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The impact of apple peel polyphenols on intestinal and mitochondrial functions in experimental colitis

par

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Ce mémoire est intitulé:

The impact of apple peel polyphenols on intestinal and mitochondrial functions in experimental colitis

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Dr Prevost Jantchou, Président rapporteur

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Résumé

Contexte:

L'inflammation et le stress oxydatif (OxS) participent à la pathogenèse de la colite ulcéreuse (CU). Nos résultats récents montrent que les polyphénols de la pelure de pomme (DAPP) jouent un rôle clé dans la prévention de la maladie.

Objectifs:

Évaluer les effets préventifs et curatifs du DAPP sur la CU et démontrer leur impact sur la dysfonction mitochondriale.

Méthode:

Une induction de l'inflammation intestinale a été effectuée chez des souris par administration du dextran sulfate sodium (DSS). Des doses de DAPP (200 et 400 mg/kg/j) ont été administrées par gavage pendant 10 jours afin d'évaluer les effets préventifs et curatifs, respectivement, sur l'Inflammation et le OxS au niveau intestinal ainsi que sur les fonctions mitochondriales.

Résultats:

Le DSS a provoqué une perte de poids, un raccourcissement du côlon, une augmentation du stress oxydant, niveaux de malondialdéhyde et une inflammation documentée par l'infiltration des cellules inflammatoires, la myéloperoxydase et les cytokines inflammatoires. D'autre part, le DSS a induit des désordres au niveau de la biogenèse (PGC1α) et des fonctions de la mitochondrie : diminution de l'ATP, altération des enzymes antioxydantes (SOD2 et GPX1), augmentation de l'apoptose (Bcl 2, Bax et Cytochrome C), et des défauts de l'intégrité de l'ADN (baisse d'OGG1). Cependant, le DAPP a amélioré significativement l'inflammation et le stress oxydant de l'intestin tout en corrigeant les aberrations mitochondriales.

Conclusions:

Les polyphénols ont la capacité d'agir sur le stress oxydant et le profil inflammatoire de l'intestin ainsi que sur le dysfonctionnement mitochondrial. Ils pourraient donc intervenir efficacement dans la CU.

Mots-clés : Stress oxydatif, Mitochondrie, Inflammation, maladies inflammatoires intestinales, polyphénols

Abstract

Background:

We have recently shown that dysregulation of redox-sensitive signaling pathways and oxidative damage to biological structures are major contributors to experimental ulcerative colitis. We also demonstrated the powerful anti-oxidant and anti-inflammatory actions of dietary apple peel polyphenols (DAPP) in the intestine.

Objectives:

As mitochondria are major sources and target of free radicals, as well as exhibit various important cellular functions, we evaluated their roles in intestinal colitis and their responses to DAPP.

Methods:

Induction of intestinal inflammation in C57BL6 mice was performed by administration of 3% dextran sulfate sodium (DSS). Two different doses of DAPP (200 and 400 mg/kg/day) were administered by gavage for 10 days (before and during DSS administration) to examine the preventive and curative effects, respectively, on inflammation and oxidative stress (OxS) in the intestine, and on mitochondrial functions.

Results:

DSS caused a significant weight loss, shortening of the colon, increased OxS (noted by lipid peroxidation), and raised inflammation (verified by infiltration of inflammatory cells, up-regulation of MPO, and elevated TNF-α and COX2 protein expression). Furthermore, DSS induced perturbations in mitochondrial biogenesis, as reflected by alterations of the transcription factor PGC1α and mitochondrial function characterized by diminished Adenosine-5'-Triphosphate (ATP) production, lowered antioxidant defense (GPx and SOD2), amplified apoptosis (as illustrated by the high expression of Cytochrome C and AIF), and defects in DNA integrity (high 8-OHdG). However, DAPP administration improved macroscopic parameters (e.g. weight loss, colon shortening) and reduced DSS-induced clinical signs. DAPP showed an evident capability of reducing inflammation (as noted by decreased

TNF-α, iNOS, COX-2 and AP-1) and OxS (as shown by reduced malondialdehyde, hydrogen peroxide levels and increased GPx) in DSS mice. Our findings also revealed that DAPP partially corrected mitochondrial dysfunction related to redox homeostasis, fatty acid β-oxidation, ATP synthesis, apoptosis and regulatory mitochondrial transcription factors (PGC1α, PPAR*γ* and Nrf-2).

Conclusion:

DAPP have the ability to act on intestinal OxS, inflammation and mitochondrial dysfunction, thereby alleviating colitis progression via the modulation of cellular energy, OxS, antioxidant capacity, apoptosis and mtDNA integrity.

Keywords: Mitochondria, mitochondrial function and dysfunction, inflammatory bowel disease, inflammation, Crohn's disease, experimental colitis, oxidative stress, polyphenols, prevention, treatment.

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List of abbreviations

ACADL	Acyl-CoA dehydrogenase
AIF	Apoptosis-inducing factor
ATP	Adenosine-5'-Triphosphate
Bcl2	B-cell lymphoma 2
BAX	Bcl-2-associated X
Ca2+	Calcium
CAT	Catalase
CD	Crohn's Disease
COX	Cyclooxygenase
CRC	Colorectal Cancer
Cyt	Cytochrome
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulfate Sodium
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FA	Fatty Acids
IBD	Inflammatory Bowel Disease
IECs	Intestinal Epithelial Cells
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
GI	Gastrointestinal
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
MAMs	Mitochondria-associated membranes
MAPKs	Mitogen-activated protein kinases
MDA	Malondialdehyde
MPTP	Mitochondrial permeability transition pore

Mt	Mitochondria
mtTFA	Mitochondrial transcription factor A
NF-κB	Nuclear factor-kappa B
Nrf2	Nuclear factor erythroid 2 related factor
OxS	Oxidative Stress
8-OHdG	8-hydroxy-deoxy-guanosine
OGG1	8-oxo DNA Glycosylate
OXPHOS	Oxidative Phosphorylation
PGC-1α	Peroxisome Proliferation Activator Receptor γ-coactivator 1α
PTP	Permeability transition pore
ROS	Reactive Oxygen Species
SCFA	Short chain fatty acid
SOD	Superoxide Dismutase
TNF-α	Tumor Necrosis Factor- Alpha
UC	Ulcerative colitis

To my lovely mother, Sherry, and best brother and sister (Sina and Betsabeh)

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1 INTRODUCTION

1.1 Introduction to Inflammatory Bowel Diseases

1.1.1 Pathophysiology and Epidemiology of Inflammatory Bowel Diseases

Inflammatory bowel disease (IBD) is one of the common types of intestinal inflammatory diseases. It describes a group of disorders that attack the intestine and cause intestinal cells to become swollen and inflamed. In other words, IBD is a series of immunoinflammatory disorders of the gastrointestinal (GI) tract, which mainly include two main disorders: Crohn's disease (CD) and ulcerative colitis (UC). Additional rare forms of IBD are observed such as collagenous colitis and intractable colitis [1, 2].

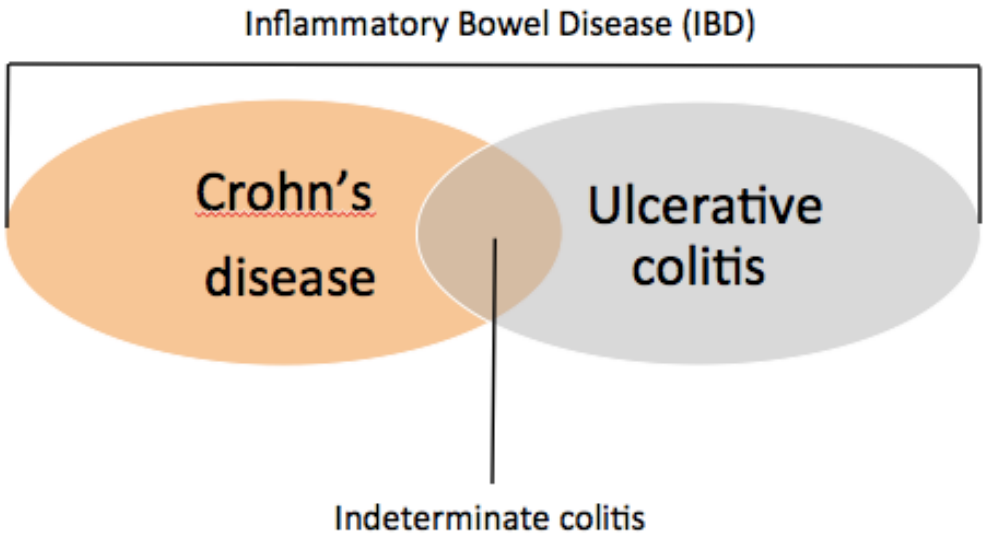

Figure 1. IBD subclasses. Taken from Rubin et al. [1]

While the two main forms of IBD, UC and CD, are not as common as heart disease or cancer, they cause significant morbidity and financial burden on the health care system. Studies have reported that the prevalence of IBD in adults in the United States is more than 200 cases per 100,000 with the total number of 1 to 1.5 million IBD patients [3, 4]. The prevalence of IBD in the past few years in Canada has been 150-250 cases per 100,000, and hospitalization due to UC and CD has been evaluated at 50.6 and 50.1 cases per 100,000, respectively [5, 6].

Recently, research has shown that IBD is more prevalent in developed countries. Lifestyle related factors such as nutrition, exposure to pollution and chemicals, low physical activity, and decreased exposure to sunlight are associated with the increased prevalence of IBD [7]. Other studies revealed that the risk of succumbing to recently diagnosed severe and long term cases of IBD is increasing. Moreover, risk of colorectal cancer (CRC) has recently raised to 20% per year compared to 0.5% reported previously, especially in cases of UC [8].

IBD is a multifactorial chronic, recurring and inflammatory disease of the GI tract commonly presenting clinical symptoms of diarrhea, bloody stools, abdominal pain and weight loss, and the development of an inflammatory response [9].

In animal models of colitis, the same common macroscopic symptoms are monitored. This includes body weight loss, fecal bleeding, stool consistency (e.g., diarrhea, liquid), shortening of the colon, and change of intestinal microbiota [10, 11]. In addition, experimental study by Islam et al. reported the severe histological changes of murine intestinal tissues with DSS-induced colitis, which are similar to human colitis, including

augmented thickness of the muscle layer, lymphocyte infiltration, mucosal erosion, loss of crypt structure, ulcer formation and also serious intestinal microbiota modifications [12].

Genetics and possible genetic heterogeneity play a fundamental role in IBD but the precise nature of the genetic implication is extremely complex, and it is probable that the cooperation of certain genes acts as a responsible modulator. Recently, the genetic study by Nell et al. has demonstrated the importance of environmental factors and specifically the gut microbiota in the occurrence of CD and UC [13].

1.1.2. CD and UC: Pathology, signs and Symptoms

Crohn's and colitis are known to cause major health problems in the human body and are characterized by abnormal chronic inflammation and immune-mediated injury to the GI tract. Although the main triggers of IBD disorders are not clearly identified, recent studies have reported that, generally, the complex relations between genetic determinants (e.g. IBD1, IBD2…), unfavourable environmental factors (smoking, diet, stress, drug, social status, education), dysregulated immune response and qualitatively and quantitatively unusual gut microbiota could be the main determinants in the pathogenesis of CD and UC as shown in Fig. 2.

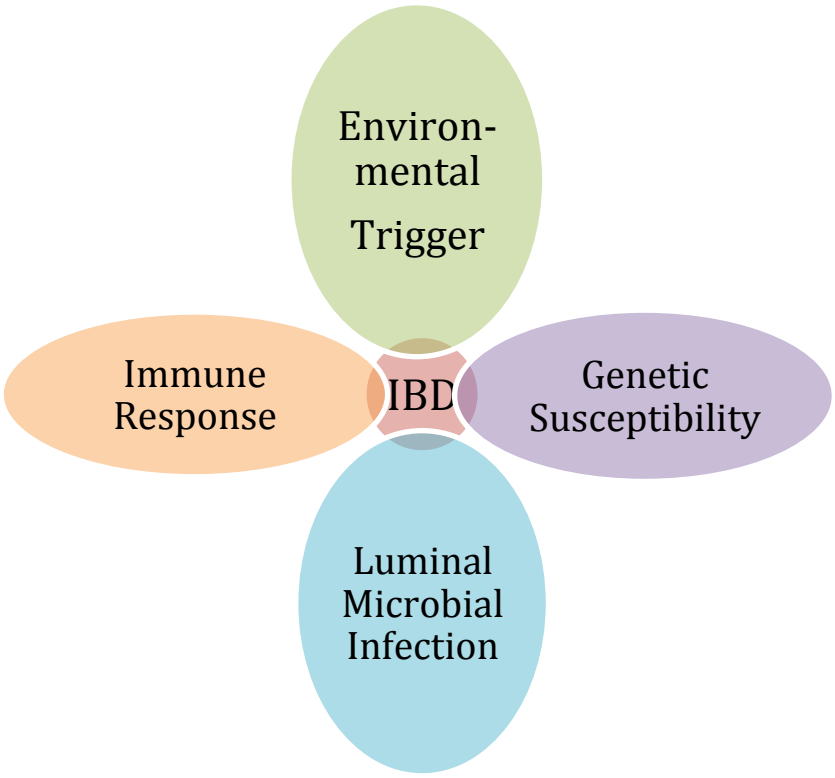

Figure 2. The common pathogenic factors in IBD. Taken from de Souza et al. [15]. This image shows that IBD can develop due to the body's distorted and changed immune response, changes in intestinal microbiota, a complicated interplay of genetic predisposition and environmental factors.

High levels of oxidative stress (OxS), mitochondrial DNA mutations and dysfunction in mitochondrial calcium (Ca2+) pathway can fundamentally cause or affect intestinal inflammation directly or indirectly, thereby increasing the risk of IBD as briefly described in Fig.3.

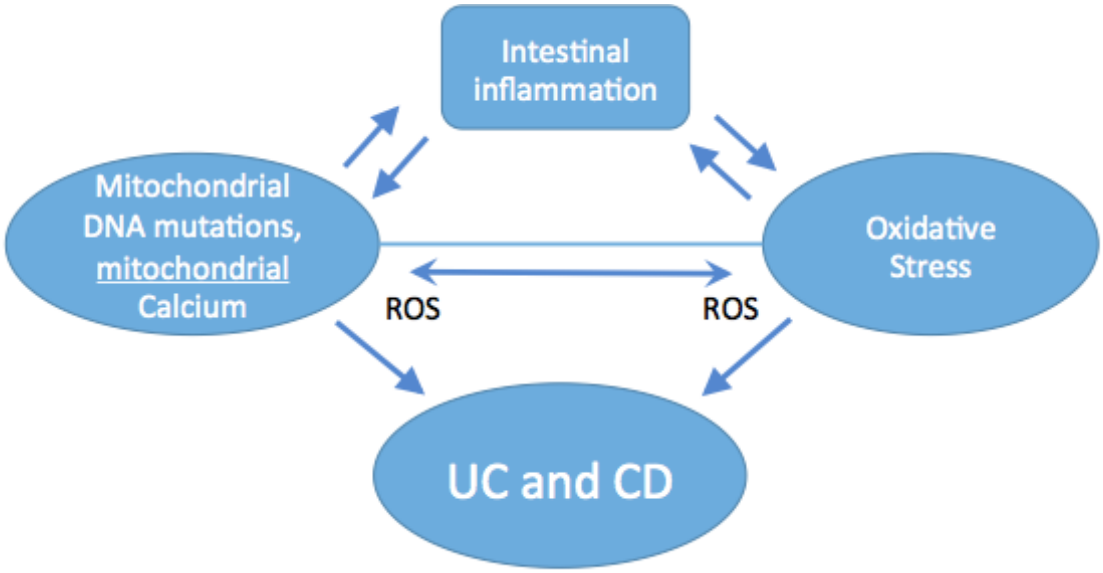

Figure 3. Risk factors involved in the pathogenesis of UC and CD.

CD and UC have been studied in parallel, as their common symptoms, structural damage and treatments are very similar. However, it is known that they are characterized by two separate pathophysiological patherns.

In CD, all parts of the GI tract, from the mouth to anus could be affected, but the disease generally affects the distal part of the small intestine or ileum. On the other hand, UC only causes colonic inflammation and affects part or all of the colon and the rectum [1]. Generally, both CD and UC patients present similar clinical symptoms (e.g. diarrhea, abdominal pain, GI bleeding, and weight loss). Duerr et al. reported that genetic risk factors play a major role in the prevalence of IBD and risk of IBD development is increased by 5-30% in families of affected individuals. Interestingly, Tysk et al. found that the risk of phenotypic concordance in monozygotic twins with CD renged from 50-75% in the second twin. However, they did not detect the same relation in UC patients [16, 17]. Duerr et al. also discovered that patients with CD have more family history of

this disorder compared to UC patients. In both studies, the authors suggested that other risk factors, including environmental factors, play an important role in UC and CD pathogenesis.

In addition, many researchers have looked of the probability of CRC, a side effect of long-term chronic inflammation, in UC and CD patients. They reported that probability of CRC in UC patients increased from 2% after 10 years of disease to 18% after 30 years compared to patients with CD that showed 2.9% risk of CRC at 10 years, which increased to 8.3% after 30 years of disease [18-20].

1.1.3. Manifestations of IBD

1.1.3.1. Intestinal dysfunction in intestinal inflammation

Recent studies reported multiple pathogenic factors that are involved in the prevalence of IBD for both CD and UC. Some of these pathogenic factors are:

1. Qualitatively and quantitatively abnormal gut microbiota
2. Largely dysregulated immune response.

Chassaing's study shows that such qualitative and quantitative changes in composition of the gut microbiota occur at the microbiological level in both CD and UC patients by reducing the complexity of commensal bacteria [21]. Former studies indicated the presence of high levels of immune reactivity against microbial antigens in patients with IBD. Murdoch et al. reported serum antibodies against microbial antigens in CD patients [22]. In addition, Andoh and his team documented the existence of high levels of

abnormal gut microbiota in UC patients compared to CD patients [23]. Additionally, Halstensen's results pointed out that an antibody-mediated immune (autoimmune) response in UC patients could act as a pathogenic mechanism for epithelial impairment and accompanying inflammation [24].

The mucous layer is known to be the first line of protection against injurious agents in the gut lumen in healthy cells. In both forms of IBD, intestinal mucosa and intestinal epithelial cells (IECs) could be injured, resulting in intestinal permeability changes. IECs could be the main target for injury in IBD patients and these alterations may decrease humoral protective properties [25]. The study of Bjarnason et al. showed that intestinal permeability in patients with active CD is increased, causing unusual infiltration (by measuring the urine excretion of 51-chromium-labeled ethylenediaminetetraacetate) [26]. Importantly, intestinal permeability disorder is directly related to higher risk of acute cases of IBD [27]. For example, Pineton et al. demonstrated that infiltration of both neutrophils and macrophages into the intestinal mucosa is present in patients with chronic intestinal inflammation and disorders of IECs [28]. Moreover, antigen production is important for immunoregulation of healthy intestinal cells and different reports suggested the existence of possible abnormalities of IEC antigen-presenting capacity in IBD. Turner reported a direct relationship between intestinal permeability and IBD immunopathogenesis [29]. As adequate balance in the regulation of intestinal mucosal barrier is essential for normal cell function in the absorption of nutrients and waste secretion, any disruption of the mucosal barrier function can change the absorption of nutrients and microbial products that may lead to inflammation, as is the case in IBD. In addition, reactive oxygen species (ROS) produced by these activated inflammatory cells

induce oxidative damage in intestinal mucosa creating an imbalance in the redox status, which exacerbates inflammation in IBD [30].

1.1.3.2. Markers of inflammation and OxS in IBD

As mentioned previously, unusual infiltration of macrophages into the intestinal mucosa and disorders of IECs triggered by the increasing intestinal epithelial permeability is present in intestinal chronic inflammation (IBD) [31]. ROS generated by these activated inflammatory cells could cause oxidative damage in intestinal mucosa. In addition, some studies present OxS as the most probable cause for the pathogenesis of human IBD [32, 33] OxS is an imbalance between anti-oxidant defense and the production of ROS such as superoxide, hydrogen peroxide, hydroxyl radical and peroxynitrite disturb the redox homeostasis. Intermediate levels of ROS cause apoptosis, while imbalance due to overproduction of ROS and free radicals can decrease antioxidant defense causing necrosis and is harmful to cellular biological processes and tissue functions [34].

Erichsen et al. and Rezaie et al. revealed that ROS levels vary under different conditions that could act by activating biological mediators (pro-oxidants or antioxidants). Low ROS levels were observed under physiological conditions whereas high ROS concentrations were noted under pathophysiological conditions. High concentration of ROS can oxidize nucleic acids (nuclear and mitochondrial DNA), or cause lipid peroxidation and protein damage [35, 36]. On the other hand, previous results demonstrate that low ROS concentrations, provoked by the regulation of cell signaling cascades (signaling

pathways) and defense arising from endogenous and exogenous antioxidants, could be beneficial on cell health [37] as shown in Fig.4.

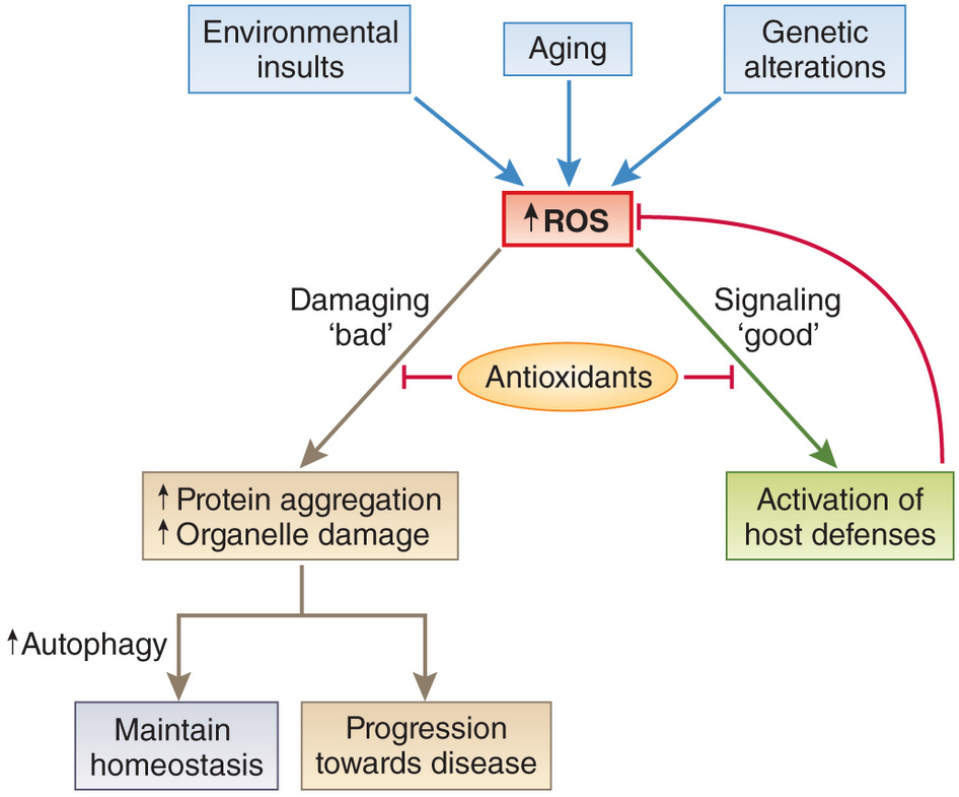

Figure 4. Beneficial and harmful biological ROS pathways. Adapted from Kawagishi and Finkel [38]. The high levels of ROS can provoke host defense pathways that cause decreased ROS levels. On the other hand, continued high levels of ROS can cause intracellular injury by stimulating autophagic clearance of damaged proteins or organelles.

Free radicals having contact with iron can potentially develop into intestinal inflammation by producing hydroxyl radicals [39]. In fact, non-enzymatic pathways

implicating transition metals leads to the production of reactive nitrogen species in UC [40, 41]. Kruidenier et al. also confirmed that OxS metabolite-mediated damage plays a role in the pathophysiological mechanisms of intestinal inflammation [42]. Patients with active CD are characterized not only by inflammation, but also by OxS markers along with decreased antioxidant status. Oxidative parameters can decrease to normal values when the disease is controlled and the patient is clinically stable [43]. The exaggerated ROS can directly damage the IECs while provoking the generation of several proinflammatory mediators, such as interleukin-1β (IL-1β), tumour necrosis factor alpha (TNF-α), IL-8 and IL-6 [44, 45]. In turn, these inflammatory mediators generate hydrogen peroxide and OxS in the IBD.

Interestingly, it has been reported that the pro-inflammatory cytokine TNF-α provokes the production of mitochondrial oxidants (ROS) [46]. Consequently, these abnormal levels of ROS and proinflammatory cytokines work as activators of transcription factors like nuclear factor- kappa B (NF-κB). In turn, stimulation of NF-κB can promote further TNF-α production. These pathways lead to an abnormal cycle of extreme production of OxS [47, 48], which can cause a malfunctioning of the intestinal epithelial barrier. Prevention of NF-κB activity can preserve intestinal membrane integrity [45, 49], while NF-κB activation may upregulate oxidant-induced inducible nitric oxide synthase (iNOS) with damaging consequences such as cytoskeletal oxidation-nitration and monolayer dysfunction [45] as shown in Fig.5.

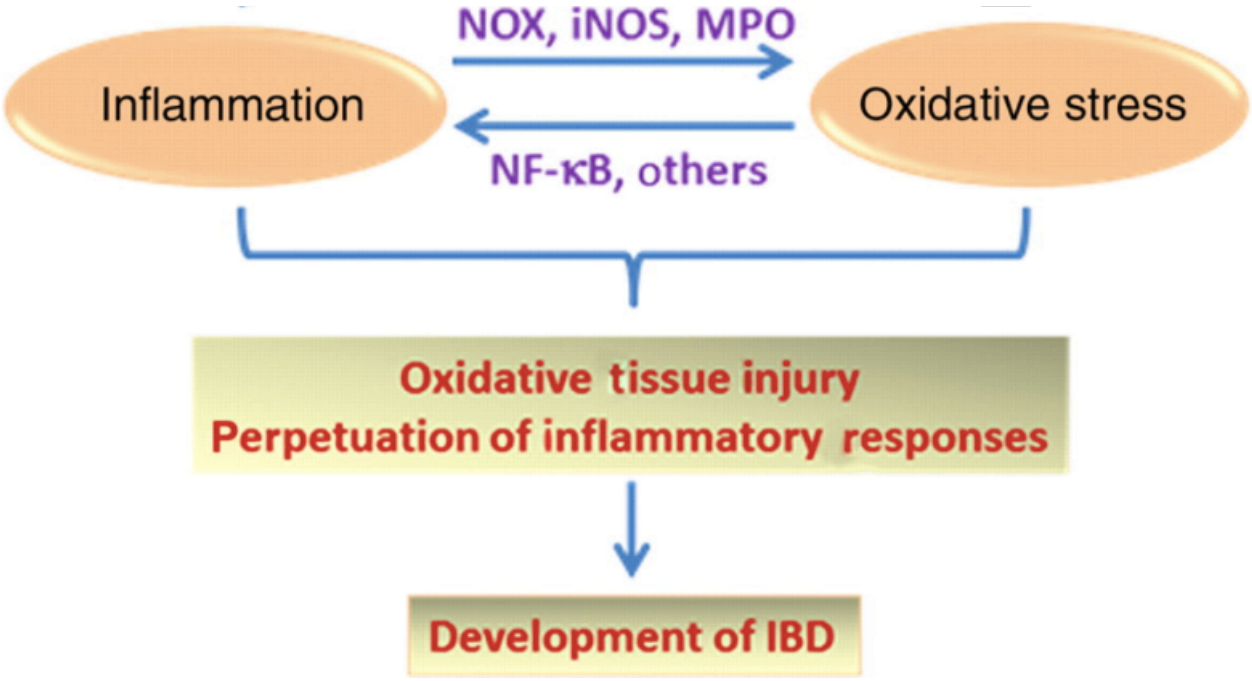

Figure 5. Role of some markers of OxS and inflammation in IBD. Adapted from Zhu and Li [50]. As shown, the interaction between inflammation and OxS triggers ROS/RNS-generating systems with harmful consequences for patients with IBD.

Pro-inflammatory cytokine TNF-α may also locate in the mitochondria and affect its gene expression [46]. Moreover, the expression of cytochrome (Cyt) C oxidase and Cyt b mRNA could be stopped due to mitochondrial contact with TNF-α. The resulting decreased expression of Cyt C oxidase and Cyt b mRNA may even decrease the regulation of mitochondrial biogenesis [51]. Disturbances in the modulation of NF-κB alter the control of prostaglandin metabolism with an impact on inflammation and cancer [52]. The cyclooxygenase (COX) is a fundamental enzyme responsible for the conversion of arachidonic acid to prostaglandins [53]. The two identified forms of the COX enzyme, COX-1 and COX-2, are expressed in many cells and tissues. COX-2 is triggered by

proinflammatory cytokines at the site of inflammation [54]. The role of COX-2 in the production of excessive inflammatory mediators such as prostaglandins has been studied. This inflammatory mediator mediates pain, assists in inflammatory activities and damages the integrity of the colon.

Importantly, nonsteroidal anti-inflammatory drugs and certain other anti-inflammatory agents act via the inhibition of the COX enzymes [54]. Similarly, the anti-inflammatory activities of bee venom could inhibit COX-2 expression and block powerful pro-inflammatory cytokines and other indicators of the inflammatory process (TNF- α, and IL-1β) [55]

In IBD, oxygen radicals, especially superoxide and hydroxyl radicals are responsible for cell and tissue damage [56]. ROS within peripheral leukocytes cause DNA damage and a reduction in plasma antioxidant defence, observed in both CD and UC patients. One important marker for lipid peroxidation is malondialdehyde (MDA). It increases in plasma [57] and colonic biopsies [42] of CD patients. Two other non-invasive markers of lipid peroxidation such as breath output (CO2) and pentane excretion (urine) were increased in CD patients and were correlated with disease activity [58].

In general, endogenous and exogenous antioxidant defense scavenges free radicals and prevents OxS. For example, catalase, glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-s-transferase, superoxide dismutase (SOD), and dietary antioxidants (e.g. vitamines C, E, A) can control free radical-induced tissue injury by preventing the formation of radicals or supporting their disintegration [32, 35, 59-61]. Some studies show that the levels of principal antioxidants in the intestinal mucosa from CD patients are mostly decreased [35, 60, 61].

1.2. Mitochondria

Mitochondria are cytoplasmic organelles found in most eukaryotic cells with aerobic respiration, except in the red blood cells, and they are key players in cellular respiration. Mitochondrial population, size, mass and composition are diverse in different cells and, in fact, they correlate with baseline cellular metabolic state [62]. Mitochondria hold their own genetic material and metabolic system [63]. In healthy cells, mitochondria are about 1-10 μm long, situated near the nucleus, and the main producers of energy [64]. This energy is produced in the form of adenosine-5'-triphosphate (ATP) by a process called oxidative phosphorylation (OXPHOS) and is consumed by the cells for different required biological processes (e.g. cellular metabolism) [65].

1.2.1. Mitochondrial structure

Mitochondria have a rod shaped structure and are found in all plants, animals, fungi, and most protists. Most cells have a large mitochondrion but some active cells have hundreds or even more mitochondria depending on the levels of cellular metabolic activity. As shown in Fig.6, the mitochondria have two protein-containing phospholipid membranes: the inner and outer membranes [66, 67], which both have their important distinct functions.

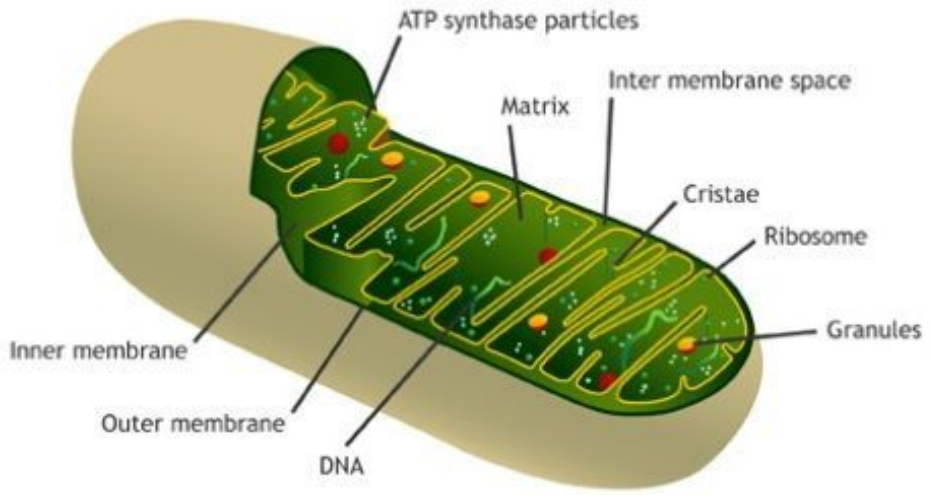

Figure 6. Schematic mitochondrial structure. Taken from The New Zealand Biotechnology Learning Hub, 2009.

The outer mitochondrial membrane covers the entire organelle, is permeable to small molecules, ions, and energy molecules like ATP and ADP molecules, and it acts as a main signaling center in all animal and human cells [68]. On the other hand the inner membrane has a more complex structure and is strictly impermeable. It is composed of crucial proteins such as enzymes that are involved in the main mitochondrial functions. The inner membrane is folded into structures called cristae, which increase surface area for cellular respiration and ATP production [69, 70]. The small space between the inner and outer membrane is called the intermembrane space while the space within mitochondria is called mitochondrial matrix, which holds, in humans, several copies of double-stranded DNA coding for many vital proteins (e.g. elements of complexes I, III,

and IV and the ATP synthase complex), 2 RNAs, 22 transfer Ribonucleic Acids and 2 Ribosomal RNAs that are required for mitochondrial DNA translation [26]. The RNA polymerase, the mitochondrial transcription factor A (mtTFA) and two mitochondrial transcription factors, mtTFB1 and mtTFB2 are part of the process of the transcription of mitochondrial genome [71]. The mitochondrial genome (mt genome) contains six main genome types that are classified in several ways such as the size, shape of DNA molecule, number of encoded genes, presence of cryptogenes, and editing of primary transcripts [72]. Some cellular metabolic processes taking place in the mitochondrial matrix include fatty acid beta-oxidation (FA β-oxidation), tricarboxylic acid cycle, heme-synthesis, and iron-sulfur cluster formation [73]. Different mitochondrial enzymes, found in the matrix, participate in most of these metabolic processes [74].

1.2.2. Mitochondrial function

Mitochondria are multifunctional organelles that interact with other organelles. Receiving, integrating and transmitting signals are part of their regulation. It is important to mention that the different structural compartments of the mitochondria play important roles in the way mitochondria function. The mitochondria are known to be the main powerhouse of the cell by participating in several cellular metabolic activities and specific functions such as ATP production though FA β-oxidation, and OXPHOS, regulation of apoptosis, ROS generation, and Ca2+ homeostasis [75-77]. In addition,

there are five main mitochondrial functions noted to be essential for the dynamics of mitochondrial structure and function. These functions are mitochondrial biogenesis (controling mitochondrial population), mitophagy process (through which the cell removes damaged mitochondria), mobility of mitochondria, redox signaling and the regulation of Ca2+ signaling, which influences oxidative metabolism and apoptosis [78].

1.2.2.1 Fatty Acid Beta-oxidation

As mentioned before, one of the functions of mitochondria is the production of energy by FA β-oxidation, another non-direct mechanism of ATP production [79]. This process takes place in the matrix of mitochondria and provides energy in the absence of glucose catabolism. Before long chain FA can enter the mitochondria, the cytoplasmic enzyme acyl-CoA synthetase activates FAs by binding them to coenzyme A. Acyl-CoA molecules are then able to enter the carnitine cycle in order to be converted into acyl-carnitine, thereby transiting through mitochondrial membranes prior to reconverting into acyl-CoA in the mitochondrial matrix. Short- and medium-chain FAs can enter into mitochondria directly. In the β-oxidation cycle, Acyl CoA is oxidized into acetyl-CoA and then integrates the citric acid cycle (Krebs cycle) to produce more ATP [80] as shown in Fig.7. Two other final products of the β-oxidation cycle, NADH and FADH2, are used in the electron transport chain of the OXPHOS system [79, 81].

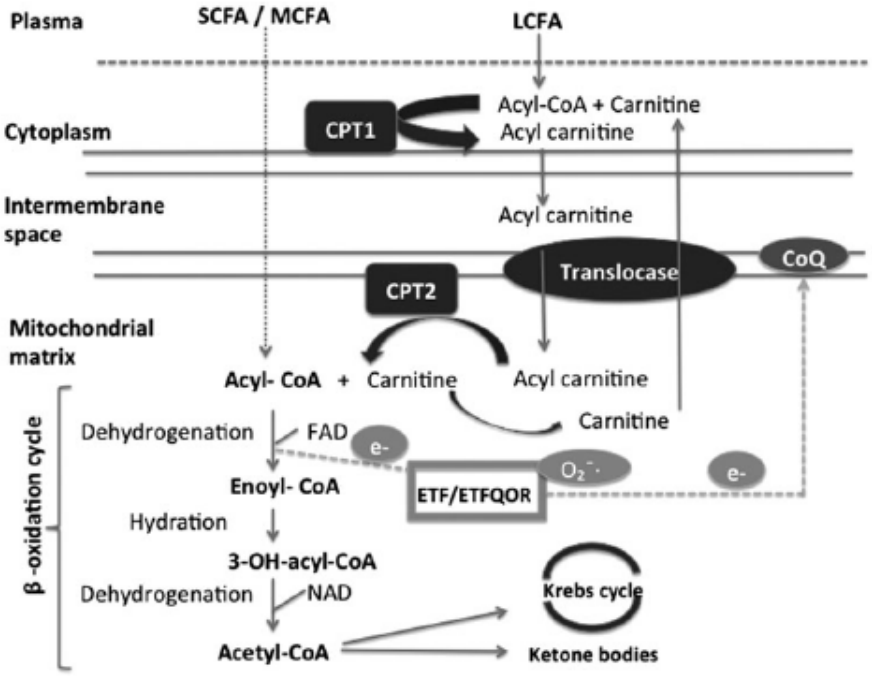

Figure 7. Fatty acid β-oxidation. Taken from Kleme et al. [74].

The electrons that are also produced by the β-oxidation cycle are transfered by electron transfer flavoprotein: ubiquinone oxidoreductase system from acyl-CoA dehydrogenases (ACADL) to an ubiquinone pool (coenzyme Q) located in the inner membrane of mitochondria [82] to participate in different complexes of the respiratory chain (RC) to produce more ATP.

1.2.2.2 Glycolysis, Krebs cycle, Electron Transport Chain Complexes (OXPHOS) and ATP production

Overall, ATP production occurs by three different pathways that consist of anaerobic glycolysis, Citric acid cycle (Krebs cycle) and mitochondrial OXPHOS in the matrix of mitochondria [83].

Glycolysis and citric acid cycle are the catabolic pathways that breakdown glucose and other fuel molecules to produce energy. Glycolysis takes place in cytosol and starts with breaking down glucose into two molecules of pyruvate. Pyruvate is then oxidized to carbon dioxide in the citric acid cycle that occurs in the mitochondrial matrix. The electrons that are produced in glycolysis and the citric acid cycle are carried and transferred via NADH to the electron transport chain [84] to produce more ATP. In addition, a final way that mitochondria contribute to ATP production is by electron transport on the inner membrane through a chain of multiprotein complexes (I-IV), two mobile carriers (coenzyme Q and Cyt C) and ATP synthase. Electron donors (i.e. NADH, FADH2) transfer the electrons that are required for these vital complexes. In the phase of electron transfer (mitochondrial OXPHOS), electrons received from electron donors are transmitted to O2 through complexes-I, -III, and -IV. Furthermore, as shown in Fig.8, this electron production causes a proton gradient in the mitochondrial inner membrane along with the formation of water and ATP that is synthesized through complex V (ATP synthase) [85-87].

Under normal conditions, mitochondrial respiratory system produces more than 95% of ATP [88] consumed by the cells for different biological processes such as cellular metabolism, steroid metabolism, amino acid biosynthesis and FA oxidation [65].

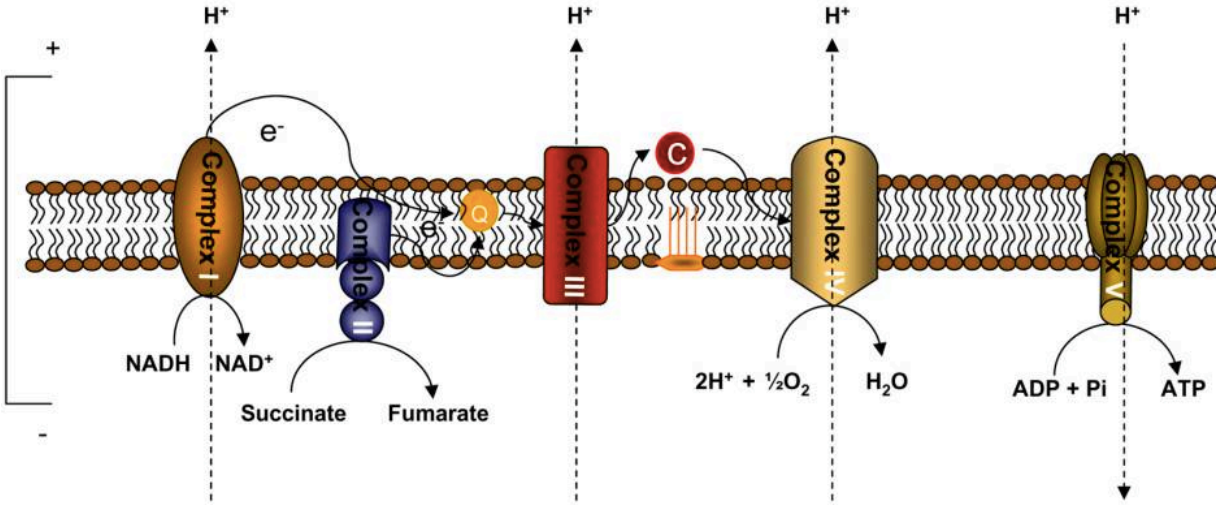

Figure 8. Mitochondrial site and role of the RC complexes. Taken from Bayir and Kagan [89]

1.2.2.3 Apoptosis regulation

In animal and human cells, apoptosis is known as a normal and healthy pathway that is otherwise referred to as programmed cell death in all steps of cell development. There are two different mechanisms for the apoptotic pathways: the mitochondrial pathway and the non-mitochondrial pathway. In recent years, numerous apoptosis related factors of the mitochondrial apoptotic pathway have been discovered [89]. They integrate different types of endogenous and exogenous pro-apoptotic signals from other organelles, including the nucleus, cytosol and lysosomes, as well as exogenous factors such as certain viral proteins [90].

During apoptosis, specific proteins are released from the mitochondria to promote cell death as is the case for the pro-apoptotic Cyt C, a protein localized in the intermembrane

space of the mitochondria in healthy cells, is activated in inflammation and is essential to respiration [90-92]. Cyt C is a small electron carrier protein that assists in cell energy production by transferring electrons from complex III to complex IV. Maintenance of Cyt C inside mitochondria is vital due to the fact that its release into the cytosol can result in apoptosis [93].

Another apoptogenic factor present in the intermembrane space of the mitochondria in normal cells is apoptosis-inducing factor (AIF) that initiates apoptosis by activating a caspase-independent pathway. AIF is a positive natural regulator of apoptosis by inducing DNA fragmentation and chromatin condensation [91]. In the normal cells, AIF's main role is to protect mitochondrial membrane permeability and support OXPHOS [94]. As studies demonstrated, both Cyt C and AIF apoptogenic mitochondrial proteins are released into the cytoplasm causing a cascade of pathways (activation of caspase 9 leading to changes in caspase 3, 6 or 7 activity), which activate the mitochondria-mediated apoptotic pathway and finally drive apoptosis [90] as illustrated in Fig.9.

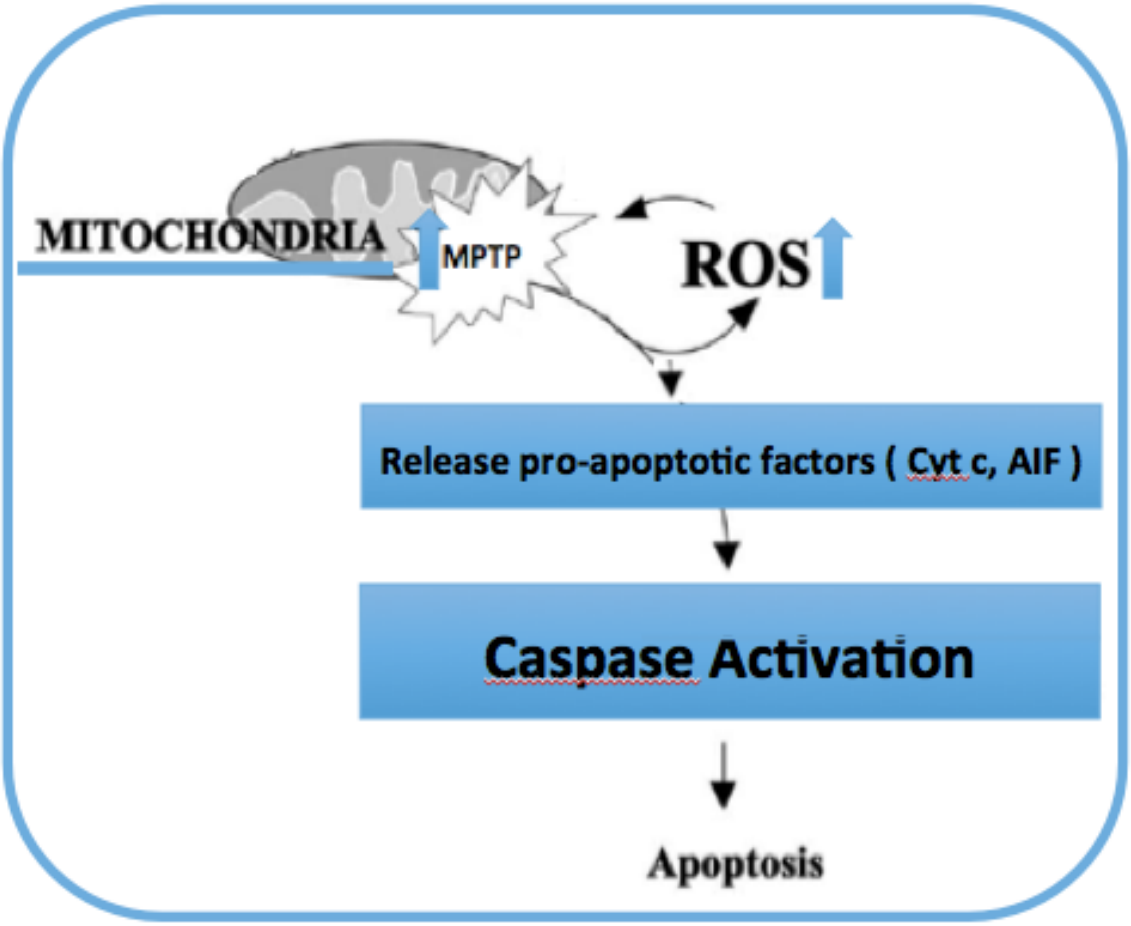

Figure 9. Graphic picture of role of mitochondrial permeability transition pore (MPTP) opening in apoptosis. Adapted from Saenz et al. [95]

Other major players of apoptosis are members of B-cell lymphoma 2 (Bcl-2) proteins, the imbalance of which may favor the apoptotic pathways in both healthy and compromised cells [90]. The anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax are the most important members of this family. They are normally located in the cytosol and they are the main regulators of the mitochondrial apoptosic process by controlling mitochondrial permeability [96]. Bcl-2 proteins are coded by the nuclear genome and

remain in the space between the inner and outer membranes of mitochondria (intermembrane space). In physiological healthy conditions, anti-apoptotic Bcl-2, the protein bound to the outer membrane, inhibits mitochondrial apoptosis. On the other hand, when the pro-apoptotic Bcl-2-associated X (Bax) protein is translocated to the mitochondria, it causes apoptosis [90, 96]. In addition, the ratio of pro- and anti-apoptotic Bcl-2 family proteins (Bax/Bcl2) establishes the sensitivity of cells to many apoptotic factors, as shown in schematic Fig.10 [91, 97].

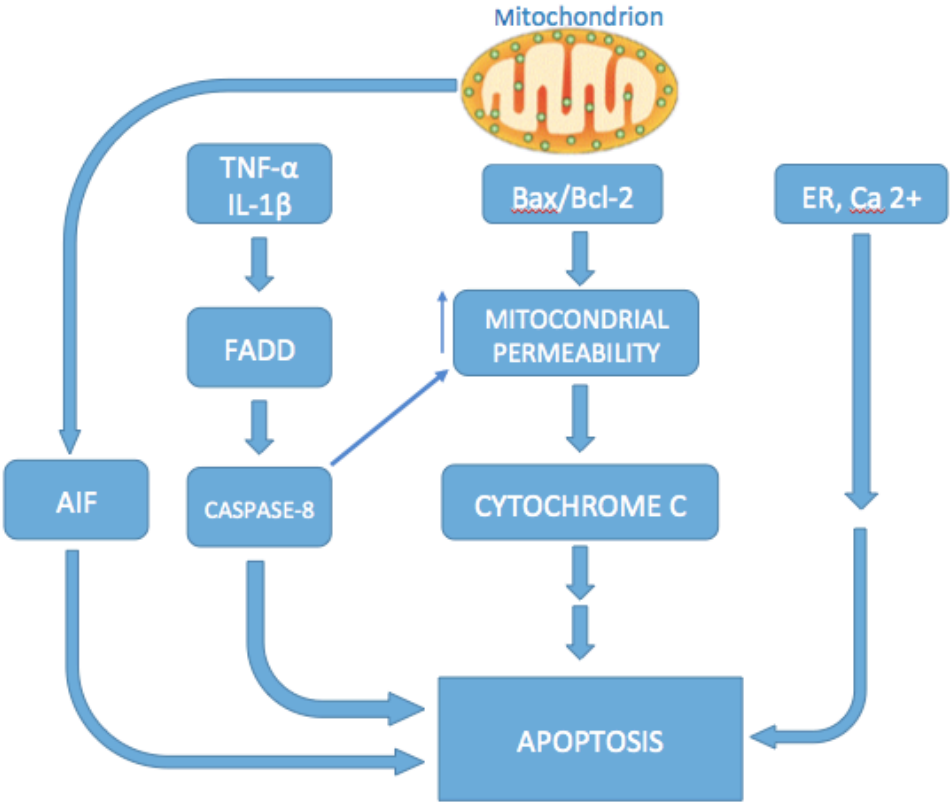

Figure 10. The roles of pro- and anti-apoptotic factors in mitochondrial permeability and apoptosis. Adapted from Kwak [98]. This image demonstrates that overexpression of pro-apoptotic Bcl-2 family proteins releases Cyt C, AIF and some caspases from mitochondria. Similar situation pertains to Ca2+ channels activity and abnormal concentration of

mitochondrial Ca2+ can cause apoptosis and cell death.

1.2.2.4 ROS generation and detoxification

In addition to energy production and regulation of programmed cell death, another important function of mitochondria is the generation of ROS, which have vital roles in cell signalling and homeostasis. Mitochondria are responsible for producing more than 90% of total ROS found in cells [99].

ROS can be generated by mitochondria as a result of normal electron transport and also during mitochondrial dysfunction. This occurs when electrons escape from the electron transport chain to generate superoxide anions. Several other proteins that participate in glycolysis, mitochondrial electron transport, β-oxidation and the Krebs cycle can produce superoxide, hydrogen peroxide and other ROS. These proteins include CI, CII, CIII, dihydro-orotate dehydrogenase, pyruvate dehydrogenase, aconitase, 2-oxoglutarate dehydrogenase and Sn-glycerol-3-phosphate dehydrogenase. Furthermore, other mitochondrial proteins such as monoamine oxidases and p66shc/ Cyt C may contribute to ROS production [100]. ROS overproduction can lead to OxS by causing oxidative deterioration of proteins, lipids and DNA.

In healthy cells, some endogenous systems of antioxidant enzymes, including SOD, GPx, GR, and catalase, act as the primary defence mechanism, keeping ROS at physiological levels [101]. These enzymes can help remove excess ROS. SOD1 enzyme is located in the intermembrane space and cytosol, SOD2 in mitochondrial matrix and SOD3 in extracellular matrix. All these enzymes assist in the conversion of superoxide to

hydrogen peroxide [102]. Subsequently, GPx, another enzyme involved in detoxification, decays hydrogen peroxide and lipoperoxides that participate in lipid peroxidation. Toxic hydrogen peroxide can also be decomposed to water and O2 by the catalase enzyme [103].

1.2.2.5 Ca2+ homeostasis

Ca2+ channels are responsible for controlling Ca2+ release from the endoplasmic reticulum (ER) in response to biochemical signals. Also, mitochondrial Ca2+ is the principal functional regulator of mitochondrial ATP output and cellular ATP needs [77, 104]. OxS can result in higher level of mitochondrial Ca2+ and thus increased cytosolic Ca2+ [105]. In this case, Ca2+ overload can cause over-production of ROS, which forces apoptosis [106-109] by MPTP opening and Cyt C release. ER are linked by mitochondria-associated membranes (MAMs) and the communication between these two organelles can take place through Ca2+ signaling.

Some important functions of mitochondria depend on the MAMs membrane that controls the transport of metabolites and proteins. Furthermore, this membrane helps transfer lipids and mitochondrial RC complex products that participate in OXPHOS and ATP production [66, 110].

1.2.3. Mitochondrial dysfunction

Mitochondrial form and function in healthy cells are important for producing various healthy metabolic products in different cells. However, numerous recent studies have

focused on mitochondrial dysfunctions in association with various diseases/disorders including diabetes, cancer, muscular disorders, cardiomyopathy, deafness, lactic acidosis, and skeletal disorders. Furthermore, it is thought that mitochondrial dysfunction may participate in inflammatory pathogenesis [111].

Several studies have reported that changes in mitochondrial structure and/or function may lead to mitochondrial dysfunction and this has been observed in the intestinal Caco-2/15 cell line [112] and animal models [113]. Likewise, Rodenburg et al. study confirmed that the IECs from an animal model of colitis demonstrated unusual mitochondrial structure [113]. Morphological changes in mitochondrial form and function were found in human intestinal cells isolated from patients with IBD. These mitochondria were swollen and had abnormal cristae [114, 115]. Additionally, the study by Nazli et al. reported that patients with intestinal inflammation presented abnormal mitochondrial structure in their intestine [116]. As shown in Fig.11, in inflammatory conditions, the mucus layers as well as the antimicrobial peptides are diminished and the intestinal epithelial lumen is exposed to intestinal microbiota and luminal antigens. Mitochondria become irregular with a reduction of ATP production and an increase in ROS formation [78].

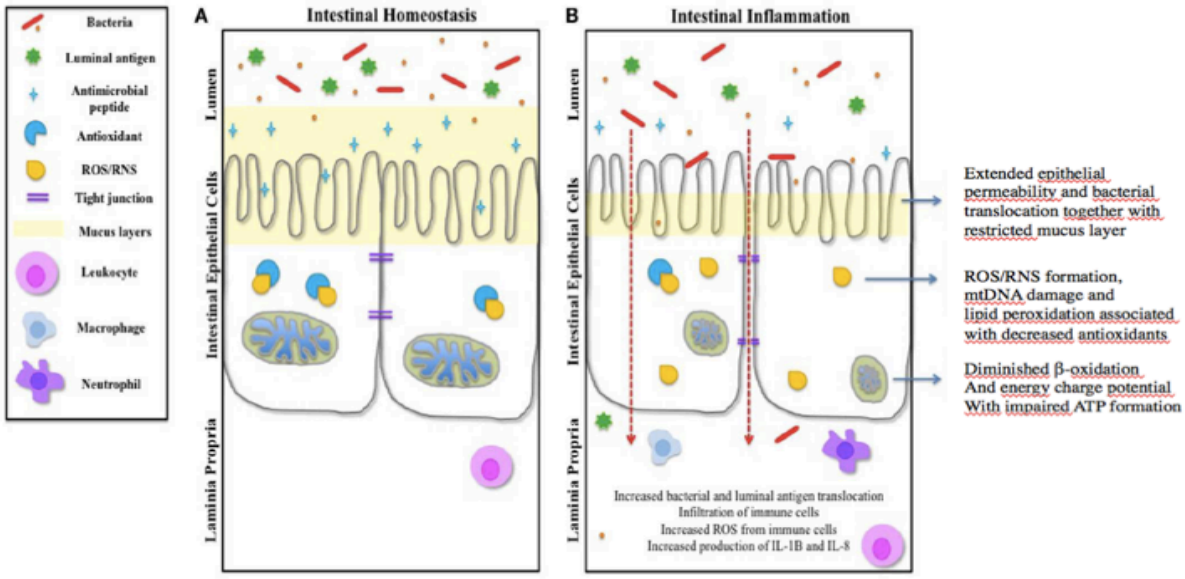

Figure 11. Intestinal homeostasis disorders in mitochondrial dysfunction. Adapted from Novak and Mollen [78]. (A) In homeostatic intestine, healthy mucus protects the epithelial cells from lumen contents, and antimicrobial peptides are produced and released as well. The mitochondria are perfectly shaped and the tight junctions prevent luminal antigens from crossing the epithelial barrier. Cellular antioxidants control basal ROS and leukocytes protect the lamina propria. (B) In inflammatory conditions, the mucus layers as well as the antimicrobial peptides are diminished and the intestinal epithelial lumen is exposed to intestinal microbiota and luminal antigens. Mitochondria become irregular with a reduction of ATP production and increase in ROS formation.

1.2.3.2. Mitochondrial respiratory chain (ATP production) and β-oxidation in IBD

In order to understand the mechanisms of mitochondrial dysfunction in diverse disease processes, researchers have focused on mitochondrial pathways, including RC and ATP production, ROS generation, and autophagy. Many studies have shown that assembly complications or structural problems, which can occur in the process of ATP production within five multiprotein complexes (complexes I to V) and in the mobile electron carriers (coenzyme Q and Cyt C), would cause mitochondrial dysfunction and thus reduce ATP generation. In addition, serious disorders in the OXPHOS complex pathways can induce a series of complications, including electron leakage, increased formation of toxic OxS, and highly abnormal production of apoptotic factors. Furthermore, these abnormalities may finally cause oxidative stress, cell death and degeneration of the related tissues [65, 117]. Accordingly, several studies have shown decreased levels of ATP in the intestinal cells of animal models with intestinal inflammation and in patients with IBD (compared to healthy condition) [118-120]. Bar et al. examined mucosal OXPHOS activities and levels of ATP in mice and suggested that mice with higher mucosal ATP levels show less inflammation in experimental colitis. This study also showed that the colonic tissues in mice with higher mucosal ATP levels had higher levels of NF-κB. Increased NF-κB activity in colonic tissues may help to preserve mucosal barrier in IBD [121].

Butyrate, a short chain fatty acid (SCFA), is the preferred energy source for colonic epithelial cells and highly significant for maintenance of normal mucosal function. This short carbon FA is metabolized through the β-oxidation pathway occurring within mitochondria. Results from the experimental colitis in mice (with DSS) [122] and the UC in human studies [123] revealed that butyrate oxidation is impaired in the colonic mucosa

in UC. Also morphological changes in the mitochondria can affect β-oxidation of SCFA in inflamed IECs [124]. De Preter et al. demonstrated reduced butyrate oxidation rate in active UC patients by evaluating their colonic biopsies compared with healthy controls [125]. More recently, another study by De Preter et al. indicated that butyrate uptake and its colonic metabolism in UC were significantly diminished compared with healthy controls and this was due to the reduced expression of butyrate transporter and enzymes participating in the β-oxidation pathway of butyrate [126].

1.2.3.3. Oxidative stress and mitochondrial dysfunction in IBD

Various components and molecular functions within mitochondria can become targets for oxidative impairment. Indeed, OxS occuring in mitochondria can cause serious intracellular impairments such as alterations of mitochondrial membrane function [30, 127]. As mentioned before, mitochondria are key players in the control of OxS due to their production of free radicals. However, they are able to balance this ROS production with their antioxidant defense system. Although the molecular mechanisms of mitochondrial intestinal injury in IBD are not completely clear, many studies have demonstrated that the presence of OxS, overproduction of colonic oxidants and imbalance between ROS production with mitochondrial antioxidant defense system can play a major role in these damages and disease processes.

Reed et al. demonstrated that a high level of ROS in the mitochondria may lead to an increased permeability of the outer mitochondrial membrane via the opening of the

MPTP. This permeability also leads to a decreased mitochondrial membrane potential [90].

Increased levels of mitochondrial OxS and defects in the intestinal epithelium were noted in mice with experimental colitis [128] and in patients with IBD [119, 129].

Furthermore, other studies demonstrated that ROS produced by mitochondria can cause damage to mitochondrial DNA bases that result in an impairment in coding of complexes I, III, and IV and the ATP synthase complex protein involved in energy production for healthy cells and tissues [130, 131]. 8-oxoguanine (8-oxoG) is a mutagenic base by-product generated by exposure to ROS in human and murine models. Healthy cells produce different repair enzymes and 8-oxo DNA glycosylase (OGG1) is a primary enzyme responsible for cell protection by removing 8-oxoG from mitochondrial DNA backbone [71]. The study of Chang et al. demonstrated that the augmentation of 8-hydroxy-deoxy-guanosine (8-OHdG), provoked by the absence of repair via OGG1, amplifies mutations and mitochondrial dysfunction, thereby resulting in the initiation of apoptotic cell death [132].

Damage of 8-OHdG from both CD and UC patients was examined by measuring the levels of 8-OHdG in the blood as a marker of OxS. The results showed that free radicals causing mitochondria DNA (mtDNA) damage within peripheral leukocytes increased and plasma antioxidant defenses decreased [7, 61]. In addition, D'Inca et al. observed that colorectal biopsies from UC patients showed oxidative mtDNA damage that was measured by production of 8-OHdG [133]. Also, Lih-Brody et al. have shown the dysfunction of intestinal homeostasis, DNA modifications and damage due to overconcentration of 8-OHdG in the inflamed part of the bowel of CD patients [134].

Overall, mtDNA damage can cause many serious problems for cells due to increased ROS production and disrupted electron transport chain [84]. In this regard, mutated mtDNA can generate increased OxS in the mtDNA of damaged cells due to higher production of superoxide radicals [135, 136]. Accordingly, mitochondrial proteins could be potential biomarkers in the pathways of several human diseases, and mitochondria may become a promising target for antioxidant-delivery strategies.

1.2.3.4. Additional mechanisms for apoptosis and mitochondrial dysfunction in IBD

An abnormal MPTP opening can liberate Cyt C into the cytosol. Unusual release of Cyt C with some other mitochondrial pro-apoptotic proteins could be the immediate signal of apoptosis causing mitochondrial dysfunctions [137] as shown in schematic Fig. 12.

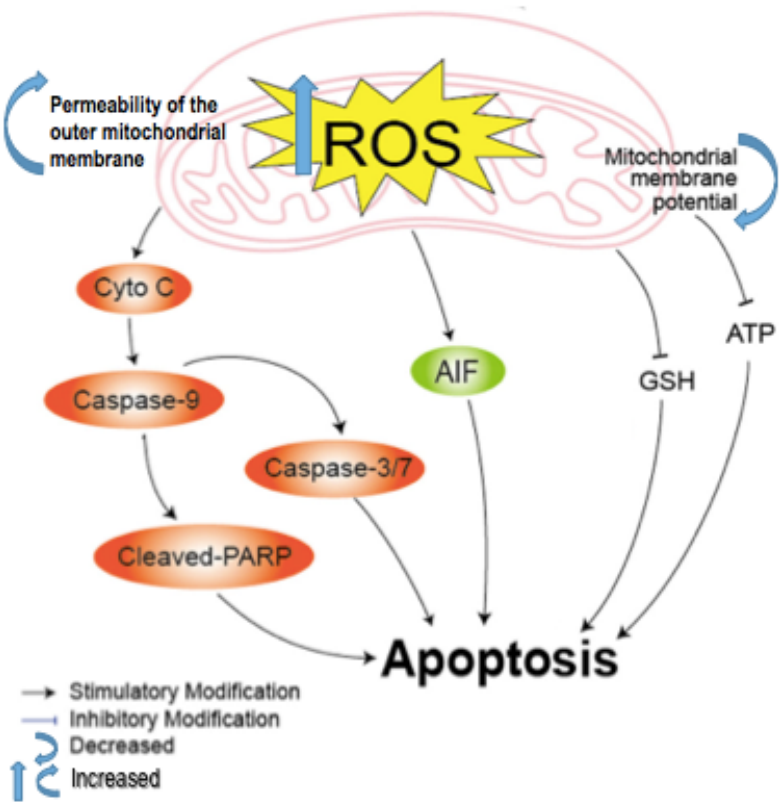

Figure 12. Schematic image of mitochondrial ROS's roles in apoptosis. Adapted from Qiu et al. [137]. This image demonstrates that increased ROS leads to increased permeability of the outer mitochondrial membrane and decreased mitochondrial membrane potential. This may cause apoptosis by activating a series of pro-apoptotic pathways.

During apoptosis, specific proteins are released from the mitochondria to promote cell death.

One of the main pro-apoptotic proteins activated in inflammation injury is Cyt C, a protein that is localized in the intermembrane space of the mitochondria in healthy cells and is essential to respiration [90, 91]. An abnormal MPTP opening can cause necrosis or apoptosis in cells. This translocation of Cyt C from the mitochondria to the cytosol starts

activating a series of caspases. Because of this cascade of caspases, the apoptotic signals release and finally provoke caspase-dependent apoptosis with activation of caspase-9, which then activates caspase-3. Unusual release of Cyt C with some other mitochondrial pro-apoptotic proteins could be the immediate signal of apoptosis causing mitochondrial dysfunctions [92].

MPTP malfunction due to high levels of ROS also initiates the release of AIF into the nucleus in the mature soluble form (AIFsol), where it provokes nuclear apoptosis in a caspase-independent manner and finally causes DNA damage [138]. High levels of ROS and increased expression of pro-apoptotic Bcl-2 family proteins, including Bax, can also cause cell death [90, 91]. In a study by Arab et al, to evaluate colonic apoptosis, the mRNA expression of Cyt C, Bax, Bcl-2 and caspase-3 was analyzed in trinitrobenezene sulphonic acid induced colitis in rats. This study showed increased mRNA expression of caspase-3, the pro-apoptotic Cyt C and Bax with downregulation of Bcl-2 [139].

In another study by Taha et al, the effect of oxidative stress on mitochondrial dysfunction in IBD was studied in the Caco-2/15 cell line. In this study Caco-2/15 cells were exposed to iron-ascorbate, which produces oxygen radicals and can contribute to lipid peroxidation in IBD. The result of this study indicated that the level of AIF and Cyt C proteins increased in Caco-2/15 cells after iron-ascorbate administration compared with the control group [112].

1.2.3.5. Ca2+ homeostasis and mitochondrial dysfunction in IBD

As mentioned before, Ca2+ levels and trafficking in mitochondria (channels activity and

Ca2+ signaling) can be affected by ROS concentration, pro-inflammatory cytokines and also by the anti-apoptotic protein Bcl-2. Recently, Pinton et al. have suggested that the anti-apoptotic protein Bcl-2 could reduce ER Ca2+ levels, hence reducing mitochondrial Ca2+ uptake [77]. Alteration in ROS concentration can affect Ca2+ channel activity and change the concentration of mitochondrial Ca2+. Also, malfunctions in Ca2+ signaling and Ca2+ concentrations may lead to increased risk of cell damage and death [77]. Qureshi et al. noted that disorders in intracellular Ca2+ mobilization could cause increased NF-κB activity and result in dysmotility of colonic smooth muscles in murine models of experimental colitis [140]. In an investigation by Di Sabatino et al., it has been found that a reduction in pro-inflammatory cytokine release can occur by prevention of mitochondrial Ca^{2+} overload in inflamed intestinal cells. In addition, prevention of mitochondrial Ca2+ overload by activation of Ca2+ channel inhibitors can result in diminished mitochondrial ROS accumulation, increased mitochondrial energy production and correction of mitochondrial oxidative stress-mediated disorders [141].

1.3. Therapeutic intervention: antioxidants targeting mitochondrial dysfunction

1.3.1. Antioxidant and anti-inflammatory agents

A phenolic antioxidant is a molecule with a strong capacity to reduce or stop the oxidation of lipids and other molecules and protect them against free radicals. In addition,

antioxidants play the main role in stabilizing or disabling free radicals before they become dangerous by attacking the closest cells and cellular components [142].

In recent years, flavonoids and other polyphenols, the so called bioactive molecules with antioxidant properties, have become the subject of many studies to determine their properties to prevent or cure oxidative damage caused by OxS, nitrogen species metabolism and other markers for oxidative damage [143, 144]. Many studies have shown that, in healthy and pathologic cell conditions, the endogenous antioxidants such as SOD, catalase [145] and GPx enzymes play an important role in keeping the balance between oxidative damage pro-oxidants and endogenous defense mechanisms. Non-enzymatic antioxidant substances are also responsible for the control of ROS overload and cell damages [146, 147].

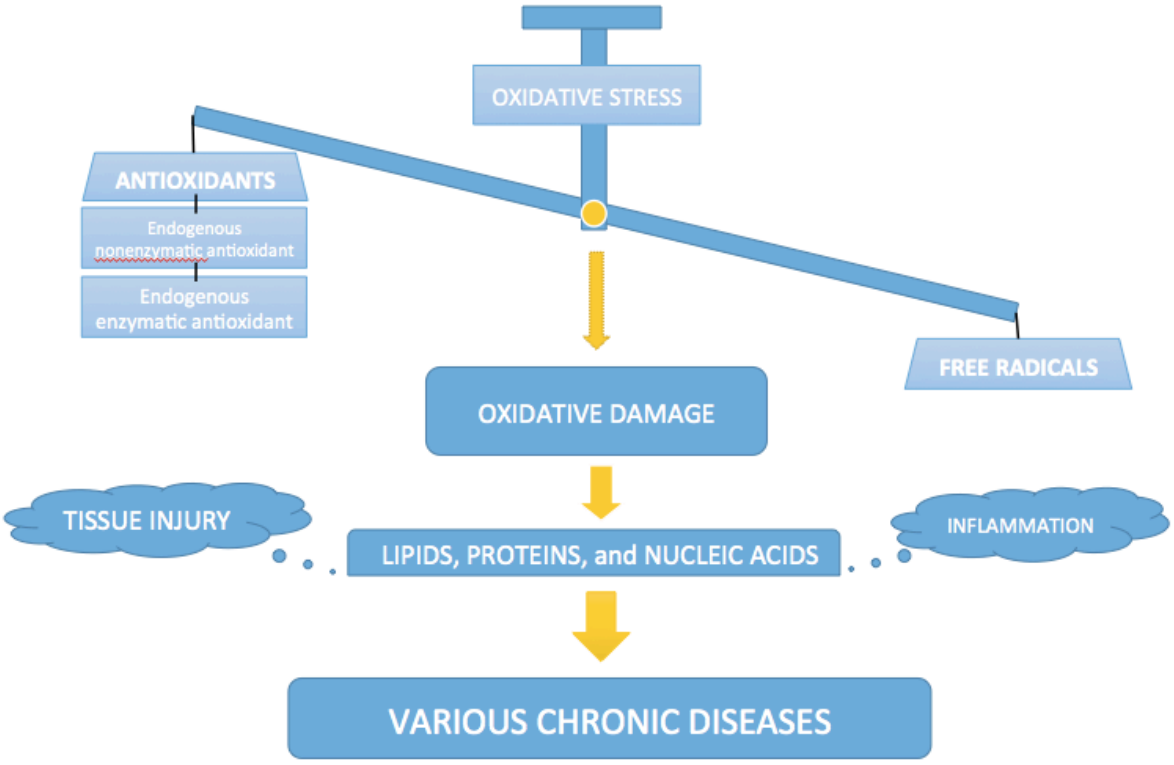

Figure 13. Schematic illustration of oxidants and antioxidants imbalance, oxidative damage, and chronic diseases. Adapted from Arulselvan et al. [148].

Endogenous enzymatic and non-enzymatic antioxidants also have an important role in decreasing OxS [149]. Recently, several studies have observed that natural antioxidants from several plant sources, including flavonoids and phenolic compounds play a preventive role in protecting against the generation of free radicals. In addition, flavonoids and phenolic compounds act as anti-inflammatory factors by inhibiting two main signalling pathways including NF-κB and mitogen-activated protein kinases (MAPKs) that play a part in production of different proinflammatory mediators [149].

1.3.2. Polyphenols

1.3.2.1. Structural diversity and dietary sources of polyphenols

Polyphenol compounds are most commonly found in fruits, vegetables, cereals, and beverages [150]. These components have a simple structure and hold one or more benzene rings with 2 or more hydroxyl groups. In addition, polyphenols are considered the most available antioxidants in the human daily diet with important biological properties, including anti-inflammatory and immunomodulatory features. Presently, elegant studies have shown that more than five hundred different polyphenols are regularly found in daily foods while also providing important information about polyphenol consumption and bioavailability [151]. Dietary polyphenols from different sources, including apples have become a focus of research due to their potential

therapeutic effects and preventive properties against several chronic diseases, including cardiovascular disorders, degenerative diseases, diabetes, osteoarthritis and GI diseases.

Polyphenols are classified into diverse groups according to their different chemical structures, degree of oxidation, amplitude of polymerization and substitutions of the basic skeleton [152]. The two principal subgroups of polyphenols are flavonoids and non-flavonoids, and each subgroup is divided into six different subclasses [151]. The two subgroups are categorized by the existence of different numbers of phenolic rings, along with two or more hydroxyl substitutions. In particular, the flavonoids contain two benzene rings linked by a linear three-carbon chain to form and produce different kinds of flavonoid subclasses. This characterization is based on the oxidation state of the central pyran ring in flavonoid molecules. Some common groups of flavonoids are flavones, isoflavones, flavonols, flavanones, flavanols, anthocyanins, and anthocyanidins. The nonflavonoids contain phenolic acids, lignans and stilbenes [152, 153].

“Aglycone”, the simple phenolic structure of polyphenols could be attached with carbohydrates and organic acids to produce “glycone” structures. Simple phenolic structure of polyphenols can also be attached with other polyphenols to produce “polymers”.

Polyphenols from food with simple phenolic compounds such as gallic acid, ellagic acid, catechin, eugenol, vanillin, caffeic acid, ferulic acid, apigenin, quercetin, gingerol, kaempferol, myricetin, resveratrol, rutin, naringenin, and cyaniding are factors that modify and prevent inflammatory response, as well as alter gut microbiota [154, 155].

1.3.2.2. Biological functions of polyphenols

Polyphenols play a key role in cell health through their antioxidant actions through two different pathways, ‘‘ROS-removing level’’ and ‘‘ROS formation level’’. The first mechanism shown in Fig.14 constitutes a direct pathway for ROS-inhibition. The ‘‘ROS-removing level’’ can act through 3 different mechanisms: ROS-scavenging (electron/hydrogen transfer), production of ROS-removing enzymes (e.g. SOD, catalase, GPx) and production of endogenous antioxidant-synthesizing enzymes (e.g. glutathione synthase). Furthermore, the second antioxidant mechanism of polyphenols is a direct action of polyphenols that inhibits the metal-dependent formation of free radicals (iron and copper) and decreases or controls ROS-forming enzymes (e.g., XO, NOX, LOX, MAO, iNOS) as shown in Fig.14.

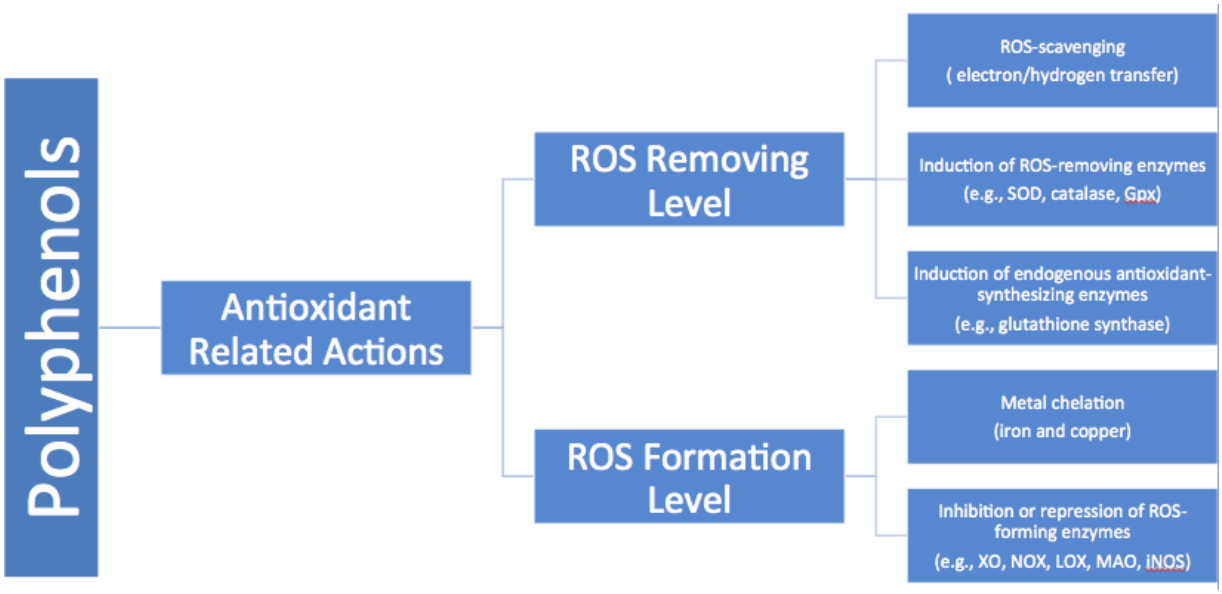

Figure 14. Classification of the polyphenols antioxidant related action. Adapted from Sandoval et al. [156]

The focus of this thesis is on the antioxidant and anti-inflammatory actions of polyphenols given their potential benefits in IBD. [157, 158].

1.3.2.3. Apple polyphenols

Apples are one of the richest sources of polyphenols. In recent years, different studies have shown that daily intake of apple polyphenols and apple-derived polyphenolic products may inhibit or decrease chronic diseases including OxS-associated disorders, cardiovascular diseases and arthritic diseases. Furthermore, they have shown to preserve the gut from drug damage [159].

Apple polyphenols contain diverse structural models, such as hydroxycinnamic acids, flavonols (quercetin glycosides), dihydrochalcones (phloretin glycosides), chlorogenic acid, procyanidins, dihydrochalcones and flavan-3-ols (oligomericpro-cyanidins). The most prevalent groups of polyphenols present in apples are chlorogenic acid, procyanidins, and dihydrochalcones [160, 161].

Several preclinical studies have demonstrated that apple polyphenols have the potential to protect or treat peptic ulcer by numerous intracellular and molecular activities [11, 162]. Graziani et al. have observed that apple polyphenols inhibit expression of COX-2 mRNA and protein in gastric tissue. These polyphenols decreased the peroxidation of lipids in the tissue and caused a moderation in the level of MDA in gastric mucosa. In addition, apple polyphenols protected against xanthine-xanthine oxidase-induced damage and indomethacin-induced oxidative injury in human gastric epithelial cells by improving the antioxidant potential and preventing lipid peroxidation [11]. These results from human studies illustrate the powerful effects of polyphenols in reducing the risk of several chronic illnesses (e.g., cardiovascular disease, neurodegenerative disorders, diabetes, cancers, osteoarthritis, and GI diseases) or improving their symptoms. In this thesis our focus is to demonstrate the preventive and/or therapeutic potential of apple polyphenols in the management of IBD.

1.3.3. Apple polyphenols in intestinal inflammation

In different inflammatory diseases, including IBD, there is an increased expression of pro-inflammatory chemokines, cytokines, cell adhesion agents, and enzymes. In addition,

different studies have shown that both CD and UC are lifelong disorders that cause different challenges for the scientific and medical communities because of several unsolved problems, such as dramatically growing prevalence of IBD, absence of a cure, severe drug side effects, unresponsiveness to medical treatments, direct and indirect treatment costs and socio-economic burden as shown in Fig. 15 [163-165]. Recently, the illustration of biological and beneficial effects of polyphenols, in particular, apple polyphenols in the management of inflammation has captivated the attention and interest of researchers. These studies looked at the molecular mechanisms of action of the different natural elements [161, 166-168].

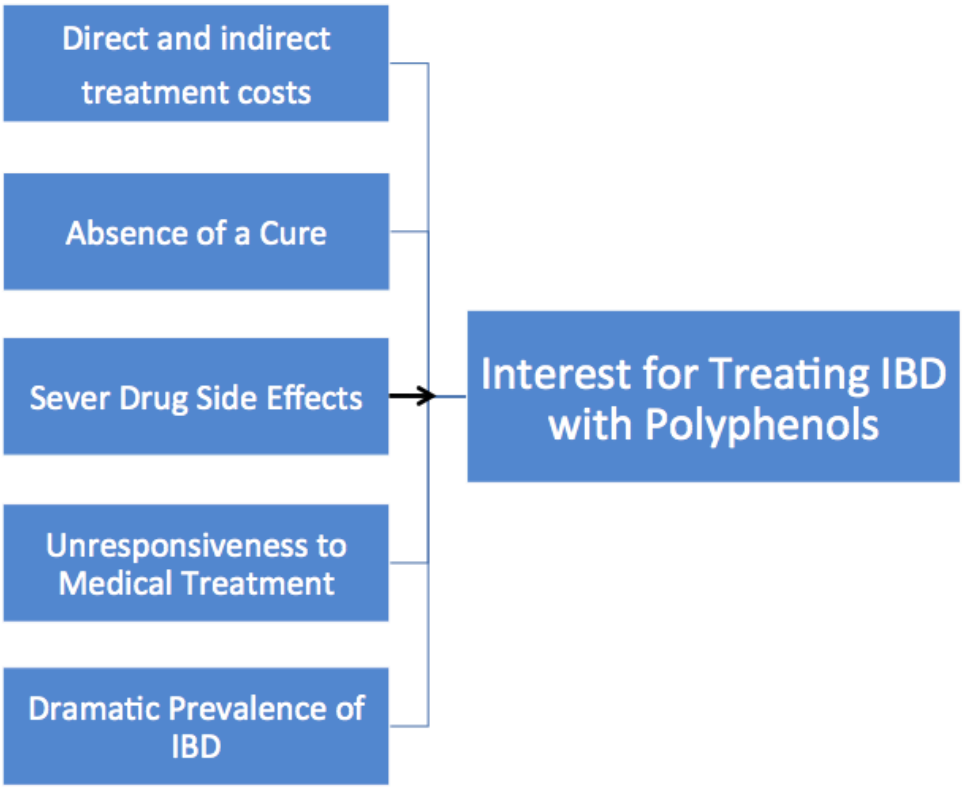

Figure 15. Schematic description of main reasons for treating IBD patients with polyphenols. Taken from Kawalec et al. [163-165].

The preclinical study by Jung et al. demonstrated that apple polyphenols can inhibit the expression of pro-inflammatory cytokines and enzymes in human colonic epithelial cells by the prevention of interferon-gamma-inducible protein-10, IL-8-promoter, and of NF-kB dependent signal transduction [161, 169]. Furthermore, apple polyphenols have also shown beneficial health effects in animal models of colitis by modulating the expression of MAPK, a signalling protein family. The modulation of this signalling pathway by polyphenols causes down-translation and down-transcription of proteins that are involved in the inflammatory response [128, 157].

Experimental aspirin-induced gastric ulcer presented gastroprotective effects of dietary intake of apple polyphenols. These natural polyphenols caused up-regulation of GSH in gastric mucosa of rats. As we know, GSH in gastric mucosa has antioxidant activity. Also, apple polyphenols elevate the expression of glutathione-S-transferase P1 that acts as an enzymatic antioxidant in gastric tissue. In Paturi's study, they also found that apple polyphenols cause overexpression of mucin-2 and trefoil factor-2 genes, which assist in the protective function of the stomach barrier and help protect the gastric mucosa [162].

2 RESEARCH PROJECT

2.1. Hypothesis

We hypothesize that:

1. Dried apple peel powder (DAPP) has the potential to prevent or reduce OxS and inflammation via the modulation of mitochondrial functions or/and structure in experimental colitis.

2. The resulting beneficial actions may contribute to the preservation of intestinal epithelium and homeostasis.

2.2. Objectives

As mitochondria are the major source and target of free radicals, while exhibiting various important functions, the aims of our study are to determine:

- Alterations in prooxidant/antioxidant balance and the status of inflammatory factors in association with intestinal microbiota and in response to DSS-induced colitis;
- The contribution of DAPP to prevention and treatment of experimental colitis by fighting OxS, inflammation and mitochondrial derangements;
- The mechanisms of action of DAPP by examining various crucial transcription factors.

3 ARTICLE 1

Apple peel polyphenols: A key player in the prevention and treatment of experimental inflammatory bowel disease

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Key words: Polyphenol; Peroxidation; Mitochondria; Inflammation

Running Head: Antioxidant and anti-inflammatory effects of apple polyphenols

Abbreviations: DAPP, dried apple peel powder; AIF, apoptosis-inducing factor; ATP, adenosine triphosphate; CD, Crohn's disease; COX-2, cyclooxygenase-2; DAI, disease activity index; DP, degree of polymerization; DSS, dextran sulfate sodium; FA, fatty acid; GPx, glutathione peroxidase; IBD, inflammatory bowel diseases; IL-6, interleukin-6; MDA, malondialdehyde; MPO, myeloperoxidase; NF-kB, nuclear factor-kappa B; Nrf-2, nuclear factor erythroid 2-related factor 2; OGG1, 8-oxoG-DNA glycosylase; OxS, oxidative stress; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1-a; PGE2, prostaglandin E2; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

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ABSTRACT

Diets rich in fruits and vegetables may reduce oxidative stress and inflammation via several mechanisms. These beneficial effects may be due to their high polyphenol content. The aims of the present study are to evaluate the preventive and therapeutic aspects of polyphenols in dried apple peel extract (DAPP) on intestinal inflammation while elucidating the underlying mechanisms and clinical benefits. Induction of intestinal inflammation in mice was performed by oral administration of the inflammatory agent DSS at 2.5% for 10 days. Physiological and supraphysiological doses of DAPP (200 and 400 mg/kg/day, respectively) were administered by gavage for 10 days pre- and post-DSS treatment. DSS-mediated inflammation caused weight loss, shortening of the colon, dystrophic detachment of the epithelium, and infiltration of mono- and polymorphonuclear cells in the colon. DSS induced an increase in lipid peroxidation, a down-regulation of antioxidant enzymes, an augmented expression of MPO and COX2, an elevated production of PGE2 and a shift in mucosa-associated microbial composition. However, DAPP normalized most of these abnormalities in preventive or therapeutic situation in addition to lowering inflammatory cytokines while stimulating antioxidant transcription factors and modulating other potential healing pathways. The supraphysiological dose of DAPP in therapeutic situation also improved mitochondrial dysfunctions. Relative abundance of *Peptostreptococcaceae* and *Enterobacteriaceae* bacteria was slightly decreased in DAPP-treated mice. In conclusions, DAPP exhibit powerful anti-oxidant and anti-inflammatory actions in the intestine and are associated with the regulation of cellular signaling pathways and changes in microbiota composition. Evaluation of preventive and therapeutic effects of DAPP may be clinically feasible in individuals with intestinal inflammatory bowel diseases.

INTRODUCTION

Inflammatory bowel diseases (IBD), clinically manifested as Crohn's disease (CD) and ulcerative colitis (UC), represents a widespread, chronic and debilitating set of disorders characterized by aberrant inflammation and immune-mediated injury to the gastrointestinal tract (1). Despite the recent advances in the fields of IBD genetics and mucosal immunology, our understanding of the pathogenesis of IBD remains fragmentary. The most widely accepted comprehensive hypothesis encompasses three main factors: genetic predisposition, environmental influences and the loss of homeostasis between the intestinal microbiome and host immunity (2, 3). A disruption of immune tolerance to commensal bacteria and deregulation of the intestinal microbiota balance (dysbiosis) may result in gut inflammation, epithelial dysfunction and enhanced mucosal permeability (4). Although genome-wide association studies and meta-analyses have resulted in the discovery of an ever-increasing number of genetic susceptibility loci (5-7), the findings can only explain ~25% of the predictable heritability risk of CD. In addition, the complex, as yet poorly understood interaction of the aforementioned factors is ultimately believed to induce chronic relapsing of intestinal inflammation leading to the various clinical phenotypes of disease expression. Noteworthy, among environmental risk factors, diet is the most commonly implicated, as epidemiological studies have shown that different dietary macro- and micronutrients may play a role in CD (8).

IBD significantly impacts on quality of life and accounts for a substantial financial burden to our health care system and to society (9, 10). Patients often require lifelong medication and the conventional treatment for IBD involves the use of corticosteroids, immunosuppressive agents, anti-tumour necrosis factor (TNF) antibodies and antibiotics (8-10). Some of these agents have been associated with the risks of infection and malignancy (11, 12), as well as numerous side effects (13-15). Moreover, not all patients are responsive to drug therapy. On the other hand, due to its perceived natural and healthy properties, pediatric and adult patients with IBD are increasingly using complementary and alternative medicine (16-19). It may reach 60% in adult IBD populations from North America and Europe (20). However, scientific evidence regarding their efficacy or safety remains inadequate, and the majority of studies have produced inconsistent results (21). If in the late 90's, the nutritional spotlight focused on the antioxidant capabilities of carotenoids, vitamins, and minerals, growing strong support has more recently been devoted to dietary polyphenols, secondary plant metabolites, ubiquitously present in fruits and vegetables, which represents a significant class within the family of phytonutrients. This rising scientific interest stems from their antioxidant capacity and their role in the prevention of certain cancers, diabetes and cardiovascular diseases (22-25). The resurgence of interest in the last decade is also aroused by their anti-inflammatory effects (26, 27). Although protective or preventive anti-inflammatory properties of a number of polyphenol species have been noted in IBD, the mechanisms of action underlying their health-promoting effects remain largely speculative. In addition, the quite unrealistic doses that cannot be reached in a common diet and the mode of administration (i.e., intraperitoneal) have prevented their mainstream medical recommendation (28).

The major aim of the present study was to investigate the preventive and therapeutic capacity of polyphenols isolated from dried apple peel extract (DAPP) in IBD-related disorders. We chose this fruit since the apple ranks second for the total polyphenol concentrations and had the highest portion of free polyphenols when compared to other fruits (16). Furthermore, 20% of polyphenols consumed from fruits are from apples in Canada and the United States (Statistics Canada, and Economic Research Service, United States Department of Agriculture, Food Availability, compilation MAPAQ). We also employed polyphenols from apples given their reported bioactivity and their presumed protecting role in reducing the risk of chronic diseases such as cancer, type II diabetes, cardiovascular disease, pulmonary disease and asthma (29). We especially selected DAPPs since they are more diversified and concentrated than those of the flesh (30). Indeed, in addition to the rich phenolic content (phloretin glycosides, phloridzin, and chlorogenic acid) in peel and fresh apples, the peel contains additional flavonoids that are not found within the fresh (30).

To examine the preventive and therapeutic effects of DAPP, we employed the dextran sulfate sodium (DSS)-induced colitis mouse, a highly valuable and the most frequently animal model employed to investigate IBD pathogenesis in view of its simplicity, wide applicability and various advantages when compared to other animal models of colitis (31). It exhibits numerous characteristics that are similar to human colitis, including inflammatory response progression, clinical signs (diarrhea, occult blood, gross rectal bleeding, shortening of the colon), histopathological changes of the intestine, and modifications of intestinal microbiota (31). In the present investigation, we examined the DAPP effects by focusing on the induction of endogenous antioxidant status, neutrophil infiltration, eicosanoid-generating enzymes, cytokine-induced inflammatory response and gastrointestinal integrity protection. Insights into the mechanisms were gained by analyzing mitochondrial functions, transcription factors activation, transcriptomic signature and microbiota profiling in connection with the pathophysiological conditions.

MATERIALS AND METHODS

DAPP extraction

The phenolic compounds of apples (80 % McIntosh and 20% Northern Spy blend, Cortland, Empire, Ida Red, Jonagold and Spartan) were extracted by a method similar to that reported previously by Liu's laboratory (30, 32, 33). Noteworthy, all the experiments were carried out using the same batch (e.g. one single extraction) of DAPP, helping to ensure comparative and correlative studies.

LC-MS analysis of DAPP extract

A reversed phase LC-MS method has been developed to separate and identify the mass and chemical structure of phenolic compounds derived from DAPP extract by ultra-performance liquid chromatography system (UPLC) coupled to a QuanTOF mass spectrometer (MS/MS QTOF) equipped with an ESI source (UPLC-ESI-MS/MS QTOF) as well described previously (34).

Animals

Male C57BL6 mice (7-8 week of age) were purchased from Charles River (Senneville, Montreal) and housed in individual cages at 24 ± 1 °C under a 12-h light/dark cycle with free access to a standard laboratory chow diet (2018 Teklad Global, containing 18.6% protein, 3.5% carbohydrates and 6.2% fat for 3.1 kcal/g, Harlan Laboratories, Indianapolis, IN). Experiments were performed during the light phase of cycle. Animal experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of experimental animals and were approved by the Institutional Animal Care Committee of the Sainte-Justine UHC Research Center. Several batches of animals, each with 5-6 animals/group, were used to complete all experiments.

Induction of DSS-induced colitis and collection of biological specimens

After a one-week adaptation under standard conditions, the mice were randomly divided into six groups. All mice received water and were fed *ad libitum* throughout the whole experimental period (20 days) with the chow diet (Controls, CTL). Colitis was induced by adding 2.5% (w/v) DSS (average molecular weight 36 000-50 000, MP Biomedicals) in the drinking water for 10 days (Supplementary Figure S1). The conditions of DSS administration (2.5% for 10 days) were chosen to induce a mild-to-moderate colitis that could be more amenable to nutritional prevention or therapy (35, 36).

In addition to the noncolitis CTL group, the five DSS colitis groups were distributed as follows: DSS colitis group received only the vehicle (water used to administer DAPP) by oral gavage; the preventive groups were orally treated by gavage with 200 mg/kg/day DAPP (DAPP-200/DSS) or 400 mg/kg/day DAPP (DAPP-400/DSS) starting 10 days before colitis induction and maintained post induction; and the therapeutic groups were orally treated by gavage with 200 mg/kg/day DAPP (DSS/DAPP-200) or 400 mg/kg/day DAPP (DSS/DAPP-400) starting with colitis induction for 10 days. Fresh DAPP solution was provided daily and the DSS solution was replaced every day. Similarly, body weight and food/water intake were measured on a daily basis. Thereafter, mice were fasted overnight, weighed and anesthetized. Blood samples were collected on EDTA by cardiac

puncture and plasma was separated by centrifugation at 3000 x g for 20 min at 4 °C. The peritoneal cavity was then opened and the colon from the ileocecal junction to the rectum was rapidly excised, measured (length and weight), cut open lengthwise and the content removed with saline for the analysis of mucosa-associated microbiota, flash-frozen, and stored at −80°C for experiments. In several experiments, prior to freezing, cross-sections of distal colon were fixed in 10% neutral buffered formalin for histological examination.

Disease activity index

The severity of DSS-induced colitis was evaluated using the disease activity index (DAI), which combines scores for the mean values of body weight loss as well as stool consistency and occult blood (37). The body weight loss was determined by calculating the percentage of weight change from the start of DSS administration until sacrifice (0 = none; 1 = 1-5%; 2 = 5-10%; 3 = 10-20% and 4 = >20%). The stool consistency and occult blood scores were determined using the scoring systems (0 = normal; 1 = moist/sticky stool; 2 = soft stool; 3 = soft stool with mild diarrhea; 4 = diarrhea only) and (0 = no blood; 1 = minimal blood in stool; 2 = mild blood in stool; 3 = evidence blood in stool and 4 = only blood), respectively.

Histological analyses

As mentioned, at the end of the experimental period, a specimen of the colon was fixed in 10% neutral buffered formalin, dehydrated in gradient ethanol series and embedded in paraffin. For histological evaluation, 5 µm-thick tissue sections were stained with hematoxylin phloxine saffron and examined under an optic microscope (ZEISS IMAGER A1 microscope) by a pathologist who was unaware of the experimental protocol. The pictures were taken using a digital camera at 10X and 20X resolution. To quantitatively compare the severity of lesions between mouse treatments, tissues were assessed for injury and inflammation using a semi-quantitative scoring system (38). Sections were scored as focal (<10% of specimen), multifocal (10–50% of specimen), or diffuse (>50% of specimen) for mucosa, submucosa and muscularis. The muscularis damage was evaluated in a blind manner by estimating the amount of myocytes, vacuoles, necrosis and infiltration of cells (maximum score = 9). The submucosa damage was evaluated by estimating the infiltration of mononuclear, polymorphonuclear and eosinophilic cells (maximum score = 3). The characterization of the damage of the surface mucosal epithelium was evaluated by erosion, necrosis, exulceration, and loss of the mucus layer (maximum score = 12). All scores on the individual parameters together could results in a total score ranging from 0 to 24.

Myeloperoxidase activity assay

Tissue samples from distal colon were used to determine myeloperoxidase (MPO) activity. Tissue homogenates were centrifuged at 5000 x g at 4°C for 15 min and MPO activity in the supernatant was analyzed by ELISA (Hycult biotech, Uden, the Netherlands). All the samples of the six mouse groups were analyzed on the same plate at 450 nm.

Lipid peroxidation

Estimation of lipid peroxidation was assessed by measuring the content of malondialdehyde (MDA) in tissue samples of the distal colon by HPLC as described previously (34).

Endogenous antioxidant enzyme activities

Total superoxide dismutase (SOD) activity was determined as described previously. For glutathione peroxidase (GPx) activity, aliquots of distal colon were added to a PBS buffer containing 10 mM GSH, 0.1 U G-Red and 2 mM NADPH with 1.5% H_2O_2 to initiate the reaction as described previously (39).

Prostaglandin E2 determination

Tissue samples from the distal colon (fixed amount 10 mg) were homogenized in 1 mL of 0.1M phosphate pH 7.4 containing 1 mM EDTA and centrifuged at 5 000 x g at 4^{o}C for 15 min. A dilution of sample supernatant 1:3000 was assayed. Prostaglandin E2 (PGE2) levels were measured by ELISA (Arbor Assay, Michigan, USA). The intensity of the generated color was detected at the 450 nm wavelengths using a microtiter plate reader (EnVision Multilabel Plate Readers, PerkinElmer). Concentrations were estimated from the absorbance of the calculated standard curve.

RNA isolation, reverse transcription and quantitative PCR analyses

Total RNA was isolated from distal colonic tissues from the 6 mouse groups using QIAzol Lysis Reagent (QIAGEN, Maryland, USA) according to the manufacturer's instructions. Precipitated total RNA was then resuspended in RNAse-free sterile water and further purified through sequential precipitation with lithium chloride and sodium acetate to prevent the inhibitory effect of DSS on polymerase enzymatic activity (40). The RNA quality was assessed by agarose gel electrophoresis, and cDNAs were synthesized from 400 ng of RNA using the Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Gene expression was analysed by quantitative RT-PCR (qRT-PCR) according to our previous studies (41, 42).

Immunoblot analysis

Tissue samples from the distal colon were homogenized in cold PBS buffer with 4 mM EDTA and 10 µL protease inhibitor cocktail (leupeptin, pepstatin, PMSF, ALV and BHT). The Bradford assay (Bio-Rad, Mississauga, ON) was used to determine the protein concentration. Proteins were denatured in sample buffer containing SDS and ß-mercaptoethanol, separated on a 7.5 % SDS-PAGE and electroblotted onto Hybond nitrocellulose membranes (Amersham, Baie D'Urfé, QC).(41) Specific binding sites of the membranes were blocked using defatted milk proteins followed by the addition of one of the following primary antibodies: 1/1000 polyclonal anti-COX-2 (70 kDa, Novus, Oakville, ON); 1/10000 polyclonal anti-NF-kB (65 kDa, Santa Cruz Biotechnology, Santa Cruz, CA); 1/5000 polyclonal anti-IkB (39 kDa, Cell Signaling, Beverly MA); 1/5000 polyclonal anti- tumor necrosis factor (TNF)-a (26 kDa, R&D, Canada); 1/5000 monoclonal anti-interleukin (IL)-6 (25 kDa, R&D, Canada), 1/1000 polyclonal anti-Nrf2 (68 kDa, Abcam, MA, USA); 1/1000 polyclonal anti-PGC-1a (92 kDa, Abcam, MA, USA); 1/1000 polyclonal anti-OGG1 (39 kDa, Novus Biologicals); 1/1000 monoclonal anti-cytochrome C (15 kDa, Novus Biologicals); 1/1000 anti-inducing factor (AIF) (67

kDa, Abcam); 1/1000 peroxisome proliferator-activated receptor (PPAR)γ (55 kDa, Santa Cruz); 1/40000 monoclonal anti-b-actin (42 kDa, Sigma, MO, USA).

The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody (Jackson Laboratory, Bar Harbor, Maine). The β-actin protein expression was determined to confirm equal loading. Molecular size markers (Fermentas, Glen Burie, Maryland) were simultaneously loaded on gels. Blots were developed and the protein mass was quantitated by densitometry using an HP Scanjet scanner equipped with a transparency adapter and the UN-SCAN-IT gel 6.1 software.

Measurement of ADP and ATP levels

ADP and ATP contents were measured using an ADP/ATP bioluminescent assay kit from BioAssay Systems (Hayward, CA, USA) as described previously (43, 44). Values for mitochondria were then normalized with regard to the protein content.

Microarray screening and data analysis

Transcripts were assayed by microarray (45) and the probes were generated from RNA isolated from 4 mouse groups (CTL, DSS, DAPP-200/DSS, and DSS/DAPP-400, n=3 for each group). The 12 samples were processed at the microarray platform of the Princess Margaret Genomics Centre (Toronto, ON). Illumina mouse whole genome WG-6 expression beadchips were screened, analyzed and quantile normalized via the Princess Margaret Genomics Centre (data are accessible through Gene Expression Omnibus (GEO)**** and are all MIAME compliant). For each gene, samples bearing a coefficient of variation larger than 10% (representing less than 1% of all samples) were not considered in the statistical analysis and a Kruskal-Wallis analysis (P<0.05) was used to identify genes expressed differentially (using TMEV 4.9 software).

Ingenuity Pathway Analysis (IPA) analysis

Functional analyses were performed using IPA (Ingenuity Systems Inc., Redwood City, CA, USA) to identify functional pathway enrichment (45) involved in the preventive and therapeutic effects of polyphenols in apple peel on intestinal inflammation. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base. IPA used Fisher's exact test to calculate a p-value, which gave the likelihood that the set of genes in this pathway could be explained by chance alone.

16S rRNA gene amplicon sequencing using the Illumina MiSeq platform

A total of 36 gut mucosal samples consisting of pooled material of two or three mice from the same group were kept at -80°C until being processed. Bacterial DNA of these samples was extracted using a mechanical lysis of bacterial cells (Bead-beater) combined with silica-based column purification kit as directed by the manufacturer (ZR Fecal DNA Minprep; Zymo Research, USA). Total extracted DNA was quantified with a Qubit (Life Technologies, USA) and DNA purity was assessed using a ND-1000 Nanodrop (Nanodrop Technologies, USA). The 16S rRNA sequencing was performed according to a previously described method (46). The V3-V4 region of the 16S rDNA gene was amplified by PCR using primers Bakt_341F (5'-CCTACGGGNGGCWGCAG-3') and

Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') adapted to incorporate the transposon-based Illumina Nextera adapters (Illumina, USA) and a sample barcode sequence allowing multiplexed paired-end sequencing. PCR mixtures contained 1X Q5 buffer (NEB), 1X Q5 Enhancer (NEB), 200 µM dNTP (VWR International, Canada), 0.2 µM of forward and reverse primer (Integrated DNA Technologies, USA), 1 U of Q5 (NEB) and 1 µL of template DNA in a 50 µL reaction. The PCR cycling conditions consisted of an initial denaturation of 30 s at 98°C, followed by a first set of 15 cycles (98°C for 10 s, 55°C for 30 s and 72°C for 30 s), then by a second step of 15 cycles (98°C for 10 s, 65°C for 30 s and 72°C for 30 s) and final elongation of 2 min at 72°C before cooling to 4°C forever. PCR products were purified using 35 µL of magnetic beads (AxyPrep Mag PCR Clean up kit; Axygen Biosciences, USA) per 50 µL PCR reaction. Amplifications were controlled on a Bioanalyzer 2100 using DNA 7500 chips (Agilent Technologies, USA). Samples were pooled at an equimolar ratio, the pool was repurified as described before and checked for quality on a Bioanalyzer 2100 using a DNA high sensitivity chip. The pool was quantified using picogreen (Life Technologies, USA) and loaded on a MiSeq system (Illumina, USA). High-throughput sequencing was performed at the IBIS (Institut de Biologie Intégrative et des Systèmes - Université Laval). The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra/) under the study accession number GSE71920.

Bioinformatics of 16S rRNA gene amplicons

After demultiplexing by the MiSeq platform, raw sequence data obtained from the 40 sequenced samples were processed using the QIIME software package (version 1.9.0) (47). Firstly, paired-end reads obtained for each sample were joined on the overlapping ends, then resulting sequences were filtered to remove low read quality score (phred <25). Forward and reverse primers were trimmed from the filtered sequences; reads with at least one reverse primer mismatch or where the reverse primer was not found were discarded. USEARCH 61 (version 6.1.544) (48) was used to check and filter chimeras from the dataset. Out of a total of 40 original samples, two samples were removed from the dataset because of too low read count. After those filtering steps, we characterized a total of 602 836 sequences for the 38 remaining samples with an average of 15 864 ± 4459 reads per sample. Final amplicon mean length varied between 441-465 bp for all samples.

16S rRNA sequences that successfully passed the pre-processing steps and presenting ≥97% nucleotide sequence identity were binned into OTUs (Operational Taxonomic Units) using USEARCH 61 (version 6.1.544) (48) with an open-reference methodology. Reads that did not hit the Greengenes reference database (August 2013 release) (49) during the closed-reference step were subsequently clustered *de novo* at 97% identity threshold. Taxonomic assignment of representative OTU sequence form each cluster was then performed against the Greengenes reference database using the naive Bayesian RDP classifier at default parameters (50). Singleton OTUs (cluster with a unique sequence occurring only once among all reads) and OTUs with a number of sequences <0.005% of total number of sequences (51) were discarded at this step. A subsampling depth of 6412 reads (smallest amount of sequences originally found among our 38 metagenomic samples) was chosen to rarefy the OTU tables used in the downstream analyses. OTUs

that were unclassified at the genus level with the Greengenes database were further investigated with the RDP classifier against the RDP database (version September 17, 2014) (52) using a minimum bootstrap cut-off of 50% (53).

Statistical analysis

All values are expressed as mean ± SEM. Data were analyzed by using a one-way analysis variance and the two-tailed Student's *t* test using the Prism 5.01 (GraphPad Software) and the differences between the means were assessed post-hoc using Tukey's test. Statistical significance was defined as $P<0.05$.

The software PC-ORD (version 6; MjM Software, USA) was used to perform a principal coordinates analysis (PCoA) in order to illustrate differences between taxonomic profiles of metagenomic samples at the genus level. This ordination method combined with Bray-Curtis distance measure is well suited for species abundance data avoiding the assumption of linear relationship between variables. STAMP (Statistical Analysis of Metagenomic Profiles, version 2.0.9; Parks & Beiko, 2014) was employed to perform two-way comparisons of taxonomic distributions (at the genus level) between metagenomic samples (e.g. CTL vs DSS-treated), using the White's non-parametric t-test associated with the bootstrap method for calculating confidence intervals (nominal coverage of 95%). A Storey FDR approach was used to indicate the percentages of false positives (reported by q-values) that should be expected among all significant taxonomic units illustrated on bar plots. In order to visualize the relationships between OTUs and samples, a heatmap was constructed with STAMP using the Kruskal-Wallis H-test combined with the Tukey-Kramer test as post-hoc analysis. Multiple test correction for the heatmap analysis was performed with the Storey FDR approach. For all analyses performed within STAMP, only specific families or genera of interest were considered and features (taxa) with a q-value >0.05 were discarded.

RESULTS

Profile of phenolic compounds of DAPP

A reversed phase LC-MS method has been developed in order to separate and identify masses and chemical structures of polyphenolic compounds contained in the DAPP extract. Flavonoids figured among the major polyphenol classes: they were identified on the basis of their common structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle. Representative extracted ion chromatograms of identified DAPP polyphenolic compounds, using accurate mass measurement, are shown in Supplementary Figure S2. The DAPP was essentially constituted of hydroxycinnamic acids (Supplementary Figure S2A), dihydrochalcones (Supplementary Figure S2B), flavan-3-ols (Supplementary Figure S2C), and flavonols (Supplementary Figure S2D). The hydroxycinnamic acids were composed of isomers of coumaric acids, p-coumaroylquinic acids and caffeoylquinic acids while we found phloretin and phloridzin as dihydrochalcones. The accurate mass measurement revealed that the flavan-3-ol subclass was constituted of (+)-catechin, (-)-epicatechin, (+)-catechin 3-O-gallate, and (+)-catechin 3-O-glucose (Table 1). Noteworthy, in the DAPP extract, flavonols constituted the dominant subclass of flavonoids and were present as a mixture of aglycone and glycosylated quercetin (Supplementary Figure S2D and Table 1).

Effects of DAPP on the severity of DSS-induced colitis in mice

Mice exposed to oral administration of 2.5% DSS over 10 days was characterized by sustained weight loss (Figure 1A), abnormal stool consistency (Figure 1B) and bloody diarrhea (Figure 1C), which presented a significant increase in DAI (Figure 1D). To assess the preventive and therapeutic effects of polyphenols, the mice were treated with DAPP before or during induction and development of colitis at two different doses (200 and 400 mg/kg/day). All DAPP animal groups exhibited significantly reduced DAI scores compared with the DSS colitis group without the DAPP treatment (Figure 1D). As the colon length is inversely correlated with the severity of colitis and is considered to be an indirect marker of inflammation, we examined colon shrinking. While the DSS group had significantly shorter colons relatively to the control group (Figures 1E and 1F), the preventive (200 mg) and the therapeutic 400 mg/kg/day doses of DAPP displayed the most significantly beneficial effect on colon shortening reduction compared with the group on DSS alone (Figures 1E and 1F).

No histopathological changes were observed in colons from the control group, (Figure 2A). In contrast, the histopathological examination of the distal colon of mice treated with DSS showed mucosal ulceration, which was marked by an increased thickness of the muscle layer, loss of crypt structure, multifocal inflammatory cell infiltration into submucosal, severe denudation of the surface epithelium (erosion) and mucodepletion of glands (Figure 2B). Except for the group with the DAPP-400/DSS, DAPP significantly decreased the microscopic inflammation score for DSS-induced colitis in the other groups (Figure 2G). For example, mice pretreated with 200 mg/kg/day of DAPP showed less structural damage and inflammatory cell infiltration without a significant effect on muscle layer thickness compared to the DSS group without DAPP treatment (Figure 2D). Moreover, the epithelium in various areas remained intact and the mucin layer was clearly visible with stain cells, suggesting the beginning of a reepithelization and healing

process. Noteworthy, the high 400 mg/kg/day therapeutic DAPP dose resulted in near-normal colonic histology (Figure 2E) whereas the mice treated with the high 400 mg/kg/day preventive DAPP dose still showed several severe ulcers (Figure 2F). The colonic lesions were evaluated by a semi-quantitative scoring system that assesses the muscularis, submucosa and surface mucosal epithelium damages. Representation of total microscopic damage score demonstrated that preventive and therapeutic dose of 200 mg/kg/day of DAPP decreased colonic lesions (Figure 2G). However, therapeutic dose of 400 mg/kg/day of DAPP had the lowest total microscopic damage score (Figure 2G).

Effects of DAPP on colonic leukocyte involvement in DSS-induced colitis in mice

The colonic MPO activity, as a marker of inflammatory cell infiltration, was significantly increased in the colitis group (25-fold) compared to control mice (Figure 2H). All DAPP groups showed a reduction in the degree of polymorphonuclear neutrophil infiltration. The mice treated with the preventive 200 mg/kg/day dose of DAPP showed only a trend of decrease in MPO activity in distal colonic tissue

Effects of DAPP on Lipid Peroxidation in the distal colon of DSS-induced colitis mice

Compared to the control group, DSS significantly increased lipid peroxidation in the distal colon (Figure 3A). When mice were pretreated with 200 or 400 mg/kg/day DAPP, DSS-induced lipid peroxidation was prevented. On the other hand, only the highest therapeutic dose of DAPP (400 mg/kg/day) was able to totally counteract lipid peroxidation occurrence.

Mechanisms for the action of DAPP on oxidative stress

As failure of antioxidant defense may favor the induction of OxS, we examined endogenous antioxidant enzymes and found that the treatment with DSS alone caused a significant augmentation in the SOD activity compared to the CTL group (Figure 3B). However, the preventive (200 mg/kg/day) or therapeutic dose of 400 mg/kg/day of DAPP was the most efficient conditions for maintaining SOD activity quite comparable to that of the CTL group (Figure 3B). Only a slight decrease was noted in the other DAPP-400/DSS group. Under these conditions, GPx activity was down regulated by DSS (Figure 3C), and restored by the same preventive and therapeutic doses.

We examined the transcription factor Nrf2 that regulates antioxidant genes expression. While DSS-induced colitis down regulated the gene (50%) and protein (30%) expression of Nrf2 (Figures 3D and 3E), and the preventive DAPP dose of 200 mg/kg/day was capable of inhibiting the decrease of Nrf2 gene expression in DSS-treated mice (Figure 3D). Apart from the therapeutic dose of 200 mg/kg/day, the other groups (DAPP-400/DSS and DSS/DAPP-400) showed a particularly favorable influence on protein expression of Nrf2 (Figure 3E).

Effects of DAPP on inflammatory markers in the distal colon of DSS-induced colitis mice

As Cyclooxygenase-2 (COX-2) produce excessive inflammatory mediators, which are detrimental to the integrity of the colon and contribute to the development of intestinal damage, we evaluated its protein expressions. As shown in Figure 4A, the COX-2 protein expression in the DSS group was higher than in CTL mice. Preventive and therapeutic

DAPP treatments were able to lessen the exaggerated induction of COX-2 protein expression noted in DSS mice. To confirm these findings, we assessed the prostaglandin E2 (PGE2) that is a major downstream mediator of COX-2. PGE2 concentrations were significantly decreased by preventive and therapeutic DAPP treatments in DSS mice (Figure 4B). Therefore, DAPP is able to down regulate the COX-2-PGE2 pathway that is of high pharmacological interest for IBD.

To further evidence the anti-inflammatory capacity of DAPP, we assessed its influence on cytokines expressions (Figure 5). While the DSS resulted in severe inflammatory response as indicated by strong increases in colonic levels of the proinflammatory TNF-α and IL6 as compared to the CTL group, DAPP strongly antagonized this inflammation magnitude given the marked drop of the colonic mRNA content and protein expression of these cytokines. Interestingly, the TNF-α gene expression was decreased only in the group of mice that received 400 mg/kg/day of DAPP (Figure 5A).

Mechanisms for the action of DAPP on transcription factors

As the ubiquitous eukaryotic transcription factor NF-κB is mainly responsible for regulating the induction of pro-inflammatory cytokines, we examined the potential of DAPP to inhibit its activation in mouse colonic samples inflamed with DSS (Figures 5C). In fact, treatment with DAPP caused a significant inhibition of NF-κB/IκB ratio (Figure 5F). Noteworthy, the effect was more pronounced with the 200 mg/kg/day and therapeutic 400 mg/kg/day doses (Figure 5F).

We also evaluated the modulation of two crucial transcription factors: peroxisome proliferator-activated receptor γ Coactivator-1α (PGC-1α) and peroxisome proliferator-activated receptor (PPAR)γ. The levels of PGC-1α (Figures 6A and 6B) and PPARγ (Figures 6C and 6D) were down regulated in the DSS group comparatively to the CTL group. Whereas the therapeutic 200 mg/kg/day dose of DAPP was inefficient in enhancing PGC-1α mRNA and protein levels in DSS-treated mice, a significant trend of increase was noted in the other groups. With regard to PPARγ, an increase was noted in all the groups except for the preventive DAPP-400/DSS group.

Effect of DAPP on mitochondrial functions in mice with DSS-induced colitis

As the ADP/ATP ratio is generally considered a key parameter in mitochondrial energy metabolism and respiration, we established its status (Figure 7A) in purified mitochondria from the distal colons. The ADP/ATP ratio for the DSS group increased 4-fold compared to the control group, but the used therapeutic dose of 400 mg/kg/day reduced significantly this ADP/ATP ratio induction.

AIF is located in the inter-membrane space of mitochondria and is involved in initiating a caspase-independent pathway of apoptosis by causing DNA fragmentation and chromatin condensation. Furthermore, when colonic cell death is triggered by an apoptotic stimulus like DSS, cytochrome (Cyt) C is released into the cytosol and contributes to caspase-dependent pathway of apoptosis. Western blot analysis revealed an increase in the AIF and Cyt C protein masse in the mitochondrial preparation following DSS-induced colitis (Figures 7B and 7C). However, the therapeutic and preventive dose of 400 mg/kg/day was able to restore their normal levels.

The base excision repair pathway is primarily responsible for removing 8-OHdG from mitochondrial DNA. In human and murine model, 8-OHdG is repaired by 8-oxoguanine

DNA glycosylase (OGG1), an enzyme that recognizes and hydrolyses the aberrant base from the DNA backbone. As illustrated in Figure 7D, the DSS treatment resulted in a significant reduction in OGG1 protein mass as compared with CTL group. However, the therapeutic and preventive dose of 400 mg/kg/day prevented the decline in OGG1 expression.

Group comparisons using a score summation index

To compare the outcomes under the different conditions, we employed a score summation index (Supplementary Table S1). The total score was calculated by summing the various effects of DAPP in the different animal groups. As higher scores indicate greater improvements of the DSS group in response to polyphenol administration, we could identify two distinctive groups that exhibit the best outcomes, i.e. the preventive group with 200 mg/kg/day and the therapeutic group with 400 mg/kg/day. Therefore, we selected these two groups to conduct a microarray analysis of colonic gene expression and to examine the effect of the polyphenol treatment on the modulation of the gut microbiota.

Functional pathways involved in the effect of DAPP in mice with DSS-induced colitis.

To further determine the effect of DAPP in mice with DSS-induced colitis, we established colonic gene expression profiles for each group (CTL, DSS, DAPP-200/DSS, and DSS/DAPP-400, n=3 for each group) using Illumina mouse whole genome WG-6 expression beadchips. Statistical analyses revealed that 2152 genes were differentially expressed between each group (Supplementary Table S2). Significantly differentially expressed genes were subjected to IPA software. Comparative analysis of the cellular, molecular, physiological and metabolic functions was performed and classified according to statistical significance in the variation of the expression of genes in DSS/DAPP-400 and DAPP-200/DSS. Supplementary Tables S3 and S4 list 86 and 89 categories for DSS/DAPP-400 and DAPP-200/DSS, respectively, which were sorted according to their statistical significance as the negative logarithm of *P*-values calculated by IPA. Plotting the negative logarithm of p-values calculated by IPA for each of the functional categories found in the DSS/DAPP-400 group against the negative logarithm of *P*-values of the corresponding categories found in the DAPP-200/DSS group allows visualization of functions that are most relevant to each treatment. In Figure 8, we plotted the most significant 25 functional categories in each group, and found that 14 of them were altered in both groups. Among the shared canonical pathways we identified “IL6 signalling”, “Atherosclerosis signalling”, “LXR/RXR activation”, “Acute phase response signalling” and “IL10 signalling”, demonstrating that the DAPP treatment was able to modulate several inflammatory pathways. Interestingly, our results also evidenced that the preventive rather than the therapeutic treatment exclusively regulated some biological functions. For instance, preventive effects modulated “PPAR signalling”, “Fc epsilon RI signalling” and “Dendritic maturation” while “Gap junction signalling”, “FcγRIIB signalling in B lymphocytes” and “Epithelial adherents junctions signalling” were modulated by therapeutic treatment, suggesting differential beneficial effects depending on the treatment period.

Impact of DSS-induced colitis and DAPP treatment on the gut microbiota

In order to assess the effect of DAPP on gut dysbiosis induced by DSS, we performed a 16S rRNA metagenomic analysis of 38 gut mucosal collections from the CTL, DSS-induced colitis (DSS), preventive (DAPP+DSS) and therapeutic (DSS+DAPP) groups. As no significant difference in gut bacterial composition was found between metagenomic samples from mice treated with different doses (200 and 400 mg/kg/day) of DAPP, only therapeutic DAPP administration at the 400 mg/kg/day dose was considered for further metagenomic analyses.

The PCoA plot (Figure 9A) indicates the correlation between bacterial communities and the different treatments administered in mice. A clear shift from the CTL to all the treated animals groups (DSS, DSS+DAPP, DAPP+DSS) occurred along the first (and the most influential) component. The genera *Parabacteroides*, *Ruminococcus*, and more specifically *Barnesiella* and *Anaerostipes*, were strongly associated with the healthy state, while *Akkermansia* and *Mucispirillum* genera were more abundant in the metagenomes of treated mice; in particular, the families *Peptostreptococcaceae* and *Enterobacteriaceae* were significantly correlated with treated animal samples. In addition, the Figure 9B illustrates that those two bacterial families were absent from the control metagenomic sample, and consequently, only represented in the groups where DSS was administered; conversely, the genus *Anaerostipes* was only found in the CTL group. The relative abundance of *Akkermansia* and *Mucispirillum* was significantly more important in the metagenomes of treated animals than in the CTL group. The proximity between the CTL and the DSS+DAPP treated mice in the PCoA plot is slightly greater than the one observed between the DSS and CTL groups, likely indicating a more pronounced bacterial profile similarity between the therapeutic and healthy metagenomes.

Associations between gut bacterial composition and treated mice groups were further investigated by pairwise comparisons of gut bacterial profiles with respect to treatment (Figure 9B). *Peptostreptococcaceae* and *Enterobacteriaceae* families were clearly overrepresented in the DSS metagenome, whereas the mean proportion of *Barnesiella* was significantly higher in the CTR group, along with *Parabacteroides* and *Ruminococcus*. Sequences assigned to *Peptostreptococcaceae* and *Enterobacteriaceae* were more abundant in the metagenomes of DSS+DAPP and DAPP+DSS groups in comparison with the CTLs, but to a less extent than in the DSS-treated mice (Figure 10). Interestingly, reads classified as *Peptostreptococcaceae* was less numerous in the metagenome of therapeutics DAPP-treated mice than in the DAPP+DSS mice when compared to the CTR group. The genus *Mucispirillum* was overrepresented in both DSS+DAPP and DAPP+DSS metagenomes compared to the CTLs, while the mean proportion of sequences attributed to *Akkermansia* was higher in the metagenome of the preventive DAPP-treated animals only.

DISCUSSION

IBD constitute a major health problem in the Western world. As no cure exists and no definitive therapies are available for this chronic inflammatory disorder, it becomes urgent and crucial to develop novel strategies with high efficacy. Multiple scientific groups studying functional nutrients have identified polyphenolic compounds among complementary and alternative medicines. However, the full spectrum of biological activities and the mechanisms of actions have rarely been reported. Therefore, in this study, we address the following issues: Does oral administration of DAPP protect against sustained oxidative stress and inflammation, two characteristic features of IBD? Is DAPP efficient in preserving mitochondrial bioenergetics and functions known to be affected in IBD? Can DAPP trigger central transcription factors that are master regulators of antioxidant, anti-inflammatory and mitochondrial cytoprotective mechanisms? What is the transcriptomic signature that can be disclosed by microarray analysis of colonic specimens in response to DAPP treatment on a mouse model of induced colitis? What are the microbiota changes elicited by DSS-induced intestinal injury and are they restored by DAPP administration? Is DAPP beneficial for prevention and management?

We found that DAPP treatment showed an improvement of weight loss, diarrhea and bloody stools at a macroscopic level, but it also improved the histology at a microscopic level given the reduction of inflammatory cell invasion into colonic tissue. Our findings also support this alleviation of inflammation in response to DAPP supplementation as noted by the reduced expression of pro-inflammatory cytokines (TNF-α) and eicosanoids (PGE2). Consistently, DSS-mediated oxidative stress and mitochondrial dysfunctions were ameliorated given the substantial fall in MDA and the ADP/ATP ratio along with the strengthening of antioxidant defense. The limited oxidative and inflammatory magnitude, displayed following DAPP administration, may be due to the regulation of transcription factors and nuclear receptors as well as to the modifications of the microbiota composition and transcript profiling. In this context, we also observed a slight decrease in the relative abundance of *Peptostreptococcaceae* and *Enterobacteriaceae* in DAPP-treated mice compared to DSS-induced colitis group, suggesting that polyphenolic compounds exert an antioxidant effect on the mucosal inflammatory milieu. Our results highlight the potential of using DAPP to prevent and treat IBD via numerous mechanisms.

In our study, special attention was given to the analysis of the composition of polyphenols as their concentration and antioxidant activity may vary depending on food processing and seasonal differences. Using the high-resolution of UPLC-ESI-MS/MS QTOF, we evidenced the presence of flavonols (aglycone and glycosylated quercetin), flavan-3-ols [(+)-catechin and (-)-epicatechin], dihydrochalcones (phloretin and phloridzin) and hydroxycinnamic acids, which represent the major flavonoid subclasses that exhibited anti-inflammatory and antioxidant activities in our previous studies (34).

Noteworthy, not only we have used in the present study apple peels that constitute a good source of phenolic compounds (30, 32), but the concentrations of phenolic extract administered to our mouse model approximates the total extractable phenolic content

of 100 g of fresh apple widely consumable by humans (~ 357 mg/100 g fresh apple) (54, 55). In addition, the phenolic doses used in this study in mice are easily attainable in humans by applying the US Food and Drug Administration's guidelines to establish the human equivalent dose based on body surface area (56). We also doubled the DAPP dose to 400 mg to examine whether large quantities of polyphenols can have more desirable outcomes especially in the therapeutic mode.

The main reason for the administration of the two concentrations of polyphenols (200 and 400 mg/kg/day) is not to examine the dose response, but rather to understand how mice with DSS-induced experimental UC respond to physiological and supraphysiological doses. We reasoned that the physiological concentration (200 mg/kg/day) may better suit preventive action while the 400 mg/kg/day dose may work better in therapeutic intervention given the numerous and serious implemented health abnormalities to counteract. According to our results, this hypothesis proved true. In this context, it is important to mention that our data converge with previous studies. For example, flavonoids such as epigallocatechin gallate (EGCG), a powerful natural anti-inflammatory substance, inhibited acetic acid-induced colitis in rats at a dose of 50 mg/kg/day (57), but higher doses exacerbated inflammation (58). In this study, the authors concluded: '' It may be prudent for individuals with IBD to avoid excessive doses of supplemental EGCG for colitis management until further evidence of its safety and efficacy in human intervention studies.'' Similar to EGCG, green tea polyphenols at doses <0.5% inhibited DSS-induced colitis, while doses of 0.5% to 1% fortified colitis symptoms and mortality (59-61). Another example is represented by genistein that a dose of 20 mg/kg reduced survival relative to the non-treated DSS-control group whereas lower concentrations (2 and 10 mg/kg) were more beneficial (62).

Evaluation of the preventive and therapeutic effects of DAPP showed a significant improvement of the DSS-induced clinical manifestations, as evidenced by the alleviation of body weight loss, diarrhea and fecal bleeding. In addition, supplementation of DAPP ameliorated colon shortening and colonic histological damages in the DSS-induced colitis mouse model. It also lessened neutrophils influx into the colonic tissue since the MPO activity was decreased, which evidenced the role of DAPP in restraining the induction of neutrophils recruitment. Moreover, DAI scores were markedly lower in the DAPP groups than in the DSS group. Taken together, these data demonstrate the preventive and therapeutic efficacy of DAPP against colitis development. Interestingly, the maximal preventive effect of DAPP was mostly achieved at the dose of 200 mg/kg/day but not with curative doses employed in our study.

A large number of experimental and clinical data suggests that chronic intestinal inflammation may be the result of a sustained overproduction of pro-inflammatory cytokines (63, 64). As inflammatory mediators like TNF-α and IL-6 play critical roles in the pathogenesis of murine colitis, we analyzed their expression by qPCR and Western blot. Consistent with previous reports (65-70), the levels of these pro-inflammatory cytokines in DSS-induced mice were found positively correlated to the severity of colitis in the current study. Furthermore, DAPP significantly provided suppressive effects on intrinsic TNF-α and IL-6 elevations under experimental colitis condition, which probably

halts the pathological progression of pro-inflammatory cascade. To delineate the mechanism of DAPP anti-inflammatory action, we assessed the expression of nuclear NF-κB that functions in the transcription regulation of numerous genes (71) and appeared remarkably elevated in colonic tissues of animals with colitis (72, 73). Estimation of NF-κB and its inhibitory molecule IκB (capable of preventing the translocation of NF-κB to the nucleus for the transcription activation of pro-inflammatory target genes) showed the capacity of DAPP to reverse NF-κB increase and cytoplasmic IκB degradation in mice with DSS-induced colitis. In fact, DAPP weakened NF-κB p65 signals while enhancing the expression of the inhibitory subunit IκB-α, which led to the decline of the NF-κB/ IκB ratio. Our results are in accordance with the anti-inflammatory effects of DAPP in intestinal epithelial Caco-2/15 cells (34) and are also consistent with previous reports that illustrate the efficiency of apple polyphenols to inhibit NF-κB activation in lipopolysaccharide/IFNγ-induced inflammation in human cell lines (74) and HLA-B27 transgenic rats (75).

Up-regulation of certain proteins such as COX-2 is also implicated in immune dysregulation characterizing IBD (76). Its induction results in an excessive inflammatory response, which may affect colon mucosa integrity and contributes to the development of intestinal damage (77). The anti-inflammatory activity of DAPP was further confirmed by measuring colonic COX-2 levels. As highlighted by our findings, DAPP supplementation was able to mediate COX-2 down-regulation and to limit the formation of its PGE2 product in parallel with the noted TNF-α and IL-6 blocking as well as MPO reduction (as an index of the neutrophils' presence and activation), thereby proving the high efficacy in ameliorating the acute colitis stage. The preventive and therapeutic benefits of DAPP supplementation are likely to stem from a global anti-inflammatory effect probably via the control of NF-κB signal transduction pathway (78).

As largely reported, IBD is initiated and perpetuated by a combination of deregulated immune response and imbalance between the production of free radical and antioxidant defense (79, 80). Given the close correlation between the activity of free radicals in the gut and DAI severity, indicating the significance of oxidative stress in the inflammatory process, we have assessed the impact of DAPP on lipid peroxidation that is responsible for many of the damaging reactions in the cell by causing membrane leakiness and breakdown (81). Our findings clearly support the protective role of DAPP in the regulation of oxidative stress since it significantly averted the elevation of MDA. Notably, our data also emphasize the indirect antioxidant effect of DAPP through the induction of endogenous protective antioxidative SOD and GPx enzymes through activation of Nrf2, which orchestrates the transcription of antioxidant genes and up-regulation of cytoprotective proteins (82).

Structurally abnormal mitochondria have been observed in tissue from patients with gut inflammation (83) and in epithelial monolayers treated with prooxidants (44). Although there are limited data on the role of mitochondria in colitis, there is a growing interest in targeting mitochondria-derived oxidative stress to reduce epithelial barrier dysfunction and colitis (84). Our findings show the effectiveness of DAPP to enhance mitochondrial ATP generation while lowering apoptosis as evidenced by the fall in AIF and Cyt C

protein expression in DSS-treated mice. Furthermore, DAPP was able to counteract the DSS-induced drop of OGG1, an enzyme that recognizes and hydrolyzes the aberrant nucleic bases from the DNA backbone. Our data are consistent with previous investigations showing that polyphenols are able to protect against the loss of mitochondrial membrane potential, the increment in lipid peroxidation and the fall in cellular ATP induced by indomethacin in Caco-2 cells (85). Mechanistically, this mitochondrial regulation by DAPP may be mediated via the increase of PGC-1α that is a master modulator of mitochondrial biogenesis and a transcriptional regulator of cellular energy processes, including OXPHOS and fatty acid oxidation (86-88). It is tempting to speculate that DAPP may have salutary effects via the induction of PGC-1α on mitochondrial dysfunctions since quercetin was recently reported to enhance the expression of PGC-1α in conjunction with mitochondrial biogenesis and functions (89).

In comparison with the CTL group, DSS treatment induced a reduction in the proportion of *Parabacteroides* and *Barnesiella* phylotypes, both belonging to the *Porphyromonadaceae* family *(*Bacteroidales order). *Parabacteroides* sp. was found to be at higher levels in healthy controls than in UC or irritable bowel syndrome patients (90). Similarly, higher levels of *Barnesiella* phylotype were correlated with lower activity levels of colitis in IL-deficient mice (91). More interestingly, an increase of the relative abundance of *Enterobacteriaceae* and *Peptostreptococceae* was also observed in DSS-induced colitis. The cytotoxic effects of DSS on gut epithelium have been associated with an increase of mucus release, intestinal permeability, and acute barrier damage. The loss of barrier function allows the translocation of pro-inflammatory microbial products (92, 93). Then, the induced intestinal inflammation could be responsible for the shift in microbial composition observed in mice with DSS treatments, including those with DAPP. Lupp *et al*. found that host-mediated inflammation alone is sufficient to perturb the composition of the intestinal microbiota in DSS-induced colitis (94) The host-mediated inflammation and oxidant-mediated injury also change the environmental conditions in the gut (e.g. oxygen availability), which may affect proliferation of some phylotypes capable of adapting to these conditions. Specifically, facultative anaerobes such as *Enterobacteriaceae* and *Peptostreptococceae* may be favored over obligate anaerobes such as *Porphyromonadaceae* (*Barnesiella* and *Parabacteroides*) and *Anaerostipes* from increased oxygen availability or resistance to reactive oxygen species produced during inflammation (95).

Mucin-degrading bacteria *Akkermansia muciniphila* and *Mucispirillum* were found to be more numerous in the DSS-treated mice. In agreement with our results, Berry *et al*. observed an increase *in Verrucomicrobiaceae* (mainly *Akkermansia* spp.) and *Deferribacteracae* (mainly *Mucispirillum* spp.) during acute inflammation (95). In DSS-induced colitis, *Mucispirillum* and *Akkermansia* could benefit from the degradation of host-derived mucus secretions. Accordingly, mucus composition and secretion are altered during inflammation while mucolytic bacteria and mucolytic activity are increased in IBD mucosa (95). Interestingly, the higher level of *Akkermansia* found in the DAPP-treated animals (compared to the CTL group) under preventive conditions, could be interpreted as a prebiotic-like effect of DAPP. This may reflect a partial restoration towards a healthier gut microbiota noticed in these animals by restraining growth of

facultative anaerobes. In this context, a substantial rise of *Akkermansia* has recently been associated with improved metabolic state in mice on high-fat/high-sucrose fed in response to a polyphenol-rich cranberry extract (46).

In the present study, we also noted a slight decrease of relative abundance of *Peptostreptococceae* and *Enterobacteriaceae* in DAPP groups (especially in the curative mode), suggesting that DAPP phenolic compounds exerted an antioxidant effect on the mucosal inflammatory milieu. However, the link between the partial correction of bacterial dysbiosis and colitis amelioration in the DSS + DAPP group has not been clearly established. The slight shift in bacterial composition compared to DSS group could only reflect improved integrity of the intestinal mucosa and decreased inflammation. Additional experiments using fecal transplantation of caecal content from DAPP-treated animals into DSS mice should be carried out in order to demonstrate that colitis improvement observed in DAPP-treated mice represents a cause-effect relationship between DAPP and restoration of gut flora.

In conclusion, the present investigation provides *in vivo* evidence that administration of DAPP lessens the intestinal oxidative stress magnitude via the modulation of the NRF2 that functions as a master switch and regulates a cascade of antioxidant genes containing the Antioxidant Response Element (ARE) sequence. The findings also show the modulation of intestinal inflammatory response by DAPP through the inhibition of neutrophil infiltration and deactivation of the NF-κB signaling pathway. These advantageous effects could be attributed, at least partially, to the regulatory role of DAPP in mitochondrial functions, messenger RNA phenotype and microbiota that is essential for optimal health. Overall, these positive modifications were associated with significant benefits such as reduction of bleeding, improvement in stool consistency, improved histological appearance, decreased weight loss, and protection from colon shortening, which indicate that polyphenols may represent potent tools for preventing and treating IBD. Noteworthy, this preclinical study will help define whether improvement of gut dysbiosis by DAPP, in association with the severity of mucosal injury or inflammation may constitute a promising treatment option in IBD as suggested previously (96). Finally, our study is an appropriate answer to the critically important scientific question seeking whether polyphenols are as effective as people believe.

CLINICAL PERSPECTIVES

Apple peel polyphenols may represent efficient functional foods capable of exerting beneficial actions on intestinal disorders such as inflammatory bowel diseases.

AUTHORS CONTRIBUTION

EL conceived and designed the study. MCD, PYR, NH, CG, ATS, NP, TV, and ET conducted the experiments. MCD, PYR, ATS, TV, NP, ET and EL analyzed and interpreted the data. MCD, DR, YD, AF, ED, AM, JFB, DA and EL contributed to the writing of the paper, EL approved the final version of the manuscript.

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REFERENCES

1. Rubin DC, Shaker A, Levin MS. Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. Front Immunol. 2012;3:107.
2. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet. 2007;369(9573):1627-40.
3. Podolsky DK. Inflammatory bowel disease. NEnglJMed. 2002;347(6):417-29.
4. Danese S, Fiocchi C. Etiopathogenesis of inflammatory bowel diseases. World J Gastroenterol. 2006;12(30):4807-12.
5. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447(7145):661-78.
6. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science. 2006;314(5804):1461-3.
7. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012;491(7422):119-24.
8. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. Am J Gastroenterol. 2011;106(4):563-73.
9. Bernstein CN, Loftus EV, Jr., Ng SC, Lakatos PL, Moum B. Hospitalisations and surgery in Crohn's disease. Gut. 2012;61(4):622-9.
10. Rocchi A, Benchimol EI, Bernstein CN, Bitton A, Feagan B, Panaccione R, et al. Inflammatory bowel disease: a Canadian burden of illness review. Can J Gastroenterol. 2012;26(11):811-7.
11. Lichtenstein GR, Rutgeerts P, Sandborn WJ, Sands BE, Diamond RH, Blank M, et al. A pooled analysis of infections, malignancy, and mortality in infliximab- and immunomodulator-treated adult patients with inflammatory bowel disease. Am J Gastroenterol. 2012;107(7):1051-63.
12. Toruner M, Loftus EV, Jr., Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, et al. Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology. 2008;134(4):929-36.
13. Dear KL, Compston JE, Hunter JO. Treatments for Crohn's disease that minimise steroid doses are associated with a reduced risk of osteoporosis. Clin Nutr. 2001;20(6):541-6.
14. Sarzi-Puttini P, Ardizzone S, Manzionna G, Atzeni F, Colombo E, Antivalle M, et al. Infliximab-induced lupus in Crohn's disease: a case report. Dig Liver Dis. 2003;35(11):814-7.
15. Steenholdt C, Svenson M, Bendtzen K, Thomsen OO, Brynskov J, Ainsworth MA. Acute and delayed hypersensitivity reactions to infliximab and adalimumab in a patient with Crohn's disease. J Crohns Colitis. 2012;6(1):108-11.
16. Fernandez A, Barreiro-de AM, Vallejo N, Iglesias M, Carmona A, Gonzalez-Portela C, et al. Complementary and alternative medicine in inflammatory bowel disease patients: frequency and risk factors. Dig Liver Dis. 2012;44(11):904-8.
17. Lakatos PL, Czegledi Z, David G, Kispal Z, Kiss LS, Palatka K, et al. Association of adherence to therapy and complementary and alternative medicine use with

demographic factors and disease phenotype in patients with inflammatory bowel disease. J Crohns Colitis. 2010;4(3):283-90.
18. Rawsthorne P, Clara I, Graff LA, Bernstein KI, Carr R, Walker JR, et al. The Manitoba Inflammatory Bowel Disease Cohort Study: a prospective longitudinal evaluation of the use of complementary and alternative medicine services and products. Gut. 2012;61(4):521-7.
19. Weizman AV, Ahn E, Thanabalan R, Leung W, Croitoru K, Silverberg MS, et al. Characterisation of complementary and alternative medicine use and its impact on medication adherence in inflammatory bowel disease. Aliment Pharmacol Ther. 2012;35(3):342-9.
20. Hilsden RJ. Seeking the ultimate bowel preparation for colonoscopy: is the end in sight? Can J Gastroenterol. 2011;25(12):655-6.
21. Ng SC, Lam YT, Tsoi KK, Chan FK, Sung JJ, Wu JC. Systematic review: the efficacy of herbal therapy in inflammatory bowel disease. Aliment Pharmacol Ther. 2013;38(8):854-63.
22. Gonzalez-Vallinas M, Gonzalez-Castejon M, Rodriguez-Casado A, Ramirez de MA. Dietary phytochemicals in cancer prevention and therapy: a complementary approach with promising perspectives. Nutr Rev. 2013;71(9):585-99.
23. Khurana S, Venkataraman K, Hollingsworth A, Piche M, Tai TC. Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients. 2013;5(10):3779-827.
24. Munir KM, Chandrasekaran S, Gao F, Quon MJ. Mechanisms for food polyphenols to ameliorate insulin resistance and endothelial dysfunction: therapeutic implications for diabetes and its cardiovascular complications. Am J Physiol Endocrinol Metab. 2013;305(6):E679-E86.
25. Tangney CC, Rasmussen HE. Polyphenols, inflammation, and cardiovascular disease. Curr Atheroscler Rep. 2013;15(5):324.
26. Chu AJ. Antagonism by bioactive polyphenols against inflammation: a systematic view. Inflamm Allergy Drug Targets. 2014;13(1):34-64.
27. Gupta SC, Tyagi AK, Deshmukh-Taskar P, Hinojosa M, Prasad S, Aggarwal BB. Downregulation of tumor necrosis factor and other proinflammatory biomarkers by polyphenols. Arch Biochem Biophys. 2014;559:91-9.
28. Romier B, Schneider YJ, Larondelle Y, During A. Dietary polyphenols can modulate the intestinal inflammatory response. Nutr Rev. 2009;67(7):363-78.
29. Boyer J, Liu RH. Apple phytochemicals and their health benefits. Nutr J. 2004;3:5.
30. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem. 2003;51(3):609-14.
31. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol. 2014;104:Unit.
32. Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. Nature. 2000;405(6789):903-4.
33. He X, Liu RH. Phytochemicals of apple peels: isolation, structure elucidation, and their antiproliferative and antioxidant activities. J Agric Food Chem. 2008;56(21):9905-10.

34. Denis MC, Furtos A, Dudonne S, Montoudis A, Garofalo C, Desjardins Y, et al. Apple peel polyphenols and their beneficial actions on oxidative stress and inflammation. PLoS One. 2013;8(1):e53725.
35. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology. 1994;107(6):1643-52.
36. Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. Gastroenterology. 1995;109(4):1344-67.
37. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest. 1993;69(2):238-49.
38. Cooney RM, Warren BF, Altman DG, Abreu MT, Travis SP. Outcome measurement in clinical trials for Ulcerative Colitis: towards standardisation. Trials. 2007;8:17.
39. Precourt LP, Marcil V, Ntimbane T, Taha R, Lavoie JC, Delvin E, et al. Antioxidative properties of paraoxonase 2 in intestinal epithelial cells. Am J Physiol GastrointestLiver Physiol. 2012;303(5):G623-G34.
40. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. BMC Res Notes. 2013;6:360.
41. Levy E, Spahis S, Garofalo C, Marcil V, Montoudis A, Sinnet D, et al. Sar1b transgenic male mice are more susceptible to high-fat diet-induced obesity, insulin insensitivity and intestinal chylomicron overproduction. J Nutr Biochem. 2014;25(5):540-8.
42. Levy E, Ben Djoudi Ouadda A, Spahis S, Sane AT, Garofalo C, Grenier E, et al. PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. Atherosclerosis. 2013;227(2):297-306.
43. Denis MC, Desjardins Y, Furtos A, Marcil V, Dudonne S, Montoudis A, et al. Prevention of oxidative stress, inflammation and mitochondrial dysfunction in the intestine by different cranberry phenolic fractions. Clin Sci (Lond). 2015;128(3):197-212.
44. Taha R, Seidman E, Mailhot G, Boudreau F, Gendron FP, Beaulieu JF, et al. Oxidative stress and mitochondrial functions in the intestinal Caco-2/15 cell line. PLoS One. 2010;5(7):e11817.
45. Veilleux A, Mayeur S, Berube JC, Beaulieu JF, Tremblay E, Hould FS, et al. Altered intestinal functions and increased local inflammation in insulin-resistant obese subjects: a gene-expression profile analysis. BMC Gastroenterol. 2015;15:119.
46. Anhe FF, Roy D, Pilon G, Dudonne S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut. 2015;64(6):872-83.
47. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.

48. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-1.
49. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72(7):5069-72.
50. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261-7.
51. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing Nat Methods. 2013;10(1):57-9.
52. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42(Database issue):D633-D42.
53. Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, et al. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One. 2009;4(8):e6669.
54. Liu RH, Eberhardt MV, Lee CY. Antioxidant and antiproliferative activites of selected New York apple cultivars . . NYFruit Q. 2001;9:15-7.
55. Podsedek A, Wilska-Jeszka J, Anders B, Markowski J. Compositional characterisation of some apple varieties. European Food Research and Technology. 2000;210:268-72.
56. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2008;22:659-61.
57. Ran ZH, Chen C, Xiao SD. Epigallocatechin-3-gallate ameliorates rats colitis induced by acetic acid. Biomed Pharmacother. 2008;62(3):189-96.
58. Guan F, Liu AB, Li G, Yang Z, Sun Y, Yang CS, et al. Deleterious effects of high concentrations of (-)-epigallocatechin-3-gallate and atorvastatin in mice with colon inflammation. Nutr Cancer. 2012;64(6):847-55.
59. Barnett MP, Cooney JM, Dommels YE, Nones K, Brewster DT, Park Z, et al. Modulation of colonic inflammation in Mdr1a(-/-) mice by green tea polyphenols and their effects on the colon transcriptome and proteome. J Nutr Biochem. 2013;24(10):1678-90.
60. Inoue H, Akiyama S, Maeda-Yamamoto M, Nesumi A, Tanaka T, Murakami A. High-dose green tea polyphenols induce nephrotoxicity in dextran sulfate sodium-induced colitis mice by down-regulation of antioxidant enzymes and heat-shock protein expressions. Cell Stress Chaperones. 2011;16(6):653-62.
61. Inoue H, Maeda-Yamamoto M, Nesumi A, Tanaka T, Murakami A. Low and medium but not high doses of green tea polyphenols ameliorated dextran sodium sulfate-induced hepatotoxicity and nephrotoxicity. Biosci Biotechnol Biochem. 2013;77(6):1223-8.
62. Sakai T, Furoku S, Nakamoto M, Shuto E, Hosaka T, Nishioka Y, et al. Soy isoflavone equol perpetuates dextran sulfate sodium-induced acute colitis in mice. Biosci Biotechnol Biochem. 2011;75(3):593-5.

63. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene. 1999;18(49):6853-66.
64. Papadakis KA, Targan SR. Current theories on the causes of inflammatory bowel disease. Gastroenterol Clin North Am. 1999;28(2):283-96.
65. Urushima H, Nishimura J, Mizushima T, Hayashi N, Maeda K, Ito T. Perilla frutescens extract ameliorates DSS-induced colitis by suppressing proinflammatory cytokines and inducing anti-inflammatory cytokines. Am J Physiol Gastrointest Liver Physiol. 2015;308(1):G32-41.
66. Dou W, Zhang J, Ren G, Ding L, Sun A, Deng C, et al. Mangiferin attenuates the symptoms of dextran sulfate sodium-induced colitis in mice via NF-kappaB and MAPK signaling inactivation. Int Immunopharmacol. 2014;23(1):170-8.
67. Cui L, Feng L, Zhang ZH, Jia XB. The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-kappaB pathway activation. Int Immunopharmacol. 2014;23(1):294-303.
68. Gotardo EM, Ribeiro Gde A, Clemente TR, Moscato CH, Tome RB, Rocha T, et al. Hepcidin expression in colon during trinitrobenzene sulfonic acid-induced colitis in rats. World J Gastroenterol. 2014;20(15):4345-52.
69. Xu H, Lai W, Zhang Y, Liu L, Luo X, Zeng Y, et al. Tumor-associated macrophage-derived IL-6 and IL-8 enhance invasive activity of LoVo cells induced by PRL-3 in a KCNN4 channel-dependent manner. BMC Cancer. 2014;14:330.
70. Pervin M, Hasnat MA, Lim JH, Lee YM, Kim EO, Um BH, et al. Preventive and therapeutic effects of blueberry (Vaccinium corymbosum) extract against DSS-induced ulcerative colitis by regulation of antioxidant and inflammatory mediators. J Nutr Biochem. 2016;28:103-13.
71. Visekruna A, Joeris T, Seidel D, Kroesen A, Loddenkemper C, Zeitz M, et al. Proteasome-mediated degradation of IkappaBalpha and processing of p105 in Crohn disease and ulcerative colitis. J Clin Invest. 2006;116(12):3195-203.
72. Andresen L, Jorgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J. Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. Gut. 2005;54(4):503-9.
73. Dong WG, Liu SP, Yu BP, Wu DF, Luo HS, Yu JP. Ameliorative effects of sodium ferulate on experimental colitis and their mechanisms in rats. World J Gastroenterol. 2003;9(11):2533-8.
74. Jung M, Triebel S, Anke T, Richling E, Erkel G. Influence of apple polyphenols on inflammatory gene expression. Mol Nutr Food Res. 2009;53(10):1263-80.
75. Castagnini C, Luceri C, Toti S, Bigagli E, Caderni G, Femia AP, et al. Reduction of colonic inflammation in HLA-B27 transgenic rats by feeding Marie Menard apples, rich in polyphenols. Br J Nutr. 2009;102(11):1620-8.
76. Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology. 1998;115(2):297-306.
77. Sanchez-Fidalgo S, Cardeno A, Villegas I, Talero E, de la Lastra CA. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. Eur J Pharmacol. 2010;633(1-3):78-84.
78. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol. 2002;2(10):725-34.

79. Levy E, Rizwan Y, Thibault L, Lepage G, Brunet S, Bouthillier L, et al. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. Am J Clin Nutr. 2000;71(3):807-15.
80. Lih-Brody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, et al. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. Dig Dis Sci. 1996;41(10):2078-86.
81. Alzoghaibi MA. Concepts of oxidative stress and antioxidant defense in Crohn's disease. World J Gastroenterol. 2013;19(39):6540-7.
82. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem. 2002;277(45):42769-74.
83. Nazli A, Yang PC, Jury J, Howe K, Watson JL, Soderholm JD, et al. Epithelia under metabolic stress perceive commensal bacteria as a threat. Am J Pathol. 2004;164(3):947-57.
84. Wang A, Keita AV, Phan V, McKay CM, Schoultz I, Lee J, et al. Targeting mitochondria-derived reactive oxygen species to reduce epithelial barrier dysfunction and colitis. Am J Pathol. 2014;184(9):2516-27.
85. Carrasco-Pozo C, Gotteland M, Speisky H. Apple peel polyphenol extract protects against indomethacin-induced damage in Caco-2 cells by preventing mitochondrial complex I inhibition. J Agric Food Chem. 2011;59(21):11501-8.
86. Scarpulla RC. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. Gene. 2002;286(1):81-9.
87. Scarpulla RC. Nuclear control of respiratory chain expression by nuclear respiratory factors and PGC-1-related coactivator. Ann NY Acad Sci. 2008;1147:321-34.
88. Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. 2008;88(2):611-38.
89. Rayamajhi N, Kim SK, Go H, Joe Y, Callaway Z, Kang JG, et al. Quercetin induces mitochondrial biogenesis through activation of HO-1 in HepG2 cells. Oxid Med Cell Longev. 2013;2013:154279.
90. Ubeda C, Bucci V, Caballero S, Djukovic A, Toussaint NC, Equinda M, et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. Infect Immun. 2013;81(3):965-73.
91. Ye J, Lee JW, Presley LL, Bent E, Wei B, Braun J, et al. Bacteria and bacterial rRNA genes associated with the development of colitis in IL-10(-/-) mice. Inflamm Bowel Dis. 2008;14(8):1041-50.
92. Kitajima S, Takuma S, Morimoto M. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. ExpAnim. 1999;48(3):137-43.
93. Mueller C, Macpherson AJ. Layers of mutualism with commensal bacteria protect us from intestinal inflammation. Gut. 2006;55(2):276-84.
94. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007;2(2):119-29.
95. Berry D, Schwab C, Milinovich G, Reichert J, Ben MK, Decker T, et al. Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. ISMEJ. 2012;6(11):2091-106.

96. Yoshioka Y, Akiyama H, Nakano M, Shoji T, Kanda T, Ohtake Y, et al. Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice. Int Immunopharmacol. 2008;8(13-14):1802-7.

Table 1: Polyphenols identification from DAPP extract

Polyphenol class	Polyphenol subclass	Polyphenol name	Formula	Ion Formula [M-H]-	Experimental mass (m/z)	Theoretical mass (m/z)	Diff.ppm (<5ppm)
Phenolic acids	Hydroxycinnamic acid	Coumaric acids	$C_9H_8O_3$	$C_9H_7O_3$	163.0390	163.0395	-3.1
		Coumaroylquinic acids	$C_{16}H_{18}O_8$	$C_{16}H_{17}O_8$	337.0928	337.0923	1.5
		Caffeoylquinic acids	$C_{16}H_{18}O_9$	$C_{16}H_{17}O_9$	353.0878	353.0872	1.7
Flavonoids	Dihydrochalcone	Phloretin	$C_{15}H_{14}O_5$	$C_{15}H_{13}O_5$	273.0769	273.0763	2.2
		Phloridzin	$C_{21}H_{24}O_{10}$	$C_{21}H_{23}O_{10}$	435.1282	435.1291	-2.1
	Dihydroflavonol	Dihydroquercetin	$C_{15}H_{12}O_7$	$C_{15}H_{11}O_7$	303.0520	303.0505	4.9
		Dihydroquercetin 3-O-rhamnoside	$C_{21}H_{22}O_{11}$	$C_{21}H_{21}O_{11}$	449.1100	449.1084	3.6
	Flavanol	(+)-Catechin	$C_{15}H1_4O_6$	$C_{15}H_{13}O_6$	289.0709	289.0712	-1.0
		(-)-Epicatechin					
		(+)-Catechin 3-O-gallate	$C_{22}H_{18}O_{10}$	$C_{22}H_{18}O_{10}Na$	465.0798*	441.0822	-4.9
		(+)-Catechin 3-O-glucose	$C_{21}H_{24}O_{11}$	$C_{21}H_{23}O_{11}$	451.1223	451.1240	-3.8
	Flavonol	Quercetin	$C_{15}H_{10}O_7$	$C_{15}H_9O_7$	301.0336	301.0348	-4.0

	Quercetin 3-O-arabinoside		$C_{20}H_{18}O_{11}$	$C_{20}H_{17}O_{11}$	433.0721	433.0771	-1.2
	Quercetin 3-O-xyloside						
	Quercetin 3-O-rhamnoside		$C_{21}H_{20}O_{11}$	$C_{21}H_{19}O_{11}$	447.0941	447.0927	3.1
	Quercetin 3-O-galactoside		$C_{21}H_{20}O_{12}$	$C_{21}H_{19}O_{12}$	463.0894	463.0876	3.9
	Quercetin 3-O-glucoside						
	Quercetin 3-O-galactoside-7-O-rhamnoside		$C_{27}H_{30}O_{16}$	$C_{27}H_{29}O_{16}$	609.1471	609.1456	2.5
	Quercetin 3-O-rhamnosyl-galactoside						
	Quercetin 3-O-rutinoside						

Experimental mass measurement and empirical formula calculation for phenolic acids and flavonoids. A good agreement between the theoretical and the experimental m/z values was obtained for all compounds examined (< 5ppm). Separations were performed on an ultra-performance liquid chromatography system (UPLC) coupled to a QuanTOF mass spectrometer (MS/MS QTOF) equipped with an ESI source (UPLC-ESI-MS/MS QTOF). The UPLC-ESI-MS/MS QTOF system consisting of a Waters-ACQUITY UPLC with an Synapt G2-Si High Definition mass spectrometer (Waters, MA, USA).* adduct sodium of $C_{22}H_{18}O_{10}$.

FIGURE LEGENDS

Figure 1 **Effects of DAPP on macroscopic damage in mice with DSS-induced colitis.** The severity of the colitis was determined by the disease activity index score (D), which combines scores for the body weight change (A) and feces condition (stool consistency (B) and fecal bleeding (C). The body weight change was determined by calculating the percentage of weight change relative to the starting weight before DSS treatment (0 = none; 1 = 1-5%; 2 = 5-10%; 3 = 10-20% and 4 = >20%). The fecal condition score was determined using two parameters: stool consistency (0 = normal; 1 = moist/sticky stool; 2 = soft stool; 3 = soft stool with mild diarrhea; 4 = diarrhea only) and fecal bleeding (0 = no blood; 1 = minimal blood in stool; 2 = mild blood in stool; 3 = evidence blood in stool and 4 = only blood). After sacrificing the mice, the colon length was measured between the colocecal junction and the rectum (E and F). In addition to the noncolitis control group (CTL), the five DSS colitis groups were distributed as follows: DSS colitis group received only the vehicle (water used to administer DAPP) by oral gavage; the preventive groups were orally treated by gavage with 200 mg/kg/day DAPP (DAPP-200/DSS) or 400 mg/kg/day DAPP (DAPP-400/DSS) starting 10 days before colitis induction; and the therapeutic groups were orally treated by gavage with 200 mg/kg/day DAPP (DSS/DAPP-200) or 400 mg/kg/day DAPP (DSS/DAPP-400) starting with colitis induction for 10 days. The results shown are representative of three independent experiments with 5 to 6 mice per group. ***P<0.001 vs. CTL group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. DSS group.

Figure 2 **Effects of DAPP on microscopic damage and myeloperoxidase in mice with DSS-induced colitis.** Histological characterization was assessed by histological sections of distal colonic mucosa and tissue samples from distal colon were used to determine myeloperoxidase (MPO) concentration (H) from the six groups of mice (as described in Supplementary Figure 1) stained with hematoxylin phloxine saffron: (A) control group (CTL); (B) colitis group (DSS), (C) DSS/DAPP-200, (D) DAPP-200/DSS, (E), DSS/DAPP-400 and (F) DAPP-400/DSS. The pictures were taken using a digital camera at 10X (pictures B, C and F) or 20X (pictures A, D and E) resolution. Colon sections were scored for DSS-induced colonic inflammation and tissue injury (G) as described in Materials and Methods. The results shown are representative of three independent experiments with 5 to 6 mice per group. ***P<0.001 vs. CTL group; $^{\#\#}P$<0.01 vs. DSS group.

Figure 3 **Effects of DAPP on oxidative stress markers in mice with DSS-induced colitis.** Estimation of lipid peroxidation was assessed by measuring malondialdehyde (MDA) by HPLC (A). The activity of superoxide dismutase (SOD, B), glutathione peroxidase (GPx, C) was then measured. Gene and protein expression of the transcription factor Nrf2 (D and E) were determined by qPCR and Western blot respectively. The results from the six groups of mice (as described in Supplementary Figure 1) shown are representative of three

independent experiments with 5 to 6 per group. *P<0.05 vs. CTL group; $^{\#}P$<0.05 $^{\#\#}P$<0.01 $^{\#\#\#}P$<0.001 vs. DSS group.

Figure 4 **Regulatory effects of DAPP on cyclooxygenase 2 and prostaglandin E2 in mice with DSS-induced colitis.** Protein expression of COX-2 (A) was determined by Western blotting while PGE_2 (B) was determined by enzymatic immunoassay. The results from the six groups of mice (as described in Supplementary Figure 1) shown are representative of three independent experiments with 5 to 6 mice per group. *P<0.05, ***P<0.001 vs. CTL group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. DSS group.

Figure 5 **Effects of DAPP on proinflammatory cytokines in mice with DSS-induced colitis.** Gene and protein expression of the inflammatory markers TNF-a (A and D), IL-6 (B and E) and the transcription factors NF-kB (C and F) were determined by qPCR and Western blot respectively. The ratio NF-kB/IkB was then calculated. The results from the six groups of mice (as described in Supplementary Figure 1) shown are representative of three independent experiments with 5 to 6 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs. CTL group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. DSS group.

Figure 6 **Regulator effects of DAPP on peroxisome proliferator-coactivator-1 (PGC1-α) and -activated receptor (PPAR) γ in mice with DSS-induced colitis.** The mitochondrial transcription and translation were evaluated by gene and protein expression of PCG1-α (A and B), PPAR-γ (C and D), determined by qPCR and Western blot respectively. The results from the six groups of mice (as described in Supplementary Figure 1) shown are representative of three independent experiments with 5 to 6 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs. CTL group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. DSS group.

Figure 7 **Effects of DAPP on mitochondrial functions in mice with DSS-induced colitis.** Mitochondrial ADP/ATP ratio (A) was measured by luciferase driven bioluminescence while the protein expression of AIF (B), Cyt C (C) and OGG1 (D) in mitochondria was determined by Western blot. The results from four groups of mice (as described in Supplementary Figure 1) shown are representative of three independent experiments with 5 to 6 mice per group. **P<0.01, ***P<0.001 vs. CTL group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs. DSS group.

Figure 8 **Comparative analysis of functional enriched pathways between preventive and therapeutic DAPP treatments**. The negative logarithm of p-values (Fisher's test), calculated by IPA, for each of the most significant 25 functional categories overrepresented in DSS/DAPP-400 was plotted against those modulated in DAPP-200/DSS. ***Insert***: Venn diagram showing the 155 canonical pathways between DSS/DAPP-400 and DAPP-200/DSS. Thresholds (dotted lines) denote the p=0.05 [-Log (0.05) =1.3]. **B)** List of the most significant twenty-five functional categories for DSS/DAPP-400 and DAPP-200/DSS represented in A with their corresponding genes.

Figure 9 **Principal Coordinates Analysis (PCoA) plot and Heatmap of bacterial distribution of different communities.** Figure 9A depicts the PCoA plot illustrating the clustering of sampled gut mucosal communities at the genus level with respect to treatment (CTL, DSS, DSS+DAPP, DAPP+DSS).

Samples are plotted as colored points and bacterial families or genera of interest as black vectors. This analysis was based on Bray-Curtis similarities of the log-transformed OTU abundances. Randomization test (999 permutations) indicates that the first two PCO axes are significant. The percent of variation explained for each axis is given in brackets. OTU, operational taxonomic unit. * Family group with unclassified genus. Figure 9B shows the Heatmap of the relative abundance (%) of selected bacterial taxa that were found in the control (CTL) and treated animal metagenomic samples (DSS, DSS+DAPP, DAPP+DSS). Each row corresponds to a family or genus of interest and each column to a mucosal metagenomics sample. The color scale represents the sample affiliation to its respective treatment, while the gray scale represents the abundance level (%). * Family group with unclassified genus.

Figure 10 **Pairwise mean proportional differences (%) calculated at the genus level between the control (CTL) and the treated animal metagenomic samples (DSS, DSS+DAPP, DAPP+DSS).** The bar graphs on the left side display the mean proportion of sequences assigned to each selected family or genus. The dot plots on the right side show the bacterial genera that were overrepresented in one sample or another. Whiskers denote calculated 95% CIs. Only features of interest with a q-value of >0.05 are represented. * Family group with unclassified genus.

ARTICLE 2

APPLE PEEL POLYPHENOLS REDUCE MITOCHONDRIAL DYSFUNCTION IN MICE WITH DSS-INDUCED ULCERATIVE COLITIS

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Running title: Improvement of mitochondrial dysfunction in colitis by polyphenols

Keywords: Inflammatory bowel diseases, oxidative stress, inflammation, mitochondrial function, treatment.

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ABSTRACT

Scope: Inflammatory bowel diseases (IBD) are multifaceted and relapsing immune disorders, which necessitate long-term dependence on powerful drugs. As the use of natural product-based therapies has emerged as a promising intervention, the present study aimed to further characterize dried apple peel powder (DAPP) mechanisms of action, and evaluate the preventive and curative effects of DAPP on mitochondrial functions in a murine model. **Methods**: Induction of intestinal inflammation in mice is performed by oral administration of the DSS at 2.5% for 10 days. Doses of DAPP (200 or 400 mg/kg/day) were administered by gavage for 10 days pre- and during-DSS. **Results:** DAPP limited DSS-induced histological lesions, improved macroscopic parameters and attenuated clinical signs. Concomitant with the reduction of massive infiltration of inflammatory cells, DAPP displayed a robust potential of counteracting inflammation and oxidative stress in DSS mice. Moreover, DAPP partially restored mitochondrial abnormalities related to size, density, redox homeostasis, fatty acid β-oxidation, ATP synthesis, apoptosis and regulatory mitochondrial transcription factors. **Conclusions:** Our findings demonstrate the preventive and therapeutic impact of DAPP on experimental colitis while underlying the role of mitochondria. They also suggest that this natural DAPP product may represent an interesting candidate for further studies on the prevention/treatment of IBD.

INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of chronic polygenic and debilitating immune-mediated conditions with idiopathic Crohn's disease and ulcerative colitis (UC) as the two main forms. The etiology of these recurring and incurable disorders remains obscure although the latter are generally considered to result from complex interactions between genetic determinants, unfavorable environmental factors and dysregulated gut microflora [1, 2]. Consequently, the loss of tolerance characterizing the intestinal immune system towards the microbiota leads to continual immune activation, mucosal damage and uncontrolled long-lasting inflammation. The dramatically growing prevalence of IBD, absence of a cure, severe drug side effects, unresponsiveness to medical treatments and socio-economic burden pose special challenges for the scientific and medical community [3-5].

Reactive oxygen species (ROS) play an important role in IBD. They are released in large amounts from phagocytic cells isolated from the inflamed bowels of patients with IBD in response to various stimuli [6-8]. ROS disturb intestinal homeostasis by causing DNA modifications and damage as noted by the significantly increased concentration of 8-hydroxyguanine (8-OHdG) in the inflamed part of the bowel of Crohn's patients [9]. The cellular imbalance between the reduced antioxidant levels and elevated ROS concentrations results in enhanced oxidative stress (OxS) [10-12]. The implication of OxS in IBD pathogenesis has been evidenced particularly by the attenuation of mucosal inflammation in response to CuZnSOD in Crohn's patients [13] or by the beneficial effects of the antioxidant 5-aminosalicylic acid that inhibits lipid peroxidation [14]. Undoubtedly, the detailed

understanding of the mechanisms responsible for OxS is crucial for the development of effective prevention strategies and adequate intervention tools in IBD.

Mitochondria are the primary cellular sources and sensitive targets of ROS [15]. It is quite understandable to detect mitochondrial dysfunctions in patients with IBD. Inflated ROS may damage mitochondrial proteins, lipids and DNA, thereby resulting in abnormal respiration and mitochondrial dysfunction, culminating in IBD expansion [16-18]. Accordingly, high mitochondrial lipid peroxidation along with deficient bioenergetics have been detected in the colonic epithelial cells of patients with UC [19]. Ultrastructural examination of the colonic mucosa in UC has shown the presence of abnormal mitochondria while biochemical analyses have revealed decreased activity of mitochondrial Complex II and acetoacetyl CoA thiolase (the enzyme catalyzing the final step in butyrate oxidation) in both the inflamed and non-inflamed mucosa of patients with UC. Therefore it has been proposed that mitochondrial derangements are likely a primary defect to mucosal damage [19-21]. In line with these observations, genetic deletion of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α), the primary regulator of mitochondrial biogenesis, renders mice more susceptible to dextran sodium sulfate (DSS) colitis [22]. Altogether, most of these data indicate that dysregulation of OxS and mitochondrial dysfunction contributes to mucosal inflammation.

Polyphenols are natural compounds in plants, vegetables, fruits, olive oil, and wine. They captivate the attention and interest of scientists in view of their numerous biological properties including antioxidant, anti-inflammatory, antineoplastic, antiaging and antimicrobial activities [23]. Therefore, polyphenolic compounds have the robust potential to prevent or lessen the risks of diseases and the onset of disease progression by modulating several biological pathways [24]. Recently, we have shown that exposure of mice to DSS resulted in increased

disease activity index, mucosal ulcerations, immune cell infiltration, lipid peroxidation and colonic inflammatory markers [25]. Our findings evidenced a significant improvement of all these harmful features in response to polyphenols contained in dried apple peel extract (DAPP). The present study aims at extending our understanding on the pathways controlling OxS and inflammation in experimental colitis by focusing especially on the implication of the mitochondria and the response to DAPP.

MATERIALS AND METHODS

Experimental animal

Male C57BL6 mice (aged 6 weeks, weighing 17-20 gr) were purchased from Charles River (Montreal). They were housed in individual cages at 24 ± 1^{o}C and exposed to a 12-h light/dark cycle with free access to tap water and to a standard laboratory chow diet (2018 Teklad Global, containing 18.6% protein, 3.5% carbohydrates and 6.2 % fat for 3.1 kcal/g, Harlan Laboratories, Indianapolis, IN) for 20 days. Experiments were performed during the light phase of the cycle. Animal experiments were conducted according to the Canadian Council on Animal Care guidelines for the care and use of experimental animals and were approved by the Institutional Animal Care Committee of the Sainte-Justine UHC Research Center.

Induction of colitis and administration of DAPP

After a one-week acclimation to their environment, mice were randomly allocated to six groups: (1) control mice (CTL) without colitis treated with water (as a vehicle) by oral gavage; (2) mice receiving DSS only (DSS mice) and administered the vehicle throughout the experimental period; (3) DSS mice receiving 200 mg/kg/day DAPP as a preventive treatment (DAPP-200/DSS); (4) DSS mice receiving 400 mg/kg/day DAPP as a preventive treatment (DAPP-400/DSS); (5) DSS mice receiving 200 mg/kg/day DAPP as a therapeutic treatment (DSS/DAPP-200); and (6) DSS mice receiving 400 mg/kg/day DAPP as a therapeutic treatment (DSS/DAPP-400) as well described in our previous paper [25].

Colitis was induced by adding 3% (w/v) DSS (average molecular weight 36 000-50 000, MP Biomedicals) in the drinking water for 10 days. The preventive group received DAPP 10 days before colitis induction while the therapeutic group was given DAPP 10 days after colitis

induction. DAPP (AppleActiv™, Leahy Orchards Inc.) was prepared as previously reported [25]. Body weight and food/water intake were measured on a daily basis.

Macroscopic and histological analysis of the colon

In all mice, weight, presence of bloody stool, gross stool consistency and disease activity index were determined as previously described [25].

Western blot analysis

Homogenate, mitochondrial and nuclear protein extracts from colonic tissues were electroblotted onto Hybond nitrocellulose membranes following separation on a 7.5 % SDS-PAGE. The immunoblot was incubated with blocking solution (defatted milk proteins) at room temperature and incubated overnight with a primary antibody. Blots were washed and incubated with a species-specific horseradish peroxidase-conjugated secondary antibody. Molecular size markers were simultaneously loaded on gels along with the β-actin protein to confirm equal loading. Blots were developed and the protein mass was quantified by densitometry using an HP Scanjet scanner equipped with a transparency adapter and the UN-SCAN-IT gel 6.1 software as previously reported [26, 27].

OxS and antioxidant defense

Estimation of lipid peroxidation was assessed by malondialdehyde (MDA) using a fluorescence detector HPLC as described previously [28]. The quantification of hydrogen peroxide (H_2O_2) levels was determined using the fluorimetric hydrogen peroxide assay kit (Sigma). Endogenous antioxidants were evaluated by Western blots as previously reported [29].

Inflammation magnitude

Samples of colonic mucosa were homogenized, centrifuged and their content of tumor necrosis factor (TNF)-α, inducible oxide synthase (iNOS) and cyclooxygenase-2 (COX2) proteins in the supernatant was measured by Western blotting as previously described [25].

Isolation of mitochondrial organelles and functional studies

The mitochondrial fraction was isolated from colonic samples by differential centrifugation according to our previously reported method [25, 30]. Briefly, after homogenization in sucrose extraction buffer (0,25M, pH 7,38) and light centrifugation (1000 g x 10 min at 4^{o}C), the supernatant was ultracentrifuged twice (10000 g x 10 min at 4^{o}C) to pellet mitochondria. Total mitochondrial proteins were quantified using the Bradford assay. ATP content was determined using the ATP bioluminescent assay kit from BioAssay Systems (Hayward, CA, USA) [30, 31]. Apoptosis was evaluated by analyzing the protein expression of antiapoptotic Bcl2 and proapototic Bax, Cytochrome C (Cyt C) and apoptosis-inducing factor (AIF) by Western blotting. Mitochondrial antioxidant defense was assessed by measuring the protein expression of superoxide dismutase 2 (SOD2) and the major DNA glycosylase, 8-oxoguanine glycosylase (OGG1 that is responsible for removing the most abundant form of oxidative DNA damage) by Western blotting. The mitochondrial biogenesis master regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and its downstream transcription nuclear erythroid-2-p45-related factor-2 (NRF-2), as well as nuclear factor- kappa B (NF-kB) and activator protein-1 (AP-1) were also assessed by Western blot. Finally, β-oxidation, the major mitochondrial pathway for fatty acid (FA) degradation, was assessed by suspending colonic samples with U-C^{14} palmitic acid solution at 37^{o} and evaluating $^{14}CO_2$ and [^{14}C]-acid-soluble metabolites (ASM) at the end of a 2 h-incubation period in the presence of hydroxid benzothenium as per our previous method [32]. The data were confirmed by analyzing the

expression of the enzymes carnitine palmitoyltransferase 1 (CPT1, that controls mitochondrial β-oxidation) and long chain acyl-CoA dehydrogenase (LCAD, that catalyzes the initial step of long FA β-oxidation) by Western blotting.

Mitochondrial density and morphology

After removal of the colon, fresh tissue samples were immediately fixed in a 2% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4), post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in Epon. One-μm-thick sections were stained with toluidine blue to verify the orientation prior the ultrathin sectioning. Ultrathin sections were cut using an Ultracut S ultramicrotome (Leica) and mounted on nickel carbon-formvar coated grids. Uranyl acetate and lead citrate stained sections were imaged using a Philips CM100 electron microscope (FEI). Digital micrographs were captured using an AMT XR80 CCD digital camera at x34000 magnification. As described previously [33], individual mitochondria were manually traced using in ImageJ (NIH) to quantify the following morphological and shape descriptors: area (μm^2), perimeter (μm), circularity ($4\pi\cdot$(surface area/perimeter2)), Feret's diameter, longest distance (μm) between any two points within a given mitochondrion, aspect ratio (major axis)/(minor axis); a measure of the "length to width ratio" and form factor (perimeter)/($4\pi\cdot$surface area); a measure sensitive to the complexity and branching aspect of mitochondria.

Statistical analysis

All values are expressed as mean ± SEM. Unless otherwise specified in the legends of the figures, data were analyzed by one-way variance analysis and two-tailed Student *t* test using

Prism 5.01 (GraphPad Software) and differences between means were assessed post-hoc using Tukey's test. Statistical significance was defined as $P<0.05$.

RESULTS

DAPP Reduces DSS-Induced Damage

Mice with DSS-induced colitis are one of the most commonly used animal models of IBD, however validation was necessary before evaluating the impact of preventive and therapeutic DAPP. Therefore, as illustrated in **Fig. 1**, symptomatic and histopathological features, similar to those seen in human UC, were obtained up to 10 days following DSS administration. Monitoring body weight loss, fecal bleeding, stool consistency and colon shortening in DSS-induced colitis revealed a significant attenuation in response to DAPP treatment. In line with these macroscopic symptom findings, DAPP improved inflammation-associated histological changes triggered by DSS. This was noted by the reduction of lymphocyte infiltration, mucosal erosion, crypt damages and ulcer formation. Altogether, these observations highlight the preventive and therapeutic efficacy of DAPP in ameliorating colonic mucosal injury as evidenced by macroscopic and histopathological examinations (**Fig. 2**).

DAPP decreases the expression of pro-inflammatory factors

Because significant piling of inflammatory cells was seen in histological preparations of DSS-treated animals, we assessed the protein expression of pro-inflammatory biomarkers such as TNF-α, COX-2 (involved in arachidonic acid metabolism) and iNOS (catalyzing NO formation). Indeed, per os treatment of mice with DSS enhanced the protein expression of all these pro-inflammatory proteins relative to untreated mice, whereas DAPP administration significantly reduced these increased levels (**Fig. 3**). Therefore, DAPP are compounds with potential anti-inflammatory properties in adverse situations such as inflammation-associated colitis.

DAPP decreases the expression of oxidative markers

The implication of OxS in the complex IBD pathophysiology is widely accepted; therefore, its occurrence was assessed by determining MDA levels in the mouse groups. HPLC analysis showed a significant elevation of MDA in the distal colon of DSS-treated mice compared with that of the healthy control mice (**Fig. 4A**). Nevertheless, its magnitude was restrained by the preventive 200 mg/kg/day and the therapeutic 400 mg/kg/day doses of DAPP, likely through the beneficial alterations in the endogenous antioxidant GPx-1 (**Fig. 4B**). Then, we determined the protein expression of NRF-2 transcription factor since this is one of the critical regulators of intracellular redox homeostasis. In fact, disruption of NRF-2 protein expression by DSS was prevented or mitigated by DAPP. Thus, DAPP alleviated NRF-2 abrogation, which raised antioxidant defense mechanisms, thereby contributing to the reduction in DSS-induced (**Fig. 4C**).

DAPP interferes with the mitochondrial formation of OxS

As mitochondria are considered the main quantitative source of OxS, we measured the influence of DSS and DAPP on the mitochondrial H_2O_2 content in the different mouse groups. Intra-mitochondrial H_2O_2 metabolite and SOD2 protein expression were elevated in the DSS exposed group compared to the control mice (**Fig. 5**). Moreover, DSS mice having undergone preventive or therapeutic DAPP gavage exhibited a marked reduction in H_2O_2 and SOD2 protein expression. The focus was also placed on 8-oxoguanine DNA glycosylase 1 (OGG1), the most important enzyme involved in DNA repair via the removal of 8-oxoguanine elicited by OxS. While DSS altered OGG1, DAPP upregulated its expression. Therefore, DAPP administration slowed down mitochondrial OxS in DSS-induced colitis.

DAPP lessens Mitochondria-dependent cell death program

As the pivotal role of mitochondria in the execution of cell death is well established, apoptosis was assessed by estimating the protein expression of anti-apoptotic Bcl2 and the pro-apoptotic Bax, Cyt C and AIF transcription factor. While DSS treatment resulted in significantly decreased Bcl2 and increased Bax, as well as Cyt C and AIF transcription factor, DAPP displayed the capacity to limit apoptosis (**Fig. 6**).

DAPP modulates FA β-oxidation

One of the most important functions of the mitochondria is the β-oxidation of FAs to maintain body energy homeostasis. Accordingly, as illustrated in **Fig.** 7, the degradation of [^{14}C]-palmitate to [^{14}C]-CO_2 and acid-soluble metabolites (ASM) was significantly affected following DSS treatment. However, the preventive and therapeutic DAPP supplementation resulted in a less dramatic decline of [^{14}C]-palmitate conversion into CO_2 and acid-soluble metabolites, indicating enhanced total β-oxidation in DSS-induced colitis. We reasoned that the expression of key mitochondrial regulatory enzymes such as carnitine palmitoyltransferase-1 (CPT-1) and ACADL should present the same trend. Consistent with the findings of [^{14}C]-palmitate β-oxidation to CO_2 and ASM, a low protein expression of CPT-1 and ACADL was observed in the DSS-treated mice, whereas DAPP narrowed their down-regulation (**Fig. 8A-8B**). Overall, our data underline the ability of DAPP to positively modulate the β-oxidation.

DAPP enhances the mitochondrial energetic competence to produce ATP

The production of ATP molecules is instrumental in energy homeostasis. We therefore evaluated changes in mitochondrial ATP concentrations in the distal colon. Decreased levels were detected in mice with DSS-induced colitis compared to control mice, but DAPP supplementation attenuated this ATP decline (**Fig. 8C**).

DAPP is able to induce an increase in PGC1α and PPAR γ protein expression in DSS-induced colitis

To further understand the mechanisms behind the modulatory role of DAPP, the protein expression of PGC-1α was measured. This transcription factor regulates mitochondrial biogenesis as well as protein composition and function. The data in **Fig. 9** indicates the decline of PGC-1α in mice with DSS-induced colitis compared with control mice. However, improvement was noted with DAPP supplementation. Since PGC1a acts as a transcriptional co-activator of nuclear receptors and other transcription factors regulating mitochondrial biogenesis, we analyzed the expression of PPARγ that plays an important role in maintaining the intestinal immune balance and may exert anti-inflammatory effects in UC [34, 35]. The same phenomenon was observed in the other transcription factors NF-kB and AP-1 (**Fig. 10**).

DAPP attenuates DSS-induced alteration in mitochondrial morphology

It is now widely accepted that changes in mitochondrial morphology can greatly impact mitochondrial function and vice versa. Considering the protective effects of DAPP against DSS-induced mitochondrial dysfunction, we investigated whether DSS and DAPP treatments altered mitochondrial morphology on TEM images. As can be seen in **Fig. 11**, DSS-induced colitis decreased mitochondrial density. Interestingly, our data suggest that DAPP supplementation partially protected from this DSS-induced reduction in mitochondrial content since no differences between control and DAPP treated animals were observed. Although no effect of DSS on mitochondrial area was observed, mitochondria from DSS-treated mice displayed changes in multiple indicators of mitochondrial morphology, which is compatible with a reduction in mitochondrial complexity and an increase in mitochondrial fragmentation (i.e. increase in circularity and roundness, decrease in aspect ratio and form factor).

Interestingly, DAPP supplementation protected from DSS-induced alteration in mitochondrial morphology. The increase in mitochondrial area and perimeter seen in the preventive DAPP administration vs control animals also suggests that DAPP could stimulate mitochondrial fusion.

DISCUSSION

Mitochondria regulate vital functions necessary in maintaining overall cellular homeostasis. Despite the role of mitochondria in OxS, energetics, apoptosis, epithelial junction integrity and innate immune response, all known to be implicated in IBD, only limited information is available on the link between mitochondrial biological behavior and IBD pathogenesis. In addition, the impact of key bioactive polyphenols in the management of IBD and especially on mitochondrial functions has not been fully explored. This represents an important task since many current pharmacological drugs are not fully satisfactory, and the development of more effective therapeutic agents is greatly needed. To reinforce and deepen knowledge on the role of DAPP in intestinal inflammation, we chose the DSS-induced murine colitis that closely mimics the changes observed in the colonic epithelium of UC patients. Following validation of the DSS model, our experiments evidenced the beneficial and protective influence of DAPP while providing an insight and underlying some of the mechanisms responsible for the preventive and therapeutic actions of these functional foods. In particular, our findings stress the attenuation of DSS-induced mitochondrial dysfunction by DAPP.

As Bcl2 family proteins have emerged as critical regulators of mitochondria-mediated apoptosis by functioning as either a promoter (e.g. Bax) or an inhibitor (e.g. Bcl2) of the cell death process [36, 37], we evaluated the expression of Bax and Bcl2. The inflammatory condition caused by the DSS chemical agent was accompanied by an increase in the expression of pro-apototic Bax along with a decrease in anti-apoptotic Bcl2 within the mitochondria. This resulted in an increase in the ratio of Bax/Bcl2, which could elicit the apoptotic process. We confirmed the exaggerated apoptosis by examining the protein expression of Cyt C and AIF by western blotting. These proteins are normally located in the

space between the mitochondrial inner and outer membranes [38] and released into the cytoplasm to enhance the intrinsic apoptosis signaling pathways [39]. Concomitantly with the high Bax/Bcl2 ratio, the levels of Cyt C and AIF were raised significantly. Collectively, our observations clearly indicate a DSS-induced cell death program, mechanistically mediated by mitochondrial pathways. To further identify the bioactivity of DAPP, we analyzed its ability to prevent or inhibit this mitochondria-dependent apoptotic pathway. We found that treatment of DSS-induced colitis mice with DAPP led to a consistent rise of anti-apoptotic Bcl2 and decline in pro-apototic Bax, Bax/Bcl2 ratio, Cyt C and AIF. Overall, our data support that DAPP constitutes an effective functional nutrient that could prevent and treat mitochondrial dysfunction caused by DSS. However, additional efforts are certainly needed to clarify the underlying mechanisms. For now we may suggest that the presence of DAPP retains Bcl_2 in the cytosol, complexed with Bax, thereby avoiding apoptosis by impeding Bax translocation to the mitochondria. Without DAPP, Bax may form a channel for the exit of Cyt C and AIF with a concomitant opening of mitochondrial permeability transition pore (MPTP) and a collapse of mitochondrial membrane potential [40, 41]. Importantly, inhibition of MPTP opening has been identified as a promising therapeutic target in preventing cell damage [42].

Lipid metabolism largely depends on mitochondria to generate cellular energy. We argued that if DSS-induced colitis affects mitochondrial dysfunction, FA β-oxidation would be affected. To test this hypothesis, colonic tissue specimens were incubated with [U-^{14}C]-palmitate. There was a significant reduction in ^{14}C–palmitate–derived CO_2 and ASM release from the intestinal explants of DSS-induced colitis. Accordingly, butyrate metabolism was reported to be defective in the colonic mucosa of UC patients and an animal model of colitis [43-45]. However, the addition of DAPP (within the designated preventive and curative conditions) to

DSS-induced colitis mice led to an enhanced production of $^{14}CO_2$ and ASM. One potential explanation for the dysfunction of mitochondrial FA oxidation in response to DSS may lie in the faulty expression of CPT1a (a rate-limiting enzyme located on the mitochondrial outer membrane and controlling mitochondrial entry of long chain FA) and/or ACADL (a key enzyme participating in FA oxidation). As anticipated, the mass of the two enzymes was markedly decreased in mice with DSS-induced colitis, an effect highly counteracted by DAPP supplementation. Similarly, DAPP partially restored colonic ATP decline mediated by DSS administration. These results show that the polyphenol-rich extract enhances mitochondrial FA import and oxidation. This suggests that DAPP can be regarded as a robust functional food capable of ameliorating intestinal inflammation most likely through facilitating mitochondrial FA β-oxidation and promoting energy production.

PGC-1α acts as a central regulator of mitochondrial biogenesis and energy metabolism [46, 47]. It exhibits the potential to bind to various downstream transcriptional nuclear respiratory factors such as PPARγ, NRF-2 and mitochondrial DNA transcription factor A (mtTFA), to regulate mitochondrial proliferation and function [48, 49]. This master co-transcriptional regulation factor also controls ROS metabolism by activating NRF-2 and triggering antioxidant factors, thereby disclosing high effectiveness in inducing mitochondrial antioxidant expression [50]. To provide just a few examples, PGC-1α mediated the upregulation of mitochondrial antioxidant enzyme activity in cultured vascular endothelial cells [51], as well as in brain [50] and heart [52] tissues. A significant drop of PGC-1α was recorded in mice with DSS-induced colitis, which explains diverse mitochondrial dysfunctions related to mitochondrial density, respiration, ATP generation and pro-oxidant/antioxidant balance. By contrast, DAPP succeeded in reversing this trend. Noteworthy, the raised NRF-2

expression in these mice, in response to DAPP, might have contributed to the greater antioxidant defense, but studies are required to determine the distinct contribution of PGC1α and PGC1α-dependent Nrf2 expression. Additionally, PGC1α may act through its downstream target PPARγ, whose elevation in response to DAPP supplementation may produce the observed beneficial mitochondrial actions [53]. Finally, it is important to mention that PGC1α-mediated mtTFA, which was found stimulated by polyphenols in our previous studies [31], may have contributed to the improved functions associated with DAPP supplementation.

Mitochondrial DNA (mtDNA) damage resulting from increased OxS is emerging as an important factor in the pathogenesis of various disorders. A prevalent lesion that occurs in mtDNA damage is the formation of 8- hydroxy-2'-deoxyguanosine (8-OHdG), which can cause mutations and mitochondrial dysfunction, while triggering apoptotic cell death if not repaired properly by 8- oxoguanine DNA glycosylase (OGG1) [54]. Here, we report that the colonic tissues of DSS-induced colitis mice have decreased OGG1 protein levels. This was associated with the increased levels of 8-OHdG noted in our previous studies [30]. Exposure to DAPP resulted in a partial correction of OGG1 in agreement with the diminished 8-OHdG. We therefore conclude that DAPP-dependent antioxidant strengthening influenced mtDNA repair process; thereby preventing oxidant-mediated mitochondrial dysfunction and apoptotic cell death.

Once exclusively considered as bean-shaped structures, mitochondria are now recognized as dynamic organelles capable of changing their morphology through fusion and fission processes while harbouring complex morphology, e.g. elongated and branched structures. It is also now widely established that mitochondrial morphology and function are intertwined, with mitochondrial fragmentation being often associated with mitochondrial dysfunctions, whereas

mitochondrial fusion and the concomitant increase in complexity are frequently associated with improved mitochondrial function [55]. Here, we report that DSS treatment, in addition to reducing mitochondrial density, is associated with a mitochondrial fragmentation in combination with a reduction in mitochondrial complexity (increased in circularity, decreased in aspect ratio and form factor values). This decrease in mitochondrial complexity is in line with the DSS-induced mitochondrial dysfunction we report herein. Importantly, DAPP treatment completely abolished the effects of DSS on mitochondrial morphology. Therefore, our results suggest that impairment of mitochondrial dynamics (fusion and fission processes) in enterocytes might be involved in the etiology of DSS-induced colitis. They also suggest that DAPP might positively influence mitochondrial dynamics. Further studies are now required to assess the potential role of impaired mitochondrial dynamics in IBD.

In conclusion, our preclinical studies have emphasized the positive effects of DAPP on ameliorating the severity of DSS-induced colitis via the reduction of OxS and inflammation. In particular, we showed that DAPP may influence mitochondrial functions in colonic cells, including pro-oxidant/antioxidant balance, β-oxidation, cell death, and ATP production, which are all fundamental processes critical for the maintenance of cell homeostasis and integrity. As DAPP markedly decreased chemically induced colitis through these specific mechanisms implicated in IBD, they may be highly effective in protecting against the chronic inflammation that underlies IBD in humans.

AUTHORS CONTRIBUTION

EL conceived and designed the study. PYR, JL, CG, NP, JPLG, GG conducted the experiments. SS, ED and EL analyzed and interpreted the data. PYR, JL, SS, EL contributed to the writing of the paper. PYR, SS, YD, DR, DS, GG, JFB, AM, EL approved the final version of the manuscript.

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REFERENCES

[1] de Souza, H. S., Fiocchi, C., Immunopathogenesis of IBD: current state of the art. *Nat. Rev. Gastroenterol. Hepatol.* 2016, *13*, 13-27.

[2] Malik, T. A., Inflammatory Bowel Disease: Historical Perspective, Epidemiology, and Risk Factors. *Surg. Clin. North. Am.* 2015, *95*, 1105-1122, v.

[3] Kawalec, P., Mikrut, A., Lopuch, S., Systematic review of the effectiveness of biological therapy for active moderate to severe ulcerative colitis. *J. Gastroentero.l Hepatol.* 2014, *29*, 1159-1170.

[4] Kawalec, P., Indirect costs of inflammatory bowel diseases: Crohn's disease and ulcerative colitis. A systematic review. *Arch. Med. Sci.* 2016, *12*, 295-302.

[5] Huoponen, S., Blom, M., A Systematic Review of the Cost-Effectiveness of Biologics for the Treatment of Inflammatory Bowel Diseases. *PLoS One.* 2015, *10*, e0145087.

[6] Nielsen, O. H., Ahnfelt-Ronne, I., Involvement of oxygen-derived free radicals in the pathogenesis of chronic inflammatory bowel disease. *Klin. Wochenschr.* 1991, *69*, 995-1000.

[7] Grisham, M. B., Oxidants and free radicals in inflammatory bowel disease. *Lancet* 1994, *344*, 859-861.

[8] Kitahora, T., Suzuki, K., Asakura, H., Yoshida, T.*, et al.*, Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease. *Dig. Dis. Sci.* 1988, *33*, 951-955.

[9] Lih-Brody, L., Powell, S. R., Collier, K. P., Reddy, G. M.*, et al.*, Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig. Dis. Sci.* 1996, *41*, 2078-2086.

[10] Buffinton, G. D., Doe, W. F., Altered ascorbic acid status in the mucosa from inflammatory bowel disease patients. *Free Radic. Res.* 1995, *22*, 131-143.

[11] Grisham, M. B., MacDermott, R. P., Deitch, E. A., Oxidant defense mechanisms in the human colon. *Inflammation* 1990, *14*, 669-680.

[12] Kruidenier, L., Kuiper, I., Lamers, C. B., Verspaget, H. W., Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J. Pathol.* 2003, *201*, 28-36.

[13] Emerit, J., Pelletier, S., Likforman, J., Pasquier, C., Thuillier, A., Phase II trial of copper zinc superoxide dismutase (CuZn SOD) in the treatment of Crohn's disease. *Free Radic. Res. Commun.* 1991, *12-13 Pt 2*, 563-569.

[14] Ahnfelt-Ronne, I., Nielsen, O. H., Christensen, A., Langholz, E.*, et al.*, Clinical evidence supporting the radical scavenger mechanism of 5-aminosalicylic acid. *Gastroenterology* 1990, *98*, 1162-1169.

[15] Yue, C., Yang, Y., Zhang, C., Alfranca, G.*, et al.*, ROS-Responsive Mitochondria-Targeting Blended Nanoparticles: Chemo- and Photodynamic Synergistic Therapy for Lung Cancer with On-Demand Drug Release upon Irradiation with a Single Light Source. *Theranostics* 2016, *6*, 2352-2366.

[16] Kohli, R., Pan, X., Malladi, P., Wainwright, M. S., Whitington, P. F., Mitochondrial reactive oxygen species signal hepatocyte steatosis by regulating the

phosphatidylinositol 3-kinase cell survival pathway. *J. Biol. Chem.* 2007, *282*, 21327-21336.

[17] Serviddio, G., Bellanti, F., Vendemiale, G., Altomare, E., Mitochondrial dysfunction in nonalcoholic steatohepatitis. *Expert. Rev. Gastroenterol. Hepatol.* 2011, *5*, 233-244.

[18] Tessari, P., Coracina, A., Cosma, A., Tiengo, A., Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutr. Metab. Cardiovasc. Dis.* 2009, *19*, 291-302.

[19] Santhanam, S., Rajamanickam, S., Motamarry, A., Ramakrishna, B. S.*, et al.*, Mitochondrial electron transport chain complex dysfunction in the colonic mucosa in ulcerative colitis. *Inflamm. Bowel. Dis.* 2012, *18*, 2158-2168.

[20] Sifroni, K. G., Damiani, C. R., Stoffel, C., Cardoso, M. R.*, et al.*, Mitochondrial respiratory chain in the colonic mucosal of patients with ulcerative colitis. *Mol. Cell. Biochem.* 2010, *342*, 111-115.

[21] Santhanam, S., Venkatraman, A., Ramakrishna, B. S., Impairment of mitochondrial acetoacetyl CoA thiolase activity in the colonic mucosa of patients with ulcerative colitis. *Gut* 2007, *56*, 1543-1549.

[22] Cunningham, K. E., Vincent, G., Sodhi, C. P., Novak, E. A.*, et al.*, Peroxisome Proliferator-activated Receptor-gamma Coactivator 1-alpha (PGC1alpha) Protects against Experimental Murine Colitis. *J. Biol. Chem.* 2016, *291*, 10184-10200.

[23] Kaulmann, A., Bohn, T., Bioactivity of Polyphenols: Preventive and Adjuvant Strategies toward Reducing Inflammatory Bowel Diseases-Promises, Perspectives, and Pitfalls. *Oxid. Med. Cell. Longev.* 2016, *2016*, 9346470.

[24] Alissa, E. M., Ferns, G. A., Functional foods and nutraceuticals in the primary prevention of cardiovascular diseases. *J. Nutr. Metab.* 2012, *2012*, 569486.

[25] Denis, M. C., Roy, D., Yeganeh, P. R., Desjardins, Y., *et al.*, Apple peel polyphenols: a key player in the prevention and treatment of experimental inflammatory bowel disease. *Clin. Sci (Lond)*. 2016, *130*, 2217-2237.

[26] Kleme, M. L., Sane, A. T., Garofalo, C., Levy, E., Targeted CFTR gene disruption with zinc-finger nucleases in human intestinal epithelial cells induces oxidative stress and inflammation. *Int. J. Biochem. Cell. Biol.* 2016, *74*, 84-94.

[27] Ntimbane, T., Mailhot, G., Spahis, S., Rabasa-Lhoret, R., *et al.*, CFTR silencing in pancreatic beta-cells reveals a functional impact on glucose-stimulated insulin secretion and oxidative stress response. *Am. J. Physiol. Endocrinol. Metab.* 2016, *310*, E200-212.

[28] Denis, M. C., Furtos, A., Dudonne, S., Montoudis, A., *et al.*, Apple peel polyphenols and their beneficial actions on oxidative stress and inflammation. *PLoS One* 2013, *8*, e53725.

[29] Veilleux, A., Grenier, E., Marceau, P., Carpentier, A. C., *et al.*, Intestinal lipid handling: evidence and implication of insulin signaling abnormalities in human obese subjects. *Arterioscler. Thromb. Vasc. Biol.* 2014, *34*, 644-653.

[30] Taha, R., Seidman, E., Mailhot, G., Boudreau, F., *et al.*, Oxidative stress and mitochondrial functions in the intestinal Caco-2/15 cell line. *PLoS One* 2010, *5*, e11817.

[31] Denis, M. C., Desjardins, Y., Furtos, A., Marcil, V., *et al.*, Prevention of oxidative stress, inflammation and mitochondrial dysfunction in the intestine by different cranberry phenolic fractions. *Clin. Sci (Lond)*. 2015, *128*, 197-212.

[32] Montoudis, A., Seidman, E., Boudreau, F., Beaulieu, J. F., *et al.*, Intestinal fatty acid binding protein regulates mitochondrion beta-oxidation and cholesterol uptake. *J. Lipid. Res.* 2008, *49*, 961-972.

[33] Leduc-Gaudet, J. P., Picard, M., St-Jean Pelletier, F., Sgarioto, N.*, et al.*, Mitochondrial morphology is altered in atrophied skeletal muscle of aged mice. *Oncotarget* 2015, *6*, 17923-17937.

[34] Debril, M. B., Renaud, J. P., Fajas, L., Auwerx, J., The pleiotropic functions of peroxisome proliferator-activated receptor gamma. *J. Mol. Med (Berl)*. 2001, *79*, 30-47.

[35] Hontecillas, R., Bassaganya-Riera, J., Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis. *J. Immunol.* 2007, *178*, 2940-2949.

[36] Adams, J. M., Cory, S., The Bcl-2 protein family: arbiters of cell survival. *Science* 1998, *281*, 1322-1326.

[37] Chao, D. T., Korsmeyer, S. J., BCL-2 family: regulators of cell death. *Annu. Rev. Immunol.* 1998, *16*, 395-419.

[38] He, Q., Zhou, W., Xiong, C., Tan, G., Chen, M., Lycopene attenuates inflammation and apoptosis in post-myocardial infarction remodeling by inhibiting the nuclear factor-kappaB signaling pathway. *Mol. Med. Rep.* 2015, *11*, 374-378.

[39] Shao, D., Kan, M., Qiao, P., Pan, Y.*, et al.*, Celecoxib induces apoptosis via a mitochondriadependent pathway in the H22 mouse hepatoma cell line. *Mol. Med. Rep.* 2014, *10*, 2093-2098.

[40] Tsujimoto, Y., Shimizu, S., Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* 2007, *12*, 835-840.

[41] Bernardi, P., Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol. Rev.* 1999, *79*, 1127-1155.

[42] Javadov, S., Karmazyn, M., Escobales, N., Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. *J. Pharmacol. Exp. Ther.* 2009, *330*, 670-678.

[43] Roediger, W. E., The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 1980, *2*, 712-715.

[44] Chapman, M. A., Grahn, M. F., Boyle, M. A., Hutton, M.*, et al.*, Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. *Gut* 1994, *35*, 73-76.

[45] Ahmad, M. S., Krishnan, S., Ramakrishna, B. S., Mathan, M.*, et al.*, Butyrate and glucose metabolism by colonocytes in experimental colitis in mice. *Gut* 2000, *46*, 493-499.

[46] Arany, Z., He, H., Lin, J., Hoyer, K.*, et al.*, Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle. *Cell. Metab.* 2005, *1*, 259-271.

[47] Puigserver, P., Wu, Z., Park, C. W., Graves, R.*, et al.*, A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 1998, *92*, 829-839.

[48] Wu, Z., Puigserver, P., Andersson, U., Zhang, C.*, et al.*, Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999, *98*, 115-124.

[49] Jornayvaz, F. R., Shulman, G. I., Regulation of mitochondrial biogenesis. *Essays Biochem.* 2010, *47*, 69-84.

[50] St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J. M.*, et al.*, Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006, *127*, 397-408.

[51] Valle, I., Alvarez-Barrientos, A., Arza, E., Lamas, S., Monsalve, M., PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. *Cardiovasc. Res.* 2005, *66*, 562-573.

[52] Lu, Z., Xu, X., Hu, X., Fassett, J.*, et al.*, PGC-1 alpha regulates expression of myocardial mitochondrial antioxidants and myocardial oxidative stress after chronic systolic overload. *Antioxid. Redox. Signal.* 2010, *13*, 1011-1022.

[53] Liang, H., Ward, W. F., PGC-1alpha: a key regulator of energy metabolism. *Adv. Physiol. Educ.* 2006, *30*, 145-151.

[54] Cheng, Y., Ren, X., Gowda, A. S., Shan, Y.*, et al.*, Interaction of Sirt3 with OGG1 contributes to repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress. *Cell Death Dis.* 2013, *4*, e731.

[55] Picard, M., Taivassalo, T., Gouspillou, G., Hepple, R. T., Mitochondria: isolation, structure and function. *J. Physiol.* 2011, *589*, 4413-4421.

FIGURE LEGENDS

Figures 1&2 Effects of DAPP on DSS-induced colitis. The disease activity index, scores for the body weight change, stool consistency, fecal bleeding, and the microscopic damage determined the severity of the colitis. Colon sections were scored for DSS-induced colonic inflammation and tissue injury as described in Materials and Methods.

The body weight change was determined by calculating the percentage of weight change relative to the starting weight before DSS treatment (0 = none; 1 = 1-5%; 2 = 5-10%; 3 = 10-20% and 4 = >20%). The fecal condition score was determined using two parameters: stool consistency (0 = normal; 1 = moist/sticky stool; 2 = soft stool; 3 = soft stool with mild diarrhea; 4 = diarrhea only) and fecal bleeding (0 = no blood; 1 = minimal blood in stool; 2 = mild blood in stool; 3 = evidence blood in stool and 4 = only blood). The pictures were taken using a digital camera at 20X resolution. The results shown are representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 3 Effects of DAPP on proinflammatory cytokines in mice with DSS-induced colitis. Protein expression of the inflammatory markers was determined by Western blot as described in Materials and Methods. The results shown are representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 4 Effects of DAPP on oxidative stress markers in mice with DSS-induced colitis. Estimation of lipid peroxidation was assessed by measuring the

malondialdehyde (MDA) by HPLC (A). The protein expression of glutathione peroxidase (GPx) (B) and the transcription factor Nrf2 (C) were determined by Western blot as described in Materials and Methods. The results shown are representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 5 **Effects of DAPP on mitochondrial oxidative stress in mice with DSS-induced colitis.**

Intra-mitochondrial H_2O_2 metabolite (A) was determined by using the fluorimetric hydrogen peroxide assay kit. The protein expression of superoxide dismutase (SOD2) (B) and 8-oxoguanine DNA glycosylase 1 (OGG1) (C) were determined by Western blot as described in Materials and Methods. The results shown are representative of three independent experiments with n=8 mice/group. *P<0.05, **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 6 **Effects of DAPP on mitochondrial apoptosis in mice with DSS-induced colitis.**

Protein expression of anti-apoptotic Bcl2 (A) and the pro-apoptotic Bax (B), Cyt C (D) and AIF transcription factor (E) were verified by Western blot as described in Materials and Methods. Moreover, the Bax/Bcl2 ratio was calculated (C). The results shown are representative of three independent experiments with n=8 mice/group. *P<0.05, **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 7 **Effects of DAPP on mitochondrial fatty acid oxidation in mice with DSS-induced colitis.**

Mitochondrial pathway for fatty acid (FA) degradation was determined by suspending colonic tissues with U-^{14}C palmitic acid solution and by calculating $^{14}CO_2$ (A) and [^{14}C]-acid-soluble metabolites (ASM) (B) as described in Materials and Methods. The results shown are representative of three independent experiments with n=8 mice/group. *P<0.05, **P<0.01 vs. CTL group; $^{\#}$P<0.05 vs. DSS group.

Figure 8 **Impact of DAPP on the expression of two main mitochondrial enzymes in mice with DSS-induced colitis.**

The expression of the enzymes carnitine palmitoyl transferase 1 (CPT1) (A) and long chain acyl-CoA dehydrogenase (LCADL) (B) were analyzed by Western blot as described in Materials and Methods. Also, mitochondrial ATP generation (C) was measured using the ATP bioluminescent assay kit. The results shown are representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. DSS group.

Figure 9 **Regulatory effects of DAPP on peroxisome proliferator-coactivator-1 (PGC1-α) and -activated receptor (PPAR) γ in mice with DSS-induced colitis.**

The expression of transcription factor peroxisome proliferator-coactivator-1 (PGC1-α) (A) and -activated receptor (PPAR)γ (B) were illustrated by Western blot as described in Materials and Methods. The results shown are

representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; #P<0.05, ##P<0.01 vs. DSS group.

Figure 10 Effects of DAPP on nuclear factor- kappa B (NF-kB) and activator protein-1 (AP-1) in mice with DSS-induced colitis.

Protein expression of the transcription factor NF-kB (A) and pro-inflammatory factor AP-1 (B) were measured by Western blot as described in Materials and Methods. The results shown are representative of three independent experiments with n=8 mice/group. **P<0.01 vs. CTL group; #P<0.05, ##P<0.01 vs. DSS group.

Figure 11 Effects of DAPP on mitochondrial morphological structure and density.

Representative TEM images of enterocytes from control and DSS-treated mice (A), as well as from mice treated with DAPP before (DAPP-200/DSS) or after (DSS/DAPP-400) treatment. These images were used for the quantification of mitochondrial volume density and parameters related to mitochondrial mitochondrial morphology (B). For each group, mitochondrial density was quantified using multiple images collected from 2 different animals. Mitochondrial area (C), perimeter (D), circularity (E), roundness (F), form factor (G) and aspect ratio (H) were obtained by manually tracing mitochondria on TEM images obtained from 2 different mice per group (control: n=95; DSS: n=53; DAPP-200/DSS: n=72; DSS/DAPP-400: n=71). The graph in J represents, for each group, the percentage of mitochondria with simple (i.e. mitochondria with aspect ratio and factors inferior to the 25th percentile of control values), complex (i.e. mitochondria with aspect ratio and factor above

the 75th percentile of control values) and intermediate (i.e. neither simple nor complex). Due to the fact that data in (C-H) did not pass the D'Agostino & Pearson omnibus normality test, data in these graphs we analyzed by Kruskal-Wallis test followed by the Dunn's post-hoc test. *$P < 0.05$.

4 DISCUSSION

The interaction between inflammation and oxidative stress is associated with the evidence of their concomitant manifestation in IBD [50]. IBD is a chronic inflammatory disease that causes significant health problems worldwide. Currently there is no definite cure available for IBD that can threaten patients' life, affect their quality of life and impose great financial burden. In these conditions, many scientists and clinicians attempt to discover functional foods without secondary effects.

In fact, the use of functional nutrients such as polyphenolic compounds as alternative medicines have been recognized by different scientific groups, but their function and mechanisms of action in the management of IBD and especially on mitochondrial functions have not been clearly studied. As mitochondria are highly important for the maintenance of homeostasis in all tissue cells, we examined their biological functions and responses to DAPP administration in experimental colitis.

The present studies have highlighted abnormal mitochondrial OxS and dysfunction in intestinal epithelium of mice with DSS-induced colitis, which mimics tissues from patients with gut inflammation (IBD) [116] or epithelial monolayers treated with powerful prooxidants [112]. Our aims were also to extend and unravel the role of DAPP in intestinal inflammation by focusing on mitochondria.

Previous studies indicated that different polyphenols could inhibit OxS and inflammation pathways [170-172]. In this context, we chose DAPP, which is a rich source of polyphenols that revealed anti-inflammatory and antioxidant activities in our previous study [169]. In this context, it is noteworthy to note that other researchers proposed apple peels as a valuable source of phenolic compounds [173, 174]. Importantly, two different concentrations of

polyphenols (200 and 400 mg/kg/day) were used in our experiments in order to evaluate how mice with DSS-induced experimental colitis react to physiological (200 mg/kg/day) and supra-physiological (400 mg/kg/day) doses [175].

Special attention was given in our investigation to the assessment of the regulatory DAPP effects on intestinal integrity and on the mechanisms of mitochondrial dysfunction in the DSS-induced colitis model. We first found that DAPP supplementation slowed body weight loss, diminished diarrhoea and prevented bloody stools at a macroscopic level. In addition, this treatment improved colon shortening and colonic histological damages at a microscopic level by reducing inflammatory cell invasion into colonic tissues. DAPP also decreased neutrophil infiltration in the colonic tissue as noted by the reduced colonic myeloperoxidase activity, which represents a valuable marker of inflammatory cell infiltration in DSS-induced colitis mice.

Our results demonstrated that DSS animals were characterized by an overproduction of pro-inflammatory cytokines such as TNF-α and IL-6, which are implicated in the pathogenesis of colitis [176, 177]. Accordingly, previous reports showed that the levels of these pro-inflammatory cytokines are directly correlated to the severity of colitis [178, 179]. Interestingly, our studies clearly indicate that the influence of DAPP results in reduced inflammation by blocking the expression of pro-inflammatory cytokines (TNF-α and IL-6), which may prevent or stop the pathological progression of pro-inflammatory cascade such as NF-*κ*B signal transduction pathway. Our results are clearly consistent with Li's study by showing the capacity of DAPP treatment to moderate NF-*κ*B signals, while augmenting the expression of the inhibitory subunit IκB and then leading to the reduction of the NF-*κ*B /IκB ratio [180].

Interestingly, the disruption in NF-κB modulation can change prostaglandin metabolism with an effect on inflammation and cancer occurrence in IBD [52]. Prostaglandins participate in inflammatory activities and damage the integrity of the colon. Also, in inflammatory conditions, proinflammatory cytokines cause the increased of COX-2, one of main forms of the COX enzymes. COX-2 is capable to transform arachidonic acid into prostaglandins [53]. Our findings reveal that DAPP treatment is significantly effective to facilitate the down-regulation of COX-2, as a protein implicated in immune dysregulation in IBD [181], while limiting the production of PGE2, a main downstream agent of COX-2 and consequently ameliorating DSS-induced colitis. The down-regulation of the COX-2-PGE2 pathway by preventive and therapeutic DAPP supplementation can be of high interest for IBD. We also measured the expression of Bcl2 family proteins, as main regulators of mitochondria-mediated apoptosis, such as Bax, a promoter of the cell death process, and Bcl2, an inhibitor of the cell death process and their ratio of Bax/Bcl2 [182, 183]. In DSS-induced colitis, we observed that the inflammation raised pro-apototic Bax while it limited the production of anti-apoptotic Bcl-2 in the mitochondria, thereby causing the rise in the ratio of Bax/Bcl2 and amplifying the process of apoptosis. To recognize the severity of apoptosis, we also measured the protein expression of Cyt C and AIF. Cyt C and AIF proteins are normally located in the space between the mitochondrial inner and outer membranes [184] but, in inflamed conditions, they move into the cytoplasm to increase the principal apoptosis signaling pathways [185]. Our findings show the high levels of AIF and Cyt C protein expression in DSS-treated mice, concomitantly with the increased ratio of Bax/Bcl2. Remarkably, our findings confirm the effectiveness of DAPP in raising anti-apoptotic Bcl2 while provoking fall in pro-apoptotic Bax, Bax/Bcl2 ratio, Cyt C and AIF in DSS-treated mice. Further studies are certainly needed

to illuminate the underlying mechanisms. These results may suggest that DAPP can maintain Bcl2 in junction with Bax that are both normally located in the cytosol and thus it may prevent apoptosis pathway by inhibiting Bax to enter mitochondria. In the inflammation situation without DAPP, the binding of Bax to mitochondria may create a pathway for the release of Cyt C and AIF into the cytoplasm simutaneously with the opening of MPTP, which may decrease the mitochondrial membrane potential [186]. DAPP could be used as a therapeutic factor by prohibiting MPTP opening and thus controlling cell death [187].

Knowing that FA catabolism occurs in mitochondria to produce cellular energy, we wanted in the present study to determine whether β-oxidation would be affected as a result of the mitochondrial dysfunction observed in DSS-induced colitis. Therefore, we incubated colonic tissue with [U-^{14}C]-palmitate to assess palmitate conversion to CO2 and ASM. We observed that in DSS-induced colitis mice, CO2 and ASM generation significantly declined. On the other hand, DAPP supplementation resulted in increased production of CO2 and ASM and thus improving total β-oxidation. Decrease in FA catabolism was also demonstrated by other studies that showed significant reduction in butyrate oxidation in experimental colitis model and colonic mucosa of UC patients [122, 188]. The defective expression of two important enzymes such as carnitine palmitoyltransferase 1a, an enzyme residing on the mitochondrial outer membrane and regulating mitochondrial entry of long chain FA, and ACADL, a vital enzyme for FA β-oxidation, may be the reason for the dysfunction of mitochondrial FA β-oxidation. Our data showed a significant lowering in the expression of both enzymes in DSS-induced colitis mice, whereas the protective and therapeutic role of DAPP resulted in increased expression of both mitochondrial enzymes to restore normal levels.

Our studies showed decreased mitochondrial ATP concentrations in colonic tissues of DSS-induced colitis mice as well as a diminished level of the ratio of ADP/ATP, a key parameter in mitochondrial energy metabolism and respiration. DAPP supplementation partially attenuated the decrease in ATP and ADP/ATP ratio. Therefore, the present results in experimental colitis confirm previous studies in which polyphenols can improve the loss of mitochondrial membrane potential, correct the increase in lipid peroxidation and the fall in cellular ATP induced by indomethacin in Caco-2 cells [189]. Consequently, one possible explanation for improving intestinal inflammation is probably via the stimulation of mitochondrial FA β-oxidation and elevating ATP production pathways in DSS-induced colitis mice.

PGC-1α plays a key role as a main modulator of mitochondrial biogenesis and a transcriptional regulator of cellular energy activities, including OXPHOS and FA oxidation [86, 190]. This principal transcription factor improves ROS metabolism by provoking the expression of Nrf-2 [191]. The activation of Nrf2 can then cause the production of endogenous protective antioxidative SOD and GPx enzymes, which could initiate the up-regulation of cytoprotective proteins and also the transcription of antioxidant genes [192]. In addition, PGC-1α can bind to additional transcriptional nuclear factors, including PPARγ and mtTFA to control mitochondrial proliferation and function [191, 193]. Our results demonstrated a significant fall in PGC-1α level in mice with DSS-induced colitis with a concomitant reduction in the expression of Nrf2, which may have caused mitochondrial disorders relevant to pro-oxidant/antioxidant disequilibrium. However, the antioxidant effect of DAPP polyphenols was able to correct the mitochondrial status by upregulating the expression of PGC-1α and Nrf2 in DSS mice, which collectively improved the antioxidant defense by ameliorating the expression of SOD and GPx enzymes. Our findings also confirmed the down-

regulation of transcriptional nuclear factor PPARγ in experimental colitis, while DAPP preventive and therapeutic treatment restored its expression. It is likely that the action of DAPP on PGC-1α is related to the modifications of PPARγ.

Increased level of OxS can result in mtDNA damage, which is an important factor in the pathogenesis of different disorders. The high production of 8-OHdG is one of the widespread injuries that happen when mtDNA is damaged. Conversely, OGG1 can correct the production of 8-OHdG. The rise of 8-OHdG, due to the lack of repair by OGG1, provokes mutations, mitochondrial dysfunction and finally initiates apoptotic cell death [132]. Our findings suggest that the expression of OGG1 protein in experimental colitis mice was decreased in correlation with augmented production of 8-OHdG. In contrast, DAPP showed the ability to counteract the DSS-induced fall of OGG1. Consequently, the powerful antioxidant properties of DAPP can affect the mtDNA repair pathways and may prevent mitochondrial dysfunction.

Mitochondria are known to be vital organelles that can change their morphology via fusion and fission processes. Importantly, under normal conditions, mitochondrial function depends on fusion process of mitochondria. Since mitochondrial morphology and function are interconnected, any changes in mitochondrial morphology can seriously affect mitochondrial function. It is known that mitochondrial fragmentation is usually correlated with mitochondrial dysfunctions. In our second study we observed diminished mitochondrial density related to mitochondrial fragmentation and decreased mitochondrial complexity (increased circularity, decreased aspect ratio and form factor values) in DSS induced mice. However, DAPP treatment was able to completely correct the effects of DSS on mitochondrial morphology. Therefore, our results suggest that DAPP treatment has a potential to impact mitochondrial morphology and consequently protect against mitochondrial dysfunction in IBD.

5 CONCLUSIONS

These preclinical studies demonstrate the importance of mitochondria as a major source and target of oxidative stress. We showed that the intake of DAPP in an experimental colitis mouse model is beneficial in the preventive and therapeutic management of DSS-induced colitis. These beneficial effects of DAPP were achieved through the improvement of mitochondrial dysfunctions by decreasing OxS and inflammation.

Our findings indicate that administration of DAPP could affect microbiota and mitochondrial functions (i.e. proxidant/antioxidant balance, ATP production, β-oxidation and cell death) in inflamed bowel cells, which represent vital functions and processes in the protection of cell homeostasis and integrity; finally leading to optimal health.

In addition, these modifications lead to other improvements related to bleeding, stool consistency, histological aberrations, weight loss and colon shortening. This further reveals the powerful preventive and therapeutic effects of apple polyphenols in managing, treating and improving IBD. In conclusion, our findings show amelioration of chronic inflammation in mouse IBD, in particular, improvement of mitochondrial dysfunctions by specific antioxidant mechanisms triggered by DAPP. Future clinical research is needed to determine if DAPP would have the same effect in protecting against chronic inflammation in human IBD.

6 REFERENCES

1. Rubin DC, Shaker A, Levin MS. Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. *Front Immunol* 3 107 (2012).
2. Rahimi R, Nikfar S, Abdollahi M. Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: a meta-analysis. *World J Gastroenterol* 19(34), 5738-5749 (2013).
3. Kappelman MD, Rifas-Shiman SL, Kleinman K *et al*. The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States. *Clin Gastroenterol Hepatol* 5(12), 1424-1429 (2007).
4. Loftus EV, Jr. The burden of inflammatory bowel disease in the United States: a moving target? *Clin Gastroenterol Hepatol* 5(12), 1383-1384 (2007).
5. Halpin SJ, Ford AC. Prevalence of symptoms meeting criteria for irritable bowel syndrome in inflammatory bowel disease: systematic review and meta-analysis. *Am J Gastroenterol* 107(10), 1474-1482 (2012).
6. Button LA, Roberts SE, Goldacre MJ, Akbari A, Rodgers SE, Williams JG. Hospitalized prevalence and 5-year mortality for IBD: record linkage study. *World J Gastroenterol* 16(4), 431-438 (2010).
7. Hanauer SB. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 12 Suppl 1 S3-9 (2006).
8. May D, Pan S, Crispin DA *et al*. Investigating neoplastic progression of ulcerative colitis with label-free comparative proteomics. *J Proteome Res* 10(1), 200-209 (2011).
9. Rigoli L, Caruso RA. Inflammatory bowel disease in pediatric and adolescent patients: a biomolecular and histopathological review. *World J Gastroenterol* 20(30), 10262-10278 (2014).
10. Spadea L, Balestrazzi E. Treatment of vascular retinopathies with Pycnogenol. *Phytother Res* 15(3), 219-223 (2001).
11. Graziani G, D'argenio G, Tuccillo C *et al*. Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. *Gut* 54(2), 193-200 (2005).
12. Islam MS, Murata T, Fujisawa M *et al*. Anti-inflammatory effects of phytosteryl ferulates in colitis induced by dextran sulphate sodium in mice. *Br J Pharmacol* 154(4), 812-824 (2008).
13. Nell S, Suerbaum S, Josenhans C. The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol* 8(8), 564-577 (2010).
14. Malik TA. Inflammatory Bowel Disease: Historical Perspective, Epidemiology, and Risk Factors. *Surg Clin North Am* 95(6), 1105-1122, v (2015).
15. De Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 13(1), 13-27 (2016).
16. Duerr RH. The genetics of inflammatory bowel disease. *Gastroenterol Clin North Am* 31(1), 63-76 (2002).
17. Tysk C, Lindberg E, Jarnerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 29(7), 990-996 (1988).

18. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 48(4), 526-535 (2001).
19. Feagins LA, Souza RF, Spechler SJ. Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. *Nat Rev Gastroenterol Hepatol* 6(5), 297-305 (2009).
20. Canavan C, Abrams KR, Mayberry J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment Pharmacol Ther* 23(8), 1097-1104 (2006).
21. Chassaing B, Darfeuille-Michaud A. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 140(6), 1720-1728 (2011).
22. Murdoch TB, Xu W, Stempak JM *et al*. Pattern recognition receptor and autophagy gene variants are associated with development of antimicrobial antibodies in Crohn's disease. *Inflamm Bowel Dis* 18(9), 1743-1748 (2012).
23. Andoh A, Imaeda H, Aomatsu T *et al*. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol* 46(4), 479-486 (2011).
24. Halstensen TS, Das KM, Brandtzaeg P. Epithelial deposits of immunoglobulin G1 and activated complement colocalise with the M(r) 40 kD putative autoantigen in ulcerative colitis. *Gut* 34(5), 650-657 (1993).
25. Gebbers JO, Otto HF. Alterations of the intestinal mucosal block in ulcerative colitis and Crohn's disease--immunological and ultrastructural findings, and considerations of the pathogenesis. *Klin Padiatr* 197(4), 341-348 (1985).
26. Bjarnason I, O'morain C, Levi AJ, Peters TJ. Absorption of 51chromium-labeled ethylenediaminetetraacetate in inflammatory bowel disease. *Gastroenterology* 85(2), 318-322 (1983).
27. Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 341(8858), 1437-1439 (1993).
28. Pineton De Chambrun G, Colombel JF, Poulain D, Darfeuille-Michaud A. Pathogenic agents in inflammatory bowel diseases. *Curr Opin Gastroenterol* 24(4), 440-447 (2008).
29. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 9(11), 799-809 (2009).
30. Kruidenier L, Verspaget HW. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther* 16(12), 1997-2015 (2002).
31. Bernotti S, Seidman E, Sinnett D *et al*. Inflammatory reaction without endogenous antioxidant response in Caco-2 cells exposed to iron/ascorbate-mediated lipid peroxidation. *Am J Physiol Gastrointest Liver Physiol* 285(5), G898-906 (2003).
32. Young IS, Woodside JV. Antioxidants in health and disease. *J Clin Pathol* 54(3), 176-186 (2001).
33. Wendland BE, Aghdassi E, Tam C *et al*. Lipid peroxidation and plasma antioxidant micronutrients in Crohn disease. *Am J Clin Nutr* 74(2), 259-264 (2001).
34. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39(1), 44-84 (2007).

35. Rezaie A, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci* 52(9), 2015-2021 (2007).
36. Erichsen K, Ulvik RJ, Nysaeter G *et al*. Oral ferrous fumarate or intravenous iron sucrose for patients with inflammatory bowel disease. *Scand J Gastroenterol* 40(9), 1058-1065 (2005).
37. Di Meo S, Reed TT, Venditti P, Victor VM. Harmful and Beneficial Role of ROS. *Oxid Med Cell Longev* 2016 7909186 (2016).
38. Kawagishi H, Finkel T. Unraveling the truth about antioxidants: ROS and disease: finding the right balance. *Nat Med* 20(7), 711-713 (2014).
39. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat Res* 145(5), 532-541 (1996).
40. Simmonds NJ, Rampton DS. Inflammatory bowel disease--a radical view. *Gut* 34(7), 865-868 (1993).
41. Amini-Shirazi N, Hoseini A, Ranjbar A *et al*. Inhibition of tumor necrosis factor and nitrosative/oxidative stresses by Ziziphora clinopoides (Kahlioti); a molecular mechanism of protection against dextran sodium sulfate-induced colitis in mice. *Toxicol Mech Methods* 19(2), 183-189 (2009).
42. Kruidenier L, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 201(1), 28-36 (2003).
43. Maor I, Rainis T, Lanir A, Lavy A. Oxidative stress, inflammation and neutrophil superoxide release in patients with Crohn's disease: distinction between active and non-active disease. *Dig Dis Sci* 53(8), 2208-2214 (2008).
44. Sandborn WJ, Hanauer SB, Rutgeerts P *et al*. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 56(9), 1232-1239 (2007).
45. Banan A, Zhang LJ, Shaikh M, Fields JZ, Farhadi A, Keshavarzian A. Novel effect of NF-kappaB activation: carbonylation and nitration injury to cytoskeleton and disruption of monolayer barrier in intestinal epithelium. *Am J Physiol Cell Physiol* 287(4), C1139-1151 (2004).
46. Haddad JJ. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cell Signal* 14(11), 879-897 (2002).
47. Amasheh M, Grotjohann I, Amasheh S *et al*. Regulation of mucosal structure and barrier function in rat colon exposed to tumor necrosis factor alpha and interferon gamma in vitro: a novel model for studying the pathomechanisms of inflammatory bowel disease cytokines. *Scand J Gastroenterol* 44(10), 1226-1235 (2009).
48. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 22(6), 1115-1126 (1997).
49. Carrier JC, Aghdassi E, Jeejeebhoy K, Allard JP. Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. *Int J Colorectal Dis* 21(4), 381-387 (2006).
50. Zhu H, Li YR. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med (Maywood)* 237(5), 474-480 (2012).
51. Cogswell PC, Kashatus DF, Keifer JA *et al*. NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. *J Biol Chem* 278(5), 2963-2968 (2003).

52. Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. *Cancer Lett* 267(2), 204-215 (2008).
53. Park YS. [COX-2 inhibitors in inflammatory bowel disease: friends or foes?]. *Korean J Gastroenterol* 50(6), 350-355 (2007).
54. Seibert K, Masferrer JL. Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor* 4(1), 17-23 (1994).
55. Nam KW, Je KH, Lee JH *et al*. Inhibition of COX-2 activity and proinflammatory cytokines (TNF-alpha and IL-1beta) production by water-soluble sub-fractionated parts from bee (Apis mellifera) venom. *Arch Pharm Res* 26(5), 383-388 (2003).
56. Allgayer H. Clinical relevance of oxygen radicals in inflammatory bowel disease--facts and fashion. *Klin Wochenschr* 69(21-23), 1001-1003 (1991).
57. Levy E, Rizwan Y, Thibault L *et al*. Altered lipid profile, lipoprotein composition, and oxidant and antioxidant status in pediatric Crohn disease. *Am J Clin Nutr* 71(3), 807-815 (2000).
58. Soto J, Sacristan JA, Alsar MJ. [The use of topical chloramphenicol]. *Rev Clin Esp* 191(6), 336-337 (1992).
59. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 16 33-50 (1996).
60. Dincer Y, Erzin Y, Himmetoglu S, Gunes KN, Bal K, Akcay T. Oxidative DNA damage and antioxidant activity in patients with inflammatory bowel disease. *Dig Dis Sci* 52(7), 1636-1641 (2007).
61. D'odorico A, Bortolan S, Cardin R *et al*. Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. *Scand J Gastroenterol* 36(12), 1289-1294 (2001).
62. Kunz WS. Different metabolic properties of mitochondrial oxidative phosphorylation in different cell types--important implications for mitochondrial cytopathies. *Exp Physiol* 88(1), 149-154 (2003).
63. Wang CH, Wang CC, Wei YH. Mitochondrial dysfunction in insulin insensitivity: implication of mitochondrial role in type 2 diabetes. *Ann N Y Acad Sci* 1201 157-165 (2010).
64. Mitchell P, Moyle J. Chemiosmotic hypothesis of oxidative phosphorylation. *Nature* 213(5072), 137-139 (1967).
65. Moran M, Moreno-Lastres D, Marin-Buera L, Arenas J, Martin MA, Ugalde C. Mitochondrial respiratory chain dysfunction: implications in neurodegeneration. *Free Radic Biol Med* 53(3), 595-609 (2012).
66. Perkins GA, Frey TG. Recent structural insight into mitochondria gained by microscopy. *Micron* 31(1), 97-111 (2000).
67. Strauss M, Hofhaus G, Schroder RR, Kuhlbrandt W. Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J* 27(7), 1154-1160 (2008).
68. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. *Cell* 148(6), 1145-1159 (2012).
69. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell* 120(4), 483-495 (2005).
70. Sjostrand FS. Electron microscopy of mitochondria and cytoplasmic double membranes. *Nature* 171(4340), 30-32 (1953).

71. Kondo S, Toyokuni S, Tanaka T *et al*. Overexpression of the hOGG1 gene and high 8-hydroxy-2'-deoxyguanosine (8-OHdG) lyase activity in human colorectal carcinoma: regulation mechanism of the 8-OHdG level in DNA. *Clin Cancer Res* 6(4), 1394-1400 (2000).
72. Kolesnikov AA, Gerasimov ES. Diversity of mitochondrial genome organization. *Biochemistry (Mosc)* 77(13), 1424-1435 (2012).
73. Clark GM, Tong YC, Dowell RC. Clinical results with a multichannel pseudobipolar system. *Ann N Y Acad Sci* 405 370-376 (1983).
74. Kleme ML, Levy E. Cystic fibrosis-related oxidative stress and intestinal lipid disorders. *Antioxid Redox Signal* 22(7), 614-631 (2015).
75. Drew B, Leeuwenburgh C. Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria of Fischer-344 rats with age and caloric restriction. *Am J Physiol Regul Integr Comp Physiol* 285(5), R1259-1267 (2003).
76. Kwong JQ, Beal MF, Manfredi G. The role of mitochondria in inherited neurodegenerative diseases. *J Neurochem* 97(6), 1659-1675 (2006).
77. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R. Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. *Oncogene* 27(50), 6407-6418 (2008).
78. Novak EA, Mollen KP. Mitochondrial dysfunction in inflammatory bowel disease. *Front Cell Dev Biol* 3 62 (2015).
79. Larrey D. [Mitochondrial liver diseases]. *Gastroenterol Clin Biol* 25(4 Suppl), B117-122 (2001).
80. Bartlett K, Eaton S. Mitochondrial beta-oxidation. *Eur J Biochem* 271(3), 462-469 (2004).
81. De Lonlay P, Djouadi F, Bonnefont JP, Saudubray JM, Bastin J. [Mitochondrial beta-oxidation of fatty acids: an essential metabolic pathway of muscular function]. *Arch Pediatr* 9 Suppl 2 175s-178s (2002).
82. Ramsay RR, Steenkamp DJ, Husain M. Reactions of electron-transfer flavoprotein and electron-transfer flavoprotein: ubiquinone oxidoreductase. *Biochem J* 241(3), 883-892 (1987).
83. Lasserre JP, Dautant A, Aiyar RS *et al*. Yeast as a system for modeling mitochondrial disease mechanisms and discovering therapies. *Dis Model Mech* 8(6), 509-526 (2015).
84. Webb EL, Rudd MF, Sellick GS *et al*. Search for low penetrance alleles for colorectal cancer through a scan of 1467 non-synonymous SNPs in 2575 cases and 2707 controls with validation by kin-cohort analysis of 14 704 first-degree relatives. *Hum Mol Genet* 15(21), 3263-3271 (2006).
85. Shoubridge EA. Cytochrome c oxidase deficiency. *Am J Med Genet* 106(1), 46-52 (2001).
86. Scarpulla RC. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. *Gene* 286(1), 81-89 (2002).
87. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 552(Pt 2), 335-344 (2003).
88. Benard G, Faustin B, Passerieux E *et al*. Physiological diversity of mitochondrial oxidative phosphorylation. *Am J Physiol Cell Physiol* 291(6), C1172-1182 (2006).

89. Bayir H, Kagan VE. Bench-to-bedside review: Mitochondrial injury, oxidative stress and apoptosis--there is nothing more practical than a good theory. *Crit Care* 12(1), 206 (2008).
90. Reed JC. Mechanisms of apoptosis. *Am J Pathol* 157(5), 1415-1430 (2000).
91. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 281(5381), 1309-1312 (1998).
92. Koehler CM, Beverly KN, Leverich EP. Redox pathways of the mitochondrion. *Antioxid Redox Signal* 8(5-6), 813-822 (2006).
93. Eleftheriadis T, Pissas G, Liakopoulos V, Stefanidis I. Cytochrome c as a Potentially Clinical Useful Marker of Mitochondrial and Cellular Damage. *Front Immunol* 7 279 (2016).
94. Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 84(2-3), 131-141 (2002).
95. Saenz GJ, Hovanessian R, Gisis AD, Medh RD. Glucocorticoid-mediated co-regulation of RCAN1-1, E4BP4 and BIM in human leukemia cells susceptible to apoptosis. *Biochem Biophys Res Commun* 463(4), 1291-1296 (2015).
96. Schendel SL, Reed JC. Measuring pore formation by Bcl-2 family proteins. *Methods Enzymol* 322 274-282 (2000).
97. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* 274(4), 2225-2233 (1999).
98. Kwak HB. Effects of aging and exercise training on apoptosis in the heart. *J Exerc Rehabil* 9(2), 212-219 (2013).
99. Bratic I, Trifunovic A. Mitochondrial energy metabolism and ageing. *Biochim Biophys Acta* 1797(6-7), 961-967 (2010).
100. Liemburg-Apers DC, Willems PH, Koopman WJ, Grefte S. Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. *Arch Toxicol* 89(8), 1209-1226 (2015).
101. Dai DF, Chiao YA, Marcinek DJ, Szeto HH, Rabinovitch PS. Mitochondrial oxidative stress in aging and healthspan. *Longev Healthspan* 3 6 (2014).
102. Dan Dunn J, Alvarez LA, Zhang X, Soldati T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. *Redox Biol* 6 472-485 (2015).
103. Insani EM, Eyherabide A, Grigioni G, Sancho AM, Pensel NA, Descalzo AM. Oxidative stability and its relationship with natural antioxidants during refrigerated retail display of beef produced in Argentina. *Meat Sci* 79(3), 444-452 (2008).
104. Murchison D, Griffith WH. Mitochondria buffer non-toxic calcium loads and release calcium through the mitochondrial permeability transition pore and sodium/calcium exchanger in rat basal forebrain neurons. *Brain Res* 854(1-2), 139-151 (2000).
105. Castro J, Bittner CX, Humeres A, Montecinos VP, Vera JC, Barros LF. A cytosolic source of calcium unveiled by hydrogen peroxide with relevance for epithelial cell death. *Cell Death Differ* 11(4), 468-478 (2004).
106. Patergnani S, Suski JM, Agnoletto C *et al*. Calcium signaling around Mitochondria Associated Membranes (MAMs). *Cell Commun Signal* 9 19 (2011).
107. Denton RM. Regulation of mitochondrial dehydrogenases by calcium ions. *Biochim Biophys Acta* 1787(11), 1309-1316 (2009).

108. Szabadkai G, Rizzuto R. Participation of endoplasmic reticulum and mitochondrial calcium handling in apoptosis: more than just neighborhood? *FEBS Lett* 567(1), 111-115 (2004).
109. Bianchi K, Rimessi A, Prandini A, Szabadkai G, Rizzuto R. Calcium and mitochondria: mechanisms and functions of a troubled relationship. *Biochim Biophys Acta* 1742(1-3), 119-131 (2004).
110. Arco AD, Satrustegui J. New mitochondrial carriers: an overview. *Cell Mol Life Sci* 62(19-20), 2204-2227 (2005).
111. Vafai SB, Mootha VK. Mitochondrial disorders as windows into an ancient organelle. *Nature* 491(7424), 374-383 (2012).
112. Taha R, Seidman E, Mailhot G *et al*. Oxidative stress and mitochondrial functions in the intestinal Caco-2/15 cell line. *PLoS One* 5(7), e11817 (2010).
113. Rodenburg W, Keijer J, Kramer E, Vink C, Van Der Meer R, Bovee-Oudenhoven IM. Impaired barrier function by dietary fructo-oligosaccharides (FOS) in rats is accompanied by increased colonic mitochondrial gene expression. *BMC Genomics* 9 144 (2008).
114. Soderholm JD, Yang PC, Ceponis P *et al*. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology* 123(4), 1099-1108 (2002).
115. Delpre G, Avidor I, Steinherz R, Kadish U, Ben-Bassat M. Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis. *Am J Gastroenterol* 84(9), 1038-1046 (1989).
116. Nazli A, Yang PC, Jury J *et al*. Epithelia under metabolic stress perceive commensal bacteria as a threat. *Am J Pathol* 164(3), 947-957 (2004).
117. Ishii N. Role of oxidative stress from mitochondria on aging and cancer. *Cornea* 26(9 Suppl 1), S3-9 (2007).
118. Roediger WE. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet* 2(8197), 712-715 (1980).
119. Schurmann G, Bruwer M, Klotz A, Schmid KW, Senninger N, Zimmer KP. Transepithelial transport processes at the intestinal mucosa in inflammatory bowel disease. *Int J Colorectal Dis* 14(1), 41-46 (1999).
120. Kameyama J, Narui H, Inui M, Sato T. Energy level in large intestinal mucosa in patients with ulcerative colitis. *Tohoku J Exp Med* 143(2), 253-254 (1984).
121. Bar F, Bochmann W, Widok A *et al*. Mitochondrial gene polymorphisms that protect mice from colitis. *Gastroenterology* 145(5), 1055-1063 e1053 (2013).
122. Ahmad MS, Krishnan S, Ramakrishna BS, Mathan M, Pulimood AB, Murthy SN. Butyrate and glucose metabolism by colonocytes in experimental colitis in mice. *Gut* 46(4), 493-499 (2000).
123. Duffy MM, Regan MC, Ravichandran P *et al*. Mucosal metabolism in ulcerative colitis and Crohn's disease. *Dis Colon Rectum* 41(11), 1399-1405 (1998).
124. Halestrap AP, Dunlop JL. Intramitochondrial regulation of fatty acid beta-oxidation occurs between flavoprotein and ubiquinone. A role for changes in the matrix volume. *Biochem J* 239(3), 559-565 (1986).
125. De Preter V, Geboes KP, Bulteel V *et al*. Kinetics of butyrate metabolism in the normal colon and in ulcerative colitis: the effects of substrate concentration and

carnitine on the beta-oxidation pathway. *Aliment Pharmacol Ther* 34(5), 526-532 (2011).
126. De Preter V, Arijs I, Windey K *et al*. Impaired butyrate oxidation in ulcerative colitis is due to decreased butyrate uptake and a defect in the oxidation pathway. *Inflamm Bowel Dis* 18(6), 1127-1136 (2012).
127. Asin-Cayuela J, Gustafsson CM. Mitochondrial transcription and its regulation in mammalian cells. *Trends Biochem Sci* 32(3), 111-117 (2007).
128. Skyberg JA, Robison A, Golden S *et al*. Apple polyphenols require T cells to ameliorate dextran sulfate sodium-induced colitis and dampen proinflammatory cytokine expression. *J Leukoc Biol* 90(6), 1043-1054 (2011).
129. Santhanam S, Rajamanickam S, Motamarry A *et al*. Mitochondrial electron transport chain complex dysfunction in the colonic mucosa in ulcerative colitis. *Inflamm Bowel Dis* 18(11), 2158-2168 (2012).
130. Poulsen HE, Prieme H, Loft S. Role of oxidative DNA damage in cancer initiation and promotion. *Eur J Cancer Prev* 7(1), 9-16 (1998).
131. Fukui H, Moraes CT. The mitochondrial impairment, oxidative stress and neurodegeneration connection: reality or just an attractive hypothesis? *Trends Neurosci* 31(5), 251-256 (2008).
132. Cheng Y, Ren X, Gowda AS *et al*. Interaction of Sirt3 with OGG1 contributes to repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress. *Cell Death Dis* 4 e731 (2013).
133. D'inca R, Cardin R, Benazzato L, Angriman I, Martines D, Sturniolo GC. Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia. *Inflamm Bowel Dis* 10(1), 23-27 (2004).
134. Lih-Brody L, Powell SR, Collier KP *et al*. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 41(10), 2078-2086 (1996).
135. Howell N, Elson JL, Chinnery PF, Turnbull DM. mtDNA mutations and common neurodegenerative disorders. *Trends Genet* 21(11), 583-586 (2005).
136. Balcavage WX, Mattoon JR. Properties of Saccharomyces cerevisiae mitochondria prepared by a mechanical method. *Biochim Biophys Acta* 153(3), 521-530 (1968).
137. Qiu M, Chen L, Tan G *et al*. A reactive oxygen species activation mechanism contributes to JS-K-induced apoptosis in human bladder cancer cells. *Sci Rep* 5 15104 (2015).
138. Boujrad H, Gubkina O, Robert N, Krantic S, Susin SA. AIF-mediated programmed necrosis: a highly regulated way to die. *Cell Cycle* 6(21), 2612-2619 (2007).
139. Arab HH, Al-Shorbagy MY, Abdallah DM, Nassar NN. Telmisartan attenuates colon inflammation, oxidative perturbations and apoptosis in a rat model of experimental inflammatory bowel disease. *PLoS One* 9(5), e97193 (2014).
140. Qureshi S, Song J, Lee HT, Koh SD, Hennig GW, Perrino BA. CaM kinase II in colonic smooth muscle contributes to dysmotility in murine DSS-colitis. *Neurogastroenterol Motil* 22(2), 186-195, e164 (2010).
141. Di Sabatino A, Rovedatti L, Kaur R *et al*. Targeting gut T cell Ca2+ release-activated Ca2+ channels inhibits T cell cytokine production and T-box transcription factor T-bet in inflammatory bowel disease. *J Immunol* 183(5), 3454-3462 (2009).

142. Nijveldt RJ, Van Nood E, Van Hoorn DE, Boelens PG, Van Norren K, Van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 74(4), 418-425 (2001).
143. Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Arch Biochem Biophys* 476(2), 107-112 (2008).
144. Urquiaga I, Leighton F. Plant polyphenol antioxidants and oxidative stress. *Biol Res* 33(2), 55-64 (2000).
145. Hodnick WF, Duval DL, Pardini RS. Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids. *Biochem Pharmacol* 47(3), 573-580 (1994).
146. Bhatia S, Shukla R, Venkata Madhu S, Kaur Gambhir J, Madhava Prabhu K. Antioxidant status, lipid peroxidation and nitric oxide end products in patients of type 2 diabetes mellitus with nephropathy. *Clin Biochem* 36(7), 557-562 (2003).
147. Steer P, Millgard J, Sarabi DM *et al*. Cardiac and vascular structure and function are related to lipid peroxidation and metabolism. *Lipids* 37(3), 231-236 (2002).
148. Arulselvan P, Fard MT, Tan WS *et al*. Role of Antioxidants and Natural Products in Inflammation. *Oxid Med Cell Longev* 2016 5276130 (2016).
149. Ravipati AS, Zhang L, Koyyalamudi SR *et al*. Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. *BMC Complement Altern Med* 12 173 (2012).
150. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev* 2(5), 270-278 (2009).
151. Visioli F, De La Lastra CA, Andres-Lacueva C *et al*. Polyphenols and human health: a prospectus. *Crit Rev Food Sci Nutr* 51(6), 524-546 (2011).
152. Tsao R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* 2(12), 1231-1246 (2010).
153. Cardona F, Andres-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuno MI. Benefits of polyphenols on gut microbiota and implications in human health. *J Nutr Biochem* 24(8), 1415-1422 (2013).
154. Daglia M. Polyphenols as antimicrobial agents. *Curr Opin Biotechnol* 23(2), 174-181 (2012).
155. Uncapher RP, Holder HG. Megaduodenum in the adult; report of a case. *Am J Roentgenol Radium Ther Nucl Med* 77(4), 634-638 (1957).
156. Sandoval-Acuna C, Ferreira J, Speisky H. Polyphenols and mitochondria: an update on their increasingly emerging ROS-scavenging independent actions. *Arch Biochem Biophys* 559 75-90 (2014).
157. Castagnini C, Luceri C, Toti S *et al*. Reduction of colonic inflammation in HLA-B27 transgenic rats by feeding Marie Menard apples, rich in polyphenols. *Br J Nutr* 102(11), 1620-1628 (2009).
158. Yoshioka Y, Akiyama H, Nakano M *et al*. Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice. *Int Immunopharmacol* 8(13-14), 1802-1807 (2008).
159. Hyson DA. A comprehensive review of apples and apple components and their relationship to human health. *Adv Nutr* 2(5), 408-420 (2011).
160. Vrhovsek U, Rigo A, Tonon D, Mattivi F. Quantitation of polyphenols in different apple varieties. *J Agric Food Chem* 52(21), 6532-6538 (2004).

161. Jung M, Triebel S, Anke T, Richling E, Erkel G. Influence of apple polyphenols on inflammatory gene expression. *Mol Nutr Food Res* 53(10), 1263-1280 (2009).
162. Paturi G, Butts CA, Bentley-Hewitt KL, Mcghie TK, Saleh ZS, Mcleod A. Apple polyphenol extracts protect against aspirin-induced gastric mucosal damage in rats. *Phytother Res* 28(12), 1846-1854 (2014).
163. Kawalec P. Indirect costs of inflammatory bowel diseases: Crohn's disease and ulcerative colitis. A systematic review. *Arch Med Sci* 12(2), 295-302 (2016).
164. Kawalec P, Mikrut A, Lopuch S. Systematic review of the effectiveness of biological therapy for active moderate to severe ulcerative colitis. *J Gastroenterol Hepatol* 29(6), 1159-1170 (2014).
165. Huoponen S, Blom M. A Systematic Review of the Cost-Effectiveness of Biologics for the Treatment of Inflammatory Bowel Diseases. *PLoS One* 10(12), e0145087 (2015).
166. Neicheva A, Kovacheva E, Marudov G. Determination of organophosphorus pesticides in apples and water by gas-liquid chromatography with electron-capture detection. *J Chromatogr* 437(1), 249-253 (1988).
167. Han JY, Cho KH, Lee DH *et al*. Phase II study of irinotecan plus cisplatin induction followed by concurrent twice-daily thoracic irradiation with etoposide plus cisplatin chemotherapy for limited-disease small-cell lung cancer. *J Clin Oncol* 23(15), 3488-3494 (2005).
168. Femia AP, Luceri C, Bianchini F *et al*. Marie Menard apples with high polyphenol content and a low-fat diet reduce 1,2-dimethylhydrazine-induced colon carcinogenesis in rats: effects on inflammation and apoptosis. *Mol Nutr Food Res* 56(8), 1353-1357 (2012).
169. Denis MC, Furtos A, Dudonne S *et al*. Apple peel polyphenols and their beneficial actions on oxidative stress and inflammation. *PLoS One* 8(1), e53725 (2013).
170. Piberger H, Oehme A, Hofmann C *et al*. Bilberries and their anthocyanins ameliorate experimental colitis. *Mol Nutr Food Res* 55(11), 1724-1729 (2011).
171. Sugimoto K, Hanai H, Tozawa K *et al*. Curcumin prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. *Gastroenterology* 123(6), 1912-1922 (2002).
172. Kim M, Murakami A, Miyamoto S, Tanaka T, Ohigashi H. The modifying effects of green tea polyphenols on acute colitis and inflammation-associated colon carcinogenesis in male ICR mice. *Biofactors* 36(1), 43-51 (2010).
173. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. *J Agric Food Chem* 51(3), 609-614 (2003).
174. Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. *Nature* 405(6789), 903-904 (2000).
175. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 22(3), 659-661 (2008).
176. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18(49), 6853-6866 (1999).
177. Papadakis KA, Targan SR. Current theories on the causes of inflammatory bowel disease. *Gastroenterol Clin North Am* 28(2), 283-296 (1999).
178. Urushima H, Nishimura J, Mizushima T, Hayashi N, Maeda K, Ito T. Perilla frutescens extract ameliorates DSS-induced colitis by suppressing proinflammatory cytokines and

inducing anti-inflammatory cytokines. *Am J Physiol Gastrointest Liver Physiol* 308(1), G32-41 (2015).
179. Pervin M, Hasnat MA, Lim JH *et al*. Preventive and therapeutic effects of blueberry (Vaccinium corymbosum) extract against DSS-induced ulcerative colitis by regulation of antioxidant and inflammatory mediators. *J Nutr Biochem* 28 103-113 (2016).
180. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2(10), 725-734 (2002).
181. Singer, Ii, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology* 115(2), 297-306 (1998).
182. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 281(5381), 1322-1326 (1998).
183. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 16 395-419 (1998).
184. He Q, Zhou W, Xiong C, Tan G, Chen M. Lycopene attenuates inflammation and apoptosis in post-myocardial infarction remodeling by inhibiting the nuclear factor-kappaB signaling pathway. *Mol Med Rep* 11(1), 374-378 (2015).
185. Shao D, Kan M, Qiao P *et al*. Celecoxib induces apoptosis via a mitochondriadependent pathway in the H22 mouse hepatoma cell line. *Mol Med Rep* 10(4), 2093-2098 (2014).
186. Tsujimoto Y, Shimizu S. Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* 12(5), 835-840 (2007).
187. Javadov S, Karmazyn M, Escobales N. Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. *J Pharmacol Exp Ther* 330(3), 670-678 (2009).
188. Chapman MA, Grahn MF, Boyle MA, Hutton M, Rogers J, Williams NS. Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. *Gut* 35(1), 73-76 (1994).
189. Carrasco-Pozo C, Gotteland M, Speisky H. Apple peel polyphenol extract protects against indomethacin-induced damage in Caco-2 cells by preventing mitochondrial complex I inhibition. *J Agric Food Chem* 59(21), 11501-11508 (2011).
190. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 116(3), 615-622 (2006).
191. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. *Essays Biochem* 47 69-84 (2010).
192. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* 277(45), 42769-42774 (2002).
193. Wu Z, Puigserver P, Andersson U *et al*. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98(1), 115-124 (1999).