The Combined Effects of Arsenite and Ethanol on Brain Endothelial Cells and Microglial Cells

By Siying Li

Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

____

Chair: Dr. Honglian Shi

____

Dr. Jackob Moskovitz

____

Dr. Adam Smith

Date Defended: 18 July 2019

The thesis committee for Siying Li certifies that this is the approved version of the following thesis:

The Combined Effects of Arsenite and Ethanol on Brain Endothelial Cells and Microglial Cells

Chair: Dr. Honglian Shi

Date Approved: 25 July 2019

Abstract

Arsenic (As) is a natural compound widely distributed in air, water, and soil. Drinking ground water is the major source of As exposure. As exposure causes many health issues, including nausea, vomiting, pain, diarrhea, cancer and neurotoxicity [1]. Alcohol drinks may also contain As because grapes and rice which are used in making wine and beer, take up As from soil, water, and fungicides containing As. Emerged evidence showed that ethanol (EtOH) also impairs neurological functions [2]. However, the combined toxic effects of As and EtOH on the brain is still unclear. Our long-term goal is to understand the effects of As combined with EtOH on the blood brain barrier (BBB).

The BBB controls molecule exchange between peripheral and cerebral compartments [3]. Alterations of the BBB are a critical risk factor of pathology and progression of different neurological diseases [4]. Many studies have shown that As as well as EtOH induced BBB abnormalities [5, 6]. Since brain endothelial cells play a crucial role in the BBB, we used Rat Brain Endothelia (RBE4) cells to investigate the combined toxic effects of As and EtOH on the BBB.

Overproduction of reactive oxygen species (ROS) results in destruction of cellular structures, lipid, and proteins [7]. Previously, our lab showed that As increased endothelial cell permeability through a ROS-vascular endothelial growth factor pathway in mouse brain vascular endothelial cells (bEnd 3 cells) [8]. Others have shown that EtOH also impairs the barrier function and junctional organization of human brain microvascular endothelial cell monolayer [9]. In neurons, EtOH-induced ROS mainly come from damaged mitochondria

[10]. Since mitochondria are major source of ROS generation, we proposed that As-EtOH-combined treatment impairs the BBB through ROS released by damaged mitochondria.

Mitochondrial oxidative stress affected microglia-associated neurodegenerative diseases through their role as pro-inflammatory molecules and modulators of pro-inflammatory processes [11]. BBB disruption is mediated by neuroinflammation which is associated with increase in pro-inflammatory cytokines [12]. Research showed that inflammatory mediators control BBB permeability through regulating the structural components [13]. As induced cytotoxicity in brains via regulation of oxidative stress and TNF-a associated inflammatory pathways [14]. Alcohol consumption enhanced oxidative and inflammatory stress, resulting in cognitive deficit [15]. Since microglial cells are the main effectors in the inflammatory process of the central nervous system [16], we used microglial cells (BV2) to investigate the combined effects of As and EtOH on microglia.

Our results showed that As and EtOH increased RBE4 cell monolayer permeability. As-EtOH combined treatment increased the permeability more than As or EtOH treatment alone. RBE4 cells and BV2 cells showed an increase in ROS by the combined treatment. Mitochondrial ROS generation was increased by the combined treatment of As and EtOH but reduced by antioxidant Tempol in RBE4 cells and microglia BV2 cells. The combined treatment of As and EtOH decreased mitochondrial bioenergetics (mtBE) in RBE4 cells but increased by antioxidant Tempol. BV2 cells viability decreased as As or EtOH concentration increased and further decreased by the combined treatment. In conclusion, our results suggest that the combined treatment of As and EtOH induced toxicity on both endothelial cells and microglial cells via increased oxidative stress induced by mitochondrial dysfunction.

Acknowledgements

First, I would like to thank my advisor Dr. Honglian Shi who gives me the opportunity to join his lab and learn from him. It is my advisor who gives me support not only in research and study but also in my life. I am really appreciated that he always gives me valuable advises when I face some problems. His kindness and generosity help me to be confident to deal with difficulties properly. From the beginning to the end of my two years master journey, my gratitude to my advisor persisted.

Second, I would like to thank my committee members Dr. Jackob Moskovitz and Dr. Adam Smith who have given me valuable suggestions and feedback regarding my proposal and thesis. Their passion for research and abundance professional knowledge influenced me to be better.

Additionally, I would like to thank Dr. Justin Douglas at Core Research Resource Labs who trained us on Electron Paramagnetic Resonance. Also I want to thank my lab member Mohammed Almutairi for teaching me in lab and in life through the past two years. And, I want to extend my thanks to my previous lab member Jiani Chen for a whole year wonderful company both in lab and life.

What's more, I would like to thank all the faculty in Pharmacology & Toxicology Department for teaching and guiding me on study and research. Also, I am grateful to have my friends in Pharmacology & Toxicology Department who helped me a lot in the past two years. Last but not least, I really thank my parents who show their love and support unconditionally.

Table of Contents

Abstract ..... **iii**

Acknowledgements ..... **v**

List of Figures ..... **ix**

List of Tables ..... **x**

List of Abbreviations ..... **xi**

Chapter 1 Introduction **1**

1.1 Introduction of Arsenic (As) 1

1.1.1 As Exposure 1

1.1.2 As Toxicity 1

1.2 Introduction of Ethanol (EtOH) 4

1.3 As and EtOH 6

1.4 Introduction of the BBB 7

1.4.1 BBB 7

1.4.2 Brain Endothelial cells 7

1.5 Introduction of Oxidative stress 8

1.5.1 Oxidative stress and ROS 8

1.5.2 Oxidative stress Affects the BBB 9

1.5.3 Oxidative Stress and Mitochondrial Dysfunction 10

1.5.4 Antioxidant 11

1.6 Introduction of Inflammation 11

1.6.1 Microglial cells 11

1.6.2 Inflammation Affects the BBB 12

1.6.3 Inflammation and oxidative stress.. 14

1.7 Statement of purpose 14

Chapter 2 Materials and Methods..**18**

2.1 Reagents 17

2.2 Methods 19

2.2.1 Cell Culture and Treatment 19

2.2.2 Paracellular Permeability Assay 20

2.2.3 mtBE Assessment 20

2.2.4 Electron Paramagnetic Resonance (EPR) Spectroscopy 21

2.2.5 MitoSOX Live Cell Staining 21

2.2.6 MTT Assay 22

2.2.7 Statistical Analysis 22

Chapter 3 Results...**23**

3.1 As and EtOH Increased Permeability of RBE4 cell Monolayer 23

3.2 As and EtOH Increased ROS Production in RBE4 Cells 26

3.3 As and EtOH Induced Mitochondrial ROS Production in RBE4 Cells 29

3.4 As and EtOH changed mtBE in RBE4 cells 32

3.5 Antioxidant Decreased As-EtOH-Induced Mitochondrial ROS Production in RBE4 cells 35

3.6 Antioxidant decreased As-EtOH-Induced mtBE Loss in RBE4 cells 38

3.7 As and EtOH induced cell death in BV2 cells 41

3.8 The combined treatment of As and EtOH induced cell death in BV2 cells 44

3.9 As and EtOH Increased ROS Production in BV2 Cells 47

3.10 As and EtOH Induced Mitochondrial ROS Production in BV2 Cells 50

3.11 Antioxidant Decreased As-EtOH-Induced Mitochondrial ROS Production in BV2 cells 53

Chapter 4 Discussion 56

References 66

List of Figures

Figure 3.1 As and EtOH increased RBE4 cell monolayer permeability 24

Figure 3.2 As and EtOH increased ROS Production in RBE4 cells 27

Figure 3.3 As and EtOH increased mitochondrial ROS production in RBE4 cells 30

Figure 3.4 As and EtOH affected mtBE in RBE4 cells 33

Figure 3.5 Antioxidant decreased As-EtOH-induced mitochondrial ROS production in RBE4 cells 36

Figure 3.6 Antioxidant reduced As-EtOH-induced mtBE loss in RBE4 cells 39

Figure 3.7 As and EtOH induced BV2 cells death 42

Figure 3.8 The combined treatment of As and EtOH induced BV2 cells death 45

Figure 3.9 As and EtOH Increased ROS Production in BV2 Cells 48

Figure 3.10 As and EtOH increased mitochondrial ROS production in BV2 cells 51

Figure 3.11 Antioxidant decreased As-EtOH-induced mitochondrial ROS production in BV2 cells 54

Figure 4.1 The combined effects of As and EtOH 65

List of Tables

Table 1.1.2 Recent Animal studies on As neurotoxicity...3

Table 1.1.3 Recent Animal studies on EtOH neurotoxicity...5

Table 2.1 List of Major Reagents...18

List of Abbreviations

•OH	Hydroxyl Radical
As	Arsenic
BBB	Blood-Brain Barrier
CYP	Cytochrome P450
EPR	Electron Paramagnetic Spectroscopy
EtOH	Ethanol
FCCP	Cyanide p-(trifluoromethoxy) Phenylhydrazone
MMPs	Matrix Metallopeptidase
MRC	Maximal Respiratory Capacity
mtBE	Mitochondrial Bioenergetics
O_2^-	Superoxide Anion
OCR	Oxygen Consumption Rate
OD	Optical Density
PT	Polyethylene Terephthalate
RBE4	Rat Brain Endothelial cells

ROS	ROS
Tempol	4-hydroxy-2, 2, 6, 6,-tetramethylpiperidine-N-oxyl
TJs	Tight Junctions
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Chapter 1 Introduction

1.1 Introduction of Arsenic (As)

1.1.1 As Exposure

As is a naturally occurring substance that can be found in air, water and soil. There are two forms of As, organic and inorganic. As combined with carbon which is referred to organic As is mainly found in seafood such as fish and shellfish. While As combined with other elements such as chlorine which is called inorganic As is more toxic than the organic form. Inorganic forms of As were used in pesticides, fungicides, paint pigment, wood preservations, etc. Although there is some limitations of As uses nowadays, the remained As still exists in the environment.

As exists in several oxidation states, with As trioxide and As pentoxide being the most common forms. The major source of As exposure is groundwater used for drinking water in many countries such as Vietnam and Bangladesh [17-19]. According to the World Health Organization (WHO), at least 140 million people in 50 countries have been drinking water containing As at levels above the provisional guideline value of 10µg/L. The introduction of As into drinking water occurs as a result of its natural geological presence in local bedrock which is known in Bangladesh [20]. It is obvious that As exposure is a worldwide problem.

1.1.2 As Toxicity

Exposure to organic and inorganic As is associated with cancer and non-cancer effects in nearly every organ in the human body. Acute As poisoning results in nausea, vomiting, colicky abdominal pain, profuse watery diarrhea, excessive salivation and even death (lethal

dose: >100mg) [21]. Chronic As exposure affects a broad range of organs and system including skin, development processes, nervous system, respiratory system, cardiovascular system, liver, kidney, bladder, immune system and endocrine system [22]. Chronic exposure to As in drinking water caused skin lesions which is common symptom of As poisoning [23, 24]. In addition, As exposure results in different kinds of cancer such as skin cancer, lung cancer, bladder cancer and prostate cancer [25-28].

As exposure also affects the central nervous system, but it has not been extensively explored like As carcinogenic toxicity. For example, in Mexico, children who were exposed to As showed cognitive problems such as decreased intelligence quotients (IQ) scores, long-term memory, linguistic abstraction, attention span and visuospatial organization [29, 30]. Chronic As poisoning patients had psychiatric manifestations including depression, anxiety and depression [31]. Different animal models also showed neurotoxicity after As exposure. (**Table 1.1.2**). Although the neurotoxic effects of As are wildly reported, the cellular and molecular mechanisms of As neurotoxicity have not well studied. Therefore, more research is needed to understand these mechanisms.

Table 1.1.2 Recent Animal studies on As neurotoxicity

As Dose	**Exposure Duration**	**Animal Model**	**Finding**	**Reference**
20mg/L of iAs	1, 15, 90 days	CD1 mice	Spatial memory loss	Ramos et al. 2015 [32]
0, 50, 500 ppb As	4 hours	Zebrafish	Harmful effects on the developing embryo	Beaver et al. 2017 [33]
15µg/L As_2O_3	6 days	Rats	Induced autism-like behavior and affects frontal cortex neurogenesis	Zhou H et al. 2018 [34]
85ppm $NaAsO_2$	10 days	C3H mice	Developmental As exposure affected social behavior	Htway et al. 2019 [35]
0, 23.6, 47.7, 71.0 ppm arsenate	17, 21 days	Sprague Dawley rats	Developmental neurotoxicity	Moore et al. 2019 [36]
25 ppm As	16 days	Rats	Impairments in neurobehavioral functions	Saritha et al. 2019 [37]

1.2 Introduction of Ethanol (EtOH)

Alcohol has historically, and continues to, hold an important role in social engagement and bonding for many. However, it is also known that alcohol drink leads to many health issues. In 2014, the World Health Organization reported that alcohol contributed to more than 200 diseases and injury-related health conditions.

According to the 2015 National Survey on Drug Use and Health, 86.4 percent of people ages 18 or older reported that they drank alcohol at some point in their lifetime; 26.9 percent of people ages 18 or older reported that they engaged in binge drinking in the past month in the United States. The Substance Abuse and Mental Health Service Administration defines binge drinking as 5 or more alcoholic drinks for male or 4 or more alcoholic drinks for females on the same occasion on at least 1 day in the past month. Alcohol use disorder has a life time prevalence of nearly one in three individuals in the United States [38].

Alcohol affects many parts of the human body including brain, heart, liver, pancreas, and immune system, which can cause many health problems such as cancer, cardiovascular diseases, liver diseases, depression and brain injury [39-42]. When alcohol is consumed, around 33% of it gets absorbed immediately into the blood. Once in the bloodstream, EtOH easily diffuses into the brain and leads to neurotoxicity. Different animal models were used to study neurotoxicity of EtOH. (**Table 1.1.3**).

Table 1.1.3 Recent Animal studies on EtOH neurotoxicity

EtOH Dose	Exposure Duration	Animal Model	Finding	Reference
56% v/v Wine	Twice daily for 4 weeks	Sprague-Dawley rats	Metabolite content and axonal fiber change in pontine	Luo et al. 2017 [43]
1% EtOH (water)	1 hour	Zebrafish	Anxiety-like behavior and oxidative damage in the brain	Mocelin et al. 2018 [2]
4g/kg/day i.p, 12% v/v EtOH	6 days	Sprague-Dawley rats	Early-EtOH exposure altered dorsal hippocampus	Swart et al. 2018 [44]
4.8, 9.6, 14.4 g/kg EtOH v/v	3, 14 days	Sprague-Dawley rats	Metabolism alteration in brain	Hsieh et al. 2018 [1]
6%, 12% EtOH (water)	2 months	Wistar Han rats	Neurophysiological transformations in dorsal striatal circuits	Lagstrom et al. 2019 [45]

1.3 As and EtOH

Alcohol drink may be contaminated with As as a result of poor quality management. Grapes and rice which are traditionally used to made wine and beer, take up As from soil, water, and fungicides containing As [46]. The filtering process used to remove sediment from beer and wine could contaminate alcohol drinks with potentially dangerous heavy metals such as As. Researchers collected 65 representative wines from the top four wine producing states in the U.S.A. and found that all samples contain a level of As that exceeds the U.S.A. Environment Production Agency exposure limit which is less than 10 parts per billion in drinking water [47]. Dangerously high levels of As have been found in drinking water wells in more than 25 states in the United States, potentially exposing 2.1 million people to drinking water with As concentration more than 10μg/L [48]. Although the number of people who exposed to both As and EtOH at the same time around the world is still not clear, there is high prevalence of co-exposure of As and EtOH.

The combined treatment of As and EtOH induced toxicity in cancer and liver disease. For example, in colon cancer cells, EtOH enhances low-dose As induced tumor angiogenesis which is related to intracellular ROS generation, NASPH oxidase activation, and upregulation of PI3K/Akt and hypoxia-inducible factor 1 alpha signaling [49]. Not only the combined treatment with As and EtOH affects cancer, but also liver. In zebrafish, inorganic As increased the unfolded protein load in endoplasmic reticulum by directly acting as a reducing agent and indirectly by disrupting the redox balance through ROS generation, which potentiate the effect of EtOH to cause fatty liver disease [50]. Therefore, ROS generation is associated with the combined effects of As and EtOH. However, the cellular effects of As and EtOH co-exposure in the brain still needs to be studied.

1.4 Introduction of the Blood Brain Barrier (BBB)

1.4.1 BBB

The blood –brain barrier shields the brain from toxic substance. It tightly controls molecule and cell exchange between cerebral and peripheral compartments [3]. The precise control of central nervous system homeostasis allows for proper neuronal function and also protects the neural tissue from toxins and pathogens [51]. Alterations of the BBB are an important component of pathology and progression of different neurological diseases including traumatic brain injuries, Alzheimer's disease, stroke, epilepsy and multiple sclerosis [4].

Both organic As and inorganic As have been reported to cause impairment of the BBB. As-containing hydrocarbons, a subgroup of arsenolipids enhances the permeability of the in vitro BBB which facilitates the transfer of toxicants into the brain [52]. Developmental As exposure significantly alters tight junction proteins, resulting in increased BBB permeability [5]. Besides As, EtOH also affects the BBB. Chronic alcohol drinking augmented and dysregulated the lipopolysaccharide-induced BBB abnormalities [6]. However, the effects of combined treatment of As and EtOH in the brain is still unclear.

1.4.2 Brain Endothelial cells

The BBB constitutes a neurovascular unit formed by microvascular endothelial cells, pericytes and astrocytes [53]. BBB endothelial cells display stringent tight junctions and which formed by tight junction proteins such as Occludin, Claudin [54]. The tight junction constitutes the barrier both to the passage of ions and molecules through the paracellular

pathway and to the movement of proteins and lipids between the apical and the basolateral domains of the plasma membrane [55]. Therefore, brain endothelial permeability plays a crucial role in the BBB.

In this research, Rat Brain Endothelial (RBE4) cells were used to study the effects of combined treatment of As and EtOH on brain endothelial. Previously, our lab showed that As increased endothelial cell permeability through a ROS-vascular endothelial growth factor pathway in mouse brain vascular endothelial cells [8]. Animal study showed that As-fed mice showed microvascular dysfunction with increased vascular leakage [56]. Not only As affects endothelial cells but also EtOH. Long term EtOH treatment leads to downregulation of tight junction proteins and loss of structural integrity in human cerebral microvascular endothelial cells [57]. However, the combined effects of As and EtOH on the brain have not been fully understood.

1.5 Introduction of Oxidative Stress

1.5.1 Oxidative Stress and ROS

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen and nitrogen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. ROS contains unpaired electrons which increase the chemical reactivity of an atom or molecule, for example, hydroxyl radical (•OH) and superoxide anion (O^-_2). Two endogenous sources of ROS are the mammalian CYP-dependent microsomal electron transport system and the mitochondrial electron transport chain.

Oxidative stress causes toxic affects through damaging the cellular components including proteins, lipids and DNA, which is associated with different diseases. Studies showed that oxidative stress is associated with neurodegenerative diseases, cardiovascular diseases and cancer. Clinical and preclinical studies indicate that neurodegenerative diseases are characterized by high levels of oxidative stress biomarkers and by lower levels of antioxidant defense biomarkers in the brain [58]. Oxidative stress accounts for impaired endothelial function, a process which promotes atherosclerotic lesion or fatty streaks formation [59]. Increased ROS production has been detected in various cancers and has been shown to have several roles in activating pro-tumorigenic signaling, enhancing cell survival and proliferation [60].

1.5.2 Oxidative Stress Affects the BBB

The brain, with its high oxygen consumption and lipid-rich content, is highly susceptible to oxidative stress which involved in both neurodegenerative and neuropsychiatric disorders. ROS trigger a variety of molecular cascades that increase BBB permeability and later alter brain morphology, causing neuroinflammation and neuronal death [61].

One of the effects of oxidative stress on the brain is altering the BBB which plays an essential role in protecting the brain. Oxidative stress contributes to enhanced BBB permeability in blast-induced traumatic brain injury [62]. Studies also showed that increased endothelial oxidative stress has a focal role in psychostimulant-induced BBB dysfunction by endothelial activation and regulation of BBB tight junction function [63]. However, inhibition of ROS could effectively protect the silica nanoparticles induced BBB

dysfunction including tight junction loss of brain microvessel endothelial cells [64]. Anti-oxidant oleuropein protects intracerebral hemorrhage induced disruption of the BBB through alleviation of oxidative stress [65].

1.5.3 Oxidative stress and Mitochondrial Dysfunction

The mitochondria is responsible for producing energy (adenosine triphosphate) by consuming oxygen. In normal condition, 1-5% oxygen is converted to ROS in mitochondria, thus the major production of intracellular oxidative stress is from mitochondria [66]. The electron transport chain which occurs in the inner membrane of the mitochondria produce adenosine triphosphate for cells with the involvement of different mitochondrial complexes. In fact, 0.2-2% of the electrons in the mitochondrial electron transport chain directly leak out and interact with oxygen to produce superoxide or hydrogen peroxide, which mainly located at mitochondrial complex I and complex III [67].

Actually, mitochondria not only is the source of ROS but also is the target of oxidative stress. ROS damage the mitochondrial proteins/enzymes, membranes, and DNA, which leads to the interruption of energy generation and other essential functions in mitochondria [68]. It has been known that mitochondria dysfunction is associated with numerous neurodegenerative disorders including Parkinson’s disease, Alzheimer’s disease, Huntington’s disease and cerebral stroke [69]. Recently, researchers pay more and more attention on mitochondrial biology and several novel avenues in drug designing against neurodegenerative diseases [70].

1.5.4 Antioxidant

Antioxidants are substances that help to prevent the harmful effects of excessive ROS activity, and combat or delay cell damage. Several antioxidants have been exploited in recent years for their actual or supposed beneficial effects against oxidative stress, such as vitamin E, flavonoids, and polyphenols [71]. Animal experiments have proved that the use of antioxidants is a beneficial therapeutic strategy of the treatment of diabetic retinopathy which is one of the most common microvascular complications of diabetes, but more data are required from clinical trials [72].

Tempol (4-hydroxy-2, 2, 6, 6,-tetramethylpiperidine-N-oxyl) is a redox-cycling nitroxide water-soluble superoxide dismutase mimetic that has been reported to be an effective antioxidant in detoxifying ROS both in cell culture and animal studies [73]. Research showed that a Tempol has a protective effect against post-traumatic stress disorder induced short- and long- term memory impairment through prevention of alteration in oxidative stress in the hippocampus [74]. In addition, Tempol inhibits superoxide anion-induced inflammatory pain in mice from molecular levels to behavioral alterations [75]. In this research, we will use Tempol as antioxidant to rescue the effect of As, EtOH and As with EtOH in cell models.

1.6 Introduction of Inflammation

1.6.1 Microglial cells

A number of nervous system disorders are characterized by a state of inflammation in which members of the innate immune system, mostly notably microglia acting as single

entities and in unison produce inflammatory molecules that play major roles [76]. Activated microglia were found surrounding lesions of various neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, muscular degeneration and necrosis, are involved in the removal of necrotic neurons [16].

Microglial pro-inflammatory activation is associated with increased cytokine/chemokine expression, oxidative stress, which in turn leads to neuroinflammation which damages critical support cells such as brain endothelial cells that from the BBB [77]. Methylprednisolone sodium succinate, a well-known immunosuppressive agent, significantly suppressed activation of microglia and reduced permeability of the BBB in post-intracranial hemorrhage mice [78]. On the other hand, disruption of the BBB changes in blood flow, introduction of pathogens in the sensitive central nervous system niche, insufficient nutrient supply, and abnormal secretion of cytokines or endothelial receptors are reported to prime and attract microglia [79].

In this research, BV2 microglial cells will be used to study the effects of combined treatment of As and EtOH on microglia.

1.6.2 Inflammation Affects the BBB

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells or irritants, and is a protective response involving immune cells, blood vessels, and molecular mediators. There is increasing recognition that inflammation plays a critical role in the BBB alteration. Systemic inflammation leads to both disruptive BBB change (modification of tight junctions, endothelial damage) and non-disruptive change which occurs at the molecular level [80].

Microglia are known to express IL-1 7A and IL-1 7C receptors, which upon intracellular signaling can trigger the release of pro-inflammatory cytokines and a wide range of chemokines involved in leukocyte trafficking such as Monocyte Chemoattractant Protein 1 (MCP-1) [81]. MCP-1 was shown to increase BBB permeability by binding BBB-EC-expressed chemokine C-C motif receptor 2, which in turn activates small GTPase Rho and Rho kinase to trigger the reorganization of the actin cytoskeleton and redistribution of TJ proteins [82].

Pro-inflammatory cytokines stimulate gene expression of adhesion molecules such as Intercellular Adhesion Molecule-1(ICAM-1) [83]. ICAM-1 induced loss of tight junction proteins occluding and ZO-1 from cerebral vascular endothelium during neutrophil-induced BBB breakdown in vivo [84]. Increase of soluble inflammation mediators such as Vascular Endothelial Growth Factor (VEGF) activates several membrane receptors that converge in cellular pathways hallmark of inflammation leading to phosphorylation of tight junction proteins [85]. Anti-inflammatory effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor reduced BBB permeability restricting the diffusion of molecular tracers and the migration of immune cells across the BBB [86].

Activated microglia produced ROS which further contribute to increased BBB permeability by activating the PI3K/AKT pathway and by decreasing the expression of VE-cadherin, occluding and claudin-5 in BBB-ECs [87]. Microglia can also increase BBB permeability through secretion of Matrix Metallopeptidase (MMPs) [81]. MMPs induce degradation of tight junction proteins, breakdown of the extracellular matrix and subsequent loss of BBB integrity [88].

1.6.3 Inflammation and oxidative stress

Increasing evidence showed an important relationship between inflammation and oxidative stress. Oxidative stress is central in the pathology both as an initiator of vascular and renal inflammation and the consequence of inflammatory responses and cytokines [89]. At the site of inflammation, inflammatory cells such as neutrophils, monocytes, and lymphocytes trigger the release of various enzymes, including ROS, pro-inflammatory cytokines and other chemical mediators inducing oxidative stress [90]. Deregulation of the mitochondria electron transport chain, with increased mitochondrial ROS levels, was observed in inflammatory bowel disease patients and decreasing mitochondrial ROS ameliorated colitis [91]. Various inflammatory stimuli such as excessive ROS produced in the process of oxidative metabolism and some natural or artificial chemicals have been reported to initiate the inflammatory process resulting in synthesis and secretion of pro-inflammatory cytokines.

1.7 Statement of purpose

As is a toxic metalloid that affects almost every organ including the brain. It is widely distributed throughout the environment especially in the groundwater. After As exposure, human beings across all age develop neurological and cognitive dysfunction, including learning and memory deficits as well as mood disorders [92, 93]. EtOH is also a common toxic substance affects the brain. In addition, EtOH is associated with transient neurological dysfunction as well as deficits in cognitive and motor domains [94, 95]. Recently, researchers have found that combined treatment of As and EtOH increases toxicity in

cancer and liver disease [50, 96], but the effects of the combined treatment on brain are still unclear.

The BBB regulates the movement of ions, molecules and cells between the blood and the brain. It protects the neural tissue from toxins and pathogens [51]. Disruption of the BBB leads to neurological diseases and contributes to cognitive changes [97]. Since the properties of the BBB are largely manifested with the endothelial cells which form the walls of the blood vessels [51], rat brain endothelial cells (RBE4 cells) are used to investigate the effects of As and EtOH on the BBB.

Previously, our lab showed that As induced mouse brain endothelial cell permeability via oxidative stress that resulted from imbalance between the generation and detoxification of ROS [8]. Actually, oxidative stress induces BBB dysfunction though various pathways including mitochondrial alterations and direct mediation of BBB compromise such as tight junction modification [98]. Recent studies have shown that EtOH induces oxidative stress in the brains of animal models such as rat and zebrafish [99, 100]. Since brain endothelial cells contain a large number of mitochondria which is the major source of ROS generation, mitochondria may be a major target of As and EtOH neurotoxicity on the BBB.

Oxidative stress is central in the pathology both as an initiator of vascular inflammation and the consequence of inflammation responses and cytokines [89]. There is an increasing recognition that inflammation plays a critical role in the BBB alteration. Elevated levels of pro-inflammatory cytokines leads to down-regulation of both junctional proteins and junctional complexes, ultimately contributing to endothelial cell permeabilization [101]. Microglial pro-inflammatory activation is associated with increased

cytokine expression, oxidative stress, which damages critical support cells such as brain endothelial cells [77]. Therefore, in this study, microglial cells (BV2 cells) were used to investigate the combined effects of As and EtOH on the BBB.

Thus, we hypothesize that the combined treatment of As and EtOH induces toxic effects on both brain endothelial cells and microglial cells. To test this hypothesis, we will study the effects of As and EtOH on Rat Brain Endothelial Cells (RBE4 cells) and Microglial cells (BV2 cells) with the following specific aims:

Aim 1: To determine the effects of As and EtOH on brain endothelial cells. After As and EtOH treatment, FITC-dextran leakage assay will be performed to assess the permeability of RBE4 cell monolayer. In addition, EPR and MitoSOX live cell staining will be performed to detect oxidative stress and mitochondria oxidative stress in treated RBE4 cells. Also, mtBE assessment will be used to detect mitochondria dysfunction in treated RBE4 cells. We expect that the co-treatment increased RBE4 cell monolayer permeability, oxidative stress, mitochondria oxidative stress and induced mitochondria dysfunction more than either As or EtOH treatment alone.

Aim 2: To determine the effects of As and EtOH on microglial cells. After As and EtOH treatment, MTT assay will be performed to investigate the effects of As and EtOH on the BV2 cells viability. In addition, EPR and MitoSOX live cell staining will be performed to detect oxidative stress and mitochondria oxidative stress in treated BV2 cells. We expect that the co-treatment increased cell death, oxidative stress, mitochondria oxidative stress more than either As or EtOH treatment alone.

The proposed studies will help us to gain more information regarding the mechanistic effects of As and EtOH induced toxicity on endothelial cells and microglial cells, which gives some support to find a potential therapeutic approach to prevent or reduce the combined toxicity of As and EtOH.

Chapter 2 Materials and Methods

2.1 Reagents

Table2.1 List of Major Reagents

Name	Company	Catalog Number
Collagen I	CORNING	354236
Sodium As	SIGMA	71287
TEMPO	SIGMA	176141
FITC-DEXTRAN	SIGMA	FD40S
XF Assay Medium	Seahorse Bioscience	102365-100
Oligomycin	SIGMA	75351
FCCP	SIGMA	C2929
Rotenone	SIGMA	R8875
Antimycin A	SIGMA	A8674
CMH	Enzo Life Science	ALX-430-117-M010
MitoSOX	FISHER	M36008

2.2 Methods

2.2.1 Cell Culture and Treatment

Rat brain endothelial cells (RBE4) were provided by Dr.Michael Aschner from the Albert Einstein College of Medicine. RBE4 cells were grown in T25 flask coated with 50µg/ml rat tail collagen I (354236) within passage 20. The media of RBE4 cells consists of 44% alpha-MEM (12571-063; Invitrogen), 44% Ham's F-10 Nutrient (11550-043; Invitrogen), 10% fetal bovine serum, Geneticin and penicillin. RBE4 cells were kept in humidified incubator maintained in 37°C and 5% CO_2. RBE4 cells media was replaced every 2 days. 2 days after seeding, RBE4 cells were treated with 5µM As, 80mM EtOH, and As combined with EtOH.

Microglial cells (BV2) were obtained from Dr. Russell Swerdlow's lab at The University of Kansas Medical Center. BV2 cells were grown in T25 flask coated with Poly-D-lysine hydrobromide (P0899; Sigma). The media of BV2 cells consists of 90% DMEM (SH30022.01; HyClone), 10% fetal bovine serum and penicillin. BV2 cells were kept in humidified incubator maintained in 37°C and 5% CO_2. BV2 cells media was replaced every 2 days. 2 days after seeding, BV2 cells were treated with As, EtOH, and As combined with EtOH. As concentrations are 100nM, 200nM, 300nM. EtOH concentrations are 100mM, 200mM, 300mM.

2.2.2 Paracellular Permeability Assay

RBE4 cells were seeded in collagen I coated polyethylene terephthalate (PT) cell inserts (2×10^5 cells/cm^2). The luminal compartments were filled with 300µl media and the abluminal compartments were filled with 600µl media. RBE4 cells were treated with As, EtOH, As with EtOH for 1, 3, 6 days. To determine the permeability of RBE4 cell monolayer, FITC-labeled dextran (MW 40,000, 1mg/ml) were added to the upper chamber and incubated for 3 hours. After incubation, 50µl of medium from the outside of the insert was taken and measure at the excitation wavelength of 492nm and the emission wavelength of 520nm by a fluorescent multi-mode microplate reader.

2.2.3 mtBE Assessment

Seahorse XF96 Analyzer was used to detect mtBE in RBE4 cells. RBE4 cells were seeded in XF96-well plates (50.000 cells per well) and incubated overnight. Then RBE4 cells were treated with 5µM As, 80mM EtOH, and 5µM As combined with 80mM EtOH for 1 day. After treatments, XF assay medium (containing 5µM glucose, 1mM sodium pyruvate) was used to wash RBE4 cells twice. Before the experiment, RBE4 cells with XF assay medium were incubated without CO_2 at least 1 hour. The sensor cartridge was loaded with 20µg/ml oligomycin, 2µM FCCP, 1µM rotenone with 1µM antimycin-A and XF assay medium. Once the sensor cartridge was equilibrated, the calibration plate was replaced with the cell plate. The Seahorse Analyzer using a 3 minute mix cycle to oxygenate the medium followed by 4 minute measurement of the OCR.

2.2.4 Electron Paramagnetic Resonance Spectroscopy (EPR)

EPR instrument (Bruker BioSpin) was adopted for measuring superoxide in RBE4 cells and BV2 cells. After one day treatment of As, EtOH, As with EtOH, RBE4 cells and BV2 cells were collected to prepare EPR samples. RBE4 cells or BV2 cells from 3 wells in 6-well plates were trypsinized and collect for EPR measurement. Collected cells were treated with 1mM CMH for 3 hours. Then ice cold PBS was used to wash cells and store the sample at -80°C for analysis.

Collect the sample to 50µL glass capillary tubes and use the Bruker e-scan EPR spectrometer to analyze the sample. Spectrometer settings were as follows: sweep width: 100G; microwave frequency: 9.75 GHz; modulation amplitude: 1G; conversion time: 5.12ms; time constant: 5.12ms; receiver gain: 2×102; number of scans: 30. Quantification of the EPR signal intensity was determined by comparing the intensity of the recorded middle derivative EPR peak of each sample.

2.2.5 MitoSOX Live Cell Staining

RBE4 cells were seeded at low density on Lab-Tek eight-well chamber slides and treated with As, EtOH, As with EtOH for one day. Mitochondrial ROS generation in treated RBE4 cells was measured by MitoSOX following the manufacture instructions. After treatment, RBE4 cells were washed with PBS and incubated with medium containing 2.5µM MitoSOX for 20min. Next, cells were washed with PBS three times and incubated with 0.5µg/ml Hoechst 33342 in HBSS for 5min. Image were taken by Leica DMI4000 B microscope. At least two images from each well were collected and there were two wells in each treatment. Images were collected from 3 independent experiments and the

fluorescence intensity was calculated using Image J software. The corrected cell fluorescence intensity is calculated by integrated density minus area of cells multiply by mean fluorescence of background. Around 12 cells were counted for each images. In each experiment, different treatments of fluorescence intensity divided by the mean of fluorescence intensity in control groups to get the percentage of control.

2.2.6 MTT Assay

BV2 cells (1×10^4 cells/well) were seeded on Falcon 96-well culture plate coated with Poly-D-lysine hydrobromide and treated with 100, 200, 300nM As and 100, 200, 300mM EtOH; 100nM As combined with 50mM EtOH for one day. MTT solution was prepared at 5mg/ml in PBS and was filtered through a 0.2µm filter. After removing old media in each well, 20µL of MTT were added into each well. Cells were incubated for 4 hours at 37°C with 5% CO_2, 95% air and complete humidity. After 2-4 hours, the MTT solution was removed and replaced with 200µL of DMSO. The plate was further incubated for 10-30min, and the optical density (OD) of the wells was determined using a plate reader at a test wavelength of 570 nm and a reference wavelength of 630nm.

2.2.7 Statistical Analysis

All data were collected from three independent experiments. One-way ANOVA was used to determine overall significance difference. Dunnett's multiple comparisons test was applied to determine the significant difference in different groups compared to control. T-test was used to determine the significant between two groups. All data are presented as mean ± SEM. Differences were considered statistically significant at $P<0.05$.

Chapter 3 Results

3.1 As and EtOH Increased Permeability of RBE4 Cell Monolayer.

Previously, our lab showed that 5µM As increased permeability of mouse brain vascular endothelial cell bEnd3 monolayer without inducing cell death [8]. Study of Shiu showed that 68mM alcohol increased the permeability of human brain microvascular endothelial cell monolayer without affecting cell viability [102]. However, the combined effects of As and EtOH on the endothelial cell permeability is still unknown.

Rat brain endothelial (RBE4) cell monolayer permeability was assessed by FITC-Dextran leakage assay which is widely used to determine endothelial cell monolayer permeability. After RBE4 cells growing to confluent monolayer on culture inserts, the cells were treated with 5µM As, 80mM EtOH, or both compounds for 6 days.

As **Figure 3.1** shown, the combined treatment of As and EtOH increased RBE4 cell monolayer permeability more than either As or EtOH treatment alone. After one day treatment, As or EtOH did not alter the permeability but the combined treatment significantly induced hyper-permeability of the RBE4 cell monolayer (*$P<0.05$). Although EtOH at 80mM did not significantly alter the permeability after one day treatment, EtOH significantly increased the permeability after 6 day treatment (*$P<0.05$). The combined treatment significantly increased the permeability compared to As which had stronger effect than EtOH after 3 and 6 days treatment (#$P<0.05$).

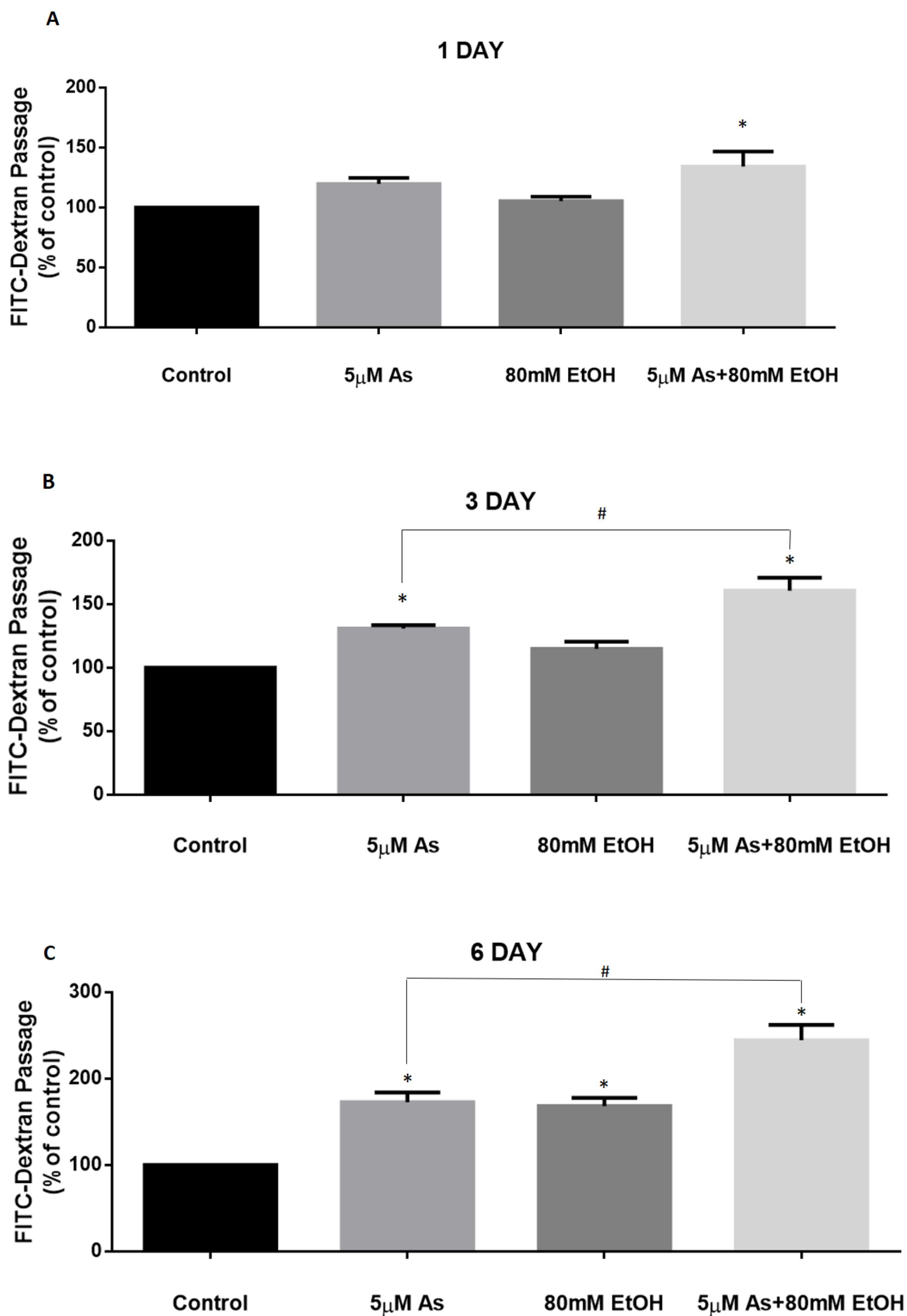

A
1 DAY
FITC-Dextran Passage (% of control)
200
150
100
50
0
*
Control
5μM As
80mM EtOH
5μM As+80mM EtOH
B
3 DAY
#
FITC-Dextran Passage (% of control)
200
150
100
50
0
*
*
Control
5μM As
80mM EtOH
5μM As+80mM EtOH
C
6 DAY
#
FITC-Dextran Passage (% of control)
300
200
100
0
*
*
*
Control
5μM As
80mM EtOH
5μM As+80mM EtOH

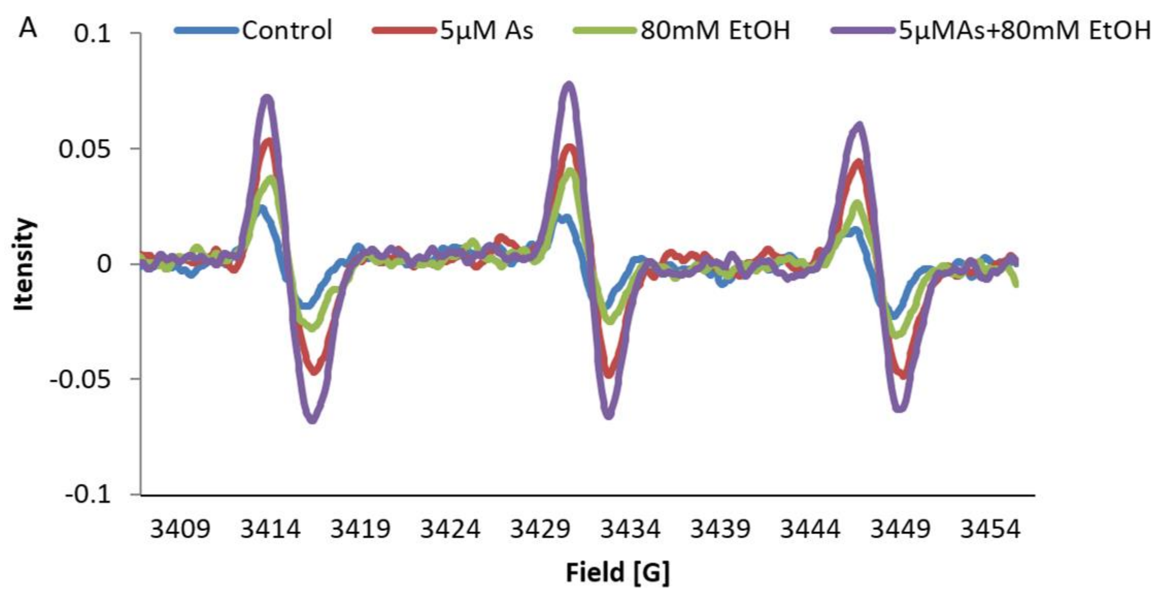
Figure 3.1 As and EtOH increased RBE4 cell monolayer permeability. Fluorescence of FITC-Dextran leaked from inserts to plate wells was assessed to determine the permeability after 1, 3, and 6 day treatment. Data was normalized to the control. **A)** 1 day treatment. One-way ANOVA result showed there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that, compare to the control, the combined treatment significantly increased FITC-Dextran passage (*$p<0.05$). **B)** 3 day treatment. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that, compared to the control, As and the combined treatment significantly increased FITC-Dextran passage but not EtOH (*$p<0.05$). And T-test showed that the combined treatment significantly increased permeability compared to As treatment (#$p<0.05$). **C)** 6 day treatment. One-way ANOVA result showed there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that, compared to the control, As, EtOH, the combined treatment all significantly increased FITC-Dextran passage (*$p<0.05$). And T-test showed that the combined treatment significantly increased permeability compared to As treatment (#$p<0.05$). Results were presented as mean ±SEM. n=3.

3.2 As and EtOH Increased ROS Production in RBE4 Cells.

Study of Lei demonstrated that antioxidant had an inhibitory effect on As-EtOH-induced Cyclooxygenase-2 expression in colorectal cancer calls, indicating that the responsive signaling pathways from co-exposure to As and EtOH relate to ROS generation [96]. However, more studies are still needed on the combined effects of As and EtOH on ROS generation in brain endothelial cells.

It is known that increased oxidative stress is associated with the increased permeability of the BBB [61]. To determine whether As and EtOH induced oxidative stress in RBE4 cells, ROS levels in RBE4 cells were measured after one day treatment of 5µM As, 80mM EtOH, and As combined with EtOH. Electron paramagnetic resonance spectroscopy was used to determine the production of oxygen-derived free radical within treated RBE4 cells.

As **Figure 3.2 B** shown, the combined treatment of 5µM As and 80mM EtOH significantly increased EPR signal in RBE4 cells but not As or EtOH treatment alone (*$P<0.05$). Therefore, co-exposure of As and EtOH significantly increased ROS generation more than either As or EtOH treatment alone.


A
Control
5μM As
80mM EtOH
5μMAs+80mM EtOH
0.1
0.05
0
-0.05
-0.1
Itensity
3409
3414
3419
3424
3429
3434
3439
3444
3449
3454
Field [G]

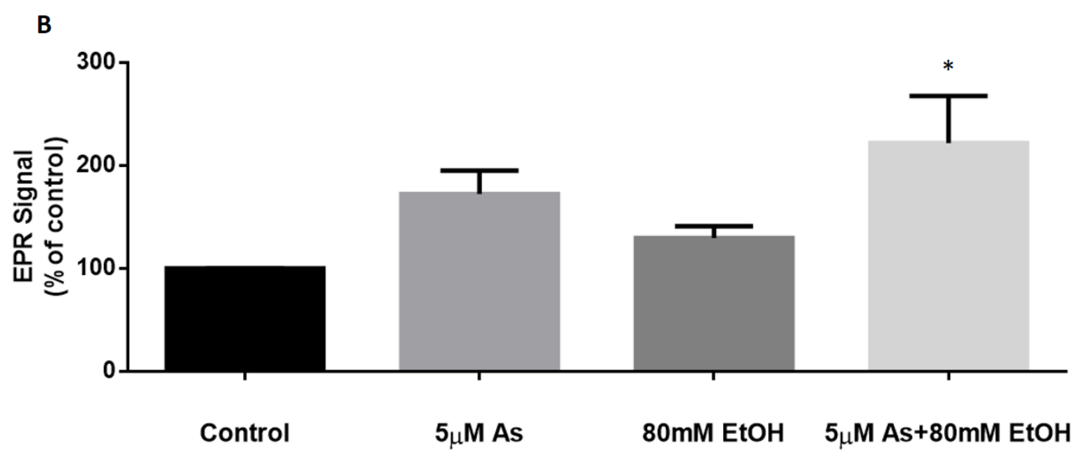

B
300
200
100
0
EPR Signal
(% of control)
*
Control
5μM As
80mM EtOH
5μM As+80mM EtOH

Figure 3.2 As and EtOH increased ROS Production in RBE4 cells. A) EPR spectral profiles of ROS production from RBE4 cells after one day treatment with 5µM As, 80mM EtOH, and As combined with EtOH. To detect levels of ROS, cell pellets were incubated with the spin probe CMH. **B)** Quantification of the EPR signal intensity was determined by comparing the intensity of the recorded middle-derivative EPR peak of each sample. Data were normalized to the control. As One-way ANOVA result showed that there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that the combined treatment significantly increased EPR intensity compared to control (*$p < 0.05$). Results were presented as mean ±SEM. n=3.

3.3 As and EtOH Induced Mitochondrial ROS Production in RBE4 Cells.

Study of Kathryn showed that As and EtOH upregulated the genes of mitochondrial-associated endoplasmic reticulum membranes and a regulator of oxidative protein folding and endoplasmic reticulum redox homeostasis in the liver of zebrafish [50]. However, it is still unknown on the combined effect of arsenic and ethanol on mitochondria in brain endothelial cells.

One of the sources of ROS generation is mitochondria. To investigate whether As and EtOH induced mitochondrial oxidative stress in RBE4 cells, live cell imaging of MitoSOX oxidation was used to detect mitochondrial oxidative stress in RBE4 cells after one day treatment of 5µM As, 80mM EtOH, and As combined with EtOH.

As **Figure 3.3A** shown, the combined treatment of As and EtOH increased the red fluorescence of MitoSOX in RBE4 cells compared to control more than As or EtOH treatment alone. This effect was confirmed in **Figure 3.3 B**, the combined treatment significantly induced mitochondrial oxidative stress but not As or EtOH treatment alone ($*P<0.05$).

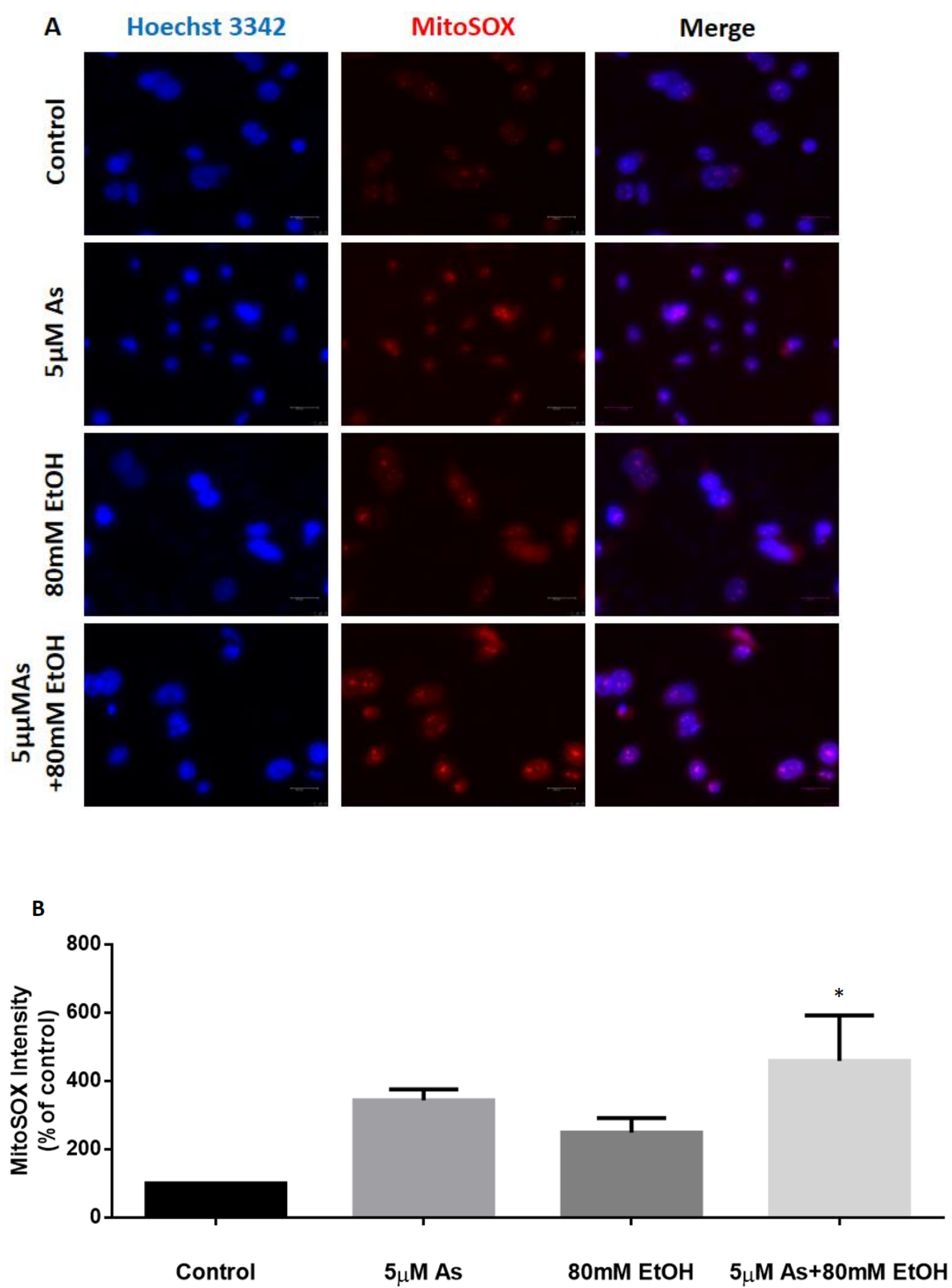
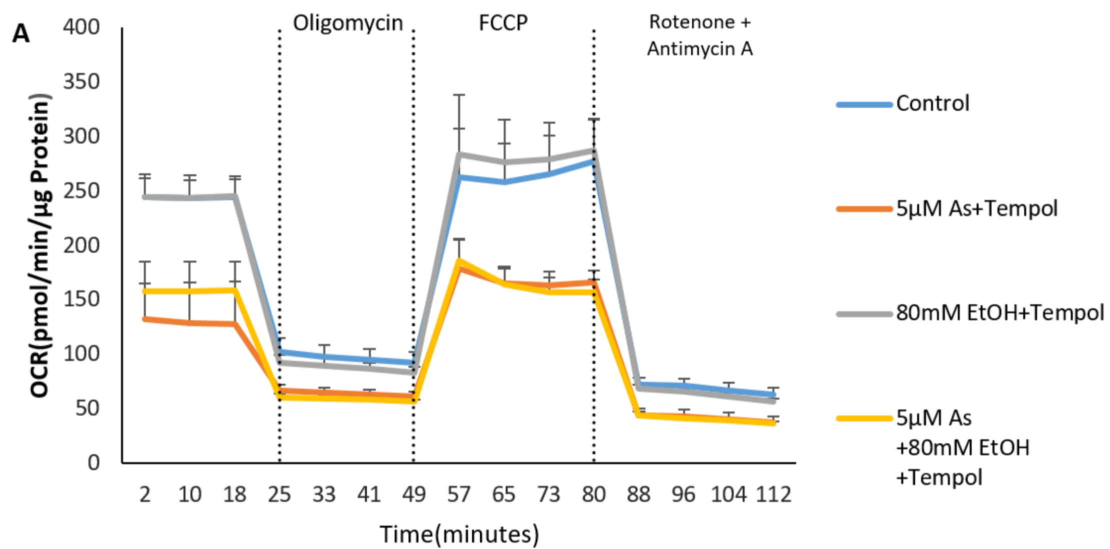

A
Hoechst 3342
MitoSOX
Merge
Control
5µM As
80mM EtOH
5µµMAs
+80mM EtOH
B
MitoSOX Intensity
(% of control)
800
600
400
200
0
*
Control
5µM As
80mM EtOH
5µM As+80mM EtOH

Figure 3.3 As and EtOH increased mitochondrial ROS production in RBE4 cells. RBE4 cells were incubated with MitoSOX and Hoechst 33342 to label mitochondrial superoxide radical (red) and nuclei (blue) respectively. A) As and EtOH increased mitochondrial ROS production. Scale bar, 20µm, objective, 40X B) Quantification of MitoSOX staining intensity. The mean fluorescence intensity per image was calculated and averaged over three images by Image J software. Around 12 cells were counted for each images. In each experiment, different treatments of fluorescence intensity divided by the mean of fluorescence intensity in control groups to get the percentage of control. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that the combined treatment significantly increased MitoSOX intensity compared to the control (*$p < 0.05$).

3.4 As and EtOH affect mtBE in RBE4 cells.

In cells and organs with high-energy demand, there is a high density of mitochondria that produce ROS as a by-product of mitochondria respiration, because the electron transport chain is not 100% efficient, which makes electrons escape from electron transport chain complexes and reduce oxygen to produce ROS [103]. To investigate the effects of the combined treatment of As and EtOH on mtBE in RBE4 cells, Seahorse extracellular flux analyzer was used for real-time measurements of oxygen consumption and provide an overall assessment of cellular mtBE.

The ATP-linked respiration was estimated by the decrease in OCR followed by oligomycin, an ATP synthase inhibitor. As shown in **Figure 3.4A**, Basal and ATP-linked OCR of the combined treatment with As and EtOH decreased compared to the control. Residual OCR remaining after oligomycin is from uncoupled respiration. As shown in **Figure 3.4A**, proton leak OCR after the combined treatment with As and EtOH decreased compared to control. Maximal Respiratory Capacity (MRC) induced by FCCP, reflects the rate of maximal electron transport and substrate oxidation achievable in the absence of limits imposed by the inner mitochondrial membrane proton gradient. In **Figure 3.4 B**, the decrease in MRC in RBE4 cells after the combined treatment of As and EtOH suggested that mitochondrial electron transport chain were impaired by As and EtOH.


A
OCR(pmol/min/µg Protein)
400
350
300
250
200
150
100
50
0
Oligomycin
FCCP
Rotenone +
Antimycin A
2 10 18 25 33 41 49 57 65 73 80 88 96 104 112
Time(minutes)
Control
5µM As+Tempol
80mM EtOH+Tempol
5µM As
+80mM EtOH
+Tempol

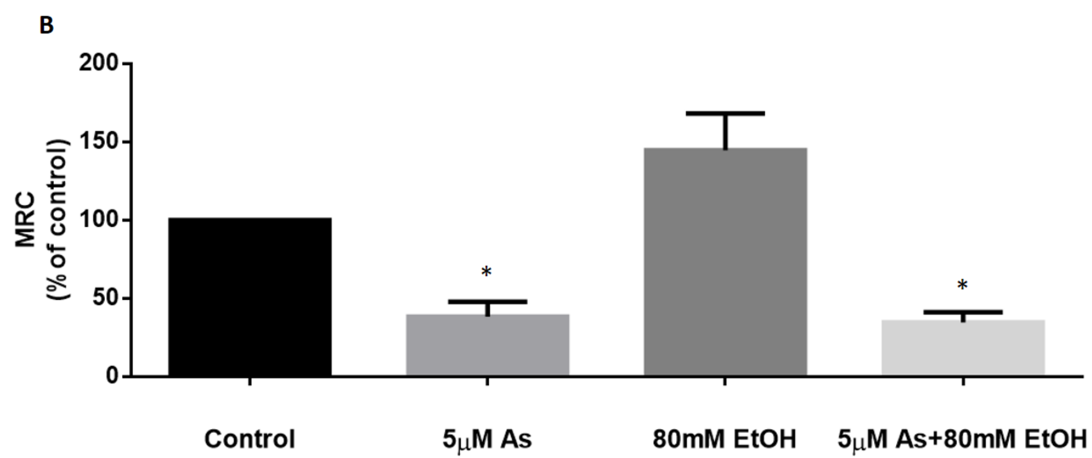

B
MRC
(% of control)
200
150
100
50
0
*
*
Control
5µM As
80mM EtOH
5µM As+80mM EtOH

Figure 3.4 As and EtOH affect mtBE in RBE4 cells. After one day treatment of As, EtOH, As with EtOH, oxygen consumption was measured. OCR was manipulated with injections of oligomysin, FCCP, rotenone with antimycin-A. **A)** Representative bioenergetics profile of RBE4 cells treated with 5µM As, 80mM EtOH and the combined treatment for one day. **B)** Quantification of MRC. MRC was achieved by adding FCCP which is protonophore. Data were normalized by the control. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that As and the combined treatment significantly decreased MRC (* $p < 0.05$). Results were presented as mean ±SEM. n=3.

3.5 Antioxidant Decreased As-EtOH-Induced Mitochondrial ROS Production in RBE4 cells.

Previously, our data showed that the combined treatment of 5µM As and 80mM EtOH significantly increased both cellular ROS and mitochondrial oxidative stress. To determine whether antioxidant can decrease As-EtOH-induced mitochondrial oxidative stress in RBE4 cells, superoxide anion radical scavenger Tempol (500µM) was added with 5µM As, 80mM EtOH, and the combined two in RBE4 culture media. After one day treatment, live cell imaging of MitoSOX oxidation was used to detect mitochondrial oxidative stress in RBE4 cells.

As **Figure 3.5A** shown, all three treatment slightly increased the red fluorescence of MitoSOX in RBE4 cells compared to control. In addition, as shown in **Figure 3.5 B**, there is no significant effect of all three treatments on mitochondrial oxidative stress compared to the control.

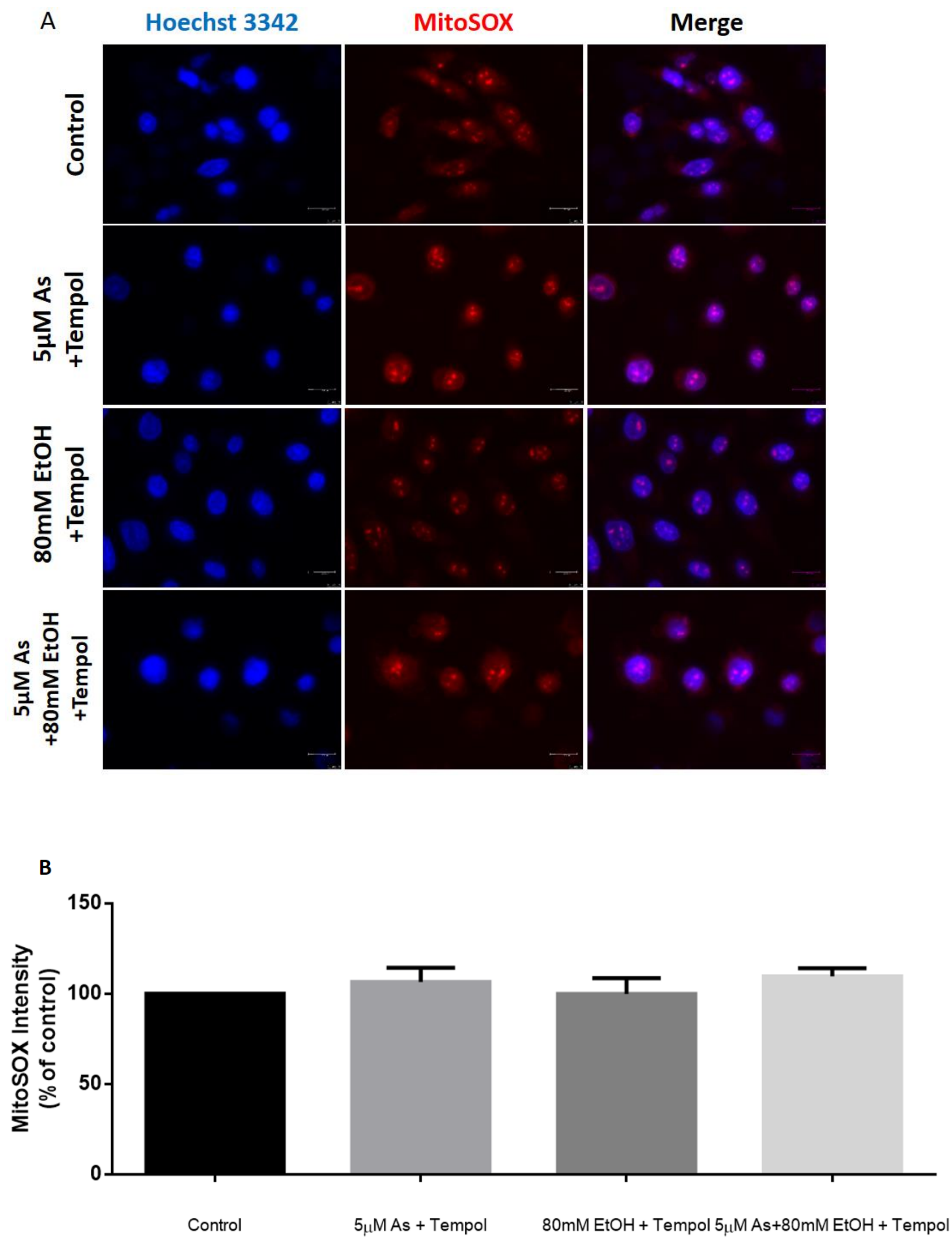
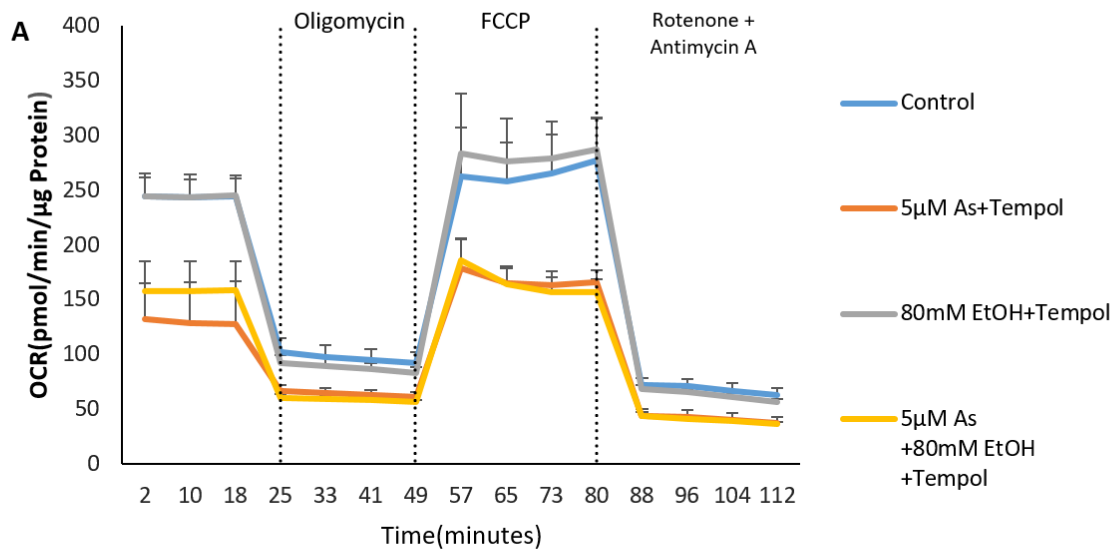

A
Hoechst 3342
MitoSOX
Merge
Control
5μM As +Tempol
80mM EtOH +Tempol
5μM As +80mM EtOH +Tempol
B
MitoSOX Intensity (% of control)
150
100
50
0
Control
5μM As + Tempol
80mM EtOH + Tempol
5μM As+80mM EtOH + Tempol

Figure 3.5 Antioxidant decreased As-EtOH-induced mitochondrial ROS production in RBE4 cells. RBE4 cells were incubated with MitoSOX and Hoechst 33342 to label mitochondrial superoxide radical (red) and nuclei (blue) respectively. **A)** Antioxidant Tempol decreased As-EtOH-induced mitochondrial ROS production. Scale bar, 20μm, objective, 40X **B)** Quantification of MitoSOX staining intensity. The mean fluorescence intensity per image was calculated and averaged over three images by Image J software. Around 12 cells were counted for each images. In each experiment, different treatments of fluorescence intensity divided by the mean of fluorescence intensity in control groups to get the percentage of control. As One-way ANOVA result shown, there were no statistically significant difference between group means. Results were presented as mean ± SEM. n=3.

3.6 Antioxidant decreased As-EtOH-Induced mtBE Loss in RBE4 cells.

Tempol which promotes the metabolism of many ROS has been shown to preserve mitochondria against oxidative damage[104]. After confirming that antioxidant Tempol can decrease As-EtOH-induced mitochondrial oxidative stress, we next to investigate whether antioxidant can also reduce As-EtOH-induced mitochondrial dysfunction in RBE4 cells.

Previously, our data showed that the combined treatment of 5μM As and 80mM EtOH decreased maximum respiratory capacity in RBE4 cells, which is around 30%. As shown in **Figure 3.6 B**, antioxidant Tempol significantly increased As-EtOH-induced decrease in maximum respiratory capacity, which is around 60% (#P<0.05). Although Tempol did not significantly increased As-induced MRC decrease, there is a trend for Tempol to increase MRC. Interestingly, Tempol had a trend to decreased EtOH-increased MRC from around 140% reduced to around 120%.


A
Oligomycin
FCCP
Rotenone +
Antimycin A
OCR(pmol/min/µg Protein)
400
350
300
250
200
150
100
50
0
2 10 18 25 33 41 49 57 65 73 80 88 96 104 112
Time(minutes)
Control
5µM As+Tempol
80mM EtOH+Tempol
5µM As
+80mM EtOH
+Tempol

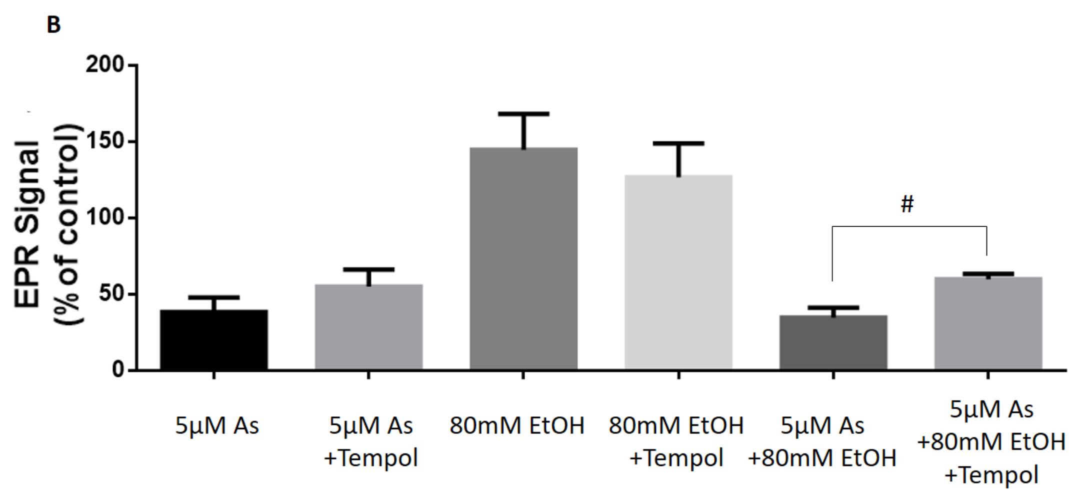
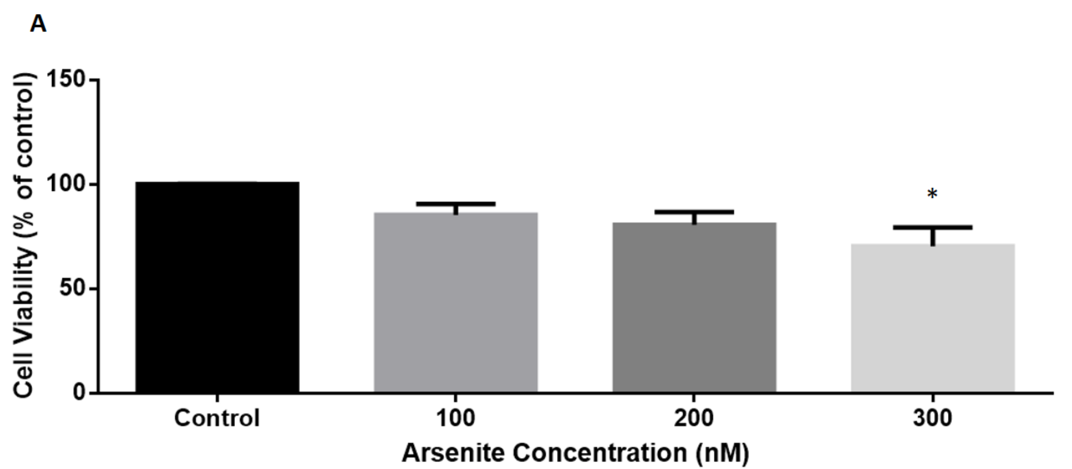

B
EPR Signal
(% of control)
200
150
100
50
0
#
5µM As
5µM As
+Tempol
80mM EtOH
80mM EtOH
+Tempol
5µM As
+80mM EtOH
5µM As
+80mM EtOH
+Tempol

Figure 3.6 Antioxidant reduced As-EtOH-induced mtBE loss in RBE4 cells. After one day treatment of 5µM As with 500µM Tempol, 80mM EtOH with 500µM Tempol, and As combined with EtOH and Tempol, oxygen consumption was measured. OCR was manipulated with injections of oligomysin, FCCP, rotenone with antimycin-A. **A)** Representative bioenergetics profile of RBE4 cells treated 500µM Tempol, 80mM EtOH with 500µM Tempol, and As combined with EtOH, Tempol for one day. **B)** Quantification of MRC. MRC was achieved by adding FCCP which is protonophore. Data were normalized by the control. Compared with the **Figure 3.4B**. T-test showed that Tempol significantly increased the combined treatment induced decreased in MRC (#$p< 0.05$). Results were presented as mean ±SEM. n=3.

3.7 As and EtOH induced cell death in BV2 cells.

Microglial cells are phagocytic cells of the central nervous system and have been proposed to be a primary component of the innate immune response and maintain efficient central nervous system [105]. To investigate the effects of As and EtOH on the cell viability of microglia BV2 cells, MTT assay was performed after one day treatment of 100, 200, 300nM As and 100, 200, 300mM EtOH.

As shown in **Figure 3.7 A**, after one day treatment, cell viability of microglia BV2 cells decreased as As concentration increased. As significantly induced BV2 cells death at 300nm, demonstrating a concentration-dependent effect (*$P<0.05$). **Figure 3.7 B** showed that EtOH induced microglia BV2 cells death with an increase in EtOH concentration. EtOH at 300mM significantly decrease BV2 cells viability (*$P<0.05$). The higher concentration of EtOH the lower BV2 cells survival. Therefore, both As and EtOH can induce microglia BV2 cells death.


A
Cell Viability (% of control)
150
100
50
0
*
Control
100
200
300
Arsenite Concentration (nM)

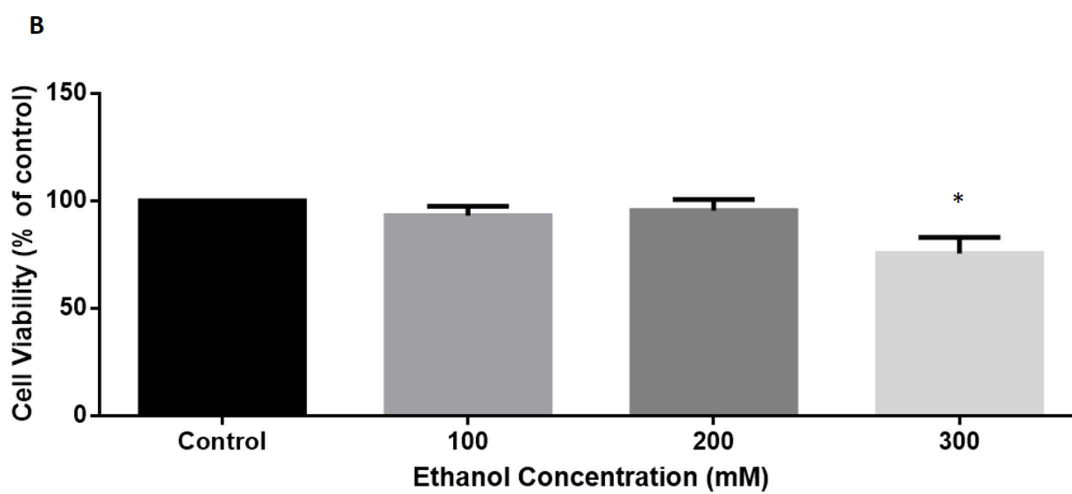

B
Cell Viability (% of control)
150
100
50
0
*
Control
100
200
300
Ethanol Concentration (mM)

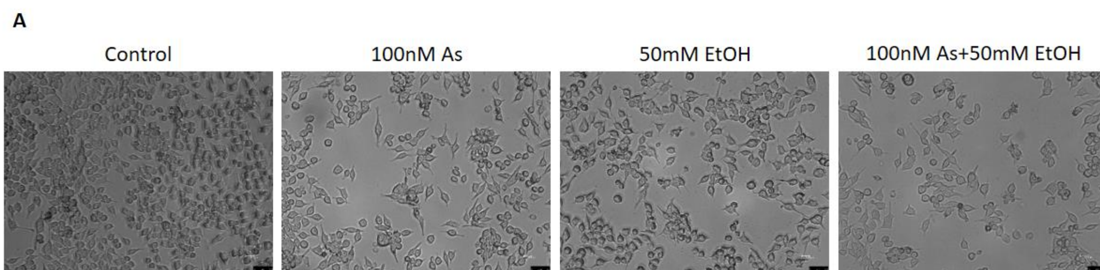
Figure 3.7 As and EtOH induced BV2 cells death. A) After one day treatment of 100, 200, 300nM As, BV2 cells viability decreased as As concentration increased. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that 300nM As significantly decreased BV2 cells viability. (*P<0.05) **B)** After one day treatment of 100, 200, 300mM EtOH, BV2 cells viability decreased as EtOH concentration increased. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that 300mM EtOH significantly decreased BV2 cells viability (*P<0.05). Results were presented as mean ±SEM. n=3.

3.8 The combined treatment of As and EtOH decreased cell viability in BV2 cells.

As demonstrated above, our data showed that As and EtOH caused death of microglia BV2 cells. We further investigate the effects of combined treatment of As and EtOH on BV2 cells. BV2 cells were treated with 100nM As, 50mM EtOH, and As combined with EtOH for one day. After treatment, MTT assay will be performed to detect the combined effects of As and EtOH on BV2 cells.

As shown in **Figure 3.8A**, there were fewer BV2 cells in As, EtOH, and especially the combined treatment than control. The combined treatment decreased BV2 cells number more than As or EtOH treatment alone. In **Figure 3.8 B**, MMT assay result confirmed the combined effects of As and EtOH on BV2 cells viability. The combined treatment of As and EtOH significantly decreased BV2 cells viability but not As or EtOH treatment alone (*$P<0.05$).

Therefore, the combined treatment of As and EtOH can induce microglial cells death more than either As or EtOH treatment alone.


A
Control
100nM As
50mM EtOH
100nM As+50mM EtOH

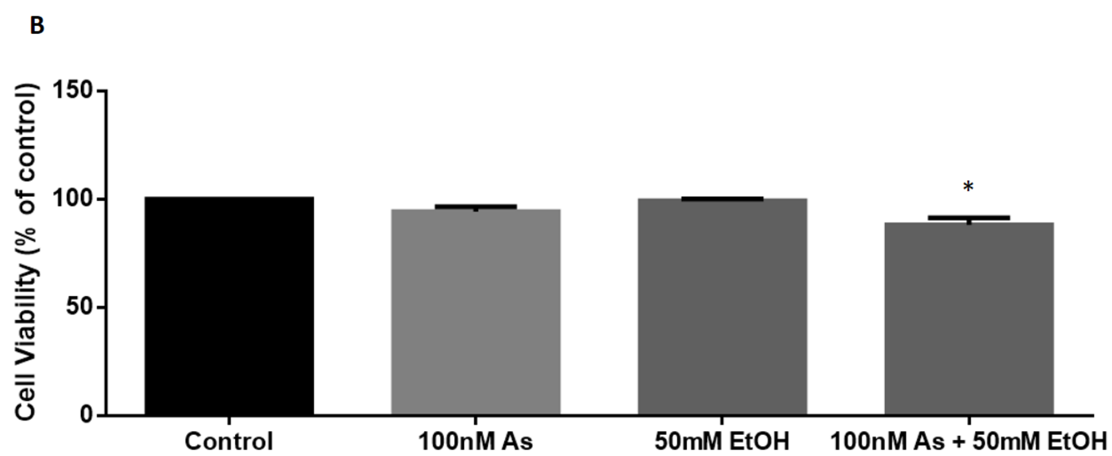
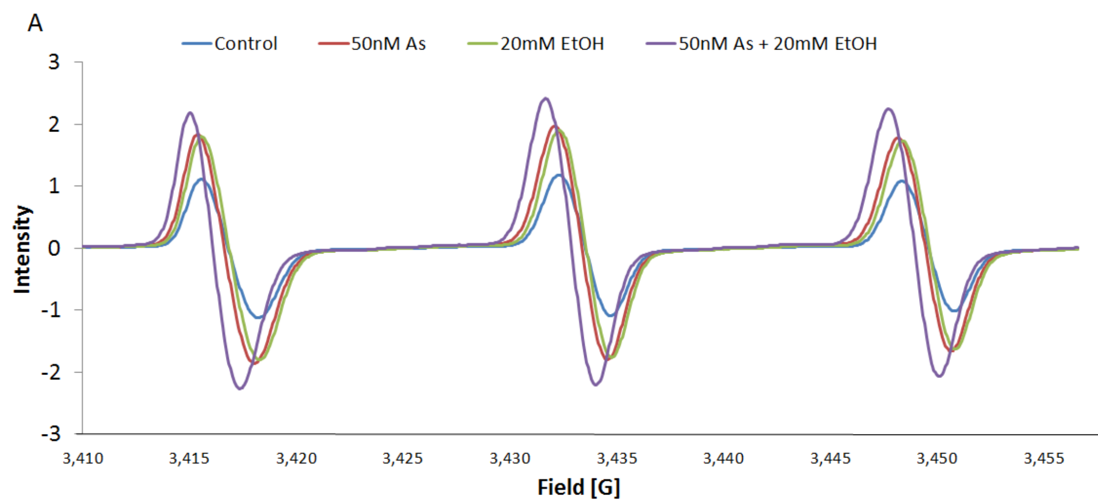

B
Cell Viability (% of control)
150
100
50
0
*
Control
100nM As
50mM EtOH
100nM As + 50mM EtOH

Figure 3.8 The combined treatment of As and EtOH induced BV2 cells death. A) After one day of 100nM As, 50mM EtOH, and combined two treatment, images of treated BV2 cells were collected. Scale bar, 20µm, objective, 10X **B)** After one day of 100nM As, 50mM EtOH, and combined two treatment, BV2 cells viability decreased in the combined treatment in MTT assay. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that the combined two treatment significantly decreased BV2 cells viability compared to the control (*$P<0.05$). Results were presented as mean ±SEM. n=3.

3.9 As and EtOH Increased ROS Production in BV2 Cells.

Microglia detect and respond to a diverse array of stimuli in the brain, including environmental toxins, where the pro-inflammatory response activation state is characterized by the upregulation of pro-inflammatory mediators and the production of ROS [106]. To investigate the effects of As and EtOH on microglia, electron paramagnetic resonance spectroscopy was used to determine the production of ROS within treated microglia BV2 cells.

As **Figure 3.9 A** shown, both 50nM As and the combined treatment with 50nM As and 20mM EtOH increased EPR signal compared to control. And as **Figure 3.9 B** shown, As and the combined treatment significantly induced EPR signal compared to control (*$P<0.05$). Therefore, the combined treatment of As and EtOH can induce intracellular ROS generation and induce microglia pro-inflammatory response activation.


A
Control
50nM As
20mM EtOH
50nM As + 20mM EtOH
Intensity
3
2
1
0
-1
-2
-3
3,410
3,415
3,420
3,425
3,430
3,435
3,440
3,445
3,450
3,455
Field [G]

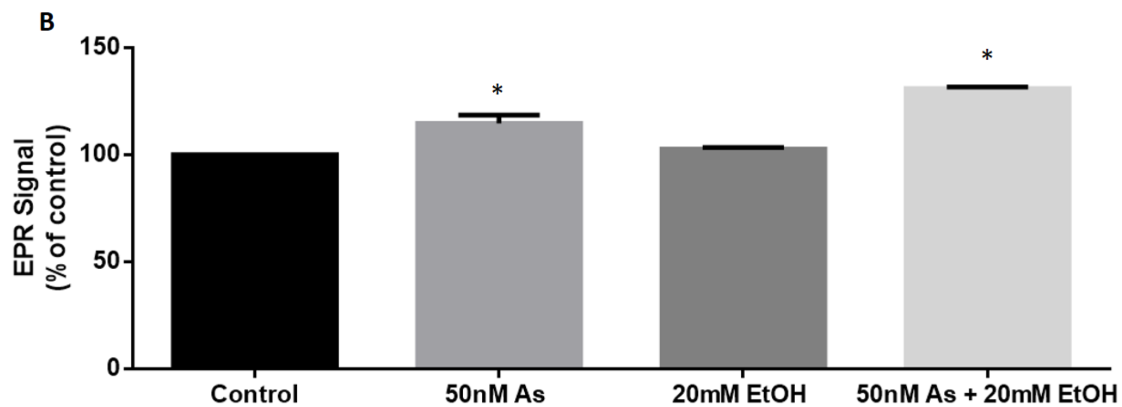

B
EPR Signal
(% of control)
150
100
50
0
*
*
Control
50nM As
20mM EtOH
50nM As + 20mM EtOH

Figure 3.9 As and EtOH Increased ROS Production in BV2 Cells. To detect levels of ROS, cell pellets were incubated with the spin probe CMH. **A)** EPR spectral profiles of ROS production from BV2 cells after one day treatment with 50mM As, 20mM EtOH, and the combined two. **B)** Quantification of the EPR signal intensity was determined by comparing the intensity of the recorded middle-derivative EPR peak of each sample. Data were normalized to the control. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that the combined treatment significantly increased EPR intensity compared to the control (*$p < 0.05$). Results were presented as mean ±SEM. n=2.

3.10 As and EtOH Induced Mitochondrial ROS Production in BV2 Cells.

Mitochondrial ROS have been proposed as important regulators of the inflammatory response in the innate immune system which is associated with microglia activation [11]. To investigate the effects of As and EtOH on mitochondrial ROS in microglial cells, live cell imaging of MitoSOX oxidation was used to detect mitochondrial oxidative stress in microglia BV2 cells after one day treatment of 50nM As, 20mM EtOH, and As combined with EtOH.

As **Figure 3.10A** shown, the combined treatment increased the red fluorescence of MitoSOX in BV2 cells compared to As or EtOH treatment alone. In addition, as shown in **Figure 3.10 B**, the combined two treatments significantly increased MitoSOX intensity in BV2 cells but not As or EtOH treatment alone (*$P<0.05$). Therefore, the combined treatment of As and EtOH can increase mitochondrial oxidative stress more than either As or EtOH treatment alone.

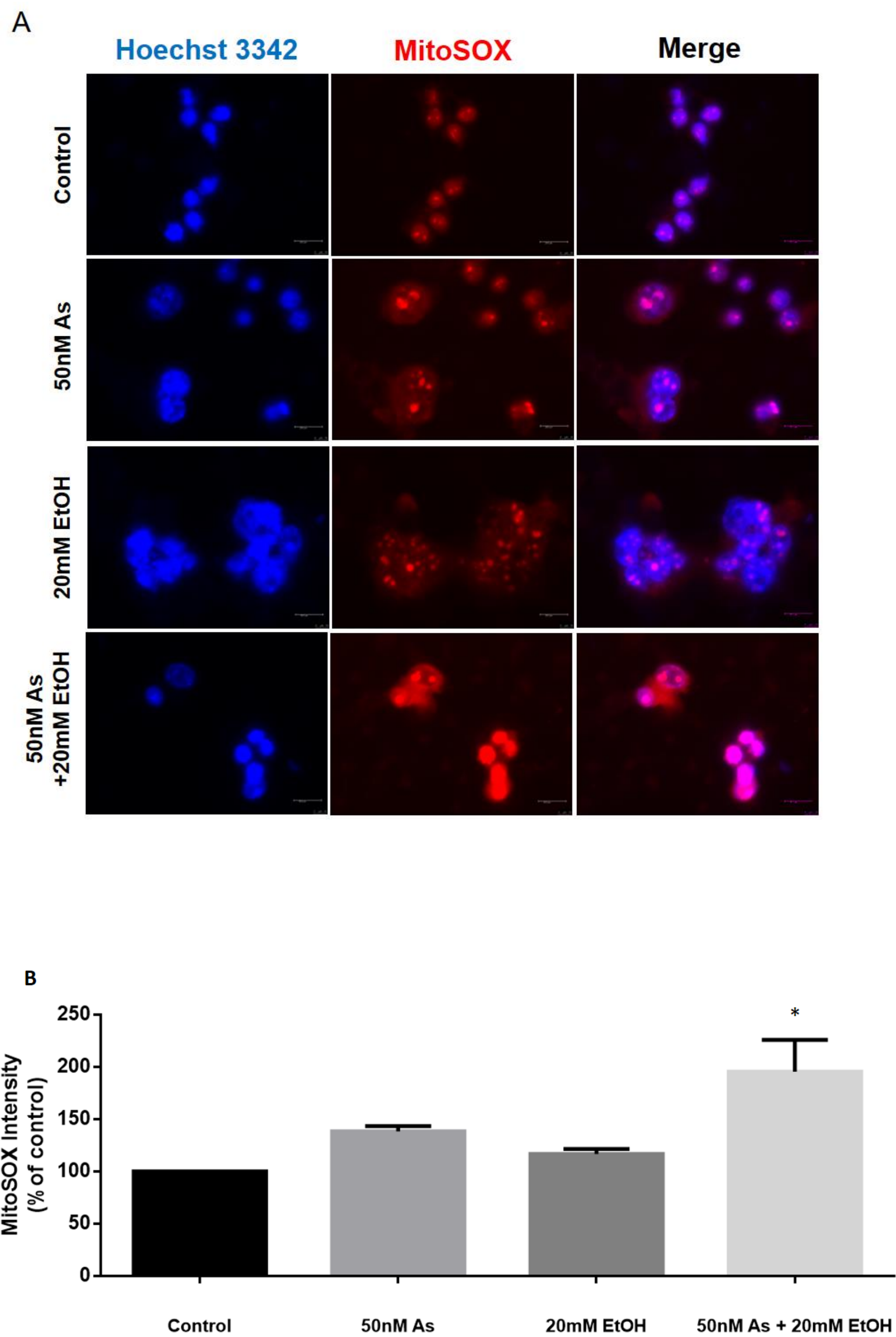

A
Hoechst 3342
MitoSOX
Merge
Control
50nM As
20mM EtOH
50nM As
+20mM EtOH
B
MitoSOX Intensity
(% of control)
250
200
150
100
50
0
*
Control
50nM As
20mM EtOH
50nM As + 20mM EtOH

Figure 3.10 As and EtOH increased mitochondrial ROS production in BV2 cells. After one day of 50nM As, 20mM EtOH, and As combined with EtOH treatments, BV2 cells were incubated with MitoSOX and Hoechst 33342 to label mitochondrial superoxide radical (red) and nuclei (blue) respectively. **A)** As and EtOH increased mitochondrial ROS production. Scale bar, 20μm, objective, 40X **B)** Quantification of MitoSOX staining intensity. The mean fluorescence intensity per image was calculated and averaged over three images by Image J software. Data were normalized by the control. As One-way ANOVA result shown, there were statistically significant difference between group means. Dunnett's multiple comparisons test showed that the combined treatment significantly increased MitoSOX intensity compared to the control (*$p < 0.05$). Results were presented as mean ±SEM. n=3.

3.11 Antioxidant Decreased As-EtOH-Induced Mitochondrial ROS Production in BV2 cells.

Previously, our data showed that the combined treatment of 50nM As and 20mM EtOH significantly increased both cellular ROS and mitochondrial oxidative stress. To determine whether antioxidant can rescue As-EtOH-induced mitochondrial oxidative stress in microglia BV2 cells, superoxide anion radical scavenger Tempol (500μM) was added with 50nM As, 20mM EtOH, and As combined with EtOH in BV2 cells culture media. After one day treatment, live cell imaging of MitoSOX oxidation was used to detect mitochondrial oxidative stress in BV2 cells.

As **Figure 3.11A** shown, all three treatments slightly increased the red fluorescence of MitoSOX in BV2 cells compared to control. In addition as shown in **Figure 3.11 B**, there is no significant effect of all three treatments on mitochondrial oxidative stress compared to the control. Therefore, antioxidant can rescue the effect of As, EtOH, As with EtOH on mitochondrial oxidative stress in microglial cells.

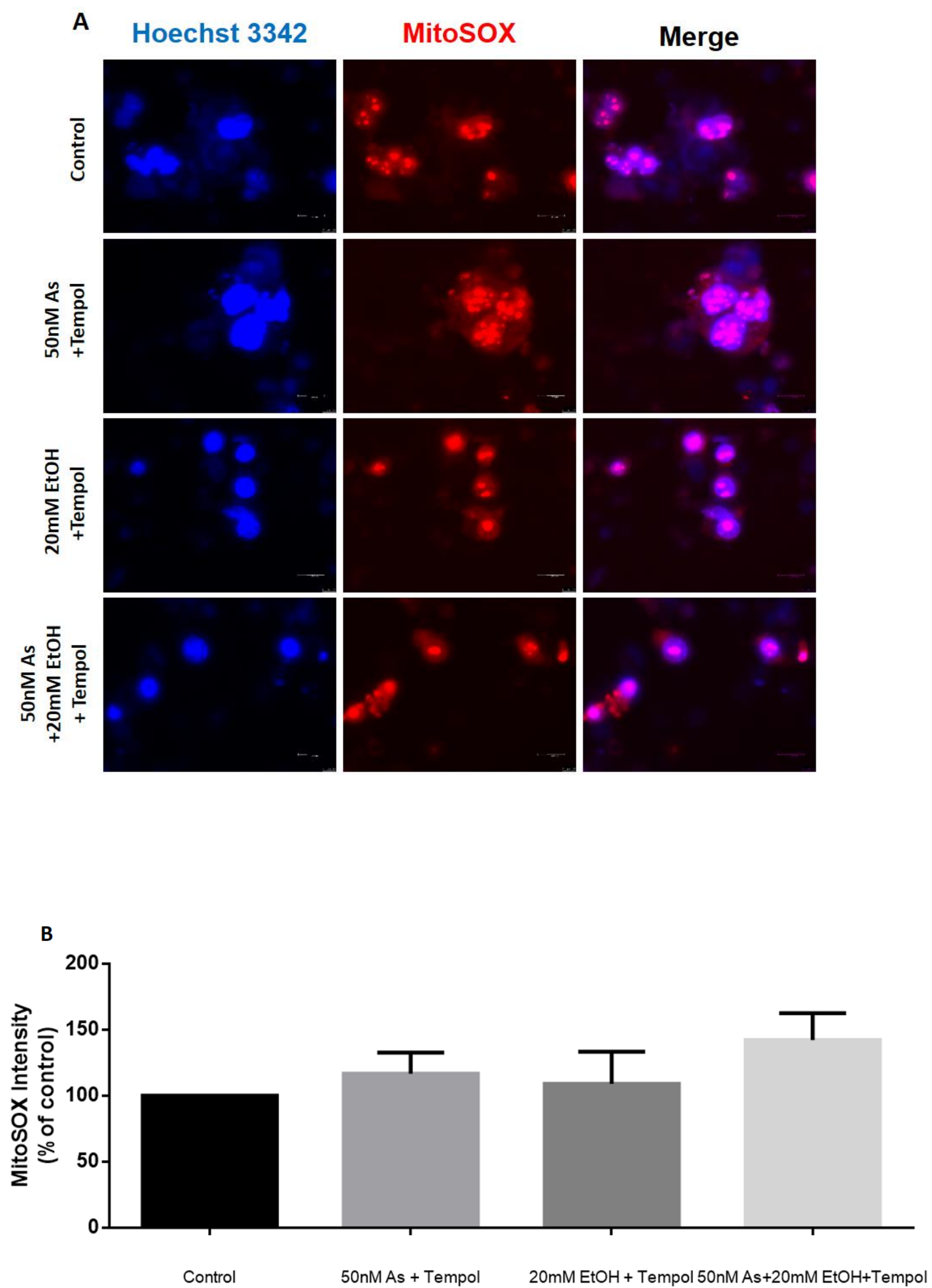

A
Hoechst 3342
MitoSOX
Merge
Control
50nM As
+Tempol
20mM EtOH
+Tempol
50nM As
+20mM EtOH
+ Tempol
B
MitoSOX Intensity
(% of control)
200
150
100
50
0
Control
50nM As + Tempol
20mM EtOH + Tempol
50nM As+20mM EtOH+Tempol

Figure 3.11 Antioxidant decreased As-EtOH-induced mitochondrial ROS production in BV2 cells. After one day treatment of antioxidant Tempol with 50nM As, 20mM EtOH, and the combined two, BV2 cell were incubated with MitoSOX and Hoechst 33342 to label mitochondrial superoxide radical (red) and nuclei (blue) respectively. **A)** Antioxidant Tempol decreased As-EtOH-induced mitochondrial ROS production. Scale bar, 20μm, objective, 40X **B)** Quantification of MitoSOX staining intensity. The mean fluorescence intensity per image was calculated and averaged over three images by Image J software. Around 12 cells were counted for each images. In each experiment, different treatments of fluorescence intensity divided by the mean of fluorescence intensity in control groups to get the percentage of control. As One-way ANOVA result shown, there were no statistically significant difference between group means. Results were presented as mean $\pm$ SEM. n=3.

Chapter 4 Discussion

Grapes and rice which are traditionally used to made wine and beer, take up As from soil, water, and fungicides containing As [46]. The filtering process used to remove sediment from beer and wine could contaminate alcohol drinks with potentially dangerous heavy metals such as As. Subsequently, this can cause people to be exposed to alcohol and As together. The combined treatment of As and EtOH induced toxicity in cancer and liver disease. For example, in colon cancer cells, EtOH enhances low-dose As induced tumor angiogenesis which is related to intracellular ROS generation, NASPH oxidase activation, and upregulation of PI3K/Akt and hypoxia-inducible factor 1 alpha signaling [49]. In zebrafish, inorganic As increased the unfolded protein load in endoplasmic reticulum by directly acting as a reducing agent and indirectly by disrupting the redox balance through ROS generation, which potentiate the effect of EtOH to cause fatty liver disease [50]. Therefore, ROS play an important role on the combine effects of As and EtOH. However, the effects of As and ethanol co-exposure on the brain is still unclear. In this study, we focused on the combined effects of As and EtOH on brain endothelial cells and microglia cells. We hypothesized that As combined with EtOH induced toxicity on the blood brain barrier more severe than either As or ethanol treatment alone.

The vasculature is often affected by, and engaged in, the disease process, resulting in hyperpermeable vessels which further promotes disease propagation [107]. It is believed that recovery of the normal vasculature requires diminishing this hyperpermeable state [108]. In this study, we have tested the effects of As and EtOH on the permeability of brain endothelial cells which mainly regulate the vasculature in the BBB by using FITC-Dextran leakage assay.

Previously, our lab showed that 5, 10, 15, 20 µM As increased permeability of the mouse brain vascular endothelial cells (bEnd3) monolayer through a ROS-vascular endothelial growth factor pathway [8]. In the current studies, we chose 5µM As which is in the range of As concentration in the environment. For example, in Nepal, the concentration of As in tube wells water is range from around 100µg/L (1.33µM) to 800µg/L (10.64µM) in 2018 [109].

In addition to As, EtOH is also associated with the permeability of the endothelial cells. EtOH actions on the endothelial transient receptor potential channels could affect intracellular Ca2+ and Mg2+ dynamics, which mediate leukocyte adhesion to endothelial cells and endothelial permeability at the BBB, thus altering immune and inflammatory response [110].

In our study, we chose 80mM EtOH which is in the concentration range (5-200mM) used in many previous publications [111, 112]. Our results showed that both As and EtOH induced hyper-permeability of RBE4 cell monolayer and the combined treatment further increased the permeability. Although endothelial cells play an important role in BBB permeability, the effects of As and EtOH on these cells have not been well studied. Therefore, more research is needed to gain more information about the effect of the As and EtOH combined treatment on the permeability of the BBB in vivo.

Dysfunction of the BBB, which is induced by oxidative stress is associated with loss of neurons, altered brain functions such as impaired consciousness, memory, and motor impairment by up-regulating expression of cell adhesion molecules on the surface of the brain vascular endothelium [113]. In addition, oxidative stress is an important factor

contributing to endothelial dysfunction and is linked with increased ROS production and decreased availability of antioxidants [114]. Research showed that oxidative stress induced vascular endothelial growth factor can activate a series of steps leading to internalization of vascular-cadherin and breakdown the adherent junctions between endothelial cells [115]. In this study, we proposed that the combined treatment of As and EtOH induced oxidative stress leading to hyperpermeability of the brain endothelial.

Previously, our lab found that As elevated cellular ROS generation in mouse brain vascular endothelial cells bEnd3, and antioxidant NAC, Tempol can rescue this effect [8]. Research showed that EtOH intake induced an increase in ROS generation in freshly isolated endothelial cells from EtOH-treated rats [116]. To investigate the combined effects of As and EtOH on endothelial oxidative stress generation on rat brain endothelial cells, electron paramagnetic resonance spectroscopy was used to determine the production of oxygen-derived free radical within treated RBE4 cells. Our results showed that the combined treatment of 5μM As and 80mM EtOH increased oxidative stress higher than either As or EtOH treatment alone. Since oxidative stress plays an important role on the BBB, it is necessary to determine the source of ROS.

The brain is a high energy consuming organ that requires about 20% of body basal oxygen to fulfill its function [117]. In cellular level, energy is mainly provided via oxidative phosphorylation taking place within mitochondrial. When mitochondria provide energy for cell survival, ROS are also formed through this process. In normal condition, 1-5% oxygen is converted to ROS in mitochondria, which makes mitochondria is one of the major sources of intracellular oxidative stress [66].

Research showed that As treated rats exhibited about 35% increase in 2’, 7’ dichlorofluorescin diacetate signal fluorescence comparing to controls, suggesting that higher mitochondrial ROS accumulation after As treatment in rat brains [118]. In addition, synaptosomes from alcohol-treated mice showed an increase in MitoSOX fluorescence median compared with control group, suggesting EtOH induced mitochondrial oxidative stress in synaptosomes [119]. Therefore, we proposed that the combined treatment of As and EtOH induced mitochondrial oxidative stress on brain endothelial cells.

To investigate the combined effects of As and EtOH on mitochondrial ROS generation on rat brain endothelial cells, live cell imaging of MitoSOX oxidation was used to detect mitochondrial oxidative stress in RBE4 cells after one day treatment of 5μM As, 80mM EtOH, and the combined two. Our results showed that the combined treatment significantly induced mitochondrial oxidative stress more than either As or EtOH treatment alone. Since mitochondria dysfunction can cause mitochondrial oxidative stress, next we focus on the combined effects of As and EtOH on mitochondria bioenergetics in brain endothelial cells.

Mitochondrial function is impaired during the production of ROS, which is currently considered as a critical role in the development and progression of disease. In addition, cellular oxygen consumption is increasingly recognized as a fundamental measure of mitochondrial function. In this study, Seahorse XF96 Analyzer was used to detect the OCR of rat brain endothelial cells after As and EtOH treatment.

Basal OCR reflects coupled mitochondrial respiration as well as uncoupled consumption of oxygen to form ROS at mitochondrial and non-mitochondrial enzymatic sites. Our results showed that treatment with As decreased the basal OCR compared to the

control. The basal OCR is depends on the availability of the substrate such as glucose and pyruvate involving nutrient conditions. As can enter mitochondrial via aquaglyceroporins, where it can bind and inhibit numerous enzymes involved in energy production including pyruvate [120]. Research showed that hepatocyte basal respiration can be greatly increased by addition of pyruvate [121]. Therefore, alteration in nutrient conditions such as decrease in pyruvate level may be considered as one of phenomena that explain why As decreased the basal OCR.

Coupled and uncoupled respiration can be distinguished by examining the effect of an inhibition of ATP synthase (oligomycin) and Complex III (antimycin A). While maximal OCR, provoked by addition of a mitochondrial uncoupling agent such as carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), provides an index of energetic reserve capacity. The decreased maximal OCR indicates mitochondrial dysfunction. Our results show that the combine treatment of As and EtOH decreased MRC indicating mitochondrial dysfunction. Interestingly, our results showed that EtOH trend to increased MRC compared to the control but antioxidant trend to decrease this effect. Previously, our data showed that EtOH trend to induce endothelial hyperpermeability, cellular oxidative stress, and mitochondrial ROS generation. Therefore, excessive increased in mitochondrial MRC may also indicates mitochondrial dysfunction.

Microglial cells are a specialized population of macrophages that are found in the central nervous system. As the resident macrophage cells, they act as the first and main of active immune defense in the central nervous system. The BBB constituted by an extensive network of endothelial cells together with neurons and glial cells, including microglia, forms the neurovascular unit. The crosstalk between these cells guarantees a proper

environment for brain function. Therefore, microglial cells play an important roles in the BBB.

In the second part of our study, we used mouse brain microglial cells (BV2 cells) to investigate the combined effects of As and EtOH on the microglia. BV2 cells are a useful cell line because they can be maintained in culture, yet they keep many of the functions and features that microglia express in vivo [122]. To detect the effects of As and EtOH on the cell viability of BV2 cells, MTT assay was performed. Our results showed that both As and EtOH decreased BV2 cells viability and the combined treatment can further induce BV2 cells death.

After As and EtOH getting into the blood stream, As and EtOH need to pass the brain endothelium formed by brain endothelial cell first in order to enter the brain. Brain microglia which is the major sources of pro-inflammatory cytokines expression in the brain can then interact with As and EtOH that successfully passed from brain endothelium. Our results showed that mouse brain microglial cells are more susceptible to As and EtOH than rat brain endothelial cells, which matches the real condition. Study of Singh showed that 500nM As and IFN-γ increased pro-inflammatory cytokines expression in microglia cell line (N9) [123]. Previously, our lab showed that 5μM As increased permeability of mouse brain vascular endothelial cell (bEnd3) monolayer [8]. EtOH increased superoxide anion generation in cultured neonatal hamster microglia and this effect was maximal at 20mM [124]. EtOH at 200Mm decreased tight junction proteins expression in human cerebral microvascular endothelial cells[57].

Activated microglia migrate and release pro-inflammatory cytokines that contribute to disruption of the BBB, neuronal loss, and enhanced ROS production [125]. To investigate the combined effects of As and EtOH on ROS generation on microglia BV2 cells, electron paramagnetic resonance spectroscopy was used to determine the production of oxygen-derived free radical within treated BV2 cells. Our results showed that the combined treatment of As and EtOH significantly increased ROS more than either As or EtOH treatment alone. Therefore, As and EtOH can activate microglial cells, which is a hallmark of brain pathology.

In addition, similar to rat brain endothelial cells, As and EtOH induced mitochondrial oxidative stress while antioxidant can rescue this effect. However, targeted delivery of antioxidant to mitochondrial has failed to translate into clinical success due to their nonspecific cellular localization, poor transport properties across multiple biological barriers, and associated side effects [126]. Since the function of the mitochondria is complex, more study still needed to find a way to rescue mitochondrial oxidative stress in clinical use.

A study showed that ROS can lead to different molecular cell death mechanisms including necrosis and apoptosis [127]. Mild oxidative stress may activate biological response that can induce apoptosis, while the accumulation of high levels of ROS may promote necrosis instead [128]. In addition to the well-established role of the mitochondria in energy metabolism, regulation of cell death has recently emerged as a second major function of these organelles[129]. In a neuroblastoma cell line, oxidative stress induced necrotic cell death via mitochondria dependent burst of ROS [130]. During apoptosis, mitochondrial membrane permeability increases and the release into the cytosol of pro-

apoptotic factors including procaspase, caspase activator and caspase independent factors such as apoptosis-inducing factor leads to the apoptotic phenotype [131]. Cytochrome c released from mitochondria, that triggers caspase activation, appears to be largely mediated by direct or indirect ROS action [132]. Our results showed the combined treatment of As and EtOH induced microglia cells death and antioxidant reduced mitochondria oxidative stress. Further studies are needed to investigate the role of mitochondrial oxidative stress on As-EtOH-induced microglia cells death.

In this study, we investigated the combined effects of As and EtOH on the BBB by using brain endothelial cells and microglial cells. However, the components of the BBB are complicated. Pericytes are multi-functional mural cells of the microcirculation that wrap around the endothelial cells that line the capillaries and venules. Astrocytes are essential for the formation and maintenance of the BBB and the formation of strong tight junctions. In vertebrates, the BBB formation is coordinated by interactions between neurons, glial cells and endothelial cells [133]. The limitation of our study is that it only focused on endothelial cells and microglial cells and there are other BBB cellular components else. Therefore, more studies are needed to investigate the effects of As and EtOH combined treatment on the BBB not only in endothelial cells and microglial cells. In addition, there is still a gap between in vitro and in vivo study. Animal studies are needed to investigate the effects of As and EtOH co-exposure on the BBB.

In summary, our present results showed the combined effects of the As and EtOH on the brain endothelial cells and microglial cells (**Figure 4.1**). We have identified that the combined treatment of As and EtOH induced mitochondrial oxidative stress more than either As or EtOH treatment alone in RBE4 cells and BV2 cells, while antioxidant Tempol

can reduce this effect. The increased in mitochondria oxidative stress is associated with increased in ROS. Our results showed that both As and EtOH increased ROS level in endothelial cells and microglial cells. We investigated the permeability of endothelial cells and found that As with EtOH significantly increased RBE4 cells permeability. In conclusion, the combined treatment of As and EtOH induced toxicity in both brain endothelial cells and microglial cells.

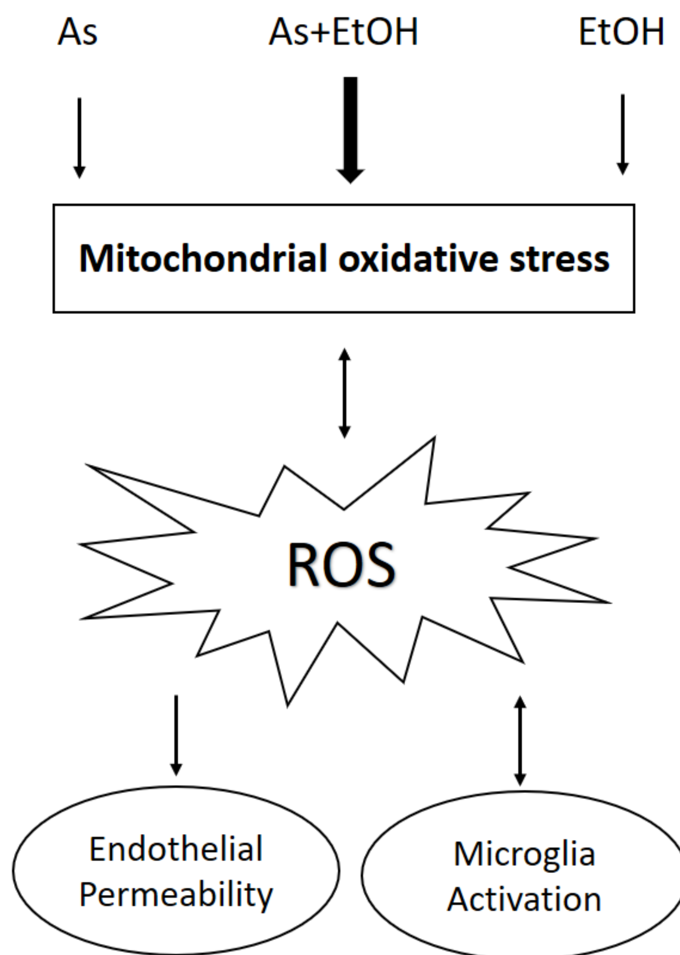


Figure 4.1 The combined effects of As and EtOH

References

1. Hsieh, Y.J., et al., *Effects of the Acute and Chronic Ethanol Intoxication on Acetate Metabolism and Kinetics in the Rat Brain.* Alcohol Clin Exp Res, 2018. **42**(2): p. 329-337.
2. Mocelin, R., et al., *Behavioral and Biochemical Effects of N-Acetylcysteine in Zebrafish Acutely Exposed to Ethanol.* Neurochem Res, 2018. **43**(2): p. 458-464.
3. Gosselet, F., *[Modelling of the blood-brain barrier].* Med Sci (Paris), 2017. **33**(4): p. 423-431.
4. Blanchette, M. and R. Daneman, *Formation and maintenance of the BBB.* Mech Dev, 2015. **138 Pt 1**: p. 8-16.
5. Manthari, R.K., et al., *Arsenic-Induced Autophagy in the Developing Mouse Cerebellum: Involvement of the Blood-Brain Barrier's Tight-Junction Proteins and the PI3K-Akt-mTOR Signaling Pathway.* J Agric Food Chem, 2018. **66**(32): p. 8602-8614.
6. Singh, A.K., et al., *Effects of chronic ethanol drinking on the blood brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection.* Alcohol Alcohol, 2007. **42**(5): p. 385-99.
7. Islam, M.T., *Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders.* Neurol Res, 2017. **39**(1): p. 73-82.
8. Bao, L. and H. Shi, *Arsenite induces endothelial cell permeability increase through a reactive oxygen species-vascular endothelial growth factor pathway.* Chem Res Toxicol, 2010. **23**(11): p. 1726-34.
9. Alves, N.G., S.Y. Yuan, and J.W. Breslin, *Sphingosine-1-phosphate protects against brain microvascular endothelial junctional protein disorganization and barrier dysfunction caused by alcohol.* Microcirculation, 2019. **26**(1): p. e12506.
10. Luo, J., *Autophagy and ethanol neurotoxicity.* Autophagy, 2014. **10**(12): p. 2099-108.
11. Park, J., et al., *Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-kappaB pathways.* Neurosci Lett, 2015. **584**: p. 191-6.
12. Dohgu, S., et al., *Monomeric alpha-synuclein induces blood-brain barrier dysfunction through activated brain pericytes releasing inflammatory mediators in vitro.* Microvasc Res, 2019. **124**: p. 61-66.
13. Almutairi, M.M., et al., *Factors controlling permeability of the blood-brain barrier.* Cell Mol Life Sci, 2016. **73**(1): p. 57-77.
14. Saha, S., et al., *Ameliorative role of genistein against age-dependent chronic arsenic toxicity in murine brains via the regulation of oxidative stress and inflammatory signaling cascades.* J Nutr Biochem, 2018. **55**: p. 26-40.
15. Chen, Q. and P. Hu, *Proanthocyanidins prevent ethanol-induced cognitive impairment by suppressing oxidative and inflammatory stress in adult rat brain.* Neuroreport, 2017. **28**(15): p. 980-986.
16. Xu, L., D. He, and Y. Bai, *Microglia-Mediated Inflammation and Neurodegenerative Disease.* Mol Neurobiol, 2016. **53**(10): p. 6709-6715.
17. Agusa, T., et al., *Human exposure to arsenic from drinking water in Vietnam.* Sci Total Environ, 2014. **488-489**: p. 562-9.

18. Andrew, A.S., et al., *Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population.* Environ Health Perspect, 2008. **116**(4): p. 524-31.
19. Serre, M.L., et al., *An application of the holistochastic human exposure methodology to naturally occurring arsenic in Bangladesh drinking water.* Risk Anal, 2003. **23**(3): p. 515-28.
20. Garelick, H., et al., *Arsenic pollution sources.* Rev Environ Contam Toxicol, 2008. **197**: p. 17-60.
21. Ratnaike, R.N., *Acute and chronic arsenic toxicity.* Postgrad Med J, 2003. **79**(933): p. 391-6.
22. Naujokas, M.F., et al., *The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem.* Environ Health Perspect, 2013. **121**(3): p. 295-302.
23. Wei, B., et al., *The relationships between arsenic methylation and both skin lesions and hypertension caused by chronic exposure to arsenic in drinking water.* Environ Toxicol Pharmacol, 2017. **53**: p. 89-94.
24. Hore, S.K., et al., *Detecting arsenic-related skin lesions: experiences from a large community-based survey in Bangladesh.* Int J Environ Health Res, 2007. **17**(2): p. 141-9.
25. Roh, T., et al., *Low-level arsenic exposure from drinking water is associated with prostate cancer in Iowa.* Environ Res, 2017. **159**: p. 338-343.
26. Kuo, C.C., et al., *The Association of Arsenic Metabolism with Cancer, Cardiovascular Disease, and Diabetes: A Systematic Review of the Epidemiological Evidence.* Environ Health Perspect, 2017. **125**(8): p. 087001.
27. Hsu, K.H., et al., *Dose-Response Relationship between Inorganic Arsenic Exposure and Lung Cancer among Arseniasis Residents with Low Methylation Capacity.* Cancer Epidemiol Biomarkers Prev, 2017. **26**(5): p. 756-761.
28. Oberoi, S., et al., *Global burden of cancer and coronary heart disease resulting from dietary exposure to arsenic, 2015.* Environ Res, 2019. **171**: p. 185-192.
29. Calderon, J., et al., *Exposure to arsenic and lead and neuropsychological development in Mexican children.* Environ Res, 2001. **85**(2): p. 69-76.
30. Roy, A., et al., *Association between arsenic exposure and behavior among first-graders from Torreon, Mexico.* Environ Res, 2011. **111**(5): p. 670-6.
31. Sen, D. and P. Sarathi Biswas, *Arsenicosis: Is it a Protective or Predisposing Factor for Mental Illness?* Iran J Psychiatry, 2012. **7**(4): p. 180-3.
32. Ramos-Chavez, L.A., et al., *Neurological effects of inorganic arsenic exposure: altered cysteine/glutamate transport, NMDA expression and spatial memory impairment.* Front Cell Neurosci, 2015. **9**: p. 21.
33. Beaver, L.M., et al., *Combinatorial effects of zinc deficiency and arsenic exposure on zebrafish (Danio rerio) development.* PLoS One, 2017. **12**(8): p. e0183831.
34. Zhou, H., et al., *Postnatal low-concentration arsenic exposure induces autism-like behavior and affects frontal cortex neurogenesis in rats.* Environ Toxicol Pharmacol, 2018. **62**: p. 188-198.
35. Htway, S.M., et al., *Effects of Developmental Arsenic Exposure on the Social Behavior and Related Gene Expression in C3H Adult Male Mice.* Int J Environ Res Public Health, 2019. **16**(2).

36. Moore, C.L., et al., *Developmental neurotoxicity of inorganic arsenic exposure in Sprague-Dawley rats.* Neurotoxicol Teratol, 2019. **72**: p. 49-57.
37. Saritha, S., et al., *Effects of combined arsenic and lead exposure on the brain monoaminergic system and behavioral functions in rats: Reversal effect of MiADMSA.* Toxicol Ind Health, 2019. **35**(2): p. 89-108.
38. Grant, B.F., et al., *Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III.* JAMA Psychiatry, 2015. **72**(8): p. 757-66.
39. O'Keefe, J.H., et al., *Alcohol and cardiovascular health: the dose makes the poison...or the remedy.* Mayo Clin Proc, 2014. **89**(3): p. 382-93.
40. Homman, L.E., et al., *The longitudinal relationship of alcohol problems and depressive symptoms and the impact of externalising symptoms: findings from the Belfast Youth Developmental Study.* Soc Psychiatry Psychiatr Epidemiol, 2019.
41. Seesen, M., et al., *High blood alcohol concentration associated with traumatic brain injury among traffic injury patients during New Year festivals in Thailand.* Traffic Inj Prev, 2019: p. 1-7.
42. Nivukoski, U., et al., *Liver enzymes in alcohol consumers with or without binge drinking.* Alcohol, 2019.
43. Luo, J., et al., *Pontine Changes in Metabolites and Axonal Fibres of Rats Following Four-week Alcohol Exposure: In Vivo Diffusion Tensor Imaging and 1h-magnetic Resonance Spectroscopy Study at 7.0 T.* Alcohol Alcohol, 2017. **52**(2): p. 145-150.
44. Swart, P.C., et al., *Early ethanol exposure and vinpocetine treatment alter learning- and memory-related proteins in the rat hippocampus and prefrontal cortex.* J Neurosci Res, 2017. **95**(5): p. 1204-1215.
45. Lagstrom, O., et al., *Voluntary ethanol intake produces sub-region specific neuroadaptations in striatal and cortical areas of Wistar rat.* Alcohol Clin Exp Res, 2019.
46. Engel, R.R., et al., *Vascular effects of chronic arsenic exposure: a review.* Epidemiol Rev, 1994. **16**(2): p. 184-209.
47. Wilson, D., *Arsenic Content in American Wine.* J Environ Health, 2015. **78**(3): p. 16-22.
48. Ayotte, J.D., et al., *Estimating the High-Arsenic Domestic-Well Population in the Conterminous United States.* Environ Sci Technol, 2017. **51**(21): p. 12443-12454.
49. Wang, L., et al., *Ethanol enhances tumor angiogenesis in vitro induced by low-dose arsenic in colon cancer cells through hypoxia-inducible factor 1 alpha pathway.* Toxicol Sci, 2012. **130**(2): p. 269-80.
50. Bambino, K., et al., *Inorganic arsenic causes fatty liver and interacts with ethanol to cause alcoholic liver disease in zebrafish.* Dis Model Mech, 2018. **11**(2).
51. Daneman, R. and A. Prat, *The blood-brain barrier.* Cold Spring Harb Perspect Biol, 2015. **7**(1): p. a020412.
52. Muller, S.M., et al., *Effects of arsenolipids on in vitro blood-brain barrier model.* Arch Toxicol, 2018. **92**(2): p. 823-832.
53. Bhowmick, S., et al., *Impairment of pericyte-endothelium crosstalk leads to blood-brain barrier dysfunction following traumatic brain injury.* Exp Neurol, 2019. **317**: p. 260-270.
54. Yazdani, S., et al., *Endothelial cell barriers: Transport of molecules between blood and tissues.* Traffic, 2019.

55. Gonzalez-Mariscal, L., et al., *Tight junction proteins.* Prog Biophys Mol Biol, 2003. **81**(1): p. 1-44.
56. Chen, S.C., et al., *Microvascular dysfunction with increased vascular leakage response in mice systemically exposed to arsenic.* Cardiovasc Toxicol, 2014. **14**(3): p. 222-31.
57. Yu, H., et al., *Long-term exposure to ethanol downregulates tight junction proteins through the protein kinase Calpha signaling pathway in human cerebral microvascular endothelial cells.* Exp Ther Med, 2017. **14**(5): p. 4789-4796.
58. Niedzielska, E., et al., *Oxidative Stress in Neurodegenerative Diseases.* Mol Neurobiol, 2016. **53**(6): p. 4094-4125.
59. Ooi, B.K., B.H. Goh, and W.H. Yap, *Oxidative Stress in Cardiovascular Diseases: Involvement of Nrf2 Antioxidant Redox Signaling in Macrophage Foam Cells Formation.* Int J Mol Sci, 2017. **18**(11).
60. Moloney, J.N. and T.G. Cotter, *ROS signalling in the biology of cancer.* Semin Cell Dev Biol, 2018. **80**: p. 50-64.
61. Salim, S., *Oxidative Stress and the Central Nervous System.* J Pharmacol Exp Ther, 2017. **360**(1): p. 201-205.
62. Kuriakose, M., et al., *Synergistic Role of Oxidative Stress and Blood-Brain Barrier Permeability as Injury Mechanisms in the Acute Pathophysiology of Blast-induced Neurotrauma.* Sci Rep, 2019. **9**(1): p. 7717.
63. Sajja, R.K., S. Rahman, and L. Cucullo, *Drugs of abuse and blood-brain barrier endothelial dysfunction: A focus on the role of oxidative stress.* J Cereb Blood Flow Metab, 2016. **36**(3): p. 539-54.
64. Liu, X., B. Sui, and J. Sun, *Blood-brain barrier dysfunction induced by silica NPs in vitro and in vivo: Involvement of oxidative stress and Rho-kinase/JNK signaling pathways.* Biomaterials, 2017. **121**: p. 64-82.
65. Shi, J., et al., *Oleuropein protects intracerebral hemorrhage-induced disruption of blood-brain barrier through alleviation of oxidative stress.* Pharmacol Rep, 2017. **69**(6): p. 1206-1212.
66. Bhat, A.H., et al., *Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight.* Biomed Pharmacother, 2015. **74**: p. 101-10.
67. Zhao, R.Z., et al., *Mitochondrial electron transport chain, ROS generation and uncoupling (Review).* Int J Mol Med, 2019.
68. Bhatti, J.S., G.K. Bhatti, and P.H. Reddy, *Mitochondrial dysfunction and oxidative stress in metabolic disorders - A step towards mitochondria based therapeutic strategies.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(5): p. 1066-1077.
69. Li, P.A., X. Hou, and S. Hao, *Mitochondrial biogenesis in neurodegeneration.* J Neurosci Res, 2017. **95**(10): p. 2025-2029.
70. Chakrabarty, S., et al., *Mitochondria in health and disease.* Mitochondrion, 2018. **43**: p. 25-29.
71. Pizzino, G., et al., *Oxidative Stress: Harms and Benefits for Human Health.* Oxid Med Cell Longev, 2017. **2017**: p. 8416763.
72. Li, C., et al., *Oxidative Stress-Related Mechanisms and Antioxidant Therapy in Diabetic Retinopathy.* Oxid Med Cell Longev, 2017. **2017**: p. 9702820.
73. Cai, J., et al., *Antioxidant Drug Tempol Promotes Functional Metabolic Changes in the Gut Microbiota.* J Proteome Res, 2016. **15**(2): p. 563-71.

74. Alzoubi, K.H., A.M. Rababa'h, and O.N. Al Yacoub, *Tempol prevents post-traumatic stress disorder induced memory impairment.* Physiol Behav, 2018. **184**: p. 189-195.
75. Bernardy, C.C.F., et al., *Tempol, a Superoxide Dismutase Mimetic Agent, Inhibits Superoxide Anion-Induced Inflammatory Pain in Mice.* Biomed Res Int, 2017. **2017**: p. 9584819.
76. Skaper, S.D., *Impact of Inflammation on the Blood-Neural Barrier and Blood-Nerve Interface: From Review to Therapeutic Preview.* Int Rev Neurobiol, 2017. **137**: p. 29-45.
77. Mehrabadi, A.R., et al., *Poly(ADP-ribose) polymerase-1 regulates microglia mediated decrease of endothelial tight junction integrity.* Neurochem Int, 2017. **108**: p. 266-271.
78. Cheng, S., et al., *Methylprednisolone sodium succinate reduces BBB disruption and inflammation in a model mouse of intracranial haemorrhage.* Brain Res Bull, 2016. **127**: p. 226-233.
79. Dudvarski Stankovic, N., et al., *Microglia-blood vessel interactions: a double-edged sword in brain pathologies.* Acta Neuropathol, 2016. **131**(3): p. 347-63.
80. Varatharaj, A. and I. Galea, *The blood-brain barrier in systemic inflammation.* Brain Behav Immun, 2017. **60**: p. 1-12.
81. Lecuyer, M.A., H. Kebir, and A. Prat, *Glial influences on BBB functions and molecular players in immune cell trafficking.* Biochim Biophys Acta, 2016. **1862**(3): p. 472-82.
82. Stamatovic, S.M., et al., *Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase.* J Cell Sci, 2003. **116**(Pt 22): p. 4615-28.
83. Satoh, S., et al., *Proinflammatory cytokines and endotoxin stimulate ICAM-1 gene expression and secretion by normal human hepatocytes.* Immunology, 1994. **82**(4): p. 571-6.
84. Dietrich, J.B., *The adhesion molecule ICAM-1 and its regulation in relation with the blood-brain barrier.* J Neuroimmunol, 2002. **128**(1-2): p. 58-68.
85. Hurtado-Alvarado, G., et al., *Blood-Brain Barrier Disruption Induced by Chronic Sleep Loss: Low-Grade Inflammation May Be the Link.* J Immunol Res, 2016. **2016**: p. 4576012.
86. Ifergan, I., et al., *Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: relevance to multiple sclerosis.* Ann Neurol, 2006. **60**(1): p. 45-55.
87. Rochfort, K.D., et al., *Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions.* PLoS One, 2014. **9**(7): p. e101815.
88. Freeman, L.R. and J.N. Keller, *Oxidative stress and cerebral endothelial cells: regulation of the blood-brain-barrier and antioxidant based interventions.* Biochim Biophys Acta, 2012. **1822**(5): p. 822-9.
89. Guzik, T.J. and R.M. Touyz, *Oxidative Stress, Inflammation, and Vascular Aging in Hypertension.* Hypertension, 2017. **70**(4): p. 660-667.
90. Miller, M.W., et al., *Oxidative Stress, Inflammation, and Neuroprogression in Chronic PTSD.* Harv Rev Psychiatry, 2018. **26**(2): p. 57-69.
91. Aviello, G. and U.G. Knaus, *ROS in gastrointestinal inflammation: Rescue Or Sabotage?* Br J Pharmacol, 2017. **174**(12): p. 1704-1718.
92. Liu, J., et al., *Assessment of relationship on excess arsenic intake from drinking water and cognitive impairment in adults and elders in arsenicosis areas.* Int J Hyg Environ Health, 2017. **220**(2 Pt B): p. 424-430.

93. Tyler, C.R. and A.M. Allan, *The Effects of Arsenic Exposure on Neurological and Cognitive Dysfunction in Human and Rodent Studies: A Review.* Curr Environ Health Rep, 2014. **1**: p. 132-147.
94. Fama, R., et al., *Neurological, nutritional and alcohol consumption factors underlie cognitive and motor deficits in chronic alcoholism.* Addict Biol, 2017.
95. Raike, R.S., et al., *Stress, caffeine and ethanol trigger transient neurological dysfunction through shared mechanisms in a mouse calcium channelopathy.* Neurobiol Dis, 2013. **50**: p. 151-9.
96. Wang, L., et al., *Ethanol enhances arsenic-induced cyclooxygenase-2 expression via both NFAT and NF-kappaB signalings in colorectal cancer cells.* Toxicol Appl Pharmacol, 2015. **288**(2): p. 232-9.
97. Rosenberg, G.A., *Neurological diseases in relation to the blood-brain barrier.* J Cereb Blood Flow Metab, 2012. **32**(7): p. 1139-51.
98. Pun, P.B., J. Lu, and S. Moochhala, *Involvement of ROS in BBB dysfunction.* Free Radic Res, 2009. **43**(4): p. 348-64.
99. Agostini, J.F., et al., *Cholinergic System and Oxidative Stress Changes in the Brain of a Zebrafish Model Chronically Exposed to Ethanol.* Neurotox Res, 2018. **33**(4): p. 749-758.
100. Brocardo, P.S., et al., *The Effects of Ethanol Exposure During Distinct Periods of Brain Development on Oxidative Stress in the Adult Rat Brain.* Alcohol Clin Exp Res, 2017. **41**(1): p. 26-37.
101. Rochfort, K.D. and P.M. Cummins, *The blood-brain barrier endothelium: a target for pro-inflammatory cytokines.* Biochem Soc Trans, 2015. **43**(4): p. 702-6.
102. Shiu, C., et al., *HIV-1 gp120 as well as alcohol affect blood-brain barrier permeability and stress fiber formation: involvement of reactive oxygen species.* Alcohol Clin Exp Res, 2007. **31**(1): p. 130-7.
103. Pung, Y.F., et al., *The role of mitochondrial bioenergetics and reactive oxygen species in coronary collateral growth.* Am J Physiol Heart Circ Physiol, 2013. **305**(9): p. H1275-80.
104. Wilcox, C.S., *Effects of Tempol and redox-cycling nitroxides in models of oxidative stress.* Pharmacol Ther, 2010. **126**(2): p. 119-45.
105. Wang, X., et al., *Role of hypoxiainducible factor1alpha in autophagic cell death in microglial cells induced by hypoxia.* Mol Med Rep, 2017. **15**(4): p. 2097-2105.
106. Taetzsch, T., et al., *Redox regulation of NF-kappaB p50 and M1 polarization in microglia.* Glia, 2015. **63**(3): p. 423-40.
107. Claesson-Welsh, L., *Vascular permeability--the essentials.* Ups J Med Sci, 2015. **120**(3): p. 135-43.
108. Park-Windhol, C. and P.A. D'Amore, *Disorders of Vascular Permeability.* Annu Rev Pathol, 2016. **11**: p. 251-81.
109. Mueller, B. and S.J. Hug, *Climatic variations and de-coupling between arsenic and iron in arsenic contaminated ground water in the lowlands of Nepal.* Chemosphere, 2018. **210**: p. 347-358.
110. Chang, S.L., et al., *Ethanol's Effects on Transient Receptor Potential Channel Expression in Brain Microvascular Endothelial Cells.* J Neuroimmune Pharmacol, 2018. **13**(4): p. 498-508.

111. Lamichhane, T.N., et al., *Ethanol Induces Enhanced Vascularization Bioactivity of Endothelial Cell-Derived Extracellular Vesicles via Regulation of MicroRNAs and Long Non-Coding RNAs.* Sci Rep, 2017. **7**(1): p. 13794.
112. Abdul Muneer, P.M., et al., *Ethanol impairs glucose uptake by human astrocytes and neurons: protective effects of acetyl-L-carnitine.* Int J Physiol Pathophysiol Pharmacol, 2011. **3**(1): p. 48-56.
113. Chodobski, A., B.J. Zink, and J. Szmydynger-Chodobska, *Blood-brain barrier pathophysiology in traumatic brain injury.* Transl Stroke Res, 2011. **2**(4): p. 492-516.
114. Shrestha, B., et al., *Differential arterial and venous endothelial redox responses to oxidative stress.* Microcirculation, 2018. **25**(7): p. e12486.
115. Ulker, E., et al., *Ascorbic acid prevents VEGF-induced increases in endothelial barrier permeability.* Mol Cell Biochem, 2016. **412**(1-2): p. 73-9.
116. Hipolito, U.V., et al., *Vitamin C prevents the endothelial dysfunction induced by acute ethanol intake.* Life Sci, 2015. **141**: p. 99-107.
117. Grimm, A. and A. Eckert, *Brain aging and neurodegeneration: from a mitochondrial point of view.* J Neurochem, 2017. **143**(4): p. 418-431.
118. Prakash, C., M. Soni, and V. Kumar, *Biochemical and Molecular Alterations Following Arsenic-Induced Oxidative Stress and Mitochondrial Dysfunction in Rat Brain.* Biol Trace Elem Res, 2015. **167**(1): p. 121-9.
119. Karadayian, A.G., et al., *Free radical production and antioxidant status in brain cortex non-synaptic mitochondria and synaptosomes at alcohol hangover onset.* Free Radic Biol Med, 2017. **108**: p. 692-703.
120. Luz, A.T., et al., *Arsenite Uncouples Mitochondrial Respiration and Induces a Warburg-Like Effect in Caenorhabditis elegans.* Toxicol Sci, 2016. **154**(1): p. 195.
121. Korzeniewski, B., M.E. Harper, and M.D. Brand, *Proportional activation coefficients during stimulation of oxidative phosphorylation by lactate and pyruvate or by vasopressin.* Biochim Biophys Acta, 1995. **1229**(3): p. 315-22.
122. Henn, A., et al., *The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation.* Altex, 2009. **26**(2): p. 83-94.
123. Singh, V., et al., *Sneaky Entry of IFNgamma Through Arsenic-Induced Leaky Blood-Brain Barrier Reduces CD200 Expression by Microglial pro-Inflammatory Cytokine.* Mol Neurobiol, 2019. **56**(2): p. 1488-1499.
124. Colton, C.A., J. Snell-Callanan, and O.N. Chernyshev, *Ethanol induced changes in superoxide anion and nitric oxide in cultured microglia.* Alcohol Clin Exp Res, 1998. **22**(3): p. 710-6.
125. Ding, X., et al., *Activated microglia induce the production of reactive oxygen species and promote apoptosis of co-cultured retinal microvascular pericytes.* Graefes Arch Clin Exp Ophthalmol, 2017. **255**(4): p. 777-788.
126. Sharma, A., et al., *Targeting Mitochondrial Dysfunction and Oxidative Stress in Activated Microglia using Dendrimer-Based Therapeutics.* Theranostics, 2018. **8**(20): p. 5529-5547.
127. Valencia, A. and J. Moran, *Reactive oxygen species induce different cell death mechanisms in cultured neurons.* Free Radic Biol Med, 2004. **36**(9): p. 1112-25.
128. Silva, J.P. and O.P. Coutinho, *Free radicals in the regulation of damage and cell death - basic mechanisms and prevention.* Drug Discov Ther, 2010. **4**(3): p. 144-67.

129. Orrenius, S., *Reactive oxygen species in mitochondria-mediated cell death.* Drug Metab Rev, 2007. **39**(2-3): p. 443-55.
130. Choi, K., et al., *Oxidative stress-induced necrotic cell death via mitochondira-dependent burst of reactive oxygen species.* Curr Neurovasc Res, 2009. **6**(4): p. 213-22.
131. Fleury, C., B. Mignotte, and J.L. Vayssiere, *Mitochondrial reactive oxygen species in cell death signaling.* Biochimie, 2002. **84**(2-3): p. 131-41.
132. Simon, H.U., A. Haj-Yehia, and F. Levi-Schaffer, *Role of reactive oxygen species (ROS) in apoptosis induction.* Apoptosis, 2000. **5**(5): p. 415-8.
133. Banerjee, S. and M.A. Bhat, *Neuron-glial interactions in blood-brain barrier formation.* Annu Rev Neurosci, 2007. **30**: p. 235-58.