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ANTIOXIDANT AND MITOCHONDRIAL MECHANISMS OF PROTECTION BY ANTHOCYANINS AND RESVERATROL AGAINST MIXED MICELLE-INDUCED OXIDATIVE STRESS AND MONOLAYER PERMEABILITY IN CACO-2 EPITHELIAL CELLS

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By

Maryam Ershad Langroodi

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University of Saskatchewan

Saskatoon, Saskatchewan S7N 2Z4

Canada

ABSTRACT

Intestinal epithelial cells form important barriers that protect the body from biotic and abiotic or dietary stresses. Thus, maintaining the health and functionality of enterocytes, and their progenitors, is critically important to support barrier integrity and avoid entrance of non-nutrient substances that can lead to endotoxemia and inflammation. Dietary polyphenols have potential antioxidant and other bioactive effects on intestinal epithelial cells, and their possible role in improving mitochondrial function to provide sufficient energy for the cells to combat stressful conditions has not gained much attention.

In my thesis, Caco-2 intestinal epithelial cells exposed to mixed micelles (MM) was used as a model of high dietary fat consumption to induce cellular stress, and the molecular aspects of protection by anthocyanins and the polyphenol resveratrol were investigated.

Exposure of the Caco-2 cells to MM induced cytotoxicity, oxidative stress and mitochondrial dysfunction, and increased differentiated cell monolayer permeability. In protection against intracellular and mitochondrial generation of reactive oxygen species (ROS), an anthocyanin-rich bilberry extract (ARBE) was more effective than resveratrol, and also protected against differentiated cell monolayer permeability to a larger extent, whereas resveratrol only protected for a short time (Chapter 3). Resveratrol, however, protected more than ARBE against a MM-induced decrease in mitochondrial content and expression of mRNA for proteins related to mitochondrial biogenesis (such as PGC-1α, and TFAM), although ARBE protected against declines in mitochondrial function such as basal respiration, spare capacity and ATP production (Chapter 4). In investigations of a possible protective interactions of ARBE and resveratrol against MM-induced intracellular ROS, cytotoxicity and mitochondrial dysfunction, little evidence was found except a significantly greater protection by the combination against intracellular ROS at the lowest concentration (0.1 μM) (Chapter 5). Comparing pure anthocyanins (cyanidin and cyanidin-3-glucoside (C3G)) and their phenolic derivatives 2,4,6-trihydroxybenzaldehyde (THB) and protocatechuic acid (PCA), showed that PCA equally decreased intracellular ROS, and the derivatives acted stronger than the pure anthocyanins in reducing mitochondrial superoxide after 24 h MM challenge (Chapter 6). At shorter times (2-12 h), however, cyanidin, THB and PCA, but

not the glycosides C3G or ARBE protected against MM-induced mitochondrial superoxide formation (Chapter 6).

In conclusion, different polyphenols were all able to protect against MM-induced oxidative stress in the *in vitro* Caco-2 cell model. In general, anthocyanins increased mitochondrial efficiency, that is higher ATP generation as well as lower ROS production, and resveratrol induced mitochondrial content. The effects of anthocyanins and resveratrol were not synergistic however, and some of the effects of the anthocyanins could be due to their phenolic metabolites/degradation products. Further studies investigating an *in vivo* model of protective effects of polyphenols in increased intestinal permeability due to high dietary fat consumption could enlighten our understanding of how these compounds contribute to health.

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DEDICATION

This dissertation is dedicated to my dear husband who I am grateful to have and has patiently been beside me taking each step with me during this journey and to my dearest, beautiful and patient children Amir Mojtaba and Aala who have made me a stronger person. Last but not least this work is dedicated to my loyal and very supportive family my dad, mom and sister who have always been encouraging and supportive in any kind and kept me going to accomplish this commission.

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LIST OF ABBREVIATIONS

ΔΨm	Mitochondrial membrane potential
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ARBE	Anthocyanin-rich bilberry extract
ATP	Adenosine triphosphate
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
cDNA	Complementary DNA
CLA	Conjugated linoleic acid
COX-2	Cyclooxygenase 2
CS	Citrate synthase
Cy	Cyanidin
C3G	Cyanidin-3-glucoside
DCA	Deoxycholic acid
DCF	2',7'-dichlorofluorescein
DCFH-DA	Dichloro-dihydrofluorescein diacetate
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis (2-nitrobenzoic acid)
ECAR	Extracellular acidification rate
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-phenylhydrazone
FITC-dextran	Fluorescein isothiocyanate–dextran
FOXO3	Forkhead box O3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GI	Gastrointestinal
GP_X	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S transferase
HBSS	Hank's balanced salt solution
IL-1β	Interleukin 1β
IMS	Intermembrane space
iNOS	Inducible nitric oxide synthase
LCFA-CoA	Long-chain fatty acyl-coenzyme A
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MitoSOX	Mitochondrial superoxide
MM	Mixed micelles
Mn-SOD	Manganese-superoxide dismutase
mRNA	Messenger RNA
MPT	Mitochondrial permeability transition
MTATP6	Mitochondrially encoded ATP synthase F_o subunit 6
MTCO1	Mitochondrially encoded cytochrome c oxidase I
MTCYB	Mitochondrially encoded cytochrome b
mtDNA	Mitochondrial DNA
MTDR	MitoTracker deep red
MTND1	Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa b
NO	Nitric oxide
NRF-1	Nuclear respiratory factor
OA	Oleic acid

OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PA	Palmitic acid
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-γ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription PCR
SD	Standard deviation
SDC	Sodium deoxycholate
SOD	Superoxide dismutase
SGLT1	Sodium dependent glucose transporter1
SIRT1	Sirtuin 1
SRB	Sulforhodamine b
TCA	Trichloroacetic acid
TEER	Transepithelial electrical resistance
TFAM	Mitochondrial transcription factor A
TG	Triglycerides
THB	2,4,6-trihydroxybenzaldehyde
TMRE	Tetramethyl rhodamine ethyl ester
TNFα	Tumor necrosis factor alpha
UC	Ulcerative colitis
UCDA	Ursodeoxycholic acid
UCP	Uncoupler protein
UPS	Unfolding protein response

CHAPTER 1 INTRODUCTION

1.1. Introduction

Increased dietary fat consumption in developed and developing countries has contributed to many chronic diseases (Lichtenstein et al., 1998; Hoffman et al., 2004). Part of the pathogenesis of high fat intake is the result of its influence on the gastrointestinal tract. Consumption of high amounts of fat can induce functional disruption of the gut barrier leading to an increase in intestinal permeability (Kvietys et al., 1991; König et al., 2016; Bischoff et al., 2014) and exposure to microbial components (such as bacterial endotoxins) that are known to trigger inflammation and metabolic disease through endotoxemia (Bischoff et al., 2014; Moreira et al., 2012; Murakami et al., 2016), as well as increase the risk of intestinal cancer (la Vecchia, 1992; Beyaz et al., 2016). Therefore, maintaining the function and integrity of the gut is an important factor to prevent diet-related diseases (Y Lee, 2013).

Several mechanisms are responsible for the disruption of intestinal cell function and integrity which result from excessive fat intake including a rise in bile acid secretion which can irritate the gut (Stenman et al., 2103), increased cytokine production and inflammation in the gut barrier (Y Lee, 2013), and changes in intestinal microbiota abundance and composition (Moreira et al., 2012). In several tissues, high dietary fat can induce changes in mitochondria and impair mitochondrial function (Turner et al., 2007; Sparks et al., 2005; Wang et al., 2014; Novak and Mollen, 2015). A high fat diet has been reported to increase mitochondrial workload in intestinal mucosa through an increase of proteins involved in mitochondrial β-oxidation, components of the respiratory chain and in oxidative energy metabolism (Wiśniewski et al., 2014). This increased mitochondrial respiratory workload, due to high dietary fat, is associated with an elevation in mitochondrial reactive oxygen species (ROS) production, which can often lead to impaired mitochondrial function. Increased oxidative stress and impaired ATP production has been demonstrated in intestinal diseases such as inflammatory bowel disease (Novak and Mollen, 2015). Mitochondria are likely to have an essential role in preserving the integrity of the intestinal barrier as they are

the major source of ATP production. If the increase in energy demand is not met due to mitochondrial malfunction, tight junctions are disrupted (Moreira et al., 2012), and intestinal permeability increases (Mandel et al., 1993; Madsen et al., 1995).

This dysfunction can be further exacerbated by lack of protective agents (Ames et al., 1993; Lui et al., 2002), including mitochondrial antioxidants that function to prevent ROS-induced damage. The damage inflicted by mitochondrial ROS on lipids, proteins and DNA adversely impacts mitochondrial efficiency and reduces mitochondrial ATP production (Kalogeris et al., 2014). This can result in a cascade of events leading to mitochondrial permeability transition, cytochrome C release, and apoptosis (Crimi and Esposti, 2011). Mitochondria that suffer this fate can no longer contribute to the ATP output needed to combat the increased workload, leading to a weakening of the gut barrier and allowing bacteria antigens to penetrate to systemic sites. This penetration triggers inflammation, which, when chronic, represents a risk factor for metabolic disease.

Taken together, we reason that improvements in cellular ATP production at the level of the intestinal epithelial cells, can confer improved resistance to dietary stressors. This may be achieved by either increasing the number of mitochondria per cell, increasing their capacity, improving their efficiency, or decreasing mitochondrial oxidative stress and damage due to mitochondrial overwork. Increasing the capacity and efficiency of mitochondria while reducing ROS generation and apoptosis are potential mechanisms by which dietary agents may confer heightened resistance to toxic challenges.

An increasing body of evidence suggests that polyphenols such as anthocyanins (Lila, 2004) and resveratrol (Habauzit and Morand, 2012) have a beneficial impact on a variety of diseases. Anthocyanins are especially known for their antioxidant and anti-inflammatory effects (Chrubasik et al., 2010), which support their anticancer and anti-diabetes actions (Dai et al., 2009; Kong et al., 2003; Neto, 2007; Thomasset et al., 2009). Unlike resveratrol and some flavonoids whose protective bioactivities associate with activation of SIRT1, anthocyanins are reported to not be effective in activating this enzyme (Howitz et al., 2003), an important effector of mitochondrial biogenesis, suggesting that the protection by anthocyanins acts through a different mechanism. A previous study from our laboratories suggest a novel mechanism by which anthocyanins may protect: anthocyanidins (the aglycone forms) were found to preferentially accumulate in

mitochondria compared to other flavonoids (Peng, 2012), wherein they may offer mitochondria localized antioxidant activity. Resveratrol has been known to possess a wide range of bioactivities including antioxidant, anti-inflammatory, anti-aging and anticancer effects (Gambini et al., 2015). The main mechanism by which resveratrol has shown protective effects is through its ability to increase mitochondrial biogenesis, by activation of SIRT1 (Csiszar et al., 2009; Kelkel et al., 2010; Price et al., 2012). The induction of mitochondrial biogenesis by resveratrol is accompanied by an increase in mitochondrial mass, mitochondrial DNA content, components of the respiratory chain, and increased expression of nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Szabo, 2009).

Limited studies have focused on understanding the effects of polyphenols on systemic metabolic diseases from a gut-derived perspective. Moreover, there is lack of information about protective effects of polyphenols in preventing the mitochondrial damage induced by excess fat intake and thereby avoidance of gut permeability. We postulate that resisting the adverse effect of excess fat will require an increase in energy output at the level of the small intestine, as this tissue represents a key barrier that is designed to prevent inflammation-inducing gut-derived antigens, such as LPS, from entering the blood stream (Baumgart and Carding, 2007; Lochmiller and Deerenberg, 2000). Thus, we reason that if cells can combat the physical stress of excess fat exposure by improving cellular bioenergetics (i.e. ATP production), they are more likely to maintain barrier integrity and prevent entry of gut-derived antigens. We hypothesize that anthocyanins, by acting as mitochondrial antioxidants, may help neutralize potentially cytotoxic free radicals arising from oxidative phosphorylation. Resveratrol, on the other hand, by increasing mitochondrial biogenesis and function, may increase the amount of ATP available to manage the heightened energy demands of stressful conditions. Due to different mechanisms of action, we also hypothesize that anthocyanins and resveratrol may interact together to provide protection.

Only a few studies have examined the protective effect of anthocyanins and resveratrol on the function and integrity of intestinal epithelial cells and tight junctions (Li et al., 2014a; Shin et al., 2011; Haggard et al., 2017; Ling et al., 2016; Wang et al., 2016). Also, among studies investigating the protective effect of resveratrol on intestine and related diseases, most have focused on anti-inflammatory and to a lower extent the antioxidant (Moura et al., 2015) effects of this compound. However, the protection of intestinal cell mitochondria afforded by these polyphenols has not been

addressed in previous studies. Considering the importance of mitochondria in supplying the energy required to regulate metabolism and function of cells, research should be conducted to determine whether polyphenols act in the small intestine to support mitochondrial function in a manner shown in many other tissues and cell types (Lagoa et al., 2011; Pajuelo et al., 2012; Xie et al., 2012b; Skemiene et al., 2015; Zhao et al., 2015; Csiszar et al., 2009; Price et al., 2012; Onyango et al., 2010; Higashida et al., 2013). Concentrations of polyphenols used in this study were chosen to be relevant to the amount present in the gut lumen. Since the gut represents one of the first anatomical sites of exposure, gut luminal concentrations of polyphenols can achieve very high concentrations (µM) relevant to those used in *in vitro* experiments to show benefit. Polyphenols such as anthocyanins and resveratrol may increase mitochondrial efficiency and spare capacity, enabling the production of sufficient ATP when demand increases. The consequence of such improvements may be better maintenance of the gut barrier when challenged, thereby preventing epithelial disruption, intestinal barrier impairment and inflammation (Pearson et al., 2008).

In this study, we used Caco-2 intestinal epithelial cells exposed to MM prepared from a combination of free fatty acids and bile acid as a model of dietary lipid-induced intestinal stress to investigate the mechanisms involved and the possible protective effects of anthocyanins and resveratrol. We therefore measured the effects of an anthocyanin-rich bilberry extract (ARBE), resveratrol and MM on intracellular and mitochondrial ROS generation, indices of mitochondrial function and respiration, mitochondrial biogenesis, and expression of genes involved in oxidative stress and inflammation, and intestinal epithelial monolayer permeability. Since some effects of anthocyanins *in vivo*, and *in vitro*, may be due to their phenolic metabolites/breakdown products, we also compared the effects of cyanidin, cyanidin-3-glucoside (C3G) and the phenolic products protocatechuic acid (PCA) and 2,4,6-trihydroxybenzaldehyde (THB).

1.2. Hypothesis

The main hypothesis of my study was that anthocyanins and their monophenolic derivatives protocatechuic acid (PCA) and trihydroxybenzaldehyde (THB), and resveratrol act by different

mechanisms (antioxidant effect or inducing mitochondrial biogenesis) to protect against MM-induced oxidative stress, mitochondrial dysfunction and monolayer permeability in Caco-2 cells. The detailed hypotheses were that:

1) MM increase cellular oxidative stress, decrease intestinal cell mitochondrial function, and disrupt intestinal epithelial integrity.

2) ARBE and resveratrol blunt MM-induced mitochondrial and intracellular ROS generation, by different mechanisms, and protect against increased monolayer epithelial permeability.

3) ARBE and resveratrol increase mitochondrial function and content in order to produce sufficient ATP to combat the stress induced by MM.

4) Resveratrol, by increasing mitochondria biogenesis, and anthocyanins, by reducing mitochondrial oxidative stress, together productively interact to resist the toxic effects of MM challenge.

5) The phenolic breakdown/digestion products of anthocyanins, such as protocatechuic acid (PCA), and 2,4,6-trihydroxybenzaldehyde (THB) contribute to the reduction of intracellular ROS and cytotoxicity by anthocyanins and anthocyanin rich bilberry extract.

1.3. Objectives

The specific objectives were to determine:

1. The extent to which ARBE and resveratrol inhibit the pro-oxidant activities of MM in Caco-2 cells, and to identify the mechanisms involved.

2. The ability of ARBE and resveratrol to increase mitochondrial biogenesis and ameliorate oxidative stress and mitochondrial damage induced by MM in Caco-2 cells, and to confirm the mechanisms involved.

3. The interaction and possible synergistic effects of resveratrol and ARBE to preserve mitochondrial function and reduce oxidative stress in Caco-2 cells

4. Whether anthocyanin breakdown/digestive products and pure anthocyanins contribute to the protective effects of anthocyanins against MM-induced ROS generation in Caco-2 intestinal cells.

The first three hypotheses will be addressed in Chapters 3 and 4. The fourth hypothesis will be addressed in Chapter 5. The fifth hypothesis will be addressed in Chapter 6.

CHAPTER 2 LITERATURE REVIEW

2.1. The Intestinal Barrier in Health and Disease

The intestinal epithelium is the inner layer of the intestinal mucosa lining the lumen of the gastrointestinal tract. This layer is a self-renewing folded monolayer including several cell types in the crypts and villi (López-Posadas et al., 2017). The crypts contain proliferative cells. Stem cells located in the base of the crypts provide renewal of the intestinal epithelium. While moving upwards, the stem cells feed into progenitors, also known as transit-amplifying cells through asymmetric division. These highly prolific cells have tumorigenic potential (Ma et al., 2016; Phesse &Clarke, 2009), thus, the intestinal cells are susceptible to cancer (Ma et al., 2016; Phesse &Clarke, 2009). Over 4-5 days, these cells migrate up the villi and undergo differentiation to replace aged enterocytes that are shed at the villi tips.

The intestinal epithelium creates an impermeable barrier which acts selectively in the transport of molecules between the lumen and the systemic circulation. This selective functionality is necessary for mucosal barrier function, and changes in paracellular permeability may cause alteration in intestinal absorption and secretion (reviewed by Arrieta et al., 2006 and Bjarnason et al., 1995). Intestinal permeability may also allow translocation of foreign molecules across or between the intestinal cells via transcellular or paracellular transport, respectfully. Paracellular transport is regulated via tight junction proteins, and dysfunction of these proteins or changes in the structure of enterocytes can increase intestinal permeability and uncontrolled translocation of different substances. Increased intestinal permeability is a potentially pathological condition in which the intestinal barrier becomes permeable to undigested food, toxins and bacteria. This condition is associated with increased bacterial endotoxin translocation, oxidative stress and chronic inflammation (Stenman et al., 2012; Bischoff et al., 2014). Impairment of the gut barrier is noted in diseases such as steatohepatitis, fatty liver disease and diabetes (Dai and Wang, 2015) and different factors, including the Western diet, can trigger this impairment (Bischoff et al., 2014).

Inflammation associated with increased intestinal permeability is postulated to increase the risk of many diseases (Bischoff et al., 2014). Gut barrier dysfunction and increased inflammatory

bacterial lipopolysaccharide (LPS) in the circulation has been noted in several diseases such as fatty liver disease, steatohepatitis and diabetes (Stenman et al., 2012; Stenman et al., 2013). LPS does not initiate gut barrier dysfunction however, if the barrier is disrupted, LPS may aggravate the damage (Hanson et al., 2011). Systemic inflammation has a close link to plasma endotoxemia induced by intestinal permeability (Cani et al., 2007; Cani et al., 2008).

2.2. Health Issues of a High Fat Diet

Diet plays a major role in the occurrence of disease and studies have established the link between different nutrients and chronic diseases. Lipids are essential and should be consumed moderately to provide essential fatty acids and lipid soluble vitamins for the body (Thomson & Dietschy, 1981); however, excessive intake may lead to a variety of diseases such as metabolic syndrome, cardiovascular disease, diabetes, cancer, etc. A high fat diet also increases the risk of obesity, which, in turn, is associated with many diseases including cardiovascular disease, insulin resistance and cancer (Schwingshackl & Hoffmann, 2013; Lam et al., 2012; Winer et al., 2016). Epidemiological studies (Miller et al., 1983; Giovannucci & Willett, 1994) as well as *in vivo* studies have shown high dietary fat (Rao et al., 2001) especially saturated fat intake promotes colorectal cancer. In addition to the effects of the fat, the bile acids that emulsify them can contribute to the carcinogenicity (Bernstein et al., 2005). The amount of fat along with type of fat in the diet both have a significant role in either promoting health or contributing to disease risk. A meta-analysis performed by Schwingshackl & Hoffmann (2013) showed that a high fat diet (>30% total energy intake), high in saturated fatty acid (SFA) (>10% total energy intake) is associated with increased plasma cholesterol and low density lipoprotein (LDL) and is considered a major risk factor for coronary vascular disease. The Dietary Guidelines for Americans suggests fat intake of 20-35% daily energy (Nutrition and Your Health: Dietary Guidelines for Americans, 2010).

2.2.1. High Fat Diet and Intestinal Permeability

The gut barrier is one of the most important barriers that protect the body and organs against foreign molecules and damage. Therefore, maintaining the function and integrity of the gut is an important factor in order to prevent diet-related diseases (Y Lee, 2013).

Several studies have identified mechanisms by which high fat diet triggers intestinal permeability and disease risk. One mechanism is through the induction of oxidative stress in the endoplasmic reticulum (ER) of intestinal goblet cells and activation of inflammatory signaling (Gulhane et al., 2016). The mucin glycoprotein, Muc2, is produced via intestinal goblet cells and is susceptible to misfolding which activates the unfolding protein response (UPS) in an attempt to restore homeostasis. Failure to restore the misfolding induces ER stress and triggers an inflammatory response and apoptosis, which compromises mucosal barrier integrity (Gulhane et al., 2016).

Another mechanism of fat-induced intestinal permeability is via secretion of bile acids. Bile acids are necessary for absorbing fats and fat-soluble nutrients, and for biliary secretion of lipids and xenobiotics. Also, the conversion of cholesterol to bile acids is important for maintaining cholesterol homeostasis. However, accumulation of bile acids in ileal lumen may damage the intestinal epithelium (Craven et al., 1986; Milovic et al., 2002). Bile acids have also been shown to alter Muc2 structure (Shekels et al., 1996; Song et al., 2005). In addition, toxic bile acids or disorders in bile acid metabolism can induce inflammation and cholestatic liver disease, and contribute to dyslipidemia (Ferrebee and Dawson, 2015). Dietary fat affects bile acids by decreasing the proportion of the less membrane disturbing ursodeoxycholic acid (UDCA) vs. the more membrane disturbing deoxycholic acid (DCA), which can impair intestinal barrier function (Stenman et al., 2012; Stenman et al., 2013). Stenman et al. (2013) showed that a diet containing 1-3 mM DCA for 10 weeks, increased intestinal permeability 1.5-fold compared to a normal diet in mice. Also, 3 mM DCA induced epithelial barrier disruption *in vitro*. In another study, DCA induced disruption of the epithelial barrier, increased prostaglandin E2 (PGE2) in the jejunum, and intestinal inflammation in mice, indicating a connection between permeability and inflammation (Bernstein et al., 2007).

A stress such as high fat diet also increases intestinal permeability when ATP levels decline as a result of mitochondrial dysfunction induced by rise in mitochondrial oxidant production (Yu et al., 2014). Decline in ATP levels associate with disruption of tight junction proteins, as actin polymers that are used to support cell structure and a tether to protein complexes that comprise the tight junction are dependent on ATP. Alterations that result in depolymerization (i.e. ATP loss) will destabilize both the shape of the cell and the tight junctions that are required to maintain the barrier (Shen and Turner, 2005), leading to their disassembly (Zheng and Cantley, 2007). The dependency

of tight junctions, and barrier integrity, on adequate ATP for actin polymer integrity, reinforces the concept that energy metabolism is an important determinant of a healthy gut.

Moreover, an important complication associated with excess fat ingestion is modification of composition of gut microbiota, which together with impaired tight junctions may increase systemic exposure to microbial antigens, such as LPS (Moreira et al., 2012). LPS, a cell wall component of gram-negative bacteria and is a potent stimulus for activation of the inflammatory response. In an article by Cani et al. (2007) it is stated that entrance of LPS into the circulatory system from the gut in response to high dietary fat intake provokes "metabolic endotoxemia", which results in a host response involving release of inflammatory cytokines. Increased circulating levels of LPS due to gut barrier dysfunction has been noticed in several diseases such as fatty liver disease, steatohepatitis and diabetes (Stenman et al., 2013). LPS contains a negatively charged lipid A component in the molecular structure that enables these molecules to bind the highly cationic apolipoprotein B present in chylomicrons and other lipoproteins (VLDL, LDL) and apolipoprotein A-1 present in HDL (Vreugdenhil et al., 2001). Also, excessive chylomicron formation as a result of a high fat diet may induce aggregation of these lipoproteins in the intercellular space of enterocytes, thus, causing an increase in local pressure and deterioration of the tight junctions between the enterocytes or membrane rupture on the basal side (Moreira et al., 2012).

Furthermore, and perhaps most importantly, the inflammatory response induced by a high fat diet associates with increased cytokine release from enterocytes and mast cells (Y Lee, 2013) (reviewed by Moreira et al., 2012). Inflammatory cytokines released from mast cell activation include TNF-α (Y Lee, 2013), IL-1b, IL-4 and IL-13 (Moreira et al., 2012). Because TNF-α decreases phosphorylation of occludin, an essential tight junction protein, occludin becomes detached, thus disrupting the functionality and structure of tight junction protein complex and increasing intestinal permeability (Ma et al., 2004; Cui et al., 2010b; Novak and Mollen, 2015; Lee et al., 2015).

The monolayer of enterocytes that comprise the gut wall represent a critically important barrier that protects the body from antigens that can provoke inflammatory responses linked to heightened disease risk. Since food first comes in contact with the GI system, it is very important to know the beneficial and adverse properties of dietary factors in regard to enterocytes. Improving the strength

of the gut through provision of dietary agents that support mitochondrial metabolism may be a promising approach in preventing or alleviating detrimental effects of high dietary fat.

2.3. The Intestinal Epithelial Caco-2 Cell Line as a Gut Barrier Model

Caco-2 cells, a human colon adenocarcinoma cell line, have been extensively used as a model for intestinal permeability. Under appropriate culture conditions, these cells differentiate and form monolayers with morphology and functional properties of mature enterocytes (Sambuy et al., 2005). Differentiated cells resemble epithelial cells in the small intestine (Pinto et al., 1983) as they are polarized, possess apical and basolateral surfaces (Sambuy et al., 2005; Fogh et al., 1977), express microvilli on the apical side and form tight junctions between neighboring cells (Pinto et al., 1983).

While some studies utilize fully differentiated Caco-2 cells, other studies use undifferentiated or partially differentiated Caco-2 cells to study protection of intestinal epithelial cells against different stresses. In the case of undifferentiated cells, the cells could be considered to be similar to intestinal progenitor cells, and the stresses interfere by inducing mitochondrial dysfunction, thus influencing their ability to differentiate and form a barrier. In studies on stresses to undifferentiated Caco-2 cells, they are usually first grown to confluence over 24 h so are not actively dividing.

Several studies have shown protective effects of polyphenols in differentiated or undifferentiated Caco-2 cells. In differentiated Caco-2 cells for example, studies have shown protection by flavonoids (Erlejman et al., 2006; Chen and Kitts, 2017; Cremonini et al., 2017), phenolic acids (Liang et al., 2016) and polyphenol-rich extracts (Denis et al., 2013, 2015) against different stresses. In undifferentiated Caco-2 cells, protection against different stresses has been shown with flavonoids (Elisia and Kitts, 2008; Gonzales et al., 2016), resveratrol (Panaro et al., 2012; De Maria et al., 2013), phenolic compounds (Pacheco-Ordaz et al., 2018), and polyphenol-rich extracts (Yazheng and Kitts, 2012; D'evoli et al., 2013 Muto et al., 2015).

The presence of tissue specific genes ability to differentiate and produce a polarized membrane, and expression of growth factors receptors, digestive enzymes and nutrient transporters that characterize normal enterocytes, make Caco-2 cells a suitable *in vitro* model for the major cell type comprising the gut barrier. In addition, Caco-2 cells express a variety of cytochrome P450

enzyme isoforms and phase 2 enzymes such as UDP-glucuronosyltransferase, sulfotransferase and glutathione-S-transferase. Expression of these enzymes, which are designed to protect the body by inactivating foreign, potentially toxic, dietary components, is also characteristic of normal enterocytes (Meunier et al., 1995). Although culture conditions may influence the properties of these cells, several studies have indicated that Caco-2 cells resemble the characteristics and properties of intestinal cells better than other carcinoma cells lines (Matsumoto et al., 1990). Tsuzuki (2007) also showed Caco-2 cells are appropriate models to investigate absorption of dietary lipids in human intestine as they maintain the cellular machinery involved in lipid absorption, triglyceride (TG) resynthesis, and chylomicron secretion. Caco-2 cells also express glycosidases (Klumperman et al., 1991; Mizuma et al., 2005; Arafa, 2009; Henry-Vitrac et al., 2006) which like normal enterocytes remove the sugar moieties from dietary flavonoids and release the flavonoid aglycone. Thus, in general, Caco-2 cells provide an appropriate model for investigating effect of dietary components on the barrier function of this organ.

2.4. Lipid-induced Mitochondrial Dysfunction

Mitochondria are the power house of cells and contribute to cell metabolism by producing ATP from dietary energy sources through oxidative phosphorylation (Martel et al., 2013). Therefore, organs with higher energy demand, such as the GI tract, liver, brain and muscle, are more vulnerable to the effects of mitochondrial dysfunction (Reviewed by Labbé et al., 2014; Ernster and Schatz, 1981; Nunnari and Suomalainen, 2012).

Exposure of intestinal cells to high dietary fat may induce mitochondrial dysfunction both by the physical stress of fatty acids and bile acids on membranes of the cell, and the metabolic disturbance of the excess fatty acids. Disruption of the plasma and mitochondrial membrane structure by bile acids can trigger mitochondrial-mediated apoptosis (Perez and Briz, 2009). Also, exposure of mitochondria to free fatty acids has long been known to produce mitochondrial uncoupling (Vaartjes and van den Bergh, 1978; Borst et al., 1962), which decreases the capacity of mitochondria to produce ATP. Moreover, the excess supply of NADH-provided electrons from β-oxidation may exceed mitochondrial oxidative capacity, resulting in increased reduction of the ETC and consequent ROS generation (Liesa and Shirihai, 2013; Maassen et al., 2007). In turn, the mitochondrial ROS can adversely impact mitochondrial efficiency by damaging mitochondrial

lipids and other components (Paradies et al., 2010). The resulting oxidative stress that damages the mitochondrial components and reduces the ability of the mitochondrial electron transport chain to couple oxygen consumption to ATP production may trigger the mitochondrial permeability transition, cytochrome C release and apoptosis (Crimi and Esposti, 2011). The decreased cell viability can then disrupt tight junction proteins and increase permeability of the cell monolayer (Gogvadze et al., 2006).

Processes which help maintain healthy and efficient mitochondria include mitochondrial biogenesis and mitophagy. The former produces new mitochondria and the latter removes damaged mitochondria and is triggered via loss of membrane potential (Ashrafi and Schwarz, 2013; Basit et al., 2017; Ploumi et al., 2017; Ding & Yin, 2012). Disruption in either process plays an important role in mitochondrial related diseases (Ding & Yin, 2012), and nutrient deficiencies aggravate this condition resulting in increased mitochondrial ROS generation. Below, studies on effects of fat on mitochondrial function will be reviewed in detail.

2.4.1 Effect of Lipids on Mitochondrial Function and Gene Expression

Several studies suggest excessive intake of dietary fat leads to pathological conditions associated with decreased mitochondrial number and function (reviewed by Rohrbach, 2009; Ritov et al., 2005; Liu et al., 2009; Mabalirajan and Ghosh, 2013). In one study by Yang et al. (2012), 0.2 mM saturated fatty acid (sodium palmitate bound to bovine serum albumin, as an established model of inducing insulin resistance *in vitro*) in C2C12 myoblast cells induced mitochondrial dysfunction. A decrease in oxygen consumption and mitochondrial membrane potential, an increase in mitochondrial electron transport chain subunits (complexes IV and V) (probably due to a compensatory mechanism) and an increase of UCP2 and UCP3 was noted after 24 h of palmitate treatment (Yang et al., 2012).

Very limited studies have investigated the effects of dietary lipid on mitochondrial function in intestinal cells and/or intestinal tissue. In one study (Chapkin et al., 2002) feeding omega-3 polyunsaturated fat to rats increased the susceptibility of colonocyte mitochondria to damage from ROS, facilitating colonocyte apoptosis. Polyunsaturated fatty acids (PUFAs) are prone to oxidation and can compromise mitochondrial function. Another study (Eya et al., 2015) revealed dietary

lipid fed to fish affected expression of different mitochondrial and nuclear genes related to the electron transport chain of the intestine, as well as liver and muscle, by either decreasing or increasing gene expression.

Also, of interest are effects of high saturated fat common in Western diets on mitochondrial lipid metabolism. A high fat diet modifies expression of carnitine palmitoyl transferase-1 (CPT1) in different tissues such as muscle. CPT1 is located in the outer mitochondrial membrane and controls long-chain fatty acyl-coenzyme A (LCFA-CoA) entry into mitochondria, where LCFA-CoA undergoes β-oxidation to provide energy (Turner et al., 2007). The study by Turner et al. (2007) indicated that a high saturated fat diet (D12451, 45% total calorie from fat) fed to C57BL/6J mice for 5 or 20 weeks increased muscle peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and thereby increased mitochondrial metabolism and fatty acid oxidative capacity, uncoupling protein 3 (UCP 3), and mitochondrial respiratory chain subunits. However, Sparks et al. (2005) showed a decrease in genes related to mitochondria complexes (I, II, III and IV), oxidative phosphorylation and PGC-1α in skeletal muscle of C57B1/6J mice fed a high saturated fat diet (D12451) for 3 weeks. The reason for conflicting data on effect of fat on PGC-1α may be due to the composition of the diet, duration of the feeding or the muscle groups examined (i.e., quadriceps versus gastrocnemius bundle). In addition, some studies focus on mRNA expression for PGC-1α, but the gene may not reflect the protein levels as it is regulated by modification after translation (Turner et al., 2007). In another study on muscle (Gomes et al., 2012), a high fat diet (60%) for 12 weeks in Sprague Dawley rats induced mitochondrial dysfunction, decreasing oxygen consumption and mitochondrial ATP by decreasing succinate dehydrogenase, cytochrome C oxidase and citrate synthase activity.

Some studies also showed effects of high saturated fat on mitochondria of liver. A study conducted by Martel et al. (2013) using several models (liver of patients with fatty liver disease, ob/ob mice, mice fed a high fat diet and *in vitro* models of lipotoxicity), showed increased mitochondrial membrane permeability and swelling. In another study by Lin et al. (2005), dietary saturated fat (58% fat- D12331) in C57/Bl6J mice induced an increase in expression of PGC-1β and alteration of lipid metabolism in liver leading to increased lipogenesis, hypertriglyceridemia and hypercholesterolemia. PGC-1β also stimulated expression of mitochondrial genes such as cytochrome C and β-ATPase in liver (Lin et al., 2005). Thus, saturated fat induced a stress that

increased mitochondrial biogenesis. PGC-1β like PGC-1α increases mitochondrial biogenesis and respiration in differentiated myotubes and hepatocytes (Lin et al., 2003; St-Pierre et al., 2003)

In summary, both saturated and unsaturated fat affect mitochondrial gene expression and function. Although conflicting results exist on the effects of lipids on mitochondrial function, PUFAs seem to increase ROS generation in mitochondria and saturated fat has shown to decrease mitochondrial efficiency by decreasing oxygen consumption, increasing uncoupling proteins and decreasing mitochondrial respiratory chain subunits. However, other studies as mentioned above show lipids actually increased mitochondrial metabolism and fatty acid oxidative capacity. Some of the inconsistencies in the results may be explained by the timing and dosimetry of such exposures, differences in various tissues, or perhaps induction of early compensatory mechanisms, that mask the detrimental effects observed later on, but the reason for other disagreements is not known.

2.4.2 Effect of Lipids on Mitochondrial ROS Generation

Mitochondria are major sources of ROS production and complex I and III are the main sites of superoxide anions (reviewed by Turrens, 2003). ROS generated from complex I mainly enter the mitochondrial matrix whereas ROS produced by complex III enter either the matrix or IMS (Turrens, 2003). Such ROS associates with increase in DNA mutations, inefficient mitochondrial function, loss of ATP generation and aging.

Fatty acids impair ATP production in mitochondria by inducing oxidative stress and ROS generation in mitochondria. Schönfeld & Wojtczak (2007), for example, showed free fatty acids (FFA) can impair ATP generation by inhibiting electron transport and increasing mitochondrial oxidant production or induce depolarization of the inner membrane due to uncoupling (Schönfeld & Wojtczak, 2007; Korshunov et al., 1997). Both saturated and unsaturated fat in the diet increase mitochondrial ROS generation in different tissues. In one study, dietary unsaturated fat (25% of total fat) increased lipid peroxidation and membrane alteration in the heart of Wistar rats (Diniz et al., 2004). Another study showed high dietary fat (D12492 60% lard) for 17-19 weeks induced increased ROS and decreased ATP production in liver and heart mitochondria in C57BL/6 mice (Yu et al., 2014) with changes in the liver being more pronounced. Wang et al. (2014) found that C57BL/6J mice fed a high fat diet for 12 months showed an increase in mitochondrial ROS

generation and oxygen consumption in visceral and subcutaneous fat. In general, the results of different studies show that mitochondrial ROS generation increases when mitochondria are exposed to increased dietary fats.

2.4.3 Mitochondria and Intestinal Permeability

Mitochondria dysfunction is considered a common factor in many diseases; however, it has not been studied in the gastrointestinal tract to a great extent. Intestinal cells in general require a high amount of energy for normal function and maintaining the intestinal barrier has been estimated to require 40% of the body's energy expenditure (Bischoff et al., 2014); Thus, inadequate mitochondrial ATP generation in the gut barrier may lead to pathological conditions.

In the review of Novak and Mollen (2015), the authors mention that patients with intestinal diseases such as IBD have disrupted intestinal cell mitochondrial respiration, reduced levels of ATP and increased intestinal permeability. Inadequate ATP has been related to pathologies affecting high energy requiring tissues (Desler et al., 2012).

There is evidence that impaired mitochondrial function may lead to increased intestinal permeability. For example, drugs (non-steroidal anti-inflammatory drugs) can impair mitochondrial function and concomitantly increase intestinal permeability (reviewed by Bjarnason, 1995). Also, in a study by Rodenburg et al. (2008), non-digestible fructo-oligosaccharides (FOS) (60 g/Kg diet) increased intestinal permeability (mainly colon) via altering energy metabolism and depleting ATP in colonic epithelial cells. As a compensating mechanism, an increase in expression of mitochondrial genes such as 5 complexes of mitochondrial oxidative phosphorylation and also enzymes of the tricarboxylic acid (TCA) cycle was observed following FOS consumption.

In addition, tight junctions and the cytoskeleton in small intestine enterocytes control the transport of luminal contents in an ATP dependent manner, thus, disturbed cellular metabolic function and ATP depletion may disrupt the barrier permeability (Mandel et al., 1993; Kennedy et al., 1998; Salzman et al., 1995; Unno et al., 1996). In a study by Madsen et al. (1995) male Lewis rats given the antibiotic FKS06 for 6 weeks had increased intestinal permeability that corresponded with decreased intestinal cell ATP production and mitochondrial respiration. The authors conclude that

decreased mitochondrial activity and ATP production led to increased intestinal permeability (Madsen et al., 1995). In a study with Caco-2 intestinal epithelial cells (Dickman et al., 2000), infection of the cells with rotavirus, which lead to a drop in mitochondrial respiration and cellular ATP, was reported to impair tight junctions and increased paracellular permeability.

Because mitochondria have an important role in intestinal cell barrier function (Henke & Jung, 1993; Mandel et al., 1993; Madsen et al., 1995), we reason that improvements in cellular bioenergetics at the level of the intestinal epithelial cell can confer improved resistance to dietary stressors, through ATP-dependent maintenance of epithelial tight junction integrity and overall cell function. This improvement may be achieved by maintaining ATP production during stressful exposures through either an increase in the number of mitochondria per cell or by reduction in the oxidative damage provoked by stress-induced mitochondrial overwork. Mitochondrial antioxidants may serve such a purpose. Increasing mitochondrial biogenesis and efficiency are two potential mechanisms by which dietary agents may confer heightened resistance to toxic challenges, which can prevent increase in ROS generation, and impairment in barrier integrity. Polyphenols, potentially through their ability to increase the capacity and efficiency of the mitochondria may inhibit the effect of stressors thereby preventing intestinal barrier impairment and organ dysfunction.

2.5. Anthocyanins

Berries have a variety of phytochemicals such as polyphenols, stilbenes, tocopherols, and carotenes. Among polyphenols, anthocyanins are of great interest (Patterson, 2008). Anthocyanins are flavonoid compounds with polyphenolic groups responsible for the pigmentation of plants ranging from pink to purple (Pervin et al., 2014). The color of anthocyanins depends on the chemical structure and the presence of copigments (Mistry et al., 1991).

Anthocyanins have two aromatic rings connected via three carbon oxonium heterocycle, referred to as a chromane ring, which is attached to an aromatic ring in the 2 position. Fig. 2.1 displays the basic structure of anthocyanins (Bueno et al., 2012).

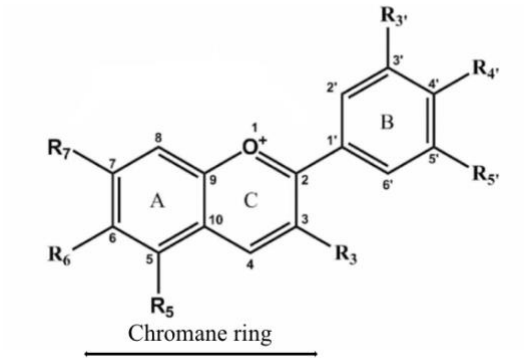


Figure 2.1. *Basic structure of anthocyanin.*

Anthocyanins are naturally present in glycosylated (usually at the 3 position) forms of polyhydroxyl or polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium) cation (Mazza and Miniati, 1993). Identified anthocyanins differ in number of hydroxyl or methoxyl groups, and number and position of the sugar groups. The most common sugar groups are glucose, galactose, arabinose, rhamnose and xylose. The aglycone form of an anthocyanin, called an anthocyanidin, is rarely found in nature. Approximately 17 anthocyanins are found in nature and 6 are the most common (Fig. 2): cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (reviewed by Prior & Wu, 2006; Del Rio et al., 2013). In addition, gut microflora metabolize anthocyanins to phenolic acid and aldehyde derivatives. Two major derivatives are protocatechuic acid (PCA) and 2,4,6-trihydroxybenzaldehyde (THB) (Faria et al., 2014; Seeram et al., 2001). PCA is a dihydroxybenzoic acid, a type of phenolic acid and is structurally similar to gallic acid, caffeic acid, vanillic acid, and syringic acid, all of which are well-known antioxidant compounds (Kakkar and Bais, 2014). Figure 2 shows some of the main anthocyanins, anthocyanidins and derivatives of anthocyanins.

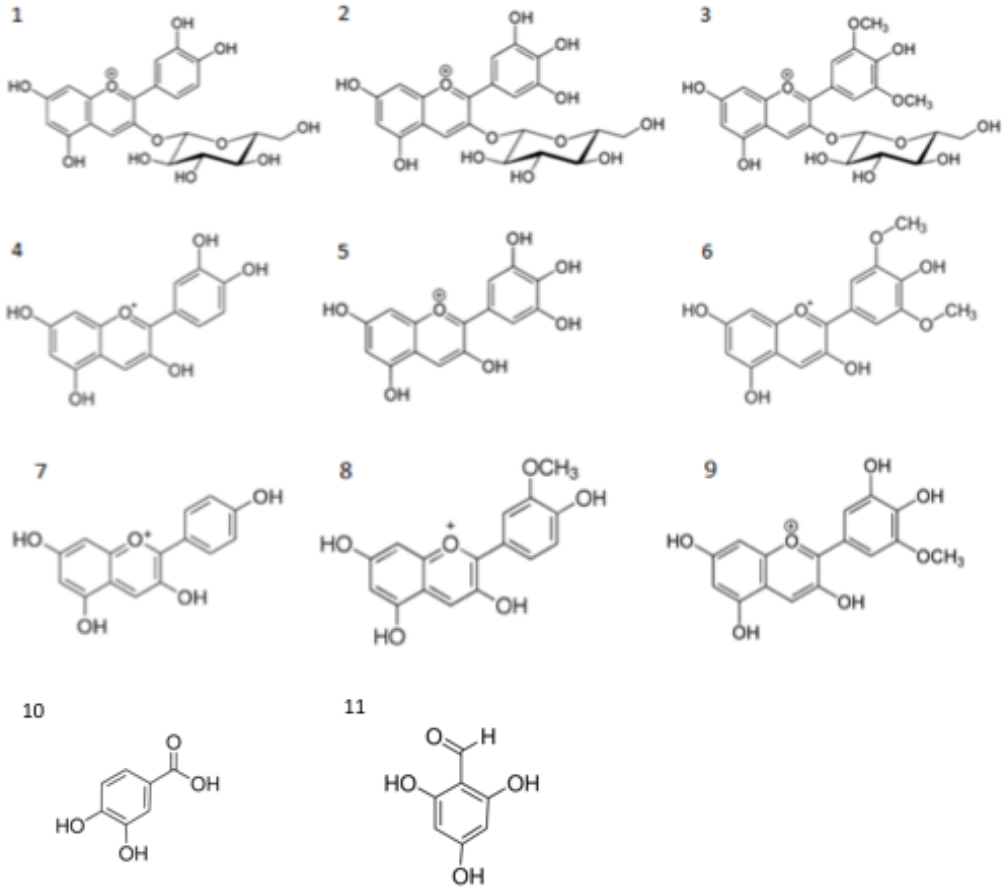


Figure 2.2. *Structures of anthocyanins.* **(1)** cyanidin 3-β-D-O-glucoside (C3G), **(2)** delphinidin 3-β-D-O-glucoside (C3G), **(3)** malvidin 3- β-D-O-glucoside (M3G), **(4)** cyanidin, **(5)** delphinidin, **(6)** malvidin, **(7)** pelargonidin, **(8)** peonidin, **(9)** petunidin, **(10)** protocatechuic acid, **(11)** 2,4,6-trihydroxybenzaldehyde.

The transformation of anthocyanins in aqueous solution under different pH has been reviewed by He and Giusti (2010). Four major anthocyanin forms exist in equilibria: the red flavylium cation, the colorless carbinol pseudobase, the colorless chalcone, and the blue quinonoidal base. The primary form of anthocyanidin at pH <2 is the red flavylium cation. Hydration of the flavylium cation at the C-2 position occurs at pH 3 to 6 which forms a colorless chalcone pseudobase. In

neutral and slightly acidic pH the neutral quinonoidal base is formed which has a purple to violet color and at alkaline pH (8-10) the blue color ionized quinonoidal base is generated.

2.5.1. Amount of Anthocyanin in Foods

Anthocyanins are the largest group of water soluble pigments in plants. These compounds are usually associated with fruits; however, flowers, vegetable roots, tubers, bulbs, legumes and cereals also contain these compounds (reviewed by Bridle & Timberlake, 1997). Derivatives and metabolites of anthocyanins such as protocatechuic acid (PCA) are found in olives and white grapes (Semaming et al., 2015). Berries are among the richest sources of anthocyanins. Canada grows two different types of blueberries, wild (low-bush) blueberries (i.e. *Vaccinium angustifolium*) and cultivated (high-bush) blueberries (*Vaccinium corymbosum*) and is also the second biggest producer of cranberries. Canada also produces saskatoon berries (Amelanchier alnifolia) which is a deep blue/purple berry grown on a tree (Patterson, 2008). Bilberries (*Vaccinium myrtillus L*.) are mainly grown in Europe, and high-bush blueberries (*Vaccinium corymbosum L*.) are primarily cultivated in North America (Moze et al., 2011). Low-bush "wild" blueberries (*Vaccinium angustifolium*) contain a higher amount of anthocyanins and have higher antioxidant capacity compared with high-bush blueberry (Vaccinium corymbosum) (Kalt et al., 1999).

More than 600 anthocyanins have been discovered (He et al., 2006; Liu et al., 2018), and the glycoside form of 6 aglycone forms are mostly distributed in foods (Wu et al., 2006). Among anthocyanin rich foods, black raspberry and black currant contain mainly glycosides of cyanidin and delphinidin respectively, blueberries contain mainly malvidin and petunidin, cranberries contain mainly peonidin, red radish have a high amount of pelargonidin (Wu et al., 2006) and bilberry is the main source of cyanidin and delphinidin (Pervin et al., 2014). The study of Wu et al. (2006) also found that in general, monoglycosylated forms of anthocyanins are present to a greater extend compared to di and tri-glycosylated forms and acetylated anthocyanins are present in minor amounts in foods.

In the study by Wu et al. (2006), 100 different foods were tested for anthocyanin content. From the total 100 foods tested, 24 had anthocyanins and the main anthocyanins were glycosides of

cyanidin, delphinidin and malvidin. The amount of anthocyanin found in foods tested ranged between 0.7-1480 mg/100 g of fresh weight. According to Moze et al. (2011), the amount of anthocyanins in bilberries is 1210.3 mg /100 g fresh weight and in blueberries is 212.4 mg/100 g fresh weight. Likewise, Bornsek et al. (2012) state that fresh bilberry and blueberry contain 1122.9±27.4 mg and 262.2±12.0 mg anthocyanins expressed as C3G/100 g fresh weight respectively. Thus, the anthocyanin content of bilberry was more than 4 times of the blueberry extract. Also, Morais et al. (2016) have reported 200 g of eggplant or black grapes provides approximately 1500 mg anthocyanins, 100 g of berries provides approximately 500 mg of these compounds and 100 g of Brazilian berries such as açai (*Euterpe oleracea Mart.*) and juçara (*Euterpe edulis Mart.*), can provide between 239 and 409 mg of anthocyanins. The most abundant anthocyanin in food is cyanidin (50%) and then pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%) and malvidin (7%) (Reviewed by Morais et al., 2016).

A 1-y-old red wine contains anthocyanins ranging from 40 to 1269 mg/L and the anthocyanin content decreases about 60% in 4-y-old bottled wine (Pojer et al., 2013). Wu and others (2006) also estimated that a glass of wine contains approximately 20 to 35 mg of anthocyanins.

Based on NHANES in 2001-2002 the total amount of anthocyanin intake for each individual was estimated to be 12.5 mg/day. The daily ingestion of anthocyanins in the United States is estimated to be more than 200 mg/day (Kühnau, 1976). However, Pojer et al. (2013) in their review state that this amount was overestimated because of inaccurate food data intake. The estimated daily intake of anthocyanins in Finland is 82.5 mg/d (Heinonen, 2007), which contains 44.7% cyanidin glycosides (Wu et al., 2006). The average daily intake of anthocyanins for men in Holland and Italy have been reported to be 19.83 and 64.88 mg/day respectively and for women 18.73 mg/day in Spain and 44.08 mg/day in Italy (Morais et al., 2016). In the Netherlands, the average intake of flavonoids (mainly quercetin, kaempferol, myricetin, apigenin, and luteolin) in 4,112 adults tested was 23 mg/day of all flavonoids combined and the most important flavonoid was quercetin with 16 mg mean daily intake (Hertog et al., 1993). In another study, the total flavonoid intake in 93,600 women included in the Nurses' Health Study II (NHSII) was estimated to be (58–643 mg/day), whereas anthocyanin intakes ranged from 2 to 35 mg/d (Cassidy et al., 2013). In a clinical trial studying 12 healthy subjects, 7 mL/kg body weight consumption of acai after overnight fasting

resulted in a peak of 2321 ng/L 2.2 h after the consumption in the form of pulp and of 1138 ng/L 2.0 h after juice (Mertens-Talcott et al., 2008).

2.5.2. Absorption and Metabolism of Anthocyanins

There are several studies about anthocyanin ingestion and the extent of their absorption. Marazzoni (1991) was the first to mention 400 mg/kg body weight of anthocyanin resulted in 2.5 mg/L in plasma after 15 min. Anthocyanins appear in the blood relatively fast (about 6-20 min) after ingestion and reach peak levels after 20-60 min (Pojer et al., 2013). However, according to a meta-analysis by Manach et al. (2005), after being metabolized via microbiota in the intestine and having a rapid biotransformation, the average time for anthocyanins to reach the highest concentration in human plasma was 90 min.

Although many studies (Talavéra et al., 2004; Talavéra et al., 2003; McGhie & Walton, 2007) have investigated the absorption and bioavailability of anthocyanins, the rate of absorption, metabolism and distribution among cells and tissues remain unclear (Faria et al., 2009). Two major sites of anthocyanin absorption include the stomach and small intestine mainly the jejunum (Talavéra et al., 2004; Talavéra et al., 2003). However, in a review, Morais et al. (2016) indicate high concentration of anthocyanins are found in the distal intestine, cecum and colon because most absorption occurs in the lower GI tract. Approximately 85% of blueberry anthocyanins reach the colon where they are largely degraded to phenolic acids via microbiota for further absorption (Reviewed by Morais et al., 2016).

Studies have reported that only less than 1% of total anthocyanin intake is absorbed and excreted in the urine (in the native form as well as metabolites) (Miyazawa et al., 1999). Approximately 69% of anthocyanins disappear in the GI tract 4 h after ingestion which may be due to degradation and metabolism of the parent compound and formation of phenolic acids and aldehydes (Aura et al., 2005; Keppler and Humpf, 2005; Tsuda et al., 1999).

The concentration of anthocyanins in the systemic circulation ranges from nM to low μM (Pojer et al., 2013). Several studies indicate anthocyanin glycosides (stable at low pH) are absorbed intact from the stomach and small intestine by a saturable mechanism indicating a carrier involved in the absorption, then circulated and excreted via urine unmodified (Youdim et al., 2000; Faria et al.,

2009; Miyazawa et al., 1999; Pojer et al., 2013). Likewise, Bornsek et al. (2012) believe anthocyanins as hydrophilic compounds are not transported through the cellular wall by simple diffusion indicating there might be a transporter at the cellular surface for these compounds. However, others signify cleavage of the sugar group of anthocyanins by bacteria or intestinal enzymes to form anthocyanidins (Keppler and Humpf, 2005; Faria et al., 2009). Anthocyanidins, which are lipophilic compounds, diffuse across the epithelial membrane passively (Del Rio et al., 2013) and are converted to their metabolites (sulfate or glucuronide conjugates) before entrance to the circulatory system (Keppler and Humpf, 2005; Del Rio et al., 2013). In any case the bioavailability of anthocyanins is very low, ranging from 1.7% to 3.3% (Marczylo et al., 2009). Anthocyanins pass the blood-brain barrier, the blood-retina barrier, and apart from the GI tract have been detected in vascular endothelial cells, liver, kidney, prostate glands (Pojer et al., 2013).

In detail, after ingestion of flavonoids the glycosylated form is converted to the aglycone form by lactase phlorizin hydrolase (LPH) present in the brush border of the small intestine epithelial cells. The aglycone form enters the enterocytes passively. Cytosolic β-glucosidase (CBG) is another alternative hydrolytic step within the epithelial cells. In order for the CBG to act within the cells, the glycosylated flavonoid must enter the cell via a transporter (possibly active sodium-dependent glucose transporter 1 (SGLT1)) (Del Rio et al., 2013). However, other studies have shown SGLT1 is not responsible for flavonoid transportation and in fact glycosylated flavonoids may inhibit the glucose transporter (Kottra & Daniel, 2007). After entrance into enterocytes and before entering the circulatory systems, the aglycone forms of flavonoids are metabolized by phase II enzymes and form sulfate, glucuronide, and/or methylated metabolites by sulfotransferases (SULTs), uridine-5′-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyltransferases (COMTs) respectively (Talavera et al., 2004). After entrance in the portal vein, the metabolites may enter the liver and undergo further phase II metabolism and enter the enterohepatic circulation and be recycled to the intestinal lumen (De Rio et al., 2013). Some of the metabolites might return to the intestinal lumen via members of the adenosine triphosphate-binding cassette (ABC) family of transporters, namely multidrug resistance protein (MRP). GLUT2 (glucose transporter) and MRP-3 are involved the efflux of metabolites from the basolateral membrane to the portal vein (De Rio et al., 2013). Anthocyanins have shown to increase facilitative glucose transporter 2 (GLUT2) expression but facilitative glucose transporters 5 (GLUT5) and sodium-dependent

glucose transporter 1 (SGLT1) remained unchanged (Faria et al., 2009). While anthocyanins increase GLUT2, the transport of glucose is decreased. Faria et al. (2009) propose that anthocyanins compete with glucose for the same transporter in the small intestine affecting glucose uptake. In addition, anthocyanins are found both intact and as metabolites in blood and several tissues such as bile, brain, liver, kidney, heart, testes and lung in rats and mice. Figure 2.3 shows possible mechanisms involved in absorption of polyphenols (Del Rio et al., 2013).

Polyphenolic glucuronide, methyl, and sulfate metabolites are treated as xenobiotics and are excreted via the kidneys (Del Rio et al., 2013). Methylation of anthocyanins occurs mainly in kidney tubules via COMT. The methylated form may be excreted or transported back to the blood by a bidirectional carrier (Pojer et al., 2013). Conjugated anthocyanins are mainly excreted via urine but may also be recycled to the intestine via enterohepatic circulation and excreted in the feces (Pojer et al., 2013). Glycosylated anthocyanins resistant to LPH or CBG which are not absorbed in the small intestine are excreted via the colon where the conjugating moieties are cleaved by the microbiota and the anthocyanidins are digested to breakdown products and phenolic acids and hydroxycinnamates, including protocatechuic acid (PCA) and 2,4,6-trihydroxybenzaldehyde (THB) from cyanidin (Kay et al., 2009). These phenolic metabolites of anthocyanins are readily bioavailable and likely provide many of the systemic benefits of dietary anthocyanins (Kay et al., 2017; Kay et al., 2009).

In contrast, several studies show the presence of intact anthocyanins or their metabolites in different tissues. For example, Cooke et al. (2006) detected the intact form of anthocyanin in liver and kidney, the aglycone form in urine samples and methylated and glucuronide metabolites in urine and intestine of mice consuming Mirtoselect (anthocyanin rich bilberry extract). In mice on a C3G-supplemented diet, the liver and kidney had glucuronide and methylated metabolites and cyanidin was found in the intestinal mucosa. Cooke et al. (2006) showed that total anthocyanin levels in intestinal mucosa was 43 ng and 8.1 µg, after oral ingestion of 0.3% of dietary C3G or Mirtoselect for 12 weeks, respectively, equivalent to 9 mg/day anthocyanin (extract) per Apc mouse. In another study by Felgines et al. (2009) in rats consuming blackberry anthocyanin-enriched diet for 12 days, the bladder had the highest level of anthocyanins, C3G and the monoglucuronide were found in the prostate, testes and heart, and native C3G and methylated derivatives were present in adipose tissue. With intravenous administration, Ichiyanagi et al.

(2009), showed 400 mg/kg bilberry extract (153.2 mg/kg as anthocyanins) resulted in anthocyanin recovery of 30.8 and 13.4%, in urine and bile respectively during the first 4 h and primary anthocyanins in liver and kidney were O-methyl anthocyanins such as peonidin, malvidin, and other O-methyl anthocyanins derived from delphinidin, cyanidin, and petunidin-glycosides. Moreover, several studies have indicated protective effects of anthocyanins against cognitive disorders, however there are conflicting data on whether anthocyanins cross the blood brain barrier (reviewed by Prior and Wu, 2006; Del Rio et al., 2013).

The rate of absorption of anthocyanins depends on their chemical structure and stability. In an attempt to investigate the fate of anthocyanins in the small intestine, Talavera et al. (2004) found anthocyanin glycosides to be partially unstable at intestinal pH (2-9% degradation in the perfusate over 45 min at 37 °C at pH 6.6). The chemical structure of the anthocyanins influences its stability, with delphinidin glycosides being the least stable. Nevertheless, the anthocyanins were efficiently absorbed by the small intestine (Talavera et al., 2004). The glycosidic group of anthocyanins affected absorption from the small intestine. Cyanidin-3-glucoside (C3G) was absorbed the most from the intestine (compared to other sugar moiety groups attached such as cyanidin-3-galactoside and cyanidin-3-rutinoside) among purified anthocyanins and extracts. The presence of methyl groups in the structure of anthocyanins such as malvidin glycosides decreased absorption of these compounds.

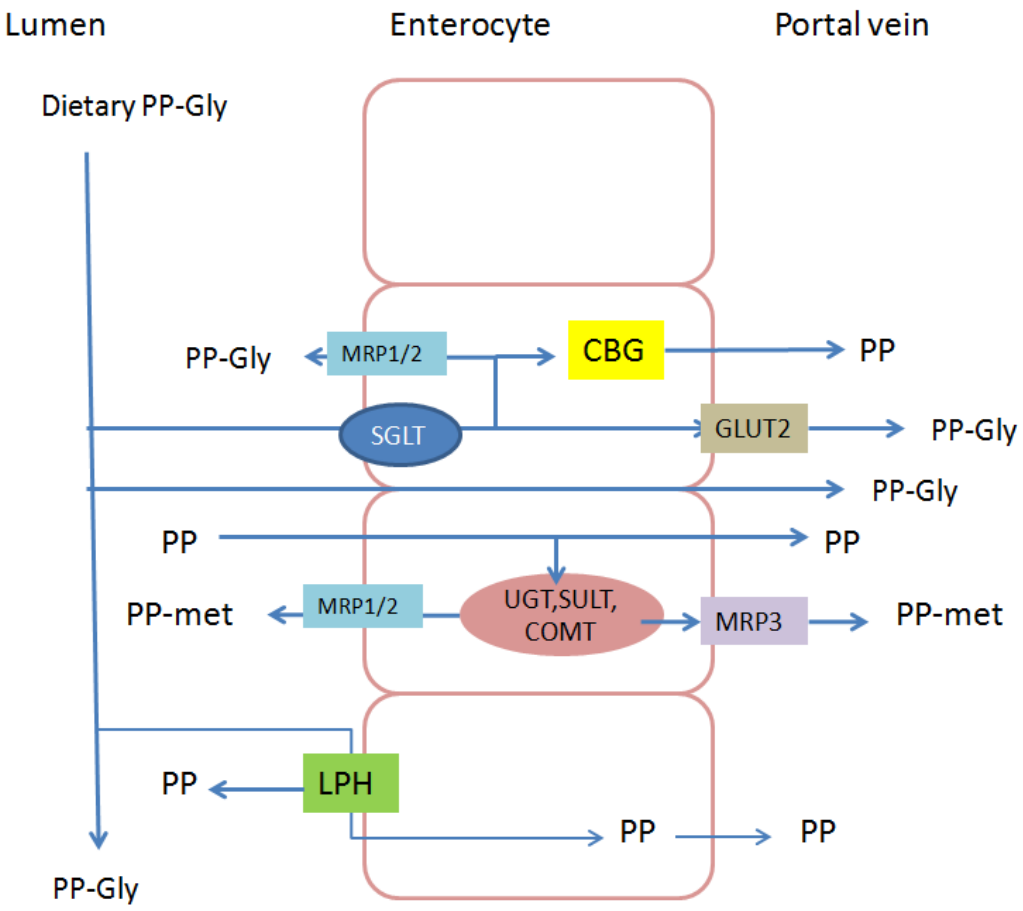


Figure 2.3. *Possible mechanism for absorption and metabolism of (poly) phenolic compounds in the small intestine.* CBG, cytosolic β-glucosidase; COMT, catechol-O-methyl transferase; GLUT2, glucose transporter; LPH, lactase phlorizin hydrolase; MRP1-2–3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenols sulfate/glucuronide/methyl metabolites; SGLT1, sodium dependent glucose transporter; SULT, sulfotransferase; UGT, uridine-5′-diphosphate glucuronosyltransferase (adapted from Del Rio et al., 2013). In the small intestine, LPH in the brush border cleaves the glucose molecule to produce the aglycone form which enters the enterocytes passively. Cytosolic β-glucosidase (CBG) is another alternative hydrolytic step within the epithelial cells. Sodium-dependent glucose transporter 1 (SGLT1) is possibly responsible for actively transporting the glycone form into the intestinal cells. After entrance into enterocytes, the aglycone forms of anthocyanins are

metabolized by phase II enzymes and form sulfate, glucuronide, and/or methylated metabolites by SULT, UGT, and COMT, which along with the glycoside or aglycone form may enter the portal vein.

2.5.2.1. Effect of Food and Enzymes on Anthocyanin Metabolism

Other studies have focused on the effects of digestive enzymes and food composition on bioavailability, absorption and metabolism of anthocyanins (reviewed by Bohn, 2014). Dietary factors such as fiber, divalent minerals, and protein-rich meals decrease polyphenol bioavailability. Flavonoids may decrease phase II metabolism and excretion and thus enhance polyphenol bioavailability. High dietary fat and MM may increase absorption, trans epithelial transportation and bioavailability of polyphenols. In contrast polyphenols may bind to digestive proteins, limiting lipolysis and increasing intestinal bulk and thus reducing availability of polyphenols (Bohn, 2014).

Additionally, several studies have investigated the effect of food on absorption and bioavailability of anthocyanins (reviewed by Pojer et al., 2013; Prior and Wu, 2006). Alcohol, for example, decreases absorption and transport of anthocyanins or only slows down the absorption rate without affecting the bioavailability. Total urinary anthocyanin excretion following a similar single dose of anthocyanin from red wine and red grape juice was significantly higher in the latter. However, Faria et al. (2009), suggested ethanol may increase both absorption and transportation of anthocyanins through intestinal epithelial cells.

2.5.2.2. Effect of Anthocyanin on Intestinal Microbiota and of Gut Microbiota on Anthocyanin Metabolism

Most studies investigating the effect of anthocyanins on gut health have been limited to the effect of these compounds on gut microbiota and how intestinal microbiota and other endogenous factors or exogenous factors such as the food matrix may affect anthocyanin degradation, metabolism and bioavailability. Several studies have shown that polyphenols modulate the gut microbiota balance by increasing growth of beneficial bacteria and inhibiting pathogen bacteria and thus help maintain a healthy gut (Cardona et al., 2013; Moraise et al., 2016). Anthocyanin-rich berries have been shown to increase bacterium species such as Bifidobacterium (Morais et al., 2016) and have a cytotoxic effect on *Helicobacter pylori* (Zafra-stones et al., 2007). In a review article, Morais et

al. (2016) mention anthocyanins to reduce inflammation associated with intestinal microbiota and LPS, which has a key role among modification of gut microbiota, intestinal permeability and inflammation.

Anthocyanins are also metabolized to anthocyanidins by *Bifidobacterium sp.* and *Lactobacillus sp.* through β-glucosidase which may affect colonization of these bacteria in the intestine and induce an anti-inflammatory effect (Morais et al., 2016). These effects may result in improved intestinal barrier and decreased translocation of bacterial toxins such as LPS to the blood. Thus, anthocyanins by modulating intestinal microbiota may act as therapeutic agents in controlling inflammation that may lead to chronic diseases.

Anthocyanins are extensively modified by intestinal microflora and the glycoside moiety is hydrolyzed within 20 min to 2 h of incubation with microflora depending on the type of the sugar group (Pojer et al., 2013). Some studies show that anthocyanins reaching the colon are extensively biotransformed by the gut microflora, decreasing bioavailability of the anthocyanin but providing potentially bioactive degradation products. For example, Keppler and Humpf (2005) showed that gut microflora hydrolyzes the 3-glycoside moiety of anthocyanin, causing the conversion of anthocyanins to aglycones, which are then converted to phenolic degraded products followed by methylation of the phenolic acid and aldehyde derivatives (Keppler & Humpf, 2005). According to this article, these derivatives may produce the protective effect of anthocyanins (Keppler and Humpf, 2005). In a study by Hanske et al. (2013), human microbiota associated (HMA) rats in comparison with germ free (GF) rats had 3 times higher feces excretion of C3G phenolic degradation products and selected human gut bacteria were shown to rapidly degrade C3G. These phenolic metabolites may undergo further metabolism by phase II enzymes in intestine, liver and kidney. Some of the antioxidant activity of anthocyanins may be due to these metabolites (Pojer et al., 2013).

2.5.3. Anthocyanin Localization in Mitochondria

Anthocyanins are of great interest because their chemical structure suggest that they could serve as mitochondrial membrane antioxidants. Anthocyanins are generally hydrophilic, but the polyphenolic structure creates a hydrophobic character to compounds and the aglycones are even

more hydrophobic. The logarithm of octanol-water partition coefficient is an indicator of hydrophobicity, the partition coefficients (K_{ow}) at pH 7.0 for several anthocyanins have been determined ranging from 0.21 for cyanine (cyanidin-3,5-O-diglucoside) and 25.54 for pelargonidin with the aglycone having a much higher value compared to the glycosylated form (Müller et al., 2005). This hydrophobicity of anthocyanin aglycones may drive the compound into the membranes of mitochondria. Considering the fact that the mitochondrial matrix has a pH value of 7.7, the pH of intermembrane space (IMS) is 6.8 (Santo-Domingo & Demaurex, 2012) and the pH of cytosol is 7.2, we hypothesize that the presence of anthocyanin in the acidic environment of the IMS creates some oxonium form of the anthocyanin aglycone, which would be pulled toward the matrix-side of the inner membrane by the mitochondrial membrane potential. Previous work in our laboratory (Peng, 2012) showed that cyanidin was taken up by rat liver mitochondria, partially dependent on the mitochondrial membrane potential, to a much greater extent than C3G, quercetin, or quercetin-3-glucoside. The presence of an anthocyanidin in the inner membrane creates with its free radical scavenging activity a protective action against damage that could affect mitochondrial activity and maintenance of the gut barrier.

2.5.4. Anthocyanins and Mitochondrial Biogenesis

To our knowledge studies on the effect of anthocyanins on mitochondrial biogenesis have been very limited. Lu and colleagues (2012) documented that 200 mg/kg body weight purple sweet potato color, rich in anthocyanins administered to mice promotes NRF-1 mediated mitochondrial biogenesis in the hippocampus of the mice without effecting PGC-1α expression. A recent study by Tang et al. (2015) showed purified anthocyanins from bilberry and black currant prevented depletion of mitochondrial content and damage of mitochondrial biogenesis in mice with non-alcoholic steatohepatitis (NASH) possibly through its effects on the AMPK/PGC-1α signaling pathways. Another study by Benn et al. (2014) showed black current extract increased energy expenditure and mitochondrial biogenesis related gene expression including PPAR-α and mitochondrial transcription factor A in obese mice. In these *in vivo* studies, however, it is not clear if the effects are due to anthocyanins themselves or their metabolites. In a study by Howitz et al. (2003), anthocyanins themselves did not activate SIRT1 but their phenolic acid derivatives activated this enzyme.

2.5.5. Protective Effects of Anthocyanins

An increasing body of evidence suggests that flavonoids and in particularly anthocyanins have beneficial impacts on a variety of human conditions. There are several conflicting hypotheses on how anthocyanins exert their protective effects, whether the properties are from the aglycone or glycosylated form, or from the metabolites or polyphenolic degradation products. As mentioned, according to Miyazawa et al. (1999), the glycoside form is responsible for the bioavailability of anthocyanins in the plasma of humans, with no conjugated metabolites or aglycones being detected. However, Cooke et al. (2006) indicate the effect of anthocyanins on intestinal adenoma may be partly due to the anthocyanidin form, as the aglycones and their glucuronide and methyl metabolites were detected in the intestinal mucosa. Regardless, anthocyanins in extracts or purified forms have been reported to confer many beneficial effects.

Anthocyanins have gained particular interest because of their antioxidant effects. Anthocyanins and especially C3G are strong antioxidants and several *in vitro* and *in vivo* models have shown protective effects of these compounds against damage induced by oxidative stress (Elisia and Kitts, 2008; Serraino et al., 2003; Cooke et al., 2006; Miyazawa, 1999). Other protective effects of anthocyanins include anticancer (Renis et al., 2008), inhibiting oxidative mitochondrial DNA damage, increasing antioxidant enzymes (Pervin et al., 2014), antimicrobial, anti-inflammatory (Chrubasik et al., 2010), chemoprevention, neuroprotection, cardiovascular protection, as well as amelioration of diabetes (Dai et al., 2009; Kong et al., 2003; Neto, 2007; Thomasset et al., 2009).

2.5.5.1. Antioxidant Effects of Anthocyanins

Reactive oxygen species are regularly produced in the body and they have a main role in immune function. However, excess ROS induces cellular damage and can lead to diseases such as inflammation, cancer, and cardiovascular disease. An imbalance between ROS generation and detoxification produces a condition of oxidative stress which can trigger cell damage, modify biological function, and ultimately cause cell death (Taha et al., 2010).

Despite their low absorption, anthocyanins are well known for their high antioxidant activity (Manach et al., 2005). These compounds have higher antioxidant activity compared with dietary nutrients such as vitamin C and E. The antioxidant activity of berries such as blackberries, red

raspberries, black raspberries and strawberries is directly proportionate to the anthocyanin content (Castaneda-Ovando et al., 2009). The antioxidant capacity of anthocyanins is mainly due to the phenolic structure. Anthocyanins are potent antioxidants that scavenge free radicals by donating or transferring electrons from hydrogen atoms to free radicals with unpaired electrons and acting as reducing agents in electron transfer reaction pathways and thus protect against oxidative damage (He and Giusti, 2010; Morais et al., 2016).

The antioxidant ability of anthocyanins depends on several factors such as the number of hydroxyl groups (Fukumoto and Mazza, 2000) on rings B and C, which are responsible for the radical scavenging activity of anthocyanins (Rice-Evans et al., 1996). Other factors include the oxonium ion on the C ring (which makes it a more potent and hydrogen−donating antioxidant), glycosylation, methylation, and acetylation. These two factors are mainly associated with the free radical scavenging activity of anthocyanins (Khoo et al., 2017).

In general, the strength of antioxidant activity of anthocyanins depends on the substitution groups on the B ring as follows −OH > −OCH3 >> −H (Rossetto and others 2007), and thus the potency of antioxidant activities is in the order of delphinidin > petunidin > malvidin = cyanidin > peonidin > pelargonidin (Rahman et al., 2006).

Some of the antioxidant effects of anthocyanins are direct such as scavenging free radicals and ferric reducing antioxidant activity (Giovanelli & Buratti, 2009) by donating a hydrogen atom or electron to reactive oxygen species. Other effects may be indirect such as increasing the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) and increasing glutathione (Pojer et al., 2013). Together these antioxidant effects inhibit oxidations such as lipid peroxidation (Rice-Evans et al., 1996; Mazza et al., 2002) and LDL oxidation (Heinonen, 2007).

The antioxidant activity of anthocyanins has been compared to well-known antioxidants in several papers. As Rice-Evans et al. (1996) and de Beer et al. (2002) have mentioned in their comparison of antioxidant activity of different polyphenols to trolox, anthocyanins have trolox equivalent antioxidant activity (TEAC) near the value of well-known antioxidants such as epicatechin-3-gallate and epigallocatechin-3-gallate, which is approximately 5 times that of trolox. Also, the

antioxidant activity of 3-glucosides of delphinidin, petunidin, and malvidin, were shown to be 2 to 2.5 times that of ascorbic acid (Garcia-Alonso, and others 2004). It has been shown by Fukumoto and Mazza (2000) that a higher number of hydroxyl groups on rings B and C in anthocyanins results in increased antioxidant and radical scavenging activity (Rice-Evans et al., 1996; Hou et al., 2003; Hou et al., 2004) whereas glycosylation decreases antioxidant activity of anthocyanins (Fukumoto & Mazza, 2000). This may be due to the fact that glycosylation reduces the ability for delocalization of electrons (Mazza et al., 2002).

The mitochondrial electron transport chain (ETC) is a major source of ROS, and superoxide ($O_2^{\bullet-}$) is the primary ROS produced. In the normal and steady state, there is only a minimum leakage of electrons from the ETC complexes. However, during stress conditions only a fraction of electrons reach complex IV from complex III. Leakage of electrons to oxygen results in formation of $O_2^{\bullet-}$ or hydroperoxyl radical ($HO_2^{\bullet}$) which mainly takes place from Q_i and Q_o sites of complex III which results in release of superoxide both into the mitochondrial matrix and into the IMS (Anand & Tikoo, 2013)), with the later a site of superoxide protonation to form hydroperoxyl radical. Anthocyanins react with both superoxide or hydroperoxyl radical (Alov et al., 2015) and thereby decrease lipid peroxidation of the inner membrane and inhibit the disruption of the membrane, preventing cytochrome C release to the cytosol and apoptosis. Since protons are abundant in the IMS, protonation of superoxide to hydroperoxyl radical ($O_2^{\bullet-} + H \rightarrow HO_2^{\bullet}$) might take place. The hydroperoxyl radical is more likely to react with lipids both because of its hydrophobicity compared to superoxide which is hydrophilic and its higher reactivity in comparison to superoxide. Whether anthocyanins react with $O_2^{\bullet-}$ or $HO_2^{\bullet}$, lipid peroxidation can be prevented. In addition, $O_2^{\bullet-}$ in the mitochondrial matrix inhibits aconitase (a TCA enzyme that catalysis conversion of citrate to isocitrate) (Longo et al., 2000), which disrupts the TCA cycle and interferes with the production of TCA-derived NADH, the key reductant to the mitochondrial electron transport chain. However, Mn-SOD a superoxide dismutase in the mitochondria matrix protects against mitochondria-derived superoxide/hydroperoxyl radial by converting superoxide to hydrogen peroxide which is then converted to water and oxygen via catalase (Fukai & Ushio-Fukai, 2011). Anthocyanins can increase Mn-SOD and other antioxidant enzymes (Bártíková et al., 2013; Khoo et al., 2017; Li et al., 2014b), which represents another mechanism by which they help to prevent mitochondrial damage that adversely impacts mitochondrial efficiency.

Investigations in several *in vitro* models have demonstrated protective effects of anthocyanins against damage induced by oxidative stress (Elisia and Kitts, 2008; Serraino et al., 2003). Elisia and Kitts (2008) studied the protective effects of anthocyanins in crude blackberry extract against peroxyl radical-induced oxidative damage and cytotoxicity in Caco-2 cells. Anthocyanin pretreatment at concentrations of 0.02–50 μg/mL were not toxic, decreased ROS in a dose dependent manner and reduced apoptosis. The oxidant scavenging effect was mostly due to C3G. Another example by which anthocyanins reduce oxidative stress, is via inhibition of lipid peroxidation which was shown in liver microsomes (Narayan et al., 1998).

Another study (Bornsek et al., 2012) investigated antioxidant effects of anthocyanins in crude bilberry and blueberry extract in different mammalian cell lines such as human epithelial colon cancer (Caco-2), human hepatocarcinoma (HepG2), human vascular endothelial (EA.hy926), and rat vascular smooth muscle (A7r5) cells. Anthocyanin rich bilberry and blueberry extracts (0.25-50 μg/L anthocyanin equivalents) decreased ROS in all cell types. In this study, very low amounts of anthocyanin (1 nM) used in these *in vitro* models, were comparable to the concentrations found in plasma after achievable oral doses and showed antioxidant activity (confirming the high potency of the antioxidant bioactivities of these compounds). In this study, anthocyanin rich bilberry extract showed higher antioxidant effect compared to anthocyanin rich blueberry extract, consistent with the higher concentration of anthocyanins in bilberry versus blueberry fruit. The difference in potency may also be due to higher proportions of cyanidin and delphinidin in bilberries, whereas blueberries have higher amounts of malvidin (Bornsek et al., 2012). In addition, several *in vivo* studies have been conducted to investigate antioxidant activity of anthocyanins. Pervin et al. (2014) showed that oral consumption of grape skin anthocyanin (GSA) at 50 mg/kg body weight for 30 days increased SOD, CAT and GPx in serum, liver and brain in female BALB/c mice. In another *in vivo* study in vitamin E-deficient rats (Ramirez-Tortosda and Andersen, 2001), an anthocyanin containing diet increased plasma antioxidant capacity and lowered hepatic lipid hydroperoxides and 8-hydroxy-2-deoxyguanosine, as compared to the untreated group. In a study on indomethacin-induced gastric mucosa damage in rats (Valcheva-Kuzmanova et al., 2005), oral pre-treatment with anthocyanin rich juice from chokeberry (*Aronia melanocarpa*) ameliorated gastric oxidative stress (malondialdehyde levels) and mucosal lesions.

Antioxidant effects of anthocyanins have also been observed in humans. In a study by Mazza et al. (2002) a high fat meal with 1.2 g total anthocyanin from freeze-dried low bush blueberries, increased the antioxidant activity in the serum, which was correlated to the serum concentration of anthocyanins. From the total 25 anthocyanins present in glycosylated and acetylated forms, 19 were detected in the serum after oral ingestion. Also, in a study by Weisel et al. (2006), consumption of an anthocyanin/phenolic-rich mixed berry juice for 4 weeks decreased several indices of oxidative stress in plasma and urine of human subjects.

The major anthocyanin metabolites and naturally occurring phenolic compounds PCA and THB, have also shown antioxidant and ROS scavenging properties in different *in vitro* studies by decreasing lipid peroxidation and increasing the scavenging of hydrogen peroxide (H_2O_2), inhibiting superoxide ($O_2^{\bullet-}$) and H_2O_2 production, restoring glutathione (GSH) related enzymes and by improving mitochondrial function and inhibiting DNA fragmentation (Semaming et al., 2015). Similarly, *in vivo* studies have revealed the antioxidant effect of PCA by inducing endogenous antioxidant enzymes, and decreasing ROS formation in liver, heart, kidney, and brain (reviewed by Semaming et al., 2015; Kakkar and Bais, 2014). Kim et al. (2006) compared the antioxidant activity of THB to 5 other antioxidants including α-tocopherol, rosmarinic acid, butylated hydroxytoluene, and butylated hydroxyanisole, concluding that THB had higher antioxidant activity. Huyut et al. (2017) also showed THB with IC_{50} value of 12.80 μg/mL had higher radical scavenging effect compared to flavonoid compounds.

2.5.5.2. Anti-Inflammatory Effect of Anthocyanins

Another well-known aspect of anthocyanins is their anti-inflammatory property (Reviewed by Pojer et al., 2013). Inflammation occurs as a natural response to injury, trauma and stimulants and is associated with initiation, progression and development of cancer. Inflammation occurs when cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandins and is characterized by vasodilation, increased capillary permeability, and migration of monocytes to the damage tissue. Anti-inflammatory effects of anthocyanins are revealed by inhibition of COX-2 and NF-κB activity (Pojer et al., 2013).

Several studies have shown anti-inflammatory effects of anthocyanins in several cell types. *In vitro* studies have shown that anthocyanins are able to suppress COX and NF-κB expression, decrease COX-1 and COX-2 activity and reduce MAPK activation (which directs the cellular response to stimuli such as mitogens and inflammatory cytokines) and inhibit prostaglandin synthase (reviewed by Pojer et al., 2013). In Caco-2 BBe1/THP-1 co-culture cell model, anthocyanins of purple carrot and potato, inhibited IL-8 and TNF-α secretion and NF-κB, and MAPK mediated inflammatory cellular signaling cascades (Zhang et al., 2017). *In vivo*, blueberry anthocyanins have shown to decrease inflammatory parameters, and red wine anthocyanins to inhibit TNF-α induced inflammation in human endothelium (reviewed by Pojer et al., 2013). Piberger et al. (2011) showed protective effects of supplementation of anthocyanins in an acute and chronic model of colitis in Balb/c mice. In this study, oral administration of anthocyanin-rich bilberry extract reduced secretion of IFN-γ and TNF-α from mesenteric lymph node cells and decreased cytokine secretion, inflammation, and apoptosis in colonic epithelial cells. IFN-γ can decrease AMP-activated protein kinase (AMPK) and induce disruption of epithelial barrier function (Scharl et al., 2009). In another study, Pereira et al. (2017) showed anthocyanin-rich fraction obtained from Portuguese blueberries (*Vaccinium corymbosum* L.) for 8 days, induced anti-inflammatory properties via inhibition of COX-2 expression and down regulation of iNOS and increased antioxidant defenses in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis rat model. As a result, anthocyanins, by decreasing inflammatory cytokines may have promising therapeutic effects in patients with inflammatory bowel disease.

Other studies have also shown the anthocyanin degradation product PCA to inhibit inflammation in different tissues. Several studies have shown PCA to inhibit inflammation *in vitro* and *in vivo* by regulating NF-κB and MAPK activation and lowering inflammatory cytokines (reviewed by Semaming et al., 2015; Kakkar and Bais, 2014).

2.5.5.3. Protective Effects of Anthocyanins in Intestinal Cells

Considering that anthocyanins act both as antioxidants and anti-inflammatory agents, these compounds may offer benefit in a wide variety of diseases associated with oxidative stress and inflammation including those observed in the intestine. Most studies on the beneficial effects of these compounds have focused mainly on the anti-inflammatory effect in intestine related diseases

in concentrations higher than what we have applied (Abouaf-Tabet et al., 2014). Also, as mentioned earlier, anthocyanins modulate growth of beneficial intestinal bacteria acting as prebiotics (Faria et al., 2014). Moreover, the biotransformation of anthocyanins by gut microbiota into metabolites that may have greater or different biological activity and bioavailability may contribute to health benefits of these compounds to the host (Ozdal et al., 2016).

Little information is available on protective effects of anthocyanins on diseases related to the intestine and damage induced by excess fat intake such as gut permeability and dysfunction. In a review by Yang et al. 2017, several mechanisms by which polyphenols and anthocyanins may induce protective effects in intestinal epithelial cells were proposed. Possible regulatory pathways include inhibition of nuclear factor-kB (NF-kB) signaling, inhibition of mitogen-activated protein kinases (MAPK), and reduction of ROS (Yang et al. 2017). As shown in the study of Li et al. (2014a), an anthocyanin rich extract from raspberries inhibited the NF-κB signaling and MAPK in a dextran sulfate sodium (DSS)-induced colitis mouse model and improved barrier function.

A few articles have reported protective effects of anthocyanins in differentiated or undifferentiated Caco-2 cells. Using undifferentiated Caco-2 cells, a purified anthocyanin extract from blackberries was shown to suppress peroxide radical-induced intracellular ROS and apoptosis (Elisia and Kitts, 2008). In a study with differentiated and undifferentiated Caco-2 cells (D'evoli et al., 2010), an extract from anthocyanin-rich strawberries, to a greater extent than conventional strawberries, significantly increased the antioxidant activity in the membrane fraction of differentiated Caco-2 cells, and inhibited proliferation of undifferentiated cells. In another study by these authors (D'evoli et al., 2013), an anthocyanin-rich extract from red chicory leaf increased membrane antioxidant activity and protected against t-butyl hydroperoxide-induced intracellular ROS generation in differentiated Caco-2 cells, and inhibited proliferation of undifferentiated cells. In a study on monolayer barrier integrity of differentiated Caco-2 cells (Cremonini et al., 2017), pure glucosides of cyanidin and delphinidin (but not of malvidin, peonidin and petunidin) as well as anthocyanin-rich extracts from different berries protected against TNF-α induced monolayer barrier permeability, and inhibited NF-κB activation and myosin light chain phosphorylation.

2.5.5.4. Protective Effects of Anthocyanins against Cancer

Another aspect of anthocyanin protection is the effect against cancer through several mechanisms (reviewed by Pojer et al., 2013) such as anti-angiogenesis, antiproliferation (selectively on cancer cells), induction of apoptosis, cell cycle arrest, inhibition of DNA oxidative damage, induction of phase II enzymes, and inhibition of COX-2 enzyme. Anthocyanidins inhibit cell proliferation better than anthocyanins by inhibiting the MAPK pathway (Pojer et al., 2013). Anthocyanins induce apoptosis both by the extrinsic pathway (inducing Fas and Fas ligand) and the intrinsic pathway (decreasing the mitochondrial membrane potential and increasing release of cytochrome C) in cancer cells. Most studies however, have used higher concentrations of anthocyanins for this purpose (Prior and Wu, 2006; Pojer et al., 2013)

A few *in vivo* and *in vitro* experiments on anticancer effects of anthocyanins have focused on cancer in the GI tract as this organ receives high concentrations of anthocyanins. Cooke et al. (2006) showed Mirtoselect (bilberry anthocyanin extract) or C3G decreased the number of intestinal adenomas in mice. In another study, Renis et al. (2008) showed that cyanidin (Cy) and C3G (25-200 µM) for 24 h induced an anticancer effect via ROS dependent and ROS independent activities respectively in Caco-2 cells.

2.6. Resveratrol

Another polyphenol of interest from grapes and some other berries is resveratrol. Resveratrol (3,5,4'-trihydroxystilbene) was first discovered by Mitio Takaoka (1940) who obtained resveratrol from the roots of Veratrum Grandiflorum. Plants containing resveratrol have been used medicinally for over 2000 years (reviewed by Gambini et al., 2015). This molecule consists of two phenolic rings attached by a double bond, forming the cis and trans isoforms of resveratrol (Figure 1.3). The trans form is the most stable and naturally abundant form. Resveratrol is produced in plants as a mechanism to resist stresses such as parasite and fungal infection, and UV irradiation (Gambini et al., 2015).

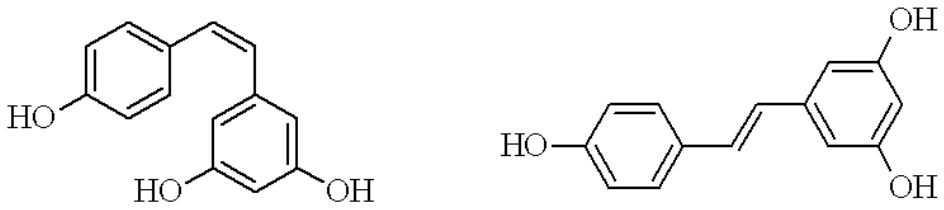


Figure 2.4. *Chemical structures of cis- and trans-resveratrol.*

2.6.1. Amount of Resveratrol in Food

Numerous sources of resveratrol have been described, several of which are found in our diet. Grapes, peanuts, mulberries, and blueberries are all-natural sources of resveratrol (Aggarwal & Bhardwaj 2004). For most people this polyphenol is associated with red wine. This compound was made popular by the suggestion that it may increase longevity, and contribute to the French paradox, a phenomenon, in which French people despite consuming a high fat diet (mainly saturated), have a relatively low rate of heart disease. The concentration of resveratrol varies greatly among the different sources. The concentration of trans-resveratrol (trans-3,5,4′-trihydroxystilbene) in red wine is reported to be as high as 20 mg/L (Waterhouse and Teisse`dre, 1997). The amount of the glucoside form of trans-resveratrol, called trans-piceid, may be more than 10 times the aglycone form in red wine (Ribeiro de Lima et al., 1999). The concentration of resveratrol in several sources is presented in table 1.1.

Table 2.1. Concentration of resveratrol in different sources.

Source	**Concentration of resveratrol**	**Reference**
Red wine	Non-detectable levels to 62.65 nmol/L (average of 8.3 ± 7.4 nmol/L)	(Stervbo et al., 2007)
Italian red wine	8.63 to 24.84 μmol/L	(Wang et al., 2002)
Concord grape products	1.560 to 1042 nmol/g	(Wang et al., 2002)

Peanuts	130 to 613 pmol /g	(Sanders et al., 2000)
Highbush blueberries from Michigan	140.0 +/- 29.9 pmol/g	(Lyons et al., 2003)
Bilberries from Poland	71.0 +/- 15.0 pmol/g	(Lyons et al., 2003)

2.6.2. Absorption and Metabolism of Resveratrol

There are many studies on the effect of resveratrol in many health aspects; however, the mechanism by which health effects are induced are not fully understood, possibly because our knowledge of the metabolic fate is incomplete. Resveratrol is rapidly absorbed and metabolized to different metabolites with different biological activities. Thus, understanding the extent of metabolism of resveratrol is necessary to understand the role of resveratrol.

Several studies have investigated absorption of resveratrol *in vitro* and *in vivo*. Trans-resveratrol is highly absorbed by Caco-2 cells and the absorption increases in a concentration dose-dependent manner (Kaldas et al., 2003; Teng et al., 2012). In Caco-2 cells, trans-resveratrol is transported via passive diffusion due to the small and non-polar structure of this molecule (Walle et al., 2006) and the glycosidic form (trans-piceid) uses an energy dependent pathway. Therefore, the uptake and absorption of trans-resveratrol from the apical membrane is faster than trans-piceid. Although the carrier for trans-piceid is not yet known, sodium-glucose transporter 1 (SGLT1) does not seem to be involved in the transport of this compound. Some articles (Planas et al., 2012) have indicated resveratrol is transported through intestinal cells via ATP-dependent binding cassette (ABC) transporters.

In Caco-2 cells, transpiceid is deglycosylated to trans-resveratrol after 30 min of incubation, and conjugation (glucuronidation, sulfation) is associated with the amount that is taken up by the cell (Henry et al., 2005). Efflux data for Caco-2 cells shows higher amount of trans-resveratrol present in the cells compared to trans-piceid, and multidrug-related protein 2 (MRP2) located on the apical side of cells seems to be involved in their efflux. The absorption of resveratrol by Caco-2 cells is direction independent and mainly through transepithelial diffusion. Transport of resveratrol was nonlinear with time, which suggests resveratrol being metabolized. Two metabolites of the phase

II biotransformation enzymes were detected in Caco-2 cells, resveratrol-3-sulfate and resveratrol-3-glucuronide, the former being produced to a greater extent (Walle et al., 2004).

Some other studies have looked at absorption and metabolism of resveratrol. In a study by Wenzel et al. (2005), perfusion of resveratrol in the small intestine of rat showed that about 46% of resveratrol remains in the lumen whereas 2% is located in the enterocytes and 21% reached the vascular side. The majority of resveratrol at the vascular side is the glucuronide form and most of the sulfate conjugate is secreted to the lumen. However, Teng et al. (2012) showed after first pass metabolism in the intestine, most of the resveratrol was detected as sulfated metabolites compared to glucuronidated forms, and the majority of these metabolites were present in the vascular side of the intestine.

Human studies on resveratrol absorption have also been conducted. In one study, human subjects received an oral dose of 25 mg (110 μM) and a 0.2 mg (0.8 μM) intravenously (IV) of ^{14}C-labled resveratrol to determine the absorbance, bioavailability and metabolism of the molecule (Walle et al., 2004). Results showed resveratrol was highly absorbed orally (approximately 70%) but underwent metabolism to a great extent and only a trace amount of resveratrol was found intact in the systemic circulation. The plasma concentration of resveratrol metabolites after a single oral dose was 2 and 1.3 μM after 1 and 6 h, respectively. The bioavailability of intact resveratrol was almost zero due to metabolism of this compound.

In general, the oral absorption of resveratrol is 75% in humans (reviewed by Walle, 2011; Gambini et al., 2015). Transport in the intestine occurs by transepithelial diffusion for resveratrol and via transmembrane transporters for resveratrol metabolites (Walle, 2011). After deglycosylation of piceid, resveratrol is metabolized in the colon by gut bacteria to dihydroresveratrol which is partially absorbed or further metabolized and excreted via urine (Gambini et al., 2015). Despite high absorption of resveratrol, the bioavailability of intact resveratrol to the systemic circulation is quite low (Walle, 2011).

First pass metabolism (small intestine and liver) is involved in deglycosylation and conjugation of resveratrol (Németh et al., 2003). The glycoside moiety is cleaved via β-glucosidase in human small intestine or liver (Day et al., 1998). Resveratrol is then metabolized by phase II enzymes in

the liver to its metabolites which may be recycled to intestine via enterohepatic circulation. Major metabolites of resveratrol as mentioned include the glucuronide and sulfate forms (Figure 1.4) (Gambini et al., 2015). Resveratrol is found in three forms (glucuronide, sulfate or free) in the systemic circulation; the free form is attached to LDL or albumin which allows resveratrol to enter target cells that have receptors for these molecules (Delmas et al., 2011).

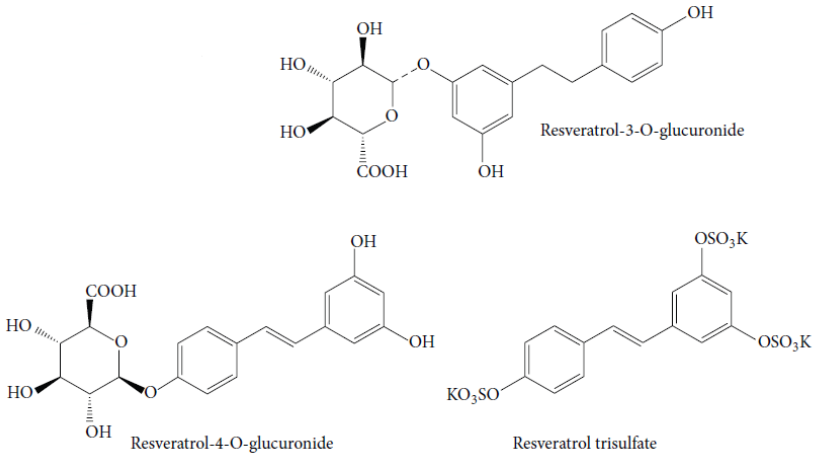


Figure 2.5. *Resveratrol metabolites.*

2.6.2.1. Effect of Food on resveratrol

Cells of the intestinal tract are likely exposed to unmetabolized resveratrol while the metabolites of resveratrol reach cells of other tissues. A few studies have investigated the effects of food on resveratrol absorption. In one study, the bioavailability of resveratrol from red wine was not different when consumed with a low or high fat diet (Vitaglione et al., 2005). Vitaglione et al. (2005) mention the type of food does not impact resveratrol bioavailability but rather the inter-individual variability in absorption is the main factor that determines its bioavailability. One study showed that when consumed with food, the absorption of resveratrol supplement was delayed but not reduced (Vaz-da-Silva et al., 2008). Another study on resveratrol supplement absorption showed that alcohol or quercetin had no effect, but fat decreased the bioavailability of resveratrol (la Porte et al., 2010). More studies on impact of dietary factors on resveratrol absorption and bioavailability should be conducted.

2.6.2.2. Effects of Gut Microbiota on Resveratrol

While resveratrol is mainly metabolized in intestinal and hepatic cells, intestinal microbiota has also been shown to metabolize resveratrol. The study by Bode et al. (2013), indicates the bioconversion of trans-resveratrol, mainly by two intestinal microbiota (*Slackia equolifaciens* and *Adlercreutzia equolifaciens*), into three metabolites of trans-resveratrol, and suggests that different metabolites produced may have different physiological activities. The three trans-resveratrol metabolites detected were dihydroresveratrol, 3,4′-dihydroxy-trans-stilbene and 3,4′-dihydroxybibenzyl (lunularin).

2.6.3. Resveratrol and Mitochondrial Biogenesis

One of the most important reported effects of resveratrol is its ability to increase mitochondrial capacity. Several studies suggest different mechanisms by which resveratrol acts to increase mitochondrial biogenesis through activation of PGC-1α. PGC-1α is a key regulator of mitochondrial biogenesis and function (Howitz et al., 2003; Price et al., 2012, Denu, 2012). PGC-1α also has an important role in regulating metabolism by controlling expression of genes involved in oxidative phosphorylation, fatty acid oxidation, lipid transport, and gluconeogenesis and thus regulates cellular energy metabolism towards a more oxidative and less glycolytic state (Finck et al., 2006; Lee et al., 2006; Liang and Ward, 2006).

The first mechanism of resveratrol effects on mitochondrial biogenesis, is activation of PGC-1α through SIRT1, which among other targets, deacetylates PGC-1α. Among polyphenols that activate SIRT1, resveratrol is reported to be the most potent (Howitz et al., 2003; de Boer et al., 2006; Lagouge et al., 2006). Csiszar et al. (2009) demonstrated that 10 μM resveratrol activates SIRT 1 and increases the content of mitochondria in cultured human coronary arterial endothelial cells. According to Kelkel et al. (2010), resveratrol strongly affects SIRT1 and although Denu et al. (2012) argue there are some doubts in this area, Price et al. (2012) verified the role of SIRT1 in mediating the effects of resveratrol on mitochondria. Resveratrol also increases mitochondrial biogenesis via activation of AMP-activated protein kinase (AMPK), which activates PGC-1α through phosphorylation (Price et al., 2012, Denu, 2012). This kinase is a sensor of low ATP levels, and is an important factor in revealing functions of resveratrol. Although some studies have

suggested a direct effect of resveratrol on AMPK and an indirect effect via SIRT1, Lan et al., (2017) have implicated resveratrol SIRT1 activation in producing AMPK activation. In any case, PGC-1α is activated (Denu, 2012) which orchestrates the program to expand mitochondria. Finally, another mechanism in which resveratrol activates PGC-1α to induce mitochondrial biogenesis, is through nitric oxide (NO) (López-Lluch et al., 2008). Resveratrol induced endothelial NO synthase (eNOS) through activation of SIRT1 has shown to increase mitochondrial biogenesis in endothelial cells (Szabo, 2009). There are controversies in molecular mechanisms of resveratrol health benefits. The debate whether resveratrol affects SIRT1 directly or through AMPK still exists (Hu et al., 2011) and also the mechanism in which SIRT1 is involved in resveratrol induced activation of AMPK is unknown. Mechanisms of *in vivo* effects of resveratrol in regard to the role of SIRT1 in different diseases are areas for further study (Chung et al., 2010). It was proposed by Howitz et al. (2003) that effects of polyphenols such as resveratrol may be because they mimic the energy deficiency response mediated by sirtuins similarly that produced by calorie restriction.

Regardless of the pathway by which resveratrol exerts its effects this polyphenol increases mitochondrial mass, mitochondrial DNA content, and components of the respiratory chain as well as inducing nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Szabo, 2009). Resveratrol also modulates mitochondrial activity by changing the fuel source from glucose to lipid (López-Lluch et al., 2008).

2.6.4. Protective Health Benefits of Resveratrol

Resveratrol has been known for its wide range of bioactivity and the aglycone form seems to have greater biological effect compared to the glycoside (Varache-Lembe`ge et al., 2000). As mentioned earlier, resveratrol is well known for its effect in increasing mitochondrial biogenesis (Denu, 2012; Howitz et al., 2003; Csiszar et al., 2009). Other properties of resveratrol include antioxidant (Rubiolo et al., 2008), anti-inflammatory (Yu et al., 2008; Pearson et al., 2008) and chemopreventive effects (De Maria et al., 2013; Gambini et al., 2015). In addition to inducing mitochondrial biogenesis, mechanisms behind health benefits of resveratrol include antioxidant, modulating lipoprotein metabolism, inhibiting platelet aggregation (Soleas et al., 1997) and acting as a vasorelaxing agent (Jager & Nguyen-Duong, 1999).

2.6.4.1 Antioxidant Effects of Resveratrol

Oxidative stress is an underlying cause of many diseases such as diabetes, cardiovascular disease and aging. Recently, natural antioxidants have been studied extensively. Resveratrol as an antioxidant molecule and protectant agent against oxidative stress, inflammation, and cancer has gained major attention. Resveratrol decreases oxidative stress through several mechanisms.

Resveratrol may induce antioxidant effects by increasing antioxidant enzymes. Rubiolo et al. (2008) showed increased antioxidant enzyme levels including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S transferase (GST), and NADH:quinone oxido-reductase in primary hepatocyte culture after 24 h (less so after 48 h). Also, NRF-2, a transcriptional factor that regulates expression of antioxidant and phases II enzymes, was increased in the cells by resveratrol at a concentration of 50 µM. Ingles et al. (2014) also showed dietary relevant concentrations of resveratrol (1, 20, 100, 500 nM) for 48 h also decreased H_2O_2 by inducing catalase and manganese superoxide dismutase (Mn-SOD) in MCF-7 cells through the PTEN/Akt pathway.

As a direct-acting antioxidant, resveratrol has moderate activity. Sobotková et al. (2009) indicated that resveratrol has the same amount of antioxidant power as trolox in scavenging hydroxyl radical during platelet aggregation. In another measure of trolox-equivalent antioxidant activity (TEAC), resveratrol was twice as potent as trolox, but less than half as potent as quercetin or cyanidin (Rice-Evans et al., 1996). Moreover, Çelik et al. (2010) showed that during plasmid DNA damage induced by idarubicin, resveratrol unlike quercetin had little protective effect. Some other studies have shown direct antioxidant effects of resveratrol. For example, resveratrol at 0.1 mM for 30 min, partially decreased lipid peroxidation and nitration in human blood platelets treated with 0.1 mM peroxynitrite (Olas et al., 2008), and resveratrol at 10-30 µM attenuated ROS and H_2O_2 levels in aortic smooth muscle cells treated with oxidized LDL (Liu and Liu, 2014). However, most studies showing direct antioxidant effects of resveratrol require relatively high concentrations.

A few *in vitro* studies have demonstrated the protective effects of resveratrol by decreasing mitochondrial ROS generation either directly (Zini et al., 1999) or by activating SIRT1 (Xu et al., 2012). In this regard, Xu et al. (2012) showed protective effects of resveratrol on mitochondria in high glucose (30 mM) induced oxidative damage in mesangial cells. Pretreatment with 10 µM

resveratrol reversed the decrease in mitochondrial complex III activity, decreased mitochondrial ROS generation, and stimulated mitochondrial SOD (Mn-SOD) activity. Resveratrol also increased ATP production, protected mtDNA, and decreased hyperpolarization of the mitochondrial membrane potential ($\Delta\Psi m$) all by activating SIRT1 in these cells. In a study on isolated rat brain mitochondria, Zini et al. (1999) found that resveratrol is capable of dose-dependently decreasing the superoxide anion production, and to inhibit complex III activity by competing with coenzyme Q.

A few studies have shown antioxidant effects of resveratrol in animal models. Protective effects of intraperitoneally injected resveratrol were observed against secondary spinal cord injury in rats (Yang and Piao, 2003) and of intravenously injected resveratrol from ischemia reperfusion-induced spinal cord injury in rabbits (Kiziltepe et al., 2004). In a study with rats, oral delivery of resveratrol for 3 weeks was able to prevent muscle and bone alteration after disuse by inhibiting oxidative stress, decreasing lipid peroxidation and increasing antioxidant enzymes (Jackson et al., 2010).

2.6.4.2 Anti-Inflammatory Effects of Resveratrol

Several studies have indicated beneficial effects of resveratrol against inflammatory cytokine production (Sun et al., 2006; Pearson et al., 2008; Sánchez-Fidalgo et al., 2010). For example, 24 h resveratrol treatment at 10, 50 and 100 μM decreased TNF-α-mediated matrix metallopeptidase 9 (MMP-9) expression and invasion of human hepatocellular carcinoma cells. Resveratrol inhibited nuclear factor κB (NF-κB) signaling pathway in HepG2 cells, thus potentially preventing inflammation (Yu et al., 2008). A study by Fouad et al. (2013) also showed 48 h resveratrol (100 μM) treatment decreased leptin expression and increased insulin expression in Caco-2 cells. Leptin activates nuclear factor-kappa B (NF-κB) which induces damage to the epithelial monolayer and induces an immune response. In a human study (Tomé-Carneiro et al., 2013), daily consumption of 350 mg grape extract plus 8.1 mg resveratrol supplement for a duration of one year decreased peripheral blood mononuclear cells KLF2, NF-κB, Ap-1, JUN, ATF-2, and CREBBP (inflammation related transcription factors) and increased anti-inflammatory serum adiponectin in patients with coronary heart disease.

2.6.4.3 Protective Effect of Resveratrol in Intestinal Cells

Understanding the molecular mechanisms of resveratrol could offer promise in determining its use to prevent/control conditions such as increased intestinal permeability. Resveratrol has been shown to have inhibitory effects against intestinal permeability by several mechanisms such as alleviating the inflammatory response.

Studies about protective effects of resveratrol on intestinal cells (especially in regard to high fat diet) are very limited and most have investigated effects at high concentrations of this polyphenol (Cui et al., 2010a). In a study by Singh et al. (2010), resveratrol at a dose of 10, 50, and 100 mg/kg body weight given by oral gavage to C57BL/6 mice was protective against DSS-induced colitis, and also attenuated the increase of serum pro-inflammatory cytokines, increased lymphocyte SIRT1 expression, and decreased lymphocyte COX-2 expression, consistent with its anti-inflammatory effects. Another study (Garcia et al., 2012) revealed a possible protective effect of resveratrol in colon smooth muscle cells from Lewis rats (a model of Crohn's disease). Fifty and 100 μM resveratrol treatment for 24 h decreased the intestinal smooth muscle cell number, which was associated with an increased number of cells in the S phase, induction of cell cycle arrest and apoptosis. Thus, resveratrol by preventing the smooth muscle cell hyperplasia could be considered as a treatment for Crohn's disease (Garcia et al., 2012). In a study by Carrasco-Pozo et al. (2013) resveratrol was able to ameliorate indomethacin-induced disruption in Caco-2 cells and inhibited the increased permeability of the monolayer.

A few studies have investigated effects of resveratrol on Caco-2 cell differentiation. De Maria et al. (2013) showed 100 μM resveratrol or 240 μM polydatin (a glucoside precursor of resveratrol) inhibited proliferation and shifted undifferentiated Caco-2 cells to differentiated cells which then undergo programmed cell death. However, Wolter et al. (2002) showed resveratrol (50 μM) for 24 or 48 h did not affect differentiation of Caco-2 cells but intensified the differentiating effect of butyrate.

2.6.4.4 Protective Effect of Resveratrol Against Cancer

Resveratrol has been shown to be effective as a potential cancer therapeutic agent. Studies have shown resveratrol to confer anticancer effects in cultured human cancer cells from several tissues such as colon, skin, breast, prostate, liver and pancreas (reviewed by Walle et al., 2004).

A few studies have looked at the effects of resveratrol on colon cancer cells. Accumulation of resveratrol and its metabolites (mainly dihydroresveratrol) in intestinal epithelial cells may contribute to their chemopreventive effects in this site (Walle et al., 2004). De Maria et al. (2013) showed that resveratrol and its natural precursor (the glucoside piceid) together at concentrations of 100 and 240 μM for 24 h in Caco-2 cells acted synergistically to induce antiproliferative effects, induced oxidative stress and increase cell cycle arrest, differentiation, and apoptosis. Fouad et al. (2013) also showed anticancer effects of resveratrol on HCT116 and Caco-2 cells at half maximal inhibitory concentrations (IC_{50}) of 130 and 50 μM for 48 and 72 h, respectively. Resveratrol also decreased glycolytic enzymes (pyruvate kinase and lactate dehydrogenase) and increased citrate synthase, indicating that it induced the cancer cells to shift toward oxidative phosphorylation for energy production and ATP generation, as well as activating the intrinsic apoptosis pathway by increasing the Bax/Bcl2 ratio. Other metabolites, including resveratrol sulfate, may also contribute to the chemopreventive effect in other tissues such as the breast and prostate.

2.7. Synergistic Effect of Anthocyanins and Resveratrol

Many researchers have been interested in studying the synergistic effects of a combination of substances with complementary mechanisms, which may produce more efficient, beneficial and less toxic effects compared to that of an individual compound at high concentrations. Examples of research on combinations of chemopreventive and chemotherapeutic agents are those of synthetic drugs with functional foods (Suganuma et al., 2001; Murakami et al., 2003), and phytochemicals (Murakami et al., 2003; de Kok et al., 2008; Kowalczyk et al., 2010; Mikstacka et al., 2010). These studies investigate the additive or synergistic effects of these compounds.

Beneficial effects of resveratrol and anthocyanins have only been studied alone, but not in combination except in foods such as grapes and red wine that contain them both, among other compounds. Combination of polyphenols or other compounds present in foods at concentrations

that are both functionally important and achievable, could represent a practical means of preventing/ameliorating several diseases when compared to individual polyphenols in higher concentrations.

It should be noted that with dietary delivery of anthocyanins, the effects could be due to the parent compound or the metabolites such as protocatechuic acid or gallic acid (Forester et al.,2012), and the same concern also applies for resveratrol. However, this might be less of a concern in intestinal epithelial cells, as these cells are directly exposed to these polyphenols at µM concentrations, comparable to those of *in vitro* studies (Tsuda, 1999). Thus, the polyphenols may exert their beneficial effect in the intestine, where such concentrations are readily achievable.

Considering different effects of these polyphenols, they may have synergistic or additive beneficial effect on the gut barrier. Although they may act by different mechanisms, antioxidative and anti-inflammatory effects are major protective pathways of both polyphenols. A major difference in the activities of anthocyanins and resveratrol is in their effects on Sir2/SIRT1. As mentioned resveratrol is well known to activate SIRT1 and increase mitochondrial biogenesis (Han et al., 2007; Denu, 2012). However, Howitz et al. (2003) originally showed that unlike resveratrol, anthocyanins had no effect on or even inhibited SIRT1.

However, there are some gaps in this field that should be addressed, and more studies are needed to confirm and extend these potential mechanisms. Specific areas of interest for this study are to define the mechanisms by which resveratrol and anthocyanins act on mitochondria of Caco-2 cells to protect against fat induced oxidative stress and inhibit paracellular permeability of epithelial monolayers.

2.8. Transition to CHAPTER 3

In order to develop a suitable model for investigating protective effects of ARBE and resveratrol in MM-exposed Caco-2 cells, preliminary experiments were conducted to optimize for time and concentration of MM and polyphenol exposure. Details on the composition of the ARBE are shown in Appendix 8.A, and the results of the experiments are shown in Appendix 8.B.

In CHAPTER 3, we report investigations using this model on the abilities of ARBE and resveratrol to protect against MM-induced oxidative stress and to protect a Caco-2 cell monolayer against MM-induced permeability.

CHAPTER 3 COMPARISON OF ANTHOCYANINS AND RESVERATROL IN PROTECTING INTESTINAL CACO-2 EPITHELIAL CELLS AGAINST LIPID MICELLE-INDUCED OXIDATIVE STRESS AND MONOLAYER PERMEABILITY

3.1. Abstract

Excess dietary fat, and the bile acids used for their absorption, can impair intestinal barrier integrity which may lead to increased permeability, entrance of luminal contents and microbe derived-LPS, and inflammation. Dietary polyphenols in polyphenol-rich foods such as berries can reach the intestine in relatively high concentrations and may have important effects on intestinal epithelial cells to help prevent oxidative stress and strengthen the intestinal barrier. However, little is known about the relative protection of intestinal epithelial cells by different polyphenols, and the mechanisms involved. In a Caco-2 cell model of dietary fat-induced intestinal epithelial cell cytotoxicity, we investigated potential protective effects of anthocyanin-rich bilberry extract (ARBE) and resveratrol. Exposure of the Caco-2 intestinal epithelial cells to MM, composed of bile salts and fatty acids, increased intracellular and mitochondrial ROS generation, decreased cell viability, increased expression of mRNA for Mn-SOD and the pro-inflammatory cytokine TNF-α, and increased cell monolayer permeability. Treatment with ARBE or resveratrol at a concentration providing 20 μM polyphenol, strongly protected against the MM-induced rise in intracellular ROS generation and expression of Mn-SOD and TNF-α. ARBE but not resveratrol decreased mitochondrial superoxide generation. In experiments designed to assess barrier integrity, exposure to MM decreased transepithelial electrical resistance (TEER) of differentiated Caco-2 cells by approximately 46% after 3 h and by more than 50% after 9 h, indicating increased monolayer permeability. Resveratrol gave a short-term improvement up to 6 h following MM challenge, but then lost its protective effect beyond this time point. Protection by ARBE increased with time and by 9 h MM-induced impairment was largely reversed, with TEER values reaching 82% of control. This study showed that ARBE and resveratrol, at concentrations relevant to dietary intake, increase the ability of intestinal cells to combat oxidative stress and protect against intestinal cell monolayer permeability induced by MM. The results suggest a role for polyphenol rich foods in conferring

resistance to excess dietary fat through protection against oxidative stress in intestinal epithelial cells, with implications for health and disease in people consuming a Western diet.

Abbreviations

ANOVA, analysis of variance; **ARBE**, anthocyanin-rich bilberry extract; **ATP**, adenosine triphosphate; **cDNA**, complementary DNA; **CLA**, conjugated linoleic acid; **COX-2**, cyclooxygenase 2; **DCF**, 2',7'-dichlorofluorescein; **DCFH-DA**, 2',7'-dichlorodihydrofluorescein diacetate; **DMEM**, Dulbecco's modified essential medium; DMSO, dimethyl sulfoxide; **DNA**, deoxyribonucleic acid; **DTNB**, 5,5′-dithiobis (2-nitrobenzoic acid); **FBS**, fetal bovine serum; **FITC-dextran**, fluorescein isothiocyanate–dextran; **HBSS**, Hank's balanced salt solution; **IL-1β**, Interleukin1β; **iNOS**, Inducible nitric oxide synthase; **LDL**, low density lipoprotein; **LPS,** lipopolysaccharide; **MAPK**, mitogen-activated protein kinase; **MitoSOX**, mitochondrial superoxide; **MM**, mixed micelle; **Mn-SOD**, manganese-superoxide dismutase; **mRNA,** messenger RNA; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB**, nuclear factor kappa b; **NO**, nitric oxide; **OA**, oleic acid; **PA**, palmitic acid; **PCR**, polymerase chain reaction; **ROS**, reactive oxygen species; **RT-qPCR**, quantitative reverse transcription PCR; **SDC**, sodium deoxycholate; **SD**, standard deviation; **SIRT1**, sirtuin 1; **SRB**, sulforhodamine b; **TCA**, trichloroacetic acid; **TEER**, transepithelial electrical resistance; **TNF-α**, tumor necrosis factor-α**.**

3.2. Introduction

Among the concerns of a Western-style diet are detrimental effects of the high fat content on the intestinal epithelial cells and impairment of the gut barrier (Park et al., 2016; Y Lee, 2013; Hamilton et al., 2015). Disruption of the gut barrier and increased intestinal permeability may result in release of bacterial lipopolysaccharide (LPS) into the circulatory system, which can provoke inflammation and metabolic disease (Cani et al., 2007; Bischoff et al., 2014; Moreira et al., 2012; Murakami et al., 2016). Therefore, it is suggested that maintaining the function and integrity of the gut is an important factor in preventing Western diet-related diseases (Y Lee, 2013).

The effects of dietary fats on the intestinal microbiota and intestinal inflammation are well documented, in addition, dietary fats and bile acids, required to emulsify dietary fat in the small intestine, may trigger tumorigenesis and barrier dysfunction in the epithelial cells. Possible mechanisms by which dietary fats and bile acids produce intestinal epithelial cell cytotoxicity include increased oxidative stress and mitochondrial dysfunction. Although necessary for fat absorption, bile acids may be cytotoxic to intestinal cells producing intracellular oxidative stress and mitochondria-mediated apoptosis (Barrasa et al., 2013). High dietary fat intake may also produce mitochondrial changes and has been reported to increase mitochondrial workload in intestinal mucosa through an increase of proteins involved in mitochondrial β-oxidation, components of the respiratory chain and oxidative energy metabolism (Wiśniewski et al., 2014). The impact of this increased workload on intracellular and mitochondrial ROS generation however, is unknown in intestinal epithelial cells. Mitochondrial ROS can unfavorably impact mitochondrial efficiency such that it can no longer contribute to the ATP output needed to support the increased workload (Murphy, 2009). Together these changes may decrease intestinal cell viability and disrupt the intestinal barrier due to lack of sufficient ATP. Oxidative stress can also induce production of inflammatory cytokines (Hussain et al., 2016), leading to increased inflammation of the intestine.

Anthocyanins and anthocyanin-rich berries are especially known for their antioxidant and anti-inflammatory effects (Chrubasik and Chrubasik, 2010), and their presence in the intestinal tract at relatively high levels may confer protection to intestinal cells against oxidative stress. While many studies have investigated the interactions of anthocyanins with the gut microbiota (Morais et al.,

2016), only a few have studied the effect of anthocyanins on the function and integrity of intestinal epithelial cells. In one study (Li et al., 2014a) an anthocyanin rich extract from raspberries inhibited intestinal damage in a dextran sulfate-induced colitis mouse model and improved barrier function. In an *in vitro* model of celiac disease using Caco-2 intestinal epithelial cells challenged with a toxic wheat peptide, an anthocyanin rich extract protected against cytotoxicity and decreased production of inflammatory cytokines (Haggard et al., 2017). Another study showed that low concentrations (0.25-1 μM) of anthocyanins (O-glucosides of cyanidin and delphinidin) or 5 μg/mL of an anthocyanin-rich plant extract for 30 min inhibited tumor necrosis factor-alpha (TNF-α)-induced permeability of Caco-2 cells partly through inhibiting the NF-κB pathway (Cremonini et al., 2017).

Resveratrol is another polyphenol that is present together with anthocyanins in berries such as blueberries, bilberries and grape skin, and might confer protection to intestinal cells. For example, resveratrol may inhibit chemically induced cancer in the intestinal tract (Bishayee, 2009). Many studies in animal models and a few in humans have shown resveratrol to improve symptoms and state of inflammatory bowel disease, and to decrease leukocyte infiltration and inflammatory markers such as MAPK, NF-κB, and COX-2 (reviewed by Nunes et al., 2017, Shi et al., 2017, Abouaf-Tabet et al., 2014). Resveratrol has also been shown to suppress ulcerative colitis and colon cancer in mice, which is associated with a decrease in inflammation and inflammatory stress markers such as TNF-α (Cui et al., 2010). Another study showed resveratrol increased SIRT1 gene expression in immune cells of the colon in mice and reduced NF-κB activation and inflammation (Singh et al., 2010). In a study of mice with *Toxoplasma gondii*-induced acute ileitis, oral resveratrol treatment protected against acute inflammation in the small intestine by down-regulating the immune response, improved maintenance of intestinal barrier function, and prevented systemic bacterial translocation and mortality (Bereswill et al., 2010).

In the current study, we used Caco-2 intestinal epithelial cells exposed to MM prepared from fatty acids (oleic acid and palmitic acid) and bile acids as a model of dietary fat-induced intestinal stress to investigate the mechanisms involved and the possible protective effects of anthocyanin-rich bilberry extract (ARBE) and resveratrol. We therefore measured the effects of MM, ARBE and resveratrol on intracellular and mitochondrial ROS generation, expression of genes involved in oxidative stress and inflammation, cell viability, and epithelial monolayer permeability.

3.3. Materials and Methods

Dulbecco's modified essential medium (DMEM) and Hank's balanced salt solution (HBSS) were purchased from Life Technologies (Waltham, MA, USA). Fetal bovine serum, penicillin/streptomycin mixture, sodium oleate, sodium palmitate, sodium deoxycholate, phosphatidyl choline (egg, chicken), oxaloacetate (OAA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetyl CoA and resveratrol were obtained from Sigma-Aldrich (St. Louis, MO, USA). ARBE was kindly provided by Indena, Indena S.p.A. (Detail information about ARBE provided in Appendix 8.A). The ARBE contains 36% anthocyanins (w/w), and 20 mM stock solutions of ARBE calculated as mM cyanidin-3-glucoside (C3G) equivalents (eq) per g of dry extract and resveratrol were prepared in dimethyl sulfoxide (DMSO) and kept at -20 ^{o}C. RNAeasy mini kit for isolating RNA was purchased from Qiagen (MD, USA). VILO cDNA synthesis kit, Trizol reagent, and Power SYBR Green PCR master mix for the polymerase chain reaction were obtained from Invitrogen, (NY, USA). Caco-2 cells were obtained from the American Type Culture Collection (MD, USA).

3.3.1. Cell Culture Conditions

Cell cultures were maintained at 37 ^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Briefly Caco-2 cells were cultured in T-75 flasks Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25 mmol/L glucose, 2 mmol/L glutamine, 100 mmol/L nonessential amino acids, and 1% penicillin/streptomycin (100 units/mL penicillin, and 100 mg/mL streptomycin) as described by Natoli et al. (2012). When 70-80% confluent, Caco-2 cells were detached with trypsin, harvested and used for different experiments. Prior to an experiment, cells were seeded at 5-10$\times 10^4$ cells per well in 96 well plates and grown to confluence over 24 h in DMEM with 10% FBS at 37°C, 5% CO_2. Thus, cells are not proliferating but are in the process of differentiation, so are not considered fully differentiated cells. On the day of an experiment, the medium was replaced with DMEM containing 2% FBS.

FBS is a necessary component for optimal cell growth in *in vitro* cell culture media. However, the albumin in FBS may interact with lipids such as palmitate (Francis, 2010) and disrupt the micelle structure. Thus, different concentrations of FBS in DMEM were tested in order to find a suitable

concentration to prevent micelle disruption and provide proteins and growth factor for growth of cells as well as to provide optimal condition in which the treatments would induce their effects. 0 % FBS stressed the cells and at 10% FBS treatments were not effective. 2% FBS medium was adequate to prevent deprivation of Caco-2 cells from proteins and growth factors in FBS and also allow MM integrity and ability to affect the cells.

3.3.2. Mixed Micelle Preparation

In our study, MM were composed of fatty acids (oleic acid and palmitic acid- two major dietary fatty acids), phospholipid and deoxycholate. Similarly, in a study by Shilnikov et al. (1987) MM was formed by hydrophobic chain of fatty acids (C9 and more) being incorporated with sodium deoxycholate. To prepare MM, 1.4 mg sodium palmitate and 1.54 mg sodium oleate were dissolved in methanol, and 15 mg phosphatidyl choline dissolved in chloroform was added. The lipids were dried under nitrogen and then 12.4 mg of sodium deoxycholate dissolved in PBS was added to the mixture to obtain a ratio of lipid to sodium deoxycholate of 1:1. The mixture was rotated continuously in dry ice to freeze. The tube was then placed in a lyophilizer for 48 h, and dried samples were stored at -20 °C until the day of an experiment. On the day of the experiment 1 mL of the medium without FBS was added to the dried sample to produce 60 mM lipid and the content was aliquoted into 10 micro-tubes each containing 100 µL, and frozen for future experiments. For each experiment a final concentration of 0.4 mM lipids as MM was added to the cell culture medium.

3.3.3. Treatment with mixed micelles and polyphenols

Caco-2 cells seeded at 3-7×10^4 cells/well were cultured in 96-well optical-bottom plates and were grown to confluence in DMEM with 10% FBS for 24 h. A 20 mM stock solution of ARBE (considered as mM cyanidin-3-glucoside (C3G) equivalents (eq) per g of dry extract) and resveratrol were prepared in DMSO. On the day of an experiment, cells in 2% FBS media were pretreated for 2 h with ARBE (1.25, 2.5, 5, 10, 15, 20 µM), as per Sergent et al. (2005), or for 5 h with resveratrol (1.25, 2.5, 5, 10, 15, 20 µM), as per Xu et al. (2012). In order to avoid a toxic effect of DMSO, the final concentration of DMSO in the medium was at most 0.1% (for the highest concentrations of ARBE and resveratrol) and lower concentrations of ARBE and resveratrol were

made via serial dilution with DMEM to decrease the amount of DMSO exposure to cells. To control for any possible effects of DMSO, the highest concentration used in the preparation of the ARBE or resveratrol solutions (0.1%) was added to control cells. Thus, the control group was no treatment (no MM or polyphenols), plus the DMSO through all experiments. After pre-treatment with polyphenols, cells were challenged with 0.4 mM MM for 24 h in 2% FBS media. The effects of MM, resveratrol and ARBE were assessed as described in the following sections.

3.3.4. Measurements of intracellular reactive oxygen species (ROS) and mitochondrial superoxide generation

The levels of intracellular ROS were measured with the fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Aranda et al., 2013; Renis et al., 2008). This probe diffuses through the cell membrane to the cytosol where it is enzymatically hydrolyzed by intracellular esterases which cleave off the acetate groups converting it into DCFH and trapping it inside the cells. The probe is then oxidized in the presence of ROS (mainly by hydrogen peroxide (H_2O_2)) to fluorescent 2',7'-dichlorofluorescein (DCF). Caco-2 cells were seeded at 5-10×10^4 cells per well in a 96-well microplate in DMEM with 10% FBS and grown to confluence for 24 h. After treatments with MM and polyphenols, 20 μM DCFH-DA was added and the cells were incubated at 37 °C for 2 h. Then media was replaced with fresh DMEM and the fluorescence signal of each well was measured by a Synergy H1 plate reader (BioTek Instruments, VT, USA,) at excitation 488 nm, and emission 528 nm. The results are reported relative to control values. Data have been corrected for fluorescence from the probe added to DMEM with no cells and were normalized to adherent protein content estimated by SRB and expressed as percent of the control group.

Mitochondrial generation of superoxide was measured with MitoSOX Red. MitoSOX Red is a fluorogenic dye that targets mitochondria in live cells and when oxidized by superoxide, produces a red fluorescence signal. For measurement of mitochondrial superoxide by MitoSOX via plate reader, cells were incubated with 5 μM MitoSOX for 30 min at 37 °C in the dark, cells were then washed, and the fluorescence was measured at excitation and emission wavelengths of 510 and 580 nm. Antimycin A, added to control cells 30 min prior to incubation with MitoSOX, was used as a positive control. Data for MitoSOX experiments were normalized to SRB data and expressed as percent of the control group.

3.3.5. qPCR

3.3.5.1. RNA isolation from Caco-2 cells:

Total RNA was isolated from Caco-2 cells after the treatments to determine relative mRNA expression for each treatment. Trizol reagent was used for cells lysis and RNAeasy mini kit was used according to the manufacturer's instructions. Caco-2 cells were plated at 5×10^5 cells per well in 6-well plates. After reaching confluence, medium was replaced, and cells were given the treatments with ARBE or resveratrol and MM. After treatments, cells were then washed with HBSS and 1 mL of Trizol was added to each well for 3 min to lyse the cells. Cell lysates were then transferred to a RNase free Eppendorf tube (1.5 mL) and 200 µL chloroform was added to each sample and samples were vortexed for 10 sec. After incubating at room temperature for 3 min, the lysates were vortexed again and centrifuged at 12,000 g for 10 min at 4 ^{o}C. The clear supernatant was carefully removed and transferred to a new Eppendorf tube and 500 µL of isopropanol was added to each sample. After incubation for 5 min at room temperature, each sample was applied to a spin column and centrifuged at 4 ^{o}C and 8000 ×g for 15 sec. The flow-through was discarded and 350 µL RW1 buffer (provided within the kit) was added to each sample and centrifuged again at 4 ^{o}C and 8000 ×g for 15 sec. In order to remove any DNA contamination, 80 µL (1X) DNase (Invitrogen, USA) was added to each sample followed by incubation at room temperature for 15 min. Samples were washed again with 350 µL RW1 buffer and centrifuged. The flow-through was discarded and samples were washed twice with 500 µL RPE buffer (provided with the kit) and centrifuged again. Samples were then spin-dried for 1 min and the spin columns were transferred to a new RNase free microtube. After adding 40 µL of nuclease free water (Invitrogen, USA), each spin column was centrifuged at 8000 ×g for 1 min at 4 ^{o}C. The flow-through containing purified total RNA was used to measure concentration and purity of the acquired RNA using a Nano drop spectrophotometer (BioRad, USA) and the A260/A280 absorbance ratio. All extracted RNAs had an A260/A280 absorbance ratio of 2-2.1 indicating highly pure RNA (within the acceptable ratio of 1.8-2.1). Samples were then stored at -80 ^{o}C until they were used for synthesis of complementary DNA.

3.3.5.2. Synthesis of complementary DNA (cDNA)

A VILO cDNA synthesis kit (Invitrogen, USA) and a Thermocycler (BioRad, USA) were used for reverse transcription of 2 μg total RNA to cDNA. In order to acquire cDNA, the thermal cycling was performed as follows: one cycle at 25 ºC for 10 min, one cycle at 42 ºC for 60 min (for cDNA synthesis), and one cycle at 85 ºC for 5 min (to inactivate the DNA polymerase). The reaction was performed in 20 μL samples. The purity and concentration of the synthesized DNA was measured using a Nano drop spectrophotometer (BioRad, USA) and the A260/A280 absorbance ratio. All extracted cDNA had an A260/A280 absorbance ratio of 1.8-2.2 indicating highly pure cDNA. Samples were then stored at -80 ºC until analysis by PCR.

3.3.5.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed using a Quant Studio12K Flex Real-time PCR system (Thermofisher Scientific, CA, USA) using SYBR Green master mix (Life Technologies, NY, USA) according to the manufacturer's protocol. Amplification was carried out in 15 μL reactions containing 7 μL SYBR Green master mix, 2 μL forward and reverse primers each, 2 μL nuclease free water and 2 μL cDNA. cDNA samples were diluted 20 times before use according to the VILO cDNA synthesis kit protocol to remove PCR inhibitory effects of reverse transcription. All primers were provided by Integrated DNA Technologies (IDT) (ON, Canada). Table 3.1 shows primer sequences used in the current study. The experimental protocol consisted of the following programs: One cycle at 95 ºC for 5 min (for enzyme activation), amplification and annealing including 40 cycles at 95 ºC for 15 sec and 60 ºC for 30 sec. Melting curves of all PCR products were evaluated to confirm the amplification and primer quality. A comparative method ($2^{-\Delta\Delta CT}$) was used to analyze the relative expression of genes of interest. β-actin and GAPDH were both used as housekeeping genes which both yielded similar data. All data were normalized to β-actin. Two independent experiments were conducted with 3 wells of cells per treatment condition in each experiment.

Table 3.1. Nuclear and mitochondrial encoded mRNA measured via qPCR in Caco-2 cells.

Gene	Species	Primer sequence (5'-3')	Cycles

			(Ct values)
Mn-SOD[α]	Human	Sense: GGA GAT GTT ACA GCC CAG ATA G Antisense: CGT TAG GGCTGA GGTTTG T	19
TNFα[ß]	Human	Sense: GGA TGG ATG GAG GTG AAA GTA G Antisense: TGA TCC TGA AGA GGA GAG AGA A	33

[α] Manganese-dependent superoxide dismutase (Mn-SOD)
[ß] Tumor necrosis factor-alpha

3.3.6. Cell viability

3.3.6.1. Resazurin Cytotoxicity Assay

Resazurin or Alamar Blue has been extensively used as a probe to measure cell viability and metabolic activity based on aerobic respiration (Rampersad, 2012). Resazurin (blue and nonfluorescent) is reduced in mitochondria via NADH or NADPH to resorufin (pink and highly fluorescent) in viable cells (Aleshin et al., 2015). Resorufin production correlates with the degree to which cells respire and are metabolically active. The fluorescence intensity of resorufin was measured via microplate reader. Briefly, Caco-2 cells were seeded at 5-10×10^4 cells per well in a 96-well, flat-bottomed microplate in a final volume of 0.2 mL DMEM and incubated at 37 °C in a 5% CO_2 environment for 24 h to allow attachment and growth to confluence. On the day of an experiment, cells were pretreated with ARBE or resveratrol in media containing 2% FBS for 2 or 5 h, respectively, and then challenged with MM for 24 h. After the treatment, 44 μM resazurin was added to each well and cells were incubated at 37 °C in 5% CO_2 for 2 h. The fluorescence was measured at a wavelength excitation of 530 nm and emission of 590 nm with a Synergy H1, plate reader (BioTek Instruments, VT, USA,). Data have been corrected for probe added to DMEM with no cells and the results are reported as percent of the control group.

3.3.6.2. Forward and side scattering of Caco-2 cells using flow cytometry

In order to identify morphologically normal cells for analysis of fluorescent probes used to assess mitochondrial numbers/density, forward and side scattering of light by the Caco-2 cells was

measured by flow cytometry. Forward scatter measures cell size and shape and is proportional to the diameter of a cell and side scatter provides information about cellular complexity and granularity. After seeding 2.5×10^5 cells in each well of a 24-well plate and growing to confluence for 24 h, cells were pretreated with 20 µM ARBE or resveratrol for 2 or 5 h. Cells were then challenged with 0.4 mM MM. DMSO was added to untreated cells to control for any effect of DMSO, in which the polyphenols were solubilized. After 24 h, the cells were washed twice with HBSS and trypsinized. After adding medium and centrifuging at 1,000 ×g for 5 min, supernatant was discarded, and the pellet was suspended in PBS. Then, 0.5 mL of cell suspension was transferred to a flow cytometry tube and forward and side scattering was determined using a FACS system. Figure 3.1 displays arbitrary FACS sorting gates for Caco-2 cells based on their size and granularity. The cells in the P1 area are considered apoptotic cells while cells in gated and P2-P4 area are considered viable cells with varying degrees of health.

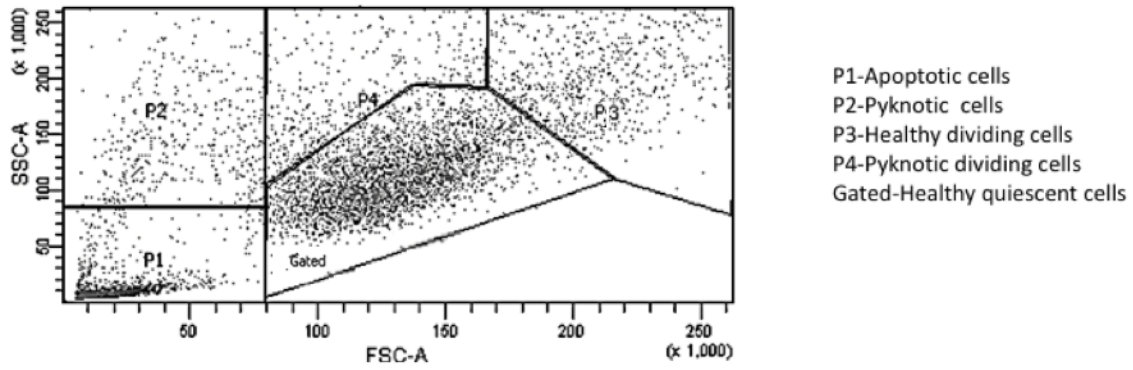


Figure 3.1. *FACS sorting gates for Caco-2 cells.*

3.3.6.3. Sulforhodamine B assay

The sulforhodamine B (SRB) assay was performed to estimate cytotoxicity in Caco-2 cells based on the measurement of the amount of cellular protein content remaining after toxic challenge. SRB is a bright pink dye with two sulfonic groups that in mild acidic conditions, binds to amino-acid residues of proteins in cells that have been fixed to tissue culture plates by trichloroacetic acid (TCA). The amount of cellular protein is relative to the number of attached cells (Vichai and

Kirtikara, 2006). After seeding 50,000 Caco-2 cells in 96 well-plates and growing to confluence for 24 h in 5% CO_2 at 37 °C, cells were pretreated with ARBE or resveratrol for 2 or 5 h, respectively. Cells were then challenged with 0.4 mM MM for 24 h. Then cells were fixed in 100 μM trichloroacetic acid (TCA) at 10% final concentration and kept at 4 °C for 1 h. After washing the plate 4 times with running water followed by air-drying the plate, 100 μL of 0.057% (wt/vol) SRB solution was added to each well and the plate was kept at room temperature for 30 min. Afterwards, wells were rinsed with 1% acetic acid to remove unbound dye. Plates were allowed to dry at room temperature, and 10 mM Tris base solution (pH 10.5) was added to each well to solubilize protein-bound dye. Absorbance was measured at 510 nm using a microplate reader.

3.3.7. Transepithelial Electrical Resistance (TEER)

Transepithelial electrical resistance is a quantitative technique that measures the integrity of tight junctions and is an indicator of cell permeability in an *in vitro* model of epithelial cell barrier integrity (Anderson, 2001; Lo et al., 1999; Chen et al., 2015). The electrical resistance is measured in ohms (Srinivasan et al., 2015). In this assay, the electrical impedance is measured and a continuous current is applied to the cells on both transcellular and paracellular paths (Powell, 1981) which when the barrier is disrupted, enables current to increase. Thus, high TEER values reflect an intact barrier whereas a decrease in the TEER value corresponds to an increase in permeability of the Caco-2 cell monolayer (Xiao et al., 2013). The apical and basolateral membrane create the transcellular resistance while paracellular resistance is mainly created by cell-cell contacts through the formation of tight junctions, which significantly affect epithelial resistance (Anderson, 2001; Lo et al. 1999). Therefore, the TEER values reflect the integrity of the Caco-2 cell monolayer (Chen et al., 2015). For these measurements 1.5×10^5 Caco-2 cells per well were seeded on collagen coated membrane transwell inserts (4 mm diameter inserts, 3 μm pore size; Corning, USA) with 500 μL culture medium added to the inserts (apical side) and 1.5 mL medium to the basolateral chamber. Cells were allowed to differentiate for 21 days before the experiment (Aspenström-Fagerlund et al., 2009). The culture medium was changed every other day after 7 days of reaching a confluent state and also changed in both the apical and basolateral sides after each measurement. The electrical resistance of confluent and differentiated cells was measured by TEER using an epithelial voltmeter EVOM (EMD Millipore millicell volt-ohm meter, USA). A pair of chopstick electrodes was used - one placed in the apical and the other in

the basolateral chamber to estimate TEER. Measurements were performed every two-three days to assess the integrity of the Caco-2 monolayer until cells were differentiated as judged by the TEER values reaching a plateau above 1500 Ω cm^2. Experiments were performed on day 22-23. Aeraseal sealing film was placed on the transwell on the day of the experiment to allow uniform air and CO_2 exchange of all wells. On the day of an experiment, DMEM containing 2% FBS was added to the wells and cells were allowed to adapt to the new condition for 1 h before the experiment. Then treatments in 2% FBS media were added to the apical side (2 h or 5 h for ARBE and resveratrol pretreatment, respectively, before adding 1.5 mM MM) and TEER measurements were performed every 1.5 h afterwards. TEER values were expressed as % relative to control cells.

Paracellular movement through the Caco-2 cell monolayer was also measured by the amount of FITC-dextran (4 kDa) appearing in the basolateral side. Prior to treatments FITC-dextran at the final concentration of 100 μg/mL was added to the apical compartment. After each TEER measurement, 100 μL of the medium in the basolateral compartment was diluted with 100 μL of HBSS 1× in a 96 well plate and fluorescence was measured at λ_{ex}: 485 nm and λ_{em}: 530 nm in a microplate reader.

3.3.8. Statistical analysis

Results were expressed as means and standard deviations. Each experiment was repeated at least twice with 3 wells of cells per treatment condition. Data are represented as percent of untreated cells. Statistical analysis was performed using one-way or two-way ANOVA followed by Tukey's post hoc test using GraphPad Prism (Version 7; La Jolla, CA, USA). Differences were considered statistically different at $P < 0.05$.

3.4. Results

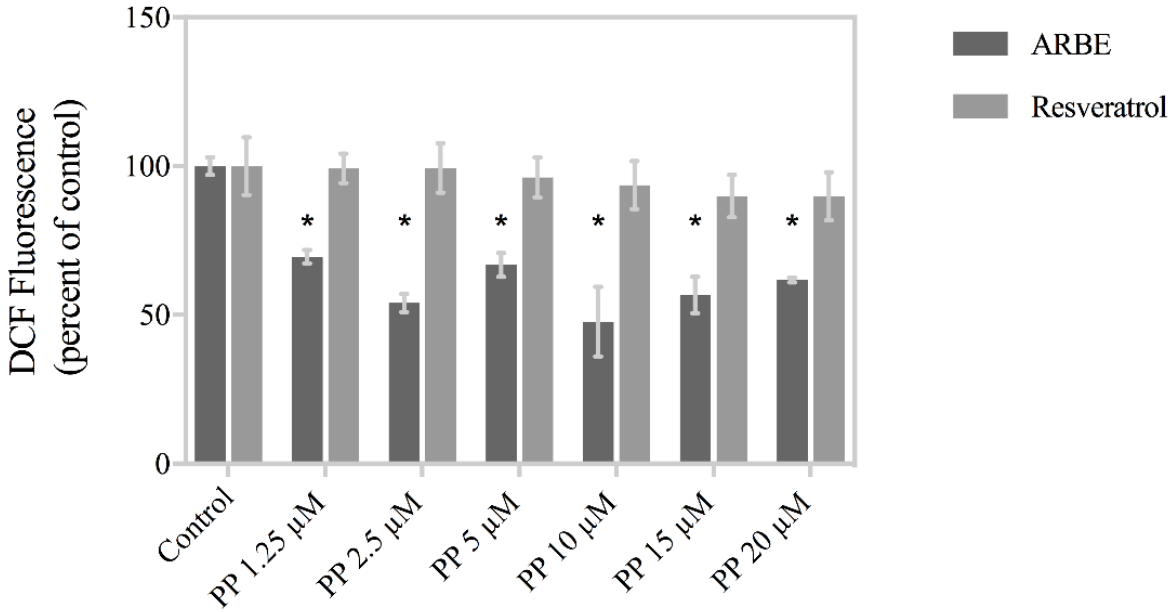
3.4.1. Oxidative stress

To establish whether the polyphenol treatments alone increased or decreased intracellular ROS generation, Caco-2 cells were treated with different concentrations of ARBE or resveratrol (1.25-20 μM) for 26 and 29 h respectively. The measurements of DCF fluorescence showed that ARBE

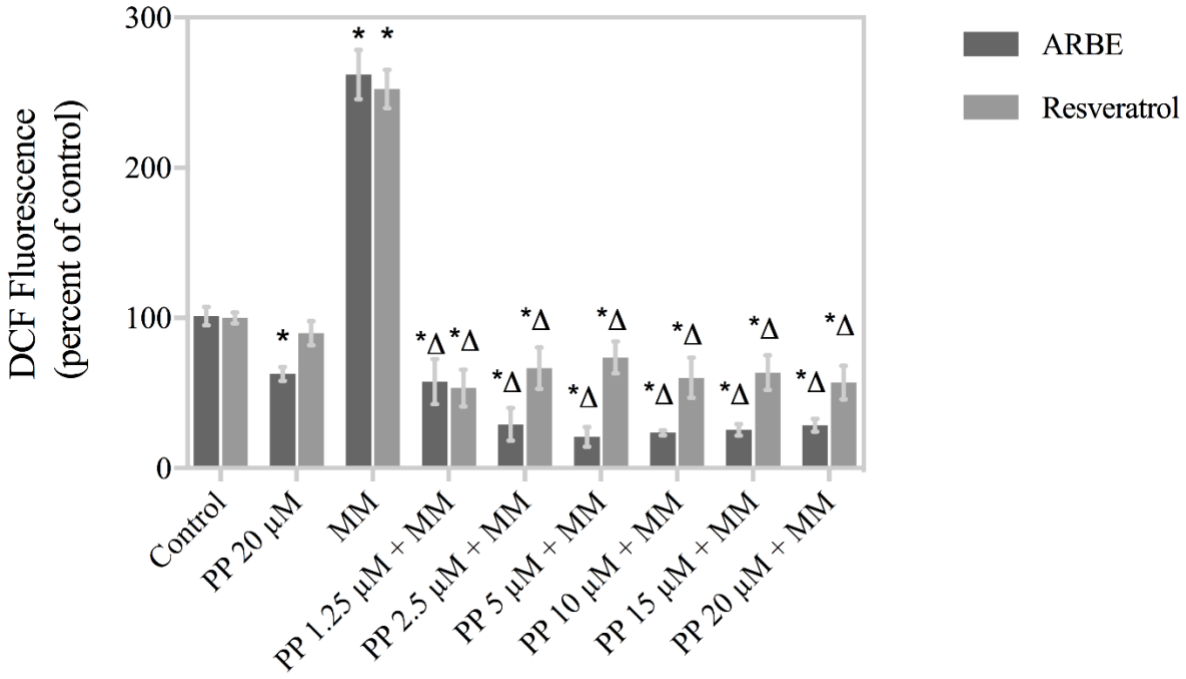
at all doses significantly decreased basal intracellular ROS generation, while resveratrol had no effect (Fig. 3.2.A).

Challenge of Caco-2 cell monolayers with 0.4 mM MM for 24 h increased DCF fluorescence by 150% (Figure 3.2.B) ($P < 0.001$). All concentrations of ARBE and resveratrol completely prevented the ROS production induced by MM, with ARBE acting slightly stronger than resveratrol. DCF fluorescence microscopic images of Caco-2 cells (Appendix Fig. 8.B.6), shows high fluorescent intensity of DCF in cells challenged with MM compared to control cells and cells treated with ARBE or resveratrol alone. Pretreatment with ARBE or resveratrol clearly shows reduced DCF fluorescence compared to MM consistent with the quantitative data produced by the plate reader assay. Increasing the concentrations of ARBE from 1.25 to 2.5 µM gave a slight further increase in the antioxidant effect of ARBE. Correcting for any differences in the number of cells by dividing the DCF fluorescence by that of SRB, showed an even larger increase in ROS generation by MM on a per adherent cell protein basis, which was completely prevented by ARBE or resveratrol (Figure 3.2.C). The antioxidant effects of ARBE and resveratrol were evident at concentrations as low as 1.25 µM, and ARBE produced DCF fluorescence values that were significantly less than control cells in the absence of MM.

A.



B.


DCF Fluorescence
(percent of control)
300
200
100
0
ARBE
Resveratrol
Control
PP 20 μM
MM
PP 1.25 μM + MM
PP 2.5 μM + MM
PP 5 μM + MM
PP 10 μM + MM
PP 15 μM + MM
PP 20 μM + MM

C.

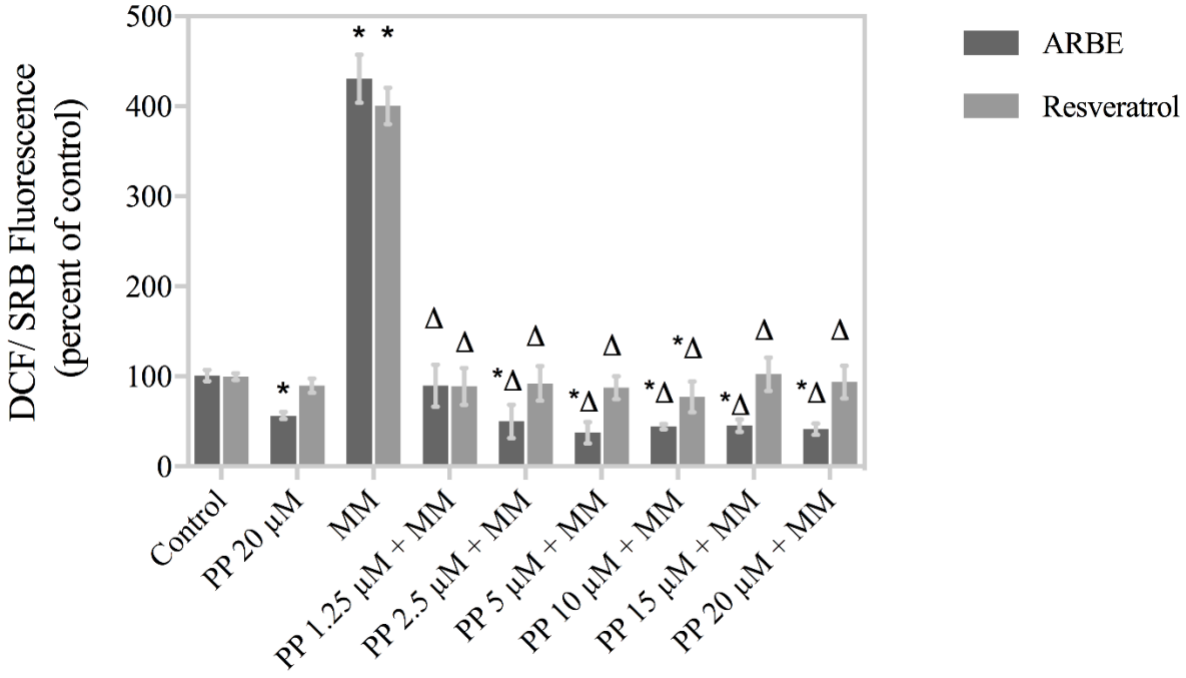

DCF/ SRB Fluorescence
(percent of control)
500
400
300
200
100
0
ARBE
Resveratrol
Control
PP 20 μM
MM
PP 1.25 μM + MM
PP 2.5 μM + MM
PP 5 μM + MM
PP 10 μM + MM
PP 15 μM + MM
PP 20 μM + MM

Figure 3.2. *Effects of MM, polyphenols (PP) such as ARBE and resveratrol on intracellular ROS production.* Caco-2 cells were treated with ARBE or resveratrol at 20 µM for 2 or 5 h respectively prior to adding 0.4 mM MM and incubating for an additional 24 h. After treatments, cells were then incubated with DCFH-DA for 2 h and ROS generation was measured via DCF fluorescence (AU) using a microplate reader. Control cells had no MM or polyphenols and had 0.1% DMSO. **(A)** Absence of MM ($P_{ARBE} < 0.0001$; $P_{Resveratrol} = 0.2972$). **(B)** Presence of MM ($P_{MM} < 0.0001$, $P_{ARBE} = 0.0022$, $P_{Interaction} < 0.0001$; ($P_{MM} < 0.0001$, $P_{Resveratrol} = 0.0018$, $P_{Interaction} < 0.0001$). **(C)** ROS generation per attached cell protein content measured with SRB ($P_{MM} < 0.0001$, $P_{ARBE} < 0.0001$, $P_{Interaction} < 0.0001$; $P_{MM} < 0.0001$, $P_{Resveratrol} < 0.0001$, $P_{Interaction} < 0.0001$). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant difference between groups in the experiments shown in A was tested with one-way ANOVA and in B and C with two-way ANOVA followed by Tukey's multiple comparisons test.

3.4.2. Mitochondrial superoxide generation

The fluorogenic probe MitoSOX was used to assess the effect of MM and polyphenols on mitochondrial superoxide generation. Figure 3.3 shows MitoSOX fluorescence intensity of Caco-2 cells pretreated with ARBE or resveratrol for 2 h or 5 h respectively, followed by 24 h challenge with 0.4 mM MM. Challenge with MM increased MitoSOX fluorescence compared to control by 97% ($P < 0.0001$). ARBE pretreatment significantly decreased MM-induced mitochondrial ROS generation relative to the MM condition by 24%. However, resveratrol was not able to protect against mitochondrial superoxide generation.

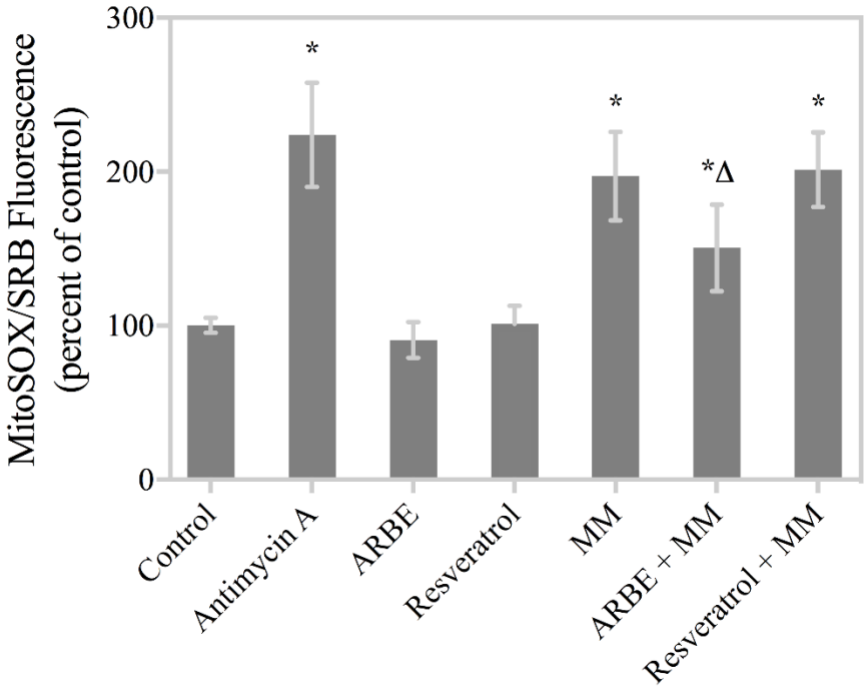


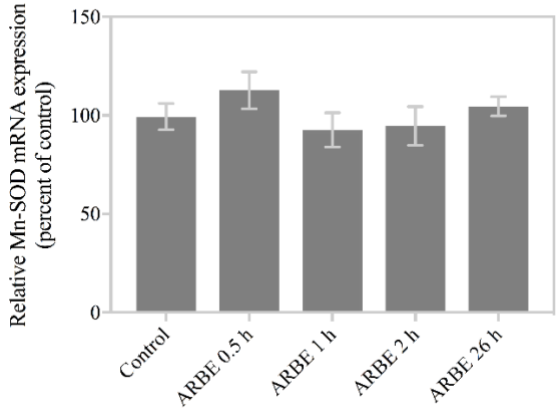
Figure 3.3. *Effects of MM and polyphenols on mitochondrial superoxide generation.* Fluorescence intensity of MitoSOX was analyzed in Caco-2 cells treated with ARBE or resveratrol as described in Figure 3.2. MitoSOX fluorescence intensity of Caco-2 cells pretreated with ARBE for 2 h or resveratrol for 5 h before MM challenge ($P_{MM} < 0.0001$, $P_{ARBE} = 0.0002$, $P_{Interaction} = 0.0336$; $P_{MM} < 0.0001$, $P_{Resveratrol} = 0.0075$, $P_{Interaction} = 0.0045$). Antimycin A was added 30 min prior to addition of MitoSOX. Data were measured by plate reader and normalized to attached-cell protein content measured via SRB. Values represent means ± SD of three independent experiments with three wells of cells for each treatment. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from the MM group ($P<0.05$). The significant difference between groups were tested with two-way ANOVA followed by Tukey's multiple comparisons post hoc test. The statistical analysis was done separately for ARBE and MM and for resveratrol

and MM; the two factors for the analysis were ARBE and MM and also resveratrol and MM, respectively.

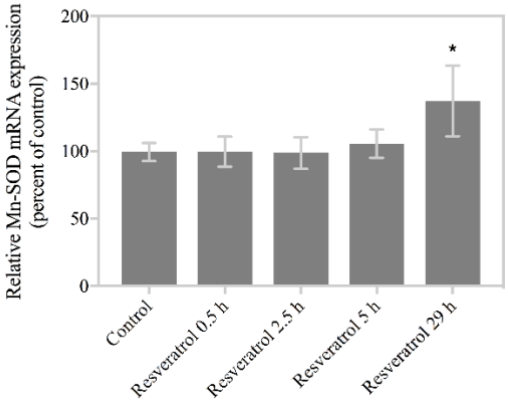
3.4.3. Effect of treatments on Mn-SOD and TNF-α gene expression

As shown in Figure 3.4.A and B, among ARBE or resveratrol treatment at different time points, only resveratrol after 29 h had a significant effect on Mn-SOD mRNA expression, increasing it by 37%. Also, challenging Caco-2 cells with 0.4 mM MM for 24 h increased Mn-SOD mRNA expression by 23% ($P < 0.05$) and ARBE and resveratrol pretreatment prevented this increase. ARBE alone had no effect on Mn-SOD mRNA expression and resveratrol alone increased Mn-SOD mRNA expression in these experiments (Fig. 3.4.C) ($P = 0.010$).

A.



B.



C.

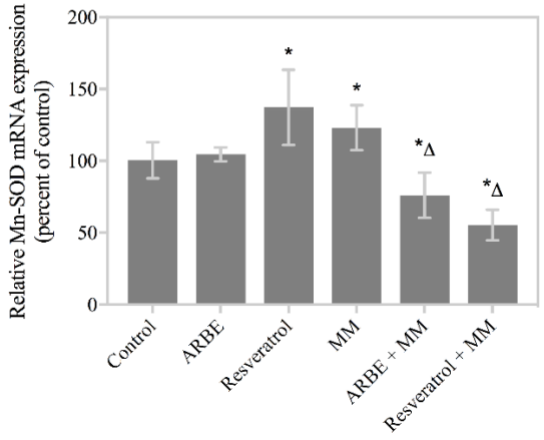


Figure 3.4. *Effects of MM and polyphenols on expression of Mn-SOD mRNA.* **(A)** At different time points after exposure to 20 µM ARBE alone (P =0.0184). **(B)** At different time points after exposure to 20 µM resveratrol alone (P =0.0001). **(C)** After 2 h exposure to 20 µM ARBE or 5 h of 20 µM resveratrol pretreatment and 24 h challenge with 0.4 mM MM as described in Figure 3.2. (P_{MM} =0.6236, P_{ARBE} =0.0019, $P_{Interaction}$ =0.0004; P_{MM} =0.0011, $P_{Resveratrol}$ =0.0569, $P_{Interaction}$ <0.0001). Data are normalized to β-actin and presented as percent of control cells. Values are the means ± SD (two independent experiments with three wells of cells for each treatment). * Mean value significantly different from the control group ($p < 0.05$). $^{\Delta}$ Mean value significantly different from the MM group ($P < 0.05$). The significant difference between conditions in A and B were tested with one-way ANOVA and in C with two-way ANOVA followed by Tukey's multiple comparisons post hoc test.

As shown in Figure 3.5.A, ARBE alone after 2 and 24 h exposure significantly decreased expression of TNF-α mRNA by 32% and 63%, respectively, compared to the control condition (Fig. 3.5.A). With resveratrol, it initially (after 30 min) decreased TNF-α expression, but by 1 and 2 h exposure expression levels were comparable to control, and after 29 h produced a 73% increase in TNF-α expression (Fig. 3.5.B). Also, MM induced a 497% increase in expression of TNF-α mRNA ($P < 0.0001$). Both ARBE and resveratrol inhibited this increase by 30% ($P < 0.0001$) and 24% respectively. ($p = 0.005$) (Fig 3.5.C). In these experiments, ARBE or resveratrol themselves had no significant effect compared to control cells (Fig. 3.5.C).

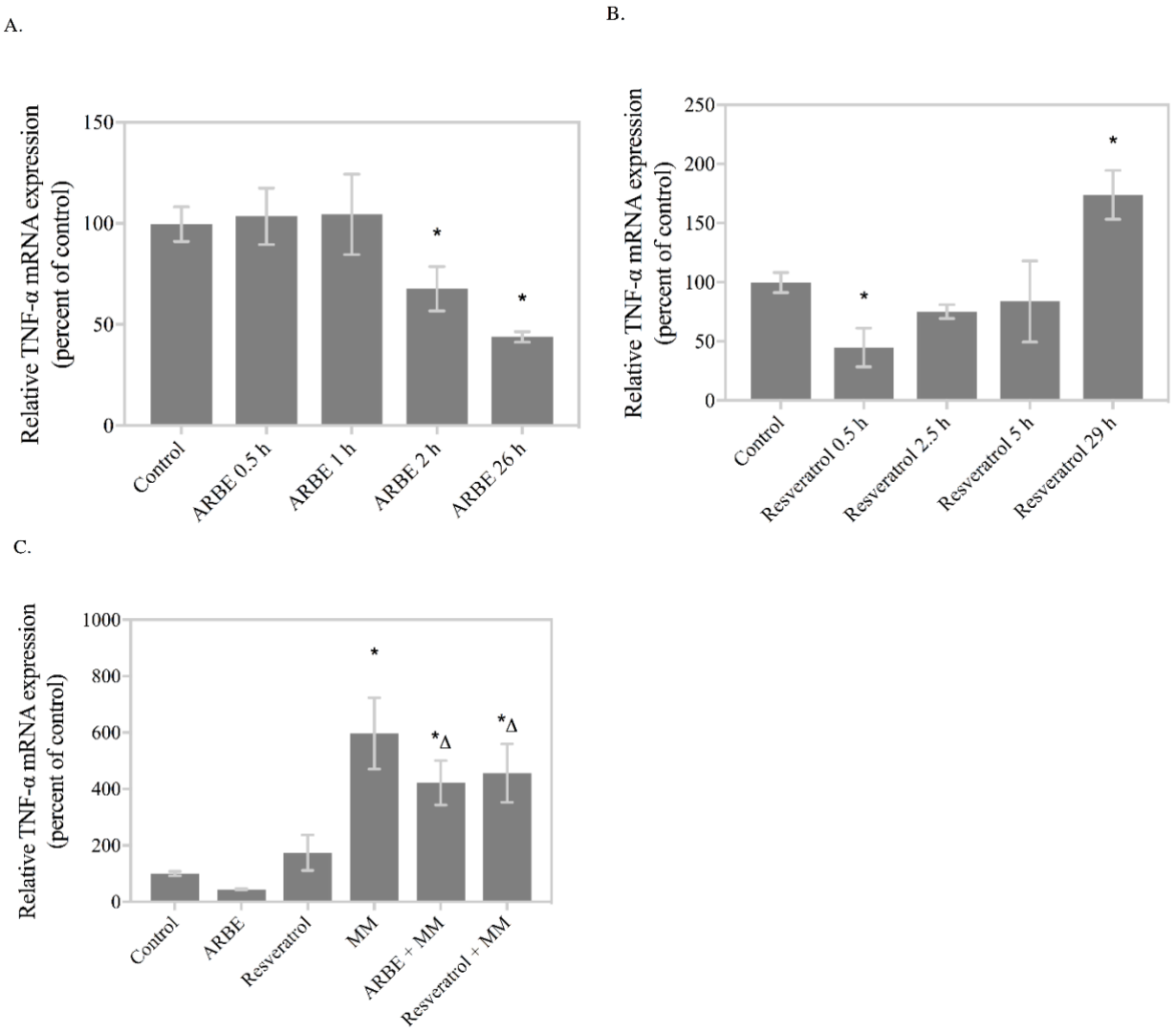


Figure 3.5. *Effects of MM and polyphenols on expression of TNF-α mRNA.* **(A)** At different time points after exposure to 20 µM ARBE alone ($P < 0.0001$). **(B)** At different time points after exposure to 20 µM resveratrol alone ($P < 0.0001$). **(C)** After 2 h exposure to 20 µM ARBE or 5 h of 20 µM resveratrol pretreatment and 24 h of 0.4 mM MM as described in Figure 3.2. ($P_{MM} < 0.0001$, $P_{ARBE} < 0.0001$, $P_{Interaction} < 0.0001$; $P_{MM} < 0.0001$, $P_{resveratrol} = 0.0302$, $P_{Interaction} = 0.0005$). (B) Data were normalized to β-actin and are presented as percent of control cells. Values are the means ± SD (two independent experiments with three wells of cells for each treatment). * Mean value significantly different from the control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from the MM group ($P < 0.05$). The significant difference between conditions in A and B were

tested with one-way ANOVA and in C with two-way ANOVA followed by Tukey's multiple comparisons post hoc test.

3.4.4. Cell viability/metabolic activity

The effect of treatments on health and morphology of Caco-2 cells were assessed via light microscopic imaging. Images show clusters of cells and cell loss (empty space) as well as deteriorated morphology of cells induced by MM compared to control cells (Fig. 3.6.A & B). ARBE, especially at higher concentrations (≥10 μM) and resveratrol at concentrations higher than 5 μM, appeared to prevent the toxic effect of MM on cells, with morphology and distribution of the cells similar to control cells. Lower doses of ARBE (≤5 μM) appeared to partially prevent the toxic effects of MM on cell morphology (e.g. patchiness/loss of cells). However, at the lower concentrations of ARBE and resveratrol, accumulation and granulation of cells were still evident (Fig. 3.6.A & B). The visual cues from these light microscopic images suggested a protective effect of the polyphenol pretreatments, prompting us to follow up these observations with quantitative measures.

With the resazurin assay, measurements of resorufin fluorescence showed that ARBE or resveratrol alone did not affect cell viability/metabolic activity, except for slight increases observed with ARBE at 2.5 and 15 μM (Fig. 3.6.C). In experiments with MM, resorufin fluorescence showed a 35% decrease in metabolic activity in cells challenged with MM compared to control cells (Fig. 3.6.D). This decrease however was not affected by pretreatment with resveratrol or ARBE.

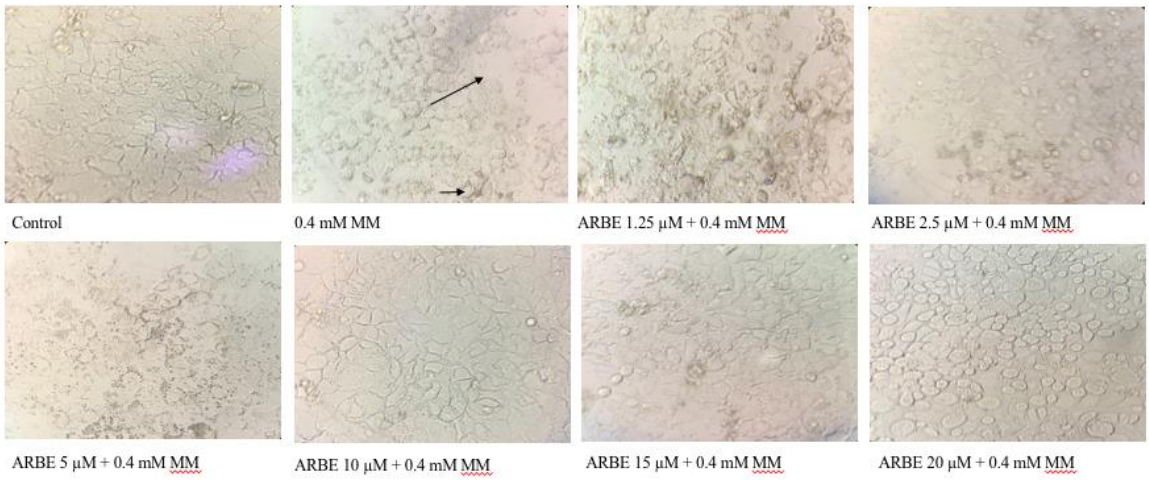
In order to further investigate effects of MM and polyphenols on cell viability, we used flow cytometry measurements of forward and side scatter of light by Caco-2 cells under the different treatment conditions. The scatter plots displayed in Figures 3.6.E and 3.6.F showed that treatment with MM noticeably decreased forward scatter and increased side scatter, indicating decreased cell size and increased cell granularity reminiscent of cells undergoing apoptosis. Treatments with ARBE and resveratrol both ameliorated the light scattering effect of MM exposure. ARBE

treatment alone also slightly increased forward scattering of Caco-2 cells compared to control cells.

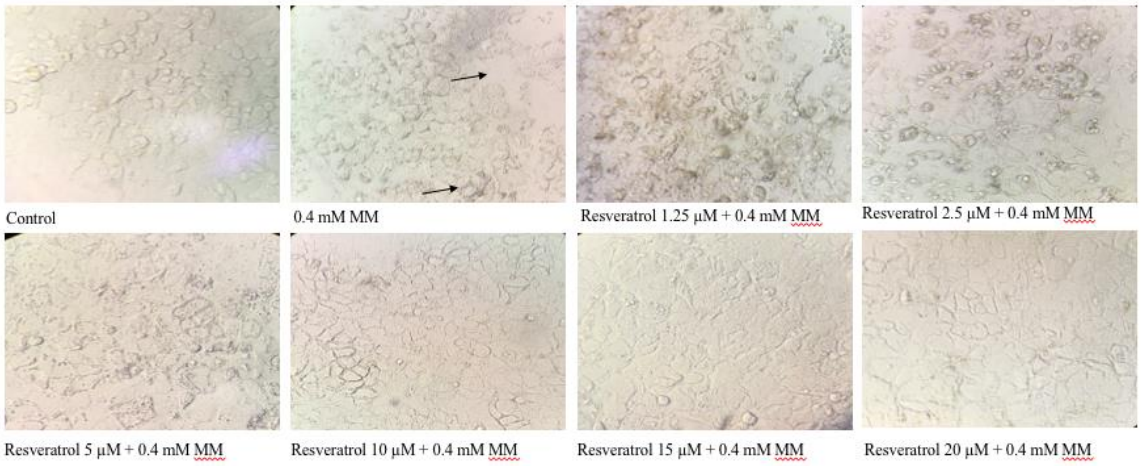
Flow cytometry analyses comparing the percentage of viable and apoptotic cells (Fig. 3.6.G), showed that MM increased the percentage of apoptotic cells (by 67%) and decreased the percentage of viable cells (by 28%) compared to control cells. Both ARBE and resveratrol pretreatment maintained more viable cells and decreased apoptotic cells by 27% and 31%, respectively, compared to cells challenged with MM alone. ARBE treatment alone decreased apoptotic cells by 39% compared to control cells.

As a further measure of viability (or cytotoxicity), the effects of MM on the density of adherent Caco-2 cells was measured via the SRB assay, which indicated an MM-induced decrease in viable cells (39.5% decrease compared to control cells) (Fig. 3.6.H). With this assay, ARBE or resveratrol blunted the MM-induced decline in adherent protein content at the highest dose (20 µM) of ARBE ($P = 0.0018$) and at the intermediate (5 and 10 µM) doses of resveratrol ($P < 0.0001$).

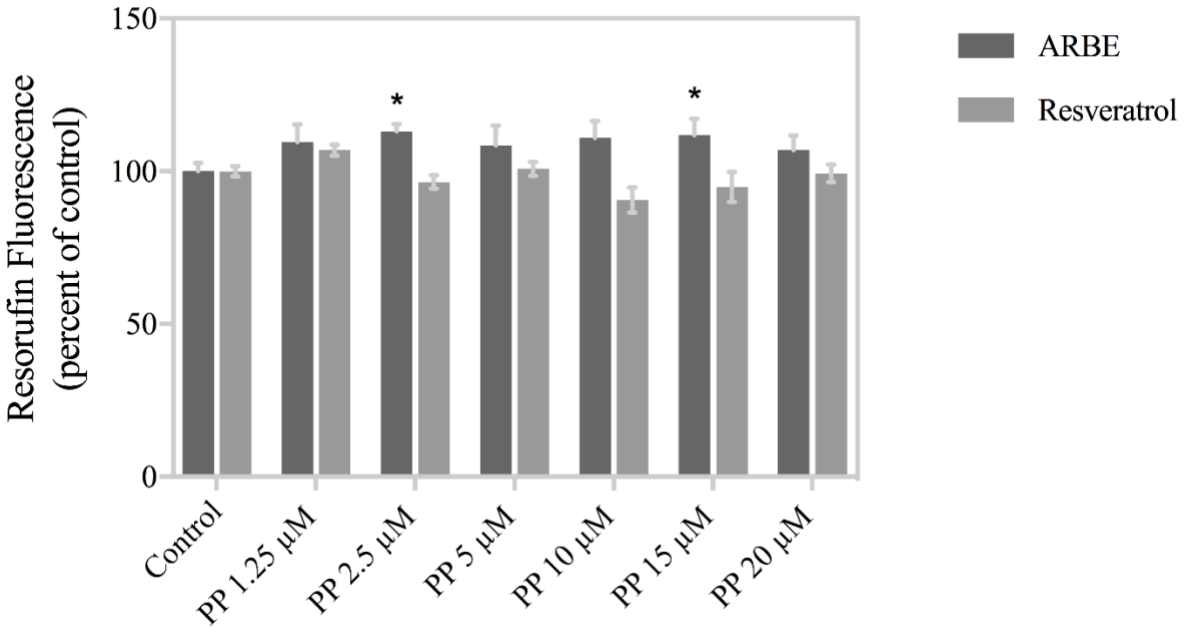
A.



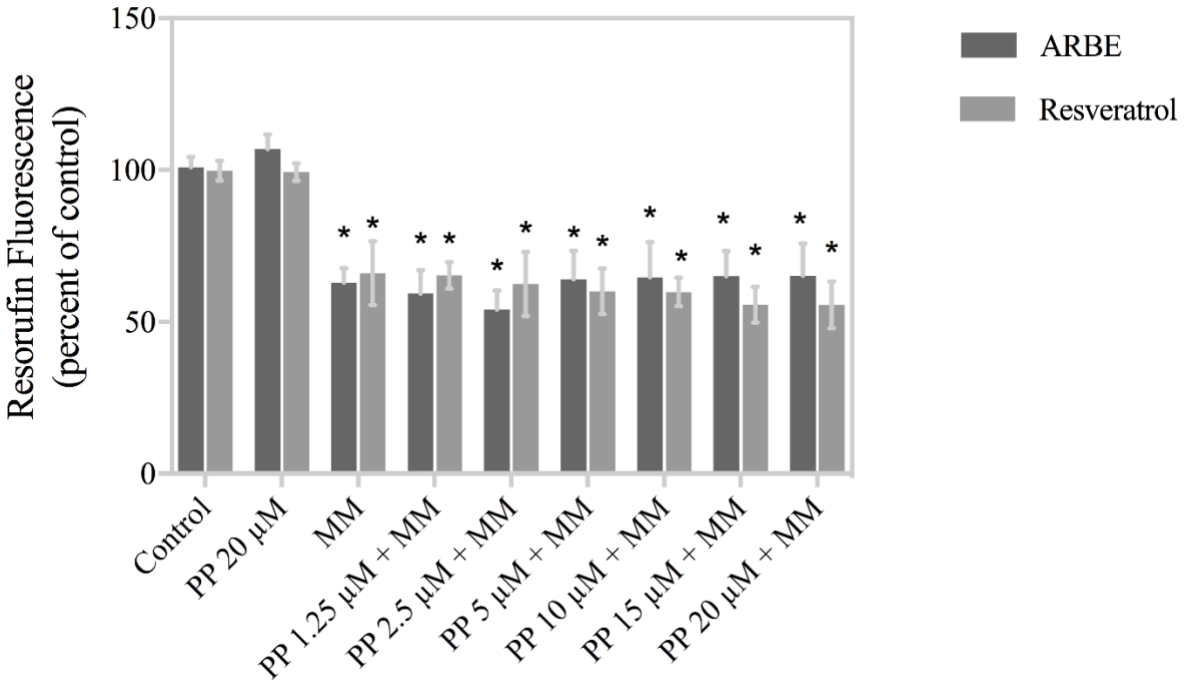
B.


Control
0.4 mM MM
Resveratrol 1.25 µM + 0.4 mM MM
Resveratrol 2.5 µM + 0.4 mM MM
Resveratrol 5 µM + 0.4 mM MM
Resveratrol 10 µM + 0.4 mM MM
Resveratrol 15 µM + 0.4 mM MM
Resveratrol 20 µM + 0.4 mM MM

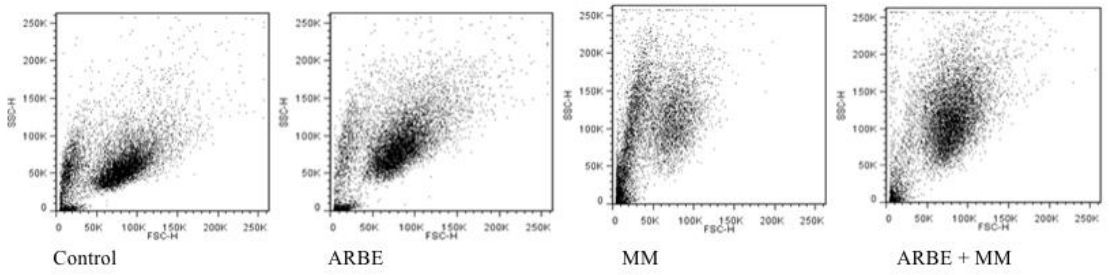
C.


Resorufin Fluorescence
(percent of control)
150
100
50
0
*
*
ARBE
Resveratrol
Control
PP 1.25 µM
PP 2.5 µM
PP 5 µM
PP 10 µM
PP 15 µM
PP 20 µM

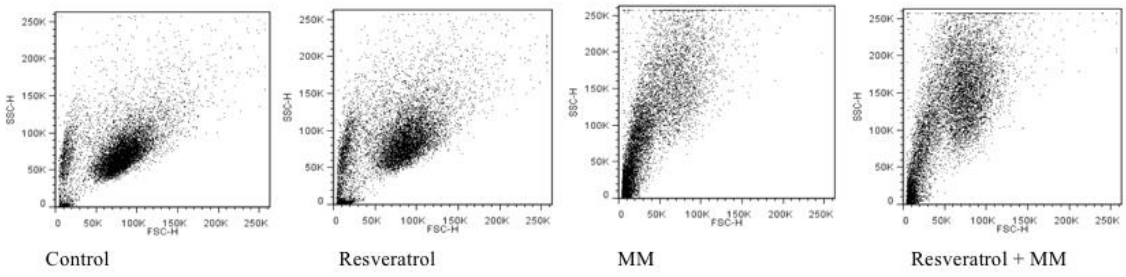
D.


Resorufin Fluorescence
(percent of control)
150
100
50
0
ARBE
Resveratrol
Control
PP 20 µM
MM
PP 1.25 µM + MM
PP 2.5 µM + MM
PP 5 µM + MM
PP 10 µM + MM
PP 15 µM + MM
PP 20 µM + MM

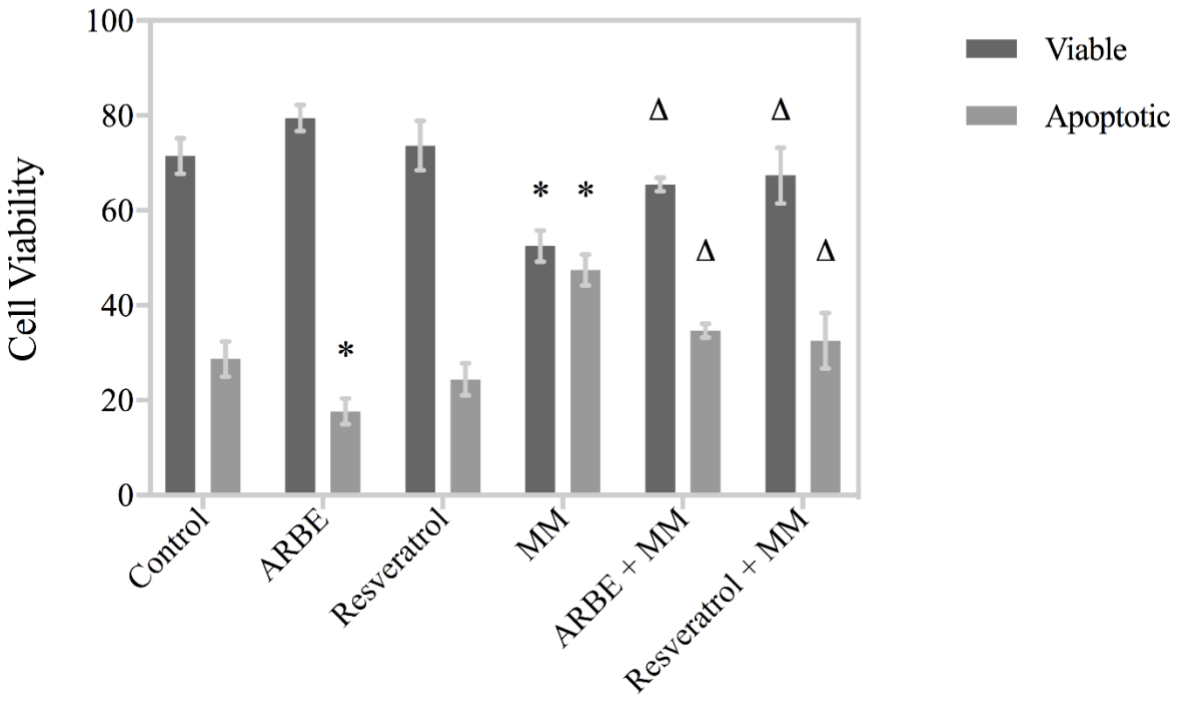
E.


SSC-H
FSC-H
Control
ARBE
MM
ARBE + MM

F.


SSC-H
FSC-H
Control
Resveratrol
MM
Resveratrol + MM

G.


Cell Viability
100
80
60
40
20
0
Control
ARBE
Resveratrol
MM
ARBE + MM
Resveratrol + MM
Viable
Apoptotic

H.

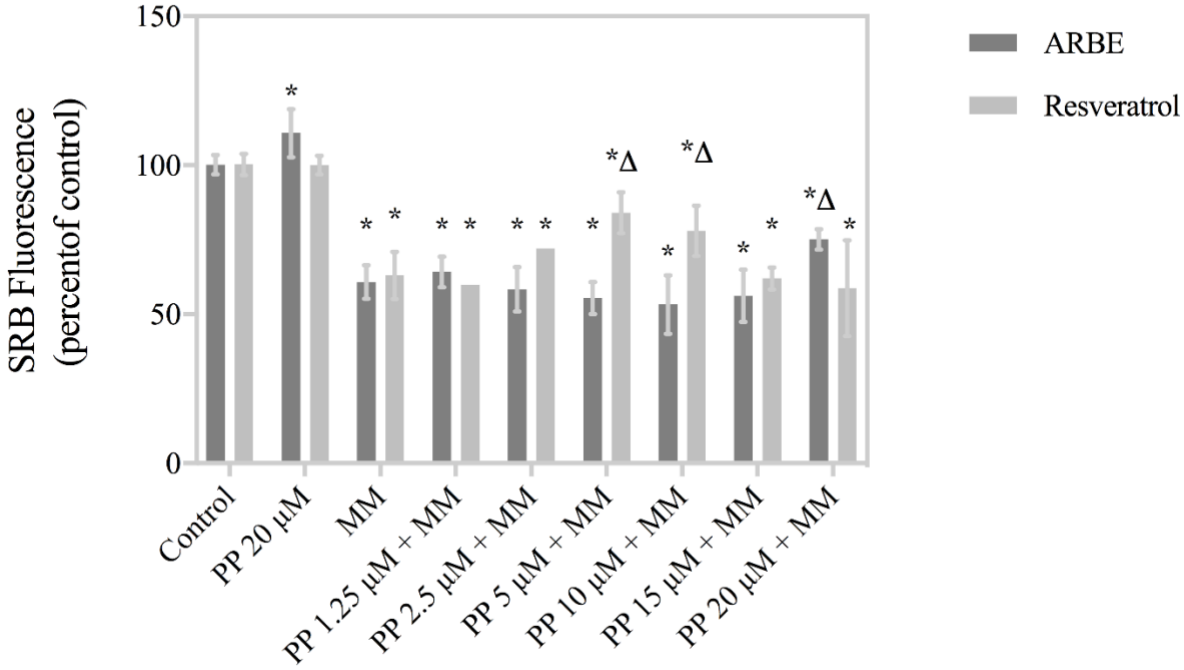

SRB Fluorescence (percentof control)
150
100
50
0
Control
PP 20 μM
MM
PP 1.25 μM + MM
PP 2.5 μM + MM
PP 5 μM + MM
PP 10 μM + MM
PP 15 μM + MM
PP 20 μM + MM
ARBE
Resveratrol

Figure 3.6. *Effect of MM and polyphenols on cell morphology, viability, and density.* **(A and B)** Light microscopic images showing the effects of MM and ARBE/resveratrol on cell health and morphology. Images were captured using a 40x objective lens. **(C)** Resazurin measurements of cell viability after treatment with different concentrations of ARBE or resveratrol alone for 26 or 29 h respectively (P_{ARBE} =0.0239; $P_{Resveratrol}$ =0.4694). **(D)** Resazurin measurements of cell viability in the presence of MM. (P_{MM} =0.4415, P_{ARBE} <0.0001, $P_{Interaction}$ =0.0831; P_{MM} =0.0018, P_{ARBE} <0.0001, $P_{Interaction}$ =0.2240). **(E and F)** Scatter plots of forward and side scattering of a total of 10,000 Caco-2 cells for each sample. **(G)** Percent of cells determined as viable or apoptotic when analyzed by flow cytometry. (Viable: P_{MM} <0.0001, P_{ARBE} =0.0011, $P_{Interaction}$ =0.2958; Viable: P_{MM} =0.0016, $P_{Resveratrol}$ =0.0914, $P_{Interaction}$ =0.0896; Apoptotic: P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ =0.6276; P_{MM} <0.0001, $P_{resveratrol}$ =0.0049, $P_{Interaction}$ =0.0818). **(H)** Adherent cell density measured by the SRB assay after pre-treatment with different concentrations of ARBE or resveratrol for 2 or 5 h, respectively, followed by 24 h challenge with 0.4 mM MM (P_{MM} <0.0001, P_{ARBE} =0.0003, $P_{Interaction}$ =0.5135; P_{MM} <0.0001, $P_{Resveratrol}$ =0.9624, $P_{Interaction}$ =0.9979). Values are the means ± SD (3 independent experiments with three wells of cells for each treatment). * Mean value significantly different from the control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from the MM group ($P < 0.05$). The significant difference between conditions in D, G, H was tested with two-way ANOVA and in C with one-way ANOVA followed by Tukey's multiple comparisons post hoc test.

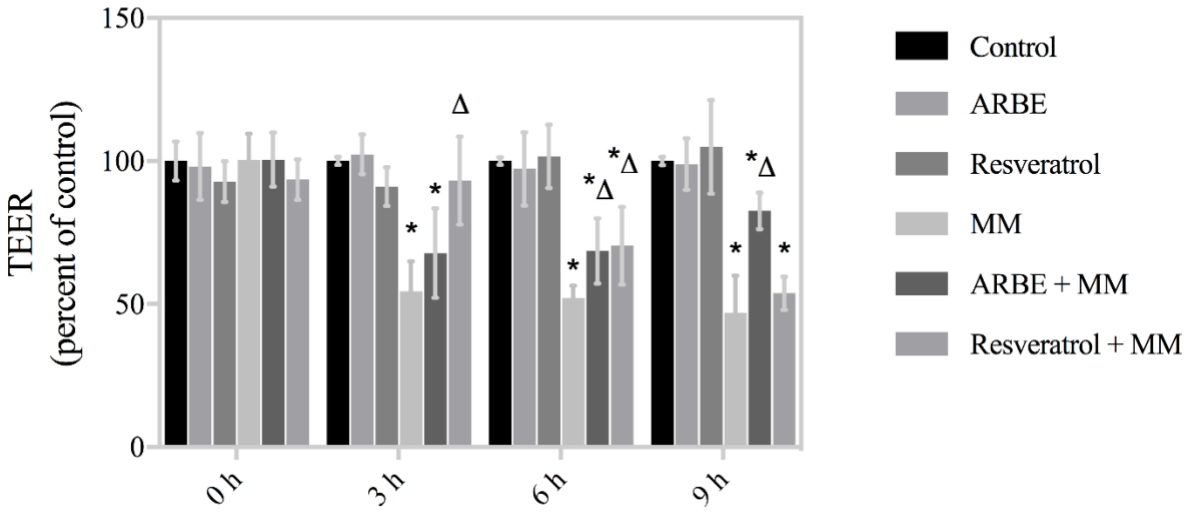
3.4.5. Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was used to measure the effects of different treatments on the integrity of a Caco-2 cell monolayer. Initially, a dose response was performed to determine the best dose of MM to induce recoverable permeability (Appendix Fig 8.B.7). Findings showed that 1.5 mM MM was a suitable concentration. Exposure of Caco-2 cells to 1.5 mM MM resulted in decrease of TEER (by 46%) after 3 h and by more than 50% after 9 h (Fig. 3.7.A), indicating loss of barrier integrity and increase in permeability. ARBE or resveratrol alone at 20 μM did not affect TEER. ARBE and resveratrol showed different abilities to protect against the decrease in TEER induced by MM. ARBE did not protect initially, but significantly reversed the MM-induced

decrease in TEER value at 6 and 9 h, reaching 82% of control at 9 h. Resveratrol initially protected against the decrease in TEER at 3 h after MM exposure, but this protection decreased at 6 h and was lost at 9 h. (TEER values for control group was recorded as approximately 1800-2100 via EVOM and chopsticks)

Fluorescein isothiocyanate (FITC)-labeled dextran was used as a measure of paracellular permeability and served as another measure of epithelial barrier integrity. Data were consistent with the observations from the TEER experiment, and showed an increase in fluorescence intensity of FITC-dextran applied to the apical compartment in the basal compartment from cell monolayers treated with MM. The fluorescent signal increased over 9 h of MM challenge to 252% of control. ARBE inhibited the increase in FITC-dextran transportation at 6 and 9 h after MM challenge by 63% and 67% compared to that produced by MM challenge alone, respectively. Similar to the effects of ARBE, resveratrol pretreatment showed protection at 3 h and 6 h after MM exposure of 37% and 50% compared to MM treated group, respectively. However, this protective effect of resveratrol was not sustained, as both TEER and FITC dextran experiments showed that resveratrol did not induce protection against monolayer permeability by 9 h of MM exposure (Fig. 3.7.B).

A.



B.

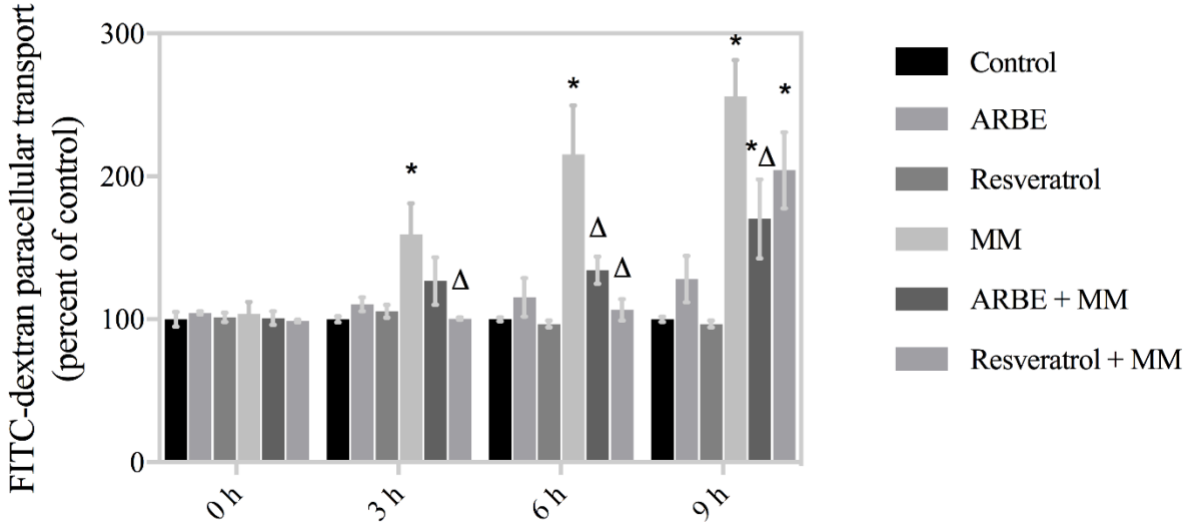


Figure 3.7. *Effects of ARBE, resveratrol and MM on transepithelial electrical resistance of a differentiated Caco-2 cell monolayer.* Differentiated Caco-2 cells grown on a Transwell membrane for 21 days were treated with 20 μM ARBE for 2 h or 20 μM resveratrol for 5 h prior to addition

of 1.5 mM MM and incubation for an additional 9 h. **(A)** Effects of the treatments on cell permeability were measured by transepithelial electrical resistance (TEER) (0 h: P_{MM} =0.6861, P_{ARBE} =0.7991, $P_{Interaction}$ =0.7489; P_{MM} =0.8656, $P_{Resveratrol}$ <0.0295, $P_{Interaction}$ <0.9379; 3 h: P_{MM} <0.0001, P_{ARBE} =0.0581, $P_{Interaction}$ =0.1725; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0002, $P_{Interaction}$ <0.0001; 6 h: P_{MM} <0.0001, P_{ARBE} =0.0501, $P_{Interaction}$ =0.0081; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0079, $P_{Interaction}$ <0.0225; 9 h: P_{MM} <0.0001, P_{ARBE} =0.0012, $P_{Interaction}$ =0.0006; P_{MM} <0.0001, $P_{Resveratrol}$ =0.1730, $P_{Interaction}$ <0.8264) and **(B)** FITC dextran paracellular transport (0 h: P_{MM} =0.9606, P_{ARBE} =0.8041, $P_{Interaction}$ =0.2331; P_{MM} =0.8300, $P_{Resveratrol}$ <0.5290, $P_{Interaction}$ <0.2828; 3 h: P_{MM} =0.0005, P_{ARBE} =0.19581, $P_{Interaction}$ =0.0207; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0074, $P_{Interaction}$ <0.0023; 6 h: P_{MM} <0.0001, P_{ARBE} =0.0156, $P_{Interaction}$ =0.0015; P_{MM} =0.0005, $P_{Resveratrol}$ =0.0011, $P_{Interaction}$ <0.0017; 9 h: P_{MM} <0.0001, P_{ARBE} =0.0262, $P_{Interaction}$ =0.0005; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0372, $P_{Interaction}$ <0.0586). Measurements were done every 1.5 h after addition of MM. Bars represent means ± SD of 4 independent experiments with 3 treatment wells in each experiment for TEER and 3 independent experiments with 3 treatment wells in each experiment for FITC-dextran. * Mean value significantly different from the control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant difference between groups were tested with two-way ANOVA followed by Tukey's multiple comparisons post hoc test.

3.5. Discussion

The objectives of this study were to determine the effects of MM on intestinal epithelial cell oxidative stress, cytotoxicity and barrier function, and the abilities of ARBE and resveratrol to induce protective effects. Caco-2 cells were used as a model of intestinal epithelial cells due to their ability to hydrolyze MM to fatty acids (Spalinger et al., 1998), to de-glycosylate and metabolize polyphenols (Klumperman et al., 1991; Mizuma et al., 2005; Arafa, 2009; Henry-Vitrac et al., 2006), and to differentiate into a functional epithelial cell monolayer with barrier function (Lea, 2015) in ways similar to those produced by normal enterocytes.

Our data showed that challenge of Caco-2 cell monolayers with MM induces oxidative stress by increasing both intracellular and mitochondrial ROS generation by approximately 300% and 100%, respectively. Suppressing ROS generation can both prevent mitochondrial dysfunction and

increase mitochondrial efficiency, enabling the cell to maintain its ability to produce ATP and better resist stressful challenges that can increase permeability.

Comparison of the effects of ARBE and resveratrol on MM-induced increases in intracellular ROS and mitochondrial superoxide showed some differences between these two classes of polyphenols. Both polyphenols, even at the lowest concentration, completely prevented MM-induced intracellular ROS generation measured by DCF fluorescence, with ARBE acting slightly stronger. This antioxidant effect of ARBE showed a modest dose-dependence at the lower concentrations, whereas all concentrations of resveratrol acted similarly in reducing ROS levels. Possible explanations for the dose effect are that ARBE may be taken up to a lesser extent or require metabolic conversion (e.g. deglycosylation) to a more active species present in a lower concentration. Nevertheless, ARBE gave slightly stronger protection than resveratrol, and decreased intracellular ROS to levels below that in control cells.

The antioxidant effects of ARBE may be due to its direct antioxidant activity or due to blunting of ROS generation. In our results ARBE per se was also shown to decrease intracellular ROS generation compared to control cells while resveratrol alone, in the absence of MM challenge, did not suppress intracellular ROS. This may indicate that a stress must be present for resveratrol to exert measurable effects as an antioxidant. In agreement with our study, Xie et al. (2012) also showed that cyanidin-3-glucoside alone or as a pretreatment 30 min before exposure to glycated LDL for 24 h, decreased intracellular oxidative stress in cultured porcine aortic endothelial cells. Similar to our results employing MM, the lipoprotein exposure induced a 2.5-fold increase in ROS generation. Also, Elisia and Kitts (2008) showed that a purified blackberry anthocyanin extract protected Caco-2 cells against peroxyl radical (AAPH)-induced oxidative damage and cytotoxicity. Bornsek et al. (2012) showed anthocyanin rich bilberry extract, as used in our study, to significantly decrease AAPH-induced intracellular ROS generation in Caco-2 cells, and other cell types exposed to a radical initiating agent. Several other studies have shown anthocyanin rich extracts decrease intracellular ROS in Caco-2 cells exposed to different oxidative stresses (D'evoli et al., 2013; Tarozzi et al., 2006; Deiana et al., 2012; Schantz et al., 2010).

Resveratrol is a relatively weak direct-acting antioxidant compared to anthocyanins (Fukui et al., 2010; Rice-Evans et al., 1996), and its effects on intracellular ROS are usually attributed to effects

on metabolic pathways and expression of antioxidant enzymes. With hepatocytes for example, antioxidant effects against peroxide-induced intracellular and mitochondrial ROS and oxidative stress, were associated with activation of AMPK and NRF-2, which helped maintain mitochondrial function and increased expression of antioxidant enzymes (Shin et al., 2009; Rubiolo et al., 2008). Wang and colleagues (2016) also showed in Caco-2 cells exposed to H_2O_2, resveratrol treatment decreased intracellular ROS and improved intestinal epithelial cell monolayer barrier function. These effects were associated with increased expression and activity of superoxide dismutase and heme oxygenase-1.

In measurements of mitochondrial superoxide induced by MM, ARBE and resveratrol produced different effects. ARBE treatment decreased mitochondrial superoxide compared to MM exposure alone, but to a lower extent than it did for intracellular ROS. A possible explanation for a lesser effect on mitochondrial superoxide than intracellular ROS may be the amount of anthocyanin reaching different parts of the cell. Anthocyanins in ARBE are in the glycosylated form, and as reviewed by Kamiloglu et al. (2015), anthocyanins unlike other flavonoids could be transported through Caco-2 cells in the glycone form. Therefore, the amount of the more lipophilic aglycone form that reaches the mitochondria membranes after being deconjugated in intestinal cells may be much lower than the amount of glycoside presented to the cell. Also, anthocyanins decompose to phenolic products in cell culture media such as DMEM (Kuntz et al., 2015; Woodward et al., 2009), which over time would decrease the amount of anthocyanin entering the cell. According to the study of Peng (2012), an anthocyanin aglycone (anthocyanidin) can accumulate in mitochondria to a greater extent than the glycone or other flavonoids, in part, due to the mitochondrial membrane potential providing a negative charge in the matrix, which serves to attract positively charged oxonium forms of the anthocyanin species. While low amounts of the aglycone form in mitochondria may have limited the effect on mitochondrial superoxide compared to the effect on intracellular ROS, ARBE still substantially decreased mitochondrial superoxide. No previous studies have shown an effect of anthocyanins on mitochondrial superoxide in intestinal cells. Resveratrol treatment on the other hand, had no effect on mitochondrial superoxide.

We also evaluated the effect of MM and polyphenols on expression of mRNA for Mn-SOD, the mitochondrial antioxidant enzyme that scavenges superoxide (Candas and Li, 2014). Oxidative

stress increases the expression of Mn-SOD (Candas and Li, 2014), so the increased expression of Mn-SOD by MM is consistent with the MM-induced increases in intracellular ROS and mitochondrial superoxide. Therefore, the Caco-2 cells appear to increase Mn-SOD expression to compensate for the increase in oxidative species. While ARBE alone had no effect on expression of Mn-SOD, resveratrol alone increased mRNA expression of this enzyme (by 37% after 29 h). Resveratrol is known for its ability to induce mitochondrial biogenesis and may show this effect by increasing the quantity of mitochondria. In addition, resveratrol has been reported to directly stimulate complex I activity and increase oxidative stress and Mn-SOD expression in old mice (Gueguen et al., 2015). This complex is one of the major sites of ROS generation in mitochondria and therefore may be another reason why resveratrol triggers antioxidative defense systems such as Mn-SOD.

In the presence of MM however, both ARBE and resveratrol decreased Mn-SOD compared to MM-challenged cells. This effect is consistent with the strong inhibition of MM-induced intracellular ROS by both ARBE and resveratrol, such that the cells may not require increased Mn-SOD to combat the oxidative stress. The results also show that the decreases in MM-induced intracellular ROS and, in the case of ARBE, mitochondrial superoxide, are not due to increased expression of Mn-SOD. This effect of ARBE could possibly be due to a direct antioxidant effect of anthocyanins by scavenging the superoxide radical (Saint-Cricq de Gaulejac et al., 1999).

Increased ROS generation is an important signaling process and may induce inflammation (Mittal et al., 2014). Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine involved in the inflammatory response and plays an important role in apoptosis (Chen et al., 2008b) and thereby can contribute to increased intestinal permeability (Cui et al., 2010b; Novak and Mollen, 2015) by promoting the dismantling of tight junctions (Gibson, 2004; Cui et al., 2010b). Due to the role of inflammation in increasing intestinal permeability it was important to assess how MM affects expression of an inflammatory cytokine such as TNF-α.

Our findings show that MM exposure strongly increased (by 497%) the expression of mRNA for TNF-α. This increase may be due to the MM-induced increase in ROS generation induced by MM. Other studies (Cani et al., (2007) have also shown that high dietary fat increases expression of inflammatory cytokines such as TNF-α and IL-1β in visceral adipose tissue via enabling entry of

gut-derived LPS. In addition to provoking inflammation, TNF-α can itself have detrimental effects on cells, and exposure to TNF-α was shown previously to increase paracellular permeability of a differentiated Caco-2 cell monolayer (Cui et al., 2010b).

ARBE and resveratrol on the other hand greatly protected against MM-induced TNF-α expression, indicating that both polyphenols possess anti-inflammatory properties. Our data are consistent with the study of Lila et al. (2004), which showed anti-oxidant and anti-inflammatory effect of anthocyanins. Denis et al. (2015) also showed cranberry phenolic compounds (250 μg/mL) decreased oxidative stress and inflammation and improved mitochondrial function in Caco-2 cells. Other studies have also shown anti-inflammatory effect of anthocyanins in Caco-2 cells as demonstrated by decreased NF-κB, inhibition of NO secretion, and reduction of pro-inflammatory cytokines and adhesion molecules (reviewed by Kamiloglu et al., 2015; Jung et al., 2015; Taverniti et al., 2014). However, Graf et al. (2013) reported that a physiological concentration (15 mg/day) of anthocyanin during a treatment with blueberry and grape juice had no effect on gut associated lymphoid tissue and lymphocytes derived from blood and spleen (referred to as systemic immune system) in Fischer rats. Our results showed resveratrol to strongly decrease MM-induced TNF-α expression. Panaro et al., (2012) also showed resveratrol to prevent LPS-induced activation of inflammatory signaling in Caco-2 cells, which was associated with inhibition of both IκB degradation and expression of iNOS.

Interestingly, in our study, ARBE and resveratrol gave contrasting effects on expression of TNF-α when given alone (absence of MM) to Caco-2 cells. ARBE alone decreased TNF-α mRNA expression in Caco-2 cells by 32% in short term (after 2 h) and by 63% in long term (26 h) treatments. Resveratrol in contrast decreased TNF-α mRNA initially (after 0.5 h), but this effect was lost during the intermediate time points, only to increase by 73% at the 29 h time point. These effects of ARBE and resveratrol alone on TNF-α were similar to their effects on Mn-SOD and suggest that resveratrol acts as a stressor with longer term exposure.

Effects of MM and polyphenols on cellular viability were evaluated to gain more insight on the protection of polyphenols against MM-induced cytotoxicity to Caco-2 intestinal epithelial cells. Light microscopic images showing MM-induced cell patchiness and changes in morphology suggested impaired cell viability with exposure to MM. These images show Caco-2 cells pretreated

with ARBE or resveratrol especially at higher concentration (>10 µM), were relatively healthy and had similar morphology to control cells. Both polyphenols prevented the appearance of empty patches and granulation of Caco-2 cells exposed to MM. In quantitative measures of cell viability, MM substantially decreased resorufin and SRB signals (approximately 34 and 35% respectively) indicating a cytotoxic effect of MM. These measures reflect metabolic activity and number of attached cells, respectively. Increased ROS generation, as observed with exposure to MM, can disrupt biomembrane composition and mediate intracellular signaling cascades (Radi et al., 2002), leading to loss of cellular function and ultimately triggering apoptosis (Xuan et al., 2014). Thus, decreased resazurin reduction and SRB seen in cells exposed to 0.4 mM MM suggest decreased viability of Caco-2 cells induced by oxidative stress.

Although the SRB data suggested some protective effect of ARBE at 20 µM and resveratrol at intermediate concentrations (5 and 10 µM), the resorufin data failed to indicate any protective effect of the polyphenols against MM-induced cytotoxicity. ARBE or resveratrol treatment per se also did not affect the resorufin signal. Resorufin, a product of the metabolic conversion of resazurin by cellular reducing equivalents (i.e. NADH, NADPH, and FADH2) reflects viability, but since interpretation can be confounded when metabolism changes in cell populations of different treatments, thus is a crude marker of viability. While microscopic images and SRB data of cells pretreated with ARBE and resveratrol before MM exposure showed improvements, this was not reflected in increased resorufin signal. This could either indicate quiecent metabolic activity due to stability of the metabolic function in cells pretreated with polyphenols, or that the effect of MM is too strong for the antioxidants to prevent the overall effect on cell viability by this measure. Also, the method used to evaluate cell viability might be an explanation, similar to the study of Elisa & Kitts (2008), in which protective effects of anthocyanins against radical (AAPH) induced cell toxicity in Caco-2 cells were evident by the CellTiter-Glo assay (which quantifies ATP) whereas protection was not observed with the MTT assay. This could possibly imply that ROS produced by MM may not initially target mitochondrial function but rather affect cell membrane stability and function (Elisia & Kitts, 2008).

As another measure, we evaluated the proportion of viable and apoptotic cells by flow cytometry. FACS data showed that MM challenge decreased forward scatter and increased side scatter, indicating increased apoptosis of the cells.

Our data comply with the study of Tan et al. (2013) where MM with lower than 0.2 mM sodium deoxycholate (SDC) did not affect cell viability; however, MM containing 0.4 mM sodium deoxycholate decreased cell viability and affected the Caco-2 monolayer. In addition, Poli et al. (2004) showed lipid exposure increased changes in the biomembrane composition ultimately leading to loss of cellular functionality and viability. In line with the images, SRB and FACS data also suggest that ARBE and resveratrol treatments ameliorated the cytotoxic effect of MM. The FACS data showed that both polyphenols decreased the proportion of apoptotic cells and increased the proportion of viable cells compared to MM alone.

As an indication of the functional effects of MM and polyphenols on intestinal epithelial cell barrier integrity, we measured transepithelial electrical resistance (TEER) of differentiated Caco-2 cell monolayers. The TEER measurement is a sensitive method that assesses the integrity of tight junctions and paracellular permeability of the Caco-2 cell monolayer (Xiao et al., 2013; Srinivasan et al., 2015). In the current experiments, MM challenge decreased TEER, and thus increased the monolayer permeability. Dietary fat has been shown to damage intestinal cells leading to leaky gut (Zhang and Yang, 2016). The increased permeability may be due to MM-induced perturbations of surface fatty acids on integrity of the apical membrane. Fatty acids may interact with cell membranes causing release of calcium ions from the intracellular pool and thus disrupt tight junctions (Aspenström-Fagerlund et al., 2009). In addition, fatty acid exposure to Caco-2 cells affects several properties of the monolayer such as osmolality, critical micelle concentration and paracellular absorption (Lindmark et al., 1995). Several studies have shown effects of fatty acids on TEER in Caco-2 cells. A study by Aspenström-Fagerlund et al. (2009) showed that the two fatty acids common in foods, oleic acid (15, 30 mM) and DHA (5, 10 mM) for 90 min decreased TEER and increased aluminum absorption in Caco-2 cells. In another study Aspenström-Fagerlund et al. (2007) showed that both fatty acids increased paracellular absorption of mannitol and cadmium in Caco-2 cells as a result of fatty acid induced increase in permeability. Droke et al. (2003) showed 1 mM oleic acid for 8 h increased paracellular movement of iron in Caco-2 cells due to an increase in permeability reflected by a decrease in TEER. Roche et al. (2001) found exposure of Caco-2 cells to lower concentrations (0.05 mM) of linoleic acid, cis-9 CLA, or trans-10 CLA for 21 days increased paracellular permeability. Sawai et al. (2001) also showed 2 mM

oleic acid or palmitic acid for 2 h decreased TEER by 21% and 23%, respectively, which was weaker than the effect of lysophosphatidyl choline (94%).

Our data showed that pretreatment with ARBE before MM, lead to maintenance of a higher TEER value compared to cells challenged with MM alone, indicating less permeability and higher integrity of these cells. Thus, ARBE protects against disruption of the barrier induced by MM. The study of Shin et al. (2011) also showed anthocyanins to increase TEER in HCT-116 colon cancer cells grown on a membrane compared to control cells. The effect of anthocyanins may be due to inhibition of the NF-κB pathway (Cremonini et al., 2017). The results of our study suggest that this protective effect may occur by decreasing MM-induced intracellular ROS and mitochondrial superoxide.

In our study, resveratrol pretreatment showed only short-term protection against MM-induced permeability. After 6 h there was mild protection which was lost after 9 h of MM challenge. Carrasco-Pozo et al. (2013), showed resveratrol treatment at much higher concentrations than what we applied (438 μM) protected against the decrease in TEER induced by indomethacin. Also, resveratrol (15 mg/kg body weight (BW)/day) protected against a high-fat/sucrose diet (HFS) in Wistar rats, which was associated with alterations in mRNA expression of tight junction proteins and inflammation-associated genes (Etxeberria et al., 2015). It is not clear in the current study why the protective effect of resveratrol decreased and was lost by 9 h. Although resveratrol decreased MM-induced ROS generation, cytotoxicity and TNF-α production, and in the short term was effective in blunting the drop in TEER, its effects on MM-induced parameters might not be strong or sustained enough to inhibit the decrease of TEER in the long term (9 h).

In summary, MM increased intracellular and mitochondrial oxidative stress, induced expression of the pro-inflammatory cytokine TNF-α and decreased intestinal epithelial cell barrier integrity in a Caco-2 cell model. ARBE, and resveratrol were able to protect against MM-induced oxidative stress, inflammation and permeability in this model with ARBE being more protective compared to resveratrol. The results suggest that both dietary polyphenols have the potential to protect against oxidative stress and inflammation caused by challenge with dietary lipids, a major component of Western diets, and help maintain the integrity of the intestinal barrier to prevent related disease.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interests.

3.6. Transition to CHAPTER 4

In CHAPTER 3, we focused on antioxidant effects of anthocyanins and resveratrol against oxidative stress and epithelial barrier permeability induced by MM as a model of high dietary fat intake. In the next chapter we investigated effects of MM, ARBE and resveratrol on mitochondrial biogenesis and function to distinguish possible protective mechanisms.

CHAPTER 4 MITOCHONDRIAL MECHANISMS IN PROTECTION BY ANTHOCYANINS AND RESVERATROL AGAINST MIXED MICELLE-INDUCED BIOENERGETIC DYSFUNCTION IN CACO-2 INTESTINAL EPITHELIAL CELLS

4.1. Abstract

Consumption of excess dietary fat imposes a stress on intestinal cells, increasing their demand for ATP to combat the stress. Healthy and efficient mitochondria have a critical role in maintaining barrier function and preventing pathological conditions related to increased intestinal permeability. While there is evidence supporting the protective effects of polyphenols on mitochondrial health and function in some tissues, a possible beneficial effect of dietary polyphenols on intestinal mitochondria has not gained much attention. This study investigated potential protective effects of anthocyanin-rich bilberry extract (ARBE) and resveratrol, representing two classes of polyphenols found in purple grapes and other berries, on bioenergetic stress induced in intestinal epithelial cells by exposure to mixed lipid-bile acid micelles. Exposure of intestinal epithelial Caco-2 cells to MM used as a model of ingested fat exposure to gut epithelia, decreased mitochondrial membrane potential, mitochondrial content and expression of mRNA for proteins involved in mitochondrial biogenesis and respiration (MTND1, MTCYB, MTCO1, MTATP), and parameters of mitochondrial respiratory function. Treatment with ARBE protected against the decreases in mitochondrial membrane potential and parameters of mitochondrial function but had little effect on indices of mitochondrial content and biogenesis. Resveratrol, in contrast, increased mitochondrial content and genes involved in mitochondrial biogenesis but did not significantly protect against MM-induced declines in mitochondrial membrane potential and mitochondrial respiratory function. The results suggest that anthocyanins and resveratrol act by different mechanisms, and that anthocyanins can help protect intestinal epithelial barrier function against dietary lipid-induced mitochondrial dysfunction in a manner that does not require mitochondrial biogenesis.

Abbreviations

ΔΨm, mitochondrial membrane potential; **A/R**, antimycin A/rotenone; **AMPK**, AMP-activated protein kinase; **ANOVA**, analysis of variance; **ARBE**, anthocyanin-rich bilberry extract; **ATP**, adenosine triphosphate; **CCCP**, carbonyl cyanide 3-chlorophenylhydrazone; **cDNA**, complementary DNA; **CS**, citrate synthase; **DMEM**, Dulbecco's modified essential medium; **DMSO**, dimethyl sulfoxide; **DNA**, deoxyribonucleic acid; **DTNB**, 5,5′-dithiobis (2-nitrobenzoic acid); **ECAR**, extracellular acidification rate; **ETC**, electron transport chain; **FBS**, fetal bovine serum; **FCCP**, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; **HBSS**, Hank's balanced salt solution; **KO**, knock out; **LPS**, lipopolysaccharide; **MM**, mixed micelles; **Mn-SOD**, manganese-superoxide dismutase; **MPT**, mitochondrial permeability transition; **mRNA**, messenger RNA; **MTATP6**, mitochondrially encoded ATP synthase F_o subunit 6; **MTCO1**, mitochondrially encoded cytochrome c oxidase I; **MTCYB**, mitochondrially encoded cytochrome b; **mtDNA**, mitochondrial DNA; **MTDR**, mitotracker deep red; **MTND1**, mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1; **NADH**, nicotinamide adenine dinucleotide; **NAD(P)H**, nicotinamide adenine dinucleotide phosphat**e; NF-κB**, nuclear factor kappa b; **NRF-1**, nuclear respiratory factor-1; **OCR**, oxygen consumption rate; **OXPHOS**, oxidative phosphorylation; **PBS**, phosphate buffered saline; **PCR**, polymerase chain reaction; **PGC-1α**, peroxisome proliferator-activated receptor gamma coactivator 1α; **PPAR**, peroxisome proliferator-activated receptor; **ROS**, reactive oxygen species; **SD**, standard deviation; **SIRT1**, sirtuin 1; **SRB**, sulforhodamine b; **ROS**, reactive oxygen species; **RT-qPCR**, quantitative reverse transcription PCR; **TCA**, tricarboxylic acid; **TFAM,** transcription factor A, mitochondrial; TMRE, tetramethlyrhodamine ethyl ester; **TNFα**, tumor necrosis factor alpha; **UCP**, uncoupling protein

4.2. Introduction

Intestinal cells create a barrier for the body to prevent entrance of undigested food and luminal contents into the systemic circulation. Mitochondria have a significant role in providing ATP to maintain tight junction protein complexes that are needed to preserve the integrity of the intestinal barrier. Alteration in mitochondrial morphology, disruption in mitochondrial function and decreased ATP production can disrupt the intestinal barrier leading to increased permeability (Novak and Mollen, 2015). Increased fat consumption has contributed to many chronic diseases (Lichtenstein et al., 1998; Hoffman et al., 2004), and part of the adverse effects of high fat intake may be its influence on the gastrointestinal tract. Consumption of high amounts of fat can induce functional disruption in the gut barrier leading to increased intestinal permeability and endotoxemia (Bischoff et al., 2014; Kvietys et al., 1991; Moreira et al., 2012; Murakami et al., 2016; Novak and Mollen, 2015). Therefore, maintaining the function and integrity of the gut is an important factor in preventing diet-related diseases (Y Lee, 2013).

High dietary fat also induces changes in mitochondria and impairs mitochondrial function by creating a stress that imposes an increased demand to produce more ATP in the affected intestinal mucosa and which associates with an increase in oxidative phosphorylation and proteins involved in mitochondrial β-oxidation (Wiśniewski et al., 2014). Increased mitochondrial workload is associated with rise in mitochondrial oxidant production which can eventually impair mitochondrial function, disrupt tight junction proteins and induce loss of gut barrier integrity (Moreira et al., 2012). Mitochondrial oxidative stress and impaired ATP production have also been demonstrated in intestinal diseases such as inflammatory bowel disease (Novak and Mollen, 2015).

An increasing body of evidence suggests beneficial properties of the dietary polyphenols, such as anthocyanins and resveratrol. Anthocyanins are especially well-known for their antioxidant and anti-inflammatory effects (Chrubasik and Chrubasik, 2010; Dai et al., 2009; Neto, 2007; Thomasset et al., 2009; Lila, 2004), and are shown to protect mitochondria in several tissues by acting as antioxidants, regulating energy metabolism and inhibiting mitochondrial dysfunction (Xie et al., 2012a; Skemiene et al., 2015; Zhao et al., 2015). Unlike resveratrol, anthocyanins do not directly activate SIRT1 (Howitz et al., 2003) or increase mitochondrial biogenesis and, therefore, may act by a different mechanism such as ROS scavenging or direct effects on the

respiratory chain such as acting as electron acceptors at complex I, thus bypassing ischemia-induced inhibition and increasing ATP generation (Skemiene et al., 2015). Resveratrol has also been known for its wide range of bioactivity including antioxidant, anti-inflammatory, anti-aging and anticancer effects (Gambini et al., 2015). The effect of this polyphenol in inducing mitochondrial biogenesis, at least, in part, through activation of SIRT1, is well established (Csiszar et al., 2009; Kelkel et al., 2010; Price et al., 2012; Lagoa et al., 2011). Effects of resveratrol on mitochondrial biogenesis include increases in mitochondrial mass, mitochondrial DNA content, and components of the respiratory chain, which are stimulated by induction of nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Szabo, 2009). Many studies have investigated protective effects of resveratrol and polyphenols in different tissues by inducing increased numbers and function of mitochondria (Csiszar et al., 2009; Price et al., 2012; Onyango et al., 2010; Higashida et al., 2013). Among studies investigating the protective effect of resveratrol on intestine and related diseases, most have focused on its anti-inflammatory and antioxidant enzyme-inducing effects (Moura et al., 2015).

Limited studies have focused on understanding the effects of polyphenols on systemic metabolic diseases from a gut perspective. Moreover, there is lack of information about protective effects of polyphenols in preventing the impairments in mitochondrial function due, in part, to mitochondrial damage caused by excess fat intake, and thereby avoiding gut permeability. We postulate that resisting the adverse effect of excess fat will require an increase in energy output at the level of the small intestinal barrier, as this tissue represents a major anatomical site from which lipopolysaccharide (LPS or endotoxin) can enter the blood stream to incite inflammation. Enabling improvements in mitochondrial function, bioenergetic capacity and biogenesis could lead to a strengthened gut wall and enhance resistance to stressors that could facilitate entry of LPS or other unwanted antigens and incite chronic inflammation.

This study investigated the effect of these polyphenols, at concentrations that can be readily achieved by diet, on the mitochondrial and barrier functions of Caco-2 intestinal epithelial cells. We reason that improvements in cellular bioenergetics at the level of the intestinal epithelial cells, can confer improved resiliency to dietary stressors. This may be achieved by either increasing the number of mitochondria per cell or improving their efficiency, both of which can lead to increased ATP production. We therefore measured the effects of ARBE, resveratrol and MM on

mitochondrial biogenesis, gene expression and indices of mitochondrial function and respiration in Caco-2 cells challenged with MM as a model for excess dietary fat intake

4.3. Materials and methods

A standardized anthocyanin rich bilberry extract (Mirtoselect®) was kindly provided by Indena S.p.A. and was used as a standardized preparation including C3G (detailed information about ARBE provided in Appendix 8.A). Fetal bovine serum (FBS), penicillin/streptomycin mixture, sodium oleate, sodium palmitate, sodium deoxycholate, phosphatidyl choline (egg, chicken), oxaloacetate (OAA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetyl CoA, protease inhibitor cocktail, and resveratrol were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified essential medium (DMEM) and Hank's balanced salt solution (HBSS) was acquired from Life Technologies (Waltham, MA, USA). VILO cDNA synthesis kit, Trizol reagent, and Power SYBR Green PCR master mix for the polymerase chain reaction were purchased from Invitrogen (NY, USA), and RNAeasy mini kit for isolating RNA was purchased from Qiagen (MD, USA). Caco-2 cells were obtained from the American Type Culture Collection (MD, USA).

4.3.1. Caco-2 Cell Culture

Caco-2 cells were grown as described by Natoli et al. (2012) in T75 flasks containing DMEM supplemented with 10% FBS, 25 mmol/L glucose, 2 mmol/L glutamine, 100 mmol/L nonessential amino acids, and 1% penicillin/streptomycin (100 units/mL penicillin, and 100 mg/mL streptomycin) and maintained at 37 ^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded at 5-10×10^4 cells per well in 96 well plates and grown to confluence over 24 h in DMEM with 10% FBS. On the day of an experiment, the medium was replaced with DMEM containing 2% FBS.

4.3.2. Mixed Micelle Preparation

In order to prepare MM, 1.4 mg sodium palmitate and 1.54 mg sodium oleate were dissolved in methanol. 15 mg phosphatidyl choline was dissolved in chloroform and added to the fatty acid mixture. Lipids were dried under nitrogen and 12.4 mg of sodium deoxycholate dissolved in PBS

was added to the solution. The mixture was rotated continuously in dry ice to provide a frozen film in the tube, which was then placed in a lyophilizer for 48 h to dry. Dried samples were then stored at -20 °C until the day of an experiment. On the day of the experiment 1 mL of the DMEM without FBS was added to the dried sample and vortexed to produce 60 mM lipid and the content was aliquoted in 10 micro-tubes each containing 100 µL for future experiments. For each experiment one tube was added to cell culture medium to produce a final concentration of 0.4 mM lipids.

4.3.3. Treatment with mixed micelles and polyphenols

Caco-2 cells were cultured and grown to confluence in 96-well optical-bottom plates. Stock solutions of 20 mM ARBE and resveratrol in DMSO were prepared and maintained in a -20 °C freezer. ARBE contains 36% anthocyanins (considered as mM C3G equivalents per g of dry extract) and a 20 mM stock solution of ARBE was prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in medium was at most 0.1% (for the highest concentration of ARBE and resveratrol that is 20 µM) and lower concentrations of polyphenols were made via serial dilution to decrease the amount of DMSO exposure to cells. To control for any toxic effect of DMSO, the highest concentration (0.1%) was added to control cells. On the day of an experiment, cells were pretreated for 2 h with ARBE (Sergent et al., 2005) or for 5 h with resveratrol (Xu et al., 2012) at different concentrations (1.25, 2.5, 5, 10, 15, 20 µM) in 2% FBS media. After pre-treatment with polyphenols cells were challenged with 0.4 mM MM for 24 h in 2% FBS media, and the effects on different parameters were assessed.

4.3.4. Mitochondrial membrane potential assessment

The mitochondrial membrane potential ($\Delta\Psi m$) was determined based on the fluorescence intensity of tetramethlyrhodamine ethyl ester (TMRE) staining. TMRE is a cationic fluorescent dye which due to its positive charge and lipophilicity is attracted and driven to the negatively charged mitochondrial matrix where it accumulates in an inner membrane potential dependent manner. TMRE does not accumulate when the mitochondrial membrane potential is disrupted, as in the case of depolarized or inactive mitochondria (Farkas et al., 1989). Caco-2 cells (seeded at approximately 5×10^4 cells per well) were grown to confluence in 96-well plates in DMEM medium with 10% FBS. On the day of an experiment, the medium was changed to DMEM medium with

2% FBS. Cells were treated with polyphenols and MM as described previously, then washed with PBS and incubated with TMRE (100 nM) for 30 min at 37 °C in the dark in the incubator. Excess dye was removed by carefully washing with HBSS and cell-associated fluorescence was measured with a Synergy H1 plate reader at an excitation wavelength of 549 nm and an emission wavelength of 575 nm. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 50 μM), which collapses the mitochondrial membrane potential was added to control cells at the same time of TMRE and used as control for depolarized mitochondria. Data were expressed as percent of control. The mitochondrial membrane potential of cells stained with TMRE was also captured by a Zeiss Axiovert imager.

4.3.5. RT-PCR

4.3.5.1. RNA isolation from Caco-2 cells

Total RNA was isolated from Caco-2 cells pretreated with 20 μM ARBE for 2 h or resveratrol for 5 h and then challenged with MM (0.4 mM) for 24 h, to determine relative mRNA expression after each treatment. Trizol reagent was used for cells lysis and RNAeasy mini kit was used according to the manufacturer's instructions. Caco-2 cells were plated at 5×10^5 cells per well in 6-well plates. After reaching confluence, medium was replaced, cells were pretreated with ARBE or resveratrol and then challenged with MM. After treatments, cells were then washed with HBSS and 1 mL of Trizol was added to each well for 3 min to lyse the cells. Cell lysates were then transferred to an RNase free Eppendorf tube (1.5 mL) and 200 μL chloroform was added to each sample and samples were vortexed for 10 sec. After incubating at room temperature for 3 min, the lysates were vortexed again and centrifuged at 12,000 g for 10 min at 4 ºC. The clear supernatant was carefully removed and transferred to a new Eppendorf tube and 500 μL of isopropanol was added to each sample. After incubation for 5 min at room temperature, each sample was applied to a spin column and centrifuged at 4 ºC and 8000 ×g for 15 sec. The flow-through was discarded and 350 μL RW1 buffer (provided within the kit) was added to each sample and centrifuged again at 4 ºC and 8000 ×g for 15 sec. In order to remove any DNA contamination, 80 μL (1X) DNase (Invitrogen, Grand Island, NY, USA) was added to each sample followed by incubation at room temperature for 15 min. Samples were washed again with 350 μL RW1 buffer and centrifuged. The flow through was discarded and samples were washed twice with 500 μL RPE (provided with the kit) buffer and centrifuged again. Samples were then spin-dried for 1 min and the spin columns were transferred

to a new RNase free microtube. After adding 40 µL of nuclease free water (Invitrogen, USA), each spin column was centrifuged at 8000 ×g for 1 min at 4 °C. The flow-through containing purified total RNA was used to measure concentration and purity of the acquired RNA using a Nano drop spectrophotometer (BioRad, USA) and the A260/A280 absorbance ratio. All extracted RNAs had an A260/A280 absorbance ratio of 2-2.1 indicating highly pure RNA (within the acceptable ratio of 1.8-2.1). Samples were then stored at -80 °C until they were used for synthesis of complementary DNA.

4.3.5.2. Synthesis of complementary DNA (cDNA)

A VILO cDNA synthesis kit (Invitrogen, USA) and a Thermocycler (BioRad, USA) was used for reverse transcription of 2 µg total RNA to cDNA. In order to acquire cDNA, the thermal cycling was performed as follows: one cycle at 25 °C for 10 min, one cycle at 42 °C for 60 min, and one cycle at 85 °C for 5 min (to inactivate the DNA polymerase). The reaction was performed in 20 µL samples. The purity and concentration of the synthesized DNA was measured using a Nano drop spectrophotometer (BioRad, USA) and the A260/A280 absorbance ratio. All extracted cDNA had an A260/A280 absorbance ratio of 1.8-2.2 indicating highly pure cDNA. Samples were then stored at -80 °C until analysis by PCR.

4.3.5.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed using a Quant Studio12K Flex Real-time PCR system (Thermofisher Scientific, CA, USA) using SYBR Green master mix (Life Technologies, NY, USA) according to the manufacturer's protocol. Amplification was carried out in 15 µL reactions containing 7 µL SYBR Green master mix, 2 µL forward and reverse primers each, 2 µL nuclease free water and 2 µL cDNA. cDNA samples were diluted 20 times before use according to the VILO cDNA synthesis kit protocol to remove PCR inhibitory effects of reverse transcription. All primers were provided by Integrated DNA Technologies (IDT) (ON, Canada). Table 4.1 shows primer sequences used in the current study. The experimental protocol consisted of the following programs: One cycle at 95 °C for 5 min (for enzyme activation), amplification and annealing including 40 cycles at 95 °C for 15 sec and 60 °C for 30 sec. Melting curves of all PCR products were evaluated to confirm the amplification and primer quality. A comparative

method ($2^{-\Delta\Delta CT}$) was used to analyze the relative expression of genes of interest. β-actin and GAPDH were both used as housekeeping genes which both yielded similar data. All data expressed were normalized to β-actin. Two independent experiments were conducted with 3 wells of cells per treatment condition in each experiment.

Table 4.1. Nuclear and mitochondrial gene encoded mRNA measured via qPCR in Caco-2 cells

Gene	Species	Primer sequence (5'-3')	Cycles (Ct values)
PGC-1α[å]	Human	Sense: TGA ACT GAG GGA CAGTGA TTT C Antisense: CCC AAG GGT AGCTCA GTT TAT C	23
NRF-1[ß]	Human	Sense: GTATCT CAC CCT CCA AAC CTA AC Antisense: CCA GGATCA TGC TCT TGT ACT T	24
TFAM[∂]	Human	Sense: ATA GGC ACA GGA AAC CAGTTA G Antisense: GCA GAA GTC CAT GAG CTG AAT A	24
MTND1[f]	Human	Forward: CTT AGCTCT CAC CAT CGCTCT T Reverse: AGATTG TTT GGG CTA CTG CTC G	19
MTCYB[˙]	Human	Forward: GAT CCT CCA AAT CAC CAC AGG AC Reverse: GGA GGATAATGC CGATGT TTC AG	17
MTCO1[˚]	Human	Forward: GAG CTG GGC CAG CCA GGC AA Reverse: GGA AAC GCC ATATCG GGG GCA	15
MTATP6[ˆ]	Human	Forward: GCC GCA GTA CTG ATC ATT CTA TTT C Reverse: TCG GTT GTT GAT GAG ATA TTT GGA	14

[å]Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α)

ß Nuclear respiratory factor 1 (NRF-1)
∂ Transcription factor A, mitochondrial (TFAM)
ƒ Mitochondrially Encoded NADH:ubiquinone Oxidoreductase Core Subunit 1(MTND1)
˙ Mitochondrially Encoded Cytochrome B (MTCYB)
˚ Mitochondrially Encoded Cytochrome C Oxidase I(MTCO1)
ˆ Mitochondrially Encoded ATP Synthase Membrane Subunit 6 (MTATP6)

4.3.6. Flow cytometry analysis

Cells (2.5×10^5 cells/mL) were analyzed by flow cytometry on a FACSDiva (BD Biosciences, San Jose, CA, USA). Before analysis, cells were gated to perform measurements on morphologically normal single living cells. The fluorogenic MitoTracker Deep Red FM (MTDR) were used to determine mitochondrial content. For measuring mitochondrial content, after washing and centrifugation at 1,000 ×g for 5 min, cells were stained with 25 nM MTDR for 30 min at room temperature. The fluorescence emission for MTDR was also detected and analyzed by flow cytometry (excitation wavelength 635 nm, emission filter 661/16 nm). Data were collected for 10,000 cells per group and were normalized to and expressed as percent of the control group.

4.3.7. Citrate synthase activity

Measurements of citrate synthase activity were performed as per Eigentler et al. (2015). In brief, cells were seeded at 2.5×10^5 cells/well in 24 well plates. Caco-2 cells were pretreated with ARBE for 2 h or resveratrol for 5 h and then challenged with 0.1 or 0.4 mM MM for an additional 24 h. After the treatment, cells were washed twice with ice-cold PBS then trypsinized and pelleted at 1,000 ×g for 5 min. Cells were then lysed using lysis buffer containing 150 mM sodium chloride, 10 mM Tris, 0.2% Triton X-100, 0.3% NP-40, 0.2 mM sodium vanadium oxide and protease inhibitor cocktail, pH 7.7 (comprised of aprotinin, bestatin, E-64, leupeptin, pepstatin A) (Fouad et al., 2013). The cell lysates were centrifuged at 13,200 ×g for 15 min at 4 ℃ and the supernatant was then stored at -80 ºC until the day of measurement. For measurement of citrate synthase activity different components of the assay were added and formation of the yellow product 5-thio-2-nitrobenzoic acid at 412 nm was measured spectrophotometrically after incubation on ice for 10 min. Protein content was determined by the Bradford method and enzyme activity was normalized to protein content and data was expressed as percent of control cells.

4.3.8. Measurements of mitochondrial function

Mitochondrial respiration was assessed in a Seahorse XFe96 Extracellular Flux Analyzer (Seahorse analyzer) (Seahorse Bioscience, North Billerica, MA) using an XF Cell Mito Stress Test Kit to assess mitochondrial function. This kit measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The Seahorse XFe96 Extracellular Flux Analyzer also measures different aspects of mitochondrial function including basal and maximal respiration, spare capacity, proton leak and ATP production. Measurements were based on oxygen dependent quenching of fluorescent sensors. Caco-2 cells were cultured on Seahorse XFe96 provided micro plates at a seeding density of 1.0×10^4 cells/well in 10% FBS media. After 24 h, FBS concentration was lowered to 2%, the experimental treatments were performed, and oxygen consumption measured. A dose response of several concentrations of MM (0.5, 0.1, 0.2, 0.3, 0.4 mM) for 24 h was assessed and a dose of 0.1 mM MM was demonstrated as the best for inducing stress while preventing complete collapse of OCR (Appendix Fig. 8.B.8). For experiments designed to test the effects of polyphenols, the cells were pretreated either with 20 μM ARBE for 2 h or resveratrol for 5 h, and incubated for an additional 24 h with or without 0.1 mM MM. The sensor cartridge was hydrated with calibration buffer 1 day prior to the experiment as described in the kit instructions. After the experimental treatments media were replaced with DMEM XF buffer medium (supplemented with 25 mM glucose, 1 mM L-glutamine, 2 mM sodium pyruvate and 1% FBS), pH 7.4, and incubated at 37 °C. Cells were then incubated in a non-CO_2 incubator at 37 ºC for 1 h before running on the Seahorse XFe96 Extracellular Flux Analyzer.

Baseline measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were taken before injection of reagents and chemicals (1 μM oligomycin, 1 μM FCCP, and 0.5 μM antimycin A/rotenone (A/R)). These substances were injected through ports of the Seahorse Flux Pak cartridges. Oligomycin was injected to measure loss of ATP production. FCCP was added to measure maximal respiration and complex I and III inhibitors rotenone/ antimycin A (0.5 μM) were added to determine non-mitochondrial respiration. A measurement showing the different components of respiration evaluated by the Seahorse XFe96 Extracellular Flux Analyzer is shown in Figure 4.1 (Seahorse manual).

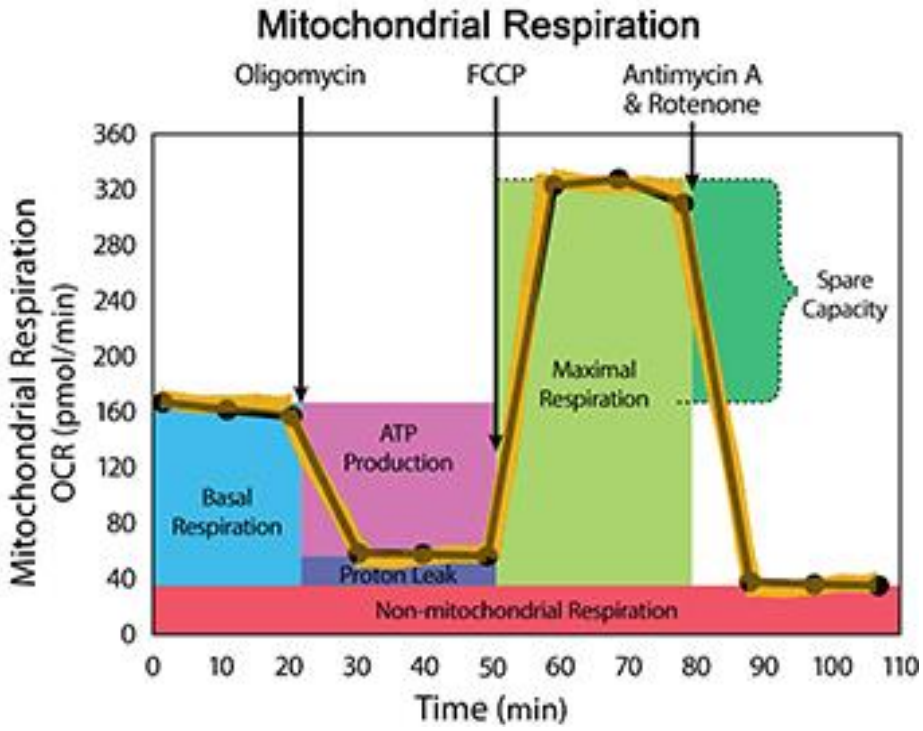


Figure 4.1. *The main parameters of mitochondrial function that influence the oxygen consumption rate (OCR)*: basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. *Note.* Reprinted https://www.agilent.com/en/products/cell-analysis/mitochondrial-respiration-xf-cell-mito-stress-test. Reprinted with permission (Appendix 8C).

Basal respiration indicates the oxygen consumption under basal, non-stressed conditions. Oligomycin was injected to the wells to inhibit ATP synthase. Inhibition of mitochondrial ATP production by oligomycin creates a dependence of cells on glycolysis to provide ATP. FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) is a mitochondrial uncoupling agent (Beeson et al., 2010) that dissipates the proton gradient, which causes an increase in oxygen consumption that is correlated with the capacity of mitochondria to generate ATP. Thus, this uncoupler of the proton gradient enables one to determine the mitochondrial bioenergetic capacity of the population of cells measured, including its "spare respiratory capacity". Cells that consume more oxygen in response to FCCP possess a greater potential to create more ATP when coupled.

Spare respiratory capacity is defined as the difference between maximal and basal respiration and is the ability of substrate supply and electron transfer to provide enough ATP when energy demand increases above basal needs. Higher spare capacity and ATP production indicates increased mitochondrial efficiency, conferring heightened ability to combat the energy demands of a stressor. Rotenone and antimycin A block respiration and mitochondrial ATP production by inhibiting the transfer of electrons through complex I and III, and, therefore, prevent the reduction of oxygen, the terminal electron acceptor, at complex IV. Finally, proton leak is determined by subtracting the OCR due to ATP production from that due to basal respiration after addition of oligomycin to block ATP production. The OCR reading after oligomycin treatment is due to protons leaking back across the membrane (e.g. through uncoupling proteins) as well as non-mitochondrial oxygen consumption pathways. While OCR measurements in combination with these various inhibitors (i.e. oligomycin, FCCP, antimycin A/rotenone) denote different parameters of mitochondrial respiratory functions, the measurement of the extracellular acidification rate (ECAR) is used as an index of glycolysis by specifying lactate production through the measurement of pH changes. OCR and ECAR were measured in the Seahorse XFe96 extracellular flux analyzer over 3 h. Titration of FCCP was performed initially to determine the appropriate concentration to induce maximal respiration and the final concentration of oligomycin, FCCP, and rotenone/antimycin A of 1, 1, and 0.5 μM were used, respectively.

After measuring OCR and ECAR the amount of cell protein of each well was determined by the Bradford assay and cell protein was used to normalize respiration rate in each well. At least 4 replicates of each treatment were measured, and the experiments were repeated independently 3 times.

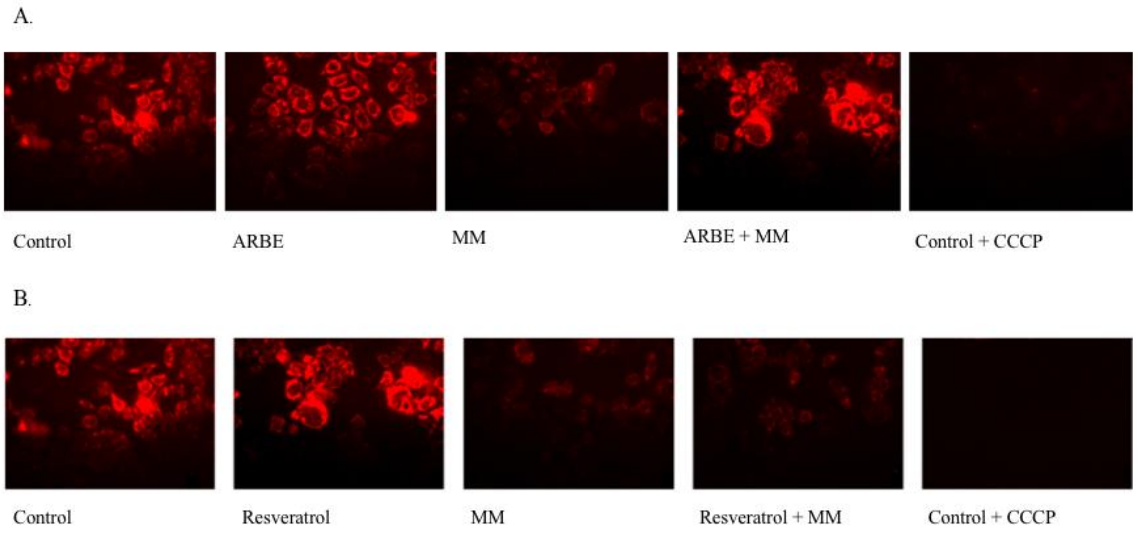
4.3.9. Statistical analysis

Data were represented as means ± standard deviations and normalized to percent of control cells. Each experiment was repeated at least two times in triplicate. Data were analyzed using two-way ANOVA or one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism (Version 7; La Jolla, CA, USA). Differences were considered statistically different at $P < 0.05$.

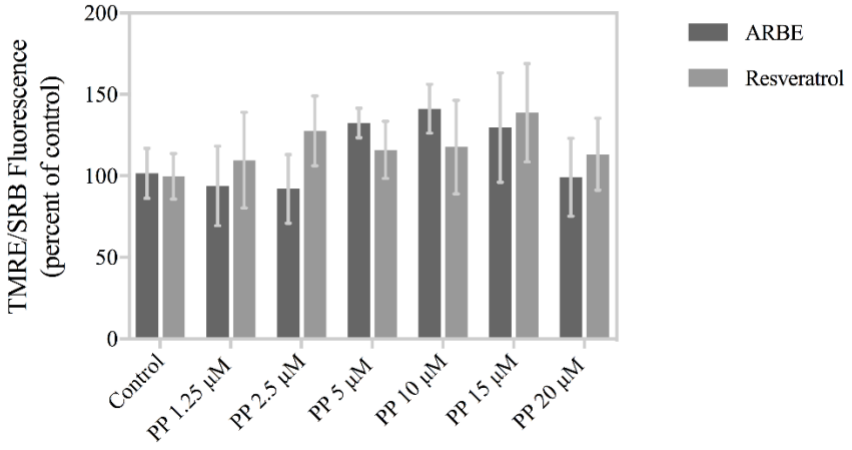
4.4. Results

4.4.1. Mitochondrial membrane potential

To investigate the effect of MM, ARBE and resveratrol on mitochondrial membrane potential (ΔΨm), Caco-2 cells were pretreated with ARBE and resveratrol for 2 or 5 h, respectively, prior to adding 0.4 mM MM for 24 h. Fluorescence images via ZEISS Axiovert, displayed in Figure 4.2.A & B, show that MM decreased the TMRE signal, indicating loss of mitochondrial membrane potential (Fig. 4.2.A & B). As shown in Figure 4.2A and B, ARBE prevented this decrease at 20 μM, while resveratrol failed to protect against loss of TMRE, indicating that the latter did not preserve mitochondrial membrane potential following MM challenge. To quantify the effect of both polyphenols on ΔΨm, the fluorescent signal of TMRE was also measured via plate reader. Treatments with ARBE or resveratrol alone did not significantly affect TMRE fluorescence indicating that ΔΨm was not altered by either compound (Fig. 4.2.C). Challenging cells with 0.4 mM MM decreased TMRE fluorescence intensity to approximately 60% of control values (Fig. 4.2.D). Data showed that pretreatment with different concentrations of ARBE (1.25-20 μM) for 2 h prevented the MM-induced decrease of TMRE fluorescence at concentrations below 15 μM. Compared to ARBE, resveratrol only protected against loss of mitochondrial membrane potential at 1.25 μM (Fig. 4.2.D).



C.



D.

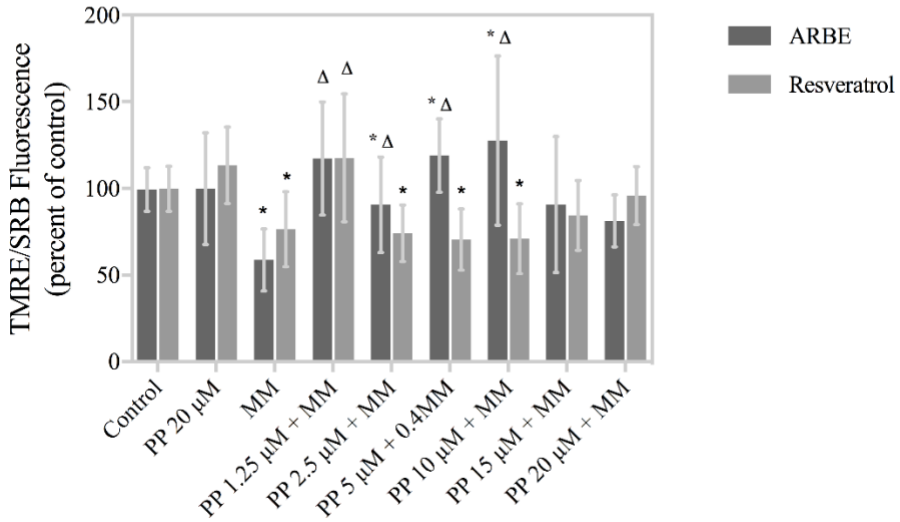


Figure 4.2. *Effects of MM, ARBE and resveratrol on mitochondrial membrane potential (ΔΨm).* Mitochondrial membrane potential was measured after treatment with ARBE or resveratrol at 20 µM alone or for 2 or 5 h respectively prior to adding 0.4 mM MM and incubating for an additional 24 h and staining with TMRE for 30 min using a micro plate reader. **(A & B)** Images of TMRE fluorescence captured at 40× objective lens using a ZEISS Axiovert Fluorescent Cell Imager showing effects of MM and ARBE or resveratrol on mitochondrial membrane potential. **(C)** Absence of MM (P_{ARBE} =0.0004; $P_{Resveratrol}$ =0.1997). **(D)** Presence of MM (P_{MM} <0.0001, P_{ARBE}

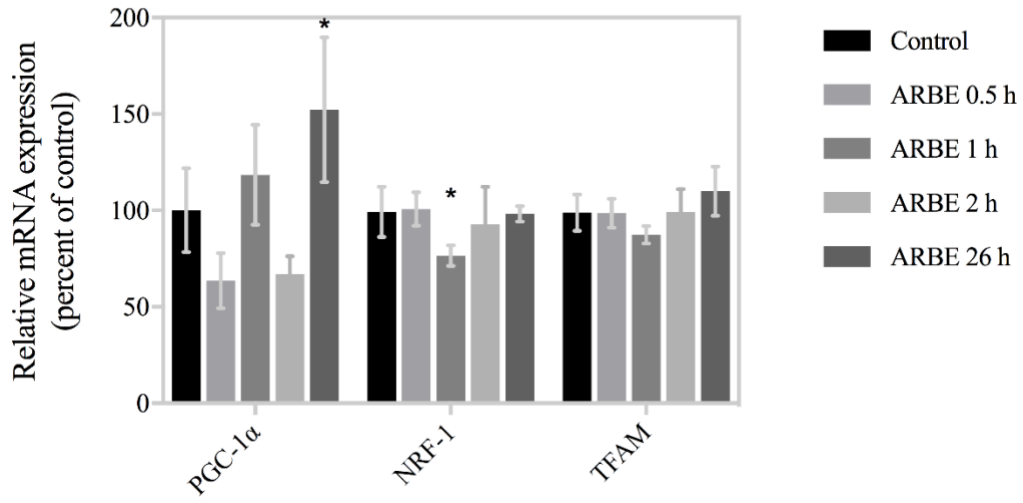
=0.0004, $P_{Interaction}$ <0.0001; P_{MM} =0.1771, $P_{Resveratrol}$ =0.0004, $P_{Interaction}$ =0.0362). Values are expressed as means ± SD of 4 independent experiments with 4 wells of cells in each experiment. * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). The significant difference between groups in C was tested with one-way ANOVA and in D with two-way ANOVA followed by Tukey's multiple comparisons test.

4.4.2. Effect of polyphenol treatments on expression of genes involved in mitochondrial biogenesis

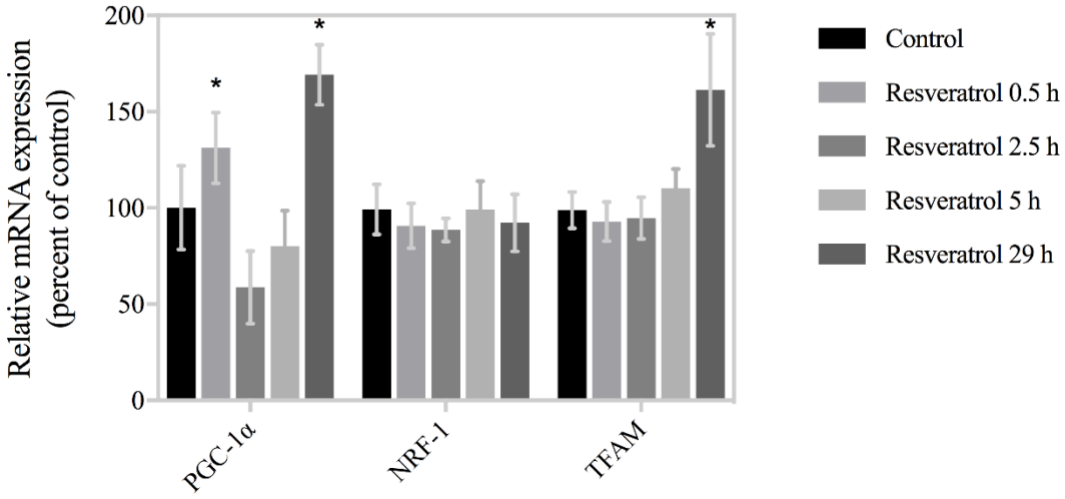
Caco-2 cells were exposed to ARBE or resveratrol (20 μM) alone for different durations to compare the short-term and long-term effect of both polyphenols on expression of three main transcription factors involved in mitochondrial biogenesis (Fig. 4.3.A & B). ARBE only produced a significant increase in PGC-1α mRNA expression after 26 h, and a 24% decrease in NRF-1 mRNA after 1 h. ARBE treatment had no effect on TFAM mRNA expression at any time point. With resveratrol, short term treatment produced a slight (30%) increase in PGC-1α mRNA after 30 min, while treatment for 29 h produced 69% and 61% increases in mRNA for PGC-1α and TFAM, respectively (Fig. 4.3.B).

In addition, the effects of 0.4 mM MM and 20 μM ARBE or resveratrol on mRNA expression of genes involved in mitochondrial biogenesis are displayed in Figure 4.3.C Both ARBE and resveratrol alone increased PGC-1α mRNA expression by 40% and 69% respectively. MM had no effect on expression of PGC-1α, while ARBE or resveratrol both in the presence of MM significantly increased PGC-1α mRNA expression (by 40% and 64% compared to control). MM or polyphenol treatments did not affect NRF-1 mRNA expression. MM challenge decreased TFAM mRNA expression by 25% and resveratrol treatment alone induced a 61% increase in TFAM mRNA expression compared to the control group (Fig. 4.3.C).

A.


Relative mRNA expression
(percent of control)
200
150
100
50
0
*
*
PGC-1α
NRF-1
TFAM
Control
ARBE 0.5 h
ARBE 1 h
ARBE 2 h
ARBE 26 h

B.


Relative mRNA expression
(percent of control)
200
150
100
50
0
*
*
*
PGC-1α
NRF-1
TFAM
Control
Resveratrol 0.5 h
Resveratrol 2.5 h
Resveratrol 5 h
Resveratrol 29 h

C.

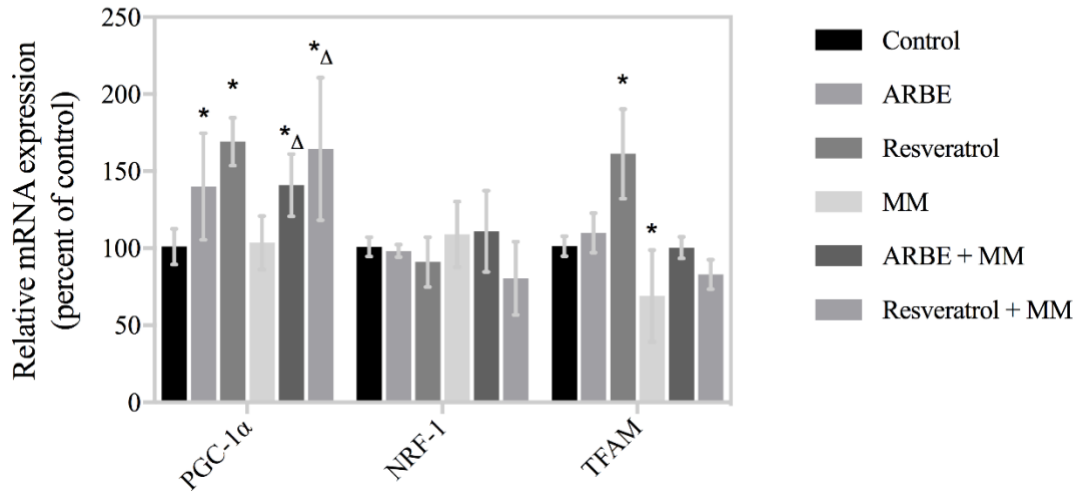


Figure 4.3. *Effects of MM, ARBE and resveratrol on expression of mRNA encoding transcription factors involved in mitochondrial biogenesis.* **(A)** After pretreatment of Caco-2 cells with 20 µM ARBE (PGC-1α: P <0.0001; NRF-1: P <0.0075; TFAM: P <0.0114) or **(B)** 20 µM resveratrol for different time points (PGC-1α: P <0.0001; NRF-1: P <0.4658; TFAM: P <0.0001). **(C)** After pretreatment with 2 h of ARBE or 5 h of resveratrol prior to 24 h MM exposure (PGC-1α: P_{MM} =0.8142, P_{ARBE} =0.0003, $P_{Interaction}$ =0.9917; PGC-1α: P_{MM} =0.9246, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ =0.7683; NRF-1: P_{MM} =0.1687, P_{ARBE} =0.9619, $P_{Interaction}$ =0.7560; NRF-1: P_{MM} =0.8654, $P_{Resveratrol}$ =0.0198, $P_{Interaction}$ =0.2300; TFAM: P_{MM} =0.0041, P_{ARBE} =0.0059, $P_{Interaction}$ =0.0932; TFAM: P_{MM} <0.0001, $P_{Resveratrol}$ =0.0006, $P_{Interaction}$ =0.0180). Data are normalized to β-actin and presented as percent of untreated cells. Values are the means ± SD for two independent experiments with three wells of cells for each treatment group. * Mean value significantly different from the control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P < 0.05). The significant difference between groups in A and B was tested with one-way ANOVA and in C was tested with two-way ANOVA followed by Tukey's multiple comparisons test.

4.4.3. Effect of treatments on expression of mitochondrial DNA (mtDNA) encoded respiratory complex subunits

As indicated in Figure 4.4, challenge of Caco-2 cells with 0.4 mM MM for 24 h markedly decreased expression of mtDNA-encoded genes related to the respiratory complex (I, III, IV, V) subunits (Fig. 4.4). ARBE alone had no effect, while resveratrol treatment alone increased MTCO1 expression by 56% compared to untreated cells and had no effect on other complex subunits. However, ARBE or resveratrol treatment in MM challenged cells had no protective effect on expression of MTND1, MTCYB, MTCO1 and MTATP6 (Fig. 4.4).

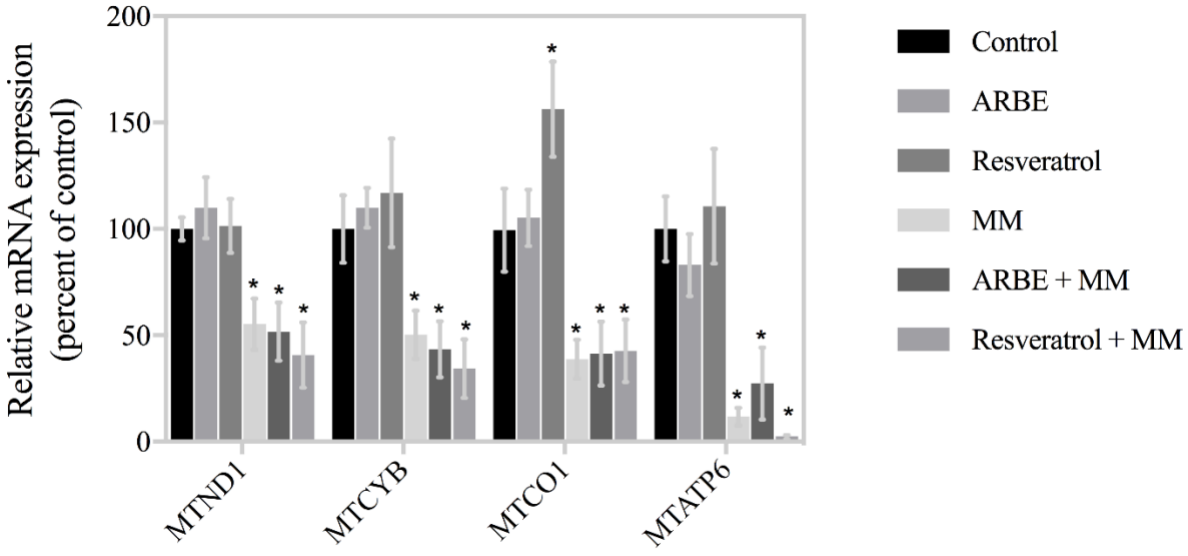


Figure 4.4. *Effects of MM, ARBE and resveratrol and MM on expression of mRNA for mitochondrial DNA encoded respiratory complex subunits.* Cells were treated with ARBE or resveratrol for 26 and 29 h, respectively, or pretreated for 2 h with ARBE or 5 h with resveratrol (20 µM) followed by 24 h MM (0.4 mM) exposure. MTND1 (complex I subunit), MTCYB (complex III subunit), MTCO1 (complex IV subunit) and MTATP (complex V subunit). (MTND1: P_{MM} <0.0001, P_{ARBE} =0.5000, $P_{Interaction}$ =0.1629; MTND1: P_{MM} <0.0001, $P_{Resveratrol}$ =0.16780, $P_{Interaction}$ =0.0953; MTCYB: P_{MM} <0.0001, P_{ARBE} =0.9655, $P_{Interaction}$ =0.0406; MTCYB: P_{MM} <0.0001, P_{ARBE} =0.7438, $P_{Interaction}$ =0.0892; MTCO1: P_{MM} <0.0001, P_{ARBE} =0.4547, $P_{Interaction}$ =0.7835; MTCO1: P_{MM} <0.0001, $P_{Resveratrol}$ =0.0008, $P_{Interaction}$ =0.0028; MTATP6: P_{MM} <0.0001, P_{ARBE} =0.9026, $P_{Interaction}$ =0.0091; MTATP6: P_{MM} <0.0001, $P_{Resveratrol}$ =0.9545, $P_{Interaction}$ =0.1434). Data were normalized to β-actin and presented as percent of untreated control cells. Values are the means ± SD (two independent experiments with three wells of cells for each treatment group). * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). The significant difference between groups was tested with two-way ANOVA followed by Tukey's multiple comparisons test).

4.4.4. Mitochondrial content

The auto fluorescence of the Caco-2 cells made it difficult to measure the mitochondrial content with MitoTracker Green. Therefore, MitoTracker Deep Red (MTDR) was used. As displayed in Figure 4.5, ARBE or resveratrol treatment alone had no effect on MTDR fluorescence compared to control cells. MM slightly decreased the signal although not significantly and only resveratrol pretreatment blunted the effect of MM and increased mitochondrial content by nearly 100% compared to MM challenge (Fig. 4.5).

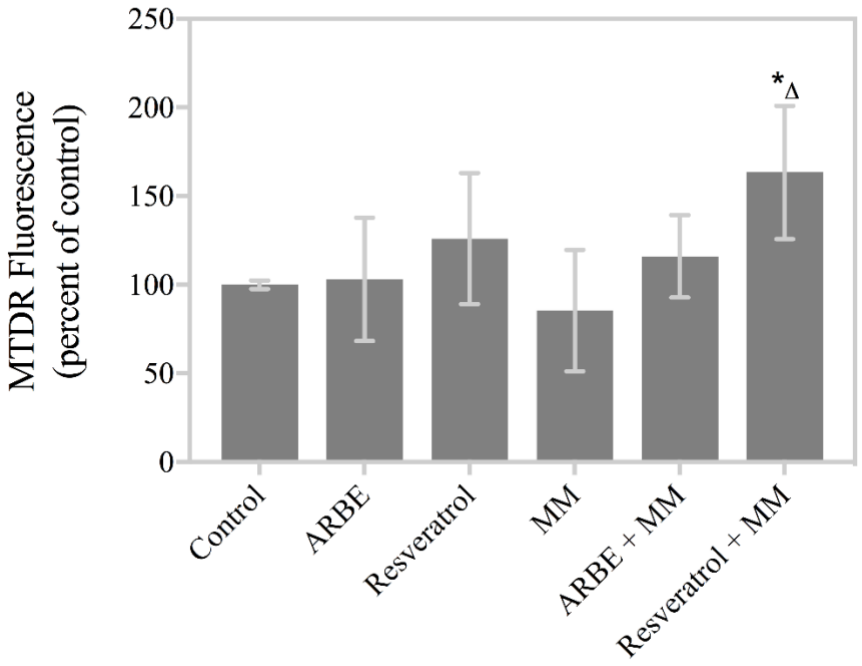
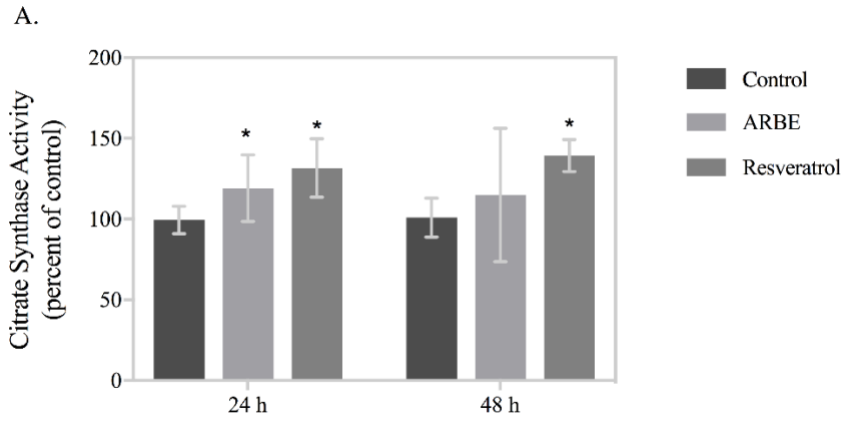


Figure 4.5. *Effect of MM, ARBE and resveratrol on mitochondrial content.* Mitochondrial content was measured using flow cytometry of MTDR fluorescence measured in Caco-2 cells pretreated with 20 µM ARBE or resveratrol alone for 26 or 29 h, respectively, or 2 h ARBE or 5 h Resveratrol pretreatment before MM challenge (P_{MM} =0.9385, P_{ARBE} =0.1097, $P_{Interaction}$ =0.1853; P_{MM} =0.2873, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ =0.0191). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. * Mean value

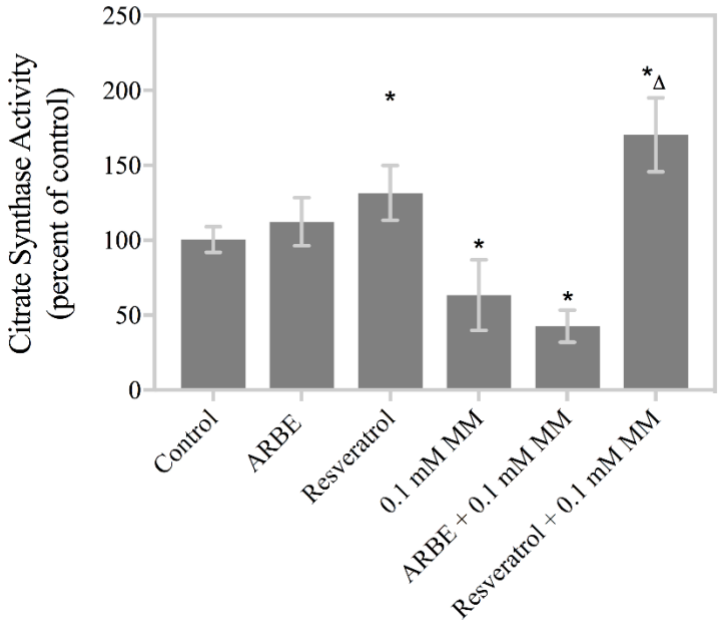
significantly different from the control group (P <0.05). $^{\Delta}$ Mean value significantly different from the MM group (P <0.05). The significant difference between groups was tested with two-way ANOVA followed by Tukey's multiple comparisons test.

4.4.5. Citrate synthase activity

Citrate synthase (CS) activity was assessed as an independent marker of mitochondrial content. The activity of citrate synthase (CS) was measured after 24 and 48 h treatment of Caco-2 cells with 20 μM ARBE or resveratrol alone. ARBE increased the activity of this enzyme by 19% after 24 h and resveratrol showed an increase after both 24 h and 48 h by 31% and 39% respectively (Fig. 4.6.A), compared to the control group. As shown in Figure 4.6.B, challenge with 0.1 mM MM for 24 h decreased CS by 36%. While ARBE pretreatment in the presence of MM decreased the activity of this enzyme further, resveratrol pretreatment increased the activity of citrate synthase by 70% in the presence of MM (Fig. 4.6.B). Also, the effects of ARBE and resveratrol pretreatment on the activity of CS was assessed in Caco-2 cells challenged with 0.4 mM MM. In these experiments, resveratrol alone increased mitochondrial content by 36%, MM decreased CS activity by 41% and pretreatment with resveratrol increased CS activity 70% compared to MM challenged cells (Fig. 4.6.C).



B.



C.

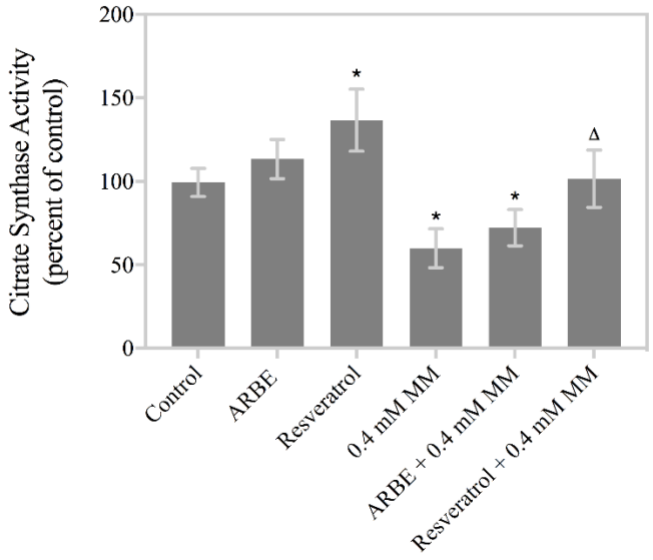


Figure 4.6. *Effects of MM, ARBE and resveratrol on mitochondrial content measured by citrate synthase activity.* The activity of citrate synthase (CS) in cells after treatments was measured in cell protein extracts and normalized to protein content measured via Bradford assay. **(A)** After treatment with 20 µM ARBE or resveratrol alone for 24 h (P =0.0055) and 48 h (P =0.0247), **(B)** After pretreatment with 20 µM ARBE or resveratrol for 2h or 5 h respectively, followed by

challenge with 0.1 mM MM for 24 h (P_{MM} <0.0001, P_{ARBE} =0.5774, $P_{Interaction}$ =0.0485; P_{MM} =0.4306, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ =0.0052) or **(C)** After treatments as in B but with 0.4 mM MM (P_{MM} <0.0001, P_{ARBE} =0.0028, $P_{Interaction}$ =0.8292; P_{MM} <0.0001, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ =0.7020). Values represent means ± SD of four independent experiments with three replicate wells for A and three independent experiments with three replicate wells for B and C. * Mean value significantly different from control group (P<0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). The significant difference between groups in A was tested with one-way ANOVA and in B and C with two-way ANOVA followed by Tukey's multiple comparisons test.

4.4.6. Mitochondrial function

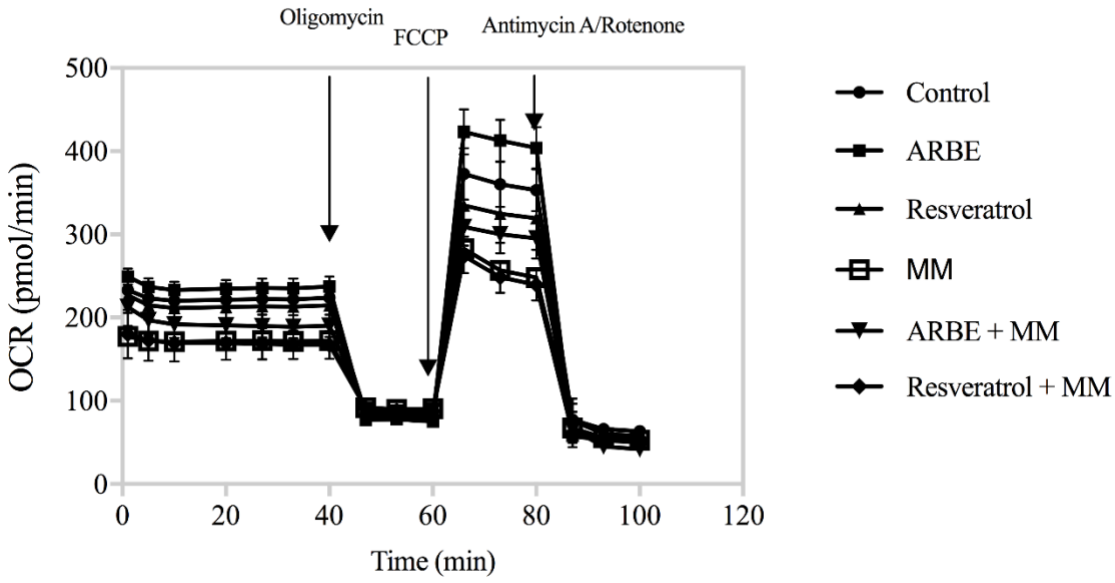
Although CS activity is a well-known indicator of mitochondrial content, it is important to consider other endpoint measures in a complicated organelle such as mitochondria. Thus, measuring mitochondrial oxygen consumption and parameters of oxidative phosphorylation is the key indicator of mitochondrial function (Lanza and Nair, 2009). Mitochondrial function based on oxygen consumption were measured by different aspects; basal respiration, ATP production, spare capacity and proton leak using a Seahorse XFe96 Extracellular Flux Analyzer and the effects of MM, ARBE and resveratrol on these functions in Caco-2 cells was assessed.

Initially, a dose response of MM concentrations (0.5, 0.1, 0.2, 0.3, 0.4 mM) for 24 h was assessed and 0.1 mM MM was identified as the best concentration for inducing a recoverable stress and decline in mitochondrial respiration in this set of experiments (data shown in Appendix-Fig. 8.B.8). Higher concentrations of MM almost completely inhibited mitochondrial oxygen consumption.

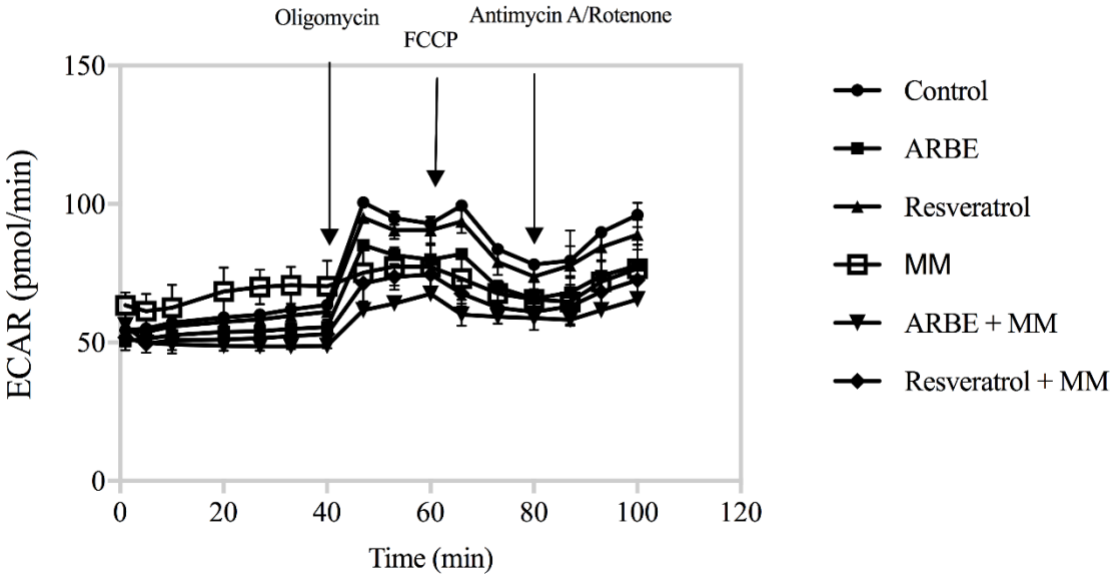
Figures 4.7.A & B show representative OCR and ECAR traces measured using the Seahorse XFe96 Extracellular Flux Analyzer. Protein content was measured via the Bradford assay and wells from all groups had similar amounts of protein with no significant differences between groups.

The different mitochondrial functions were quantified, and the results are shown in Figure 4.7.C-F. As seen in Figures 4.7.C, both polyphenols alone increased basal respiration compared to control cells. Exposure to 0.1 mM MM for 24 h significantly decreased basal respiration (29%), ATP production (43%) and spare capacity (33%) and also increased proton leak (64%) compared to control cells (Fig. 4.7.C-F). ARBE pretreatment inhibited the decrease in basal respiration and ATP production and increased these values compared to MM by 28% and 24%, respectively, and also increased spare capacity of Caco-2 cells by 42% compared to MM challenged cells. Resveratrol did not significantly protect against the effects of MM on these aspects of mitochondrial function.

A.


Oligomycin
FCCP
Antimycin A/Rotenone
OCR (pmol/min)
500
400
300
200
100
0
0
20
40
60
80
100
120
Time (min)
Control
ARBE
Resveratrol
MM
ARBE + MM
Resveratrol + MM

B.


Oligomycin
FCCP
Antimycin A/Rotenone
ECAR (pmol/min)
150
100
50
0
0
20
40
60
80
100
120
Time (min)
Control
ARBE
Resveratrol
MM
ARBE + MM
Resveratrol + MM

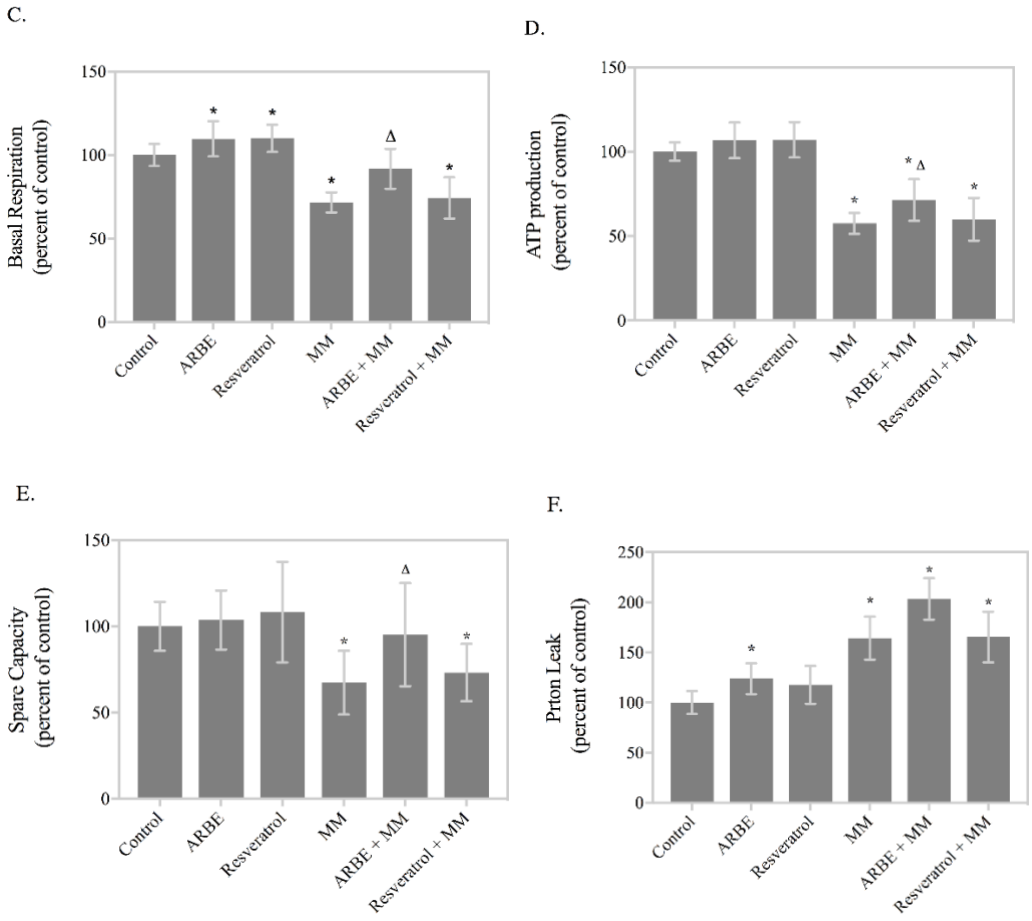


Figure 4.7. *Effects of MM, ARBE and resveratrol treatments on mitochondrial functions.* Caco-2 cells grown in Seahorse plates were treated with 20 µM ARBE for 2 h or 20 µM resveratrol for 5 h prior to adding 0.1 mM MM and incubating for 24 h. Oxygen consumption rate (OCR) was then measured for 3 h with additions of respiratory agents at selected time points and data were normalized to number of protein measured via Bradford assay. **(A)** OCR and **(B)** ECAR measured using the Seahorse XFe96 Extracellular Flux Analyzer and oligomycin (1 µM), FCCP (1 µM) and A/R (0.5 µM) injected sequentially as shown. **(C)** Basal respiration, (P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ =0.0319; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0039, $P_{Interaction}$ =0.0963). **(D)** ATP production (P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ =0.1472; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0450, $P_{Interaction}$ =0.32743). **(E)** Spare Respiratory capacity (P_{MM} =0.0005, P_{ARBE} =0.0066, $P_{Interaction}$ =0.0345; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0708, $P_{Interaction}$ =0.6206). **(F)** proton leak (P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ =0.0102; P_{MM} <0.0001, $P_{Resveratrol}$ =0.0631, $P_{Interaction}$ =0.1045). Data represent means ± SD of 3 independent experiments with 4 wells of cells in each experiment. * Mean value significantly different from the control group (P <0.05). $^{\Delta}$ Mean value significantly

different from the MM group ($P < 0.05$). The significant difference between groups were analyzed with two-way ANOVA followed by Tukey's multiple comparisons test.

4.5. Discussion

Mitochondria play a critical role in regulating cellular energy homeostasis and cell death. Decreased mitochondrial function and depletion of ATP disrupts tight junction proteins and induces loss of enterocyte integrity promoting increased permeability both in *in vitro* (JanssenDuijghuijsen et al., 2017) and *in vivo* (Novak and Mollen, 2015) studies. In our previous study (Chapter 3), we showed that MM increased the permeability of a Caco-2 cell monolayer by inducing oxidative stress and cytotoxicity and ARBE and resveratrol protected against loss of integrity to different extents. In the current study, we focused on the protective effect of ARBE and resveratrol on mitochondrial biogenesis and function in a Caco-2 cell model using MM as a model of high fat consumption.

In this model, MM significantly decreased mitochondrial membrane potential by more than 50%. The mitochondrial membrane potential ($\Delta\Psi m$) is created by pumping protons at complexes I, III, and IV in the IMS of mitochondria. Impaired mitochondrial respiratory complex activity or proton leak (caused by uncouplers or opening of the mitochondrial permeability transition pore) causes loss of mitochondrial membrane potential (Sivitz and Yorek, 2010). This impairment may be due to damage of mitochondria from MM-induced perturbations, including increases in ROS generation as mentioned in our previous study (Chapter 3). Reactive oxygen species can disrupt membrane integrity and affect cell signaling pathways to directly or indirectly cause collapse of the mitochondrial membrane potential, decreasing the amount of ATP production and triggering mitochondrial associated apoptosis (Radi et al., 2002; Joshi and Bakowska, 2011); hence, loss of membrane potential is a characteristic of cellular apoptosis. Several mechanisms have been proposed as the underlying cause of the decline in mitochondrial membrane potential. One might be due to mitochondrial overwork due to the irritant effects of MM and the excessive energy demands that are required to respond to this challenge. This could lead to increase in mitochondrial superoxide production, oxidative stress-induced damage and permeability of the inner mitochondrial membrane resulting in dissipation of the proton gradient and reduced accumulation

of TMRE in the mitochondria (Paixão et al., 2011). In addition, this challenge could be accompanied by the triggering of the mitochondrial permeability transition (MPT), a mitochondrial inner membrane process (Heiskanen et al., 1999), that is known to increase in response to mitochondrial superoxide (Kowaltowski et al., 2001). Excessive mitochondrial superoxide production could damage mitochondrial membrane lipids (Kowaltowski et al., 2001) and electron transport chain components (Ghezzi and Zeviani, 2012) leading to impaired electron transport chain activity, reduced proton gradient and lower mitochondrial membrane potential. Finally, the fatty acids of the MM may act as an uncoupler and cause depolarization of the mitochondrial membrane, reducing membrane potential and causing loss of mitochondrial function along with increasing ROS generation, release of cytochrome C, and ultimately the triggering of the apoptotic process (Wang et al., 2014). As shown in Chapter 3, MM decreased cell viability of Caco-2 cells.

This study also showed ARBE to significantly inhibit the decrease in mitochondrial membrane potential induced by MM especially at lower concentration (≤10 µM), while only the lowest dose of resveratrol (1.25 µM) inhibited MM-induced depolarization. The effect of ARBE was possibly due to the antioxidant effect of anthocyanins present in ARBE. Caco-2 cells have glycosidase activity (Klumperman et al., 1991; Mizuma et al., 2005; Arafa, 2009; Henry-Vitrac et al., 2006), which could convert anthocyanins to the more lipid soluble anthocyanidin aglycones and allow them to accumulate in mitochondria. As Khoo et al., (2017) mention, the oxygen radical absorbance capacity (ORAC) value of anthocyanidin is higher than anthocyanin and addition of sugar group at position C-3 in the heterocyclic C-ring decreases the antioxidant activity. Moreover, the proton rich environment of the mitochondrial IMS can convert the neutral and hydrophobic anthocyanidin aglycone to the corresponding positively charged oxonium species. The mitochondrial membrane potential dependent concentration of the oxonium aglycone species within the inner mitochondrial membrane, may enable this antioxidant to blunt mitochondrial ROS and damage (Peng 2012). Similar to our results, Chen et al. (2016) showed in Caco-2 cells that raspberry-derived anthocyanins attenuated acrylamide-induced ROS and decline in mitochondrial membrane potential. Other studies have also shown protective effects of anthocyanins against decreases in mitochondrial membrane potential in RAW macrophages exposed to lipopolysaccharide (Bognar et al., 2013). Studies have mentioned different effects of resveratrol

on mitochondrial membrane potential. The study of Ferretta et al. (2014) is in agreement with our results showing that resveratrol treatment in primary fibroblast cultures from two patients with early-onset Parkinson disease had no effect on mitochondrial membrane potential in control and in-patient fibroblast despite increasing mitochondrial biogenesis and ATP production. However, Wang et al. (2014), indicated that 0.06% resveratrol in the diet was able to prevent the decrease in $\Delta\Psi m$ in regulatory T-cells of mice fed a high fat diet (45%) for 20 weeks.

To investigate whether MM, ARBE and resveratrol influence cellular bioenergetic signaling pathways, we measured the effects on expression of mRNA for factors involved in mitochondrial biogenesis. Mitochondrial biogenesis can include growth of pre-existing mitochondria and production of new mitochondria from division, a complex process that involves both the nuclear and mitochondrial genomes (Sastre-Serra et al., 2012). Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) is an important modulator of mitochondrial biogenesis and function. It activates several transcription factors such as NRF-1 and NRF-2 and thereby modulates expression of nuclear genes encoding mitochondrial biogenesis as well as activating transcription and replication of the mitochondrial genome via activation of TFAM (Jornayvaz and Shulman, 2010; Wu et al., 1999). Thus, we evaluated MM challenge in cells pretreated with polyphenols on mRNA expression of PGC1-α, NRF-1 and TFAM in the Caco-2 cells. Our study only showed MM to decrease TFAM significantly compared to control. MM induced expression of TNF-α (mentioned in Chapter 3), which might explain the negative effect of MM on mitochondrial biogenesis as shown in decreasing TFAM and citrate synthase activity. TNF-α has shown to downregulate eNOS thereby decreasing mitochondrial biogenesis in adipocytes and muscle satellite cells of mice (Valerio et al., 2006). In addition, Sente et al., (2016) showed TNF-α decreases mitochondrial metabolism and biogenesis by decreasing Forkhead box O3 (FOXO3) and PGC-1α, respectively. Several studies have noted high fat diet decreases PGC-1α expression in different tissues such as liver (Barroso et al., 2017), cerebellum (Vervaecke, 2013), skeletal muscle (Crunkhorn et al., 2007; Koves et al., 2005). Likewise, Sparks et al. (2005) showed high fat diet decreases genes involved in mitochondrial biogenesis and mitochondrial oxidative phosphorylation in skeletal muscle of humans and rodents. Consistent with our results, the study of McAinch et al. (2003) showed that a high fat diet for 8 weeks had no effect on muscle PGC-1α

expression in Sprague-Dawley rats. No previous studies have been reported however, on the effects of dietary fat on the expression of mitochondrial biogenesis factors in intestinal epithelium.

ARBE increased PGC-1α mRNA expression in the presence of MM. Exposure of ARBE alone also increased PGC-1α expression after 26 h, but not at earlier time points (0.5-2 h). It should be noted, however, that PGC-1α activity is also controlled post translationally by deacetylation. Dietary studies in mice with anthocyanin rich extracts from bilberries and blackcurrants have shown increased expression of PGC-1α as well as other mitochondrial biogenesis factors in liver and muscle (Ling et al., 2016; Benn et al., 2014), but previous studies have not shown an effect in intestinal epithelial cells. Ling et al. (2016), showed that in mice, provision of anthocyanin rich extracts from bilberries and blackcurrants (100 mg/1 kg diet) for 16 weeks increased activation of phosphorylated AMP-activated protein kinase (p-AMPK) and PGC-1α, promoting activation of AMPK/PGC-1α signaling axis in high fat diet induced non-alcoholic fatty liver disease, thus activating mitochondrial biogenesis and activity as well as inducing mitochondrial antioxidant activity and increasing fatty acid oxidation. Also, in mice, provision of a polyphenol-rich blackcurrant extract supplementation of (0.1% wt) in a high-fat/high-cholesterol diet for 12 weeks increased mRNA expression of genes involved in energy expenditure and mitochondrial biogenesis, including PPAR-α, PPAR-δ and mitochondrial transcription factor A in skeletal muscle (Benn et al., 2014).

Resveratrol alone in our experiments increased PGC-1α and TFAM compared to control cells and resveratrol along with MM exposure also increased PGC-1α expression. Many dietary studies have shown resveratrol to increase levels of de-acetylated PGC-1α, and other mitochondrial biogenesis factors, in different tissues (e.g. Baur et al., 2006; Csiszar et al. 2009; Kim et al., 2014). Several studies have shown that resveratrol increases mitochondrial biogenesis through de-acetylating and activating PGC-1α, although the mechanisms behind this effect are controversial (Higashida et al., 2013; Kim et al., 2014; Tang et al., 2016). We didn't study activation of PGC-1α, but we did find that resveratrol increased its mRNA expression in Caco-2 cells.

Although MM did not much affect expression of the mitochondrial biogenesis signaling factors, we found that it markedly decreased expression of mtDNA-encoded subunits of the respiratory chain (mtND1, MTCYB, MTCO1, MTATP). This downregulated expression of mtDNA-encoded

subunits may be due to MM-induced uncoupling of mitochondrial oxidative phosphorylation. However, neither ARBE nor resveratrol protected against this effect. In the liver of mice, García-Ruiz et al. (2014) showed that a high fat diet contributes to mitochondrial dysfunction by decreasing expression of mtDNA-encoded mitochondrial respiratory subunits and mitochondrial biogenesis, changes that were associated with oxidative stress and inflammation. In another study on cultured HepG2 hepatocytes, Garcia-Ruiz et al. (2015) also showed that exposure to 200 µM saturated fatty acids such as palmitic acid and stearic acid for 24 h reduced OXPHOS complexes and mtDNA-encoded complex subunits. The former due to nitro-oxidative stress, as measured by 3-nitrotyrosylation of mitochondrial proteins, including OXPHOS subunits, and their degradation and the later due to oxidative damage of mtDNA caused by exposure to saturated fatty acids.

Since mitochondrial biogenesis and function are designed to provide adequate ATP for cellular function, both are expected to play a critical role in maintaining intestinal barrier integrity and preventing increased permeability, the effect of ARBE and resveratrol on mitochondrial content was measured using MTDR fluorescence and citrate synthase activity. MTDR is taken up by active mitochondria and is retained in the mitochondria by reaction with protein thiols. Citrate synthase is a mitochondrial matrix enzyme, and the activity of this enzyme is widely used as an indicator of mitochondrial content. Challenge of Caco-2 cells with MM did not produce any change in cellular MTDR fluorescence. MTDR measures the fluorescence of this thiol-reactive probe when attached to mitochondria but does not provide information about the function of the mitochondria. Also, since MTDR fluorescence was measured by flow cytometry, information is limited to the mitochondria of cells judged to be viable and healthy. This judgement is based on user-defined gates corresponding to the range of light scattering properties of normal cells. Nevertheless, while ARBE had no effect on estimates of mitochondrial content based on MTDR fluorescence in the absence or presence of MM, resveratrol pretreatment before MM challenge increased MTDR fluorescence compared to MM challenge alone. This result suggests that resveratrol increases mitochondrial content in MM challenged cells.

Citrate synthase (CS) is the first enzyme of the tricarboxylic acid (TCA) cycle and the activity of this enzyme reflects cellular mitochondrial activities, thus it is useful in assessing the impact of treatment on mitochondrial content. With measurements of cellular CS, MM at 0.1 and 0.4 mM decreased the CS activity by 36% and 41%, respectively, suggesting a decrease in mitochondrial

TCA cycle activity. Although it has not been studied in intestinal epithelial cells, some studies have supported the concept of high fat diet decreasing mitochondrial function and content in liver or muscle (De la Pena et al., 2017; Vial et al., 2011; Nadal-Casellas et al., 2010). De la Pena et al. (2017) showed in male Sprague Dawley rats that provision for six weeks of a high fat diet rich in polyunsaturated fatty acids results in lower muscle mitochondrial content compared to high fat diet rich in monounsaturated fatty acid. However, other studies have noted a high fat diet to increase muscle mitochondrial markers (Miller et al., 1984; Turner et al., 2007; Hancock et al., 2008). As mentioned in the review study by Kakimoto and Kowaltowski (2016), considering the source and duration of fat intake as well as rodent strain or initial age provides no evident explanation for this discrepancy.

While ARBE had no effect on the 0.1 or 0.4 mM MM-induced decrease in CS activity, resveratrol completely prevented the decreases (and with 0.1 mM MM increased the CS activity to a level significantly higher than control cells). Resveratrol alone also increased CS activity in all of the experiments. Xie et al. 2012, also showed that 30 μM cyanidin-3-glucoside in the presence or absence of glycated LDL for 12 h did not affect CS activity in cultured vascular endothelial cells. In *in vitro* studies with rabbit bladder mucosa (Venugopal et al., 2010; Francis et al., 2014), acute treatment with 1 mg/mL grape suspension (containing anthocyanins, catechins, resveratrol, quercetin, flavans and simple phenolics as well as sugars) for 30 min protected against a hydrogen peroxide-induced decrease of citrate synthase activity while resveratrol did not, suggesting that components other than resveratrol were responsible for the antioxidant effect. In contrast to anthocyanins, studies showing effects of resveratrol on mitochondrial biogenesis markers such as citrate synthase are ample. Several studies have shown the effect of resveratrol in increasing mitochondrial markers such as CS in cultured cells or in different animal tissues. In studies on primary fibroblasts, from patients with Parkinson's or mitochondrial diseases or healthy controls (Ferretta et al., 2014; De Paepe et al. 2014), 25 or 100 μM resveratrol for 48 h increased citrate synthase activity, along with oxidative respiration and complex II and complex IV enzyme activities. In animal studies, dietary supplementation with resveratrol has been shown to increase markers of mitochondrial number and function in different tissues, although the effects on CS activity have been inconsistent (Baur et al., 2006; Robb et al., 2008; Olesen et al., 2014).

While the activity of citrate synthase is one well-known indicator of mitochondrial function, this enzyme activity alone may not accurately mirror the function of a complex organelle such as mitochondria. The effects of ARBE and resveratrol on citrate synthase activity prompted a more in depth investigation of their effects on mitochondrial oxidative consumption, a key indicator of mitochondrial function (Lanza and Nair, 2009). Therefore, Seahorse XFe96 Extracellular Flux Analyzer was employed to measure oxygen consumption rates, a more direct indicator of mitochondrial function.

The Seahorse XFe96 Extracellular Flux Analyzer was used to measure different aspects of mitochondrial function including basal respiration, spare capacity, proton leak and ATP production. High dietary fat consumption increases mitochondrial workload by increasing β-oxidation and ROS generation which can lead to disruption in mitochondrial function (Murphy, 2009; Wajner and Amaral, 2015). As seen in our study, MM challenge decreased mitochondrial function by decreasing basal respiration, ATP production, spare capacity and increasing proton leak. These changes in OCR induced by MM indicates decreased oxidative phosphorylation and cells challenged with MM may therefore rely more on glycolysis as the alternative fuel source. Increased reliance on glycolysis was also shown by the elevation in basal ECAR with exposure to MM. As shown in Chapter 3, microscopic images of MM challenged cells show that cells are viable but stressed. Since MM can induce uncoupling of mitochondrial respiration from ATP production, less ATP is produced for each mole of oxygen consumed, therefore leading the cells to compensate by deriving additional energy from glycolysis. Exposure to MM also may impair electron transport in the ETC chain by preventing electron transport, most likely by inhibiting complex I and III (Schönfeld & Wojtczak, 2007), thus producing a higher amount of ROS. A decreased expression of mitochondrial respiratory complex subunits as well as a decrease in the number of healthy and functional mitochondria can result in increased ROS generation and lead to increased intestinal permeability (Liu et al., 2009; Guo et al., 2013; Raza et al., 2011; Bratic & Trifunovic, 2010). Thus, increasing mitochondrial function may reduce ROS production and protect against intestinal permeability.

The measurements of Caco-2 cell mitochondrial functions with the Seahorse analyzer showed that ARBE, but not resveratrol, protected against MM-induced decreases in basal respiration, spare capacity and mitochondrial ATP production. Since resveratrol was stronger than ARBE at

inducing indices of mitochondrial biogenesis (PGC-1α, MTDR and citrate synthase activity), this result suggests that ARBE protected mitochondrial function by other mechanisms. The results in Chapter 3 showed that ARBE but not resveratrol decreased MM-induced mitochondrial superoxide, which may be one possible explanation for the protection by ARBE against MM-induced mitochondrial function declines. Alone, both ARBE and resveratrol slightly increased basal respiration. Also, both ARBE and resveratrol in the presence of MM significantly decreased ECAR compared to MM at 40 min (prior to adding oligomycin) indicating cells relying more on oxidative respiration and less on glycolysis for ATP production supporting the idea that these compounds modulate mitochondrial function.

Interestingly, ARBE in the presence of MM further increased proton leak, to a level almost 100% higher than in untreated cells, and ARBE alone also slightly increased proton leak. The mechanism behind the effect of ARBE on proton leak requires further investigation.

The results might suggest an uncoupling effect, but ARBE alone did not decrease mitochondrial ATP production or membrane potential, and ARBE partially prevented the decrease induced by MM on these parameters. One possible explanation is that the anthocyanins produce some uncoupling but also participate in the electron transfer chain (as described below) such that membrane potential and ATP synthesis are not compromised. For example, other studies have shown anthocyanins to directly influence mitochondrial respiratory chain activities. Skemiene et al. (2015) showed that delphinidin-3-O-glucoside and cyanidin-3-O-glucoside (10-40 μM) increased complex I activity in the absence or presence of coenzyme Q1 in ischemia-damaged mitochondria in mitochondria isolated from normal rat hearts and rat hearts subjected to ischemia for 45 min. Both anthocyanins restored the function of the mitochondrial respiratory chain by serving as electron acceptors at NADH dehydrogenase to transport electrons, increased state 3 respiration and restored ischemia-depleted ATP levels in ischemia-damaged mitochondria of rat heart. Likewise, Boušová et al. (2015) also demonstrated that an anthocyanin-rich cranberry extract enriched diet (2%) for 28 days increased hepatic NAD(P)H:quinone oxidoreductase activity, as well as other antioxidant enzymes in erythrocytes and small intestine of mice in a model of monosodium glutamate-induced obesity.

Although our data showed that resveratrol increases mitochondrial biogenesis, no effect of this polyphenol on mitochondrial function was noted. Intestinal cells have a low amount of mitochondria (approximately 21-42 per cell) (Jeynes and Altmann, 1975), moreover, Caco-2 cells used in this study are transformed cells, which might be an explanation as to why resveratrol treatment was not able to measurably increase mitochondrial function in these cells.

The effects of MM, ARBE and resveratrol on mitochondrial functions after 12 and 48 h exposure were also assessed (Appendix Fig. 8.B.9 & 10). After 12 h pretreatment, resveratrol was only able to protect against decrease in spare capacity induced by MM. After a total of 48 h (24 h pretreatment plus another 24 h treatment with MM), ARBE and resveratrol treatments did not have protective effect on mitochondrial respiration functions.

In conclusion, MM decreased mitochondrial function in Caco-2 intestinal epithelial cells by depolarizing the mitochondrial membrane potential and decreasing mitochondrial biogenesis and respiration. The resulting decrease in cellular ATP can disturb the monolayer as actin polymerase requires ATP to attach cells and disrupts tight junction proteins (Chen et al., 2008a) triggering a widening of the paracellular space and increasing permeability (Novak and Mollen, 2015). Maintaining the intestinal barrier requires a high amount of energy (Bischoff et al., 2014). In addition, stress exposure increases demand for ATP and increased electron transport chain activity associated with this increase in mitochondrial workload may result in an unmanageable increase in ROS production, possibly leading to intestinal permeability (Sun et al., 2016; Jornayvaz & Shulman, 2010; Guo et al., 2013). Our data showed ARBE and resveratrol improved mitochondrial bioenergetics via different effects; in particular ARBE increased mitochondrial function and membrane potential and resveratrol increased mitochondrial biogenesis. Figure 4.8 provides a summary of how polyphenols may inhibit the effect of MM in inducing oxidative stress, inflammation and increased permeability of the Caco-2 cell model.

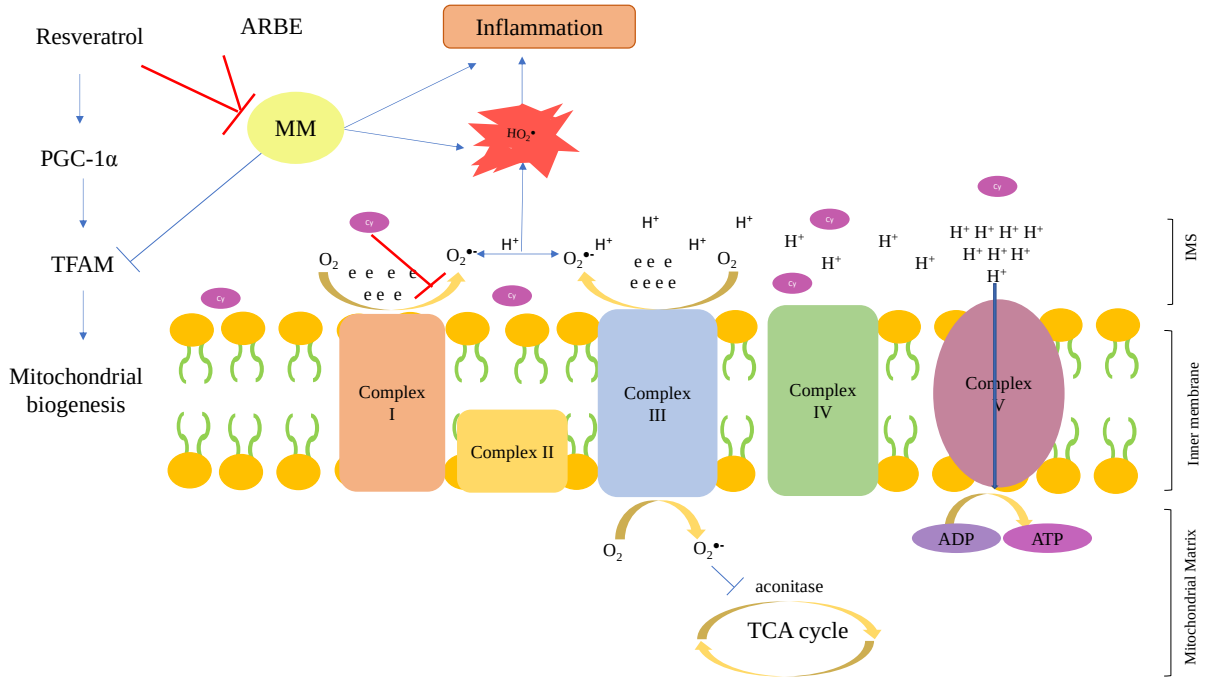


Figure 4.8. *Schematic representation of the postulated effects of polyphenols on blunting MM-induced ROS production and inflammation, and on mitochondrial biogenesis in Caco-2 cells.* MM-induced stress decreases mitochondrial biogenesis, membrane potential and function and mechanisms may include ROS production and inadequate ATP production. Polyphenols protected against MM-induced oxidative stress via different pathways to blunt the increased permeability of Caco-2 cells. While both polyphenols decrease intracellular ROS and expression of the inflammatory cytokine TNF- α, anthocyanins in ARBE act stronger in preventing MM-induced mitochondrial superoxide generation and loss of mitochondrial membrane potential and increase mitochondrial function and ATP production. Resveratrol on the other hand acts to increase mitochondrial content via increasing gene expression of PGC-1α and TFAM.

ADP, adenosine diphosphate; ARBE, anthocyanin rich bilberry extract; ATP, adenosine triphosphate; Cy, cyanidin; MM, mixed micelles; PGC-1α; peroxisome proliferator-activated receptor gamma coactivator 1; TCA, tricarboxylic acid; TFAM, mitochondrial transcription factor A.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interests.

4.6. Transition to CHAPTER 5

Chapters 3 and 4 investigated protective effects of anthocyanins and resveratrol against lipid micelle induced oxidative stress, inflammation, monolayer permeability and mitochondrial dysfunction. In Chapter 5 we investigate our third hypothesis that anthocyanins and resveratrol may act synergistically to protect against oxidative stress while supporting cell viability and mitochondrial function.

CHAPTER 5 TEST OF INTERACTION BETWEEN RESVERATROL AND ANTHOCYANIN RICH BILBERRY EXTRACT IN PROTECTING AGAINST LIPID MICELLE-INDUCED OXIDATIVE STRESS IN CACO-2 CELLS

5.1. Abstract

Resveratrol and anthocyanins represent two main classes of polyphenols in red wine and berries that have gained interest due to their strong protective and biological effects. Due to their different properties and mechanisms of action, we evaluated the interaction and a possible synergistic effect of these polyphenols in Caco-2 cells challenged with MM as a model of intestinal epithelial cells exposed to a high fat diet. In preliminary experiments, all dose combinations of resveratrol and ARBE strongly protected against MM-induced intracellular ROS. A significant decrease in intracellular ROS between each of the polyphenols at 0.1 µM with the combination using half the concentration was noted. The dose combination of 2.5 µM resveratrol + 15 µM ARBE was the only combination that protected (by 28%) against MM-induced cytotoxicity. The effect of this combination on cytotoxicity was slightly greater than an additive effect of either polyphenol alone, but the interaction wasn't significant. With Seahorse XFe96 Analyzer measurements of effects on mitochondrial functions, however, this combination gave no significant protection against MM-induced impairments, although ARBE alone gave a mild (19%) protection against the decline in basal respiration. Thus, our results did not show that resveratrol and ARBE have synergistic effects in preventing MM-induced cellular oxidative stress, cytotoxicity or mitochondrial dysfunction in Caco-2 cells.

Abbreviations

ANOVA, analysis of variance; **ARBE**, anthocyanin-rich bilberry extract; **ATP**, adenosine triphosphate; **DCF**, 2',7'-dichlorofluorescein; **DMEM**, Dulbecco's modified essential medium; DMSO, dimethyl sulfoxide; **ECAR**, extracellular acidification rate; **FBS**, fetal bovine serum; **FCCP**, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; **MM**, mixed micelles; **OCR**,

oxygen consumption rate; **ROS** reactive oxygen species, **SD**, standard deviation; **SRB**, sulforhodamine b; **UC**, ulcerative colitis

5.2. Introduction

Since diet contains many different phenolic compounds, the possibility exists for compounds that exert different protective mechanisms to produce a synergistic protection when provided in combination. Both resveratrol and anthocyanins, used in our study, possess several beneficial effects such as antioxidant, anti-inflammatory, anti-aging and anti-cancer properties. In addition, the combination of polyphenols found in food at concentrations relative to dietary intake may be more efficient and less toxic compared to an individual compound. With regards to anthocyanins and resveratrol, foods such as berries and red wine contain much more anthocyanins than resveratrol (Pour Nikfardjam et al., 2006; Borowska et al., 2009). The gastrointestinal tract receives a relatively high amount of polyphenols compared to other organs. Thus, these compounds are more likely to present a beneficial effect in the GI tract and protect from related diseases. Several *in vivo* and *in vitro* studies have investigated the synergistic effects of phytochemicals in fruits and vegetables (Liu, 2003), the combination of phytochemicals and drugs (Prabhakar and Doble, 2009) or different polyphenols in ameliorating different diseases (González-Castejón and Rodriguez-Casado, 2011; Kim et al., 2010).

Studies on protective effect of polyphenols such as resveratrol and anthocyanins on intestine are limited; this is especially surprising considering their reported benefits on supporting mitochondrial function and reducing oxidative stress. It is known that resveratrol acts by increasing the number and function of mitochondria (Gibellini et al., 2015; Lagouge et al., 2006), and in our previous study on intestinal Caco-2 cells it increased mitochondrial content and mitochondrial biogenesis factors (Chapter 4). On the other hand, the protective bioactivities of anthocyanins do not appear to rely on inducing the expansion of mitochondria, as they are shown to not activate the mitochondrial biogenesis-inducing enzyme SIRT1 (Howitz et al., 2003). Consistent with this finding, our previous study in Caco-2 cells did not increase mitochondrial content or biogenesis. Anthocyanins however are strong direct-acting antioxidants compared to resveratrol (Rice-Evans et al., 1996) and due to their lipophilicity and their ability to assume a positive charge at slightly acidic pH (4-5) (Khoo et al., 2017) they are taken up by mitochondria to a greater extent than other

flavonoids (Peng, 2012). In contrast, resveratrol has an antioxidant activity that appears to be principally due to its effects on metabolic pathways and the expression of antioxidant enzymes (Fukui et al., 2010). Anthocyanins that appear to bioconcentrate inside mitochondria may help reduce free radicals formed during oxidative phosphorylation which adversely affect mitochondrial function. In our previous studies (Chapters 3 and 4) we found ARBE, but not resveratrol, decreased mitochondrial superoxide and protected against MM-induced mitochondrial dysfunction. Resveratrol, on the other hand, is a well-known inducer of mitochondrial biogenesis, whose ability to expand mitochondrial capacity may productively interact with the mitochondrial antioxidant activities of ARBE to produce protection that exceeds that of either alone. Consequently, both polyphenols might operate complementarily to provide synergistic or additive protective benefits, potentially resulting in improvements in mitochondrial integrity and function and protection of intestinal cells against stress induced impairments. Thus, we investigated possible protective interaction of both bioactive compounds in Caco-2 cells challenged with MM.

5.3. Materials and Methods

5.3.1. Cell Culture Conditions

See section 3.3.1

5.3.2. Mixed Micelle Preparation

See section 3.3.2

5.3.3. Treatment with mixed micelles and polyphenols

Caco-2 cells seeded at 3-7×10^4 cells/well were cultured in 96-well optical-bottom plates and were grown to confluence. In combination experiments, cells were pretreated initially with resveratrol and after 3 h ARBE was added for another 2 h, thus cells were exposed to resveratrol and ARBE for a total of 5 h and 2 h, respectively. In order to avoid a toxic effect of DMSO, the highest final concentration of DMSO in the medium was 0.1%. To control for any possible effects of DMSO, the highest amount (0.1%) was added to control cells. After pre-treatment with polyphenols cells

were exposed to 0.4 or 0.1 mM MM for 24 h in 2% FBS media. The effects of MM, resveratrol and ARBE were assessed as in the following sections.

5.3.4. Measurements of intracellular reactive oxygen species (ROS)

See section 3.3.4

5.3.5 Measurements of intracellular viability via sulforhodamine B assay

See section 3.3.6.3

5.3.6. Measurement of mitochondrial function

See section 4.3.8.

5.3.7. Statistical analysis

See section 3.3.8.

5.4. Results

5.4.1. Oxidative Stress

Previously (Chapter 3) we showed concentrations from 1.25-20 µM of ARBE anthocyanins or resveratrol alone completely prevented MM-induced intracellular ROS in Caco-2 cells (Fig. 3.2.C). As confirmed experimentally (results not shown) any further interaction of the two compounds to prevent ROS generation was not observed at these concentrations. In the current set of experiments, lower doses of ARBE and resveratrol (0.1, 0.5, 1 µM) were tested, alone and in combination, to identify concentrations at which the combination of both polyphenols might interact to blunt intracellular ROS production beyond either alone. In the absence of MM, all doses and combinations of polyphenol decreased intracellular ROS by 13-24% compared to control cells (Fig. 5.1.A). As seen in Figure 5.1.B, MM induced a 4-fold increase in ROS generation compared to control cells. All concentrations and combinations of ARBE and resveratrol decreased MM-induced intracellular ROS by 29-54%. At the tested concentrations of the polyphenols there was

slightly increased inhibition of ROS generation by the combination (at some concentrations tested) compared to ARBE or resveratrol alone (Fig. 5.1.B).

For 0.1 µM of polyphenols, there was a significant decrease in intracellular ROS by using each of the polyphenols in combination at half of the concentration when either was used in isolation. The two-way ANOVA showed a significant interaction between ARBE and resveratrol, meaning that the combined effect was less than additive. In 0.5, and 1 µM the relative combination had only decreased DCF/SRB significantly compared to ARBE alone.

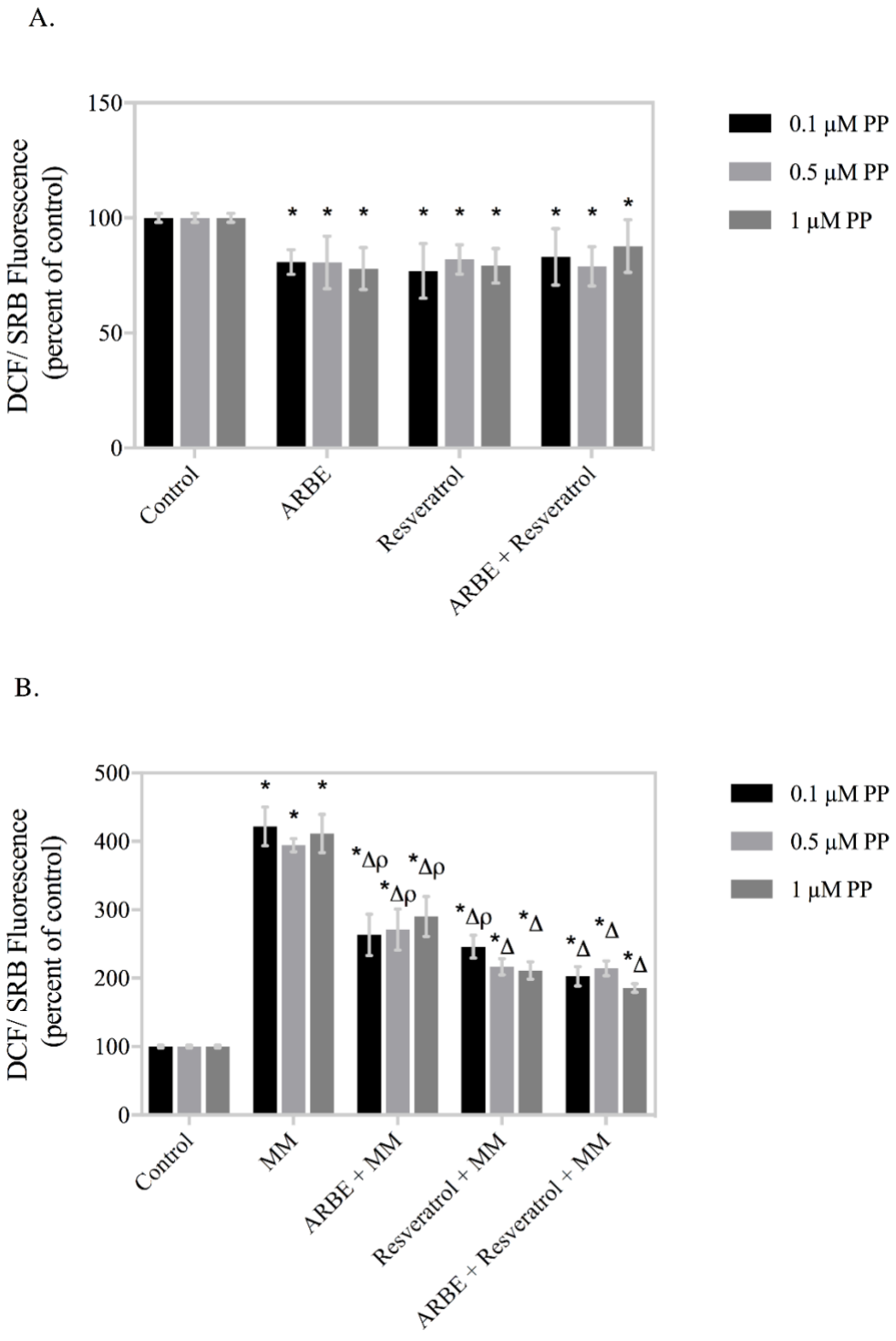

Figure 5.1. *Evaluation of possible interaction between ARBE and resveratrol on MM-induced intracellular ROS.* Caco-2 cells were treated with different concentrations of resveratrol for 3 h before adding ARBE and incubation for another 2 h prior to challenge with 0.4 mM MM for an

additional 24 h. **(A)** Absence of MM (with ARBE or resveratrol alone or in combination at half concentration each with resveratrol for 3 h prior to adding ARBE for an additional 26 h) (PP 0.1 µM: P_{ARBE} =0.0356, $P_{Resveratrol}$ =0.0015, $P_{Interaction}$ =0.0002; PP 0.5 µM: P_{ARBE} =0.0002, $P_{Resveratrol}$ = 0.0006, $P_{Interaction}$ =0.0037; PP 1 µM: P_{ARBE} =0.0271, $P_{Resveratrol}$ =0.0659, $P_{Interaction}$ <0.0001). **(B)** Presence of MM (PP 0.1 µM: P_{ARBE} <0.0001, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ <0.0001; PP 0.5 µM: P_{ARBE} <0.0001, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ <0.0001; PP 1 µM: P_{ARBE} <0.0001, $P_{Resveratrol}$ <0.0001, $P_{Interaction}$ =0.0002). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. The significant difference between groups in A, B and D was tested with two-way ANOVA and in C with one-way ANOVA, followed by Tukey's multiple comparisons test. * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). $^{\rho}$ Mean value significantly different from the relative combination (ARBE + Resveratrol + MM) group (P <0.05).

5.4.2. Cytotoxicity

At the low concentrations (0.1, 0.5 and 1 µM) used to test protective interaction against cytotoxicity (Figs. 5.1.A&B), individual or combinations of ARBE and resveratrol had no effect on cell viability compared to control cells (Fig 5.2.A) and did not protect against MM-induced cytotoxicity (Fig 5.2.B).

In comparing the higher concentrations of ARBE and resveratrol, the combination of 15 µM ARBE + 2.5 µM resveratrol protected against cytotoxicity induced by MM by approximately 29% (Fig 5.2.C). Therefore, possible synergism/protective interaction of the two polyphenols on MM-induced cytotoxicity was tested. While the combination was protective, neither polyphenol alone protected against the cytotoxic effect of MM (Fig 5.2.D). The analysis of the results in the presence of MM did not show a significant synergistic interaction between ARBE and resveratrol, however, as the protection was approximately additive.

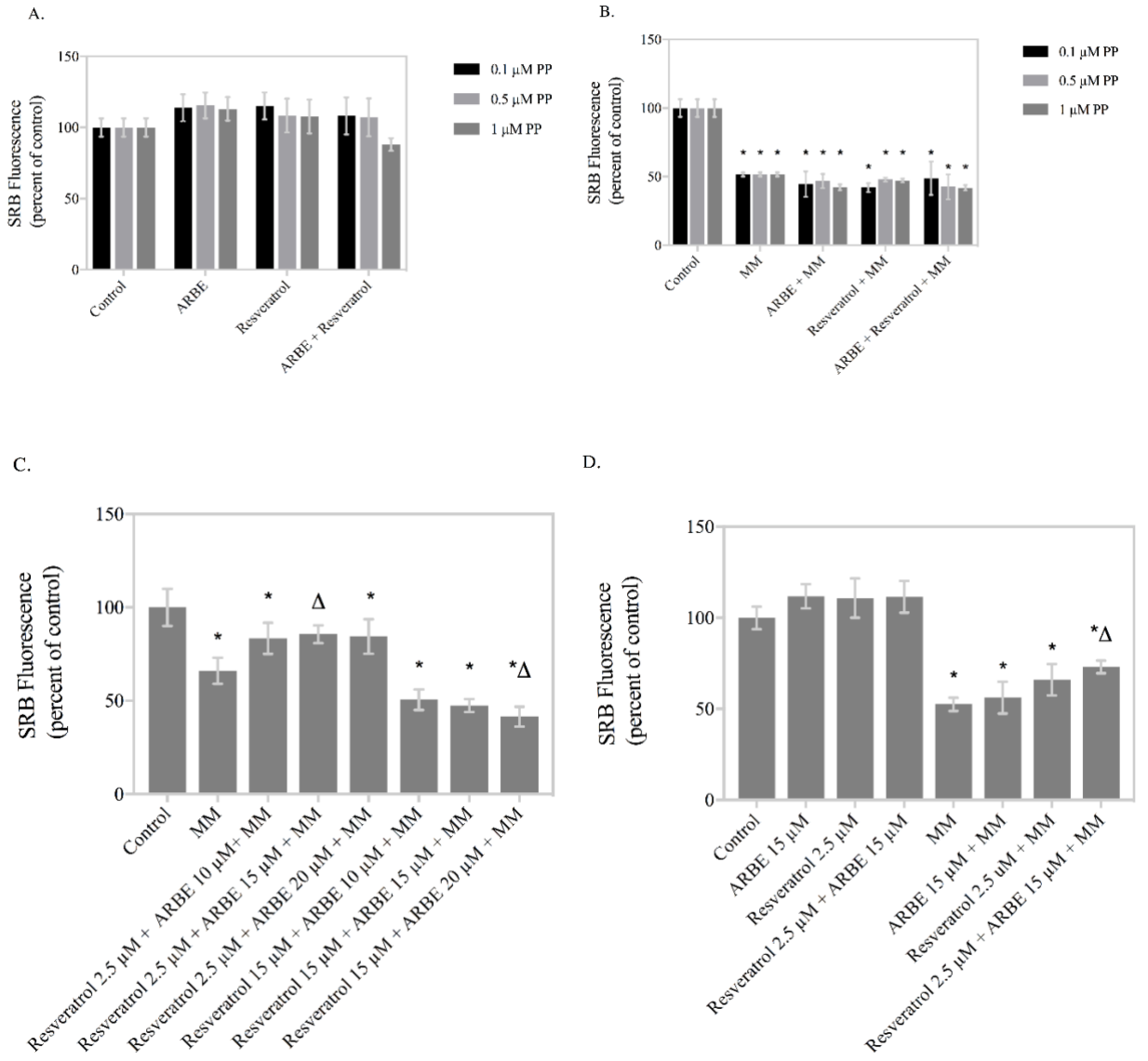

Figure 5.2. *Evaluation of possible protective interactions of ARBE and resveratrol on MM-induced cytotoxicity.* Adherent cell protein content, a surrogate measure of cell viability, was measured by the SRB assay after pre-treatment with resveratrol and ARBE as described in Figure 5.1 followed by 24 h challenge with 0.4 mM MM. **(A)** Absence of MM at 0.1 – 1.0 µM polyphenol (PP) (with ARBE or resveratrol alone or in combination at half concentration each) (PP 0.1 µM: P_{ARBE} =0.3334, $P_{Resveratrol}$ =0.1866, $P_{Interaction}$ =0.0063; PP 0.5 µM: P_{ARBE} =0.0690, $P_{Resveratrol}$ =0.9957, $P_{Interaction}$ = 0.0329; PP 1 µM: P_{ARBE} 0.4443, $P_{Resveratrol}$ =0.0522, $P_{Interaction}$ =0.0008). **(B)**

Presence of MM at 0.1 – 1.0 µM polyphenol (PP) (for the results in the presence of MM, PP 0.1 µM: P_{ARBE} =0.9855, $P_{Resveratrol}$ =0.5516, $P_{Interaction}$ =0.1522; PP 0.5 µM: P_{ARBE} =0.1607, $P_{Resveratrol}$ =0.2522, $P_{Interaction}$ =0.9477; PP 1 µM: P_{ARBE} =0.0007, $P_{Resveratrol}$ =0.0801, $P_{Interaction}$ =0.1179). **(C)** Higher concentrations of ARBE and resveratrol followed by MM exposure (one-way ANOVA P <0.0001). **(D)** Pretreatment with ARBE or resveratrol, alone or in combination, followed by MM exposure for 24 h (for the results without MM, P_{ARBE} =0.2344, $P_{Resveratrol}$ =0.3099, $P_{Interaction}$ =0.2700 and for the results with MM: P_{ARBE} =0.2085, $P_{resveratrol}$ =0.0057, $P_{Interaction}$ =0.3961). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. The significant difference between groups was tested with two-way ANOVA in A, B, and D, and one-way ANOVA in C, followed by Tukey's multiple comparisons test. * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05).

5.4.3. Mitochondrial Function

We then proceeded to explore the effect of the most effective combination concentrations (15 µM ARBE and 2.5 µM resveratrol) on mitochondrial functions in Caco-2 cells challenged with 0.1 mM MM as measured by the Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). The combination had no protective effect on different parameters of mitochondrial function, including basal respiration, ATP production, spare capacity and proton leak (Fig.5.3.A-D). In the presence of MM, ARBE alone, however, did significantly blunt (by 28%) the MM-induced decrease in basal respiration (Fig. 5.3.A). ARBE in the presence of MM also produced a significant further increase in proton leak compared to MM (Fig. 5.3.D).

In addition, pretreatment with polyphenols before shorter exposures to MM (12 h) or longer exposures to MM (48 h), except for a decrease in proton leak after 48 h exposure of polyphenols, did not show any protective effect on any parameter of the mitochondrial function (data shown in Appendix 8.B.9 and 8.B.10).

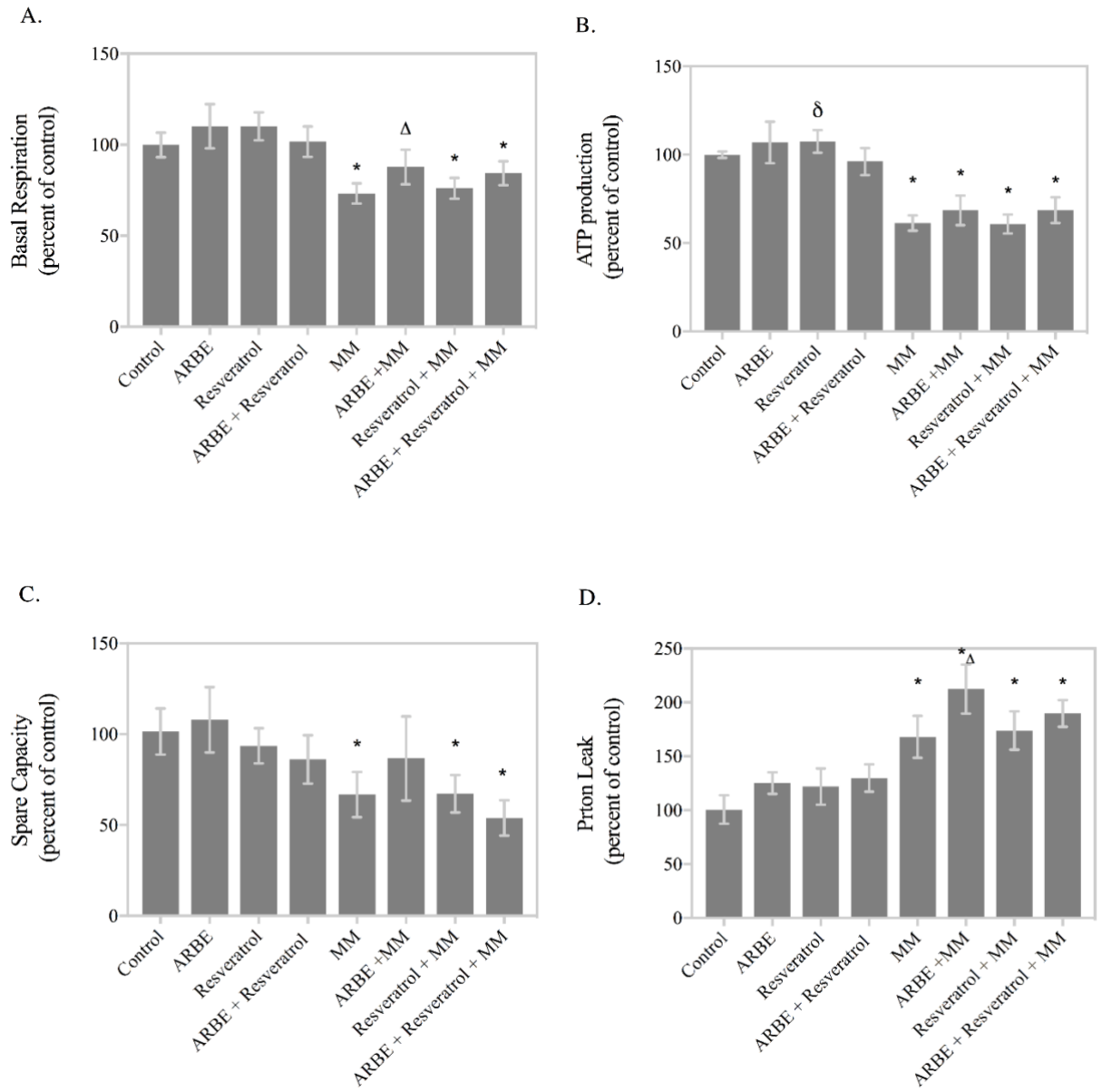

Figure 5.3. *Evaluation of the possible protective interaction of ARBE and resveratrol treatment on MM-induced mitochondrial dysfunction.* Caco-2 cells grown in Seahorse plates were treated with 15 µM ARBE and/or 2.5 µM resveratrol as described in Figure 5.1 prior to adding 0.1 mM MM and incubating for 24 h. Oxygen consumption rate (OCR) was then measured for 3 h with additions of respiratory agents at selected time points and data were normalized to number of protein content, as measured by the Bradford assay. **(A)** Basal respiration (for the results without MM, P_{ARBE} =0.7610, $P_{Resveratrol}$ =0.7918, $P_{Interaction}$ =0.0050 and for the results with MM: P_{ARBE} =0.0001, $P_{resveratrol}$ =0.9199, $P_{Interaction}$ =0.2365). **(B)** ATP production (for the results without MM,

P_{ARBE} =0. 4150, $P_{Resveratrol}$ =0.5732, $P_{Interaction}$ =0.0023 and for the results with MM: P_{ARBE} =0.0074, $P_{resveratrol}$ =0.9221, $P_{Interaction}$ =0.8926). **(C)** Spare respiratory capacity (for the results without MM, P_{ARBE} =0. 9217, $P_{Resveratrol}$ =0.0057, $P_{Interaction}$ =0.1699 and for the results with MM: P_{ARBE} =0.6158, $P_{resveratrol}$ =0.0188, $P_{Interaction}$ =0.0158). **(D)** Proton leak (for the results without MM, P_{ARBE} =0.0022, $P_{resveratrol}$ =0.0114, $P_{Interaction}$ =0.0993 and for the results with MM: P_{ARBE} =0.0009, $P_{resveratrol}$ =0.3041, $P_{Interaction}$ =0.0865). Data represent means ± SD of 3 independent experiments with 4 replicate wells in each experiment. The significant difference between groups were with two-way ANOVA followed by Tukey's multiple comparisons test. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). $^{\delta}$ Mean value significantly different from the relative combination (ARBE + Resveratrol) group ($P < 0.05$).

5.5. Discussion

In order to test our fourth hypothesis, a possible protective interaction between resveratrol and anthocyanins against MM-induced stress in Caco-2 cells was investigated. In previous chapters we document that anthocyanins and resveratrol protect Caco-2 cells via different mechanisms and to different extents. A combination of different polyphenols can provide benefits by targeting several pathways and lowering the dose and toxicity of each compound (Singh et al., 2016), thus this chapter focuses on the potential protective interaction of ARBE and resveratrol against MM-induced oxidative stress in Caco-2 cells.

Previous results showed ARBE anthocyanins act as strong antioxidants, preserve mitochondrial membrane potential and better maintain mitochondrial function, while resveratrol increased mitochondrial content within Caco-2 cells, and both protected against monolayer permeability but to different extents and duration. Thus, we hypothesized that by combining the effects of the two polyphenols, possessing bioactivities that lead to increase in mitochondrial capacity or better maintenance of mitochondria efficiency afforded by mitochondrial antioxidant activity, mitochondrial function and ATP production will be better maintained following challenge than by either polyphenol alone.

Our data showed all combinations of ARBE and resveratrol used protected against intracellular ROS production. Among the lower concentrations tested, the combination of 0.05 μM ARBE + 0.05 μM resveratrol had a stronger antioxidant effect than either one alone at 0.1 μM (Fig. 5.1.B), suggesting a mild synergism. The lower concentrations of ARBE and resveratrol (0.1-1 μM) or their combination did not show any protective effect against cytotoxicity of Caco-2 cells induced by MM. However, among different concentrations tested, the combination of 15 μM ARBE + 2.5 μM resveratrol resulted in a 40% protection against cytotoxicity, this protection was additive. Similar to our study, Elisia and Kitts (2008), showed anthocyanin-rich extracts from crude blackberry (0.8, 1.6, 3.1, 6.3, 12.5 and 25 μg/mL for 24 h) inhibited peroxyl radical induced oxidative damage and associated cytotoxicity in Caco-2 cells. In measurements of mitochondrial function, the combination of 15 μM ARBE + 2.5 μM resveratrol did not protect, although ARBE alone protected against the MM-induced decline in basal respiration.

Very few studies have considered a potential interaction between anthocyanins and resveratrol. In a review by Kristo et al. (2016) antioxidant and anticancer effect of berries, rich in flavonoids and resveratrol, was demonstrated to involve the induction of several pathways that participate in the attenuation of ROS generation in HepG2-C8 cells. Other authors have suggested a synergism of resveratrol and flavonoids in the health promoting effects of whole fruits such as grapes, a major source of both compounds (Liu et al., 2003; Liu et al 2004; Singh et al., 2015, 2016). As mentioned by Liu et al. (2004), the synergistic effect of a complex mixture of phytochemicals in whole foods such as fruits may be responsible for the antioxidant protective effects which cannot be replicated by single antioxidants. In a review, Singh and colleagues (2015) mention whole grape products seem to be a better choice and more promising approach for prevention of diseases however, individual compounds such as resveratrol has been shown to be effective for therapies of diseases (Berman et al., 2017).

In conclusion, numerous studies have demonstrated protective effects of grapes and polyphenol-rich foods, such as activating antioxidant enzyme system, preventing lipid peroxidation and DNA damage, promoting wound healing and supporting anti-inflammatory effects (Choi et al., 2010; Nayak et al., 2010; Rho and Kim, 2006; Janiques et al., 2014), that could be due to combined and protective interactive effects of different polyphenols. In addition, most studies with individual polyphenols have used concentrations far beyond what human cells would encounter (Scalbert et

al., 2005), thus making it hard to relate it to human studies. In our study we used low doses of both resveratrol and anthocyanins relevant to what the enterocytes might be exposed to when consuming fruit in the diet. Our study found protective effects of different dose combinations of ARBE and resveratrol in reducing intracellular ROS generation and preventing MM-induced cytotoxicity. Further studies investigating effects of anthocyanins and resveratrol in combinations found in whole foods are needed to show if it could be an appropriate strategy for improving mitochondrial and bioenergetic efficiency in cells.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interests.

5.6. Transition to CHAPTER 6

Chapters 3 and 4 showed the relative abilities of ARBE and resveratrol to protect Caco-2 cells against lipid micelle induced oxidative stress and mitochondrial dysfunction, while Chapter 5 showed that ARBE and resveratrol had no synergistic effects. In Chapter 6 we investigated the protective effects of phenolic anthocyanin derivatives in comparison with pure anthocyanins and anthocyanins present in bilberry extract, to determine the extent to which they may account for protective effects of anthocyanins.

CHAPTER 6 COMPARISON OF ANTIOXIDANT EFFECTS OF ANTHOCYANINS AND THEIR PHENOLIC BREAKDOWN PRODUCTS AGAINST LIPID MICELLE INDUCED ROS GENERATION IN CACO-2 CELLS

6.1. Abstract

Dietary anthocyanins are well known for their health benefits, including protection of the intestinal barrier against oxidative stress and inflammation (observed in previous chapters), but the extent to which these effects are from the anthocyanins parent compound or from phenolic digestion/breakdown products is unclear. In an *in vitro* model of intestinal stress from high fat consumption in the form of MM exposed to Caco-2 intestinal epithelial cells, the relative protective effects of cyanidin (Cy), cyanidin-3-glucoside (C3G), the phenolic breakdown/digestion products, protocatechuic acid (PCA), and 2,4,6-trihydroxybenzaldehyde (THB) and an anthocyanin rich bilberry extract (ARBE) on intracellular and mitochondrial ROS generation and cell cytotoxicity were assessed. MM challenge for 24 h decreased adherent cell density by ~45% and increased both intracellular and mitochondrial ROS generation per adherent cells by more than 250% and 100%, respectively. Treatment with all polyphenols at 1-20 µM significantly decreased MM-induced intracellular ROS, with the order of potency being ARBE and C3G > CY > PCA > THB. At 20 µM the polyphenols decreased MM-induced intracellular ROS by 45-85%, with ARBE giving the strongest and THB the weakest protection. With MM-induced mitochondrial superoxide generation after 24 h, the breakdown compounds, PCA and THB, and also ARBE significantly inhibited (by ~35%) while the pure anthocyanins did not. In the absence of MM however, Cy and C3G, along with THB decreased mitochondrial superoxide compared to control cells. At earlier time points (2, 4, 8, and 12 h) of MM exposure, Cy decreased MM-induced mitochondrial superoxide (by 21%-33%) while C3G or ARBE did not. Among the polyphenols tested, only ARBE and PCA significantly protected Caco-2 cells against MM-induced cytotoxicity, inhibiting the loss in adherent cell density after 24 h by 35% for both. This study distinguishes different forms of anthocyanins and phenolic breakdown products as potent intracellular ROS scavengers in Caco-2 enterocytes and suggests that PCA may provide some of the protective effects of dietary anthocyanins on intestinal epithelial cells.

Abbreviations

ANOVA, analysis of variance; **ARBE**, anthocyanin rich bilberry extract; **Cy**, cyanidin; **C3G**, cyanidin-3-glucoside; **DCFH-DA**, 2',7'-dichlorodihydrofluorescein diacetate; **DNA**, deoxyribonucleic acid; **GPx**, glutathione peroxidase; **GSH**, glutathione; **GST**, glutathione S-transferase; **LDL**, low-density lipoprotein levels; **MitoSOX**, mitochondrial superoxide; **MM**, mixed micelles; **PCA**, protocatechuic acid; **ROS**, reactive oxygen species; **SD**, standard deviation; **SRB**, sulforhodamine b; **THB**, 2,4,6-trihydroxybenzaldehyde

6.2. Introduction

Anthocyanins are well known for their antioxidant and protective effects in many conditions, making them key candidates for disease treatment and prevention (Elisia and Kitts, 2008; Cooke et al. 2006; Renis et al., 2008; Pervin et al., 2014; Dai et al., 2009; Kong et al., 2003; Neto, 2007; Thomasset et al., 2009). These flavonoids are present in different plants especially berries and are responsible for their red, purple, and blue color (Vitaglione et al., 2007). Studies have demonstrated several biological properties for anthocyanins, including antioxidant, anti-obesity, cardiovascular-protective, and anti-inflammatory activities (Prior and Wu, 2006). However, the protective effects of anthocyanins may not be directly due to the anthocyanin itself, which is poorly absorbed and highly metabolized and transformed mainly via large intestine bacteria to phenolic products (Borges et al., 2007). Intestinal metabolism of anthocyanins includes deglycosylation, opening of the anthocyanidin heterocycle and ring fission of the C-ring to produce phenolic acid and aldehyde derivatives (Keppler and Humpf, 2005; Aura et al., 2005). Cyanidin-3-glucoside (C3G) is one of the major and most abundant anthocyanins and when ingested, the glucose moiety is cleaved via glucosidase in the GI tract to produce cyanidin (Cy), which is further metabolized via gut bacteria to produce the phenolic acid and aldehyde derivatives protocatechuic acid (PCA) and 2,4,6-trihydroxybenzaldehyde (THB), both of which have higher bioavailability (Keppler and Humpf, 2005). Some articles argue that these phenolic metabolites are responsible for the protective effects of anthocyanins (Forester and Waterhouse, 2010). In addition, *in vitro* studies with anthocyanins are complicated by their instability in cell culture media, especially of the aglycone form, which over time break down to phenolic products such as PCA and THB (Kay et al., 2009; Kern et al., 2009; Seeram et al., 2001).

Studies investigating and comparing the antioxidant activity of anthocyanins and their phenolic metabolites on intestinal cells are limited. In an *in vitro* model, the antioxidant protection of pure anthocyanins (cyanidin, cyanidin-3-glucoside), phenolic products of anthocyanin breakdown products (PCA and THB) (Fig. 6.1) and anthocyanins present in an anthocyanin-rich bilberry extract (ARBE), against intracellular ROS and mitochondrial superoxide generation induced by MM in Caco-2 cells were compared.

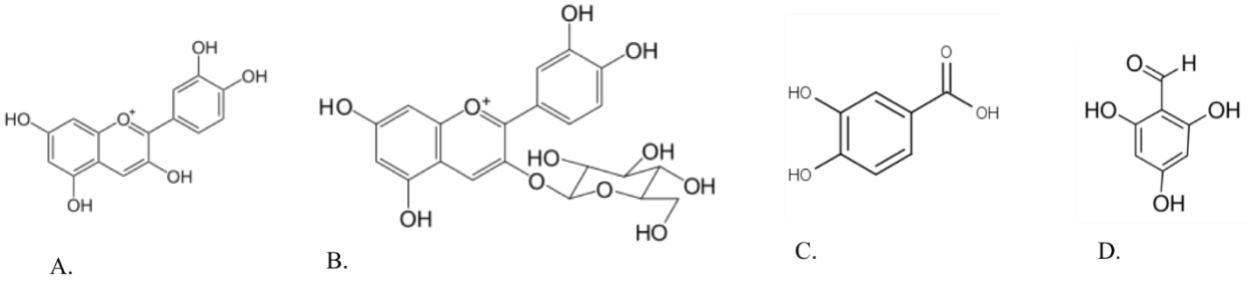


Figure 6.1. *Images of anthocyanins and their phenolic derivatives.* **(A)** Cyanidin, **(B)** Cyanidin-3-glucoside, **(C)** Protocatechuic acid (PCA), **(D)** trihydroxybenzaldehyde (THB)**.**

6.3. Materials and Methods

See section 3.3.

6.3.1. Cell culture condition

Caco-2 cells were incubated with anthocyanins cyanidin (Cy) or cyanidin-3-glucoside (C3G), Protocatechuic acid (PCA), trihydroxybenzaldehyde (THB) or anthocyanin rich bilberry extract (ARBE) for 2 h prior to being exposed to MM for 24 h.

6.3.2. Mixed Micelle preparation

See section 3.3.2.

6.3.3. Treatment with mixed micelles and polyphenols

See section 3.3.3.

6.3.4. Measurements of intracellular reactive oxygen species (ROS) and mitochondrial superoxide generation

See section 3.3.4.

6.3.5. Measurement of cellular viability

See section 3.3.6.

6.3.6. Statistical analysis

See section 3.3.8.

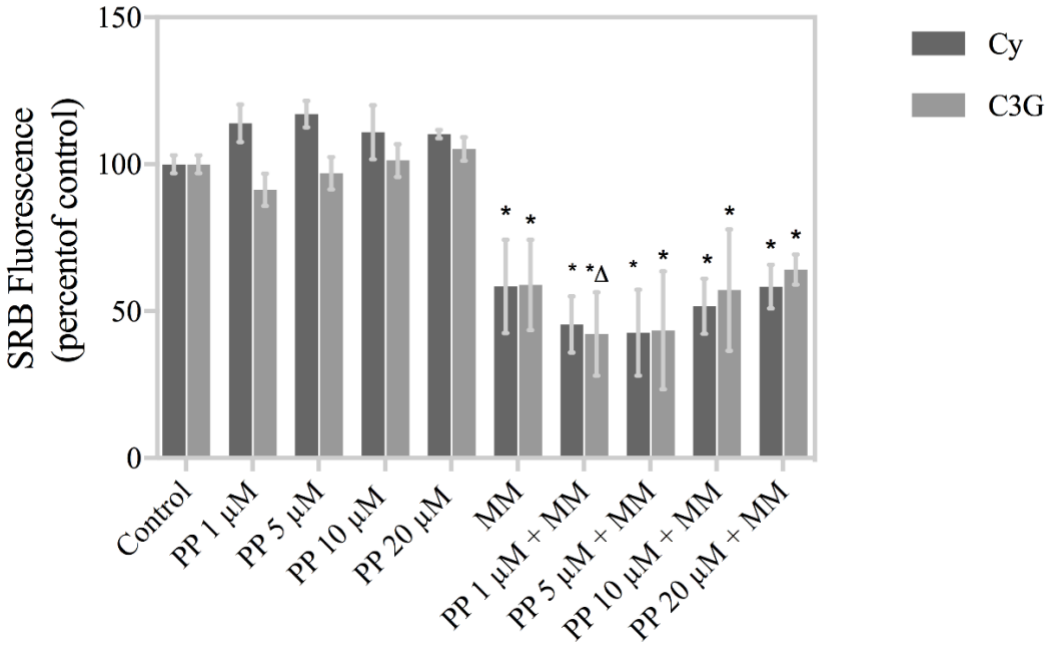
6.4. Results

6.4.1. Dose response of pure anthocyanins in protecting against MM-induced cytotoxicity and intracellular ROS

Initially, different concentrations of anthocyanins and phenolic derivatives were assessed to determine the effect on cellular viability and oxidative stress. To exclude the possibility of toxicity, adherent cell density via SRB was measured in cells treated with the anthocyanins alone. No cytotoxicity was noted for cells treated with the anthocyanins (Fig. 6.2.A). Challenge with MM for 24 h significantly decreased SRB and lower dose of C3G (1 µM) further decreased the SRB value. Cy and higher dose of C3G however, had no effect on the decreased cell density induced by MM (Fig. 6.2.A).

In the absence of MM, all concentrations of Cy and doses of C3G higher than 1 µM decreased intracellular ROS generation compared to control cells (Fig. 6.2.B). With treatments for 2 h before MM exposure for 24 h, Cy and C3G dose-dependently decreased intracellular ROS compared to the MM challenged Caco-2 cells.

A.


SRB Fluorescence (percentof control)
150
100
50
0
Cy
C3G
Control
PP 1 μM
PP 5 μM
PP 10 μM
PP 20 μM
MM
PP 1 μM + MM
PP 5 μM + MM
PP 10 μM + MM
PP 20 μM + MM

B.

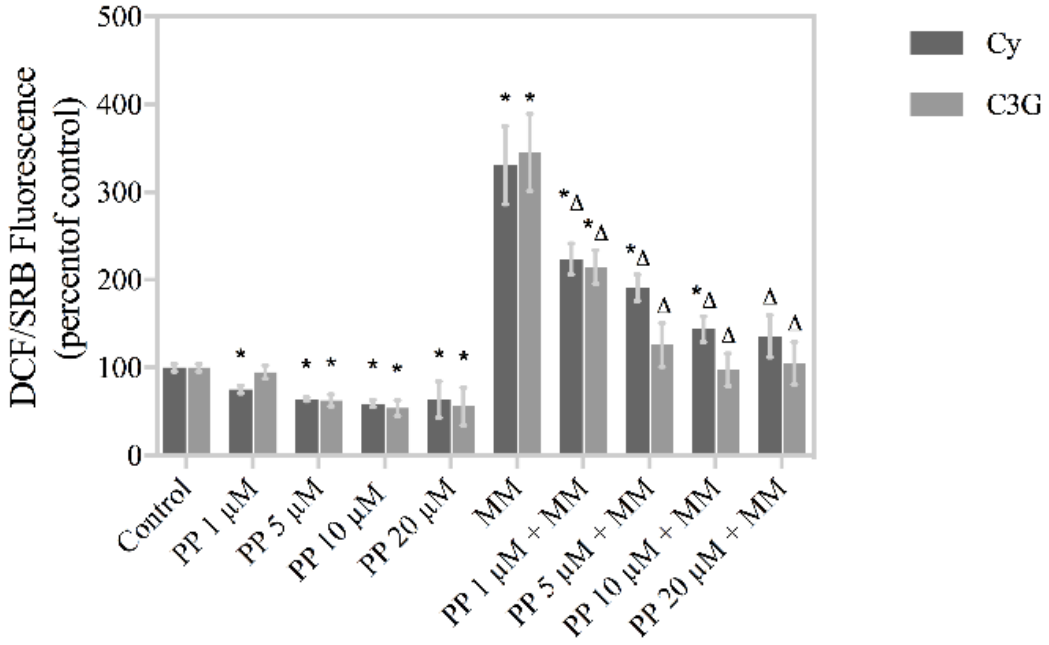

DCF/SRB Fluorescence (percentof control)
500
400
300
200
100
0
Cy
C3G
Control
PP 1 μM
PP 5 μM
PP 10 μM
PP 20 μM
MM
PP 1 μM + MM
PP 5 μM + MM
PP 10 μM + MM
PP 20 μM + MM

Figure 6.2. *Effects of MM, Cy and C3G on adherent cell density and intracellular ROS production.* Caco-2 cells were treated with Cy or C3G at different concentrations for 2 h prior to adding 0.4 mM MM and incubating for an additional 24 h. After treatments, cells were incubated with DCFH-DA for 2 h, then washed with PBS and intracellular ROS was measured via DCF fluorescence (AU) using a microplate reader. After measurement of DCF fluorescence, the SRB assay was performed to determine the amount of adherent cell protein in each well. **(A)** Adherent cell protein measured by the SRB assay after treatments with different concentrations of anthocyanins with or without MM exposure (PP 1 µM: P_{MM} <0.0001, P_{Cy} =0.8950, $P_{Interaction}$ =0.0016; P_{MM} <0.0001, P_{C3G} =0.0045, $P_{Interaction}$ =0.3333; PP 5 µM: P_{MM} <0.0001, P_{Cy} =0.8819, $P_{Interaction}$ =0.0006; P_{MM} <0.0001, P_{C3G} =0.1863, $P_{Interaction}$ =0.4633; PP 10 µM: P_{MM} <0.0001, P_{Cy} =0.1466, $P_{Interaction}$ =0.1437; ; P_{MM} <0.0001, P_{C3G} =0.6846, $P_{Interaction}$ =0.8820; PP 20 µM: P_{MM} <0.0001, P_{Cy} =0.8819, $P_{Interaction}$ =0.0006; P_{MM} <0.0001, P_{C3G} =0.2866, $P_{Interaction}$ =0.9977). **(B)** Intracellular ROS generation per adherent cell protein after treatment with Cy or C3G with or without MM exposure (PP 1 µM: P_{MM} <0.0001, P_{Cy} <0.0001, $P_{Interaction}$ =0.0004; P_{MM} <0.0001, P_{C3G} <0.0001, $P_{Interaction}$ <0.0001; PP 5 µM: P_{MM} <0.0001, P_{Cy}<0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{C3G} <0.0001, $P_{Interaction}$ <0.0001; PP 10 µM: P_{MM} <0.0001, P_{Cy}<0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{C3G} <0.0001, $P_{Interaction}$ <0.0001; PP 20 µM: P_{MM} <0.0001, P_{Cy}<0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{C3G} <0.0001, $P_{Interaction}$ <0.0001). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant differences between groups was tested with two-way ANOVA followed by Tukey's multiple comparisons test.

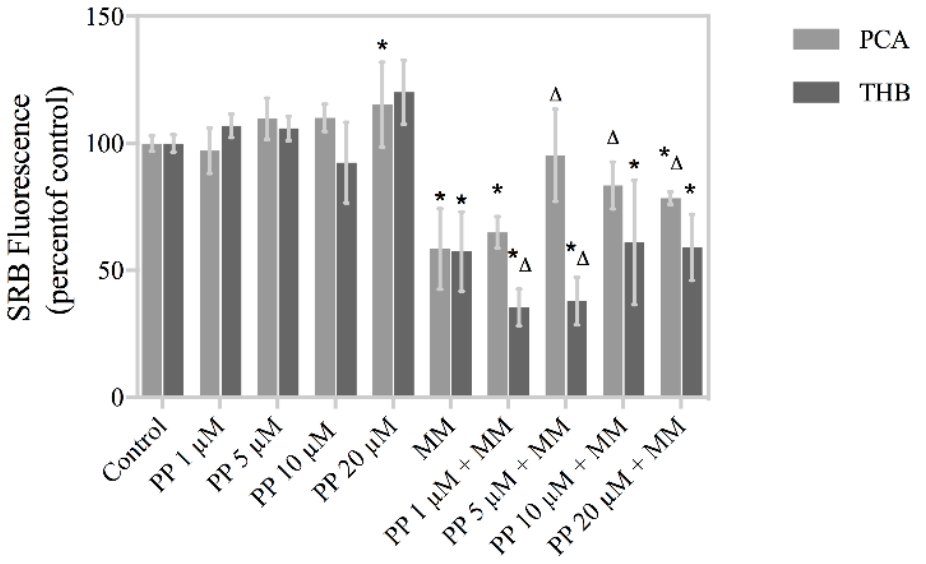
6.4.2. Dose response of phenolic derivatives in protecting against MM-induced cytotoxicity and intracellular ROS

The same experiments were also performed for the phenolic derivatives, PCA and THB. Neither compound alone affected cell content compared to control cells (Fig. 6.3.A). In the presence of MM however, the lower concentrations of THB (1 and 5 µM) further decreased cell viability compared to MM challenged cells but this effect was not seen in higher dose of THB (10 and 20

μM). With PCA, 5, 10 and 20 μM inhibited MM-induced cell loss by 63, 43, and 34%, respectively.

Experiments on intracellular ROS generation showed that PCA alone at concentrations 10 and 20 μM decreased intracellular ROS compared to control cells, whereas THB produced this antioxidant effect only at 20 μM (Fig. 6.3.B). All concentrations of PCA strongly protected against MM-induced intracellular ROS. THB was less potent but gave dose-dependent protection by up to 57%.

A.



B.

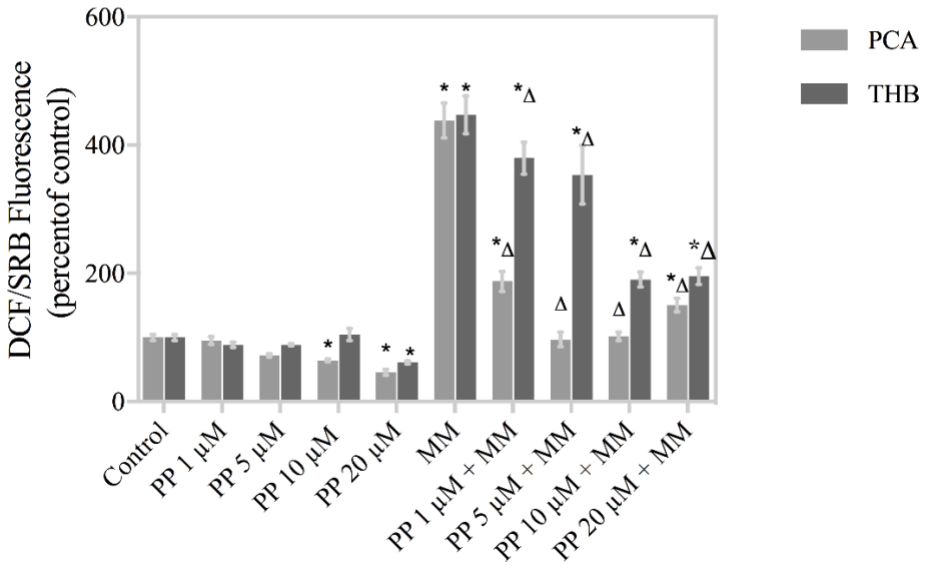


Figure 6.3. *Effects of MM, PCA and THB on adherent cell density and intracellular ROS production.* Experiments were conducted as in Fig. 6.2 with PCA or THB at different

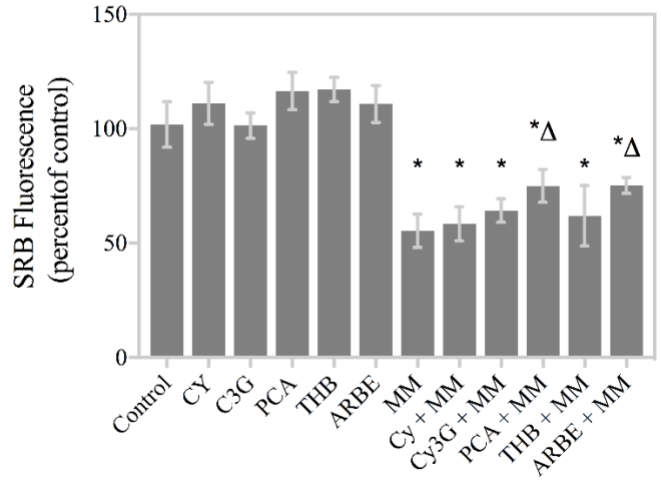
concentrations. **(A)** Adherent cell protein measured by the SRB assay via plate reader after treatment with different dose of PCA or THB with or without MM exposure (PP 1 µM: P_{MM} <0.0001, P_{PCA} =0.7215, $P_{Interaction}$ =0.3628; P_{MM} <0.0001, P_{THB} =0.1285, $P_{Interaction}$ =0.0063; PP 5 µM: P_{MM} <0.0001, P_{PCA} =0.0005, $P_{Interaction}$ =0.0256; P_{MM} <0.0001, P_{THB} =0.2004, $P_{Interaction}$ =0.0009; PP 10 µM: P_{MM} <0.0001, P_{PCA} =0.0025, $P_{Interaction}$ =0.1568; P_{MM} <0.0001, P_{THB} =0.6782, $P_{Interaction}$ =0.3888; PP 20 µM: P_{MM} <0.0001, P_{PCA} =0.0763, $P_{Interaction}$ =0.1548; P_{MM} <0.0001, P_{THB} =0.0248, $P_{Interaction}$ =0.0304). **(B)** ROS generation per adherent cell protein after treatment with PCA or THB with or without MM exposure (PP 1 µM: P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{THB} =0.002, $P_{Interaction}$ =0.0048; PP 5 µM: P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{THB} =0.0002, $P_{Interaction}$ =0.0020; PP 10 µM: P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.00018; P_{MM} <0.0001, P_{THB} <0.0001, $P_{Interaction}$ <0.0001; PP 20 µM: P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.00018; P_{MM} <0.0001, P_{THB} <0.0001, $P_{Interaction}$ <0.0001). Values represent means ± SD of three independent experiments with three wells of cells per treatment condition. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant differences between groups was tested with two-way ANOVA followed by Tukey's multiple comparisons test.

6.4.3. Adherent cell density, oxidative stress, and mitochondrial superoxide generation

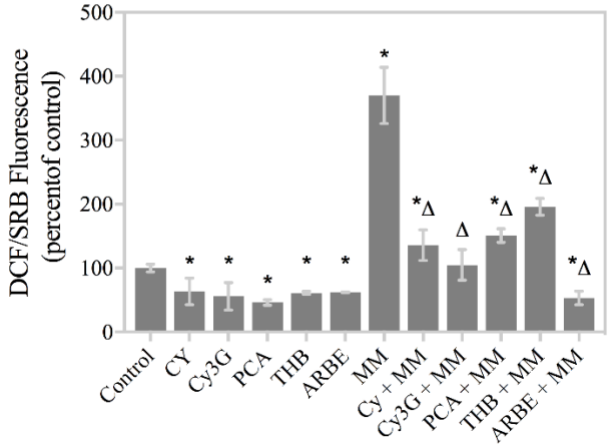
Next, we compared protective effects of 20 µM of the different polyphenols and an anthocyanin rich bilberry extract (ARBE) on adherent cell density (Fig 6.4.A), intracellular and mitochondrial ROS generation (Fig 6.4.B&C). Comparing adherent cell density of Caco-2 cells pretreated with polyphenols for 2 h prior to MM exposure for 24 h, showed that only PCA and ARBE significantly inhibited MM-induced cell loss to the extent of 34 and 36% respectively (Figure 6.4.A). All polyphenols decreased intracellular ROS compared to control cells and all strongly (45-85 %) inhibited MM-induced intracellular ROS, with ARBE being the most and THB the least protective (Fig 6.4.B).

Pure anthocyanins (Cy and C3G) along with THB deceased mitochondrial superoxide compared to control cells (Fig. 6.4.C). With 24 h MM exposure, the phenolic derivatives (PCA and THB) and ARBE inhibited the increase of mitochondrial superoxide by 32-35%, while Cy and C3G did not significantly protect.

A.



B.



C.

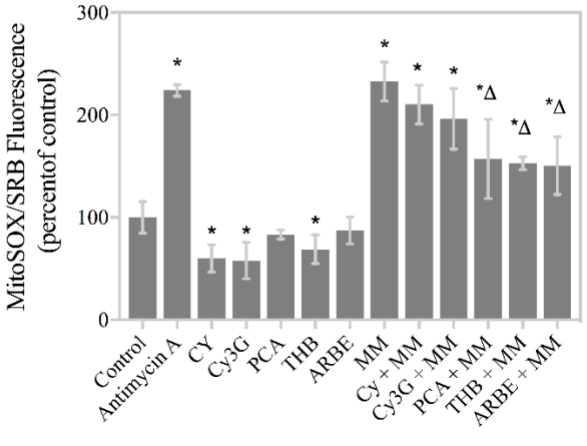


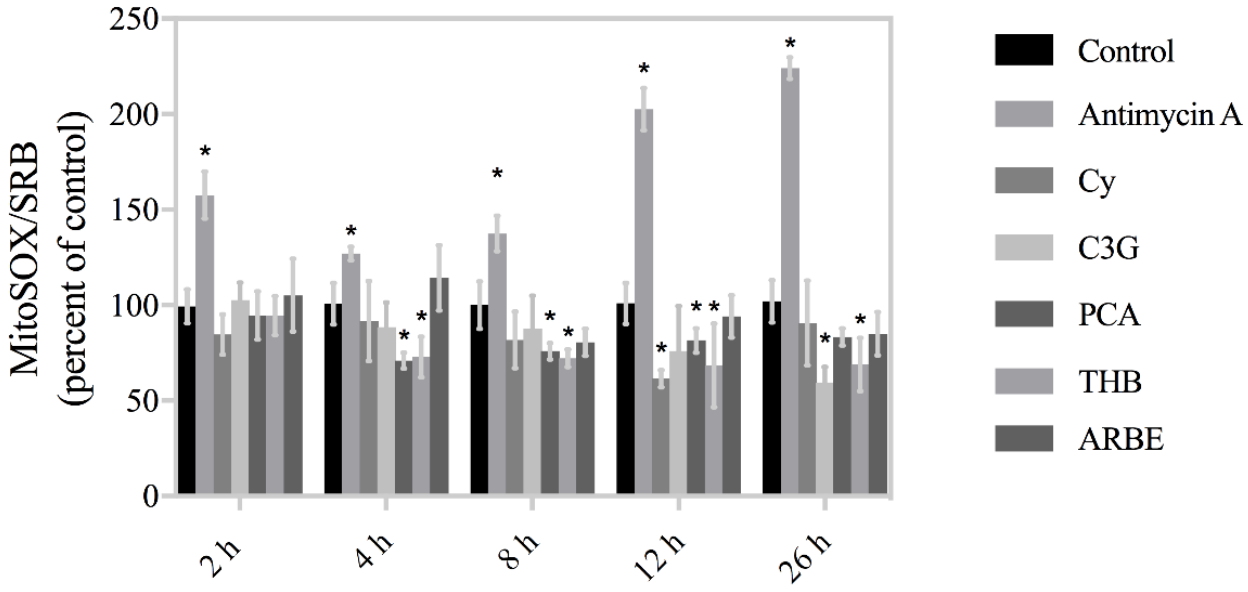
Figure 6.4. *Effects of MM and polyphenols on intracellular ROS and mitochondrial superoxide generation*. Caco-2 cells were treated with polyphenols at 20 µM for 2 h prior to adding 0.4 mM MM and incubating for an additional 24 h. **(A)** Adherent cell density was measured by the SRB assay (P_{MM} <0.0001, P_{Cy} =0.0016, $P_{Interaction}$ =0.0967; P_{MM} <0.0001, P_{C3G} =0.0004, $P_{Interaction}$ =0.32513; P_{MM} <0.0001, P_{PCA} =0.1302, $P_{Interaction}$ =0.4784; P_{MM} <0.0001, P_{THB} =0.0452, $P_{Interaction}$ =0.0744; P_{MM} <0.0001, P_{ARBE} =0.0003, $P_{Interaction}$ =0.2046). **(B)** Intracellular ROS generation measured via DCFH-DA (P_{MM} <0.0001, P_{Cy} <0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{C3G} <0.00, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.0001; P_{MM} <0.0001, P_{THB} <0.0001, $P_{Interaction}$ =0.0005; P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ <0.0001). **(C)** Mitochondrial superoxide generation measured with MitoSOX Red (P_{MM} <0.0001, P_{Cy} <0.0001, $P_{Interaction}$ <0.1259; P_{MM} <0.0001, P_{C3G} <0.00, $P_{Interaction}$ <0.6992; P_{MM} <0.0001, P_{PCA} <0.0001, $P_{Interaction}$ <0.0006; P_{MM} <0.0001, P_{THB} <0.0001, $P_{Interaction}$ =0.0013; P_{MM} <0.0001, P_{ARBE} <0.0001, $P_{Interaction}$ <0.0001). Antimycin A was added 30 min prior to addition of MitoSOX. Data were normalized to protein content measured via SRB. Values represent means ± SD of three independent experiments with three wells of cells in each treatment condition. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant differences between groups were tested with two-way ANOVA followed by Tukey's multiple comparisons post hoc test.

6.4.4. Protective effect of anthocyanins against short time MM exposure induced mitochondrial superoxide generation

Since the anthocyanins Cy and C3G had no effect on MM-induced increase in mitochondrial superoxide, and due to the relative instability of Cy in cell culture media, the effects of anthocyanins on mitochondrial superoxide were compared after shorter time periods (2, 4, 8 and 12 h) of MM challenge (Fig. 6.5). After 4 h incubation with polyphenols, PCA and THB alone decreased mitochondrial superoxide compared to control cells and this effect remained until 26 h, although PCA lost the antioxidant effect by 26 h. Cy and C3G decreased mitochondrial superoxide compared to control cells after 12 h and 26 h respectively.

MM increased mitochondrial superoxide generation after 2 h by 53% and increasingly by up to 149% at 8 h, after which MM-induced mitochondrial superoxide remained steady by 12 h MM exposure and then declined to a 68% increase at 24 h. At these earlier time points (from 2-12 h), Cy inhibited MM-induced mitochondrial superoxide (by 23-32%), but protection was lost by 24 h. The phenolic derivatives of anthocyanin (PCA and THB) were able to maintain mitochondrial superoxide near control levels until 4 h after MM exposure. Although the antioxidant power of both phenolic compounds decreased substantially after 4 h, THB maintained its antioxidant effect until 24 h after MM challenge. PCA appeared to slightly lose its antioxidant effect after 8 h but regained its protective effect by 12 h of MM exposure. C3G and ARBE pretreatment did not protect before 24 h, but ARBE protected after 24 h.

A.


MitoSOX/SRB
(percent of control)
250
200
150
100
50
0
2 h
4 h
8 h
12 h
26 h
Control
Antimycin A
Cy
C3G
PCA
THB
ARBE

B.

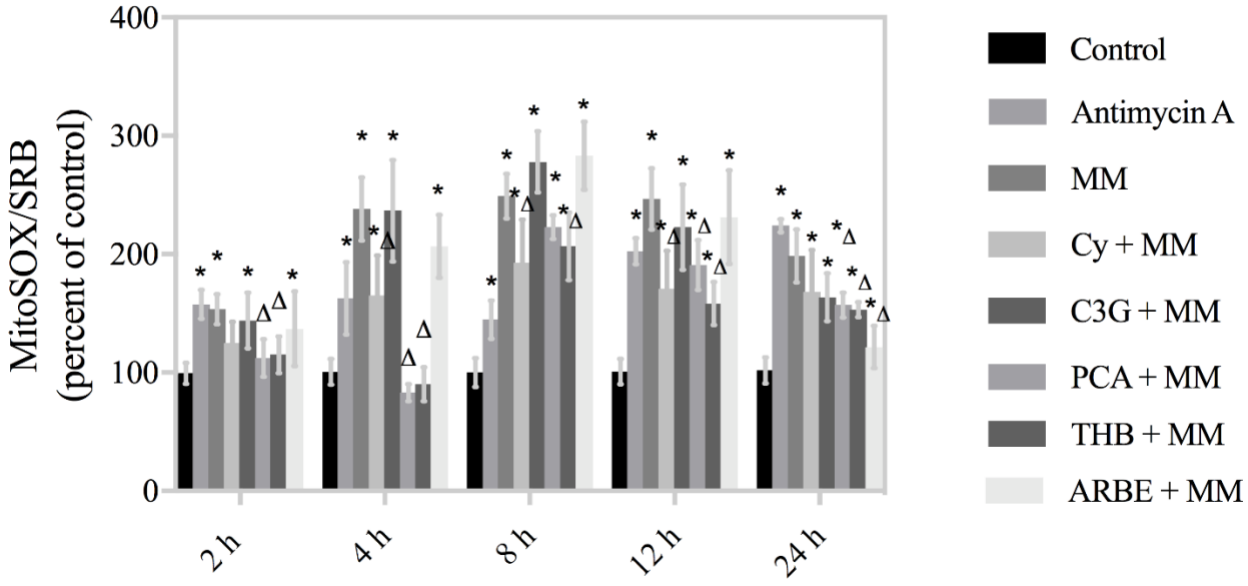

MitoSOX/SRB
(percent of control)
400
300
200
100
0
2 h
4 h
8 h
12 h
24 h
Control
Antimycin A
MM
Cy + MM
C3G + MM
PCA + MM
THB + MM
ARBE + MM

Figure 6.5. *Short term effects of MM and polyphenols on mitochondrial superoxide generation*. Mitochondrial superoxide generation was measured in Caco-2 cells treated with polyphenols with or without MM challenge as described in Figure 6.2. MitoSOX fluorescence intensity of Caco-2 cells was measured via plate reader after 30 min incubation with MitoSOX. **(A)** Absence of MM with polyphenol for 26 h. ($P_{2\,h}$=0.0348; $P_{4\,h}$<0.0001; $P_{8\,h}$=0.0004; $P_{12\,h}$=0.0021; $P_{24\,h}$<0.0499). **(B)** Presence of MM ($P_{2\,h}$<0.0001; $P_{4\,h}$<0.0001; $P_{8\,h}$<0.0001; $P_{12\,h}$<0.0001; $P_{24\,h}$<0.0001). Antimycin A was added 30 min prior to addition of MitoSOX. Data were normalized to attached-cell mass measured via SRB. Values represent means ± SD of three independent experiments with three wells of cells in each treatment condition. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant differences between groups were tested with one-way ANOVA followed by Tukey's multiple comparisons post hoc test.

6.5 Discussion

Dietary anthocyanins are well-known to mitigate oxidative stress, these compounds are absorbed in the GI tract but undergo extensive metabolism and phase II transformation and due to their relatively low bioavailability, previous studies have suggested that at least some of the protective properties are mediated via their phenolic metabolites. Two major derivatives of anthocyanins include protocatechuic acid (PCA) and 2,4,6-trihydroxybenzaldehyde (THB) (Forester and Waterhouse, 2010). Anthocyanin derivatives such as PCA could be found in higher amounts (Azzini et al., 2010) and remain longer in target tissue compared to the parent compound, strengthening the suggestion of the derivatives being the bioactive compounds responsible for their protective effects (Tsuda et al., 1999).

We have previously reported the protective and antioxidant effect of anthocyanin-rich bilberry extract (ARBE) against MM-induced ROS generation in Caco-2 cells. Here we compare the antioxidant effect of ARBE with pure anthocyanins, in both glycosylated and aglycone form, and the main phenolic metabolites PCA and THB. The different anthocyanins, as well as PCA and THB all strongly decreased MM-induced intracellular ROS after 24 h of MM exposure. Possible

mechanisms for this protective effect may be direct antioxidant activities and activation of signaling pathways that induce antioxidant enzymes. As mentioned in the review by Smith et al. (2016), phenolic derivatives of Cy and C3G produced via gut microbiota can activate the Nrf-2 system to induce antioxidant enzymes. While anthocyanidins, and to a lesser extent, anthocyanin glycosides, were previously shown to induce antioxidant enzymes in cultured cells (Shih et al., 2007), Cy, and more slowly C3G, degrade over time to PCA and THB in cell culture media (Kay et al., 2009), so it is not clear if the effects are due to the parent anthocyanin or the phenolic derivatives. A previous study in cultured CHO cells showed that THB but not C3G or ARBE could activate the Nrf-2 system (Kropat et al., 2013). Similarly, dietary anthocyanins have been shown to increase mitochondrial biogenesis in mice (Tang et al., 2015; Matsukawa et al., 2017) and cultured C2C12 cells (Matsukawa et al., 2017), but it is unknown if this effect is due to their phenolic derivatives.

While all forms of anthocyanins evaluated in this study significantly protected against increased intracellular ROS generation induced by 24 h exposure to MM, only ARBE, PCA and THB protected against increased mitochondrial superoxide generation at this time point. Although, they did not protect against 24 h MM-induced mitochondrial superoxide generation, the pure anthocyanins Cy and C3G alone, as well as THB did decrease mitochondrial superoxide compared to control cells. This might indicate that the amount of Cy and C3G that reached or was retained in the mitochondria at this time point was sufficient to decrease endogenous mitochondrial superoxide but insufficient to exert their effect with MM exposure.

Though Cy did not inhibit MM-induced mitochondrial superoxide generation after 24 h MM exposure, along with the phenolic derivatives, it did decrease MM-induced mitochondrial superoxide at earlier time points up to 12 h. In other words, in an acute oxidative stress situation, Cy is a more potent antioxidant, but its antioxidant effect declined beyond this treatment period. A possible reason is that at early time points Cy was taken up by mitochondria to a greater extent than anthocyanin glycosides, as observed by Peng (2012), but by 24 h Cy had degraded within the medium and/or had been metabolized or lost from mitochondria, thus reducing its protective effect.

Our study also showed that after 24 h, ARBE protected Caco-2 cells in these aspects tested, including ROS generation, mitochondrial superoxide and cytotoxicity induced by MM more

strongly than other anthocyanins tested. This result suggests that the different anthocyanins, or some other components in ARBE provide stronger effects than Cy or C3G. Other studies have also shown protective effects of the ARBE Mirtoselect® in different tissue cells, although very few have looked at effects on intestine. The study of Riva et al. (2017) showed anthocyanins in Mirtoselect® (containing 36% anthocyanins) had higher bioavailability and thus a greater protective effect compared with a highly purified anthocyanin rich extract containing 89% anthocyanins, supporting the possible reason in our study that the specific anthocyanins or other components of Mirtoselect® contributed to the protection. Another study also showed Mirtoselect® supplementation to be more protective in a variety of retinopathy conditions after 6 months than another bilberry extract (obtained by the dilution with maltodextrin) (Gizzi et al., 2016). In this study, nuclear magnetic resonance and HPLC analyses showed differences in contents of both extracts, the generic bilberry extract having a higher content of anthocyanidins, fiber, maltodextrins and maltose and lower amount of fructose, glucose and organic acids compared to Mirtoselect® (Gizzi et al., 2016). Another study also showed that a combination of polyphenols found in *Beta vulgaris var. cicla* seeds induced higher chemoprotective effect compared with its single components in both human colon cancer cells and normal human fibroblasts (Gennari et al., 2011). Moreover, as Gizzi et al. (2016) and Keith et al. (2005) mention, medicine is more effective as a synergy of diverse components rather than one compound and many properties of extracts are not present when divided into individual compounds. Although the anthocyanin component of Mirtoselect® is considered the main bioactive fraction, it is unknown whether other bioactive components alone or in combination are accountable for the observed effects (Morrison et al., 2015).

Our study showed that in Caco-2 cells, both PCA and THB protected against intracellular and mitochondrial ROS generation, and their effect in reducing mitochondrial superoxide after 24 h was greater than the anthocyanins. Also, PCA protected against MM-induced cytotoxicity. Being a major anthocyanin metabolite and naturally occurring phenolic acid, PCA has also shown to possess antioxidant and ROS scavenging properties in different *in vitro* studies (Rafiei et al., 2017; Li et al., 2011) including those that demonstrate decreased lipid peroxidation and increased scavenging of H_2O_2, decreased oxidized low-density lipoprotein levels (LDL), inhibition of superoxide and H_2O_2 production, restoration of glutathione (GSH)-related enzymes, improvement

in mitochondrial function and inhibition of DNA fragmentation. Similarly, *in vivo* studies have revealed indirect antioxidant effects of PCA including the induction of endogenous antioxidant enzymes that associate with decreases in ROS formation in liver, heart, kidney, and brain (reviewed by Semaming et al., 2015).

Our results also demonstrate that Cy and C3G display antioxidant effects as shown in the inhibition of MM-induced intracellular ROS generation, findings that are consistent with the study of Song et al. (2013), who demonstrated antioxidant activity of C3G in human MDA-MB-231 cells. In this study, C3G reduced ROS generation, inhibited glutathione (GSH) depletion and decreased the activities of glutathione peroxidase (GPx) and glutathione S-transferase (GST). Although pure anthocyanins did not show mitochondrial superoxide protective effect after 24 h MM challenge, our data showed short term decrease in mitochondrial superoxide production by Cy as well as by the corresponding phenolic derivatives.

In general, while the anthocyanins and phenolic derivatives tested in this study all protected against intracellular ROS generation induced by MM in Caco-2 cells, they showed some differences with regard to effects on mitochondrial superoxide production. Although the pure anthocyanins, Cy, did not protect against long term (24 h) MM-induced mitochondrial ROS generation, it was noted that Cy was effective when MM challenge was shorter. This study showed anthocyanins and their derivatives have different potential and mechanisms in protecting against MM-induced stress in Caco-2 cells.

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Conflict of interest

The authors declare no conflicts of interests.

CHAPTER 7 GENERAL DISCUSSION AND CONCLUDING REMARKS

7.1. General discussion

The main goal of this thesis was to determine the relative protective effects of anthocyanins and resveratrol in amounts easily achievable by diet and their mechanisms in reducing the stress induced by MM as a model of high fat diet in intestinal epithelial cells. Due to the prevalence of high dietary fat consumption, we investigated the effects of MM on intestinal epithelial cells and mechanisms of how these two classes of polyphenols in fruits can ameliorate the damaging effects of MM and prevent impairment to the barrier integrity. Intestinal cells are the inside barriers that separate the body from the outside, thus maintaining their health is essential to prevent entrance of toxins and provide a healthy body. In addition, since the gut represents one of the first anatomical sites of exposure, gut luminal concentrations of polyphenols can achieve very high levels (µM) relevant to those used experimentally to show benefit. This project elucidates the different mechanisms by which polyphenols induce their protective effects in a model of high fat exposure in Caco-2 intestinal epithelial cells.

The intestine is composed of both differentiated and undifferentiated cells, with the latter needing differentiation to mature enterocytes to avoid cancer and produce the epithelial barrier. Thus, in our study, both undifferentiated and differentiated Caco-2 cells were used as a model of intestinal epithelial cells. A major focus of our study was protective effects of polyphenols on mitochondria of Caco-2 cells. Functional mitochondria are necessary for providing energy that is needed to maintain the integrity of the intestinal barrier (JanssenDuijghuijsen et al., (2017). Cancer cells tend to derive energy from glycolytic pathways even in the presence of oxygen, due to the Warburg effect which mitochondrial oxidative phosphorylation is suppressed (Liberti and Locasale, 2016). By increasing mitochondrial biogenesis and providing efficient as well as functional mitochondria the energy source could shift toward aerobic pathways, thus blunting the Warburg effect and allowing differentiation to enterocytes of the epithelial barrier.

In the work described in CHAPTER 3, the protective effect of anthocyanins rich bilberry extract was compared to resveratrol in a Caco-2 cell model of MM-induced oxidative stress. This study showed MM increased both intracellular and mitochondrial ROS in addition to inducing

inflammatory cytokine production, cytotoxicity and loss of Caco-2 cell monolayer integrity. Both polyphenols notably decreased ROS generation, inflammatory cytokine (TNFα) production and cytotoxicity induced by MM. In order to investigate the mechanisms involved, effects of polyphenol treatment and MM challenge on mRNA expression of Mn-SOD and mitochondrial superoxide generation were assessed. Both polyphenols decreased the effect of MM on Mn-SOD, however only ARBE was shown to decrease mitochondrial superoxide generation. In general, ARBE seemed to be a stronger antioxidant compared to resveratrol and had a greater effect in maintaining monolayer integrity and decreasing permeability.

Part of the mechanism of MM-induced cytotoxicity may be alterations in mitochondrial content and function that can reduce ATP production and therefore disrupt the intestinal barrier and increase intestinal permeability. The connection between lack of sufficient ATP production and epithelial permeability has been demonstrated in diseases such as inflammatory bowel disease (Novak and Mollen, 2015). Mitochondrial ROS adversely affects mitochondrial productivity and reduces mitochondrial ATP by damaging mitochondrial lipids and other mitochondrial components, which can lead to further increases in ROS generation and loss of ATP producing efficiency. This may ultimately lead to a cascade of events causing mitochondrial permeability transition, release of cytochrome C, and apoptosis (Crimi and Esposti, 2011). Thus, considering the vital role of mitochondria, in CHAPTER 4 we compared the protective effects of ARBE and resveratrol against MM-induced bioenergetic dysfunction in intestinal epithelial cells focusing on mechanisms involving this organelle. We found that MM induced loss of mitochondrial membrane potential, decreased mitochondrial content, as well as increased mitochondrial dysfunction. Our results indicated that ARBE prevented loss of mitochondrial membrane potential and improved different aspects of mitochondrial function such as basal respiration, spare capacity and ATP production, whereas resveratrol had a greater effect in increasing number and content of mitochondria along with increasing expression of genes involved in mitochondrial biogenesis such as PGC-1α and TFAM. Increasing efficiency and/or biogenesis of mitochondria enables this organelle to supply sufficient ATP when needed, which helps cells combat stressful conditions. Therefore, maintaining healthy mitochondria is vital for cell survival and function, and important in protecting intestinal epithelial cells against the stress of high dietary fat.

The previous chapters showed anthocyanins to mainly decrease oxidative stress and improve mitochondrial efficiency and function and resveratrol to increase mitochondrial biogenesis. Increasing mitochondrial function accompanied with low oxidative stress and higher capacity afforded by mitochondrial biogenesis enables ATP production to support many cellular functions, including the formation and maintenance of tight junction protein complexes that are responsible for intactness of the gut barrier. Protecting this critical site may prevent systemic exposure to gut-derived endotoxin and the physiological alterations of such exposure, namely chronic inflammation. Thus, ARBE and resveratrol, by supporting mitochondrial energy metabolism and improving barrier function, may prevent inflammation without invoking blockade of inflammatory pathways that are critical for the body's defense. Preventing entry of gut-derived endotoxin by dietary component/extracts such as resveratrol and ARBE may offer an advantage, as many anti-inflammatory agents interfere with immune activation.

In CHAPTER 5, we investigated possible synergistic effects of the combination of ARBE and resveratrol in decreasing MM-induced oxidative stress in Caco-2 cells. Our results only showed an additive cyto-protective effect of the combination of higher concentrations of polyphenols tested, and a slightly greater antioxidant effect among the lower concentrations of both polyphenols. Although the previous results showed different protective effects of ARBE and resveratrol in regard to mitochondrial function, the combination of both polyphenols did not protect against MM-induced loss of mitochondrial efficiency and function. While Liu (2004) and others suggest that the synergistic effect of a complex mixture of phytochemicals in fruits as whole food are responsible for the antioxidant protective effects, the current experiments did not provide strong support for a synergism between ARBE and resveratrol. Although anthocyanins are well-known for their antioxidant and protective effects, due to extensive metabolism and transformation in the GI tract, it is still unknown whether the protective effects are due to the parent compound or the breakdown products. In CHAPTER 6, we compared protective effects of pure anthocyanins and two of the most common breakdown/digestive products of anthocyanins (THB and PCA), and ARBE. Our results showed all forms decreased intracellular ROS induced by MM in Caco-2 cells; however, after 24 h only the derivatives (THB and PCA) as well as ARBE decreased mitochondrial superoxide. This result may indicate entrance of the derivatives into the mitochondria. Also, the protective antioxidant effect of ARBE may be due to other bioactive compounds within the extract, such as smaller amounts of other phenolic compounds (Appendix 8A). As Morrison et al. (2015)

mention, although the anthocyanin component of Mirtoselect is considered the main bioactive fraction, it is unknown whether other bioactive components alone or in combination are accountable for the observed effects. Since the pure anthocyanins did not show protection against mitochondrial ROS, and cyanidin is relatively unstable in cell culture media, we measured shorter times points after anthocyanin and MM treatment. The data showed that MM increased mitochondrial superoxide over time to a peak after 8 h, and among the pure anthocyanins, only Cy along with the derivatives (PCA and THB) protected against mitochondrial superoxide up until 12 h post MM exposure. This effect of Cy was lost after 24 h of MM challenge, while the antioxidant effect of both derivatives extended to 24 h post MM challenge. The results suggest that Cy may enter mitochondria initially, but then be lost or converted to other forms over 24 h.

7.2. Concluding remarks

In conclusion, MM as a model of the adverse effects of a high fat diet, induced oxidative stress and mitochondrial dysfunction in Caco-2 cells, and ARBE and resveratrol protected against the oxidative stress via different mechanisms. All polyphenols tested greatly decreased intracellular ROS, anthocyanins in the form of extract or the breakdown metabolites were able to decrease mitochondrial superoxide generation. ARBE and resveratrol also both greatly decreased TNF-α expression. However, there were differences in mechanisms of protection induced via ARBE compared to resveratrol. ARBE decreased mitochondrial superoxide and increased efficiency of mitochondria evidenced by improvement of different aspects of mitochondrial function such as basal respiration, spare capacity and ATP production. However, resveratrol seemed to induce its protective effects via inducing mitochondrial content and genes related to mitochondrial biogenesis.

7.3. Limitations of the study

Some limitations and gaps for this study include:

1. This study used an *in vitro* model of intestinal barrier function to investigate the disturbances created by MM challenge, and the protection afforded by ARBE/resveratrol: *In vitro* studies have value for investigating molecular mechanisms of cause and effects and give good reproducibility; however, with each cell line there are some limitations compared to an *in vivo*

model. For example, although Caco-2 cells have been extensively used for models of cell permeability and transport, they lack some features of human intestinal epithelial cells such as mucus synthesis and secretion. Thus, they do not simulate the composition of an epithelial layer with different types of cells (Hidalgo, 1996). Mucus acts as a barrier for lipophilic compounds and thus has an important role in protecting against intestinal permeability and lack of this layer is likely to result in an over estimate of the permeability-stimulating effects of challenges at a given concentration. In addition, Caco-2 cells lack some of the transporters and carriers present in human intestinal epithelium. Lack of intestinal microbiota and their effects on lipid and polyphenols absorption is another limitation of this study.

2. The activity of the mitochondrial respiratory complexes as well as nuclear mRNA of respiratory complex subunits has not been measured: Although ARBE or resveratrol did not show any effect on mRNA expression of mitochondrial respiratory complex subunits, measuring the effect of different polyphenols on activity of mitochondrial respiratory complexes in addition to evaluating the effect of polyphenols on expression of nuclear mRNA of mitochondrial respiratory complexes could give better insight into the effect of polyphenols on these subunit as post translational changes may occur and transcript abundance may not correspond to the activity of proteins or enzymes.

3. Measure protein level of deacetylated PGC-1α since post translational changes may occur and gene expression may not completely indicate activity of the protein and also measure SIRT1 activity since resveratrol may affect its activity.

4. Lack of knowledge about phase II metabolites of resveratrol and ARBE: Both ARBE and resveratrol are prone to metabolism via phase II enzymes within the Caco-2 cell line. Thus, the proportion of inactive conjugated metabolites and active metabolites are unknown which may cause underestimation of the effect of both polyphenols or might influence their relative protection.

5. Our preliminary experiments showed 0.4 mM MM to be a suitable concentrations to induce stress by decreasing cell viability and inducing intracellular ROS which could be protected via the polyphenols used; however, Seahorse experiments showed this concentration of MM to irreversibly decrease mitochondrial function and oxygen consumption. Thus, 0.4 mM MM might be too strong for mitochondria to tolerate. (Access to the Seahorse instrument was possible only

later in our experiments, thus the dose of MM in prior experiments was not based on Seahorse data).

7.4. Future directions

For future studies, investigating the protective effect of different polyphenols in decreasing oxidative stress and improving mitochondrial function and thereby protecting the intestinal barrier in an *in viv*o experimental model of increased intestinal permeability due to high dietary fat consumption would be useful in confirming the observations of our *in vitro* study. Investigating genomics or proteomics of the intestinal epithelial cells in rodents pretreated with different polyphenols prior to consumption of a high fat diet will give insights about genes and proteins affected in a condition of intestinal permeability, such as measuring expression of other inflammatory cytokines, measuring inflammatory proteins via ELISA, as well as measuring activity of SIRT1, along with activity of mitochondrial respiratory complexes. Also, investigating the effect of dietary lipids on mitochondrial content, biogenesis, and function and discovering the importance of efficient and functional mitochondria in providing sufficient ATP in order to prevent the effects of a high fat diet on intestinal permeability and related disease such as insulin resistance in an *in vivo* model is a great future direction. In addition, performing research on specific effects of polyphenols on mitochondrial biogenesis as well as determining how polyphenols can increase intestinal epithelial mitochondrial efficiency with increasing duration of intake may provide valuable insight on how regular consumption of polyphenol antioxidants can help prevent chronic diseases via protecting the gut barrier. Comparing effects of the parent compound to its derivatives is important to distinguish the active species involved.

Other future directions could use different *in vitro* models. Due to the limitations of Caco-2 cell models, a co-cultivation of Caco-2 and HT-29MTX goblet cells could better resemble normal intestine and give better insights of what would happen in *in vivo* studies (Walter et al., 1996). In addition, investigating and evaluating effects of MM and polyphenols on polarized epithelial cells and comparing the results with pre-differentiated Caco-2 cells could provide more information on differences between human intestinal epithelial cells and *in vitro* cell lines. Since human intestinal epithelial cells are composed of both differentiated (villi) and undifferentiated cells (stem cells and progenitors), comparing effects of MM and polyphenols on fully differentiated cells, which rely

more on mitochondria compared to stem cells (Wanet et al., 2015; Ito and Suda, 2014), provides a more complete insight as to what might occur in human intestine. Effects of polyphenol pretreatment and co-treatment of polyphenols with lipid exposure to intestinal epithelial cells in *in vivo* or *in vitro* studies can be compared in order to determine the preventive or treatment effect of polyphenols. In addition, further elucidating and comparing the effect of individual anthocyanins as well as resveratrol, breakdown products and anthocyanins in whole food products on mitochondrial function and biogenesis would give insights about importance of consumption of polyphenols as supplements or food products.

In addition, as mentioned, anthocyanidins (the aglycone forms) were found to accumulate in mitochondria compared to other flavonoids (Peng, 2012). Further investigating the localized site (near the inner membrane), transport studies and providing means of entry of the unstable compound to enter mitochondria will provide great insights on how the aglycone form of anthocyanins induce their protective effect possibly, in part, through prevention of mitochondrial superoxide generation and intracellular ROS.

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CHAPTER 8 APPENDICES AND SUPPORTING DOCUMENTS

8.1. Appendix A-Details about ARBE

Appendix 8A.1. ARBE-Average composition

indena 20139 Milano - Viale Ortles, 12 - Tel. 02.57496.1 - Fax 02.57404620

BILBERRY (VACCINIUM MYRTILLUS) DRY EXTRACT

AVERAGE COMPOSITION

Anthocyanins	about	36	%
Anthocyanidins	about	0.2	%
Flavonols and Phenolic Acids	about	4	%
Monoglycosidic Flavonoids	about	2	%
Monomeric Proanthocyanidins	about	1.3	%
Dimeric Proantocyanidins	about	0.4	%
Polymeric Proanthocyanidins	about	10	%
Carbohydrates and Aliphatic Organic Alcohols	about	29	%
Water	about	3	%
Sulphated Ash	about	0.8	%
Residual Organic Solvents	about	0.1	%
Insoluble Residue in Water	about	7	%
Total	**about**	**94**	**%**

Data from Report IdB 17/86/LA (Dec. 17, 1986) and average Indena Production 2000 – 2008.

Dr Alberto Griffini
Customer Service & Technical Coordination Manager

Settala (MI), October 8, 2009

Manufacturer's Address:
INDENA S.p.A.
Via Don Minzoni, 6
20090 SETTALA (MI), Italy

Indena spa - Cap. sociale Euro 14.245.000 - Codice Fiscale e Registro delle Imprese di Milano n. 00712180165
Partita IVA n. 04411780150 - R.E.A. Milano n. 1048296 - C/C postale n. 13777206 - http://www.indena.com
Stabilimento: 20090 Settala (MI) - Via Don Minzoni, 6 - Tel. 02.95413.1 - Fax 02.95413.640
Direzione e coordinamento ex art. 2497 C.C. e segg.: IdB Holding spa

Appendix 8A.2. ARBE-Technical data Sheet

indena

Technical Data Sheet

PRODUCT NAME	**BILBERRY (VACCINIUM MYRTILLUS) ETHANOLIC EXTRACT 36%**
TRADENAME	**MIRTOSELECT®**
PRODUCT CODE	**9042202**

IDENTIFICATION AND COMPOSITION

CAS Number	Vaccinium myrtillus extract: 84082-34-8
EC Number	Vaccinium myrtillus extract: 281-983-5
Composition	Bilberry fresh frozen fruits dry extract, refined and standardized 100%
Excipients/Other Components	None
Antioxidants/Preservatives	None
Active material Assay	HPLC Assay of Anthocyanins >=36.0% HPLC Assay of Anthocyanidins <=1.0%
Appearance	Dark red-violet powder
Starting Herbal material	*Vaccinium myrtillus* L. - Ericaceae
English Common Name	Bilberry
Part of the plant	Fruit
Extraction solvent	Ethanol and Water
Drug to Extract Ratio (native)	80 – 130 : 1

PACKAGING, SHELF LIFE AND STORAGE

Internal	Food grade polyethylene bag closed with wrap-it tie
Intermediate	Thermosealed multilayer bag
External (for 5 Kg size)	Polypropylene pail
External (for 25 Kg size)	Kraft Type cardboard drum
Storage conditions	Store in well closed containers, protected from light-moisture-heat

* refer to MSDS section 7.

Shelf life	3 years

IONIZING RADIATION	This product and its ingredients are not irradiated / treated with ionizing radiation according to Dir. 1999/2/EC and 1999/3/EC and their amendments
NANOMATERIALS	This product does not contain nanomaterials according to Reg. (EC) 1169/2011 and its amendments
PACKAGING	The packaging in direct contact with the product complies with the provisions of Reg. (EC) 1935/2004
COUNTRY OF ORIGIN	ITALY (EU)

NUTRITIONAL INFORMATION

Energy Value	128 540		kcal/100 g kJ/100 g
Protein	0.98	(*)	g/100 g
Total Fat	1.08		g/100 g
• **Saturated Fatty Acids**		11.42%	
• **Monounsaturated Fatty Acids**		20.70%	
• **Polyunsaturated Fatty Acids**		67.88%	
Total Dietary Fiber	2.6		g/100 g
• **Soluble**	2.3		g/100 g
• **Insoluble**	0.3		g/100 g
Total Reducing Sugars, after inversion	28.2		g/100 g
Sugars Composition (HPLC)			
Glucose	13.88		g/100 g
Galactose	< 0.01		g/100 g
Fructose	13.07		g/100 g
Lactose	< 0.01		g/100 g
Saccharose (Sucrose)	0.040		g/100 g
Maltose	< 0.01		g/100 g
Maltotriose	< 0.01		g/100 g
Iron	0.96		mg/100 g
Phosphorous	29.6		mg/100 g
Magnesium	44.9		mg/100 g
Potassium	261		mg/100 g
Sodium	11.2		mg/100 g
Zinc	0.428		mg/100 g
Moisture	2.33		g/100 g
Ash	0.82		g/100 g

(*): N * 6.25

Ref. 09/000427809

The above information must be considered indicative due to the natural variation in the composition of the starting herbal material.
The above information is given in accordance with Regulation (EC) 1169/2011 on food information to consumers.
The energy values may differ per region because of different calculations.

8.2. Appendix B. Development of the model and experimental conditions

Appendix 8B.1. Determining percent of FBS in DMEM

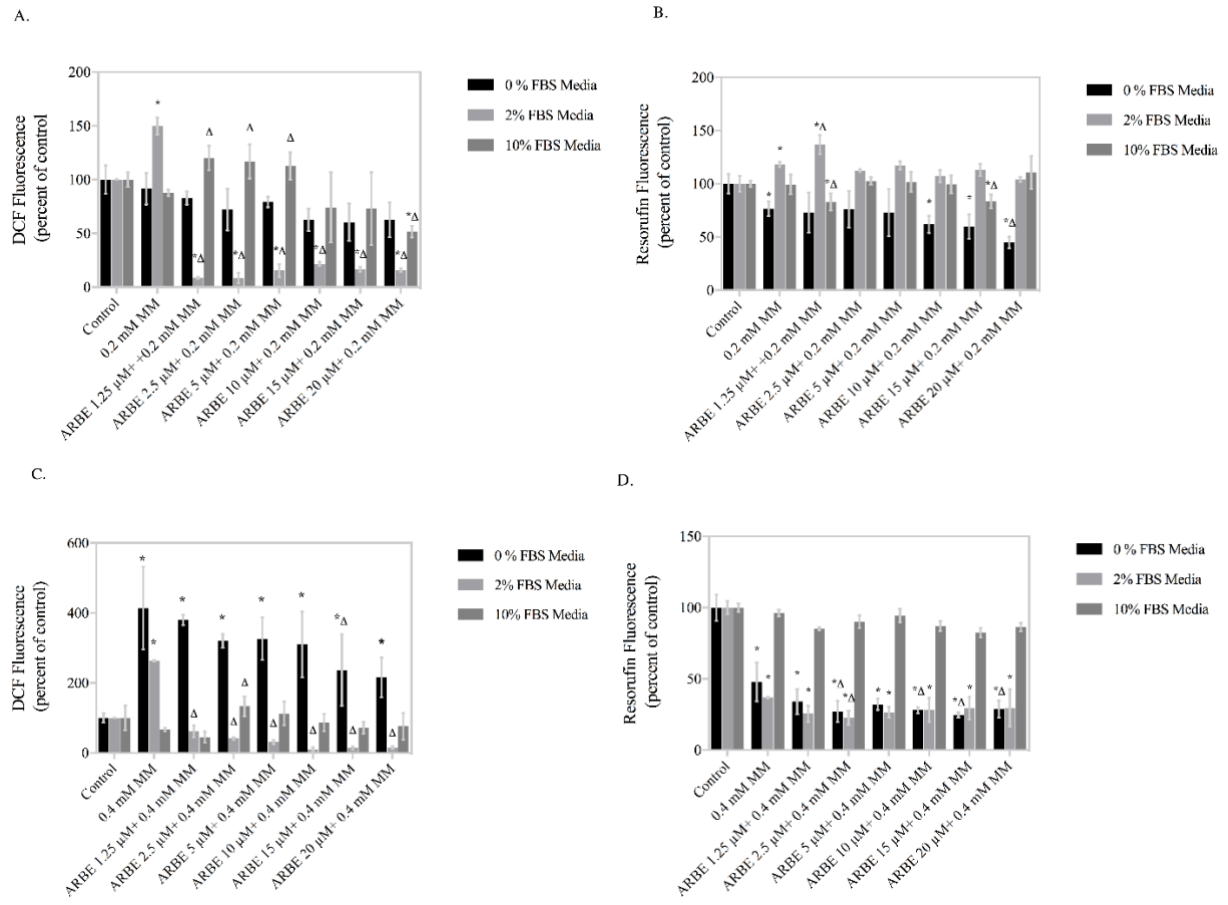


Figure 8.B.1. *Effects of MM and ARBE on intracellular ROS production and cell viability.* After growing to confluence for 24 h, Caco-2 cells were treated with 1.25-20 µM ARBE for 2 h prior to adding MM and incubating for an additional 24 h. After treatments, cells were incubated with DCFH-DA or resazurin for 2 h. **(A)** ROS generation measured via DCF fluorescence using a microplate reader after pretreatment with ARBE followed by challenge with 0.2 mM MM. **(B)** Cell viability in Caco-2 cells after pre-treatment with ARBE for 2 h followed by challenge with 0.2 mM MM for 24 h measured via quantifying resorufin fluorescence using a microplate reader. **(C)** ROS generation measured via DCF fluorescence after pretreatment with ARBE and 0.4 mM MM challenge. **(D)** Cell viability measured after ARBE pretreatment and 0.4 mM MM challenge via microplate reader. Values represent means ± SD of two independent experiments with three

replicate wells. * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). The significant difference between groups was tested with one-way ANOVA followed by Tukey's post hoc test.

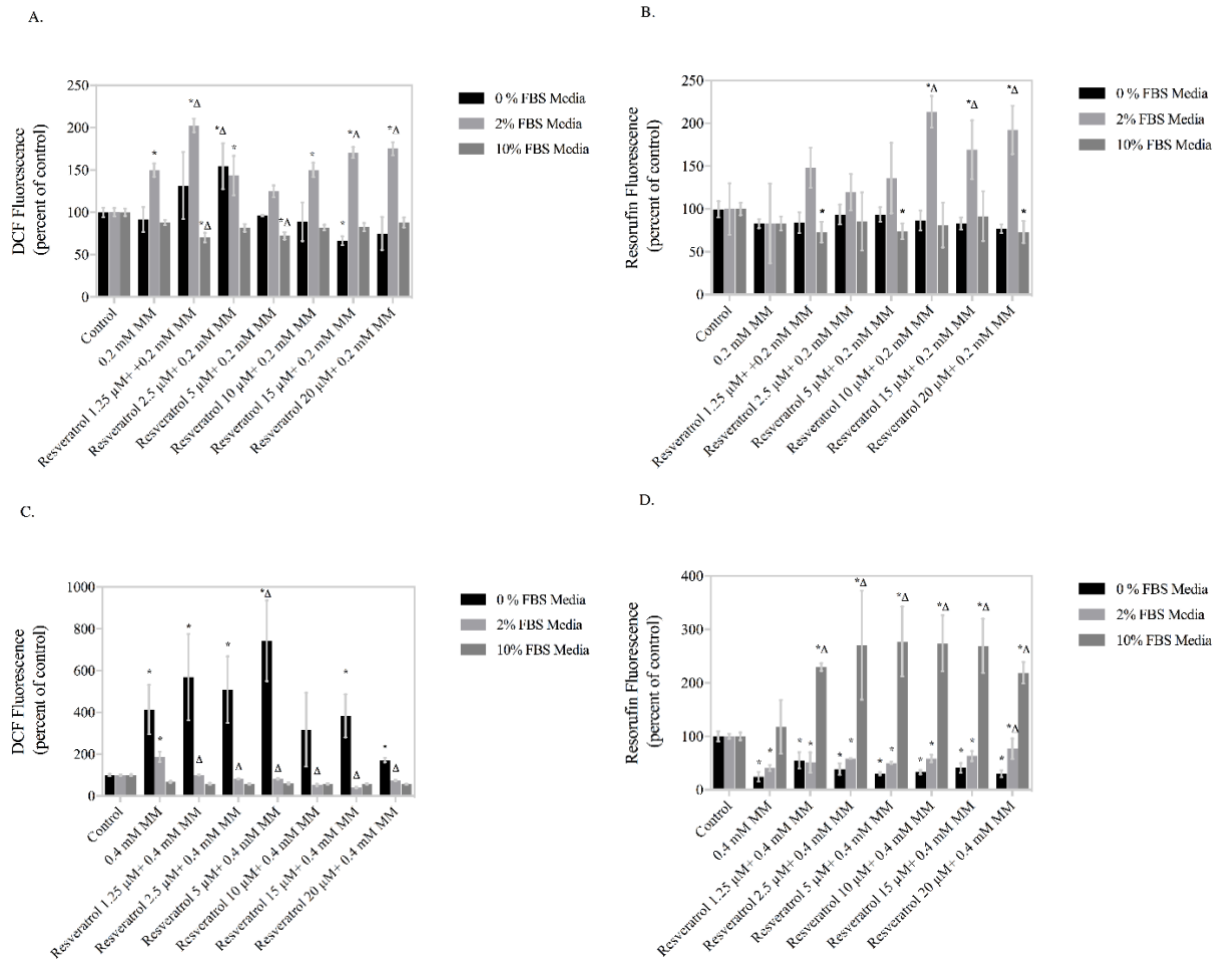


Figure 8.B.2. *Effects of MM and resveratrol on intracellular ROS production and cell viability.* Caco-2 cells were treated with 1.25-20 µM resveratrol for 2 h prior to adding MM and incubating for 24 h. After treatments, cells were then incubated with DCFH-DA or resazurin for 2 h. **(A)** ROS generation measured via DCF fluorescence using a microplate reader after pretreatment with resveratrol followed by 0.2 mM MM treatment. **(B)** Cell viability in Caco-2 cells after pre-treatment with resveratrol for 2 h followed by treatment with 0.2 mM MM for 24 h measured by quantifying resorufin fluorescence using a microplate reader. **(C)** ROS generation measured via DCF fluorescence using a microplate reader after pretreatment with resveratrol and 0.4 mM MM exposure. **(D)** Cell viability measured after resveratrol pretreatment and 0.4 mM MM treatment

via microplate reader. Values represent means ± SD of two independent experiments with three replicate wells. * Mean value significantly different from control group (P <0.05). $^{\Delta}$ Mean value significantly different from MM group (P <0.05). The significant difference between groups was tested with one-way ANOVA followed by Tukey's post hoc test.

Appendix 8B.2. Determining dose of MM

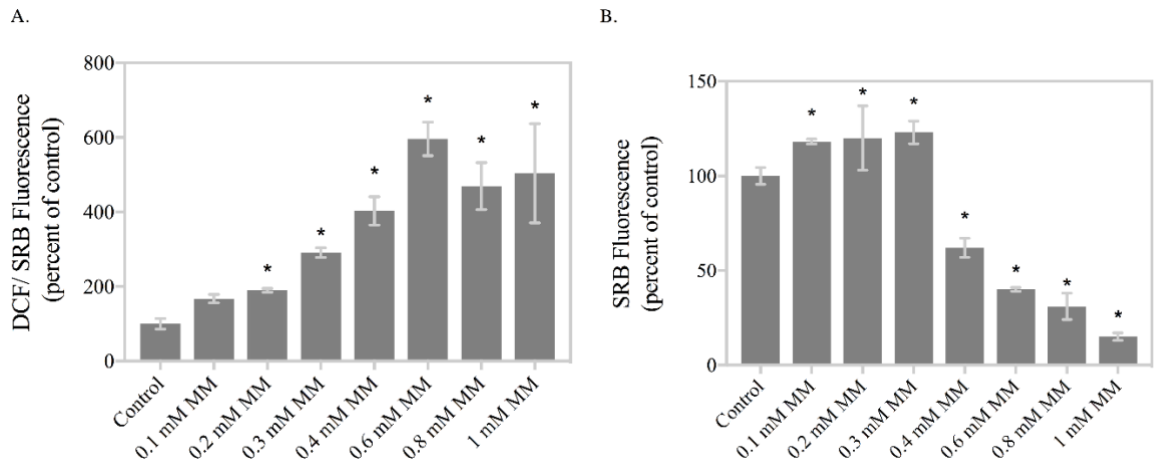


Figure 8.B.3. *Effects of doses of MM on intracellular ROS production and cell viability.* Caco-2 cells were treated with 0.1-1 mM MM for 24 h in 2% FBS media. After treatments, cells were incubated with DCFH-DA or resazurin for 2 h. **(A)** ROS generation measured via DCF fluorescence using a microplate reader after treatment with doses MM. **(B)** Cell viability in Caco-2 cells after treatment with MM measured by quantifying resorufin fluorescence using a microplate reader. Values represent means ± SD, four biological replicates with 3 technical replicates per experiment. * Mean value significantly different from control group (P <0.05). The significant difference between groups was tested with one-way ANOVA followed by Tukey's post hoc test.

Appendix 8B.3. Determining pretreatment duration of ARBE

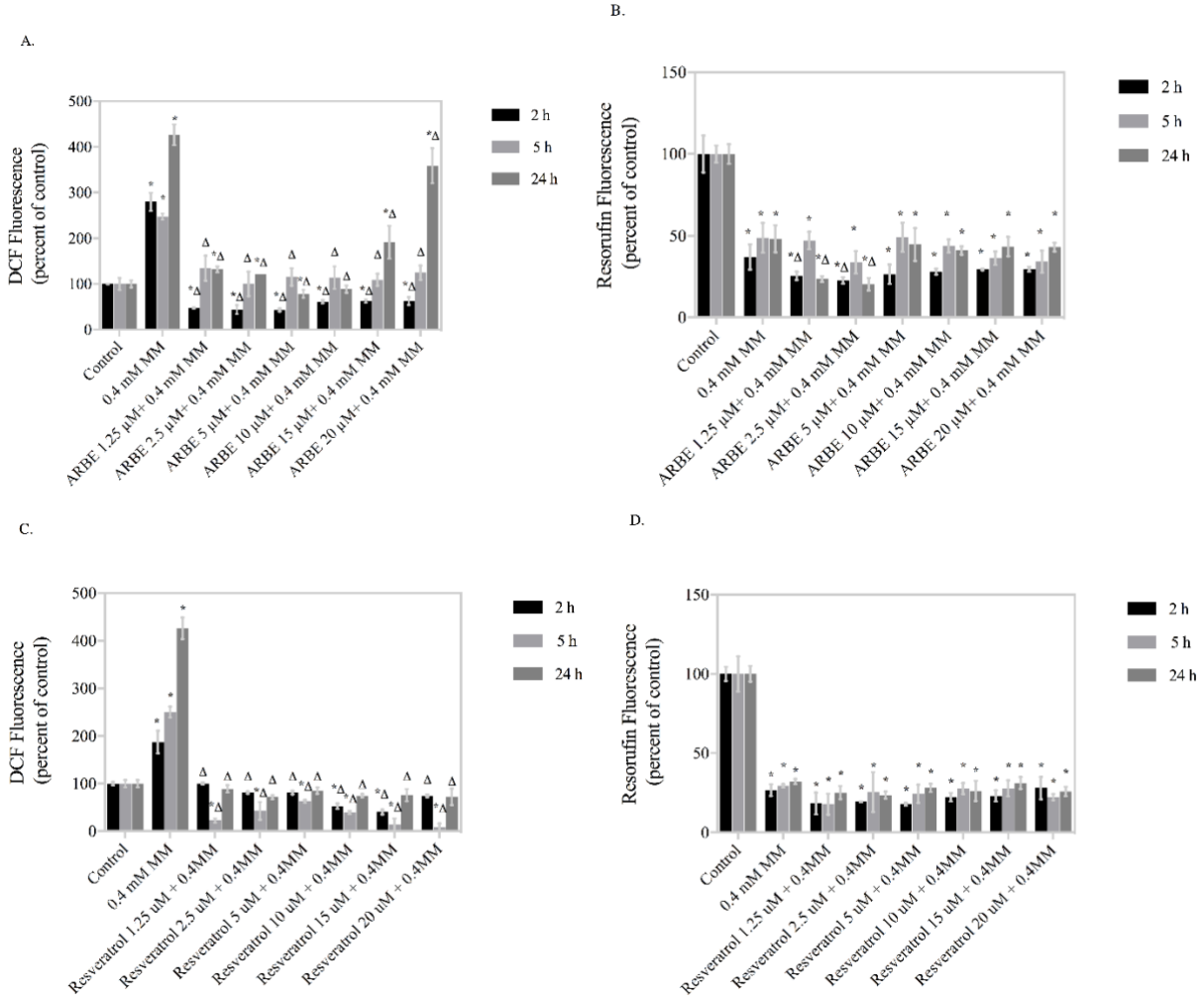
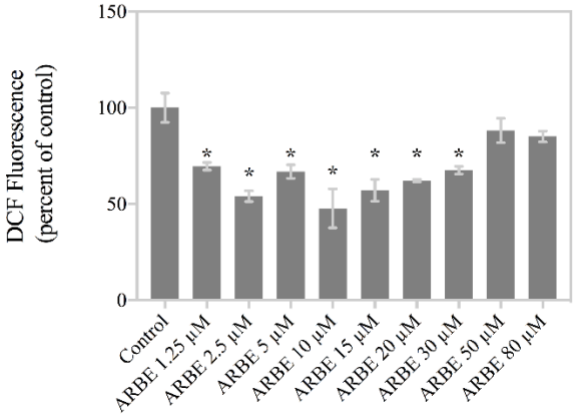


Figure 8.B.4. *Effects of different duration of ARBE and resveratrol pretreatment before MM exposure on intracellular ROS production and cell viability.* Caco-2 cells were treated with 1.25-20 µM ARBE or resveratrol for 2, 5 or 24 h prior to adding mM MM and incubating for 24 h. After treatments, cells were then incubated with DCFH-DA or resazurin for 2 h. **(A)** ROS generation measured via DCF fluorescence using a microplate reader after different duration of pretreatment with ARBE followed by 0.4 mM MM treatment. **(B)** Cell viability in Caco-2 cells after pre-treatment with ARBE for 2, 5 or 24 h period followed by treatment with 0.4 mM MM for 24 h measured by quantifying resorufin fluorescence using a microplate reader. **(C)** ROS generation measured via DCF fluorescence using a microplate reader after different duration pretreatment with resveratrol before 24 h exposure with 0.4 mM MM. **(D)** Cell viability measured

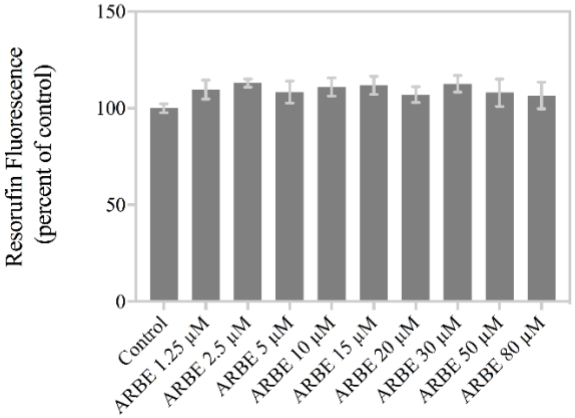
after resveratrol pretreatment for 2, 5 or 24 h then 0.4 mM MM treatment via microplate reader. Values represent means ± SD of two independent experiments with three replicate wells. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant difference between groups was tested with one-way ANOVA followed by Tukey's post hoc test.

Appendix 8B.4. Determining pretreatment duration of Resveratrol

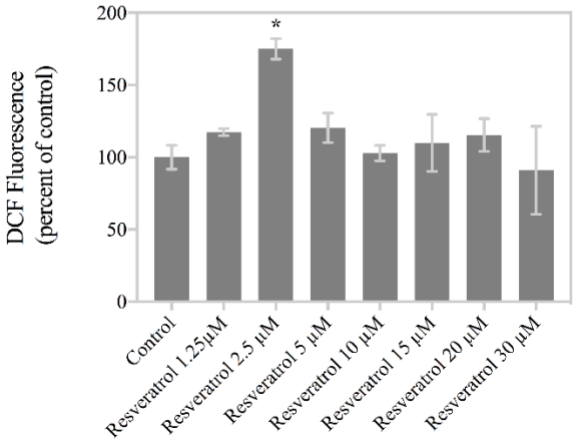
A.


DCF Fluorescence (percent of control)
150
100
50
0
Control
ARBE 1.25 µM
ARBE 2.5 µM
ARBE 5 µM
ARBE 10 µM
ARBE 15 µM
ARBE 20 µM
ARBE 30 µM
ARBE 50 µM
ARBE 80 µM

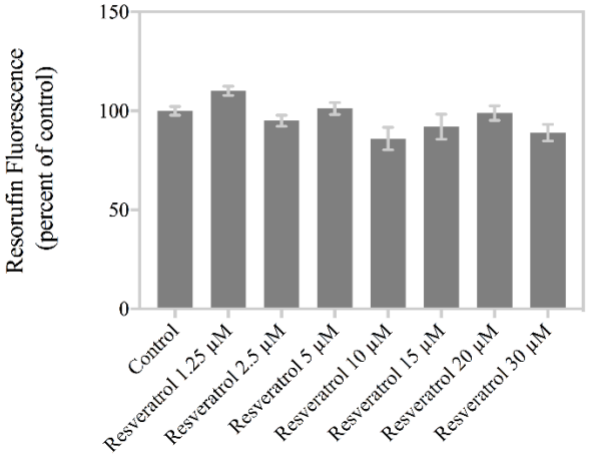
B.


Resorufin Fluorescence (percent of control)
150
100
50
0
Control
ARBE 1.25 µM
ARBE 2.5 µM
ARBE 5 µM
ARBE 10 µM
ARBE 15 µM
ARBE 20 µM
ARBE 30 µM
ARBE 50 µM
ARBE 80 µM

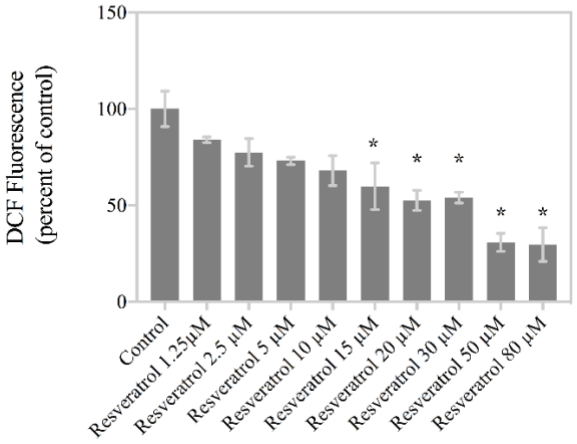
C.


DCF Fluorescence (percent of control)
200
150
100
50
0
Control
Resveratrol 1.25µM
Resveratrol 2.5 µM
Resveratrol 5 µM
Resveratrol 10 µM
Resveratrol 15 µM
Resveratrol 20 µM
Resveratrol 30 µM

D.


Resorufin Fluorescence (percent of control)
150
100
50
0
Control
Resveratrol 1.25 µM
Resveratrol 2.5 µM
Resveratrol 5 µM
Resveratrol 10 µM
Resveratrol 15 µM
Resveratrol 20 µM
Resveratrol 30 µM

E.


DCF Fluorescence (percent of control)
150
100
50
0
Control
Resveratrol 1.25µM
Resveratrol 2.5 µM
Resveratrol 5 µM
Resveratrol 10 µM
Resveratrol 15 µM
Resveratrol 20 µM
Resveratrol 30 µM
Resveratrol 50 µM
Resveratrol 80 µM

F.

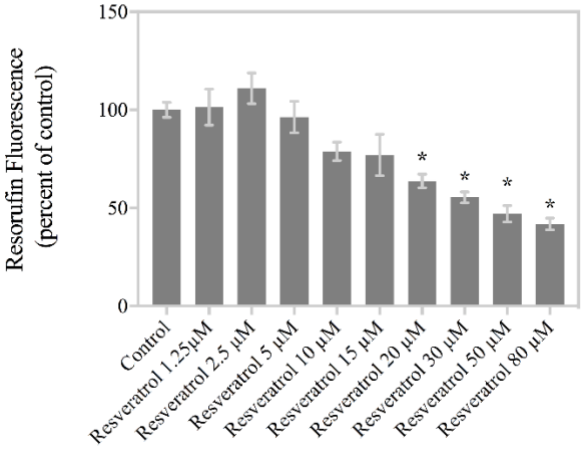

Resorufin Fluorescence (percent of control)
150
100
50
0
Control
Resveratrol 1.25µM
Resveratrol 2.5 µM
Resveratrol 5 µM
Resveratrol 10 µM
Resveratrol 15 µM
Resveratrol 20 µM
Resveratrol 30 µM
Resveratrol 50 µM
Resveratrol 80 µM

Figure 8.B.5. *Effects of doses of ARBE and resveratrol on intracellular ROS production and cell viability.* Caco-2 cells were treated with 1.25-80 µM ARBE for 26 h or resveratrol for 30 or 48 h in 2% FBS media. After treatments, cells were incubated with DCFH-DA or resazurin for 2 h. **(A)** ROS generation measured via DCF fluorescence using a microplate reader after treatment with different doses of ARBE. **(B)** Cell viability in Caco-2 cells after treatment with ARBE measured by quantifying resorufin fluorescence using a microplate reader. **(C)** ROS generation after treatment with 1.25-30 µM resveratrol for 30 h. **(D)** Cell viability measured after resveratrol treatment for 30 h via microplate reader. **(E)** ROS generation after treatment with 1.25-80 µM resveratrol for 48 h. **(F)** Cell viability measured after resveratrol treatment for 48 h via microplate reader. Values represent means ± SD of two independent experiments with three replicate wells. * Mean value significantly different from control group ($P < 0.05$). The significant difference between groups was tested with one-way ANOVA followed by Tukey's post hoc test.

Appendix 8B.5. Determining dose of MM for TEER experiment

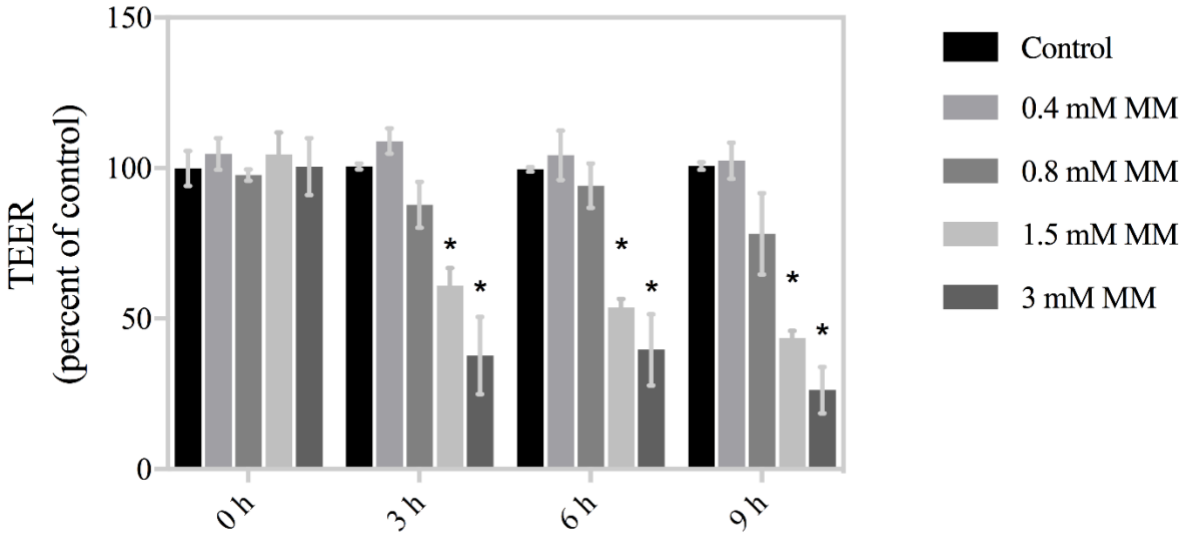
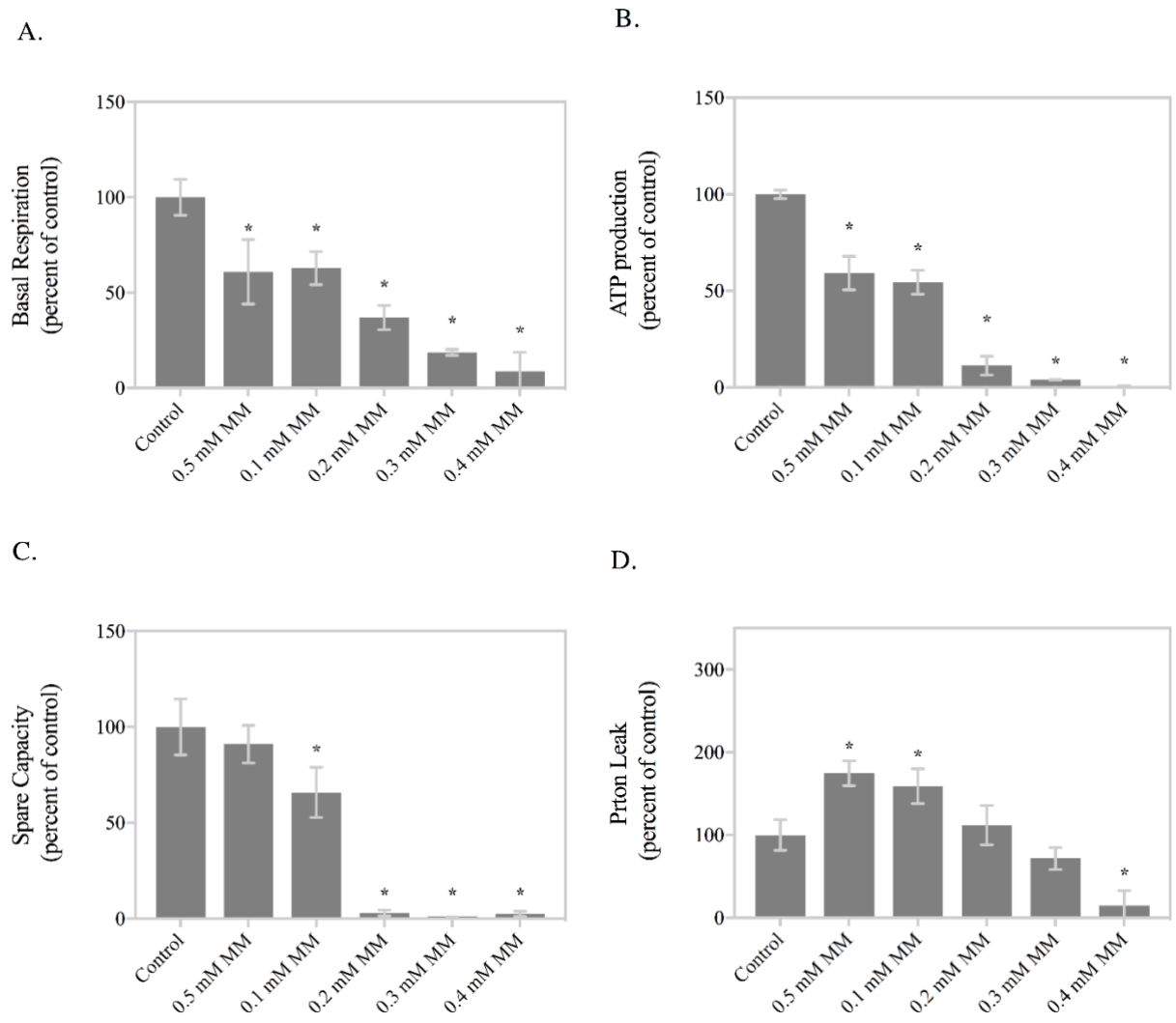


Figure 8.B.6. *Effects of doses of MM on transepithelial electrical resistance of a differentiated Caco-2 cell monolayer*. Caco-2 cells grown on a membrane for 21 days were treated with different doses of MM for 9 h. Measurements were done every 1.5 h after addition of MM. Bars represent means ± SD of 2 independent experiments with 3 treatment wells in each experiment * Mean value significantly different from the control group ($P < 0.05$). The significant difference between groups were tested with one-way ANOVA followed by Tukey's multiple comparisons post hoc test.

Appendix 8B.6. Mitochondrial function



E.

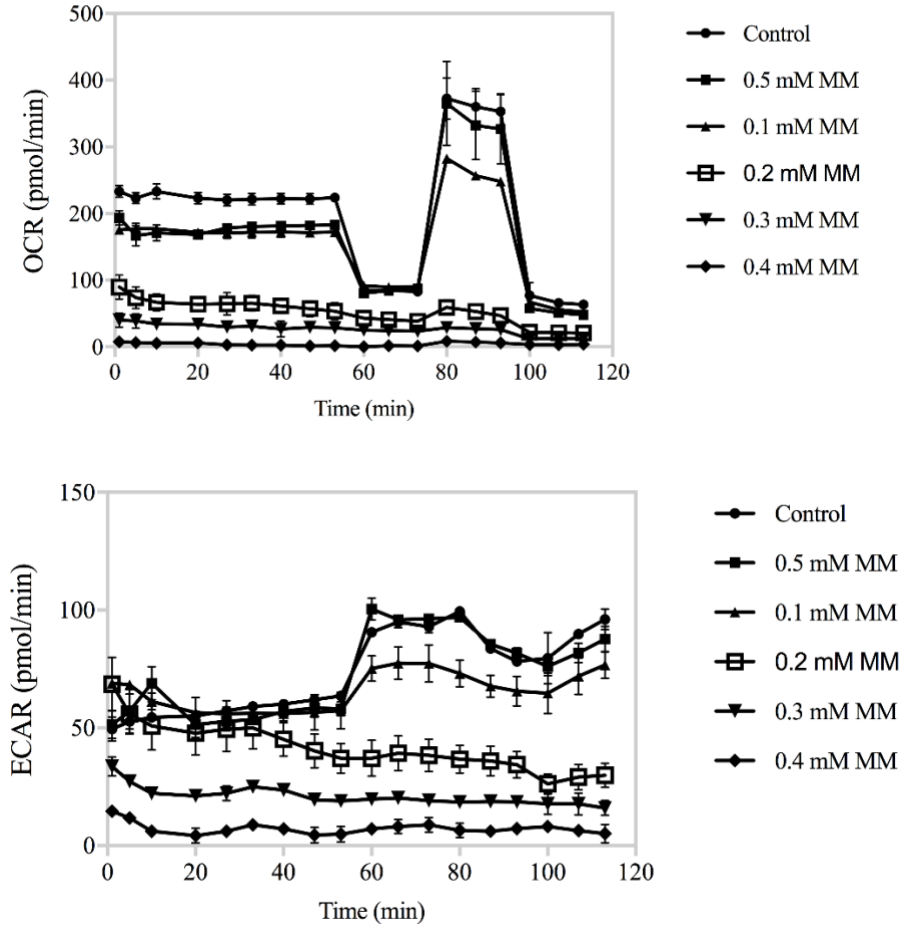


Figure 8.B.7. *Effects of doses of MM on mitochondrial function.* Caco-2 cells grown in Seahorse plates were treated with different doses of MM for 24 h. Oxygen consumption rate (OCR) was then measured for 3 h with additions of respiratory agents at selected time points. **(A)** Basal respiration **(B)** ATP production **(C)** Spare respiratory capacity and **(D)** proton leak. **(E)** OCR and **(F)** ECAR measured using the Seahorse XFe96 Extracellular Flux Analyzer and oligomycin (1 μM), FCCP (1 μM) and A/R (0.5 μM) injected sequentially as shown. Data represent means ± SD of 4 independent experiments with 3 replicate wells in each experiment. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant difference between groups were with one-way ANOVA followed by Tukey's multiple comparisons test.

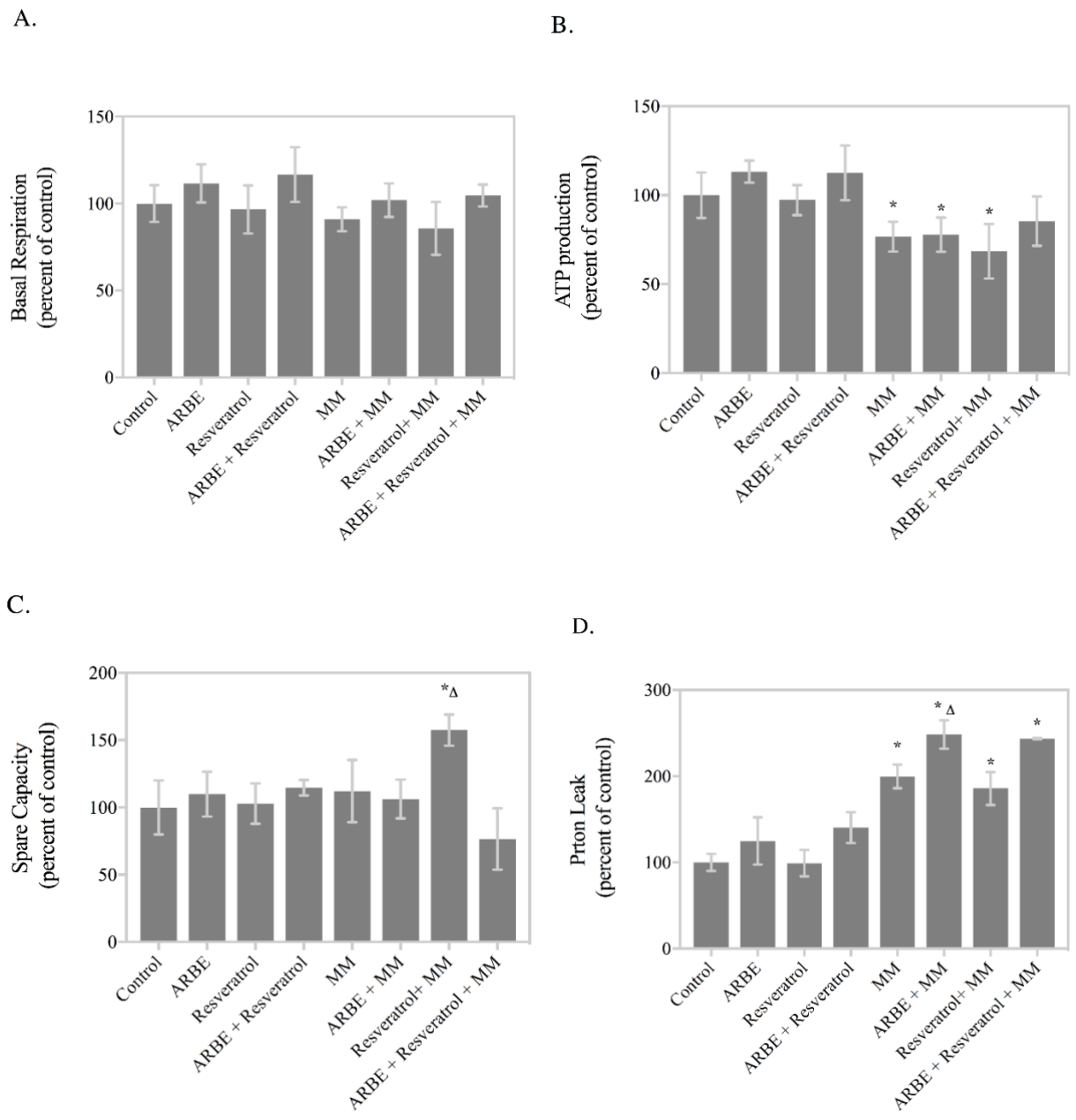


Figure 8.B.8. *Effects of MM and 12 h pretreatment with ARBE and resveratrol on mitochondrial function.* Caco-2 cells grown in Seahorse plates were treated with 20 µM ARBE or 20 µM resveratrol for 12 h prior to adding 0.1 mM MM and incubating for 24 h. Oxygen consumption rate (OCR) was then measured for 3 h with additions of respiratory agents at selected time points. **(A)** Basal respiration, (P_{MM} =0.0065, $P_{Polyphenol}$ =0.0004, $P_{Interaction}$ =0.9935). **(B)** ATP production (P_{MM} <0.0001, $P_{Polyphenol}$ =0.0024, $P_{Interaction}$ =0.4776), **(C)** Spare Respiratory capacity (P_{MM} 0.2879, $P_{Polyphenol}$ =0.0013, $P_{Interaction}$ <0.0001) and **(D)** proton leak (P_{MM} <0.0001, $P_{Polyphenol}$

<0.0001, $P_{Interaction}$ =0.0496). Data represent means ± SD of 2 independent experiments with 4 replicate wells in each experiment. * Mean value significantly different from control group (p <0.05). $^{\Delta}$ Mean value significantly different from MM group (p <0.05). The significant difference between groups were with two-way ANOVA followed by Tukey's multiple comparisons test.

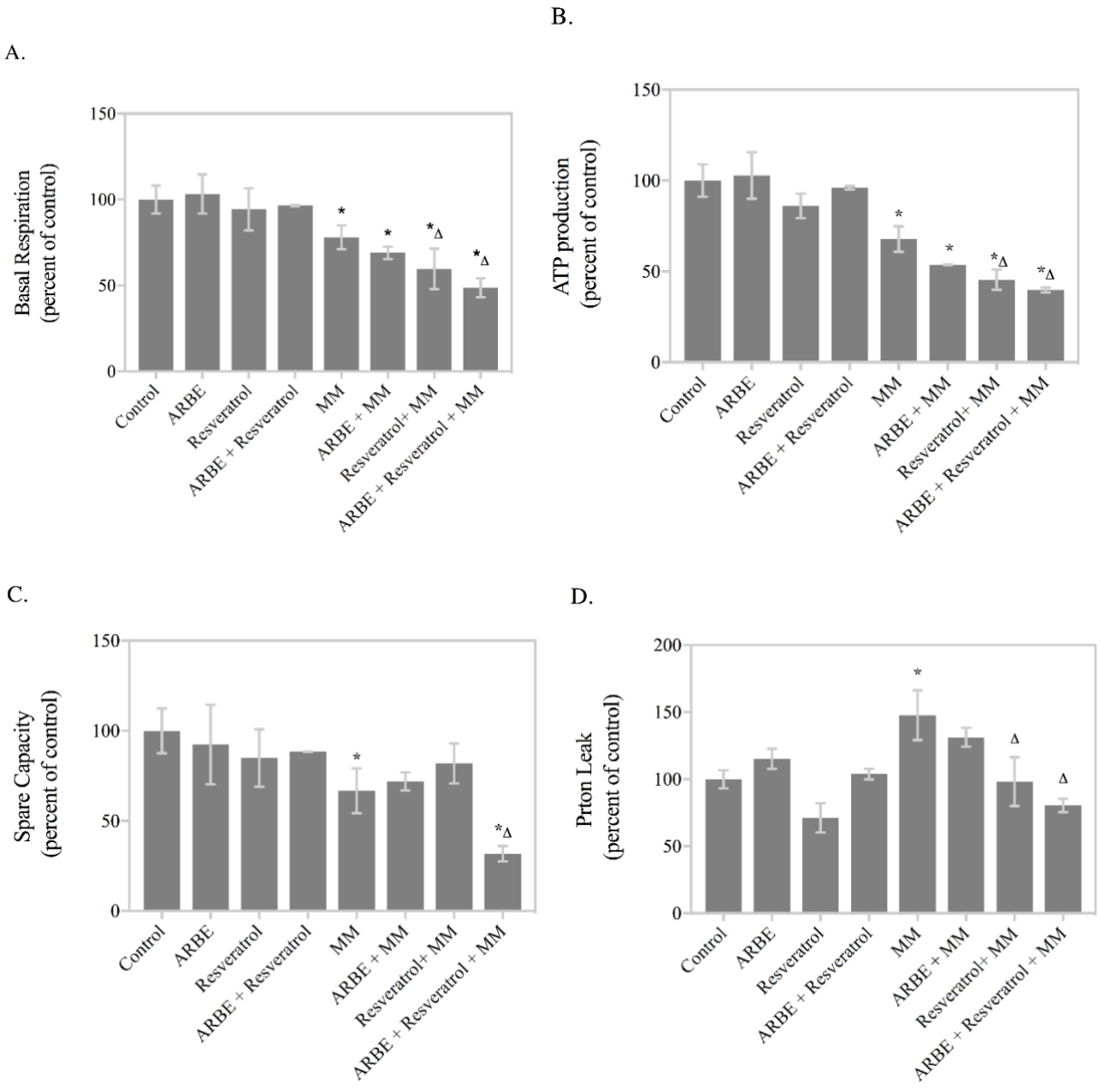


Figure 8.B.9. *Effects of MM and 24 h pretreatment with ARBE and resveratrol on mitochondrial function.* Caco-2 cells grown in Seahorse plates were treated with 20 µM ARBE or 20 µM resveratrol for 24 h prior to adding 0.1 mM MM and incubating for 24 h. Oxygen consumption rate (OCR) was then measured for 3 h with additions of respiratory agents at selected time points. **(A)** Basal respiration, ($P_{MM} < 0.0001$, $P_{Polyphenol} = 0.0033$, $P_{Interaction} = 0.0711$). **(B)** ATP production ($P_{MM} < 0.0001$, $P_{Polyphenol} = 0.0002$, $P_{Interaction} = 0.0557$) **(C)** Spare Respiratory capacity ($P_{MM} = 0.0486$, $P_{Polyphenol} < 0.0001$, $P_{Interaction} = 0.0200$) and **(D)** proton leak ($P_{MM} = 0.0487$, $P_{Polyphenol} < 0.0001$, $P_{Interaction} = 0.0068$). Data represent means ± SD of 2 independent experiments with 4 replicate wells in each experiment. * Mean value significantly different from control group ($P < 0.05$). $^{\Delta}$ Mean value significantly different from MM group ($P < 0.05$). The significant difference between groups were with two-way ANOVA followed by Tukey's multiple comparisons test.

8.3. Appendix C. Permission for Seahorse graph

Agilent Technologies, Inc.
Intellectual Property Group
5301 Stevens Creek Blvd., MS1A-PB
Santa Clara, California 95051

303 588 7502 telephone
janet.hajek@agilent.com
www.agilent.com

Janet Shih Hajek
Corporate Counsel

May 25, 2018

Via email to: maryamershad@yahoo.com

Maryam Ershad Langroodi
College of Pharmacy and Nutrition
University of Saskatchewan
110 Science Place
Saskatoon, SK S7N 5CP
Canada

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Janet Shih Hajek
Corporate Counsel
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