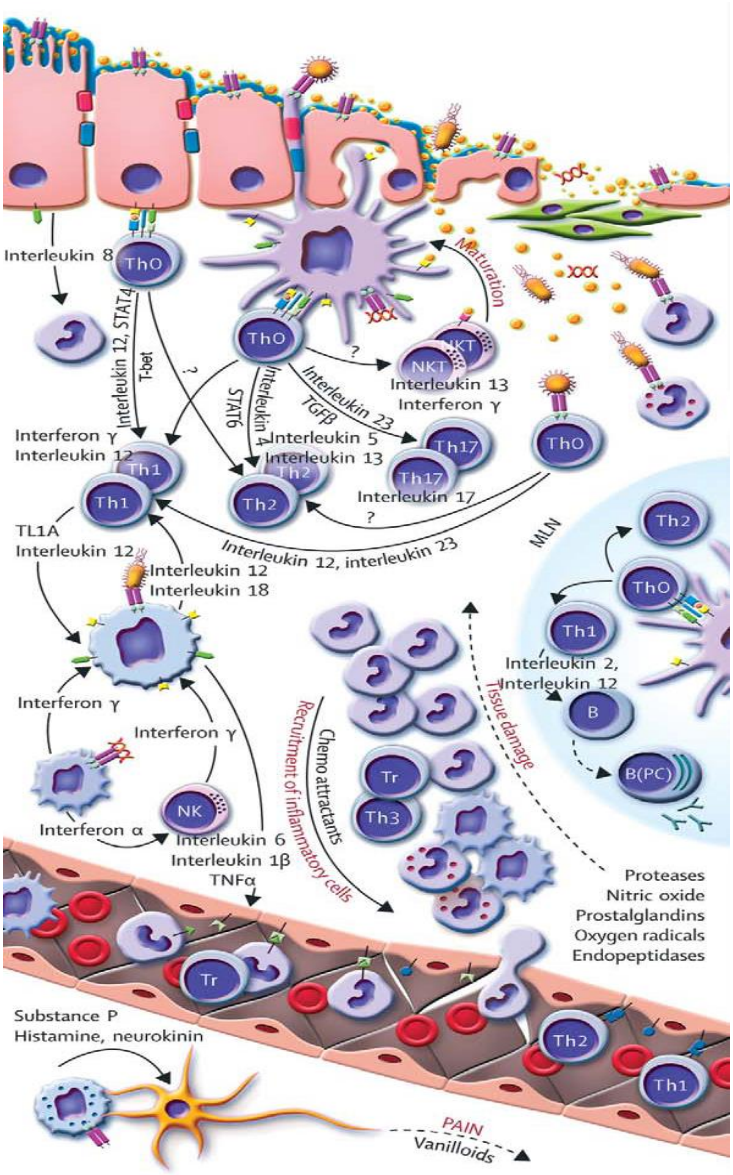


Figure 4. Intestinal immune system in inflammatory bowel disease (Baumgart et al, 2007).

Each T-cell subset secretes specific cytokines that are able to differentially modulate inflammatory reactions. Lymphocytes Th1 are the main mediators of type1 immunity. They act against intracellular pathogens by means of intense phagocyte activity and produce IL-2, IFNγ, TNFα and tissue-infiltrating Th17 cells; the same cytokines amplify this response by inducing macrophages to release further amounts of TNFα, IL-6, and IL-1β. Th1 cells and related cytokines are predominant in active CD. The expressions of IL-1β, IL-6, and TNFα are increased in the affected mucosa of patients with active IBD. Conversely, the same patients showed decreased levels of IL-2 and IFNγ in the peripheral blood cells (Raddatz D et al., 2005). Among the various cytokines produced during the Th1 response, the overexpression of TNFα undoubtedly plays a key role in the induction of intestinal damage, especially in CD patients. This molecule can trigger pro-inflammatory and survival pathways, as well as the apoptotic process. Its effect probably depends on the amount and time of cell exposure to the cytokine, the type of receptor that binds TNFα, and the sequence of events that take place on ligand binding. Regulation of NF-κB activity appears to be fundamental in determining the specific cellular response to TNFα (Rangamani P et al., 2007). Furthermore, the use of TNFα blockers in treating CD has recently generated considerable interest. TNF-α therefore links the innate and the adaptive immune responses, and has a crucial importance in the pathogenesis of IBD, as demonstrated by the impressive results of clinical trials with biologic agents targeting TNF-α (Macdonald et al., 2012). Cytokines produced by Th2 cells are selectively increased in the inflamed mucosa of UC. These cells synthesize IL-4, IL-5, IL-10, and IL-13, and are required for humoral response (Neuman MG, 2007). Th2 lymphocytes support the synthesis of all types of immunoglobulins, by secreting IL-4 and IL-5, and inhibit Th1- activated macrophages by secreting IL-4, IL-10, and IL-13. IL-4 and IL-10 are considered anti-inflammatory cytokines (Heller F et al., 2005). Treg cell subsets are activated by the two anti-inflammatory cytokines IL-10 and TGFβ, mainly those produced by dendritic cells (Coombes JL et al., 2007). IL-10 and TGFβ have long

been known as the two main cytokines involved in the negative regulation of inflammation and immune response, thus contributing to the maintenance of gut homeostasis. The protective role of IL-10 in IBD had already been demonstrated in an IL-10 knock-out (KO) mouse model of colitis, which required gut microbial intervention to develop inflammation. This gave augmentation to the idea that IL-10 could be involved in restricting the mucosal immune response to the enteric flora (Kühn R et al., 1993). IL-10 inhibits pro-inflammatory cytokine expression and nitric oxide (NO) generation in macrophages. It has been observed that IL-10 activates different cell signaling pathways in order to exert its anti-inflammatory action. In particular, it promotes proliferation in selected immune cells through JAK/STAT3 or PI3K/Akt pathways. IL-10 can also inhibit TNFα production and hemeoxygenase-1 induction in macrophages through the activation of p38 MAPK pathway (Paul G et al., 2012). The TGFβ cytokine family is recognized as a key regulator of cell proliferation, differentiation, and apoptosis in the intestinal mucosa, as well as of the control of immunological self-tolerance, inducing Treg activation by up-regulating FoxP3 (Zhou L et al., 2008). IBD is a classic chronic disease process in which inflammatory cells, cytokines, chemokines, and intestinal flora interact, until they induce a net imbalance of the redox equilibrium of colonic mucosa, with the consequent development of an ‘‘oxy-radical overload’’ (Ullman TA et al., 2011). RNS and ROS have been demonstrated to up-regulate a number of different genes involved in adaptive and innate immune responses in the gastrointestinal tract. NO steady-state levels in the bowel mucosa during the development of inflammatory diseases may be significantly altered because of marked changes in both the synthesis and the disposition of this reactive chemical species. Enhanced recruitment of phagocytic cells, followed by the increased inducible NO synthase (iNOS) concentration, which occur in IBD affected intestinal mucosa, are accompanied by peculiar modifications of the local endothelium, expressing both iNOS and endothelial NO synthase (eNOS). iNOS appears to play a physiological role protecting against leukocyte adhesion (Binion DG et al., 2000). Indeed, a significant infiltration by neutrophils and increase in MPO levels was observed in the inflamed lamina propria of humans with UC in close approximation to the epithelia

(Kruidenier et al. 2003). iNOS-derived NO stimulates TNF-α production in the middle and distal colon, which promotes the infiltration of neutrophils for example through stimulation of synthesis of intracellular adhesion molecule (ICAM) and P-selectin, therefore leading to colonic tissue damage (Yasukawa K et al. 2012). Neutrophil recruitment and activation of key transcriptional signalling pathways like NF-κB and AP-1 augment the inflammatory response and tissue damage (Brennan et al. 1995). When activated, NF-kB translocates to the nucleus, binds DNA and subsequently activates gene expression. The activated genes involved in mucosal inflammation include cytokines IL-6, IL-8 IL-1β, IL-10, TNF-α and ICAM (Yasukawa K et al. 2012). Regarding, ROS comprise many species, that is, superoxide (O_2^-), hydroxyl radical (HO^-), and H_2O_2. Various studies have demonstrated that ROS are implicated in determining mucosal injury in UC (Pravda J, 2005). Gene expression profiles in the three different mouse models of colitis revealed alterations in the expression of important genes involved in the regulation of H_2O_2 production in the intestinal tissue (te Velde AA et al., 2008). Furthermore, the mucosal barrier dysfunction has been related to disorganization of the cytoskeleton in epithelial cells, due to excessive ROS generation. An excess of ROS causes massive DNA damage to accumulate. An important source of oxidative DNA damage is the mitochondria, because of the central role they play in energy oxidative cell metabolism. Mitochondrial DNA is particularly exposed to ROS attack, due to its localization, close to the intracellular electron transport chain in the inner mitochondrial membrane, and to the absence of protective histones (Nishikawa M et al., 2005). Moreover, lipid oxidation products play a role in IBD (Rezaie A et al., 2007). In recent years, accelerating research has drawn attention to another redox-sensitive transcription factor, namely nuclear factor erythroid 2-related factor (Nrf2), that is known to play a key role in the regulation of several genes coding for primary antioxidant and/or type 2 detoxifying enzymes (Kobayashi M et al., 2005). Nrf2 may contribute to maintaining the homeostasis of the intestinal mucosa, by preventing and/or counteracting excess production of ROS within the intestinal epithelium and the lamina propria, as occurs in IBD. Further, Nrf2 appears to negatively modulate pro-inflammatory reactions occurring in the gut mucosa, through antioxidant

and, possibly, non-antioxidant mechanisms. Direct evidence of the Nrf2-dependent negative regulation of gut inflammation was obtained in the DSS-induced mouse model of colitis using Nrf2 wild-type and KO animals. Colitis induced in $Nrf2^{+}/^{+}$ mice after 6–7 days of 1% DSS oral administration caused the loss of about one/third of mucosal crypts. When Nrf2 KO mice underwent the same treatment, the loss of crypts was significantly greater at about two thirds. This increased vulnerability of $Nrf2^{-}/^{-}$ mice was associated with a net lower expression of antioxidant/ phase II detoxifying enzymes, such as hemeoxygenase-1 and GSH-S-transferase Mu-1. However, even more importantly, the expression of genes coding for key pro-inflammatory molecules, such as IL-1, IL-6, TNFα, iNOS, and COX-2, was significantly increased in the colonic mucosa of $Nrf2^{-}/^{-}$ mice versus the wildtype mice (Khor TO et al., 2008). With regard to oxidative stress, a biochemical condition that significantly exacerbates the expression of IBD, NADPH oxidase (NOX) is considered one of the main cellular sources of ROS; using a mouse model of LPS-induced sepsis, Nrf2 has recently been shown to be essential for the physiological regulation of enzyme's activity. According to the researchers involved, the transcription factor down-regulates the activity of protein kinase C, which is a redox-sensitive kinase, as well as reduces the consequent activation of NOX, by increasing intracellular levels of antioxidants, in particular of GSH. This would at least partly explain why Nrf2 deficient mice display increased generation, as well as increased steady-state levels, of ROS (Kong X et al., 2010). Disruption of Nrf2 activity was shown to allow an exaggerated production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα, to take place; on the contrary, activation of this transcription factor efficiently down-regulated the expression of key chemokines, such as MCP-1 and IL-8; adhesion molecules, such as VCAM-1, metalloprotease-9, as well as down-regulated COX-2-dependent inflammatory reactions (Kim J et al., 2010).

3.5 Parkinson's disease

Parkinson's disease (PD), first described as a clinical entity in 1817 by James Parkinson, is the second most common neurodegenerative disorder after Alzheimer's disease and is expected to impose an increasing social and economic burden on people (Moore et al., 2005). PD is considered one of the major neurological disorders that affect the population over 65 years of age and it is affected more than 1.5% of the global population. A majority of PD cases is idiopathic (90-95%) (Schapira et al, 2011), but there are also familial forms of PD that involve mutations in a number of genes such as α-synuclein mutations and aggregation, parkin, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), DJ-1, phosphatase and tensin homolog-inducible kinase 1 (PINK1), leucine-rich repeat kinase2 (LRRK2), and glucocerebrosidase (GBA) (Houlden et al, 2012). The mechanism by which mutation of these genes lead to degeneration of the nigral neurons have shed light to understanding of the pathophysiology of PD (Houlden et al, 2012). Various evidences suggested that the oxidative stress and reactive oxygen species (ROS) played an important role in the etiology and/or progression of disease (Dalle-Donne I et al., 2005). In both idiopathic and genetic cases of PD, oxidative stress is thought to be the common underlying mechanism that leads to cellular dysfunction and demise. PD is associated with the selective loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and DA levels in the corpus striatum of the nigrostriatal DA pathway in the brain. This loss of DA causes a deregulation in the basal ganglia that leads to the appearance of motor symptoms such as bradykinesia, resting tremor, rigidity, and postural instability as well as non-motor symptoms such as sleep disturbances, depression, and cognitive deficits (Rodriguez-Oroz et al., 2009). Dopamine is an essential neurotransmitter; it is a catechol, a good metal chelator and a potential electron donor. Dopamine coordinates metals such as Cu^{2+} and Fe^{3+}, reduces the oxidation state of the metal, and subsequently generates production of H_2O_2, setting up conditions for Fenton chemistry (Barnham K et al., 2004). The exact etiology of PD still remains elusive and the precise mechanisms that cause this disease remain to be identified (Obeso et al., 2010). At the cellular level, PD can be related to an excess production of reactive oxygen

species (ROS), to an alteration in catecholamine metabolism, to a modifications in mitochondrial electron transporter chain (METC) function or to enhancement of iron deposition in the SNpc. The failures of normal cellular processes that occur in relation to the aging process are also believed to contribute to the increased vulnerability of DA neurons (Schapira et al, 2011; Rodriguez et al., 2014). Mutations of genes (Kieburtz et al., 2013; Trinh et al, 2013), mitochondrial dysfunction, neuroinflammation and environmental factors are increasingly appreciated as key determinants of dopaminergic neuronal susceptibility in PD, and are a feature of both familial and sporadic forms of the disease (Ryan et al., 2015). The neuropathological diagnosis of PD requires the detection of marked dopaminergic neuronal loss in the SNpc and the presence of Lewy bodies, that are eosinophilic inclusions consisting of a dense core surrounded by a pale-staining of radiating filaments. The role of Lewy body in PD is still not well known, however, the discovery, that misfolded α-synuclein is a major component of the radiating filaments and is also present in neuronal processes as Lewy neurites, gave rise to ideas about the role in the neuronal loss, bringing awareness to consider PD as synucleinopathy (Taschenberger et al., 2012). The principal protein component of intracellular inclusion bodies is α-synuclein, which is ubiquitously expressed in the brain; mutations of α-synuclein (A30P and A53T) contribute to familial forms of the disease (Gasser T et al., 2001). Moreover in the neurons of the substantia nigra there is an accumulation of neuromelanin: It is a dark brown pigment that accumulates metal ions and it is known that neuromelanin consists primarily in the products of dopamine redox chemistry (Zecca L, 2003). Theoretically, the progressive neurodegeneration of PD could be produced by chronic neurotoxin exposure or by limited exposure initiating a down self-perpetuating cascade of deleterious events. The finding that people intoxicated with MPTP develop a syndrome nearly identical to PD is a prototypic example of how an exogenous toxin can mimic the clinical and pathological features of PD.

3.6 Motor and non-motor symptoms

Impaired motor function is classically used to make a clinical diagnosis of PD. The main features are bradykinesia (slowness of movement), rigidity, tremor, and postural instability with an asymmetric onset spreading to become bilateral with time. Other motor features include gait and posture changes that manifest as festination (rapid shuffling steps with a forward-flexed posture when walking), speech and swallowing difficulties, and a mask like facial expression and micrographia (Jankovic J, 2008). A good response to dopaminergic medication is confirmatory of the diagnosis. Recently it has become recognized as a more complex illness encompassing both motor and non-motor symptoms (NMS), such as depression, sleep disturbance, sensory abnormalities, autonomic dysfunction, and cognitive decline (Langston JW, 2006). NMS affect all patients with PD, the frequency of which increases with disease severity, with late-stage patients exhibiting 6-10 NMS. NMS create the biggest demand on clinical resources, they are poorly diagnosed and treated and they are the major determinant of disease outcome, increasing disability, poor quality of life, and entry into long-term care (Chaudhuri KR et al., 2009). The causes of motor dysfunction in PD are reasonably well understood, the cause of NMS in PD remains poorly researched and they may largely relate to pathology outside of the basal ganglia. The most important feature of NMS is that some, for example, olfactory deficits, constipation, rapid-eye-movement sleep, behavior disorder, and depression, may precede the onset of motor symptoms by many years (although they can occur at the same time as motor symptoms or follow the onset of motor abnormalities) (Muzerengi S et al., 2007). NMS may in the future be used for the early diagnosis of PD, enabling neuroprotective strategies to be introduced at an early stage, and studies of large populations of apparently normal older individuals are ongoing at this time to enable such early detection to occur (Stern MB et al., 2012).

3.7 Epidemiology

The prevalence of PD in industrialized countries is about 0.3% of the population. This rises with age from 1% in those over 60 years of age to 4% of the population over 80. The age at which there is the appearance of PD is approximately 60 years; however, 10% of cases are classified as young onset, occurring between 20 and 50 years of age, which may represent a distinct disease group. Cross-cultural variations in the prevalence of PD are potentially interesting from an etiological point of view, as they might result from differences in environmental exposures or distribution of susceptibility genes (Van Den Eeden et al., 2003). PD is more prevalent in men than in women, with reports of ratios of 1.1:1 to almost 3:1 being quoted (Schrag A, et al., 2000), which may be attributable to the protective effects of estrogen in women (Popat RA et al., 2005). About 25‑40% of the patients with PD eventually develop dementia, due to spread of degeneration and Lewy bodies to the cerebral cortex and limbic structures. Epidemiological studies have reported that the risk of dementia is 1.7‑5.9 times higher in patients with PD than in healthy people (Emre, 2003).

3.8 Genetics of PD

Investigation of familial PD has so far revealed at least 17 autosomal dominant and autosomal recessive gene mutations responsible for variants of the disease (Houlden H et al., 2012). These include α-synuclein mutations and triplication, parkin, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), DJ-1, phosphatase and tensin homolog-inducible kinase 1(PINK1), leucine-rich repeat kinase 2 (LRRK2), and glucocerebrosidase (GBA). Of these parkin and LRRK2 are probably the most common genetic link to young-onset and late-onset PD, respectively, whereas GBA mutations maybe the most common risk factor. Autosomal dominant mutations are typified by mutations in α-synuclein and LRRK2. Although α-synuclein mutations are only rarely encountered, their discovery led directly to α-synuclein being identified as a major component of Lewy bodies and Lewy neurites and the labeling of PD as a synucleinopathy, sustaining much of the consensus on the final stages of neuronal loss in PD being related to altered protein aggregation (Goedert M, 2001).

Mutations in LRRK2 seem to alter kinase/GTPase of this mixed lineage-like kinase found in cytoplasm and outer membrane of mitochondria (Gupta A et al., 2008). LRRK2 interacts with another PD-related protein, parkin, and mutant LRRK2 induces apoptotic cell death in cultured neurons. Autosomal loss of function mutations include those in the ubiquitin E3 ligase parkin, which in combination with the ubiquitin-conjugating enzyme causes the attachment of ubiquitin as a marker on proteins destined for destruction by the proteasome. Additionally mutations in the mitochondrial PINK1 protect cells from mitochondrial stress/dysfunction, and the rarest mutation, in the redox-sensitive chaperone DJ-1, protects cells against oxidative stress (Gupta A et al., 2008). More recently, interest in the role of mitochondria has restricted from genetic investigations in familial PD. Notably mutations in α synuclein, parkin, PINK1, and DJ-1 and perhaps LRRK2 have been associated with altered mitochondrial function (Schapira AH et al., 2011) (Figure 5).These mutations can lead to altered protein localization in mitochondria in PD, abnormalities in mitochondrial structure and function, and a decrease in complex I assembly and activity. Loss of function of DJ-1, but also parkin and PINK1, decreases mitochondrial protection against oxidative stress, which in turn increases mitochondrial dysfunction. Other important role for parkin and PINK1 is in the turnover of mitochondria by autophagy, specifically mitophagy; they act in tandem to regulate this process. This may be critically important in PD, in which autophagy seems impaired, reducing the ability of cells to remove damaged mitochondria.

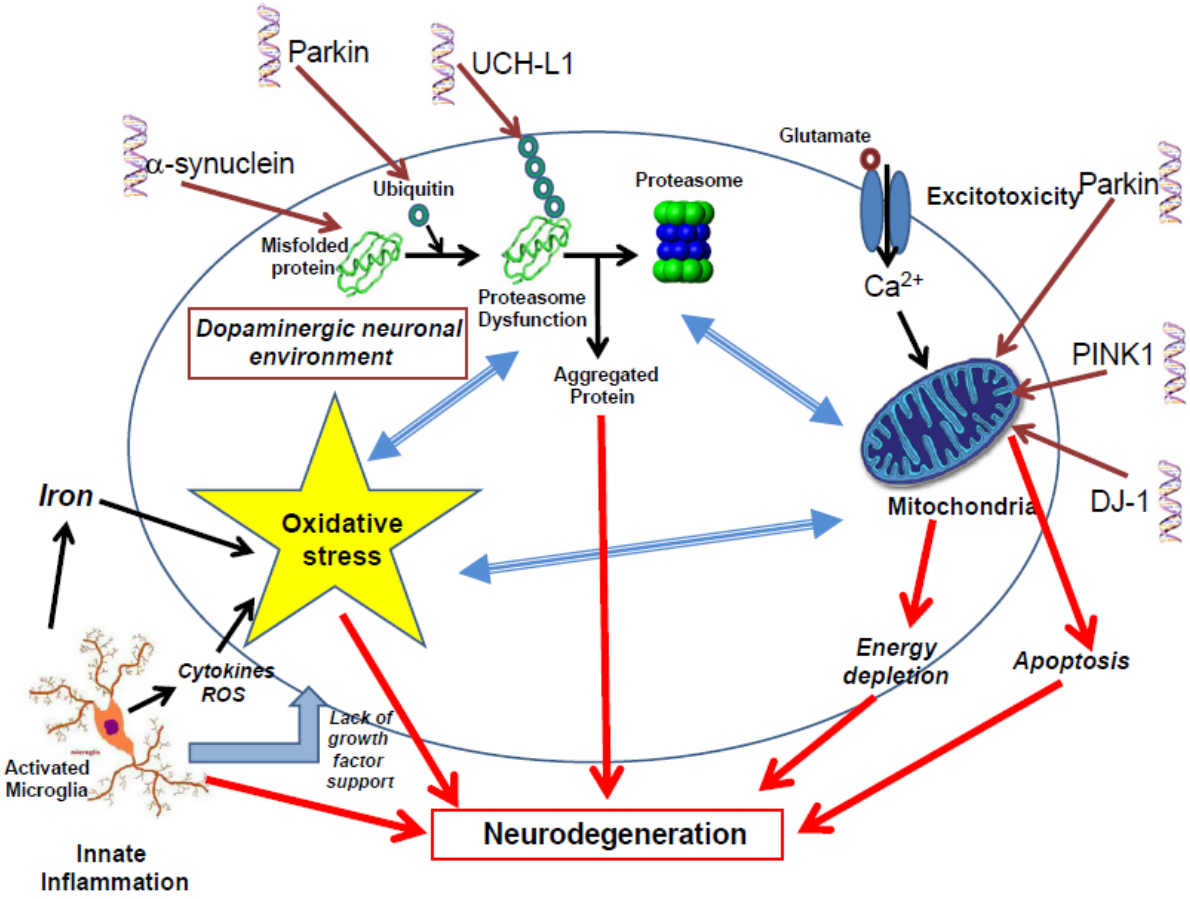


Figure 5: Key molecular mechanisms that are widely accepted to contribute to the neurodegenerative process in dopaminergic neurons in the substantia nigra in PD (Dexter et al, 2013).

3.9 Oxidative stress and neuroinflammation in PD

Major causes of neurodegeneration are mitochondrial impairment, oxidative stress and neuroinflammation. In this context it is interesting to note that although the adult human brain constitutes only about 2% of body weight, it consumes about 20% of the body's oxygen and glucose for the production of energy in the form of adenosine triphosphate (ATP) (Purdon AD et al., 2002). Thus, this organ is particularly exposed to the consequences of mitochondrial energy failure. The anti-oxidant defenses of the brain such as catalase, superoxide dismutase, glutathione, and glutathione peroxidase are relatively scarce, and under oxidative stress conditions result in lipid peroxidation and the generation of toxic products (Liu X et al., 2008). One of the sources of oxidative stress is the neurotransmitter DA; it is synthesized from tyrosine by tyrosine hydroxylase and aromatic amino acid decarboxylase, following this, DA is stored in synaptic vesicles, excess

cytosolic DA is easily oxidized both spontaneously and enzymatically to produce DA quinone. The DA quinone species are capable of covalently modifying cellular nucleophiles, including low molecular weight sulfhydryls such as GSH and protein cysteine residues, whose normal functions are important for cell survival. Notably, DA quinone has been shown to modify a number of proteins whose dysfunctions have been linked to PD pathophysiology, such as α-synuclein, parkin, DJ-1, SOD2 and UCH-L1. DA quinone covalently modifies α-synuclein monomer and promotes the conversion of α-synuclein to the cytotoxic protofibril form (Conway KA et al., 2001). Parkin is also covalently modified by DA and becomes insoluble, that leads to inactivation of its E2 ubiquitin ligase activity. Catechol-modified parkin has been detected in the substantia nigra but no in other regions of the human brain, and parkin insolubility is observed in PD brain (LaVoie MJ et al., 2005). DA quinone has also been shown to cause inactivation of the DA transporter (DAT) and tyrosine hydroxylase (TH) (Kuhn DM et al., 1999). In addition, it leads to mitochondrial dysfunction and swelling of brain mitochondria (Lee CS et al., 2002). Furthermore, DA quinone can cyclize to become the highly reactive aminochrome, whose redox-cycling leads to generation of superoxide and depletion of cellular NADPH, and which polymerizes to form neuromelanin. Neuromelanin in turn can exacerbate the neurodegenerative process by triggering neuroinflammation (Zecca L et al., 2008). Therefore, neuromelanin is the dark insoluble polymer produced from DA oxidation and confers the dark pigmentation to the substantia nigra. Insoluble extraneuronal neuromelanin granules have been observed in patients of juvenile PD (Ishikawa et al, 1998) and idiopathic PD, as well as those with MPTP-induced parkinsonism (Langston et al, 1999). Addition of neuromelanin extracted from PD brain to microglia culture caused increases in nitric oxide (Wilms et al, 2003). Intracerebral injection of neuromelanin caused strong microglia activation and a loss of DAergic neurons in the substantia nigra (Zecca et al, 2008). Neuromelanin appears to remain for a very long time in the extracellular space (Langston et al, 1999) and thus thought to be one of the molecules responsible for inducing chronic neuroinflammation in PD. Furthermore, hydrogen peroxide is generated during DA metabolism by monoamine oxidase and is

subsequently converted to the highly reactive hydroxyl radical in the presence of transition metal ions (Halliwell B, 1992), contributing to oxidative stress. Besides ROS, evidence also exists for the involvement of reactive nitrogen species (RNS) in mediating nitrosative stress (Malkus KA et al., 2009). RNS are generated by the quick reaction of superoxide with nitric oxide (NO), which results in the production of large amounts of peroxynitrite ($ONOO^-$) (Szabo C et al., 2007). NO is produced by NO synthase (NOS), which has three isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS) identified in neurons, and inducible NOS (iNOS) identified in glial cells (Hirsch EC et al., 2003). NO is present within cells and in the extracellular space surrounding dopaminergic neurons produced by either nNOS or iNOS (Tieu K et al., 2003). Additionally, with gliosis, activated glial cells expressing iNOS could contribute to increasing the levels of NO (Mander P et al., 2005). NO inhibits several enzymes including complexes I and IV of the mitochondrial electron transport chain, leading to ROS generation. It also reacts with proteins to form S-nitrosothiols thus altering their function, and with lipids causing their lipid peroxidation (Carr AC et al., 2000). Neuronal loss in PD is associated with chronic neuroinflammation, which is controlled primarily by microglia (Barcia et al., 2003), the resident innate immune cells and the main immune responsive cells in the central nervous system, and, to a lesser extent, by astrocytes and oligodendrocytes (Perry, 2012). Microglial reaction has been found in the SN of sporadic PD patients (McGeer PL et al., 1988) as well as familial PD patients (Yamada T et al., 1993) and in the SN and/or striatum of PD animal models elicited by MPTP (O'Callaghan JP et al., 1990). Microglia are activated in response to injury or toxic insult as a self-defensive mechanism to remove cell debris and pathogens. Additional activation of microglia may be due to the release of aggregated proteins from neurons into the extracellular space (Roodveldt et al., 2008). Extracellular a-synuclein is phagocytized by microglia (Zhang et al., 2005), and aggregated, nitrated, and oxidized forms of a-synuclein have been found to induce microglial activation (Zhang et al., 2005). When activated, they release free radicals such as nitric oxide and superoxide, which can in turn contribute to oxidative stress in the microenvironment. Over-activated and/or chronically activated state of microglia causes excessive

and uncontrolled neuroinflammatory responses, leading to a self-perpetuating vicious cycle of neurodegeneration (Qian L et al., 2010). Studies have demonstrated that microglia are activated in PD and increased levels of pro-inflammatory mediators such as TNF α, interleukin-1beta (IL-1β), IL-6, iNOS and cyclo-oxygenase-2 (Cox-2) are found in the striatum and in the substantia nigra (Tansey et al., 2007). Another type of cells present in CNS are astrocytes, they are one of the two primary types of macroglia (Sofroniew, 2005). They comprise nearly 35% of the total CNS cell population and like microglia are found in all regions of the CNS. Histologically, astrocytes can be visualized by immunolabeling with antisera specific for glial fibrillary acidic protein (GFAP), S100b or the astrocyte specific glutamate transporters, GLT1 and GLAST (Walz, 2000). Astrogliosis and astroglial scar formation (usually monitored only by increased GFAP immunoreactivity) have been found to be nearly as common a response to CNS injury and dysfunction as microgliosis (Liberto et al, 2004). Recent studies have highlighted the significant role that oxidative stress and impairment of antioxidant defense mechanisms play as major contributors to disease pathogenesis (Lin MT et al., 2006). Nrf2 is a transcription factor in the phase II antioxidant and xenobiotic response pathway and is termed a 'master regulator' of expression for many antioxidant and detoxification pathway genes (Alfieri A et al., 2011). At basal levels, NRF2 is constitutively degraded in the cytoplasm by its antagonist, KEAP1 (Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1). KEAP1 forms a complex with CUL3-RBX1 and regulates NRF2 through targeted ubiquitination and subsequent degradation. The mechanism of action for NRF2 begins upon exposure to oxidative stress, xenobiotics, or electrophilic compounds. This causes the modification of cysteine-151 of KEAP1 and subsequent stabilization and translocation of NRF2 to the nucleus where it binds to the antioxidant response element (ARE) of its target genes (Alfieri A et al., 2011). NRF2 activity and expression also significantly decrease with age, the most common predisposing factor for PD (Suh JH et al., 2004). In addition, other molecules directly or indirectly regulated by NRF2 have been strongly linked

with PD; these include glutathione, heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1) (Todorovic M et al., 2015) (Figure 6).

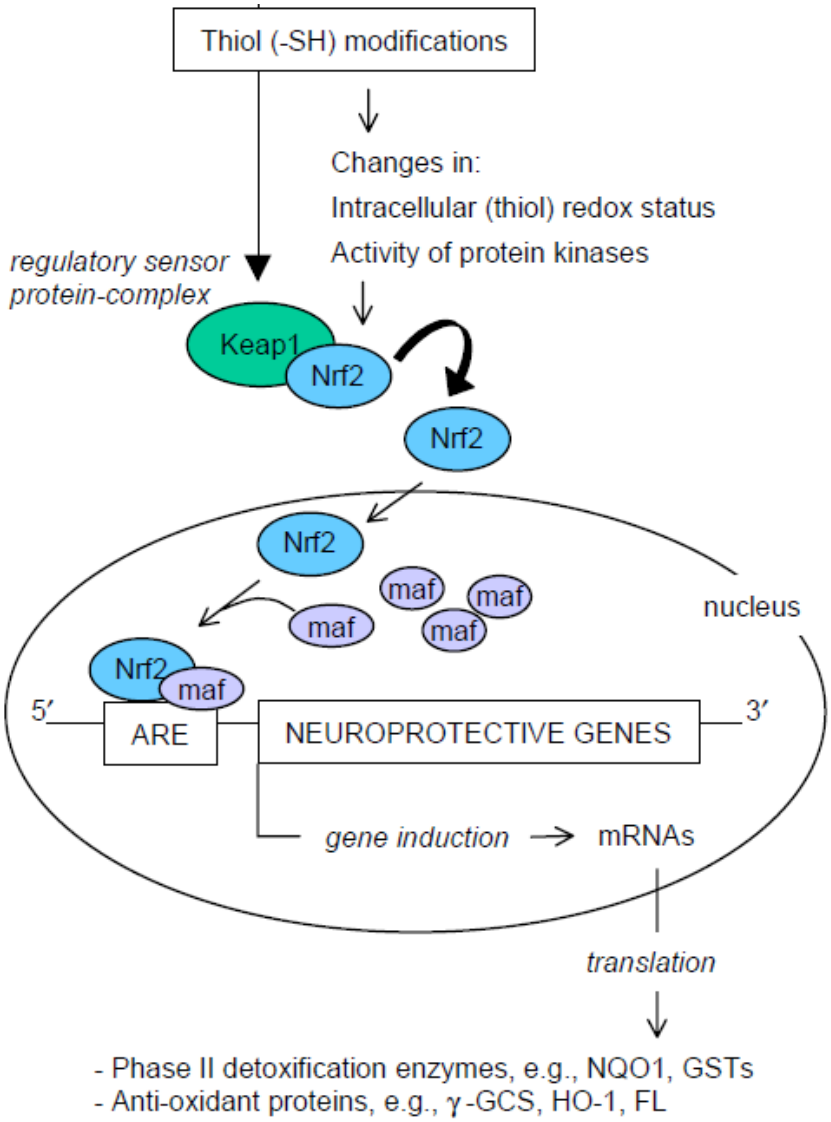


Figure 6: Nrf2's pathway (Drukarch et al, 2001).

The master regulator of the cellular antioxidant defense system, the Nrf2-ARE pathway is a logical target to examine for neuroprotection against misfolded proteins induced pathology. Activation of the Nrf2-ARE pathway has been shown to be protective against the toxic forms of αSyn in several studies. In SK-N-SH neuroblastoma cells, ferrous iron promotes αSyn aggregation through inhibiting Nrf2 pathway (He et al., 2013). αSyn aggregation exacerbates ferrous iron-induced oxidative damage, mitochondrial impairment and apoptosis. Overexpression of Nrf2 downstream gene HO-1 is able to reverse the toxicity. In a Drosophila model of PD with αSyn, it was found that

transgenic activation of Nrf2 by overexpression of Nrf2 or Maf-S and knockdown of Keap1 could delay the αSyn-mediated dopaminergic neuron loss and motor dysfunction (Barone et al 2011). Consistently, genetic deletion of Nrf2 enhances αSyn toxicity delivered via an adeno-associated viral vector and exaggerates αSyn/p-αSyn accumulation in dopaminergic neurites and gliosis in vivo (Lastres-Becker et al., 2012). αSyn treatment activates the Nrf2 and NF-κB pathway and promotes proinflammatory cytokine production, as well as phagocytosis in vitro. Nrf2 deficiency enhances the inflammatory response and lowers the capability of phagocytosis in primary microglial cells.

Chapter 4

Aim of thesis

Inflammation causes a wide variety of physiological and pathological processes. Although the pathological aspects of many types of inflammation are well appreciated, their physiological functions are mostly unknown. The classic instigators of inflammation are at one end of a large range of adverse conditions that induce inflammation, and they trigger the recruitment of leukocytes and plasma proteins to the affected tissue site. As described previously in this thesis, FAEs showed a lot beneficial effects in many pathologies such us psoriasis and MS. Therefore, the purpose of this thesis was to investigate the effect of DMF in some of the main inflammatory disease in both peripheral tissue and central nervous system. In the first instance, the effects of DMF on the inflammatory response and colon injury caused by intra-colonic administration of dinitrobenzene sulfuric acid (DNBS) to normal mice that displays human CD-like features. To demonstrate that DMF exerts this beneficial therapeutic effect by interfering with neutrophilic infiltration and release of pro-inflammatory mediators (e.g. ROS, TNF-α) we studied the effects of DMF on: the degree of colonic injury after DNBS, neuthrophil infiltration, cytokines expression, the rise of oxidative stress as well as the expression of MMP-9 and -2 caused by DNBS in the colon; involvement of NF-κB and Nrf-2 pathways and barrier dysfunction in human intestinal epithelial cells. Later, it was decided to study the effects of DMF in Parkinson's disease-induced neuroinflammation. To show how DMF exerts neurotherapeutic action NFκB-Nrf2 dependent, we studied the effects of DMF both *in vivo* and *in vitro* models. The *in vivo study* aimed to study the neuronal degeneration of dopaminergic tract induced by MPTP; behavioral impairments, α-synuclein-induced neurodegeneration; antioxidant response activation and the anti-neuroinflammatory activity. The *in vitro* study aimed to study DMF mechanism of action in SH-SY5Y cells by using Nrf-2 antagonist.

Chapter 5

Materials and methods colitis

5.1 *In vitro studies*

5.1.2 Cell Culture

The human intestinal colorectal adenocarcinoma cell line, Caco2, was cultured in Dulbecco's modified Eagle's medium. Culture media contained 10% fetal bovine serum (FBS), 2 mM L–glutamine, 100 U/ml penicillin, 100mg/ml streptomycin and 1% non-essential amino acids. Cells were cultured at 37°C in a humidified 5% CO2 and 95% filtered air and the culture medium was replaced every 2 days. After washing in phosphate buffered saline (PBS), cells were trypsinized with 0.25% trypsin-EDTA at 37°C for 5 min., centrifuged at 1000 ×g for 3 min and then re-suspended in the appropriate medium. Cell viability was determined by trypan blue staining. Cell line was purchased from ATCC (Rockville, MD, USA). For Caco2, experiments were initiated on days 14–15 after seeding and 3–7 days after confluence. Previous studies in our laboratory have shown this is a time at which the cells begin to express alkaline phosphatase activity and are in an early stage of differentiation, corresponding to the upper crypt-lower villus stage of differentiation. H_2O_2 (Sigma Aldrich, Milan, Italy) 500 μM was added to culture medium in presence or absence of 10 μM DMF (Sigma Aldrich, Milan, Italy) dissolved in PBS. Proteins were extracted for western blot analyses. The concentration of DMF used in this study was chosen on the basis of viability test performed in our laboratory (data not shown) and on the basis of recent literature data (Bernadais et al., 2013).

5.1.3 Western Blot Analysis

Cell lysates were collected after 6 h and proteins were extracted for western blot analysis, it was performed as previously described (Siracusa et al., 2015). The membrane was incubated overnight

at 4°C with: anti-HO-1 (1:1000; StressGen Biotech), or anti-ZO-1 (1:1000; Cell Signaling), or anti-occludin (1:500; ThermoFisher). To ascertain that blots were loaded with equal amounts of protein lysate, they were also incubated with the antibody β-actin (1:500; Santa Cruz Biotechnology). Signals were detected as described before (Siracusa et al., 2015).

5.2 *In vivo studies*

5.2.1 Animals

IL-10KO mice and corresponding WT controls were purchased from Jackson Laboratories (Charles River, Italy).Male adult CD1 mice (25-30 g, Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light, 12-h dark cycle. The animals were acclimated to their environment for two weeks under standard conditions, with free access to tap water and standard rodent diet. The study was approved by the University of Messina Review Board for the care of animals. All animal experiments were carried out with the accordance with the national law on animal protection (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA).

5.2.2 Experimental Groups

CD1 mice were randomly divided into the following groups (n = 6 for each group):

1. Sham+vehicle group: vehicle solution (saline) was administered by oral gavage for 4 days;

2. Sham+dimethylfumarate (10mg/kg): DMF (10mg/kg) was administered by oral gavage for4 days;

3. Sham+dimethylfumarate (30mg/kg): DMF (30mg/kg) was administered by oral gavage for4 days;

4. Sham+dimethylfumarate (100mg/kg): DMF (100mg/kg) was administered by oral gavage for 4 days;

5. DNBS + vehicle: vehicle (saline) was administered by oral gavage for 4 days starting from 3h after the administration of DNBS;

6. DNBS + DMF (10mg/kg): DMF was administered as by oral gavage at 10mg/kg every 24 h,starting from 3h after the administration of DNBS;

7. DNBS + DMF (30mg/kg): DMF was administered as by oral gavage at 30mg/kg every 24 h, starting from 3h after the administration of DNBS;

8. DNBS + DMF (100mg/kg): DMF was administered as by oral gavage at 100mg/kg every 24 h, starting from 3h after the administration of DNBS.

The dose of DMF (10, 30 and 100 mg/kg) used here were based on previous dose-response and time-course studies by our laboratory.

Moreover, as therapeutic approach, we evaluated the therapeutic activity of DMF (100 mg/kg) for 7 weeks on 9-week-old IL-10KO. IL-10KO mice spontaneously develop a Th1-dependent chronic enterocolitis shortly after birth that is fully established at 8-10 weeks of age (Cannarile L et al., 2009).

5.2.3 Induction of experimental colitis

Colitis was induced by intrarectal administration with a very low dose of DNBS (4 mg per mouse) by using a modification of the method first described in rats (Sturiale et al., 1999). In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were anesthetized by Enflurane. 2, 4, 6-Dinitrobenzene sulfonic acid, DNBS, (4 mg in 100 µl of 50% ethanol) was injected into the rectum through a catheter inserted 4.5 cm proximally to the anus. Vehicle alone (100 µl of 50% ethanol) was administered in control experiments (sham).

Thereafter, the animals were kept for 15 min in a trendelenburg position to avoid reflux. After colitis and sham-colitis induction, the animals were observed for 4 days. At the end of the experiment period, animals were weighed, killed, and the colon was removed, opened along the anti-mesenteric border, rinsed, weighed, and processed for histology, immunohistochemistry, cell separation, and Western blot analysis. Colon damage (macroscopic damage score) was evaluated and scored by 2 independent pathologists as described previously 17 according to the following criteria: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers with no significant inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending greater than 1 cm along the length of the colon; and 5–8, one point is added for each centimeter of ulceration beyond an initial 2 cm.

5.2.4 Histological examination

After fixation at room temperature in buffered formaldehyde solution (10% in phosphate buffered saline), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey). Thereafter, 7 μm sections were de-paraffinized with xylene, stained with haematoxylin-eosin, observed with a Axostar Plus equipped with AxioCamMRc (Zeiss, Milan, Italy) and studied using an Imaging computer program (AxioVision, Zeiss, Milan, Italy). The following morphologic criteria were considered as reported by Cannarile et al. 2: score 0, no damage; score 1 (mild), focal epithelial edema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; and score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage. Colon damage (sections n= 6 for each animals) was scored by 2 independent pathologists blinded to the experimental protocol.

5.2.5 Myeloperoxidase Activity

At 4 days after intracolonic injection of DNBS, the colon was removed and weighed. The colon was analyzed for myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a previously method described (Mullane KM et al., 1985). Each piece of tissue was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer pH 7 and centrifuged for 30 min at 20,000 × g at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37 °C and was expressed in U/g wet tissue.

5.2.6 Thiobarbituric acid- reactant substances measurement

As a marker of lipid peroxidation, thiobarbituric acid-reactant substances measurement was determined, as previously described in the colon tissue at 4 days after DNBS administration. Thiobarbituric acid-reactant substances were calculated by comparison with OD532 of standard solutions of 1, 1, 3, 3-tetramethoxypropan 99% malondialdehyde bis (dymethylacetal) 99% (MDA) (Sigma, Milan). The absorbance of the supernatant was measured by spectrophotometer at 532 nm.

5.2.7 Western blot analysis for manganese superoxide dismutase (Mn-SOD), IκB-α, NF-κB, Nrf-2, IL-1β, TNF-α, MMP-9, MMP-2

Tissue or cellular samples from the terminal colon were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 × g for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1

mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 μm leupeptin, 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000 g at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. The expression of Mn-SOD, IκB-α,IL-1β, TNF-α, MMP-9 and MMP-2, was quantified in cytosolic fraction from colon tissues collected 4 days after DNBs-induced colitis. NF-κB and Nrf-2 expression were quantified in nuclear fraction from colon tissues collected 4 days after DNBs-induced colitis. The filters were blocked with 1 × PBS, 5% (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs anti-MnSOD (1:500; Millipore),or anti IκB-α (1:1000; Santa Cruz Biotechnology), or anti-IL-1β (1:500; Santa Cruz Biotechnology), or anti-MMP-9 (1:500; Millipore) or anti-MMP-2 (1:500; Millipore), or anti-NF-κB (1:1000; Santa Cruz Biotechnology), or Nrf-2 (1:500; Santa Cruz Biotechnology), or anti-TNF-α (1:250; Abcam) in 1 × PBS, 5% w/v non fat dried milk, 0.1% Tween-20 (PMT) at 4 °C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) 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 1:1000 Sigma–Aldrich Corp.) 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 (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA). The relative expression of the protein bands of Mn-SOD (~24 kDa), IκB-α (~37 kDa), NF-κB p65 (~65 kDa), IL-1β (~31 kDa), TNF-α (~17kDa), MMP-9 (~92 kDa), MMP-2 (~72 kDa), and Nrf-2 (~61kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM), and standardized for densitometric analysis to β-actin or lamin A/C levels. A preparation of commercially available molecular weight markers (Precision Plus Protein Standard, Bio-Rad,

Hercules, CA, USA), consisting of proteins of molecular weight 10–250 kDa, was used to define molecular weight positions and as reference concentrations for each molecular weight.

5.2.8 Immunohistochemical localization of ICAM-1, P-Selectin and TNF-α

At 4 days after DNBS administration, colon tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 7 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (Vector Laboratories, Burlingame, CA), respectively. Sections were incubated overnight with: 1) purified goat polyclonal antibody directed towards P-selectin (Santa Cruz Biotechnology, C-20:sc-6941, 1:200 in PBS, v/v) or 2) purified hamster anti-mouse ICAM-1 (CD54) (AbDSerotec, MCA1371Z, 1:100 in PBS, w/v) or 3) purified goat polyclonal antibody directed towards TNF-α (Santa Cruz Biotechnology, C-20:sc-6941, 1:200 in PBS, v/v). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vector Laboratories, Burlingame, CA).

5.3 Materials

All compounds were obtained from Sigma–Aldrich (Milan, Italy). All chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

5.4 Statistical evaluation

The authors performed three distinct experiments with six animals per groups for each experiment. All values in the figures and text are expressed as mean ± standard deviation (SD) of N observations. For the in vivo studies N represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least 3 experiments performed. The results were analyzed by one way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

Chapter 6

Materials and methods Parkinson's disease

6.1 *In vivo studies*

6.1.1 Animals

Male CD1 mice (Envigo, Italy, 30-34g) were accommodated in a controlled environment and supplied with standard rodent chow and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1°C with a 12-h light, 12-h dark cycle. The study was recognized by the University of Messina Review Board for the care of animals. All 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 USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA).

6.1.2 MPTP-induced Parkinson's disease and treatment

Eight-week-old male CD1 mice were treated with MPTP. For MPTP intoxication, mice received four intraperitoneal injections (i.p.) of MPTP (20 mg/kg; Sigma, St. Louis, MO) in saline at 2 hours intervals in 1 day: the total dose per animal is 80 mg/kg. For DMF treatment (Sigma Aldrich) (10, 30 and 100 mg/kg in 10 % carboxymethylcellulose, CMC) mice received by oral gavage (o.s) DMF starting 24 hours after the first MPTP injection and continuing through 7 additional days after the last administration of MPTP. Mice were anesthetized by ketamine and xylazine (2.6 and 0.16 mg/kg body weight respectively) administered i.p. and then decapitated with large bandage scissors, their brains were harvested, sectioned and processed. The dose of MPTP (20 mg/kg) used here was based on previous in vivo studies (Esposito et al., 2012), while doses of DMF used here were based on our previous study on colitis experiment (Casili et al., 2015) and dose response and time-course studies by our laboratory.

6.1.3 Experimental group:

Animals were randomly distributed into the following groups:

Group 1: Sham = vehicle solution (saline) was administrated during the first day, as MPTP protocol, i.p (N=20).

Group 2: Sham + DMF 10 mg/kg = DMF solution was administrated by oral gavage for 7 days (N=20).

Group 3: Sham + DMF 30 mg/kg =DMF solution was administrated by oral gavage for 7 days (N=20).

Group 4: Sham + DMF 100 mg/kg=DMF solution was administrated by oral gavage for 7 days (N=20).

Group 5: MPTP + vehicle = MPTP solution was administrated during the first day, as MPTP protocol, i.p and vehicle solution (saline + CMC) was administrated by oral gavage for 7 days after the last administration of MPTP (N=20).

Group 6: MPTP + DMF 10 mg/kg = MPTP and DMF solution was administrated by oral gavage for 7 days after the last administration of MPTP (N=20).

Group 7: MPTP + DMF 30 mg/kg = MPTP and DMF solution was administrated by oral gavage for 7 days after the last administration of MPTP(N=20).

Group 8: MPTP + DMF 100 mg/kg = MPTP and DMF solution was administrated by oral gavage for7 days after the last administration of MPTP (N=20).

Experimental data regarding 2, 3, 4 groups are related only to histological evaluation because we observed that DMF administration didn't demonstrate any toxicity, improvement any difference in mice respect to sham controls, as observed in revised Figure 1. Furthermore, MPTP+DMF 100

mg/kg group was only subjected to histological and behavioral analysis because this group had the same effect of the DMF 30mg/kg group, so we decided to continue to analyze only DMF 10 and 30 mg/kg treatments.

6.1.4 Cytosolic and nuclear extracts from midbrain and Western Blot analysis

Tissue samples from the brain were processed as previous described (Esposito et al., 2012). The expression of manganese superoxide dismutase (Mn-SOD), hemeoxygenase 1 (HO-1), neuronal nitric oxide synthase (nNOS), IκB-α, cyclooxygenase 2 (COX-2), Interleukin (Il)-1β, CD11β, ionized calcium-binding adapter molecule 1 (Iba-1) and α-synuclein was quantified in cytosolic fraction from brain tissues. Nuclear factor erythroid 2- related factor 2(Nrf-2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), expressions were quantified in nuclear fraction from brain tissues. The filters were probed with specific Abs anti-MnSOD (1:500; Millipore) or anti-HO-1 (1:500; Santa Cruz Biotechnology), or anti-nNOS (1:500; Cell Signaling), or anti-NF-κB (1:500; Santa Cruz Biotechnology), or anti IκB-α (1:500; Santa Cruz Biotechnology), or anti-COX-2 (1:500; Cayman), or anti-Il-1β (1:500 Santa Cruz Biotecnology), or anti-CD11β (1:500; Biorad Antibodies), or anti-Iba-1 (1:500; Santa Cruz Biotechnology) or anti- α-synuclein (1:500; Santa Cruz Biotechnology), or Nrf-2 (1:500; C-20 sc-722, Santa Cruz Biotechnology) in 1 × PBS, 5% w/v non fat dried milk, 0.1% Tween-20 at 4 °C, overnight. 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) 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, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDoc^{TM}XRS+software and standardized to β-actin and lamin A/C levels. Images of blot signals (8 bit/600 dpi resolution) were imported to analysis software (Image Quant TL, v2003).

6.1.5 Histology

Brain tissues were taken 8 days after MPTP injection. Tissue section were stained with Hematoxylin/Eosin (H&E) and studied using light microscopy connected to an Imaging system (AxioVision, Zeiss, Milan, Italy) as previously described (Siracusa et al., 2015). Histological assessment was made by blinded observation and slides were scored for severity of pathological profiles after H&E staining using a semiquantitative 5-point rating: 0 = no pathology; 1 = mild pathology; 2 = moderate pathology; 3 = severe pathology; 4 = more severe pathology (Siracusa et al., 2015).

6.1.6 Behavioral testing

Behavioral assessments on each mouse were made 7 days after MPTP injection.

6.1.6a Pole test

The test consists of an about 50 cm high, gauze-taped pole (1cm in diameter). Mice are placed with their head upwards right below the top. Were considered two parameters: the time until the animals have turned by 180°, and the time until they have descended to the floor (Sedelis et al., 2001).

6.1.6b Open field test

The test consists of an open field boxes (80x80 cm) and mice had been placed into for a period of 5 minutes. In the test is observed the animal's activity (Rial et al., 2014).

6.1.6c Rotarod test

The rotarod treadmill (Accuscan, Inc., Columbus, OH, USA) provided a motor balance and coordination assessment. For testing, the animals were subjected to three trials and the average score on these three trials was used as the individual rotarod score, as previous described (Campolo et al., 2013).

6.1.7 Immunohistochemical localization of TH, DAT, Nitrotyrosine, MAP-2, NGF, α-synuclein

We performed immunohistochemical localization as previously described (Esposito et al., 2012). Sections were incubated overnight with anti-tyrosine hydroxylase (anti-TH) polyclonal antibody (Millipore, 1:250 in PBS, v/v), anti-dopamine transporter (anti-DAT) antibody (Santa Cruz Biotechnology, 1:100 in PBS, v/v), anti-nitrotyrosine antibody (Millipore, 1:250 in PBS, v/v), anti microtubule associated protein 2 (MAP-2) antibody (millipore, 1:250), anti-nerve growth factor (anti-NGF) antibody (1:250, Santa Cruz Biotechnology), anti-α-synuclein antibody (1:50, Santa Cruz Biotechnology). Immunohistochemical photographs were assessed by densitometric analysis using Imaging Densitometer (AxioVision, Zeiss, Milan, Italy).

6.1.8 Stereological nigral dopamine neuron counts

Unbiased counting of TH^+ dopaminergic neurons within the SN was performed as previously described (Lee et al., 2012). Every fourth free floating section was incubated with polyclonal rabbit anti-TH (1:400, Millipore) overnight and processed with the ABC method (Vector Laboratories, Burlingame, CA). The stereologist was blind to the treatment received. For each mouse brain, five selected representative sections of the SNpc were analyzed with Stereo Investigator software (Microbrightfield, Williston, VT).

6.1.9 Immunofluorescence staining of Nrf-2 and NeuN

Sections were processed for immunofluorescence staining as previous described (Siracusa et al., 2015). Sections were incubated with rabbit anti-Nrf-2 (1:100, Santa Cruz Biotechnology) or mouse monoclonal anti-neuronal nuclei (anti-NeuN) (1:100, v/v Millipore) antibody in a humidified chamber for O/N at 37°C. Sections were observed and photographed at x20 magnification using a Leica DM2000 microscope (Leica). All images were digitalized at a resolution of 8 bits into an array of 2560×1920 pixels. Optical sections of fluorescence specimens were obtained using a HeNe

laser (543 nm), a laser UV (361–365 nm) and an argon laser (458 nm) at a 1-min, 2-s scanning speed with up to 8 averages; 1.5-μm sections were obtained using a pinhole of 250. Contrast and brightness were established by examining the most brightly labeled pixels and applying settings that allowed clear visualization of structural details while keeping the highest pixel intensities close to 200. The same settings were used for all images obtained from the other samples that had been processed in parallel. Digital images were cropped and figure montages prepared using Adobe Photoshop 7.0 (Adobe Systems; Palo Alto, CA).

6.1.10 Measurement of reduced glutathione and oxidized glutathione

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in the substantia nigra and cerebral cortex by the GSH assay kit (Cayman) using enzymatic recycling, as previously described (Lee et al., 2013). Absorbance was measured at 405 nm. GSH concentration of each sample was calculated as nmol/mg protein.

6.2 *In vitro studies*

6.2.1 Cell Culture

Neuroblastoma SH-SY5Y cells (ATCC® CRL-2266 ™) were cultured as previously described (Siracusa et al., 2015). In preliminary experiments to assess cell viability, $3x10^4$ cells were plated in a volume of 150 μl in 96-well plates. Increasing concentrations of DMF (1- 10- 30-50-100 μM) were used to determine the effective concentration with minimal cytotoxicity. The concentrations chosen was DMF 1, 10 and 30 μM. In another set of experiments $8x10^5$ cells were plated and incubated with retinoic acid (100 nM) for 24 h to induce differentiation. After this time, cells were pre-treated for 2 h with DMF at a concentration of 1, 10 and 30 μM (based on previous 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric cell viability assay), followed by addition of MPTP to a final concentration of 3 mM. The protective concentration chosen was

DMF 30μM, considering that DMF 1 and 10 μM were not protective following MPTP damage. Then, trigonelline (TR), as Nrf-2 inhibitor, was pre-incubated, at a concentration of 1 μM, as previous described (Rizza et al., 2015), for 30 min before treatment with DMF 30μM. The concentration of MPTP (3mM) used was based on previous *in vitro* studies (Siracusa et al., 2015). After 24 h cell lysates were prepared for Western blot analysis.

SH-SY5Y cultures were divided into 4 groups:

1. Control group (Ctr): differentiated cells were cultured with normal medium;

2. MPTP group: differentiated cells were treated with 3mM MPTP;

3. MPTP+DMF 30μM group: differentiated cells were treated with DMF 30 μM for 2h before addition of 3 mM MPTP;

4. MPTP+DMF+TR group: differentiated cells were pretreated with DMF30 μM for 2h and TR 1 μM for 30 m, before addition of 3 mM MPTP;

6.2.2 Western Blot Analysis

Western blot analysis was performed as previously described (Siracusa et al., 2015). The membrane was incubated overnight at 4°C with: anti-iNOS (1:500; Trasduction) and anti-Mn-SOD (1:500; Millipore). To ascertain that blots were loaded with equal amounts of protein lysate, they were also incubated with the antibody β-actin (1:500; Santa Cruz Biotechnology). Signals were detected as described before (Siracusa et al., 2015).

6.3 Materials

Unless otherwise stated, all compounds were acquired from Sigma-Aldrich. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9%NaCl, Baxter, Milan, Italy).

6.4 Statistical evaluation

All values in the figures and the text are calculated as mean ± SEM. Results revealed in the figures are representative of at least 3 experiments made on different in vivo experimental days. The results were examined by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons. A p value of less than 0.05 was considered significant.

Chapter 7

Results colitis

7.1 Effects of DMF treatment on the degree of colitis

No histological alteration was observed in the colon tissue from sham CD1 mice (Fig. 7A, see macroscopic score 7G; 8A, see histological score 8F). Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon and rectum showed presence of mucosal congestion, erosion and hemorrhagic ulcerations (Fig. 7B, see macroscopic score 7G). The histopathological features included a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-injected mice (Fig. 8B, see histological score 8F). DMF reduced the extent and severity of the macroscopic (Fig. 7C, 7D and 7E, respectively, see macroscopic score 7G) and histological signs of colon injury (Fig. 8C, 8D and 8E, see histological score 8F) with significant beneficial effects at dose of 100 mg/kg ($p < 0.01$) (Fig. 7E, 8E). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a reduction in body weight (compared with the sham groups of mice) (Fig. 7F). DMF treatment reduced the loss of body weight with significant effects at dose of 100 mg/kg ($p < 0.01$) (Fig. 7F).

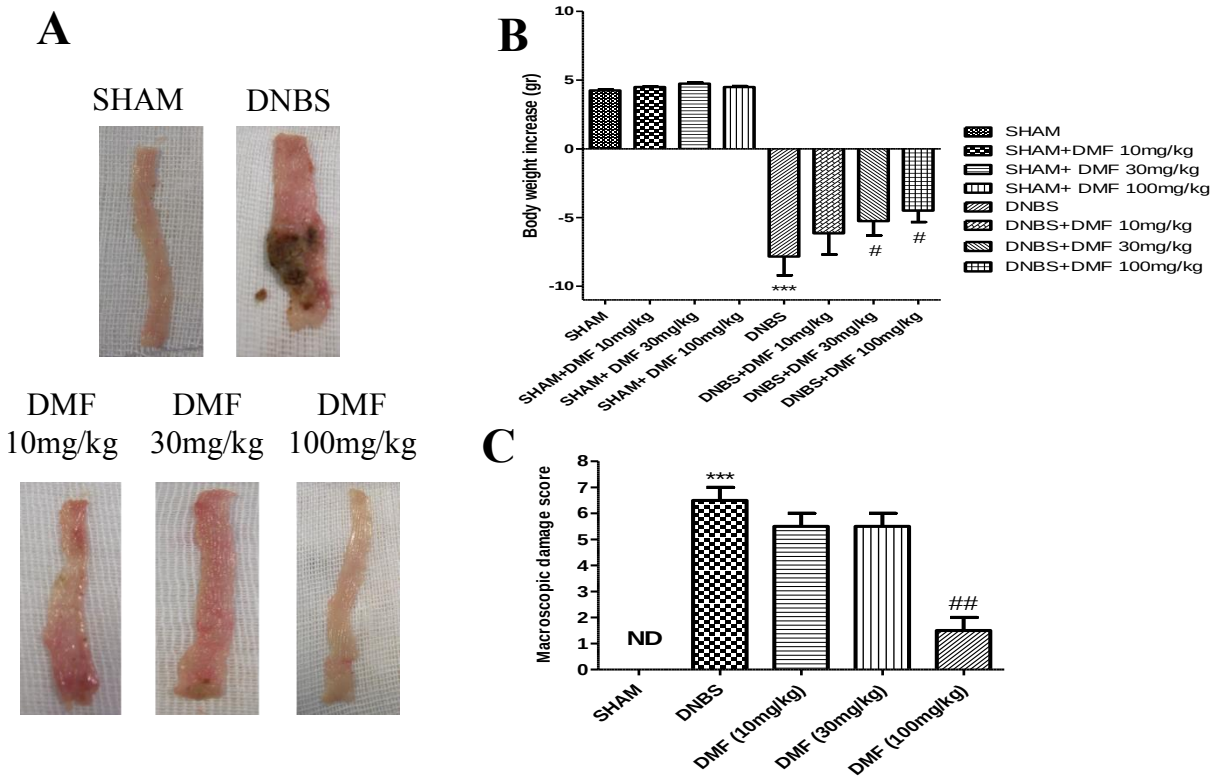


Figure 7: Effects of DMF treatment on clinical expression of DNBS-induced colitis, body weight and macroscopic damage score changes. Colon tissues from sham-treated mice, colon tissues from DNBS-treated mice at 4 days post DNBS administration, colon tissues collected from DNBS-treated mice which have received DMF at 10, 30 and 100 mg/kg treatment (A). The macroscopic damage score (C) was made by two independent observers. 4 days after DNBS administration was observed a significant reduction in body weight increase (B). Treatment with DMF at 100 mg/kg significantly reduced the increase in body weight loss (A) and in macroscopic damage score (C). Data are means ± SEM of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS. ND: not detectable.

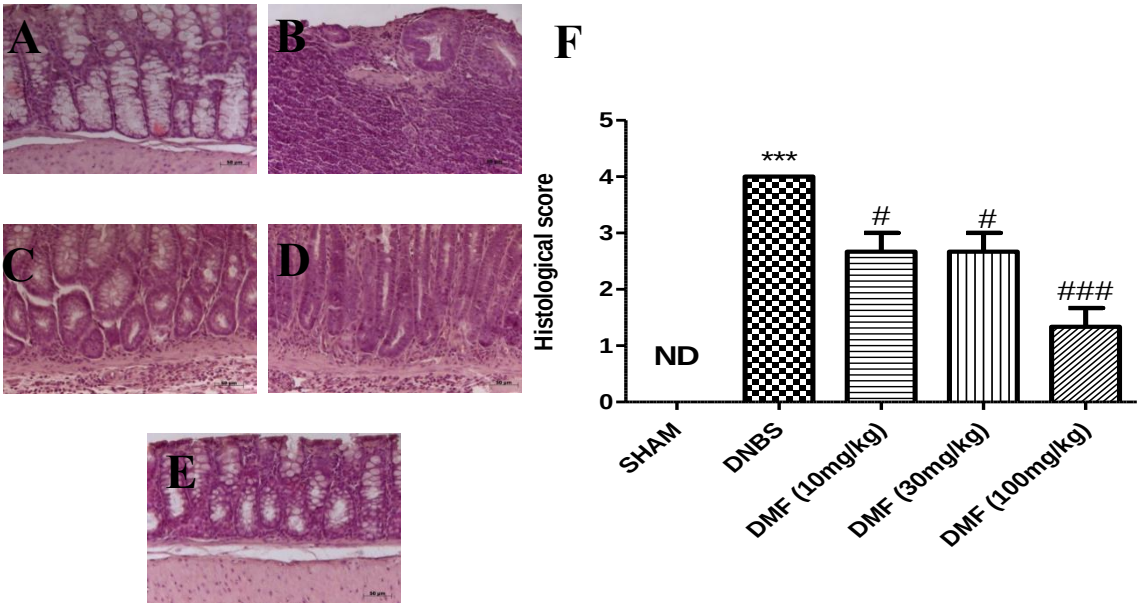


Figure 8: Effects of DMF treatment on colon injury and histological score. No histological and macroscopic alterations were observed in the colon tissue from sham-treated mice (A, F). Mucosal injury was produced after DNBS administration characterized by absence of epithelium and a massive mucosal and submucosal infiltration of inflammatory cells (B, F). The histopathological features of colon tissues from DNBS-injected mice at 4 days post DNBS administration showed severe signs of colon injury (B, F). Treatment with DMF reduced the severity of colon inflammation with statistically significant effects at dose of 100 mg/kg (E, F) compared to lower doses of 10 and 30 mg/kg (C, D and F). Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS; ND: not detectable.

IL-10 KO mice spontaneously develop a Th1-dependent chronic enterocolitis shortly after birth that is fully established at 8–10 weeks of age. Therefore, we evaluated the therapeutic activity of DMF (100 mg/kg) on 9-week-old IL-10KO mice with fully established colitis. As Figure 9 shows, 7 weeks of treatment with DMF (100mg/kg) significantly reduced the activity of colitis, as assessed by colonic MPO activity (panel D), histologic damage scores (panel E), and colonic TNF-α levels (panel F) at week 16.

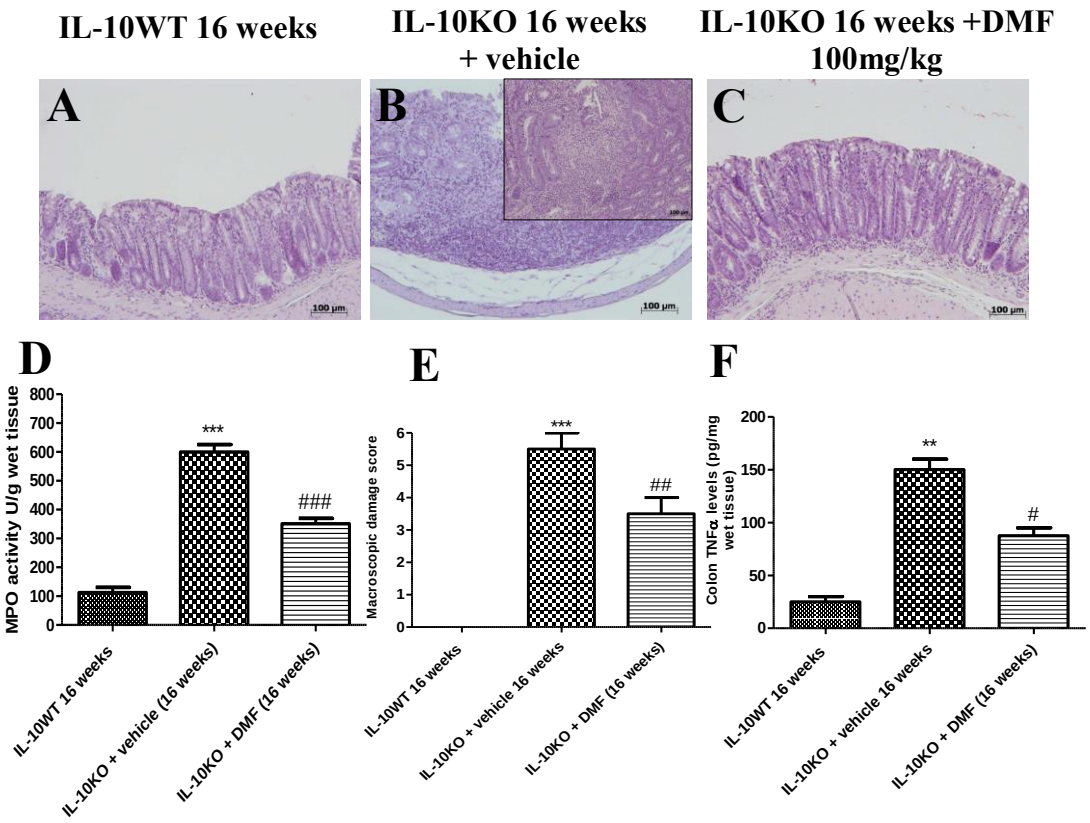


Figure 9: Effects of DMF treatment in IL-10KO 16 weeks mice. Effect of DMF administration on colonic MPO activity (D), macroscopic damage score (E) and colonic TNF-α levels (F). Treatment with DMF at 100 mg/kg significantly reduced macroscopic damage score in IL-10KO 16 weeks mice (C and E) compared to vehicle (B and E). MPO activity was significantly increased in IL-10KO 16 weeks DNBS-treated mice in comparison to IL-10WT 16 weeks mice (D); treatment with DMF at 100 mg/kg significantly reduced the colon MPO activity in IL-10KO 16 weeks mice. Colonic TNF-α levels were increased after DNBS injection in IL-10KO 16 weeks mice compared to IL-10WT 16 weeks mice (F); treatment with DMF at 100 mg/kg significantly reduced the colonic TNF-α levels in IL-10KO 16 weeks mice (F). Data are means ± SD of 6 mice for each group. *P <0. 01 vs IL-10WT 16 weeks mice; #P <0.01 vs IL-10KO 16 weeks mice + vehicle.

7.2 DMF reduced lipid peroxidation after DNBS administration and regulated Mn-SOD expression in colon tissues

The colitis caused by DNBS was also characterized by an increase in lipid peroxidation. Four days after DNBS administration, thiobarbituric acid-reactant substances (TBARs) levels were measured

in the colon tissues as an indicator of lipid peroxidation. A significant increase in TBARs levels (Fig. 10A) was observed in the colon tissues collected from DNBS-treated mice 4 days after the induction of experimental colitis when compared with sham-treated mice. TBARs were significantly attenuated by DMF (Fig. 10A). Reactive oxygen species, either directly or via the formation of lipid peroxidation products, may play a role in enhancing inflammation. To test whether DMF modulates the oxidative process we analyzed the colon expression of anti-oxidant enzyme Mn-SOD. A basal expression of Mn-SOD was observed in the colon tissues from sham-treated mice, while DMF significantly reduced colon Mn-SOD (Fig. 10B). DMF treatment (100mg/kg) significantly increased Mn-SOD expression ($p < 0.01$) (Fig. 10B).

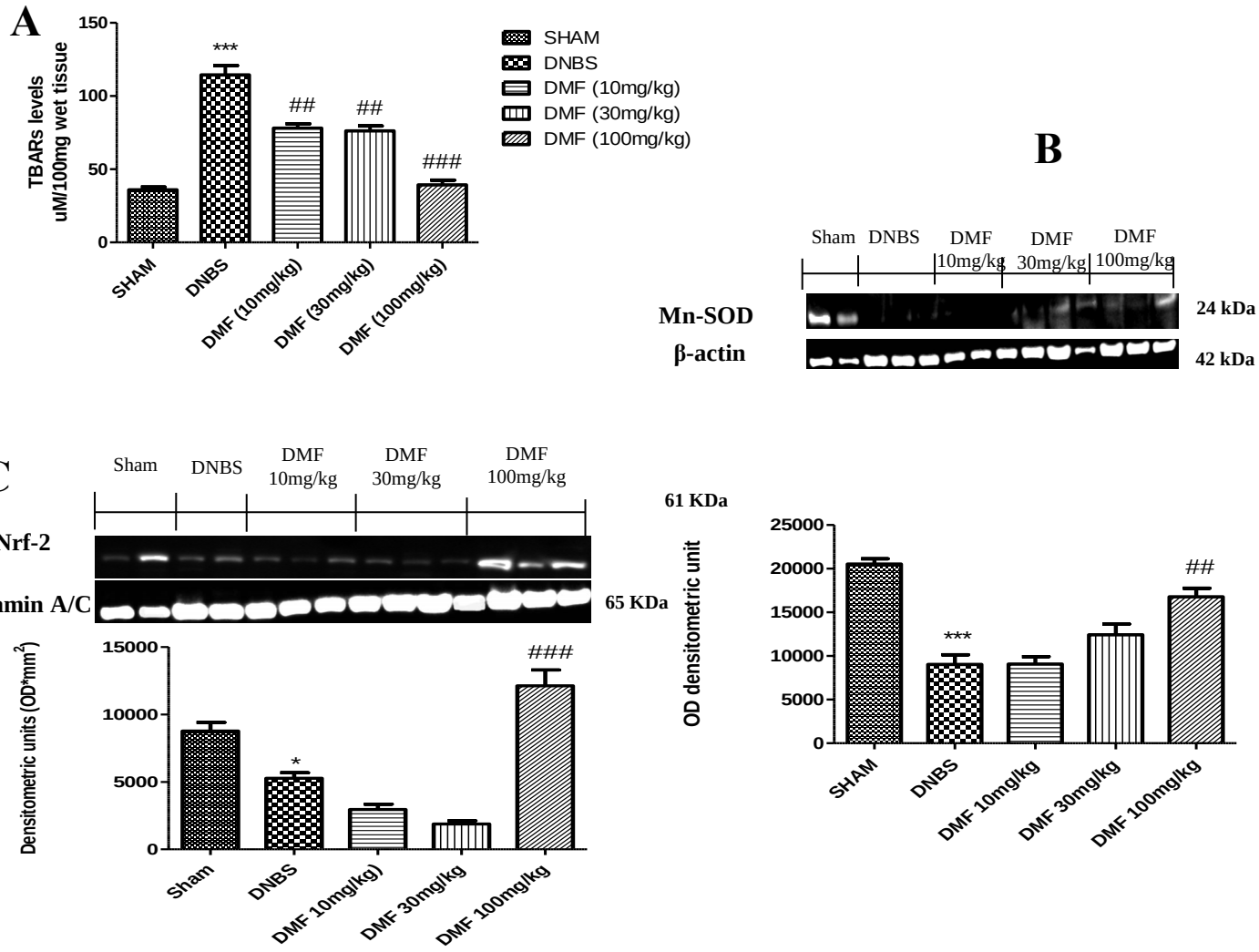


Figure 10: Effects of DMF treatment on lipid peroxidation. A significant increase in thiobarbituric acid-reactant substances (TBARs) (A) were observed in the colon tissues collected at 4 days after DNBS administration when compared with sham-treated mice. TBARs were significantly attenuated by DMF treatment (A). In addition, Mn-SOD and Nrf-2 expression was measured by Western blot analysis. Mn-SOD and Nrf-2 expression was reduced in colon

tissue from DNBS injected mice compared to sham groups (B and C). Treatment with DMF (100 mg/kg) demonstrated increased Mn-SOD and Nrf-2 levels (B and C). The relative expression of the protein bands was standardized for densitometry analysis to b-actin levels. Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS.

7.3 Effects of DMF on IκB-α degradation and NF-κB p65 traslocation

Most inflammatory mediators are controlled by NF-κB transcription factor, which is kept inactive by IκB-α. By Western blot analysis, we evaluated IκB-αin colon cytosolic extract and NF-κB p65 expression in colon nuclear extract. A basal level of IκB-α was detected in the colon tissues from sham-treated mice, whereas in DNBS-injured mice IκB-α expression was substantially reduced (Fig. 11A). IκB-α degradation were prevented in the colon tissues collected from DMF 100mg/kg treated mice (Fig. 11A). Moreover, NF-κB p65 translocation was also significantly increased after DNBS instillation compared to the sham-treated mice (Fig. 11B). A significant reduction in NF-κB p65 nuclear expression was observed in the tissues from DMF treated mice (Fig. 11B), significantly only at higher doses.

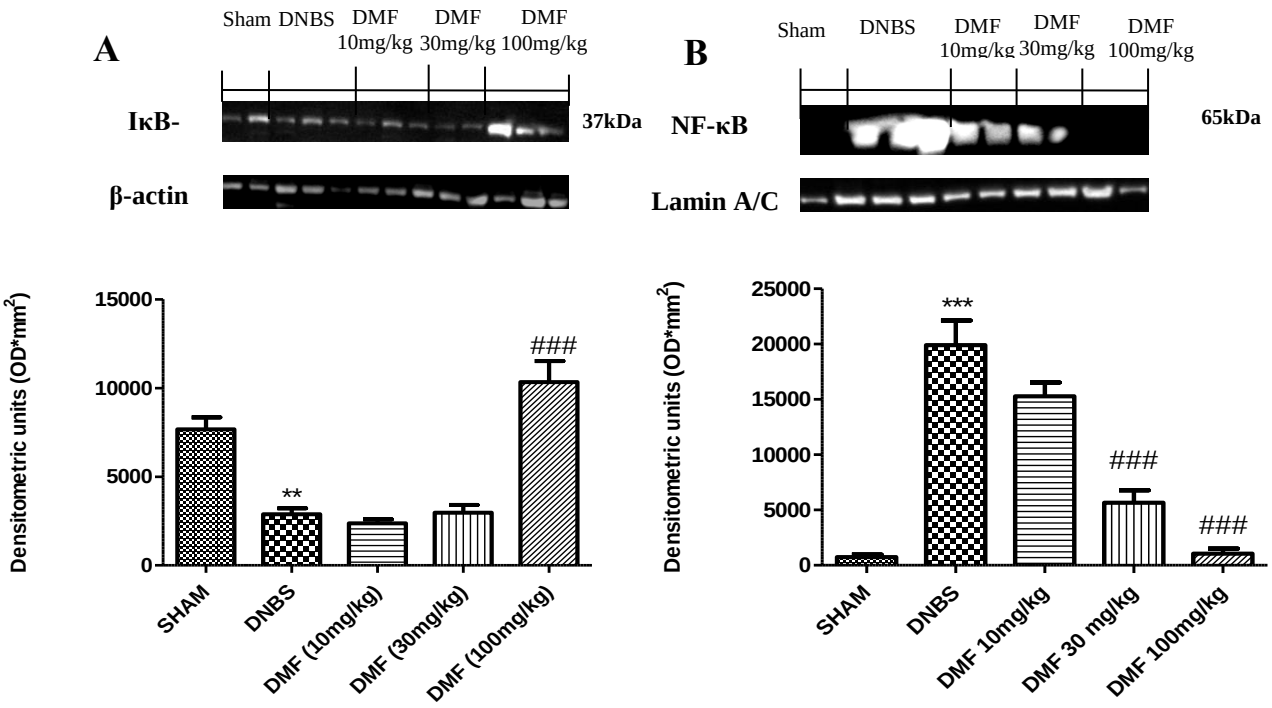
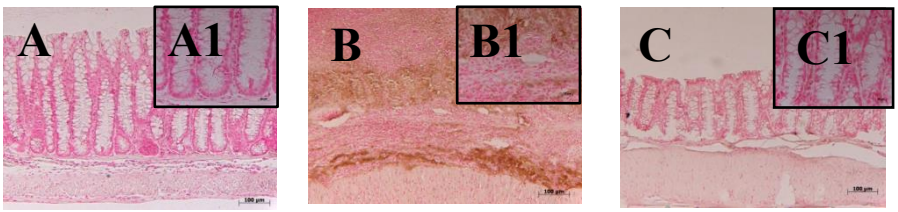
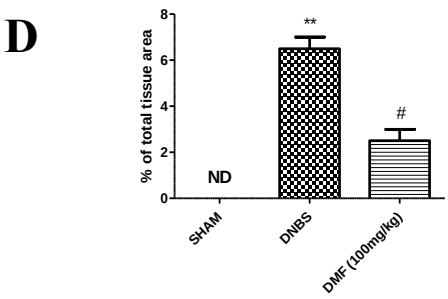


Figure 11: Effects of DMF on IκB-α degradation and NF-κB p65 translocation. By Western blot analysis NF-κB p65 levels in the colon nuclear fractions were increased after DNBS injection compared to the sham-treated mice (B). DMF treatment (100 mg/kg) reduced the levels of NF-κB p65 (B). Basal expression of IκB-α was detected in colon samples from sham-treated animals, whereas IκB-α levels were substantially reduced in colon tissues obtained from vehicle-treated animals after DNBS injection (A). DMF treatment (100 mg/kg) prevented DNBS-induced IκB-α degradation (A). The relative expression of the protein bands was standardized for densitometry analysis to b-actin and laminin levels. Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS.

7.4 Effect of DMF treatments on TNF-α and IL-1β expression

To test whether DMF modulates the inflammatory process we analyzed the colon expressions and levels of pro-inflammatory cytokines TNF-α and IL-1β. A substantial increase in TNF-α (Fig. 12B, B1) and IL-1β expression (Fig. 12E) was found in the colon tissues collected 4 days after DNBS administration. DMF treatment reduced in a dose dependent manner colon expression of IL-1β (Fig. 12E) in DNBS-injected mice and lowered the expression of TNF-α (Fig. 12C, C1) with an important effect at dose of 100 mg/kg ($p < 0.01$).





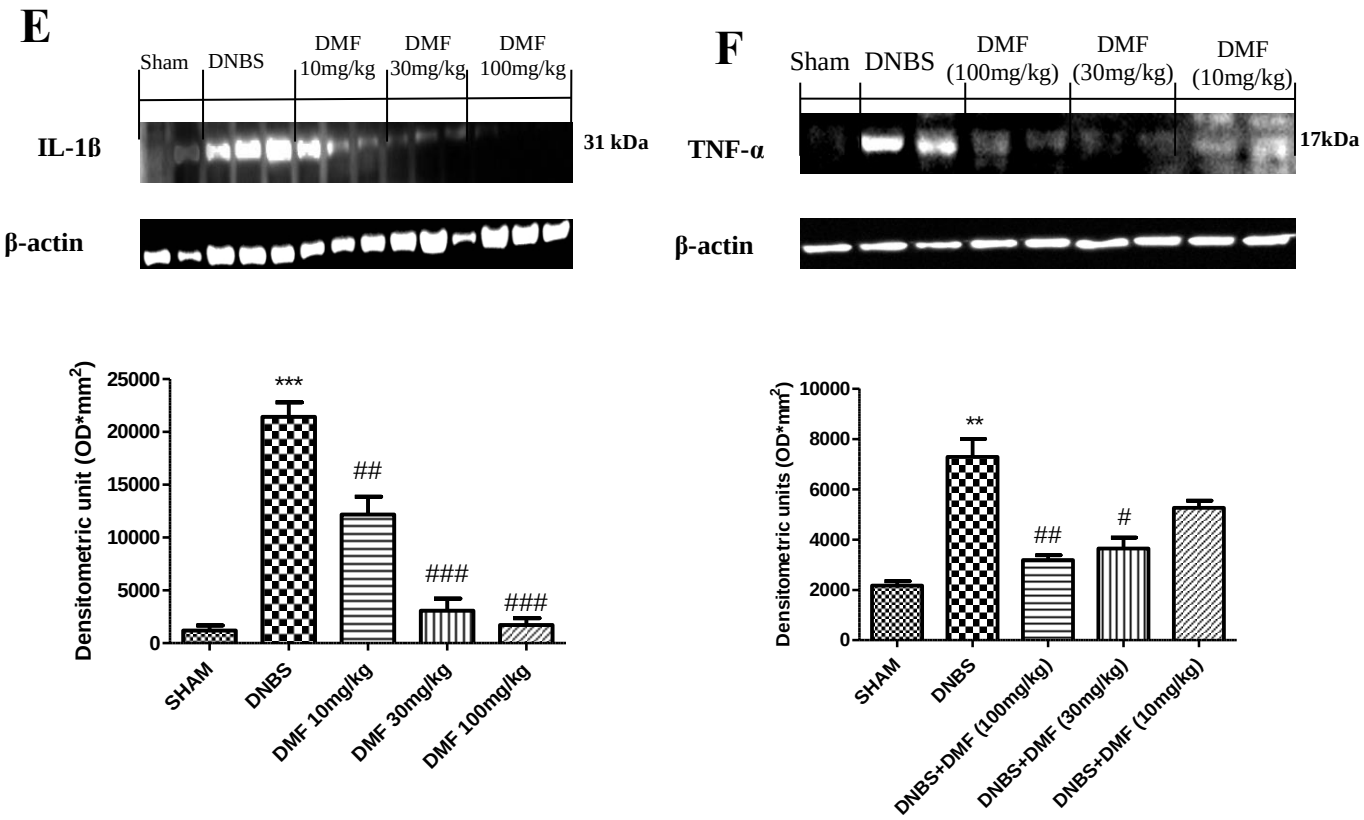


Figure 12: Effects of DMF treatment on colon levels of cytokine production. Immunohistochemical analysis for TNF-α showed positive staining localized in the inflammatory cells in the injured area from DNBS-injected animals (B, B1 and D) compared to the sham-treated mice (A, A1 and D). Treatment with DMF reduced the increase of TNF-α in the colon after DNBS administration with statistically significant effects at dose of 100 mg/kg (C, C1 and D). IL-1β and TNF-α levels in the colon were increased after DNBS injection compared to the sham-treated mice (E and F). DMF treatment significantly reduced the levels of IL-1β and TNF-α at dose of 100 mg/kg (E and F). Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS; ND: not detectable.

7.5 DMF modulated ICAM-1 and P-selectin expression and reduced MPO activity

In this study we also evaluated the intestinal expression of ICAM-1 and P-selectin that contribute to cell recruitment during colon inflammation. Positive staining for ICAM-1 (Fig. 13B, B1) and for P-selectin (Fig. 13E, E1) was substantially ($p < 0.01$) in the vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrated inflammatory cells in damaged tissues from DNBS-injected mice. Treatment with DMF at 100 mg/kg reduced the staining for ICAM-I (Fig. 13C, C1) and for P-selectin (Fig. 13F, F1) in the colon tissues collected from DNBS-injected mice ($p < 0.01$). The colitis caused by DNBS was also characterized by an increase in MPO activity ($p < 0.01$), an indicator of the neutrophils accumulation in the colon (Fig. 13H). This was consistent with observations made with light microscopy that evidenced colon that

DNBS-mice contained a large number of neutrophils. On the contrary, DMF significantly reduced in the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Fig. 13H).

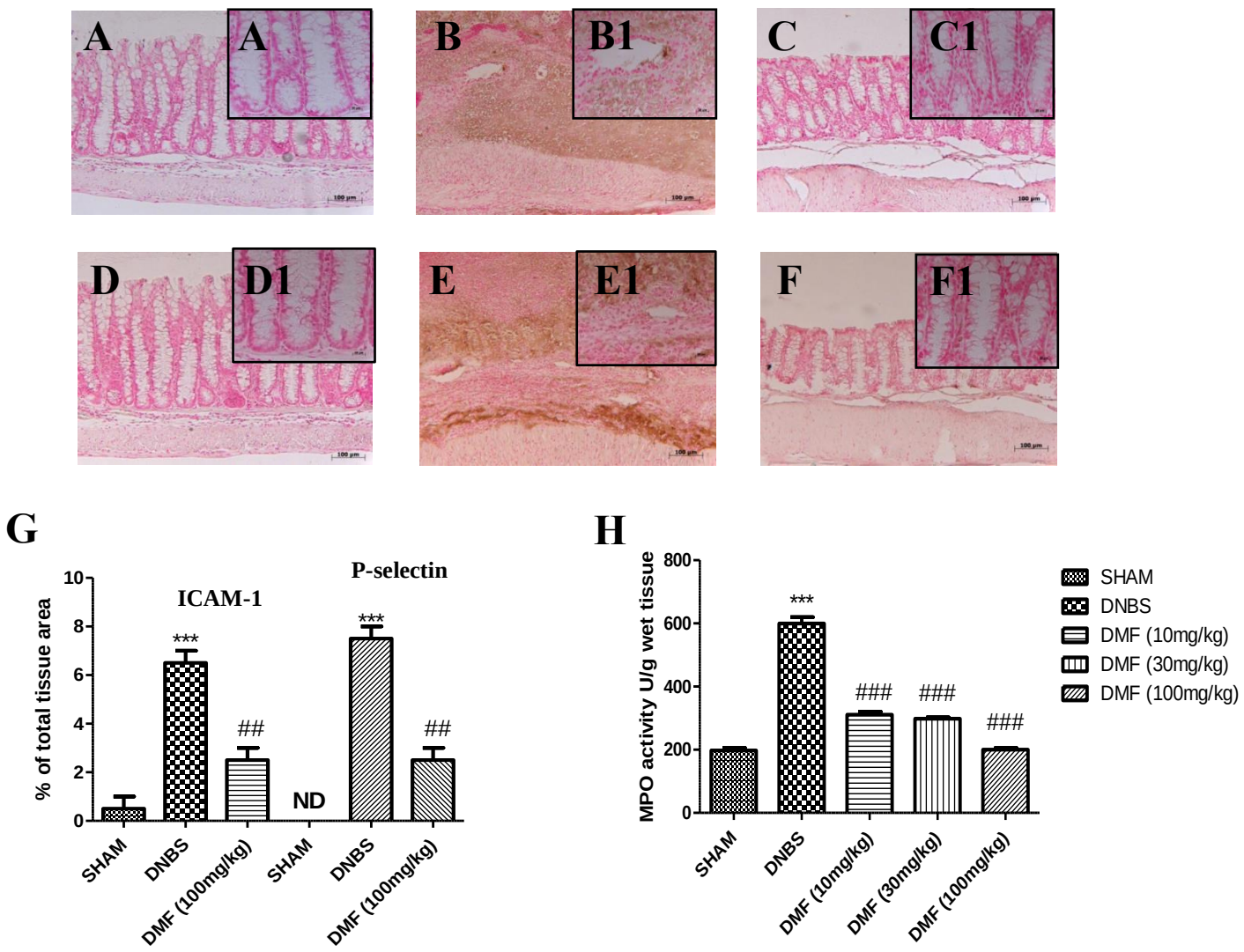


Figure 13: Effects of DMF treatment on immunohistochemical localization of ICAM-1, P-selectin and on Myeloperoxidase (MPO) activity. No positive staining for ICAM-1 and P-selectin was observed in the colon tissues from sham-treated mice (A, A1 and D, D1). On the contrary immunohistochemical analysis for ICAM-1 (B, B1 and G) and P-selectin (E, E1 and G) showed positive staining localized in the inflammatory cells in the injured area from DNBS-injected animals. The intensity of the positive staining for ICAM-1 (C, C1 and G) and P-selectin (F, F1 and G) was markedly reduced in tissue section obtained from DNBS-injected animals which have been treated with DMF (100 mg/kg). MPO activity was significantly increased in DNBS-treated mice in comparison to sham-treated mice (H). Treatment with DMF at 100 mg/kg significantly reduced the colon MPO activity (H). Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS; ND: not detectable.

7.6 Effect of DMF treatment on MMPs expression in colon tissues

To assess whether colon injury is associated with the alterations in expression of secreted MMP-9 and MMP-2, mice were sacrificed at 4 days after DNBS administration and colon were subjected to Western blot analysis. In this study we found a basal level of total MMP-9 and MMP-2 from sham mice (Fig. 14A, 14B). A significant up-regulation of MMP-9 and MMP-2 expression was observed in the colon tissues from DNBS-treated mice and DMF treatment at 10-30-100 mg/kg significantly prevented the increase of DNBS induced MMP-9and MMP-2 expression ($p < 0.01$) (Fig.14A, 14B).

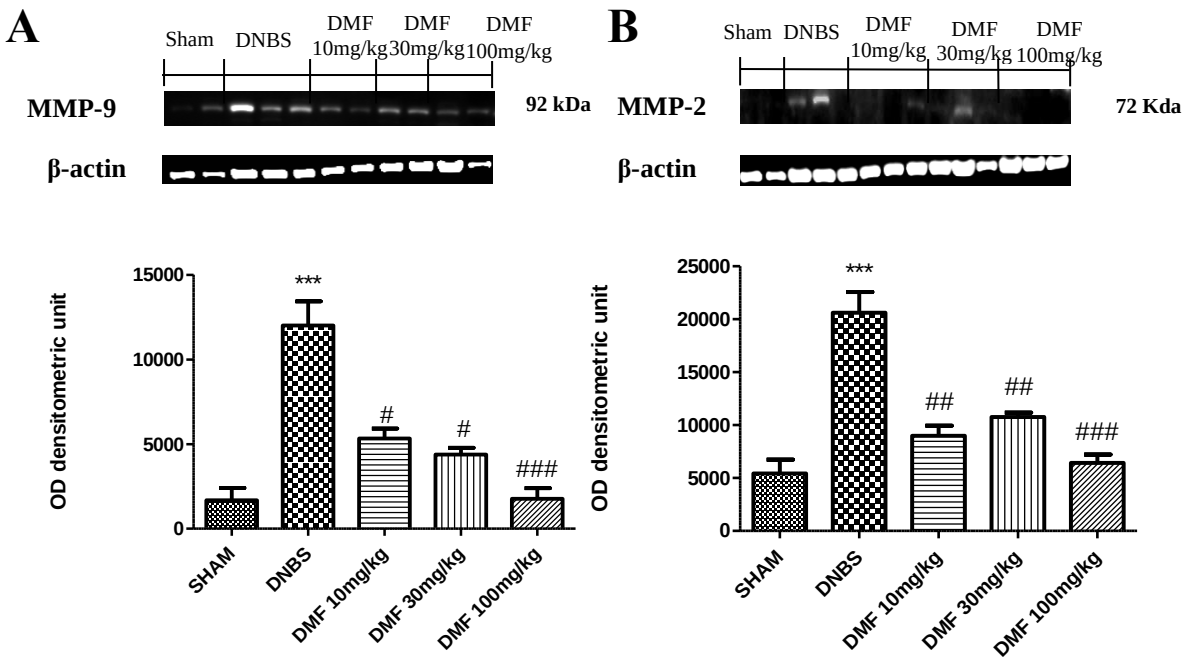


Figure 14: Effects of DMF treatment on MMP-9 and MMP-2 activity. In contrast to sham-treated mice, colon tissues extracts from DNBS-treated mice showed an induction of MMP-9 and MMP-2 (A and B). DMF at dose of 100mg/kg treatment reduced the degree of activation of MMP-9 and MMP-2 (A and B). Data are means ± SD of 6 mice for each group. *P < 0.01 vs. SHAM; #P < 0.01 vs. DNBS.

7.7 Effect of DMF on H_2O_2-induced barrier dysfunction on human intestinal epithelial cells

After administration of H2O2 (500μM), occludin and ZO-1 proteins were significantly restored by 10μM DMF in Caco-2 (Figure 15AA1, 15BB1). Moreover, the protection of human intestinal epithelial cells seem to be dependent by HO-1 pathway (Figure 15C,C1).

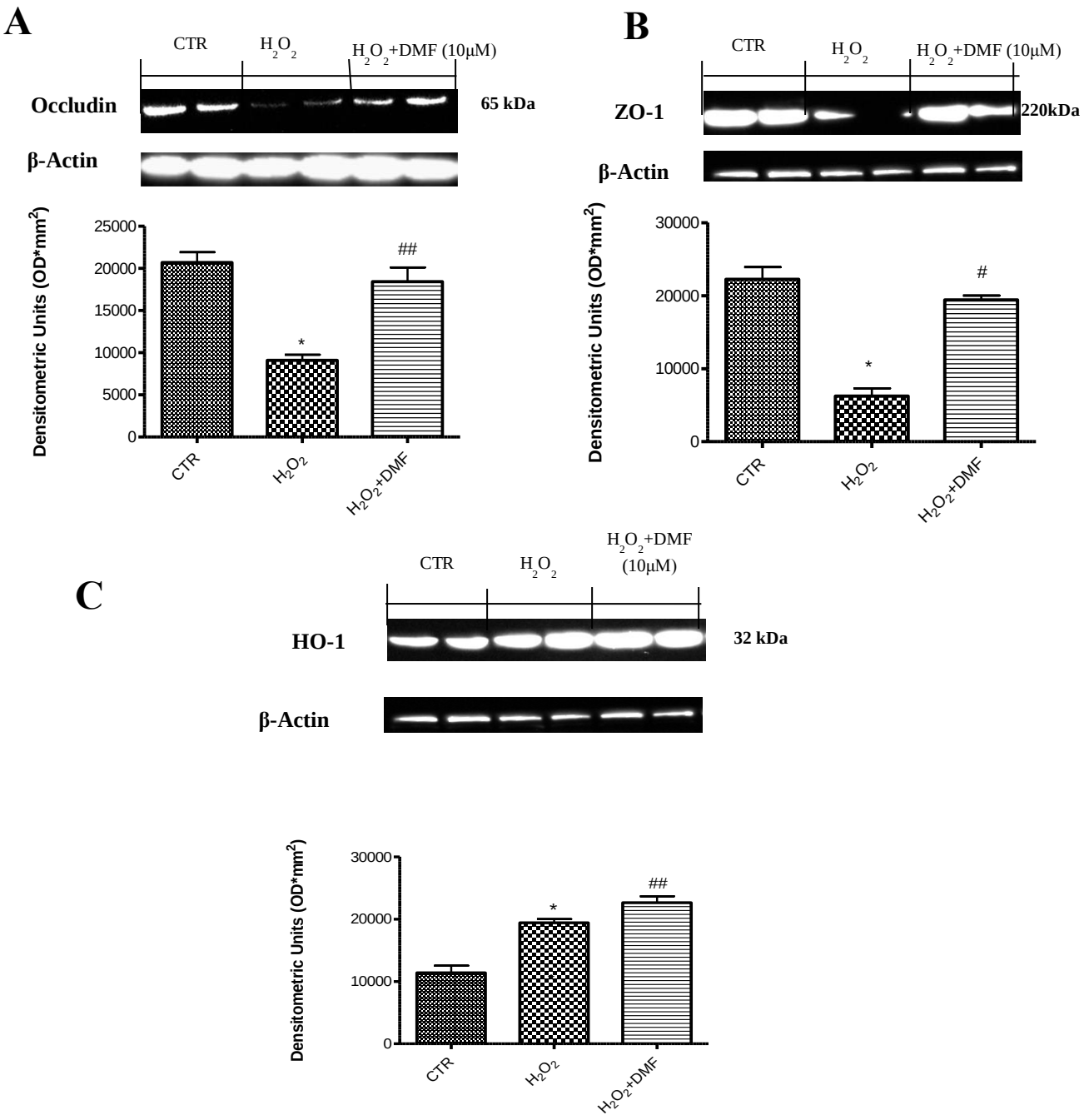


Figure 15: Effects of DMF treatment on Occludin, ZO-1 and HO-1 expression in Caco-2 cells. By Western blot analysis, Occludin and ZO-1 levels were lowered after H_2O_2 stimulation compared to control group (A and B). DMF treatment (100 mg/kg) increased the levels of Occludin and ZO-1 in Caco-2 cells (A and B). HO-1 levels were increased in Caco-2 cells after H_2O_2 stimulation and DMF treatment (100 mg/kg) respect to basal expression in control group (C).

Chapter 8

Results Parkinson's disease

8.1 DMF treatment reduced the neuronal degeneration of dopaminergic tract induced by MPTP administration

PD predominantly strikes dopaminergic tract, so we evaluated the severity of neuronal degeneration of midbrain 8 days after MPTP intoxication. MPTP-injured mice were characterized by nigrostriatal dopaminergic degeneration, translated into neuronal loss in the substantia nigra pars compacta (Figure 16E and 16E1, see histological score Figure 16J) respect to physiological neuronal structure observed in control mice (Figure 16A,16A1 see histological score Figure 16J) and in DMF alone group (Figure 16B and 16B1, 16C and 16C1, 16D and 16D1, see histological score Figure 16J). DMF treatment, at the dose of 10 mg/kg, partly reduced the alteration of the dopaminergic tract (Figure 16F and 16F1, see histological score Figure 16J), restoring neuronal loss at the dose of 30 mg/kg and 100mg/kg (Figure 16G and 16G1, 16H and 16H1 respectively, see histological score Figure 16J).

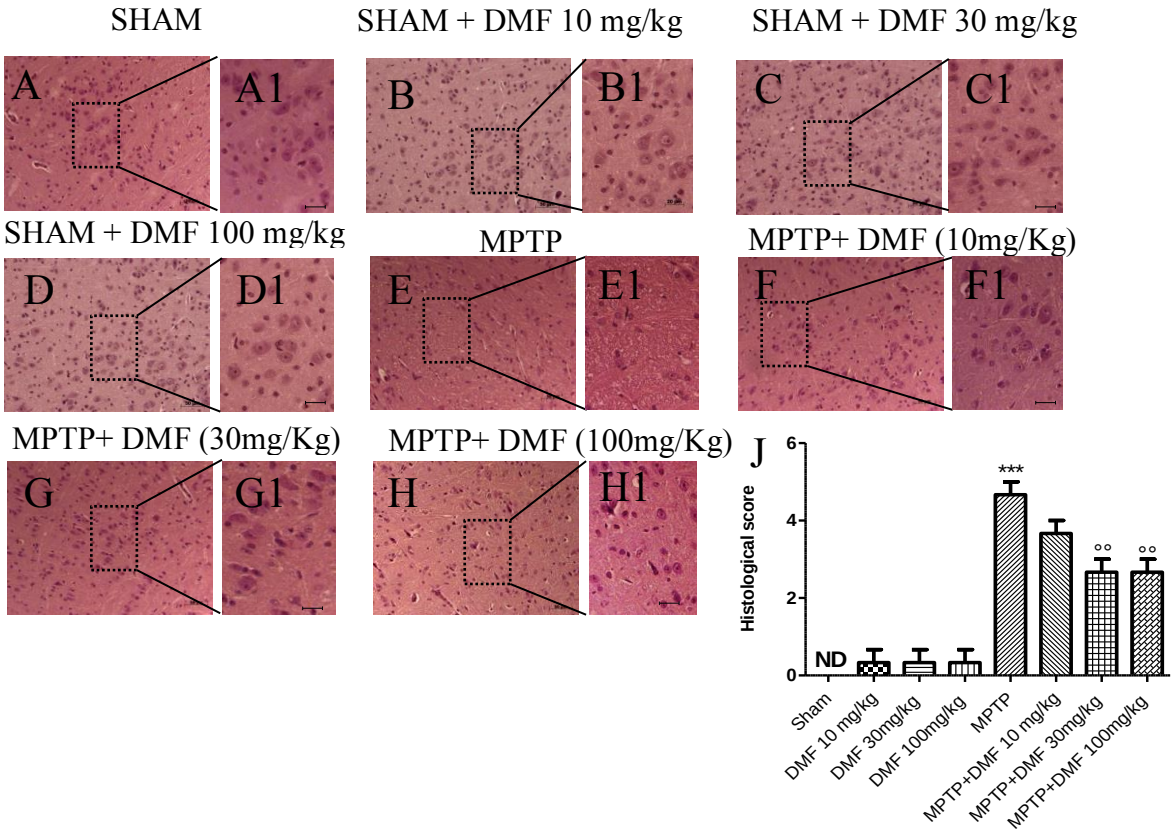


Figure 16: Effects of DMF treatment on neuronal degeneration of dopaminergic tract in the midbrain at 8 days after MPTP administration. MPTP-injured mice were characterized by nigrostriatal dopaminergic degeneration, translated into neuronal loss in the substantia nigra pars compacta (E, E1) respect to physiological neuronal structure observed in control mice (A, A1) and in DMF alone groups (B and B1, C and C1, D and D1 respectively). DMF treatment, at the dose of 10 mg/kg, partly reduced the alteration of the dopaminergic tract (F, F1), restoring neuronal loss at the dose of 30 and 100 mg/kg (G and G1, H and H1 respectively). See histological score (J). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. ***P <0.001 vs. Sham; °°P < 0.01 vs. MPTP.

8.2 DMF treatment reduced behavioral impairments induced by MPTP administration

The behavioral deficits in the MPTP-induced mouse model of PD are useful for investigating the relationship between dopaminergic neuron degeneration and recovery processes, particularly in motor activity. The pole test was applied to assess whether the MPTP-induced mouse model successfully induced bradykinesia and to analyze the therapeutic effects of DMF at different doses. “Total time” and “Time to turn” significantly increased following injection of MPTP compared with the control group (Figure 17A and 17B). DMF treatment, on the seventh day after MPTP administration, significantly reduced “Total time” and “Time to turn” comparably in all doses (10, 30 and 100 mg/kg) (Figure 17A and 17B), suggesting a significative reduction of bradykinesia.

Furthermore, the rotarod test was applied to assses motor function. At 7 days after MPTP intoxication, mice displayed a range of impairments

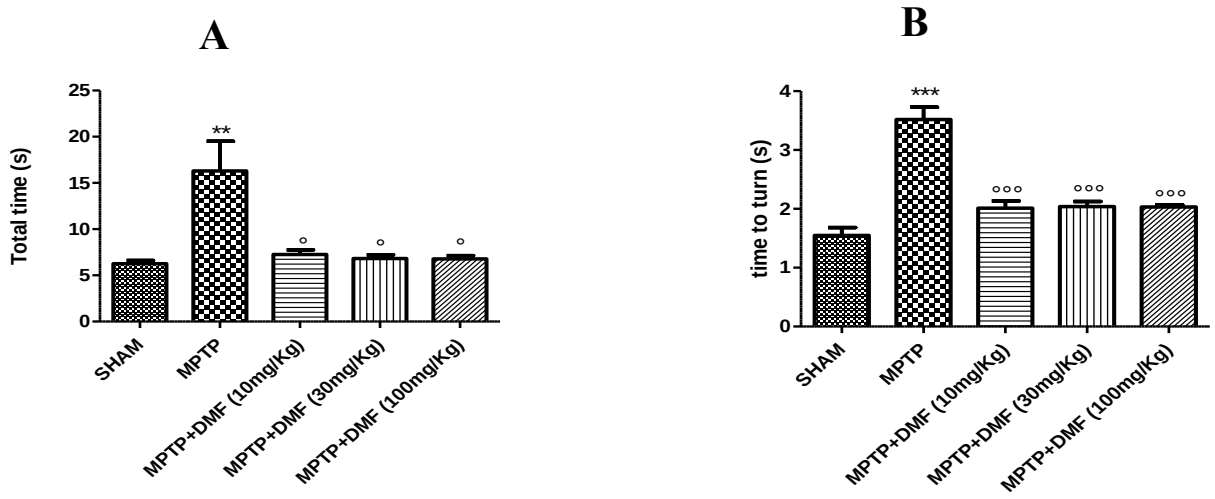


Figure 17: Effects of DMF treatment on behavioral impairments and emotional state induced by MPTP administration. "Total time" and "Time to turn" significantly increased following injection of MPTP compared with the control group (A and B respectively). DMF treatment, on the seventh day after MPTP administration, significantly reduced "Total time" and "Time to turn" equally at all three doses (10, 30 and 100 mg/kg) (A and B respectively). Values are indicated as the mean±SEM. **P< 0,01 and ***P< 0.001 vs. Sham; °P <0.05 and °°°P< 0.001 vs. MPTP.

in locomotor tasks (Figure 18C) respect to control mice (Figure 18C). DMF treatment, at all three doses, improved latency, compared to injured mice (Figure 18C).

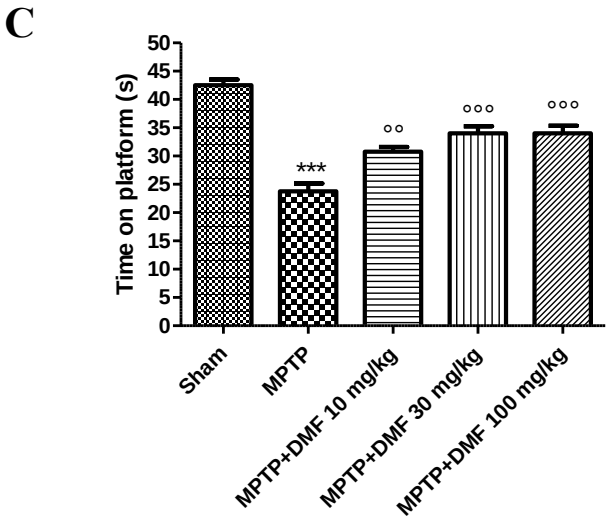


Figure 18: Moreover, MPTP-injured mice showed significant impairments in motor deficits as revealed by significantly shortened time to stay on rotating rod respect to control group (C), while DMF treatment significantly increased the residence time (C). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. ***P< 0.001 vs. Sham; °°P<0.01 and °°°P< 0.001 vs. MPTP.

8.3 Effects of DMF treatment on emotional state

To determine whether DMF treatment influences emotional-behavioral disorders in mice after MPTP intoxication, we assessed open field test. MPTP-lesioned mice showed a pronounced increase in thigmotaxis when compared to sham mice, as indicated by less time spent in the center of the open field (Figure 19A) and reduced frequency of line crossing (Figure 19B). However, there were no significant improvements observed in DMF-treated mice as compared to MPTP injured mice (Figure 19A and 19B).

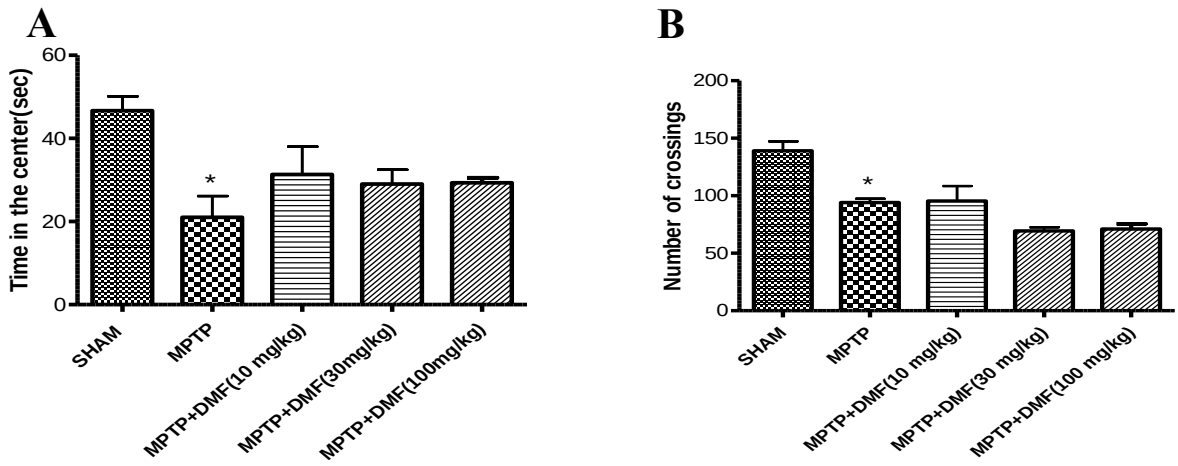


Figure 19: Also, in MPTP-lesioned mice decreased the time spent in the center of the open field (A) and the frequency of line crossing (B) respect to control group (A and B respectively). However, there were no significant improvements observed in DMF treated mice as compared to MPTP injured mice (A and B respectively). Values are indicated as the mean±SEM. *P<0.05 vs. Sham.

8.4 DMF prevented striatal dopaminergic neurons lost and dopamine depletion from MPTP toxicity

PD can be considered as a tyrosine hydroxilase (TH)-deficiency syndrome of the striatum, in fact TH catalyses the formation of L-DOPA, the precursor for dopamine. So, to determine whether DMF might protect against MPTP-induced loss of striatal DA neurons, midbrain sections were stained for TH immunoreactivity. We observed an important loss of TH-positive cells in MPTP-injected animals at 8 days after intoxication (Figure 20B and 20B1, see densitometric analysis Figure 20J). DMF treatment, at the dose of 10 mg/kg, showed a significant protection from MPTP-induced TH cells death (Figure 20C and 20C1, see densitometric analysis Figure 20J), while, at the

higher dose of 30mg/kg, DMF preserved density of TH-positive cells population (Figure 20D and 20D1, see densitometric analysis Figure 20I) in comparable manner to control mice (Figure 20A and 20A1, see densitometric analysis Figure 20I). The integrity of nigral dopaminergic neurons was assessed by stereological counting of TH positive neurons (Figure 20J). In MPTP-injured mice there was about 75% loss of TH-positive neurons (Figure 20J) respect to control mice (Figure 20J); DMF treatment restored almost completely the neuronal count in a dose dependent manner (Figure 20I). Moreover, being the loss of striatal dopamine transporter (DAT) a significant sign in PD, in order to better study the effects of DMF treatment on the dopamine pathway, we evaluated the levels of DAT by immunistochemical analysis. We assessed that MPTP injection produced an important loss of DAT positive staining (Figure 20F and 20F1, see densitometric analysis Figure 20K) compared to sham group (Figure 20E and 20E1, see densitometric analysis Figure 20K). DMF treatment improved the severe reductions of DAT positive staining in dose dependent manner (Figure 20G and 20G1, 20H and 20H1 respectively, see densitometric analysis Figure 20K).

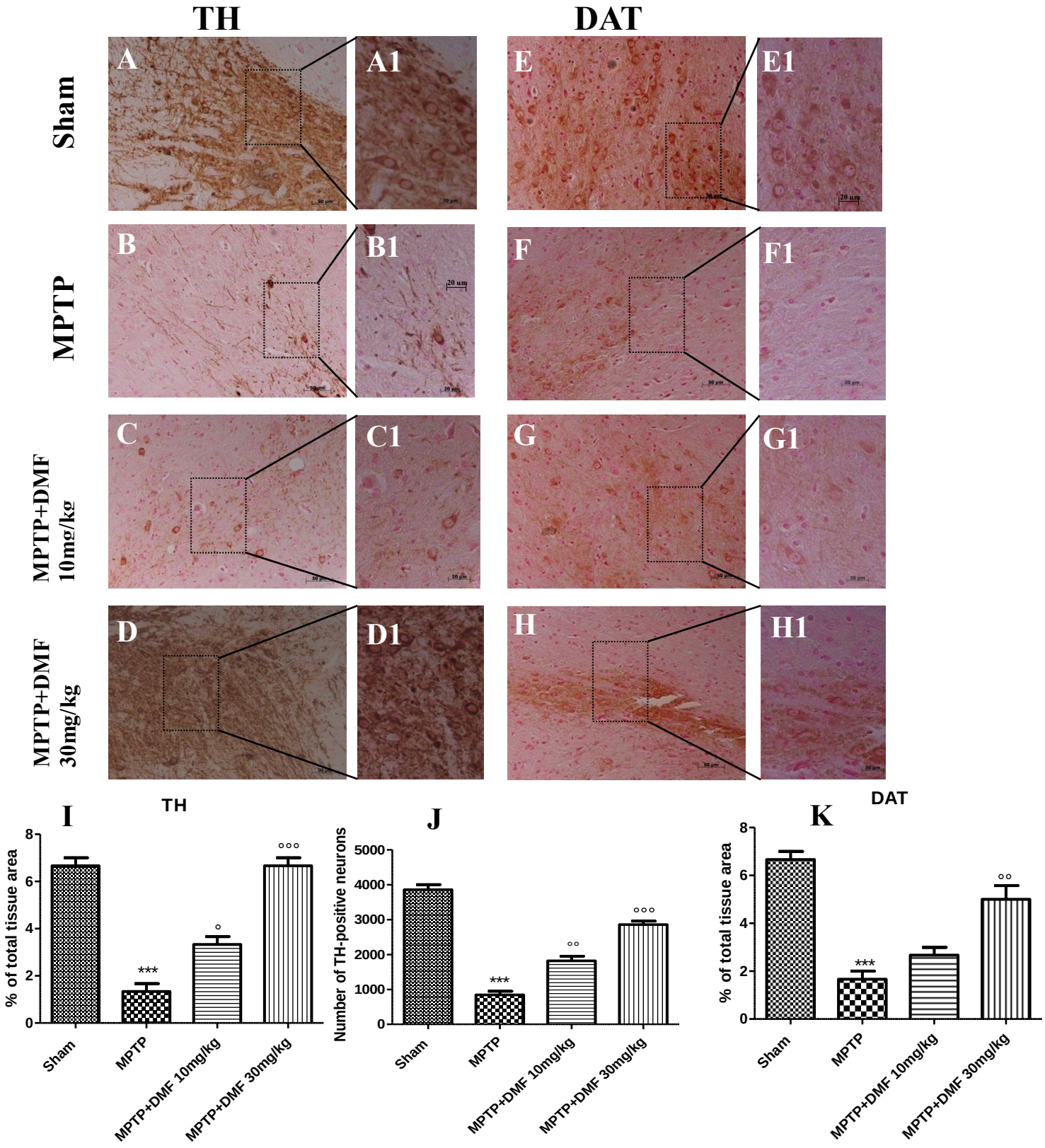


Figure 20: DMF prevented striatal dopaminergic neurons lost and dopamine depletion from MPTP toxicity. We observed an important loss of TH-positive cells in MPTP-injected animals at 8 days after intoxication (B, B1 and I). DMF treatment, at the dose of 10 mg/kg, showed a positive TH staining (C, C1 and I) that increased at the higher dose of 30 mg/kg (D, D1 and I) in comparable manner to control mice (A, A1 and I). Furthermore, stereological counting of TH-positive neurons in sections of the substantia nigra showed the protective effect of DMF treatment (J). Instead, MPTP injection produced an important decrease of DAT expression (F, F1and K) compared to sham (E, E1 and K). DMF treatment improved the sever reductions in DAT positive staining, in dose dependent manner (G and G1, H and H1 respectively and K). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. ***P< 0.001 vs. Sham; °P <0.05, °°P< 0.01 and °°°P<0.001 vs. MPTP.

8.5 Protective effects of DMF on α-synuclein - induced neurodegeneration

To test the ability of DMF to counteract α-synuclein-induced degeneration, focusing on the protective potential of this compound in PD, we assessed the expression of α-synuclein by western blot and immunohistochemical analysis. Particularly, it is known that α-synuclein dimerization can accelerate the formation of neurotoxic aggregates and amyloid fibrils which can be crucial to comprehend PD pathology at the molecular level (Hashimoto et al., 1999). We observed an ascending accumulation of α-synuclein monomer, dimer and oligomer in MPTP-injured mice (Figure 21A, 21B and 21C), as compared to control mice, in which there was a constant baseline expression of α-synuclein (Figure 21A, 21B and 21C). DMF, at the dose of 10 mg/kg, was not able to contrast dimer and oligomer α-synuclein aggregates compared to monomer (Figure 21A, 21B and 21C). Conversely, DMF, at the dose of 30 mg/kg, decreased significantly α-synuclein aggregates (Figure 21A, 21B and 21C). Also, by immunohistochemical analysis on midbrain section of MPTP-injected animals at 8 days after intoxications, we observed a protective effect of DMF, dose dependent manner, respectively at the dose of 10 mg/kg (Figure 21F and 21F1, see densitometric analysis Figure 21H) and at the dose of 30 mg/kg (Figure 21G and 21G1, see densitometric analysis Figure 21H). In MPTP-injured mice, we observed a significant α-synuclein accumulation induced neurodegeneration (Figure 21E and 21E1, see densitometric analysis Figure 21H) compared to control midbrain sections (Figure 21D and 21D1, see densitometric analysis Figure 21H).

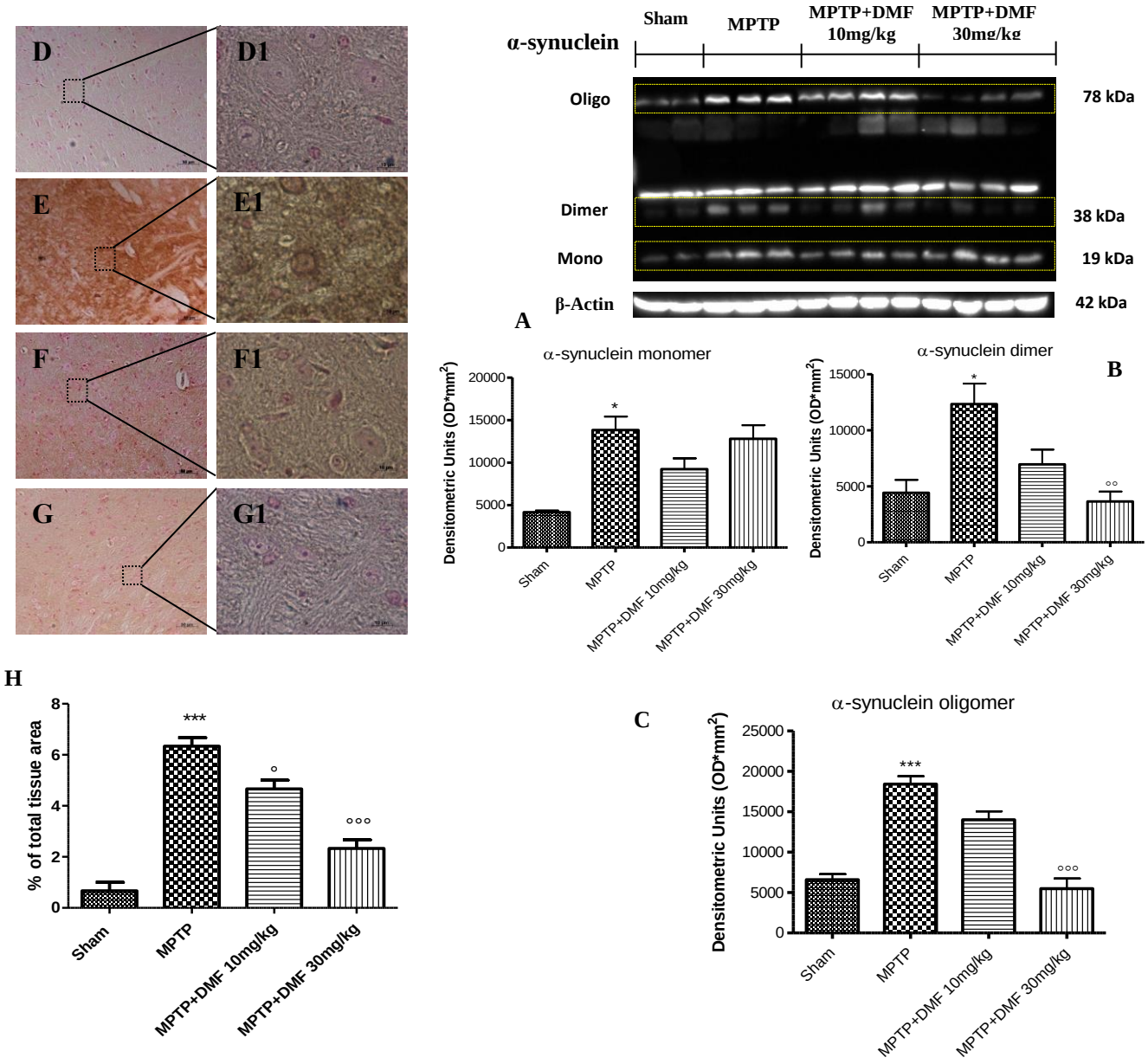
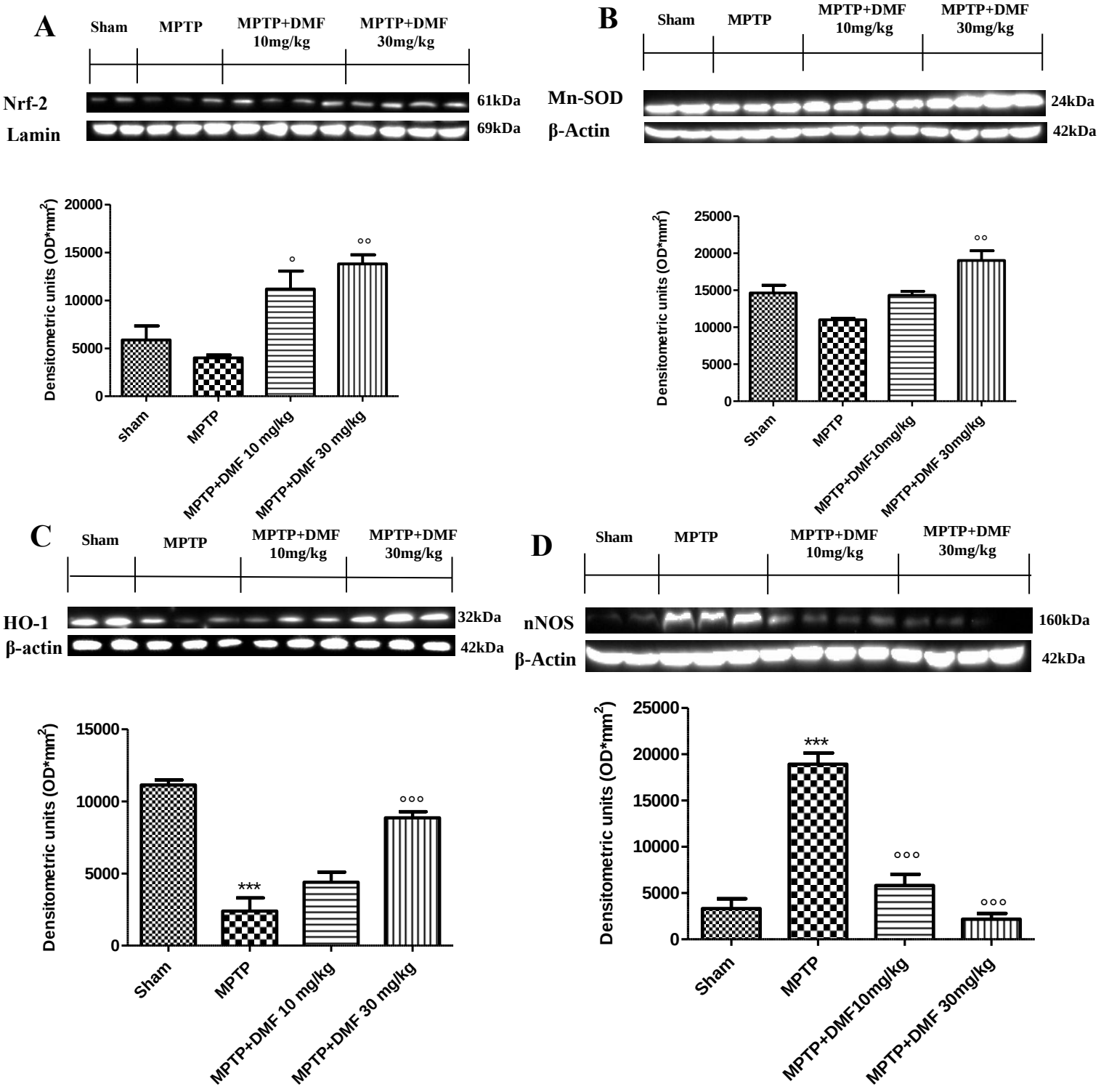


Figure 21: Protective effects of DMF on α-synuclein - induced neurodegeneration. We observed an ascending accumulation of α-synuclein monomer, dimer and oligomer in MPTP-injured mice (A, B and C), as compared to control mice, in which there was a constant baseline expression of α-synuclein (A, B and C). DMF, at the dose of 10 mg/kg, contrasted α-synuclein monomer accumulation, but its protective action decreased in respect of the dimeric and oligomeric forms (A, B and C). Conversely, protective effect of DMF, at the dose of 30 mg/kg, was lower against the monomeric form, that is less toxic, increasing significantly against dimeric and oligomeric forms (A, B and C). Data are means ± SD of 10 mice for each group. Also, we observed a protective effect of DMF, dose dependent manner in α-synuclein staining (F and F1, G and G1 respectively and H). In MPTP-injured mice, we observed a significant α-synuclein accumulation (E, E1 and H) respect to its basal level observed in control mice (D and H). Images are shown at 20X and 100x resolution. Values are indicated as the mean±SEM. *P<0.05 and ***P< 0.001 vs. Sham; °°P < 0.01 and °°°P< 0.001 vs. MPTP.

8.6 Effects of DMF treatment on antioxidant response activation

In PD, oxidative stress plays a key role in determining neuronal damage. Nrf-2 is a transcription factor with strong antioxidant effect which protects neurons from the damages induced by ROS, thus we evaluated the effect of DMF on Nrf-2 pathway by western blot analysis. The expression of Nrf-2 levels showed a tendency to decrease following MPTP administration as compared to control mice (Figure 22A). DMF treatment caused an up-regulation, dose dependent, of Nrf-2 levels (Figure 22A). Also, regarding antioxidant enzymes expression, in MPTP-injured mice, we observed a greater decreasing in HO-1 levels than Mn-SOD levels (Figure 22C and 22B). Interestingly, treatment with DMF, at the dose of 30 mg/kg, increased Mn-SOD expression (Figure 22B), while, at the dose of 10 mg/kg, Mn-SOD expression was compared to control mice expression (Figure 22B). Instead, DMF treatment , at the dose of 10 mg/kg, increased HO-1 expression (Figure 22C) but not significantly, compared to DMF treatment at the dose of 30 mg/kg (Figure 22C) that reported HO-1 expression to control values (Figure 22C). More, to investigated the additional properties of DMF associated with oxidative stress, we measured the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in the brains of mice. MPTP intoxication resulted in a significant decrease of GSH/GSSG ratio in the substantia nigra (Figure 22J) respect to the levels in control mice (Figure 22J). Instead, DMF treatment, especially at the higher dose, significantly increased GSH/GSSG ratio (Figure 22J).Furthermore, as nitrotyrosine (NT) is an indicator of cell damage, the activation of inflammation and NO production, to understand the role of DMF on nitrosative stress, we evaluated NT by immunohistochemical analysis and nNOS expression by western blot analysis. NT-immunopositive cells were observed in MPTP-injured mice (Figure 22F and 22F1, see densitometric analysis Figure 22I) as compared to sham mice (Figure 22E and 22E1, see densitometric analysis Figure 22I). Treatment with DMF protected by nitrosative stress NT-induced in dose dependent manner (Figure 22G and 22G1, 22H and 22H1 respectively, see densitometric analysis Figure 22I). Also, a significant increase in nNOS expression was observed in

MPTP-injected mice as compared to controls (Figure 22D). DMF treatment, especially at the dose of 30mg/kg, significantly reduced the expression of nNOS (Figure 22D).



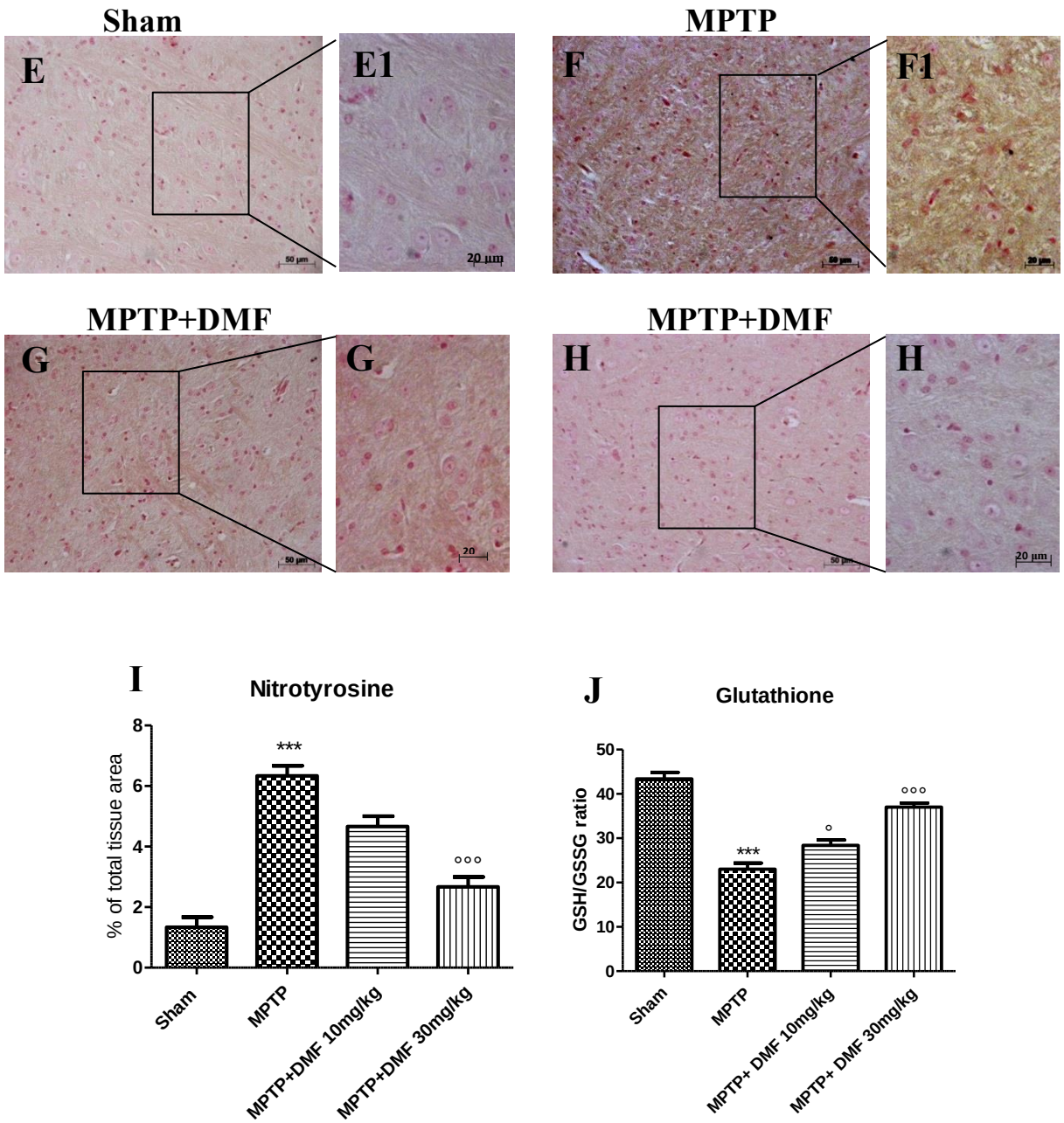


Figure 22: Effects of DMF treatment on antioxidant response activation. The expression of Nrf-2 levels showed a tendency to decrease following MPTP administration as compared to control mice (A). DMF treatment caused an up-regulation, dose dependent, of Nrf-2 levels (A). Also, regarding antioxidant enzymes expression, in MPTP-injured mice, we observed a greater decreasing in HO-1 levels than Mn-SOD levels (C and B). Interestingly, treatment with DMF, at the dose of 30 mg/kg, increased Mn-SOD expression (B), while, at the dose of 10 mg/kg, Mn-SOD expression was compared to control mice expression (B). Instead, DMF treatment, at the dose of 10 mg/kg, increased HO-1 expression (C) but not significantly, compared to DMF treatment at the dose of 30 mg/kg that reported HO-1 expression to control values (C). Also, the increase of nNOS expression was more pronounced in MPTP-injected mice (D), while DMF treatment, especially at the dose of 30 mg/kg, reduced nNOS levels as compared to sham (D). Furthermore, by immunohistochemical analysis, a positive immunostaining for NT was found in MPTP-injected mice at 8 days after intoxication (F, F1 and I) compared to control mice (E, E1 and I). DMF prevented the MPTP-induced increased expression of NT immunoreactive cells in a dose dependent manner (G and G1, H and H1 respectively and I). More, the reduced/oxidized glutathione (GSH/GSSG) was determined, observing that, the decline in the ratio of GSH/GSSG following MPTP intoxication was almost completely prevented by DMF treatment at the dose of 30 mg/kg (J). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. **P< 0.01 and ***P<0.001 vs. Sham; °P< 0.05, °°P < 0.01 and °°°P< 0.001 vs. MPTP.

8.7 DMF treatment rescued neurons from oxidative stress-induced cell death

To investigate whether Nrf2 plays a protective role on rescue neurons from oxidative stress-induced cell death, midbrain sections were double stained with antibodies against Nrf-2 (red) and NeuN (green), as NeuN is an useful DNA-binding neuronal specific nuclear protein, that decreases in PD. The results showed that compared with the sham group (Figure 23B), MPTP administration caused a decrease in NeuN+ positive cells (Figure 23F), while DMF restored neuronal population in a dose dependent manner (Figure 23H and 23N). Basal levels of Nrf-2 were observed in control mice (Figure 23A) respect to MPTP-injury mice (Figure 23E). DMF enhanced Nrf-2 levels in MPTP-treated mice in a dose dependent manner (Figure 23I and 23M). The yellow spots indicate the co-localization between Nrf-2 and NeuN (Figure 23L, 23P and 23Q). Reported images are representative of triplicate experiments.

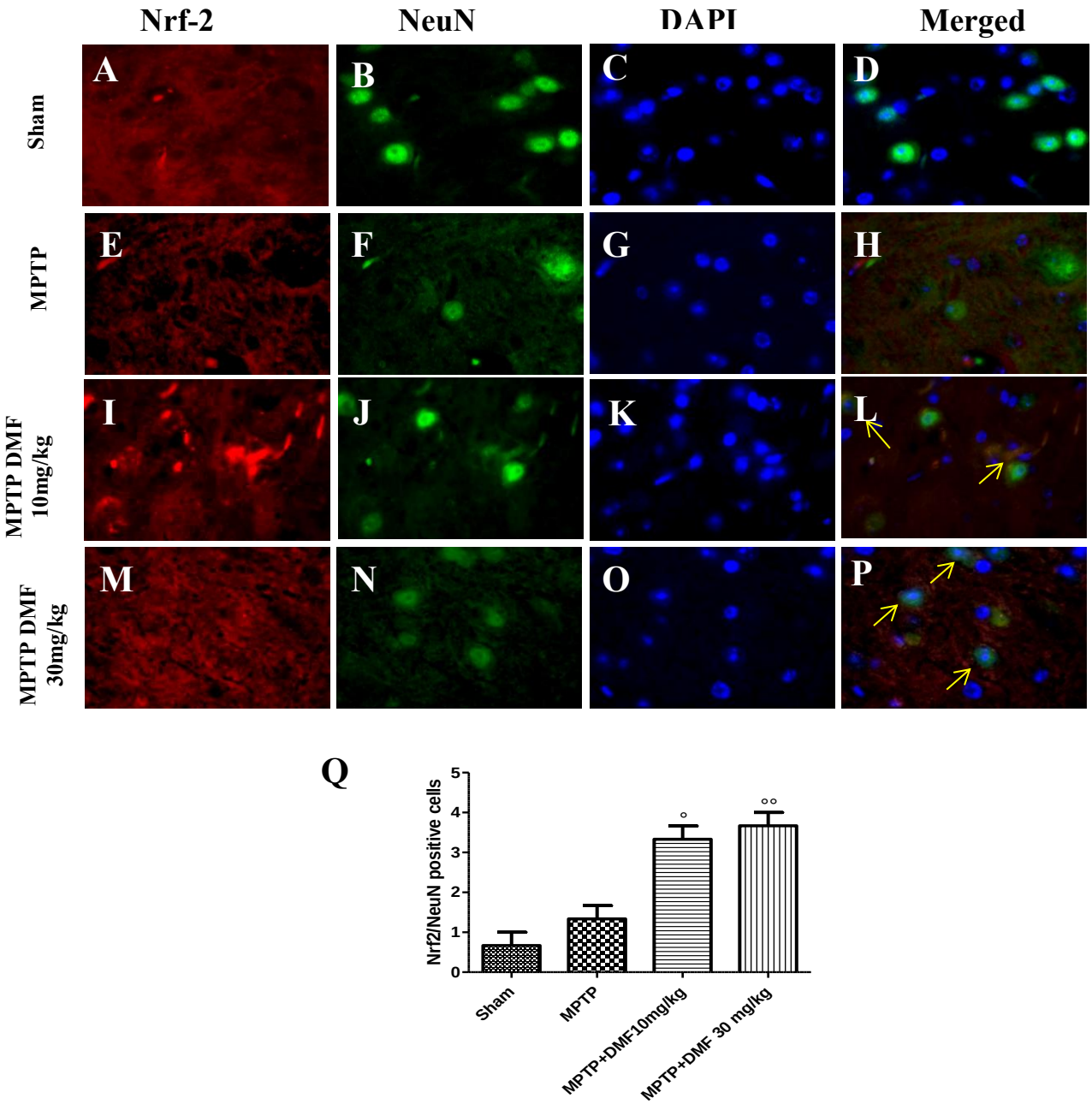
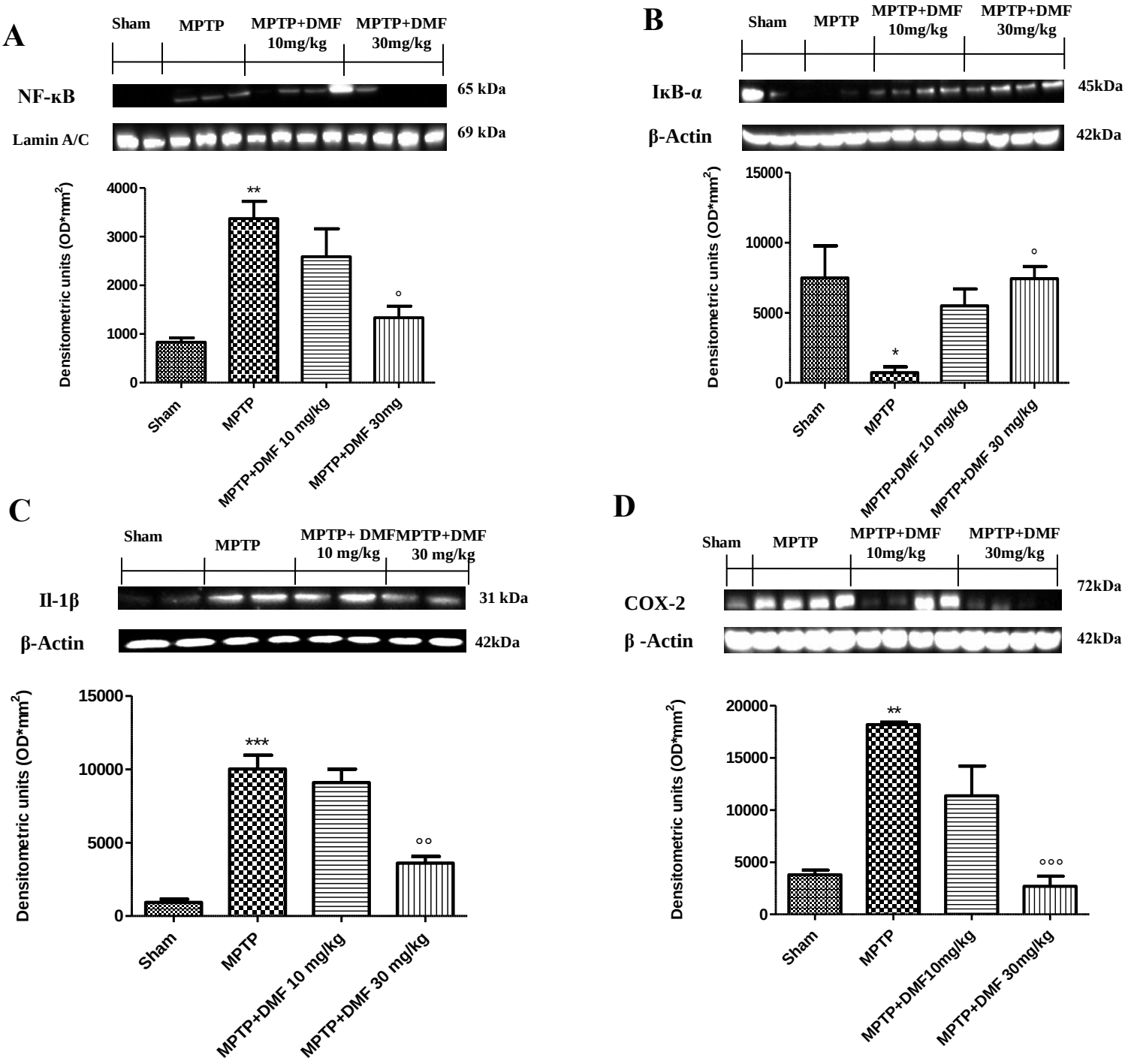


Figure 23: Neuronal protection of DMF treatment on oxidative stress. Midbrain sections were double stained with antibodies against Nrf-2 (red) and NeuN. NeuN immunoreactivity was reduced in MPTP-injured mice (F and Q) respect to control group (B and Q). DMF treatment increased NeuN positive staining at the dose of 10 mg/kg (K and Q) and 30 mg/kg (N and K). Instead, Nrf-2 immunoreactivity was basal in sham group (A and Q), increasing in MPTP-injured mice (E and Q). DMF, at both doses, reinforced antioxidant response increasing Nrf-2 positive staining (I, M and Q). Yellow spots revealed a high Nrf-2/NeuN co-localizations (L, P and Q). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. °P< 0.05 and °°P< 0.01 vs. MPTP.

8.8 DMF treatment exerted anti-inflammatory effects

To evaluate the anti-neuroinflammatory activity by which DMF treatment may attenuate the development of PD, we assessed the expression of NF-κB, IκB-α, Il-1β, Cox-2, CD11β and Iba-1 by western blot analysis in the midbrain homogenates at 8 days after MPTP administration. NF-κB pathway has a key role in MPTP-induced dopaminergic damage, so we evaluated the levels of NF-κB and IκB-α expression by western blot analysis. Treatment with MPTP resulted in a significant increase in NF-κB DNA-binding activity in the substantia nigra as compared to control mice (Figure 24A). DMF treatment, at the dose of 10mg/kg, attenuated the NF-κB nuclear traslocation in MPTP-injured mice, while, at the dose of 30 mg/kg, NF-κB DNA-binding activity is considerably reduced (Figure 24A). Conversely, IκB-α degradation was very lowered in MPTP-injured mice as compared to sham mice (Figure 24B), while DMF treatment increased, in a dose dependent manner, IκB-α cytosolic activity (Figure 24B). Also, our findings revealed significantly higher Il-1β and Cox-2 expression in MPTP-injected mice as compared to control mice (Figure 24C and 24D respectively); MPTP-induced up-expression of Il-1β was considerably reduced by DMF treatment at the dose of 30 mg/kg (figure 24C), while Cox-2 expression was completely blocked by treatment with higher dose (30 mg/kg) (Figure 24D). Furthermore, to evaluate microglial expression and activation, we assessed the levels of CD11β and Iba-1 expression by western blot analysis. In MPTP-injured mice, we observed a numerous microglial population (Figure 24F), with plenty of activated cells (Figure 24E) respect to control mice (Figure 24E and 24F); DMF treatment, at both doses, significantly reduced microglial expression and activation (Figure 24F and 24E respectively).


A
Sham
MPTP
MPTP+DMF 10mg/kg
MPTP+DMF 30mg/kg
NF-κB
65 kDa
Lamin A/C
69 kDa
Densitometric units (OD*mm2)
4000
3000
2000
1000
0
**
°
Sham
MPTP
MPTP+DMF 10 mg/kg
MPTP+DMF 30mg
B
Sham
MPTP
MPTP+DMF 10mg/kg
MPTP+DMF 30mg/kg
IκB-α
45kDa
β-Actin
42kDa
Densitometric units (OD*mm2)
15000
10000
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°
Sham
MPTP
MPTP+DMF 10 mg/kg
MPTP+DMF 30 mg/kg
C
Sham
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MPTP+ DMF 10 mg/kg
MPTP+DMF 30 mg/kg
Il-1β
31 kDa
β-Actin
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MPTP+DMF 30mg/kg

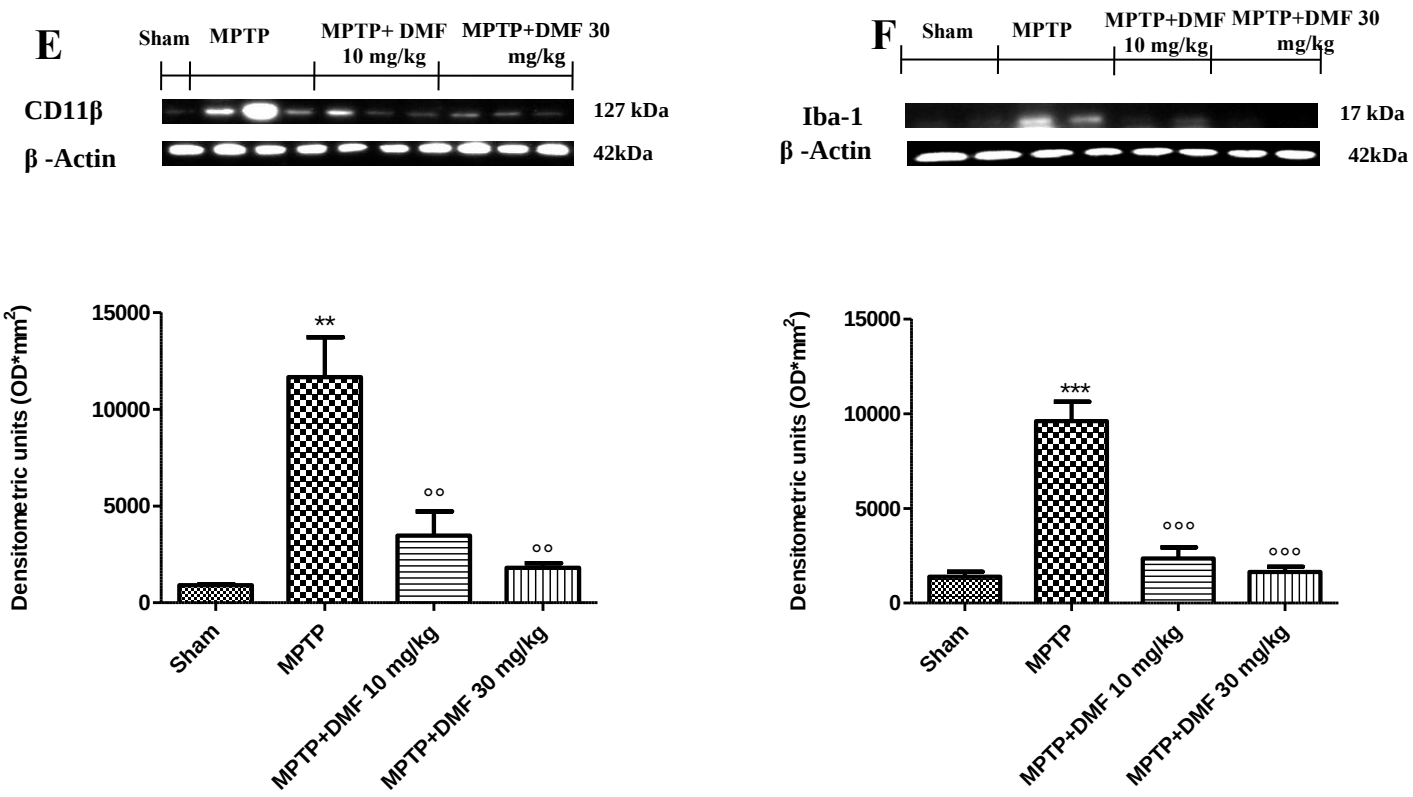

Figure 24: Anti-inflammatory effects of DMF. MPTP-injured mice showed a significant increase in NF-κB DNA-binding activity in the substantia nigra as compared to control mice (A). DMF treatment, at the dose of 10mg/kg, attenuated the NF-κB nuclear traslocation in MPTP-injured mice, considerably reduced at the dose of 30 mg/kg (A). Conversely, IκB-α degradation was very lowered in MPTP-injured mice as compared to sham (B), while DMF treatment increased, in a dose dependent manner, IκB-α cytosolic activity (B). Furthermore, we observed a significant expression of Il-1β in MPTP-injured mice (C), greatly reduced by DMF treatment at higher dose (C). Also, we revealed significantly higher Cox-2 expression in MPTP-injected mice as compared to control mice (D). MPTP- induced up-expression of Cox-2 was partly reduced by DMF treatment at the dose of 10 mg/kg, while it was completely blocked by treatment with higher dose (30 mg/kg) (D). Instead, MPTP intoxication caused a significant increase of microglia population (F) with its consequent activation (E), respect to control mice (E and F). DMF treatment significantly reduced the presence and the activation of microglial cells at both doses (F and E). Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. *P< 0.05,**P< 0.01 and ***P<0.001 vs. Sham; °P< 0.05, °°P< 0.01 and°°°P< 0.001 vs. MPTP.

8.9 DMF reduced the alteration of MAP-2 and restored neurotrophic factors levels after MPTP intoxication

In addition, to understand the effect of DMF treatment on microtubule assembly stabilization in neuronal dendrites, we evaluated MAP-2 expression levels by immunohistochemical analysis. MAP-2 is a cytoskeleton protein mainly localized in neuronal dendrites that stabilizes microtubule assembly and mediate their interactions with other neuronal cell components, whose expression decreases in PD. We observed that MPTP administration produced a significant decrease in MAP-2 expression (Figure 25B and B1, see densitometric analysis Figure 25E) compared to control mice (Figure 25A and 25A1, see densitometric analysis Figure 25E). DMF treatment was more protective in MPTP-injured mice at the dose of 30 mg/kg, (Figure 25D and 25D1, see densitometric analysis Figure 25E) than lower dose of 10 mg/kg (Figure 25C and 25C1, see densitometric analysis Figure 25E). Also, we assessed the role of DMF treatment in restoring neurotrophic factors levels after MPTP intoxication, through NGF expression levels by immunehistochemical analysis. NGF is a neurotrophic factor, that regulates the development and maintenance of the sympathetic and sensory nervous systems, whose expression is lower in PD. In midbrain section collected at 8 days after MPTP, NGF expression levels were significantly reduced (Figure 25G and 25G1, see densitometric analysis Figure 25J), in comparison to sham mice (Figure 25F and 25F1, see densitometric analysis Figure 25J). Treatment with DMF significantly restored NGF expression in dose dependent manner (Figure 25H and 25H1, 25I and 25I1, see densitometric analysis Figure 25J).

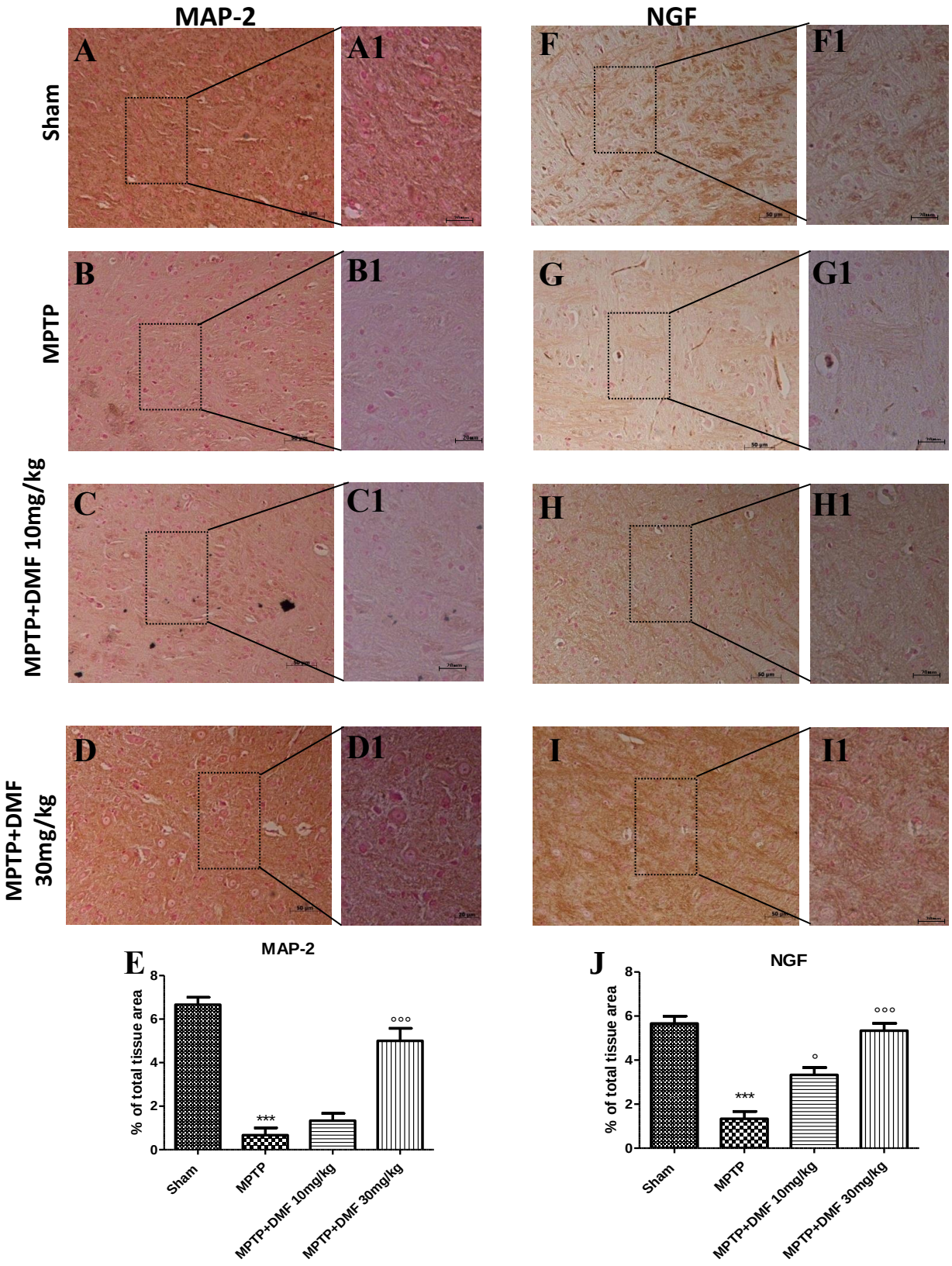

Figure 25: Effects of DMF on microtubule stabilization and neurotrophic factors levels after MPTP intoxication. MPTP administration produced a significant decrease in MAP-2 expression (B, B1 and E) compared to control mice (A, A1 and E). DMF treatment was very protective in MPTP-injured mice at the dose of 30 mg/kg, (D, D1 and E), but not in the lower dose (C, C1 and E). Also, in midbrain section collected at 8 days after MPTP, NGF expression levels were significantly reduced (G, G1 and J), compared to sham mice (F, F1 and J). Treatment with DMF significantly restored NGF expression in dose dependent manner (H and H1, I and I1 and J).Data are means ± SD of 10 mice for each group. Values are indicated as the mean±SEM. ***P< 0.001 vs. Sham; °P<0.05 and °°°P< 0.001 vs. MPTP.

8.10 The protective effects, NF-κB/Nrf-2 mediated, of DMF treatment *in vitro*

To evaluate the effect of DMF treatment on cell viability, a preliminary study was made incubating SH-SY5Y cells with increasing concentrations of DMF (1-10-30-50-100 μM). Cell viability was assessed after 24 h and only DMF at the concentrations of 1-10 and 30μM lacked cytotoxicity (Figure 26A). Incubation of cells with 3 mM MPTP significantly reduced viability compared to the control group. Instead, pre-treatment with DMF 30 μM, 2 h before MPTP, significantly reduced cell death compared to MPTP group and to the DMF pre-treatment at the concentrations of 1 and 10 μM (Figure 26B), demonstrating that DMF 30 μM was the most effective dose. Furthermore, to confirm *in vitro* that DMF protective effect is Nrf-2 dependent, SH-SY5Y cells were stimulated with MPTP 2 h after pretreatment with DMF 30μM. Moreover, SH-SY5Y cells were also stimulated with the Nrf-2 antagonist TR 1μM, 30 min before MPTP. We observed that TR notably inhibited DMF protective effect on cell viability compared to MPTP+DMF 30μM group (Figure 26C).

In addition, in order to corroborate, *in vitro*, that the anti-inflammatory and antioxidant effects of DMF treatment are NF-κB/Nrf-2 mediated, we evaluated the action of DMF treatment in the regulation of iNOS and Mn-SOD expression in SH-SY5Y cells. Western blot analysis demonstrated that iNOS expression was significantly higher (fourfold increase) in the MPTP group compared to control, while pre-treatment with DMF at the concentration of 30 μM lowered (84%) iNOS expression (Figure 24D). Also, the presence of TR 1μM, in the MPTP+DMF+TR group, abolished DMF protective effect, increasing by three times iNOS expression (Figure 24D). Conversely, Mn-SOD expression decreased (85%) after MPTP stimulation, while DMF treatment reported Mn-SOD at control levels (Figure 26E); incubation with TR 1μM increased SHSY-5Y susceptibility to MPTP damage, lowering Mn-SOD expression (Figure 26E) and strengthening, also *in vitro*, the thesis that Nrf2 inhibition antagonizes the protective effect of DMF.

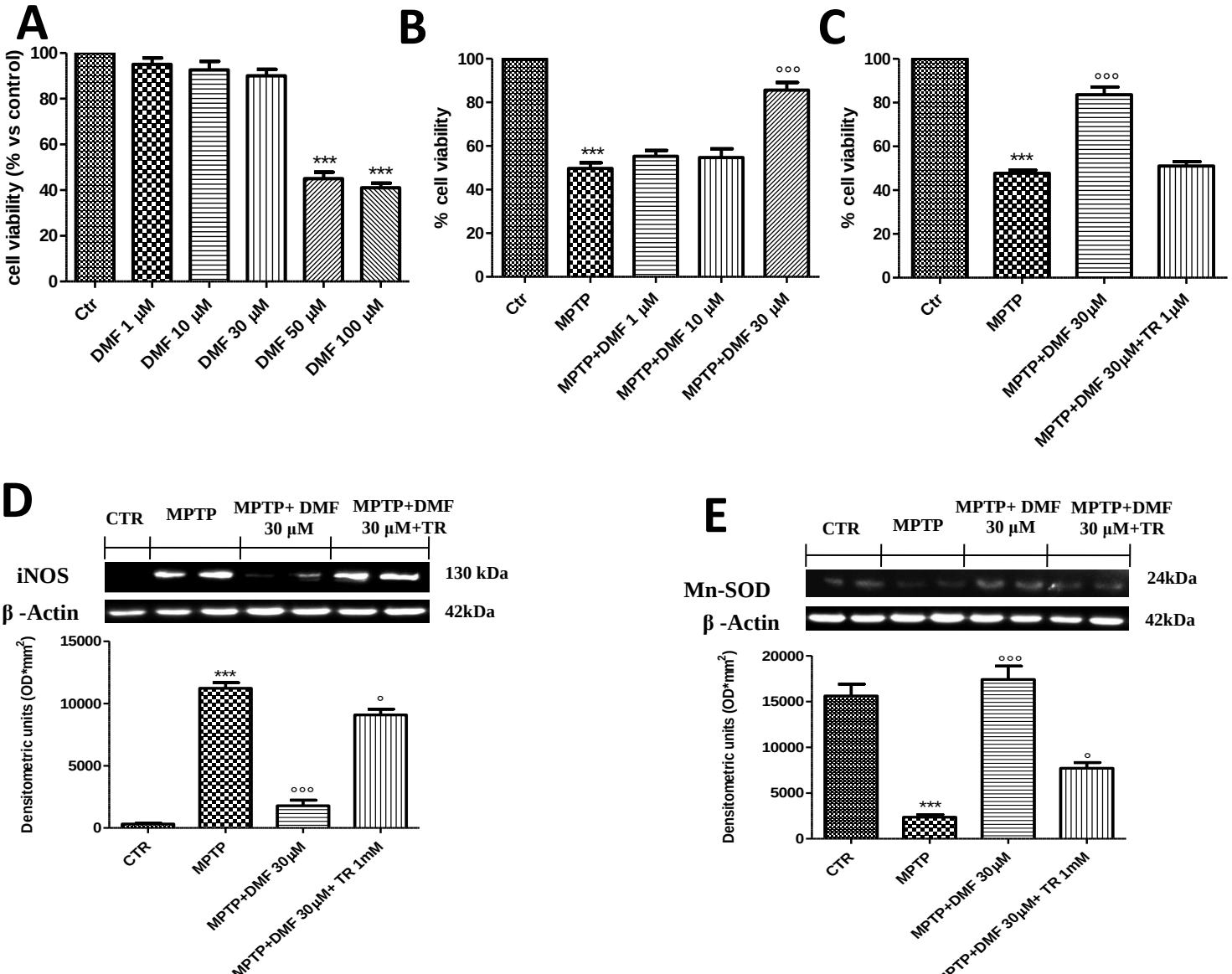

Figure 26: The anti-inflammatory an anti-oxidant effects, Nrf-2 mediated, of DMF treatment on survival of retinoic acid-differentiated SH-SY5Y cells. Cell death was assessed 24 h after treatment with the indicated concentrations of DMF treatment (1-10-30-50-100 µM), underling that only DMF 1-10-30 µM lacked cytotoxicity (A). Stimulation of cells with MPTP significantly reduced viability compared to the control group (Ctr); only pretreatment with DMF 30 µM significantly limited this cell death (B). Moreover, incubation with TR 1 µM at the same time with DMF 30 µM antagonized DMF treatment life-saving effect (C). Western blot analysis demonstrated that iNOS expression was significantly higher in the MPTP. Pre-treatment with DMF 30 µM lowered iNOS expression to control levels (D), but in MPTP+DMF+TR group, the presence of TR abolished DMF protective effect (D). Moreover, Mn-SOD expression was decreased in cells stimulated with MPTP respect to control group, augmenting following DMF 30 µM pre-treatment (E). TR impeded DMF protective action (E). Data and figures are representative of at least three independent experiments. (A) ***p 0.001 *vs* Ctr; (B) ***p 0.001 *vs* Ctr and °°°p<0.001 *vs* MPTP; (C) ***p 0.001 *vs* Ctr and °°°p<0.001 *vs* MPTP; (D)***p 0.001 *vs* Ctr and °p<0.05 and °°p<0.001 *vs* MPTP; (E) ***p 0.001 *vs* Ctr and °p<0.05 and °°p<0.001 *vs* MPTP.

Chapter 9

Discussion

Inflammation is a protective response by the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue (Medzhitov, 2008). Inflammation is caused by various factors such as microbial infection, tissue injury. The inflammatory cascade is a complex network of immunological, physiological, and behavioral events that are coordinated by cytokines, immune signaling molecules. For inflammatory diseases different medications are used, among which are used FAE;Since about use of the FAE is known that DMF is the most pharmacologically effective molecule and different studies have shown anti-inflammatory action of DMF (Miljkovic et al., 2015) and also that DMF activates the Nrf-2 system (Sandberg et al., 2014). Nrf2's activity, it is regulated at the molecular level by KEAP1 that sequesters and poly-ubiquitinates Nrf2 in the cytosol, leading to constitutive degradation (Nguyen et al., 2009). Electrophilic and oxidative stress changes the interaction between Nrf2 and KEAP1, resulting in the stabilization and translocation of Nrf2 to the nucleus. Similarly, DMF interaction with KEAP-1 results in the stabilization and translocation of Nrf2 that regulate cytoprotective genes associated with the phase II antioxidant response such as HO-1, NQO-1 and glutathione synthesizing enzymes. Thanks to its characteristics the DMF was approved by the FDA as an oral medication for the treatment of multiple sclerosis (Gold et al., 2013) and psoriasis (Mrowietz et al., 2013). Furthermore the DMF was tested *in vivo* and *in vitro*, for different curative properties, in autoimmune myocarditis (Meili-Butz et al., 2008), in HIV-associated neurocognitive disorders (Cross et al., 2011) and for others biomedical applications (Das et al., 2015). Recent experimental studies have shown that DMF exerts beneficial effects in preclinical models of neuroinflammation and neurodegeneration (Scannevin et al., 2012), protecting SH-SY5Y cells against 6-OHDA-induced neurotoxicity through Nrf-2 dependent mechanisms (Jing et al., 2015). Therefore, the aim of this study is to investigate, in the first

instance, the effects of DMF on the inflammatory response and colon injury caused by intra-colonic administration of DNBS to normal mice that displays human CD-like features and then to show that DMF exert a neurotherapeutic action NFκB-Nrf2 dependent in a model of Parkinson's disease, inducing in normal mice by administration of MPTP, that cause a disease with the same human symptoms. IBDs are characterized by inflammation of intestinal and colonic mucosa in response to the dysregulated immune system. Indeed, because of their relapsing bouts and chronic course, both Crohn's disease (CD) and ulcerative colitis (UC), can progress to fibrosis, resulting in pharmacologically uncontrollable alterations, which can be resolved by disabling surgical resections (Zhang et al., 2015; Rieder et al., 2012). Novel therapeutic approaches, including preliminary experience with biological therapies directed at TNF-α and other cytokines, adhesion molecules, growth factors, and probiotics have been reviewed (Hanauer, 2004). Particularly, TNFα-targeted therapies are a class of medication that has revolutionized the treatment of these diseases and the quality of life for patients but it also poses risk of developing various side effects including infections, exacerbation of some neurological manifestations, cutaneous lesions or induces antibody production (Dumitrescu et al., 2015). The first novel compound for IBDs to be introduced was Infliximab (Remicade®, Centocor®) directed at TNF-α (Bickston, 2007).

In this study we show that DMF attenuated DNBS-induced colitis in mice At the histological level, we observed that epithelial disruption was statistically significant less in mice treated with DMF. Furthermore, DMF-treated mice are more resistant to DNBS induced colitis with an important resolution of the macroscopic and histological signs of the inflammatory process. Also, we observed that DMF significantly attenuated body weight loss. Also, we have taken into account IL10KO mice that spontaneously develop a Th1-dependet chronic enterocolitis immediately after birth that is fully established at 8-10 weeks of age. We showed that in 9-week-old IL10KO mice with fully established colitis, after 7 weeks of treatment with DMF (30mg/kg) significantly reduced the degree of colitis as assessed by histologic damage score, by MPO activity and TNF-α levels

after treatment with DMF. Oxidative stress also plays a significant role in the pathogenesis of IBD. The intestinal mucosa is vulnerable to oxidative stress from constant exposure to ROS generated by the lumen contents (Grisham, 1994). It can cause cell damage either directly or through altering signaling pathways. Recently, has been demonstrated that DMF reduced ROS production and, also, oxidative stress (Wang et al., 2015). Particularly, De-Hyung at al. showed that application of DMF leads to stabilization of Nrf2, activation of Nrf2-dependent transcriptional activity and abundant synthesis of detoxifying proteins (Lee et al., 2012). Furthmore, endogenous antioxidants such as superoxide dismutase (SOD) are normally able to counteract oxidative stress in the intestinal mucosa (Mandalari et al., 2011). The present study confirmed the increase in-oxidized membrane phospholipids in the colon tissues from DNBS-injected mice; also we have shown that treatment with DMF reduced lipid peroxidation or MDA levels and increased the expression of anti-oxidant enzyme Mn-SOD. Several experimental evidences have clearly suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in the inflammation associated with experimental colitis. However, recent findings suggest that NF-κB has not only pro-inflammatory but also tissue-protective functions (Spehlmann et al., 2009). Regulation of NF-κB activity appears to be fundamental in determining the specific cellular response to TNFα (Rangamani P et al., 2007). Previously, many studies were demonstrate that DMF inhibits NF-κB by decreasing its phosphorylation and its entry to the nucleus (Peng et al., 2012). Thus, we reported here that DNBS administration caused an increase in NF-κB nuclear translocation and IκB-α degradation in the colon inflamed tissues at 4 days, whereas DMF reduced NF-κB translocation and inhibited the IκB-α degradation. Importantly, it was observed that DMF inhibits the TNFα-induced nuclear entry of NF-κB (Loewe et al., 2002) and degradation of IkB-α (Seidel et al., 2010). The present study confirmed the expression of TNF-α and IL-1β in the colon tissues from DNBS-injected mice and the treatment with DMF reduced these inflammatory cytokines levels. There is a consistent evidence that during acute and chronic colitis the sustained production of pro-inflammatory cytokines play a key role in the adherence and infiltration of

leukocytes to endothelial cells, thus maintaining the chronic inflammation into the cecal and colonic interstitium (Kawachi et al., 2000). TNF-α's production, in the middle and distal colon, promotes the infiltration of neutrophils for example through stimulation of synthesis of intracellular adhesion molecule (ICAM) and P-selectin, therefore leading to colonic tissue damage (Yasukawa K et al. 2012). In the present study, we confirmed that DNBS administration induced the appearance of P-selectin on the endothelial vascular wall and up-regulated the surface expression of ICAM-1 on endothelial cells. Treatment with DMF abolished the expression of P-selectin and the up-regulation of ICAM-1 without effecting constitutive levels of ICAM-1 on endothelial cells. The absence of an increased expression of the adhesion molecule in the colon tissue from DMF-treated mice correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme MPO and with the attenuation of the colon tissue damage as evaluated by histological examination. It has been demonstrated that NF-κB regulates the expression of MMPs that are considered to be an important component in the progression of inflammation (Gold et al., 2013).There is also growing evidence that MMP-9 and -2 can be implicated in the pathophysiology of several intestinal inflammatory disorders. In fact, MMPs can be released from almost all connective tissue cells present in the bowel in response to inflammatory stimuli including colitis (Medina et al., 2006). The involvement of MMPs in inflammatory processes was demonstrated by studies in animal models that induced CD or UC and in cell cultures (Silosi et al., 2014). Recently, different studies have been demonstrated that DMF decreases matrix metalloproteinase expression (Yamazoe et al., 2009) and activity (Kunze et al., 2015). In the present study, we confirmed the expression of MMP-9 and -2 in DNBS-injected mice, while the treatment with DMF reduced the degree of expression of MMP-9 and -2. Nrf-2 and HO-1 protein expressions were up-regulated in those mice treated with DMF and the activation of NF-κB pathway was drastically ameliorated. Moreover, data showed the positive correlation of the antioxidant activity with the mechanism involved in the physiologic maintenance of the integrity and function of the intestinal epithelium. In fact, in this study occludin and ZO-1 proteins were significantly restored by 10μM DMF in Caco-2

after administration of H_2O_2. In recent findings, it is seen that the Nrf2 activation is related to the interaction with NF-κB (Nair et al., 2008).

It has been shown that DMF exerts many beneficial effect in CNS, in many pathologies such us MS, PD. PD is the second most common progressive neurodegenerative disorder, characterized by both motor and non-motor system manifestations, resulting from a pathophysiologic loss or degeneration of dopaminergic neurons in the SNpc of the midbrain and the development of neuronal Lewy bodies (Beitz, 2014). Recent years have attested an increase of medical therapies for treatment of PD, including pharmacotherapy, non-pharmacological alternative approaches and innovative surgical interventions (Agundez et al., 2013). Despite these advances in the treatment of Parkinson's disease, an effective therapy has not yet been found (Suchowersky et al., 2006). Although the pathogenesis of neuronal degeneration in PD is not yet fully understood, it is known that oxidative stress is common underlying mechanism that leads to cellular dysfunction and demise in many several neurodegenerative diseases, including PD (Yamazaki et al., 2015). Nrf-2 signaling cascade is a promising pathway for medical purpose by transcriptional modulation of both inflammation and oxidative stress, whereby there is an increasing clinical interest in using Nrf-2 activators in inflammatory diseases (Kaidery et al., 2013). We showed that DMF exerted a neurotherapeutic action NFκB-Nrf2 dependent in a mouse model of Parkinson's disease. It is clearly demonstrated that mice treated with the dopaminergic neurotoxin MPTP can develop a variety of behavioral deficits (Sedelis et al, 2001), resulting in a variety of symptoms concerning motor control such as akinesia, rigidity, tremor, gait and posture disturbances that indicate a longitudinal decrease in locomotor performance (Klemann et al, 2016). In this study, for the first time, resorting to pole test and rotarod test, was demonstrated that DMF significantly improves locomotor agility, stability and latency, for all doses. Also, since non-motor features of PD represent important challenges in the clinical management of this pathology and a degenerated nigrostriatal circuit plays a role in anxiety-like behaviors in animal models of PD (Santiago et al., 2010), it was assessed

whether DMF may affects not only sensorimotor performance, but also emotional aspects. We highlighted that DMF did not significantly interfere with emotional behavior component, suggesting a possible role of dopamine medication for DMF, because non-motor features of PD usually do not respond to dopaminergic treatment (Chaudhuri et al., 2006). For *in vivo* experiments have been used three different doses of DMF at 10, 30, 100 mg/kg, however, since by histological and behavioral studies, DMF 30 mg/kg showed an effective dose similar to DMF 100 mg/kg, we continued this experimental study using only 30 mg/kg as the highest dose to avoid possible side effects. Also, by histological evaluation, we observed that, in the sham+DMF (at three different doses) groups, DMF administration didn't demonstrate any toxicity, improvement o any difference respect to sham controls mice; so we continued this experiment excluding DMF alone groups. The decline in dopamine level, hallmark of PD, has been thought to arise solely from the severe loss of dopaminergic neurons in the nigrostriatal pathway. However, the dopamine deficits in the affected regions of the brain significantly exceed the loss of dopaminergic neurons (Ara et al., 1998) suggesting that dopamine synthesis is impaired before cellular demise. In fact, studies on experimental models of PD demonstrated that the reduction in dopamine metabolism-related markers such as TH and DAT is far greater than the loss of neuronal cell bodies (Jackson-Lewis et al., 1995). In this study, we demonstrated that acute treatment with DMF, especially at the dose of 30 mg/kg, protects against MPTP-induced loss of TH^+ neurons in the SNC. Also, despite DAT expression depends on the striatal extracellular dopamine concentration (Zhang et al., 2015), we observed that DMF treatment, at the higher dose, increased the dopamine release. MPTP-induced neuronal degeneration was associated with the redistribution of α-synuclein from its normal synaptic location to aggregates in degenerating neuronal cell bodies, the early stages of Lewy bodies formation (Kowall et al., 2000). Also, under pathologic conditions, α-synuclein proceed from monomers to inclusions trough multistep process leading to the formation of soluble oligomer species of α-synuclein that cause neuronal toxicity (Kalia et al., 2013), but DMF treatment, especially at the dose of 30 mg/kg, significantly reduced α-synuclein-positive neurons, mainly

inhibiting the formation of dimers and oligomers. Furthermore, it has been reported that oxidative stress affects α-synuclein, causing the oxidative damaged α-synuclein to mimic some of the abnormal behaviors of mutant α-synuclein (Hashimoto et al., 1999). Nrf-2 is a key regulator of endogenous inducible defense systems in the body that, in response to oxidative stress, translocates to the nucleus and binds to specific DNA sites termed "anti-oxidant response elements" (Sandberg et al., 2014). The combination of properties of fumarate and our data suggest that DMF up-regulated the Nrf-2 transcriptional system, dysregulated in brains of individuals suffering from neurodegenerative diseases, such as PD, playing a major role in neuronal cell and tissue defense against oxidative stress. It is known that Nrf-2 induces phase II detoxifying and antioxidant enzymes (Hashimoto et al., 1999) as HO-1 and Mn-SOD proteins, working together to contest oxidative stress and inflammation. In fact, our study showed that DMF treatment, via Nrf-2 pathway, up-regulates Mn-SOD and HO-1 expression, conferring resistance against neurodegenerative insults. Our findings provide new insights on the biochemical properties of DMF as Nrf2/HO-1/Mn-SOD activators that are relevant for the control of inflammatory processes in neuronal cells. The anti-oxidative pathway activated by DMF involves downstream glutathione (GSH) regulation; it has been observed that during PD, there is a further reduction in GSH levels within the SNpc, so GSH depletion is the first indicator of oxidative stress during PD progression (Bharath et al., 2002). DMF treatment, through Nrf2-dependent regulation of GSH redox, is essential for neuronal cell survival during oxidative stress in PD disease. Indeed, DMF treatment resulted in an increased Nrf-2/NeuN immunoreactivity in neuronal subpopulations that highlights a protective effect of preservation of morphologically intact neurons in the striatum mediated by DMF. Neuronal loss in PD is associated with chronic neuroinflammation, which is controlled primarily by microglia, the resident innate immune cells and the main immune responsive cells in the central nervous system. Microglial reaction has been found in the SN of sporadic PD patients (McGeer PL et al., 1988) as well as familial PD patients (Yamada T et al., 1993) and in the SN and/or striatum of PD animal models elicited by MPTP (O'Callaghan JP et al., 1990). In this study

we suggested that DMF treatment, through Nrf-2 activation, might attenuate microglial expression and activation, modulating Iba-1 and CD11β levels. Microglia become persistently activated and preserves elevated production of both cytokines and reactive oxygen species in PD. Recent findings have linked activation of the Nrf-2 system to anti-neuroinflammatory effects (Linker et al., 2011); furthermore, mediators of neuroinflammation are extensively involved in the dopaminergic neurodegeneration, COX-2 enzyme and Il-1β cytokine up regulation has widely been documented in the dopaminergic neurodegeration in the PD pathophysiology (Teismann et al., 2003) as mediates neuronal damage presumably by forming excessive amount of harmful prostanoids and free radicals. DMF treatment, mainly at the major dose, reported COX-2 and Il-1β to baseline levels, highlighting the role of DMF to mediate neuronal damage by forming excessive amount of harmful prostanoids and free radicals (Nogawa et al., 1997). In recent findings, it is seen that the Nrf2 activation is related to the interaction with NF-κB (Nair et al., 2008). The relation between Nrf-2 and NF-κB is not well characterized but the identification of NF-κB binding sites in the promoter region of the Nrf-2 gene suggests a link between these two regulators of inflammatory processes. Thus DMF treatment in mouse model Parkinson disease, mostly at the dose of 30 mg/kg, we have shown that inhibited the nuclear translocation of NF-κB and promoting the transcription of IκB-α, represents a functional system to regulate neuro-inflammation in response to oxidative stress in the brain. However, oxidative stress is intimately linked not only to inflammation, but also to other components of neurodegenerative process, such as nitrosative stress (Yokoyama et al., 2008). Various isoforms of the nitric oxide (NO) producing enzyme nitric oxide synthase (NOS) are elevated in PD indicating a critical role for NO in the pathomechanisms, considering that increased expression of all NOS isoforms in astrocytes and neurons contributes to the synthesis of peroxynitrite which leads to generation of nitrotyrosine (Iravani et al., 2002). We demonstrated that DMF treatment can be considered a selective inhibitor of nNOS, producing dose dependent protection against nitrotyrosine increase MPTP induced. Then, DMF treatment significantly down regulated the MPTP-induced oxidative and nitrative stress indicating its useful antioxidant

properties against MPTP insults. To confirm, in vitro, that the anti-inflammatory and antioxidant effects of DMF are NF-κB/Nrf-2 dependent, we evaluated DMF capacity to protect against cell death after MPTP stimulation in SH-SY5Y cells, highlighting that DMF treatment loses its effectiveness in presence of Nrf-2 inhibitor TR. TR, a natural coffee extract, significantly reduces Nrf2 nuclear protein levels at low concentrations (0.0001–1 mM) (Arlt et al., 2013). Thereby, DMF mediated Nrf-2 upregulation could be a possible tool to be exploited in order to counteract PD progression. PD, as many others neurodegenerative conditions, is associated with low production neurotrophins, such as NGF, that migrates into the brain at the site of disease, contributing to resolution of neuroinflammation (Lim et al., 2011) and decreased expression of MAP-2 in the striatum and substantia nigra (Muramatsu et al., 2003). We demonstrated that DMF treatment also rehabilitates brain function by direct binding to dendritic brain microtubule-associated protein MAP2 and to NGF receptors.

The obtained results have showed that DMF present many beneficial effects in many inflammatory disorders that affect both peripheral tissues and CNS. Moreover, it has been clarified the essential involvement of NF-κB/Nrf-2 pathways important for DMF effects. Taken together, the data presented in this study suggest that DMF could represent a target for therapeutic intervention in inflammatory disorders.

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