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Mitochondrial mechanisms in benzo[a]pyrene-induced carcinogenesis and chemoprevention by polyphenols

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By

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ABSTRACT

Naturally occurring polyphenols in fruits and vegetables have been shown to exhibit anticancer characteristics. Although some roles of polyphenols in cancer prevention have been previously described, an involvement of mitochondrial mechanisms has not been well-studied. Also, while mitochondrial dysfunction has been identified in several cancer cells and is correlated with poor prognosis, less is known about the involvement of mitochondrial changes in carcinogenesis and neoplastic transformation. In an *in vitro* model of cancer initiation and promotion using Bhas 42 fibroblasts, we investigated the involvement of mitochondrial changes induced by benzo[a]pyrene (B[a]P) and possible roles of different polyphenols in preventing carcinogenesis and neoplastic transformation, through inhibiting oxidative stress, inducing mitochondrial biogenesis, and ameliorating mitochondrial dysfunction.

Bhas 42 mouse fibroblast cells were pre-treated with 5 μM polyphenols (resveratrol, quercetin, catechin, cyanidin, cyanidin-3-glucoside (C3G), and berberine) for 2h for most experiments followed by treatment with 4 μM B[a]P for 12h, 24h and 72h. Different experiments including measuring intracellular reactive oxygen species (ROS), mitochondrial superoxide, gene expression, mitochondrial content, and neoplastic transformation were conducted.

B[a]P induced oxidative stress by increasing intracellular ROS and mitochondrial superoxide generation, as well as induced UCP2 expression compared to untreated cells. Most of the polyphenols prevented these effects; however, only anthocyanins (cyanidin and C3G) and berberine decreased B[a]P-induced mitochondrial superoxide generation. B[a]P induced neoplastic transformation almost 5-fold while resveratrol and quercetin inhibited this effect and resveratrol had the strongest effect, inhibiting by 75%. B[a]P also decreased mitochondrial content, as well as decreased SIRT1 activity, ERRα expression, and expression of some mitochondrial respiratory subunits (NDUFS8, ATP5A1, and CYB). All polyphenols increased at least one of these factors with different effectiveness. B[a]P exposure also produced mitochondrial dysfunction, decreased mitochondrial membrane potential (MMP) and ATP content by 25% and 28%, respectively, while some polyphenols such as resveratrol and quercetin completely prevented B[a]P-induced mitochondrial dysfunction. The increased mitochondrial biogenesis by resveratrol corresponded with decreased ROS generation and can be suggested as a plausible mechanism by

which resveratrol inhibited B[a]P-induced neoplastic transformation more strongly than other studied polyphenols.

The study showed that B[a]P impaired mitochondrial biogenesis and induced mitochondrial dysfunction, oxidative stress, and neoplastic transformation, whereas different polyphenols protected against these effects, with resveratrol showing the most robust effects. The results shed new light into mitochondrial mechanisms by which polyphenols may prevent cancer initiation and progression.

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DEDICATION

This dissertation is dedicated to my husband Hossein, for all love, encouragements, and supports he has given me. This work is also dedicated to my kind and supportive parents whom without them I would not be able to accomplish this milestone. I am also dedicating this work to the beloved new member of our family, Ryan, who has fulfilled our life with joy, happiness, and love.

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LIST OF ABBREVIATIONS

8-OH-G	8-Hydroxyguanine
AhR	Aryl hydrocarbon receptor
AIF	Apoptosis inducing factor
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
AP-1	Activating potein-1
ARE	Antioxidant response element
B[a]P	Benzo[a]pyrene
BPDE	Benzo[a]pyrene 7,8 diol 9,10 epoxide
COX	Cyclooxygenases
COX2	Cyclooxygenase-2
C3G	Cyanidin-3-glucoside
CYP450	Cytochrome P450 drug metabolizing enzymes
DISC	Death-inducing signaling complex
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
ERRα	Estrogen-related receptor α
ETC	Electron transfer chain
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1α
HK2	Hexokinase2
HNE	4-hydroxy-2-nonenal
IAP	Inhibitors of apoptosis proteins
IARC	International Agency for Research on Cancer
ICDH	Isocitrate dehydrogenase
iNOS	Inducible nitric oxide synthase
JCRB	Japanese Collection of Research Bioresources Cell Bank
Keap 1	Kelch-like-ECH-associated protein 1
MAO	Mitochondrial bound monoamine oxidase
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MMP	Mitochondrial membrane potential
MPT	Mitochondrial permeability transition
MTC	Monocarboxylic transporters
mtDNA	Mitochondrial DNA
MTG	MitoTracker Green

nDNA	Nuclear DNA
NRF1	Nuclear respiratory factor-1
NRF2	Nuclear respiratory factor-2
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PAH	Polycyclic aromatic hydrocarbons
PDK	Pyruvate dehydrogenase kinase
PGC-1α	PPARγ coactivator-1α
PI3K	Phosphoinositide 3-kinase
PRC	PGC-related coactivator
ROS	Reactive oxygen species
SGLT1	Sodium-dependant glucose transporter 1
SOD	Superoxide dismutase
SRB	Sulforhodamine B
STAT3	Signal transducer and activator of transcription 3
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor a
TMRE	Tetramethylrhodamine ethyl ester, perchlorate
TRAIL	TNF-related apoptosis inducing ligands
UCP2	Uncoupling protein 2
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor

CHAPTER 1: LITERATURE REVIEW

1.1. Background

Cancer is one of the most common chronic diseases worldwide. Although current therapies such as chemotherapy, radiation, surgery, and immune therapy contribute to decreased cancer mortality, still cancer is still considered as the second fatal disease after heart disease in people less than 85 years (Yao et al., 2011). Canadian cancer statistics reported that 206,200 Canadians would be diagnosed with cancer in 2017 and 80,800 people would die of cancer in 2016 (Nuttall et al., 2017).

Although current chemotherapy drugs are successful for cancer treatment, they usually are accompanied with adverse side effects. Moreover, due to the high expenses of cancer treatment for society and the mental stress of cancer disease on patients and their families, cancer prevention has attracted the interest of researchers for many years. Using medicines, vitamins, and other agents to prevent or delay the process of carcinogenesis is called chemoprevention. Chemopreventive agents may protect against carcinogenesis by several mechanisms including inhibiting carcinogen activation/mutagenesis, oxidative stress and inflammation. Chemotherapeutic agents may also inhibit cancer progression by inhibiting cell growth and cell proliferation or inducing apoptosis. Polyphenols have been suggested to be chemopreventive/chemotherapeutic agents in the last two decades and have shown promising results against carcinogenesis. In the current review, first the hallmarks of cancer and mechanisms involved in carcinogenesis are discussed and then it is narrowed to the involvement of mitochondrial dysfunction including role of oxidative stress and mitochondrial biogenesis in cancer development. A major question addressed in this thesis is whether a carcinogen such as benzo[a]pyrene (B[a]P) produces neoplastic transformation through inducing mitochondrial dysfunction and resultant oxidative stress. The second major question is the extent to which different polyphenols can inhibit neoplastic transformation through mitochondrial mechanisms.

1.2. The hallmarks of cancer

To dictate malignant transformations, cells need to acquire 10 essential capabilities which are common among all types of human tumors: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless cell proliferation, sustained angiogenesis,

metastasis, inflammation, dysregulation of energy metabolism, genome instability and mutations, and evading immune destruction (Hanahan and Weinberg, 2011). These ten hallmarks make a comprehensible image of neoplastic transformation and tumor cell functions.

However, there are other mechanisms including mitochondrial dysfunction that can be involved in transformation as well as tumor survival through ROS production and cell signaling pathways. Although oxidative stress due to mitochondrial dysfunction is not one of the cancer hallmarks, it has been identified in many cancer cells and can contribute to tumorigenesis through inducing or involving other hallmarks such as evasion of apoptosis, limitless cell proliferation, metastasis, dysregulation of energy metabolism, inflammation, and mutations (Ward and Thompson, 2012). Due to these major roles of mitochondria in cancer development, the focus of this review is the underlying mitochondrial mechanisms in carcinogenesis and neoplastic transformation. To review mitochondrial mechanisms involved in cancer development we first explain the three stages of carcinogenesis, initiation, promotion, and progression.

1.3. Cancer initiation, promotion and progression

Carcinogenesis is a multi-stage process divided into three stages, initiation, promotion, and progression (Pitot, 1993; Weston and Harris, 2003). The first stage of carcinogenesis is triggered with one or two mutations leading to irreversible genetic damage. Formation of DNA adducts by carcinogens, which may cause oncogene activation or tumor-suppressor gene inactivation, can be a cancer initiator. The promotion stage is a reversible step with additional adaptive changes and clonal expansion. This stage involves proliferation of the cells with mutations from the initiation stage that increases risk of further gene mutations and malignant conversion. The progression stage comprises transformation of preneoplastic cells to the neoplastic and malignant phenotype. This irreversible stage requires further genetic mutations and cell proliferation. In this stage, malignant cells acquire characteristics over time to become aggressive and metastatic. The main characteristic of the progression stage is uncontrolled cell growth and proliferation as well as inclination to the genome instability. To convert premalignant cells to malignant cells, the frequent exposure to the carcinogen is more significant than dose of exposure (Weston and Harris, 2003).

Mitochondrial dysfunction has been shown in a variety of cancers and can be involved in all three stages of carcinogenesis (Higuchi, 2012; Vyas et al., 2016). In the next sections, we discuss roles

of mitochondria in cancer development through different mechanisms such as oxidative stress and energy metabolism.

1.4. Mitochondrial dysfunction and carcinogenesis

Mitochondria are considered as the powerhouse of cells and are responsible for the synthesis of most a cell's required ATP. Mitochondria are involved in several metabolic functions including ATP production by oxidative phosphorylation and metabolism of carbohydrates, amino acids, and lipids (Modica-Napolitano and Singh, 2004). On average, cells contain several hundred mitochondria; however, some cells that have a larger demand for ATP such as nerve cells and muscle cells may contain thousands of mitochondria (Cole, 2016).

Mitochondria play important roles in cancer cells through multiple mechanisms such as ROS generation, resistance to apoptosis, altered energy metabolism, and mtDNA mutations (Księżakowska-Łakoma et al., 2014). Mitochondrial changes can occur at any of the stages of carcinogenesis and neoplastic transformation (Vyas et al., 2016). As previously hypothesized, mitochondrial genome mutations by carcinogens may increase ROS generation and tumorigenesis (Bandy and Davison, 1990). ROS generation causes more mutations and can activate cell signalling pathways that contribute to inflammation and cell proliferation. Mechanisms by which mitochondria may develop carcinogenesis such as mutations in the mitochondrial genome, impaired energy metabolism, and oxidative stress are reviewed in the following sections.

1.4.1 Mitochondrial mutations

Several types of cancers express mutations in mtDNA genes (Chattopadhyay et al., 2016; Cruz-Bermúdez et al., 2015; de Araujo et al., 2015). mtDNA is more susceptible to mutations than nuclear DNA due to its proximity to the ETC, which is the main source of ROS, poor DNA repair systems, and lack of histones which protect DNA from impairment (Gogvadze et al., 2008). mtDNA mutations lead to oxidative phosphorylation (OXPHOS) dysfunction and play a crucial role in carcinogenesis. In cancer cells, mtDNA mutations were shown to occur in mitochondrial-encoded respiratory subunits of complexes including complex I (7 mitochondrial-encoded subunits), complex III (1 mitochondrial-encoded subunit), complex IV (3 mitochondrial-encoded subunits), and complex V (2 mitochondrial-encoded subunits) (Tan et al., 2014). Moreover,

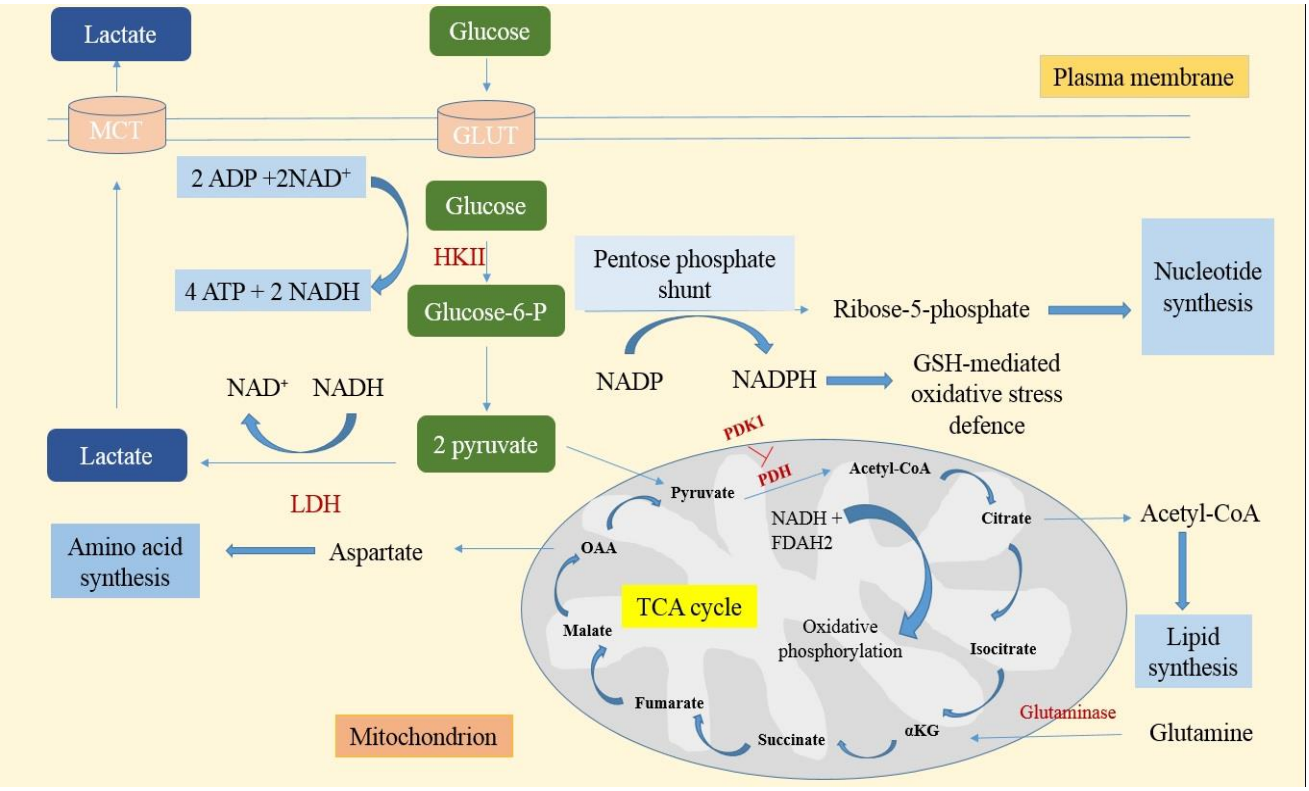
mutations in mitochondrial genes encoding 2 rRNA and 22 tRNA also have effects on the functioning of the respiratory chain. The displacement (D)-loop is a non-encoding nucleotide sequence of the mitochondrial genome that controls mtDNA replication and transcription. Mutations in this part of mtDNA also lead to ETC dysfunction by influencing mRNA generation and mtDNA duplication (Fliss et al., 2000).

An analysis of mtDNA genes of 921 tumors showed that 56% of tumors had at least one mutation in mtDNA with 28% belonging to complex I and 35% belonging to the D-loop (Tan et al., 2014). For other mutations, 5.8%, 12.7%, and 3.8 % involved complexes III, IV, and V, respectively. Mutations in complex I were correlated with ROS generation, DNA damage and tumor progression. However, extensive mutations in complex I seem to have negative effects on carcinogenesis. Mutated mtDNA that causes mild oxidative phosphorylation dysfunction may stimulate tumorigenesis more than normal mtDNA or markedly mutated mtDNA that causes cell death (Cruz-Bermúdez et al. (2015). Therefore, it appears that a threshold level of mitochondrial function is required to support tumorigenesis.

1.4.2. Energy metabolism in cancer cells

The idea that cancer is principally a metabolic disease was developed in the 1920s by Otto Warburg. Warburg showed that tumor cells choose the glycolytic pathway under normal oxygen conditions rather than oxidative phosphorylation to generate ATP. He hypothesized that this switching in energy metabolism is as a result of mitochondrial dysfunction (Warburg, 1956). However, several studies have demonstrated that mitochondrial dysfunction is not the main contributor of this shifting energy. Despite functional mitochondria, cancer cells prefer to provide their energy from aerobic glycolysis. The rate of ATP production in glycolysis is higher due to higher speed and less enzymatic steps (Guppy et al., 1993), which is an advantage in the cancer phenotype. Other advantages for enhanced glucose uptake to support aerobic glycolysis include (Cairns et al., 2011; Gatenby and Gillies, 2004) (Figure 1.1): 1) Due to rapid cell proliferation and inconsistent distance from blood vessels, fluctuation in oxygen levels could be fatal for tumor cells if energy is provided through oxidative phosphorylation; 2) ATP production by oxidative phosphorylation may limit the speed of glycolytic flux. Entrance of pyruvate to mitochondria and its oxidation by the highly regulated enzyme, pyruvate dehydrogenase, is regulated by free levels of acetyl-CoA and the NAD^+/NADH ratio. However, proliferating cells express high levels of

lactate dehydrogenase A, which rapidly converts pyruvate to lactate that is easily secreted (and regenerates NAD^+ for glycolysis); 3) Lactic acid, an end product of glycolysis, provides a favorable condition for tumor invasion and represses anticancer immune system effectors. Moreover, the excess of lactic acid produced by hypoxic cells undergoes monocarboxylate transporter (MTC1 and MTC2) uptake into the cell with subsequent conversion to pyruvate and used as fuel for mitochondrial metabolism. This cooperation between hypoxic and normoxic cells avoids glucose limitation and supports cell survival for cancer cells; 4) Glucose-6-phosphate in glycolysis can enter the pentose phosphate pathway and produce NADPH. NADPH is consumed to regenerate glutathione and synthesize lipids and amino acids, major building blocks needed for cell proliferation; and 5) The major role of glycolysis in tumor cells is to provide substrates for anabolic reactions. For instance, ribose-5-phosphate from the pentose phosphate pathway is used to synthesize nucleic acids. Besides, pyruvate can enter to a truncated tricarboxylic acid (TCA) cycle where citrate is exported to cytosol and cleaved to oxaloacetate and acetyl-CoA. Acetyl-CoA in the cytosol is used to synthesize fatty acids and cholesterol. Oxaloacetate is reduced to malate which then enters the matrix and converts into oxaloacetate while generating NADH (Kroemer and Pouyssegur, 2008). Furthermore, other TCA cycle intermediates are used to synthesize biomolecules. For instance, oxaloacetate and α-ketoglutarate are used to synthesize nonessential amino acids. Taken together, elevated glycolysis in cancer cells contributes to intermediates which sustain cell proliferation, the pivotal and ultimate role of glycolysis. Moreover, the TCA cycle also plays a key role in proliferating cells is to provide intermediates for biosynthesis.



Adapted from Vander Heiden et al., 2009.

Figure 1.1. Energy metabolic pathways in cancer cells

This schematic illustrates cellular ATP production in cancer cells. Glucose enters into the glycolytic pathway and provides pyruvate. Pyruvate either enters into the mitochondrial Krebs cycle and produces ATP or is transformed to lactate and leaves the cell. The Krebs cycle also provides intermediate substances for lipid and nucleotide synthesis required for cell proliferation in cancer cells. HKII: hexokinase II; LDH: lactate dehydrogenase; PDK1: pyruvate dehydrogenase kinase 1; PDH: pyruvate dehydrogenase.

1.4.2.1. Molecular basis of aerobic glycolysis

In neoplastic transformation, a major change in energy metabolism is a switch to aerobic glycolysis. Aerobic glycolysis is a short and fast pathway of transforming glucose to lactate in the state of oxygen deprivation (Jones and Bianchi, 2015) or mitochondrial dysfunction. In cancer cells, mitochondrial dysfunction or rapid cell proliferation and insufficient angiogenesis, which causes lack of oxygen in cells, drive them to provide their ATP from aerobic glycolysis pathway instead of the mitochondrial oxidative phosphorylation pathway (Gatenby and Gillies, 2004). In this section we discuss mitochondrial basis of aerobic glycolysis in cancer cells.

Activation of phosphoinositide 3-kinase (PI3K), is one of the main changes in cancer cells. The PI3K/Akt pathway is highly activated in tumor cells and is a major regulator of glycolysis. In normal cells, PI3K is negatively controlled by the phosphatase and tensin homolog (PTEN) which loses its activation in cancer cells. PI3K is activated by growth factors after binding to their receptors on the surface of cells. Activation of PI3K leads to activation of Akt and mTOR. mTOR increases expression of amino acid transporters and genes related to protein synthesis (DeBerardinis et al., 2008). Akt increases expression of glucose transporters and glycolytic pathway enzymes such as hexokinase and phosphofructokinase, through regulation of genes and activation of enzymes (DeBerardinis et al., 2008; Wallace, 2012). Activation of the PI3K/Akt pathway increases glucose consumption by tumor cells and results in 90% conversion of pyruvate to lactate.

Activation of the PI3K/Akt pathway also stimulates hexokinase II (HK2) binding to the mitochondrial surface by its phosphorylation (Wallace, 2012). HK2 is the predominant isoform of four different HK enzymes and is over-expressed isoform in tumor cells. In cancer cells, HK2 binds to the outer mitochondrial membrane protein, voltage dependent anion channel (VDAC) (Wallace, 2012). The bound VDAC-HK2 provides three favorable conditions for tumor cells to sustain cell proliferation and survival. First, glucose-6-phosphate (G-6-P), the product of HK2, inhibits the activity of this enzyme. However, binding to mitochondria protects HK2 from inhibition by G-6-P and increases the glycolysis rate. Second, the adenine nucleotide translocator (ANT) exchanges ATP synthesized in the matrix with ADP in the cytosol through VDAC. By binding to VDAC, HK2 gains this ability to access ATP that is transferred by ANT (Mathupala et al., 2009; Shoshan-Barmatz et al., 2010). Third, VDAC plays a role in apoptosis by interacting with pro-apoptotic and anti-apoptotic proteins, such as BCL-2 family proteins. HK2 association with VDAC leads to interaction of BCL-XL (an anti-apoptotic protein) with VDAC and inhibition of outer mitochondrial membrane permeability, cytochrome *c* release, and apoptosis (Chiara et al., 2008). Therefore, over-expressed HK2 in cancer cells is a strong anti-apoptotic effector and results in cell survival.

1.4.2.2. Role of HIF-1α in aerobic glycolysis

Hypoxia-inducible factor 1 (HIF1) is a transcription factor that is over-expressed in fast growing cancer cells under hypoxia and regulates cell proliferation, glycolysis and energy metabolism, as

well as apoptosis, angiogenesis, and metastasis (Masoud and Li, 2015). HIF1 consists of 2 subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed in normoxic conditions, thus the activity of HIF1 is determined by expression of HIF-1α. While HIF-1α expression in normoxic conditions is negligible and its half-life is 5 minutes, its half-life under hypoxic conditions becomes 30 minutes (Salceda and Caro, 1997). In addition to hypoxia, the level of HIF-1α is increased by activation of some oncogenes such as H-RAS and inactivation of tumor suppressors such as p53 and PTEN (Zundel et al., 2000). Mutation or down-regulation of TCA cycle enzymes such as succinate dehydrogenase and fumarate dehydrogenase cause a condition called pseudo-hypoxia in which tumor cells behave as if they are under a hypoxic condition. Moreover, ROS is one of the activators of HIF-1α by diminishing enzymes that hydroxylate and inactivate HIF-1α (Movafagh et al., 2015).

HIF-1α mediates the glycolytic pathway in cancer cells by regulating enzymes and transporters. HIF-1α up-regulates glucose transporters including GLUT1 and GLUT3 and glycolytic enzymes such as HK2, phosphofructokinase, aldolase, enolase, pyruvate kinase and lactate dehydrogenase (Semenza, 2010). HIF-1α regulates the mitochondrial enzyme, pyruvate dehydrogenase kinase (PDK). PDK phosphorylates and inhibits activity of pyruvate dehydrogenase, the rate-limiting enzyme that transfers pyruvate to acetyl-CoA in the Krebs cycle (Papandreou et al., 2006). Up-regulation of PDK by HIF-1α therefore decreases pyruvate oxidation and oxidative phosphorylation. Given its key role in regulation of the glycolytic pathway, HIF-1α over-expression in tumor cells is correlated with poor survival in patients with many kinds of cancers such as lung, breast, stomach, and esophagus (Marin-Hernandez et al., 2009).

1.4.3. Mitochondrial biogenesis

Mitochondrial biogenesis is a biological condition where biomolecules such as proteins, lipids, and nucleotides are synthesized and imported to the existing mitochondria which is accompanied by mitochondrial DNA replication (Jornayvaz and Shulman, 2010; Lee and Wei, 2005; Ventura-Clapier et al., 2008). These events result in mitochondrial division and increased number of healthy and functional mitochondria. In addition to increased mitochondrial number, mitochondrial biogenesis can sometimes involve increases in mitochondrial mass through increased expression of mitochondrial proteins and membrane lipid biosynthesis (Carelli et al., 2015; del Mar Blanquer-Rosselló et al., 2017). In this condition, the size of mitochondria is increased and mitochondrial

respiratory subunits become more functional with elevated mitochondrial oxygen consumption. The majority of mitochondrial proteins are encoded by nuclear DNA (~ 1500) and only 13 proteins are encoded by the mitochondrial genome; however, these mtDNA-encoded proteins play important roles in oxidative phosphorylation and are considered as essential proteins. Cells and mitochondria contain multiple copies of mtDNA, which through mutations to different copies can produce a heteroplasmy of more or less functional copies (Carelli et al., 2015; Van Gisbergen et al., 2015). Through clonal expansion, mutated forms can predominate and contribute to development of mitochondria-related disease.

To increase mitochondrial biogenesis and mitochondrial function, expression of a wide range of genes regulated by a group of coactivators and transcription factors are required (Figure 1.2). Nuclear respiratory factor-1 (NRF-1) is a transcription factor which increases expression of mitochondrial target genes such as oxidative phosphorylation and mitochondrial transporter genes (Jornayvaz and Shulman, 2010). It also regulates expression of TFAM, which induces mtDNA replication. The transcriptional activity of NRF-1 is determined by PPARγ coactivator-1α (PGC-1α), PPARγ coactivator-1β (PGC-1β), and PRC (PGC-related coactivator). Phosphorylation mainly by PGC-1α increases translocation of NRF-1, DNA binding and transcriptional activity (Fernandez-Marcos and Auwerx, 2011). Nuclear respiratory factor-2 (NRF-2), is another transcription factor that induces mitochondrial biogenesis genes by increasing expression of mitochondrial genes (Dinkova-Kostova and Abramov, 2015).

Estrogen-related receptors (ERRs) are a group of nuclear transcription factors that regulate mitochondrial genes including components of oxidative phosphorylation and the TCA cycle (Eichner and Giguère, 2011). Among three ERRs, ERRα particularly has an important role in mitochondrial biogenesis and is regulated by PGC-1α. In addition to increasing expression of ERRα, PGC-1α physically binds to this receptor and activates it. The major role of ERRα in mitochondrial biogenesis is induction of NRF-2. As discussed above, NRF-2 is a transcription factor that regulates nuclear-encoded genes that are responsible for mitochondrial proteins such as those in oxidative phosphorylation and mitochondrial importers (Schreiber et al., 2004)

PGC-1α, a well-known transcriptional coactivator, binds to transcription factors and enhances transcriptional activity. Animal studies have demonstrated that transgenic expression of PGC-1α leads to induction of mitochondrial mass, mitochondrial genes, mitochondrial respiratory function

and exercise performance (Lin et al., 2002; Viscomi et al., 2011). In contrast, lack of PGC-1α leads to mitochondrial dysfunction. PGC-1α activity is regulated at the posttranslational level via phosphorylation and deacetylation by AMP-activated protein kinase (AMPK) and SIRT1, respectively (Fernandez-Marcos and Auwerx, 2011). AMPK is a sensor of energy deficiency that is regulated by the AMP:ATP ratio, which is increased under caloric restriction conditions. Briefly, animal studies have shown that treating with AMPK activators increases PGC-1α and NRF-1 activity as well as mitochondrial biogenesis (Bergeron et al., 2001; Terada et al., 2002). Recently, targeting AMPK activation as a possible tumor suppressor in cancer prevention and treatment has emerged as a promising and novel cancer therapy (Li et al., 2015; Luo et al., 2010). AMPK activation inhibits tumorigenesis through several mechanisms such as regulating energy metabolism (e.g. mTOR), inhibiting inflammation, and activating tumor suppressor genes (e.g. p53) (Faubert et al., 2015; Li et al., 2015).

In addition to AMPK, PGC-1α is also activated by SIRT1. SIRT1 is a NAD^+-dependant deacetylase and is another sensor of caloric restriction that deacetylates and activates PGC-1α and thereby induces expression of mitochondrial genes (Ventura-Clapier et al., 2008). While the role of SIRT1 in cancer has been greatly studied for more than a decade, its role as a tumor suppressor or tumor promotor is still controversial. Tumorigenic or anti-tumorigenic roles of SIRT1 depend on the type and stage of cancers as well as signaling pathways that SIRT1 is involved in (Deng, 2009; Lin and Fang, 2013). Analysis of a cancer patient gene expression database shows decreased SIRT1 gene expression in different types of tumors including breast cancer, prostate cancer, and hepatic carcinoma suggesting that SIRT1 acts as a tumor suppressor (Wang et al., 2008). However, an anti-tumorigenic role of SIRT1 through mitochondrial biogenesis pathways is not well-understood.

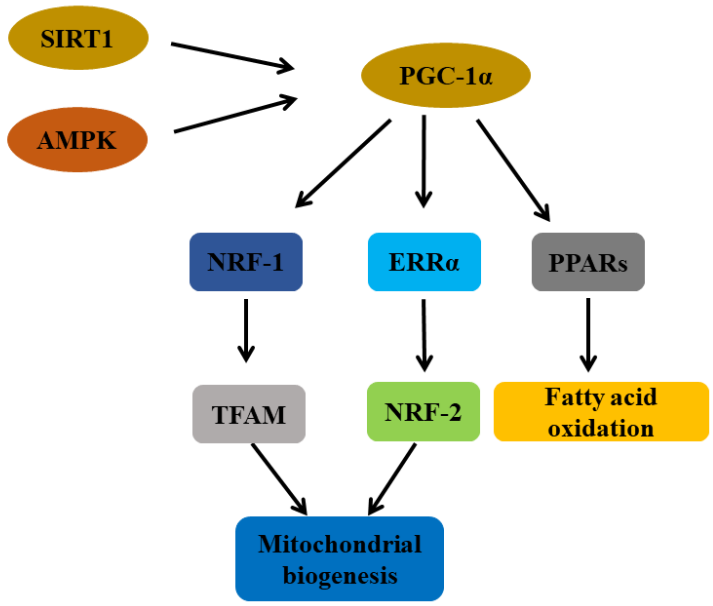


Figure 1.2. Regulation of mitochondrial biogenesis by different signaling factors

SIRT1 and AMPK activate PGC-1α by de-acetylation and phosphorylation, respectively. PGC-1α increases transcriptional activity of NRF1 and ERRα and their downstream effectors, TFAM and NRF2 and consequently induce mitochondrial biogenesis.

A few studies have shown a role of mitochondrial biogenesis in cancer development; however, the results are conflicting. Some studies implicate impaired mitochondrial biogenesis or function in tumorigenesis through abolished oxidative phosphorylation and elevated aerobic glycolysis (Formentini et al., 2010; Roberts and Thomas, 2013; Wang and Moraes, 2011; Yadava et al., 2013). Some other studies report increased mitochondrial biogenesis in some tumors particularly in invasive and metastatic tumors (LeBleu et al., 2014; Toki et al., 2010). In these studies, the mitochondria could be abnormal due to mutations and increased oxidative damage (Lee et al., 2013). Moreover, increased mitochondrial biogenesis in the last stages of cancer can be an adaptive response for detoxification of excessive ROS.

1.5. Role of oxidative stress in carcinogenesis

1.5.1. Overview on oxidative Stress

Oxidative stress is defined as an imbalance between oxidants and antioxidants resulting in an increase in intracellular ROS (Barber and Harris, 1994; Betteridge, 2000; Klaunig and

Kamendulis, 2004). There are two sources of ROS production, endogenous and exogenous. Mitochondrial oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes, and leukocytes are endogenous sources of ROS, whereas pollution, radiation, and redox cycling compounds are exogenous sources of ROS (Phaniendra et al., 2015).

While most oxygen molecules are reduced to water during oxidative phosphorylation, approximately 1-5% of electrons leak from the ETC by reacting with oxygen at sites other than cytochrome oxidase and form ROS, primarily superoxide anion ($O_2^{\bullet-}$). Superoxide anion can be converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). In the presence of reduced metal ions such as iron, hydrogen peroxide reacts with the reduced ion through Fenton and Haber-Weiss reactions to yield hydroxyl radicals ($OH^{\bullet}$). Hydroxyl radicals have a short half-life because they are highly reactive and react rapidly with lipids, proteins, carbohydrates and nucleic acids (Turrens, 2003).

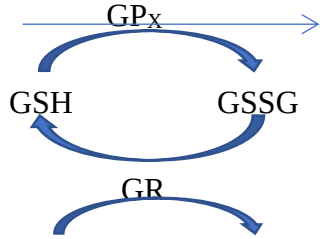
In normal cells, antioxidant defences are capable of counterbalancing generated ROS. Endogenous antioxidant defences are principally enzymatic antioxidants including superoxide dismutases (Mn-SOD and CuZn-SOD), glutathione peroxidase (GP_X), glutathione reductase (GR), and catalase. CuZn-SOD (SOD1) and Mn-SOD (SOD2) are located in the cytosol and mitochondrial matrix, respectively, and oxidize/reduce superoxide anions to oxygen and hydrogen peroxide (reactions [1] and [2]) (Birben et al., 2012; Ighodaro and Akinloye, 2017).

$$O_2^{\bullet-} + SOD\ (M^{(n+1)+}) \longrightarrow O_2 + SOD\ (M^{n+}) \qquad [1]$$

$$O_2^{\bullet-} + 2H^{+} + SOD\ (M^{n+}) \longrightarrow SOD\ (M^{(n+1)+}) + H_2O_2 \qquad [2]$$

Glutathione peroxidase (GPx) is localized in the cytosol and mitochondria and breaks down hydrogen peroxide to water while oxidizing GSH to GSSG. The GSSG is then reconverted to GSH by glutathione reductase (GR) using NADPH as a source of reducing equivalents (reactions [3] and [4]) (Miyamoto et al., 2003; Mytilineou et al., 2002).

$H_2O_2 \xrightarrow{GPx} 2H_2O$ [3]



[4]

Catalase, which is located in peroxisomes and to a lesser extent in other cell compartments, removes hydrogen peroxide by converting it to water and oxygen (reaction [5]) (Heck et al., 2010).

$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$ [5]

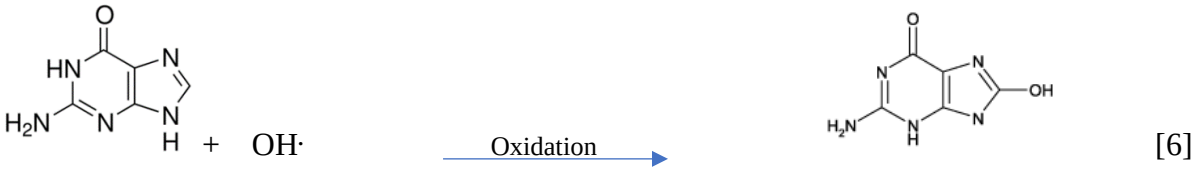
Glutathione is the most abundant thiol in mammalian cells and exists in two chemical forms, reduced (GSH) and oxidized (GSSG). In normal cells, the level of GSH is 10-100-fold higher than GSSG (Zitka et al., 2012). However, in a state of oxidative stress the level of the oxidized form increases and the level of GSH as a cofactor of glutathione peroxidase decreases. The increased level of GSSG is due to the activity of glutathione peroxidase to remove hydrogen peroxide. Glutathione reductase is a NADPH-dependant enzyme responsible for converting GSSG to GSH (Forman et al., 2009).

1.5.2. Oxidative damage to biomolecules and influence of ROS on cell signalling

Oxidative stress is one of the main triggers of cancer initiation and promotion which mediates carcinogenesis through direct and indirect pathways. It has been estimated that in every single cell, approximately 1×10^5 oxidative lesions are formed per day (Klaunig and Kamendulis, 2004). Endogenous and exogenous ROS can cause permanent modifications and genome instability in nuclear and mitochondrial DNA. ROS-mediated DNA damage induces single or double strand DNA breaks, as well as purine, pyrimidine, and deoxyribose modifications. DNA damage leads to mutations, induction or inhibition of transcripts, and replication arrest.

8-Hydroxyguanine (8-OH-G), an oxidized product of guanosine in DNA, is considered one of the important biomarkers of oxidative stress and carcinogenesis found in urine and leukocytes (Irie et al., 2005). Smoking, tobacco, radiation, and other exogenous sources of ROS induce both urinary

and (Irie et al., 2005) leukocyte 8-OH-G. Reaction [6] illustrates guanine oxidation forming 8-OH-G.



[6]

Guanine (G) 8-Hydroxyguanine (8-OH-G)

In addition to DNA, free radicals have the ability to react with fatty acids and produce mutagenic products. In the lipid peroxidation process, free radicals react with polyunsaturated fatty acids and produce malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). MDA can react with DNA bases guanine, cytosine, and adenine and form DNA adducts, which are mutagenic (Valko et al., 2006).

In addition to the ability of ROS to affect key cellular biomolecules or produce intermediates such as MDA that impairs both nuclear and mitochondrial DNA to cause irreversible changes and mutations, ROS contributes to neoplastic transformation through cell signalling pathways. ROS contributes to a series of cell signals which ultimately accelerate the process of cancer initiation and promotion. ROS has effects on cell growth and proliferation, angiogenesis, metastasis, and apoptosis via activating mitogen activated protein kinase (MAPK), NF-κB, and activator protein-1 (AP-1), among other signaling proteins (Waris and Ahsan, 2006).

NF-κB is a transcription factor that is overexpressed in cancer cells and regulates cell proliferation and survival. Translocation of NF-κB to the nucleus up-regulates the expression of many anti-apoptotic gene products including of the Bcl-2 family. Bcl-2 family proteins bind to mitochondria to inhibit apoptosis and are associated with the survival of cancer cells. Other NF-κB-induced gene products (eg. cyclin D1, c-myc and COX-2) are associated with tumor proliferation (Aggarwal and Gehlot, 2009). Also, elevated NF-κB in tumors is correlated with drug resistance (Baldwin, 2001). The NF-κB system is a redox-mediated sensor for ROS which is dose-dependently up-regulated by exposure of cells to hydrogen peroxide (Li and Karin, 1999). In its inactive form, NF-κB binds to its inhibitor protein IκB located in the cytosol. Once IκB becomes phosphorylated, it liberates NF-κB to translocate to the nucleus. The IκB kinase (IκK) that phosphorylates IκB is activated by

upstream kinases such as protein kinase D1 (PKD1). The mechanism of how ROS activates NF-κB-inducing kinases is through inhibition of phosphatase activity through oxidation of critical thiols in phosphatases (Morgan and Liu, 2011). Activation of IκK by upstream kinases leads to proteosomal degradation of IκB and translocation of NF-κB from cytosol to nucleus. Thus, in an indirect pathway, ROS activates NF-κB via inducing IκB degradation away from NF-κB. NF-κB activation also induces production of pro-inflammatory cytokines such as TNF-α and IL-1. Therefore, induction of pro-inflammatory cytokines by ROS is another oxidative NF-κB-inducing signaling pathway (Gloire et al., 2006).

Another important signaling pathway involved in tumor progression is the MAPK pathway, which consists of three kinases ERK, JNK, and p38. Studies show that ROS regulate activity of MAPKs resulting in progression of tumors (Liou and Storz, 2010). For example, it has been shown in cancer cells that ROS activate ERK, a cell growth and proliferation regulating kinase (Liou and Storz, 2010). ROS also activate AP-1 either directly or through the MAPK pathway (Wu, 2006). The AP-1 heterodimer is a transcription factor downstream of MAPK related to cell proliferation and cell survival (Thannickal and Fanburg, 2000). AP-1 proteins including c-Jun, stimulate cell proliferation by up-regulation of Cyclin D proteins and regulators of G1 to S phase transition that are often overexpressed in cancers (Shaulian and Karin, 2001).

1.5.3. Uncoupling proteins and oxidative stress in cancer cells

Uncoupling protein 2 (UCP2) is a key protein involved in oxidative stress in cancer cells. UCP2 belongs to the family of uncoupling proteins acting as a mitochondrial anion transporter. Uncoupling proteins increase the permeability of the inner membrane to protons, which accelerates electron flow to cytochrome oxidase (which reduces oxygen without producing ROS) in the ETC and thereby lowers ROS production. During electron flux within the ETC, H^+ ions are pumped into the inter membrane space and produce the mitochondrial membrane potential (MMP), which ultimately leads to formation of ATP. During transfer, some of the electrons escape from the ETC by reducing oxygen to generate superoxide. Formation of ROS by mitochondria induces expression of UCP2, a sensor and suppressor of mitochondrial ROS generation (Baffy, 2010; Casteilla et al., 2001).

ROS is a vital component for cancer progression due to ability to induce further genomic instability and ROS signaling pathways. On the other hand, an excessive amount of ROS could be toxic and leads to cell death. In cancer cells, overexpression of UCP2 acts as an adaptive response to protect cancer cells from excessive ROS (Derdak et al., 2008). Moreover, UCP2 is involved in carcinogenesis through several other mechanisms including inhibiting OXPHOS and inducing glycolysis, increasing cell proliferation, cell invasion, cell transformation, and cachexia resulting in chemoresistance and poor prognosis of cancer (Derdak et al., 2008; Pons et al., 2015).

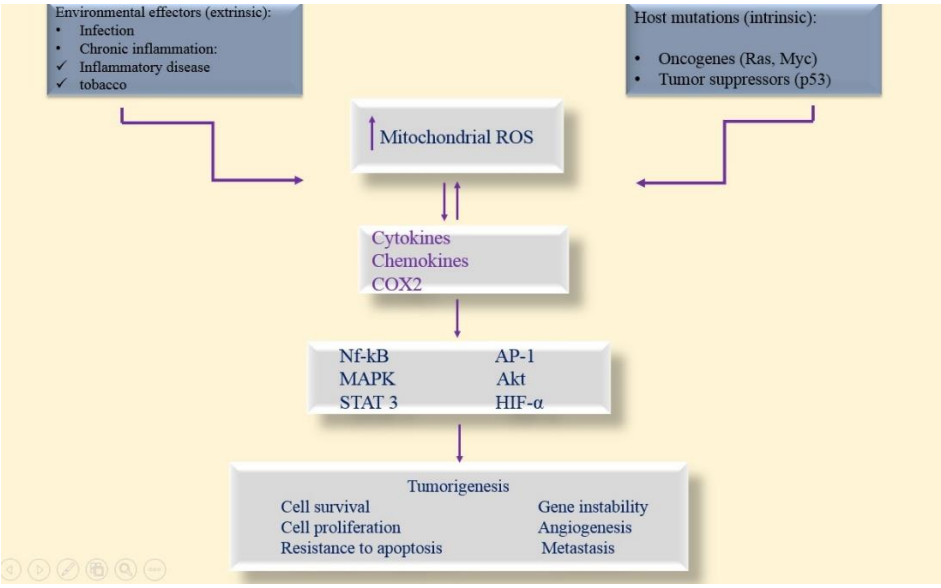
1.6. Inflammation and carcinogenesis

Experimental and epidemiological studies show a strong association between cancer and chronic inflammation (Grivennikov et al., 2010; Mantovani et al., 2008; Shacter and Weitzman, 2002). On the other hand, anti-inflammatory therapies show effectiveness in cancer prevention and treatment (Gonda et al., 2009). The pioneering idea of inflammation-associated cancer was established by Virchow in the 19th century. He observed that tumors often progressed in the inflammation settings and inflammatory biomarkers were found in tumor biopsy samples (Balkwill and Mantovani, 2001). Since then, clear associations between inflammatory chronic disease and cancer have been shown such as Crohn and ulcerative colitis with colon cancer and chronic pancreatitis with pancreatic cancer have been confirmed (Ekbom et al., 1990). The main component in inflammation-associated cancer is ROS and there is a cross-talk between ROS and inflammation. In inflammation, leukocytes and mast cells are recruited to the damage site and lead to a "respiration burst" as a result of increased consumption of oxygen for ROS generation. Moreover, inflammatory cells secrete mediators such as chemokines and cytokines that recruit more inflammatory cells contributing to more ROS generation (Reuter et al., 2010).

Inflammation can be triggered by extrinsic factors (e.g. tobacco, infection) or intrinsic factors such as ROS and mutations in oncogenes and tumor suppressor genes (e.g. Ras, Myc, p53). Mutated oncogenes and tumor suppressor genes can induce inflammation by targeting nuclear and mitochondrial genes, for example p53, and increasing mitochondrial ROS production (Vafa et al., 2002) (Kamp and Weitzman, 2011). ROS produce a cascade of signal transductions (Figure 1.3) by inducing a group of transcription factors involved in inflammation including NF-kB, a signal transducer and activator of transcription 3 (STAT3), HIF-1α, and activator protein 1 (AP-1), as well as enzymes such as cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS)

(Reuter et al., 2010). COX2 and iNOS stimulate expression of inflammatory cytokines (TNF-α and IL-6) and chemokines (IL-8). Therefore, a vicious circle (interplay between ROS and inflammation) leads to enhanced DNA damage, gene instability, and cell proliferation that promotes cancer development (Coussens and Werb, 2002; Perwez Hussain and Harris, 2007).

In addition to DNA damage, inflammation increases cell survival and cell proliferation in cancer cells via ROS cell signaling. One of the important ways that ROS induce cell survival is through activation of Akt and inhibition of PTEN, the phosphatase inhibiting PI3K-dependant Akt activation (Azad et al., 2009). Akt induces cell survival through several routes such as inducing cyclin D1 and degradation of cyclin dependant-kinase (Cdk) inhibitor, activating NF-κB, inhibiting apoptosis, and elevating the glycolytic pathway (Manning and Cantley, 2007; Plas and Thompson, 2005). In addition to Akt, ROS regulates NF-κB, MAPK, and AP-1 and induce genes associated with cell survival, cell proliferation, angiogenesis, and cell invasion (Reuter et al., 2010).



Adapted from Kamp & Weitzman, 2011.

Figure 1.3. Association between ROS and inflammation-related cancer

In cancer cells, mitochondrial ROS leads to the expression of inflammatory mediators such as cytokines and chemokines and activates a variety of transcription factors and enzymes including NF-κB, AP-1, and MAPK. In turn, increased inflammation in tissues induces cell mitochondrial ROS production. The interplay between ROS and inflammation contributes to tumorigenesis through several mechanisms including increased cell survival, cell proliferation, mutations, angiogenesis, and metastasis.

1.7. Apoptosis

Programmed cell death, known as apoptosis, is a critical event to maintain normal development and it is a balance between cell proliferation and cell death that leads to normal physiological process. Any imbalance between the rates of cell proliferation and apoptosis results in cancer progression (Indran et al., 2011). Imbalance can result from dysfunction in the two major pathways of apoptosis, extrinsic (receptor-mediated pathway) and intrinsic (mitochondria-mediated pathway) (Danial and Korsmeyer, 2004).

The receptor-mediated pathway is an engagement between death receptors, located on the surface of the plasma membrane, and their ligands (Scaffidi et al., 1998). Death receptors that are involved in this pathway are the tumor necrosis factor (TNF) superfamily such as CD95 (fas), and TNF-related apoptosis inducing ligands (TRAIL). Upon ligand binding, death signals are transferred from the surface to intracellular signaling pathways via a cytoplasmic death domain (Ashkenazi and Dixit, 1998). Activation of death receptors by their ligands leads to formation of a group of proteins known as death-inducing signaling complex (DISC). Activation of pro-caspase 8 by DISC activates the apoptosis execution phase by triggering caspase 3, a downstream effector of caspase 8 (Elmore, 2007).

In the intrinsic pathway, stimuli such as ROS, radiation, viral infections, toxins, hypoxia, and hyperthermia cause changes in the mitochondrial inner membrane that leads to opening of the mitochondrial permeability transition (MPT) pores, loss of the mitochondrial membrane potential and release of two major groups of pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al., 2004). The first group consisting of cytochrome *c* and Smac/DIABO (second mitochondria-derived activator of caspases) activates a caspase-dependant pathway. Cytochrome *c* activates Apaf-1 leading to activation of procaspase 9 and formation of a cytochrome *c*/Apaf-1/caspase 9 complex referred to as the apoptosome. The apoptosome complex subsequently activates caspase 3 (execution phase) (Chinnaiyan, 1999; Hill et al., 2004). Smac/DIABLO deactivates IAP (inhibitors of apoptosis proteins), and thus induces apoptosis by arresting activity of IAP (Schimmer, 2004; van Loo et al., 2002). The second group of pro-apoptotic proteins, apoptosis inducing factor (AIF) and endonuclease G function in a caspase-independent pathway. Following opening of the MPT pore and collapse of the mitochondrial membrane potential, this group of proteins are released to the cytosol, translocated to the nucleus

and results in DNA fragmentation without caspase involvement. However, this is a late event and it occurs only when the cell is committed to die (Joza et al., 2001).

1.7.1. The Bcl-2 family of proteins

Apoptosis is highly regulated by the Bcl-2 family, a group of proteins divided into pro-apoptotic (eg, Bax, Bak, and Bad) and anti-apoptotic (eg, Bcl-2 and Bcl-XL) proteins. The Bcl-2 family proteins function through effects on the mitochondrial outer membrane. The pro-apoptotic proteins such as Bax and Bak lead to formation of a transmembrane channel across the mitochondrial inner and outer membrane and release of cytochrome *c* and AIF, while Bcl-2 and Bcl-XL prevent formation of pores and apoptosis and promotes cell survival (Kluck et al., 1997; Yang et al., 1997) (Adams and Cory, 2007; Yang et al., 1997). In the normal situation, Bax is localized in cytosol in an inactive form, but, in response to death stimuli, it is translocated to mitochondria and causes mitochondrial membrane permeabilization and release of pro-apoptotic proteins to the cytosol (Westphal et al., 2011). In contrast, Bak is localized in the mitochondrial membrane in an inactive form and it becomes activated in response to death stimuli (Gustafsson and Gottlieb, 2007). The fate of the cell is greatly depending on a balance between function of these two groups of proteins.

Many cancer cells overexpress anti-apoptotic Bcl-2 proteins or have mutations in pro-apoptotic Bcl-2 proteins, which increase resistance to apoptosis and may cause drug resistance (Adams and Cory, 2007; Murphy et al., 2000; Yip and Reed, 2008). Some chemotherapy drugs (drug-induced apoptosis) act through the BCL-2 family. Such drugs might change conformation of Bax or induce expression of Bax, Bak, and Bad through up-regulation of the p53 gene (Bellosillo et al., 2002; Zhang et al., 2000).

In the previous sections we discussed several aspects of carcinogenesis such as hallmarks of cancer, mitochondrial dysfunction, energy metabolism in cancer cells, inflammation, and apoptosis. In the next section we discuss the group of chemicals called polycyclic aromatic hydrocarbons (PAH) which include potent carcinogens such as B[a]P.

1.8. Polycyclic aromatic hydrocarbons

Humans are exposed to mixtures of chemicals through several routes such as cigarette smoke, pollution produced by vehicle exhaust and factories, pharmaceuticals and foodstuffs. Although exposures usually happen at low concentrations, they may occur over a prolonged period of time

(Jarvis et al., 2014). A major family of chemicals to which humans are exposed are polycyclic aromatic hydrocarbons (PAH), which consists of more than 1500 chemicals (Kim et al., 2013). PAHs encompass two or more bonded aromatic rings which can be found in native form or substituted forms (e.g. oxygenated, methylated, or nitrated). PAHs are ubiquitous pollutants that are yielded in an incomplete pyrolytic process. Pyrolysis occurs as a result of decomposition of organic chemicals at the high temperatures and in the absence of oxygen (Jarvis et al., 2014).

Humans are exposed to PAHs via inhalation, dermal absorption and ingestion. Studies have shown association between PAHs exposure and chronic diseases including cancer, cardiovascular disease and respiratory diseases (Boffetta et al., 1997; cancer, 2010; Mumtaz et al., 1996; Peters et al., 2001; Pope III et al., 2002). The correlation between sources of PAHs and cancer has been known since 1775 when British surgeon Sir Percival Pott demonstrated that there is an association between scrotal cancer and exposure of chimney sweepers to soot (Poirier, 2004). Although the carcinogenic potency of PAHs varies, the International Agency for Research on Cancer (IARC) categorizes these chemicals as possible carcinogens in both individual and mixture forms (Orasche et al., 2012). One of the most potent carcinogens in the PAH family is benzo[a]pyrene (B[a]P), which is assigned as a standard to assess potency of other PAHs (Boström et al., 2002; Pufulete et al., 2004).

1.8.1. Benzo [a] pyrene

Benzo[a]pyrene is classified a as a group 1 carcinogen by the International Agency for Research on Cancer (IARC). B[a]P is yielded from fusion of benzene with pyrene as the by-products of incomplete combustion of organic compounds at high temperatures between 300ºC to 600ºC. Burning wood, diesel exhaust, cigarette smoke, and grilled foods are the main sources of B[a]P. B[a]P exposure occurs through three major routes: skin exposure, dietary intake, and inhalation and compromises numerous organs including lung, liver, stomach, esophagus, spleen, and skin (Melchini et al., 2011). Cigarettes and air pollution are 2 major sources of B[a]P inhalation which are associated with lung and skin cancers. By analysis of smoke of cigarette samples conducted in the USA cigarette market between 1995 and 2000, inhalation of B[a]P by cigarettes is estimated to be ~ 9 ng per cigarette (Swauger et al., 2002). Moreover, studies show that B[a]P pollution in a developing country is higher than a developed country, 0.38 ng/m^3 in La Plata (Argentina) versus

0.1 ng/m^3 in Berlin; however, pollution and exposure to PAHs in developed countries still remains as a great issue (Fromme et al., 2004; Rehwagen et al., 2005).

Exposure to dietary sources of B[a]P is one of the major routes of B[a]P uptake (~ 97% for non-smokers) and is highly correlated with gastrointestinal cancers such as colorectal and gastric cancers (Lee and Shim, 2007; Vasiluk et al., 2008). Studies have demonstrated that B[a]P content in food is affected by the type of cooking process (grilling, frying, boiling, and broiling) and the time of cooking (Lee and Shim, 2007). In an epidemiological study, Kazerouni et al. (2001), found that grilled/barbequed steak (4.86 ng/g), grilled/barbequed chicken with skin (4.57 ng/g), and burger (1.52 ng/g) had highest concentrations of B[a]P. Grilled/barbequed meat and bread/grain/cereal contributed 21% and 29% of the total B[a]P intake respectively, while single items of bread/grain/cereal group had lower concentrations of B[a]P (0.5 ng/g). Several studies have shown association between grilled/barbequed meat consumption which contain high level of B[a]P and risk of colon, pancreatic, esophagus, and gastric cancers (Anderson et al., 2005; Cross et al., 2011; Sinha et al., 2005).

1.8.1.1. Carcinogenic effects of B[a]P

Binding of B[a]P metabolites to DNA and formation of bulky adducts in DNA is the major mechanism of B[a]P-induced mutagenesis and carcinogenesis (Conney, 1982). To be converted to a carcinogen, B[a]P first needs to be metabolized to its activated metabolites by cytochrome P450 enzymes including CYP1A1, CYP1A2, and CYP1B1, that are principally involved in B[a]P activation (Shimada and Fujii-Kuriyama, 2004; Shimada et al., 1996). B[a]P is a ligand for the aryl hydrocarbon receptor (AhR), a transcription factor regulated by aromatic hydrocarbons and involved in expression of P450 enzymes (Baird et al., 2005; Nebert et al., 2004). B[a]P activation consists of a three-step enzymatic mechanism that is initiated with conversion of B[a]P to B[a]P-7,8-epoxides by P450 enzymes, followed by conversion to B[a]P-7,8-diols by epoxide hydrolase. Ultimately, B[a]P-7,8-diols are catabolized to the B[a]P-7,8-diol-9,10-epoxide metabolites by P450 enzymes (Figure 1.4) (Conney, 1982). An additional metabolic pathway identified in lung but not liver microsomes, is auto-oxidation of B[a]P radical cation metabolites resulting in formation of quinones (Briede et al., 2004). As well as being electrophiles that form DNA adducts,

the B[a]P quinones may undergo redox-cycling which ultimately results in production of superoxide and other ROS.

Formation of bulky DNA adducts by B[a]P metabolites results in mutagenesis, ROS generation, and carcinogenesis. Following reaction with DNA, B[a]P causes mutations in oncogenes and tumor suppressor genes (Baird et al., 2005). Mutations in tumor suppressor genes such as p53 that occurs in a variety of cancer cells and contribute to DNA damage, uncontrolled cell cycle regulation, and ultimately carcinogenesis, have been observed after exposure to PAHs (Hainaut et al., 1998). Mutated pro-oncogene Ras is associated with many types of cancers (Prior et al., 2012), and has also been observed after exposure with PAHs (Gray et al., 2001). In addition to impairing nDNA, B[a]P also has effects on mtDNA and causes permanent DNA damage and mutations (Backer and Weinstein, 1980; Backer and Weinstein, 1982; Jung et al., 2009). Studies have demonstrated that mitochondria are the major target of B[a]P due to lack of histones that compact and protect nDNA from damage (Suliman et al., 2004). Furthermore, base excision repair (BER) has been detected in mtDNA but nucleotide excision repair (NER), which is responsible to repair bulky DNA damage, has not been observed in mtDNA (Berneburg et al., 2006). Therefore, mitochondria are more susceptible to bulky DNA damage by B[a]P and could be affected 40- to 90-fold more than nDNA (Pavanello et al., 2013). Some evidence shows that B[a]P causes loss of mitochondrial membrane potential, decrease in ATP generation, changes in mitochondrial morphology, and induction of mitochondrial-dependant apoptosis (Huc et al., 2006; Ko et al., 2004; Li et al., 2003; Xia et al., 2004; Zhu et al., 1995).

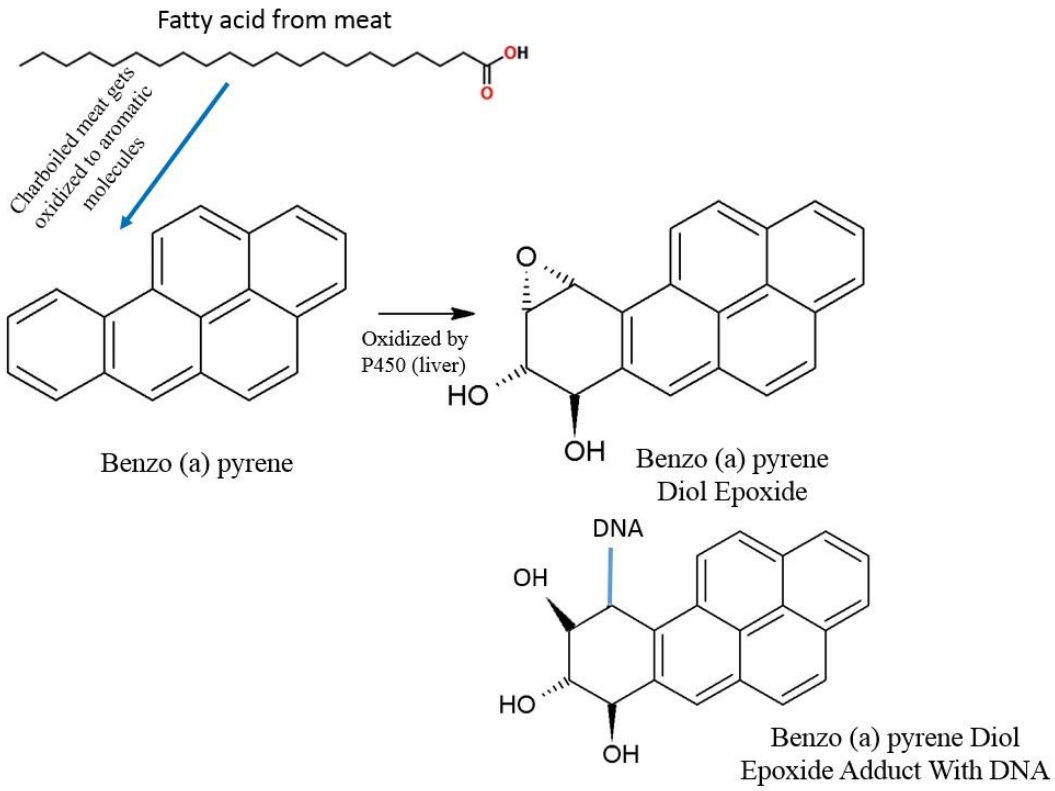


Figure 1.4. B[a]P metabolism via cytochrome P450 enzyme to a reactive epoxide and reaction with DNA to produce DNA adducts

Adapted from Bui et al., 2009.

1.8.1.2. Benzo [a] pyrene and mitochondrial dysfunction

Evidence shows inhibitory effects of B[a]P on mitochondrial biogenesis and mitochondrial function. In human bronchial epithelial cells (16HBE), administration of 16 μM B[a]P for 48h decreased mRNA expression and protein levels of NRF-1 and TFAM, two important transcription factors regulating mitochondrial biogenesis (Zhang et al., 2014). In a later study, in the F258 rat liver epithelial cell line, treatment of 1 μM and 50 nM B[a]P for 24h, 48h, and 72h shifted energy metabolism from OXPHOS to glycolysis (Hardonnière et al., 2016). In an animal study, oral administration of B[a]P in different doses (2.5, 5 and 10 mg/kg body weight) significantly depleted antioxidant enzymes (e.g. SOD, CAT, GPx, GR) and Krebs cycle's enzymes such as isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase and alpha-ketoglutarate dehydrogenase in liver, lung, brain, stomach and kidney of ICR mice (Ji et al., 2016). Taken together, these studies suggest that B[a]P induces mitochondrial dysfunction and inhibits

mitochondrial biogenesis by stimulating oxidative stress, altering mitochondrial enzymes, switching energy to glycolysis, and decreasing mitochondrial biogenesis regulatory biomarkers.

1.9. Polyphenols

Polyphenols are a large group of phytochemicals naturally occurring in plants and are found in a wide range of plant foods and beverages including fruit, vegetables, cocoa, cereal, tea, wine, beer, and coffee, and are characterized by their hydroxylated phenyl groups (Cardona et al., 2013). Over 8,000 polyphenols have been identified and of these more than 4,000 are flavonoids. It has been estimated that total dietary intake of flavonoids in the US population is 210 mg per day (98% from diet and 2% from supplements) (Yao et al., 2011).

Polyphenols range from simple molecules such as phenolic acids to highly polymerized molecules such as tannins (Dai and Mumper, 2010). Polyphenols are responsible to protect plants from ultraviolet radiation damage, to deter aggression by pathogens and parasites, and to contribute to a plant's color. Based on their chemical structures including the number and site of hydroxyl groups, the numbers of phenolic rings, and substituting groups, polyphenols are classified to several groups and subgroups (Figure 1.5).

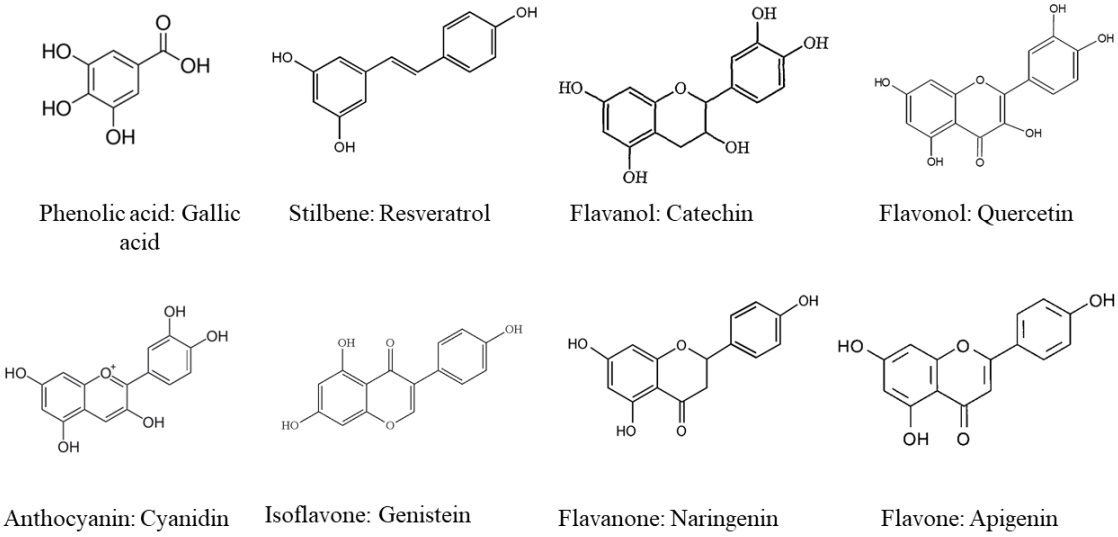


Figure 1.5. Chemical structure of some major polyphenols

1.9.1. Classification of polyphenols

Polyphenols can be classified into two large groups: flavonoids and non-flavonoids (phenolic acids, tannins, stilbenes, and lignans) (Dai and Mumper, 2010). Flavonoids are a major group of polyphenols with a C6-C3-C6 general structure backbone and consist of six classes: flavan-3-ols (monomeric and proanthocyanidins), flavonols, anthocyanins, isoflavones, flavanones, and flavones. Most of the flavonoids except flavan-3-ols exist in the glycoside form in plants (Thilakarathna and Rupasinghe, 2013).

Flavan-3-ols of the flavonoid class are found as monomers such as catechin, epicatechin, gallocatechin, epigallocatechin, and epigallocatechin gallate. Monomeric flavan-3-ols are mostly found in tea (*Camellia sinensis*) and fruits (except citrus). Green tea, cocoa, apples (particularly apple peel), berries, grapes, cherries, plums, and red wine are the best sources of catechin and epicatechin. Catechin and epicatechin can be polymerized to form proanthocyanidins that possess high anti-oxidative ability and readily bind to metal ions (Thilakarathna and Rupasinghe, 2013).

The flavonols are ubiquitous in the human diet and foods such as apples, grapes, berries, onions, red wine, tea, broccoli, and kale are main sources. Quercetin, kaempferol, and myricetin are examples of flavonols (Thilakarathna and Rupasinghe, 2013).

Anthocyanins are the major constituents of natural pigments, blue, purple, and red in fruits, vegetables, grains, and flowers. They are abundant in cherries, berries, purple and red grapes, pomegranates, apples, red wine, cabbages, red onion, and radishes. The colors of anthocyanins are influenced by different factors including pH (blue in basic and red in acidic conditions), degree of hydroxylation or methylation of the aromatic rings, and glycosylation pattern (Tsao, 2010). The anthocyanins are principally found in the glyosidic form in plants and commonly referred to as anthocyanins, while the aglycones are termed anthocyanidins. Cyanidin, delphinidin, malvidin, and pelargonidin are the major anthocyanidins out of a total of 31 monomeric anthocyanidins (Andersen and Markham, 2005). However, there are over 500 anthocyanins depending on the substitute groups can be find as hydroxylated, methylated, and glycosylated with different units of sugar (McCallum et al., 2007).

The leguminous plant family particularly soybean are the best sources of isoflavones. Genistein, daidzein, and biochanin A are major isoflavones found in soybean. Isoflavones have phytostrogenic effects and can bind to estrogen receptors (D Archivio et al., 2007); therefore, they may have an impact on hormone-dependent cancer prevention (Sarkar and Li, 2003). As for the flavanones, hesperetin and naringenin are examples and are mainly found in citrus fruits. Flavones are a less common class of flavonoids and can be found in vegetables such as parsley and celery. Apigenin and luteolin are two compounds belonging to flavones (Thilakarathna and Rupasinghe, 2013).

The non-flavonoids consist of the phenolic acids, stilbenes, and others. Phenolic acids are a group of dietary non-flavonoids divided into derivatives of benzoic acid such as gallic aid and derivatives of cinnamic acid such as caffeic and ferulic acid. Caffeic acid is the most abundant phenolic acid in fruits and vegetables and a major polyphenol in coffee. Ferulic acid is an abundant phenolic compound found in cereals (D Archivio et al., 2007).

Stilbenes are a less common group of polyphenols presented in low quantities in the human diet. Resveratrol is the best representative of stilbenes produced by plants in response to stress conditions such as infection by pathogens (Delmas et al., 2006). Resveratrol is found in over 70 species of plants particularly in grapes. Grape skin is a rich source of resveratrol (50-100 g/kg) which contributes to the high amount of resveratrol in wine and grape juice (up to 7 mg aglycones /L and 15 mg glycosides/L)(D Archivio et al., 2007). Extensive data show an effective inhibitory role of resveratrol in three stages of cancer, initiation, promotion and progression (Athar et al., 2007).

There are other non-flavonoid polyphenols found in the human diet considered as important compounds to human health. Among them, ellagic acid, a dimer of gallic acid, and derivatives found in berries, eg., raspberry and strawberry; lignans found in flax seeds, sesame, and grains; and curcumin found in turmeric (Tsao, 2010).

1.9.2. Bioavailability and metabolism of polyphenols

A definition of bioavailability is the proportion of the food or nutrient that is digested, absorbed, enters the circulation and has an active effect (D Archivio et al., 2007). As xenobiotics that undergo

extensive presystemic metabolism, polyphenols have low bioavailability which varies among different classes of polyphenols (Cardona et al., 2013). Furthermore, the absorption of polyphenols varies and depends on molecular weight and degree of structural complexity (polyphenols with low molecular weight such as monomeric or dimeric are readily absorbed in small intestine while polyphenols with higher molecular weight such as polymeric or oligomeric reach the colon almost unchanged)(Appeldoorn et al., 2009). Approximately 5-10% of total polyphenol intake is absorbed in the small intestine and 90-95% of total intake reaches the colon. The colonic microbiota extensively break down the polyphenolic parent structures into smaller and absorbable phenolic molecules (Cardona et al., 2013). The major focus of the previous studies had been on the bioavailability and activities of the polyphenolic parent compounds while today a growing number of studies investigate the biological activities of their metabolites (Forester and Waterhouse, 2010; Tsuda, 2018). For example, the bioavailability of anthocyanins can be as low as 0.1%; however, the bioavailability of its colonic degradation products and metabolites (eg., protocatechuic acid, syringic acid, vanillic acid) can reach as high as 10% and more in the blood (Czank et al., 2013).

In the small intestine, flavonoids are hydrolyzed to aglycones as a result of the action of lactase phloridzin hydrolase and β-glucosidases (located on the brush border of small intestine). An alternative enzyme to hydrolyze flavonoids exists in epithelial cells known as cytosolic *β*-glucosidase (Day et al., 2000; Gee et al., 2000). For the CBG-catalyzed hydrolysis route, flavonoids in the glycoside form need to enter epithelial cells through the activity of sodium-dependant glucose transporter 1 (SGLT1) (Del Rio et al., 2013). At the next step, hydrolysed flavonoids are conjugated to O-glucuronides, sulfate esters, and O-methyl esters by intestinal enzymes. Almost all polyphenols are present as conjugated forms, while aglycone forms are hardly seen in the plasma (Landete, 2012). Therefore, the form of polyphenols in plasma and tissue is different than those of present in the foods. Molecules not conjugated by the intestine can be subsequently conjugated by the liver to facilitate their excretion through urine and bile (Thilakarathna and Rupasinghe, 2013). Polyphenols that are not absorbed in the small intestine and reach the colon, are hydrolyzed to aglycones and metabolized to derivatives of benzoic acid by microflora (D Archivio et al., 2007).

There are some exceptions regarding absorption of polyphenols (D Archivio et al., 2007). For example, anthocyanins are mostly found as glycosides in the plasma due to the stability of

glycosides in comparison to aglycone forms. Moreover, glycosylation of resveratrol protects it from oxidation, and resveratrol glycoside is more soluble and more readily absorbed in the gastrointestinal tract. Glycosylation also facilitates absorption of quercetin; thus, absorption efficiency of quercetin glycoside is higher than that of the aglycone form. All polyphenols except flavanols (catechins) exist in the glycosylated forms and resist acid hydrolysis in the stomach (Gee et al., 1998). Some experimental studies carried out in rats show that some flavonoids such as quercetin are absorbed in the stomach but only in the aglycone form (Crespy et al., 2002; Piskula et al., 1999). Moreover, recent studies show that other than quercetin, anthocyanins could be absorbed in the stomach (Matuschek et al., 2006; Passamonti et al., 2005; Talavera et al., 2003).

1.9.3. Polyphenols and cancer prevention

Both *in vitro* and *in vivo* studies suggest that polyphenols have anti-cancer properties. Polyphenols are known to cause apoptosis of cancer cells *in vitro* (D'Archivio et al., 2008). With regard to cancer prevention, epidemiological studies strongly implicate protection by dietary polyphenols (Athar et al., 2007; Bishayee, 2009; Yang et al., 2009). A variety of experimental studies also show cancer preventive effects of polyphenols at early stages of carcinogenesis and neoplastic transformation (for reviews see Lin et al., 2008; Shukla and Singh, 2011; Farraz da Costa et al., 2017; Clementino et al., 2017). Polyphenols may prevent carcinogenesis and neoplastic transformation via multiple mechanisms including modulation of carcinogen metabolism, inhibition of oxidative stress and mitochondrial dysfunction, modulation of inflammation, induction of mitochondrial biogenesis, regulation of apoptosis, regulation of the cell cycle, and inhibition of angiogenesis and metastasis (del Mar Blanquer-Rosselló et al., 2017; Lin et al., 2008; Shukla and Singh, 2011).

1.9.3.1. Polyphenols and preventing cancer initiation and promotion

Polyphenols can prevent cancer initiation and promotion through various mechanisms including inhibition of the activation of oncogenes and genes involved in oxidative stress and inflammation (Lin, 2004; Villegas et al., 2008). Recent studies have shown that tea polyphenols, specifically epigallocatechin-3-gallate (EGCG), can protect against carcinogenesis by modulating epigenetic aberrations such as histone modifications, DNA methylations, and microRNAs. ECGC inhibits DNA methylation by altering activity of DNA methyltransferase enzymes, which may contribute

to its chemopreventive and chemotherapeutic effects (Bag and Bag, 2018; Henning et al., 2013). In addition to tea polyphenols, other polyphenols such as resveratrol, curcumin, and genistein have shown cancer preventive effects by altering DNA methylation levels in tumor suppressor genes (Dammann et al., 2017). Furthermore, polyphenols also prevent cancer initiation and promotion through modulation of phase I and phase II cytochrome P450 enzymes, inhibition of oxidative stress and inflammation, and reduction in DNA damage (Clementino et al., 2017; Ferraz da Costa et al., 2017).

1.9.3.2. Polyphenols and preventing cancer progression

In the last decade, anthocyanins have emerged as intriguing anti-cancer agents. For instance, delphinidin (10-50 µM) sensitized LNCaP and Du145 prostate cancer cells to TRAIL-mediated apoptosis through increasing protein expression of death receptor 5 (DR5), cleavage of histone deacetylase 3 (HDAC3), and modulation of BCL-2 family proteins (Ko et al., 2015). A bilberry extract containing 50% anthocyanins administered to patients with B cell chronic lymphocytic leukemia (B CLL) induced apoptotic pathways via activation of caspase 3 and down-regulation of BCL-2 family and UHRF1, an oncogene over-expressed in many cancer cells (Alhosin et al., 2015). Delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside were identified as exerting apoptotic effects of the bilberry extract.

Investigations have shown the impact of several polyphenols on regulation of the cell cycle in cancer. Epidemiological and experimental studies have demonstrated that curcumin prevents growth of cancer cells through cell-cycle arrest. An inhibition of G1 to S transition is triggered by curcumin (10 µM) in human colorectal carcinoma cells (HCT116) (Basile et al., 2009). Quercetin (10-75 µM) was reported to have anti-proliferative effects through cell cycle arrest in S phase in Human Papillary Thyroid Cancer (B-CPAP) Cells (Mutlu Altundağ et al., 2016). Moreover, EGCG blocked the progression of the cell cycle at the G1 phase in HepG2 cells (Huang et al., 2009). EGCG (3.125-50 µM) inhibited cell proliferation in oral squamous cell carcinoma (OSCC) cells by overexpression of B-cell translocation gene 2 (BTG2), whereas BTG2 knockdown revealed the opposite effect in xenograft animals injected with SAS cells (Lee et al., 2015). BTG2 is an important regulator of cell proliferation by inhibiting expression of cell cycle proteins such as cyclin D1 and cyclin E (Hu et al., 2012). BTG2 expression is regulated by mitogen activated protein kinase (MAPKs) superfamily including extracellular signal regulated kinases (ERKs), c-

Jun N-terminal kinases (JNKs), and p38. Polyphenols can modulate MAPKs superfamily and EGCG was shown to arrest the cell cycle at G1 phase and increase BTG2 expression through phosphorylation of p38 MAPK signaling pathway, and decrease cell proliferation via phosphorylation of ERK, JNK, and p38 pathways (Shih et al., 2016; Wang et al., 2014). As well, resveratrol and its analogs inhibited cell proliferation via modulating cyclins and cyclin-dependent kinases (CDK) in oral squamous cell carcinoma cell (Yu et al., 2016), glioblastoma (Chelsky et al., 2015), human epidermoid carcinoma (Ahmad et al., 2001), and liver carcinoma (Kuo et al., 2002).

1.9.4. Mechanisms of cancer prevention by polyphenols

1.9.4.1. Anti-oxidative effects of polyphenols

The human body contains an elaborate antioxidant defence that protects cellular components from oxidative damage. An antioxidant is defined as "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell, 1995; Sies, 1993). The antioxidants that directly react with oxidants to protect cellular compounds from oxidation are classified as enzymatic (e.g. superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic (e.g. ascorbic acid, GSH, and tocopherol) (Boots et al., 2008). In contrast to enzymatic antioxidants that catalyse oxidant removal, non-enzymatic antioxidants directly react with oxidants, donate an electron or hydrogen atom, scavenge the oxidant and are converted to a free radical in turn. Therefore, other antioxidants are needed to reduce free radicals produced from oxidation of antioxidants.

Accumulating data have shown antioxidant effects of polyphenols *in vitro* and *in vivo*. Among all polyphenols, flavonoids have been known as strong antioxidants, where the antioxidant capacity of some flavonoids is higher than vitamin E and vitamin C (Prior and Cao, 2000). The configuration and number of hydroxyl groups on the B ring is the most significant factor determining the ROS scavenging strength of flavonoids (Kumar and Pandey, 2013). However, the role of hydroxyl groups on the A and C ring also has some effects on the ROS scavenging ability of flavonoids. The *ortho*-dihydroxy (catechol) on the B ring (Figure 1.6) is the most efficient configuration to scavenge ROS, which is common in the structure of flavonoids and contributes to strong antioxidant effects compared to other polyphenols (Procházková et al., 2011).

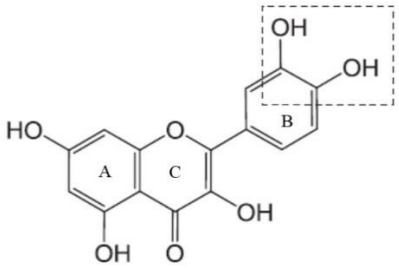


Figure 1.6. An ortho-dihydroxy structure on the B ring of flavonoids

Flavonoids protect cellular compounds from oxidative damage through multiple mechanisms. These include scavenging of ROS, chelating of metal ions, up-regulation and activation of antioxidant enzymes, direct effects on ROS generation, reduction of vitamin E radicals (α-tocopheryl), and inhibition of oxidases (Leopoldini et al., 2011; Procházková et al., 2011; Sun et al., 2009). Flavonoids work as antioxidants at two levels, either ROS removing or inhibiting ROS formation. In the first level, flavonoids remove ROS by direct scavenging or modulation of antioxidant enzyme activity, while in the second level they prevent ROS formation via metal chelating and inhibition of ROS producing enzymes (Sandoval-Acuña et al., 2014).

The ability of flavonoids to remove ROS is determined by their free radical scavenging ability as well as their ability to modulate expression of antioxidant enzymes. Polyphenols can react with free radicals such as superoxide and hydroxyl radical to scavenge ROS. This antioxidant property of polyphenols belongs to the benzene ring-bound hydroxyl groups, which can either donate a hydrogen atom or single electron to ROS. Alternatively, polyphenols are sometimes observed to up-regulate antioxidant enzymes (ROS-removing enzymes) such as superoxide dismutase, catalase and glutathione peroxidase (Na and Surh, 2008). Flavonoids up-regulate phase II enzymes through activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), resulting in up-regulation of genes controlled by the antioxidant response element (ARE) (Chiva-Blanch and Visioli, 2012; Na and Surh, 2008). Nrf2 translocation from the cytosol to the nucleus is inhibited by Kelch-like-ECH-associated protein 1 (Keap1), a cytosolic binder of Nrf2. Polyphenols directly, or as a conjugated form with GSH, can stimulate Nrf2 dissociation and translocation to nucleus. In the nucleus, Nrf2 binds to ARE to induce phase II enzymes expression (Kansanen et al., 2013).

The ability of flavonoids to inhibit ROS formation can occur through inhibition of the generation of ROS by redox-active transition metals such as iron and copper, influence of polyphenol redox

activities in mitochondrial membranes to reduce production of superoxide by complex I, II and III, (or by effects on mitochondrial biogenesis, as will be discussed later), and by inhibition of ROS-producing enzymes, such as xanthine oxidase (OX) and mitochondrial bound monoamine oxidase (MAO)(Lagoa et al., 2011; Sandoval-Acuña et al., 2014).

Several studies have investigated the effect of polyphenols on B[a]P-induced ROS and cancer. For example, quercetin (10 µM) significantly decreased ROS generation produced by co-exposure of B[a]P and UVA radiation in A539 epithelial cells (Woo et al., 2008). Catechins (epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) or epigallocatechin-3-gallate (EGCG)) significantly decreased ROS in human breast epithelial cell line MCF10A exposed to combined B[a]P and nicotine-derived nitrosamine ketone (NNK) (each 100 pmol/L) (Rathore et al., 2012). Silymarin showed protective effects against B[a]P-induced oxidative stress in peripheral blood mononuclear cells (PBMC) by modulating glutathione metabolizing enzymes and decreasing the formation of protein oxidation products and other ROS mediated damage (Kiruthiga et al. (2010)). As well, *in vivo* quercetin (25 mg/kg b/w twice a week starting one week prior to, or at the end of B[a]P administration) decreased lung and serum TBARS and reversed decreases in the levels of antioxidants (GSH, vitamin E and vitamin C) and activity of antioxidant enzymes (SOD, CAT, GPx, GST, GR) in B[a]P-induced lung cancer (Kamaraj et al., 2007). Fisetin significantly decreased lung and plasma lipid peroxidation, and restored levels of enzymatic and non-enzymatic antioxidants in B[a]P exposed animals. (Ravichandran et al., 2011).

1.9.3.4. Polyphenols and inflammation

There are extensive data showing anti-inflammatory effects of polyphenols. Polyphenols exert their anti-inflammatory roles through multiple mechanisms including (a) antioxidant and radical scavenging activities, (b) modulation of arachidonic acid metabolism (by regulating cyclooxygenase, phospholipase A2, and lipoxygenase) and nitric oxide synthase activity, and (c) modulation of gene expression and production of pro-inflammatory molecules (García-Lafuente et al., 2009).

Phagocytosis, a crucial event in inflammation performed by neutrophils and macrophages, is accompanied by superoxide anion production. In addition to ROS production, a high concentration of nitric oxide is synthesized by inducible nitric oxide synthase (iNOS) in macrophages. Nitric oxide reacts with superoxide and produces highly detrimental peroxynitrite, which has the ability

to oxidize LDL and cause irreversible damage to cell membranes and other components (Groot and Rauen, 1998; Haenen et al., 1997). Many studies have shown flavonoids to inhibit inflammation by protecting against ROS and nitric oxide generation (Cijo George et al., 2016; Pan et al., 2010).

Furthermore, many studies have shown that flavonoids have inhibitory effects on the activity of arachidonic acid metabolizing enzymes including phospholipase A2, cyclooxygenase (COX) and lipoxygenase which are involved in inflammation (García-Lafuente et al., 2009). In a cell-free assay, the impact of wines and wine constituents on COX-1, COX-2, and 5-lipoxygenase catalytic activity showed that red wine was a strong inhibitor of the activity of all three enzymes (20-94%), while white wine was identified as a strong inhibitor of 5-lipoxygenase (41-68%) but had no inhibitory effect on COX-1 and COX-2. *trans*-Resveratrol was identified as the strongest inhibitor of COX-1 and COX-2 (Kutil et al., 2014).

Gene expression regulation is one of the mechanisms by which flavonoids modulate inflammation. Flavonoids regulate cellular inflammation through several protein kinases, including protein kinase C (PKC) and MAPK, as well as transcriptional factors such as NF-κB and AP-1 that are involved in signal transduction (García-Lafuente et al., 2009). MAPKs link extracellular inflammatory signals to intracellular responses such as NF-κB. NF-κB regulates expression of multiple inflammatory mediators such as TNF-α, IL-6, IL-8, COX-2, and iNOS (Barnes and Karin, 1997). Several studies have shown an inhibitory impact of different polyphenols on the MAPK/NF-κB pathway (Gutiérrez-Venegas et al., 2014; Min et al., 2007; Nafees et al., 2015).

1.9.3.5. Anti-genotoxicity effect of polyphenols

DNA damage is a fundamental contributor to cancer by carcinogens. Carcinogens are a major cause of 4 leading cancers worldwide including lung, breast, colon and stomach cancers (Bernstein et al., 2008; Bernstein et al., 2013). Carcinogens including chemical carcinogens, ionizing radiation, ultraviolet (UV) radiation, and many pharmaceuticals attack both nuclear and mitochondrial DNA and cause several kinds of DNA lesions. The lesions may result in DNA mutations and genome instability which consequently induce tumorigenesis (Langie et al., 2015; Roos et al., 2015).

Many studies have shown anti-genotoxicity effects of different polyphenols (Azqueta and Collins, 2016; Eghbaliferiz and Iranshahi, 2016; Mazzoni et al., 2016). One of the well-studied carcinogens that polyphenols are able to protect against is B[a]P. Oral treatment with catechin for example was able to protect against B[a]P genotoxicity in mice (Shahid et al., 2016). Similarly, black soybean seed coat (4.85 µg/mL) and its main polyphenols, procyanidins and cyanidin-3-glucoside (10 µM), significantly decreased B[a]P-induced genotoxic effects in human hepatoma HepG2 cells and ICR mice. These polyphenols prevented DNA damage by decreasing micronucleus (DNA damage) and cytochrome P4501A1 (CYPA1A1) expression, as well as by enhancing glutathione S-transferases (GSTs) expression and DNA-binding activity of nuclear factor-erythroid-2-related factor 2 (Nrf2) (Zhang et al., 2013). Treatment with the polyphenol resveratrol (10-50 µM) also protected bronchial epithelial cells and animal lung (50 mg/kg/week) against bulky DNA formation and DNA damage induced by B[a]P (Berge et al., 2004; Revel et al., 2003). In addition to B[a]P, polyphenols also protect against DNA damage induced by other carcinogens such as triclocarban (TCC) (Sood et al., 2013) and arsenic (Rahman et al., 2009). In a chemoprevention study against arsenic carcinogenesis, treatment with green tea extract (10 mg/ml/100 g body weight/day for 28 days) decreased DNA fragmentation and reversed arsenic-induced DNA damage in the liver tissue of animals fed with sodium arsenite (0.6 ppm/100 g body weight/day)(Acharyya et al., 2015). Due to the increasing levels of environmental carcinogens in the last decades and their association with high prevalence of several types of cancer, the anti-genotoxicity role of polyphenols has become an intriguing area of chemoprevention. Different polyphenols show promising protective effects against several kinds of carcinogen-induced DNA damage.

1.9.3.6. Polyphenols and energy metabolisms

Several studies have shown the effects of different polyphenols on energy metabolism in cancer cells. Due to a high rate of glycolysis, cancer cells have a higher demand for glucose; thus, glucose uptake is significantly increased in cancer cells (Epstein et al., 2017). Polyphenols can inhibit glucose uptake through interfering with glucose transporters with subsequent effects on glycolysis (Moreira et al., 2013; Tan et al., 2016). This interference may involve down regulation of key glucose transporters such as glucose transporter 1 (GLUT1)(Azevedo et al., 2015). GLUT1 has been suggested as an oncogene due to its over-expression in most cancer cells (Furuta et al., 2010), and an association between over-expression of GLUT1 in cancer cells and proliferative index,

invasiveness, and poor prognosis. In addition to a direct effect on glucose transporters, polyphenols modulate energy metabolism in cancer cells through regulation of transcription factors and enzymes as well as molecular pathways involved in glycolysis (Keating and Martel, 2018). For example, HIF-1α has become an important target in cancer drug discovery, and polyphenols can down-regulate HIF-1α, and consequently suppress glycolysis (Jung et al., 2013; Wang et al., 2015; Wilson and Poellinger, 2002). Moreover, polyphenols inhibit glycolysis by regulating glycolytic enzymes such as hexokinase II (HKII) (Wang et al., 2015). For instance, EGCG, one of the most common catechins found in green tea, down-regulated protein expression of HKII in the Tca8113 and TSCCa human tongue squamous cell carcinoma cell lines and decreased glucose consumption and lactate production (Gao et al., 2015). Polyphenols can inhibit glycolysis by decreasing other glycolytic enzymes as well (Gomez et al., 2013; Takasawa et al., 2010).

Polyphenols can inhibit glycolysis also by regulating cell signaling pathways. Quercetin for example was shown to inhibit the PI3K/Akt pathway in lymphoma cells, and suppress growth (Maurya and Vinayak, 2015). The PI3K/Akt pathway is highly activated in tumor cells and is a major regulator of glycolysis. In normal cells PI3K is negatively controlled by the PTEN which loses its activation in cancer cells. Activation of the PI3K/Akt pathway increases glucose consumption by tumor cells and results in 90% conversion of pyruvate to lactate. Activation of this pathway also stimulates hexokinase II binding to the mitochondrial surface by its phosphorylation (Wallace, 2012).

Finally, in addition to glycolysis inhibition, induction of mitochondrial oxidative phosphorylation is considered a key mechanism involved in cancer prevention (Chattopadhyay et al., 2016; Keijer et al., 2011). Studies in cancer and non-cancer cells have shown that some polyphenols induce mitochondrial proteins, therefore, increase mitochondrial respiratory capacity (del Mar Blanquer-Rosselló et al., 2017; Gomes et al., 2012; Nichols et al., 2015).

1.9.3.7. Polyphenols and mitochondrial biogenesis

PGC-1α is a master regulator of mitochondrial biogenesis that regulates expression of NRF1 and EERα and subsequently TFAM and NRF2 (Jornayvaz and Shulman, 2010). Consequently, PGC-1α regulates transcription and replication of mtDNA and other genes involved in mitochondrial function. PGC-1α is deacetylated and phosphorylated by SIRT1 and AMPK, respectively

(Sandoval-Acuña et al., 2014). Accumulating evidence shows positive effects of different polyphenols on mitochondrial biogenesis (Csiszar et al., 2009; Davis et al., 2009; Grabacka et al., 2014; Lagouge et al., 2006). However, the role of polyphenols in mitochondrial biogenesis and cancer prevention is not well-studied.

Resveratrol is a well-studied polyphenol in this area and several studies have demonstrated roles of this stilbene in elevating mitochondrial biogenesis via activating the SIRT1/PGC-1α pathway. For instance, Lagouge et al., (2006) showed that dietary resveratrol (4 g/kg of food) in mice fed a high-fat diet, decreased muscle acetylated PGC-1α and increased expression and activity of PGC-1α through the SIRT1 pathway. In another study carried out by Csiszar et al., (2009), treatment of coronary arterial endothelial cells with 10 μM resveratrol significantly increased protein and activity of SIRT1, which resulted in increased mitochondrial mass as well as expression of PGC-1α, NRF-1, TFAM, and mitochondrial complexes, in a SIRT1-dependent manner. They also demonstrated their results in the aorta of type 2 diabetic mice given a 20 mg/kg dose of resveratrol for 4 weeks. Although one study in SW620 colon cancer cells showed increased mitochondrial mass and function without an increase in mtDNA (Blanquer-Rosselló et al., 2017), resveratrol has been observed to produce an increase in mtDNA copy number in other cells such as oocytes and neuroblastic PC12 cells (Peng et al., 2016; Sugiyama et al., 2015).

Quercetin and some other polyphenols also induce mitochondrial biogenesis. In mice quercetin induced expression and activity of SIRT1 and PGC-1α as well as mtDNA content and cytochrome *c* concentration in both skeletal muscle and brain (Davis et al., 2009). This was associated with improved maximal physical endurance capacity and prolonged exercise activity. Supplementation with quercetin has also been shown to increase exercise performance in humans (MacRae and Mefferd, 2006; Nieman et al., 2010), although increases in muscle markers of mitochondrial biogenesis (mtDNA, SIRT1, PGC-1α, citrate synthase, and cytochrome c oxidase) were not significant (Nieman et al., 2010). Resveratrol, quercetin, epigallocatechin gallate, genistein, daidzein, and apigenin increased mitochondrial biogenesis biomarkers such as TFAM, COX1 (complex IV subunit), CYB (complex III subunit), and mitochondrial content in a C2C12 muscle cell line (Yoshino et al., 2015).

Anthocyanins have shown different effects on mitochondrial biogenesis. Early *in vitro* studies showed that anthocyanins did not activate SIRT1 (even partially inhibiting) (Howitz et al., 2003). However, in a later *in vivo* study, purified anthocyanins from bilberry and black currant (1g purified anthocyanins per kg food) in mice with non-alcoholic fatty liver disease caused elevated mtDNA and expression of mitochondrial respiratory chain complexes, and increased activation of AMPK and expression of PGC-1α (Tang et al., 2015). In human umbilical vein endothelial cells (HUVECs), delphinidin increased expression of parameters involved in mitochondrial biogenesis (NRF1, ERRα, TFAM, and TFB2M), but did not increase mitochondrial respiration, enzyme activities or content of mtDNA (Duluc et al., 2014). The reason for the different effects of anthocyanins in different *in vitro* or *in vivo* studies is unclear. One possible explanation may be effects of anthocyanins themselves versus their breakdown products.

In summary, preventive/therapeutics roles of polyphenols have been shown in several diseases including cancer. However, preventive effects of polyphenols on mitochondrial changes such as decreasing mitochondrial biogenesis in early stages of carcinogenesis are not well studied. In the current study, we investigated the roles of different classes of polyphenols in B[a]P-induced carcinogenesis mainly through mitochondrial mechanisms.

CHAPTER 2: RATIONALE, HYPOTHESES AND OBJECTIVES

2.1. Rationale

Cancer is one of the major leading cause of mortality in Canada and is responsible for approximately 30% of annual deaths (Nuttall et al., 2016). Annually, cancer treatment imposes high expenses on society and mental stress on patients and their families. Therefore, cancer prevention has attracted the interest of researchers for many years and is considered as a possible approach to reduce risk of cancer development. Among tactics used for cancer prevention, chemoprevention is considered as a promising approach using natural or synthetic compounds to inhibit cancer initiation and promotion. The fundamental goal of prevention is to decrease cancer incidence by blocking or delaying the process of cancer development (Yao et al., 2011).

While much research has focused on mitochondria in cancer cells and in progression of transformed cells, less is known about mitochondrial changes in the early initiation and promotion stages of carcinogenesis and neoplastic transformation. Mitochondrial dysfunction is known as one of the main triggers of cancer development by initiating or exacerbating cancer hallmarks including evasion of apoptosis, abnormal metabolic pathways, genome instability, inflammation, and modulating the cell cycle and cell survival (Boland et al., 2013). Mitochondrial dysfunction also causes a shift in energy from oxidative phosphorylation to aerobic glycolysis (Warburg effect) which exacerbates cancer development (Warburg, 1956).

Accumulation of mutations in both mtDNA and nDNA-encoded mitochondrial genes as well as decreased number of mitochondria have been identified in different types of cancer (Brandon et al., 2006; Chattopadhyay et al., 2016; García-Ruiz et al., 2013; Taylor and Turnbull, 2005). Mutations in mitochondrial genes can perturb the electron transfer chain and increase reactive oxygen species (ROS) production (Bandy and Davison, 1990). The resulting mitochondrial dysfunction and cellular oxidative stress may contribute to nuclear DNA mutations and a shift in energy metabolism from oxidative phosphorylation to glycolysis. Due to the importance of mitochondrial dysfunction in carcinogenesis, targeting mitochondrial biogenesis may be important for cancer prevention.

A role for mitochondrial biogenesis in improvement of several diseases such as metabolic syndrome and cardiovascular disease has been previously reported (Ren et al., 2010). However,

not much is known regarding the role of mitochondrial biogenesis in cancer prevention. In non-cancer models, increased mitochondrial biogenesis results in induction of ROS-detoxifying enzymes and a reduced level of ROS production (Guarente, 2008; Kong et al., 2010). Decreased ROS can protect cells from further DNA damage and signal transduction that can lead to cancer development.

There are a few studies which have investigated the role of increased mitochondrial biogenesis in decreasing cancer progression (Bellance et al., 2009; Wang and Moraes, 2011), but none on its role in cancer prevention in the initiation and promotion phases of carcinogenesis. Since carcinogens that produce mitochondrial mutations may decrease the efficiency of oxidative phosphorylation and increase ROS production, elevated mitochondrial biogenesis could theoretically produce more functional mitochondria with efficient electron transfer chains (ETC) and low ROS generation, and thereby help prevent oxidative stress-mediated carcinogenesis. However, data showing the association between mitochondrial biogenesis and ROS generation in cancer prevention is lacking and needs to be elucidated.

Unrepaired DNA damage from carcinogens is a major cause of cancers worldwide including lung, breast, colon, and stomach cancers (Bernstein et al., 2013; Bernstein et al., 2008). Carcinogenic stresses including from chemicals, pharmaceuticals, ionizing radiation, ultraviolet (UV) radiation, inflammation and ROS cause damage to both nuclear and mitochondrial DNA and produce several kinds of DNA lesions. The lesions may result in DNA mutations and genome instability, which consequently result in cancer initiation and progression (Langie et al., 2015; Roos et al., 2015). Benzo[a]pyrene (B[a]P) is recognized as a group 1 carcinogen by the International Agency for Research on Cancer (IARC). Binding of B[a]P diol epoxide metabolites to DNA and formation of bulky adducts in DNA is the major mechanism of B[a]P-induced mutagenesis and carcinogenesis (Conney, 1982). In addition to nDNA, B[a]P also forms adducts in mtDNA and causes mtDNA damage and mutations (Backer and Weinstein, 1980; Backer and Weinstein, 1982; Jung et al., 2009). Studies have revealed that mitochondria are a major target of B[a]P, in part due to weak error excision repair systems (Lewis et al., 2001). Damage to mtDNA by a mutagen such as B[a]P may cause mitochondrial dysfunction which leads to aberrant ROS generation and increased aerobic glycolysis.

Experimental and epidemiological studies have demonstrated a direct association between consumption of fruits and vegetables and lowered risk of several cancers (Key, 2011; Van Duijnhoven et al., 2009). It has been estimated that approximately one-third of all cancer mortality in the US is preventable by appropriate modification of dietary habits (Liu, 2003). Therefore, using natural compounds has been suggested as an effective approach to cancer prevention. In this study, we focus on dietary polyphenols that have been of interest for their chemopreventive potential (He et al., 2008; Lall et al., 2015; Yang et al., 2008).

Polyphenols are a large group of phytochemicals naturally occurring in a wide range of plant foods and beverages including fruits, vegetables, cocoa, cereal, tea, wine, beer, and coffee, and are characterized by their hydroxylated phenyl groups (Cardona et al., 2013). Polyphenols from different groups and food sources may prevent carcinogenesis through multiple mechanisms including effects on signal transduction pathways leading to enhanced apoptosis, as well as inhibition of cell proliferation, cell invasion, angiogenesis, and metastasis (Yang et al., 2009; Zhou et al., 2016). Moreover, a great portion of anti-carcinogenesis effects of polyphenols is thought to be due to their anti-oxidant and anti-inflammatory properties (Hu, 2011).

Although antioxidant and anti-inflammatory effects of polyphenols have been widely investigated, their specific effects on mitochondria are less well known. One mitochondrial mechanism that has been investigated for anti-cancer (prevention or treatment) effects of polyphenols is induction of apoptosis (D'Archivio et al., 2008), although often this requires high concentrations. However, some other mitochondrial mechanisms by which polyphenols may prevent cancer initiation and promotion, such as effects on mitochondrial biogenesis or mitochondrial ROS generation are not well-understood. By increasing mitochondrial biogenesis and non-mutated copies of mtDNA, a polyphenol such as resveratrol may help minimize the effect of a mutagen such as B[a]P on mitochondria.

Although some polyphenols have been shown to prevent carcinogenesis in rodents and humans (Athar et al., 2007; Yang et al., 2009), it is not known which polyphenols are the most potent and through what molecular mechanisms they act to inhibit carcinogenesis. Flavonoids of interest in the current research are quercetin, found in many fruits and vegetables, catechin found in green tea and cacao, and anthocyanins (such as cyanidin, delphinidin and malvidin), found in red-purple berries. Other polyphenols of interest in the current study are the stilbene, resveratrol, notable in

grapes and red wine, and the isoquinoline alkaloid, berberine (metabolized to a polyphenol), found in the plants and berries of berberis species such as Oregon grape and barberry. According to previous studies, berberine and anthocyanins can be taken up by mitochondria to a greater extent than other polyphenols (Peng, 2012; Serafim et al., 2008). Anthocyanins also have ROS scavenging properties due to their phenolic structure (Wang and Stoner, 2008). Therefore, we hypothesize that anthocyanins and berberine may protect against carcinogenesis by decreasing mitochondria-derived oxidative stress. Other polyphenols such as resveratrol and quercetin are known as inducers of mitochondrial biogenesis (Baur, 2010; Howitz et al., 2003), and as explained above we hypothesize that the increased mitochondrial biogenesis may help decrease the mutagenic impact of B[a]P and resulting mitochondrial dysfunction and ROS generation.

Since cyanidin is a potent antioxidant and resveratrol is known as an inducer of mitochondrial biogenesis, the combination of these two polyphenols may have a combination effect on ROS generation. Therefore, we hypothesized that cyanidin and resveratrol will together decrease ROS generation more than either one alone through a direct effect on ROS generation and elevating mitochondrial biogenesis, respectively.

To investigate our hypotheses, we used Bhas 42 cells which are v-Ha-ras-transfected mouse embryo fibroblast (BALB/c 3T3 A31-1-1) cells. Bhas 42 cells are used to identify potential genotoxic and non-genotoxic carcinogenic chemicals and carcinogenesis mechanisms. Bhas 42 cells represent a multistage carcinogenesis model of tumor initiation and tumor promotion. Tumor initiator and promotor chemicals such as B[a]P and ROS induce transformation of the cells to a neoplastic phenotype (Asada et al., 2005; Sasaki et al., 2015). In the studies in this thesis therefore, I investigated in Bhas 42 cells the underlying mitochondrial mechanisms in B[a]P-induced carcinogenesis and prevention by different classes of polyphenols.

2.2. Hypotheses

The main hypothesis of this research is that different polyphenols such as resveratrol, quercetin, catechin, cyanidin, and cyanidin-3-glycoside (C3G), and the methoxyphenyl compound, berberine, will protect against B[a]P-induced cancer initiation and promotion in Bhas 42 cells with different effectiveness and mechanisms (in part through mitochondrial mechanisms). The detailed hypotheses of the study are:

1- B[a]P will cause increased mitochondrial and intracellular ROS in its process of carcinogenesis in Bhas 42 cells.
2- Berberine and anthocyanins will most effectively decrease mitochondrial ROS in B[a]P-induced neoplastic transformation.
3- Resveratrol and quercetin will prevent cancer initiation and promotion in part by inducing mitochondrial biogenesis.
4- Resveratrol and anthocyanins will cooperatively decrease oxidative stress by increasing mitochondrial biogenesis and decreasing oxidative stress.

2.3. Objectives

Using the Bhas 42 cell *in vitro* model of carcinogenesis, the three main goals are:

1- To determine the effect of B[a]P on intracellular and mitochondrial ROS generation during transformation of Bhas 42 cells.

2- To determine the relative effectiveness of the selected polyphenols and their mechanisms in protecting against B[a]P-induced changes including, oxidative stress, inflammatory mediator production, mitochondrial dysfunction, and neoplastic transformation

3- To determine the possible synergistic or cooperative antioxidant effect of resveratrol and anthocyanins on intracellular and mitochondrial ROS generation.

CHAPTER 3: POLYPHENOL INHIBITION OF BENZO[A]PYRENE-INDUCED OXIDATIVE STRESS AND NEOPLASTIC TRANSFORMATION IN AN *IN VITRO* MODEL OF CARCINOGENESIS

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This work was published as part of my PhD project. This manuscript answers some of the research questions in my PhD project, including effects on generation of intracellular ROS and mitochondrial superoxide as well as induction of neoplastic transformation by B[a]P and prevention by polyphenols.

Abbreviations

AP-1, activating protein 1; **ARE**, antioxidant response element; **B[a]P**, benzo [a]pyrene; **ETC**, electron transfer chain; **MAPK**, mitogen-activated protein kinases; **NFκB**, nuclear factor kappa b; **NQO1**, NAD(P)H quinone dehydrogenase 1; **Nrf2**, nuclear factor (erythroid-derived 2)-like 2; **PAH**, polycyclic aromatic hydrocarbons; **PBS**, phosphate-buffered saline; **ROS**, reactive oxygen species; **SOD2**, superoxide dismutase 2; **SRB**, sulforhodamine B; **TCA**, trichloroacetic acid; **TNF-α**, tumor necrosis factor-α1; **UCP2**, uncoupling protein 2.

Abstract

While dietary polyphenols are widely recognized for cancer-preventing characteristics, the relative effectiveness and mechanisms of action of different polyphenols is not clear. In the present study, we investigated the protective effects of six different polyphenols against benzo[a]pyrene (B[a]P)-induced oxidative stress and neoplastic transformation in the Bhas 42 cell carcinogenesis assay. All of the polyphenols completely prevented the increased intracellular ROS generation by B[a]P at 12h, and most inhibited after 3 days. B[a]P increased mitochondrial superoxide generation at 12h, which was inhibited by the anthocyanins and berberine. B[a]P increased expression of genes related to oxidative stress and inflammation (Nrf2, UCP2, and TNF-α) after 24h. Polyphenols strongly inhibited the increase in TNF-α and also several polyphenols inhibited the increase in UCP2. At 21 days after 72h treatment, B[a]P produced a large increase in the number of neoplastic colonies. This transformation was inhibited by resveratrol and quercetin, and strongly by resveratrol. In summary, all tested polyphenols were able to inhibit B[a]P-induced increases in markers of oxidative stress and inflammation, and to inhibit cellular transformation, with resveratrol being notable for the strongest preventive effect on cell transformation. The results support a role for dietary polyphenols in protecting against B[a]P-induced carcinogenesis.

Keywords: oxidative stress, mitochondrial reactive oxygen species, polyphenols, neoplastic transformation, benzo [a]pyrene, carcinogenesis

3.1. Introduction

Epidemiological and experimental studies indicate a role of diet in development of many cancers such as colon, breast, stomach, and prostate cancers (Boada et al., 2016; Di Sebastiano and Mourtzakis, 2014), while consumption of polyphenol-rich fruits, vegetables and beverages has been widely implicated in cancer prevention (Yang et al., 2009; Zanini et al., 2015). Two critical ways by which polyphenols may prevent cancer initiation and progression are by acting as anti-oxidant and anti-inflammatory agents (Lee and Lee, 2006).

Oxidative stress and inflammation are major contributors to cancer initiation and progression (Candido and Hagemann, 2013; Valko et al., 2006). Damage from reactive oxygen species (ROS) to biomolecules, particularly DNA, is one of the principal consequences of oxidative stress causing DNA mutations and genome instability (Kryston et al., 2011). Moreover, oxidative stress can initiate a cascade of cell signaling pathways that induce cell survival, cell proliferation, angiogenesis, and metastasis (Halliwell, 2007). One source of reactive oxygen species (ROS) in cells that has been linked to tumorigenesis is the mitochondrial electron transfer chain (Sabharwal and Schumacker, 2014). In addition, there is cross-talk between oxidative stress and inflammation, which can further accelerate the process of tumorigenesis (Candido and Hagemann, 2013).

B[a]P is a carcinogenic polycyclic aromatic hydrocarbon (PAH) found in wood and cigarette smoke and grilled foods (Kazerouni et al., 2001; Smith et al., 2000). In part of its carcinogenic mechanism, B[a]P is activated to metabolites which can form bulky DNA adducts and cause mutations. In addition, B[a]P has been observed to increase oxidative stress in cultured cells (Zhu et al., 2014) and *in vivo* (Briede et al., 2004). Mechanisms by which B[a]P may induce ROS are through formation of B[a]P quinone metabolites, which undergo redox-cycling and result in production of superoxide and other ROS (Briede et al., 2004) or through mutations or other changes to mitochondria which increase ROS generation and may persist to promote the process of neoplastic transformation (Bandy and Davison, 1990; Sabharwal and Schumacker, 2014).

B[a]P has been shown to induce neoplastic transformation in several *in vitro* systems , including in two-stage carcinogenesis transformation assays using BALB\c 3T3 cells (Li et al., 2015; Rowe et al., 2004; Sakai et al., 2003) and Bhas 42 cells (Sakai et al., 2010). The Bhas 42 assay has been developed to detect both tumor initiating and promoting activities (Asada et al., 2005). Because ROS have both initiating and promoting activities (Storz, 2005), this assay was chosen for testing

the effects of B[a]P and polyphenols on ROS generation and cellular transformation. While B[a]P has been observed to increase oxidative stress in some cell lines (Sigounas et al., 2010; Vijayaraman et al., 2012), a role for oxidative stress in B[a]P-induced transformation needs further investigation.

Several studies have reported preventive effects of different polyphenols on neoplastic transformation and carcinogenesis induced by B[a]P *in vivo* or *in vitro*. In animal studies, oral delivery of polyphenols including quercetin, catechin, naringenin, curcumin, resveratrol, and a green tea extract have shown protection against B[a]P-induced changes in lung or other tissues (Banerjee et al., 2016a; Cao et al., 2011; Kasala et al., 2015; Liu et al., 2015; Shahid et al., 2016). In *in vitro* studies of cell transformation using BALB/c 3T3 cells, protection has been reported against B[a]P from polyphenol-rich extracts of passion fruit and mango (Percival et al., 2006; Rowe et al., 2004), and against other carcinogens by a resveratrol-containing extract or quercetin (Müller et al., 2011; Sakai et al., 1990). Although these studies showed preventive roles of different polyphenols on B[a]P-induced carcinogenesis, it is not known which polyphenol is the strongest and by which mechanisms. Moreover, prevention by different polyphenols on neoplastic transformation through inhibiting oxidative stress has yet to be elucidated.

In this study, we used the Bhas 42 cell model to investigate the effects of B[a]P and different polyphenols on intracellular oxidative stress and neoplastic transformation. Objectives were to gain insight into the involvement of oxidative stress and related gene expression in neoplastic transformation by B[a]P, and to compare the protection by different polyphenols and the mechanisms involved. Therefore, we investigated the preventive effects of selected polyphenols of different classes that have shown promise as anticarcinogens in previous studies - resveratrol, berberine, and several classes of flavonoids (quercetin, catechin, and anthocyanins).

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Cyanidin and cyanidin-3-glucoside (C3G) were purchased from Extrasynthese (Genay, France). MitoSOX and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dyes were purchased from Invitrogen (Carlsbad, CA, USA). TRIzol reagent, VILO cDNA synthesis kit, and SYBR green master mix were purchased from Invitrogen (Carlsbad, CA, USA). RNAeasy mini

kit for isolating RNA was purchased from Qiagen (Germantown, MD, USA). All primers for qPCR were purchased from Integrated DNA Technology (Toronto, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (1 g/l) was purchased from Thermo Fisher Scientific (Bartlesville, OK, USA). All other reagents were purchased from Sigma-Aldrich, (St. Louis, MO, USA). The purity of polyphenols used in this study were as follows: resveratrol ≥ 99%, quercetin ≥ 95%, catechin ≥ 97%, cyanidin ≥ 96%, C3G ≥ 96%, and berberine ≥ 90%.

3.2.2. Cell culture

The Bhas 42 cells, which are v-Ha-ras-transfected mouse embryo fibroblast cells, were obtained from the JCRB cell bank (Japanese Collection of Research Bioresources Cell Bank) and were cultured in DMEM containing 5 mM glucose, 5% fetal bovine serum, and 1% penicillin/streptomycin at 5% CO_2 and 37ºC.

3.2.3. Intracellular ROS assay

The level of intracellular ROS was determined by using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). After seeding 20,000 Bhas 42 cells in a 96-well plate and growing for 24 hours, polyphenols at 5 µM were administrated in DMSO (final DMSO concentration 0.5 %). After 2h pre-treatment with polyphenols, 4 µM B[a]P was added and cells were incubated for 12-72h at 37 ºC. After this incubation, 10 µM H_2O_2 was administrated to some control wells as a positive control and incubated for 1h. Then DCFH-DA at 25 µM final concentration dissolved in DMEM without FBS was loaded to each well. After incubating for 30 min all the wells were cautiously washed twice with phosphate-buffered saline (PBS) and read at 480/20 nm excitation and 528/20 nm emission using a microplate reader (BioTek, Synergy HT). Images were captured by fluorescence microscope (Olympus IX 71) at 473/31 nm excitation and 520/35 nm emission. We also measured intracellular ROS after pre-treatment with polyphenols at 5 µM for 2h and 4 µM B[a]P for 4h followed by replacing the medium with fresh medium and measuring ROS after 72h.

3.2.4. Mitochondrial superoxide assay

The level of mitochondrial superoxide was measured using MitoSOX red fluorogenic dye. After seeding 20,000 Bhas 42 cells for 24 hours and pre-treatment with polyphenols at 5 µM for 2 hours, B[a]P at 4 µM was administrated and cells were incubated for 12 hours in 5% CO_2 at 37ºC.

Antimycin A (a cytochrome c reductase inhibitor) was added as a positive control and incubated for 30 minutes. Then, MitoSOX at 5 μM final concentration dissolved in DMEM without FBS was loaded to each well and plates were incubated for 30 minutes. Then wells were cautiously washed twice with PBS and read at 530±20 nm excitation and 590±20 nm emission using a microplate reader. Images were captured by a ZOE Cell Fluorescence Imager (Bio-Rad).

3.2.5. Cell proliferation assay

The sulforhodamine B (SRB) assay was conducted to observe the effect of polyphenols and B[a]P on proliferation of Bhas 42 cells. SRB is a bright pink dye which is used for anticancer-drug screening. Under a moderate acidic condition, SRB binds to protein residues and makes a sensitive index of cellular protein content that is linear with cell viability and cell proliferation (Vichai and Kirtikara, 2006). After seeding 20,000 Bhas 42 cells in 96 well-plates for 24 hours and pre-treatment with polyphenols at 5 μM for 2 hours, B[a]P at 4 μM was administrated and cells were incubated for 24 and 72 hours in 5% CO_2 at 37ºC. For fixation, cells were exposed to trichloroacetic acid (TCA) at 10% final concentration and kept at 4°C for 1 hour. After washing with running water, 100 μL of 0.057% (wt/vol) SRB solution was added to each well and the plate was kept at room temperature for 30 minutes. Then, wells were rinsed with 1% acetic acid to remove unbound dye. Plates were allowed to dry at room temperature, and then 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.

3.2.6. RNA extraction, RNA quantification, and cDNA preparation

To obtain RNA, $2x10^6$ cells for each treatment group were seeded in 6-well plates and incubated for 24 hours. Polyphenols at 5 μM were administrated for 2 hours, then 4μM B[a]P was added to all treated groups. The untreated group was treated with 0.5% DMSO, the same concentration of DMSO applied to other groups. After 24h exposure, cells were lysed with Trizol, 1 ml for each well. Cell lysates were transferred to microtubes and 200 μL chloroform was added to each. After centrifuging and adding 500 μL isopropanol, RNA was extracted using the RNeasy Mini Kit. A NanoVue (GE Healthcare Bio-Sciences Corp, USA) spectrometer was used to measure the amount and quality of RNA. The quality of yielded RNA was with A260/A280 ratio ~2. cDNA was

synthesized in a 20 µl reaction using the SuperScript VILO cDNA Synthesis Kit and a Bio-Rad Thermocycler.

3.2.7. Real-time polymerase chain reaction (PCR)

Real-time RT-PCR was conducted to analyze mRNA expression using the Applied Biosystems 7300 and SYBR green real-time PCR master mix, according to the manufacturer's protocol. Thermal cycling for PCR was as follows: One cycle at 95^{o}C for 5 min (for enzyme activation), followed by 40 cycles at 95^{o}C for 15 s, and at 60^{o}C for 30 s. Analysis of the results was performed by a comparative method ($2^{-\Delta\Delta CT}$) using beta actin as a reference gene. All results were normalized to the beta actin gene. Sequences of primers were used are as follows: B-actin (forward: CCT TCT TGG GTA TGG AAT CCT G, reverse: AGC ACT GTG TTG GCA TAG AG); UCP2 (forward: GGG TTC ATG CCT TCC TTT CT, reverse: GAG ATT GGT AGG CAG CCA TTA G); Nrf2 (forward: GGC TCA GCA CCT TGT ATC TT, reverse: CAC ATT GCC ATC TCT GGT TTG); SOD2 (forward: GTA GAG CCT TGC CTG TCT TAT G, reverse: AAA CCC AGA GGC ACC ATT AC); TNF-α (forward: GAG GCA CTC CCC CAA AAG, reverse: GGG TCT GGG CCA TAG AAC T).

3.2.8. Transformation assay

The transformation assay was conducted according to the protocol of Sakai et al., (2011) with slight modification. After seeding 40,000 cells in a 6-well plate and incubating them in 5% CO_2 at 37^{o}C for 24 hours, polyphenols at 5 µM were administrated to the medium. After 2h pre-treatment with polyphenols, 4 µM B[a]P was added and cells were incubated for 21 days. Medium was replaced with the fresh medium (without polyphenols or B[a]P) at days 3, 7, 11, and 14. On day 21 the cells were fixed with methanol and stained with 5% Giemsa's solution. The number of transformed foci (colonies) were scored by counting under the microscope (at least twice for each well) considering the following morphological characteristics: (a) more than 100 cells, (b) spindle-shaped cells different from the contact-inhibited monolayer cells, (c) deep basophilic staining, (d) random orientation of cells at the edge of foci, (e) dense multi-layering of cells and (f) invasive growth into the monolayer of surrounding contact-inhibited cells (Sakai et al., 2010). Colonies without these characteristics were recorded as normal colonies. For the transformation assay, two biological experiments with different batches of cells and polyphenols were conducted. In each experiment, one 6-well plate was assigned for each group.

3.2.9. Statistical analysis

Data were analysed by one-way ANOVA and significant differences between groups was determined by Tukey's post-hoc test and differences of $p<0.05$ were considered significant. Results are reported as means ± SEM of 2 - 3 independent experiments with 3 sample wells of cells per treatment condition per experiment; except for the transformation assay which was 6 sample wells of cells per treatment condition per experiment. Correlation was determined by Pearson's R.

3.3. Results

3.3.1. Effect of polyphenols and B[a]P on cell proliferation after 24h and 72h

Treatment of Bhas 42 cells with B[a]P at 4 μM gave a significant 20% increase in cell proliferation after 24h (Fig. 3.1A), but no significant effect after 72h (Fig. 3.1B). In the results after 24h, quercetin, catechin, and cyanidin blocked the effect of B[a]P by 100%. In the results after 72h the same three polyphenols plus C3G decreased cell proliferation significantly by 16-22% compared to the B[a]P alone condition.

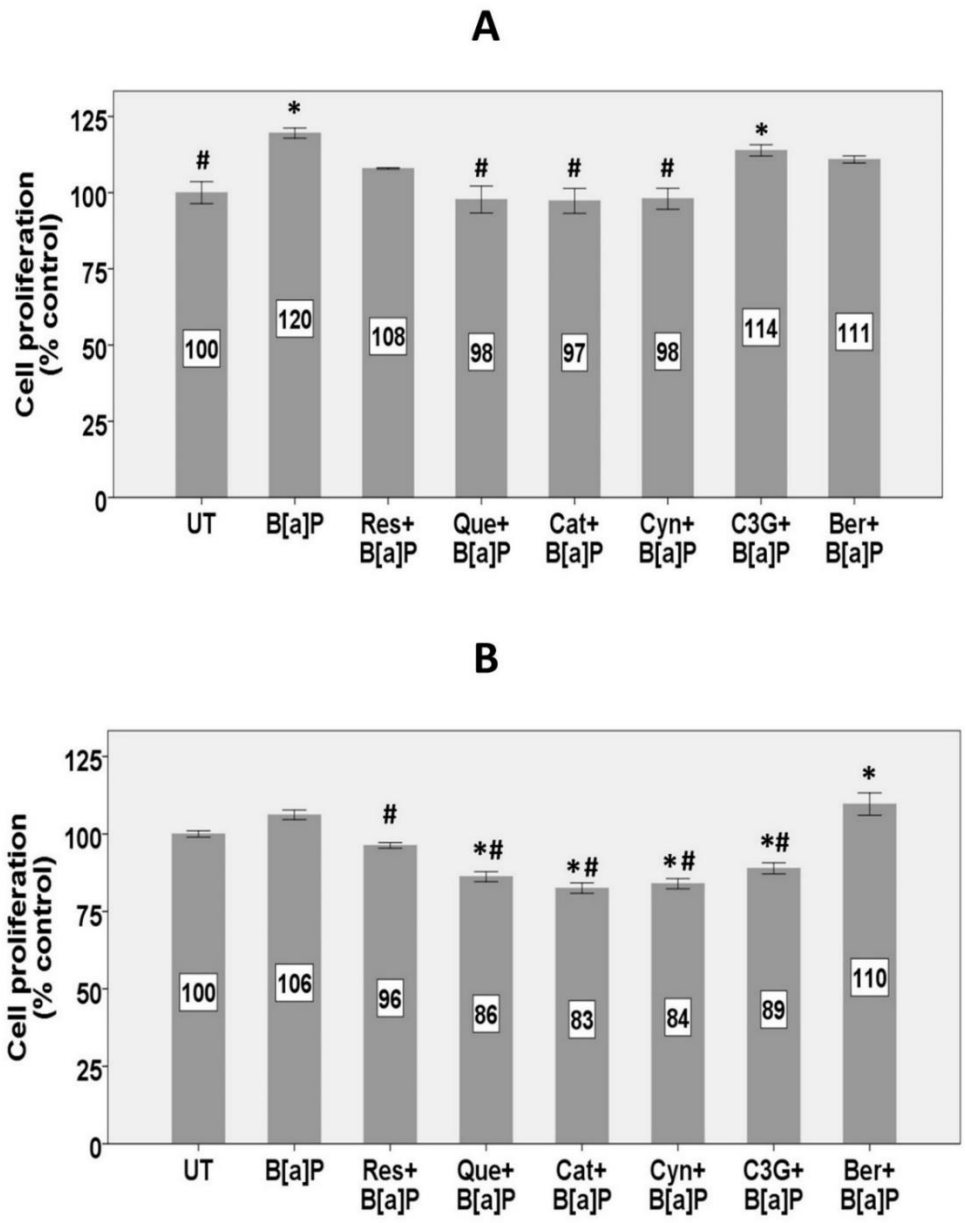
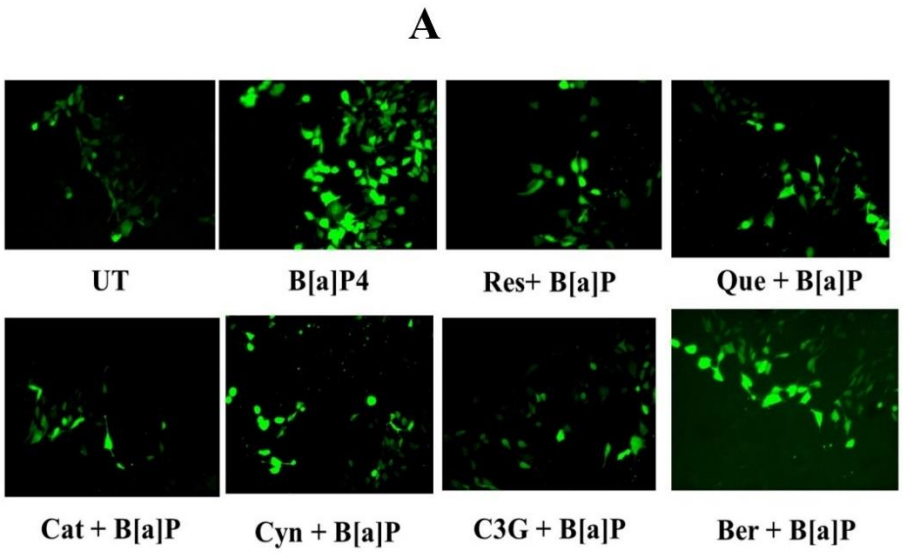


Figure 3.1. Effect of polyphenols on B[a]P-induced cell proliferation in Bhas 42 cells after 24 and 72 h.

Cell proliferation was measured in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P after (A) 24h and (B) 72h by the SRB assay. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$. UT, untreated cells; Res, resveratrol; Que, quercetin; Cat, catechin; Cyn, cyanidin; C3G, cyanidin-3-glucoside; Ber, berberine.

3.3.2. Effects of B[a]P and polyphenols on intracellular ROS and mitochondrial superoxide generation

Analysis of intracellular ROS for 12h shows that treatment of Bhas 42 cells with 4 µM B[a]P significantly increased ROS generation by 25% compared to untreated cells (Fig. 3.2A&B). The additional presence of all of the polyphenols significantly decreased the ROS levels compared to the B[a]P alone condition, to levels similar to the untreated control condition.



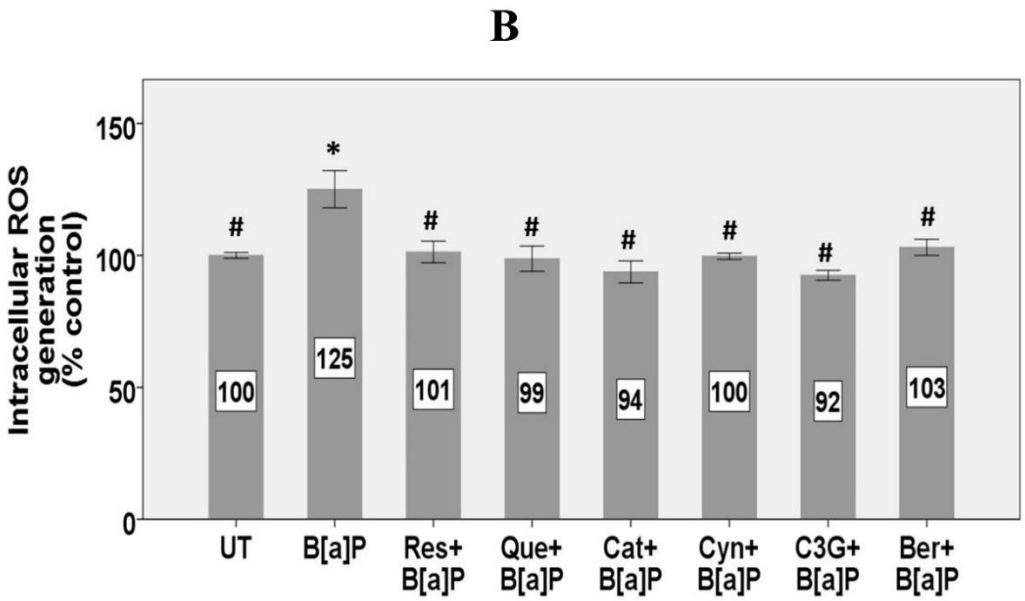


Figure 3.2. Effect of polyphenols on B[a]P-induced intracellular ROS generation after 12 h.

Intracellular ROS generation was measured using DCFH-DA, incubated for 30 min in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P for 12h. (A) Representative images of intracellular ROS generation captured by fluorescence microscope. (B) Quantification of intracellular ROS generation. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

Treatment with B[a]P for 72h induced ROS generation more than 200%, and all administrated polyphenols except cyanidin significantly inhibited this effect (Fig. 3.3A). C3G and berberine had the strongest effect, decreasing ROS by up to 76%, while resveratrol, quercetin, and catechin decreased ROS by ~ 60%. In the short-term exposure experiment, exposure to B[a]P for 4h increased ROS generation after 72h by 40% and polyphenols except resveratrol and quercetin significantly inhibited this effect (Fig. 3.3B). Cyanidin and berberine had the strongest effect and completely prevented the increase in ROS.

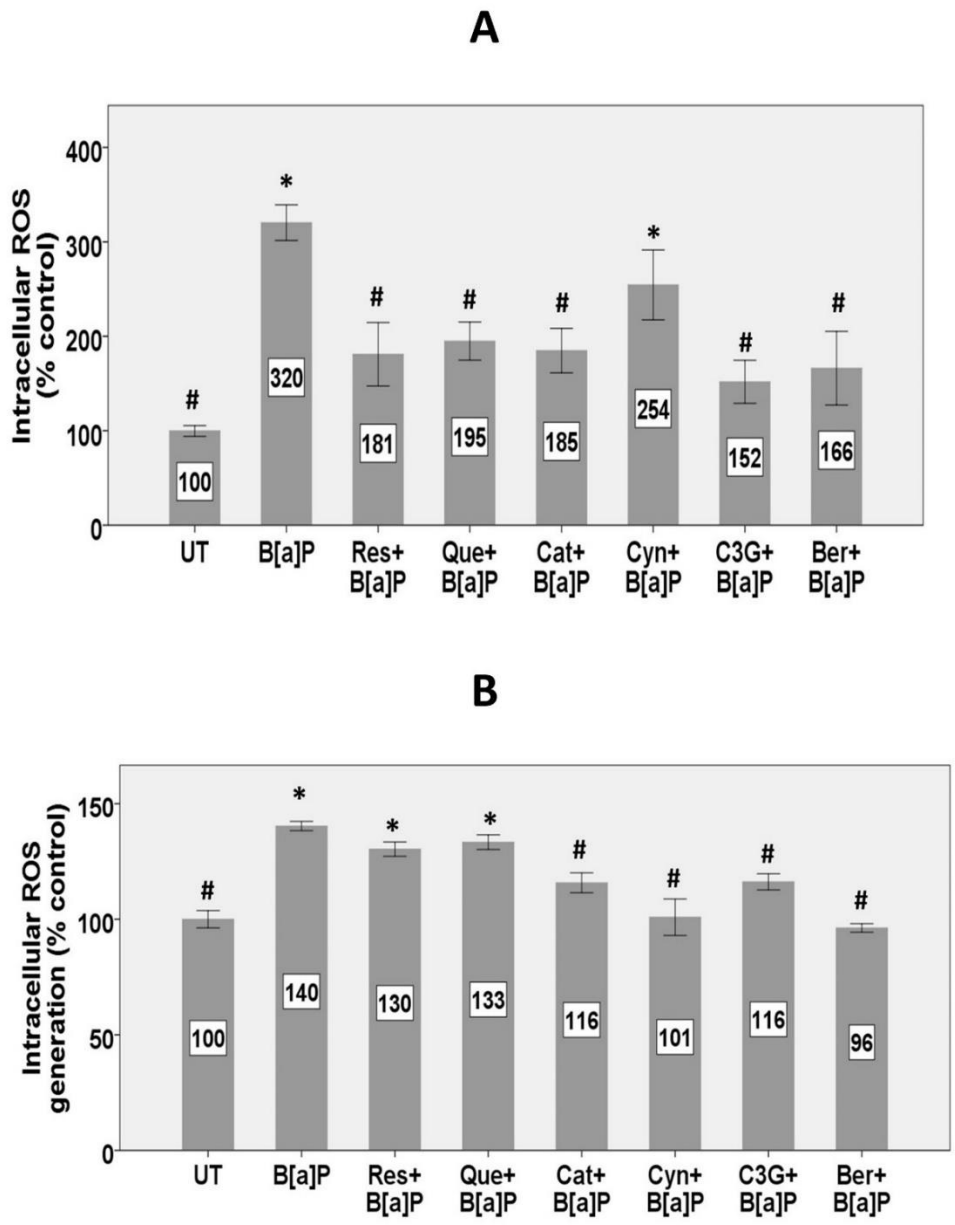
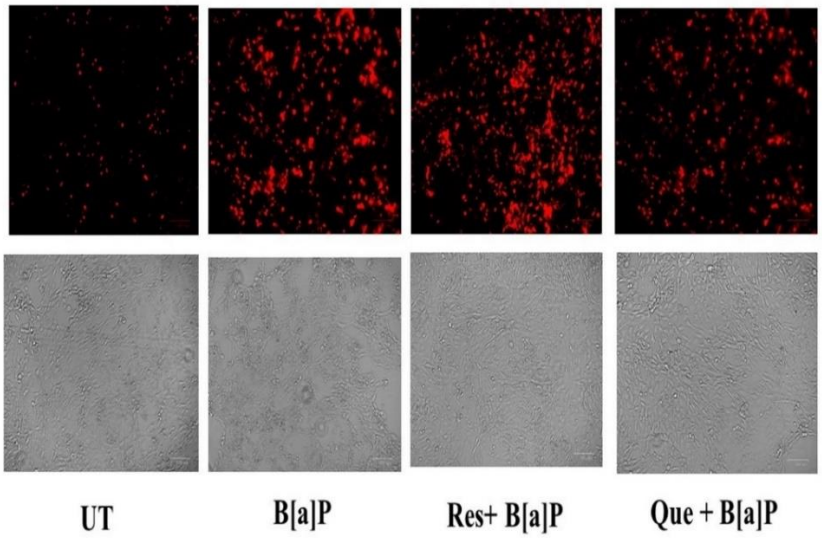


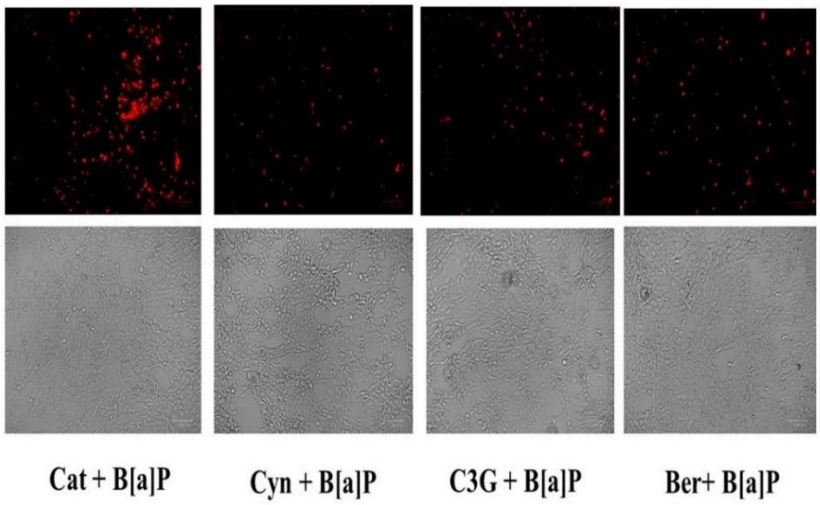
Figure 3.3. Effect of polyphenols on intracellular ROS generation with long- and short-term B[a]P exposure.

Intracellular ROS generation was measured with DCFH-DA in Bhas 42 cells after pre-incubation with polyphenols at 5 µM for 2h and (A) adding 4 µM B[a]P for 72h and (B) adding 4 µM B[a]P for 4h then replacing media with fresh media and measuring after 72h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

Treatment of Bhas 42 cells with 4 µM B[a]P significantly increased mitochondrial superoxide generation by 52% compared to untreated cells, measured 30 min after adding MitoSox probe (Fig. 3.4A&B). Among the polyphenols, cyanidin and C3G as well as berberine significantly decreased the B[a]P-induced mitochondrial superoxide by up to 73%.

A


UT
B[a]P
Res+ B[a]P
Que + B[a]P


Cat + B[a]P
Cyn + B[a]P
C3G + B[a]P
Ber+ B[a]P

B

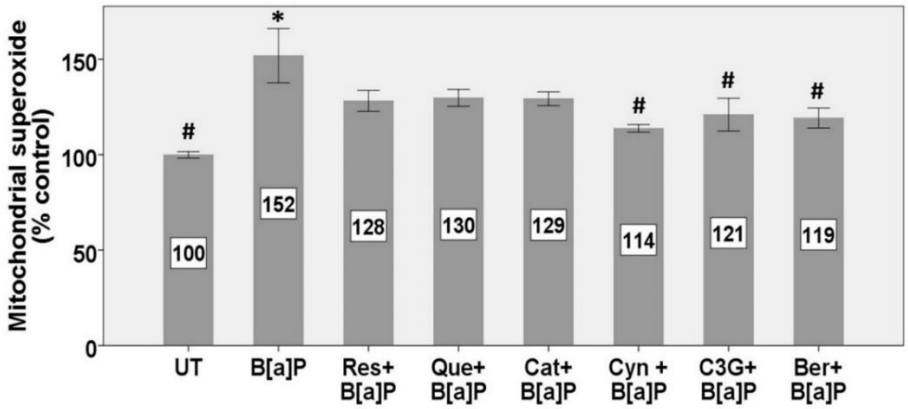

Mitochondrial superoxide (% control)
150
100
50
0
*
#
100
152
128
130
129
114
121
119
UT
B[a]P
Res+ B[a]P
Que+ B[a]P
Cat+ B[a]P
Cyn + B[a]P
C3G+ B[a]P
Ber+ B[a]P

Figure 3.4. Effect of polyphenols on B[a]P-induced mitochondrial superoxide generation after 12 h.

Mitochondrial superoxide was measured using MitoSOX Red, incubated for 30 min in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P for 12h. (A) Representative fluorescent and bright-field images of mitochondrial superoxide generation captured by ZOE Fluorescent Cell Imager. (B) Quantification of mitochondrial superoxide generation. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

3.3.3. Effects of B[a]P and polyphenols on gene expression

Treatment of Bhas 42 cells with B[a]P significantly up-regulated mRNA expression of the UCP2 gene by 70% after 24h compared to the untreated cells (Fig. 3.5A). Resveratrol, cyanidin, and berberine at 5 µM significantly inhibited B[a]P-induced UCP2 mRNA expression by 70, 87%, and 90%, respectively.

Analysis of gene expression showed that B[a]P significantly increased Nrf2 expression compared to untreated cells by 49% (Fig. 3.5B). None of the polyphenols significantly affected the expression compared to the B[a]P alone condition.

Treatment with B[a]P had no effect on expression of SOD2 (Fig. 3.5C). Among different polyphenols, quercetin and catechin increased expression of SOD2 by 68% and 63%, respectively.

Treatment of Bhas 42 cells with B[a]P increased mRNA expression of TNF-α by 269% after 24h (Fig. 3.5D). All administrated polyphenols strongly inhibited this pro-inflammatory effect, inhibiting by up to 86%.

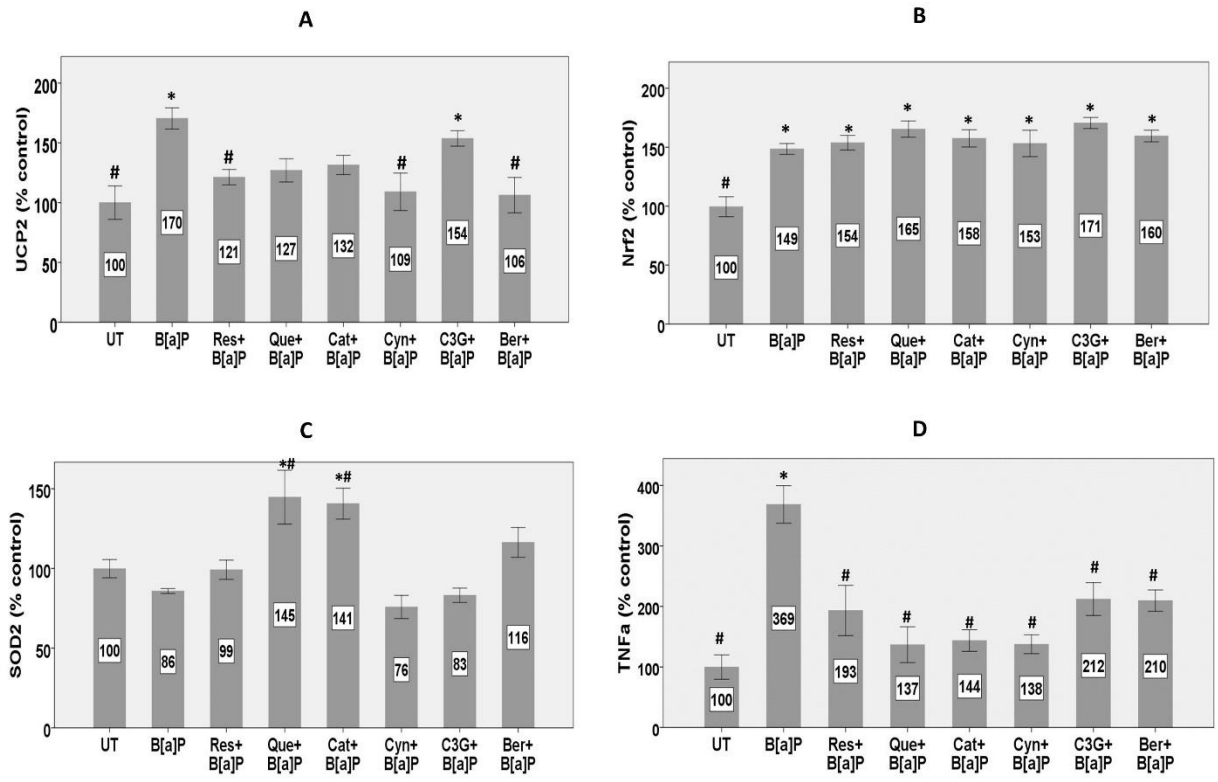


Figure 3.5. Effect of B[a]P and polyphenols on gene expression in Bhas 42 cells.

Gene expression was measured by RT-qPCR in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P for 24h. (A) UCP2, (B) NRF2, (C) SOD2, (D) TNF-α. Data are presented as a percentage of the untreated cells normalized to beta actin. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

3.3.4. Effects of B[a]P and polyphenols on neoplastic transformation

Figure 3.6A represents images of an invasive focus and different characteristics between a neoplastic focus and normal cells in the same image (the purpled ones are normal cells). Treatment of Bhas 42 cells with B[a]P for 21 days increased the number of neoplastic transformed foci (colonies) by 465% (Fig. 3.6B). Only resveratrol and quercetin significantly inhibited B[a]P-induced transformation. Resveratrol had the strongest inhibitory effect, inhibiting by 75%.

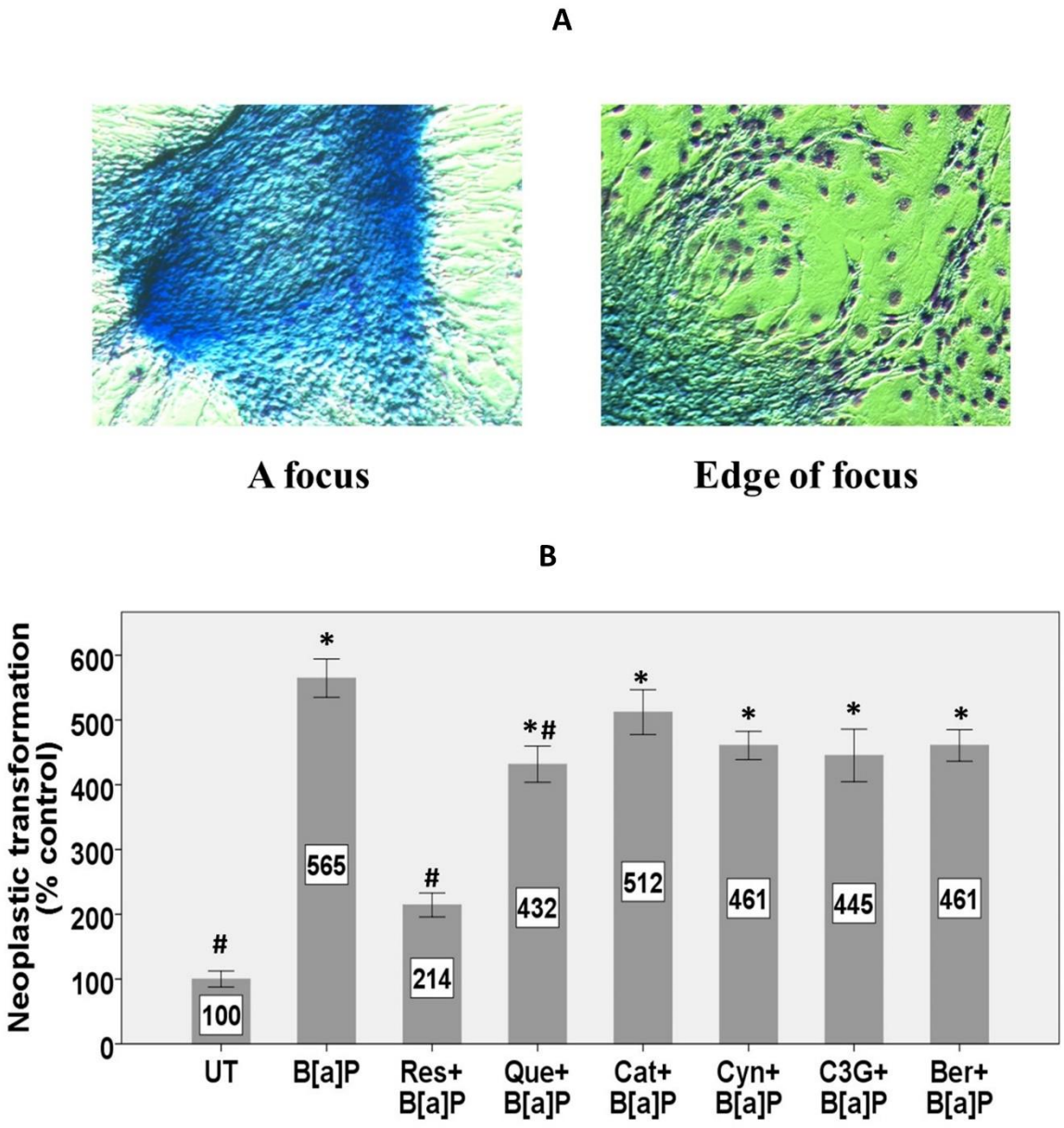


Figure 3.6. Effect of B[a]P and polyphenols on neoplastic transformation in Bhas 42 cells.

After pre-treatment with polyphenols at 5 µM for 2 h, followed by 4 µM B[a]P for 21 days. (A) Images of foci captured by fluorescence microscope at 100X and 400X magnifications. (B) Number of foci. The figure represents means ± SEM of 2 independent experiments with 6 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

3.3.5. Correlation results

Analysis of correlations between different studied biomarkers show that intracellular ROS has positive correlations with TNF-α expression (Fig. 3.7A), showing elevated oxidative stress by B[a]P induces expression of this pro-inflammatory cytokine ($r=0.46$, $p<0.01$). TNF-α expression also correlated with cell proliferation (Fig. 3.7B) ($r=0.62$, $p<0.01$). Expression of UCP2 was correlated with expression of TNF-α (Fig. 3.7C) ($r=0.47$, $p<0.01$).

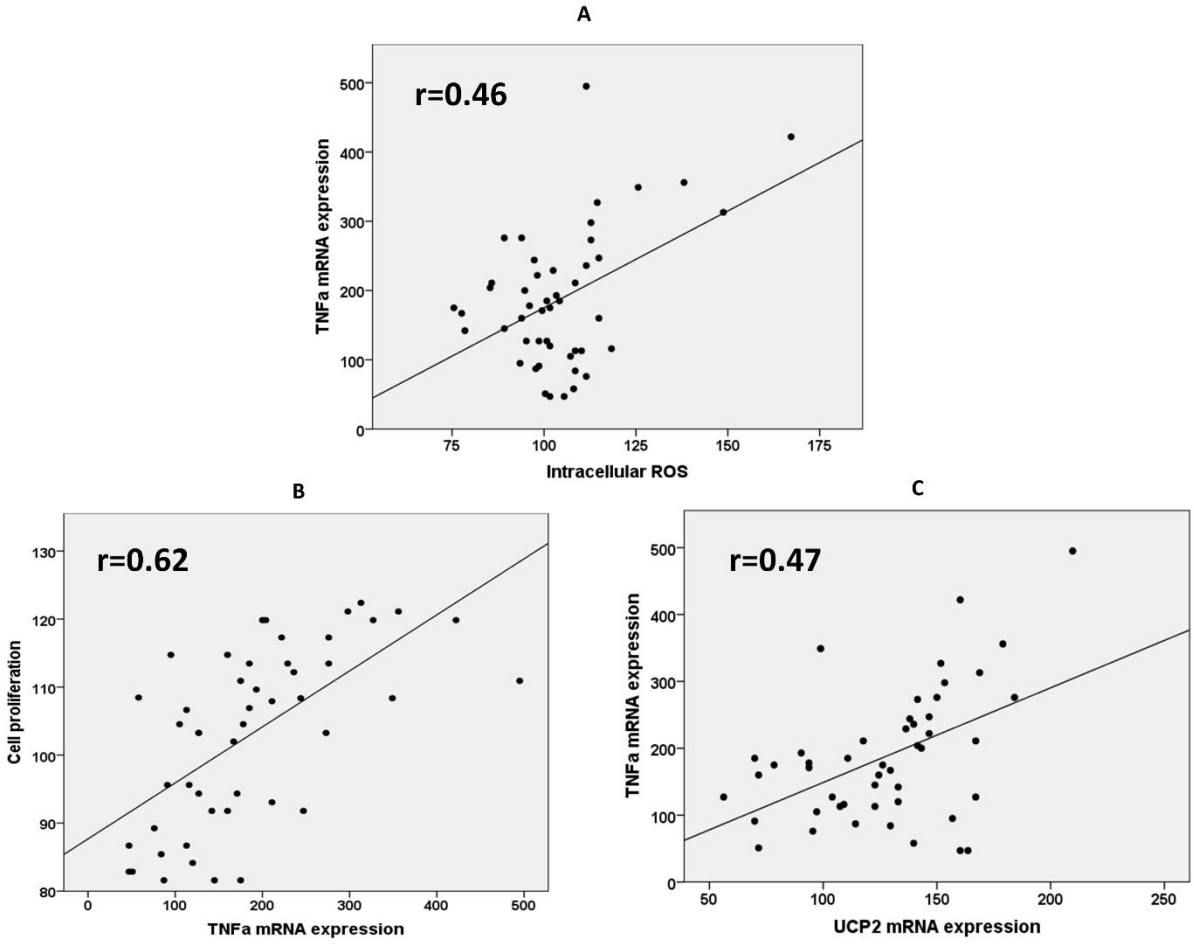


Figure 3.7. Correlations between different parameters of interest.

Correlations between (A) intracellular ROS and TNF-α mRNA expression (B) TNF-α mRNA expression and cell proliferation (C) UCP2 and TNF-α mRNA expression. The significant correlations between different parameters were tested with Pearson's R and significance was determined at $p<0.05$.

3.4. Discussion

In the current study, we used Bhas 42 cells to evaluate the effects of different polyphenols on B[a]P-induced changes in intracellular ROS generation, mitochondrial superoxide generation, gene expression (of SOD2, Nrf2, UCP2, and TNF-α), cell proliferation, and cellular transformation (formation of foci). These cells are transfected cells (BALB/c 3T3 cells transfected with the v-Ha-ras gene) and the model therefore represents one of progression to a neoplastic phenotype. This assay can detect both initiating and promoting carcinogens (Sakai et al., 2011).

After 12h, B[a]P increased intracellular ROS by 25% and after 72h, it increased intracellular ROS more than 200%. B[a]P has previously been found to produce oxidative stress *in vivo* , and in some cultured cells (Nkrumah-Elie et al., 2012). The current results show that B[a]P produces ROS generation in the Bhas 42 cell carcinogenesis system. Acting both in initiation and promotion (Valko et al., 2006), this increased ROS may have contributed to the increased neoplastic transformation observed after 21 days.

An objective of this study was to compare the ability of different polyphenols to inhibit B[a]P-induced oxidative stress and cell transformation. The polyphenols were from different classes and structures, and expectedly exert anti-oxidant effects to differing extents and by different mechanisms. However, they were all similarly effective in decreasing the B[a]P-induced intracellular ROS, eliminating the increase in detected ROS at 12h and all except cyanidin inhibiting by 57-76% at 72h. Different mechanisms by which the polyphenols might decrease the detected ROS include direct scavenging of ROS, induction of antioxidant enzymes, and decreasing the generation of ROS. Although the current study did not distinguish the mechanisms involved the net result was that the polyphenols were more similar than different in decreasing intracellular ROS.

We also evaluated the effects of B[a]P and polyphenols on mitochondrial superoxide. The mitochondrial electron transfer chain (ETC) is a major source of endogenous ROS that may participate in carcinogenesis by inducing nuclear and mitochondrial DNA mutations as well as cell signaling pathways (Sabharwal and Schumacker, 2014). Exposure of the cells to B[a]P for 12h increased mitochondrial superoxide generation by 52% compared to untreated cells. Although the mechanism behind this increased mitochondrial superoxide generation is not clear, B[a]P has previously been observed to increase mitochondrial superoxide generation in HaCaT keratinocytes

exposed *in vitro* to B[a]P for 48h (Das et al., 2016) and in oocytes after 7 day *in vivo* i.p. administration of B[a]P to mice (Sobinoff et al., 2012). Here certain of the polyphenols, anthocyanins and berberine decreased the B[a]P-induced generation of mitochondrial superoxide by 60-73%. The result with berberine is consistent with evidence showing that berberine is localised to mitochondria (Serafim et al., 2008). While not itself a polyphenol, berberine is demethylated to a phenolic metabolite in the intestine (Liu et al., 2009), and perhaps in the Bhas 42 cells. These results are also in agreement with previous studies showing mitochondrial anti-oxidative effects of berberine or its metabolites (Zhang et al., 2014; Zhang et al., 2015) and anthocyanins (Zhu et al., 2012).

One question about the B[a]P-induced ROS observed after 72h is whether the increase is due to the continual presence of B[a]P or to changes induced by B[a]P over a shorter term. To test whether a short-term exposure to B[a]P produced sustained effects on intracellular ROS generation, the cells were exposed for 4h to B[a]P (6h to polyphenols), after which the media was replaced and intracellular ROS generation was measured after 72h. With this short-term exposure, B[a]P increased generation of ROS at 72h by 40%. These data show that B[a]P produced intracellular changes that resulted in sustained ROS generation. All of the polyphenols except resveratrol and quercetin significantly inhibited this effect. These results identified a possible difference between different polyphenols. Some polyphenols, particularly cyanidin and berberine completely prevented the increase in ROS generation at 72h after short-term B[a]P exposure, suggesting that they exert acute effects against the B[a]P-induced changes. Since cyanidin and berberine were also the most effective against mitochondrial superoxide generation after 12h exposure, perhaps they most effectively inhibited B[a]P-induced changes to mitochondria. Other polyphenols such as resveratrol and quercetin were more effective when continually present for 72h.

Effects of B[a]P and polyphenols on expression of genes related to oxidative stress (Nrf2, UCP2, SOD2) and inflammation (TNF-α) were measured to gain more insights into the mechanisms involved. Nrf2 is a transcription factor that is activated and translocated to the nucleus under the presence of oxidative stress and induces expression of antioxidant enzymes (Ma, 2013). We found that B[a]P significantly increased Nrf2 gene expression by 49% compared to untreated cells. Nrf2 expression is regulated by aryl hydrocarbon receptor (AHR) activation (Miao et al., 2005). Since B[a]P is known as an activator of the AHR, it may explain the up-regulation of Nrf2 by B[a]P.

This result is consistent with a previous study that reported increased Nrf2 mRNA expression after treatment of Caco-2 cells with B[a]P (Niestroy et al., 2011). Unlike in this previous study however, where quercetin inhibited the effect of B[a]P (Niestroy et al., 2011), polyphenols had no effect on Nrf2 gene expression induced by B[a]P in the current study.

In expression of SOD2, 4μM B[a]P had no significant effect after 24 hours, while two of the polyphenols, quercetin and catechin induced it by 41-45%. SOD2 is a manganese-containing superoxide dismutase located in the mitochondrial matrix that has been strongly implicated in carcinogenesis and tumor development (Clair et al., 2005). SOD2 is regulated by different mechanisms, including mitochondrial biogenesis regulatory factors such as SIRT3 and PGC-1α. Down-regulation of SOD2 gene expression, along with mitochondrial dysfunction and increased ROS generation, by B[a]P has been previously described in human airway epithelial A549 cells (Min et al., 2011). In the current study, however, the induction of SOD2 by quercetin and catechin was not reflected in a significant decrease in the measured mitochondrial superoxide generation.

Another protein involved in modulating oxidative stress is UCP2, which is often overexpressed in cancer cells (Baffy, 2010). Formation of ROS by mitochondria induces expression of UCP2, a sensor and suppressor of mitochondrial ROS generation (Brand et al., 2004). In Bhas 42 cells, we found that B[a]P increased UCP2 expression by 70%, and some polyphenols inhibited this effect. ROS is a vital component for cancer cell progression due to inducing further genomic instability and ROS signaling pathways. On the other hand, excessive ROS could be toxic and leads to cell death. In cancer cells, overexpression of UCP2 acts as an adaptive response to protect cancer cells from excessive ROS and apoptosis (Derdak et al., 2008). Besides involvement in oxidative stress, UCP2 is involved in carcinogenesis through several other mechanisms including inhibiting oxidative phosphorylation and inducing glycolysis, increasing cell proliferation, cell invasion, cell transformation, and cachexia, and producing chemoresistance and poor prognosis of cancer (Derdak et al., 2008; Pons et al., 2015). Protecting against UCP2 expression shows that the administrated polyphenols have anti-carcinogen influences by abrogating this pathway of carcinogenesis. As a novel therapeutic target, inhibition of UCP2 has been recently suggested to be effective against cancer initiation and progression (Derdak et al., 2008). However, the role of UCP2 in cancer initiation and progression has not been well understood and more *in vitro* and *in vivo* studies are needed to elucidate other possible roles of UCP2 in cancer progression.

In the current study in Bhas 42 cells, B[a]P increased expression of TNF-α more than 200%, and all of the polyphenols, especially the flavonoids quercetin, catechin, and cyanidin, almost completely inhibited this effect. Up-regulation of TNF-α by B[a]P was shown previously in animal models (Huang et al., 2015). TNF-α plays an important role in carcinogenesis by producing inflammatory signals that induce cell survival, cell proliferation, angiogenesis, and metastasis (Wu and Zhou, 2010). The current result is consistent with previous studies in human lung fibroblasts (Chen et al., 2016) and A549 alveolar cells showing that quercetin inhibited production of pro-inflammatory cytokines such as IL-6 and TNF-α induced by B[a]P or B[a]P diol epoxide (Günay et al., 2016).

The protective effects of the polyphenols on TNF- α expression may reflect the decreases in intracellular ROS. Flavonoids exert anti-inflammatory effects by inhibiting ROS-induced activation of transcription factors such as NF-kB and activating protein 1 (AP-1) that induce expression of inflammatory cytokines such as TNF-α and interleukins (Leyva-López et al., 2016). TNF-α and ROS act in carcinogenesis in a coordinated manner (Blaser et al., 2016). Therefore, inhibiting either one abrogates the vicious cycle and its involvement in carcinogenesis.

In the current study, treatment with B[a]P mildly but significantly increased cell proliferation in Bhas 42 cells compared to untreated cells. This increased proliferation coincided with increased ROS, which at sub-lethal levels is known to stimulate cell proliferation (Reuter et al., 2010). This result is consistent with a previous observation (Burdick et al., 2003) of increased ROS generation and proliferation of breast epithelial cells exposed to B[a]P. Therefore, this process may be part of the promotion of neoplastic transformation. Among administrated polyphenols, resveratrol, quercetin, catechin, and cyanidin significantly inhibited this effect.

Ultimately, we investigated the preventive effects of polyphenols on carcinogenesis induced by B[a]P in a 21-day transformation assay. Similar to previous studies in Bhas 42 cells (Sakai et al., 2010) and other cell lines (Bersaas et al., 2016), B[a]P stimulated neoplastic transformation in Bhas 42 cells and strongly increased the number of foci (colonies) compared to untreated cells. Carcinogenicity of B[a]P involves complex mechanisms, including formation of bulky DNA adducts and gene mutations (Liu et al., 2015), induction of oxidative stress (Vijayaraman et al., 2012), inflammation (Shahid et al., 2016), cell proliferation (Burdick et al., 2003), and cell

signaling pathways (Jiao et al., 2008). A few previous studies have reported protective effects of individual polyphenols or polyphenol-rich extracts against neoplastic transformation induced by B[a]P or other carcinogens in cultured cells (Kao et al., 2007; Xue et al., 2001). Comparing different polyphenols in the current study, resveratrol and quercetin significantly decreased the number of formed foci. Resveratrol was revealed as the notably strongest polyphenol in protecting against neoplastic transformation in this assay. Since there was no difference between resveratrol and other administrated polyphenols in measurements of ROS or gene expression at earlier time points, it may have been a delayed effect of resveratrol or an effect on other non-measured parameters that produced such a strong effect in the transformation assay.

Other possible mechanisms by which resveratrol may inhibit formation of neoplastic colonies *in vitro* and tumors *in vivo* have been previously reported. These include effects on drug metabolising enzymes, such as inhibiting expression of CYP1B1and inducing NQO1expression (Lu et al., 2008) which may influence carcinogen metabolism, or effects on cell signaling such as decreasing expression of the MAPK family and increasing expression of P53 (George et al., 2011). Therefore, influences on other pathways involved in carcinogenesis is a possible reason that resveratrol showed the strongest effect in inhibiting formation of neoplastic transformation in the current study. Further investigations are needed to understand the special effect of resveratrol in preventing neoplastic transformation in the current study.

Several correlations were identified in the current study. Consistent with other studies (Reuter et al., 2010), we found a correlation between intracellular ROS and TNF-α gene expression. ROS can induce inflammation by activating NF-kB and up-regulating cytokine expression (Gloire et al., 2006). In turn, leukocytes and mast cells are recruited to the damage site and generate further ROS. Moreover, in non-inflammatory cells pro-inflammatory cytokines including TNF-α and IL-1β have been observed to increase intracellular NO and mitochondrial superoxide generation, induce damage to mitochondrial DNA, and disturb mitochondrial function (Kim et al., 2010). Therefore, ROS and inflammatory cytokines such as TNF-α are intricately involved in tumor promotion.

Furthermore, we found positive correlations between TNF-α and cell proliferation and between UCP2 and TNF-α. These correlations may reflect a common influence of increased intracellular

ROS, which can stimulate cell proliferation as well as increase cytokine expression (Reuter et al., 2010). A correlation between UCP2 and TNF-α expression was described in previous studies (Wang et al., 2016).

In conclusion, the present study revealed that polyphenols protected against oxidative stress and neoplastic transformation induced by B[a]P in Bhas 42 cells. The results suggest that dietary polyphenols may inhibit B[a]P induced oxidative stress and inflammation by protecting against increased mitochondrial superoxide generation, intracellular ROS generation, TNF-α expression, and UCP2 over-expression. Figure 3.8 illustrates a summary of the results. Together these effects help decrease cell proliferation, and neoplastic transformation induced by B[a]P. Additional studies are needed to further elucidate mechanisms by which dietary polyphenols, especially resveratrol, act as anti-carcinogens to protect against cancer initiation and progression.

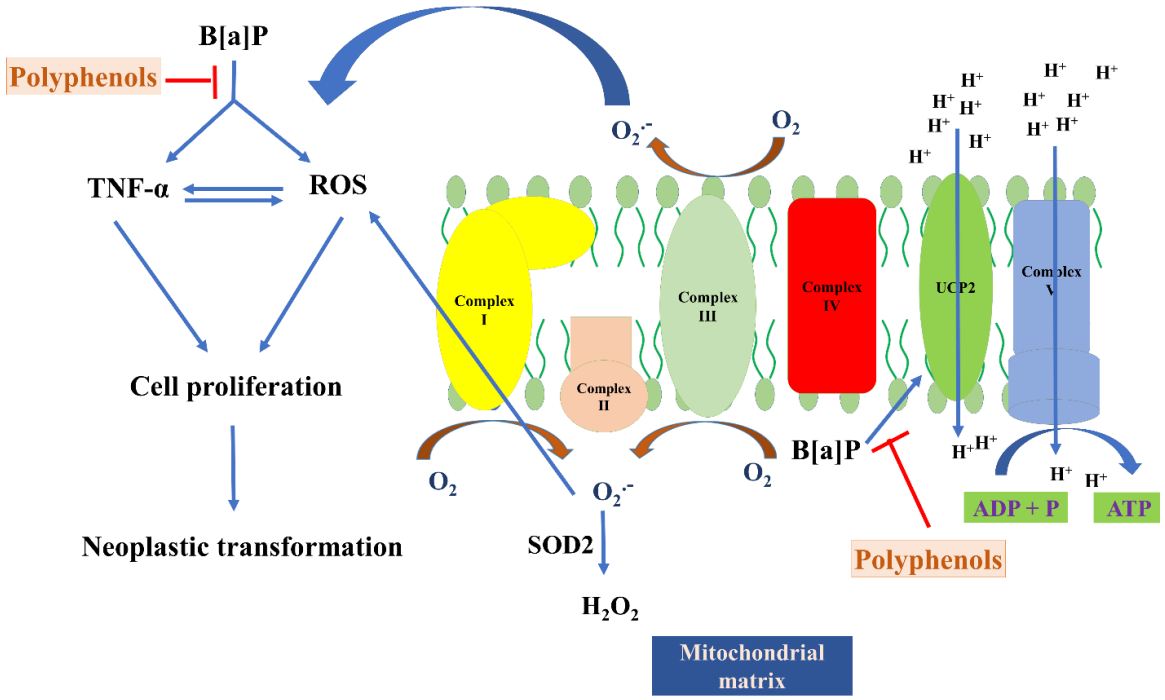


Figure 3.8. Schematic representation of molecular mechanisms by which polyphenols prevent neoplastic transformation induced by B[a]P in Bhas 42 cells.

B[a]P induces neoplastic transformation by stimulating mitochondrial and intracellular ROS production, up-regulating TNF-α and UCP2, and increasing cell proliferation. Polyphenols protected against these effects of B[a]P, up-regulated SOD2 expression, and decreased cellular transformation.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

3.5. Transition to CHAPTER 4

In CHAPTER 3 we investigated oxidative stress and cellular transformation induced by B[a]P and preventive effects of different polyphenols on B[a]P-induced oxidative stress and neoplastic transformation. The results showed that B[a]P increased intracellular ROS generation and polyphenols prevented B[a]P-induced intracellular ROS to a similar extent. B[a]P also increased mitochondrial superoxide generation, and only polyphenols with more positive charges (anthocyanins and berberine) decreased mitochondrial superoxide. B[a]P also strongly induced TNF-α expression and all polyphenols inhibited this effect. We also observed that B[a]P significantly induced neoplastic transformation in Bhas 42 cells while only resveratrol and quercetin protected against this effect of B[a]P, and resveratrol protected most strongly. One question from CHAPTER 3 is whether the observed inhibitory effects of the polyphenols may have occurred through decreased expression of cytochrome P450 drug metabolizing enzymes that activate B[a]P to mutagenic metabolites. In CHAPTER 4 I report the effects of B[a]P and polyphenols on expression of mRNA for the cytochrome P450 drug metabolizing enzymes CYP1A1 and CYP1B1.

CHAPTER 4: EVIDENCE AGAINST AN INVOLVEMENT OF ARYL HYDROCARBON RECEPTOR (AHR) IN POLYPHENOL INHIBITION OF BENZO[A]PYRENE-INDUCED OXIDATIVE STRESS AND NEOPLASTIC TRANSFORMATION

Results published in *Food and Chemical Toxicology* (2017)

(Letter to the Editor)

Food Chem Toxicol. 2017 Sep;107(Pt A):526-527. doi: 10.1016/j.fct.2017.07.028

In a letter to editor, published in the Journal of Food and Chemical Toxicology, in response to a letter regarding a possible down-regulation of P450 enzymes by polyphenols in preventing effects of B[a]P, we showed that the effects of our studied polyphenols were not through an AhR-antagonist effect.

4.1. Introduction

Some cytochrome P450 drug metabolizing enzymes (CYP450) have been identified which in metabolizing carcinogens also bioactivate them to reactive metabolites leading to DNA damage and mutations (Nebert and Dalton, 2006). CYP450 enzymes can metabolize polycyclic aromatic hydrocarbons such as B[a]P to their mutagenic metabolites which can form bulky DNA adducts and provoke neoplastic transformation. CYP1A1 and CYPB1 are two primary enzymes that are known to participate in metabolizing B[a]P to mutagenic metabolites (Harrigan et al., 2006). B[a]P exposure increases expression of these enzymes by binding to and activating the aryl hydrocarbon receptor (AhR), which induces expression of CYP1A1 and CYP1B1 (Shiizaki et al., 2017).

Extensive experimental studies have revealed the role of different polyphenols in modulating CYP enzymes. Polyphenols show positive or negative effects on CYP450 enzymes depending on their structure and the model of study. Polyphenols exert their effects through either direct interaction with CYP450 enzymes or by regulating gene expression. For instance, polyphenols can modulate gene expression through the pregnane X receptor (PXR) or AhR, two receptors that induce expression of detoxifying enzymes (Korobkova, 2015; Krizkova et al., 2008).

We previously showed that polyphenols prevent B[a]P-induced oxidative stress and neoplastic transformation in Bhas 42 cells, which could occur by different mechanisms. One plausible mechanism by which polyphenols could exert these effects is by inhibiting expression of CYP450 enzymes that activate B[a]P. There is no study which measured levels of the CYP450 enzymes in Bhas 42 cells, and data regarding effects of polyphenols on changes in these enzymes after B[a]P treatment are limited. Therefore, we measured the effects of B[a]P and polyphenols on mRNA expression of CYP1A1 and CYP1B1 in Bhas 42 cells.

4.2. Materials and Methods

4.2.1. Chemicals and reagents

See section 3.2.1

4.2.2. Cell culture

See section 3.2.2

4.2.3. RNA extraction, RNA quantification, and cDNA preparation for qPCR

See section 3.2.6

4.2.4. Real-time polymerase chain reaction (PCR)

See section 3.2.7

4.2.5. Statistical analysis

See section 3.2.9

4.3. Results

Treatment of the Bhas 42 cells with 4 μM B[a]P significantly increased mRNA expression of CYP1A1 and CYP1B1 by 393-fold and 5-fold, respectively compared to untreated cells (Fig. 4.1. A&B). None of the polyphenols decreased expression of CYP1A1, and resveratrol, quercetin, catechin, and berberine further increased expression of CYP1A1 mRNA over that induced by B[a]P alone by up to 50%. None of the polyphenols had any effect on expression of CYP1B1.

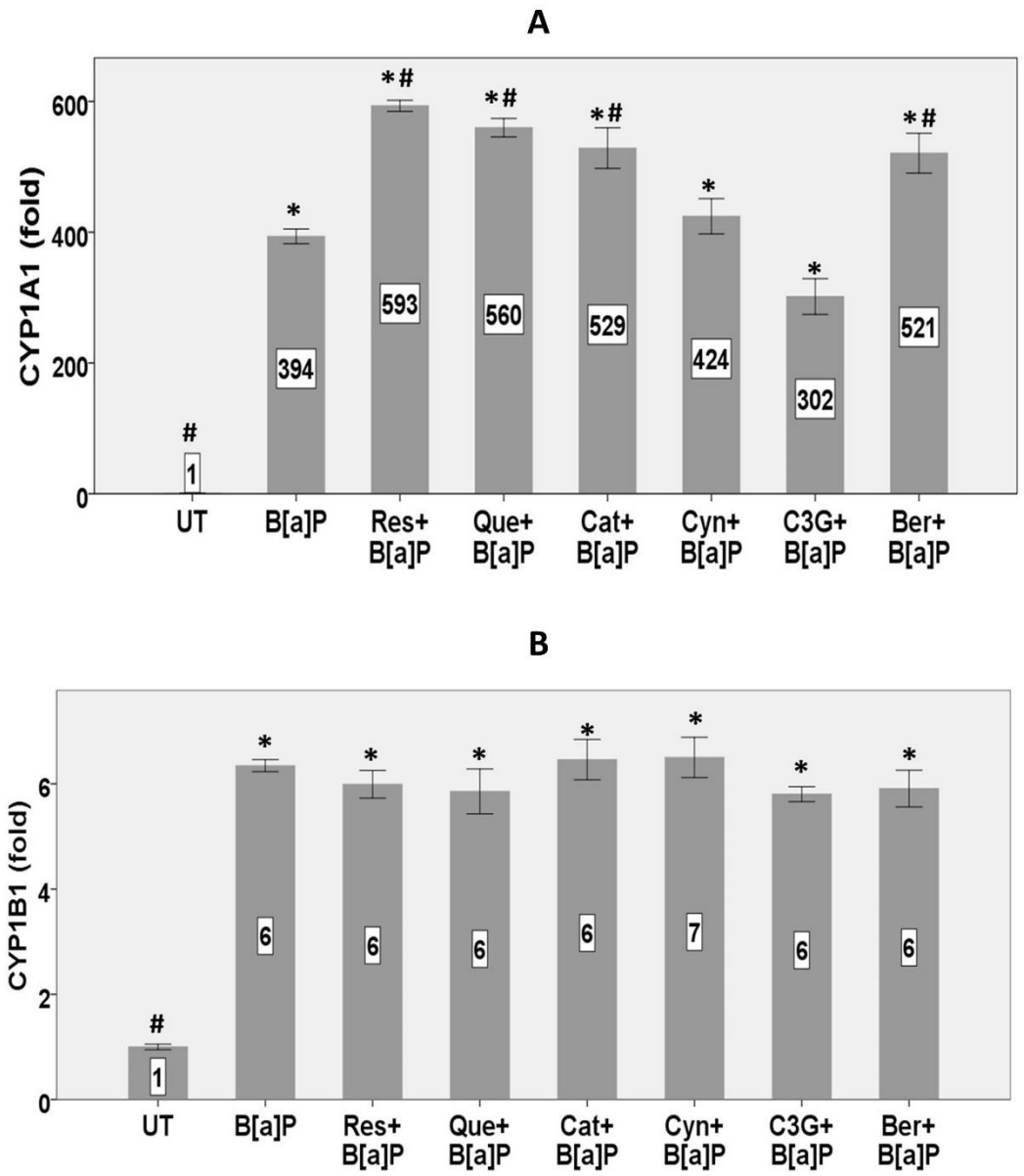

Figure 4.1. Effect of B[a]P and polyphenols on CYP1A1 and CYP1B1 gene expression in Bhas 42 cells.

CYP1A1 (**A**) and CYP1B1 (**B**) gene expression were measured by qPCR in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P for 24h. Data are presented as a percentage of the untreated cells normalized to beta actin. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

4.4. Discussion

In the current study we measured mRNA expression of CYP1A1 and CYP1B1 to understand mechanisms behind the preventive effects of polyphenols against B[a]P-induced oxidative stress and neoplastic transformation. Under the conditions of these experiments therefore, the results do not support a mechanism involving antagonism by the polyphenols of AhR activation for the observed findings. Polyphenols have been found as both agonists and antagonists of the AhR. Multiple studies have demonstrated different polyphenols to inhibit induction of CYP450 enzymes by other AhR ligands. For example, Andrieux et al. (2004) showed that resveratrol at 100 μM acted as an AhR antagonist against a novel AhR agonist and inhibited induction of CYP1A1 expression in rat primary hepatocytes. Other studies have shown resveratrol to antagonise AhR mediated CYP450 induction by dioxins (Casper et al., 1999) or B[a]P (Revel et al., 2003). However, there are some studies showing AhR-agonist activity of polyphenols. In immortalized human keratinocytes (HaCaT) and HepG2 hepatocytes, quercetin, resveratrol, and curcumin at 1, 5, and 10 μM induced AhR-mediated CYP1A1 activity (Mohammadi-Bardbori et al., 2012). Resveratrol has been shown to be an AhR agonist alone, but to antagonise CYP450 protein expression by a dioxin AhR agonist (Lee and Safe, 2001; Pastorková et al., 2017). The expression of CYP450 enzymes is regulated through several pathways including translocation of the AhR to the nucleus and binding to the response elements in the gene, and polyphenols have been suggested to affect all of these pathways. AhR-agonist or antagonist activity of polyphenols can be determined by showing the ability of direct interactions with the CYP450 enzyme, or by regulating any step of CYP450 expression process (Korobkova, 2015). In summary, we showed that some of the polyphenols of our study such as resveratrol, quercetin, catechin, and berberine augmented induction of CYP1A1 mRNA by B[a]P and all the polyphenols had no effect on the B[a]P-induced expression of CYP1B1 mRNA.

In conclusion, the results of our previous study showing preventive effect of polyphenols against B[a]P-induced oxidative stress and neoplastic transformation are not due to inhibiting induction of mRNA for enzymes involved in B[a]P metabolism and activation.

4.5. Transition to CHAPTER 5

In CHAPTER 4 we investigated a possible inhibitory effect of polyphenols on B[a]P metabolism by measuring mRNA for CYP1A1 and CYP1B1, two enzymes which primarily metabolize B[a]P to its mutagenic metabolites. We observed that not only did polyphenols not inhibit induction of these enzymes, but some of the polyphenols significantly increased the expression of CYP1A1 over that of B[a]P alone. In CHAPTER 5, to further explore mechanisms behind protective effects of polyphenols on B[a]P-induced neoplastic transformation, we investigated effects of polyphenols and B[a]P on mitochondrial biogenesis and mitochondrial dysfunction.

CHAPTER 5: INCREASED MITOCHONDRIAL BIOGENESIS BY POLYPHENOLS PROTECTS AGAINST MITOCHONDRIAL DYSFUNCTION IN BHAS 42 FIBROBLASTS EXPOSED TO BENZO[A]PYRENE

Abbreviations

B[a]P, Benzo[a]pyrene; **C3G**, cyanidin-3-glucoside; **ERRα**, estrogen-related receptor α; **ETC**, electron transfer chain; **IARC**, International Agency for Research on Cancer; **MMP**, mitochondrial membrane potential; **mtDNA**, mitochondrial DNA; **MTG**, MitoTracker Green; **nDNA**, nuclear DNA; **NRF1**, nuclear respiratory factor 1; **PAH**, polycyclic aromatic hydrocarbon; **PGC-1α**, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; **ROS**, reactive oxygen species; **TFAM**, mitochondrial transcription factor A; **TMRE**, tetramethylrhodamine ethyl ester

Abstract

Background: Polyphenols naturally occurring in fruits and vegetables have been shown to act in cancer prevention and may inhibit carcinogenesis. Changes to mitochondria have been implicated in carcinogenesis and neoplastic transformation; however, a possible mitochondrial mechanism by which polyphenols inhibit cancer initiation and promotion is largely unexplored. In this study, we investigated the effects of benzo[a]pyrene and different polyphenols on mitochondrial biogenesis and dysfunction in the Bhas 42 cell model of carcinogen-induced neoplastic transformation.

Methods: Bhas 42 cells were treated with 5 μM polyphenol (resveratrol, quercetin, catechin, cyanidin, C3G) or berberine starting 2h before exposure to 4 μM B[a]P. The effects on parameters of mitochondrial biogenesis (mitochondrial content, citrate synthase, nDNA and mtDNA-encoded respiratory subunits, transcription factors, SIRT1 expression and activity, and deacetylated PGC-1α) and function (mitochondrial membrane potential and galactose-dependent cellular ATP) were measured after 24 and 72h.

Results: Exposure to B[a]P decreased mitochondrial content by 46% after 24h and 30% after 72 h. Except for cyanidin and berberine after 72h, the polyphenols protected against these decreases, with resveratrol being the most effective (increasing the content after 72h to significantly more than cells without B[a]P exposure). In the presence of B[a]P, different polyphenols significantly increased expression of mRNA for transcription factors or enzymes involved in mitochondrial

biogenesis, for citrate synthase, and for nDNA- and mtDNA-encoded mitochondrial respiratory subunits. Resveratrol most notably increased SIRT1 activity, leading to deacetylation of PGC1α protein. Exposure to B[a]P also induced mitochondrial dysfunction as evidenced by decreased mitochondrial membrane potential (MMP) and galactose-dependent ATP level in cells after 24h by 25% and 22%, respectively. Some polyphenols, most notably resveratrol (and less so quercetin and catechin), prevented these effects on mitochondrial dysfunction.

Conclusion: The results support involvement of a mitochondrial mechanism in carcinogenesis by B[a]P, and in chemoprevention by polyphenols.

5.1. Introduction

While much research has focused on mitochondria in cancer cells and in progression of transformed cells (Boland et al., 2013; Yadava et al., 2013), less is known about mitochondrial changes in the early initiation and promotion stages of carcinogenesis and neoplastic transformation. Carcinogen-induced mutations in mtDNA and in nDNA encoding mitochondrial respiratory proteins might trigger transformation through different mechanisms such as increasing ROS generation and shifting energy from oxidative phosphorylation to aerobic glycolysis. Benzo[a]pyrene (B[a]P) is a carcinogen present in wood and cigarette smoke and in charbroiled foods, which was previously shown to damage mtDNA and cause mitochondrial dysfunction (Backer and Weinstein, 1980; Gao et al., 2010). The role of mitochondrial pathways in carcinogenesis by B[a]P however is not well-understood. In a recent study using the Bhas 42 cell *in vitro* carcinogenesis model, we found that exposure of the cells to benzo[a]pyrene caused an increase in mitochondrial and intracellular ROS generation, which was inhibited by several dietary polyphenols (Omidian et al., 2017). In addition, exposure of the Bhas 42 cells to B[a]P led to increases in transformed foci. The neoplastic transformation was inhibited by treatment with polyphenols, most effectively by resveratrol.

Mechanisms by which B[a]P and polyphenols might influence mitochondrial ROS generation and neoplastic transformation include effects on mitochondrial content and function. Some studies have shown that increased mitochondrial biogenesis helps prevent cancer cell progression to a more tumorigenic phenotype (Bellance et al., 2009; del Mar Blanquer-Rosselló et al., 2017; Wang and Moraes, 2011). Few, if any, studies have investigated mitochondrial biogenesis induction as a

possible mechanism to prevent the initiation and promotion phases of carcinogenesis. We hypothesized that by stimulating mitochondrial biogenesis and function, the number of healthy and functional mitochondria would be increased to reduce the impact of a mitochondrial mutagen. Increased mitochondrial electron transport efficiency and capacity may decrease ROS generation and help prevent a shift from oxidative phosphorylation to aerobic glycolysis.

Polyphenol-rich fruits, vegetables and beverages are of interest for possible roles in cancer chemoprevention (Gullett et al., 2010; Lambert and Elias, 2010; Ramos, 2008). While chemopreventive effects of polyphenols are often attributed to their antioxidant characteristics, other mechanisms and influences on molecular pathways, including mitochondrial biogenesis (Kim et al., 2014; Sandoval-Acuña et al., 2014) may play important roles in chemoprevention. Polyphenols such as resveratrol and quercetin have been widely reported to induce mitochondrial biogenesis and provide beneficial roles in several diseases and conditions (Csiszar et al., 2009; de Oliveira et al., 2016; Kulkarni and Cantó, 2015). In cancer treatment, targeting mitochondrial pathways with polyphenols, particularly mitochondria-mediated apoptotic pathways has been reported in many studies (Gorlach et al., 2015). However, studies investigating preventive roles of polyphenols on cancer initiation through mitochondrial mechanisms are limited.

In our previous study on *in vitro* cell transformation by B[a]P (Omidian et al., 2017) we found that several polyphenols, at a physiologically relevant dose of 5 μM (Howells et al., 2007), strongly decreased mitochondrial and intracellular ROS. While the different polyphenols were similarly effective at decreasing ROS, resveratrol was notable for giving the strongest decrease in cell transformation. However, the mechanisms by which polyphenols decreased B[a]P-induced ROS generation and neoplastic transformation needed further investigation. Regarding a potentially pivotal role of mitochondria in carcinogenesis, in the current study we further investigated the role of mitochondrial biogenesis in preventive effects of different polyphenols on B[a]P-induced mitochondrial dysfunction.

5.2. Materials and Methods

5.2.1. Chemicals and Reagents

Cyanidin and C3G were obtained from Extrasynthese (Genay, France). Primers for qPCR were obtained from Integrated DNA Technologies (Toronto, ON, Canada). MitoTracker Green, TRIzol reagent, VILO cDNA synthesis kit, and SYBR green master mix were obtained from Invitrogen (Carlsbad, CA, USA). RNAeasy mini kit for isolating RNA was obtained from Qiagen (Germantown, MD, USA). Tetramethylrhodamine ethyl ester perchlorate (TMRE) and Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (1g/l) were obtained from Thermo Fisher Scientific (Bartlesville, OK, USA). SIRT1 Direct Peptide, SIRT1 Direct NAD+, and SIRT1 Direct Developer were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). CellTiter-Glo kit was purchased from Promega, USA. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2. Cell culture

See section 3.2.2

5.2.3. RNA extraction, RNA quantification, and cDNA preparation RT-qPCR

See sections 3.2.6. and 3.2.7. Sequences of primers of interest are shown in Table 1.

Table 1. The sequences of primers used to measure the expression of genes involved in mitochondrial biogenesis and mitochondrial function

Gene	Species	Primer sequence (5'-3')
SIRT1	Mouse	FWD: GGA ACC TTT GCC TCA TCT ACA REV: CAC CTA GCC TAT GAC ACA ACT C
PGC-1α	Mouse	FWD: CGA CAG CTA TGA AGC CTA TGA G REV: CTT CTG CCT CTC TCT CTG TTT G
Citrate synthase	Mouse	FWD: TCC TGG TCG TTT GGC TTT ATC REV: GTT CCG TGC CAG AGC ATA TT
ERRα	Mouse	FWD: CTG CTT AAT CCG ATC TCC TCT C REV: GGA GCC TGC TTG GAG TTA TT
NRF1	Mouse	FWD: ACC CTC AGT CTC ACG ACT AT REV: GAA CAC TCC TCA GAC CCT TAA C
TFAM	Mouse	FWD: GTT GGA TGG CAT GGG TTT AAG REV: CTC ACG TCT CTC CTG GAT TTG
NDUFS8	Mouse	FWD: GAG TCT GAG GTG GAC ATG AAG REV: GGC TCT CGA AAG AGG TAA CTT AG
SDHb	Mouse	FWD: GCG ATT GCG GAA ATC AAG AAG REV: TTT GAC ACC AGA GTT GAC AGG
UQRCB	Mouse	FWD: GAG GCA TCA GAT CTT GCC TAA G REV: CCC ACT CTT CTC TCT CCT TTC T
COX5A	Mouse	FWD: GCT AGT GCT GTT CGC ATC TT REV: GTT GGT CTA AGT TCC TGG ATG AC
ATP5A1	Mouse	FWD: CCA AGG TAG CGT TGG TAT ATG G REV: CCC TCC TGG TCT CTG AAG TAT
MTND1	Mouse	FWD: TGC ACC TAC CCT ATC ACT C REV: ATT GTT TGG GCT ACG GCT C
MTCYB	Mouse	FWD: CTT TGG GTC CCT TCT AGG AGT CTG REV: GCT GTG GCT ATG ACT GCG AAC AG
MTCOX1	Mouse	FWD: GCT GAA GGA GAA GGA GAA G REV: ATA CAC ATA GCT CTT CTC CC
MTATP6	Mouse	FWD: CCA CAC ACC AAA AGG ACG AAC ATG A REV: CGG ACT GCT AAT GCC ATT GGT TG
b-actin	Mouse	FWD: CCT TCT TGG GTA TGG AAT CCT G REV: AGC ACT GTG TTG GCA TAG AG

5.2.4. Protein extraction, protein quantification, and Western blot

Bhas 42 cells were seeded in T25 flasks for 24h and exposed to polyphenols at 5 μM for 2h followed by treatment with 4 μM B[a]P for 24h. For protein extraction, cells were lysed in ice-cold RIPA buffer 1X (Millipore, USA) supplemented with Halt Protease Inhibitor, EDTA, and Phosphatase Inhibitor (Thermo Fisher Scientific, USA) and centrifuged for 15 minutes at 1400

rpm and 4°C. The supernatant containing protein was gently removed and used for protein quantification, SIRT1 activity measurement, and Western blots. Protein content was determined using a BCA assay kit (Thermo Fisher Scientific, USA). Results of protein quantification are shown in Appendix 1. For Western blots, protein samples were normalized and denatured by boiling at 70°C with loading buffer (2X Laemmli buffer supplemented with 10% β-mercaptoethanol) for 10 minutes. Equal amounts of protein samples (30-35 μg) were loaded per well using 8% NuPAGE Bis-Tris Pre-Caset gels (Invitrogen, USA) and then transferred to a nitrocellulose membrane using Mini Gel Tank (Life Technologies, USA). After transfer, the membrane was blocked with 5% bovine albumin serum (BSA) and Tris-buffered saline containing 0.1% Tween-20 (TBST, pH 7.6) for 1 hour at room temperature. The membrane was incubated at 4°C overnight with rabbit polyclonal primary anti-de-acetylated PGC-1α (1:1000; Abcam, USA) and anti-b-actin (1:1000; Cell Signaling Technologies, USA) antibodies. The membrane was washed with TBST three times each 5 minutes and then incubated with secondary anti-rabbit (Horseradish Peroxidase 1:2000, Santa Cruz Biotech) antibody. After several washings with TBST, the membrane was incubated with Pierce ECL Western Blotting Chemiluminescent Substrate (Thermo Fisher Scientific, USA) for 3 minutes. Protein bands were observed and quantified using an Alpha Imager Gel Imaging System (Alpha Innotech). Results were normalized to b-actin.

5.2.5. Measurement of mitochondrial membrane potential (MMP)

TMRE fluorescent dye was used to measure the MMP in Bhas 42 cells. TMRE is a positively charged dye that accumulates in active mitochondria due to the negative charge in the matrix. Therefore, higher polarized mitochondria have greater TMRE fluorescence intensity (Perry et al., 2011). After seeding 20,000 Bhas 42 cells in a 96 well optical bottom plate for 24h and pre-treatment with 5 μM polyphenols for 2h followed by treatment with 4 μM B[a]P for 24h, cells were incubated with TMRE dye for 20 minutes. Cells were gently washed twice with PBS and then the average fluorescence intensity was quantified at 540±20 nm excitation and 590±20 nm emission using a microplate reader. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 100 nM administered to control cells for 10 minutes was used as a positive control.

5.2.6. Measurement of mitochondrial content

MitoTracker Green (MTG) fluorescent dye was used to measure mitochondrial content in Bhas 42 cells. MTG stains mitochondria independently from mitochondrial membrane potential changes (Métivier et al., 1998). MTG is taken up into mitochondria where it binds to protein thiols and interacts with mitochondrial lipids to produce a fluorescent green color (Cottet-Rousselle et al., 2011). After seeding 20,000 Bhas 42 cells in a 96 well optical bottom plate for 24 hours and pre-treatment with polyphenols at 5 µM for 2 hours, B[a]P at 4 µM was administrated and cells were incubated for 24h and 48h in 5% CO_2 at 37ºC. Medium was then removed and MTG probe at 250 nM final concentration was loaded in medium without FBS. After 30 minutes incubation, cells were carefully washed twice with PBS. The average fluorescence intensity was quantified at 485±20/528±20 nm using a microplate reader.

5.2.7. Measurement of SIRT1 activity

To measure SIRT1 activity in cell extracts, 25 µL assay buffer and 5 µL samples (described in section 5.2.4 for protein extraction and quantification) of each T25 flask experiment in triplicate were added to a 96 well optical bottom plate. This measurement is based on deacetylation of SIRT1 Direct Peptide, a fluorogenic p53 protein sequence (Arg-His-Lys-Lys(ε-acetyl)-AMC) with an acetyl group, in the presence of SIRT1 co-substrate, NAD+ (Cayman Chemical, USA). The reaction was initiated by adding 15 µL of substrate solution containing SIRT1 Direct Peptide and NAD+, and 25 µL assay buffer (50 mM Tris buffer (pH 8.0), 137 mM NaCl, 2.7 mM KCl, and 1mM $MgCl_2$). The plate was covered and incubated for 45 minutes on a shaker at room temperature. In the second step, 50 µL of developer dissolved in assay buffer was added to each well to release AMC from deacetylated peptide, and the plate was incubated for 30 minutes at room temperature. The fluorescence intensity from free AMC was read at 360±40/465±40 nm using a microplate reader. Protein concentration was determined using a BCA assay kit and results of SIRT1 activity assay were normalized to protein concentration.

5.2.8. Measurement of ATP

Intracellular ATP content was measured using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, USA) based on the manufacturer's protocol. This kit is based on cell lysis and generation of a luminescence signal proportional to the amount of ATP present. Briefly, 20,000

Bhas 42 cells were seeded in a 96 well optical bottom plate in the presence of 5 mM glucose or 25 mM galactose and then incubated for 24h. Cells were treated with 5 μM polyphenols for 2h followed by treatment with 4 μM B[a]P for 24h. Antimycin A (inhibitor of complex III) at 10 μM added to control cells for 30 minutes was used as a positive control. Cells were lysed using reagent included in the kit with 3 minutes vigorous shaking and then 10 minutes standing at room temperature to stabilize luminescence signal. The luminescence was recorded using a microplate reader under standard luminescence settings.

5.3. Results

5.3.1. Effects of polyphenols and B[a]P on mitochondrial content

Figure 5.1A shows fluorescence images from a mitochondrial content experiment after 24h exposure to 4 μM B[a]P and 5 μM polyphenols. Bhas 42 cells treated with 4 μM B[a]P for 24h had lower fluorescence compared to untreated cells, while for all polyphenols mitochondrial content was similar to untreated cells. Figure 5.1B shows quantification of experiments represented in Figure 5.1A. The treatment with B[a]P decreased mitochondrial content by 46% compared to untreated cells, and all polyphenols significantly protected against this effect, with most inhibiting by 39-54%. At this time point cyanidin had the strongest effect and inhibited the B[a]P effect by 78%, resulting in a level not significantly different from control untreated cells.

To investigate the effect of the polyphenols alone on mitochondrial content, we measured their effect without B[a]P (Fig 5.1C). Resveratrol had the strongest effect and increased mitochondrial content compared to untreated cells by 62% after 24 h. Catechin and C3G also increased the content of mitochondria in the absence of B[a]P.

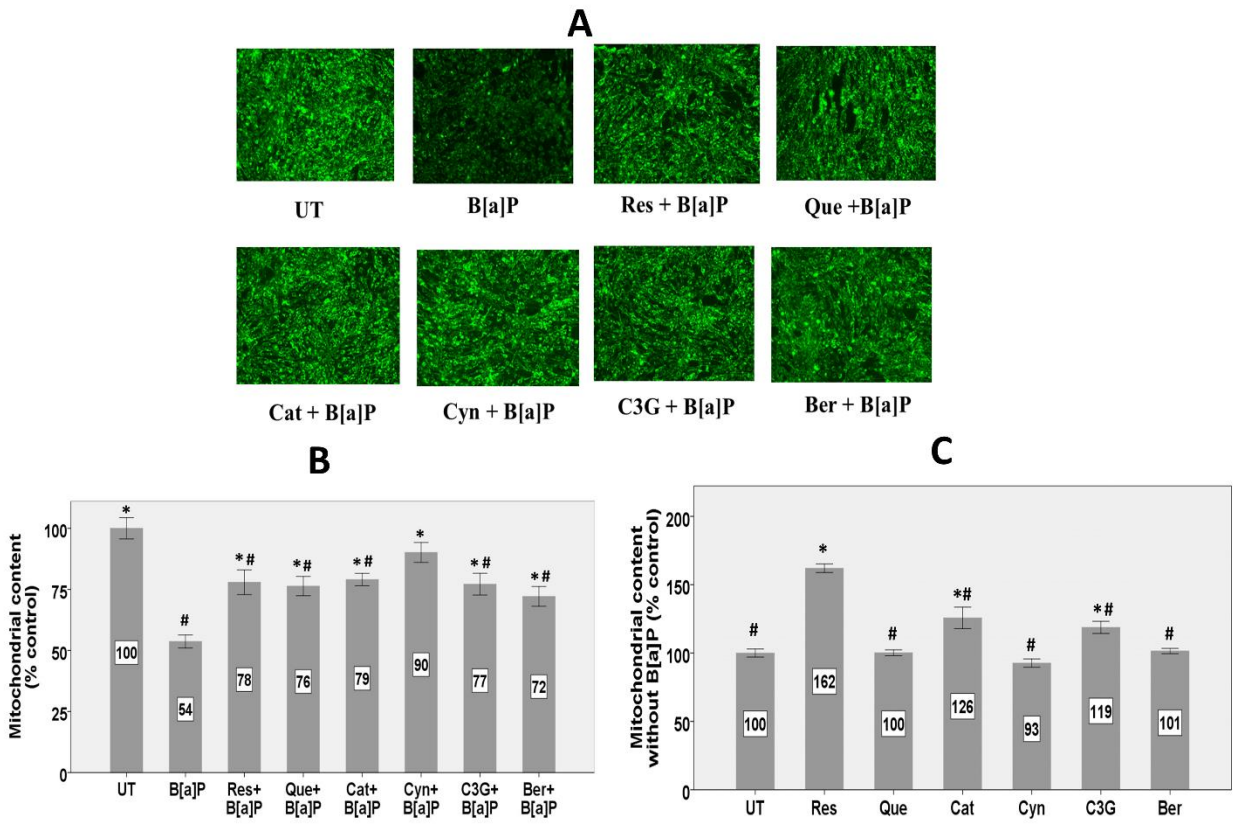

Figure 5.1. Effect of B[a]P and polyphenols on mitochondrial content after 24h.

Mitochondrial content was measured in Bhas 42 cells after treatment with polyphenols at 5 µM for 2h, adding 4 µM B[a]P and incubating for 24h, or after treatment with polyphenols alone for 24 h. (A) Representative fluorescence images (with 20X objective lens) of mitochondrial content after staining with MitoTracker Green. (B) Quantification of mitochondrial content fluorescence. (C) Mitochondrial content after treatment with 5 µM polyphenols for 24h. Data are presented as a percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different at $p < 0.05$ from the B[a]P group for graph B, and from the resveratrol group for graph C.

After 72h exposure, B[a]P decreased the mitochondrial content by 30% compared to untreated cells, and the polyphenols except cyanidin and berberine significantly inhibited the effect of B[a]P (Fig. 5.2). At this time point resveratrol had the strongest effect among administrated polyphenols and prevented the decrease in the content of mitochondria by 173%.

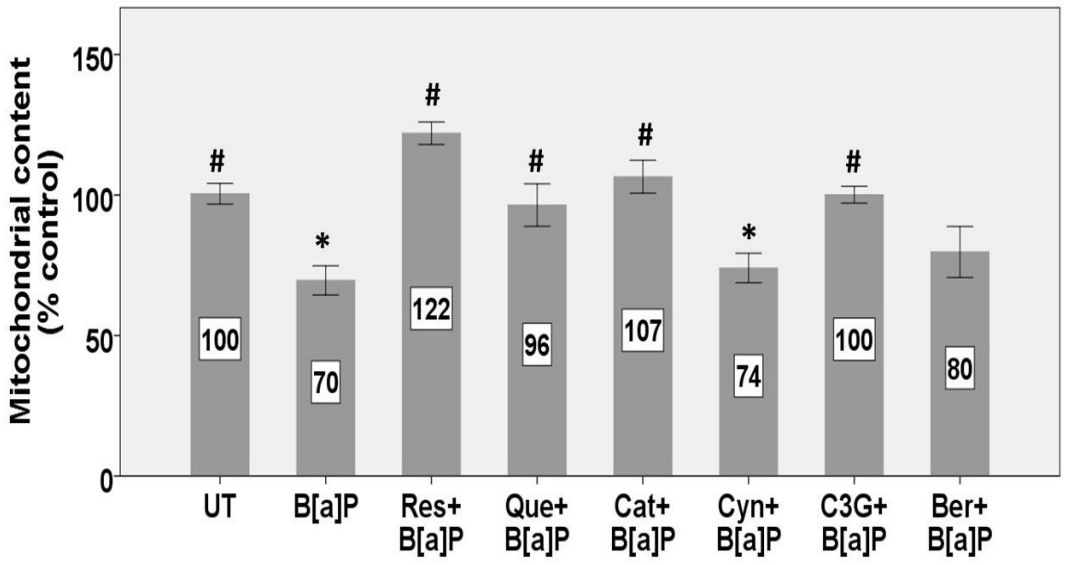

Figure 5.2. Effect of B[a]P and polyphenols on mitochondrial content after 72h.

Mitochondrial content was measured in Bhas 42 cells after treatment with polyphenols at 5 µM for 2h, adding 4 µM B[a]P and incubating for 72h. Data are presented as a percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.2. Effects of B[a]P and polyphenols on expression of mRNA for nuclear and mitochondrial DNA-encoded mitochondrial proteins, and proteins regulating mitochondrial biogenesis

5.3.2.1. Citrate synthase

We measured the level of the nDNA-encoded mitochondrial enzyme citrate synthase as an additional marker of mitochondrial content (Price et al., 2012). Treatment of the Bhas 42 cells with 4µM B[a]P for 24h had no significant effect on expression of mRNA for citrate synthase (Fig 5.3).

Among all of the polyphenols only resveratrol significantly increased expression, to a significant level 80% above untreated cells.

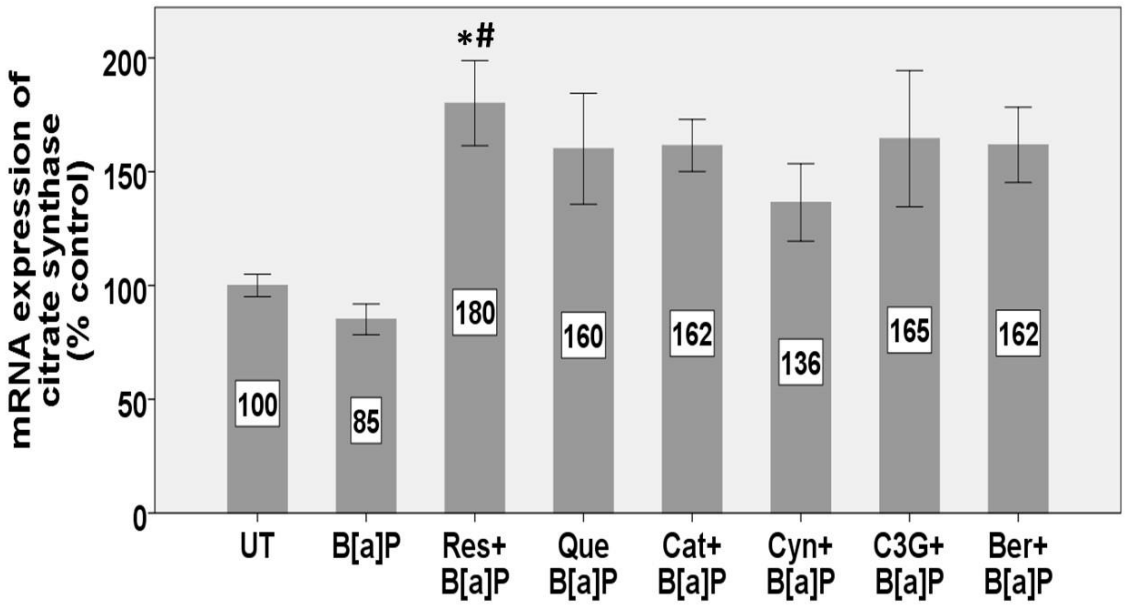

Figure 5.3. Effect of B[a]P and polyphenols on citrate synthase mRNA expression.

Citrate synthase gene expression was measured in Bhas 42 cells using qPCR after treatment with polyphenols and B[a]P for 24h. Data were normalized to beta actin and are presented as a percentage of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.2.2. nDNA-encoded respiratory subunits

The effects of B[a]P and polyphenols on expression of nuclear DNA-encoded mitochondrial respiratory subunits are shown in Figure 5.4. Treatment with B[a]P for 24h significantly decreased expression of the complex I subunit NDUFS8 and the complex V subunit ATP5A1 by 30% and 35%, respectively, but had no significant effect on other nDNA-encoded subunits. Only berberine prevented the effect on NDUFS8 by 140%, and only cyanidin inhibited the effect on ATP5A1 by 85%. Some of the polyphenols (resveratrol, quercetin, and berberine) increased expression of COX5A by up to 68% compared to B[a]P alone, and two polyphenols (cyanidin and berberine) increased expression of UQRCB by up to 43% compared to B[a]P alone. Catechin and C3G were without effect on nDNA-encoded subunits.

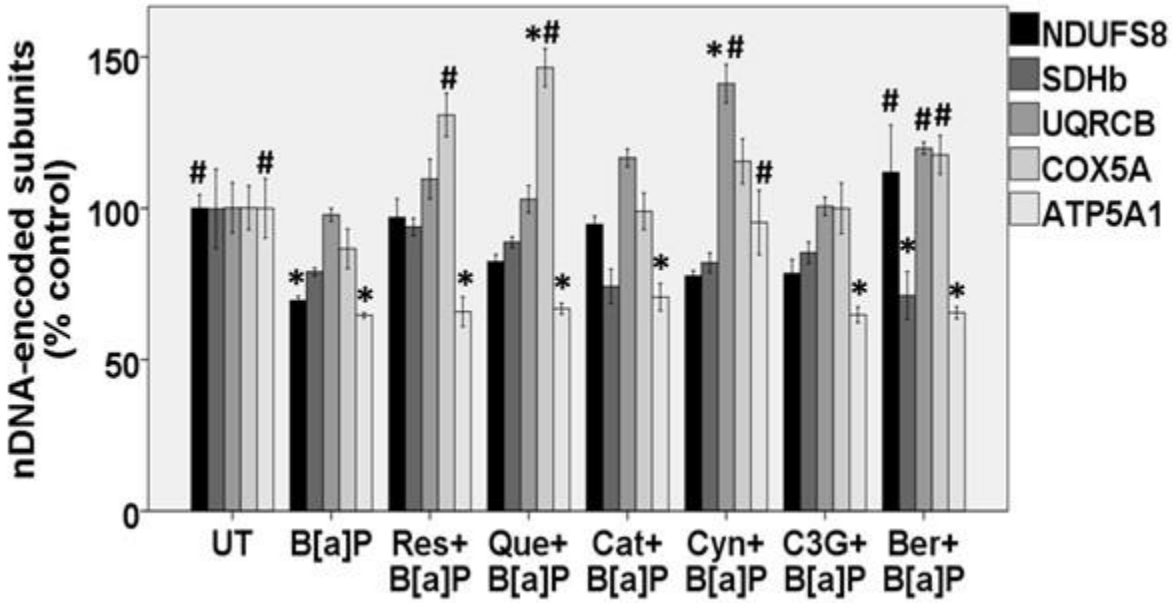

Figure 5.4. Effect of B[a]P and polyphenols on nDNA-encoded mitochondrial respiratory subunits.

nDNA-encoded mitochondrial respiratory subunits were measured in Bhas 42 cells using qPCR after treatment with polyphenols and B[a]P for 24h. Data were normalized to beta actin and are presented as a percentage of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.2.3. mtDNA-encoded respiratory subunits

The effects of B[a]P and polyphenols on expression of mitochondrial DNA-encoded respiratory subunits are shown in Figure 5.5. Treatment with B[a]P for 24h significantly decreased expression of the complex III subunit CYB by 28% but did not affect expression of other subunits. Quercetin, catechin and berberine prevented the effect of B[a]P on CYB, and catechin significantly increased the level to 40% higher than control untreated cells. Quercetin, catechin and berberine also increased expression of mRNA for ND1 compared to B[a]P alone, and significantly by up to 95% above untreated cells. Catechin and berberine significantly increased ATP6 mRNA compared to B[a]P and untreated control cells.

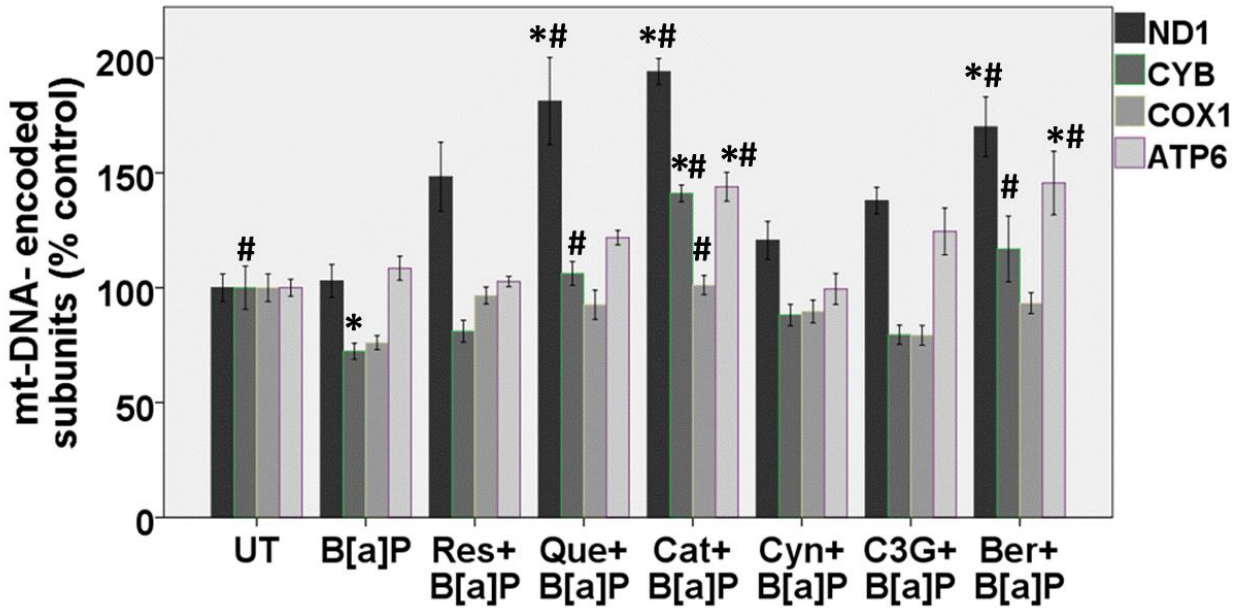

Figure 5.5. Effect of B[a]P and polyphenols on mtDNA-encoded respiratory subunits.

mtDNA-encoded respiratory subunits were measured in Bhas 42 cells using q-PCR after treatment with polyphenols and B[a]P for 24h. Data were normalized to beta actin and are presented as a percentage of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.2.4. Transcription factors (ERRα, NRF1, TFAM) involved in mitochondrial biogenesis

Exposure to B[a]P significantly decreased expression of mRNA for ERRα by 29% and had no

effect on expression of mRNA for NRF1 and TFAM compared to untreated cells (Fig. 5.6). Resveratrol, quercetin, and cyanidin completely inhibited the effect of B[a]P on ERRα and up-regulated ERRα expression to the level of untreated cells. Although B[a]P had no effect on NRF1, quercetin and berberine significantly up-regulated its expression compared to B[a]P alone to levels 50% higher than untreated cells. Among the polyphenols, only C3G significantly increased TFAM expression (by 45% compared to B[a]P alone).

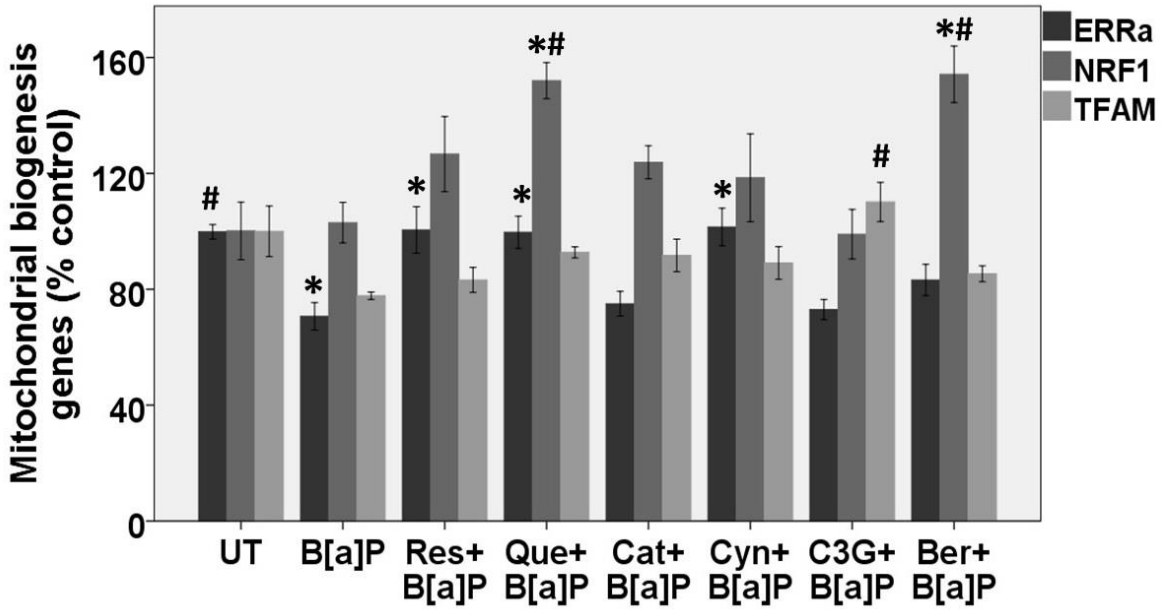

Figure 5.6. Effect of B[a]P and polyphenols on mitochondrial biogenesis transcription factors.

Mitochondrial biogenesis transcription factors gene expression were measured in Bhas 42 cells after treatment with polyphenols and B[a]P for 24h. Data are presented as a percentage of the untreated cells normalized to beta actin. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.3. Effects of polyphenols and B[a]P on mRNA expression, and enzyme activity or protein level of SIRT1 and PGC-1α

Figure 5.6A shows that treatment of Bhas 42 cells with 4 μM B[a]P for 24h significantly down-regulated SIRT1 gene expression by 37% compared to untreated cells. Polyphenols at 5 μM except resveratrol and berberine significantly prevented this effect by up to 140%. In measurements of

SIRT1 activity however, B[a]P had no significant effect (Fig. 5.6B). Although resveratrol had no significant effect on SIRT1 gene expression (Fig. 5.6A), it significantly increased SIRT1 activity compared to B[a]P alone by 22% (Fig. 5,6B). Quercetin also significantly increased SIRT1 activity by 18% compared to B[a]P alone. In spite of significantly up-regulating SIRT1 gene expression, catechin, cyanidin, and C3G did not show any significant effect on SIRT1 activity.

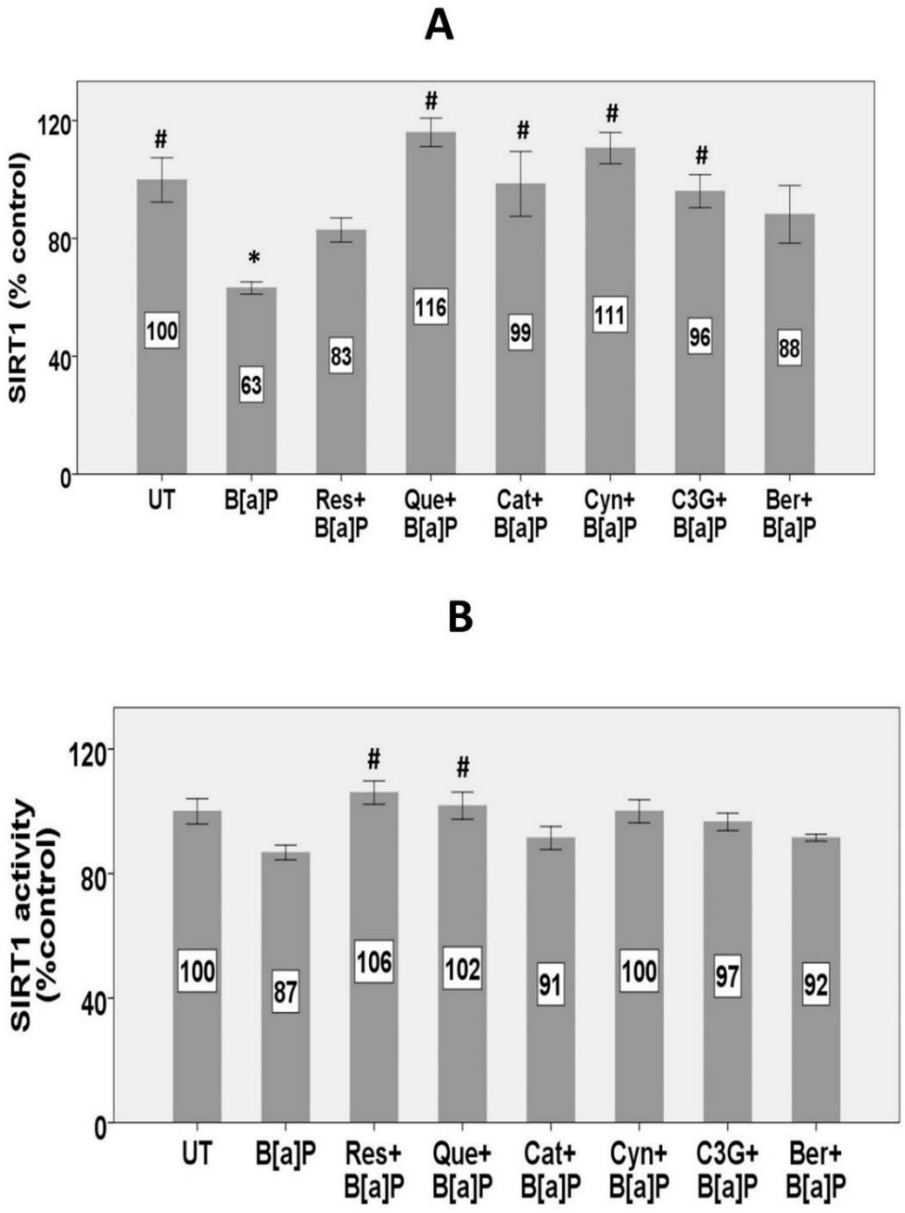

Figure 5.7. Effect of B[a]P and polyphenols on SIRT1 mRNA expression and activity.

SIRT1 gene expression and activity were measured in Bhas 42 cells after treatment with polyphenols and B[a]P for 24h. (A) SIRT1 mRNA expression, (B) SIRT1 activity. Data are presented as a percentage of the untreated cells normalized to beta actin mRNA (for gene expression) or protein (for SIRT1 activity). The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition in gene expression, and 3 independent experiments with SIRT1 activity measurements in triplicate. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

Treatment of Bhas 42 cells with 4 µM B[a]P had no significant effect on PGC-1α mRNA expression (Fig. 5.7A) or on de-acetylated protein level (Fig. 5.7B&C) compared to untreated cells. All polyphenols except resveratrol significantly increased mRNA expression of PGC-1α by up to 70% compared to the B[a]P alone condition (Fig. 5.7A). Resveratrol, however, increased the level of de-acetylated PGC-1α protein (by 135%), as did all of the polyphenols except C3G (Fig. 5.7C). The result for berberine was excluded due to a technical problem with one of the gels.

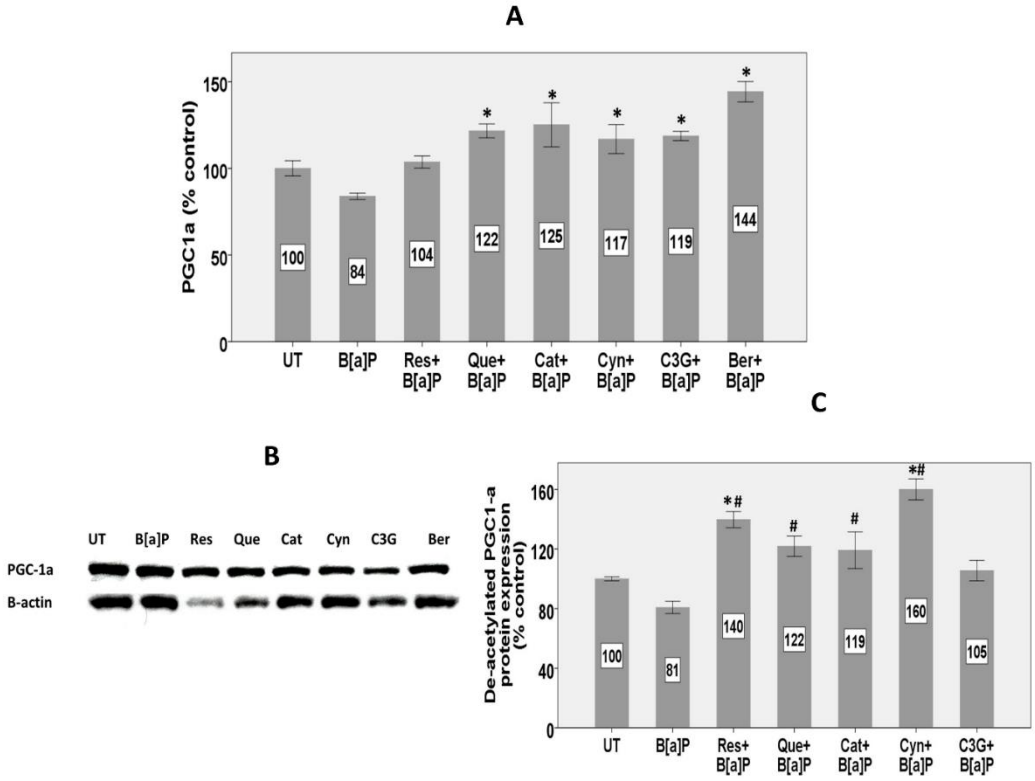

Figure 5.8. Effect of B[a]P and polyphenols on PGC-1α mRNA expression and de-acetylated PGC-1α protein level.

PGC-1α mRNA expression and de-acetylated protein levels were measured in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h and treatment with 4 µM B[a]P for 24h. (A) PGC-

1α mRNA expression, (B) Example Western blot of de-acetylated PGC-1α protein, (C) Quantified de-acetylated PGC-1α protein. Data were normalized to beta actin mRNA or protein and are presented as a percentage of the untreated cells. The figure represents means ± SEM of 2 independent experiments with 3 wells of cells per treatment condition in gene expression experiments, and 2 independent experiments in deacetylated PGC-1α measurements. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.4. Effect of polyphenols and B[a]P on mitochondrial function

To further investigate mitochondrial dysfunction, we assessed the effect of B[a]P and polyphenols on mitochondrial membrane potential (MMP), and on ATP levels in the presence of glucose or galactose.

5.3.5. Mitochondrial membrane potential

Figure 5.9A shows fluorescence images of a representative experiment evaluating the effects of B[a]P and polyphenols on MMP. Treatment of Bhas 42 cells with 4 µM B[a]P showed lower TMRE fluorescence compared to untreated cells. All polyphenols except C3G and berberine appeared to decrease the B[a]P effect and increase TMRE fluorescence. Figure 5.9B shows quantification of TMRE fluorescence. The exposure to B[a]P significantly decreased the MMP by 25%. Resveratrol had the strongest effect and significantly increased the MMP to levels 28% above untreated cells. Quercetin, catechin, and cyanidin also significantly increased the MMP compared to B[a]P alone.

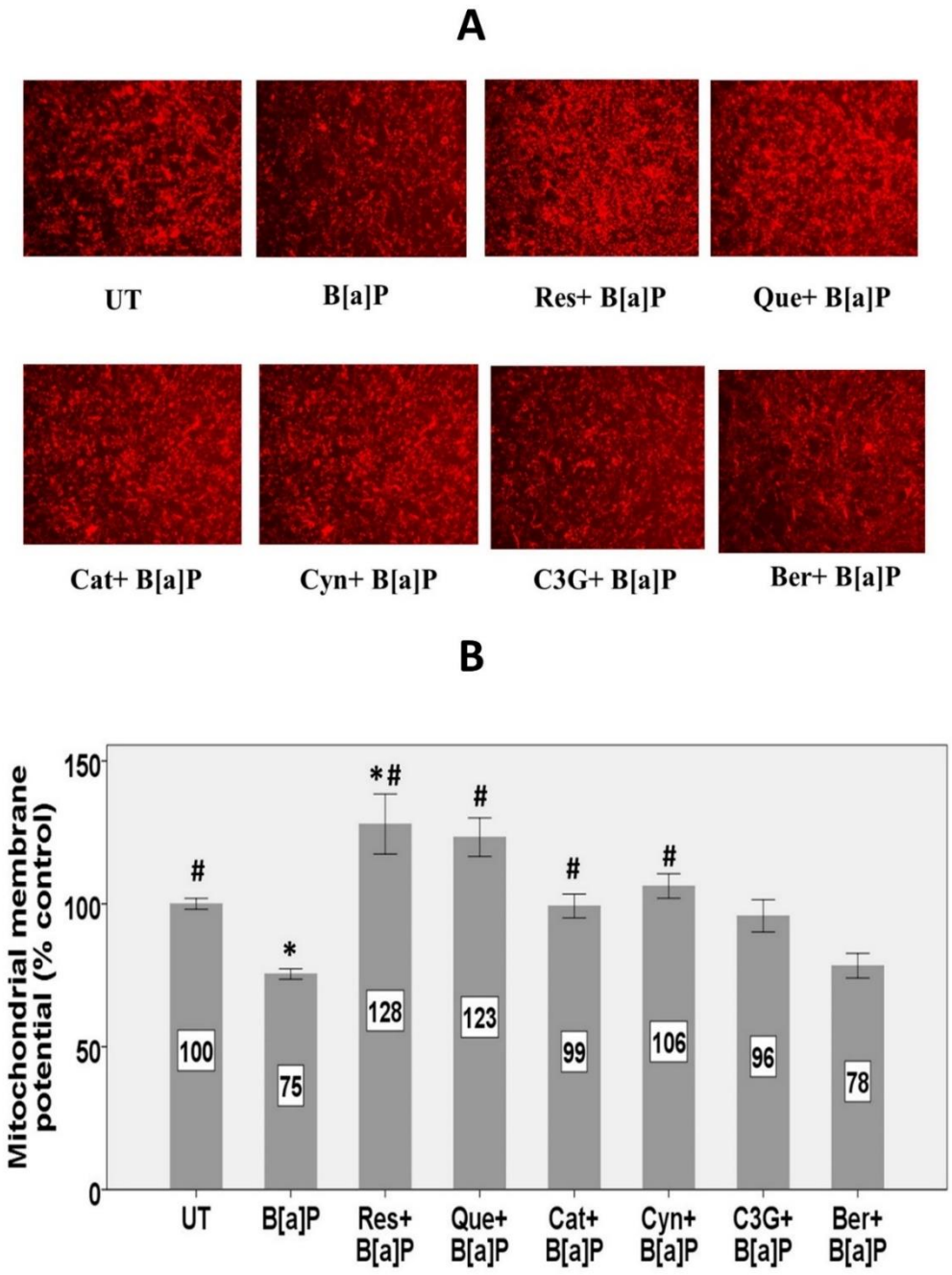

Figure 5.9. Effect of B[a]P and polyphenols on MMP.

MMP was measured using the fluorescent probe TMRE in Bhas 42 cells 24h after treatment with polyphenols and exposure to B[a]P. (A) Representative fluorescence images at 20X objective lens magnification. (B) Quantification of MMP fluorescence. Data are presented as a percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.3.6. Cellular ATP content

The effects of B[a]P and polyphenols on cellular ATP content was measured in Bhas 42 cells grown in glucose medium and grown in galactose medium which requires mitochondrial metabolism for net ATP synthesis. Exposure to B[a]P depleted cellular ATP in both the glucose and galactose medium by 28% and 22%, respectively (Fig. 5.10. A& B). In glucose medium, all of the polyphenols except cyanidin, C3G, and berberine prevented the effect of B[a]P. In galactose medium, only resveratrol protected the effect of B[a]P. In both media, resveratrol showed the strongest effect and completely protected against B[a]P-induced cellular ATP depletion. Berberine significantly decreased ATP content in the galactose medium to a level lower than that of B[a]P alone.

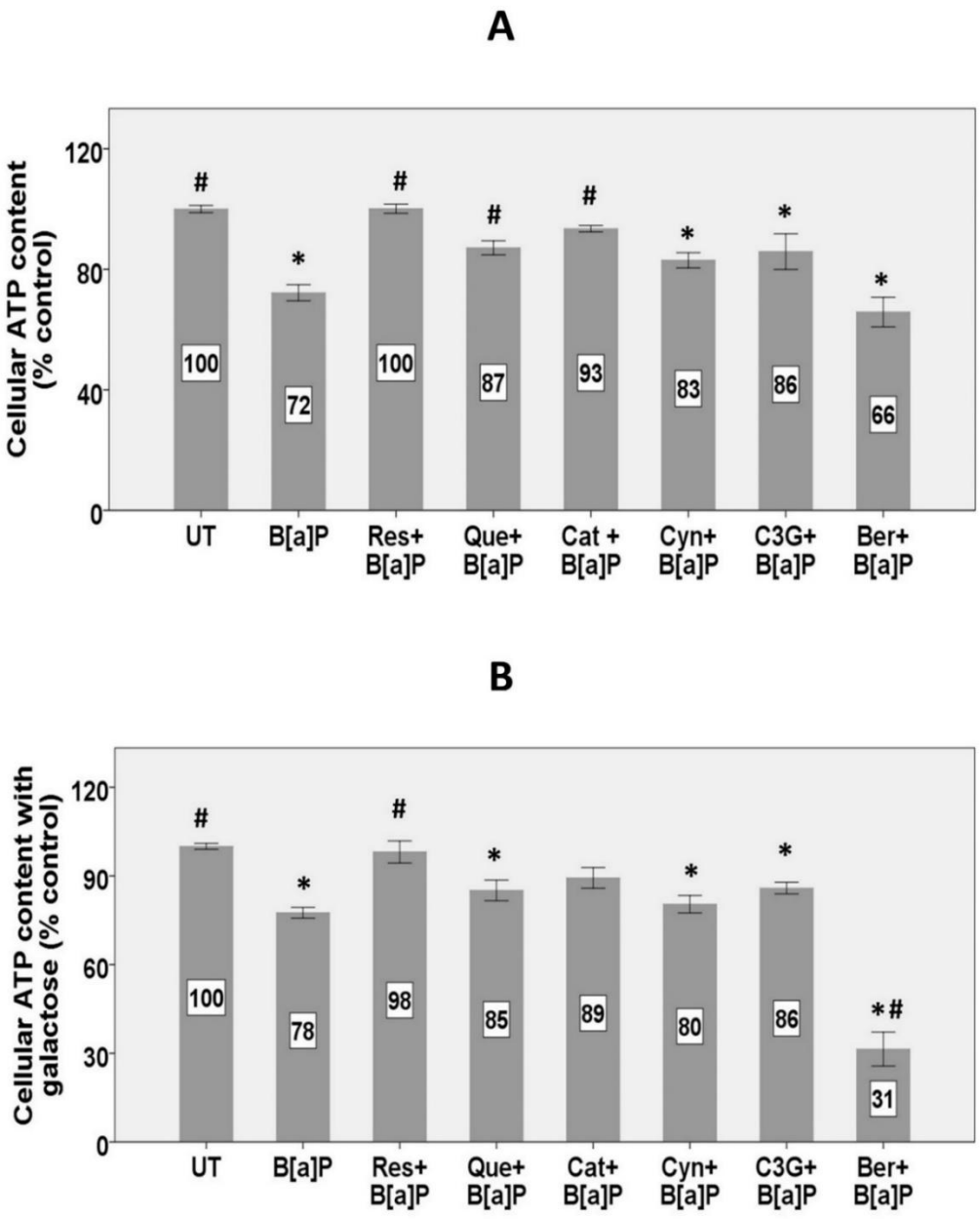

Figure 5.10. Effect of B[a]P and polyphenols on cellular ATP content.

Cellular ATP content was measured in Bhas 42 cells using a Cell Titer-Glo Assay kit after treatment with polyphenols and B[a]P for 24h as in Figure 5.1. (A) ATP content in cells grown in glucose medium, (B) ATP content in cells grown in galactose medium. Data are presented as a percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition per experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

5.4. Discussion

In the current experiments we explored the possibility that the ability of polyphenols to protect against carcinogenesis by B[a]P, as we observed previously (Omidian et al., 2017), might be through inducing mitochondrial biogenesis. By increasing the number of functional mitochondria, such an effect might diminish the impact of a mutagenic agent like B[a]P that produces mitochondrial DNA adducts and lesions / targets mitochondrial DNA to a greater extent than nuclear DNA (Backer and Weinstein, 1980).

To investigate the impact of B[a]P and polyphenols on mitochondria, we measured mitochondrial content after 24h and 72h. Exposure of Bhas 42 cells to B[a]P for 24h and 72h decreased mitochondrial content by 46% and 30%, respectively. Previous studies have shown metabolites of B[a]P to alkylate mtDNA 40-90 times greater than nuclear DNA (Backer and Weinstein, 1980)(and to deplete mtDNA (Pieters et al., 2013). The exposure to B[a]P may produce adducts that interfere with mtDNA replication or produce mutations that increase mitochondrial oxidative stress and decrease mitochondrial content through mitophagy (Van Houten et al., 2016). Such a mechanism is consistent with our previous observation that exposure of Bhas 42 cells to B[a]P increased intracellular and mitochondrial ROS generation (Figs. 3.2 and 3.3; Omidian et al, 2017).

The effects of the different polyphenols on B[a]P-induced decline in mitochondrial content at 24h and 72h distinguished some differences between polyphenols. The polyphenols all protected against the effect of B[a]P on mitochondrial content by 39-78% after 24h, with cyanidin being the most effective. The effect of cyanidin is consistent with its strong protection against mitochondrial superoxide at early time points as we observed previously (Fig. 3.4). At 72h B[a]P exposure however, cyanidin (and berberine) had lost protection, while resveratrol became the most effective. The lack of effect of cyanidin at this time point compared to C3G may be a result of its instability in the medium, whereas C3G has more stability due to having a glucoside group (Khoo et al., 2017). The differences might also be explained by different abilities to induce mitochondrial biogenesis.

Although resveratrol protected with similar potency to other polyphenols against the decreased mitochondrial content after 24h, it had the strongest effect after 72h exposure and increased number of mitochondria to levels higher than that of untreated cells. This robust long-term effect of resveratrol on inducing mitochondrial content is most likely one of the key mechanisms by

which resveratrol has shown the strongest preventive effect on B[a]P-induced neoplastic transformation (with 72h exposure) compared to other polyphenols (Fig. 3.6). Induction of mitochondrial content by resveratrol and its beneficial effects on health have been shown in several diseases and conditions such as neurodegenerative diseases, aging and exercise performance (Hart et al., 2013; Pallàs et al., 2009). However, data showing preventive roles of resveratrol or other polyphenols in carcinogenesis through mitochondrial biogenesis mechanisms are limited. In one report on SW620 colon cancer cells (del Mar Blanquer-Rosselló et al. (2017), treatment with 10 μM resveratrol for 48h induced mitochondrial mass and mitochondrial respiratory capacity, which was associated with elevated ROS production and apoptosis. In Bhas 42 cells exposed to B[a]P we showed decreased ROS production (Figure 3.2 & 3.3) as a result of increased mitochondrial biogenesis by polyphenols. Given that our study is a prevention but not a treatment study, decreasing ROS production would be beneficial.

To explore molecular mechanisms by which resveratrol and other polyphenols induced mitochondrial content, we measured effects of polyphenols and B[a]P on the expression of mRNA for citrate synthase and mitochondrial and nuclear DNA-encoded respiratory subunits. Citrate synthase is the first enzyme in the tricarboxylic acid cycle and is often used as a marker of mitochondrial content and aerobic energy metabolism (Price et al., 2012). In our study, although B[a]P had no significant effect on citrate synthase expression, resveratrol increased its expression up to a level higher than untreated cells. This result suggests a role for resveratrol in protecting against a shift to aerobic glycolysis by increasing oxidative energy metabolism. In human colorectal cancer cell lines (HCT116 and Caco2), 100 μM resveratrol increased citrate synthase activity and decreased glucose consumption which was concomitant with activation of apoptotic markers (Fouad et al., 2013). However, an effect of resveratrol on citrate synthase in anti-carcinogenesis has not been previously shown.

We also measured effects of B[a]P and polyphenols on expression of mtDNA- and nDNA-encoded respiratory subunits. Exposure to B[a]P decreased expression of nDNA-encoded complex I (NDUFS8) and V (ATP5A1) and mt-DNA-encoded complex III (CYB) subunits. Only a few studies have reported an effect of B[a]P on expression of mitochondrial complex subunits (Kamaraj et al., 2011; Qiao et al., 2015) and none of them investigated mtDNA-encoded subunits. Previous studies have shown that mutations in the nuclear and mtDNA-encoded subunits are

correlated with poor prognosis for cancer (Allalunis-Turner et al., 2006; Kulawiec et al., 2006; Moro et al., 2009). Consistent with our results, some studies reported that mutations to mtDNA increases the tumorigenicity of cultured cancer cells (Chattopadhyay et al., 2016; Cruz-Bermúdez et al., 2015; de Araujo et al., 2015; Yadava et al., 2013). In one study for example, a mutation to the mtDNA-encoded complex 1 subunit ND5 in C8T and C9T cell lines, increased tumorigenesis compared to the control 143B cells through increasing ROS production, decreasing ATP production from oxidative phosphorylation, and increasing resistance to apoptosis (Park et al., 2009). Mitochondrial subunit mutations, specifically in complexes I and III are the major mutations and sources of ROS production observed in cancer cells (Brandon et al., 2006; Sullivan and Chandel, 2014). The decrease in expression of the mtDNA-encoded complex III subunit CYB that we observed in this study, which can impair electron flow through the respiratory chain and increase ROS generation (Bandy and Davison, 1990), may have contributed to the increased ROS generation in Bhas 42 cells exposed to B[a]P that we observed previously (Omidian et al., 2017, Chapter 3). Therefore, inhibiting mitochondrial subunit mutations prevents ROS production and cancer progression. Moreover, complex V (ATP synthase) mutation is associated with shifting energy from oxidative phosphorylation to aerobic glycolysis (Warburg effect) in cancer cells (Sánchez-Cenizo et al., 2010). In our study, most polyphenols protected against these B[a]P effects and up-regulated mitochondrial complex subunits some to a level higher than untreated cells.

To further investigate an involvement of mitochondrial biogenesis in effects produced by polyphenols, we measured SIRT1, PGC-1α, and mitochondrial biogenesis-related transcription factors (NRF-1, TFAM, and ERR α). SIRT1 is a NAD^+-dependent deacetylase that deacetylates and activates PGC-1α, and thereby increases expression of genes involved in mitochondrial biogenesis (Ventura-Clapier et al., 2008). Briefly, PGC-1α co-activates transcription-inducing activity of NRF-1 which up-regulates nDNA-encoded mitochondrial proteins as well as TFAM. In turn, TFAM translocates to mitochondria and stimulates mtDNA replication and mitochondrial gene expression (Tang, 2016). PGC-1α also co-activates ERRα, a nuclear transcription factor that up-regulates genes involved in aerobic energy metabolism and mitochondrial function (Schreiber et al., 2004).

SIRT1 expression is regulated at multiple levels (Milner, 2009), and so we measured expression of both mRNA and activity. We observed that exposure of the Bhas 42 cells to B[a]P decreased

SIRT1 mRNA expression by 37% but did not significantly affect cellular SIRT1 activity. These observations conflict with those of a previous study (Lu et al., 2015) in which B[a]P exposure increased SIRT1 mRNA and protein levels in cultured human lung epithelial cells and in the lungs of mice. The reason behind these different responses to B[a]P is not clear.

The polyphenols differently affected SIRT1 mRNA expression and SIRT1 activity. Several of the polyphenols (quercetin, catechin, cyanidin and C3G) prevented the B[a]P-induced decrease in SIRT1 mRNA expression. The regulation of SIRT1 mRNA expression is complex and not well understood (Milner, 2008), and so it is difficult to know the mechanism behind this effect of the polyphenols. With measurements of SIRT1 activity, although B[a]P had no significant effect, resveratrol and quercetin increased SIRT1 activity compared to cells exposed to B[a]P alone. Resveratrol is known as a direct or indirect activator of SIRT1 (Howitz et al., 2003; Hu et al., 2011); and its ability to suppress tumorigenesis through activating SIRT1 has been previously shown (Wang et al., 2008). Quercetin also is well known as a SIRT1 activator (Baur, 2010), and induced both SIRT1 gene expression and activity. Catechin, cyanidin, and C3G increased SIRT1 gene expression however had no effect on SIRT1 activity.

To further investigate whether effects of B[a]P and polyphenols on mitochondrial content during carcinogenesis involve effects on mitochondrial biogenesis, we measured their effects on PGC-1α mRNA expression and on cellular de-acetylated PGC-1α protein levels. In addition to the anti-Warburg effect of PGC-1α in cancer prevention, it can also prevent carcinogenesis through other mechanisms such as decreased ROS production, increased anti-oxidant enzymes and reduced inflammation (Girnun, 2012). Our data show that B[a]P had no significant effect on PGC-1α gene and de-acetylated protein expression. Therefore, down-regulation of PGC-1α does not seem to be a major mechanism in the decreased mitochondrial content produced by B[a]P. All of the polyphenols, however, increased expression of either PGC-1α mRNA or de-acetylated PGC-1α protein, or both. Resveratrol was unique in having no significant effect on PGC-1α mRNA expression, but it strongly increased the level of de-acetylated PGC-1α protein. This effect on de-acetylated PGC-1α is consistent with its effect to increase SIRT1 activity, which de-acetylates PGC-1α. A role of increased PGC-1α by resveratrol and other polyphenols in protecting against other diseases has been previously reported (Krishnamoorthy and Venkatraman, 2017; Ma et al., 2017), however this effect of polyphenols on PGC-1α has not been shown in cancer prevention.

To further investigate effects on mitochondrial biogenesis, we measured expression of the transcription factors ERRα, NRF1, and TFAM. B[a]P significantly down-regulated ERRα mRNA by 29% but had no effect on NRF1 and TFAM gene expression. The effect of B[a]P on ERRα may have contributed to the decrease in mitochondrial content. The lack of effect on NRF1 and TFAM suggest that these transcription factors were not involved in the decrease caused by B[a]P. Previous studies have shown conflicting effects of B[a]P on expression of TFAM and NRF1 (Wu et al., 2016; Zhang et al., 2011). Zhang et al. (2011) showed that 12 μM B[a]P in human bronchial epithelial cells (16HBE) had no effect on mRNA expression but decreased protein expression of NRF1 and TFAM. In this study, NRF1 down-regulation by siRNA increased susceptibility of the cells to decreased mitochondrial membrane potential and ROS generation induced by B[a]P. In a subsequent study by this group, Wu et al. (2016) reported that transfection-induced overexpression of NRF1 in the 16HBE cells could attenuate the cytotoxic effect of the B[a]P metabolite benzo a pyrene-7,8-diol-9,10-epoxide (BPDE) . Although the results on effects of B[a]P on expression of mitochondrial biogenesis transcription factors are mixed, they suggest that increased expression of these factors can help protect against the detrimental effects of B[a]P on mitochondrial content and function.

The effects of the polyphenols in the presence of B[a]P on expression of mRNA for the mitochondrial biogenesis transcription factors were modest. Quercetin and berberine increased NRF1 expression to a level above that of untreated cells. Resveratrol, quercetin, and cyanidin significantly increased the expression of ERRα, and only C3G increased TFAM compared to the level in the presence of B[a]P alone.

To investigate effects of B[a]P and polyphenols on mitochondrial function, we measured their effects on MMP and ATP. Mitochondrial dysfunction as a result of gene mutations and aberrant ETC, increases ROS production and decreases MMP; therefore, MMP reflects mitochondrial status in the cells and is a biomarker of mitochondrial dysfunction (de Moura et al., 2010). Another consequence of mitochondrial dysfunction is decreased ATP content as a result of impaired ETC (Lu et al., 2009; Moiseeva et al., 2009; Wang and Moraes, 2011).

In our study, exposure to B[a]P decreased MMP and ATP levels by 22-28%, suggesting a positive association between decreased MMP and ATP depletion. Loss of MMP and ATP by exposure of

cells to B[a]P has been shown in previous studies (Jiang et al., 2011; Zhang et al., 2011). Given shifting energy from oxidative phosphorylation to aerobic glycolysis in cancer cells and increasing the rate of glycolysis to compensate energy, we also measured ATP content in the presence of galactose. Because galactose cannot provide net ATP synthesis in glycolysis, galactose medium suppresses glycolysis and forces cells to supply their energy from oxidative phosphorylation (Dott et al., 2014). Therefore, results of ATP content in the presence of galactose reflect the ATP produced from mitochondrial oxidative phosphorylation. Complete suppression of ATP by antimycin A, a complex III inhibitor, in the presence of galactose but not glucose confirmed glycolysis repression (Fig. 3C, D). B[a]P significantly depleted ATP in the presence of both glucose and galactose with the same potency. This effect of B[a]P shows that aerobic glycolysis was not able to compensate loss of ATP caused by decreased oxidative phosphorylation.

Treatments with several of the polyphenols prevented the B[a]P induced decreases in MMP and ATP. Resveratrol, quercetin, catechin, and cyanidin prevented the decrease in MMP, and resveratrol increased the MMP to a level significantly higher than untreated cells. Also, while resveratrol, quercetin and catechin alleviated the B[a]P-induced decrease in ATP in cells grown on glucose, resveratrol was the only polyphenol that prevented B[a]P-induced ATP depletion in cells grown on galactose. These effects of resveratrol are consistent with the observations that had the most consistent and often strongest effect on mitochondrial content, citrate synthase mRNA, SIRT1 activity, and de-acetylated PGC-1α. Quercetin was also notable as the next strongest polyphenol in protecting against many of these effects of B[a]P. Together the results support the hypothesis that polyphenol-induced increases in mitochondrial content and function helps to protect against the detrimental effects of B[a]P.

One notable exception among the different polyphenol derivatives tested for its effects on MMP and ATP was berberine. Berberine significantly decreased the ATP level in cells grown on galactose even more than that of B[a]P alone, and berberine did not protect against the B[a]P-induced decreases in MMP and ATP in cells grown on glucose. This effect of berberine may be related to the observation in a previous study (Yin et al., 2008) that 5 μM berberine inhibited oxygen consumption in 3T3-L1 adipocytes and L6 myotubes, which increased the AMP/ATP ratio, activated AMPK, and increased glycolysis. The results suggest that berberine may have an inhibitory effect on mitochondrial oxidative phosphorylation. Further investigations are required

to clarify the role of berberine in aerobic metabolism and B[a]P induced mitochondrial dysfunction.

5.5. Conclusions

In summary, B[a]P decreased mitochondrial content and induced mitochondrial dysfunction while different polyphenols inhibited these effects with different effectiveness. B[a]P abolished mitochondrial biogenesis by decreasing expression of mitochondrial and nuclear DNA-encoded respiratory subunits, SIRT1 activity, and expression of gene transcription factor. In addition to effect on mitochondrial proliferation, B[a]P induced mitochondrial dysfunction by decreasing MMP and ATP content. Although all pre-treated polyphenols induced mitochondrial biogenesis and decreased mitochondrial dysfunction, resveratrol showed the most robust effect in induction of mitochondrial content after 72h. However, further studies are required to investigate other molecular mechanisms of polyphenols in cancer prevention.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

5.6. Transition to CHAPTER 6

In CHAPTER 5, we investigated possible mitochondrial mechanisms that are involved in B[a]P-induced oxidative stress and neoplastic transformation and prevention by polyphenols, particularly prevention by resveratrol. In some experiments including mitochondrial content after 72h, citrate synthase mRNA expression, and ATP content, resveratrol showed the most robust protection against B[a]P exposure compared to other polyphenols.

Because resveratrol and anthocyanidins might act by different mechanisms (inducing mitochondrial biogenesis and scavenging mitochondrial ROS, respectively), we hypothesized that resveratrol and cyanidin might act synergistically to decrease B[a]P-induced mitochondrial ROS.

Results of experiments to test this hypothesis did not show any additive or synergistic effects and are shown in APPENDIX 2.

In CHAPTER 6, we further assessed the hypothesis and results in CHAPTER 5 suggesting that mitochondrial biogenesis induced by the polyphenols, especially resveratrol, results in decreased intracellular and mitochondrial ROS generation. In this chapter we measured the effects of B[a]P and polyphenols on intracellular ROS and mitochondrial superoxide generation after 24h (to complete the measurements at this time point and allow comparisons with mitochondrial content and function in CHAPTER 5), effects of polyphenols on intracellular ROS when given after exposure to B[a]P (to help distinguish direct antioxidant effects), the effects of the different polyphenols alone (in absence of B[a]P) on the mitochondrial content after 24h in Bhas 42 cells, and effects of polyphenols when given for 24h and removed prior to exposure to B[a]P for 24 and 72h on intracellular ROS and mitochondrial superoxide (to show pre-conditioning effects in the absence of continued presence of the polyphenols in the medium).

CHAPTER 6: PROMOTING MITOCHONDRIAL BIOGENESIS BY RESVERATROL, MORE STRONGLY THAN OTHER POLYPHENOLS INHIBITS ROS GENERATION IN B[A]P-INDUCED CARCINOGENESIS

Abstract

Background: Previous studies suggest a pivotal role of mitochondria in ROS production and carcinogenesis when cells are exposed to a carcinogen such as B[a]P and show potential protection by dietary polyphenols such as resveratrol. However, data to show a link between induction of mitochondrial biogenesis and cancer prevention through suppressing oxidative stress are limited. Therefore, in the present study we investigated the association between induction of mitochondrial biogenesis by natural polyphenols and B[a]P-induced ROS generation in fibroblast Bhas 42 cells.

Materials and Methods: Bhas 42 cells were treated with polyphenols before, during or after exposure to B[a]P for different times. Intracellular ROS and mitochondrial superoxide were measured using DCFH-DA and MitoSox red fluorescent dyes, respectively, and mitochondrial content was measured using MitoTracker Green fluorescent dye.

Results: In co-treatments starting 2h before B[a]P, all of the polyphenols strongly protected against B[a]P-induced intracellular ROS generation after 24h, but only the anthocyanins (cyanidin and C3G) and berberine significantly decreased B[a]P-induced mitochondrial superoxide. In post-treatments after 12h exposure to B[a]P, 12h treatment with all of the polyphenols significantly decreased B[a]P-induced intracellular ROS by 58-90%, with resveratrol giving the least and cyanidin the strongest effect. After treatment of Bhas 42 cells for 24h with the different polyphenols (in absence of B[a]P), resveratrol, catechin and C3G significantly increased mitochondrial content and resveratrol had the strongest effect, increasing the mitochondrial content by 62%. With 24h polyphenol pre-treatment and subsequent 24h and 72h exposure to B[a]P, all of the polyphenols almost completely prevented the B[a]P-induced increase in intracellular ROS after 24h exposure, and inhibited by 48-96% after 72h with resveratrol and quercetin most strongly decreasing intracellular ROS generation.

Conclusion: The present study revealed a positive association between induction of mitochondrial content and decreased B[a]P-induced ROS generation in Bhas 42 cells that may help explain the anti-carcinogenesis effect of resveratrol.

6.1. Introduction

Mitochondrial dysfunction caused by aberrant mitochondrial electron transfer chains contributes to increased mitochondrial superoxide production (Park et al., 2011; Sauer et al., 2001), and increased intracellular ROS and oxidative stress. Oxidative stress is a major contributor to tumor initiation and promotion by producing DNA mutations and genome instability, and influencing cell signalling pathways involved in cell proliferation (Visconti and Grieco, 2009). In our previous study we showed that the carcinogen B[a]P increased mitochondrial superoxide and intracellular ROS generation in Bhas 42 cells and ultimately increased the number of transformed foci (by 465%, 21 days after exposure to 4 μM B[a]P for 72h), while different polyphenols (at 5 μM) protected against these effects (Fig 3.2-3.6). Resveratrol and quercetin significantly inhibited neoplastic transformation by B[a]P, with resveratrol showing the strongest effect (inhibiting by 75%) (Fig. 3.6).

By increasing the number of healthy and functional mitochondria, we postulated that induced mitochondrial biogenesis results in decreased ROS generation due to improved function of the mitochondrial respiratory chain. There are a few studies that have shown decreased ROS generation along with induced mitochondrial biogenesis (Kong et al., 2010; Takanche et al., 2017). Kong et al. (2010), reported that overexpression of PGC-1α and its downstream SIRT3 in C2C12 myotubes increased mitochondria and decreased ROS through induction of ROS-detoxifying enzymes including glutathione peroxidase-1 and SOD2. Takanache et al. (2017) showed that, through increases in PGC-1α and antioxidant enzymes (SOD1 and SOD2), the plant natural product schisandrin C inhibited an increase in intracellular ROS and decrease in mitochondria induced by lipopolysaccharide in cultured human dental pulp cells. Conversely with cancer cells, which generally have decreased mitochondrial oxidative phosphorylation (Bellance et al., 2009), induction of mitochondrial biogenesis by a reasonably low dose of resveratrol can increase oxidative stress and promote apoptosis (del Mar Blanquer-Rosselló et al., 2017). Although a few previous studies have reported increased mitochondrial biogenesis along with decreased oxidative stress, no studies have shown this in carcinogenesis. There are many studies that have reported a

contribution of oxidative stress in tumorigenesis and extensive data have shown that decreased ROS may prevent carcinogenesis (Seifried et al., 2007; Sosa et al., 2013). However, none of them have shown whether increased mitochondrial biogenesis can prevent carcinogenesis through decreased ROS production.

The data in Chapter 5 showed protective effects of polyphenols including resveratrol, quercetin, catechin, cyanidin, C3G, and berberine against B[a]P-induced decreases in mitochondrial content and function, with resveratrol showing the strongest effect on mitochondrial content after 72h (Fig. 5.2) and on some other measures after 24h. The data in Chapter 3 showed that the polyphenols also decreased B[a]P-induced intracellular ROS generation, with similar effectiveness (Figs. 3.2 and 3.3), but that resveratrol most strongly inhibited neoplastic transformation. In the current study, we further investigated the hypothesis that induction of mitochondrial biogenesis by polyphenols prevents B[a]P-induced carcinogenesis in Bhas 42 cells partly through decreasing ROS production.

6.2. Materials and Methods

6.2.1. Chemicals and reagents

See sections 3.2.1 and 5.2.1.

6.2.2. Cell culture

See section 3.2.2.

6.2.3. Intracellular ROS measurement

The level of intracellular ROS was determined by using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). After seeding 2×10^4 Bhas 42 cells in a 96-well plate and growing for 24 hours, polyphenols at 5 μM were administrated in DMSO (final DMSO concentration 0.5 %) for 24h, medium was replaced with the fresh medium containing 4 μM B[a]P (but no polyphenol) and incubating for 24h and 72h. After these incubations, 10 μM H_2O_2 was administrated to some control wells as a positive control and incubated for 1 h. Then DCFH-DA at 25 μM final concentration dissolved in DMEM without FBS was loaded to each well. After incubating for 30 min all of the wells were cautiously washed twice with phosphate-buffered saline (PBS) and read at 480/20 nm excitation and 528/20 nm emission using a microplate reader (BioTek, Synergy HT).

Images were captured by fluorescence microscope (Olympus IX 71) at 473±31 nm excitation and 520±35 nm emission. Intracellular ROS also was measured after treating Bhas 42 cells with B[a]P first for 12 h, replacing the medium and then treating them with polyphenols for 12 h.

6.2.4. Mitochondrial superoxide measurement

The level of mitochondrial superoxide production was measured using MitoSOX red fluorogenic dye. After seeding 2×10^4 Bhas 42 cells for 24 hours followed by pre-treatment with 5µM polyphenols for 24h, the medium was replaced with fresh medium containing B[a]P (but no polyphenol) and incubating for 24h and 72h in 5% CO_2 at 37ºC. Antimycin A (a cytochrome c reductase inhibitor) was added as a positive control and incubated for 30 minutes. Then, MitoSOX probe at 5 µM final concentration dissolved in DMEM without FBS was loaded to each well and plates were incubated for 30 minutes. Then wells were cautiously washed twice with PBS and read at 530±20 nm excitation and 590±20 nm emission using a microplate reader. Images were captured by a ZOE Fluorescent Cell Imager (Bio-Rad).

6.2.5. Mitochondrial content measurement

After seeding 2×10^4 Bhas 42 cells in a 96 well optical bottom plate for 24 hours and treatment with polyphenols at 5 µM, cells were incubated for 24h in 5% CO_2 at 37ºC. Medium was then removed and MTG probe at 250 nM final concentration was loaded in medium without FBS. After 30 minutes incubation, cells were carefully washed twice with PBS. The average fluorescence intensity was quantified at 485±20 excitation and 528±20 nm emission using a microplate reader.

6.2.6. Statistical analysis

Data were analysed by one-way or two-way ANOVA where appropriate. Significant differences between groups was determined by Tukey's post-hoc test and differences of $p<0.05$ were considered significant. Results are reported as means ± SEM of 3 independent experiments with 3 sample wells of cells per treatment condition per experiment.

6.3. Results

6.3.1. Effect on intracellular ROS generation after 2h pre-incubation with polyphenols and 24h exposure to B[a]P

Analysis of intracellular ROS generation after 24h shows that treatment of Bhas 42 cells with 4 µM B[a]P significantly increased ROS generation by 80% compared to untreated cells (Fig. 6.1). All of the polyphenols completely prevented the increase in ROS generation with similar potency.

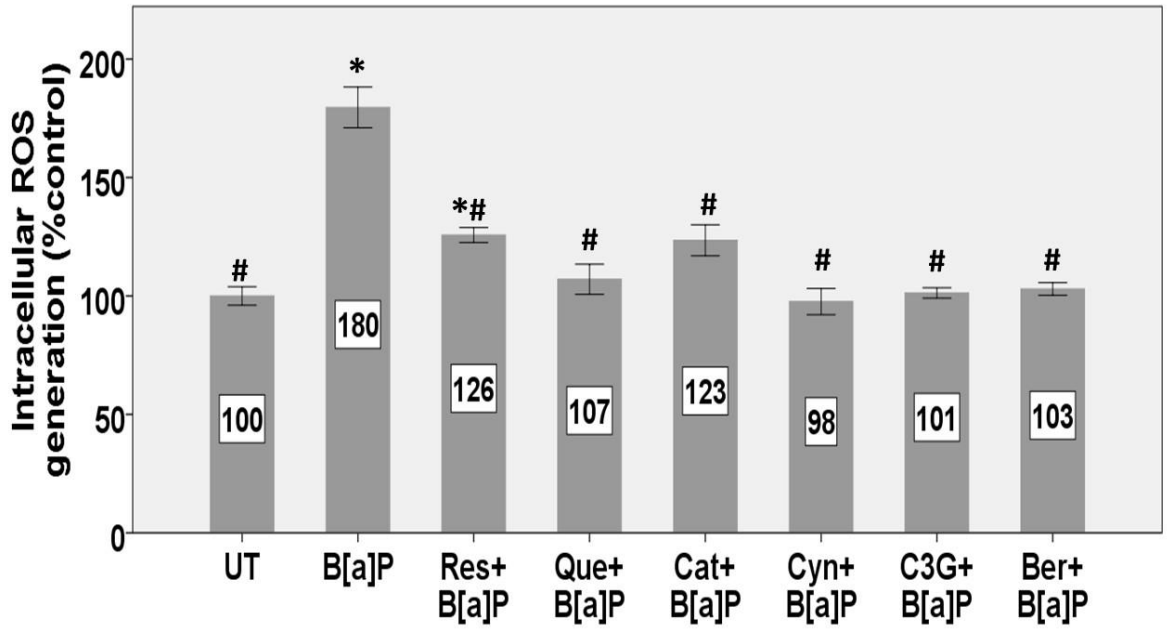

Figure 6.1. Effect of polyphenols on B[a]P-induced intracellular ROS generation after 24 h.

Intracellular ROS generation was measured using DCFH-DA in Bhas 42 cells after pre-incubation with polyphenols at 5 µM for 2h and adding 4 µM B[a]P for 24h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

6.3.2. Effect on mitochondrial superoxide generation after 2h pre-incubation with polyphenols and 24h exposure to B[a]P

Treatment with 4 µM B[a]P significantly increased mitochondrial superoxide generation after 24h by 65% compared to untreated cells (Fig. 6.2). Among the polyphenols, cyanidin, C3G, and berberine could significantly protect against this effect of B[a]P by up to 65%.

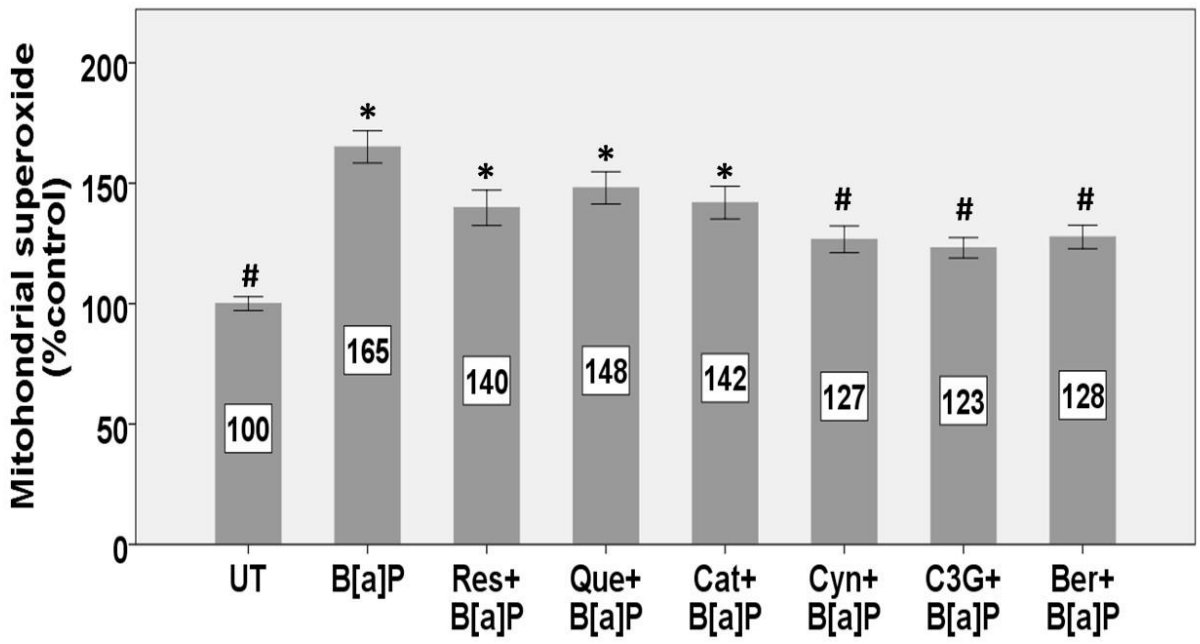

Figure 6.2. Effect of polyphenols on B[a]P-induced mitochondrial superoxide generation after 24 h.

Mitochondrial superoxide was measured using MitoSOX Red in Bhas 42 cells after pre-incubation with polyphenols at 5 µM for 2h and adding 4 µM B[a]P for 24h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

6.3.3. Effect on intracellular ROS generation after pre-exposure to B[a]P for 12h and post-treatment with polyphenols for 12h

With 12h exposure to B[a]P followed by its removal and treatment with polyphenols for another 12h, B[a]P significantly increased intracellular ROS generation by ~ 130% and all of the polyphenols significantly reversed this effect (Fig. 6.3). Cyanidin had the strongest inhibitory effect (~90%). The effect of cyanidin against B[a]P-induced intracellular ROS generation after 12h was significantly stronger than resveratrol, quercetin, and catechin.

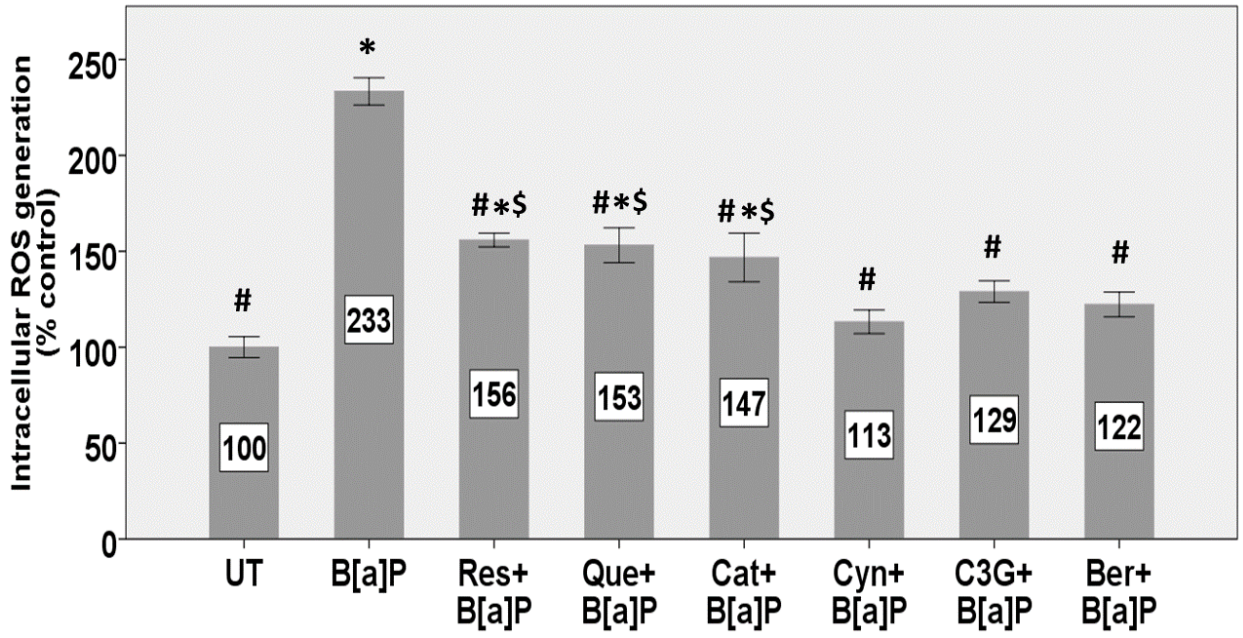

Figure 6.3. Effect of polyphenol post-treatment on B[a]P-induced intracellular ROS generation.

Intracellular ROS was measured with DCFH-DA in Bhas 42 cells after pre-treatment with B[a]P 4 µM for 12h, replacement of the medium, and treatment with 5 µM polyphenols for 12h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$. $ Significantly different from cyanidin at $p < 0.05$

6.3.4. Effect on intracellular ROS generation after 24h pre-treatment with polyphenols and 24h and 72h exposure to B[a]P

Treatment with B[a]P alone increased intracellular ROS generation ~ 90% and 130% after 24h and 72h, respectively (Fig. 6.4 A&B). With 24h pre-treatment (and removal prior to B[a]P exposure) all of the polyphenols significantly decreased intracellular ROS generation and some of them completely prevented the effect of B[a]P after 24h and 72h. After 24h exposure to B[a]P, all of the polyphenols gave similar protection; however, after 72h exposure to B[a]P, resveratrol pre-treatment had the strongest effect (95% inhibition) against B[a]P-induced intracellular ROS generation. The effect of resveratrol against B[a]P-induced intracellular ROS generation was significantly stronger than catechin, cyanidin, C3G, and berberine.

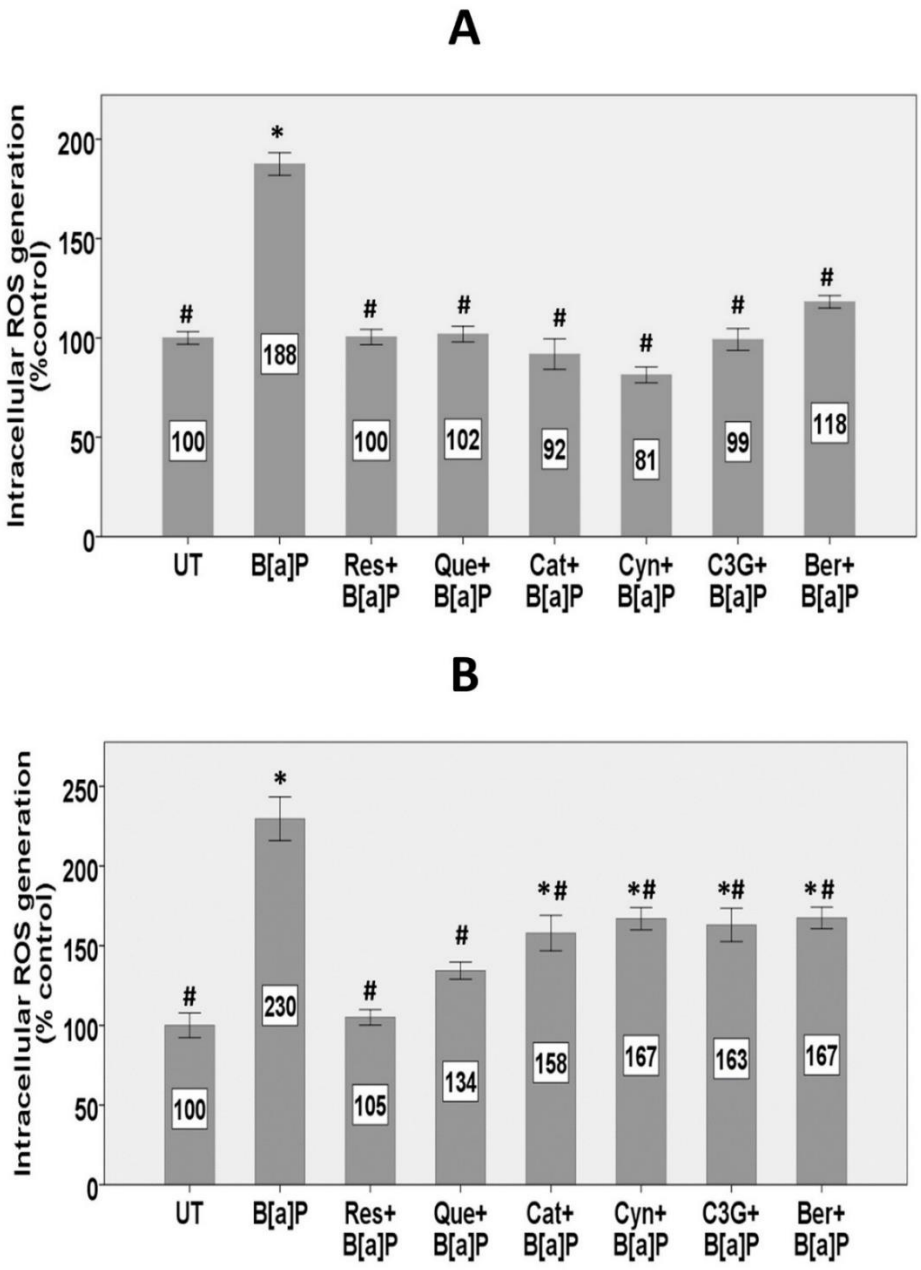

Figure 6.4. Effect of 24h pre-treatment with polyphenols on subsequent B[a]P-induced intracellular ROS generation after 24h and 72h.

Intracellular ROS generation was measured with DCFH-DA in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 24h, replacement of the medium without polyphenols and treatment with 4 µM B[a]P for (A) 24h and (B) 72h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different compared to the

B[a]P group at $p < 0.05$. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

6.3.5. Effect on mitochondrial superoxide generation after 24h pre-treatment with polyphenols and 24h and 72h exposure to B[a]P

Treatment with B[a]P increased mitochondrial superoxide generation ~ 46% and 90% after 24h and 72h, respectively (Fig. 6.5 A&B). With 24h pre-treatment (and removal prior to B[a]P exposure) all of the polyphenol pre-treatments significantly decreased mitochondrial superoxide generation after 24h and 72h by up to almost 100%. None of the effects of the polyphenols were significantly different from each other.

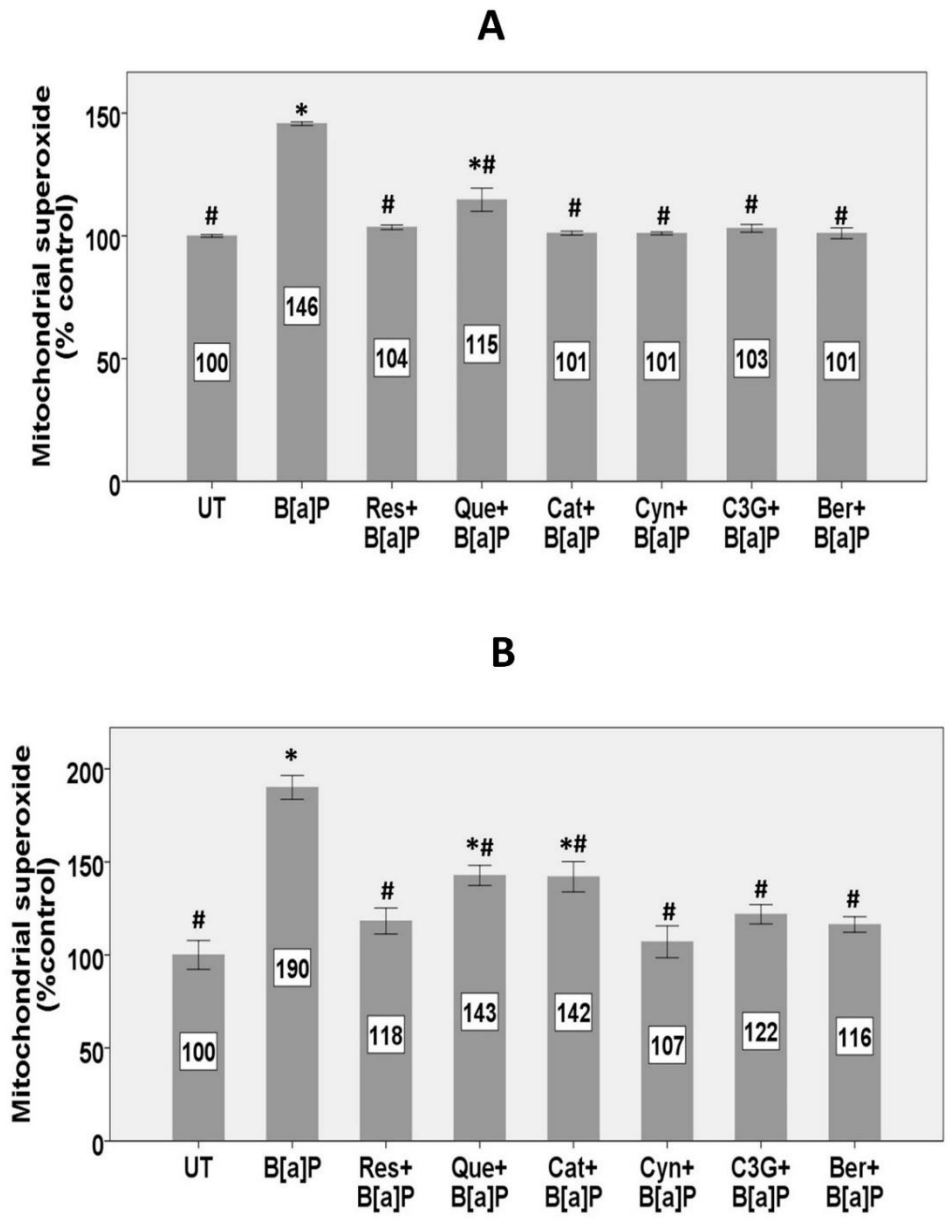

Figure 6.5. Effect of 24h pre-treatment with polyphenols on subsequent B[a]P-induced mitochondrial superoxide after 24h and 72h.

Mitochondrial superoxide was measured using MitoSOX Red in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 24h, replacement of medium without polyphenols and (A) treatment with 4 µM B[a]P for 24h and (B) treatment with 4 µM B[a]P for 72h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

6.4. Discussion

In the present study we explored the role of mitochondrial biogenesis induction by polyphenols in decreasing B[a]P-induced ROS generation. We also aimed to show whether mitochondrial biogenesis induction and associated decrease in ROS may help explain the strong effect of resveratrol on B[a]P-induced neoplastic transformation that was reported in our previous study (Omidian et al., 2017). In that study, after treatment with polyphenols and B[a]P for 72h, only resveratrol and quercetin could protect against B[a]P-induced neoplastic transformation after 21 days. Protective effects of resveratrol and quercetin against neoplastic transformation have been previously shown in different cell lines (Chen et al., 2016; Lu et al., 2008); however, studies comparing effect of resveratrol and quercetin against neoplastic transformation with other studied polyphenols are lacking. Lu et al. (2008) reported that resveratrol dose-dependently (12.5-50 µM) inhibited both 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)- and estradiol-induced neoplastic transformation in MCF-10F cells. In this study, resveratrol inhibited neoplastic transformation by decreasing CYP1B1 and increasing NQO1 expression. In a study conducted by Chen et al. (2016), quercetin at 0.5 and 2 µM inhibited IL-6 enhancement of B[a]P-induced cell transformation in human bronchial epithelial cells (HBEC). The experiments in CHAPTER 5 showed that after 72h treatment, resveratrol prevented a B[a]P-induced decrease in mitochondrial content stronger than other polyphenols (Fig. 5.2).

To investigate the effect of polyphenols alone on mitochondrial biogenesis in the current studies, we measured mitochondrial content after 24h treatment of Bhas 42 cells without B[a]P present (Fig 5.1C). Resveratrol, catechin and C3G increased mitochondrial content, however the effect of resveratrol was significantly stronger compared to the other polyphenols. Previous experimental and clinical studies have demonstrated a role of resveratrol in induction of mitochondrial biogenesis (De Paepe and Van Coster, 2017; Howitz et al., 2003); however, data comparing this effect of resveratrol with other studied polyphenols is limited.

To complete the measurements after 24h, we also measured effects of polyphenols on B[a]P-induced intracellular ROS generation after 24h. Consistent with our previous results after 12h measurement (Fig. 3.2), all polyphenols prevented against this effect of B[a]P with similar effectiveness. Therefore, the decrease in B[a]P-induced intracellular ROS at this time point was not only related to the effect of the polyphenol on mitochondrial content. It may be that the

decrease in ROS at this time point reflects both direct antioxidant effects of the polyphenols and their effects on mitochondrial content and function. Preventive effects of some of our studied polyphenols on B[a]P-induced ROS production have been previously shown (Banerjee et al., 2016b; Rathore and Wang, 2012; Woo et al., 2008). For example, in an animal study (Banerjee et al., 2016b), resveratrol (50 mg/kg of body weight) inhibited B[a]P-induced testicular dysfunction and decreased intracellular ROS production in testes tissue homogenates.

To investigate 24h pre-treatment effects of polyphenols on later B[a]P-induced intracellular ROS generation, Bhas 42 cells were exposed to 5 μM polyphenols for 24h and then medium with polyphenols was removed and 4 μM B[a]P was administrated for 24h and 72h. The purpose of this experiment was to observe whether polyphenols which induced mitochondrial biogenesis after 24h (Fig. 5.1C), would have any distinguished effect on intracellular ROS generation after subsequent 24h and 72h exposure to B[a]P. Although all polyphenols decreased ROS generation with similar effectiveness after 24h exposure to B[a]P, resveratrol and quercetin had the strongest effect on decreasing ROS generation compared to other studied polyphenols after 72h exposure to B[a]P. These strong protective effects of resveratrol and quercetin against B[a]P-induced ROS generation compared to other polyphenols therefore could be a plausible mechanism for their strongest protective affects against B[a]P-induced neoplastic transformation (Fig. 3.6).

These experiments also were designed to investigate the hypothesis that increased mitochondrial biogenesis contributed to the decreased ROS generation, and consequently prevent carcinogenesis. Taking into account the results of mitochondrial content and ROS generation, we conclude that induced mitochondrial biogenesis can be suggested as a mechanism for decreasing intracellular ROS generation by resveratrol after 24h treatment with polyphenols and 72h exposure to B[a]P. Our data are consistent with the previous studies showing association between induced mitochondrial biogenesis and decreased ROS generation (Chen et al., 2017b; Kong et al., 2010). The association between mitochondrial biogenesis and ROS generation has not been previously shown in carcinogenesis. The current results support the possibility that increasing the number of healthy mitochondria limits the impact of B[a]P-induced adducts in mtDNA on mitochondrial function.

The effects of 24h pre-treatment with polyphenols on mitochondrial superoxide generation after exposure of Bhas 42 cells to B[a]P for 24h and 72h was also investigated. After both time-points,

all polyphenols decreased mitochondrial superoxide generation with the similar potency by up to 90%. After 72h, resveratrol, cyanidin, and berberine showed the strongest effects. In the current and previous studies, we have shown that cyanidin, C3G, and berberine are the only polyphenols that protected against B[a]P-induced mitochondrial superoxide generation after 2h pre-treatment with polyphenols and 24h and 12h treatment with B[a]P, respectively. This effect of anthocyanins and berberine may be related to their abilities to accumulate in mitochondria to a greater extent than other polyphenols (Omidian et al., 2017).

To investigate treatment effects of polyphenols versus their preventive effects on B[a]P-induced intracellular ROS generation, Bhas 42 cells were pre-treated with B[a]P for 12h and then the medium containing B[a]P was removed and the cells were treated with polyphenols for another 12h. The anthocyanins and berberine showed the strongest effect in this treatment experiment. These results may be related to the strong direct antioxidant ability of anthocyanins (Kong et al., 2003), as well as the accumulation of these agents in mitochondria (Serafim et al., 2008).

In conclusion, the present study showed that one of the plausible mechanisms by which quercetin and resveratrol prevented against B[a]P-induced neoplastic transformation is through decreasing intracellular ROS generation. Moreover, this study revealed that induction of mitochondrial biogenesis by resveratrol is related to decreased B[a]P-induced intracellular ROS. With the limited amount of data in the area of mitochondrial biogenesis and cancer prevention as well as underlying mechanisms, further studies are required to elucidate related mechanisms and associations.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUDING REMARKS

7.1. General discussion

In the present study, we investigated protective effects of different polyphenols against B[a]P-induced carcinogenesis in Bhas 42 cells. Bhas 42 cells are partially transformed cells (BALB/c 3T3 cells transfected with the v-Ha-ras gene) and are used as a model of neoplastic transformation for both initiating and promoting carcinogens (Sakai et al., 2011). One of our hypotheses was that B[a]P will cause increased mitochondrial and intracellular ROS in its process of carcinogenesis in Bhas 42 cells. B[a]P has been previously shown to produce oxidative stress and oxidative DNA damage *in vivo* (Briede et al., 2004), and in cultured cells (Nkrumah-Elie et al., 2012). Oxidative stress is considered as both a trigger and consequence of carcinogenesis which contributes to DNA damage and mutations, inflammation, cell growth and proliferation, angiogenesis, and metastasis (Sabharwal and Schumacker, 2014). We showed that B[a]P at 4 μM significantly increased mitochondrial superoxide generation 42% and 65% after 12h and 24h, respectively. Increased mitochondrial superoxide by B[a]P has been shown by a few studies. Das et al. (2016) reported that B[a]P (5 μM) after 48h induced mitochondrial superoxide generation in human keratinocytes (HaCaT). Another study conducted by Sobinoff et al. (2012) showed that i.p. administration of B[a]P to mice after 7 days increased mitochondrial superoxide in oocytes. That increased mitochondrial ROS generation is a cause and consequence of mitochondrial dysfunction (Kudryavtseva et al., 2016), is consistent with our observations that B[a]P induced mitochondrial dysfunction in Bhas 42 cells. We also showed that B[a]P increased intracellular ROS generation 25%, 80%, and 220% after 12h, 24h, and 72h. Increased intracellular ROS by B[a]P has been previously shown *in vitro* and *in vivo* studies (Sang et al., 2012; Yang et al., 2017). The increased intracellular ROS generation can then promote the genome instability and changes to signaling pathways that ultimately lead to neoplastic transformation (Sabharwal and Schumacker, 2014).

We also hypothesized that berberine and anthocyanins will most effectively decrease mitochondrial superoxide in B[a]P-induced mitochondrial dysfunction. Polyphenols all inhibited intracellular ROS generation with similar effectiveness, but only positively charged polyphenols (cyanidin, C3G, and berberine) inhibited mitochondrial superoxide generation with 12h and 24h B[a]P exposure. The possible mechanism of this effect of anthocyanins and berberine, as explained

in CHAPTER 3, may be the preferential accumulation of these molecules in mitochondria, which then allows them to scavenge superoxide.

To support our results regarding carcinogenic effects of B[a]P in Bhas 42 cells, we conducted a transformation assay experiment. B[a]P stimulated neoplastic transformation in Bhas 42 cells and strongly increased the number of foci (colonies) compared to untreated cells. Carcinogenicity of B[a]P involves complex mechanisms involving formation of bulky DNA adducts and gene mutations (Lemieux et al., 2011), oxidative stress (Vijayaraman et al., 2012), inflammation (Shahid et al., 2016), cell proliferation (Burdick et al., 2003), and cell signaling pathways (Jiao et al., 2008). Some of these studies showed protective effects of individual polyphenols such as catechin (Shahid et al., 2016) and quercetin (Liu et al., 2015), and the flavonoid mixture silymarin (Vijayaraman et al., 2012). A few studies (Kao et al., 2007; Xue et al., 2001) have shown a preventive role of an individual polyphenol such as ellagic acid, or of polyphenol-rich extracts against neoplastic transformation induced by B[a]P . We found that among different polyphenols, resveratrol and quercetin significantly prevented B[a]P-induced neoplastic transformation and resveratrol showed the most robust effect. Although resveratrol had a strong effect on decreasing the number of foci induced by B[a]P, it showed similar effectiveness to the rest of the polyphenols in decreasing intracellular ROS in experiments in CHAPTER 3. Therefore, to elucidate plausible mechanisms by which resveratrol protected against B[a]P-induced neoplastic transformation, we conducted further experiments.

A possible mechanism that may explain this strong effect of resveratrol on B[a]P-induced neoplastic transformation is through decreasing cytochrome P450 drug metabolizing enzymes (CYPs) that activate B[a]P to mutagenic metabolites. We measured mRNA expression of CYP1A1 and CYP1B1, two known enzymes that metabolize B[a]P in most tissues and cells. Although some studies have reported an antagonist role of a polyphenol such as resveratrol on induction of CYPs (Andrieux et al., 2004), we showed that resveratrol, quercetin, catechin, and berberine further induced expression of CYP1A1. We conclude that the preventive effect of resveratrol on B[a]P-induced neoplastic transformation was not through inhibition of expression of CYP enzymes. Therefore, we investigated other possible mechanisms that might be involved in the inhibition of neoplastic transformation.

Due to important roles of changes to mitochondria in carcinogenesis, possible mitochondrial mechanisms by which resveratrol may have prevented B[a]P-induced neoplastic transformation were investigated. We showed that B[a]P impaired mitochondrial biogenesis and mitochondrial function whereas polyphenols protected against these effects. Although B[a]P-induced mtDNA adducts have been substantially reported (Allen and Coombs, 1980; Graziewicz et al., 2004), evidence showing effects of B[a]P on mitochondrial biogenesis are limited. In our study, exposure to B[a]P decreased the number of mitochondria by 46% and 30% after 24h and 72h, respectively. As noted in the study by Graziewicz et al., (2004), this decrease could have occurred by interference of B[a]P adducts with mtDNA replication. In another possible mechanism, Zhang et al. (2011) reported that exposure of human bronchial epithelial cells (16HBE) to B[a]P increased intracellular ROS generation, perhaps through DNA mutations, that produced mitochondrial dysfunction along with down-regulated expression of the mitochondrial biogenesis proteins NRF1 and TFAM. A few studies have investigated effects of B[a]P on mitochondrial number. For example, in a clinical study conducted on 148 non-smoking women, exposure to B[a]P from indoor and outdoor polution had inverse association with mitochondrial DNA number in leukocytes (Wong et al., 2017). Although resveratrol inhibited the decrease in mitochondrial content with similar effectiveness as other polyphenols after 24h exposure, it had the strongest effect after 72h exposure and increased the number of mitochondria to a level higher than untreated cells. These results support our third hypothesis and also may help explain the robust preventive effect of 72h treatment with resveratrol against B[a]P-induced neoplastic transformation after 21 days.

To further investigate effects of B[a]P and polyphenols on mitochondria biogenesis, other relevant biomarkers were measured. B[a]P had no effect on the expression of citrate synthase, the first enzyme in Krebs cycle and a nDNA-encoded biomarker of mitochondrial biogenesis, however resveratrol induced its expression by 80%, to a level higher than untreated cells. This result supports a role for resveratrol in induction of mitochondrial biogenesis. Lagouge et al. (2006) showed that mice fed with high fat diet and resveratrol had higher mitochondrial enzymatic activity including increased citrate synthase activity compared to the mice fed with the high fat diet alone. B[a]P also significantly decreased nDNA-encoded complex I and V subunits, and a mtDNA-encoded complex III subunit. Complexes I and III are the main sites of electron leakage and ROS production while complex V is the site of ATP production; therefore, impaired electron transfer in complexes I and III induces oxidative stress and impaired complex V shifts energy from OXPHOS

to aerobic glycolysis (Sánchez-Cenizo et al., 2010). These alterations may help explain induction of ROS generation (Fig. 3.2 & 3.4) and decreased ATP content (Fig. 5.10) induced by B[a]P. All of the polyphenols except C3G increased expression of at least one of the nDNA or mt-DNA-encoded mitochondrial subunits. Effects of these polyphenols on mitochondrial complexes in non-cancer models have been previously described (Gomes et al., 2012; Nichols et al., 2015).

Resveratrol is well known as an activator of SIRT1 (Howitz et al., 2003) and its ability to decrease tumorigenesis through activating SIRT1 has been previously shown (Wang et al., 2008). In our study, although resveratrol had no effect on SIRT1 or PGC-1α gene expression, it increased SIRT1 activity and deacetylated PGC-1α protein. We also showed that B[a]P had no significant effect on SIRT1 activity or on PGC-1α gene and deacetylated PGC-1α protein expression. However, B[a]P downregulated expression of ERRα, a transcription factor involved in mitochondrial biogenesis, while different polyphenols such as resveratrol, quercetin, and cyanidin protected against this effect.

To investigate the effects of B[a]P and polyphenols on mitochondrial function, we measured MMP and ATP content. B[a]P consistently decreased MMP and ATP while different polyphenols prevented these effects of B[a]P. In addition to decreased mitochondrial content, increased UCP2 expression by B[a]P (Fig 3.5 A) could have contributed to the decreased MMP. Other studies have shown decreased MMP and ATP in cells exposed to B[a]P. Exposure of human trophoblast cell Swan 71 to benzo(a)pyren-7,8-dihydrodiol-9,10-epoxide, an ultimate carcinogenic product of B[a]P, decreased MMP (Wang et al., 2018). B[a]P exposure also decreased cellular ATP content in immortalized human keratinocyte cell line (HaCaT) (Das et al., 2017). Resveratrol was the only polyphenol that increased the ATP content in cells grown on galactose, showing the ability of resveratrol to induce mitochondrial oxidative phosphorylation.

Resveratrol had the strongest effect on mitochondrial parameters such as mitochondrial content after 72h, citrate synthase expression and ATP content. However, these results are not ample enough to support the strong protective effect of resveratrol against B[a]P-induced neoplastic transformation. Therefore, we extended our investigations to explore the possible connection between mitochondrial biogenesis and ROS production in preventing B[a]P-induced neoplastic transformation.

In CHAPTER 6 we showed that resveratrol, catechin and C3G increased mitochondrial content without B[a]P present. Resveratrol had the strongest effect and significantly increased mitochondrial content compared to other polyphenols. To show whether prior mitochondrial biogenesis decreases the impact of B[a]P on intracellular and mitochondrial ROS production, we pre-treated with the polyphenols for 24h, removed the polyphenol-containing media, and then measured intracellular ROS and mitochondrial superoxide generation after exposure to B[a]P for 24h and 72h. We observed that 24h prior treatment with resveratrol and quercetin almost completely prevented the B[a]P-induced intracellular ROS generation after 72h exposure to B[a]P. Therefore, we can conclude that induced mitochondrial biogenesis by resveratrol is associated with decreased B[a]P-induced intracellular ROS generation. Since increased mitochondrial biogenesis increases the mtDNA copy number (Peng et al., 2016; Sugiyama et al., 2015), this likely diminished the impact of B[a]P adducts and mutations in the genes for mtDNA-encoded respiratory complexes. Therefore, the increased mitochondrial biogenesis and most strongly decreased ROS generation by resveratrol and quercetin may explain the strong effects of these polyphenols against B[a]P-induced neoplastic transformation. Pre-treatments with all of the polyphenols strongly suppressed B[a]P-induced mitochondrial superoxide generation, perhaps by direct antioxidant effects with some polyphenols (such as cyanidin) and increased mitochondrial biogenesis with others. This result suggests that not only decreasing mitochondrial superoxide, but increasing mitochondrial biogenesis is important for the strongest protective effect against neoplastic transformation.

Taken together, we showed that B[a]P exposure induces neoplastic transformation in Bhas 42 cells subsequent to decreasing mitochondrial content and function, increasing intracellular ROS and mitochondrial superoxide generation, and inducing TNF-α expression (a pro-inflammatory cytokine). Different polyphenols protected against these effects and resveratrol had the strongest effect against B[a]P-induced neoplastic transformation whereas it had similar effectiveness as other polyphenols in other experiments. These effects of polyphenols were not associated with an AhR antagonism activity. Resveratrol showed the strongest effects in some experiments including increasing mitochondrial content after 72h. In addition, we showed that induction of mitochondrial biogenesis by resveratrol lead to decreased ROS production. We conclude that the strong preventive effect of resveratrol and quercetin on B[a]P-induced neoplastic transformation is

possibly through their combined effect of increasing biogenesis of functional mitochondria and decreasing B[a]P-induced ROS generation.

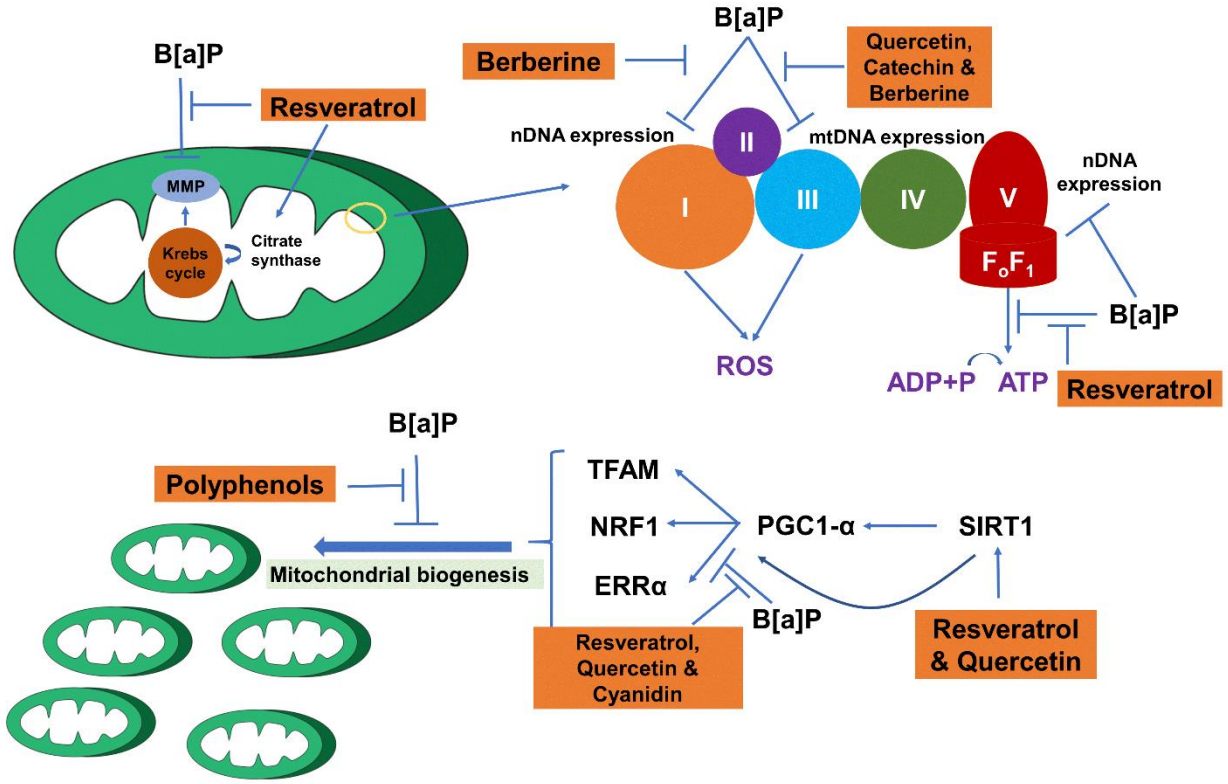

Figure 7.1. Effect of B[a]P and polyphenols on mitochondrial biogenesis and mitochondrial function.

B[a]P decreased mitochondrial content and expression of mitochondrial respiratory subunits I, III, V, and ERRα. However, different polyphenols prevented these B[a]P effects and induced mitochondrial biogenesis. B[a]P also induced mitochondrial dysfunction by decreasing levels of MMP and ATP content, while polyphenols prevented B[a]P-induced mitochondrial dysfunction.

7.2. Limitations of the study

7.2.1. Model of study

One of the limitations of this study was that we used an *in vitro* model of carcinogenesis. Although *in vitro* models are good models for identifying and testing molecular mechanisms, they do not show all the features of cancer in humans. Moreover, polyphenol bioavailability following oral consumption is typically low and highly variable, and they appear in the blood and tissues in different forms (e.g. O-glucuronides, sulfate esters, and O-methyl esters) that may impact their functional abilities. Therefore, lack of an *in vivo* model is one of our limitations.

7.2.2. Other mechanisms involved in carcinogenesis

Cancer is a complex and multifactorial disease developing through interactions between several known and unknown mechanisms. In the current study we mainly investigated mitochondrial mechanisms by which B[a]P and polyphenols may play a role in carcinogenesis. However, there are other mechanisms that need to be considered including effects on MAPK, Akt, and glycolytic pathways.

7.2.3. Mitochondrial respiratory chain activity

Although mitochondrial respiratory subunit mRNA expression can show the state of mitochondrial function to some extent, measuring mitochondrial respiratory complex activity is more reliable and accurate. Therefore, one of the limitations of our study is lack of determining respiratory chain activity. Moreover, to have a comprehensive understanding of mitochondrial function, mitochondrial oxygen consumption needs to be measured (e.g. by Seahorse XF).

7.3. Future directions

In the current study, the roles of B[a]P and polyphenols in cancer initiation and progression mainly through mitochondrial pathways was investigated. Polyphenols ameliorated decreased mitochondrial biogenesis and prevented mitochondrial dysfunction; however, resveratrol showed the strongest effect in preventing against neoplastic transformation induced by B[a]P. Although we showed that resveratrol had the most robust effect in some key experiments and probably exerts its effect by inducing mitochondrial biogenesis and decreasing ROS production, further studies are still required to elucidate anti-carcinogenetic mechanisms of resveratrol. In addition to studying

the role of mitochondrial function and biogenesis in cancer initiation and progression, other pathways involved in carcinogenesis including MAPK, Akt pathways, and glycolytic pathway (e.g. HIF-1α, glycolytic enzymes, and glucose transporters) need to be investigated. In the current study, mRNA expression of mitochondrial respiratory subunits and citrate synthase were measured. To confirm these results, activity of mitochondrial complexes and citrate synthase should be measured in future studies.

Moreover, conducting an *in vivo* study is one of the future directions. An *in vivo* evaluation is necessary to test the *in vivo* validity of preventive effects of dietary polyphenols on B[a]P-induced carcinogenesis, considering their bioavailability and metabolism. This would require chronic dietary administration of polyphenols before during and after B[a]P exposure. In such an *in vivo* study, animals could be fed with resveratrol, anthocyanin-rich extract (eg, bilberry extract), and combination of resveratrol and bilberry extract. The dose of resveratrol could be 30 mg/kg body weight throughout the experiment (added to diet). This amount is equivalent to 340 mg per day for a 70 kg human, which is far below the dose considered safe for humans (up to 5000 mg) (Boocock et al., 2007; Cui et al., 2010). The dose of bilberry extract could be 50 mg/kg body weight throughout the experiment (in the diet or water). This amount of anthocyanin extract has ~ 12 mg anthocyanins (equivalent to 200 mg in humans). The average intake of anthocyanins in the U.S. diet is estimated to be 180-215 mg (Wang & Stoner, 2008). For dietary relevance, B[a]P could be orally administrated (by gavage) with daily dose of 75 mg/kg dissolved in olive oil for 28 days (Ajayi et al., 2016; Huderson et al., 2013; Labib et al., 2012). After 16 weeks, effects on tumour incidence and pre-neoplastic lesions (eg. aberrant crypt foci) would be determined histologically, and possible mitochondrial mechanisms including mitochondrial content would be investigated in the liver, lung, and intestine of the animals. Biomarkers of interest could include inflammatory biomarkers (such as plasma and/or tissue levels of C-reactive protein, TNF-α and IL-6), liver damage biomarkers (such as alanine aminotransferase), tissue markers of oxidative stress such as TBARS and 8-oxo-dG (in mitochondrial and nuclear DNA), and tissue markers of mitochondrial content such as citrate synthase and mitochondrial respiratory complex activities, and the amount of mtDNA.

Another suggestion for future study is using *in vitro* and *in vivo* models of knockdown genes. Knockdown of mitochondrial biogenesis factors such as PGC-1α, NRF1, or TFAM can show us

the role of mitochondria in carcinogenesis induced by B[a]P. In our study, we found that B[a]P induced mitochondrial dysfunction and reduced mitochondrial biogenesis; however, contribution of mitochondria to B[a]P-induced neoplastic transformation is as yet unknown. Mitochondrial gene knockdown also can show the contribution of mitochondria to cancer prevention by different polyphenols.

Another suggestion is applying antimycin A (to increase mitochondrial superoxide) and ethidium bromide (to deplete mtDNA) for Bhas 42 transformation, and inhibition by polyphenols (maybe just resveratrol and cyanidin). By applying antimycin A, we observe whether increased mitochondrial superoxide along with impaired mitochondrial function can induce neoplastic transformation. By applying ethidium bromide, we can observe whether the absence of mitochondrial respiratory function can induce neoplastic transformation in Bhas 42 cells.

In the present study, we compared preventive roles of different polyphenols against cancer initiation and progression induced by B[a]P. Recent studies have investigated and compared roles of conjugated forms of polyphenols in prevention and treatment of several diseases. Several studies attributed beneficial effects of some polyphenols to their conjugated forms (Patel et al., 2013). Therefore, we recommend investigating role of polyphenols and their conjugated metabolites such as resveratrol-3-O-sulfate in different models of carcinogenesis.

In order to investigate the effect of B[a]P and polyphenols on mitochondrial mass versus mitochondrial biogenesis/replication, we suggest measuring the amount of mtDNA. This experiment will clarify why some polyphenols increase mitochondrial content, however, have no effect on mitochondrial respiratory subunits. To further investigate effects of B[a]P on mtDNA, we suggest measuring B[a]P-induced mtDNA adducts and 8-oxoDG.

8. REFERENCES

Acharyya, N., Ali, S., Deb, B., Chattopadhyay, S., Maiti, S., 2015. Green tea (Camellia sinensis) alleviates arsenic-induced damages to DNA and intestinal tissues in rat and in situ intestinal loop by reinforcing antioxidant system. Environmental Toxicology 30, 1033-1044.

Adams, J., Cory, S., 2007. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26, 1324-1337.

Aggarwal, B.B., Gehlot, P., 2009. Inflammation and cancer: how friendly is the relationship for cancer patients? Current Opinion in Pharmacology 9, 351-369.

Ahmad, N., Adhami, V.M., Afaq, F., Feyes, D.K., Mukhtar, H., 2001. Resveratrol causes WAF-1/p21-mediated G1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clinical Cancer Research 7, 1466-1473.

Ajayi, B.O., Adedara, I.A., Farombi, E.O., 2016. Benzo (a) pyrene induces oxidative stress, pro-inflammatory cytokines, expression of nuclear factor-kappa B and deregulation of wnt/beta-catenin signaling in colons of BALB/c mice. Food and Chemical Toxicology 95, 42-51.

Alhosin, M., León-González, A.J., Dandache, I., Lelay, A., Rashid, S.K., Kevers, C., Pincemail, J., Fornecker, L.-M., Mauvieux, L., Herbrecht, R., 2015. Bilberry extract (Antho 50) selectively induces redox-sensitive caspase 3-related apoptosis in chronic lymphocytic leukemia cells by targeting the Bcl-2/Bad pathway. Scientific Reports 5, 8996.

Allalunis-Turner, J., Ma, I., Hanson, J., Pearcey, R.G., 2006. mtDNA mutations in invasive cervix tumors: a retrospective analysis. Cancer Letters 243, 193-201.

Allen, J., Coombs, M., 1980. Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. Nature 287, 244.

Andersen, O.M., Markham, K.R., 2005. Flavonoids: chemistry, biochemistry and applications. CRC Press.

Anderson, K.E., Kadlubar, F.F., Kulldorff, M., Harnack, L., Gross, M., Lang, N.P., Barber, C., Rothman, N., Sinha, R., 2005. Dietary intake of heterocyclic amines and benzo (a) pyrene: associations with pancreatic cancer. Cancer Epidemiology and Prevention Biomarkers 14, 2261-2265.

Andrieux, L., Langouët, S., Fautrel, A., Ezan, F., Krauser, J.A., Savouret, J.F., Guengerich, F.P., Baffet, G., Guillouzo, A., 2004. Aryl hydrocarbon receptor activation and cytochrome P450 1A induction by the mitogen-activated protein kinase inhibitor U0126 in hepatocytes. Molecular Pharmacology 65, 934-943.

Appeldoorn, M.M., Vincken, J.-P., Gruppen, H., Hollman, P.C., 2009. Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. The Journal of Nutrition 139, 1469-1473.

Asada, S., Sasaki, K., Tanaka, N., Takeda, K., Hayashi, M., Umeda, M., 2005. Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells). Mutation Research/Genetic Toxicology and Environmental Mutagenesis 588, 7-21.

Ashkenazi, A., Dixit, V.M., 1998. Death receptors: signaling and modulation. Science 281, 1305.

Athar, M., Back, J.H., Tang, X., Kim, K.H., Kopelovich, L., Bickers, D.R., Kim, A.L., 2007. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicology and Applied Pharmacology 224, 274-283.

Azad, M.B., Chen, Y., Gibson, S.B., 2009. Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. Antioxidants & Redox Signaling 11, 777-790.

Azevedo, C., Correia-Branco, A., Araújo, J.R., Guimarães, J.T., Keating, E., Martel, F., 2015. The chemopreventive effect of the dietary compound kaempferol on the MCF-7 human breast cancer cell line is dependent on inhibition of glucose cellular uptake. Nutrition and Cancer 67, 504-513.

Azqueta, A., Collins, A., 2016. Polyphenols and DNA damage: a mixed blessing. Nutrients 8, 785.

Backer, J., Weinstein, I., 1980. Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative of benzo [a] pyrene. Science 209, 297-299.

Backer, J.M., Weinstein, I.B., 1982. Interaction of benzo (a) pyrene and its dihydrodiol-epoxide derivative with nuclear and mitochondrial DNA in C3h10t½ cell cultures. Cancer Research 42, 2764-2769.

Baffy, G., 2010. Uncoupling protein-2 and cancer. Mitochondrion 10, 243-252.

Bag, A., Bag, N., 2018. Tea polyphenols and prevention of epigenetic aberrations in cancer. Journal of Natural Science, Biology, and Medicine 9, 2.

Baird, W.M., Hooven, L.A., Mahadevan, B., 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environmental and Molecular Mutagenesis 45, 106-114.

Baldwin, A.S., 2001. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. The Journal of Clinical Investigation 107, 241-246.

Balkwill, F., Mantovani, A., 2001. Inflammation and cancer: back to Virchow? The Lancet 357, 539-545.

Bandy, B., Davison, A.J., 1990. Mitochondrial mutations may increase oxidative stress: implications for carcinogenesis and aging? Free Radical Biology and Medicine 8, 523-539.

Banerjee, B., Chakraborty, S., Ghosh, D., Raha, S., Sen, P.C., Jana, K., 2016a. Benzo (a) pyrene induced p53 mediated male germ cell apoptosis: synergistic protective effects of curcumin and resveratrol. Frontiers in Pharmacology 7, 245.

Banerjee, B., Nandi, P., Chakraborty, S., Raha, S., Sen, P.C., Jana, K., 2016b. Resveratrol ameliorates benzo (a) pyrene-induced testicular dysfunction and apoptosis: involvement of p38 MAPK/ATF2/iNOS signaling. The Journal of Nutritional Biochemistry 34, 17-29.

Barber, D.A., Harris, S.R., 1994. Oxygen Free Radicals and Antioxidants: A Review: The use of antioxidant vitamin supplements to scavenge free radicals could decrease the risk of disease. American Pharmacy 34, 26-35.

Barnes, P.J., Karin, M., 1997. Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine 336, 1066-1071.

Basile, V., Ferrari, E., Lazzari, S., Belluti, S., Pignedoli, F., Imbriano, C., 2009. Curcumin derivatives: molecular basis of their anti-cancer activity. Biochemical Pharmacology 78, 1305-1315.

Baur, J.A., 2010. Biochemical effects of SIRT1 activators. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1804, 1626-1634.

Bellance, N., Benard, G., Furt, F., Begueret, H., Smolkova, K., Passerieux, E., Delage, J., Baste, J., Moreau, P., Rossignol, R., 2009. Bioenergetics of lung tumors: alteration of mitochondrial biogenesis and respiratory capacity. The International Journal of Biochemistry & Cell Biology 41, 2566-2577.

Bellosillo, B., Villamor, N., López-Guillermo, A., Marcé, S., Bosch, F., Campo, E., Montserrat, E., Colomer, D., 2002. Spontaneous and drug-induced apoptosis is mediated by conformational changes of Bax and Bak in B-cell chronic lymphocytic leukemia. Blood 100, 1810-1816.

Berge, G., Øvrebø, S., Botnen, I., Hewer, A., Phillips, D., Haugen, A., Mollerup, S., 2004. Resveratrol inhibits benzo [a] pyrene–DNA adduct formation in human bronchial epithelial cells. British Journal of Cancer 91, 333-338.

Bergeron, R., Ren, J.M., Cadman, K.S., Moore, I.K., Perret, P., Pypaert, M., Young, L.H., Semenkovich, C.F., Shulman, G.I., 2001. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. American Journal of Physiology-Endocrinology and Metabolism 281, E1340-E1346.

Berneburg, M., Kamenisch, Y., Krutmann, J., Röcken, M., 2006. 'To repair or not to repair–no longer a question': repair of mitochondrial DNA shielding against age and cancer. Experimental Dermatology 15, 1005-1015.

Bernstein, C., Prasad, A.R., Nfonsam, V., Bernstein, H., 2013. DNA damage, DNA repair and cancer. New Research Directions in DNA Repair. InTech. doi 10, 53919.

Bernstein, H., Payne, C.M., Bernstein, C., Garewal, H., Dvorak, K., 2008. Cancer and aging as consequences of un-repaired DNA damage. New Research on DNA Damages. New York, NY, USA: Nova Science Publishers, 1-47.

Bersaas, A., Arnoldussen, Y.J., Sjøberg, M., Haugen, A., Mollerup, S., 2016. Epithelial-mesenchymal transition and FOXA genes during tobacco smoke carcinogen induced transformation of human bronchial epithelial cells. Toxicology in Vitro 35, 55-65.

Betteridge, D.J., 2000. What is oxidative stress? Metabolism 49, 3-8.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organization Journal 5, 9.

Bishayee, A., 2009. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. Cancer Prevention Research, 1940-6207. CAPR-1908-0160.

Blaser, H., Dostert, C., Mak, T.W., Brenner, D., 2016. TNF and ROS crosstalk in inflammation. Trends in Cell Biology 26, 249-261.

Boada, L.D., Henríquez-Hernández, L., Luzardo, O., 2016. The impact of red and processed meat consumption on cancer and other health outcomes: epidemiological evidences. Food and Chemical Toxicology 92, 236-244.

Boffetta, P., Jourenkova, N., Gustavsson, P., 1997. Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes & Control 8, 444-472.

Boland, M.L., Chourasia, A.H., Macleod, K.F., 2013. Mitochondrial dysfunction in cancer. Frontiers in Oncology 3, 292.

Boocock, D.J., Faust, G.E., Patel, K.R., Schinas, A.M., Brown, V.A., Ducharme, M.P., Booth, T.D., Crowell, J.A., Perloff, M., Gescher, A.J., 2007. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiology and Prevention Biomarkers 16, 1246-1252.

Boots, A.W., Haenen, G.R., Bast, A., 2008. Health effects of quercetin: from antioxidant to nutraceutical. European Journal of Pharmacology 585, 325-337.

Boström, C.-E., Gerde, P., Hanberg, A., Jernström, B., Johansson, C., Kyrklund, T., Rannug, A., Törnqvist, M., Victorin, K., Westerholm, R., 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environmental Health Perspectives 110, 451.

Brand, M.D., Affourtit, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Pakay, J.L., Parker, N., 2004. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radical Biology and Medicine 37, 755-767.

Brandon, M., Baldi, P., and, Wallace, D., 2006. Mitochondrial mutations in cancer. Oncogene 25, 4647-4662.

Briede, J.J., Godschalk, R.W., Emans, M.T., De Kok, T., Van Agen, E., Van Maanen, J., Van Schooten, F.-J., Kleinjans, J., 2004. In vitro and in vivo studies on oxygen free radical and DNA adduct formation in rat lung and liver during benzo [a] pyrene metabolism. Free Radical Research 38, 995-1002.

Burdick, A.D., Davis, J.W., Liu, K.J., Hudson, L.G., Shi, H., Monske, M.L., Burchiel, S.W., 2003. Benzo (a) pyrene quinones increase cell proliferation, generate reactive oxygen species, and transactivate the epidermal growth factor receptor in breast epithelial cells. Cancer Research 63, 7825-7833.

Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. Nature Reviews Cancer 11, 85.

cancer, I.a.f.r.o., 2010. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC Press, International Agency for Research on Cancer.

Candido, J., Hagemann, T., 2013. Cancer-related inflammation. Journal of Clinical Immunology 33, 79-84.

Cao, P., Vadhanam, M.V., Spencer, W.A., Cai, J., Gupta, R.C., 2011. Sustained systemic delivery of green tea polyphenols by polymeric implants significantly diminishes benzo [a] pyrene-induced DNA adducts. Chemical Research in Toxicology 24, 877-886.

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F.J., Queipo-Ortuño, M.I., 2013. Benefits of polyphenols on gut microbiota and implications in human health. The Journal of Nutritional Biochemistry 24, 1415-1422.

Carelli, V., Maresca, A., Caporali, L., Trifunov, S., Zanna, C., Rugolo, M., 2015. Mitochondria: Biogenesis and mitophagy balance in segregation and clonal expansion of mitochondrial DNA mutations. The International Journal of Biochemistry & Cell Biology 63, 21-24.

Casper, R.F., Quesne, M., Rogers, I.M., Shirota, T., Jolivet, A., Milgrom, E., Savouret, J.-F., 1999. Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. Molecular Pharmacology 56, 784-790.

Casteilla, L., Rigoulet, M., Pénicaud, L., 2001. Mitochondrial ROS metabolism: modulation by uncoupling proteins. IUBMB life 52, 181-188.

Chattopadhyay, E., De Sarkar, N., Singh, R., Ray, A., Roy, R., Paul, R.R., Pal, M., Ghose, S., Ghosh, S., Kabiraj, D., 2016. Genome-wide mitochondrial DNA sequence variations and lower expression of OXPHOS genes predict mitochondrial dysfunction in oral cancer tissue. Tumor Biology 37, 11861-11871.

Chelsky, Z.L., Yue, P., Kondratyuk, T.P., Paladino, D., Pezzuto, J.M., Cushman, M., Turkson, J., 2015. A Resveratrol Analogue Promotes ERKMAPK–Dependent Stat3 Serine and Tyrosine Phosphorylation Alterations and Antitumor Effects In Vitro against Human Tumor Cells. Molecular Pharmacology 88, 524-533.

Chen, J., Leung, P.K., Wong, H.S., Leung, H.Y., Chan, W.M., Ko, K.M., 2017a. Ursolic acid induces mitochondrial biogenesis through the activation of AMPK and PGC-1 in C2C12 myotubes: A possible mechanism underlying its beneficial effect on exercise endurance. Food & Function.

Chen, J., Wong, H.S., Leong, P.K., Leung, H.Y., Chan, W.M., Ko, K.M., 2017b. Ursolic acid induces mitochondrial biogenesis through the activation of AMPK and PGC-1 in C2C12 myotubes: a possible mechanism underlying its beneficial effect on exercise endurance. Food & Function 8, 2425-2436.

Chen, W., Padilla, M.T., Xu, X., Desai, D., Krzeminski, J., Amin, S., Lin, Y., 2016. Quercetin inhibits multiple pathways involved in interleukin 6 secretion from human lung fibroblasts and activity in bronchial epithelial cell transformation induced by benzo [a] pyrene diol epoxide. Molecular carcinogenesis 55, 1858-1866.

Chiara, F., Castellaro, D., Marin, O., Petronilli, V., Brusilow, W.S., Juhaszova, M., Sollott, S.J., Forte, M., Bernardi, P., Rasola, A., 2008. Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. PLoS One 3, e1852.

Chinnaiyan, A.M., 1999. The apoptosome: heart and soul of the cell death machine. Neoplasia 1, 5-15.

Chiva-Blanch, G., Visioli, F., 2012. Polyphenols and health: moving beyond antioxidants. Journal of Berry Research 2, 63-71.

Cijo George, V., Vijayakumaran Vijesh, V., Inoka Madumani Amararathna, D., Ayshwarya Lakshmi, C., Anbarasu, K., Ragupathi Naveen Kumar, D., Radhakrishnan Ethiraj, K., Ashok Kumar, R., P Vasantha Rupasinghe, H., 2016. Mechanism of action of flavonoids in prevention of inflammation-associated skin cancer. Current Medicinal Chemistry 23, 3697-3716.

Clair, D.S., Zhao, Y., Chaiswing, L., Oberley, T., 2005. Modulation of skin tumorigenesis by SOD. Biomedicine & Pharmacotherapy 59, 209-214.

Clementino, M., Shi, X., Zhang, Z., 2017. Prevention of polyphenols against carcinogenesis induced by environmental carcinogens. Journal of Environmental Pathology, Toxicology and Oncology 36, 87-98.

Cole, L.W., 2016. The evolution of per-cell organelle number. Frontiers in cell and developmental biology 4, 85.

Conney, A.H., 1982. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: GHA Clowes Memorial Lecture. Cancer Research 42, 4875-4917.

Cottet-Rousselle, C., Ronot, X., Leverve, X., Mayol, J.F., 2011. Cytometric assessment of mitochondria using fluorescent probes. Cytometry Part A 79, 405-425.

Coussens, L.M., Werb, Z., 2002. Inflammation and cancer. Nature 420, 860-867.

Crespy, V., Morand, C., Besson, C., Manach, C., Demigne, C., Remesy, C., 2002. Quercetin, but not its glycosides, is absorbed from the rat stomach. Journal of Agricultural and Food Chemistry 50, 618-621.

Cross, A.J., Freedman, N.D., Ren, J., Ward, M.H., Hollenbeck, A.R., Schatzkin, A., Sinha, R., Abnet, C.C., 2011. Meat consumption and risk of esophageal and gastric cancer in a large prospective study. The American Journal of Gastroenterology 106, 432-442.

Cruz-Bermúdez, A., Vallejo, C.G., Vicente-Blanco, R.J., Gallardo, M.E., Fernández-Moreno, M.Á., Quintanilla, M., Garesse, R., 2015. Enhanced tumorigenicity by mitochondrial DNA mild mutations. Oncotarget 6, 13628.

Csiszar, A., Labinskyy, N., Pinto, J.T., Ballabh, P., Zhang, H., Losonczy, G., Pearson, K., De Cabo, R., Pacher, P., Zhang, C., 2009. Resveratrol induces mitochondrial biogenesis in endothelial cells. American journal of Physiology-Heart and Circulatory Physiology 297, H13-H20.

Cui, X., Jin, Y., Hofseth, A.B., Pena, E., Habiger, J., Chumanevich, A., Poudyal, D., Nagarkatti, M., Nagarkatti, P.S., Singh, U.P., 2010. Resveratrol suppresses colitis and colon cancer associated with colitis. Cancer Prevention Research, 1940-6207. CAPR-1909-0117.

Czank, C., Cassidy, A., Zhang, Q., Morrison, D.J., Preston, T., Kroon, P.A., Botting, N.P., Kay, C.D., 2013. Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a 13C-tracer study–. The American of Clinical Nutrition 97, 995-1003.

D Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., 2007. Polyphenols, dietary sources and bioavailability. Annali-Istituto Superiore Di Sanita 43, 348.

D'Archivio, M., Santangelo, C., Scazzocchio, B., Varì, R., Filesi, C., Masella, R., Giovannini, C., 2008. Modulatory effects of polyphenols on apoptosis induction: relevance for cancer prevention. International Journal of Molecular Sciences 9, 213-228.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313-7352.

Dammann, R.H., Richter, A.M., Jiménez, A.P., Woods, M., Küster, M., Witharana, C., 2017. Impact of Natural Compounds on DNA Methylation Levels of the Tumor Suppressor Gene RASSF1A in Cancer. International Journal of Molecular Sciences 18, 2160.

Danial, N.N., Korsmeyer, S.J., 2004. Cell death: critical control points. Cell 116, 205-219.

Das, D.N., Naik, P.P., Mukhopadhyay, S., Panda, P.K., Sinha, N., Meher, B.R., Bhutia, S.K., 2017. Elimination of dysfunctional mitochondria through mitophagy suppresses benzo [a] pyrene-induced apoptosis. Free Radical Biology and Medicine 112, 452-463.

Das, D.N., Naik, P.P., Nayak, A., Panda, P.K., Mukhopadhyay, S., Sinha, N., Bhutia, S.K., 2016. bacopa monnieri-induced Protective Autophagy Inhibits Benzo [a] pyrene-mediated Apoptosis. Phytotherapy Research 30, 1794-1801.

Davis, J.M., Murphy, E.A., Carmichael, M.D., Davis, B., 2009. Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 296, R1071-R1077.

Day, A.J., Cañada, F.J., Díaz, J.C., Kroon, P.A., Mclauchlan, R., Faulds, C.B., Plumb, G.W., Morgan, M.R., Williamson, G., 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS letters 468, 166-170.

de Araujo, L.F., Fonseca, A.S., Muys, B.R., Plaça, J.R., Bueno, R.B., Lorenzi, J.C., Santos, A.R., Molfetta, G.A., Zanette, D.L., Souza, J.E., 2015. Mitochondrial genome instability in colorectal adenoma and adenocarcinoma. Tumor Biology 36, 8869-8879.

de Moura, M.B., dos Santos, L.S., Van Houten, B., 2010. Mitochondrial dysfunction in neurodegenerative diseases and cancer. Environmental and Molecular Mutagenesis 51, 391-405.

de Oliveira, M.R., Nabavi, S.M., Braidy, N., Setzer, W.N., Ahmed, T., Nabavi, S.F., 2016. Quercetin and the mitochondria: a mechanistic view. Biotechnology Advances 34, 532-549.

De Paepe, B., Van Coster, R., 2017. A Critical Assessment of the Therapeutic Potential of Resveratrol Supplements for Treating Mitochondrial Disorders. Nutrients 9, 1017.

DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., Thompson, C.B., 2008. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metabolism 7, 11-20.

Del Follo-Martinez, A., Banerjee, N., Li, X., Safe, S., Mertens-Talcott, S., 2013. Resveratrol and quercetin in combination have anticancer activity in colon cancer cells and repress oncogenic microRNA-27a. Nutrition and Cancer 65, 494-504.

del Mar Blanquer-Rosselló, M., Hernández-López, R., Roca, P., Oliver, J., Valle, A., 2017. Resveratrol induces mitochondrial respiration and apoptosis in SW620 colon cancer cells. Biochimica et Biophysica Acta (BBA)-General Subjects 1861, 431-440.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P., Tognolini, M., Borges, G., Crozier, A., 2013. Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants & Redox Signaling 18, 1818-1892.

Delmas, D., Lançon, A., Colin, D., Jannin, B., Latruffe, N., 2006. Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. Current Drug Targets 7, 423-442.

Deng, C.-X., 2009. SIRT1, is it a tumor promoter or tumor suppressor? International Journal of Biological Sciences 5, 147.

Derdak, Z., Mark, N.M., Beldi, G., Robson, S.C., Wands, J.R., Baffy, G., 2008. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. Cancer Research 68, 2813-2819.

Di Sebastiano, K.M., Mourtzakis, M., 2014. The role of dietary fat throughout the prostate cancer trajectory. Nutrients 6, 6095-6109.

Dinkova-Kostova, A.T., Abramov, A.Y., 2015. The emerging role of Nrf2 in mitochondrial function. Free Radical Biology and Medicine 88, 179-188.

Dott, W., Mistry, P., Wright, J., Cain, K., Herbert, K.E., 2014. Modulation of mitochondrial bioenergetics in a skeletal muscle cell line model of mitochondrial toxicity. Redox Biology 2, 224-233.

Duluc, L., Jacques, C., Soleti, R., Andriantsitohaina, R., Simard, G., 2014. Delphinidin inhibits VEGF induced-mitochondrial biogenesis and Akt activation in endothelial cells. The International Journal of Biochemistry & Cell Biology 53, 9-14.

Eghbaliferiz, S., Iranshahi, M., 2016. Prooxidant activity of polyphenols, flavonoids, anthocyanins and carotenoids: updated review of mechanisms and catalyzing metals. Phytotherapy Research 30, 1379-1391.

Eichner, L.J., Giguère, V., 2011. Estrogen related receptors (ERRs): a new dawn in transcriptional control of mitochondrial gene networks. Mitochondrion 11, 544-552.

Ekbom, A., Adami, H., Helmick, C., Zack, M., 1990. Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. The Lancet 336, 357-359.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic Pathology 35, 495-516.

Epstein, T., Gatenby, R.A., Brown, J.S., 2017. The Warburg effect as an adaptation of cancer cells to rapid fluctuations in energy demand. PLoS One 12, e0185085.

Faubert, B., Vincent, E.E., Poffenberger, M.C., Jones, R.G., 2015. The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator. Cancer Letters 356, 165-170.

Fernandez-Marcos, P.J., Auwerx, J., 2011. Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis–. The American Journal of Clinical Nutrition 93, 884S-890S.

Ferraz da Costa, D.C., Fialho, E., Silva, J.L., 2017. Cancer Chemoprevention by Resveratrol: The p53 Tumor Suppressor Protein as a Promising Molecular Target. Molecules 22, 1014.

Fliss, M.S., Usadel, H., Caballero, O.L., Wu, L., Buta, M.R., Eleff, S.M., Jen, J., Sidransky, D., 2000. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 287, 2017-2019.

Forester, S.C., Waterhouse, A.L., 2010. Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2, 4, 6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells. Journal of Agricultural and Food Chemistry 58, 5320-5327.

Forman, H.J., Zhang, H., Rinna, A., 2009. Glutathione: overview of its protective roles, measurement, and biosynthesis. Molecular Aspects of Medicine 30, 1-12.

Formentini, L., Martínez-Reyes, I., Cuezva, J.M., 2010. The mitochondrial bioenergetic capacity of carcinomas. IUBMB life 62, 554-560.

Fouad, M., Agha, A., Merzabani, M.A., Shouman, S., 2013. Resveratrol inhibits proliferation, angiogenesis and induces apoptosis in colon cancer cells: calorie restriction is the force to the cytotoxicity. Human & Experimental Toxicology 32, 1067-1080.

Fromme, H., Lahrz, T., Piloty, M., Gebhardt, H., Oddoy, A., Rüden, H., 2004. Polycyclic aromatic hydrocarbons inside and outside of apartments in an urban area. Science of the Total Environment 326, 143-149.

Furuta, E., Okuda, H., Kobayashi, A., Watabe, K., 2010. Metabolic genes in cancer: their roles in tumor progression and clinical implications. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 1805, 141-152.

Gao, F., Li, M., Liu, W.-B., Zhou, Z.-S., Zhang, R., Li, J.-L., Zhou, K.-C., 2015. Epigallocatechin gallate inhibits human tongue carcinoma cells via HK2-mediated glycolysis. Oncology Reports 33, 1533-1539.

Gao, M., Long, J., Li, Y., Shah, W., Fu, L., Liu, J., Wang, Y., 2010. Mitochondrial decay is involved in BaP-induced cervical damage. Free Radical Biology and Medicine 49, 1735-1745.

García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M.A., Martínez, J.A., 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflammation Research 58, 537-552.

García-Ruiz, C., Baulies, A., Mari, M., García-Rovés, P.M., Fernandez-Checa, J.C., 2013. Mitochondrial dysfunction in non-alcoholic fatty liver disease and insulin resistance: cause or consequence? Free Radical Research 47, 854-868.

Gatenby, R.A., Gillies, R.J., 2004. Why do cancers have high aerobic glycolysis? Nature Reviews Cancer 4, 891.

Gee, J.M., DuPont, M.S., Day, A.J., Plumb, G.W., Williamson, G., Johnson, I.T., 2000. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. The Journal of Nutrition 130, 2765-2771.

Gee, J.M., DuPont, M.S., Rhodes, M.J., Johnson, I.T., 1998. Quercetin glucosides interact with the intestinal glucose transport pathway. Free Radical Biology and Medicine 25, 19-25.

George, J., Singh, M., Srivastava, A.K., Bhui, K., Roy, P., Chaturvedi, P.K., Shukla, Y., 2011. Resveratrol and black tea polyphenol combination synergistically suppress mouse skin tumors growth by inhibition of activated MAPKs and p53. PloS One 6, e23395.

Girnun, G.D., 2012. The diverse role of the PPARγ coactivator 1 family of transcriptional coactivators in cancer, Seminars in cell & developmental biology. Elsevier, pp. 381-388.

Gloire, G., Legrand-Poels, S., Piette, J., 2006. NF-κB activation by reactive oxygen species: fifteen years later. Biochemical Pharmacology 72, 1493-1505.

Gogvadze, V., Orrenius, S., Zhivotovsky, B., 2008. Mitochondria in cancer cells: what is so special about them? Trends in Cell Biology 18, 165-173.

Gomes, A.P., Duarte, F.V., Nunes, P., Hubbard, B.P., Teodoro, J.S., Varela, A.T., Jones, J.G., Sinclair, D.A., Palmeira, C.M., Rolo, A.P., 2012. Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1822, 185-195.

Gomez, L.S., Zancan, P., Marcondes, M.C., Ramos-Santos, L., Meyer-Fernandes, J.R., Sola-Penna, M., Da Silva, D., 2013. Resveratrol decreases breast cancer cell viability and glucose metabolism by inhibiting 6-phosphofructo-1-kinase. Biochimie 95, 1336-1343.

Gonda, T.A., Tu, S., Wang, T.C., 2009. Chronic inflammation, the tumor microenvironment and carcinogenesis. Cell Cycle 8, 2005-2013.

Gorlach, S., Fichna, J., Lewandowska, U., 2015. Polyphenols as mitochondria-targeted anticancer drugs. Cancer Letters 366, 141-149.

Grabacka, M.M., Gawin, M., Pierzchalska, M., 2014. Phytochemical modulators of mitochondria: The search for chemopreventive agents and supportive therapeutics. Pharmaceuticals 7, 913-942.

Gray, D.L., Warshawsky, D., Xue, W., Nines, R., Wang, Y., Yao, R., Stoner, G.D., 2001. The effects of a binary mixture of benzo (a) pyrene and 7 H-dibenzo (c, g) carbazole on lung tumors and K-ras oncogene mutations in strain A/J mice. Experimental Lung Research 27, 245-253.

Graziewicz, M.A., Sayer, J.M., Jerina, D.M., Copeland, W.C., 2004. Nucleotide incorporation by human DNA polymerase γ opposite benzo [a] pyrene and benzo [c] phenanthrene diol epoxide adducts of deoxyguanosine and deoxyadenosine. Nucleic Acids Research 32, 397-405.

Grivennikov, S.I., Greten, F.R., Karin, M., 2010. Immunity, inflammation, and cancer. Cell 140, 883-899.

Groot, H.d., Rauen, U., 1998. Tissue injury by reactive oxygen species and the protective effects of flavonoids. Fundamental & Clinical Pharmacology 12, 249-255.

Guarente, L., 2008. Mitochondria—a nexus for aging, calorie restriction, and sirtuins? Cell 132, 171-176.

Gullett, N.P., Amin, A.R., Bayraktar, S., Pezzuto, J.M., Shin, D.M., Khuri, F.R., Aggarwal, B.B., Surh, Y.-J., Kucuk, O., 2010. Cancer prevention with natural compounds, Seminars in oncology. Elsevier, pp. 258-281.

Günay, E., Celik, S., Sarinc-Ulasli, S., Özyürek, A., Hazman, Ö., Günay, S., Özdemir, M., Ünlü, M., 2016. Comparison of the anti-inflammatory effects of proanthocyanidin, quercetin, and damnacanthal on benzo (a) pyrene exposed A549 alveolar cell line. Inflammation 39, 744-751.

Guppy, M., Greiner, E., Brand, K., 1993. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. European Journal of Biochemistry 212, 95-99.

Gustafsson, Å.B., Gottlieb, R.A., 2007. Bcl-2 family members and apoptosis, taken to heart. American Journal of Physiology-Cell Physiology 292, C45-C51.

Gutiérrez-Venegas, G., Contreras-Sánchez, A., Ventura-Arroyo, J.A., 2014. Anti-inflammatory activity of fisetin in human gingival fibroblasts treated with lipopolysaccharide. Journal of Asian Natural Products Research 16, 1009-1017.

Haenen, G.R., Paquay, J.B., Korthouwer, R.E., Bast, A., 1997. Peroxynitrite scavenging by flavonoids. Biochemical and Biophysical Research Communications 236, 591-593.

Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C., Montesano, R., 1998. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. Nucleic Acids Research 26, 205-213.

Halliwell, B., 1995. Antioxidant characterization: methodology and mechanism. Biochemical Pharmacology 49, 1341-1348.

Halliwell, B., 2007. Oxidative stress and cancer: have we moved forward? Biochemical Journal 401, 1-11.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646-674.

Hardonnière, K., Saunier, E., Lemarié, A., Fernier, M., Gallais, I., Helies-Toussaint, C., Mograbi, B., Antonio, S., Bénit, P., Rustin, P., 2016. The environmental carcinogen benzo [a] pyrene induces a Warburg-like metabolic reprogramming dependent on NHE1 and associated with cell survival. Scientific Reports 6.

Harrigan, J.A., McGarrigle, B.P., Sutter, T.R., Olson, J.R., 2006. Tissue specific induction of cytochrome P450 (CYP) 1A1 and 1B1 in rat liver and lung following in vitro (tissue slice) and in vivo exposure to benzo (a) pyrene. Toxicology in Vitro 20, 426-438.

Hart, N., Sarga, L., Csende, Z., Koltai, E., Koch, L.G., Britton, S.L., Davies, K.J., Kouretas, D., Wessner, B., Radak, Z., 2013. Resveratrol enhances exercise training responses in rats selectively bred for high running performance. Food and Chemical Toxicology 61, 53-59.

He, S., Sun, C., Pan, Y., 2008. Red wine polyphenols for cancer prevention. International Journal of Molecular Sciences 9, 842-853.

Heck, D.E., Shakarjian, M., Kim, H.D., Laskin, J.D., Vetrano, A.M., 2010. Mechanisms of oxidant generation by catalase. Annals of the New York Academy of Sciences 1203, 120-125.

Henning, S.M., Wang, P., Carpenter, C.L., Heber, D., 2013. Epigenetic effects of green tea polyphenols in cancer. Epigenomics 5, 729-741.

Higuchi, M., 2012. Roles of mitochondrial DNA changes on cancer initiation and progression. Cell Biology: Research & Therapy 1, 109.

Hill, M.M., Adrain, C., Duriez, P.J., Creagh, E.M., Martin, S.J., 2004. Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. The EMBO Journal 23, 2134-2145.

Howells, L.M., Moiseeva, E.P., Neal, C.P., Foreman, B.E., Andreadi, C.K., Hudson, E., Manson, M.M., 2007. Predicting the physiological relevance of in vitro cancer preventive activities of phytochemicals. Acta Pharmacologica Sinica 28, 1274-1304.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.-L., 2003. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 425, 191-196.

Hu, M.-L., 2011. Dietary polyphenols as antioxidants and anticancer agents: more questions than answers. Chang Gung Medical Journal 34, 449-460.

Hu, X., Xing, L., Jiao, Y., Xu, J., Wang, X., Han, A., Yu, J., 2012. BTG2 overexpression increases the radiosensitivity of breast cancer cells in vitro and in vivo. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics 20, 457-465.

Hu, Y., Liu, J., Wang, J., Liu, Q., 2011. The controversial links among calorie restriction, SIRT1, and resveratrol. Free Radical Biology and Medicine 51, 250-256.

Huang, C.H., Tsai, S.J., Wang, Y.J., Pan, M.H., Kao, J.Y., Way, T.D., 2009. EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK in p53 positive and negative human hepatoma cells. Molecular Nutrition & Food Research 53, 1156-1165.

Huang, H., Pan, X., Jin, H., Li, Y., Zhang, L., Yang, C., Liu, P., Liu, Y., Chen, L., Li, J., 2015. PHLPP2 downregulation contributes to lung carcinogenesis following B [a] P/B [a] PDE exposure. Clinical Cancer Research 21, 3783-3793.

Huc, L., Rissel, M., Solhaug, A., Tekpli, X., Gorria, M., Torriglia, A., Holme, J.A., Dimanche-Boitrel, M.T., Lagadic-Gossmann, D., 2006. Multiple apoptotic pathways induced by p53-dependent acidification in benzo [a] pyrene-exposed hepatic F258 cells. Journal of Cellular Physiology 208, 527-537.

Huderson, A.C., Myers, J.N., Niaz, M.S., Washington, M.K., Ramesh, A., 2013. Chemoprevention of benzo (a) pyrene-induced colon polyps in ApcMin mice by resveratrol. The Journal of Nutritional Biochemistry 24, 713-724.

Ighodaro, O., Akinloye, O., 2017. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine.

Indran, I.R., Tufo, G., Pervaiz, S., Brenner, C., 2011. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1807, 735-745.

Irie, M., Tamae, K., Iwamoto-Tanaka, N., Kasai, H., 2005. Occupational and lifestyle factors and urinary 8-hydroxydeoxyguanosine. Cancer Science 96, 600-606.

Jarvis, I.W., Dreij, K., Mattsson, Å., Jernström, B., Stenius, U., 2014. Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. Toxicology 321, 27-39.

Ji, X., Li, Y., He, J., Shah, W., Xue, X., Feng, G., Zhang, H., Gao, M., 2016. Depletion of mitochondrial enzyme system in liver, lung, brain, stomach and kidney induced by benzo (a) pyrene. Environmental Toxicology and Pharmacology 43, 83-93.

Jiang, Y., Zhou, X., Chen, X., Yang, G., Wang, Q., Rao, K., Xiong, W., Yuan, J., 2011. Benzo (a) pyrene-induced mitochondrial dysfunction and cell death in p53-null Hep3B cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 726, 75-83.

Jiao, S., Liu, B., Gao, A., Ye, M., Jia, X., Zhang, F., Liu, H., Shi, X., Huang, C., 2008. Benzo (a) pyrene-caused increased G 1–S transition requires the activation of c-Jun through p53-dependent PI-3K/Akt/ERK pathway in human embryo lung fibroblasts. Toxicology Letters 178, 167-175.

Jones, W., Bianchi, K., 2015. Aerobic glycolysis: beyond proliferation. Frontiers In Immunology 6, 227.

Jornayvaz, F.R., Shulman, G.I., 2010. Regulation of mitochondrial biogenesis. Essays in biochemistry 47, 69-84.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.-Y.M., Ravagnan, L., 2001. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410, 549-554.

Jung, D., Cho, Y., Collins, L.B., Swenberg, J.A., Di Giulio, R.T., 2009. Effects of benzo [a] pyrene on mitochondrial and nuclear DNA damage in Atlantic killifish (Fundulus heteroclitus) from a creosote-contaminated and reference site. Aquatic Toxicology 95, 44-51.

Jung, K.-H., Lee, J.H., Quach, C.H.T., Paik, J.-Y., Oh, H., Park, J.W., Lee, E.J., Moon, S.-H., Lee, K.-H., 2013. Resveratrol Suppresses Cancer Cell Glucose Uptake by Targeting Reactive Oxygen Species–Mediated Hypoxia-Inducible Factor-1α Activation. Journal of Nuclear Medicine 54, 2161-2167.

Kamaraj, S., Anandakumar, P., Jagan, S., Ramakrishnan, G., Devaki, T., 2011. Hesperidin attenuates mitochondrial dysfunction during benzo (a) pyrene-induced lung carcinogenesis in mice. Fundamental & Clinical Pharmacology 25, 91-98.

Kamaraj, S., Vinodhkumar, R., Anandakumar, P., Jagan, S., Ramakrishnan, G., Devaki, T., 2007. The effects of quercetin on antioxidant status and tumor markers in the lung and serum of mice treated with benzo (a) pyrene. Biological and Pharmaceutical Bulletin 30, 2268-2273.

Kamp, D.W., Weitzman, S.A., 2011. Chronic inflammation and cancer: the role of the mitochondria. Oncology 25, 400.

Kansanen, E., Kuosmanen, S.M., Leinonen, H., Levonen, A.-L., 2013. The Keap1-Nrf2 pathway: mechanisms of activation and dysregulation in cancer. Redox Biology 1, 45-49.

Kao, E.-S., Wang, C.-J., Lin, W.-L., Chu, C.-Y., Tseng, T.-H., 2007. Effects of polyphenols derived from fruit of Crataegus pinnatifida on cell transformation, dermal edema and skin tumor formation by phorbol ester application. Food and Chemical Toxicology 45, 1795-1804.

Kasala, E.R., Bodduluru, L.N., Barua, C.C., Sriram, C.S., Gogoi, R., 2015. Benzo (a) pyrene induced lung cancer: role of dietary phytochemicals in chemoprevention. Pharmacological Reports 67, 996-1009.

Kazerouni, N., Sinha, R., Hsu, C.-H., Greenberg, A., Rothman, N., 2001. Analysis of 200 food items for benzo [a] pyrene and estimation of its intake in an epidemiologic study. Food and Chemical Toxicology 39, 423-436.

Keating, E., Martel, F., 2018. Antimetabolic effects of polyphenols in breast cancer cells: focus on glucose uptake and metabolism. Frontiers in Nutrition 5, 25.

Keijer, J., Bekkenkamp-Grovenstein, M., Venema, D., Dommels, Y.E., 2011. Bioactive food components, cancer cell growth limitation and reversal of glycolytic metabolism. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1807, 697-706.

Key, T., 2011. Fruit and vegetables and cancer risk. British Journal of Cancer 104, 6-11.

Khoo, H.E., Azlan, A., Tang, S.T., Lim, S.M., 2017. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food & Nutrition Research 61, 1361779.

Kim, H.-S., Quon, M.J., Kim, J.-a., 2014. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. Redox Biology 2, 187-195.

Kim, J., Xu, M., Xo, R., Mates, A., Wilson, G., Pearsall, A., Grishko, V., 2010. Mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human OA chondrocytes. Osteoarthritis and Cartilage 18, 424-432.

Kim, K.-H., Jahan, S.A., Kabir, E., Brown, R.J., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. Environment International 60, 71-80.

Kiruthiga, P., Pandian, S.K., Devi, K.P., 2010. Silymarin protects PBMC against B (a) P induced toxicity by replenishing redox status and modulating glutathione metabolizing enzymes—an in vitro study. Toxicology and Applied Pharmacology 247, 116-128.

Klaunig, J.E., Kamendulis, L.M., 2004. The role of oxidative stress in carcinogenesis. Annual Review of Pharmacology and Toxicology 44, 239-267.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D., 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132-1136.

Ko, C.-B., Kim, S.-J., Park, C., Kim, B.-R., Shin, C.-H., Choi, S., Chung, S.-Y., Noh, J.-H., Jeun, J.-H., Kim, N.-S., 2004. Benzo (a) pyrene-induced apoptotic death of mouse hepatoma Hepa1c1c7 cells via activation of intrinsic caspase cascade and mitochondrial dysfunction. Toxicology 199, 35-46.

Ko, H., Jeong, M.-H., Jeon, H., Sung, G.-J., So, Y., Kim, I., Son, J., Lee, S.-w., Yoon, H.-G., Choi, K.-C., 2015. Delphinidin sensitizes prostate cancer cells to TRAIL-induced apoptosis, by inducing DR5 and causing caspase-mediated HDAC3 cleavage. Oncotarget 6, 9970.

Kong, J.-M., Chia, L.-S., Goh, N.-K., Chia, T.-F., Brouillard, R., 2003. Analysis and biological activities of anthocyanins. Phytochemistry 64, 923-933.

Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., Fang, F., Chang, Y., 2010. Sirtuin 3, a new target of PGC-1α, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS One 5, e11707.

Korobkova, E.A., 2015. Effect of natural polyphenols on CYP metabolism: implications for diseases. Chemical Research in Toxicology 28, 1359-1390.

Krishnamoorthy, R.M., Venkatraman, A.C., 2017. Polyphenols activate energy sensing network in insulin resistant models. Chemico-Biological Interactions 275, 95-107.

Krizkova, J., Burdova, K., Hudecek, J., Stiborova, M., Hodek, P., 2008. Induction of cytochromes P450 in small intestine by chemopreventive compounds. Neuro Endocrinology Letters 29, 717-721.

Kroemer, G., Pouyssegur, J., 2008. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13, 472-482.

Kryston, T.B., Georgiev, A.B., Pissis, P., Georgakilas, A.G., 2011. Role of oxidative stress and DNA damage in human carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 711, 193-201.

Księżakowska-Łakoma, K., Żyła, M., Wilczyński, J.R., 2014. Mitochondrial dysfunction in cancer. Przeglad Menopauzalny= Menopause Review 13, 136.

Kudryavtseva, A.V., Krasnov, G.S., Dmitriev, A.A., Alekseev, B.Y., Kardymon, O.L., Sadritdinova, A.F., Fedorova, M.S., Pokrovsky, A.V., Melnikova, N.V., Kaprin, A.D., 2016. Mitochondrial dysfunction and oxidative stress in aging and cancer. Oncotarget 7, 44879.

Kulawiec, M., Arnouk, H., Desouki, M.M., Kazim, L., Still, I., Singh, K.K., 2006. Proteomic analysis of proteins involved in mitochondria-to-nucleus retrograde response in human cancer cells. Cancer Biology & Therapy 5, 967-975.

Kulkarni, S.S., Cantó, C., 2015. The molecular targets of resveratrol. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1852, 1114-1123.

Kumar, S., Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. The Scientific World Journal 2013.

Kuo, P.-L., Chiang, L.-C., Lin, C.-C., 2002. Resveratrol-induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sciences 72, 23-34.

Kutil, Z., Temml, V., Maghradze, D., Pribylova, M., Dvorakova, M., Schuster, D., Vanek, T., Landa, P., 2014. Impact of wines and wine constituents on cyclooxygenase-1, cyclooxygenase-2, and 5-lipoxygenase catalytic activity. Mediators of Inflammation 2014.

Labib, S., Yauk, C., Williams, A., Arlt, V.M., Phillips, D.H., White, P.A., Halappanavar, S., 2012. Subchronic oral exposure to benzo (a) pyrene leads to distinct transcriptomic changes in the lungs that are related to carcinogenesis. Toxicological Sciences 129, 213-224.

Lagoa, R., Graziani, I., Lopez-Sanchez, C., Garcia-Martinez, V., Gutierrez-Merino, C., 2011. Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1807, 1562-1572.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. Cell 127, 1109-1122.

Lall, R.K., Syed, D.N., Adhami, V.M., Khan, M.I., Mukhtar, H., 2015. Dietary polyphenols in prevention and treatment of prostate cancer. International Journal of Molecular Sciences 16, 3350-3376.

Lambert, J.D., Elias, R.J., 2010. The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Archives of Biochemistry and Biophysics 501, 65-72.

Landete, J., 2012. Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. Critical Reviews in Food Science and Nutrition 52, 936-948.

Langie, S.A., Koppen, G., Desaulniers, D., Al-Mulla, F., Al-Temaimi, R., Amedei, A., Azqueta, A., Bisson, W.H., Brown, D., Brunborg, G., 2015. Causes of genome instability: the effect of low dose chemical exposures in modern society. Carcinogenesis 36, S61-S88.

LeBleu, V.S., O'Connell, J.T., Herrera, K.N.G., Wikman, H., Pantel, K., Haigis, M.C., De Carvalho, F.M., Damascena, A., Chinen, L.T.D., Rocha, R.M., 2014. PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. Nature Cell Biology 16, 992-1003.

Lee, B.M., Shim, G.A., 2007. Dietary exposure estimation of benzo [a] pyrene and cancer risk assessment. Journal of Toxicology and Environmental Health, part A 70, 1391-1394.

Lee, C.-H., Wu, S.-B., Hong, C.-H., Chen, G.-S., Wei, Y.-H., Yu, H.-S., 2013. Involvement of mtDNA damage elicited by oxidative stress in the arsenical skin cancers. Journal of Investigative Dermatology 133, 1890-1900.

Lee, H.-C., Wei, Y.-H., 2005. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. The International Journal of Biochemistry & Cell Biology 37, 822-834.

Lee, J.-C., Chung, L.-C., Chen, Y.-J., Feng, T.-H., Chen, W.-T., Juang, H.-H., 2015. Upregulation of B-cell translocation gene 2 by epigallocatechin-3-gallate via p38 and ERK signaling blocks cell proliferation in human oral squamous cell carcinoma cells. Cancer Letters 360, 310-318.

Lee, J.-E., Safe, S., 2001. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells1. Biochemical Pharmacology 62, 1113-1124.

Lee, K.W., Lee, H.J., 2006. The roles of polyphenols in cancer chemoprevention. Biofactors 26, 105-121.

Lemieux, C.L., Douglas, G.R., Gingerich, J., Phonethepswath, S., Torous, D.K., Dertinger, S.D., Phillips, D.H., Arlt, V.M., White, P.A., 2011. Simultaneous measurement of benzo [a] pyrene-induced Pig-a and lacZ mutations, micronuclei and dna adducts in mutaTMmouse. Environmental and Molecular Mutagenesis 52, 756-765.

Leopoldini, M., Russo, N., Toscano, M., 2011. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chemistry 125, 288-306.

Lewis, W., Copeland, W.C., Day, B.J., 2001. Mitochondrial DNA depletion, oxidative stress, and mutation: Mechanisms 0f dysfunction from nucleoside reverse transcriptase inhibitors. Laboratory Investigation 81, 777-790.

Leyva-López, N., Gutierrez-Grijalva, E.P., Ambriz-Perez, D.L., Heredia, J.B., 2016. Flavonoids as cytokine modulators: a possible therapy for inflammation-related diseases. International Journal of Molecular Sciences 17, 921.

Li, N., Karin, M., 1999. Is NF-κB the sensor of oxidative stress? The FASEB Journal 13, 1137-1143.

Li, N., Sioutas, C., Cho, A., Schmitz, D., Misra, C., Sempf, J., Wang, M., Oberley, T., Froines, J., Nel, A., 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environmental Health Perspectives 111, 455.

Li, W., Saud, S.M., Young, M.R., Chen, G., Hua, B., 2015. Targeting AMPK for cancer prevention and treatment. Oncotarget 6, 7365-7378.

Lin, J.-K., 2004. Suppression of protein kinase C and nuclear oncogene expression as possible action mechanisms of cancer chemoprevention by Curcumin. Archives of Pharmacal Research 27, 683-692.

Lin, J., Wu, H., Tarr, P.T., Zhang, C.-Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature 418, 797.

Lin, Y., Shi, R., Wang, X., Shen, H.-M., 2008. Luteolin, a flavonoid with potential for cancer prevention and therapy. Current Cancer Drug Targets 8, 634-646.

Lin, Z., Fang, D., 2013. The roles of SIRT1 in cancer. Genes & Cancer 4, 97-104.

Liou, G.-Y., Storz, P., 2010. Reactive oxygen species in cancer. Free Radical Research 44, 479-496.

Liu, R.H., 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. The American Journal of Clinical Nutrition 78, 517S-520S.

Liu, Y., Hao, H., Xie, H., Lv, H., Liu, C., Wang, G., 2009. Oxidative demethylenation and subsequent glucuronidation are the major metabolic pathways of berberine in rats. Journal of Pharmaceutical Sciences 98, 4391-4401.

Liu, Y., Wu, Y., Zhang, P., 2015. Protective effects of curcumin and quercetin during benzo (a) pyrene induced lung carcinogenesis in mice. European Review for Medical and Pharmacological Sciences 19, 1736-1743.

Lu, F., Zahid, M., Wang, C., Saeed, M., Cavalieri, E.L., Rogan, E.G., 2008. Resveratrol prevents estrogen-DNA adduct formation and neoplastic transformation in MCF-10F cells. Cancer Prevention Research 1, 135-145.

Lu, J., Sharma, L.K., Bai, Y., 2009. Implications of mitochondrial DNA mutations and mitochondrial dysfunction in tumorigenesis. Cell Research 19, 802-815.

Lu, J., Zhang, M., Huang, Z., Sun, S., Zhang, Y., Zhang, L., Peng, L., Ma, A., Ji, P., Dai, J., 2015. SIRT1 in B [a] P-induced lung tumorigenesis. Oncotarget 6, 27113.

Luo, Z., Zang, M., Guo, W., 2010. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. Future Oncology 6, 457-470.

Ma, Q., 2013. Role of nrf2 in oxidative stress and toxicity. Annual Review of Pharmacology and Toxicology 53, 401.

Ma, S., Feng, J., Zhang, R., Chen, J., Han, D., Li, X., Yang, B., Li, X., Fan, M., Li, C., 2017. SIRT1 Activation by Resveratrol Alleviates Cardiac Dysfunction via Mitochondrial Regulation in Diabetic Cardiomyopathy Mice. Oxidative Medicine and Cellular Longevity 2017.

MacRae, H.S., Mefferd, K.M., 2006. Dietary antioxidant supplementation combined with quercetin improves cycling time trial performance. International Journal of Sport Nutrition and Exercise Metabolism 16, 405-419.

Manning, B.D., Cantley, L.C., 2007. AKT/PKB signaling: navigating downstream. Cell 129, 1261-1274.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454, 436-444.

Marin-Hernandez, A., Gallardo-Perez, J.C., Ralph, S.J., Rodriguez-Enriquez, S., Moreno-Sanchez, R., 2009. HIF-1α modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. Mini Reviews in Medicinal Chemistry 9, 1084-1101.

Masoud, G.N., Li, W., 2015. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharmaceutica Sinica B 5, 378-389.

Mathupala, S.P., Ko, Y.H., Pedersen, P.L., 2009. Hexokinase-2 bound to mitochondria: cancer's stygian link to the “Warburg Effect” and a pivotal target for effective therapy, Seminars in cancer biology. Elsevier, pp. 17-24.

Matuschek, M.C., Hendriks, W.H., McGhie, T.K., Reynolds, G.W., 2006. The jejunum is the main site of absorption for anthocyanins in mice. The Journal of Nutritional Biochemistry 17, 31-36.

Maurya, A.K., Vinayak, M., 2015. Quercetin regresses Dalton's lymphoma growth via suppression of PI3K/AKT signaling leading to upregulation of p53 and decrease in energy metabolism. Nutrition and Cancer 67, 354-363.

Mazzoni, L., Perez-Lopez, P., Giampieri, F., Alvarez-Suarez, J.M., Gasparrini, M., Forbes-Hernandez, T.Y., Quiles, J.L., Mezzetti, B., Battino, M., 2016. The genetic aspects of berries: from field to health. Journal of the Science of Food and Agriculture 96, 365-371.

McCallum, J.L., Yang, R., Young, J.C., Strommer, J.N., Tsao, R., 2007. Improved high performance liquid chromatographic separation of anthocyanin compounds from grapes using a novel mixed-mode ion-exchange reversed-phase column. Journal of Chromatography A 1148, 38-45.

Melchini, A., Catania, S., Stancanelli, R., Tommasini, S., Costa, C., 2011. Interaction of a functionalized complex of the flavonoid hesperetin with the AhR pathway and CYP1A1 expression: involvement in its protective effects against benzo [a] pyrene-induced oxidative stress in human skin. Cell Biology and Toxicology 27, 371-379.

Miao, W., Hu, L., Scrivens, P.J., Batist, G., 2005. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. Journal of Biological Chemistry 280, 20340-20348.

Milner, J., 2009. Cellular regulation of SIRT1. Current Pharmaceutical Design 15, 39-44.

Min, L., He, S., Chen, Q., Peng, F., Peng, H., Xie, M., 2011. Comparative proteomic analysis of cellular response of human airway epithelial cells (A549) to benzo (a) pyrene. Toxicology Mechanisms and Methods 21, 374-382.

Min, Y., Choi, C., Bark, H., Son, H., Park, H., Lee, S., Park, J., Park, E., Shin, H., Kim, S., 2007. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-[kappa] B and p38 MAPK in HMC-1 human mast cell line. Inflammation Research 56, 210.

Miyamoto, Y., Koh, Y.H., Park, Y.S., Fujiwara, N., Sakiyama, H., Misonou, Y., Ookawara, T., Suzuki, K., Honke, K., Taniguchi, N., 2003. Oxidative stress caused by inactivation of glutathione peroxidase and adaptive responses. Biological Chemistry 384, 567-574.

Modica-Napolitano, J.S., Singh, K.K., 2004. Mitochondrial dysfunction in cancer. Mitochondrion 4, 755-762.

Mohammadi-Bardbori, A., Bengtsson, J., Rannug, U., Rannug, A., Wincent, E., 2012. Quercetin, resveratrol, and curcumin are indirect activators of the aryl hydrocarbon receptor (AHR). Chemical Research in Toxicology 25, 1878-1884.

Moiseeva, O., Bourdeau, V., Roux, A., Deschênes-Simard, X., Ferbeyre, G., 2009. Mitochondrial dysfunction contributes to oncogene-induced senescence. Molecular and Cellular Biology 29, 4495-4507.

Moreira, L., Araújo, I., Costa, T., Correia-Branco, A., Faria, A., Martel, F., Keating, E., 2013. Quercetin and epigallocatechin gallate inhibit glucose uptake and metabolism by breast cancer cells by an estrogen receptor-independent mechanism. Experimental Cell Research 319, 1784-1795.

Morgan, M.J., Liu, Z.-g., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Research 21, 103.

Moro, L., Arbini, A., Yao, J., di Sant'Agnese, P., Marra, E., Greco, M., 2009. Mitochondrial DNA depletion in prostate epithelial cells promotes anoikis resistance and invasion through activation of PI3K/Akt2. Cell Death & Differentiation 16, 571-583.

Movafagh, S., Crook, S., Vo, K., 2015. Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. Journal of cellular biochemistry 116, 696-703.

Müller, C., Ullmann, K., Steinberg, P., 2011. The grapevine-shoot extract Vineatrol30 inhibits the chemically induced malignant transformation of BALB/c-3T3 cells. Journal of Medicinal Food 14, 34-39.

Mumtaz, M., George, J., Gold, K., Cibulas, W., Derosa, C., 1996. ATSDR evaluation of health effects of chemicals. IV. Polycyclic aromatic hydrocarbons (PAHs): understanding a complex problem. Toxicology and Industrial Health 12, 742-971.

Murphy, K., Ranganathan, V., Farnsworth, M., Kavallaris, M., Lock, R., 2000. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death and Differentiation 7, 102.

Mutlu Altundağ, E., Kasacı, T., Yılmaz, A.M., Karademir, B., Koçtürk, S., Taga, Y., Yalçın, A.S., 2016. Quercetin-Induced Cell Death in Human Papillary Thyroid Cancer (B-CPAP) Cells. Journal of Thyroid Research 2016.

Mytilineou, C., Kramer, B.C., Yabut, J.A., 2002. Glutathione depletion and oxidative stress. Parkinsonism & Related Disorders 8, 385-387.

Na, H.-K., Surh, Y.-J., 2008. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. Food and Chemical Toxicology 46, 1271-1278.

Nafees, S., Rashid, S., Ali, N., Hasan, S.K., Sultana, S., 2015. Rutin ameliorates cyclophosphamide induced oxidative stress and inflammation in Wistar rats: role of NFκB/MAPK pathway. Chemico-Biological Interactions 231, 98-107.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nature Reviews Cancer 6, 947-960.

Nebert, D.W., Dalton, T.P., Okey, A.B., Gonzalez, F.J., 2004. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. Journal of Biological Chemistry 279, 23847-23850.

Nichols, M., Zhang, J., Polster, B., Elustondo, P., Thirumaran, A., Pavlov, E., Robertson, G., 2015. Synergistic neuroprotection by epicatechin and quercetin: activation of convergent mitochondrial signaling pathways. Neuroscience 308, 75-94.

Nieman, D.C., Williams, A.S., Shanely, R.A., Jin, F., McAnulty, S.R., Triplett, N.T., Austin, M.D., Henson, D.A., 2010. Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. Medicine & Science in Sports & Exercise 42, 338-345.

Niestroy, J., Barbara, A., Herbst, K., Rode, S., van Liempt, M., Roos, P.H., 2011. Single and concerted effects of benzo [a] pyrene and flavonoids on the AhR and Nrf2-pathway in the human colon carcinoma cell line Caco-2. Toxicology in Vitro 25, 671-683.

Nkrumah-Elie, Y.M., Reuben, J.S., Hudson, A.M., Taka, E., Badisa, R., Ardley, T., Israel, B.e., Sadrud-Din, S.Y., Oriaku, E.T., Darling-Reed, S.F., 2012. The attenuation of early benzo (a) pyrene-induced carcinogenic insults by diallyl disulfide (DADS) in MCF-10A cells. Nutrition and Cancer 64, 1112-1121.

Nuttall, R., Bryan, S., Dale, D., De, P., Demers, A., Ellison, L., 2017. Canadian Cancer Statistics.

Nuttall, R., Bryan, S., Dale, D., De, P., Demers, A., Ellison, L., Rahal, R., 2016. Canadian Cancer Statstics.

Omidian, K., Rafiei, H., Bandy, B., 2017. Polyphenol inhibition of benzo[a]pyrene-induced oxidative stress and neoplastic transformation in an in vitro model of carcinogenesis. Food and Chemical Toxicology, 165-174.

Orasche, J.r., Seidel, T., Hartmann, H., Schnelle-Kreis, J.r., Chow, J.C., Ruppert, H., Zimmermann, R., 2012. Comparison of emissions from wood combustion. Part 1: Emission factors and characteristics from different small-scale residential heating appliances considering particulate matter and polycyclic aromatic hydrocarbon (PAH)-related toxicological potential of particle-bound organic species. Energy & Fuels 26, 6695-6704.

Pallàs, M., Casadesús, G., Smith, M.A., Coto-Montes, A., Pelegri, C., Vilaplana, J., Camins, A., 2009. Resveratrol and neurodegenerative diseases: activation of SIRT1 as the potential pathway towards neuroprotection. Current Neurovascular Research 6, 70-81.

Pan, M.-H., Lai, C.-S., Ho, C.-T., 2010. Anti-inflammatory activity of natural dietary flavonoids. Food & Function 1, 15-31.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., Denko, N.C., 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metabolism 3, 187-197.

Park, J.S., Sharma, L.K., Li, H., Xiang, R., Holstein, D., Wu, J., Lechleiter, J., Naylor, S.L., Deng, J.J., Lu, J., 2009. A heteroplasmic, not homoplasmic, mitochondrial DNA mutation promotes tumorigenesis via alteration in reactive oxygen species generation and apoptosis. Human Molecular Genetics 18, 1578-1589.

Park, S.-H., Ozden, O., Jiang, H., Cha, Y.I., Pennington, J.D., Aykin-Burns, N., Spitz, D.R., Gius, D., Kim, H.-S., 2011. Sirt3, mitochondrial ROS, ageing, and carcinogenesis. International Journal of Molecular Sciences 12, 6226-6239.

Passamonti, S., Vrhovsek, U., Vanzo, A., Mattivi, F., 2005. Fast access of some grape pigments to the brain. Journal of Agricultural and Food Chemistry 53, 7029-7034.

Pastorková, B., Vrzalová, A., Bachleda, P., Dvořák, Z., 2017. Hydroxystilbenes and methoxystilbenes activate human aryl hydrocarbon receptor and induce CYP1A genes in human hepatoma cells and human hepatocytes. Food and Chemical Toxicology 103, 122-132.

Patel, K.R., Andreadi, C., Britton, R.G., Horner-Glister, E., Karmokar, A., Sale, S., Brown, V.A., Brenner, D.E., Singh, R., Steward, W.P., 2013. Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. Science Translational Medicine 5, 205ra133-205ra133.

Pavanello, S., Dioni, L., Hoxha, M., Fedeli, U., Mielzynska-Švach, D., Baccarelli, A.A., 2013. Mitochondrial DNA copy number and exposure to polycyclic aromatic hydrocarbons. Cancer Epidemiology and Prevention Biomarkers 22, 1722-1729.

Peng, F., 2012. Mitochondrial uptake of anthocyanidins and protection from oxidative stress. Unpublished doctoral dissertation, University of Saskatchewan, Saskatoon, Canada.

Peng, K., Tao, Y., Zhang, J., Wang, J., Ye, F., Dan, G., Zhao, Y., Cai, Y., Zhao, J., Wu, Q., 2016. Resveratrol regulates mitochondrial biogenesis and fission/fusion to attenuate rotenone-induced neurotoxicity. Oxidative Medicine and Cellular Longevity 2016.

Percival, S.S., Talcott, S.T., Chin, S.T., Mallak, A.C., Lounds-Singleton, A., Pettit-Moore, J., 2006. Neoplastic transformation of BALB/3T3 cells and cell cycle of HL-60 cells are inhibited by mango (Mangifera indica L.) juice and mango juice extracts. The Journal of Nutrition 136, 1300-1304.

Perry, S.W., Norman, J.P., Barbieri, J., Brown, E.B., Gelbard, H.A., 2011. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques 50, 98.

Perwez Hussain, S., Harris, C.C., 2007. Inflammation and cancer: an ancient link with novel potentials. International Journal of Cancer 121, 2373-2380.

Peters, A., Dockery, D.W., Muller, J.E., Mittleman, M.A., 2001. Increased particulate air pollution and the triggering of myocardial infarction. Circulation 103, 2810-2815.

Phaniendra, A., Jestadi, D.B., Periyasamy, L., 2015. Free radicals: properties, sources, targets, and their implication in various diseases. Indian Journal of Clinical Biochemistry 30, 11-26.

Pieters, N., Koppen, G., Smeets, K., Napierska, D., Plusquin, M., De Prins, S., Van De Weghe, H., Nelen, V., Cox, B., Cuypers, A., 2013. Decreased mitochondrial DNA content in association with exposure to polycyclic aromatic hydrocarbons in house dust during wintertime: from a population enquiry to cell culture. PLoS One 8, e63208.

Piskula, M.K., Yamakoshi, J., Iwai, Y., 1999. Daidzein and genistein but not their glucosides are absorbed from the rat stomach. FEBS letters 447, 287-291.

Pitot, H.C., 1993. The molecular biology of carcinogenesis. Cancer 72, 962-970.

Plas, D.R., Thompson, C.B., 2005. Akt-dependent transformation: there is more to growth than just surviving. Oncogene 24, 7435-7442.

Poirier, M.C., 2004. Chemical-induced DNA damage and human cancer risk. Nature Reviews Cancer 4, 630-637.

Pons, D.G., Nadal-Serrano, M., Torrens-Mas, M., Valle, A., Oliver, J., Roca, P., 2015. UCP2 inhibition sensitizes breast cancer cells to therapeutic agents by increasing oxidative stress. Free Radical Biology and Medicine 86, 67-77.

Pope III, C.A., Burnett, R.T., Thun, M.J., Calle, E.E., Krewski, D., Ito, K., Thurston, G.D., 2002. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. Jama 287, 1132-1141.

Price, N.L., Gomes, A.P., Ling, A.J., Duarte, F.V., Martin-Montalvo, A., North, B.J., Agarwal, B., Ye, L., Ramadori, G., Teodoro, J.S., 2012. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metabolism 15, 675-690.

Prior, I.A., Lewis, P.D., Mattos, C., 2012. A comprehensive survey of Ras mutations in cancer. Cancer Research 72, 2457-2467.

Prior, R.L., Cao, G., 2000. Antioxidant phytochemicals in fruits and vegetables: diet and health implications. HortScience 35, 588-592.

Procházková, D., Boušová, I., Wilhelmová, N., 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82, 513-523.

Pufulete, M., Battershill, J., Boobis, A., Fielder, R., 2004. Approaches to carcinogenic risk assessment for polycyclic aromatic hydrocarbons: a UK perspective. Regulatory Toxicology and Pharmacology 40, 54-66.

Qiao, C., Wei, L., Dai, Q., Zhou, Y., Yin, Q., Li, Z., Xiao, Y., Guo, Q., Lu, N., 2015. UCP2-Related Mitochondrial Pathway Participates in Oroxylin A-Induced Apoptosis in Human Colon Cancer Cells. Journal of Cellular Physiology 230, 1054-1063.

Rahman, M.M., Ng, J.C., Naidu, R., 2009. Chronic exposure of arsenic via drinking water and its adverse health impacts on humans. Environmental Geochemistry and Health 31, 189-200.

Ramos, S., 2008. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. Molecular Nutrition & Food Research 52, 507-526.

Rathore, K., Choudhary, S., Wang, H.-C.R., 2012. Green tea catechin intervention of reactive oxygen species-mediated ERK pathway activation and chronically induced breast cell carcinogenesis. Carcinogenesis 33, 174-183.

Rathore, K., Wang, H.C.R., 2012. Green tea catechin extract in intervention of chronic breast cell carcinogenesis induced by environmental carcinogens. Molecular carcinogenesis 51, 280-289.

Ravichandran, N., Suresh, G., Ramesh, B., Siva, G.V., 2011. Fisetin, a novel flavonol attenuates benzo (a) pyrene-induced lung carcinogenesis in Swiss albino mice. Food and Chemical Toxicology 49, 1141-1147.

Rehwagen, M., Müller, A., Massolo, L., Herbarth, O., Ronco, A., 2005. Polycyclic aromatic hydrocarbons associated with particles in ambient air from urban and industrial areas. Science of The Total Environment 348, 199-210.

Ren, J., Pulakat, L., Whaley-Connell, A., Sowers, J.R., 2010. Mitochondrial biogenesis in the metabolic syndrome and cardiovascular disease. Journal of Molecular Medicine 88, 993-1001.

Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radical Biology and Medicine 49, 1603-1616.

Revel, A., Raanani, H., Younglai, E., Xu, J., Rogers, I., Han, R., Savouret, J.F., Casper, R.F., 2003. Resveratrol, a natural aryl hydrocarbon receptor antagonist, protects lung from DNA damage and apoptosis caused by benzo [a] pyrene. Journal of Applied Toxicology 23, 255-261.

Roberts, E.R., Thomas, K.J., 2013. The role of mitochondria in the development and progression of lung cancer. Computational and Structural Biotechnology Journal 6, 1-11.

Roos, W.P., Thomas, A.D., Kaina, B., 2015. DNA damage and the balance between survival and death in cancer biology. Nature Reviews Cancer, 20-33.

Rowe, C.A., Nantz, M.P., DeNiera, C., Green, K., Talcott, S.T., Percival, S.S., 2004. Inhibition of neoplastic transformation of benzo [α] pyrene-treated BALB/c 3T3 murine cells by a phytochemical extract of passionfruit juice. Journal of Medicinal Food 7, 402-407.

Sabharwal, S.S., Schumacker, P.T., 2014. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? Nature Reviews Cancer 14, 709-721.

Saelens, X., Festjens, N., Walle, L.V., Van Gurp, M., van Loo, G., Vandenabeele, P., 2004. Toxic proteins released from mitochondria in cell death. Oncogene 23, 2861-2874.

Sakai, A., Kikuchi, Y., Muroi, M., Masui, T., Furihata, C., Uchida, E., Takatori, K., Tanamoto, K.-i., 2003. Overexpression of NP95 mRNA by tumor promoters in the promotion phase of a two-stage BALB/3T3 cell transformation assay. Biological and Pharmaceutical Bulletin 26, 347-351.

Sakai, A., Sasaki, K., Hayashi, K., Muramatsu, D., Arai, S., Endou, N., Kuroda, S., Poth, A., Bohnenberger, S., Kunkelmann, T., 2011. An international validation study of a Bhas 42 cell transformation assay for the prediction of chemical carcinogenicity. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 725, 57-77.

Sakai, A., Sasaki, K., Mizusawa, H., Ishidate, M., 1990. Effects of quercetin, a plant flavonol, on the two-stage transformation in vitro. Teratogenesis, Carcinogenesis, and Mutagenesis 10, 333-340.

Sakai, A., Sasaki, K., Muramatsu, D., Arai, S., Endou, N., Kuroda, S., Hayashi, K., Lim, Y.-m., Yamazaki, S., Umeda, M., 2010. A Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 702, 100-122.

Salceda, S., Caro, J., 1997. Hypoxia-inducible Factor 1α (HIF-1α) Protein Is Rapidly Degraded by the Ubiquitin-Proteasome System under Normoxic Conditions ITS STABILIZATION BY HYPOXIA DEPENDS ON REDOX-INDUCED CHANGES. Journal of Biological Chemistry 272, 22642-22647.

Sánchez-Cenizo, L., Formentini, L., Aldea, M., Ortega, A.D., García-Huerta, P., Sánchez-Aragó, M., Cuezva, J.M., 2010. Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype. Journal of Biological Chemistry 285, 25308-25313.

Sandoval-Acuña, C., Ferreira, J., Speisky, H., 2014. Polyphenols and mitochondria: an update on their increasingly emerging ROS-scavenging independent actions. Archives of Biochemistry and Biophysics 559, 75-90.

Sang, H., Zhang, L., Li, J., 2012. Anti-benzopyrene-7, 8-diol-9, 10-epoxide induces apoptosis via mitochondrial pathway in human bronchiolar epithelium cells independent of the mitochondria permeability transition pore. Food and Chemical Toxicology 50, 2417-2423.

Sarkar, F.H., Li, Y., 2003. Soy isoflavones and cancer prevention: clinical science review. Cancer Investigation 21, 744-757.

Sasaki, K., Umeda, M., Sakai, A., Yamazaki, S., Tanaka, N., 2015. Transformation assay in Bhas 42 cells: a model using initiated cells to study mechanisms of carcinogenesis and predict carcinogenic potential of chemicals. Journal of Environmental Science and Health, Part C 33, 1-35.

Sauer, H., Wartenberg, M., Hescheler, J., 2001. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cellular Physiology and Biochemistry 11, 173-186.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., Peter, M.E., 1998. Two CD95 (APO-1/Fas) signaling pathways. The EMBO Journal 17, 1675-1687.

Schimmer, A.D., 2004. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Research 64, 7183-7190.

Schreiber, S.N., Emter, R., Hock, M.B., Knutti, D., Cardenas, J., Podvinec, M., Kralli, A., 2004. The estrogen-related receptor (ERR) functions in PPAR coactivator 1 (PGC-1)-induced mitochondrial biogenesis. Proceedings of The National Academy of Sciences 17, 101.

Seifried, H.E., Anderson, D.E., Fisher, E.I., Milner, J.A., 2007. A review of the interaction among dietary antioxidants and reactive oxygen species. The Journal of Nutritional Biochemistry 18, 567-579.

Semenza, G.L., 2010. HIF-1: upstream and downstream of cancer metabolism. Current Opinion in Genetics & Development 20, 51-56.

Serafim, T.L., Oliveira, P.J., Sardao, V.A., Perkins, E., Parke, D., Holy, J., 2008. Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line. Cancer Chemotherapy and Pharmacology 61, 1007-1018.

Shacter, E., Weitzman, S.A., 2002. Chronic inflammation and cancer. Oncology (Williston Park, NY) 16, 217-226, 229; discussion 230-212.

Shahid, A., Ali, R., Ali, N., Hasan, S.K., Bernwal, P., Afzal, S.M., Vafa, A., Sultana, S., 2016. Modulatory effects of catechin hydrate against genotoxicity, oxidative stress, inflammation and apoptosis induced by benzo (a) pyrene in mice. Food and Chemical Toxicology 92, 64-74.

Shaulian, E., Karin, M., 2001. AP-1 in cell proliferation and survival. Oncogene 20, 2390.

Shih, L.-J., Lin, Y.-R., Lin, C.-K., Liu, H.-S., Kao, Y.-H., 2016. Green tea (-)-epigallocatechin gallate induced growth inhibition of human placental choriocarcinoma cells. Placenta 41, 1-9.

Shiizaki, K., Kawanishi, M., Yagi, T., 2017. Modulation of benzo [a] pyrene–DNA adduct formation by CYP1 inducer and inhibitor. Genes and Environment 39, 14.

Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and1B1. Cancer Science 95, 1-6.

Shimada, T., Hayes, C.L., Yamazaki, H., Amin, S., Hecht, S.S., Guengerich, F.P., Sutter, T.R., 1996. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. Cancer Research 56, 2979-2984.

Shoshan-Barmatz, V., De Pinto, V., Zweckstetter, M., Raviv, Z., Keinan, N., Arbel, N., 2010. VDAC, a multi-functional mitochondrial protein regulating cell life and death. Molecular Aspects of Medicine 31, 227-285.

Shukla, Y., Singh, R., 2011. Resveratrol and cellular mechanisms of cancer prevention. Annals of the New York Academy of Sciences 1215, 1-8.

Sies, H., 1993. Strategies of antioxidant defense. The FEBS journal 215, 213-219.

Sigounas, G., Hairr, J.W., Cooke, C.D., Owen, J.R., Asch, A.S., Weidner, D.A., Wiley, J.E., 2010. Role of benzo [α] pyrene in generation of clustered DNA damage in human breast tissue. Free Radical Biology and Medicine 49, 77-87.

Singh, C.K., Siddiqui, I.A., El-Abd, S., Mukhtar, H., Ahmad, N., 2016. Combination chemoprevention with grape antioxidants. Molecular Nutrition & Food Research 60, 1406-1415.

Sinha, R., Kulldorff, M., Gunter, M.J., Strickland, P., Rothman, N., 2005. Dietary benzo [a] pyrene intake and risk of colorectal adenoma. Cancer Epidemiology and Prevention Biomarkers 14, 2030-2034.

Smith, C., Perfetti, T., Rumple, M., Rodgman, A., Doolittle, D., 2000. "IARC group 2A Carcinogens" reported in cigarette mainstream smoke. Food and Chemical Toxicology 38, 371-383.

Sobinoff, A., Pye, V., Nixon, B., Roman, S., McLaughlin, E., 2012. Jumping the gun: smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. Toxicology and Applied Pharmacology 260, 70-80.

Sood, S., Choudhary, S., Wang, H.-C.R., 2013. Induction of human breast cell carcinogenesis by triclocarban and intervention by curcumin. Biochemical and Biophysical Research Communications 438, 600-606.

Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., LLeonart, M.E., 2013. Oxidative stress and cancer: an overview. Ageing Research Reviews 12, 376-390.

Storz, P., 2005. Reactive oxygen species in tumor progression. Frontiers in Bioscience 10, 1881-1896.

Sugiyama, M., KAWAHARA-MIKI, R., Kawana, H., Shirasuna, K., Kuwayama, T., Iwata, H., 2015. Resveratrol-induced mitochondrial synthesis and autophagy in oocytes derived from early antral follicles of aged cows. Journal of Reproduction and Development 61, 251-259.

Suliman, H.B., Welty-Wolf, K.E., Carraway, M., Tatro, L., Piantadosi, C.A., 2004. Lipopolysaccharide induces oxidative cardiac mitochondrial damage and biogenesis. Cardiovascular Research 64, 279-288.

Sullivan, L.B., Chandel, N.S., 2014. Mitochondrial reactive oxygen species and cancer. Cancer & Metabolism 2, 17.

Sun, Y., Xun, K., Wang, Y., Chen, X., 2009. A systematic review of the anticancer properties of berberine, a natural product from Chinese herbs. Anti-Cancer Drugs 20, 757-769.

Swauger, J., Steichen, T., Murphy, P., Kinsler, S., 2002. An analysis of the mainstream smoke chemistry of samples of the US cigarette market acquired between 1995 and 2000. Regulatory Toxicology and Pharmacology 35, 142-156.

Takanche, J., Lee, Y.H., Kim, J.S., Kim, J.E., Han, S.H., Lee, S.W., Yi, H.K., 2017. Anti-inflammatory and antioxidant properties of Schisandrin C promote mitochondrial biogenesis in human dental pulp cells. International Endodontic Journal, 438-447.

Takasawa, R., Saeki, K., Tao, A., Yoshimori, A., Uchiro, H., Fujiwara, M., Tanuma, S.-i., 2010. Delphinidin, a dietary anthocyanidin in berry fruits, inhibits human glyoxalase I. Bioorganic & Medicinal Chemistry 18, 7029-7033.

Talavera, S., Felgines, C., Texier, O., Besson, C., Lamaison, J.-L., Rémésy, C., 2003. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. The Journal of Nutrition 133, 4178-4182.

Tan, A.S., Baty, J.W., Berridge, M.V., 2014. The role of mitochondrial electron transport in tumorigenesis and metastasis. Biochimica et Biophysica Acta (BBA)-General Subjects 1840, 1454-1463.

Tan, L., Wang, W., He, G., Kuick, R.D., Gossner, G., Kueck, A.S., Wahl, H., Opipari, A.W., Liu, J.R., 2016. Resveratrol inhibits ovarian tumor growth in an in vivo mouse model. Cancer 122, 722-729.

Tang, B.L., 2016. Sirt1 and the Mitochondria. Molecules and Cells 39, 87.

Tang, X., Shen, T., Jiang, X., Xia, M., Sun, X., Guo, H., Ling, W., 2015. Purified anthocyanins from bilberry and black currant attenuate hepatic mitochondrial dysfunction and steatohepatitis in mice with methionine and choline deficiency. Journal of Agricultural and Food Chemistry 63, 552-561.

Taylor, R.W., Turnbull, D.M., 2005. Mitochondrial DNA mutations in human disease. Nature Reviews Genetics 6, 389-402.

Terada, S., Goto, M., Kato, M., Kawanaka, K., Shimokawa, T., Tabata, I., 2002. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochemical and Biophysical Research Communications 296, 350-354.

Thannickal, V.J., Fanburg, B.L., 2000. Reactive oxygen species in cell signaling. American Journal of Physiology-Lung Cellular and Molecular Physiology 279, L1005-L1028.

Thilakarathna, S.H., Rupasinghe, H., 2013. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients 5, 3367-3387.

Toki, N., Kagami, S., Kurita, T., Kawagoe, T., Matsuura, Y., Hachisuga, T., Matsuyama, A., Hashimoto, H., Izumi, H., Kohno, K., 2010. Expression of mitochondrial transcription factor A in endometrial carcinomas: clinicopathologic correlations and prognostic significance. Virchows Archiv 456, 387-393.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2, 1231-1246.

Tsuda, T., 2018. Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. Food & Function, 705-714.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. The Journal of Physiology 552, 335-344.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., Wahl, G.M., 2002. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Molecular Cell 9, 1031-1044.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions 160, 1-40.

Van Duijnhoven, F.J., Bueno-De-Mesquita, H.B., Ferrari, P., Jenab, M., Boshuizen, H.C., Ros, M.M., Casagrande, C., Tjønneland, A., Olsen, A., Overvad, K., 2009. Fruit, vegetables, and colorectal cancer risk: the European prospective investigation into cancer and nutrition. The American Journal of Clinical Nutrition 89, 1441-1452.

Van Gisbergen, M., Voets, A., Starmans, M., De Coo, I., Yadak, R., Hoffmann, R., Boutros, P.C., Smeets, H., Dubois, L., Lambin, P., 2015. How do changes in the mtDNA and mitochondrial dysfunction influence cancer and cancer therapy? Challenges, opportunities and models. Mutation Research/Reviews in Mutation Research 764, 16-30.

Van Houten, B., Hunter, S.E., Meyer, J.N., 2016. Mitochondrial DNA damage induced autophagy, cell death, and disease. Frontiers in Bioscience (Landmark edition) 21, 42.

van Loo, G., Van Gurp, M., Depuydt, B., Srinivasula, S., Rodriguez, I., Alnemri, E., Gevaert, K., Vandekerckhove, J., Declercq, W., Vandenabeele, P., 2002. The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death and Differentiation 9, 20.

Vasiluk, L., Pinto, L.J., Tsang, W.S., Gobas, F.A., Eickhoff, C., Moore, M.M., 2008. The uptake and metabolism of benzo [a] pyrene from a sample food substrate in an in vitro model of digestion. Food and Chemical Toxicology 46, 610-618.

Ventura-Clapier, R., Garnier, A., Veksler, V., 2008. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1α. Cardiovascular Research 79, 208-217.

Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols 1, 1112-1116.

Vijayaraman, K.P., Muruganantham, S., Subramanian, M., Shunmugiah, K.P., Kasi, P.D., 2012. Silymarin attenuates benzo (a) pyrene induced toxicity by mitigating ROS production, DNA damage and calcium mediated apoptosis in peripheral blood mononuclear cells (PBMC). Ecotoxicology and Environmental Safety 86, 79-85.

Villegas, I., Sánchez-Fidalgo, S., Alarcón de la Lastra, C., 2008. New mechanisms and therapeutic potential of curcumin for colorectal cancer. Molecular Nutrition & Food Research 52, 1040-1061.

Viscomi, C., Bottani, E., Civiletto, G., Cerutti, R., Moggio, M., Fagiolari, G., Schon, E.A., Lamperti, C., Zeviani, M., 2011. In vivo correction of COX deficiency by activation of the AMPK/PGC-1α axis. Cell Metabolism 14, 80-90.

Visconti, R., Grieco, D., 2009. New insights on oxidative stress in cancer. Current opinion in drug discovery & development 12, 240-245.

Vyas, S., Zaganjor, E., Haigis, M.C., 2016. Mitochondria and cancer. Cell 166, 555-566.

Wallace, D.C., 2012. Mitochondria and cancer. Nature Reviews Cancer 12, 685-698.

Wang, K., Fan, H., Chen, Q., Ma, G., Zhu, M., Zhang, X., Zhang, Y., Yu, J., 2015. Curcumin inhibits aerobic glycolysis and induces mitochondrial-mediated apoptosis through hexokinase II in human colorectal cancer cells in vitro. Anti-Cancer Drugs 26, 15-24.

Wang, L.-S., Stoner, G.D., 2008. Anthocyanins and their role in cancer prevention. Cancer Letters 269, 281-290.

Wang, P., Vadgama, J.V., Said, J.W., Magyar, C.E., Doan, N., Heber, D., Henning, S.M., 2014. Enhanced inhibition of prostate cancer xenograft tumor growth by combining quercetin and green tea. The Journal of Nutritional Biochemistry 25, 73-80.

Wang, Q., Wang, J., Hu, M., Yang, Y., Guo, L., Xu, J., Lei, C., Jiao, Y., Xu, J., 2016. Uncoupling protein 2 increases susceptibility to lipopolysaccharide-induced acute lung injury in mice. Mediators of Inflammation 2016.

Wang, R.-H., Sengupta, K., Li, C., Kim, H.-S., Cao, L., Xiao, C., Kim, S., Xu, X., Zheng, Y., Chilton, B., 2008. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell 14, 312-323.

Wang, W., Wang, R., Zhang, Q., Mor, G., Zhang, H., 2018. Benzo (a) pyren-7, 8-dihydrodiol-9, 10-epoxide induces human trophoblast Swan 71 cell dysfunctions due to cell apoptosis through disorder of mitochondrial fission/fusion. Environmental Pollution 233, 820-832.

Wang, X., Moraes, C.T., 2011. Increases in mitochondrial biogenesis impair carcinogenesis at multiple levels. Molecular Oncology 5, 399-409.

Warburg, O., 1956. On the origin of cancer. Science 123, 309-314.

Ward, P.S., Thompson, C.B., 2012. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer Cell 21, 297-308.

Waris, G., Ahsan, H., 2006. Reactive oxygen species: role in the development of cancer and various chronic conditions. Journal of carcinogenesis 5, 14.

Weston, A., Harris, C., 2003. Multistage carcinogenesis. Holland-Frei Cancer Medicine. 6th edition. Hamilton (ON): BC Decker.

Westphal, D., Dewson, G., Czabotar, P.E., Kluck, R.M., 2011. Molecular biology of Bax and Bak activation and action. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1813, 521-531.

Wilson, W.J., Poellinger, L., 2002. The dietary flavonoid quercetin modulates HIF-1α activity in endothelial cells. Biochemical and Biophysical Research Communications 293, 446-450.

Wong, J.Y., Hu, W., Downward, G.S., Seow, W.J., Bassig, B.A., Ji, B.-T., Wei, F., Wu, G., Li, J., He, J., 2017. Personal exposure to fine particulate matter and benzo [a] pyrene from indoor air pollution and leukocyte mitochondrial DNA copy number in rural China. Carcinogenesis 38, 893-899.

Woo, H.D., Kim, B.M., Kim, Y.J., Lee, Y.J., Kang, S.J., Cho, Y.H., Choi, J.Y., Chung, H.W., 2008. Quercetin prevents necrotic cell death induced by co-exposure to benzo (a) pyrene and UVA radiation. Toxicology in Vitro 22, 1840-1845.

Wu, J., Wang, Y., Wo, D., Zhang, L., Li, J., 2016. Nuclear respiratory factor 1 overexpression attenuates anti-benzopyrene-7, 8-diol-9, 10-epoxide-induced S-phase arrest of bronchial epithelial cells. Molecular Medicine Reports 13, 4372-4378.

Wu, W.-S., 2006. The signaling mechanism of ROS in tumor progression. Cancer and Metastasis Reviews 25, 695-705.

Wu, Y.-d., Zhou, B., 2010. TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. British Journal of Cancer 102, 639-644.

Xia, T., Korge, P., Weiss, J.N., Li, N., Venkatesen, M.I., Sioutas, C., Nel, A., 2004. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity. Environmental Health Perspectives, 1347-1358.

Xue, H., Aziz, R.M., Sun, N., Cassady, J.M., Kamendulis, L.M., Xu, Y., Stoner, G.D., Klaunig, J.E., 2001. Inhibition of cellular transformation by berry extracts. Carcinogenesis 22, 351-356.

Yadava, N., Schneider, S.S., Jerry, D.J., Kim, C., 2013. Impaired mitochondrial metabolism and mammary carcinogenesis. Journal of Mammary Gland Biology and Neoplasia 18, 75-87.

Yang, C.S., Ju, J., Lu, G., Xiao, H., Hao, X., Sang, S., Lambert, J.D., 2008. Cancer prevention by tea and tea polyphenols. Asia Pacific Journal of Clinical Nutrition 17, 245.

Yang, C.S., Wang, X., Lu, G., Picinich, S.C., 2009. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. Nature Reviews Cancer 9, 429-439.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129-1132.

Yang, K., Chen, C., Cheng, S., Cao, X., Tu, B., 2017. Effects of benzo (a) pyrene exposure on the ATPase activity and calcium concentration in the hippocampus of neonatal rats. International Journal of Occupational Medicine and Environmental Health 30, 203-211.

Yao, H., Xu, W., Shi, X., Zhang, Z., 2011. Dietary flavonoids as cancer prevention agents. Journal of Environmental Science and Health, Part C 29, 1-31.

Yin, J., Gao, Z., Liu, D., Liu, Z., Ye, J., 2008. Berberine improves glucose metabolism through induction of glycolysis. American Journal of Physiology-Endocrinology and Metabolism 294, E148-E156.

Yip, K., Reed, J., 2008. Bcl-2 family proteins and cancer. Oncogene 27, 6398-6406.

Yoshino, M., Naka, A., Sakamoto, Y., Shibasaki, A., Toh, M., Tsukamoto, S., Kondo, K., Iida, K., 2015. Dietary isoflavone daidzein promotes Tfam expression that increases mitochondrial biogenesis in C2C12 muscle cells. The Journal of Nutritional Biochemistry 26, 1193-1199.

Yu, X.-D., Yang, J.-l., Zhang, W.-L., Liu, D.-X., 2016. Resveratrol inhibits oral squamous cell carcinoma through induction of apoptosis and G2/M phase cell cycle arrest. Tumor Biology 37, 2871-2877.

Zamin, L.L., Filippi-Chiela, E.C., Dillenburg-Pilla, P., Horn, F., Salbego, C., Lenz, G., 2009. Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells. Cancer Science 100, 1655-1662.

Zanini, S., Marzotto, M., Giovinazzo, F., Bassi, C., Bellavite, P., 2015. Effects of dietary components on cancer of the digestive system. Critical Reviews in Food Science and Nutrition 55, 1870-1885.

Zhang, L., Bao, Y., Li, J., 2011. Nuclear respiratory factor-1 is involved in mitochondrial dysfunction induced by benzo (a) pyrene in human bronchial epithelial cells. Basic & Clinical Pharmacology & Toxicology 109, 115-122.

Zhang, L., Bao, Y., Liu, Y., Li, J., 2014. Downregulation of nuclear respiratory factor-1 contributes to mitochondrial events induced by benzo (a) pyrene. Environmental Toxicology 29, 780-787.

Zhang, L., Yu, J., Park, B.H., Kinzler, K.W., Vogelstein, B., 2000. Role of BAX in the apoptotic response to anticancer agents. Science 290, 989-992.

Zhang, P., Qiang, X., Zhang, M., Ma, D., Zhao, Z., Zhou, C., Liu, X., Li, R., Chen, H., Zhang, Y., 2015. Demethyleneberberine, a natural mitochondria-targeted antioxidant, inhibits mitochondrial dysfunction, oxidative stress, and steatosis in alcoholic liver disease mouse model. Journal of Pharmacology and Experimental Therapeutics 352, 139-147.

Zhang, T., Jiang, S., He, C., Kimura, Y., Yamashita, Y., Ashida, H., 2013. Black soybean seed coat polyphenols prevent B (a) P-induced DNA damage through modulating drug-metabolizing

enzymes in HepG2 cells and ICR mice. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 752, 34-41.

Zhou, Y., Zheng, J., Li, Y., Xu, D.-P., Li, S., Chen, Y.-M., Li, H.-B., 2016. Natural polyphenols for prevention and treatment of cancer. Nutrients 8, 515.

Zhu, H., Li, Y., Trush, M.A., 1995. Characterization of benzo [a] pyrene quinone-induced toxicity to primary cultured bone marrow stromal cells from DBA/2 mice: potential role of mitochondrial dysfunction. Toxicology and Applied Pharmacology 130, 108-120.

Zhu, W., Cromie, M.M., Cai, Q., Lv, T., Singh, K., Gao, W., 2014. Curcumin and vitamin E protect against adverse effects of benzo [a] pyrene in lung epithelial cells. PloS One 9, e92992.

Zhu, W., Jia, Q., Wang, Y., Zhang, Y., Xia, M., 2012. The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP–PKA-dependent signaling pathway. Free Radical Biology and Medicine 52, 314-327.

Zitka, O., Skalickova, S., Gumulec, J., Masarik, M., Adam, V., Hubalek, J., Trnkova, L., Kruseova, J., Eckschlager, T., Kizek, R., 2012. Redox status expressed as GSH: GSSG ratio as a marker for oxidative stress in paediatric tumour patients. Oncology letters 4, 1247-1253.

Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., 2000. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes & development 14, 391-396.

9. APPENDICES

9.1. APPENDIX 1: Protein levels quantified by BCA assay kit

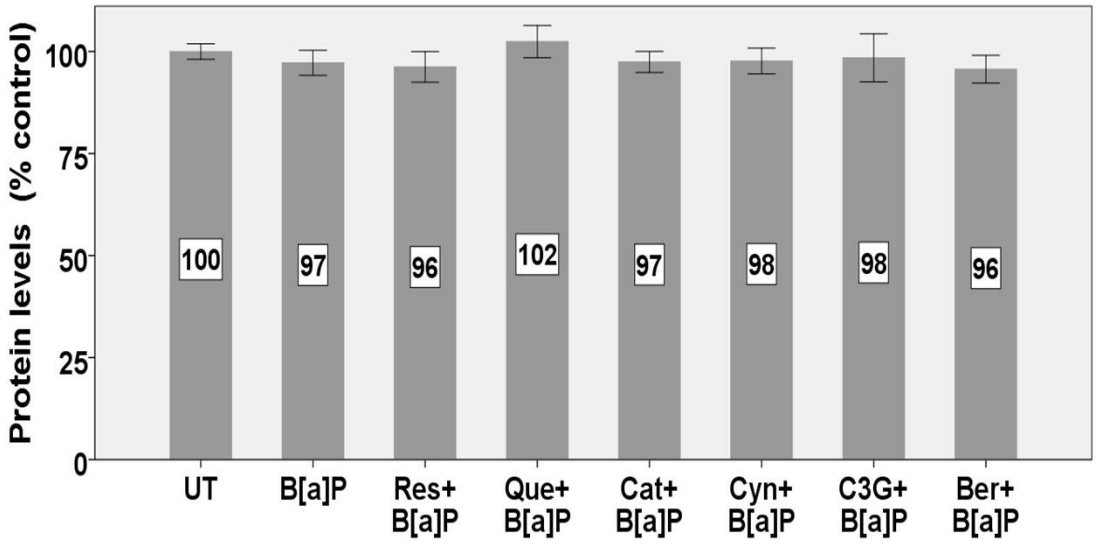

Figure 9.1. Effect of B[a]P and polyphenols on protein level.

Protein level was measured with a BCA assay kit in Bhas 42 cells after pre-treatment with polyphenols at 5 µM for 2h followed by treatment with 4 µM B[a]P for 24h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with one T25 flask of cells per treatment condition in each experiment. The significant difference between groups was tested with one-way ANOVA followed by Tukey's post-hoc test.

9.2. APPENDIX 2: Investigations into a possible synergism between cyanidin and resveratrol in decreasing B[a]P-induced intracellular and mitochondrial ROS.

9.2.1 Abstract

While resveratrol is a potent mitochondrial biogenesis inducer, flavonoids are notable antioxidants due to their more hydroxylated structure. Among flavonoids, anthocyanins are perhaps even stronger mitochondrial antioxidants due to having a more positive charge. In this study, we hypothesized that a combination of resveratrol and cyanidin decreases B[a]P-induced intracellular ROS and mitochondrial superoxide significantly more than each of them alone. Treatment with different concentrations of resveratrol and cyanidin and their combinations for 2h followed by administrating B[a]P for 12-72h showed no synergistic effects on B[a]P-induced intracellular ROS and mitochondrial superoxide generation.

9.2.2 Introduction

Resveratrol is a well-known polyphenol as a mitochondrial biogenesis inducer. Resveratrol induces mitochondrial biogenesis through activating SIRT1 (Howitz et al., 2003). So far, a few studies have reported a role of increased mitochondrial biogenesis with decreased ROS generation (Chen et al., 2017a; Kong et al., 2010; Takanche et al., 2017). In our study also, we showed that pre-treatment of Bhas 42 cells with resveratrol for 72h decreases B[a]P-induced intracellular ROS generation stronger than that of other polyphenols. We also showed that treatment of the Bhas 42 cells with resveratrol for 72h increases the content of mitochondria significantly more than that of other polyphenols. The results of our and other studies suggest that induction of mitochondrial biogenesis with resveratrol can decrease ROS generation. One possible mechanism could be that increasing number of healthy and functional mitochondria improves performance of the ETC resulting in lower production of ROS.

The antioxidative role of anthocyanins has been shown by several studies. Among anthocyanins, cyanidin is known as a potent antioxidant. The antioxidative role of anthocyanins has been attributed to the number of hydroxyl groups on the B ring (Procházková et al., 2011). Moreover, anthocyanins possess a positive charge that make them more susceptible to readily enter to and accumulate in the mitochondrial inner membrane and matrix (Peng, 2012). Also, unlike other flavonoids, anthocyanins were unable to activate SIRT1 to induce mitochondrial biogenesis (Howitz et al., 2003), although their breakdown products or metabolites may.

Although growing evidence shows synergistic effects between different polyphenols and their beneficial effects on health, a possible synergism between grape polyphenols such as resveratrol and anthocyanins in chemoprevention is not well-understood (Singh et al., 2016). Some studies have reported protective effects of resveratrol and quercetin in combination against carcinogenesis (Del Follo-Martinez et al., 2013; Zamin et al., 2009). Quercetin is a potent antioxidant and a mitochondrial biogenesis inducer as well.

Given the role of resveratrol as a mitochondrial biogenesis inducer and the potent antioxidative effects of cyanidin, in the current study, we investigated the possible synergistic effect between resveratrol and cyanidin against B[a]P-induced intracellular ROS generation and mitochondrial superoxide.

9.2.3 Material and Methods

9.2.3.1. Chemicals and reagents

See sections 3.2.1 and 5.2.1.

9.2.3.2. Cell culture

See section 3.2.2.

9.2.3.3. Intracellular ROS measurement

The level of intracellular ROS was determined using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). After seeding 2×10^4 Bhas 42 cells in a 96-well plate and growing for 24 hours, resveratrol, cyanidin and combination of resveratrol and cyanidin at different concentrations (0.1-10 µM) were administrated in DMSO (final DMSO concentration 0.5 %) for 2h followed by administrating 4 µM B[a]P for 12-72h. After these incubations, 10 µM H_2O_2 was administrated to some control wells as a positive control and incubated for 1 h. Then, medium was removed and DCFH-DA at 25 µM final concentration dissolved in DMEM without FBS was loaded to each well. After incubating for 30 min all of the wells were cautiously washed twice with phosphate-buffered saline (PBS) and read at 480/20 nm excitation and 528/20 nm emission using a microplate reader (BioTek, Synergy HT). Images were captured by fluorescence microscope (Olympus IX 71) at 473/31 nm excitation and 520/35 nm emission.

9.2.3.4. Mitochondrial superoxide measurement

The level of mitochondrial superoxide production was measured using MitoSOX Red fluorogenic dye. After seeding 2×10^4 Bhas 42 cells in a 96-well plate and growing for 24 hours, resveratrol, cyanidin and a combination of resveratrol and cyanidin at different concentrations (0.1-10 µM) were administrated in DMSO (final DMSO concentration 0.5 %) for 2h followed by administering 4 µM B[a]P for 12-72h. Antimycin A (a cytochrome c reductase inhibitor) was added as a positive control and incubated for 30 minutes. Then, the medium was removed and MitoSOX dye at 5 µM final concentration dissolved in DMEM without FBS was loaded to each well and plates were incubated for 30 minutes. Then, wells were cautiously washed twice with PBS and read at 530±20/590±20 nm using a microplate reader. Images were captured by a ZOE Fluorescent Cell Imager (Bio-Rad).

9.2.4 Results

9.2.4.1. Combination effects of resveratrol and cyanidin on intracellular ROS generation after 12h, 24h, and 72h

One of the hypotheses of this research is that the combination of resveratrol and cyanidin would give a synergistic effect in preventing initiation and promotion of B[a]P-induced carcinogenesis via increasing mitochondrial biogenesis and decreasing oxidative stress. To assess this hypothesis, Bhas 42 cells were pre-treated with combinations of equal concentrations of resveratrol and cyanidin to yield final concentrations each of 1, 5 and 10 µM and intracellular ROS was measured (Fig. 9.2). The results showed that the combination of resveratrol and cyanidin at the concentrations of 0.5 and 2.5 µM had a slightly greater effect on intracellular ROS generation compared to those of the polyphenols alone at concentrations of 1 and 5 µM. In these experiments however, resveratrol or cyanidin alone completely prevented the B[a]P-induced increase in intracellular ROS, so no significant synergistic effect was observed.

To further investigate the possible synergistic effect of resveratrol and cyanidin, intracellular ROS generation was measured after 24h and 72h with Bhas 42 cells pre-treated with the combination of equal concentrations of resveratrol and cyanidin to yield final concentrations of 1 µM, 0.4 µM, and 0.2 µM (Fig. 9.3A&B; 9.4A&B). The results showed no synergistic effect on intracellular ROS generation compared to those of polyphenols alone under these conditions.

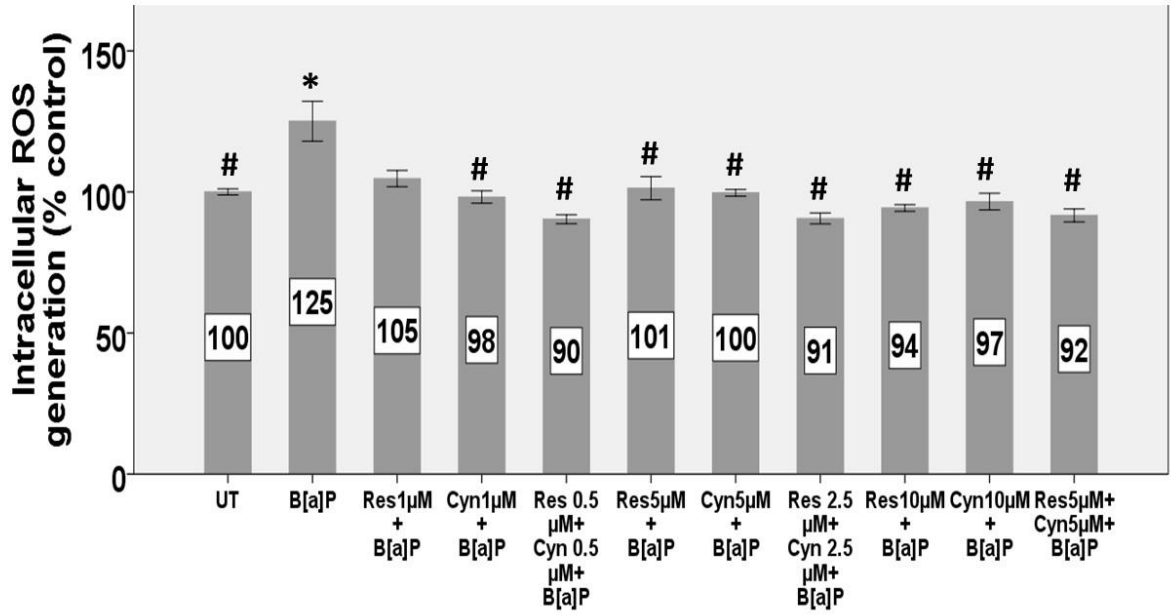

Figure 9.2. Combination effect of resveratrol and cyanidin on B[a]P-induced intracellular ROS generation after 12h.

Intracellular ROS generation in Bhas 42 cells was measured after treatment with different concentrations of resveratrol and cyanidin as well as their combination for 2h and exposure to 4 μM B[a]P for 12h, after adding the fluorogenic probe, DCFH-DA for 30 min. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with two-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

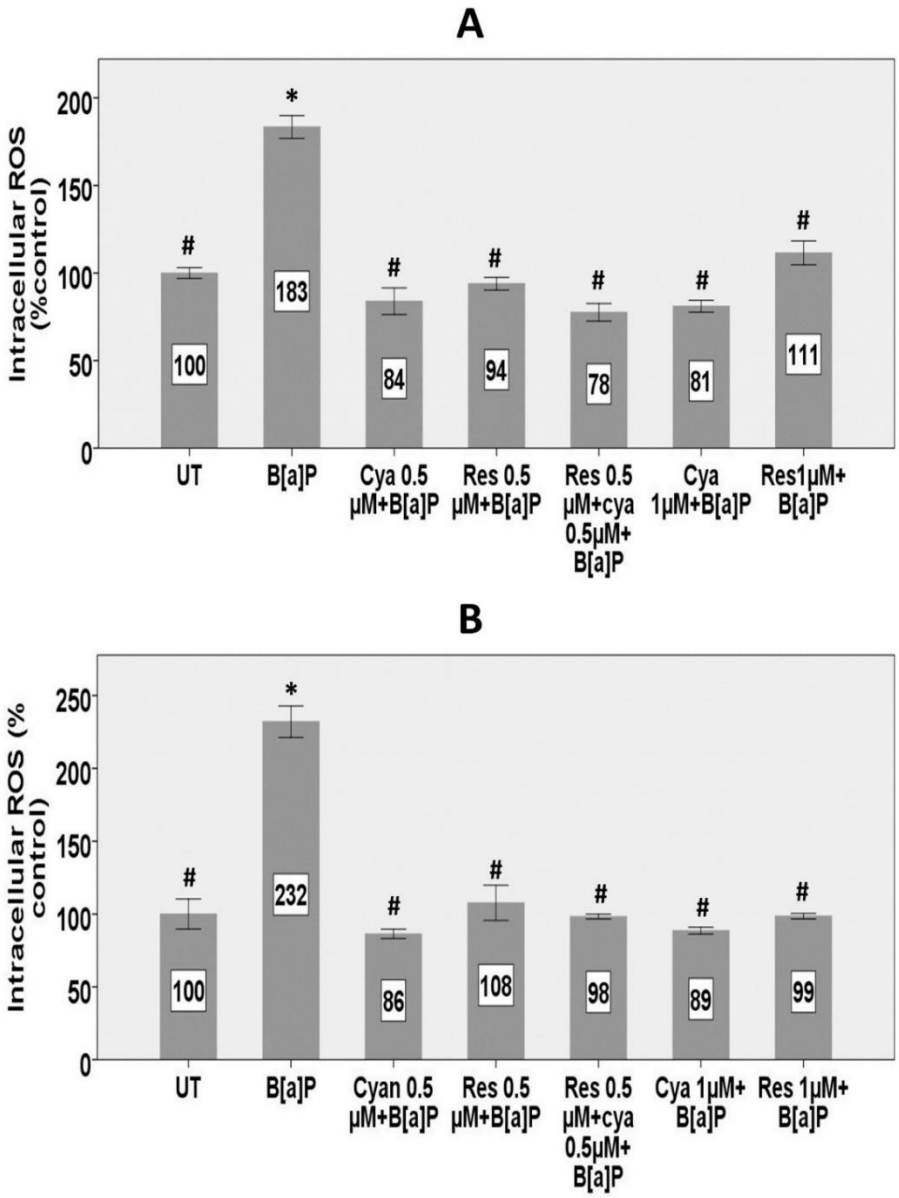

Figure 9.3. Combination effect of resveratrol and cyanidin on B[a]P-induced intracellular ROS generation after 24h and 72h.

Intracellular ROS generation in Bhas 42 cells was measured after treatment with different concentrations of resveratrol and cyanidin as well as their combination for 2h and exposure to 4 µM B[a]P for (A) 24h and (B) 72h, after adding the fluorogenic probe, DCFH-DA for 30 min. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with two-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

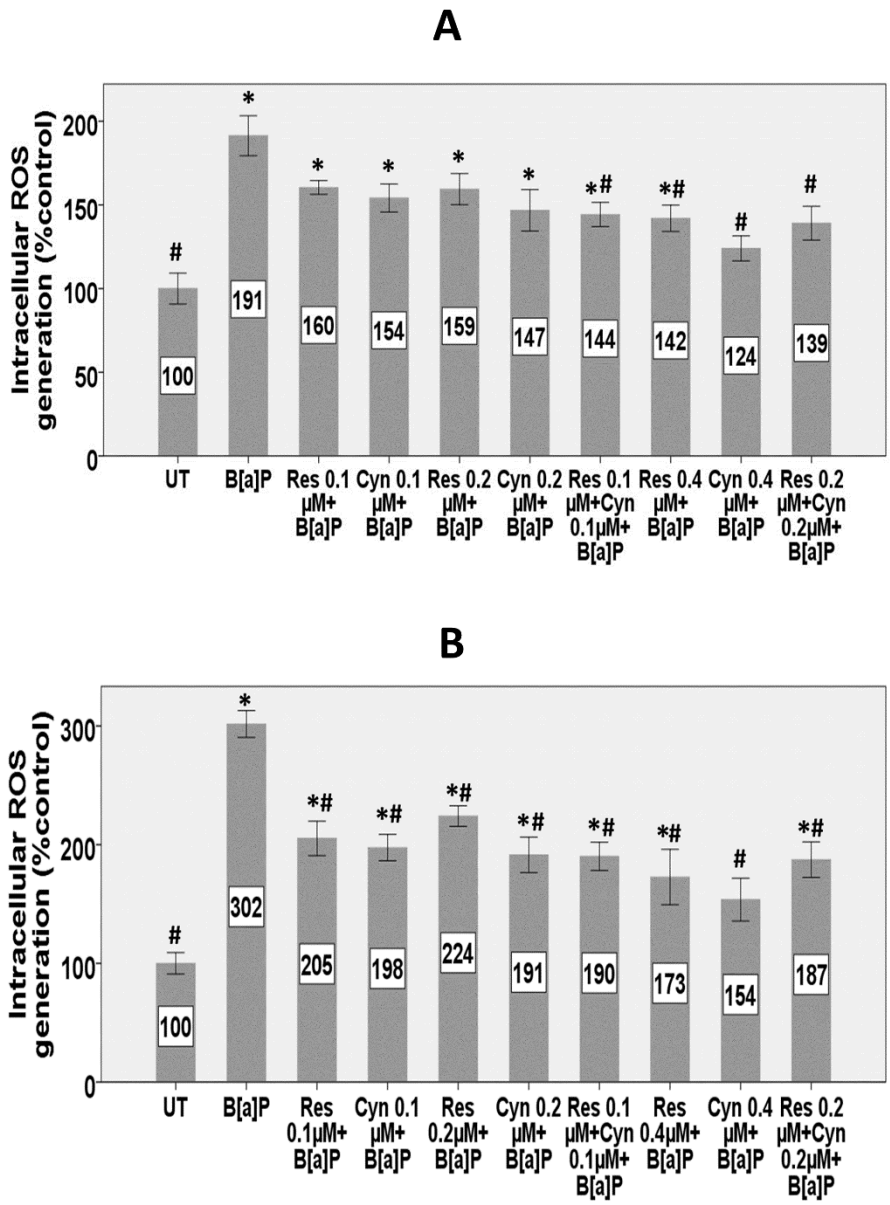

Figure 9.4. Combination effect of resveratrol and cyanidin on B[a]P-induced intracellular ROS generation after 24h and 72h.

Intracellular ROS generation in Bhas 42 cells was measured after treatment with different concentrations of resveratrol and cyanidin as well as their combination for 2h and exposure to 4 µM B[a]P for (A) 24h and (B) 72h, after adding the fluorogenic probe, DCFH-DA for 30 min. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with two-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

9.2.4.2. Combination effects of resveratrol and cyanidin on mitochondrial superoxide generation after 12h, 24h, and 72h

In measurements after 12 h, the combination of resveratrol and cyanidin in three concentrations (1, 5, 10 µM) had no synergistic effect on mitochondrial superoxide generation compared to those of polyphenols alone (Fig. 9.5). Only cyanidin alone, at all three concentrations, gave significant protection under these conditions.

To further investigate possible synergistic effects of resveratrol and cyanidin, mitochondrial superoxide generation was measured after 24h and 72h in Bhas 42 cells treated with the combination of equal concentrations of resveratrol and cyanidin to yield final concentrations of 1µM (Fig. 9.6A&B). At these time points also, the combination of resveratrol and cyanidin at 1 µM had no synergistic effect on mitochondrial superoxide generation compared to those of cyanidin alone.

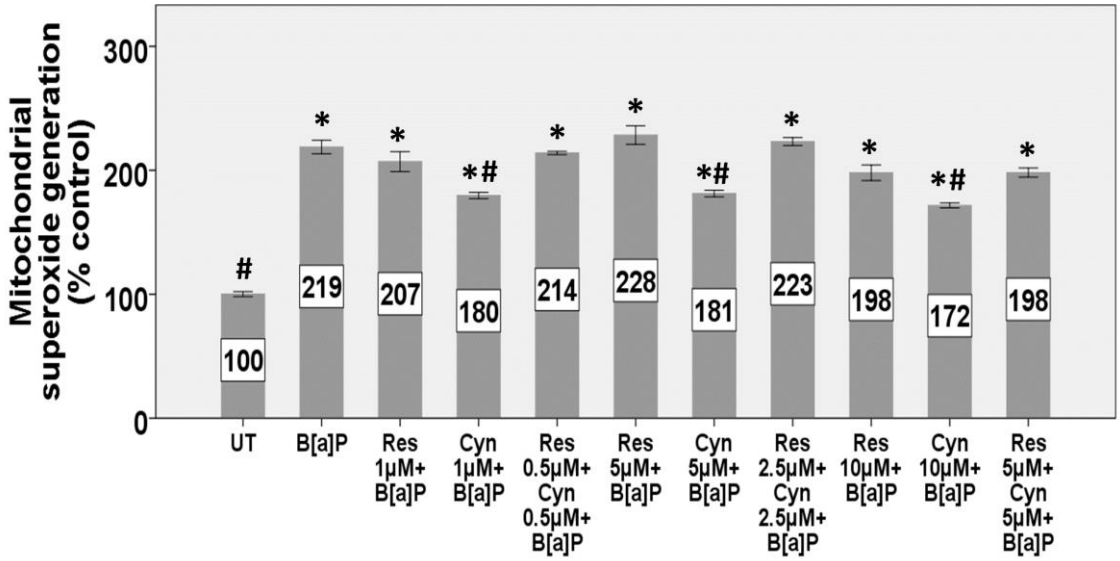

Figure 9.5. Combination effect of resveratrol and cyanidin on B[a]P-induced mitochondrial superoxide generation after 12h.

Mitochondrial superoxide generation in Bhas 42 cells was measured with MitoSOX Red after treatment with different concentrations of resveratrol and cyanidin as well as their combination for 2h and exposure to 4 µM B[a]P for 12h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with two-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.

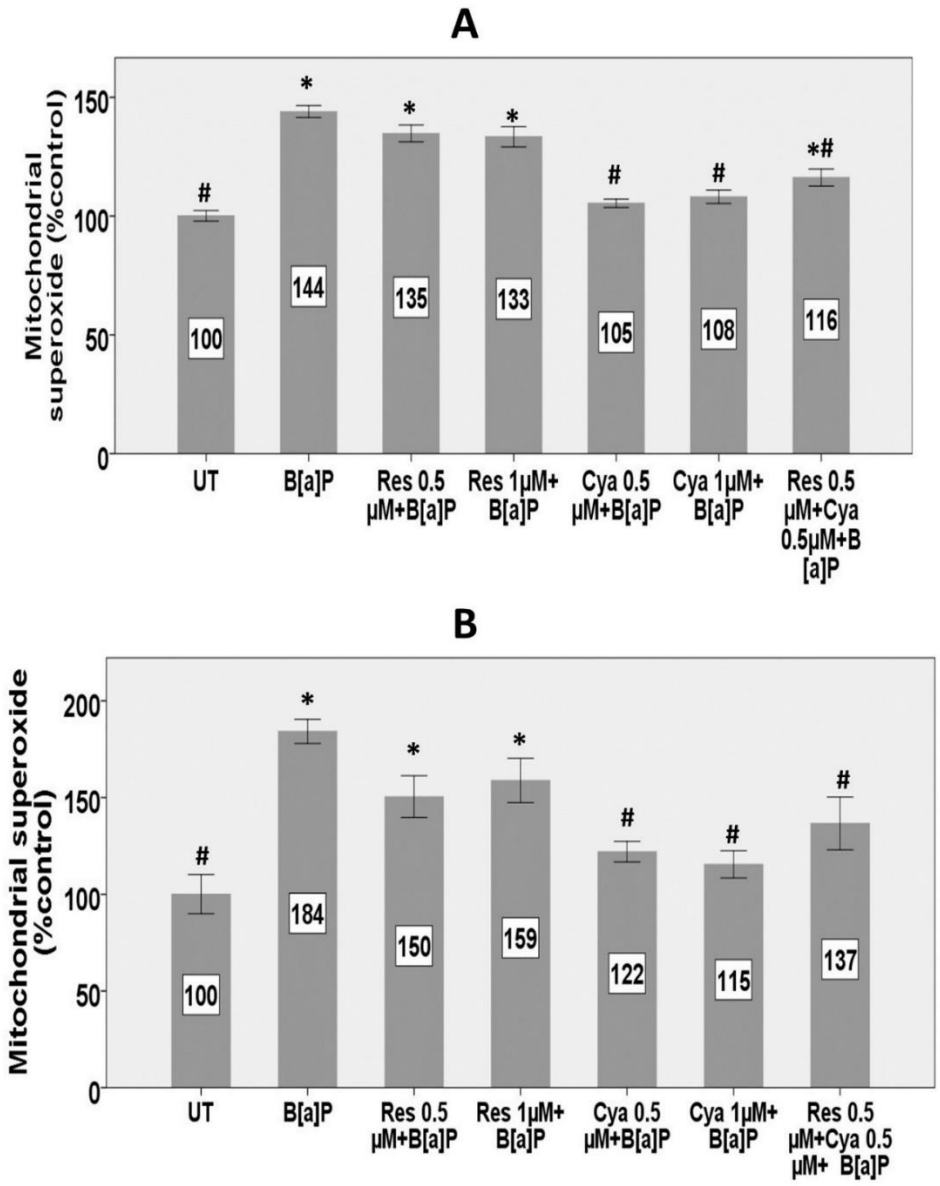

Figure 9.6. Combination effect of resveratrol and cyanidin on B[a]P-induced mitochondrial superoxide generation after 24 and 72 h.

Mitochondrial superoxide generation in Bhas 42 cells was measured using MitoSOX Red after treatment with different concentrations of resveratrol and cyanidin as well as their combination for 2h and exposure to 4 µM B[a]P for (A) 24h and (B) 72h. Data are presented as percentage of the untreated cells. The figure represents means ± SEM of 3 independent experiments with 3 wells of cells per treatment condition in each experiment. The significant difference between groups was tested with two-way ANOVA followed by Tukey's post-hoc test. * Significantly different from the UT group at $p < 0.05$. # Significantly different from the B[a]P group at $p < 0.05$.